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The effect of a complex phytase-containing enzyme preparation on the process of rye wort fermentation

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Abstract: A complex of amylases, proteases, and hemicellulases is known to enhance deep conversion of polysaccharides and proteins, especially in the processing of difficult-to-ferment raw materials, such as rye, providing grain wort with soluble carbohydrates, amino acids, and peptides. Grain is also a source of phosphorus, whose bioavailability can be increased by hydrolysing the grain with phytase-containing enzyme preparations. However, their catalytic action during the preparation of grain wort for alcohol production has hardly been studied. This study aimed to investigate the effect of a new complex phytase-containing enzyme preparation on yeast metabolism and the efficiency of rye wort fermentation. The work was carried out in the Russian Research Institute of Food Biotechnology. The Glucavamorin complex enzyme preparations derived from recombinant strains were the object of our research. The preparations differed in the activity level of the main enzyme, glucoamylase, and minor hemicellulase enzymes, as well as in the presence of phytase. The results confirmed their biocatalytic ability to efficiently hydrolyse polymers of rye grain. An increased content of hemicellulases in Glucavamorin-Xyl improved the rheological properties of rye wort. The greatest effect was achieved with the phytase-containing Glucavamorin-Ply. This preparation improved the phosphorus nutrition of yeast, which increased its biomass by 30% and decreased the level of fermentation by-products by 18–20%. Alcohol yield tended to increase and its strength reached 10.5–10.9% vol. When using a phytase-containing enzyme complex, it was possible to reduce the amount of the main enzyme, glucoamylase, without causing the key fermentation indicators to degrade.

Keywords: Rye wort, phytase, enzyme preparations, yeast, ethanol, fermentation, metabolites

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INTRODUCTION

Modern alcohol technologies are based on complex and deep processing of agricultural raw materials aimed at improving production profitability. The effectiveness of biotechnological processing is achieved by developing new biocatalysts of various action and substrate specificity. This ensures deep hydrolysis of high molecular weight polymers of grain, especially rye with its high content of non-starch polysaccharides, gum substances, and mucus.

As shown by many studies, the use of complex enzyme preparations with broad substrate specificity can increase the depth of hydrolysis of grain polymers into ethanol, especially when making concentrated grain wort [1–4]. The complex should contain amylolytic, proteolytic, and hemicellulase enzymes. Amylolytic enzymes play a part in starch conversion: α-amylase in starch dextrinization and liquefaction and glucoamylase in its saccharification). Proteases are beneficial for the generation and metabolism of alcoholic yeast, since their catalytic effect on protein enriches the wort with easily digestible amino acids assimilated by yeast [5]. Hemicellulases (β-glucanases and xylanases), catalysing the hydrolysis of non-starch polysaccharides, decrease the wort viscosity and lead to the formation of additional fermented carbohydrates due to the destruction of grain xylans and glucans. The synergic action of amylolytic, proteolytic and hemicellulase enzymes improve the quality of grain wort and its rheological properties, especially when processing difficult-to-ferment raw materials, such as rye and barley. Improved biocatalytic conversion of grain polymers intensifies alcohol

fermentation, increasing the target product (ethanol) yield and decreasing side metabolites formation.

A number of studies into the phytase effect on the processing of sorghum and corn for lager beer production showed a potential possibility of improving nutritional conditions for yeast during the fermentation of raw whole grains [6]. Some researchers noted a positive effect of phytase treatment on the embryo and fibre yield during the dry grinding of yellow dent corn [7]. A study of the phytolytic effect on the quality of wheat bread enriched with bran revealed an increase in the bioavailability of iron contained in it [8]. To make the conversion of phytin-containing raw materials more efficient, microorganism strains with phytase activity were selected and identified [9, 10]. Considerable research was conducted into the use of phytase to improve the digestibility of feed nutrients, including phytate phosphorus [11, 12]. However, there is a lack of studies into the effectiveness of phytolytic enzymes during the preparation of concentrated grain wort for alcohol production, especially from rye.

Currently, extensive studies are underway to obtain enzyme preparations based on recombinant strains of microscopic fungi using genetic engineering and mutagenesis [13–15]. The preparations contain a complex of enzymes with an increased biocatalytic capacity for xylanase, β-glucanase, and cellulase, and they can be used in the alcohol industry [16]. New highly active multienzyme preparations can contribute towards the implementation of innovative technologies for deep conversion of grain into ethanol.

Of particular interest are phytolytic enzymes. Phytase is an enzyme that breaks down phytic acid. Phytic acid in the form of myo-inositol hexaphosphoric acid or phytate (acid salt) is the main form of mineral phosphorus in plant tissues [17]. Cereal grains have a particularly high content of phytic acid [18]. Phosphorus is essential for yeast cells to grow and develop. Under anaerobic conditions, yeast assimilates phosphorus mainly in the initial period of fermentation when its consumption is 80–90% of the maximum content in yeast. Young, actively breeding yeast cells are richer in phosphorus than non-budding old cells. For example, after 6 h of fermentation, yeast cells accumulate 2.15% of phosphorus per dry matter, while this value is only 1% at the end of fermentation. Therefore, when making grain wort, it is important to enrich it with phosphorus to ensure a stable process of yeast generation and alcoholic fermentation.

Cereals are the main source of phosphorus, whose bioavailability can be enhanced by hydrolysis of grain with phytase-containing enzyme preparations. Phytate hydrolysis helps reduce the consumption of enzyme preparations, as it inhibits many enzymes and enables the release of valuable trace elements, such as calcium, magnesium, zinc, etc. [19, 20]. This way, it provides alcoholic yeast with additional nutrition.

Apart from generating the main products of fermentation, namely alcohol and carbon dioxide, yeast cells synthesise metabolites called secondary products or by-products of fermentation. The biosynthesis of by-products is associated with the cell's regulatory functions. By-products formation depends on the medium composition, the level of nitrogen, carbon, and phosphorus in the medium, the conditions of yeast cultivation and the genetic characteristics of the strain used [3, 4]. One of the ways to improve the efficiency of alcohol production is to create conditions to reduce carbohydrate expenditure for the formation of fermentation by-products through the use of media with a balanced amino acid composition. The amount of ethanol impurities can also be reduced by regulating technological processes in such a way that conditions are provided to promote ethanol synthesis with decreased formation of fermentation by-products [4]. Therefore, complex enzyme preparations contribute to a more rational use of high-molecular components of grain raw materials.

Our aim was to study the effect of a new complex phytase-containing preparation on yeast metabolism and the efficiency of rye wort fermentation.

STUDY OBJECTS AND METHODS

This research was conducted at the Department for Biotechnology of Enzyme Preparations, Yeast, Organic Acids and Biologically Active Substances of the Russian Research Institute of Food Biotechnology, a branch of the Federal Research Centre of Nutrition, Biotechnology and Food Safety.

The study objects included rye grain, the *Saccharomyces cerevisiae* 985T alcohol yeast, and complex enzyme preparations (EP), Glucavamorin Xyl and Glucavamorin Ply. The preparations were obtained in the Laboratory of New Enzyme Producers of the Russian Research Institute of Food Biotechnology from the transformants of the commercial *Aspergillus awamori* strain [21]. They differed in the activity level of the major enzyme (glucoamylase) and minor enzymes (hemicellulases).

We used the following methods to determine the catalytic activity of the enzyme preparations.

Amylolytic and glucoamylase activity was determined according to State Standard 54330-2011*. The method for determining amylolytic activity is based on the quantification of starch hydrolysed by amylolytic enzymes to dextrins of various molecular weight under standard conditions (temperature 30°C; pH 6.0 for bacterial and 4.7 for fungal α-amylase; hydrolysis duration 10 min). The method for determining glucoamylase activity is based on the quantification of glucose formed during starch hydrolysis by glucoamylase under standard conditions (temperature 30°C; pH 4.7; hydrolysis duration 10 min).

β-glucanase activity (β-GcS) was determined according to State Standard 53973-2010**. The method

* State Standard 54330-2011. Enzyme preparations for food industry. Methods for determination of amylase activity. Moscow: Standartinform; 2012.

** State Standard 53973-2010. Enzyme preparations for food industry. Methods for determination of β-glucanase activity. Moscow: Standartinform; 2011.

Table 1 Biochemical indicators of rye grain

Indicator	Content, %
Proteins	15.7
Mono- and disaccharides	1.5
Starch	56.4
Cellulose	2.2
Hemicellulose	8.4
Gum substances	2.5
β-glucan	0.18
Arabinoxylan	0.65

is based on the quantitative determination of reducing sugars resulting from β-glucanase action on β-1,4 bonds of β-glucan under standard conditions (temperature 50°C; pH 4.7; hydrolysis duration 10 min).

Cellulase activity was determined according to State Standard 55293-2012 (Enzyme preparations for food industry. Method for determination of cellulase activity. Moscow: Standartinform, 2014). The method is based on the quantitative determination of reducing sugars resulting from cellulase action on the substrate of sodium carboxymethylcellulose (CMC) at 50°C.

Xylanase activity was determined according to State Standard 55302-2012***. The method is based on the quantitative determination of reducing sugars resulting from xylanase (exoxylanase) action on β-1,4 bonds of xylan.

Phytase activity was determined according to State Standard 31487-2012****. One unit of phytase activity is the amount of enzyme that catalyses the hydrolysis of sodium phytate to form 1 μmol of inorganic phosphate per minute under standard conditions (temperature 37°C; pH 5.5; hydrolysis duration 15 min).

Rye, used as a raw material to make grain wort, was prepared by 'soft' enzymatic cooking at a water ratio of 1:3. At the mash stage, thermostable α-amylase was used for starch dextrinization at the rate of 0.5 unit/g starch. At the saccharification stage, the test samples included complex enzyme preparations, namely Glucavamorin Xyl and Glucavamorin Ply in an amount of 6–10 units/g starch each. The grain wort used as a control sample was made with commercial enzyme preparations (EP) without phytase: Glucomil L-706 as a source of glucoamylase and BrewZyme BGX as a source of xylanase, β-glucanase, and cellulase. The amount of EPs for the biocatalysis of rye wort polymers in the control sample was 10; 0.4; 0.05; and 0.2 unit/g starch for glucoamylase, xylanase, β-glucanase, and cellulase, respectively.

The wort was fermented with the *Saccharomyces cerevisiae* 985T alcohol yeast, which has thermotolerant and osmophilic properties, by the fermentation samples

*** State Standard 55302-2012. Enzyme preparations for food industry. Method for determination of xylanase activity. Moscow: Standartinform; 2013.

**** State Standard 31487-2012. Enzyme preparations. Methods of phytase enzyme activity determination. Moscow: Standartinform; 2012.

Table 2 Comparative activity of enzyme preparations derived from *Aspergillus awamori* recombinant strains and commercial preparations

Enzyme preparation	Enzyme activity, units/g				
	GlS	XS	ClS	β-GcS	PhS
Glucavamorin-Xyl	9640	810	120	285	0
Glucavamorin-Ply	7580	260	130	310	4300
BrewZyme BGX	0	3600	2000	500	0
Glucomil L-706	8000	0	0	0	0

method. The fermentation was carried out at 35°C for 72 h. The Guidelines for the Technical and Chemical Control of Alcohol Production were followed to determine the biochemical indicators of rye grain, wort concentration, the number of yeast cells, the percentage of budding cells, the content of total and residual reducing carbohydrates (RS, reducing substances), and ethanol concentration and yield [3, 5]. The composition and level of side metabolites formed during fermentation was analysed on an Agilent 6850 gas chromatograph according to State Standard 55792-2013*****.

RESULTS AND DISCUSSION

Ground rye grain, whose biochemical composition is given in Table 1, was used as a substrate for fermentation. Rye is known to be a multicomponent substrate characterised by a high content of hemicelluloses and gum substances. The studied rye grain contained 56.4% of starch, 8.4% of hemicellulose, and 2.2% of cellulose. The presence of non-starch polysaccharides complicates the process of preparing concentrated wort that has good rheological properties and contains soluble carbohydrates in a form that is accessible to yeast cells.

Therefore, new enzyme preparations of glucoamylase and hemicellulase action were tried to prepare media ensuring stable yeast generation and alcohol fermentation.

To prepare the grain for fermentation, we used the Glucavamorin complex enzyme preparations based on *A*. Awamori recombinant strains and containing xylanase, β-glucanase, and cellulase, along with amylolytic enzymes.

The Glucavamorin enzyme preparations had glucoamylase (GlS), xylanase (XS), β-glucanase (β-GcS), and cellulase (ClS) activities. Of particular interest was the Glucavamorin-Ply preparation, which additionally exhibited a high level of phytase (PhS) activity (4300 units/g). Glucavamorin-Xyl had a higher level of xylanase (810 units/g) and glucoamylase (9640 units/g) activity. The results are in Table 2.

The complex enzyme preparations were used as sources of glucoamylase and concomitant enzymes.

***** State Standard 55792-2013. Brew from food raw material. Gas-chromatographic method for determination of volatile organic admixtures. Moscow: Standartinform; 2014.

Table 3 Effects of enzyme complexes derived from recombinant strains on the concentration of rye wort and content of reducing carbohydrates

Rye wort samples	Enzyme complex and amount, unit/g			Rye wort indicators	
	Glucomil + BrewZyme (control)	Glucavamorin-Xyl	Glucavamorin-Ply	Soluble solids (SS), %	Reducing carbohydrates (RC), %
1	GlS – 10.00 XS – 0.40 ClS – 0.22 β-GcS – 0.05	–	–	26.2	15.5
2	–	GlS – 6.00 XS – 0.50 ClS – 0.08 β-GcS – 0.18	–	25.8	14.9
3	–	GlS – 8.00 XS – 0.70 ClS – 0.10 β-GcS – 0.23	–	26.1	15.3
4	–	GlS – 10.00 XS – 0.81 ClS – 0.12 β-GcS – 0.31	–	26.4	15.7
5	–	–	GlS – 6.00 XS – 0.21 ClS – 0.12 β-GcS – 0.24 PhS – 3.41	26.2	15.0
6	–	–	GlS – 8.00 XS – 0.31 ClS – 0.14 β-GcS – 0.32 PhS – 4.52	26.4	15.5
7	–	–	GlS – 10.00 XS – 0.37 ClS – 0.18 β-GcS – 0.40 PhS – 5.63	26.7	15.9

Therefore, their amount was based on the glucoamylase quantity of 6–10 units/g starch. The level of concomitant enzymes contained in the complex EPs was also monitored (Table 3).

In the control sample (No. 1) we used Glucomil, for starch saccharification, and BrewZyme, for the catalytic hydrolysis of hemicelluloses. Glucavamorin-Xyl was used in test samples No. 2, 3, and 4 in an amount of 6.0, 8.0, and 10.0 units/g starch, respectively, at the stage of saccharification at 58–60°C. Glucavamorin-Ply was used in test samples No. 5, 6, and 7 in an amount of 6.0–10.0 units/g starch.

The studies showed that the concentration of soluble solids (SS) in the rye wort prepared with various amounts of the Glucavamorin complex enzymes was 25.8–26.7%, and the content of reducing carbohydrates was 14.9–15.9%. The highest rates of reducing substances were achieved with the use of Glucavamorin-Ply. Apparently, this was due to a higher β-glucanase activity of the preparation. The catalytic action of β-glucanase contributed to the hydrolysis of grain glucans and the formation of additional reducing carbohydrates (Table 3).

Further studies showed that the quality of the grain wort made with the complex enzyme preparations affected the processes of yeast generation and alcohol fermentation.

The comparative studies into the fermentation of rye wort treated with phytase-free enzyme preparations revealed a higher efficiency of Glucavamorin-Xyl, especially in sample No. 4, where it was used at the maximum amount (10 units/g starch). As seen in Table 4, the yield of ethanol reached 65.7 cm^3/100 g starch, exceeding the rate in the control sample (65.3 cm^3/100 g starch).

Thus, the above confirmed that the synergic action of the enzymes was determined by their catalytic effect on the grain structural polymers interrelated with each other. We found that to improve the technological parameters of concentrated grain wort, it was necessary to use a complex of hemicellulase enzymes (xylanase, β-glucanase, and cellulase), along with the traditionally used amylases. The effective destruction of non-starch polymers improved the rheological properties of the rye wort, which had a positive effect on the fermentation

Table 4 Effects of enzyme complexes derived from recombinant strains on the rye wort fermentation

Rye wort samples	Enzyme complex and amount, unit/g			Fermentation indicators per 72 h				
	Glucomil + BrewZyme (control)	Glucavamorin-Xyl	Glucavamorin-Ply	Yeast, mln/cm^3		Soluble solids, g/100 cm^3	Alcohol concentration, % vol.	Ethanol yield, cm^3/100 g starch
				18 h	44 h			
1	GlS – 10.00 XS – 0.40 ClS – 0.22 β-GcS – 0.05	–	–	85	94	0.80	10.2	65.3
2	–	GlS – 6.00 XS – 0.50 ClS – 0.08 β-GcS – 0.18	–	68	83	0.74	10.2	65.3
3	–	GlS – 8.00 XS – 0.70 ClS – 0.10 β-GcS – 0.23	–	82	100	0.72	10.3	65.5
4	–	GlS – 10.00 XS – 0.81 ClS – 0.12 β-GcS – 0.31	–	100	107	0.68	10.5	65.7
5	–	–	GlS – 6.00 XS – 0.21 ClS – 0.12 β-GcS – 0.24 PhS – 3.41	108	135	0.68	10.5	65.7
6	–	–	GlS – 8.00 XS – 0.31 ClS – 0.14 β-GcS – 0.32 PhS – 4.52	115	138	0.66	10.8	65.9
7	–	–	GlS – 10.00 XS – 0.37 ClS – 0.18 β-GcS – 0.40 PhS – 5.63	120	140	0.64	10.9	66.0

process. The use of Glucavamorin-Xyl with an increased concentration of hemicellulases contributed to a slight rise in ethanol yield compared to the control sample with the same amount of glucoamylase (10 units/g starch; Table 4).

The greatest effect was achieved with Glucavamorin-Ply, which included phytase, apart from a hemicellulase complex. For example, the use of Glucavamorin-Ply in samples No. 5, 6, and 7 in amounts of 6.0; 8.0; and 10.0 units/g starch, respectively, intensified the processes of yeast generation and alcohol fermentation, which increased the ethanol yield to 66.0 cm^3/100 g and decreased the concentration level of residual carbohydrates in the mash to 0.64 g/100 cm^3 (Fig. 1, Table 4).

Such enzyme complexes contribute to a more rational use of grain components, reduce the wort viscosity, enrich the wort with nutrients, increase the physiological and fermentation activity of yeast and, as a result, accelerate the processes of yeast generation and alcohol fermentation.

The phytolytic action of the preparation had a positive effect on yeast generation and led to a higher concentration of yeast cells compared to the control (I) and those samples where a phytase-free glucoamylase EP was used (Table 2, samples No. 2–4). There was a tendency towards an increase in ethanolyield to 65.7–66.0 cm^3/100 g starch, with alcohol concentration of 10.5–10.9% vol., even though the amount of glucoamylase was reduced by 20–40% (from 10.0 to 6.0–8.0 units/g starch) in samples No. 2 and 4.

By catalysing the hydrolysis of phytic acid in the raw material, phytase contained in Glucavamorin-Ply appeared to release additional mineral phosphorus, assimilated by alcohol yeast. This improved the growth, activity and productivity of yeast cells.

We studied *Saccharomyces cerevisiae* 985T yeast cultured under anaerobic conditions on rye media with enzyme preparations that differed in the content of phytolytic enzymes. The study showed that Glucavamorin-Ply contributed to increased physiological activity of yeast cells (Fig. 1).

As seen in Fig. 1, the presence of phosphorus in the medium led to intensified yeast development, especially in the lag phase (first 18–24 h), accelerated

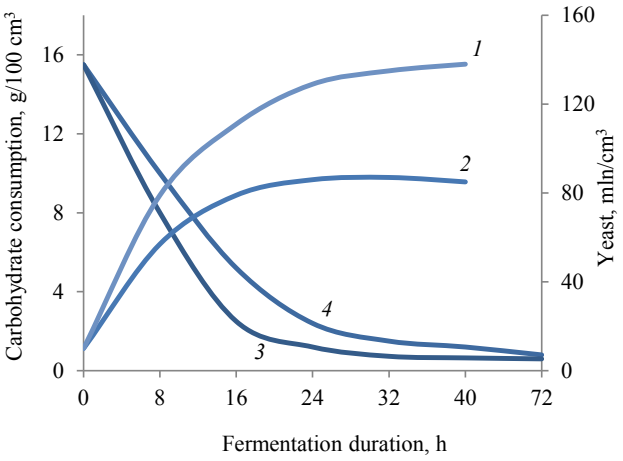

Figure 1 Yeast biomass accumulation (1, 2) and carbohydrate consumption (3, 4) patterns during grain wort fermentation using grain wort treated with phytases (1, 3) and the control sample of phytase-free grain wort (2, 4)

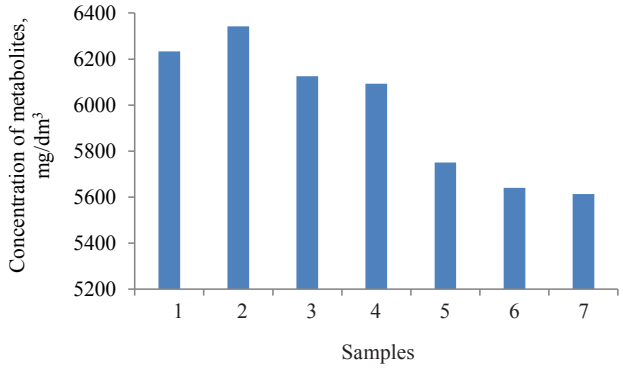

Figure 2 Formation of side metabolites when using glucoamylase preparations derived from recombinant strains of *Aspergillus awamori*

carbohydrate consumption, and increased concentration of yeast cells (1.4–1.5 times). The fermentation process was more complete, with the minimal amount of residual carbohydrates (0.64 g/100 cm^3) and the maximum ethanol yield (Table 4).

The analysis of the phytolytic effect of the enzyme preparations on the metabolism of yeast cells showed that Glucavamorin-Ply contributed to an 18–20% decrease in the formation of side metabolites accompanying ethanol synthesis, thereby improving the quality of the target product (Fig. 2).

We compared the metabolites synthesised during the fermentation of rye wort made with the glucoamylase enzyme preparations. We found that the phytase-containing Glucavamorin-Ply (sample No. 4) lowered the content of volatile substances by the end of fermentation, compared to the control (sample No. 1) and the sample with the phytase-free Glucavamorin-Xyl. It did so by reducing the synthesis of major impurities: higher alcohols, aldehydes, and esters (Fig. 3). This improved

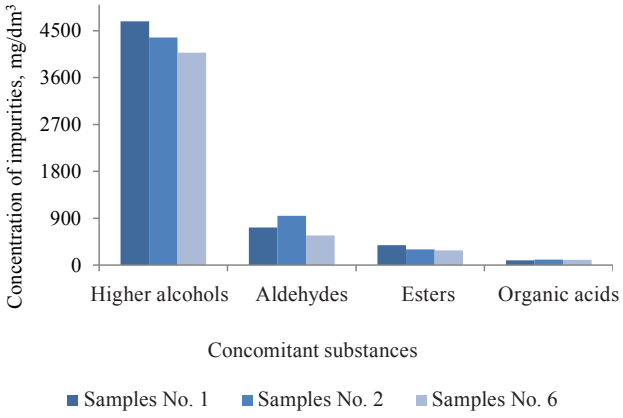

Figure 3 Composition of volatile by-products during fermentation of rye wort made with glucoamylase enzyme preparations derived from *Aspergillus awamori* recombinant strains

the organoleptic and analytical indicators of the final product, i.e. ethanol.

CONCLUSION

The study showed that the use of the Glucavamorin complex enzyme preparations, derived from *Aspergillus awamori* recombinant strains, at the stage of preparing rye wort for fermentation enhanced the efficiency of yeast generation and alcohol fermentation. The increased content of minor hemicellulase enzymes in Glucavamorin-Xyl improved the rheological properties of the rye wort and had a positive effect on the fermentation process.

The catalytic effect of the phytase-containing Glucavamorin-Ply enzyme preparation improved the phosphorus nutrition of yeast. This intensified yeast generation, increased the concentration of yeast cells in the rye wort by 30%, reduced the level of side metabolites by 18–20%, and enhanced ethanol yield. The study revealed that using a phytase-containing enzyme complex made it possible to reduce the amount of the main enzyme, glucoamylase, from 10.0 to 6.0–8.0 units/g starch without causing the key fermentation indicators to degrade.

Thus, the study confirmed that the synergic effect of enzymes with different substrate specificity on the polymers of grain raw materials enhanced the efficiency of their conversion when fermenting rye wort.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cheese whey as potential resource for antimicrobial edible film and active packaging production

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Abstract: Many cheese manufacturers still have not utilized cheese whey that damages to the environment as it is directly been drained into waters. Cheese whey can be used as active packaging material to prolong the shelf-life of food products. Fermented cheese whey contains bioactive peptides which are able to improve the functional properties of cheese whey as an antimicrobial agent. The combination of cheese whey with polysaccharides, lipid, and other additional ingredients can improve the physical characteristics of the active packaging in the form of edible film. Around 20–45% of plasticizer will expose the film formed. Cheese whey with agro-industrial waste starch-based formulation can be used as an alternative way to produce an antimicrobial edible film as an active packaging. The film has shown acceptable physical characteristics and high antimicrobial activity, which makes it possible to extend the shelf life of food products. An advanced process, for example, the use of transglutaminase enzyme and *Candida tropicalis* mutant, is also effective. The result of that is the formation of the essential compound which can improve the active packaging quality. The utilisation of cheese whey and agro-industrial waste based on starch contributes significantly to the environmental conservation.

Keywords: Whey, protein, shelf-life, packaging, antimicrobial, edible film, fermentation, environment

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INTRODUCTION

Cheese production process has significant impact on the environment. One of damaging factor is the disposal of cheese by-product. Cheese making process produces large amounts of by-product called cheese whey, which is almost 90% of used milk [1]. It implies if one batch of cheese production uses 100 L of milk, 80–90 L of cheese whey will be produced [2]. Although it is wasted, 30% of cheese whey still has been utilised as animal feed and fertiliser, while the rest has thrown away to the rivers or seas [3]. Cheese whey is able to damage the environment due to its characteristics. Cheese whey has high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which is more then 35000 and 60000 ppm, respectively [4]. Thus, 4000 L of whey from the cheese industry can damage the environment to the same extent as faecal waste from 1900 humans [5].

On the other hand, whey has a valuable chemical composition and contains 55% of total nutrients in milk [6]. Whey contains (w/w): 93.7% of water, 0.1–0.5% fat, 0.8% protein, 4.9% lactose, 0.5–0.8% ash, and 0.1–0.4% lactic acid [2]. Functionally, the beneficial effect of whey on the human health is due to immunoglobulin and glycoprotein, such as lactoferrin and transerin, as well as enzymes – lysozyme and lactoperoxidase. All of these components contribute to human immunity and have an antimicrobial activity against allergy reaction [7, 8].

Cheese whey has been utilised in various ways. About 70% of whey is processed into whey powder that can be used in pastry, ice cream, sweets, glazes, sugar dressing, jams, and melted cheese industry [3, 9]. Whey is used as a food ingredient because of its gelling, emulsifying, antimicrobial properties, good solubility, viscosity, nutritional value, as well as the ability to reduce allergenicity [2, 10]. Unfortunately, it is difficult to utilise whey for cheese manufactures [11]. One of the causes is high cost drying process of whey. Therefore, the search of alternative whey processing is of great interest.

Organic compounds of whey are a potential biomass to be utilised as bio-energy. Bioethanol can be made from whey through fermentation by using *Kluyveromyces fragilis* var *marxianus* [11–13]. Lactose,

whose content in whey is 4.5–5%, acts as a carbon source for ethanol fermentation. The fermentation also can result in various bioproducts, such as ethanol, biogas (methane), organic acids (acetic, propionic, lactic, citric, and gluconic), amino acids (glutamic, lysine, and threonine), vitamins (B_{12} and B_2), polysaccharides (gum, dextran, and gellan), lipids, enzymes (polygalacturonase), and others (calcium magnesium acetate, butanol, and glycerol) [11].

The fermentation of whey leads to other compounds which have high functional use, such as bioactive peptides. Native whey has minor bioactive compounds such as lysozyme, lactoperoxidase and lactoferrin that are reported to have antimicrobial activity towards pathogenic bacteria [7]. Fermentation causes protein hydrolysis – by a microorganism which releases bioactive peptides from protein molecules or by a digestive enzyme, such as proteases [14, 15].

Cheese whey can also be utilised as biodegradable packaging material, such as edible film. Such a film is safe to consumers and environmentally friendly. It is also expected to extend the shelf life of food products because it protects them from gases, such as oxygen, carbon dioxide, and ethylene, as well as from water loss [16–18].

The use of the edible film as a food packaging material is expected to reduce plastic waste. Annually, Indonesia produces 3.22 million metric tons of plastic waste. It is the second largest plastic waster after China that produces 8.82 million metric tons. The use of plastic has rapidly increased since the development of plastic commercialisation in the 1930s and 1940s. It reached 288 million metric tons of global plastic resin production in 2012 [19].

The high rate of population growth has caused an increase in food demand. This has resulted in an increasing use of plastic, which contributes to the economic benefits [20, 21]. Food industry still widely uses non-degradable plastic as a food packaging material. However, non-degradable plastic, such as polyethylene (PE), has an immensely slow degradation time under natural environmental conditions [22–24]. Thus, food industry indirectly affects the environment.

The utilisation of cheese whey to produce bio-products, such as edible films, would be a potential course of action to protect the environment. Cheese whey fermentation can be applied to enhance the antimicrobial effect and the packaging ability of the edible film produced in order to extend the shelf life of packaged food. The aim of this paper was to review the potential of fermented cheese whey in the produce of edible films and active packaging systems.

STUDY OBJECTS AND METHODS

The paper was written with non-research methodology based on literature reviews from various sources.

RESULTS AND DISCUSSION

Fermented cheese whey. The protein content in milk is 3.5% which is, in turn, composed of 80% of casein (α-, β-, and k-caseins) and 20% of whey proteins (β-lactoglobulin, α-lactalbumin, and others). Several proteins in milk have an antimicrobial effect as shown in Table 1 [31, 33–38]. Whey contains biological active substances, such as enzymes, trace elements, and immunoglobulins which contribute to the good health [25].

Table 1 Antimicrobial peptides in milk

Source	Protease	Peptide	Target
		Antimicrobial peptides from casein	
Bovine α_{s1}-casein	Chymosin	Caseidin	Gram-positive bacteria
	Chymosin, Chymotrypsin	Isracidin αsl-CN (f l–23)	*Staphylococcus aureus*
Bovine α_{s2}-casein	Trypsin	Casoidin-I f (150–188)	Gram-positive and gram-negative bacteria, yeast
	Chymosin	Casoidin-I f (181–207)	Gram-positive and gram-negative bacteria
β-Casein	Trypsin and chymotrypsin	β-Casein-derived peptides	*Enterococcus faecium*, *Bacillus megaterium*
		Antimicrobial peptides from whey	
β-lactoglobulin	Trypsin	β-lactoglobulin f (15–20)	Gram-positive bacteria
	Trypsin	β-lactoglobulin f (25–40)	Gram-positive bacteria
	Trypsin	β-lactoglobulin f (78–83)	Gram-positive bacteria
	Trypsin	β-lactoglobulin f (92–100)	Gram-positive bacteria
Lysozyme	Synthetic	Lysozyme D52S-Lz (from yeast in egg white)	*Staphylococcus aureus* and *Bacillus subtilis*
Lactoferrin	Pepsin	Lactoferricin B f (17–41)	*Escherichia coli*, *Listeria monocytogenes*, *viruses*, *fungi*
	Pepsin, chymosin	Lactoferricin B f (1–16)	*E. coli, Micrococcus flavus*
	Pepsin	Lactoferricin C f (14–42)	*M. flavus*
	Synthetic	Lactoferrampin/BL fampin f (268–284)	*C. albicans*, *E. coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*[40]

Table 2 Bioactive peptides derived from whey proteins

Name	Peptide sequence	Fragment	Function
α-Lactorphin	Tyr-Gly-Leu-Phe	50–53	Opioid agonist, ACE inhibition
β-Lactorphin	Tyr-Leu-Leu-Phe	102–105	Non-opioid stimulatory effect on ileum,
		10–105	ACE inhibition
β-Lactotensin	His-Ile-Arg-Leu	146–149	Ileum contraction, opioid
Serophin	Tyr-Gly-Phe-Gln-Asp-Ala	399–404	Opioid
Albutensin A	Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg	208–216	Ileum contraction, ACE inhibition
Lactoferricin	Lys-Cys-Arg-Arg-Trp-Glu-Trp-Arg-Met-Lys0Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe	17–42	Antimicrobial
Glycomacro peptide (GMP)	–	106–169	Food intake regulation

Fermented whey is known to exert antimicrobial properties. For example, *Bacillus licheniformis* can produce 3200 AU/mL of bacteriocins from 70 g/L of cheese whey. This amount can count over 4000 AU/mL if cheese whey increases up to 120.4 g/L (with initial pH of 7 and incubation temperature of 26–37°C), [26]. Bacteriocins are a bacterial peptides which are able to inhibit or kill microorganisms [27]. Other lactic acid bacteria, such as *Lactococcus lactis*, *Lactobacillus casei*, and *Leuconostoc mesenteroides*, also can produce bacteriocins in response to whey fermentation [28–30].

Besides bacteriocins, fermented whey contains bioactive peptides which also have antimicrobial activity. Bioactive peptides defined as inactive fragments of precursor protein sequences. Proteolytic enzymes can release the fragments, and they can interact with selected receptors and regulate the body's physiological function [31]. Table 2 demonstrates bioactive peptides contained in whey protein [50–55]. β-lactolobulin, α-lactoalbumin, immunoglobulin, bovine serum albumin, bovine lactoferrin, lactoperoxidase and minor proteinaceous, such as glycomacropeptide, are released from k-casein during enzymatic cheese making [32].

Along with antimicrobial effect, whey bioactive peptides act as immunomodulatory agents that regulate cell-mediated and humoral immune functions [31]. In addition, bioactive peptides inhibit angiotensin-converting enzyme (ACE) that splits angiotensin I to angiotensin II, an active peptide hormone. These peptides are able to inhibit ACE and control the increase in blood pressure [39]. Opioid peptides influence the central or peripheral nervous system that involved in hypotension, reduced appetite, fluctuating body temperature and alteration of sexual behaviour [40, 41]. Also, peptides with antioxidant activity which can protect the cell from free radicals has been detected [42].

Bioactive peptides can be released in three ways: gastrointestinal digestion (*in vivo*), fermentation (*in vivo*), and hydrolysis (*in vitro*) [31]. Release of bioactive peptides in gastrointestinal tract is the result of enzymatic action. The enzymes are pepsin, trypsin, or chymotrypsin. Pepsin, which is produced from pepsinogen by hydrochloric acid (HCl), converted protein to peptides and amino acids [43]. Other enzymes, such as alcalase and thermolysin, can also stimulate gastrointestinal digestion to produce ACE inhibitory peptides, as well as anti-bacterial, anti-oxidative, immunomodulatory, and opioid peptides [44–49].

Other ways to produce bioactive peptides are microbial fermentation and hydrolysis. For microbial fermentation, such LAB as *Lactococcus lactis* and *Lactobacillus helveticus* are used. Microbes will use distinct intracellular peptidases including endo-peptidases, amino-peptidase, di-peptidase, and tri-peptidase [33]. Hydrolysis of protein molecules can is performed by proteinases, which leads to the release of bioactive peptides. Proteinases are obtained from the secretion of the bacterial and fungal sources [31].

Bioactive peptides produced from fermented whey can be purified to enhance their functional activities. Stepwise filtration can be carried out to extract bioactive peptides after fermentation. Afterwards, the extract is centrifuged at 7000 rpm for 10 min in a refrigerated centrifuge to obtain supernatant. The supernatant is filtered through 0.45 μm and then through 0.22 μm syringe filters. Ultrafiltrate of bioactive peptides of 10 kDa and 5 kDa in size can be obtained after passing through the 10 kDa and 5 kDa MWCO membranes [56].

Edible film production. Food products usually have a short shelf-life. In order to prolong it, most of manufacturers uses food packaging. A packaging system should protect the product from contamination during handling, storage, and sale until it reaches retailers and consumers [57]. Non-degradable packaging still has widely applied by food industry. The US Environmental Protection Agency (EPA) reports that 31% of municipal solid waste (MSW) is packaging waste [58]. Edible films as a packaging material can be an effective solution of reducing waste because of their degradable characteristics [57].

The ideal edible film has high water holding ability; controls gas exchanges; inhibits solute transport, organic vapour transfers, as well as oil and fat migration; improves mechanical properties of food to simplify handling and carriage; has neutral sensory characteristics, improving sensory properties of food

products [57]. Edible films should consist of components produced mainly from edible biopolymers and food grade additives. The additives should meet safety requirements to food additives and to be at least GRAS (Generally Recognized as Safe) [59].

There are two ways to create an edible film: wet process and dry process. Wet process applies dispersion in a solvent such as water, alcohol, or mixture of water and alcohol, or other solvents. The film-forming solution is then casted and dried to obtain films. The dry process does not require any solvent. It can be produced by compression, molding, or extrusion [60, 61]. The film-making and coating processes include melting and solidification of solid fats, waxes, and resins; conservation of hydrocolloid; complex conservation of two hydrocolloids; and thermal gelation or coagulation by heating [57]. Therefore, the dry process usually needs more equipment, which results in higher cost compared to the wet process.

In edible film production, the incorporation of a certain additive is possible to form an advanced system called active packaging [62]. The additive compound enhances shelf-life and stability of the product, as well as improves its microbiological safety and sensory attributes [63]. The following additives can be used in edible films: flavouring agents, spices, antimicrobial substances, antioxidants, pigments, light absorbers, salts, etc. Antioxidants and antimicrobial additives are commonly used in order to prevent spoilage and thus enhance safety. Antimicrobial agents, being used in active packaging, can overcome the hurdles of uncontrolled migration and interaction of an active compound of various natural antioxidants used directly in food [64, 65].

Comparison of characteristics from various film bases. Edible film or even active packaging usually use polysaccharide, protein, lipid, or composite base to make a film forming solution. Thus, fermented whey can be one of multifunctional ingredients and act as a film-forming base and an antimicrobial agent.

Researchers have focused on the use of composite based films to explore the complement advantages of each component [64, 66]. A composite based film can be both one-layer and multiple-layer. The matrix of hydrophilic and hydrophobic lipid, which is called bi-layer composite system, has better functional characteristics than pure hydrocolloid films. However, one of disadvantages of bi-layers composite systems is longer preparation process. It requires two casting and two drying stages, which has made these laminated films less popular in food industry [67]. In order to enhance holding properties of active packaging, scientists have studied its mechanical properties (Table 3). These are transparency, oxygen permeability, carbon dioxide permeability, water vapor permeability, emulsion stability, and glass transition temperature.

Generally, lipid films have the less structural integrity compared to protein or polysaccharide films [68]. The use of lipids in edible films has resulted in heterogenous film structure that has an impact on discontinuities in the polymer and production of a strong emulsion matrix [69]. A composite film based on polysaccharide has the greatest mechanical properties, which allows its using in gastronomy. Along with protein added, polysaccharide film is an optimal active packaging.

Besides the film based component, the composition of the edible film is also an important factor. Plasticizer is one of substantial components to create a flexible film by reducing interaction between intermolecular starch [70]. Examples of plasticizer are polyol groups such as glycerol, xylitol, sorbitol, mannitol, and sucrose [18, 70]. Xanthan gum and carrageenan are also promising plasticizers which provide the product with strength and durability with great sensory properties. They demonstrate high stabilizing ability and resistance to water, [71]. The plasticizer is able to reduce intermolecular bonds between amylose, amylopectin, and amylose-amylopectin in the starch matrix and replace them with hydrogen bonds between plasticizer and starch. This reduces brittle and enhances flexibility [68].

Table 3 Edible film characteristics

Characteristic		Effective components for base making	Ineffective components for base making	Additional info	Source
Mechanical properties	Flexibility and texture of film	Composite, pure hydrocolloid matrix	Lipid (good in coating, bad in film)		[72]
Transparency	Lightness and colourless of film	Emulsified films with lipid	–	The higher lipid content, the less lightness	[73]
Oxygen permeability	O_2 transfer through film	Hydrocolloid, protein	Hydrophobic		[74] [75]
Carbon dioxide permeability	CO_2 transfer through film	Cellulose films, protein	Lipid (stearic acid and palmitic)	The higher lipid content, the weaker barrier	[76]
Water vapour permeability (WVP)	Moisture transfer through film	Hydrophobic (lipid)	Polysaccharide, Protein	Better at smaller and more homo-genous lipid distributed	[77] [78] [79]
Water solubility (WS)	Lower WS needed to protect food from moisture loss	Lipid	–	Lipids reduce WS	[80]

Table 4 Characteristics of composite film bases

Base	Added component	Method of making	Thickness, mm	WVP, g/m·s·Pa	Water solubility, %	Tensile strength, MPa	Elongation at break, %	Source
Native wheat starch, whey protein isolate	Glycerol	Dissolving (separately), heating at shaking (separately, 85°C, 30 min), cooling, mixing, drying (25°C, RH 40%, 24 h), peeling, storing (RH 53%, 25°C, 7 days).	0.109 ± 0.008	7.95 ± 0.33 (RH 30–100%)	10.53 ± 3.80	4.67 ± 0.19	76.26 ± 8.92	[84]
Soya protein (defatted), papaya puree (pectin)	Starch, glycerol, gelatin	Papaya puree film production (mixing PP + 0,07 ± 0,005water + starch, dissolved at 75°C, 30 min), mixing & stirring (separately, 30 min each), mixing, casting, drying (40°C, RH 23%, 18 h), peeling, storing 48 h	0.119 ± 0.002	5.55 ± 0.43 (g·mm/ m²·h·kPa)	82.26 ± 0.27	6.80 ± 0.08	22.23 ± 0.06	[85]
Almond oils, whey protein isolate	Glycerol	Dissolving WPI (250 rpm, 80°C, 30 min), cooling, mixing (13500 rpm, 5 min), casting, drying (25°C, RH 50%, 24 h), peeling, storing (RH 53%, 25°C, 48 h)	0.07 ± 0.005	11.00 ± 1.60	46.90 ± 0.69	5.40 ± 0.80	53.70 ± 7.7	[86]
Soya protein, acetem (hydrogenated soybean oil)	Vegetable glycerin, tween 60 (polyoxyethylene sorbitan monostearate) as a surfactant	Emulsification (mixing, 300 rpm, 25°C, 30 min), mixing (1 h), heating (90°C, 45 min), mixing (1300 rpm, 2 h), degassing, casting, drying (15 h, 24°C)	0.113 ± 0.008	2.70 ± 0.46 (g·mm/ m²·h·kPa)	–	2.15 ± 0.18	342.4 ± 25.2	[87]

The flexibility of the film depends on the concentration of the plasticizer in fthe ilm-forming solution. High or low concentrations would result in anti-plasticization. For example, glycerol in the amount of over 30% used in the starch-based film is the case. It will result in a decrease in such characteristics as elongation at break. It was established that strong interaction between plasticizer and other molecules blocked the macromolecular mobility [70, 81]. On the other hand, if the plasticizer concentration is too low, the film formed will be brittle and hard to handle. Generally, the optimal concentration of plasticizer is 20–45% [82].

The potential of fermented whey for active packaging composite. The composite of edible film can be made to complement each single material-based film characteristics. Protein is usually used as one of material-based because of its nutritional value [83]. The comparison of the composite edible film using protein-based is shown in Table 4. From all of the sources of protein, soya and whey have been mostly used.

As a food barrier capability, the addition of oil has resulted in a lower WVP, which is showed in a comparison of soya protein with oil and with pectin. Thereby, we can conclude that a composite protein film with oil has better barrier properties. However, the water solubility of whey protein with starch is higher than that of soya protein with pectin. In terms of mechanical properties, whey protein with starch provides a better result than soy protein with pectin. However, some other parameters cannot be compared because of the difference between the film production and the analysis method. Based on the description above, it is possible to conclude that the whey protein is more effective than soya protein to produce the edible film.

Functional characteristics of fermented whey make it promising raw material for active packaging. Bioactive peptides from fermented whey have had their ability to act as an antimicrobial agent; immuno-modulatory peptides regulate cell-mediated and humoral immune functions; ACE inhibitory peptides lower blood pressure; and opioid peptides are effective against hypotension, lack of appetite, etc., as well as exhibit antioxidant properties, protecting cells from free radicals [31].

Several bioactive peptides derived from whey protein are also known for its capability to enhance the defence towards various pathogenic bacteria and yeast. Their antimicrobial activity can inhibit the growth of such microorganisms effectively. The incorporation of bioactive peptides into film is more effective way to lower the concentration of microorganisms than direct using them in food. Thus, it allow avoiding unwanted flavours and odor of food [88].

In terms of the characteristics, a good characteristic can be achieved if the protein contained in the cheese whey is mixed with starch that contained polysaccharides. With the addition of plasticizer, this composite based film will have good mechanical properties as well as the barrier ability to prolong food shelf-life.

Advanced process to create composite fermented cheese whey film. Nowadays, an advanced process to create an edible composite is based film-forming solution. The film with cellulose, whey and sunflower oil based are stirred with Ultraturrax homogenizer at 11000 rpm for 3 min to decrease the droplet sizes of oil. As a result, the smaller droplet sizes can disperse uniformly in the hydrocolloid matrix. Therefore, the penetration of water into film will be harder, which will result in better water vapour transfer. The combination of the degassed method under vacuum (80 kPa) and a vacuum pump for 5 min will result in the film production with tensile strength of 8.59 MPa, elongation at break 35.94%, WVP 3.211 g/m·s·Pa, and transparency of 3,637 % [79].

There are several methods available to increase the stability and the quality of characteristics of active packaging. The addition of enzyme is one of methods to enhance the film quality in the complex edible film. The presence of Transglutaminase (TGase) has caused enzymatic cross-linking in P/P soluble electrostatic aggregates. Thus, TGase can strongly produce composite bioplastics by escalating the mechanical and barrier characteristics. Supramolecular structure of P/P complex as enzyme substrate is crucially influencing pH of a film-forming solution. With the addition of TGase, film characteristics at pH (pH complexation around 3.25–5.5, when soluble P/P complexes occur) create better characteristics than higher pH. The pH can significantly increase tensile strength and elongation at break, and reduce Young's modulus and WVP [89].

For the usage of fermented cheese whey in the active packaging, there is an advanced process that can be added in the fermentation process. *Candida* sp. is one of the yeast that has already found in Serro Minas, a cheese from Brazil [90]. Recently, a study for identifying the indigenous yeast that contained in homemade mozzarella whey has also found that *Candida* sp. contained in mozzarella whey and Greek fermented whey [91]. It means that *Candida* sp. is naturally contained in cheese whey and can live to ferment the whey [92]. *Candida* spp. is also known as the most massive yeasts to produce xylitol with 63–70% w/w yields. According to several studies, *C. tropicalis* mutant maximises the xylitol production, reaching 100% yields [93]. It is known that xylitol is one of sugar alcohol that can be utilized in the film production as a plasticizer [18]. Fermented cheese whey can act as an antimicrobial agent and natural plasticizer.

Prospects of the use of edible films and active packaging with cheese whey. The edible film can be an effective solution to reduce plastic waste of food packaging. Addition of several antimicrobials can also be used to prolong the shelf life to reach a proper packaging system which is similar to the plastic packaging. Thus, fermented cheese whey as a base ingredient of composite film system is able to meet this requirement. Despite some disadvantages of protein, its combination with other ingredients make is possible to obtain an excellent film with required characteristics.

Besides various modification of film manufacture, the cost in creating edible film must be taken into account. The edible film should be cost-effective compared to plastic, paper, or any other packaging that can harm the environment. Thus, advancing the edible film production is important to make film characteristics as high as characteristics of plastic packaging.

The simplified process of cheese whey fermentation using indigenous yeast can also increase the antimicrobial properties of the fermented cheese whey. In the future, advancing film manufacture process from fermented cheese whey can be one of massive ways to create modern environmentally-friendly packaging.

CONCLUSION

Cheese whey, a by-product of cheese-making process, has several functional effects, including inedible film formation. Bioactive peptides contained in native cheese whey can be enhanced by fermentation to generate high antimicrobial activity. In addition, a composite edible film can be produced from fermented whey and starch to gain good mechanical characteristics as well as a good barrier to prolong food shelf-life. The utilisation of fermented cheese whey as an edible film material allows obtaining an active packaging system with high antimicrobial activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Bioremediation of textile waste water by plant ash

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Abstract: Water is the most crucial thing to mankind, and its contamination by various agencies is posing a threat to the natural balance. In the present work, the efficiency of various adsorbents derived from plant waste to remove different dyes from aqueous solution was evaluated. Parameters for study were contact time, concentration and pH. Various combinations of plant ashes were used for the study. It was found that adsorbent prepared from the combination of orange peels, pomegranate and banana peels ashes, exhibited good adsorption capacity for methylene blue, congo red and crystal violet. All these dyes were completely removed from the aqueous solution while methyl orange was not removed. Congo red was removed completely within 40 min of contact with the adsorbent while methyl orange took 3 h to be removed to the extent of 48% only. The adsorption coefficient of congo red was found to be 2.33 while value for methylene blue and crystal violet was 1 and 1.66 respectively. The characterization of adsorbent was done by Scanning Electron Microscopy and IR spectroscopy. SEM image revealed the surface of adsorbent to be made of differential pores. From the results it became evident that the low-cost adsorbent could be used as a replacement for costly traditional methods of removing colorants from water.

Keywords: Textile waste water, orange peels, pomegranate peels, adsorption, congo red, SEM

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INTRODUCTION

Water is one of the most imperative substances on the Earth. About 75% of our body consists of water. Water is used for such a wide variety of purposes like drinking, washing, bathing, as well as in agriculture and many others industries. According to World Health Organization (WHO) data, about 85% of rural population lacks potable drinking water. Currently, the water contamination is serious problem. About 80% of diseases in First world countries are associated with stained drinking water. In Second world countries, 15 million infants die annually due to poor hygiene, polluted drinking water, and malnutrition. Chemical impurities such as heavy synthetic fertilisers, industrial metals, dyes of textile industry, and poisonous minerals can cause hazardous effect on human and animal life. Since these particles are very small in size, they can penetrate into the ground water [1].

Purification of water is a tedious process that requires a number of stages [2]. Textile goods are the necessary need of individuals, while textile industry is of immense economic importance. There are 2324 textile industries that require using a number of dyes, additional chemicals, and sizing materials [3]. Different stages of technological processes of textile dyeing industry produce huge volumes of waste water. The waste water discharged from textile mill includes a large amount of concentrated industrial dyes.

Generally, dye stuffs are complex aromatic substances that are difficult to be removed. Methods used for dye removal include flocculation, chemical coagulation, chemical oxidation, photochemical degradation, membrane filtration, adsorption, as well as aerobic and anaerobic biological degradation. However, waste after removing dyes reduces light diffusion, affecting thus aquatic plants. In turn, it may be toxic to some aquatic animals [4]. Moreover, these methods are not cost effective and environmentally friendly. None of them is effective in complete removal of dye from wastewater [4]. Dyed water not only poses aesthetic problem, but also causes serious ecological problems, for example, it significantly impacts photosynthetic.

Modern studies show that adsorption with the help of activated carbon is a very efficient method to remove various organic compounds from the waste water [5]. Numerous researchers have searched alternative

adsorbents deriving them from farming waste or natural materials to remove dyes from wastewater. Some of these alternatives are palm ash, orange peel ash, shale oil ash, pomelo (*Citrus grandis* L.) peel, fat-free soya, bottom ash, sunflower seed shells, mandarin peel, wheat husk guava leaf powder, as well as steel and fertiliser industries waste [6].

Enormous amounts of fruit peels are disposed, while they might be used in the interest of the environment. Agricultural wastes can be employed as a low-cost adsorbent for removal of dyes, such as methylene blue, crystal violet, methyl orange, and congo red, from aqueous solution [6]. Orange peel consists of a large amount of cellulose, hemi-cellulose, pectin, lignin, and other low molecular weight compounds, together with limestone. It can be used as an efficient and cost-effective bio-adsorbent for removing dyes metals and organic pollutants from industrial wastewater [7–12]. Apart from the traditional methods, there are a number of recent studies on bioremediation [16–22].

Consequently, the aim of the study was to determine the effectiveness of the combination of plant ash in removing congo red, crystal violet, methylene blue, and methyl orange dyes from aqueous solution. The parameters studied were contact time, dye concentration and pH variation.

STUDY OBJECTS AND METHODS

Materials. Glassware and apparatus used: conical flasks, a round bottom flask, a volumetric flask, funnel measuring cylinders, beakers, pipettes, a condenser, a soxhlet apparatus, an electronic weighing balance, an oven, a muffle furnace, a magnetic stirrer pH meter, and a UV-visible spectrometer.

Chemical used: $AgNO_3$, ethanol, double distilled water, methylene blue, congo red, crystal violet, and methyl orange.

Plants used: orange peels, pomegranate peels, banana peels, drumsticks, and pea pods.

Methods. To prepare peel extracts, peels of pomegranate, orange, banana, and drumstick tree obtained from local market or fruit stalls were cleaned with distilled water twice to remove dust and water-soluble impurities. After that, these were cut into small pieces, and kept for 2 days for proper drying. The dried material was powdered, and extraction was carried out in a Soxhlet apparatus using methanol as solvent.

Activated charcoal was obtained by putting the dried plant peels in the muffle furnace at 450–500°C and keeping the samples to constant weight.

The stock solution with a concentration of 0.1 g/L was prepared for different dyes. The different concentrations of the dye solutions were obtained from the stock solution by dilution method. Methylene blue, congo red, crystal violet, and methyl orange were used as adsorbates.

Kinetics study was performed as follows. 0.6 g of adsorbent was added into 250 mL conical flasks filled with 100 mL of diluted solutions (25–200 mg/L). The solutions were stirred constantly, and the concentration of dye at maximum wavelength was measured using a double beam UV-visible spectrometer. The capacity of dye adsorbed at time t, Q_t (mg/g), was calculated by the given formula:

$$Q_t = (A_0 - A_t)\, v/W \quad (1)$$

where A_t is concentration at time t, A_0 is the initial concentration, v is volume of solution, and W is the weight of adsorbent used [13].

To study the dependence of initial concentration of dyes and contact time on the degree of removing dyes, 0.6 g of each sample (orange, banana, and pomegranate ash) was added to each 100 mL flask with various dyes having different concentrations. The solution was stirred on the magnetic stirrer at room temperature. The time required for complete adsorption was determined.

RESULTS AND DISCUSSION

Different dyes, namely, methylene blue, congo red, crystal violet, and methyl orange were taken to evaluate the adsorption capacity of the adsorbent.

According to Figs. 1–4, the effectiveness of dye removal increased with an increase in time. This might be due to the better interaction between dye molecules and those of activated charcoal. It was observed that the initially dye removal occurred faster and followed first order kinetics. This was proportional to the availability of active sites, and an equilibrium between adsorption and desorption was than established.

The absorbance of methylene blue at λ_{max} (about 390 nm) decreased with increasing contact time (Fig. 1). The complete absorbance of methylene blue with the adsorbent took 60 min.

The variation of absorbance of crystal violet with time was studied by a UV-visible spectroscopy (Fig. 2). Crystal violet exhibited λ_{max} at 390 nm. It was found that the dye was completely removed after 30 min of contact with adsorbent.

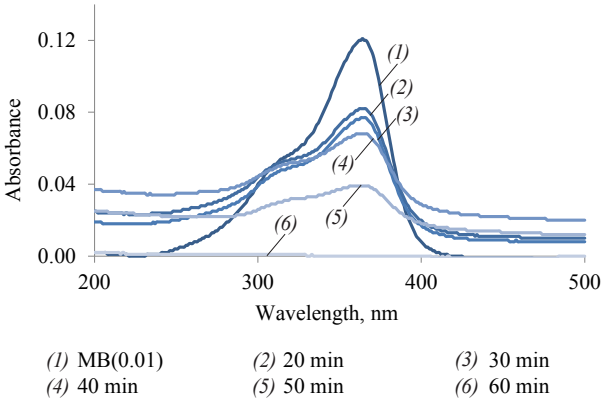


Figure 1 Absorbance of methylene blue at different contact time

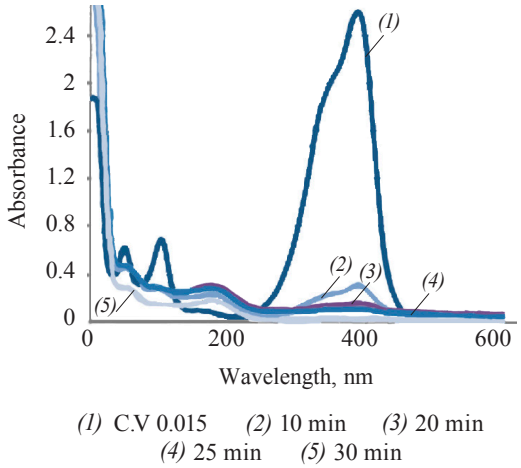


Figure 2 Absorbance of crystal violet at different contact time

Similarly, congo red was completely removed in 40 min of contact with the adsorbent (Fig. 3).

On the contrary, the adsorbent was not effective for methyl orange removal (Fig. 4). Even two hours of contact time was not enough to adsorb the dye. This could be due to the fact that methyl orange does not have any functionality that could make the Vander Waals' interaction with the adsorbent.

The successful removal of various dyes by the combination of plant ashes proved the efficacy of the combination for bioremediation of textile water. As seen from Figure 5, complete dye removal took 5 h. The textile effluent water contained a large amount of heavy metals and different kinds of dyes, so it took longer for adsorbent to absorb the colourant.

Adsorption coefficient of activated charcoal for different dyes at time t. Adsorption coefficient was calculated as the amount of dye adsorbed with one gram of the adsorbent (mg/g). Adsorption coefficient was found to be different for each dye (Table 1) because adsorption depended upon the compatibility of the dye structure with the surface and the porosity of the adsorbent. It was found that absorption capacity for

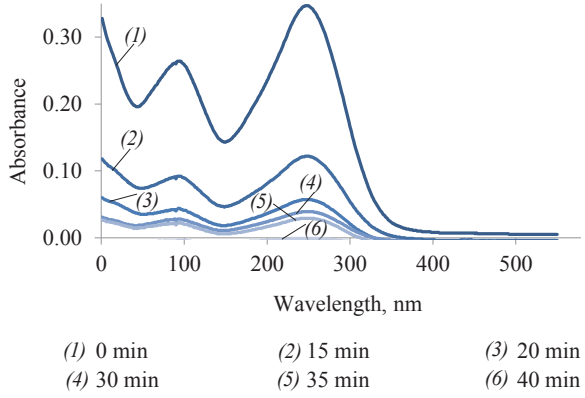


Figure 3 Absorbance of congo red at different contact time

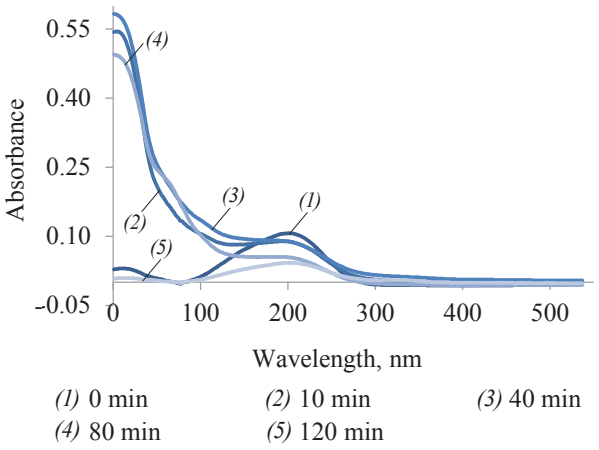


Figure 4 Absorbance of methyl orange at different contact time

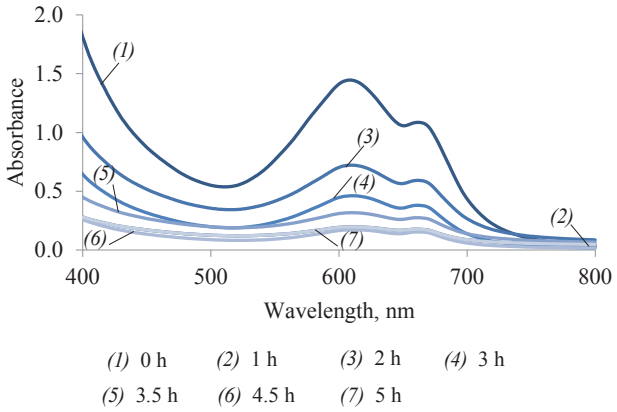


Figure 5 Absorbance of textile raw water at different time of contact

methyl orange was significantly lower, whereas that for congo red had maximum value at contact time of 30 min (Fig. 6).

Percentage of dyes adsorbed with adsorbent. The percentage of dye elimination indicated the efficiency of adsorbent (Table 2). The results made it possible to conclude that 100% of congo red was removed in 40 min, whereas the removal of only 48% of methyl

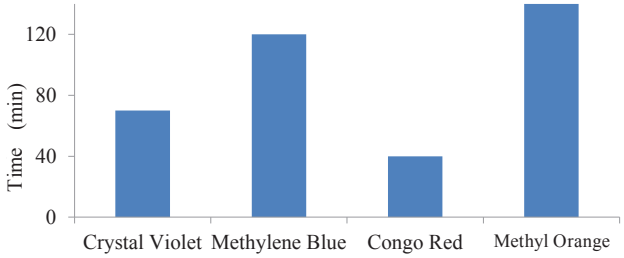
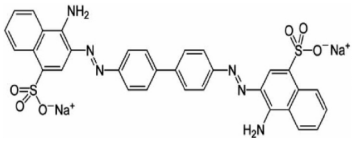
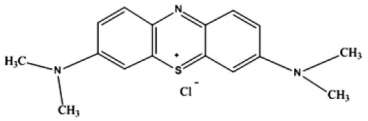
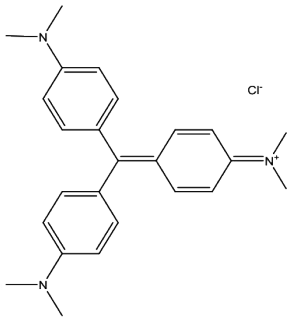


Figure 6 Contact time of different dyes at dye concentration of 0.015

Table 1 Adsorption coefficient of dyes at contact time of 30 min

Dye	Structure of dyes	Adsorption coefficient, mg/g
Congo Red		2.33
Methylene Blue		1.00
Crystal Violet		1.66

orange took 120 min. In spite of the fact that both methyl orange and congo red dyes have similar structure, the percentage of their removal from the solution differs. One of the causes for that could be the presence of two primary amine groups in congo red, which could contribute to binding of dye with adsorbent. Thus, the structure of adsorbate played a crucial role in adsorption efficacy. The efficiency of adsorption depended upon the pore size of the adsorbent. The results of the study confirmed that the structure of congo red dye was well-matched with the pore size of the adsorbent, which allowed it to exhibit fairly efficient adsorption (Table 2).

pH of dyes after treatment. The change in pH of different dye solutions was studied before and after the treatment with adsorbent. As one can see from Table 3, pH of the solution increased after the treatment. It could be due to introduction of basic component from the activated charcoal to the dye solution. Additional work could be done to find out the reason for the same.

Characteristics of the adsorbent. The adsorbent was analysed by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy

Table 2 Percentage removal of dyes

Dye	Concentration, g/L	Time, min	Dye removal, %
Congo red	0.015	40	100
Methylene blue	0.015	120	100
Crystal violet	0.015	60	100
Methyl orange	0.0025	120	48

Table 3 pH of dye solutions before and after treatment with adsorbent

Conce-ntration	Congo red		Crystal violet		Methylene blue		Methyl orange	
	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
0.015	7.34	10.90	8.50	10.00	7.70	10.30	7.57	9.74
0.01	7.30	10.06	7.50	10.12	7.43	10.25	7.50	–
0.005	7.20	10.02	7.30	10.24	7.39	10.21	7.40	–
0.0025	7.12	9.85	7.10	10.27	7.20	9.74	7.25	–

SD = ± 0.05

T_1 – before treatment; T_2 – after treatment

(SEM). The FTIR spectrum of the activated charcoal is shown in Figure 7. The various peaks were observed due to different functional groups. The peak at about 2200 cm^{-1} could be due to the presence of *sp* hybridised carbon. The peak at 1660 cm^{-1} corresponded to aromatic C=C stretching. The peak value at 3166 cm^{-1} indicated the presence of C-H group.

Surface morphology revealed the adsorbent had porous structure. This could be due to the evaporation of the chemical reagent throughout the carbonisation process, leaving the vacant spaces on the surface of the adsorbent. It is obvious from the SEM image (Fig. 8) that the adsorbent is a mixture of activated charcoal prepared from dissimilar plant material. The presence of dissimilar plant materials in the adsorbent could be accountable for elimination of broad range of dyes both cationic and anionic.

Characteristics of the adsorbent after adsorption. The FTIR analysis of adsorbent after reaction with dye showed a number of additional peaks, perhaps due to the functional groups present in the dye that was adsorbed onto the adsorbent.

FTIR spectrometry demonstrated one additional vibrational peak at 1386.61 cm^{-1}, which can be due to C-N stretching. The stretching vibration was observed at 872.88 cm^{-1} due to the presence of C-Cl bond. The C-S stretching band was observed at 572 cm^{-1}. Every new peak definited that methylene blue was adsorbed on the activated charcoal by assembling altered kinds of bonds.

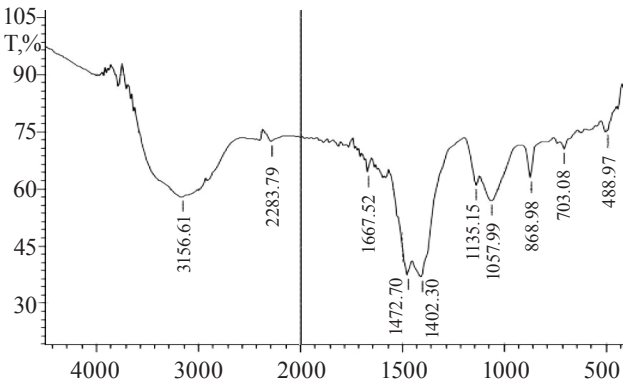


Figure 7 Infrared spectra of activated charcoal

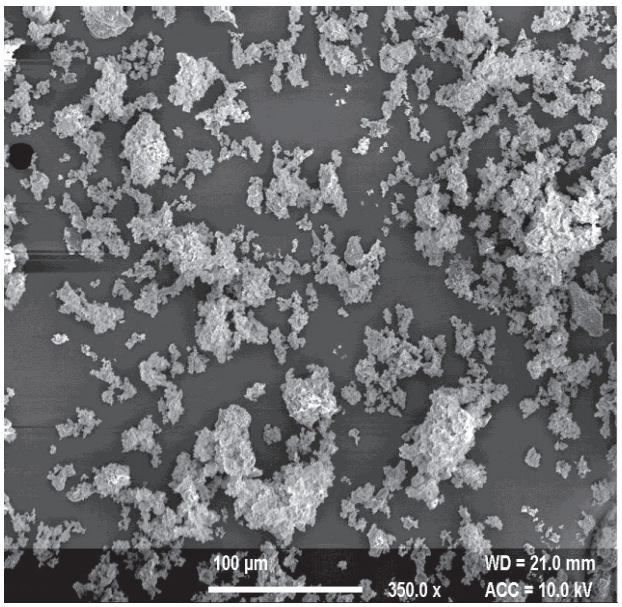


Figure 8 SEM image of the activated charcoal

The FTIR analysis of activated charcoal after adsorption of crystal violet revealed no additional peaks (Fig. 10). One of the causes can be crystal violet inserted in pores.

As for the FTIR spectra (Fig. 11) of activated charcoal with adsorbed congo red dye, three additional peaks were observed. Those were recorded at 3463, 2514, 1795, and 603 cm^{-1} that were due to N-H stretching, O-H stretching of carboxylic acid, C=O stretching, and C-C bending due to alkane, respectively. The presence of these functional groups confirms the adsorption of congo red dye on the activated charcoal.

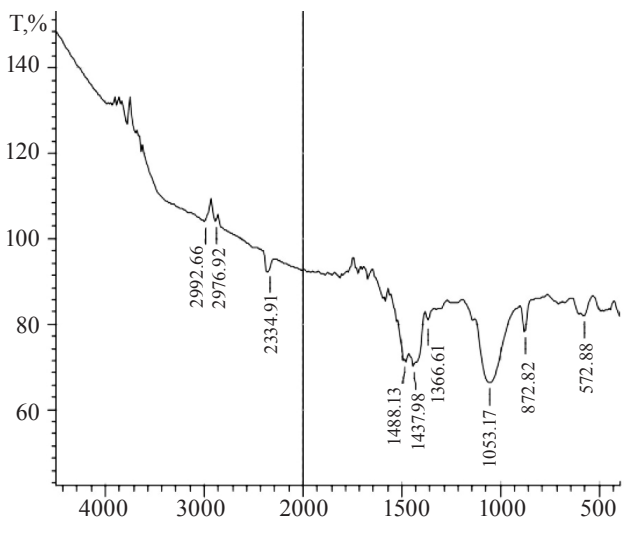


Figure 9 Infrared spectra of activated charcoal after adsorption of methylene blue

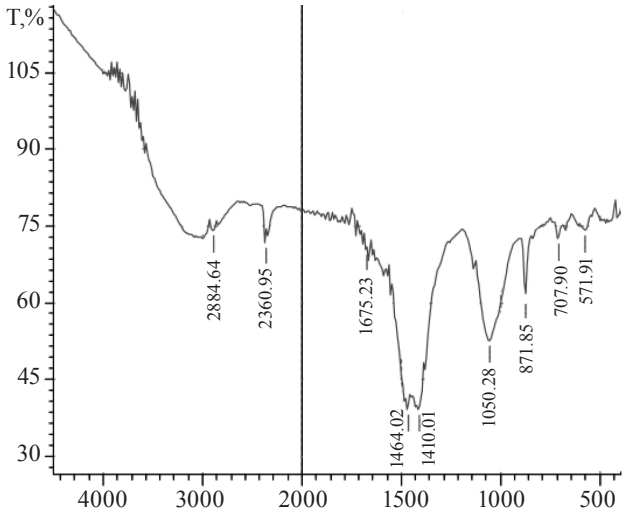


Figure 10 Infrared spectra of activated charcoal after adsorption of crystal violet

CONCLUSION

The results of this study made it possible to conclude that activated charcoal prepared from mixture of orange, banana, and pomegranate peels by carbonisation method had a great potential for removal of dyes from textile wastewater. In the present work, this adsorbent was tested on congo red, methylene blue, crystal violet, and methyl orange dyes. Studies showed that this adsorbent was effective in removing congo red, methylene blue, and crystal violet dyes from aqueous solutions, while it was not quite capable of removing methyl orange. Surface chemistry of activated carbon played an important role in dye adsorption. The type of the dye adsorbed on the adsorbent also depended on its textural

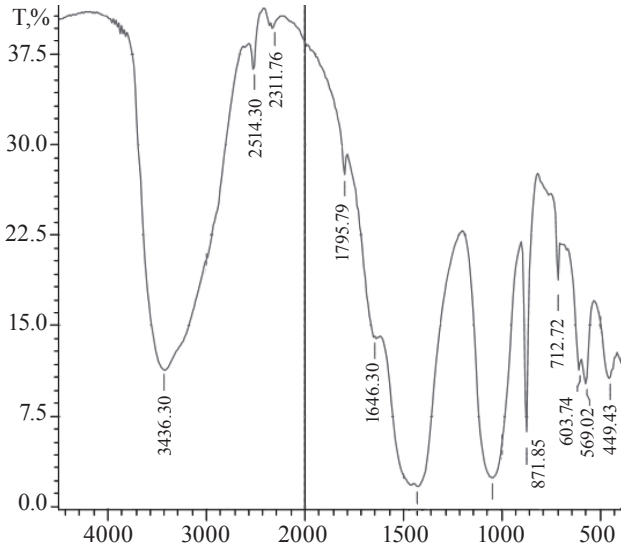


Figure 11 Infrared Spectra of activated charcoal after adsorption of congo red

properties, such as porosity and surface area. The adsorbent under study gave the best result for congo red dye. Thus, the present research developed a low-coat and environmentally friendly technology to remove dyes, as an alternative to known expensive and damaging methods.

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Thermal properties of commercial hydrobionts' tissues in the freezing process

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Abstract: The paper describes changes in thermal properties in the process of freezing of marine raw materials. The study objects were the skin of giant octopus (*Octopus dofleini* L.), pallium of Pacific squid (*Todarodes pacificus* L.), milt of Pacific herring (*Clupea pallasii* L.), and muscle tissue of Japanese cucumaria (*Cucumaria japonica* L.). The mathematical relations of the studied thermal parameters allowing the calculation of specific heat capacity, thermal conductivity coefficient and tissue density of the studied objects in the process of freezing were obtained. It was found that the change in the total specific heat capacity during the freezing of all the objects under study was of the same type: first, this figure increases due to the intensive ice formation in the tissues of hydrobionts, and then decreases due to a significant decrease in the content of the liquid aqueous phase. The values of the total specific heat capacity before the freezing of seafood were determined (kJ/kg·K): 4.26 for squid, 3.58 for milt of Pacific herring, 3.66 for octopus skin, and 3.95 for the shell of cucumaria. It was revealed that an increase in the amount of frozen out water decreased the density of samples of frozen raw materials. This was due to the high (77.4–88.9%) content of water, turning into ice, which has a lower density index. The values of hydrobionts' tissue density before freezing were obtained (ρ_0, kg/m^3): 1226.74 for squid, 1209.6 for milt of Pacific herring, 1128.55 for octopus skin, and 1031.26 for shell of cucumaria. It was established that the thermal conductivity of the hydrobiont tissue samples in the process of freezing increased with the growth of the proportion of frozen out water contained, approaching the thermal conductivity of ice. The calculated values of thermal conductivity coefficient of seafood tissue prior to freezing equal (W/m·K): 0.52 for squid, 0.47 for milt of Pacific herring, 0.63 for octopus skin, and 0.53 for cucumaria. The obtained thermal characteristics values of the objects studied are recommended for use in technical and technological calculations of aquatic biological resources cooling treatment processes.

Keywords: Hydrobionts, waste, water content, freezing, ice formation, heat capacity, thermal conductivity, density, approximation

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INTRODUCTION

Although containing a number of nutrients in their composition, some parts of commercial hydrobionts are not widely used in food production, thus being wasted while processing. These include octopus skin, which makes up to 37% by weight of raw material and is rich in caratinoids, collagen, taurine, selenium, high-limit fatty acids [1–4]. Processing of Pacific herring produces rarely used now milt (up to 12.4% by weight of raw materials), which contains nucleoproteins, including biologically active substances (deoxyribonucleic acid and ribonucleic acid), and polyunsaturated fatty acids, including ω-3 and ω-6 families [5]. Among other insufficiently used raw materials, sources are the Pacific squid and Japanese cucumaria [6, 7]. However, these commercial objects provide sources of such biologically active substances as complete protein, hexosamines, chondroitin sulfate, triterpene glycosides, and polyunsaturated fatty acids [3, 8–10]. Getting with food in the human body, they slow down the aging process and have a corrective effect on metabolic processes, thus improving the quality of life and promoting longevity.

Cryotechnology is a promising trend in the industrial processing of biologically highly valuable raw materials. The method allows obtaining concentrates with highly preserved natural properties and biological activity [11–13]. Since the resulting cryopowders, as a rule, have the properties of biologically active additives, they are often used as biological correctors in the production of various food products and

cosmetic materials, also being included in formulation compositions [14–18].

There are three main processes in cryogenic processing of raw materials of animal and plant origin: cryopreservation, cryogenic grindingб and freeze drying. Cryopreservation consists in rapid freezing of raw materials to a much lower than cryoscopic temperature, when most of the water turns into ice. It not only suppresses the activity of enzymes and the vital activity of microorganisms, but also creates favorable conditions for easier destruction of tissues during subsequent cryogenic grinding [11, 19]. By now, the process of freezing fish as a method of preservation has been widely studied, but there is lack of data on low-temperature processing of non-fish commercial objects. Also lacking are data on seafood thermal properties in the course of low-temperature processing. However, this knowledge is necessary when performing engineering calculations of processes and equipment related to cryogenic processing.

In this regard, the aim of the paper was to study the changes in thermal properties in the process of freezing raw materials of marine origin. Total specific heat capacity, thermal conductivity coefficient and density were calculated for the selected objects of study.

STUDY OBJECTS AND METHODS

The study objects were the skin of giant octopus (*Octopus dofleini* L.), pallium of Pacific squid (*Todarodes pacificus*), milt of Pacific herring (*Clupea pallasii* L.), and muscle tissue of Japanese cucumaria (*Cucumaria japonica* L.).

The amount of water in the samples, being the main factor of the freezing process, was determined by the standard method according to State Standard 7636-85 [20].

The standard software package of Microsoft Office 2007 and CurveExpert 1.4 were used for statistical data processing and graphs plotting with formula derivation.

Total specific heat capacity determination. The specific heat capacity of food products as multicomponent substances is calculated according to the law of additivity [21]:

$$c = g_1c_1 + g_2c_2 + g_3c_3 + ... + g_nc_n$$

where $c_1, c_2, c_3, ..., c_n$ are specific heat capacities of components, kJ/kg·K;
are mass fractions of the components.

Consider the body of the study object as a two-component mixture containing W parts of water and (1–W) parts of dry substances with corresponding specific heat capacities for each component c_w and $c_{d.s}$. Heat capacity of the product in the temperature range before ice formation is determined by the expression:

$$c = c_wW + c_{d.s}(1-W) \quad (1)$$

where c_w = 4.19 kJ/kg·K is water heat capacity (4.19 kJ/kg·K);
$c_{d.s}$ is specific heat capacity of dry substances in raw materials [22].

Since at negative temperatures part of the water ω in the object under study transforms into ice, whose heat capacity is c_i, the heat capacity of the frozen raw material c_{frm} is calculated by the formula:

$$c_{frm} = c_wW(1 - \omega) + c_iW\omega + c_{d.s}(1-W) \quad (2)$$

where c_i is the heat capacity of ice (2.1 kJ/kg·K).

When freezing, the heat of ice formation will be removed from the mass unit at a lower temperature, which is defined a

$$dq_\omega = L_fW\frac{d\omega}{dt} \quad (3)$$

where L_f is the specific heat capacity of ice formation (334.2 + 2.12t + 0.0042t^2 kJ/kg);
W – total water content of the sample, kg/kg.
t – temperature of frozen raw materials, °C.

If temperature change of one degree is adopted in the expression (3), the amount of heat will receive the dimension and meaning of the component of the specific total heat capacity and be recorded as:

$$q_\omega = L_fW(\omega_2 - \omega_1) \quad (4)$$

where ω_1 is the amount of frozen out water at the initial temperature;
and ω_2 is the amount of frozen out water at the final temperature.

The sum of calculated heat capacity of the frozen raw material c_{frm} and the heat of ice formation q_ω will give the total specific heat capacity:

$$c_{tot} = c_{frm} + q_\omega \quad (5)$$

Thermal conductivity coefficient determination. When the temperature drops below the cryoscopic value and the product is in the process of ice formation, its thermal conductivity increases significantly, since thermal conductivity of ice is four times greater than that of water.

The increase in thermal conductivity of the product with decrease in temperature almost ceases with the end of water freezing out, granted that further insignificant change in the thermal conductivity of ice and other components of the product is neglected. The thermal conductivity coefficient of products in the range of negative temperatures λ_{fr} depends on the amount of frozen out water and approximates to the equation [23]:

$$\lambda_{fr} = \lambda_0 + \omega\Delta\lambda \quad (6)$$

where λ_0 is the coefficient of thermal conductivity of the product before freezing, W/m·°C;

$\Delta\lambda$ is the change in thermal conductivity of the product in the temperature range from the start of freezing t_s to t_c corresponding to completion of ice formation.

Considering raw materials as a two-component mixture containing parts of water W and (1–W) parts of dry substances with respective thermal conductivity coefficients of λ_w and $\lambda_{d.s}$, the heat capacity of the product in the temperature range before ice formation is determined by the expression:

$$\lambda_m = \lambda_w W + \lambda_{d.s}(1-W)$$

where λ_w = 0,597 W/m^2·K is the coefficient of water thermal conductivity;

$\lambda_{d.s}$ = thermal conductivity coefficient of dry substances [6].

The coefficient of thermal conductivity can be calculated by the formula based on the models of Krisher [5]:

$$\lambda_f = \frac{1}{\lambda_i - \varepsilon_p(\lambda_i - \lambda_{fr})}\left[\lambda_{fr}\lambda_i + \frac{(\lambda_i - \lambda_{fr})^2}{2}(\varepsilon_p - \varepsilon_p^2)\right] \quad (7)$$

where λ_i is thermal conductivity of ice coefficient within the temperature range 273–208 K (2,22 W/m·K);

ε_p – porosity coefficient which depends on the amount of frozen out water and chemical composition.

The structure of the frozen product can be considered as a dispersed system consisting of ice pores with coefficient of thermal conductivity λ_i and a matter containing unfrozen water and dry substances with a coefficient of thermal conductivity approximately equal to λ_0 before freezing.

Porosity coefficient of the assumed structure will be determined by the expression:

$$\varepsilon_p = \frac{W\omega}{m\rho_i + W\left[\frac{\rho_i}{\rho_w} + \omega\left(1 - \frac{\rho_i}{\rho_w}\right)\right]} \quad (8)$$

where ρ_i is ice density, kg/m^3;

ρ_w is product density before freezing, kg/m^3;

m is mass fraction of dry substances in raw materials.

Taking into consideration stable weight fraction of dry substances in the process of freezing, and practically unvarying density ρ_m

$$m = \frac{1}{\rho_m} - \frac{W}{\rho_w} \quad (9)$$

Frozen raw material density determination. Consider the body of the object under study as a three-component mixture consisting of unfrozen water, ice, and dry matter. Density of the samples can thus be presented as the equation [6]:

$$\rho_{frm} = \frac{1}{\frac{g_1(1-\omega)}{\rho_1} + \frac{g_2}{\rho_2} + \frac{g_1\omega}{\rho_3}} \quad (10)$$

where g_1 is the mass fraction of water contained in the sample body;

g_2 is the mass fraction of solids contained in the sample body;

Table 1 Water content in the tissues of hydrobionts

Sample	Water content,%
Milt of Pacific herring	77.4
Pallium of Pacific squid	78.6
Skin of octopus	84.8
Japanese cucumaria	88.9

ρ_1 is water density (1000 kg/m^3);

ρ_2 is dry matter density of raw materials, kg/m^3 [21];

ρ_3 is ice density (917 kg/m^3);

ω is the amount of frozen out water.

RESULTS AND DISCUSSION

Data on water content determination in the tissues of the studied hydrobionts are given in Table 1.

The objects under study have a high water content ranging from 77.4% (in the milt of Pacific herring) to 88.9% (in the muscle tissue of the Japanese cucumaria), which corresponds to the known data [2, 3, 7, 24].

Using formula (5), we calculate the total specific heat capacity of the samples. To do this, it is necessary to determine the amount of frozen out water at different temperatures using Ryutov's formula [25]. Then we apply formulae (2) and (4) to determine the heat capacity for the selected raw material and the heat of ice formation. The resulting values of the total specific heat capacity of the raw material are depicted as graphs in Fig. 1.

Presented in Fig. 1 graphs show the relation between total specific heat capacity and the amount of frozen out water for the four studied objects. As can be seen, they are of the same type and have two distinct areas. The first one demonstrates an increase in the total specific heat capacity of seafood samples, which is associated with intensive ice formation in their tissues with a decrease in temperature and accompanying heat release. The second area is characterised by a gradual decrease in the total specific heat capacity of seafood samples. This is associated with a significant decrease in the amount of liquid aqueous phase and, accordingly, a decrease in the intensity of its transition to the crystalline form with the release of heat caused by ice formation. At the final stage, when most water is frozen out, the total specific heat capacity of the samples under study tends to the heat capacity of ice becoming one of the main factors of the further freezing process. The transition point of the total specific heat capacity from increase to decrease is reached when the amount of frozen out water gets close to 50%. The obtained values of total specific heat capacity of commercial hydrobionts' tissues are consistent with the data available in the academic literature on aquatic raw materials [25].

Approximating the curves shown in Fig. 1 with Curve Expert Professional 2.3, we get the formulae:

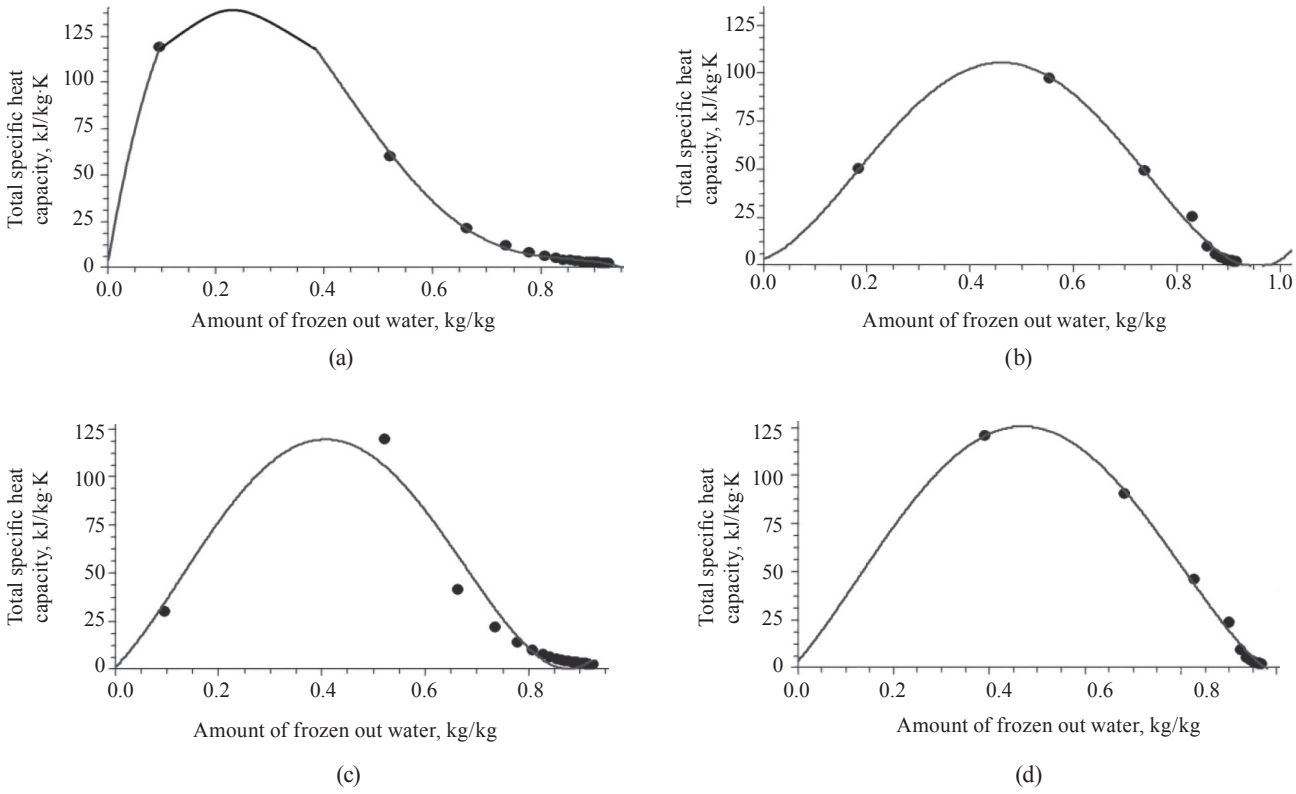

Figure 1 Relation between total specific heat capacity and the amount of frozen out water: (a) pallium of Pacific squid; (b) milt of Pacific herring; (c) octopus skin; (d) Japanese cucumaria

$$c_s = 2751.19\omega^4 - 4888.57\omega^3 + 2159.33\omega^2 + 9.05\omega + 4.26 \quad (11)$$

$$c_m = 1786.77\omega^4 - 3293.67\omega^3 + 1410.95\omega^2 + 95.48\omega + 3.58 \quad (12)$$

$$c_{os} = 2511.06\omega^4 - 4238.40\omega^3 + 1611.53\omega^2 + 149.47\omega + 3.66 \quad (13)$$

$$c_{cu} = 1140.6\omega^4 - 2110.32\omega^3 + 669.94\omega^2 + 291.58\omega + 3.95 \quad (14)$$

These formulae can be used to calculate the relation between total specific heat capacity and the amount of frozen out water for the studied raw materials with a correlation coefficient of 0.99. The free term in the obtained formulae determines the value of total heat capacity of the raw material with the amount of frozen water equal to 0. Therefore, total specific heat capacity of non-frozen seafood equals (kJ/kg·K): 4.26 for squid, 3.58 for milt of Pacific herring, 3.66 for octopus skin, and 3.95 for cucumaria shell. The values of heat capacity of non-frozen raw materials calculated, based on the standard formula (1) were as follows (kJ/kg·K): 4.06 for squid; 3.52 for milt; 4.05 for octopus skin; and 3.93 for cucumaria. The difference between the data obtained according to formulae (11–14) and (1) is 4.9, 1.7, 9.6, and 0.5% for squid, milt, octopus skin, and cucumaria, respectively. This indicates the adequacy of the derived mathematical relationships.

Using formula (7), we calculated the coefficient of thermal conductivity of the selected raw material and plotted the relation to the amount of frozen out water (Fig. 2).

Analysing the graphs in Fig. 2, we see that the dependence of the change in the thermal conductivity of the studied samples is close to linear. The thermal conductivity of the studied seafood in the process of freezing increases with the proportion of frozen out water, tending to the thermal conductivity of ice, which is almost four times greater than the thermal conductivity of water. Approximating the chart data using Curve Expert Professional 2.3, we obtain the formulae:

$$\text{for squid: } \lambda_s = 0.52 + 1.02\omega \quad (15)$$

$$\text{for milt of herring: } \lambda_m = 0.47 + 1.01\omega \quad (16)$$

$$\text{for octopus skin: } \lambda_{os} = 0.63 + 1.07\omega \quad (17)$$

$$\text{for cucumaria: } \lambda_{cu} = 0.53 + 1.54\omega \quad (18)$$

Formulae (15–18) can be used to calculate the thermal conductivity of the studied objects with a correlation coefficient of 0.99. They also allow us to determine the thermal conductivity of the test

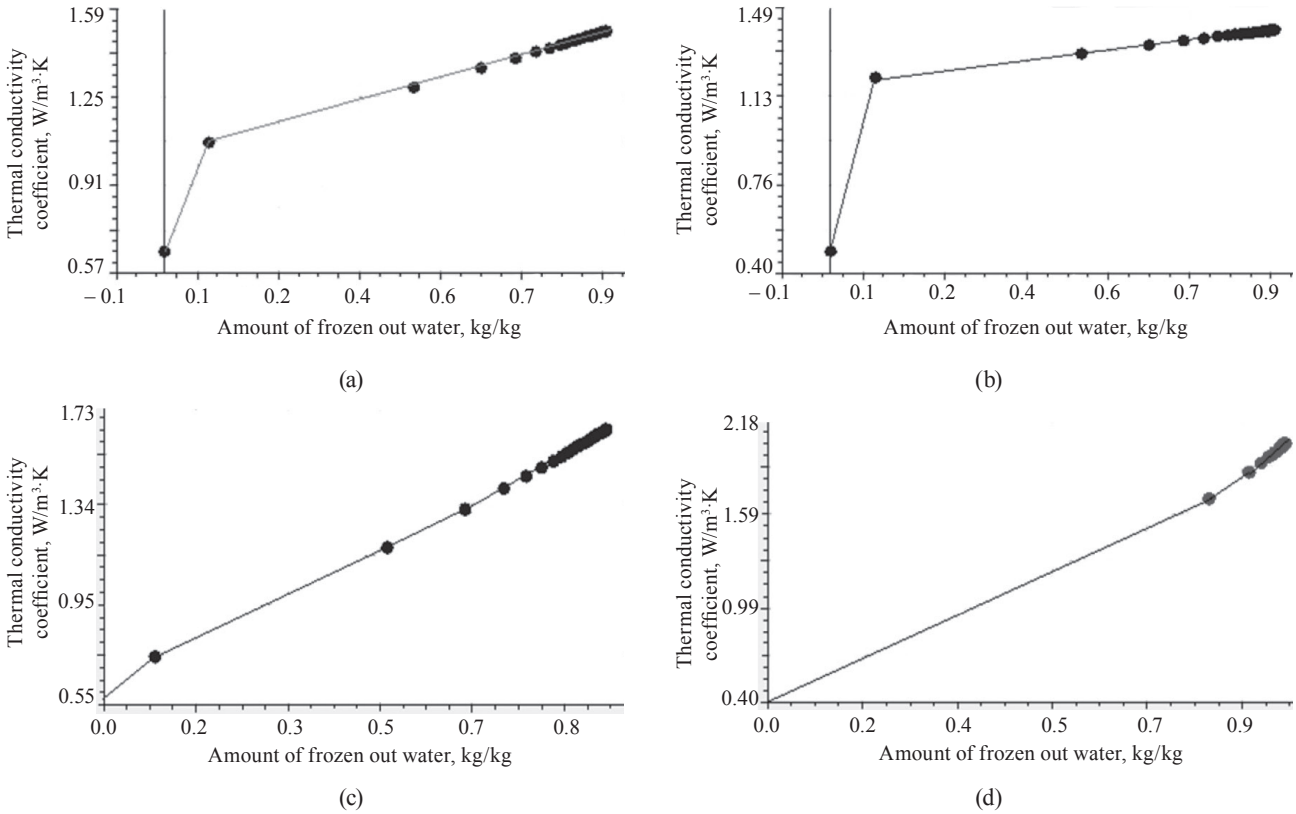

Figure 2 Relation between thermal conductivity coefficient and the amount of frozen out water for: (a) squid trunk; (b) milt of herring; (c) octopus skin; (d) cucumaria

samples before freezing, when the amount of frozen out water $\omega = 0$. The thermal conductivity coefficient of non-frozen seafood equals: squid – 0.52 W/m·K, milt of Pacific herring – 0.47 W/m·K, octopus skin – 0.63 W/m·K, cucumaria – 0.53 W/m·K. The values of thermal conductivity coefficients obtained correlate well with the data available in academic literature for fish raw materials: big-eyed tuna, Pacific cod, tilapia [26–28].

Formulae (15–18) correspond to the equation (6), which allows to conclude that for the studied samples $\Delta\lambda$ equals the following values, W/(m·K): squid – 1.02; milt of herring – 1.01; octopus skin – 1.07; cucumaria – 1.54. It is known that the value of $\Delta\lambda$ according to experimental data for food containing 70–80% of water varies within 0.928–1.16 W/m·K [23]. This range exceeds $\Delta\lambda$ of cucumaria, which can be explained by the peculiar structure and higher water content (88.9%) in its muscle tissue.

Formula (10) helps calculate the density of raw materials in the process of freezing and construct graphs of the relation between density and the amount of frozen out water (Fig. 3).

Analysing the graphs in Fig. 3 it should be noted that the considered relations are of the same type and close to linear. Density of frozen raw materials is reduced with the increase in the amount of frozen water. This happens due to the high water content in the studied objects. Water turns into ice which has a lower density index. Approximating data curves with the help of Curve Expert Professional 2.3, we get the formulae:

$$\rho_{f.m} = 1209.6 - 142.89\omega \quad (19)$$

$$\rho_{f.s} = 1226.74 - 149.08\omega \quad (20)$$

$$\rho_{f.os} = 1128.55 - 138.24\omega \quad (21)$$

$$\rho_{f.cu} = 1031.26 - 100.42\omega \quad (22)$$

These equations can be used to determine the density of the samples before freezing, with the amount of frozen water equals 0. Then the density of chilled milt of Pacific herring can be set to ρ_0 = 1209.60 kg/m^3, ρ_0 squid = 1226.74 kg/m^3, ρ_0 octopus skin = 1128.55 kg/m^3, and ρ_0 cucumaria shell = 1031.26 kg/m^3. These data correlate well with the calculated values of the density of unfrozen objects under study obtained by formula (10).

The derived formulae (19–22) can be used to calculate the relation between the density of herring milk of the Pacific, squid trunk, octopus skin, cucumaria shell and the amount of frozen out water with a correlation coefficient of 0.99. The results of calculations show that the decrease in the density of the studied hydrobionts' tissues during freezing, when the amount of frozen out water reaches, for example, 90% makes up for squid – 11.9%, milt – 9.0%, octopus – 11.0%, and cucumaria – 8.4%. It is known that during freezing the

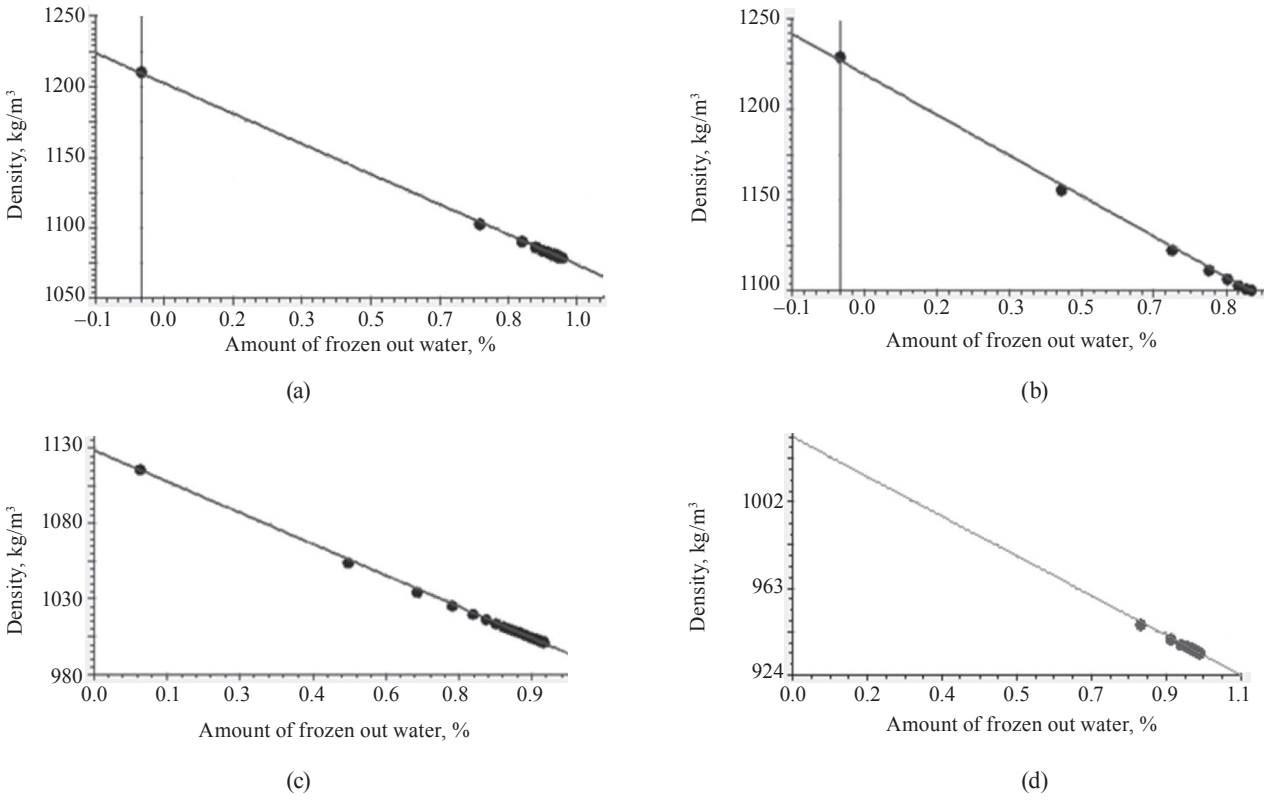

Figure 3 Relation between the density and the amount of frozen out water for: (a) milt of Pacific herring; (b) pallium of squid; (c) octopus skin; (d) cucumaria shell

density of Atlantic mackerel muscle tissue decreases by 9.3% [23].

Thus, studies of changes in thermal properties in the process of freezing Pacific squid, milt of Pacific herring, giant octopus, and muscle tissue of Japanese cucumaria were undertaken.

CONCLUSION

It was found that during freezing the change in total specific heat capacity of all the objects under study is of the same type: first, this figure increases due to the intensive ice formation in the tissues of hydrobionts, and then decreases due to a significant decrease in the content of the liquid aqueous phase in the objects under study. The transition point from growth to fall corresponds to the values of the amount of frozen water close to 50%.

The relation between the coefficient of thermal conductivity of the studied hydrobionts' tissues and the amount of frozen out water is close to linear. The thermal conductivity of tissue samples slowly increases with the proportion of frozen out water, approaching the thermal conductivity of ice.

The relation between the density index of hydrobionts' tissues in the freezing process is also close to linear. With the increase in the amount of frozen out water the density of the frozen raw material decreases, since the samples under study have a high content of water turning into the ice, which has a lower density index.

The obtained mathematical relationships of the studied thermophysical parameters also allow us to obtain the values of specific heat capacity, thermal conductivity and tissue density of fresh and chilled hydrobionts prior to freezing, when the amount of frozen out water equals zero.

The obtained digital values of total specific heat capacity, thermal conductivity, and density can be used by specialists for calculation, modeling and design of basic and derivative processes of non-fish commercial hydrobionts low-temperature processing, as well as refrigeration and process equipment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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Mechanically activated hydrolysis of plant-derived proteins in food industry

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Abstract: A poor consumption of important nutrients triggered a public interest in functional foods that contain easy-to-digest proteins. The present research features fractionation, mechanical activation, and enzymatic hydrolysis of pea protein. According to modern chemical methods, the protein content in the original pea biomass was 24.3% and its molecular weight distribution (MWD) was 5–135 kDa. Fractionation, or protein displacement, resulted in four fractions of biopolymers with different chemical composition, i.e. a different content of protein and carbohydrate molecules. The paper introduces some data on the enzymatic transformations of the substrate. A set of experiments made it possible to define the optimal conditions for the mechanical activation of pea biomass with proteolytic enzymes. The enzymes were obtained from Protosubtilin G3x, a complex enzyme preparation. When the substrate and the enzymes were mechanically activated together, it produced mechanocomposite, an intermediate product with increased reactivity. It increased the specific surface area by 3.2 times and doubled the crystallinity of the substrate. As a result, the rate and yield of the subsequent enzymatic hydrolysis increased from 18% to 61%. The study determined the capacity of the substrate in relation to the enzyme preparation. Under optimal conditions, the pea hydrolysis destroyed protein molecules within two hours. After four hours of hydrolysis, no changes were detected. A polyacrylamide gel electrophoresis revealed non-hydrolysed protein molecules with MW ≈ 20 kDa. Presumably, they corresponded with legumin, which is resistant to neutral and alkaline proteases. The resulting hydrolysates were spray-dried to test their potential use as a food component. The product obtained by spray-drying had a monomodal distribution of particle sizes of spherical shape with a diameter of 5–20 μm.

Keywords: Mechanochemistry, mechanochemical activation, mechanocomposite, plant materials, enzymatic hydrolysis, destruction of protein molecules, polypeptides, amino acids, spray-drying

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INTRODUCTION

The development and subsequent quality assessment of functional foods is one of the priorities of healthy nutrition [1]. Functional foods with a programmed chemical composition can be fortified with important nutrients and are suitable for various categories of population, e.g. athletes, lactating and pregnant women, senior citizens, children, etc. [2].

However, priority goes to gastrointestinal and allergic patients and professional athletes. Their nutrition

requires scientific approaches, since their diet should contain a complex of peptides and free amino acids, as well as simple and complex carbohydrates [3].

Foods fortified with proteins, especially those containing essential amino acids, contribute to the rapid and effective recovery of muscle tissue after intense physical exertion. Peptides and polypeptides are known to accelerate metabolic processes, hormone production, and muscle tissue growth [4].

Food intolerance and allergic reactions are another problem of modern society. Some people are allergic to products that contain proteins of animal or plant origin.

Hence, a new generation of food products with easy-to-digest nutrients remains an important objective of food industry. Modern studies confirm that plant raw materials – and legumes in particular – are suitable for isolation and modification of proteins, short peptides, and amino acids [5, 6]. These groups of compounds are widely used as dietary supplements and ingredients for functional products [7–9]. Pea protein has a better nutritional value, amino acid composition, and anti-nutrients than soybeans, beans, and other legumes [10, 11].

There are many methods to isolate protein from plant materials for subsequent hydrolysis [12–14]. However, most of them remain inefficient for enzymatic transformations of heterogeneous substrate. Preliminary mechanical activation means that raw material has to be processed in specially designed energy-stressed activator mills. The procedure makes it possible to control the reactivity of solid substrates. In addition, it increases the speed and yield of water-soluble products for commercial purposes [15, 16].

However, the process of enzymatic reactions after preliminary mechanical activation remains understudied. A series of studies on the hydrolysis of cellulose showed that the increase in specific surface area and the degree of crystallinity of the substrate affected the rate and yield of enzymatic hydrolysis [17]. In addition, it is important to study the elusive transfer of mechanochemical processes from lignocellulose to protein and starch.

The research objective was to study the mechanically activated hydrolysis of pea biomass, as well as to obtain a hydrolysate fortified with free amino acids and peptides to be used in functional foods.

STUDY OBJECTS AND METHODS

The experiment featured dry biomass of split pea seeds harvested in 2017. The peas corresponded with State Standard 6201-68*, Class I, and was produced by OOO ECO-PAK (Novosibirsk region, Russia). Before the experiment, the pea biomass was subjected to rough grinding in a knife mill to the size of ≤ 2 mm. The ground biomass was vacuum-packed, stored at room temperature, and used for further experiments.

Protosubtilin G3h was used as enzyme preparation (OOO Sibbiopharm, Berdsk, Russia). The complex was chosen for its catalytic activity and availability for further technological application. This industrially available enzyme preparation contains a complex of enzymes that consists of neutral and alkaline proteases and glycosidases, i.e. ≈ 11000 U/g of protease, ≤ 150 U/g of xylanase, ≤ 200 U/g of β-glucanase, and ≤ 300 U/g of α-amylase [18]. Protosubtilin belongs to the group of enzyme feed additives that are able to break down high-molecular proteins. This enzyme preparation is produced by *Bacillus subtilis*.

Gravimetric methods were used to assess moisture and ash content in the plant materials and processed products, respectively [19, 20].

X-ray diffraction and thermal desorption of gases were employed to measure the degree of crystallinity and specific surface area according to the methods described in [17] and [21], respectively.

The method described by Fadeeva *et al.* was used to perform the elemental analysis that made it possible to determine the quantitative protein content in the peas. After that, the protein content was determined using the nitrogen content with conversion factor of 6.25 according to the Kjeldahl method [22–24].

The mass fraction of soluble substances was determined by the method of exhaustive extraction in a Soxhlet extractor for 24 h. Distilled water was used as extractant. The yield of water-soluble substances was measured according to the reduction of the mass after the extraction.

The content of free amino acids was defined at the Centre of Mass Spectrometry Analysis (Institute of Chemical Biology and Fundamental Medicine). An optimised standard procedure was used as in [25]. A set of isotope-labelled amino acids and acyl carnitines No. 55000 (Chromsystems Instruments & Chemicals, Germany) served as internal standards and solutions. An Agilent-1200 chromatographic system with an Agilent 6410 QQQ mass spectrometer (Agilent Technologies, USA) was employed as an HPLC-MS/MS system. A quantitative analysis was performed in the mode of multiple reactions monitoring; the total analysis time was 2.5 min. The obtained data were processed using MassHunter v.1.3 software.

The molecular weight of protein molecules was measured using the *Laemmli SDS PAGE procedure* [26]. For pre-denaturation, proteins were treated with 1.4-dithiothreitol at a 1:1 ratio. After that, they were placed in a thermoshaker at 95°C (Biosan, Latvia) for 7 min. An Elf-4 power source was used to create electric field (DNA-Technology, Russia). The concentrations of polyacrylamide in the concentrating and separating gel were 4% and 18%, respectively. The pre-phoresis stage lasted 15 min. The current force was 15 mA, while during the phoresis stage it was 35 mA.

To identify the zones of proteins after the electrophoresis, they were stained with Coomassie R-250 pigment according to the procedure described by

* State Standard 6201-68. Polished pea. Specifications. Moscow: Standartinform; 2010. 3 p.

Dyballa *et al.* [27]. Protein markers were represented by Unstained protein MW marker (Thermo Fisher Scientific, USA) with protein molecular weight of 14.5–116 kDa and Unstained protein ladder (Thermo Fisher Scientific, USA) with protein molecular weight of 5–250 kDa. MultiChrom-Planar programme processed the mathematical data [28].

The fractionation of the plant material was conducted according to the method described in [29, 30]. The initial crushed pea biomass was extracted in alkaline water. A 1M solution of sodium hydroxide was added to the pre-ground pea biomass. The solution consisted of 2.5 mL of solution per 1 gram of biomass (pH 9.0). The suspensions were placed in a WSB-30 water bath at 45°C and 180 rpm for 30 min (DAIHAN Scientific, Korea). After the extraction, the soluble portion was separated by centrifugation at 6000 rpm for 20 min. The precipitate was used in the next extraction cycle under the same conditions. The extracted components were precipitated with a threefold volume of cooled ethanol and dried in a laboratory frost dryer Iney 4 (Institute for Biological Instrumentation of the Russian Academy of Sciences, Russia).

After three extraction cycles (fraction No. 4), the insoluble residue – a carbohydrate fraction – was washed twice with chilled ethanol and dried under similar conditions.

The mechanical activation of the plant material with enzymes was carried out in an RM-20 roller mill-activator (5.5 kW), which was equipped with a water cooling device (Fig. 1). The pea biomass was mixed with a dry enzyme preparation and processed in an activator at a rotor speed of 1450 rpm. The mixture of raw materials and enzymes was supplied automatically at a rate of 3 kg/h.

The spray-drying was performed in a Mini Spray Dryer B-290 (Büchi, Switzerland) in the following conditions: nozzle temperature = 110°C, cyclone temperature = 70°C, gas flow rate = 700 L/h, feed rate = 5 mL/min.

The enzymatic hydrolysis lasted 7 h at 50°C. 50 mL of distilled water was added to 15 g of initial or mechanically activated pea biomass with a certain amount of the enzyme preparation. Enzyme loading equalled 0.5–3%. Suspensions were thoroughly mixed until uniform. For enzymatic hydrolysis, the suspensions were placed in a WSB-30 water bath (DAIHAN Scientific, Korea) at 50°C and 120 rpm. After enzymatic hydrolysis, the supernatant was centrifuged at 6000 rpm for 20 min. No enzyme preparation was added to the control samples.

RESULTS AND DISCUSSION

Suitable protein plant materials were selected for the mechanoenzymatic processing to be used in functional, special, and therapeutic food products. The physical and chemical characteristics are given below. In addition, the selection was based on an analysis of the existing market for high-protein plant materials, state statistics, distribution of croppage, and percentage of various cultures in Russian regions. This approach made it possible to identify raw material with suitable physicochemical parameters, as well as to determine its prospects in subsequent processing and implementation.

Figure 2 shows a distribution diagram of croppage in Russia in 2017. The diagram was based on the data obtained from the Federal State Statistics Service [31]. Cereals and legumes clearly prevail over other cultures. Legumes are richer in protein than grains. An analysis of the distribution of croppage within the group of leguminous crops showed that a large proportion (77%) belongs to peas (Fig. 3).

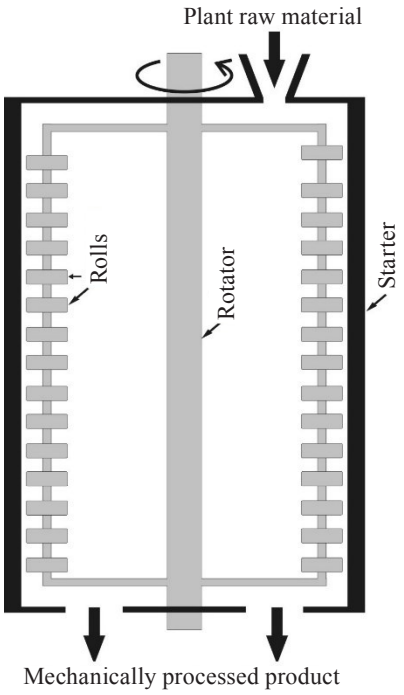

Figure 1 Scheme of the roller type activator mill RM-20

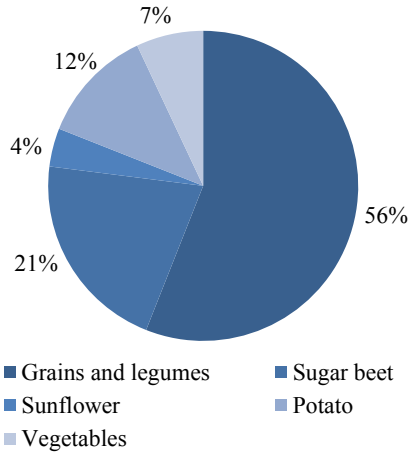

Figure 2 Croppage distribution in Russia

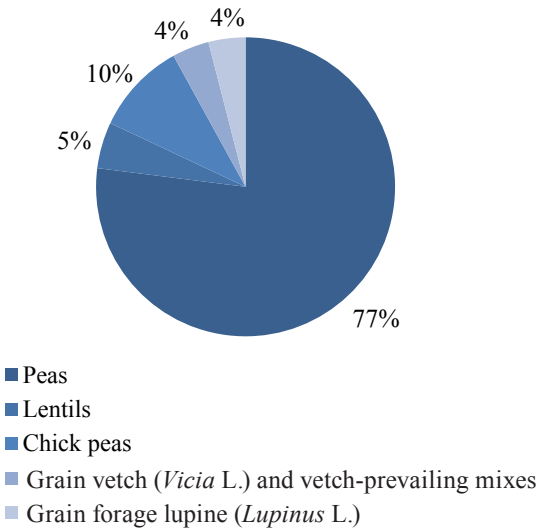

Figure 3 Percentage ratio of the croppage of legumes in Russia

Thus, legumes proved to be the most advantageous source of vegetable protein in Russia, especially peas, which contain about 25% of protein. In spite of the fact that soy contains up to 35% of protein, it was not considered in this study since it is rich in anti-nutritional substances, Moreover, it has a low consumer loyalty, which cannot be ignored in product development [5–7, 10, 32]. The protein content in peas varies greatly according to genotypic characteristics and the cultivation conditions. Leguminous proteins are poor in methionine and cysteine. This is typical of plant proteins. For instance, grain crops are poor in lysine and threonine. However, the biological value of products obtained from them can be fortified by a limiting amino acid or other types of plant materials.

The present research involved a comparative analysis of the protein content and amino acid composition together with its coefficient of imbalance and functionality in high-protein plant raw materials. Peas demonstrated the highest functionality ratio of amino acid composition (FRAAC) – 0.6, while soybeans had 0.4 and beans and lentils had 0.3. This indicated that peas possessed the optimal ratio of amino acids if compared with reference chicken egg protein.

Thus, pea biomass appeared to have a high nutritional value and a balanced amino acid composition, which made it an optimal research subject. Its physical and chemical patterns can subsequently be transferred to other types of biomass. The samples obtained after fractionation (Fig. 4) and freeze drying were analysed for the protein content in the dry product. The results are presented in Table 1. A polyacrylamide gel electrophoresis defined the molecular weight of the proteins in the fractions.

The obtained data are consistent with those already published Mession *et al.*: the pea biomass contained 23–24.4% of protein and 48–60.3% of starch [33].

Figure 4 Frozen fractionation products before freeze drying: 1–4 are numbers of corresponding fractions

Fractions No. 1 and 2 isolated from the biomass were fortified with proteins, while fraction No. 3 was fortified with proteins and carbohydrates, and fraction No. 4 – with carbohydrates.

The electrophoregram (Fig. 5) shows that fractions 1–3 contained proteins with molecular weight = 5–135 kDa, which corresponded to molecules that consisted of 50–1350 amino acid residues. The predominating molecules were those with molecular weight = 24–135 kDa (240–1350 amino acid residues). They were most likely to be sub-units of 11S-globulins [34]. Both the elemental analysis and the gel electrophoresis showed that the content of protein molecules in fraction 4 was at the level of trace amounts.

As proved by cellulose processing, enzyme preparation increases the efficiency of subsequent enzymatic hydrolysis, if added at the stage of mechanic-chemical processing [34]. The enzyme complex used in the present research had a suitable catalytic activity profile and was cheaper than its analogues, such as proteases AP1, Alcalase, Savinase, Esperase, and Neutrase (Shandong Longda Bio-Products and Novozymes).

A set of experiments made it possible to determine the effect of the conditions of mechanical activation on the subsequent enzymatic hydrolysis. The pea biomass was subjected to mechanical activation 1) without enzymes and 2) with an insufficient amount

Table 1 Protein content in the initial raw material and in the fractions

Sample	Protein content, %	Fraction content in the raw material, %
Raw material	24.3	–
Fraction No. 1	97.1	19.0
Fraction No. 2	86.7	6.5
Fraction No. 3	45.7	0.4
Fraction No. 4	Trace	74.1

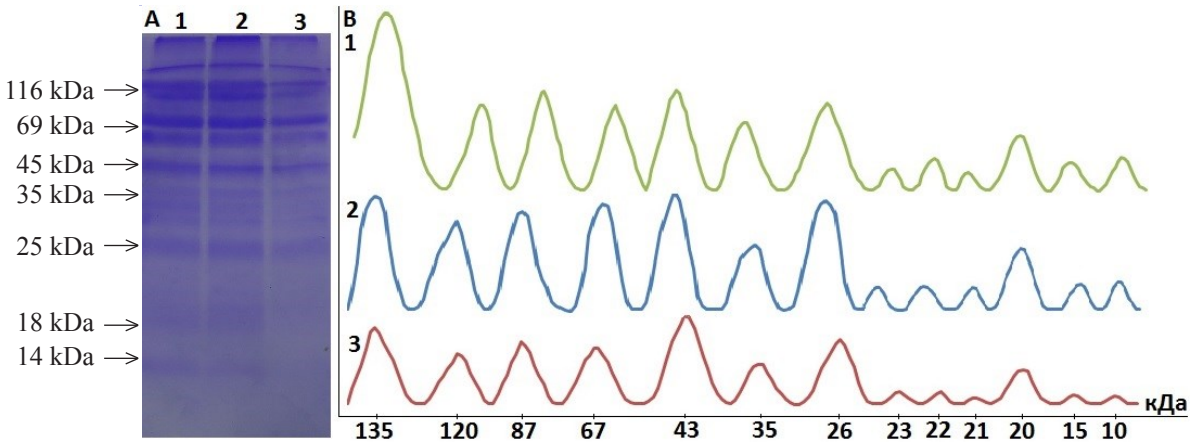

Figure 5 Electrophoregram (A) and MWD profilograms (B) of proteins in the fractions; 1, 2, and 3 – fraction numbers. Fraction No. 4 is not represented as it appeared to have no proteins in its composition

Table 2 Yield of water-soluble substances according to the processing conditions

	Extraction from the initial raw material	Extraction from the product of mechanical activation without enzymes	Product after mechanical activation (without enzymes) and hydrolysis	Product after mechanical activation with 1% of enzyme preparation after hydrolysis
Yield of water-soluble substances, %	18.0	18.5	25.1	60.6

of enzymes (1%) in relation to the substrate. The subsequent hydrolysis and complete extraction (Table 2) showed that the mechanical activation without enzymes barely increased the yield of the subsequent hydrolysis. However, the mechanical activation with enzymes increased the yield during subsequent hydrolysis from 18% to 60%, i.e. by ≥ 3 times.

The results can be explained by the fact that a simultaneous activation of substrate and enzymes produced mechanocomposite. The mechanocomposite was an intermediate solid-phase product with a high reactivity. In such mechanocomposites, enzyme particles are distributed non-diffusively, or mechanically, over the surface of the substrate, which was disordered during the activation process. Similar effects were observed in other cases of activation of food and non-food plant raw materials [35, 36]. When mechanocomposite is formed, it usually increases the rate and yield of the subsequent proteolytic and glycolytic processes. In this case, a preliminary chemical interaction preceded the mixing of the enzymes and the substrate. This interaction resulted from a significant increase in surface area, which enlarged from 0.6 to 1.9 m^2/g, and an extra disordering of the substrate structure, whose crystallinity decreased from 25% to 14%.

The conversion of enzymatic hydrolysis was studied under the same conditions, according to the substrate – enzyme ratio. The enzyme preparation was added in 0.5, 1, 2, 2.5, and 3% (Table 3).

Table 3 shows that the amount of water-soluble substances increased, as the amount of enzymes increased from 0.5% to 2%. The water-soluble substances included reducing carbohydrates, which are low molecular weight products of starch hydrolysis. When the load of the enzyme complex increased to 2.5–3%, the number of reaction products did not increase. This might have been caused by the fact that the sorption sites of the substrate were completely filled with enzymes. The situation was fully consistent with the idea that the heterogeneous stage of enzymatic hydrolysis has a limiting effect.

The polyacrylamide gel electrophoresis was used to study the changes in the molecular weight during the enzymatic hydrolysis. Figure 6 shows the electrophoregram of the proteins contained in the hydrolysate 1–7 h after the hydrolysis. The data prove that the amount of the original protein molecules significantly decreased within 7 h. As a rule, proteins degrade within 2 h. The molecular weight of the degradation products of the original polypeptide proteins revealed no significant changes after 4 h. After

Table 3 Yield of water-soluble substances and reducing carbohydrates according to the amount of enzyme preparation

Enzyme preparation, %	Yield of water-soluble substances, %	Yield of reducing carbohydrates, %
0.0	25.8	1.5
0.5	38.7	4.0
1.0	55.4	7.6
2.0	78.5	16.3
2.5	78.5	16.3
3.0	78.5	16.3

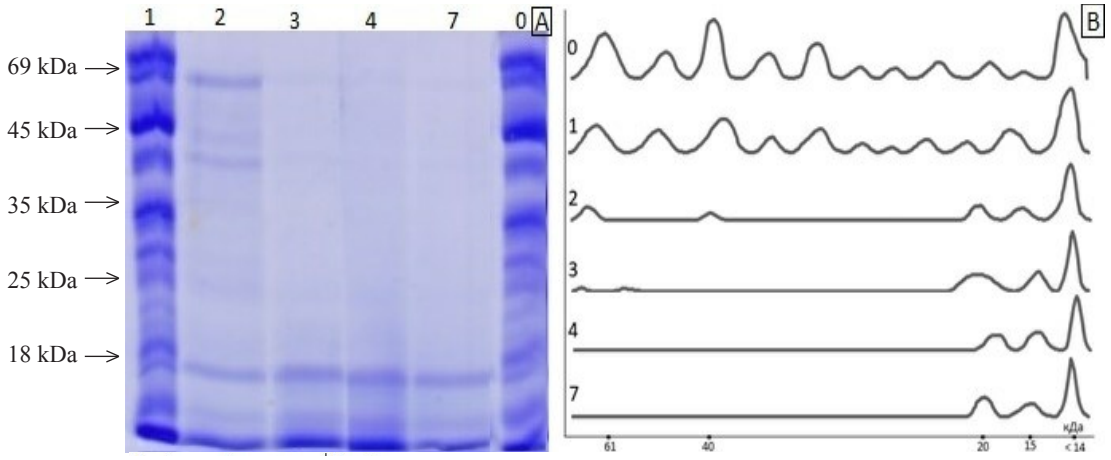

Figure 6 Electrophoregram (A) and profilogram (B) of the molecular weight distribution of proteins in the hydrolysate after 0, 1, 2, 3, 4, and 7 h

Table 4 Content of essential amino acids in the hydrolysate and in the control sample

Amino acid	Amino acid content, μg/g	
	Pea biomass extract	Hydrolysed pea biomass
Ile+Leu	515	10479
Met	110	1320
Phe	514	7681
Val	441	3921

7 h, there remained an insignificant amount of stable polypeptides with a molecular weight of ≈ 20 kDa. These polypeptides were associated with the polypeptide chain of legumin, which is resistant to neutral and alkaline proteases [33].

Thus, an enzymatic hydrolysis that exceeded 4 h is ineffective, since the number of low molecular weight polypeptides did not increase much after that time.

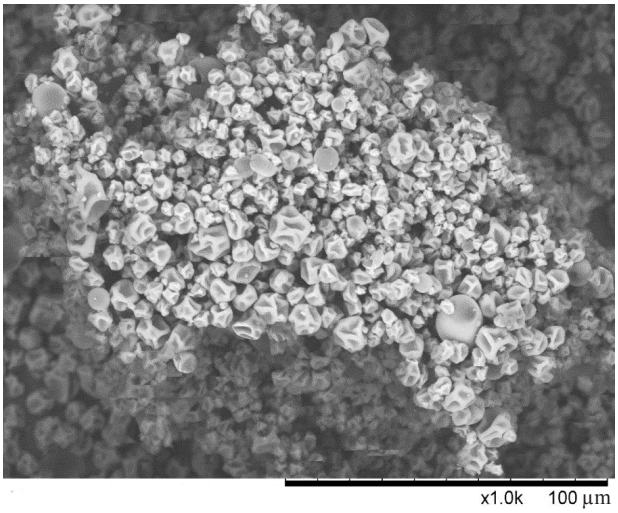

Figure 7 Scanning electron microscopy of the hydrolysate after spray-drying

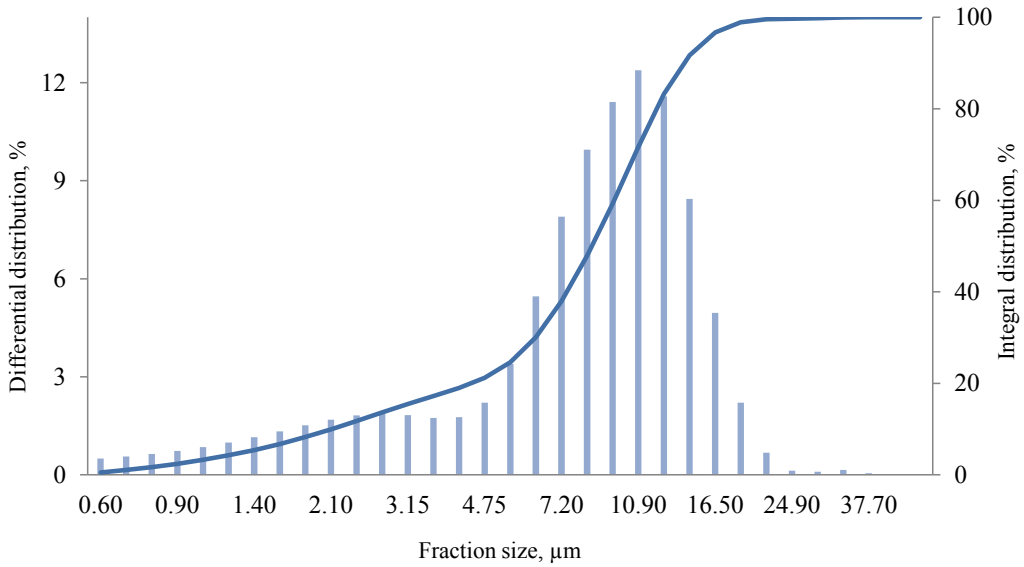

Figure 8 Granulometric composition of the hydrolysate after spray-drying

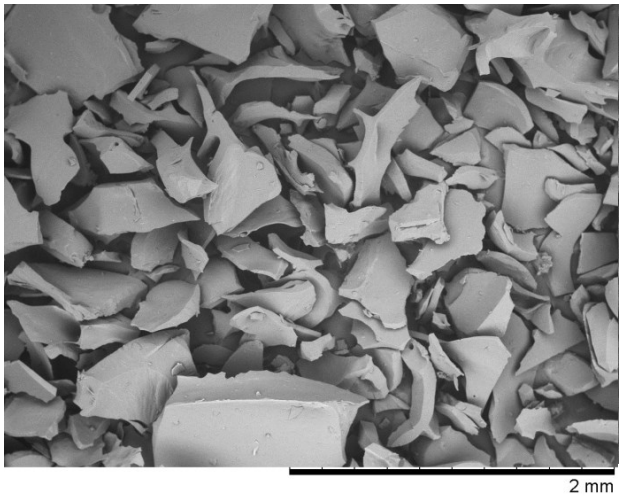

Figure 9 Scanning electron microscopy of the material after vacuum drying

A mass-spectrometric analysis of amino acids was performed to study the low molecular weight products of the enzymatic hydrolysis. Table 4 shows that the hydrolysis resulted in a significant increase in the number of essential amino acids in comparison with the control sample obtained without enzymes.

For the hydrolysates to be widely implemented, there have to be new ready-made food products with prolonged shelf life. Thus, a set of experiments on spray-drying had to be performed [37]. The spray-drying process can be easily scaled and is widely used in food industry to produce dry enzymes, foodstuffs, and unstable compounds [38–40].

The product obtained by spray drying (Figs. 7 and 8) had a monomodal particle size distribution. The main share belonged to spherical particles with a diameter of 5–20 μм. The size was associated with the characteristics of the equipment: the nozzle opening was 25 μм in diameter.

Most of the particles were concave, which made it possible to describe the mechanism of drying. Initially, a powerful inward-directed deformation removed the solvent from the surface of the drop. As a result, there formed a layer of the product. The solvent diffused the layer of the dry product, after which the particle deformed and collapsed.

In the control experiment, vacuum drying without splashing the hydrolysate resulted in the formation of a layer that was not dispersed into individual particles. An electron scanning microscopy of the ground product (Fig. 9) showed that it had a dense structure without pores. This confirms the spray-drying mechanism: the drying occurs on the surface, while the dry layer captures the solvent, and a high mechanical tension deforms the particle, giving it a concave shape.

CONCLUSION

Thus, the paper featured the process of mechanical activation and subsequent enzymatic hydrolysis of pea proteins. The original pea biomass was described using modern chemical methods. The protein content was 24.3%, and MWD was 5–135 kDa.

The fractionation produced four fractions of biopolymers with various contents of protein and carbohydrate molecules. The experiment made it possible to define the optimal conditions for the mechanical activation performed together with proteolytic enzymes. The enzymes were obtained from the complex enzyme preparation Protosubtilin G3x. When both the substrate and the enzymes were mechanically activated, it produced mechanocomposite. As a result, the specific surface area increased by 3.2 times, while the crystallinity decreased by 2 times, which raised the yield of the subsequent enzymatic hydrolysis from 18% to 61%.

During hydrolysis, protein broke down within 2 h, and there was almost no change after 4 h. The experiment detected non-hydrolysed protein molecules with a molecular weight of ≈ 20 kDa. They presumably corresponded with legumin, which is resistant to neutral and alkaline proteases.

The research involved an experiment on spray-drying of the obtained hydrolysates for their potential use as food components. The resulting product had a monomodal particle size distribution. The particles had a spherical shape with a diameter of 5–20 μ.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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Ultrasonic and microwave activation of raspberry extract: antioxidant and anti-carcinogenic properties

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Abstract: Safe and healthy nutrition has a beneficial effect on human well-being. Various foods, such as berries, are known to inhibit cancer-promoting pre-proliferative signals. Among European fruit and berry crops, raspberries demonstrate one with the widest ranges of biologically active substances. Extraction remains a reliable method of obtaining biologically active substances from plant materials. The research objective was to obtain a semi-finished raspberry product by using microwave and ultrasonic processing and to study its antioxidant, anti-carcinogenic, sensory, physico-chemical, and microbiological properties. The raspberry extracts were obtained by maceration, ultrasound treatment, and microwave processing. After that, the samples underwent a comparative analysis of their antioxidant properties. The ultrasonic method gave the best results. A set of experiments made it possible to define the optimal technological modes for the extraction process: ethanol = 50%, ultrasonic radiation = 35 kHz, temperature = 40 ± 5°C, time = 120 min, water ratio = 1:10. A set of experiments on cell cultures demonstrated that the raspberry extract was able to reduce the expression of the anti-inflammatory *COX-2*, *iNOS,* and *IL-8* genes. Hense, we recommend further studies of the effect of the raspberry extract on the induced expression of *COX-2*, *iNOS*, and *IL-8*. In addition, its anticarcinogenic properties have to be studied *in vivo*.

Keywords: Extraction of plant materials, phenolic substances, PRC-analysis, expression of anti-inflammatory genes, inhibition, ultrasound, microvaves

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INTRODUCTION

In many ways, human well-being is associated with safe and healthy food. Food safety is often understood as the absence of harmful microorganisms and chemicals, including synthetic additives, while its healthiness is often identified with naturalness and freshness [1]. The increasing demand for safe and natural food has become a major problem for food science and industry over the past decades [2]. Despite the use of various traditional and modern antibacterial agents, as well as chemical preservatives, about a third of the population of industrialised countries suffer from foodborne diseases [3]. Meanwhile, more and more consumers demand fresh, ready-to-eat, minimally processed foods that contain neither chemical preservatives nor synthetic additives [4]. For modern foods, it is not enough to be a source of energy: modern food has to be functional, e.g. to possess antioxidant or anti-carcinogenic properties. This trend has triggered multiple studies in the field of food processing, as well as an active search for alternative natural supplements with a wide spectrum of physiological properties [5].

In the process of evolution, plants developed natural mechanisms of defense against microbial infections and other harmful environmental factors. Plants are known to produce antimicrobial peptides, lectins, polyphenols, terpenoids, essential oils, and other biologically active compounds. According to some studies, phenolic substances obtained from berries can act as a new type of food components that can inhibit a wide range of pathogens, e.g. *Salmonella*, *Escherichia*, and *Staphylococcus* [6].

Cancer is a serious social problem in many parts of the world [7]. According to the World Health Organization, cancer is responsible for about 7.6 mln (13%) of the 59 mln annual deaths. By 2030, cancer mortality worldwide is expected to reach 11.8 mln per year. Most cancer deaths are caused by five basic behavioral and eating habits, including a low intake of fruits and vegetables. Bad eating habits, obesity, and sedentary lifestyle have been proven to increase the risk of oncological diseases [8]. Therefore, a healthy diet is one of the most important changes in one's lifestyle that can reduce the risk of cancer [9].

Recent epidemiological and basic studies have demonstrated the anticarcinogenic properties of fruit components. They can inhibit pro-proliferative signals that cause or stimulate the growth of tumors or cancer cells [10].

Raspberries are one of the major fruit and berry crops in Europe [11]. They can be used both fresh and processed [12].

Raspberry polyphenols consist mainly of anthocyanins and tannins [13]. Raspberries are a rich source of cyanidin glycoside. A high content of ellagitannin releases free ellagic acid during hydrolytic processes [14]. These components are responsible for about 60% of the antioxidant potential [15]. According to Landele, raspberries owe their antioxidant, antimicrobial, and anti-inflammatory properties to ellagic acid and ellagotanin [16]. Thus, raspberries and their semi-finished products can prevent many diseases, if included in foods.

Raspberries contain a wide range of phytonutrients with antioxidant, antitumor, anti-neurodegenerative, and anti-inflammatory properties [17, 18]. The following substances are responsible for the hemotherapeutic and prophylactic components: vitamins C and E, folic acid, calcium, selenium, β-carotene, α-carotene, lutein, such polyphenols as ellagic acid, ferulic acid, p-coumaric acid, anthocyanins, quercetin, and kaempferol, and such phytosterols as β-sitosterol and stigmasterol [19].

A raspberry ethanol extract suppresses cell proliferation in squamous cell carcinoma without altering cell viability and inhibits the expression of vascular endothelial growth factor. In addition, it also inhibits nitric oxide synthase activity and indicates apoptosis and terminal differentiation [20]. These data suggest that raspberry extract can be used for chemoprophylaxis in people with oral cavity dysplasia.

Solvent extraction can be used to obtain polyphenols [21]. Unfortunately, it has several drawbacks. For instance, it requires a larger amount of organic solvents and a longer extraction time. Moreover, the solvent can have a negative effect on human health and degrade the target compounds. However, there are several alternative methods that can either eliminate or reduce these disadvantages [22].

There are several factors that affect the efficiency of extraction of biologically active components from plant materials. They include technology, solvent type, time, temperature, material vs. solvent ratio, etc. However, it is technology that remains crucial. The traditional extraction technologies, such as Soxhlet extraction and maceration, require a lot of solvent, time, and energy, but are popular and effective. However, new extraction technologies are being actively introduced, e.g. ultrasonic, microwave, infrared, and fluid supercritical extractions. They are energy saving and environmentally friendly, according to one of the latest books on the extraction of biological active substances from plant and animal raw materials [23]. Still, an optimal extraction technology should be simple, safe, reproducible, inexpensive, and suitable for industrial use [24].

Ultrasonic (US) extraction is a fairly cheap method that requires minimal hardware design [25]. It destroys cell walls (lysis) and disintegrates individual cellular structures and the cell as a whole, which increases the number of components that enter the extract. US produces a mechanical effect: the solvent penetrates into the matrix of berries, thus increasing the area of the contact surface between the solid and the liquid phases [26]. Moreover, US waves can cause some undesirable chemical processes that can change the chemical composition, degrade the target compounds, and cause free radicals in gas bubbles [27]. Therefore, a set of experiments is required to define the optimal extraction conditions, i.e. time, temperature, power, and ultrasonic frequency.

Microwave (MW) radiation is another possible way to increase extraction efficiency [28]. MW radiation is a popular means of extraction, as far as low-molecular compounds from plant raw material are concerned.

The research objective was to obtain a semi-finished raspberry product using MW and US processing, as well as to study its antioxidant, anti-carcinogenic, sensory, physico-chemical, and microbiological properties.

STUDY OBJECTS AND METHODS

The experiments were performed on the premises of the Department of Technology and Catering at the Samara State Technical University (Samara, Russia). The anti-inflammatory and cytostatic, or cytotoxic, properties were determined in the N.N. Blokhin National Medical Research Oncology Center (Moscow, Russia).

The research featured a variety of fresh raspberries (*Rúbus idáeus* L.) harvested in the Samara region (53°12′N – 50°06′E) in 2017. The raspberries were provided by the Research Institute of Horticulture and Medicinal Plants 'Zhigulyovskie Sady' (Samara, Russia).

Determination of the antioxidant properties indicators.

Chemicals and reagents. The experiment used ethanol and distilled water. The Folin-Ciocalteu reagent (FCR) and the gallic acid were provided by the Fluka company (Germany). The DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium nitrite,

aluminum chloride, sodium carbonate, and linoleic acid were ordered from Sigma-Aldrich, Inc. The 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) was purchased from Fluka Chemicals (Spain). Other chemicals included hydrochloric acid, potassium chloride, acetic acid, sodium acetate, sodium phosphate, ferric chloride (II), ferric chloride (III), and ammonium rodanide.

Phenolic compounds. The content of total phenols was estimated using a modified version of the FCR method [29]. Gallic acid was used as a standard: an aqueous solution of gallic acid (200 mg in 1000 cm^3) was diluted with distilled water to obtain the concentrations appropriate for the calibration curve. The experiment involved 0.50 cm^3 of the analysed substance or standard gallic acid, 4.00 cm^3 of distilled water, 0.25 cm^3 of FCR reagent, and 0.25 cm^3 of a saturated aqueous solution of sodium carbonate. The samples were shaken and kept in the dark at room temperature for 30 min. The absorption coefficient was determined at 725 nm with a spectrophotometer. Results were expressed in mg equivalent of gallic acid per 100 g of dry weight. The experiment was performed in triplicate.

Flavonoids. The content of flavanoids was determined using a modified method described Demidova *et al.* [30]. 0.50 cm^3 of the analysed substance or standard catechin solution was put in a 10 cm^3 measuring tube. After that, 2.50 cm^3 of distilled water was added at the time zero followed by 0.15 cm^3 of a 5% aqueous solution of sodium nitrate. After 5 minutes, 0.30 cm^3 of a 10% aqueous solution of aluminum chloride was added and kept for another 5 min. The absorption coefficient was measured at 510 nm. The content of flavonoids was expressed in mg equivalent of catechin per 100 g of dry weight. The experiment was performed in triplicate.

Anthocyanins. To define the total content of anthocyanins, the absorption coefficient was measured at two different pH values (1.0 and 4.5) at 515 and 700 nm [31]. The content of anthocyanins was expressed in mg equivalent of cyanidin-3-glycoside per 100 g of dry matter. The experiment was performed in triplicate.

Antioxidant activity in the linoleic acid system. The antioxidant activity in the linoleic acid system was determined according to the method described Karabegovic [32]. 0.5 cm^3 of ethanol, 0.5 cm^3 of distilled water, 1 cm^3 of linoleic acid, and 2 cm^3 of phosphate buffer (pH 7.0) were added to 1.0 cm^3 of the analysed substance. The mixture was kept at 40°C for 120 h. Then an aliquot part (0.1 cm^3) was isolated from the mixture. After that, 9.7 cm^3 of 75% ethanol and 0.1 cm^3 of a 30% ammonium rhodanide solution were added to the aliquot and allowed to stand for 4 min. Subsequently, 0.1 cm^3 of ferric chloride (II) solution was added to the mixture (0.2 M in 3.5% of HCl). A spectrophotometer was used to measure the optical density of the mixture at 500 nm. The control sample contained all the reagents but the extract. The antioxidant activity was expressed in percent of inhibition of linoleic acid oxidation. The experiment was performed in triplicate.

Antioxidant activity by DPPH. The antioxidant properties of the samples were measured using the method described Cheigh *et al.* [33]. The method is based on the ability of the antioxidants of the raw material to bind the stable chromogen radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH). 4 mg of DPPH was dissolved in 100 cm^3 of ethanol. The aliquots were dissolved in 100 cm^3 of distilled water in the quantities of 0.05, 0.10, 0.40, 0.80, 1.00, and 5.00 cm^3. Then, 0.2 cm^3 of each solution was added to 2.0 cm^3 of the DPPH solution at 20°C and kept in the dark for 30 min. The transmittance was determined at 517 nm. The antiradical activity was expressed as the concentration of the original object in mg/cm^3, at which 50% of the radicals were bound. The experiment was performed in triplicate.

FRAP method. The restoring force of the analysed substance was determined by the FRAP method [34]. A freshly prepared FRAP solution included 10 cm^3 of acetate buffer (pH 3.6), 1 cm^3 of a 10% solution of ferric chloride (III) and 1 cm^3 of TPTZ solution (2,4,6-tripyridyl-s-triazine) (10 mmol/L TPTZ in 40 mmol/1000 cm^3 of HCl). The solution was kept at 37°C for 10 min. After that, 3.0 cm^3 of distilled water and 1 cm^3 of FRAP solution were added to the analysed substance (0.1 cm^3). The mixture was allowed to stand at 37°C for 4 min. The optical density was measured at 593 nm. The restoring force was determined according to the calibration graph and expressed in mmol of Fe^{2+}/1 kg of the raw material. The experiment was performed in triplicate.

The sensory properties of the raspberry extract were defined according to State Standard 8756.1-2017*.

The microbiological studies of the semi-finished product were performed according to State Standards 31659-2012** and State Standard 30712-2001*** in licenced testing laboratory No. ROSS RU.0001.510137.

The physical and chemical properties were determined according to State Standards 34128-2017**** and State Standards 34127-2017*****. The content of ethanol in the raspberry extract was

* State Standard 8756.1-2017. Fruit, vegetable and mushroom products. Methods for determination of organoleptic characteristics, components fraction of total mass and net mass or volume. Mocsow: Standartinform; 2017. 12 p.

** State Standards 31659-2012. Food products. Method for the detection of Salmonella spp. Mocsow: Standartinform; 2014. 21 p.

*** State Standard 30712-2001. Products of non-alcoholic industry. Methods of microbiological analysis. Mocsow: Standartinform; 2010. 11 p.

**** State Standards 34128-2017. Juice products. Refractometric method for the determination of soluble solids mass concentration. Mocsow: Standartinform; 2017. 8 p.

***** State Standards 34127-2017. Juice products. Determination of titratable acidity by method of potentiometric titration. Mocsow: Standartinform; 2017. 8 p.

determined according to State Standard ISO 2448-2013******. The experiments were performed in triplicate.

Statistical data processing. The statistical processing of the results was performed with the help of Student's t-test to determine M ± m, where M is the mean value, m is the standard error of the mean (the standard deviation √n) was defined using the Microsoft Excel software.

Determination of potential anticarcinogenic properties indicators.

Anti-inflammatory drugs are known to produce an inhibitory effect on the pro-inflammatory pathways of cells, including *COX2*, *iNOS*, and *IL-8*. It is currently considered a proven fact that these drugs exert an anticarcinogenic effect *in vivo*. That is why the present study featured these very genes and the effect of raspberry extract on them to determine the potential anticanceragenic activity of the product.

The study used HCT-116 colon cancer cell line [35]. The cells were cultured at 37°C in standard DMEM medium containing 5% fetal calf serum (PAA, Australia) and gentamicin (50 U/cm^3) (PanEko, Russia) and in 5% CO_2.

Cell viability study (MTT-test). The cells were dispersed into 96-well plates (BDMicro-FinePlus, USA). There were 3×10^3 cells in 190 µl of culture medium. After that, the cells were incubated for 24 h. Serial dilutions of raspberries were prepared on the day of the experiment. The cells were incubated with the extract for 72 h at concentrations of 0.03125–2% (v/v). Then 20 µL of the MTT reagent solution were added in the ratio of 5 mg/cm^3 (PanEko, Russia) in Hanks salt solution (PanEko, Russia). The solution was allowed to uncubate at 37°C for 2 h until it turned violet. The formazan was then dissolved in 200 µL of dimethyl sulfoxide (DMSO, PanEko, Russia) and incubated at 37°C. After the formazan crystals had completely dissolved, the optical density of the wells was measured at a wavelength of 570 nm using a MultiScan MCC 340 multiwell spectrophotometer (Labsystems, USA). The data were presented as the optical density of the experimental samples vs. that of the control sample. The optical density in the control sample was taken for 100%. The cells in the control sample were incubated in a 1% ethanol solution.

RNA isolation. The total cellular RNA was isolated using an RNA isolation kit. The RNA concentration was determined with a spectrophotometer according to the optical density of the solution at a wavelength of 260 nm. The absence of impurities in the sample was stated by the ratio of the optical density of the solution at a wavelength of 260 nm and 280 nm.

Reverse transcription reaction. Reverse transcription was used to obtain cDNA. 1 µg of RNA was mixed with 0.4 µg of random hexamer oligonucleotides, denatured at 25°C, and cooled on ice. The reverse transcription mixture included: 2 units of reverse transcriptase MMLV, a suitable buffer, 2 mM of dithiothreitol, 0.5 units of ribonuclease inhibitor, 0.5 mM of dNTP, and ≤ 20 µL of distilled water. The reaction lasted 1 h at 37°C. After that, reverse transcriptase was inactivated at 95°C for 5 min, which stopped the reaction. After adding 80 µL of distilled water, the aliquots were used for real-time PCR amplification with specific primers.

Table 1 Primer sequences

Gene	Sequence (forward/reverse), 5'-3'
RPL27	ACC GCT ACC CCC GCA AAG TG
	CCC GTC GGG CCT TGC GTT TA
COX2	CCGGGTACAATCGCACTTAT
	GGCGCTCAGCCATACAG
iNOS	CGGCCATCACCGTGTTCCCC
	TGCAGTCGAGTGGTGGTCCA
IL-8	TCCTGATTTCTGCAGCTCTGTG
	TCCAGACAGAGCTCTCTTCCAT

Quantitative real-time PCR analysis. After the reverse transcription reaction, the samples were diluted 1:10 with sterile deionised water to obtain working dilutions of cDNA. 5 µL of the cDNA working solution was added to 20 µL of the reaction mixture that contained SYBR Green Master Mix, 500 nM of the reverse primers and 500 nM of direct primers. A Bio-Rad iQ5 PCR analyser was used to perform a real-time quantitative PCR analysis. The amplification programme was as follows: 95°C – 10 min, 40 cycles (95°C – 15 s, 60°C – 30 s, 72°C – 30 s). The relative change in the expression of the mRNA was calculated using the ΔΔCt method. The ΔΔCt was determined by subtracting the average ΔCt of the control sample from the ΔCt of the experimental samples [36]. For each gene, a PCR analysis was performed in triplicate, and the melting curves were obtained for each primer pair to confirm their specificity. To analyse the melting curves, the temperature was raised from 55°C to 95°C at a pace of 0.5°C. The ribosomal protein gene L27 (*Rpl27*) was used for control.

The primers for cDNA amplification were designed using the Primer-Bank database and the Oligo 6 software [37]. Table 1 shows the primer sequences.

Statistical data processing. Statistical processing of the results performed with the help of Student's t-test to determine M ± m, where M is the mean value, m is the standard error of the mean (standard deviation √n) was defined using the Microsoft Excel software.

RESULTS AND DISCUSSION

The research compares the antioxidant properties of raspberry extracts obtained by maceration, ultrasonic treatment, and microwave processing. All the extracts were obtained using 50% ethanol, while the raw material vs. solvent ratio was 1:10 (w/v).

****** State Standard ISO 2448-2013. Fruit and vegetable products. Determination of ethanol content. Mocsow: Standartinform; 2014. 11 p.

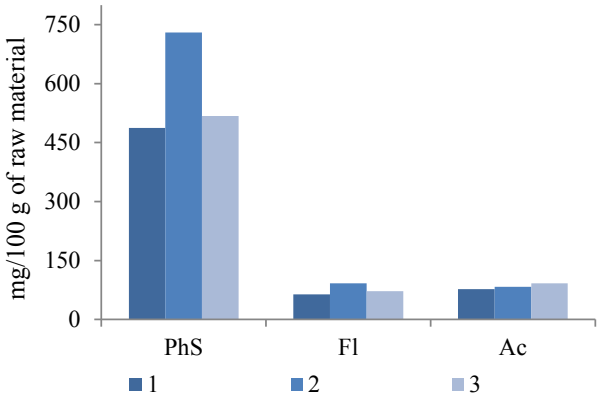

Figure 1 Total content of antioxidants in the raspberry extracts: PhS – total content of phenolic substances, mg of gallic acid/100 g of raw material; Fl – total content of flavonoids, mg of catechin/100 g of raw material; Ac – total content of anthocyanins, mg of cyanidin-3-glycoside/100 g of raw material). (1) maceration, (2) US extraction, (3) MW extraction

The maceration extract was obtained by storing the raw material and the solvent at 40°C for 120 min.

The US extract was obtained using an Elmasonic S 15H device at a frequency of 50 kHz at 40°C for 120 min.

The MW extract was obtained by using microwave irradiation with a irradiation rate of 90 W for 1 min.

Figure 1 shows the total content of phenols, flavonoids, and anthocyanins.

US radiation resulted in the biggest content of phenolic substances: it increased by 1.50 times as compared with classical maceration. MW radiation produced nothing but minor changes: the content of total phenolic substances in the extract increased by 1.06 times.

The US and MW processing also increased the extraction of flavonoids by 1.44 and 1.13 times, respectively.

All the methods showed nearly the same content of anthocyanins in the extracts.

Table 2 Antioxidant properties of the raspberry extracts

Index	Maceration	US extraction	MW extraction
Restoring force according to the FRAP method, mmol Fe^{2+}/1 kg of raw material	7.92	10.08	9.09
Antiradical activity by the DPPH method, E_{C50}, mg/cm^3	10.1	31.5	28.0
Antioxidant activity in the system of linoleic acid, % of inhibition	16.6	57.5	54.1

Thus, both US and MW methods increased the content of biologically active substances in the raspberry extracts. US extraction proved to have the greatest impact on the content of phenolic substances and flavonoids, while the content of anthocyanins remained almost the same in different types of extraction.

Table 2 demonstrates the antioxidant properties of the raspberry extracts.

The inhibitory effect of DPPH free radicals increased by 1.15 and 1.27 during MW and US extractions, respectively.

The restoring force of the US extract increased as compared with MW and maceration extracts.

In addition, US extraction increased the ability of the raspberry extract to inhibit linoleic acid by 3.46 times.

Similarly, additional treatment with US or MW radiation increased the antioxidant properties of the semi-finished products, if compared with classical maceration.

Thus, US processing is necessary to obtain a raspberry extract with a high content of physiologically active substances and high antioxidant properties.

The study introduces a optimal conditions for raspberry extract production. The new technological scheme is given in Fig. 2.

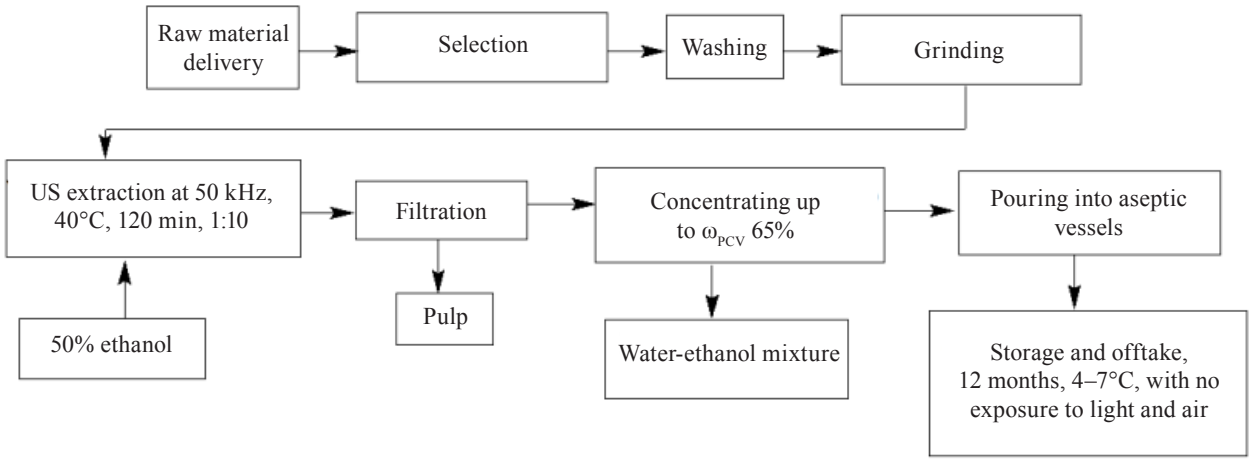

Figure 2 Procedure chart for raspberry extract production

Table 3 Properties of the raspberry extract

Indicators		Raspberry extract
Sensory properties	Appearance	Transparent liquid without residue
	Taste and aroma	Bitter-sweet, like raspberry juice
	Colour	Bright raspberry
Physical and chemical indicators	Soluble solids, %	65.0 ± 0.1
	Titratable acidity, % (expressed as malic acid)	5.50 ± 0.02
	Mass fraction of ethanol, %	< 1.0
Antioxidant properties	Total content of phenolic substances, mg of gallic acid/100 g of starting material	654.0 ± 25
	Total content of flavonoids, mg catechin/100 g of starting material	194.0 ± 13
	Total content of anthocyanins, mg cyanidin-3-glycoside/100 g of dry matter	50.81 ± 2.14
	Antiradical activity according to the DPPH method, E_{C50}, mg/cm^3	2.02 ± 0.01
	Restoring force according to the FRAP method, mmol Fe^{2+}/1 kg of starting material	22.31 ± 0.04
	Antioxidant activity in the smooth system of linoleic acid, % of berry inhibition	68.35 ± 0.07
Microbial attributes	Total visible count, CFU/g	Not detected
	Coliforms, CFU/g	Not detected
	Yeast and mould, CFU/g	Not detected

The experimental data made it possible to define the best technological modes: ethanol = 50%, US radiation frequency = 35 kHz, temperature = 40 ± 5°C, time = 120 min, raw materials vs. solvent ratio = 1:10. A circulation vacuum evaporator concentrated the extract until the content of soluble solids was 65% and the mass fraction of ethanol was ≤ 1.0%.

The extract was then analysed by sensory, microbiological, physicochemical, and antioxidant properties (Table 3).

Chronic inflammation is one of the main etiological factors that trigger certain types of cancer. As a result, some anti-inflammatory drugs, e.g. ibuprofen, have an anti-carcinogenic effect on colon cancer.

The main objective of this research was to study the anti-inflammatory properties of the raspberry extract. A set of experiments was conducted to study its effect on the expression of the genes of individual components of the anti-inflammatory pathway. A colon cancer cell line was studied by the RT-PCR method to measure the effect of non-toxic extract doses on the expression of the following genes: cyclooxygenase 2 (*COX-2*), induced NO synthase (*iNOS*) and interleukin 8 (*IL-8*) [38]. The anti-inflammatory effect of the raspberry extract indicates its potential anticarcinogenic activity.

The functional activity of the *COX-2* gene is directly related to inflammation. This gene is expressed by macrophages, synoviocytes, fibroblasts, smooth vascular muscles, chondrocytes, and endothelial cells after they have been induced with cytokines or growth factors. *COX-2*-induced prostaglandins – directly or indirectly – enhance the production of the enzyme according to the positive feedback mechanism [39]. Inhibition of *COX-2* is considered as one of the main mechanisms of the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (NSAIDs). Selective inhibition of this cyclooxygenase can minimise various side effects observed during the inhibition of cyclooxygenase 1. *COX-2* plays an important role in the development of inflammatory processes and carcinogenesis in the gastrointestinal tract. An increased *COX-2* expression was observed in 85% of gastrointestinal tumours, which also correlated with low survival. Animal studies showed that deleting *COX-2* or treating animals with selective *COX-2* inhibitors reduced the number, size, and multiplicity of tumours. *COX-2* causes tumour progression as it induces the expression of anti-apoptotic proteins of the Bcl-2 family, which leads to apoptosis resistance in the future [37].

IL-8 is known as a T-cell chemotactic factor and a neurophil activating factor (NAF) [40, 41]. It belongs to the group of chemokines, which provide chemotaxis in the area of inflammation of neutrophils, monocytes, eosinophils, and T-cells. *IL-8* possesses pronounced pro-inflammatory properties. It causes the expression of intercellular adhesion molecules and enhances neutrophil adherence to endothelial cells and subendothelial matrix proteins. Hence, it is an important mediator of inflammatory response [42]. *IL-8* is produced by macrophages, lymphocytes, epithelial cells, fibroblasts, and epidermal cells. *IL-8* also regulates pro-inflammatory angiogenesis. This cytokine enhances the expression of vascular endothelial growth factor A (VEGF-A) by endothelial cells and increases the expression of vascular growth receptor 2 (VEGFR2) [43].

iNOS expression is regulated by pro-inflammatory cytokines (tumour necrosis factor-alpha (*TNF-α*), interleukin-1β (*IL-1β*), interferon-γ (*IFN-γ*), hypoxia, oxidative stress, and, according to recent studies, by *Hsp70* heat shock protein. Inhibition of *iNOS* results from the suppression of the pro-inflammatory and proliferative pathways NF-κB and JAK-STAT [44].

The expression of these genes can denote the presence or absence of the anti-inflammatory effect of the extracts on colon cells. This research did not study the anticarcinogenic properties of the extract

Table 4 Effect of raspberry extract on cell viability of the supercritical *HCT-116*

Cell viability	IC50	IC30	IC10
Volume concentration, %	0.25 ± 0.05	0.165 ± 0.01	0.09 ± 0.03

components; however, its results may indicate the feasibility of *in vivo* experiments to determine the anticarcinogenic properties of the raspberry extract.

We performed an MTT test to define the cytotoxicity of the raspberry extract. A wide range of concentrations (0.03125–2%, v/v) showed that the raspberry extract has a cytotoxic effect on colon cancer cells *HCT-116* (Table 4).

Next, a non-toxic concentration of the raspberry extract was used to define the working concentration. It was used to study the effect of the extract on the expression level of *COX-2*, *iNOS*, and *IL-8*. Working concentrations used were 0.0625 and 0.03125% (v/v).

A PCR analysis of *COX-2* expression was performed after colon cancer cells of the HCT-116 line had undergone a proper treatment. The analysis showed that the raspberry extract had an inhibitory effect on

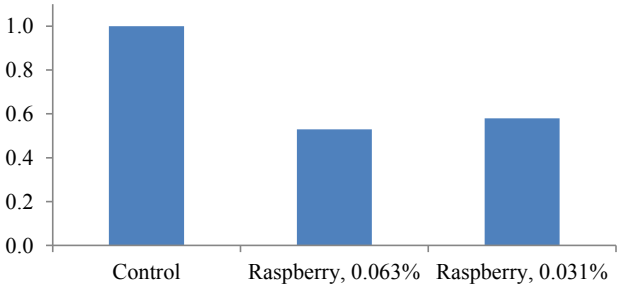

Figure 3 Effect of the raspberry extract on *COX-2* expression. The quantitative PCR analysis of COX-2 expression was performed after *HCT-116* cells had been incubated for 24 h at working concentrations (0.063% and 0.031%). The number of PCR products was assessed and normalised according to the amount of the PCR product of the *Rpl27* gene

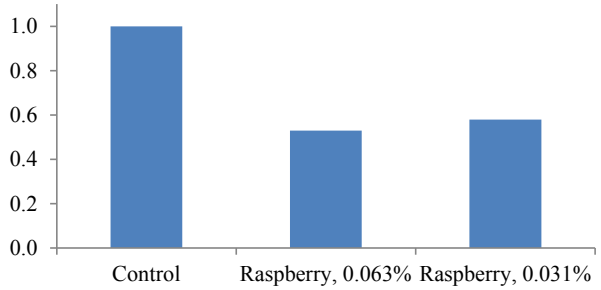

Figure 4 Effect of the raspberry extract on *iNOS* expression. The quantitative PCR analysis of *iNOS* expression was performed after *HCT-116* cells had been incubated for 24 hours at working concentrations (0.063% and 0.031%). The number of PCR products was assessed and normalized according to the amount of the PCR product of the *Rpl27* gene

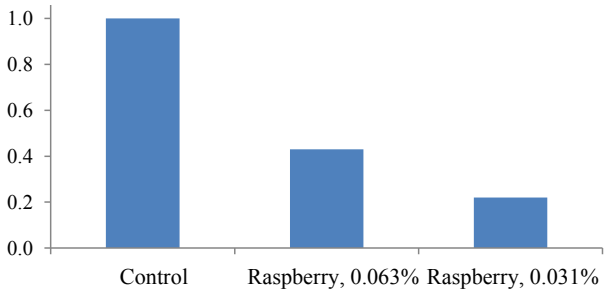

Figure 5 Effect of the raspberry extract on *IL-8* expression. The quantitative PCR analysis of *IL-8* expression was performed after *HCT-116* cells had been incubated for 24 hours at working concentrations (0.063% and 0.031%). The number of PCR products was assessed and normalized according to the amount of the PCR product of the *Rpl27* gene

the expression of this gene. The effect of the extract on *COX-2* expression depended on the dose. Figure 3 shows that when the concentration of the extract was 0.063%, *COX-2* expression fell down to 43%, i.e. by 2.3 times. When the concentration of the extract was 0.031%, it fell down to 22%, i.e. by 4.5 times.

Figure 4 shows some dependencies revealed by the analysis of *iNOS* expression. When treating the cells with the raspberry extract, both concentrations resulted in a decrease in *iNOS* expression by almost 2 times: 47% and 42% for concentrations of 0.063% and 0.031 %, respectively.

The PCR analysis showed that the raspberry extract also inhibited *IL-8* expression. When *HCT-116* cells were treated with the raspberry extract at the concentration of 0.063%, it inhibited *IL-8* expression by 54%, while the concentration of 0.031% inhibited *IL-8* expression by 42%. Figure 5 shows the effect of the raspberry extract on *IL-8* expression.

CONCLUSION

The research results made it possible to draw the following conclusions:

(1) US or MW treatment improved the extraction process and increased the content of biologically active cells and their antioxidant properties. US extraction had a greater impact on the content of phenolic substances and flavonoids, whereas the content of anthocyanins remained almost the same after different types of extraction.

(2) The experimental data made it possible to define the optimal technological parameters: ethanol = 50%, US radiation = 35 kHz, temperature = 40 ± 5°C, time = 120 min, raw materials vs. solvent ratio = 1:10.

(3) The study defined the sensory, physical, and chemical quality and safety indicators for raspberry extracts, which did not contradict with the national regulatory documentation.

(4) The raspberry extract was found able to reduce the expression of pro-inflammatory *COX-2*, *iNOS*, and *IL-8* genes. This semi-finished product can be

recommended for further studies of the effect it has on induced *COX-2*, *iNOS,* and *IL-8* expression, as well as for *in vivo* studies of its anticarcinogenic activity.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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IR-spectroscopy of polysaccharide flaxseed (*Linum usitatissimum* L.) products

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Abstract: Flax seeds are an excellent source of polyunsaturated fatty acids and high-grade protein. They are also rich in non-starch polysaccharides that are concentrated in their mucus cells. Flaxseed polysaccharides are soluble dietary fibres, which makes them an indispensable functional food ingredient. They can also serve as an additive, thus improving the structure of food, e.g. as a stabiliser, structure former, water and fat retention agent, etc. According to various researches, the functional and technological properties of polysaccharide flaxseed products are largely determined by the ratio of polysaccharide fractions and protein content, which depend on the production process. This research featured the effect of the method of obtaining flaxseed polysaccharide products on the protein content. The study employed chemical analysis and attenuated total internal reflection infrared spectroscopy (ATR-IR). The protein polysaccharide products under analysis were obtained by water extraction from two varieties of whole flax seed (Russia), under various conditions of treatment, cleaning, and fractionation. The conditions included pH, temperature, and process time. During water extraction of whole flax seeds, polypeptide-containing polysaccharide complexes were removed from the seed coats. The number, composition, and binding force between the peptide fragments and the polysaccharide matrix depended on the technological parameters of the process. The polysaccharide products were tested for total protein content. The results were consistent with the band intensity in the range of 1700–1500 cm^{-1}, where protein carbonyl groups are usually manifested.

Keywords: Flax seeds, polysaccharides, proteins, extraction, IR-spectra of polysaccharide products

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INTRODUCTION

Polysaccharides have remained in the centre of scientific attention for several decades. Previously, polysaccharides were used mainly as auxiliary substances in the pharmaceutical industry and were considered as biologically active substances. However, they are currently used as functional food ingredients and technological additives in many areas of food industry.

Polysaccharides are highly beneficial for human organism. According to various researches, polysaccharides can produce pronounced antihypoxic, expectorant, anti-inflammatory, immunotropic, enterosorbing, hepatoprotective, hypolipidymic, antitumour, and general tonic effects [1, 2].

Polysaccharides ensure the quality and texture of food products, e.g. hardness, brittleness, density, thickening, viscosity, stickiness, gel-forming ability, etc. Many food products owe their soft, fragile, swollen, or jelly-like structure to polysaccharides. Their impressive variety of functions can be explained by the structural properties of individual polysaccharides used as food additives. They act as a gelling, thickening, filling, emulsifying, swelling, or foaming agent. They can prevent crystallization and syneresis. They increase

both viscosity and the biological and nutritional value of the product. No other food additive can perform such a variety of functions.

Their structural, physicochemical, functional, and technological properties, as well as their effect on biological activity, are studied by such modern methods as nuclear magnetic resonance (NMR), infrared spectroscopy, ultraviolet imaging, etc. [3, 4].

Flax seeds are known not only as a source of polyunsaturated fatty acids and high-grade protein, but also as a source of non-starch polysaccharides that are concentrated in their mucus cells. Non-starch polysaccharides represent a group of low-digestible carbohydrates, or dietary fibre [5]. The polysaccharides of flaxseed mucus are highly soluble in water. They also reduce the glycaemic index and cholesterol. In addition, they possess a prebiotic effect [6, 7].

The polysaccharides of flaxseed mucus are a mixture of two fractions – neutral (75%) and acidic (25%) [8–10]. The neutral fraction has a high molecular weight and contains arabinoxylan, while the acidic fraction contains ramnegalacturonan. Flaxseed polysaccharide complex contains 4–20% of proteins, depending on the flax genotype and extraction conditions [10]. Proteins are bound with acidic fraction polymers by non-covalent bonds, while no protein has been found in the neutral fraction.

Flaxseed polysaccharides are a source of soluble dietary fibre, which is an indispensable functional food ingredient. They are also a technological additive that regulates the structure of food mass and can act as a stabiliser, structure former, water and fat-holding agent, etc. The functional properties that affect food systems may result from the synergistic effect they produce together with proteins [11]. According to Qian *et al*., an acidic fraction with 8% of protein showed higher emulsion properties as compared with the neutral fraction [12]. Functional and technological properties of flaxseed polysaccharide products are also determined by the ratio of fractions, which depends on the conditions of the technological process. According to Kaushik *et al*., the ratio of neutral and acidic fractions fell from 6.7 to 5.7 when the extraction temperature reached 90°C, while water absorption capacity and emulsion activity decreased [13].

Flaxseed polysaccharides have good prospects as a multifunctional food ingredient. However, there are not enough data on their component composition, functional and technological properties, and production conditions. Flax seeds of Russian varieties remain especially understudied.

The research objective was to use IR-spectroscopy to study the effect of the method of obtaining flaxseed polysaccharide products on the protein content.

STUDY OBJECTS AND METHODS

The comparative study featured polysaccharide products extracted from two Russian varieties of whole flax seeds. The first variety was industrial and corresponded with State Standard 10582-76*. The second variety was of LM-98 brand. All the products differed in extraction conditions: pH, temperature, time, and extraction rate. The technological operations included the following areas:

– neutral extraction at pH 6–7;
– acidic extraction at pH 4–5;
– fractionation of the polysaccharide complex obtained in the neutral medium;
– combined sequential extraction.

Distilled water was used to extract polysaccharides from flax seeds by constant stirring. The ratio of seed mass and solvent volume (hydromodule) was 1:20. The extract obtained was separated from the flax seeds using a 4-layer gauze filter. After that, the target product was obtained in two ways:

(1) The extract was dried in a thin layer of ≤ 0.5 cm at 60–65°C and then crushed to obtain a dry extract of polysaccharides;

(2) Polysaccharides were precipitated in a 3-fold excess of ethanol. The residue was pressed, washed with acetone, and dried in a drying cabinet at ≤ 50°C to obtain a purified polysaccharide complex.

Thus, the main study objects were the dry extract of polysaccharides (PS-extract) and a purified polysaccharide complex (PS-complex). Protein content was determined according to State Standard 13496.4-93**.

A Tensor 37 Fourier spectrometer (Bruker, Germany) was used to define the IR-spectra of ATR-IR on the surface of the dry samples. The device had an ATR diamond element. The OPUS software package had standard calibration capabilities in the frequency range of 4000–500 cm^{-1} (32 scans) in absorption format.

RESULTS AND DISCUSSION

Spectra language. Natural polysaccharides, as a rule, are not pure substances but multicomponent and diverse complexes. They may include protein and lipid components or their fragments. These fragments can be identified by vibrational spectroscopy. To give a clearer interpretation, Fig.1 illustrates the IR-spectra of three main classes of biological substances: proteins, lipids, and carbohydrates. They were obtained from dry samples of polysaccharide obtained from flax seeds, egg albumin, and linseed oil. In the spectrum of egg albumin, proteins appeared as a pair of typical bands in the range of 1680–1540 cm^{-1}. They were caused by the vibrations of the carbonyl C=O groups of Amide I and Amide II [14, 15]. The spectrum of flaxseed oil is typical of plant and animal lipids. It is clearly different from the spectrum of other substances. The narrow band at

* State Standard 10582-76. Oil flax-seed. Industrial raw material. Specifications. Moscow: Standartinform; 2010. 4 p.

** State Standard 13496.4-93. Fodder, mixed fodder and animal feed raw stuff. Methods of nitrogen and crude protein determination. Moscow: Standartinform; 2011. 15 p.

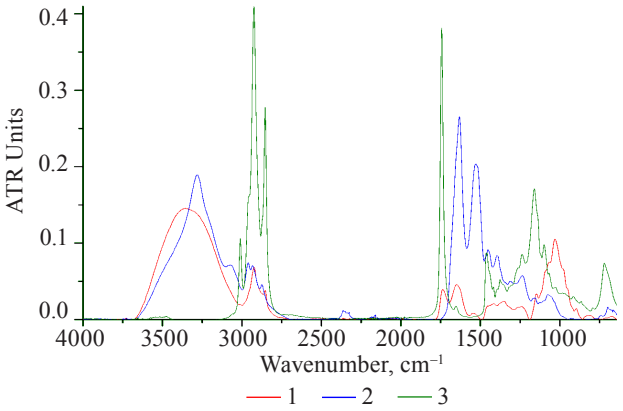

Figure 1 IR-spectra of different classes of substances: (1) dry flaxseed polysaccharide (2.4% protein), (2) dry egg albumin, (3) flaxseed oil

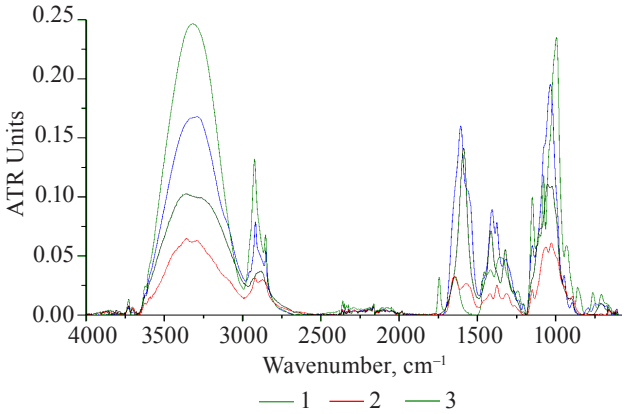

Figure 2 IR-spectra of dry samples of plant and animal polysaccharides: (1) hyaluronic acid, (2) insoluble starch, (3) fibrous cellulose, (4) chitosan

1743 cm^{-1} was caused by the vibrations of C=O groups of fatty acids in triglycerides. The trident-shaped band with a peak at 1160 cm^{-1} was due to the vibrations of C–O and C–O–C bonds of carboxylic acids.

A broad structured band with a peak at 1030 cm^{-1} is typical of polysaccharides. It partially overlapped the lipid band (1160 cm^{-1}). The spectra of all three samples contained bands in the range of 3010–2800 cm^{-1}, which reflected the vibrations of CH_n functional groups that appeared in all classes of substances. Lipids with unsaturated fatty acids in their composition were marked with stretching and bending bands of CH functional group with a CH double bond (3008 cm^{-1} and 722 cm^{-1}, respectively). Vibrations of OH groups often overlap in the range of 3400–3220cm^{-1}. These are bound water molecules and NH_n groups, which quite clearly manifest themselves in the albumin spectrum as bands of different intensity with peaks at 3250 and 3020 cm^{-1}.

The optical characteristics of various polysaccharides showed some remarkable results, if compared. Figure 2 demonstrates the spectra of dry preparations of plant and animal polysaccharides. According to the structure of all bands, animal polysaccharides differed from plant polysaccharides. However, they had a common spectral absorption region, although there can be no absolute coincidence of bands on the wave-number scale.

Hyaluronic acid is the main carbohydrate component of the mucopolysaccharides in animal connective tissue [16, 17]. In the area of protein structures (1700–1500 cm^{-1}), its spectrum showed an intense band with a shoulder on the right branch. This was due to the hyaluronic acid dimer, which had carbonyls (C=O) in the COOH group and the CONH group, a peptide bond analogue. The mucopolysaccharide spectrum showed an intense structured carbohydrate band with a peak at 1031 cm^{-1}, which was typical of plant polysaccharides.

Chitosan is obtained from chitin and is a precursor of a number of glycosaminoglycans. The chitin deacetylation reaction is incomplete [16, 17]. As a result, it contained up to 30% of residual acetyl groups bound to the amine CH_3CONH. CONH-composition is typical of hyaluronic acid. It was the presence of the CONH-composition in the structure of chitosan that explained a narrow intense band with a peak at 1640 cm^{-1} in its spectrum.

Starch and cellulose showed one and two weak bands in this region, respectively. They might result from both the natural properties of the samples and the technological features of the production process. Thus, the granules of insoluble starch are enclosed in a thin protein shell, which is partially damaged during grinding, washing, and drying. Its manifestation can be seen in the starch spectrum. To obtain soluble starch, the protein shell of starch granules is destroyed by acid treatment. Currently, starch can be obtained synthetically. This starch is identical to the natural product, except that it lacks the granular structure typical of natural plant products. Cellulose production is much more complicated and includes a greater number of chemical treatment stages.

All linear polymers, i.e. hyaluronic acid, chitosan, and cellulose, have bands with adjacent peaks, according to the analysis of the structure of the carbohydrate band in the polysaccharide spectra. Chitosan and cellulose are structural analogues [18]. The intensity of the bands with double peaks in their spectra is significantly lower than that of mucopolysaccharide. Starch is a mixture of branched and helicoid macromolecules of two polysaccharides: amylopectin and amylase. Starch is clearly separated from the group. The peak of its broad band is bathochromically shifted by 40 cm^{-1} from the peak of linear polysaccharides.

1. Products of primary and secondary flaxseed extraction in neutral medium (pH 6–7). In Minevich *et al.*, we described the kinetics of primary extraction of polysaccharides from industrial flax seeds in a neutral medium at 60°C [19]. The time interval was 5–120 min, and standard methods of chemical analysis were used as control. However, we would like to point it out again

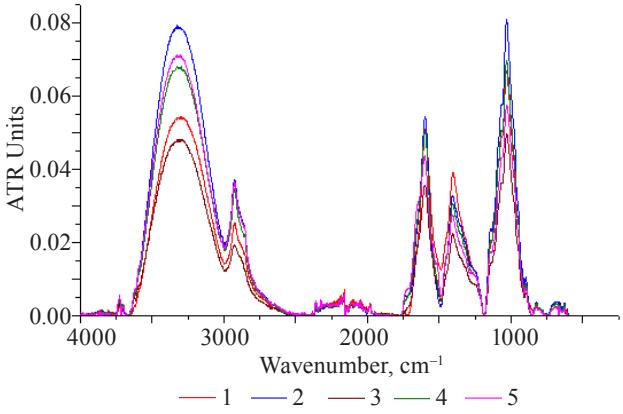

Figure 3 IR-spectra of dry extracts of industrial flax seeds according to extraction time: (1) 5 min, (2) 10 min, (3) 15 min, (4) 20 min, (5) 25 min

that the extraction process proceeds in steps. According to our own results, supported by various scientific data, the process starts with low molecular weight fractions entering the solution and ends with high molecular weight fractions doing the same. All the fractions contained various amounts of protein components.

Figure 3 shows the kinetics of extraction process studied by means of IR-spectroscopy. The intensity of the bands in the range of 1540–1680 cm^{-1} at different extraction times correlated with the total protein content measured by chemical methods. The fractions that entered the solution in the first 10 min presumably contained the maximum amount of protein substances (8.6%). The next peak in the total protein content (17%) could be explained by the fact that it was released directly from the nucleus when the seed coat swelled and partially slipped off.

Protein constituents were present in all extraction products obtained from whole flax seeds, both in the PS-extracts and in the PS-complexes. It was indicated by a broad structured band of 1540–1680 cm^{-1}. The spectra of the obtained extraction products were compared with the egg albumin spectrum (Fig. 1, curve 2). However, the results suggested that water extraction released polysaccharides with polypeptide fragments of different composition and, possibly, size. This assumption was confirmed by the increase in the fragment of this region (Fig. 4a). All the samples had a single band with variously structured peaks in the range of 1595–1605 cm^{-1}.

Only the spectrum of sample 5 (25 min) showed two clear undifferentiated bands in the form of a structured shoulder (≈ 1670 cm^{-1} and 1520 cm^{-1}) on the right and left branches of the global peak. This, in turn, could indicate a different binding force, both of the washed out polysaccharide segments and the protein components. The longer the sample contacted with the solvent, the lower the binding force.

In our previous research, we analysed this fragment of the spectra for the PS-extracts and PS-complexes obtained from industrial and LM-98 flax seeds under identical conditions (60°C, 20 min), as in Fig. 4b [19]. All samples showed structuring of the global band (1590 cm^{-1}) with the same wave numbers in varying degrees. However, it was most pronounced in the products derived from industrial flax seeds (1640 and 1540 cm^{-1}). Moreover, in all the cases under consideration, the structure of the global band was more clearly manifested

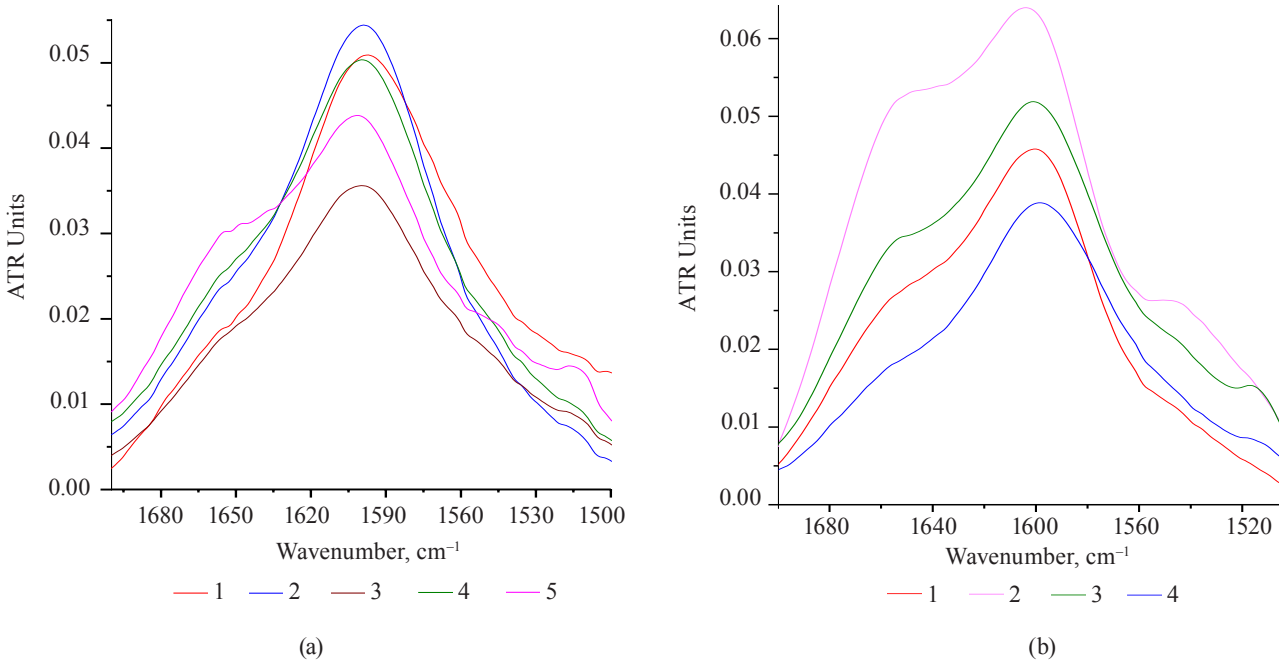

Figure 4 (a) Fragment of the spectra from Fig. 1; (b) (1) PS-complex of LM-98 flax seeds, (2) PS-complex of industrial flax seeds, (3) extract of industrial flax seeds, (4) extract of LM-98 flax seeds

Table 1 Two cycles: primary and secondary extractions from industrial flax seeds

Experiment	Primary extraction conditions	Primary extraction products (PS-complexes)	Secondary extraction conditions	Secondary extraction products (PS-complexes)	
		Protein content, %		Name	Protein content, %
I	90°C, 10 min	8.6	70°C, 60 min, precipitation in excess of ethanol	Sample A	17.0
II	22°C, 60 min	6.3		Sample B	10.2

in the position and shape of the left, high-frequency branch of the band.

In addition, differences in the structure of the bands in these samples suggested a greater variety of chemical bonds between the protein and polysaccharide in the PS-extract obtained from industrial flax seeds. In its spectrum, the right branch of the central peak revealed a clear band in the range of 1540 cm^{-1}. The band was practically absent in the spectrum of PS-extract obtained from LM-98 variety.

An analysis of the full spectra (Fig. 5) showed the following results. Dry PS-extracts obtained in the primary extraction cycle (curves 1 and 2) had similar spectra of polysaccharide bands. However, the spectrum of the PS-extract obtained from industrial flax seeds was higher throughout the entire wave range. It may indicate a higher degree of extractability of both the polysaccharide and protein components from the raw material of this variety.

Curves 1 and 3 characterized the spectra of primary and secondary PS-extracts from industrial flax seeds. Their comparison showed that they had almost identical structure of the bands: the highest peak corresponded to the position of the left shoulder, while the second, less intense peak corresponded to the position of the right shoulder. The central maximum in the spectrum of the primary PS-extract corresponded to a small right-sided shoulder in the spectrum of the secondary PS-extract. This suggested a redistribution of the contribution of fractions of different forms of polypeptide structures with a polysaccharide base. Thus, an increase in extraction cycles could trigger significant changes in the structure of the entire protein-carbohydrate complex.

PS-complexes of secondary extraction were obtained to study the possibility of an additional extraction cycle that could follow the separation of primary extracts and the re-treatment of the oil cake. Table 1 features the conditions and results of both extraction cycles. However, unlike the secondary cycle, the primary extraction had different technological parameters.

Figure 6 shows a comparative study of the spectral parameters of polysaccharide products of secondary extraction. The position of the peaks, as well as the shape and pattern of the bands show that these parameters are almost identical qualitatively. The main difference was in the intensity of the bands, which was much higher in the spectrum of sample A. This was also true for the bands of protein structures, whose intensity ratio corresponds to the results of the chemical analysis. Table 1 proves that the extraction efficiency of PS-complexes with a high content of the protein in sample A was due to the state of the oil cake after the initial treatment, i.e. temperature and time of the primary extraction.

However, both samples revealed three distinct peaks in the structure of the protein band. This experimental fact may indicate the possibility of at least three ways polypeptide fragments can be bound with the polysaccharide matrix, depending on the technological conditions of the process.

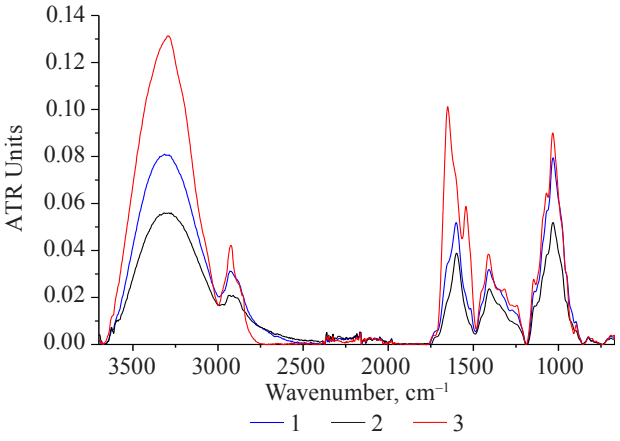

Figure 5 IR-spectra of extractions from two varieties of flax seeds: (1) primary PS-extract of industrial seeds, (2) primary PS-extract of LM-98varieties, (3) secondary PS-extract of industrial flax seeds

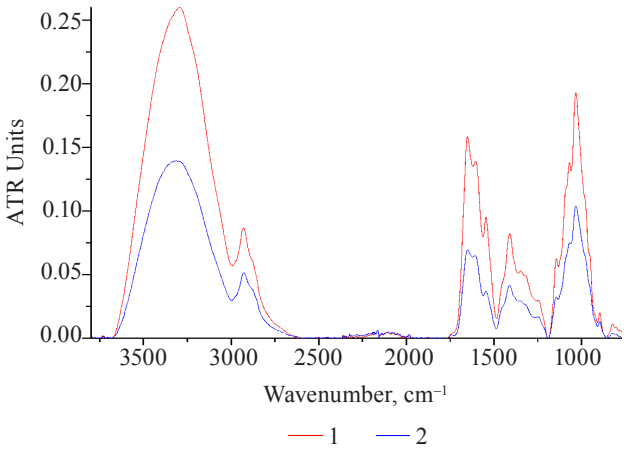

Figure 6 IR-spectra of PS-complexes of the secondary extraction of industrial flax seeds in a neutral medium: (1) 17.0, (2) 10.2% of protein

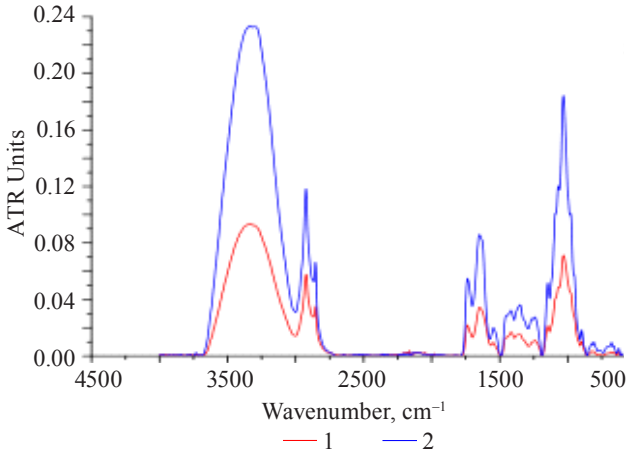

Figure 7 IR-spectra of PS-complexes of industrial flax seeds in an acidic medium: (1) fibrous, (2) amorphous

2. Flaxseed extraction in an acidic medium (pH 4–5). The extraction was carried out at 50°C for 30 min. When re-precipitated with an excess of ethanol, two products were obtained: fibrous and amorphous. A chemical analysis showed that they had the same content of total protein: 2.60–2.63%. The IR-spectra of these samples (Fig. 7) showed that the amorphous sample had a much higher intensity of the bands. The difference is associated with the features of the less dense, amorphous structure, which results from the formation of lower molecular weight segments due to partial hydrolysis and the destruction of chemical bonds.

The spectra of the samples under consideration revealed three clearly manifested peaks with similar intensity ratio in the region of protein structures. However, the central peak dominated in either case.

3. Fractionation of the PS-complex obtained by water extraction (pH 6–7). Of the two polysaccharide fractions present in the mucus of flax seeds, it is the acid fraction that contains most of the total protein to be released during extraction [12]. The PS-complex was fractionated to study the protein-polysaccharide interactions. Table 2 shows the processing steps.

Sample D was extracted from the PS-complex (sample C) in an acidic medium (pH-5). After the extraction, the residue of the PS-complex was dissolved in water, and sample E was precipitated in an excess of ethanol. A purified PS-complex (sample E) was, in fact, obtained as a result of additional purification removal of acid-soluble substances. Its protein content was 1.49%.

Table 2 Fractionation of the PS-complex and characteristics of the products obtained

Processing stages of the PS-complex (sample C)	Fractionation products of the PS complex	
	Name	Protein content, %
I 1.1 Extraction in an acidic medium pH-5, 22°C, 60 min 1.2 Precipitation with an excess of ethanol	Sample D	2.36
II 2.1 Water extraction, 50°C, 60 min 2.2 Precipitation with an excess of ethanol	Sample E	1.49

Figure 8 shows the IR spectra of the amorphous sample of the dry extract obtained by acidic extraction, PS-complex obtained by water extraction (sample C, Table 2), and the first fractionation product obtained by acidic extraction (sample D, Table 2). The obtained data showed a decrease in the spectra of the samples as the initial extract was purified. The decrease in the total protein was relatively small: 2.60–2.36%. The pattern of the bands and the position of the peaks changed greatly in the region of absorption of protein structures.

Sample D (Fig. 8) was obtained in an acidic medium, while sample E (Fig. 9) was a PS-complex (1.49% protein) purified from compounds soluble in an acidic medium. However, a comparison of their IR-spectra showed a significant rise in the entire spectrum of sample E. The intensity of the bands increased in the region of the absorption of protein components, in spite of the fact that their content in the sample decreased. The same was true for all the samples of the PS-complexes obtained from various flax seeds. In addition, the spectrum of sample E showed a distinctive single peak in the region of 1640 cm^{-1}.

Thus, the conditions of polysaccharide extraction from flax seeds and the subsequent processing of the products affect not both the content of polypeptide fragments and structural relationships with the polysaccharide matrix.

4. Combined sequential extraction. Extraction conditions have an effect on the protein content in polysaccharide products and protein-polysaccharide interrelations. To study the process, a sequential extraction of polysaccharide products from industrial flax seeds was conducted at different temperature and pH values. Table 3 shows the scheme of the experiment.

Stage I involved an aqueous extraction at room temperature for 60 min. Polysaccharide complex PSC-1

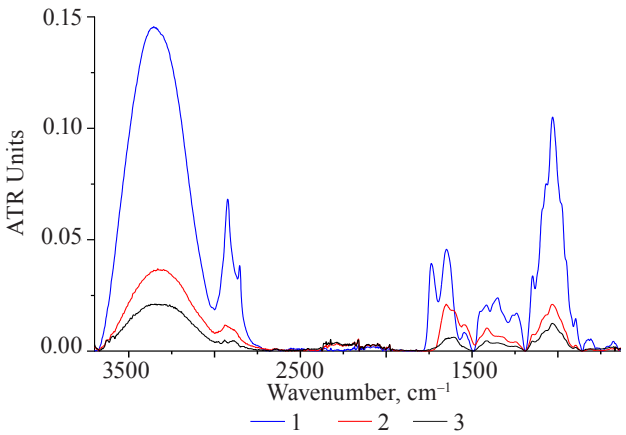

Figure 8 IR-spectra of polysaccharide products: (1) dry amorphous extract, (2) PS-complex, (3) sample D, Table 2

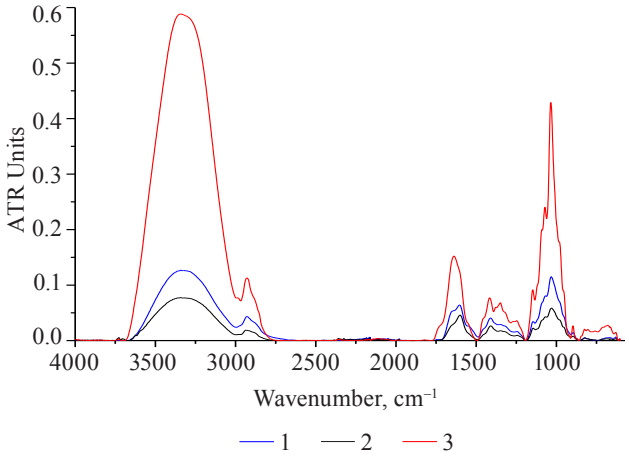

Figure 9 IR-spectra of the PS-complexes: (1) PS-complex of industrial flax seeds, (2) PS-complex of LM-98 seeds, (3) purified PS-complex obtained from sample E, Table 2

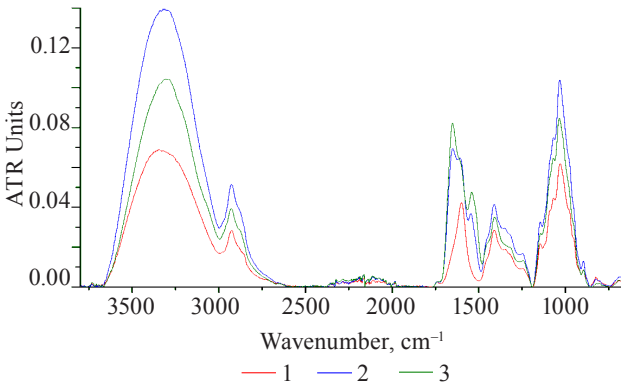

Figure 10 IR-spectra of the PS-complex samples that resulted from sequential extraction of flax seeds: (1) PSC-1 (2.4% of protein), (2) PSC-2 (10.2% of protein), (3) (PSC-3 (25.4% of protein)

was precipitated from the extract with an excess of ethanol During stage II, the seed residue (primary oil cake) was subjected to the second water extraction at 70°C for 60 min. Polysaccharide complex PSC-2 was obtained from the secondary oil cake with an excess of ethanol. The seeds that remained after the second extraction, or secondary oil cake (stage II, Table 3), were subjected to a third aqueous extraction at pH 4 and 40°C for 1 h (stage III). Polysaccharide complex PSC-3 was precipitated from the extract with an excess of ethanol. Table 3 demonstrates the total protein content in the resulting products.

For this series of samples, IR-spectra showed a logical relationship with the amount of total protein, if to analyse one band of 1750–1500 cm^{-1}, which is the structured band of stretching vibrations of C=O groups of protein components. However, this band demonstrated significant qualitative differences between the products. Samples PSC-2 and PSC-3 had two clear peaks of different intensity and a well-structured shoulder of the same intensity on the right branch of the first peak. The PSC-1 spectrum showed one narrow peak of a relatively low intensity. Its position corresponded with the shoulders at the most intense peaks of PSC-2 and PSC-3.

The general contour of the polysaccharide bands of 1030 cm^{-1} remained the same. However, their intensity in the spectra of PSC-2 and PSC-3 changed their relative position, if compared to the sequence of the curves in the protein band. PSC-2 had a more intense band. For these samples, this sequence in the arrangement of the spectral curves remained in the bands of 3500–2750 cm^{-1} and 1500–1250 cm^{-1}. These bands characterized the stretching and bending vibrations of the CH_n, NH_n, and OH groups. All the bands in the PSC-1 sample showed the lowest intensity. However, the vibration bands of these functional groups shifted to a higher frequency region, which indicated a stronger bond. It could also indicate a stronger or more compact structure of the sample.

Table 3 Conditions and results of the combined sequential extraction

Stage	Conditions	Extracted polysaccharide complex, PSC	Protein content, %
I	20°C, 60 min, pH 7, precipitation with an excess of ethanol	PSC-1	2.4
II	70°C, 60 min, pH 7, precipitation with an excess of ethanol	PSC-2	10.2
III	40°C, 60 min, pH 4 precipitation with an excess of ethanol	PSC-3	25.4

CONCLUSION

The study featured polysaccharide products obtained with different technological extraction parameters from two Russian varieties of whole flax seeds. The results make it possible to draw the following conclusions:

(1) The content of the protein component (polypeptides) in the polysaccharide extracts and their complexes was affected by the pH of the medium, temperature, time and sequence of the technological stages. An increase in the extraction temperature and a decrease in the pH of the medium contributed to a significant increase in the protein content of the polysaccharide product – by 5–10 times.

(2) The same technological parameters predetermined the proportion, the force, and the mechanism of chemical bond with the polysaccharide matrix for at least three types of polypeptide structures. This was clearly manifested in the variation of the intensity, shape, structure pattern, and the position of the peaks in the band in the range of 1700–1500 cm^{-1}. This range reflected protein components within polysaccharide complexes.

Thus, the choice of technological parameters determines the component composition of polysaccharide products during their extraction from flax seeds. The protein component affects the functional and technological properties of the products. As a result, one can obtain polysaccharide products with programmed functional properties for various food technologies.

Flaxseed polysaccharides are a dietary fibre, which makes them a physiologically necessary dietary component. Thus, flaxseed polysaccharides are both a technological additive and a biologically valuable functional ingredient. In this regard, their production may expand the range of domestic functional food ingredients.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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Degradation of β-Lactoglobulin during sourdough bread production

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Abstract: The research featured various types and strains of lactic acid bacteria (LAB) and yeast. The research objective was to study their ability to utilize β-lactoglobulin during sourdough fermentation. The present paper also described the effect of sourdough fermentation and baking on β-lactoglobulin degradation. A set of experiments with various types and strains of LAB showed that β-lactoglobulin decreased in gluten-free sourdough with 30%, 60%, and 90% of skimmed milk powder (SMP). *L.plantarum E36* demonstrated the highest biodegradation of β-lactoglobulin (by 53%) with SMP = 30%. *L.helveticus ATCC8018T* showed the lowest content of β-lactoglobulin with SMP = 60% and 90%: the content fell by 48% and 40%, respectively. The largest decrease in the content of β-lactoglobulin was observed in the sourdough with *Saccharomyces cerevisiae 17* (by 28–42%) and *Candida milleri Pushkinsky* (by 25–41%). The content of total protein increased, which was not associated with yeast biomass growth. The content was determined after fermentation in sourdoughs with SMP = 60% and 90% using a bicinchoninic acid reagent kit. The content of β-lactoglobulin in the control and experimental samples did not exceed 1 μg/g in the finished bakery products. This fact indicated a significant effect of thermal treatment on β-lactoglobulin degradation in baking. Thus, temperature processing (baking) had a greater impact on the destruction of β-lactoglobulin than enzymatic processing (fermentation).

Keywords: β-lactoglobulin, enzyme-linked immunosorbent assay, lactic acid bacteria, milk, sourdough, bread

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INTRODUCTION

These days, biochemists and food industry workers are facing an important task: they have to provide population with high-quality protein. Introducing dairy products into bakery formulae can solve the problem, since milk proteins are biologically valuable according to the content and ratio of essential amino acids. The amino acid composition of whey proteins is closest to that of human muscle tissue. Whey proteins are superior to all other animal or plant proteins in terms of essential amino and branched-chain acids, i.e. valine, leucine, and isoleucine [1–3].

However, there is the problem of people with lactose intolerance. According to the Institute of Immunology (Ministry of Health of the Russian Federation), 65% of allergic patients demonstrate intolerance to some kind of food, e.g. dairy products. This problem is especially common among children [4–7]. Therefore, dairy products as additives require a thorough research [8].

Although people of any age can digest unaltered milk proteins, cow's milk remains one of the strongest and most common allergen [6–8]. It contains about 20 proteins with different degrees of antigenicity, including those with the highest clinical relevance, such as β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), γ-globulin, and α- and β-caseins [9–11]. β-lactoglobulin is the predominant whey protein in cow's milk: 50% of whey protein and about 10% of total protein. It is considered one of the main milk allergens, while α-lactalbumin and BSA have a lower immune reactivity [12]. Sensitisation to β-lactoglobulin is caused by numerous continuous epitopes located along the entire length of its molecule [2, 12, 13].

A β-lactoglobulin molecule consists of 162 amino acid residues and has a molecular weight of about 18300 Da.

At pH 6.8–7, β-lactoglobulin can be found in milk as a dimer [14].

β-Lactoglobulin is relatively resistant to acid hydrolysis and intestinal proteases. As a result, when consumed with food, part of the protein remains intact in the gastrointestinal tract and can penetrate through the intestinal wall. Heat treatment reduces the IgE-binding ability in proportion to the degree of heating. However, new antigenic sites may form in denatured proteins. These sites were unavailable for binding in the native molecule or appeared during a chemical reaction with other food molecules. IgE obtained from patients with an allergy to β-lactoglobulin was found specific to both native and denatured proteins [2, 10].

Like any proteins, milk proteins are exposed to temperature, pressure, and enzymes. The following scheme is generally accepted for the thermal denaturation of β-lactoglobulin: deployment of protein molecules – dissociation of dimer – aggregation of denatured protein. Dimeric β-lactoglobulin reversibly dissociates into monomers at 30–55°C. At 80°C, the molecule is almost completely unfolded [11, 13]. The reversibility of β-lactoglobulin denaturation depends on the heating degree and time. After a low temperature heating, a small part of the denatured (unfolded) β-lactoglobulin molecules can restore their native structure. However, an hour at 95–97°C leads to an active aggregation of β-lactoglobulin molecules. As a result, protein denatures irreversibly. After denaturation at ≥ 70°C, the β-lactoglobulin structure can partially stabilise as the chains re-clot and disulfide bridges are formed. At 130–140°C, the disulfide bonds break, and the protein polypeptide chains deploy completely and irreversibly [2, 15–17]. Denaturation and hydrolysis of β-lactoglobulin is possible when exposed to microwave radiation [18, 19]. Denatured or hydrolysed milk proteins used in dairy mixes are known to be less allergenic [20, 21].

During baking, the temperature of the crust can reach 180–230°C, while the core crumb warms up to no more than 95°C for several minutes [22]. In this regard, the effect of the baking process on the β-lactoglobulin content in bread with dairy products remains understudied.

In fermented milk products, most milk proteins are destroyed by various microorganisms, including LAB. Prebiotic cultures of LAB are known to reduce the allergenicity of cow's milk due to the partial denaturation of allergenic proteins [24, 25].

Microorganisms play an important part in baking. For instance, fermentation process takes place in sourdough and dough. Various types of LAB are widely used in sourdough [22, 23]. Hence, it is necessary to study the effect of LAB sourdough and dough fermentation on the destruction of cow milk allergen protein. The research can result in a method of reducing the allergenicity of dairy products and creating new, safer bakery products.

Thus, the research objective was to study the effect of LAB and yeast on the destruction of β-lactoglobulin during baking.

STUDY OBJECTS AND METHODS

Effect of LAB on the β-lactoglobulin content and acidity of the sourdough. The research featured sourdough of 8 LAB strains: *Lactobacillus plantarum E36*, *Lactobacillus plantarum E4*, *Lactobacillus plantarum E1*, *Lactobacillus parabuchneri E7*, *Lactobacillus paracasei/casei E31*, *Lactobacillus paracasei E3*, *Lactobacillus acidophilus 22n2*, and *Lactobacillus helveticus ATCC 8018T*. As for the yeast strains, 8 types were employed: *Saccharomyces cerevisiae – strains L-1*, *90*, *512*, *17*, *XII*, and *Krasnodarsky*; *Candida milleri Chernorechensky*; and *Kluyveromyces marxianus Pushkinsky*. The samples were obtained from the Collection of the St. Petersburg Branch of the State Research Institute of Baking Industry (St. Petersburg, Russia) [26].

Preparing the sourdough: The nutritional mixture consisted of rice flour and SMP (30%, 60%, and 90% per 100 kg of mixture). The moisture content was 75%. The LAB culture fluid had a cell content of 108 CFU/ml cultivated in SMP for 48 h. During the first phase, it was added to a mixture of raw materials and water, stirred, and placed in a thermostat for 24 h at 30°C. The fermented sourdough was then added to the nutrient mixture in the ratio of 1:3 and allowed to ferment for 24 h at 30°C. Table 1 shows the formulae for sourdough of the propagating and production cycles. A nutritional mixture devoid of any LAB served as a control sample.

The quality of the sourdoughs was assessed according to their acidity. The acidity was determined by the common method used in baking industry. The sourdough suspension was titrated in water at H = 0.1 with NaOH solution and phenolphthalein [27].

Table 1 Formulae for sourdough with SMP and pure LAB cultures in the propagating and production cycles

Material	Raw materials in the sourdough with the content of SMP, % to dry solids					
	30	60	90	30	60	90
	Phase I of the propagating cycle			Production cycle		
LAB culture fluid, ml	10.0	10.0	10.0	–	–	–
Sourdough (Phase I of the propagating cycle), g	–	–	–	50.0	50.0	50.0
Rice flour, g	35.0	20.0	5.0	29.0	16.6	4.1
SMP, g	15.0	30.0	45.0	12.4	24.9	37.3
Water, g	121.0	121.0	121.0	108.6	108.6	108.6
Total:	181.0	181.0	181.0	200.0	200.0	200.0

Table 2 Sourdough formulae with SMP and pure cultures of yeast and LAB

Material	Raw materials in the sourdough with the content of SMP, % to dry solids		
	30	60	90
Yeast suspension, ml	10.0	10.0	10.0
Culture fluid of *L.helveticus ATCC 8018T*, ml	10.0	10.0	10.0
Rice flour, g	35.0	20.0	5.0
SMP, g	15.0	30.0	45.0
Water, g	111.0	111.0	111.0
Total:	181.0	181.0	181.0

Table 3 Sourdough formulae with SMP in propagating and production cycles

Material	Raw materials in the sourdough with the content of SMP, % to dry solids					
	30	60	90	30	60	90
	Phase I of the propagating cycle			Production cycle		
Culture fluid of *L.helveticus ATCC 8018T*, ml	10.0	10.0	10.0	–	–	–
Yeast suspension, ml: *S.cerevisiae 17*	5.0	5.0	5.0			
C.milleri Pushknsky	5.0	5.0	5.0			
Sourdough,g	–	–	–	50.0	50.0	50.0
Rice flour, g	35.0	20.0	5.0	29.0	16.6	4.1
SMP, g	15.0	30.0	45.0	12.4	24.9	37.3
Water, g	111.0	111.0	111.0	98.6	98.6	98.6
Total:	181.0	181.0	181.0	200.0	200.0	200.0

Effect of the yeast on the content of β-lactoglobulin in the sourdough. Preparing the sourdough: Yeast strains grown on malt wort slant agar (8% DS) were used to screen the allergen reducing activity of the yeast. 10 mL of yeast culture were introduced into an aqueous suspension with 10 CFU/ml cell content in the nutritional mixture (Table 2). The mixture consisted of rice flour, SMP (30%, 60%, and 90% per 100 kg of the mixture), and water. The moisture content of the mixture was 75%. To prevent the development of extraneous microflora, *L.helveticus ATCC 8018T* was added to the nutrient mixtures. The strain had been selected during the first stage of the experiment. It demonstrated the highest allergen-reducing activity.

The sourdoughs were fermented for 24 h at 30°C and then examined for acidity, temperature, and moisture content.

The effect of sourdough and dough fermentation and baking on the content of β-lactoglobulin. Laboratory baking was used to study of the effect of the technological process (fermentation and baking) on the content of β-lactoglobulin in dough and gluten-free bread.

Preparing the sourdough: The nutritional mixture consisted of rice flour and SMP (0%, 30%, 60%, 90%, and 100%). The moisture content was 75%. LAB of *L.helveticus ATCC 8018T* strain and yeast of *S.cerevisiae 17* and *C.milleri Pushkinsky* were added to the mixture in the quantities indicated in Table 3.

Preparing the dough: The dough for the control sample was kneaded from corn starch, extrusion starch, soy protein isolate, rice flour, and SMP in the amount of 3%, 6%, 9%, and 10% to the weight of the mixture. The mixture contained sugar, salt, pressed baking yeast, and vegetable oil. The moisture content was 53.5%.

The dough for the samples was prepared from the sourdough obtained at phase II of the propagating cycle (10% of the mixture in the intermediate product), corn starch, extrusion starch, rice flour, sugar, salt, pressed baking yeast, vegetable oil, and water. Table 4 shows the formulae of the dough.

The dough was poured into 250-gram moulds and allowed to rise at 35–40°C at an average humidity of

Table 4 Dough formulae

Material	Consumption of raw materials per 100 kg of the mixture with the SMP content, % to the weight of the mixture in the dough							
	Control sample				Experimental sample			
	3	6	9	10	3	6	9	10
Corn starch, g	64.2	61.2	58.2	57.2	64.2	61.2	58.2	57.2
Extrusion starch, g	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Rice flour, g	20.0	20.0	20.0	20.0	13.0	16.0	19.0	20.0
SMP, g	3.0	6.0	9.0	10.0	–	–	–	–
Sourdough, g	–				36.0			
Pressed baking yeast, g	2.5							
Vegetable oil, g	3.8							
Salt, g	0.8							
Sugar, g	2.0							
Water, g	110.6				84.7			
Total:	217.0							

80 ± 2%. After that, the samples were baked in an oven at 210°C for 18 min with a 5-second steam supply.

Preparing samples for the immunoassay and gel electrophoresis. Preceding the analysis, the samples underwent the following procedures. 9 mL of phosphate-saline buffer (PBS, pH = 7.4) was added to 1 g of the test sample (sourdough, dough, or bread). The buffer contained sodium azide to protect the samples from microorganisms. After that, a 12-hour extraction was performed using a shaker at 20 ± 1°C. After the extraction, the samples were centrifuged at 40°C and 14000 rpm in an Eppendorf Centrifuge 5417R to remove microorganisms and undissolved components. After the centrifugation, the samples were diluted 10 thousand times in a phosphate-buffered saline (20 mM phosphate, 150 mM NaCl, pH 7.2). The dilution was adapted to the concentration range defined by the test system.

The method of enzyme-linked immunosorbent assay (ELISA method) was used to measure the content of β-lactoglobulin in the sourdoughs at the onset and at the end of fermentation. The process involved antibody No. 362-beta-lactoglobulin – a set of reagents provided by OOO Hema (St. Petersburg, Russia).

Electrophoresis in a sodium dodecyl sulphate polyacrylamide denaturing gel was employed to confirm the presence of β-lactoglobulin in the sourdoughs at the onset and at the end of fermentation, as well as in the bread.

A bicinchoninic acid reagent kit (BCA, Pierce) was used to define the total protein in the sourdoughs at the onset and at the end of fermentation and in the produced bread. The disc electrophoresis was conducted in non-reducing conditions according to Laemmli method. The samples were diluted to a protein concentration of 1 mg/ml before they were applied to a 13% separating gel.

Statistics. The statistical analysis was performed using Excel software. The method of two-way ANOVA was used to compare the effects of the SMP amount and the type of strain on the content of β-lactoglobulin in the sourdoughs, dough, and bread. The research also assessed the correlation and covariance between the β-lactoglobulin content and the sourdough acidity.

The data show the confidence intervals, which prove the accuracy of the methods for determining protein content and acidity.

RESULTS AND DISCUSSION

The experiment measured the acidity in the sourdoughs based on various strains with different content of SMP. Acidity reflects the development of microorganisms in the environment. A high level of acidity improves the absorption of nutrients from the environment. High acidity values accelerate proteolysis, which is important for the destruction of protein and its constituents, including the allergenic ones. During phases I and II of fermentation, *L.acidophilus 22n2* and *L.helveticus ATCC 8018T* showed the highest titrated acidity indicators at the end of phase II (Table 5). These strains demonstrated the maximum titratable acidity with SMP = 60%.

Table 5 Effect of various LAB strains on the sourdough acidity

LAB strain in the sourdough at different SMP amounts	Titrated acidity of the sourdough, degree			
	Phase I		Phase II	
	onset	final	onset	final
	SMP = 30%			
L.paracasei E3	3.0 ± 0.3	12.9 ± 1.3	4.5 ± 0.5	13.5 ± 1.4
L.paracasei E31	3.0 ± 0.3	12.3 ± 1.2	4.2 ± 0.4	15.3 ± 1.5
L.plantarum E36	2.5 ± 0.3	7.7 ± 0.8	2.9 ± 0.3	9.8 ± 1.0
L.plantarum E4	3.0 ± 0.3	7.5 ± 0.8	2.9 ± 0.3	8.6 ± 0.9
L.parabuchneri E7	3.0 ± 0.3	6.8 ± 0.7	2.8 ± 0.3	11.0 ± 1.1
L.acidophilus 22n2	3.0 ± 0.3	15.8 ± 1.6	3.5 ± 0.4	18.5 ± 1.9
L.helveticus ATCC 8018T	3.0 ± 0.3	12.8 ± 1.3	3.3 ± 0.3	16.5 ± 1.7
L.plantarum E1	2.7 ± 0.3	9.5 ± 1.0	5.0 ± 0.5	12.2 ± 1.2
	SMP = 60%			
L.paracasei E3	3.5 ± 0.4	13.7 ± 1.4	5.3 ± 0.5	19.8 ± 2.0
L.paracasei E31	3.5 ± 0.3	14.2 ± 1.4	6.5 ± 0.7	20.4 ± 2.0
L.plantarum E36	3.0 ± 0.3	9.5 ± 1.0	3.6 ± 0.4	11.9 ± 1.2
L.plantarum E4	3.4 ± 0.3	8.2 ± 0.8	5.0 ± 0.5	11.2 ± 1.1
L.parabuchneri E7	3.8 ± 0.4	8.5 ± 0.9	4.0 ± 0.4	11.8 ± 1.3
L.acidophilus 22n2	4.5 ± 0.5	21.0 ± 2.1	6.0 ± 0.6	28.0 ± 2.8
L.helveticus ATCC 8018T	4.5 ± 0.5	17.2 ± 1.7	5.5 ± 0.6	22.5 ± 2.3
L.plantarum E1	4.1 ± 0.4	10.5 ± 1.1	4.5 ± 0.5	10.2 ± 1.0
	SMP = 90%			
L.paracasei E3	4.7 ± 0.5	13.2 ± 1.3	6.0 ± 0.6	16.2 ± 1.6
L.paracasei E31	4.2 ± 0.4	12.5 ± 1.3	5.6 ± 0.6	19.6 ± 2.0
L.plantarum E36	4.5 ± 0.5	9.5 ± 1.0	5.5 ± 0.6	17.4 ± 1.7
L.plantarum E4	4.5 ± 0.5	11.0 ± 1.1	5.3 ± 0.5	16.3 ± 1.6
L.parabuchneri E7	5.2 ± 0.5	9.1 ± 0.9	5.7 ± 0.6	17.0 ± 1.7
L.acidophilus 22n2	5.7 ± 0.6	23.9 ± 2.4	7.0 ± 0.7	27.5 ± 2.8
L.helveticus ATCC 8018T	5.5 ± 0.6	20.0 ± 2.0	6.0 ± 0.6	22.5 ± 2.3
L.plantarum E1	5.0 ± 0.5	10.5 ± 1.1	5.8 ± 0.6	15.3 ± 1.5

All the LAB strains had different effects on β-lactoglobulin (Fig. 1). The degree of β-lactoglobulin degradation decreased with the increase in the SMP concentration in the nutritional mixture, while different strains reacted differently to the increase in the SMP concentration. At SMP = 30%, the sourdough sample with *L.plantarum E36* showed the biggest drop in β-lactoglobulin content in the fermentation process – by 53%. However, at SMP = 60% and 90%, it was the *L.helveticus ATCC8018T* sample that showed the biggest drop in the content of the allergen – by 48 and 40%, respectively. In the sourdoughs, the SMP amount might have a different effect on the vital activity of lactic acid bacteria, since they normally live in silage and flour, except *L.acidophilus 22n2* and *L.helveticus ATCC8018T.*

The two-way ANOVA method gave the following results. The SMP amount had a significant effect on the β-lactoglobulin content in the sourdough after fermentation: alpha = 0.05, $P < 0.001$, F = 27.78, $F_{critical}$ = 3.63. However, the type of LAB strain factor produced no effect: alpha = 0.05, $P = 0.25$, F = 1.46, $F_{critical}$ = 2.59. A strong positive correlation and covariance was revealed between the final β-lactoglobulin content and the final acidity level of the sourdough for *L.plantarum E4* and *L.acidophilus 22n2*. The correlation coefficients were 0.99 and 0.91,

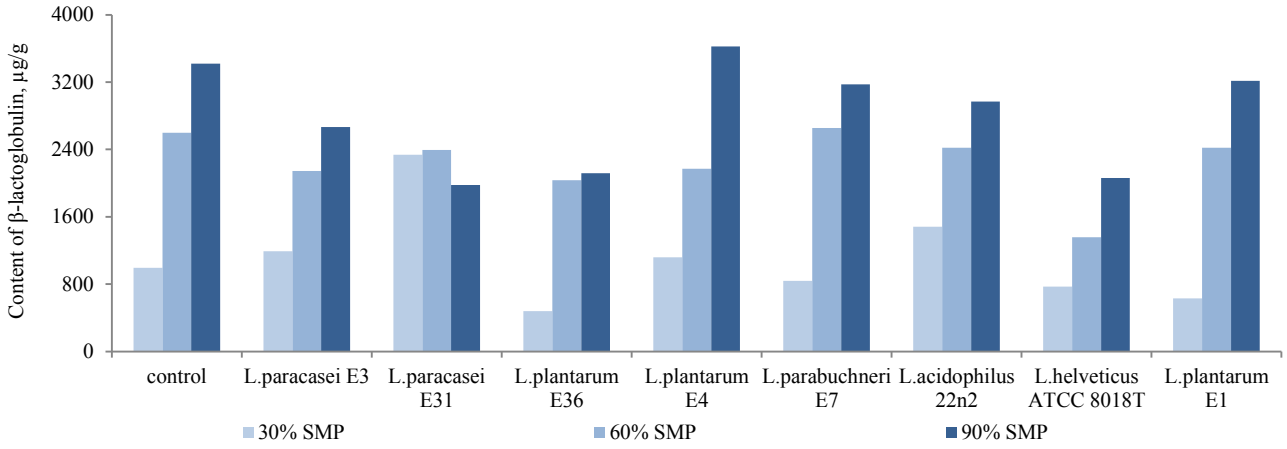

Figure 1 Content of β-lactoglobulin in the sourdoughs with various LAB strains after fermentation

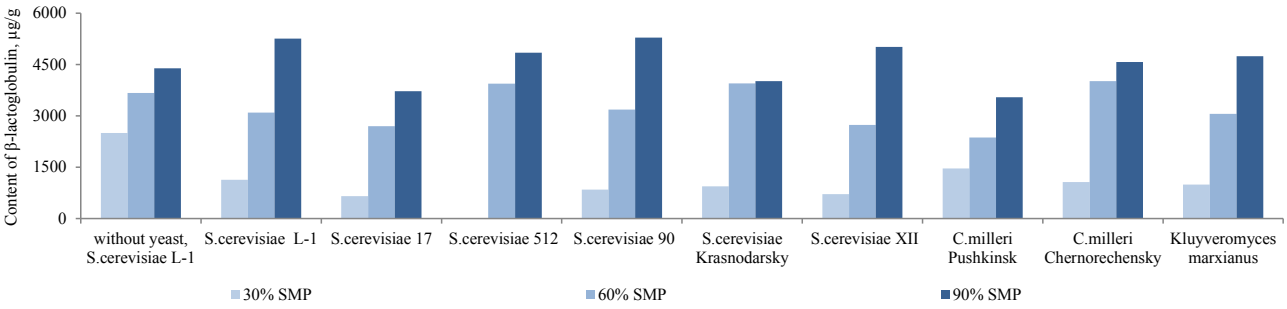

Figure 2 Content of β-lactoglobulin in the sourdoughs various yeast strains after fermentation

respectively. The covariance coefficients were 3270 and 2449, respectively. *L.paracasei E31* demonstrated a weak inverse correlation (coefficient = 0.25).

The screening of the allergen-reducing activity of various yeast strains (Fig. 2) showed that the strains produced a different effect. As for *Saccharomyces cerevisiae*, strain *17* demonstrated the highest allergen-reducing activity: the β-lactoglobulin content fell by 28–42%. As for the *Candida milleri*, it was *Pushkinsky* strain: the β-lactoglobulin content fell by 25–41%.

The two-way ANOVA method gave the following results. The SMP amount had a significant effect on the β-lactoglobulin content in the sourdough after fermentation: alpha = 0.05, $P < 0.001$, F = 93.60, $F_{critical}$ = 3.56. However, the type of yeast strain factor produced no effect: alpha = 0.05, $P = 0.37$, F = 1.17, $F_{critical}$ = 2.46.

Lactic acid bacteria strain *L.helveticus ATCC 8081T* and two yeast strains, *S.cerevisiae 17* and *C. milleri Pushkinsky*, were selected for further research, which featured the effect of fermentation and baking on the β-lactoglobulin content in sourdough, dough, and bread.

The enzyme immunoassay showed a decrease in β-lactoglobulin at the end of phases I and II by 1.4–1.8 times, if compared with its content in the nutrient mixture immediately after mixing (Fig. 3). Thus, the allergen was destroyed by the LAB enzymes.

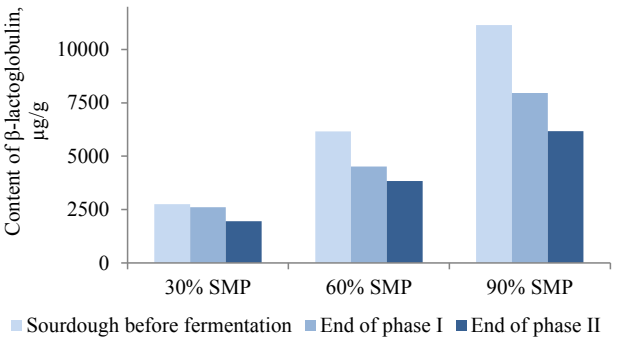

Figure 3 Content of β-lactoglobulin in the sourdough before and after fermentation at the end of phases I and II

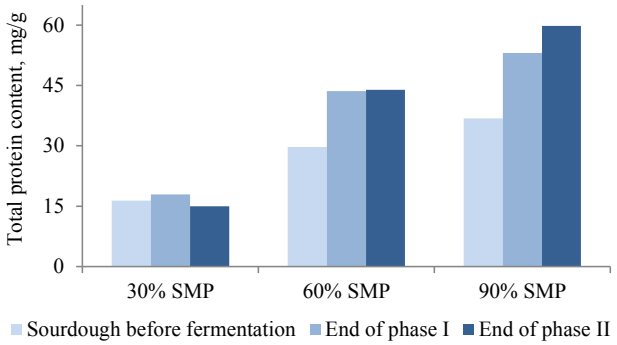

Figure 4 Total protein in the sourdough before fermentation and after phases I and II

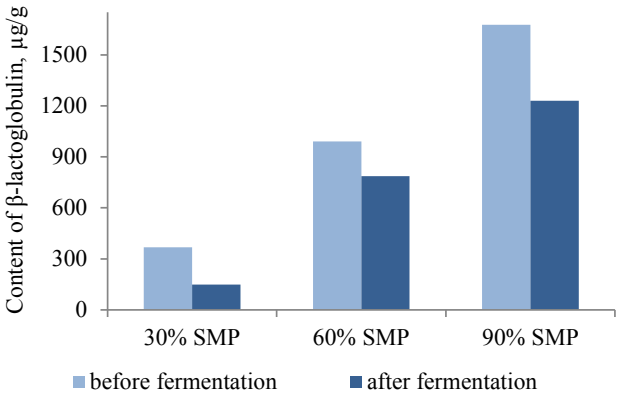

Figure 5 Content of β-lactoglobulin in the dough before and after fermentation

Despite the destruction of β-lactoglobulin, the total protein content in samples with SMP = 60% and 90% increased in the fermentation process, if compared with the initial amount (Fig. 4). The total protein content was determined using a bicinchoninic acid reagent kit. Presumably, there are two ways additional protein could appear during the experiment. First, it could increase during fermentation due to the accumulation of yeast biomass. Second, it could be released from any supramolecular or covalent complexes with other macromolecules – proteins or polysaccharides. To understand how the increase in the microbial biomass affected the increase in total protein, an experiment was conducted with sourdough based on rice flour, without SMP. In this case, the amount of total protein in the sourdough without SMP remained virtually unchanged during the fermentation. It was 3.8 mg/g before fermentation and 4.0 mg/g at the end of phase I. The increase in the total protein in the sourdoughs with SMP might have been caused by the release of the previously bound protein. It happened under the influence of yeast and LAB enzymes, not because their biomass increased.

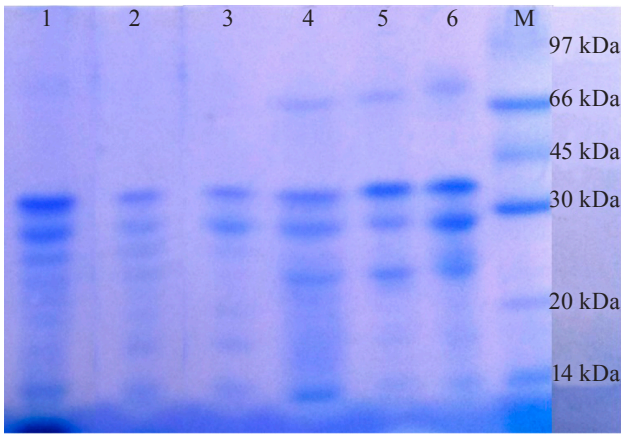

Figure 6 Electrophoregramme samples: sourdough before fermentation: SMP = 30% (1), SMP = 60% (2), SMP = 90% (3); sourdough after fermentation: SMP = 30% (4), SMP = 60% (5), SMP = 90% (6), and the marker (M)

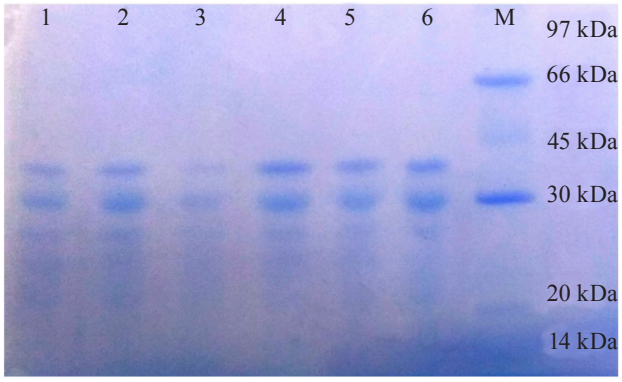

Figure 7 Electrophoregramme samples: control bread sample with SMP = 30% (1), SMP = 60% (2), SMP = 90% (3); experiment bread samples with SMP = 30% (4), SMP = 60% (5), SMP = 90% (6), and the marker (M)

The experiment revealed a decrease in β-lactoglobulin in the dough after fermentation, compared with its content immediately after kneading (Fig. 5). Due to the fact that the kneading involved pressed yeast, the decrease in β-lactoglobulin could be explained by the combined effect of fermenting microflora enzymes and industrial yeast.

As for the finished products, the content of β-lactoglobulin in the control and experimental bread samples did not exceed 1 μg/g. Hence, the temperature degradation of β-lactoglobulin proved highly efficient for bakery products.

The electrophoresis was conducted according to Laemmli's method in sodium dodecyl sulphate polyacrylamide gel with non-reducing conditions. It also confirmed a decrease in the content of β-lactoglobulin (Fig. 6 and 7). Neither blotting of polyacrylamide gel proteins to nitrocellulose, nor detection of β-lactoglobulin by antibodies from the ELISA test system gave any results. Neither of the antibodies was able to identify the antigen after electrophoresis in such conditions. That proved that the content of β-lactoglobulin in the finished products was extremely low.

Thus, the research proved that thermal treatment has a greater impact on the destruction of β-lactoglobulin than enzymatic treatment.

CONCLUSION

The research investigated the effect of various LAB and yeast strains on the β-lactoglobulin content in gluten-free sourdough with SMP. Increasing the amount of SMP had an inhibitory effect on the utilization of β-lactoglobulin by *L.plantarum E36*, *L.plantarumE1*, and *L.helveticus ATCC8018T*. The last demonstrated the highest allergen-reducing activity when SMP equalled 60% and 90% of the solid weight: β-lactoglobulin decreased by 48% and 40%, respectively. The yeast strains *Saccharomyces cerevisiae 17* and *Candida milleri Pushkinsky* showed the biggest decrease in the content of β-lactoglobulin: by 28–42% and 25–41%, respectively.

S.cerevisiae L-1, *S.cerevisiae 512*, *S.cerevisiae 90*, and *S.cerevisiae XII* demonstrated an increase in the content of β-lactoglobulin at SMP concentration of 90%. This might have been connected with a release of β-lactoglobulin, previously bound to other proteins.

The content of β-lactoglobulin in the control and experimental samples of bread did not exceed 1 μg/g, which proved a high efficiency of temperature degradation of β-lactoglobulin in the baking process. Therefore, temperature processing (baking) had a greater impact on the destruction of β-lactoglobulin than enzymatic processing (fermentation).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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The research employed microorganisms from the Collection of the St. Petersburg branch State Research Institute of a Baking Industry (St. Petersburg, Russia). The Collection is on the list of collections that deposit non-pathogenic microorganisms for government use, as approved by the Decree of the Government of the Russian Federation (June 24, 1996 No. 725-47) and the Order of the Ministry of Agriculture and Food of Russia (August 15, 1996 No. 14c).

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New technological paradigm of the Russian dairy industry: formation principles under globalisation

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Abstract: The present research employed a convergent approach and cognitive methodology to define the upgrade options in the sphere of domestic dairy industry according to the principles of the 5th technological paradigm. The principles include biological nanomembrane cluster technologies with a complete production cycle. The paper offers a forecast for the 6th technological paradigm, which presupposes picotechnology for the production of milk derivatives, such as lactose hydrolysates, lactulose, microparticulate proteins, peptides, and amino acid pool. The principle makes it possible to return secondary dairy raw materials into the technological cycle. These significant resources include low-fat milk, buttermilk, and especially whey, which can be used to produce functional foods for various population groups, as well as new generation forage resources. From the point of view of logistics, the modern dairy industry should employ a digital and robot-aided non-waste production scheme. Thus, all dairy raw materials should be considered as natural clusters according to the nature-formed biotechnological system (BTS). These clusters are lipids (fats), nitrogen-containing substances (proteins), carbohydrates (lactose), minerals (salt), biologically active substances, and water. As an idealised model of agricultural raw material, milk is extremely complex. Its chemical composition includes more than 2000 constituents and 100000 molecular structures. In addition, dairy architectonics is also extremely complex: milk is suspension, emulsion, and solution, concurrently. Finally, milk possesses unusual physicochemical, osmophoric, structural-mechanical, bio-thermodynamic, and other characteristics. We conducted a long-term systematic analysis and developed a scheme that can help the domestic diary industry to adapt safely to the new technological paradigm. The scheme takes into account various factors, such as limited traditional dairy resources, Russia's accession to the WTO, and the globalisation of the world dairy market. Our research team belongs to the leading federal scientific school 'Living Systems' (No. 7510.2010.4) developed by the North Caucasus Federal University (Russia).

Keywords: Milk, whey, nanotechnology, bioproducts, modernization, clusters

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INTRODUCTION

The current food industry as a whole, and dairy industry in particular, fits in the 5th technological paradigm, which is based on biotechnology with relics of the 4th (electricity) and the 3d (mechanics) technological paradigms [1]. The 6th technological paradigm will supposedly originate from the current 5th paradigm in 2025 [1–5]. The possible start date corresponds with Decree No. 350 issued by the President of the Russian Federation on July 21, 2016. The Decree is entitled 'On the Administration of State Science and Technology Policy for the Development of Agriculture'. It defines 2025 as the year by which raw milk production will have increased by 40%. The provisions were further specified in the Presidential Decree 'On the national goals and strategic objectives of the development of the Russian Federation for the period up to 2024' (May 7, 2018). According to Paragraph 7, almost all sectors of Russian economy are to transform to the principles of the best available technologies (BAT) by 2024.

Dairy production is an essential component of the food industry of the historically established agro-industrial sector, i.e. the milk production – dairy products – sales chain. For the new technological paradigm to take power in the current Russian dairy industry, it needs an upgrade [6, 7]. The prospective upgrade is directly related to ensuring food security and independence of the country and its regions [8].

STUDY OBJECTS AND METHODS

The research featured the paradigm of dairy raw materials, which was tested in the dairy industry on the

Table 1 Content of the main constituents in dairy raw materials, g/100 g

Constituents	Whole milk	Skimmed milk	Buttermilk	Milk whey
Milk fat	3.7	0.05	0.5	0.2
Proteins	3.3	3.3	3.3	0.9
Lactose	4.8	4.8	4.7	4.8
Mineral salts	0.7	0.7	0.7	0.6
Solids	12.5	8.8	9.1	6.5

level of Technological Platform formation. The system analysis involved the convergence methodology with elements of the cognitive approach as developed by the Russian Academy of Sciences [4].

Table 1 shows a comparative analysis of raw milk composition, cream excluded, which resulted from mechanical and biotechnological processing.

Skimmed, or rather low-fat, milk and buttermilk are protein-carbohydrate raw materials and have 50% of solids. Whey is a carbohydrate raw material with 70% of solids. Proteins, lipids (milk fat), and carbohydrates (lactose) are the main and most valuable constituents of the secondary dairy raw materials. In addition to the main constituents, skimmed milk, buttermilk, and whey also contain mineral salts, non-protein nitrogenous compounds, vitamins, enzymes, hormones, immune bodies, organic acids, etc. It means that almost all components of milk solids can be utilised, even biologically synthesized water with its memory and fullerenes-kenotrons.

Table 2 provides data on the degree of transition of dairy constituents into dairy protein-carbohydrate raw materials, or secondary dairy raw materials, as defined by the Technological Regulations.

Skimmed milk and buttermilk contain almost the entire protein, carbohydrate, and mineral complex of milk and up to 15% of milk fat. Whey contains carbohydrate complex, proteins, and mineral salts. These data should be taken into account during identification, examination, and industrial processing of secondary dairy raw materials.

Table 2 Degree of transition of the main dairy constituents into the secondary dairy raw materials

Milk constituents (100%)	Degree of transition, %		
	Skimmed milk	Buttermilk	Milk whey
Milk fat	1.4	14.0	5.5
Total protein, including	99.6	99.4	24.3
Casein	99.5	99.5	22.5
Whey proteins	99.8	99.6	95.0
Lactose	99.5	99.4	96.0
Mineral salts	99.8	99.6	98.0
Solids	70.4	72.8	52.0

Table 3 Dispersed composition of dairy clusters

Milk constituents	Size of the molecule or particle, nm	Volume of the molecule or particle, %
Water	0.1–0.2	90.10
Fat	200–10000	4.20
Casein	40–300	2.30
α-Lactoglobulin	5–20	0.30
B-Lactoglobulin	25–50	0.08
Milk sugar (lactose)	1.0–1.5	3.02
Mineral salts	0.1–1.0	0.10
BAS	0.1–100.0	0.01

Table 3 shows the sizes of the structures of the main constituents of dairy raw material.

Dairy raw materials contain all types of structural systems: ions, molecules, colloids, suspensions, and emulsions.

In the Russian Federation, the annual processing volume of dairy raw materials is 30–33 million tons. It means that secondary raw materials are estimated as 20 million tons, which is a huge reserve and available resources for the industrial upgrade. This especially concerns new-generation functional products branded by Prof. Petrovsky as 'minimum of calories – maximum of biological value' [9].

A long-term systematic analysis allowed the team of the North Caucasus Federal University (federal scientific school 'Living Systems' No. 7510.2010.4) to offer a scheme that can help the domestic diary industry to adapt safely to the new technological paradigm. The scheme takes into account various factors, such as limited traditional dairy resources (< 50%), Russia's accession to the WTO, and the globalisation of the world dairy market. The generalised scheme has nine fundamental principles, or blocks, and was published in [10]. The principles cover the whole range of dairy production from dairy raw materials to the problems of the international dairy industry, including the organisation of alternative off-season productions. This paper introduces the concept of the second principle, which involves the scaling of innovative, sustainable biological nanomembrane 'high-tech' technologies for industrial processing of dairy raw materials with the complete production cycle [11, 12].

RESULTS AND DISCUSSION

Logistically, dairy industry should implement a zero-waste production scheme that observes the following principle: waste milk is nothing but unused reserves [13]. It was 30–50 years ago that the so-called recycling plants appeared in the global dairy industry. They played a positive economic and social role: they brought in profit, gave workers two days-off, and protected the environment. For example, all large cheese-making

factories are to have skimmed milk and whey drying stations. According to the new approach to this problem, dairy constituents are to be obtained from the original milk. Raw materials of plant and animal origin are to be combined to produce such functional products (bioproducts) as pro-, pre-, and synbiotics. In addition, a significant amount of secondary dairy raw materials (milk protein-carbohydrate raw materials) enters the so-called technological cycle. As a result, the industry loses a great deal of skimmed milk, low-fat milk, buttermilk, and especially milk whey, which is responsible for half of the solids of the original raw material, while polluting water sources [14].

To update the dairy industry by implementing modern innovations, the dairy technology platform has to be revised with subsequent access to the new technological paradigm [1, 15, 16]. In the nearest future, the industry will have to comprehend and implement the large-scale high-tech options. According to Metz [11, 17, 18], the 5th technological paradigm will include food nanotechnology [19] with biomembrane and baromembrane processes aimed at the clusters of dairy constituents with the use of information and communication technologies. The European Economic Community set up the first European Institute for Food Industry (EU-IFP) (http://www.hightecheurope.com). It has three branches: Biotechnology (BIOTECH), Nanotechnology (NANOTECH), and Information and Communication Technology (ICTECH) [20].

On the industrial level, the innovative priorities of technological upgrade have the following principles.

Epistemologically, all dairy raw materials – whole and skimmed (low-fat) milk, cream, buttermilk, and whey – are constantly renewable resources. Therefore, the food obtained from them can be viewed upon as objects of rapidly developing nanotechnology. Hence, their constituents at the molecular level should be considered as naturally synthesized clusters of simple (molecules, atoms) or complex (micelles, aggregates, particles) compounds [20]. The cluster structure of the main components of dairy raw materials creates prerequisites for directional and controlled modelling, i.e. bio-technology in the 5th technological paradigm and picotechnology in the 6th.

Similarly, from the point of view of the nature-formed biotechnological system, all components of dairy raw materials can be considered as natural clusters – lipids (fat), nitrogen (proteins), carbohydrates (lactose), minerals (salt), biologically active substances, and water. As an idealised model of agricultural raw material, milk is extremely complex. Its chemical composition includes more than 2000 constituents and 100000 molecular structures. In addition, dairy architectonics is also extremely complex: it includes suspension, emulsion, and solution. Finally, milk possesses unusual physicochemical, osmophoric, structural-mechanical, bio-thermodynamic, and other characteristics.

Milk as a biotechnological system provides newborn mammals with nutrients and can serve as a basis for next-generation high-grade foods. According to its physical and chemical properties, its active acidity and osmotic pressure are close to the nutriciology of mammals. Thus, it can be of practical importance that proteins, milk sugar, and mineral salts increase the density of dairy raw materials, while milk fat reduces it.

As a biotechnological system, dairy raw materials illustrate the opinion voiced by the great Russian physiologist and Nobel Prize winner Pavlov: 'milk is an amazing food prepared by nature itself.' Structurally, this is a heterogeneous system in the form of a solution intended for direct (oral) use. It has a sufficiently high content of solids, particles (milk fat in the form of suspension or emulsion), colloids (proteins and mineral compounds), and a molecular solution (lactose, mineral salts, and BAS). Milks obtained from sheep, goats, mares, camels, and buffalos differ from cow's milk as a complex biotechnological system. This should be considered in industrial processing. The same applies to their secondary dairy raw materials, e.g. skimmed milk, buttermilk, and whey [23].

In 2007, we introduced the concept, or doctrine, of nano-, bio-, membrane, and biomembrane technologies to implement this principle. The concept was published in food industry journals and tested at food industry seminars and international summits in 2008, 2009, 2011, 2015, and 2016. Apparently, it can serve as an alternative basis for the industry upgrade [24].

The fundamental paradigm of nano-food technology in the dairy business can be confirmed by the processes of synthesis of lactose derivatives [25, 26]. For instance, the biological nanotechnology of lactose hydrolysis produces two monoses from lactose disaccharide (1 nm), i.e. glucose and galactose with a size of 0.5 nm. This solves the problem of milk intolerance. The Stavropolsky Dairy Plant (Stavropol, Russia) used this unique procedure to obtain marketable low-lactose milk under the Healthy City programme. The globally famous synthesis of lactulose at the proton level, which is pure nanotechnology, is the pride of the industry and has proved to be extremely profitable [27]. Lactulose is known to be the best prebiotic, a bifidobacteria promoter, and an ideal natural laxative. A research in its production and implementation was awarded the Prize of the Russian Federation Government on Science and Technology in 2002.

The last decade has seen a fundamentally new direction of dairy nanotechnology: whey proteins are microparticulated into nanotubes that imitate the flavour of milk fat [28]. The industry has already mastered the logistics for the formation of microgranules (nanotubes) of whey proteins from the so-called 'albumin milk'. Such products are well known abroad under the Simplesse brand [23]. This innovation has also been implemented in Russia [12, 29].

Biotechnology of dairy products is historically associated with pure cultures of microorganisms in the form of starter cultures and enzymatic catalysis that are used to obtain such fermented milk products as sour cream, cheese, cottage cheese, and dairy beverages.

High-biotech solutions have a long history in the domestic industry. Sour cream is known as traditional 'Russian cream'; sour clotted milk is a Russian biocenosis; kefir is traditionally favoured by the centenarians from the Caucasus; yogurts, or 'dairy ferments' were introduced by the famous Russian Nobel Prize winner Mechnikov as part of lactotherapy, etc. Such elements of superbiotechnologies as enzymatic catalysis and microbial synthesis have long been part of centuries-old cheese-making procedures. Unique biotechnologies of dairy industry are as sophisticated as the finest surgical operations and require the same disinfection measures. Some of them can be adapted to obtain starter cultures in controlled fermentation procedures for silage making, sauerkraut production, meat industry, as well as in medicine and veterinary. According to Luff, the 'life code' that nature and bionanotechnology give to industrially processed whey provides people with immune protection against many diseases, including various flu strain [23, 30]. As for milk lactose, Canadian veterinarian De Lookk called it the 'saviour of mankind' because it can prevent and treat salmonellosis [31].

One of the most promising biotech solutions in the dairy industry is the production of derivatives of the nitrogen-containing milk complex, namely casein and whey proteins. It deserves special consideration. It involves two separation methods: hydrolysis and proteolysis. Casein hydrolysis is well-studied and globally implemented, e.g. in pharmacy. Its biotechnology is based on the proteolysis of caseins in cheese, which determines the type and quality of products. The hydrolysis of whey proteins is of particular importance in the biomedical aspect since it is used in infant formulae, as well as in dietary and therapeutic nutrition.

The amino acid composition of whey proteins is believed to be closest to human muscle tissue [32]. They exceed all other animal and vegetable proteins according to the content of essential amino acids and branched-chain amino acids (BCAA), e.g. valine, leucine, and isoleucine. According to the FAO/WHO, the biological value of albumin and globulin, which are the main whey proteins, exceeds the ideal 100 cu ascribed to eggs. It amounts to 104 cu, which is twice as high as the biological value of wheat. This corresponds with the traditional Russian proverb that bread and milk are the best food. The digestibility of whey proteins is 98%, which is extremely high. Table 4 illustrates the data on the content of some essential amino acids according to the scale used by the FAO/WHO and their presence in whey proteins.

Table 4 Content of essential amino acids

Amino acids	Content, g/100 g of protein	
	FAO/WHO scale	Whey proteins
Isoleucine	4. 0	6.2
Leucine	7.0	12.3
Lysine	5.5	9.1
Methionine + Cystine	3.5	5.7
Phenylalanine + Tyrosine	6.0	8.2
Threonine	4.0	5.2
Tryptophan	1.0	2.2
Valine	5.0	8.7

Numerous peptides and amino acids are extremely important for human health, especially those natural polypeptide chains that can be found in dairy raw materials. They can also be obtained artificially from casein fractions and polypeptide chains of whey proteins. For instance, exomorphins are natural painkillers. They regulate the general endocrine profile of mammals and produce a soothing effect on cubs. As for β-casomorphins, they are excellent immunomodulators. 'Dairy peptides' increase the phagocytic activity of certain gastrointestinal bacteria, thus ensuring resistance to infectious diseases. For example, the Institute of Biophysics (Siberian Branch of the Russian Academy of Sciences) has synthesized lactoptin, an analogue of breast milk low molecular weight peptide. It possesses antitumor and antimetastatic properties and is absolutely safe [33]. Angiogenin (Milkang) is beneficial for blood vessels and heals wounds and burns [34]. Unfortunately, the issue remains largely understudied.

The process is extremely complex, and its results can be implemented in various ways. One can mention the studies of phenylketonuria performed at the Kemerovo Institute of Technology (University) [35]. The proprietary technology of obtaining biologically active peptides from milk proteins has been implemented on an industrial scale in England. The Molvest company (Voronezh, Russia) has started to produce dairy products with reduced antigenicity using the controlled hydrolysis of β-globulin into peptides. The technology was developed by the combined efforts of A. Bach Research Institute, the Russian Academy of Sciences, the Interindustry Scientific Center of the Siberian Research Institute of Mining Geomechanics and Surveying and the Voronezh State University of Engineering Technologies [36].

Baromembrane technology makes it possible to separate high molecular weight polydisperse liquid systems, as shown in Fig. 1. It has been adapted to dairy raw materials, especially ultrafiltration and electrodialysis [37, 38].

Figure 2 shows installations that use baromembrane methods of molecular sieve separation of whey.

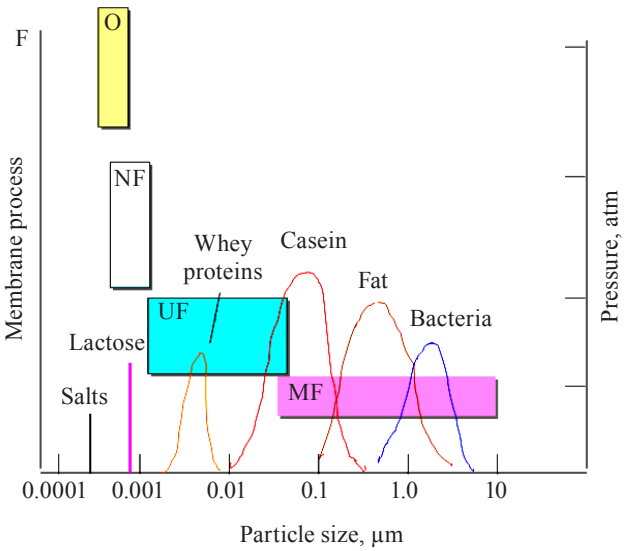


Figure 1 Diagrams of baromembrane separation of constituents of dairy raw materials: RO – reverse osmosis, NF – nanofiltration, UF – ultrafiltration, MF – microfiltration

The illustrations demonstrate the achievements and prospective scaling of the industry. As for electroflotation, sorption, desorption, and ion exchange, they are still under research and are undergoing experimental testing.

Electrodialysis desalting of whey produces up to 100000 tons annually and increases by 30% each year. Such a large-scale production has made it possible to reduce the export of solids, which were purchased from as far away as Argentina. Now it is a full sub-industry of dairy production. To demonstrate the process, Figure 3 shows the scheme of electrodialysis and devices of domestic production.

Biomembrane technology. There has been a series of long-term case studies performed by Prof. Molochnikov's team. The studies were conducted by the joint efforts of the Institute of Organoelement Compounds (Moscow) and a number of medical institutions, such as the Institute of Aviation and Space Medicine (Moscow), Ministry of Defence, Institute of Nutrition (USSR Academy of Medical Sciences), All-Union Scientific Cancer Centre (Moscow). The researches have made it possible to review and fundamentally change the approaches to raw milk processing and the composition and quality of dairy products [39–42].

The studies employed the biomembrane technology: an aqueous solution of a polysaccharide, e.g. pectin, is introduced into milk raw materials, i.e. natural or condensed skimmed milk, reconstituted milk powder, or buttermilk. Milk casein is thermodynamically incompatible with polysaccharide. As a result, casein-

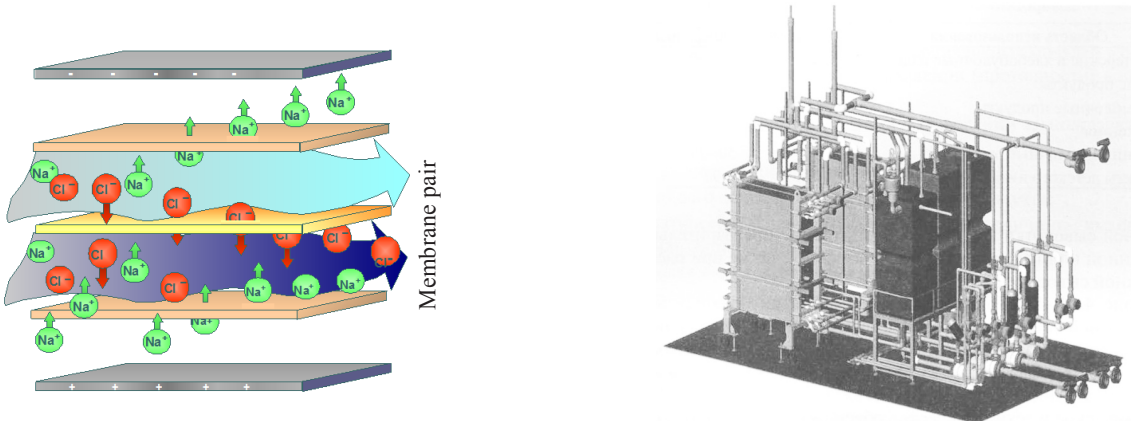
Microfiltration

Ultrafiltration

Nanofiltration

Reverse osmosis

Figure 2 Baromembrane installations for molecular sieve filtration of whey



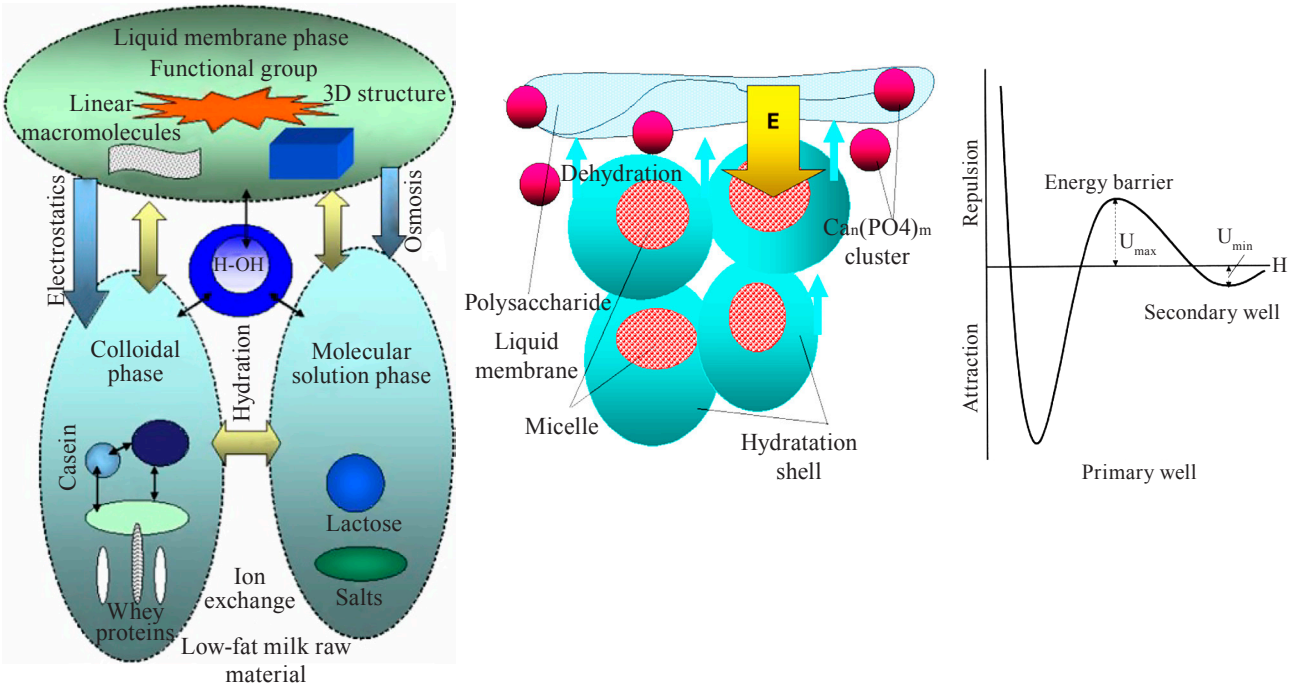
(a) (b)

Figure 3 Schematic diagram of whey demineralisation (a) and a module of Istok electrodialysis installation (b)

containing dairy raw materials spontaneously split into two liquid fractions – natural casein concentrate (NCC) and a whey-polysaccharide fraction (WPF). After that, the two liquid raw materials are separated by gravity or centrifuged. They are easily combined with the remaining dairy raw materials. The initial raw materials can be processed, and no by-products are formed, which corresponds with the principles of zero-waste technology with a closed zero-discharge production.

Figure 4 illustrates a hypothetical simulation model of the interaction between the liquid membrane (a polysaccharide solution, e.g. pectin) and the main milk constituents. The system's stability is modelled according to the DLVO theory (Derjaguin + Landau + Verwey + Overbeek) [43].

In general, basic researches in academic institutions and programme-targeted studies conducted by industry research institutes on the issue of biomembrane technology with the Bio-Ton dairy product line give every reason to revise the existing principles of production in the dairy industry. This can serve as a basis for the 6th technological paradigm in accordance with the principles of the system approach [44]. The 6th technological paradigm presupposes sono- and



(a) (b) (c)

Figure 4 Hypothetical model of the interaction between the liquid membrane and the milk constituents (a); formation of associates of the first level casein micelles (b) and the interaction energy curve according to the DLVO theory (c)

picotechnologies with a full automation under aseptic conditions, i.e. unmanned technologies.

The abovementioned data make it possible to imagine the complexity of the problems and their solutions based on modern genomics, proteomics (peptidomics), lipidomics, and genetic engineering. Their practical implementation in the form of nano-, bio-, membrane, and biomembrane technologies can shape a fundamentally new dairy science – LactoOmics [7, 45–48]. The issue has to be considered separately on the principles of cognitive approach and convergence methodology within the framework of the new technological paradigm [49].

The concept was adapted for milk whey, which was named 'universal agricultural raw material' by Prof. Lipatov [50]. The research team was represented by members of the Scientific and Educational Centre 'Membrane BioTechnologies' and the engineering BioCentre of the North Caucasus Federal University, which belong to the scientific school No. 7510.2010.4. The proposed concept of bionomembrane technologies fully complies with the principles of the 5th technological paradigm with its high-tech (1985–2025) and creates real prerequisites for the 6th technological paradigm (2025–2080). The latter will employ picotechnology and elements of artificial intelligence (neural networks) to produce food and fodders of the new generation. The concept is outlined in a manual for the new generation of industry professionals [51].

CONCLUSION

On the eve of the 6th technological paradigm and the BAT principles, the dairy industry as we know it has to be upgraded [52, 53]. The upgrade will require tremendous efforts. The principles of LactoOmics listed in [55] have to be implemented in practice [54]. Only the fundamental principles of food technology will make it possible to avoid the tragedies of such manufactured famines as Holodomor [56–60]. Now that Russia has joined the WTO, the globalisation of the world dairy market requires an adequate response.

CONFLICT OF INTEREST

The authors declare that the authenticity of the issue makes any conflict of interests impossible. The research results were only published in the Bulletin of the Russian Academy of Sciences, which was mentioned in the text.

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Use of essential oils and vacuum packaging as a way to extend shelf life of burgers from surimi

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Abstract: Essential oils are known to be a natural preservative due to their antimicrobial and antioxidant properties. The aim of this study was to evaluate an effect of thyme and cumin essential oils (EOs) in combination with air packaging and vacuum packaging on the shelf life of burgers from surimi and chicken meat. The study was conducted at 2°C for 27 days. We tested four groups of samples: (a) burgers in air package, (b) burgers with cumin and thyme EOs in air packaging, (c) burgers in vacuum packaging, and (d) burgers with cumin and thyme EOs in vacuum packaging. The greatest effect ($P < 0.001$) on the chemical and microbiological characteristics of the novel burgers displayed burgers with EOs of thyme and cumin packaged under vacuum. It can be explained by synergistic effect, which made it possible to extend the shelf life of the burgers. These results allowed us to suggest that surimi could be used as a basic ingredient in burgers production.

Keywords: Burgers, surimi, cumin essential oil, thyme essential oil, air packaging, vacuum packaging

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INTRODUCTION

Fish meat is an ideal source of animal protein which has a high nutritional value. Nowadays, consumers are interested in healthy food [1]. Nevertheless, convenience food, including burgers, has remained common all over the world [2]. Ready-to-cook fish products is becoming popular among consumers due to their high nutritional value and short time of cooking [2]. Still, to preserve its quality, fish meat should be processed properly [3].

In recent years, changing socioeconomic factors, namely, an increase in the number of employed women, have led to an increased demand for convenience products. Therefore, some efforts have been made to extend the shelf life of ready-to-eat food [4, 5].

Surimi, stabilised myofibrillar proteins of fish muscle, can be made of both sea-water and fresh-water fish. To obtain surimi, fish fillet is minced, washed by water, and stabilised by blending with cryoprotectants. A cryoprotectant mix, containing sugar, sorbitol, and phosphates, is added to the minced fish [6]. Surimi is an important ingredient for food production in many countries due to its technological properties [6].

Currently, there are a number of ways to control the growth of pathogenic microorganisms in food products. One of the ways is the use of essential oils (EOs). EOs are aromatic oily extracts obtained from different parts of plants, such as flowers, leaves, wood, bark, roots, seeds, or peel, which exhibit bactericidal or bacteriostatic properties [7]. EOs are considered as natural preservatives for raw or mildly processed food [8]. EOs have a wide spectrum of antimicrobial properties. As an antimicrobial agent, EOs destroy both the lipid bi-layer of cell membranes and enzyme systems as well as inactivate the genetic material of bacteria [9].

EOs display their antimicrobial action against pathogenic microorganisms, including gram-positive and gram-negative, as well as mold and parasites [10–14]. In addition, EOs are reported to have antioxidant properties [15–17]. Natural antioxidants have an advantage over artificial ones because of their high content in phenolic compounds as well as other active

components which can effectively inhibit oxidative reactions [17, 18].

Cumin (*Cuminum cyminum* L.) is a flowering plant in the family Apiaceae. Its seeds have been commonly used for centuries as a spice [19]. Thyme (*Zataria Multiflora* Boiss.) is an aromatic perennial evergreen herb beloning to the family *Labiateae* and used in cooking [20]. In addition, there is data on the successful use of thyme EO as an antimicrobial agent in chicken meat patties [21].

The aim of this work was to find a way to prolong the characteristics and shelf life of novel burgers made of chicken meat and surimi, as well as to investigate chemical and microbiological changes in the burgers stored at 2°C for 27 days.

STUDY OBJECTS AND METHODS

Preparation of minced chicken meat and surimi. Fresh chicken and silver carp (*Hypophthalmichthys molitrix* L.) were purchased from a local market in Ahvaz, Khuzestan Province, Iran. The chicken was minced and then kept at –18°C until used. Fresh fish was transported on ice into a laboratory, washed, beheaded, gutted, and filleted. The fillet obtained was thoroughly washed, put through a meat mincer with 4 mm diameter holes (EG-1200-EBS, Jahan Ava, Iran) for 2 min.

The minced fish was washed with a triple volume of water (4°C) and stirred for 10 min. The washed minced fish was filtered through two layers of cheesecloth and then subsequently dewatered by using a manual juicer extractor. Washing was performed three times. The third washing was carried out with 0.5% NaCl (Merck, Germany) solution. A ratio of the minced fish to NaCl was 1:3 (w/w).

After dewatering, the minced fish was mixed with cryoprotectants, i.e. sucrose 3% (Merck, Germany) and sorbitol 3% (Merck, Germany), for 60 s and frozen using a blast freezer. The surimi obtained was kept at –18°C until used.

Preparation of combined burgers and treatments. Before burgers preparation, frozen surimi and minced chicken meat were put in a refrigerator (at 4°C) at night. Meat for burgers was prepared from surimi (63%) and minced chicken meat (37%).

The meat was then blended with toasted flour, 8.2%; wheat flour, 2%; soy flour, 3%; sunflower oil, 1%; freshly grated onion, 7%; garlic powder, 1%; sodium chloride, 1%; white pepper, 0.5%; lemon juice, 1%; and sodium tri-polyphosphate, 0.3% (Merck, Germany).

All the ingredients in combination with 125 mg/L of nisin (Sigma Aldrich, England) were ground through a blender with a 5 mm plate (Gosonic, Turkey) for 4–5 min. Nisin solution, which was added to avoid the growth of *Clostridium botulinum*, was prepared by dissolving a required amount of nisin powder in sterilised 0.02N HCl solution. Burgers (25 g in weight, 50–60 mm in diameter, and 1 cm in thickness) were formed by a burger-maker according to [22].

RSM (response surface methodology) was used to optimise the formulation. The results were analysed using Design Expert 6.0.2 software, and each of the dependent variables in the form of a quadratic regression model was presented as follows:

$$y = \beta 0 + \sum_{i=1}^{k} \beta i\, Xi + \sum_{i=1}^{k} \beta ii\, Xi2 + \sum\sum_{i<j} \beta ij\, XiXj \quad (1)$$

where β0, βi, βii and βij are regression coefficients, and Xi and Xj are coded independent variables. The formula was selected based on the results of the sensory evaluation of the burgers that were stored at 2°C before testing. The test was performed with the help of RSM software.

As control samples were used burgers made without essential oils. They were objected to analyses of proximate composition and cooking characteristics. The control samples included burgers with 100, 300, and

Table 1 Composition of thyme essential oil

Number of component	Component	Retention time, min	Amount, %
1	α-Thujene	15.24	0.49
2	α-Pinene	15.42	2.28
3	Camphene	15.73	0.15
4	β-Pinene	16.37	0.52
5	3-Octanone	16.65	0.82
6	β-Myrecene	16.80	0.91
7	3-Octanol	17.00	0.20
8	α-Phellandrene	17.56	0.15
9	α-Terpinene	18.15	1.20
10	p-Cymene	20.31	16.13
11	Limonene	20.56	0.65
12	1,8-Cineole	21.76	0.92
13	β-ocimene	21.86	0.08
14	γ-Terpinene	22.07	2.43
15	Trans-sabinene hydrate	23.44	0.19
16	Linalool	24.69	6.92
17	Hotrienol	25.75	0.11
18	Borneol	26.84	0.42
19	4-Trpineol	29.16	0.81
20	α-Trpineol	30.63	0.64
21	Thymol methyl ether	32.11	1.51
22	Carvacrol methyl ether	33.13	2.64
23	Thymol	35.88	20.48
24	Carvacrol	36.23	29.61
25	Thymol acetate	37.52	0.13
26	Carvacryl acetate	39.05	0.15
27	β-caryophyllene	41.52	2.37
28	Aromadendrene	42.77	1.18
29	α-humulene	43.23	0.13
30	Allo-Aromadendrene	43.89	0.33
31	Ledene	45.05	0.56
32	Spatulenol	47.24	0.58
33	Caryophyllene oxide	47.96	1.34
Total:			98.03

Table 2 Composition of cumin essential oil

Number of components	Component	Retention time, min	Amount, %
1	β-Pinene	9.362	10.52
2	β-Myrcene	9.796	0.75
3	δ-3-Carene	10.30	0.36
4	α-Terpinene	10.523	1.20
5	o-Cymene	10.855	16.03
6	Phellandral	10.912	0.33
7	1,8-Cineole	10.975	4.90
8	γ-Terpinene	11.856	20.89
9	α-Thujene	12.640	0.21
10	Terpinene-4-ol	15.593	0.75
11	Cuminlaldehyde	17.338	38.48
12	Carvacrol	18.872	0.20
13	Trans-β-Farnesene	22.717	0.26
14	Caryophyllene oxide	25.881	0.10
15	Carotol	26.201	0.58
16	Trans-Caryophyllene	27.870	0.19
Total:			95.75

500 mg/L of both cumin essential oil (Barij Essen, Iran) and thyme essential oil (Barij essence, Iran). Based on the sensory evaluation results, an optimal concentration for each of the EOs was selected.

Tables 1 and 2 demonstrate results of the composition analysis of thyme and cumin EOs. The analysis was carried out by Barij Essence Company (Iran) by means of gas chromatography-mass spectrometry (GC-MS).

The burgers were subdivided into two groups. One group was packaged in high density polyethylene (HDPE) bags under vacuum, and another group – in bags (aerobically), six burgers in each bag. Each group included control, thyme EO and cumin EO samples. The packaged burger samples were stored at 2°C for 27 days. Microbiological and chemical evaluation of three different batches was carried out on day 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 of storage.

Table 3 Experimental design of burgers with thyme and cumin EOs in air and vacuum packaging

Sample	Packaging	
	Air packaging (AP)	Vacuum packaging (VP)
Control (without EOs)	–	–
With thyme EO, mg/L	500	500
With cumin EO, mg/L	500	500

Sensory analysis. Sensory evaluation was performed by a panel of seven experienced (laboratory-trained) judges. To optimise the fish burger formulation, the panellists were asked to evaluate taste, colour, aroma, and overall quality of burgers on a nine-point scale. The scale points were: excellent, 9; very good, 8; good, 7; acceptable, 5–6; unacceptable, 1–4 [23].

Proximate composition. Protein, moisture, ash and fat contents were measured by AOAC method [24].

Cooking characteristics. The thickness and diameter of raw burgers were estimated at room temperature. The burgers were fried in sunflower oil at 170°C for 5 min until an inner temperature of 72°C was reached [25]. Cooking yield, shrinkage and moisture retention were determined by the following equations:

$$\%\ \text{Cooking yield} = \frac{\text{cooked weight}}{\text{raw weight}} \times 100 \quad (2)$$

$$\%\ \text{Shrinkage} = \frac{(\text{raw thickness} - \text{cooked thickness}) + (\text{raw diameter} - \text{cooked diameter})}{\text{raw thickness} + \text{raw diameter}} \times 100 \quad (3)$$

$$\%\ \text{Moisture retention} = \frac{(\text{cooked weight} \times \%\ \text{moisture in cooked burger})}{\text{raw weight} \times \%\ \text{moisture in raw burger}} \times 100 \quad (4)$$

Microbiological analyses. Twenty five grams of burger sample was added into 225 mL of sterile peptone water and blended using a Stomacher lab blender (Interscience Bag Mixer, China) for 1 min. Homogenates of various concentrations were prepared for the microbial test. Cultured Plate Count Agar (PCA) (Merck, Darmstadt, Germany) was incubated at 7°C for 10 days for psychrotrophic bacteria count and at 30°C for 48 h for total viable count (TVC) [26]. Lactic acid bacteria (LAB) were determined on de Man Rogosa Sharpe Agar (MRS) (Q Lab, Canada) incubated at 30°C for 72 h [27]. Sulfite-reducing clostridia were grown on Sulphite Polymyxin Sulfadiazine Agar (Merck, Darmstadt, Germany) [28] incubated at 30°C for 48 h in a plastic anaerobic AnaeroGen sachet (Anaerobic gas pack A, Merck, Darmstadt, Germany). All microbiological analyses were performed in triplicate, and results were expressed as logarithm colony forming unit (log CFU)/g sample.

Mold and yeast were counted on Yeast Extract Agar (Merck, Darmstadt, Germany) incubated at 25°C for 5 days [29]. The experiment was performed in duplicate.

Chemical analysis. pH value was determined using a digital pH meter on the first homogenised concentration of samples (Sartorius, USA) [30]. Total volatile base nitrogen (TVB-N) content was quantified by the method of Malle and Poumeyrol, while thiobarbituric acid (TBA) amount was calculated by the method of Tsironi *et al.* [31, 32].

Peroxide value (PV) was determined according to the method described by AOAC [33]. All chemical analyses were performed in triplicate.

Statistical Analysis. Statistical analysis was carried out with the help of SPSS 19 (SPSS, 2010) software

Table 4 Average scores of sensory characteristics of burger samples (surimi percentages predicted by RSM)

Surimi, %	Colour	Taste	Aroma	Texture	Overall quality
50	7.17	6.00	7.57	5.43	6.14
100	7.57	5.14	8.29	7.71	5.43
75	7.83	5.57	7.00	6.14	5.71
0	7.85	5.71	7.14	6.86	5.71
100	7.42	5.29	8.14	7.57	5.57
0	7.71	5.57	7.28	6.71	5.85
25	7.30	5.57	8.28	5.43	5.71

Table 5 Average scores of sensory characteristics of burger samples for selecting proper concentrations of cumin and thyme EOs

Essential oil	Concentration, mg/L	Taste	Aroma
Cumin EO	100	5.71	7.71
	300	6.43	7.29
	500	6.43	8.14
Thyme EO	100	6.86	7.00
	300	7.14	7.71
	500	7.00	8.14

and one-way variance. Results were expressed as mean values and standard deviation (S.D.). Analysis of variance (ANOVA) data were subjected to determining significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Sensory analysis. Average scores of sensory characteristics were evaluated using RSM method. The results of the analysis are shown in Table 4. The optimal burger formulation was selected, which contained 63% of surimi and 37% of minced chicken meat. Also, based on average scores of sensory evaluation, a concentration of 500 mg/L for each EO was selected as optimal (Table 5).

Proximate analysis. Proximate composition was performed in burgers made without EOs before storage. Samples had moisture of 70.40% and contained 19.98% of protein, 4.27% carbohydrate, 3.35% fat, and 2.0% ash. Our results are in good agreement with those obtained by Vanitha *et al.* [34].

Cooking characteristics. The cooking characteristics of samples with no EOs were determined before storage. Cooking yield, shrinkage, and moisture retention were found to be 94.73, 10.19, and 80.98%, respectively. These data are in accordance with those of Heydari *et al.*, who measured cooking properties in camel burgers during freezer storage [25].

Microbiological analysis. Analysis of variance showed that both packaging and EOs used had a significant effect on the microbial characteristics of burgers ($P < 0.001$).

Figure 1a demonstrates changes in TVC of the burgers under study during storage. Results indicate a significant effect ($P < 0.001$) of storage time, EOs addition and packaging conditions on TVC. The maximum TVC value obtained (10^7 CFU/g) was acceptable for fresh and frozen fish [35].

The initial (day 0) TVC of burgers in air packaging, with and without the EOs, was 4.05–4.38 log CFU/g. For burgers in vacuum packaging, with or without cumin/thyme EO, these values were 4.46–4.82 log CFU/g. These results are consistent with those obtained by Cózar *et al.* for fish burgers (4 log CFU/g) and indicate a good burger quality [36]. Eventually, by day 27, TVC was 8.39–8.78 and 6.13–6.74 log CFU/g in air packaged and in vacuum packaged burgers, respectively.

As one can see in Fig. 1a, burgers with thyme EO in vacuum packaging demonstrated the least microbial growth, which indicates inhibitory properties of thyme EO. Similar results were found in an edible film containing 0.10% of oregano and 0.15% of thyme EO in fresh chicken sausages [17, 21].

Initial counts of psychrotrophic bacteria in samples in air and vacuum packaging were 4.34–4.76 log CFU/g, which reached 7.04–8.79 log CFU/g by day 27 of storage (Fig. 1b).

Kilinc *et al.* observed an increase in TVC and psychrotrophic bacteria count in sardine patties from 2.50 and 2.60 log CFU/g to 6.72 and 6.98 log CFU/g on day 7 of storage [37]. According to Pavelková *et al.*, the initial TVC value in control chicken breast was 4.72 log CFU/g, while after 18 days of storage at 4 ± 0.5°C, it was 3.68 and 4.05 log CFU/g for samples with oregano and thyme EOs in vacuum packaging [38].

In our research, thyme EO acted as a synergist to vacuum packaging, combinations of air packaging + cumin EO and air packaging + thyme EO were less effective in inhibiting microbial growth. Soni *et al.* also reported lower psychrophilic bacteria counts in chicken patties containing 0.10% of oregano and 0.15% of thyme Eos [21]. Similar results were obtained by Sharma *et al.* in fresh chicken sausages during storage [17].

This inhibitory effect was also apparently due to large amounts of phenolic substances and flavonoids in thyme and cumin EOs.

Initially, a lacto acid bacteria (LAB) amount was 3.16 log CFU/g. By the end of the storage, it was recorded to be 7.47–7.98 for burgers in air packaging and 4.15–4.40 log CFU/g for those in vacuum packaging (Fig. 1c). In [39], the initial LAB concentration in control minced goat meat was 2.75 log CFU/g, which increased to 6 log CFU/g by the end of vacuum storage at 4°C. Also, Fratianni *et al.* reported that thyme essential oil decreased total viable bacteria count and lactic acid bacterial growth in chicken breast; total microbial content reduced down to 50% compared to the control samples [40].

In the work of Pavelkova *et al.*, the LAB count in a control chicken breast fillet was within the range from 4.31 (day 3) to 2.62 log CFU/g (day 15), while the best result was observed in the vacuum packing + thyme EO

group (the highest count was 4.29 log CFU/g, on day 3, and the lowest count was 1.43 log CFU/g, on day 6) [38]. The authors found that addition of 0.20% (v/w) of thyme EO and storage of samples in vacuum allowed shelf life of the chicken breast fillet to be extended.

Clearly, it can be concluded that vacuum packaging inhibits LAB growth. Of the samples examined in this study, the vacuum packaging + thyme EO sample had the maximum impact on the LAB growth. LAB are one of the main components of meat product microflora that decreases pH of meat product through carbohydrate fermentation [41].

We found that, due to the antibacterial properties of cumin and thyme EOs, the shelf life of burgers with the EOs in vacuum packaging increased. The cause of that can be the presence of phenolic compounds such as thymol and carvacrol in thyme and cuminaldehyde in cumin.

In this study, initial mold and yeast counts were approximately 2 log CFU/g and reached 6.49–6.95 and 2.03–3.08 log CFU/g in samples stored in air and vacuum packaging, respectively (Fig. 1d). Lower mold and yeast counts in test samples compared to control indicates the presence of EOs antifungal constituents in meat products [42].

As for sulfite-reducing clostridia, they were not detected in any of the samples throughout the storage peroid.

Chemical analysis. Figure 2a demonstrates a significant decrease in pH values of control and treated samples during storage ($P < 0.001$). The initial pH value in burger samples was 6.41. By day 27, their pH values were 4.34–4.53 for all samples in air packaging and 4.71–4.98 for all samples in vacuum packaging. This decrease can be due to a reduced oxygen content as a result aerobic microflora growth and CO_2 production. Another cause of the pH decrease can be sugar contained in the burgers, which is utilised as a cryoprotectant.

According to Bingol and Ergun, pH diminishes by the end of storage [43]. They also reported that the pH of meat is influenced by various factors however the

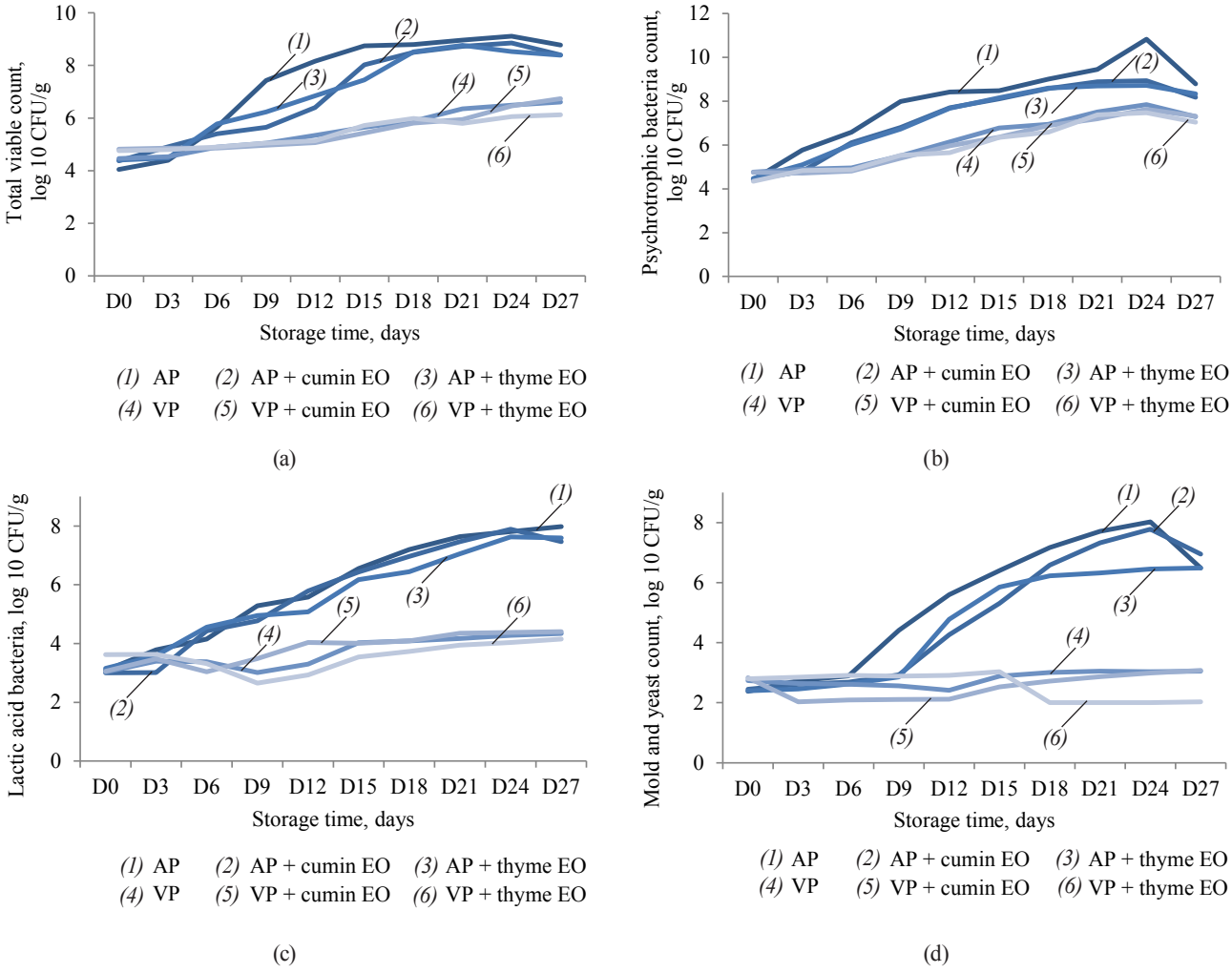

Figure 1 Effects of vacuum packaging (VP) and thyme and cumin essential oils on: (a) TVC, (b) psychrophilic count, (c) LAB, and (d) mold and yeast count in burgers stored at 2°C

major one is lactic acid bacteria growth resulted from lactic acid production. Similar results were also obtained by Soni *et al.* in regard to chicken patties stored at refrigerator temperature [21].

Total volatile base nitrogen (TVB-N) content is often used as an index to determine a degree of meat decomposition. As one can see in Figure 2b, TVB-N values of burgers increased significantly during storage ($P < 0.001$). TVN concentration was determined to be between 5 and 25 mg N/100g [44].

TVB-N content was the highest ($P < 0.001$) in samples in air packaging, which indicates that air packaging alone, even without EOs, can significantly increase TVB-N formation. Erkan investigated TVB-N in vacuum-packaged filleted hot smoked rainbow trout [45]. By day 27 of storage at 2°C, the TVB-N content increased to 33.82 and 24.16 mg/100 g flesh in untreated and treated with thyme EO samples, respectively. Also, Eskandari *et al.* reported that a TVB-N value in fish samples treated with black cumin remained below its acceptable limit by day 27 [46].

According to hygienic standards, the TVB-N acceptable limit in fish muscle is 20 mg/100 g. Thus, the results of this study demonstrated that TVB-N values in vacuum packaged samples with thyme and cumin EOs were below the limit during storage.

Fat oxidation is the main cause of fish putrefaction; an increasing amount of thiobarbituric acid (TBA) and peroxide leads to rancidity. A steady increase in TBA in burgers was observed during 27 days of storage (Fig. 2c). Vacuum packaging effectively protected the burgers from zero days, keeping TBA scores lower than 1 mg MDA/kg during the storage period. EOs in the combination with vacuum packaging displayed a positive effect on the inhibition of oxidation. Köse *et al.* found that a TBA level in surimi was acceptable up to day 15, while a TVB-N concentration reached 38.2 mg/100 g by day 13, which exceed the limit of acceptability [5].

Karabagias *et al.* reported that thyme did not protect lamb meat in air packaging from oxidation, at least not within its normal shelf life [47]. This finding is in contrast to the results of Botsoglou *et al.* who observed a three-fold reduction in a degree of lipid oxidation in turkey in air packaging [48].

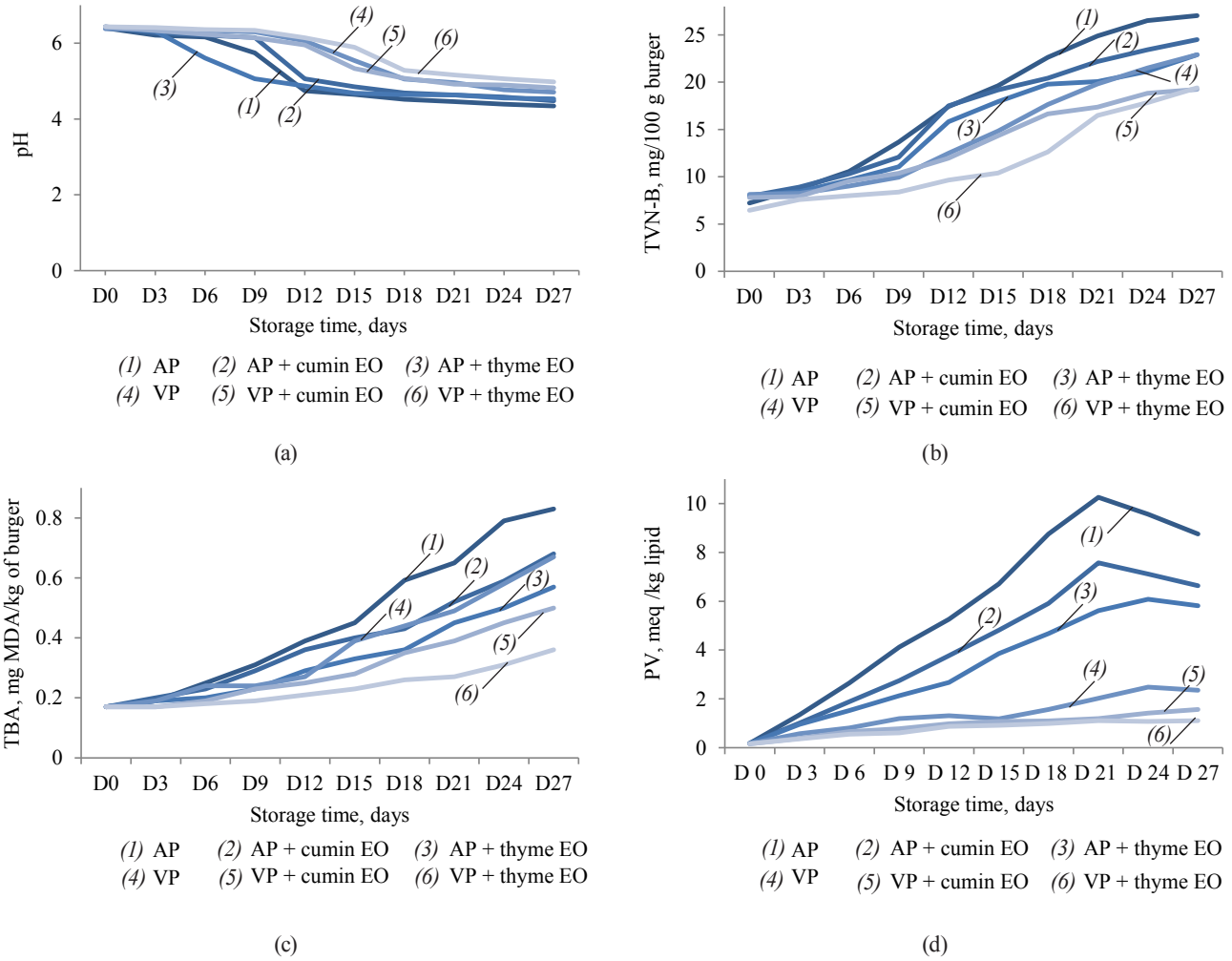

Figure 2 Effects of vacuum packaging (VP) and thyme and cumin essential oils on: (a) pH, (b) TVN-B, (c) TBA, and (d) PV of burgers at 2°C

According to Liu *et al*., TBA increased from 0.16 mg/kg (day 0) to 0.42 mg/kg (day 35) in samples stored at –1°C. In [45], the initial TBA index value for hot smoked rainbow trout fillets was 0.77 mg MDA/kg and reached 1.5 mg MDA/kg by day 27. The lower production of TBA in vacuum packing + thyme samples can contribute to the antioxidant properties of thyme oil. Soni *et al*. noticed lower TBA values in chicken patties containing 0.10% of oregano and 0.015% of thyme EOs. Jayawardana *et al*. suggested that a cause of the reduction of TBA values could be polyphenols present in EOs [49].

In our research, TBA values did not exceed the acceptable limit in all samples. Similar results were obtained by Eskandari *et al*. in fish treated with black cumin [46]. Therefore, TBA cannot be used as a reliable quality index for burgers. TBA of 2–4 mg MDA/kg indicates a good quality of fish. TBA values in this study were lower than 1 mg MDA/kg in all treatments throughout the storage period. It was apparently due to a relatively low fat content in fish (surimi).

We revealed that the antioxidant properties of cumin and thyme EOs prolonged significantly the burger shelf life. Sarıçoban and Yilmaz also confirmed the antioxidant effect of cumin and thyme on TBA, which is due to the antioxidant activity of phenolic compounds contained in different parts of plants [44]. The main compounds of cumin are gammaterpinene, 2-methyl-3-phenyl-propanal, myrtenal, and glucopyranosides [44].

Figure 2d demonstrates an effect of packaging and thyme and cumin EOs on a PV value in the burgers under study. The initial PV value was 0.16–0.18 meq/kg of lipid in all the burgers, while, by day 27, it reached 5.82–8.75 and 1.11–2.35 meq/kg of lipid in samples in air and vacuum packaging, respectively ($P < 0.001$).

In this study, PV was increasing up to day 21 of storage in all samples and then, by day 27, decreased. At the end of the storage time, PV in all vacuum-packaged samples did not reach the acceptable limit (5 meq/kg). Similar findings were obtained by Çoban and Keleştemur in catfish burger treated with thyme [50]. Such findings are an evidence of EOs inhibitory effect on microorganisms which cause burger spoilage. The reduction of PV after day 21 can be due to hydroperoxide degradation. The decay of hydroperoxides results in the formation of degradation products [51]. The reduction in PV in samples with cumin EO can be due to cumin aldehyde, which prevents lipid peroxidation [52].

CONCLUSION

We found that the shelf life of the novel burgers from surimi and minced chicken meat could be extended by using essential oils and vacuum packaging. According to the results of the microbiological analysis, the shelf life of the burgers was as follows: 9 days for burgers in air packaging, 12 days for burgers with cumin and thyme EOs in air packaging, 18 days for burgers in vacuum packaging, and 21 days for burgers with cumin and thyme EOs in vacuum packaging.

The shelf life for vacuum-packed burgers treated with thyme and cumin EOs was established as 18 days at 2°C, in compared to that for untreated burgers, which was 6 days. In addition, vacuum packaging alone was found to maintain burger freshness during 15 days.

Thus, burger shelf life was extended by 9 days for the combination of thyme/cumin EO + air packaging, 15 days for vacuum-packaged samples, and 18 days for the combination of thyme/cumin EO + vacuum packaging. Overall, the combined use of vacuum packaging and thyme/cumin EO demonstrated their synergistic effect on the shelf life of the novel burgers. These results allowed us to suggest that surimi could be successfully used as an alternative ingredient to minced meat in burgers production.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Effects of encapsulated black caraway extract and sesame oil on kolompeh quality

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Abstract: In this study, the physicochemical and sensory properties of kolompeh containing black caraway and sesame oil were investigated. Black caraway extract (BCE), encapsulated black caraway extract (EBCE), and black caraway powder (BCP) were added to kolompeh and compared to the sample without black caraway (FBC). All products contained sesame oil and were compared to control (without sesame oil). Among the samples, kolompeh with encapsulated extract demonstrated a higher oxidative stability (24.37 h), with a high IC_{50} of black caraway extract (124.1 μg·mL^{-1}). In addition, the emulsion exhibited size distribution between 3.20 and 8.51 μm, and Fourier transform infrared spectroscopy confirmed the well encapsulated extract. Gas chromatography identified oleic and linoleic acids as the main fatty acids in kolompeh with the black caraway encapsulated extract. Although, there were no significant differences in the colour parameters (L*, a* and b*) of the samples, kolompeh with EBCE had the highest score given by panelists. The control had a higher (2466 g) hardness compared to kolompeh containing EBCE (1688 g) at the end of storage. Therefore, the encapsulated extract of black caraway not only had no an adverse effect on the properties of kolompeh but also improved its quality.

Keywords: Kolompeh, black caraway extract, encapsulation, sesame oil, antioxidant activity, sensory properties

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INTRODUCTION

Kolompeh is an Iranian date-based cookie baked traditionally by local citizens, especially in Kerman, and industrially produced in Kerman and other parts of the country. This cookie has a high nutritional and energy value and includes date paste, walnut, wheat flour, butter, and eggs as the main ingredients. Pistachios or sesame powder are often used for decorating kolompeh [1].

Fats and oils, being important components of kolompeh, help soften the texture, maintain the moisture, improve the flavour, and preserve the quality of the product [2]. Their oxidation and microbial degradation leads to the reduced sensory characteristics and shelf life of the product [3]. Compounds resulted from oxidation cause rancidity. To prevent the oxidative deterioration, antioxidants have been widely used [4]. Thus, natural antioxidants, such as aromatic plants and spices, have gained their popularity in the bakery industry; they preserve bakery products from oxidation and microorganism spoilage, extend their shelf life, and have therapeutic benefits [5]. Antioxidant activity of black caraway has been proved by Kamkar [6].

In recent years, there has been a tendency to use encapsulation for improving the delivery of bioactive agents. Therefore, the application of microencapsulation in food and agricultural industries can contribute to such characteristics of food as sensory properties, especially texture, and their stability during shelf-life. In addition, encapsulation can also amend the water solubility, thermal stability, and oral bioavailability of bioactive compounds [7].

This fact stimulates the development and production of new products. Among the various methods of encapsulation, considerable research efforts have been applied to emulsion based encapsulation of different sensitive materials [8]. This method aims to improve the chemical stability during processing and storage, to protect from degradation, and to keep the release of bioactive molecules under control [9].

To our knowledge, the use of sesame oil and encapsulated extract of black caraway in kolompeh have not been studied. Thus, the purpose of this study was to evaluate an effect of black caraway and sesame oil on the physical and sensory properties of kolompeh. The kolompeh contained sesame oil and different forms of black caraway, including powder, extract, and encapsulated extract.

STUDY OBJECTS AND METHODS

Materials. Wheat flour, dates, black caraway (*Bunium persicum* L.), and flavouring ingredients were purchased from local market in Shiraz, Fars, Iran. *Saccharomyces cerevisiae* lyophilised powder (PTCC 5269) was supplied by Arya Toos Co., Mashhad, Khorasan, Iran. DPPH and other chemical reagents were obtained from Merck Co., Darmstadt, Germany.

This research was conducted at the Fars Agricultural and Natural Resources Research and Education Center.

Extraction. The extraction was carried out by the method of Upadhyay *et al.*, with some modifications [10]. Ten grams of grounded black caraway was added to 100 mL of distilled water and kept in a water bath for 45 min. Then the slurry was cooled at room temperature and filtered to obtain a clear extract for analyses.

Encapsulation of BCE. Microencapsulation of black caraway extract was performed using W/O emulsion based on the method given by Tran, with some modifications [11]. The oil phase of the W/O emulsion was prepared by adding glycerol monostearate (GMS) with HLB 3.8 (1.5 wt%) to canola oil and shaking at 4000 rpm and 70°C. The aqueous solution containing black caraway extract was heated to 40°C. The W/O emulsion (10:90) was prepared by blending the aqueous phase and the oil phase at 27000 rpm and 70°C for 2 min. Then the suspension was cooled while stirring with a magnet at 1000 rpm for 2 h and kept for 30 min to precipitate microcapsules. Finally, the suspension was centrifuged at 350 g for 10 min (4°C). The precipitate was washed with saline twice and filtered. The microcapsules obtained were stored in a refrigerator until usage.

Kolompeh preparation. Five formulations of kolompeh were developed (Table 1).

The following kolompeh samples were prepared: with 2.5% of encapsulated black caraway extract, with 0.25% of black caraway extract, with 0.4% of black caraway powder, and without (free) black caraway. All of them included sesame oil. To investigate an influence of sesame oil on the kolompeh properties, control sample was prepared with canola oil instead of sesame oil. Kolompeh was made by mixing wheat flour, yeast and oil and keeping for 30 min for proofing. Then kolompeh was formed, minced date with flavouring ingredients were put in the centre of the dough, and the samples were baked in an oven at 150°C for 30 min.

Antioxidant activity. Radical scavenging activity of black caraway extract against stable DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) was measured with a spectrophotometer. DPPH methanol solution (0.1 mmol·L^{-1}) had been prepared just before measurements. Two millilitre of the extract with different concentrations was mixed with 2 mL of 0.004% methanol solution. The samples were kept in dark room for 15 min, and then the absorbance of the solution resulted was measured at a wavelength of 517 nm. Blank sample contained 2 mL of methanol and 2 mL of DPPH solution. The experiment was conducted in triplicate. The antioxidant activity was calculated as a percentage of the radical scavenging activity [12]. Finally, the concentration of sample needed to inhibit 50% of radical scavenging activity (in mg·mL^{-1}) was appointed and demonstrated as IC_{50} value [13].

Oxidative stability. The oxidative stability measurement was performed using a Rancimat instrument (Metrohm, Herisau, Switzerland) by heating 3 g of sample at a temperature of 110°C and the air flow rate of 20 L·h^{-1}.

Particle size distribution. The mean particle size of the microcapsules was determined by dynamic light scattering technique at ambient temperature (Nano

Table 1 Kolompeh formulations

Ingredients	Samples				
	with EBCE	with BCE	with BCP	FBC	Control
Wheat flour, g	1000	1000	1000	1000	1000
Vegetable oil (combination of hydrogenated soybean and palm oil), g	–	–	–	–	500
Sesame oil, g	500	500	500	500	–
Date, g	1000	1000	1000	1000	1000
Saccharomyces cerevisiae, %	3	3	3	3	3
Flavorring ingredients, g	30	30	30	30	40
EBCE, %	2.5	–	–	–	–
BCE, %	–	0.25	–	–	–
BCP, %	–	–	0.4	–	–

EBCE is encapsulated black caraway extract
BCE is black caraway extract
BCP is black caraway powder
FBC is without (free) black caraway

Particle Analyzer Malvern 2000, Worcestershire, UK). In order to measure the particle size of the produced powder, a small quantity of powder was dissolved in 2-propanol, and then a few drops were added to the water containing reservoir of the apparatus [14].

Morphology. The morphological characteristics of encapsulated black caraway extract were determined by optical microscopy (Olympus BX51, Japan).

Fatty acid compositions. Fatty acid analysis of kolompeh samples was performed using the Alavi and Golmakani method [15]. First, fatty acids were converted to fatty acid methyl esters by shaking 60 mg oil with a mixture of 3 mL of hexane and 0.3 mL of 2 mol·L^{-1} methanolic potassium hydroxide. Then, atty acids were analysed by gas chromatography (GC) using a SP-3420 gas chromatograph (Beijing, China) coupled to a flame ionisation detector (FID) and a BPX-70 fused silica capillary column (30 m × 0.25 mm; 0.25 μm film thickness). N_2 with the split ratio of 1:10 was used as carrier gas. The temperature of the injector and the detector was 250 and 300°C, respectively. The oven temperature was increased from 140 to 200°C as follows: the temperature of 140°C was maintained for 5 min, then it was increased to 180°C by 20°C/min and remained constant for 9 min, and, finally, the temperature was increased to 200°C by 20°C/min and maintained for 3 min. Fatty acids were identified by comparing their retention times with standard values. The results were expressed as percentage of relative peak area.

Fourier transform infrared spectrometry (FTIR). FTIR spectroscopy was performed to analyse functional groups and to provide an insight into the structural characteristics of the samples. The spectrum was recorded on a Perkin-Elmer Spectrum RXI spectrophotometer (USA). All spectra were recorded at a wavelength of 4000–400 cm^{-1}.

Texture profile analysis. A CT3 4500 texture analyser (Brookfield, USA) was used to determine hardness of samples. An aluminum TA25/1000 probe was used. The samples were compressed twice (TPA test). The probe speed was considered in a compression condition of 0.5 mm·s^{-1} and a cavity depth of 5 mm. The experiments were carried out in triplicate at 25°C [16].

Colour analysis. The colour analysis was performed using a Hunter Lab model Colorflex colorimeter (USA). Lightness (L*), redness (a*), and yellowness (b*) colour parameters of kolompeh samples were obtained using Photoshop software (CS3) [17].

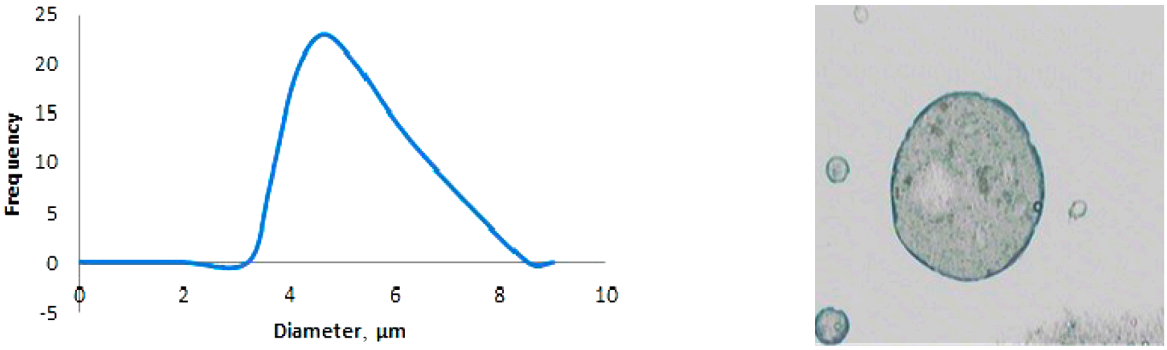
Sensory assessment. Sensory evaluation of kolompeh was conducted by thirty trained panelists with the help of a 5-point hedonic scale (5 = like extremely, 1 = dislike extremely), following the method described by Carpenter [18]. Such quality attributes as colour, aroma, flavour, tenderness, and overall acceptability were evaluated. The panelists were then served with pieces of kolompeh in individual booths under white fluorescent light, together with cold water to clean the palate between samples. The descriptors rated from 1, the lowest score, and 5, the highest one.

Statistical analysis. The data were analysed using analysis of variance (ANOVA) at $P < 0.05$. Duncan's Multiple Range test was conducted by SAS software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Antioxidant activity. According to the data, IC_{50}, or the inhibition concentration of 50% of the DPPH free radical activity of black caraway extract, was 124.10 μg·mL^{-1}, which was much higher than that of TBHQ.

Various studies had been investigated the antioxidant activity of black caraway extract. Nickavar *et al.* investigated the antioxidant properties of alcoholic extracts of seven medicinal plants belonging to *Umbelliferae* family, including black caraway [19]. The results showed that this species had the IC_{50} value of 149.9 μg·mL^{-1}, which was more than that in the present study. Another research group, Souri *et al.*, observed the IC_{50} values for black caraway extract to be 82.25 μg·mL^{-1} [20]. Also, 120.43 μg·mL^{-1} was reported by Kamkar *et al.* [6].



(a) (b)

Figure 1 Initial droplet size distribution of emulsion (a), and optical microscopy image (400×) and surface morphology of EBCE microcapsules (b)

The differences observed in the antioxidant activity of black caraway in different studies could be due to the differences in the composition of these plants. Additionally, important factors are genetics, weather, harvest season, the type of solvent used for extraction, etc. Further, there is a direct relationship between the phenol content and the antioxidant activity of medicinal plants [13].

Oxidative stability. According to the results, the induction time of black caraway encapsulated extract, black caraway extract, and black caraway powder were 24.37, 23.15, and 21.88, respectively. The kolompeh containing encapsulated extract showed better oxidative stability compared to other treatments. The high oxidative stability of samples was attributed to the antioxidant activity of black caraway extract. The higher oxidation stability of encapsulated extract showed the protective influence on the encapsulation process [21]. Though, it should be taken into consideration that canola oil is exposed to oxidation because of a high amount of unsaturated fatty acids in their composition [22].

Particle size distribution. Figure 1 demonstrates the particle size distribution of microencapsulated black caraway extract at different frequencies.

According to Fig. 1, the maximum and minimal particle sizes were 3.20 and 8.51 μm, respectively. A few investigations had been carried out in the field of canola oil as wall material of microencapsulated particles. Abraham *et al.* observed a lower particle size of emulsion based on canola oil, compared with this study (> 1μm) [23]. Mohammadi *et al.* and Davidov-Pardo *et al.* used soybean oil, phosphatidylcholine/cholesterol, soy lecithin, and grape seed oil with orange oil to encapsulate olive leaf extract, *Myrtus communis* extract, polyphenolic extract of grape seed, and resveratrol, respectively [24, 25]. According to their results, particle size was also lower than that in our research (> 0.5 μm). This difference might be due to the type and duration of encapsulation, as well as due to the rate of homogenisation, which determines particles size.

Optical microscopy. The structural characteristics of the microcapsules were depicted by optical microscope. The morphology of a microcapsule of encapsulated black caraway extract is illustrated in Fig. 1. The microcapsule had global and monotonous appearance with no aggregation. This observation was in agreement with Abraham *et al.* [23]. However, a low surfactant-to-emulsion ratio plays an important role in smoothly surface of particles, as was observed in resveratrol encapsulated with grape seed oil and orange oil [25]. Bylaitë *et al.* investigated the encapsulation properties of caraway essential oil by spray drying [26]. They used whey protein and maltodextrin as a wall material and observed some holes on surface of the sample encapsulated with whey protein concentrate. They suggested that whey protein concentrate had an inverse effect on surface dents; on the other hand, skimmed milk powder smoothes out wrinkles.

FTIR. Figure 2 plots FTIR spectra of black caraway extract and encapsulated black caraway extract at 400–4000 cm^{-1}.

According to the FTIR spectra analysis (Figs. 2a and 2b), both BCE and EBCE demonstrated bands at 3400 and 3427 cm^{-1}, which are assigned to vibration of O-H in the sugar units. The bands ranged from 3200 to 2800 cm^{-1} (3009, 2926, 2925, 2855 cm^{-1}) indicated the stretching hydrogen bands in C-H, and a broad band at 1746 cm^{-1} exhibited the C=O stretching of the ester carbonyl functional group [27]. This region is related to the triglycerides absorption bands [28]. A new band at 1608 cm^{-1} was found in the encapsulated extract, suggesting intermolecular interactions between C=C and the hydrocarbon chain of unsaturated fatty acid segments such as C18:1, C18:2 and C18:3 in canola oil [29].

We also recorded the other characteristic bands, such as 1461 and 1408 cm^{-1} (bending vibration of CH_2 and CH_3 aliphatic groups), as well as 1261, 1239, 1162, 1119, 1097, and 1053 cm^{-1} (stretching vibration of the C-O ester groups). They are in agreement with the results of Waterhouse *et al.* [30]. The last finger print region of FTIR spectra between 888 and 723 cm^{-1} wavelength frequencies was ascribed to the CH_2 rocking vibration and the out-of-plane vibration of *cis*-disubstituted olefins [28].

Overall, both samples displayed similarity in spectra. However, there were some differences between two spectra with sharp peaks at a wavenumber of 1200–1000 cm^{-1} and small absorption bands at around 850–400 cm^{-1}. They are associated to the intermolecular bonding of functional groups in polysaccharides [31].

Fatty acid compositions. Fatty acid composition of kolompeh samples is presented in Table 2.

According to the GC fatty acids profile, linoleic and oleic acids were detected as the main fatty acids in kolompeh with the extracts. The product with black caraway powder also was rich in unsaturated fatty acids, with a high amount of linoleic acid (44.15%). Similar results were observed in the sample with no black caraway, with linoleic and oleic acid content of 44.86 and 37.32%, respectively. As Egorova *et al.* reported, linoleic acid is the most important fatty acid of caraway [32].

In the control sample, the main saturated and unsaturated fatty acids were represented by palmitic acid (35.45%) and oleic acid (37.34%), while linolenic acid was found at low concentrations (19%). Further, the analysis of fatty acid profile showed lack of lauric and palmitoleic acids in kolompeh containing black caraway extract, which is in accordance with the results of Laribi *et al.* [33].

In addition, a trace of lauric acid was found in the encapsulated extract sample, which may be due to petroselinic acid contained in some types of caraway seed oil. Petroselinic acid is a main component for oleochemical processes that converts easily into lauric and adipinic acid [34]. All samples were rich in unsaturated fatty acids, compared to the saturated

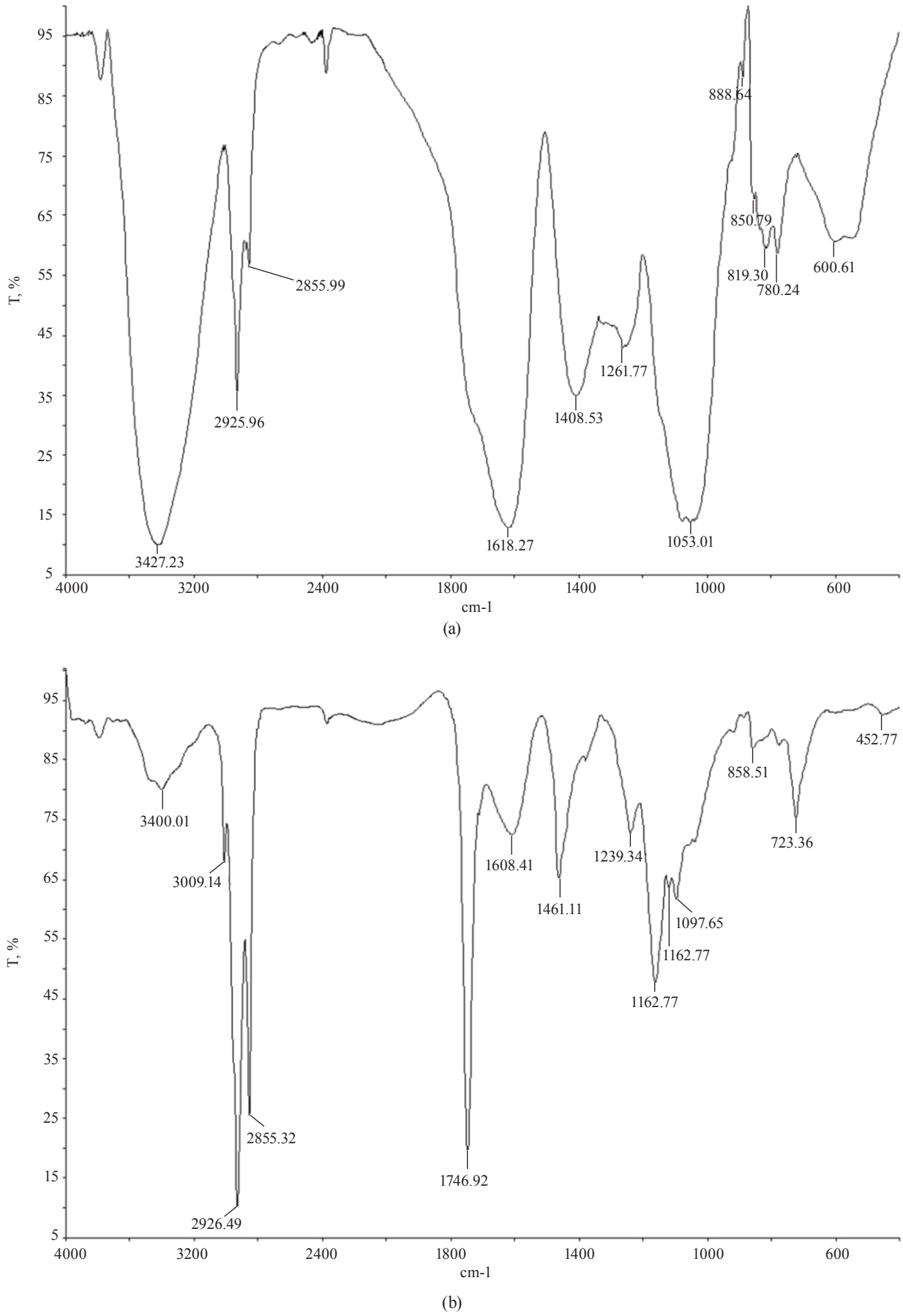


Figure 2 Fourier transform infrared spectra of BCE (a) and EBCE (b)

Table 2 Fatty acid composition of kolompeh samples

Fatty acid, %	Samples				
	with EBCE	with BCE	with BCP	FBC	Control
Lauric acid (C12:0)	1.77	–	2.31	–	–
Myrisric acid (C14:0)	2.36	2.77	0.39	1.47	1.24
Palmitic acid (C16:0)	11.12	10.73	11.58	11.64	35.45
Palmitoleic acid (C16:1)	0.18	–	–	–	–
Stearic acid (C18:0)	3.98	4.04	3.15	4.13	8.34
Oleic acid (C18:1)	36.13	37.03	37.82	37.32	37.34
Linoleic acid (C18:2)	43.38	44.99	44.15	44.86	16.76
Linolenic acid (C18:3)	0.29	0.36	0.40	0.12	0.19
Arachidonic acid (C20:0)	0.54	0.10	0.10	0.17	0.30
Behenic acid (C22:0)	0.24	0.08	0.08	0.30	0.38

EBCE is encapsulated black caraway extract
BCE is black caraway extract
BCP is black caraway powder
FBC is without (free) black caraway

analogues, which is related to sesame oil in their composition. Sesame seed oil belongs to the oleic–linoleic acid group [35]. Thus, as expected, oleic and linoleic acids were prevalent fatty acids in the kolompeh samples containing sesame oil.

Arachidonic, stearic and behenic acids were also found in trace amounts in the kolompeh containing black caraway extract and powder, which is in agreement with Nzikou *et al.* [36]. However, the samples with sesame oil were characterised by a low content of palmitic acid compared to the control.

Hardness. Figure 3 demonstrates the hardness of the samples during storage.

The results illustrated that the hardness of the products under study was decreasing during storage, except for kolompeh without black caraway, which had no significant differences in hardness. The highest and lowest hardness by the end of storage had the samples with the powder and encapsulated extract, respectively. All the samples, excluding the kolompeh with the powder, displayed lower hardness than the control. This phenomena may be related to the high density of black caraway powder [2].

In spite of the fact that the use of plant extracts in kolompeh still has not been investigated, there are data about increasing hardness of samples during storage. Budryn *et al.* mentioned that covalent interaction of polyphenols and proteins resulted in an enhancement in hardness, which was contrary to the results of this study [37]. To our opinion, there are two causes for this.

First, the presence of mono and di-glycerides in sesame oil, with their emulsifying properties, caused a reduction in the hardness of the product. Thus, they are able to make starch complex and delay staling [38]. The second cause for enhancing of hardness is related to the presence of saccharides limiting interactions between polyphenols and proteins [37]. The reducing of hardness in the kolompeh with the extract can be explained by rivalry between fibres and polyphenols and wheat starch in the dough. In addition, the sample with EBCE was softer than that with BCE because of encapsulation, which protected the sample against the direct exposure of polyphenols and starch.

Colour. Table 3 illustrates the colour properties of kolompeh samples during storage.

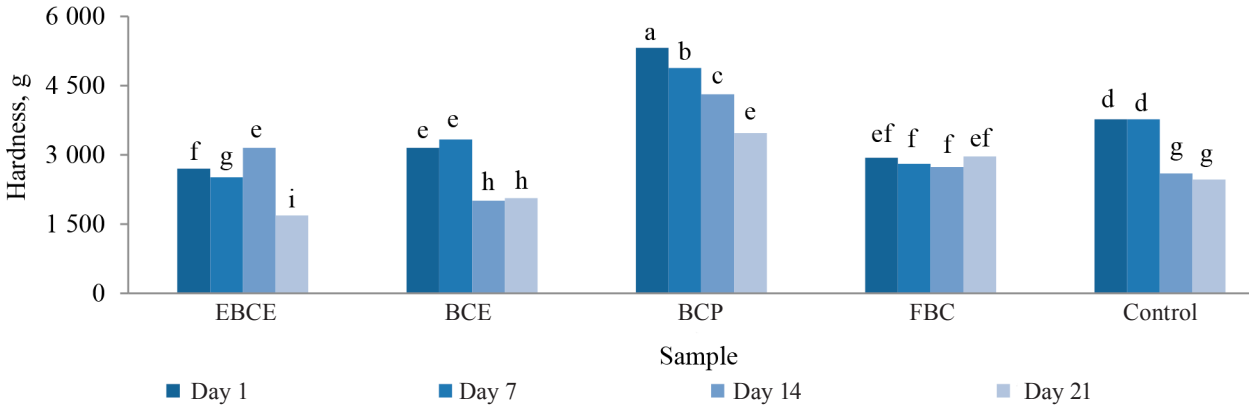


Figure 3 Hardness of kolompeh samples during storage

Table 3 Colour characteristics of kolompeh samples on day 1, 7, 14, and 21 of storage

Sample	Colour parameter	1	7	14	21
EBCE	L*	43.3 ± 52.1^{a}	53.2 ± 42.2^{a}	49.2 ± 44.9^{ab}	48.3 ± 1.3^{a}
	a*	–7.0 ± 46.6^{c}	2.0 ± 88.5^{a}	–0.0 ± 0.1^{b}	5.0 ± 22.8^{ab}
	b*	24.1 ± 23.9^{a}	27.2 ± 44.3^{ab}	33.0 ± 11.8^{b}	35.1 ± 11.1^{a}
BCE	L*	57.3 ± 88.8^{a}	65.1 ± 22.5^{a}	71.4 ± 22.3^{a}	63.3 ± 88.6^{a}
	a*	–3.0 ± 41.8^{a}	2.1 ± 88.1^{a}	1.0 ± 66.3^{ab}	5.0 ± 88.6^{a}
	b*	26.2 ± 36.2^{a}	31.1 ± 55.8^{ab}	41.2 ± 11.3^{a}	35.2 ± 11.5^{a}
BCP	L*	58.3 ± 34.8^{a}	58.3 ± 62.3^{a}	55.4 ± 87.8^{b}	45.1 ± 23.9^{b}
	a*	–5.0 ± 74.8^{b}	–3.0 ± 33.5^{b}	2.0 ± 55.3^{a}	1.0 ± 44.4^{b}
	b*	26.2 ± 30.6^{a}	24.1 ± 44.3^{b}	37.1 ± 66.5^{ab}	32.2 ± 21.1^{a}
FBC	L*	60.1 ± 94.1^{a}	74.4 ± 22.3^{a}	71.2 ± 88.8^{ab}	71.2 ± 88.5^{a}
	a*	–5.1 ± 56.2^{b}	–1.0 ± 77.1^{b}	0.0 ± 1.1^{ab}	1.0 ± 33.4^{b}
	b*	26.2 ± 19.3^{a}	30.1 ± 33.2^{ab}	32.0 ± 44.9^{b}	34.1 ± 33.5^{a}
Control	L*	43.1 ± 82.4^{a}	43.2 ± 44.1^{a}	43.1 ± 11.5^{ab}	45.0 ± 43.9^{b}
	a*	–8.0 ± 19.5^{c}	2.0 ± 33.7^{a}	0.0 ± 0.1^{b}	5.1 ± 11.8^{a}
	b*	24.0 ± 9.7^{a}	29.1 ± 44.1^{ab}	34.0 ± 22.1^{b}	33.1 ± 44.2^{a}

*Means with different letters are significantly different ($P < 0.05$). Each value is expressed as Mean ± SD. Test was conducted in triplicate

Colorimetric analysis showed that the L* value of all the samples, except BCP, increased during the storage period (21 days). The encapsulated sample had a lower lightness compared to the others, which might be probably due to a more intense yellow colour of canola oil used as a wall material in the encapsulation process [39]. In addition, as expected, the sample without carway (FBC) was lighter than the others. The diffrences were probably due to the presence of black caraway (extract or powder) in kolompeh that impacted on its lightness.

On day 14, the kolompeh with BCE had the highest L* value, while EBCE, BCE and control samples exhibited a reduction of redness. The similar results were obtained in bread fortified by cumine seeds powder by Sayed Ahmad *et al.*, who found that the lightness of bread depended on the amount of cumin [40]. The increasing in the yellowness could be related to reaction between amino acids (flour) and sugars (date), known as Maillard reaction [40].

Sensory assessment. Table 4 represents the sensory attributes of the kolompeh samples on day 1, 7, 14 and 21 of storage.

We evaluated such sensory characteristics as colour, aroma, flavour, tenderness, and general acceptability. On day 1, the kolompeh with black caraway powder had the lowest score in colour among the other samples, however no significant differences were observed between them ($P > 0.05$). The addition of black caraway (extract and powder) affected adversely the flavour, aroma, and tenderness of kolompeh.

According to the results, the sensory attributes of all samples reduced during storage. As for the kolompeh with encapsulated extract, its colour, aroma and flavour remained unchanged compared to day 1 of storage, which is a positive point of the protective effect of encapsulation. The aroma of the samples containing extract or powder of black caraway decreased by the end of storage, which was due to the loss of some volatile compounds.

In addition, the results revealed that the tenderness of kolompeh with encapsulated extract of black caraway did not change significantly during storage, and was similar to that of the control. However, considerable changes in the texture of BCE, BCP and FBC samples were observed ($P < 0.05$).

Table 4 Sensory evaluation of kolompeh samples during storage

Sensory attribute	Time, days	Sample				
		with EBCE	with BCE	with BCP	FBC	Control
Colour	1	4.6^{a}	4.0^{ab}	3.6^{b}	4.4^{a}	4.2^{a}
	7	4.1^{a}	2.2^{b}	2.6^{b}	2.4^{a}	4.0^{a}
	14	3.8^{a}	2.2^{c}	2.6^{bc}	2.8^{b}	3.8^{a}
	21	3.7^{a}	1.6^{cd}	2.0^{c}	2.4^{b}	3.6^{a}
Aroma	1	4.0^{ab}	3.6^{ab}	3.4^{b}	3.8^{ab}	4.0^{a}
	7	4.0^{a}	2.4^{b}	2.4^{b}	2.2^{b}	4.2^{a}
	14	3.7^{a}	2.4^{c}	3.0^{b}	3.0^{b}	3.6^{a}
	21	3.6^{a}	2.2^{c}	2.0^{c}	2.8^{b}	3.6^{a}
Flavour	1	4.4a	3.0b	3.0b	4.0a	4.0a
	7	4.0a	2.2b	2.2b	2.2b	4.4a
	14	3.9^{a}	2.2^{b}	2.6^{b}	2.4^{b}	3.8^{a}
	21	3.8^{a}	2.2^{cd}	2.6^{bc}	3.0^{b}	3.6^{a}
Tenderness	1	4.3^{a}	3.2^{b}	3.4^{b}	4.2^{a}	4.4^{a}
	7	4.0^{a}	2.0^{b}	2.0^{b}	2.4^{a}	4.0^{a}
	14	3.8^{a}	2.4^{b}	2.8^{b}	2.8^{b}	3.8^{a}
	21	3.8^{a}	2.0^{c}	2.2^{bc}	2.6^{b}	3.8^{a}
Overall acceptability	1	4.1^{ab}	3.4^{c}	3.6^{bc}	4.0^{ab}	4.2^{a}
	7	4.1^{a}	2.0^{b}	2.2^{b}	2.4^{b}	4.4^{a}
	14	4.0^{a}	2.4^{bc}	2.6^{b}	2.6^{b}	4.0^{a}
	21	3.7^{a}	1.8^{c}	1.8^{c}	2.6^{b}	3.6^{a}

*Means with different letters are significantly different ($P < 0.05$). Each value is expressed as Mean ± SD. Test was conducted in triplicate

Overall, the sample with encapsulated extract of black caraway demonstrated a higher score in the sensory parameters. Our results were in agreement with those of Sayed Ahmad *et al.* who fortified protein bread with cumin and caraway powder [40]. Their study showed the improvement of sensory properties in the bread, however, bitter aftertaste was felt, which was dependent on an amount of cumin and caraway powder.

CONCLUSION

In this study, we evaluated the effect of sesame oil and different forms of black caraway extract on the physicochemical and sensory properties of kolompeh. The results showed that caraway had IC_{50} of 124.1 μg·mL^{-1}. Thus, the kolompeh with encapsulated black caraway extract showed the high oxidative stability. In addition, the EBCE microcapsule had global and monotonous morphology, and FTIR spectroscopy confirmed the well encapsulated black caraway extract.

The GC results indicated that the kolompeh samples with sesame oil were rich in unsaturated fatty acids. Oleic and linoleic acid were identified as the major fatty acid in their fatty acid composition. Sesame oil and encapsulation of black caraway had a great influence on the hardness of the samples containing encapsulated extract, which had the lowest hardness among all treatments.

Furthermore, the kolompeh with black caraway encapsulated extract had lower lightness compared to the other samples, probably due to more intense yellow colour of canola oil. However, the sample without caraway was lighter than the others, which was attributed to black caraway colour. In addition, the encapsulation protected the colour, aroma, and flavour of black caraway extract.

According to the sensory assessment, the kolompeh with encapsulated extract was preferred by panelists. Nevertheless, the addition of the extract and powder of black caraway influenced adversely the flavour and aroma of kolompeh. Overall, this research revealed that black caraway extract had a considerable potential for using it as an ingredient and thus for improving the physicochemical and sensory properties of kolompeh.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Indigenous yeast with cellulose-degrading activity in napa cabbage (*Brassica pekinensis* L.) waste: Characterisation and species identification

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Abstract: Napa cabbage waste contains an organic component, cellulose, which can be utilised as an ingredient for cellulose-degrading enzyme production with the help of indigenous yeast. The aim of the research was to identify and characterise potential indigenous yeast isolated from napa cabbage waste, which has cellulose-degrading activity. Indigenous yeast were isolated and characterised using the RapID Yeast Plus System, then turbidity was used to determine the yeast total population. Indigenous yeast was grown at napa cabbage waste at 27, 37, and 40°C for three days, and cellulose-degrading activity was determined by the Dinitrosalicylic Acid (DNS) method. The potential yeast isolate with the highest cellulose-degrading activity was identified by a sequence analysis of the rRNA gene internal transcribed spacer (ITS) region with using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′). The results were compared to the GenBank database using the Basic Local Alignment Search Tools/BLAST algorithm. Three species of indigenous yeast were isolated from napa cabbage waste (S2, S6, and S8). S8, incubated at 37ºC for three days, demonstrated the highest cellulose-degrading enzyme activity (1.188 U/mL), with the average activity of 0.684U/mL. Species identification results indicated that the S8 isolate had a 100% similarity to *Pichia fermentans UniFGPF2* (KT029805.1).

Keywords: *Pichia fermentans*, temperature, cellulase enzyme, internal transcribed spacer

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INTRODUCTION

Napa cabbage (*Brassica pekinesis* L.) is one of the most cultivated agricultural products in Indonesia. In 2014 its production reached 602 468 t. The local leader in this field was the West Java province that yields 14.92 tons of napa cabbage per ha [1]. Since over 20% of napa cabbage cannot be utilised, this waste amount makes napa cabbage production inefficient [2].

Napa cabbage waste contains the same essential component, namely, polysaccharides in the form of cellulose, as napa cabbage itself. Cellulose is known to be a constituent component of plant cell walls, and it account for as much as 30–50% of total lignocellulose [3]. Currently, napa cabbage waste is used as animal feed, while its value could be increased, e.g., through production of cellulose-degrading enzymes.

Cellulose-degrading enzymes can be produced from napa cabbage waste, which is high in cellulose content, by yeast. Enzyme production by indigenous cellulolytic yeast requires optimal conditions, however, it is influenced by external factors, especially, temperature. Thus, too low temperatures can inhibit enzyme production because of the plasma membrane fluidity decrease which leads to disturbed metabolic activity [4]. On the other hand, too high temperature can damage cells and the structure of proteins, which are constituents of enzymes. The fact that temperature is an easily controlled parameter makes it possible to support yeast growth during the fermentation of napa cabbage waste for cellulose-degrading enzyme production. Therefore, the aim of this research was to characterise and identify indigenous yeast isolated from napa cabbage waste.

STUDY OBJECTS AND METHODS

The object of the research was napa cabbage waste from the Gedebage Central Market in Bandung City,

Table 1 Treatment factors

Types of yeast isolates	Incubation temperature		
	27°C	37°C	45°C
Isolate 1 (S2)	A	B	C
Isolate 2 (S6)	D	E	F
Isolate 3 (S8)	G	H	I

Indonesia. We used the following materials: Potato Dextrose Agar (PDA), Yeast and Mold Agar (YMA), Nutrient Broth (NB), Thermo Scientific RapID Yeast Plus System Kit, Carboxymethyle Cellulose (CMC), distilled water, 0.85% NaCl, DNS reagent (3.5-Dinitrosalicyclic acid), phosphate buffer solution (pH 7), gelatin, antibiotics, KH_2PO_4, and $MgSO_4$.

In our experiment we used nine treatments. Treatment factors were the type of yeast isolate and incubation temperature (Table 1). The isolation process of indigenous cellulolytic yeast from each treatment lasted for three days. The experiments were repeated three times.

The selection of the best treatment was performed based on quantitative analysis by determining the highest value of enzyme activity using Factorial Randomized Block Design. According to the results of isolation and identification of indigenous cellulolytic yeast from napa cabbage waste, descriptive analysis on the total population of yeast during the production of cellulose-degrading enzymes was conducted.

Isolation and identification of indigenous yeast. The isolation of indigenous cellulolytic yeast from napa cabbage waste was carried out using the direct plating method [5–6]. One gram of crushed napa cabbage waste was added into 0.85% NaCl, inoculated into a modified PDA (PDA with a 3% yeast extract and 10 ppm antibiotics) and then incubated at 30°C for three days. The biochemical activities of the selected isolates were characterised with the help of RapID Yeast Plus System Kit [7]. For species identification of potential indigenous yeast with the highest cellulose-degrading enzyme activity we used rRNA gene internal transcribed spacer (ITS) region. Sequence analysis was carried out using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) as forward and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as reverse. DNA amplification was performed by Macrogen Inc. The results were compared with the GenBank database using the BLAST algorithm [8].

Determination of total yeast population. Total yeast population determination was carried out by turbidimetry: 1 mL of the liquid culture was taken from the enzyme production medium followed by absorbance measurement [9]. This method is based on the spectrophotometric measurement of the total population at a wavelength of 600 nm [5].

Determination of cellulose-degrading enzyme activity. Cellulose-degrading enzyme production was carried out by the International Union's recommended method of Pure and Applied Chemistry (IUPAC) with some modifications [10]. The salt media used consisted of KH_2PO_4, Mg_2SO_4 and gelatin. The napa cabbage waste was incubated in salt media at (1:2, w/v) by adding 2% (v/v) of isolates [11]. The isolation was carried out in an incubator at 27°C, 37°C and 45°C for three days followed by stirring at 100 rpm for 60 min at room temperature. Then every 24 h the fermented solution was separated using a centrifuge to obtain crude cellulose-degrading enzymes in supernatant, where crude enzymes reacted with DNS (Dinitrosalicylic Acid) reagent. Finally, spectrophotometric analysis was carried out to obtain absorbance values which were used to determine cellulose-degrading enzyme activity. The control used was 3 mL of DNS reagent that was diluted to 25 mL by distilled water.

Table 2 Characteristics of indigenous yeast isolates

Isolate	Macroscopic characteristics
S1	Fungi, long white hyphae, aerobic, colonised
S2	Round, broken white coloured, wet, aerobic
S3	Round, broken white coloured, anaerobic
S4	Oval, broken white coloured, anaerobic
S5	Fungi, long white hyphae, aerobic, colonised
S6	Round, broken white coloured, aerobic
S7	Round, yellow, anaerobic
S8	Oval, yellow, anaerobic

RESULTS AND DISCUSSION

Characterisation of indigenous yeast. After three days of incubation, eight isolates with different characteristics were obtained (Table 2). S2, S6 and S8 isolates displayed macroscopic morphological characteristics similar to those of yeast.

Asliha and Alami state that macroscopically, yeast are round, white, with membranous colony texture, while microscopically, multilateral yeast bud and its cell size ranges from 1 to 7 µm [12]. In addition, the microscopic analysis allowed three isolates to be chosen because they had cell size classified as that of yeast. An average cell diameter of the S2, S6 and S8 was 3.87, 3.76 and 4.24 µm, respectively (Fig. 1). The selected isolates

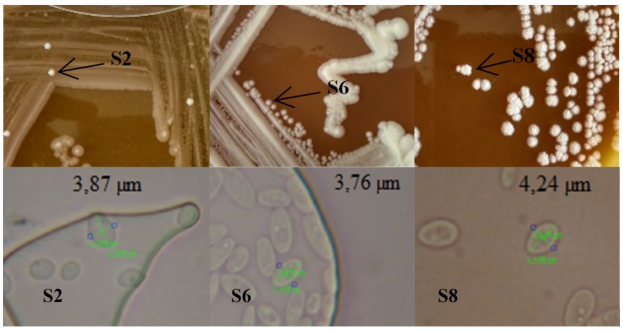

Figure 1 Macroscopic and microscopic images of selected indigenous yeast

Table 3 Identification of indigenous yeast from napa cabbage waste

Isolate	S2	S6	S8
Glucose	+	+	+
Maltose	–	–	–
Sucrose	–	–	–
Trehalose	–	–	–
Raffinose	–	–	–
Lipid	–	–	–
NAGA	–	–	–
αGlucoside	–	–	–
βGlucoside	+	–	–
ONPG	–	–	–
αGalactoside	–	–	–
βFucoside	+	–	–
PHS	–	–	–
PCHO	–	–	–
Urea	–	–	–
Prolyne	–	–	–
Histidine	+	+	+
Leucyl–Glycine	–	–	–

(+) assimilates the substrate positively; and (-) assimilates the substrate negatively

were purified, and their biochemical activities were tested using the RapID Yeast Plus System (Table 3).

The identification results are based on the biochemical properties of the isolates tested against the reacted compound. The glucose or glucoside hydrolysis ability was only shown by S2 isolates against β-Glucoside and β-Fucoside. Lopez *et al*. states that several *non-Saccharomyces* yeast could be found in soil, fruits, trees, and damaged food or drink that has glycolytic β-glucosidase activity [13]. The biochemical properties of indigenous yeast (Table 5) are also supported by Mateo *et al*., who found the glycolytic activity, especially β-glucosidase activity, on indigenous yeas [14]. It implies that glucose can be hydrolysed into acidic compounds which reduce pH until it changes the colour of the resultants. Macroscopically, two isolates identified as S6 and S8 had different characteristics. They differed from the characteristics of *Candida sp.* that has the anamorphous properties, does not have a sexual reproduction phase, and has unstable phenotypic characteristics [15]. Therefore, although the two isolates were different in form, colour, and oxygen requirements, they had the same biochemical activity.

Total population of indigenous yeasts. The results of indigenous yeasts total population determination during incubation are demonstrated in Fig. 2. During day 1 of incubation, the total population in all treatments decreased because the isolates still were in the adaptation phase in the medium. This phase is called the lag phase or the cell adaptation period of new microorganisms to the environment [16]. Nguong *et al*. states that it takes 16 h for yeast with biochemical

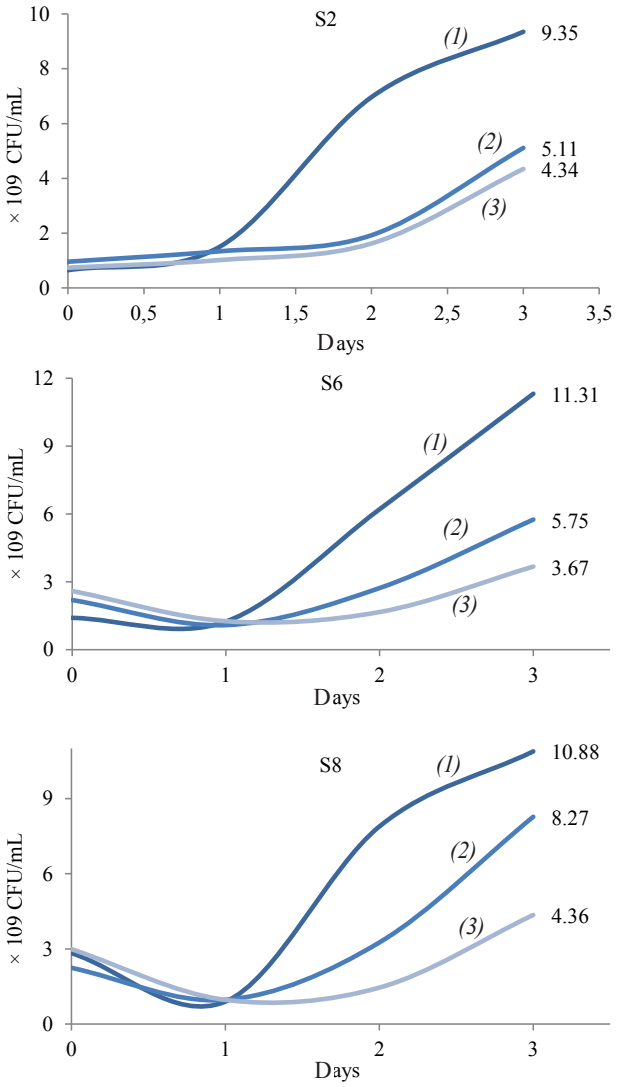

Figure 2 Total population of indigenous yeast: *(1)* 27°C, *(2)* 37°C, *(3)* 45°C

activity similar to that of the S2 isolate to adjust to a new environment [17].

After the adaptation phase, the total population of all treatments increased. The increase is the exponential growth phase, where cells of microorganisms have adapted to the environment and began to multiply so that the number of mass cells or cell density increases rapidly [16]. Spectrometric analysis preformed by Kanti *et al.* revealed that the population of indigenous yeast, such as *Candida*, *Rhodothorula*, *Pichia*, and *Debaryomyces*, began to increase from 24th h and reached a plateau by the 96th h (OD 600 nm) [5].

The highest total population was observed at the incubation temperature of 27°C in all the treatments. Mateo *et al.* state that the biochemical activity of the indigenous yeast was maximal at the temperature of 30–40°C [14]. However, the isolate that belongs to the *Hanseniaspora* genus that has biochemical activity

similar to that of the S2 isolate had the maximum biochemical activity at 28°C. Meanwhile, according to Gänzle *et al.*, a representative of the *Candida* genus with biochemical activity similar to that of S6 and S8 isolates grew rapidly at 27°C [18].

Cellulose-degrading enzyme activity. Cellulose-degrading enzyme activity of indigenous yeast is shown in Fig. 3. The highest enzyme activity produced by S2 was 0.598 U/mL at an incubation temperature of 45°C. The high temperature caused an increase in the rate of biochemical reactions, especially for indigenous yeast that has similar biochemical activity with *Hanseniaspora*. Fennema states that a high temperature affect various reactions [19]. The enzyme belongs to the group of mesozyme enzymes (in the range of 20–50°C) [20]. According to López *et al.*, the glycolytic activity (β-glucosidase) of *H.guilliermondii* at 28°C is about 0.064–2.887 U/mL [13, 21].

S6 isolates obtained at the incubation temperature of 45°C also displayed a high enzyme activity. It is

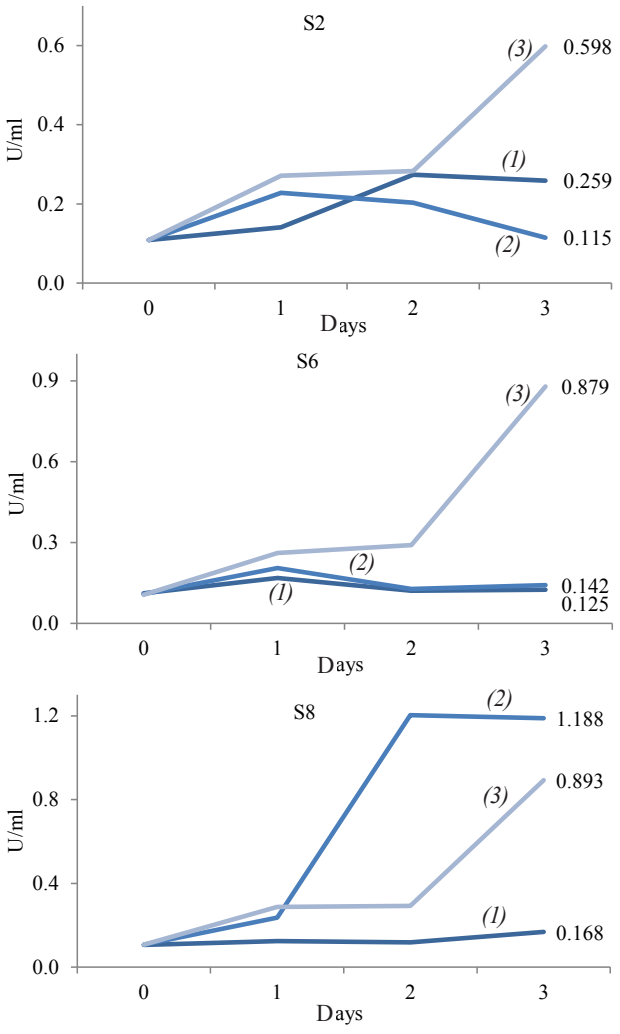

Figure 3 Cellulose-degrading enzyme activity: *(1)* 27°C, *(2)* 37°C, *(3)* 45°C

because the growth of *Candida*-like organisms occurred at the maximum temperature (40–45°C) [20]. As stated by Shuler and Kargi, enzymes are Growth-associated products, i.e. the growth of microorganisms is directly proportional to the product concentration [16]. However, the S8 isolate demonstrated the highest enzyme activity when treated at 37°C: its value was 1.203 U/mL on day1 and 1.188 U/mL on day 2.

Table 4 shows analysis of variance. F-value was greater than P_{value} probability (0.05), which indicated the presence of at least one treatment that significantly differed from the others. Hence, it required an additional test, namely, the Duncan Test.

Table 5 demonstrates the Duncan Test results. According to the data, the S8 treatment incubated at 37°C produced enzyme with an activity significantly differing from the other treatments. This is in accordance with the result of Sulman and Rehman, that *Candida*-like organisms are able to produce cellulose-degrading enzymes with the highest activity at 37°C [11]. The growth of isolates at 27°C cannot produce enzymes with high activity because energy supply from the environment is low, while at 45°C the growth of isolates is inhibited and the structure of the enzyme is denatured so that the activity is not optimal. Therefore, incubation at 37°C gives enough energy for isolates to grow without damaging the structure of the enzyme produced.

Temperature greatly influences the enzymatic activity and rigorous of yeast cell membranes, and

Table 4 Analysis of variance

Source	df	Sum of squares	Mean square	F-value	P-value
Isolate (I)	8	0.779	0.097	89.722*	2.07
Temperature (T)	3	0.539	0.108	165.668*	2.74
I*T	24	0.837	0.035	32.118*	1.67
Replication	2	0.009	0.005	4.249	3.13
Error	70	0.076	0.001		
Total:	107	2.240			

*significant

Table 5 Duncan test results

Yeast	Temperature	Average cellulose-degrading enzyme activity (U/mL)	Signifi-cance
S8	37°C	0.684	a
S8	45°C	0.395	b
S6	45°C	0.384	b
S2	45°C	0.315	c
S2	27°C	0.196	d
S2	37°C	0.160	de
S6	37°C	0.146	e
S6	27°C	0.131	e
S8	27°C	0.129	e

The treatment marked with the same sign shows no significant difference at the level of 5% according to the Duncan test

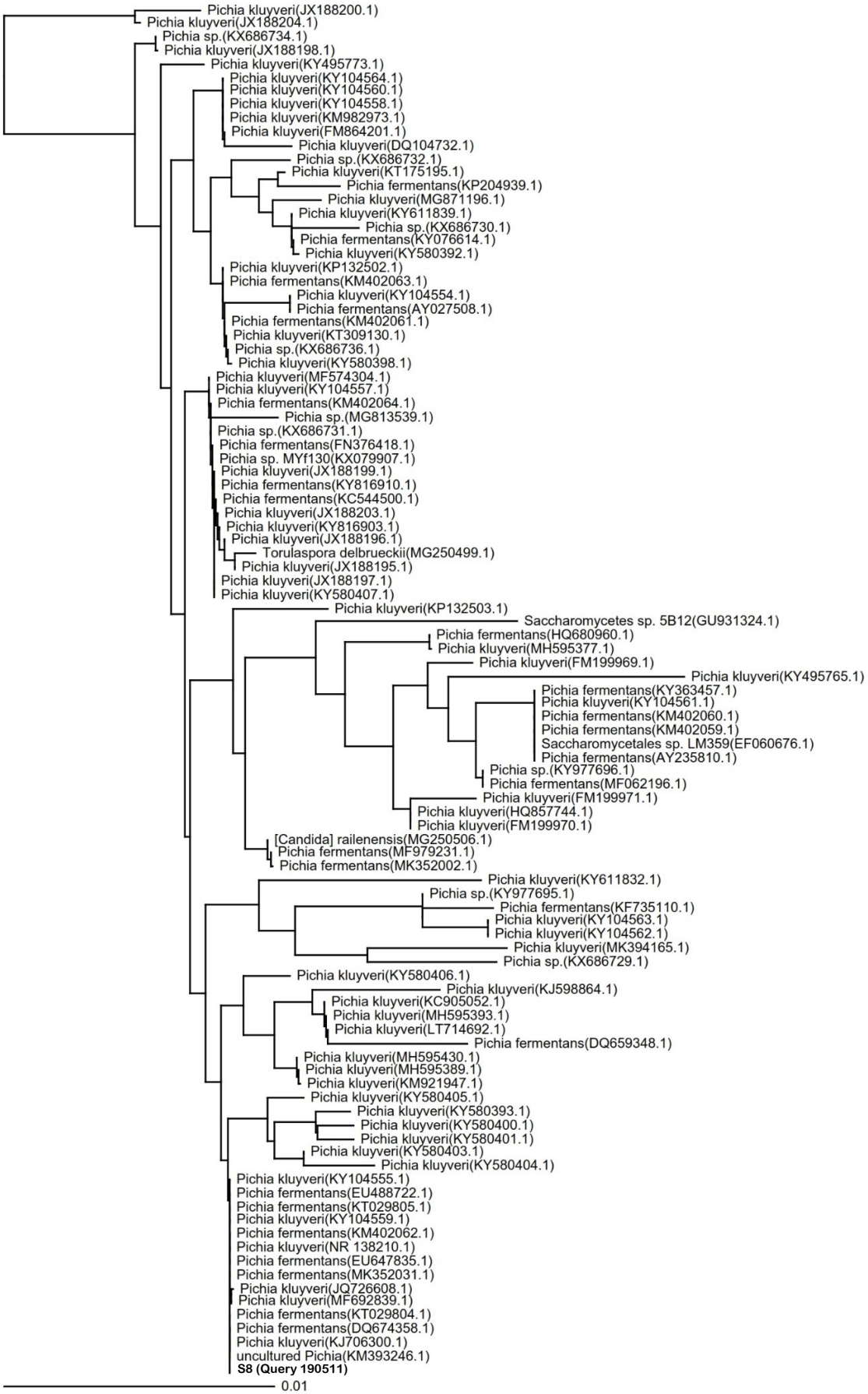

Figure 4 Phylogenetic tree of S8 isolate

higher temperature can shorten the exponential phase of yeast growth. In addition, higher temperature can cause denaturation of ribosomes and membrane fluidity problems. Thus, 30–35°C is the optimal temperature for yeast metabolism, including the enzymatic activity [22].

The difference in S2 and S8 enzyme activities was due to their different biochemical abilities. Lopez *et al.* found that *Hanseniaspora sp.*, which is similar to the S2 isolate, was able to assimilate glycerol, galactose and sucrose, unlike with *Candida sp.*, which is similar to S8 [21, 23].

The different activity of the enzyme produced by S6 and S8 could be caused by different phylogenetics between the two isolates. According to Birmeta *et al*., *Candida sp.* that was mentioned as *C. krusei* has close proximity to *P. fermentans* having certainly different biochemical ability than *C. krusei* [24]. *P. fermentans* has an anamorphic form, *Candida lambica*, but it is not uncommon to find *C. lambica* mis-identification as *C. krusei* caused by similar biochemical abilities of the yeast. Nevertheless, *C. lambica* is able to assimilate xylose, compared to *C. krusei* cannot [25]. Meanwhile, the ability of yeast to assimilate xylose has not been determined by the RapID Yeast Plus System method, so differences between *C. krusei* and *C. lambica* have not been identified.

Species identification of potential indigenous yeast with the highest cellulose-degrading activities. The identification of the S8 isolate resulted in the 100% similarity to *P. fermentans* strain *UniFGPF2* (KT029805.1). The phylogenetic tree (Fig. 4) shows that the S8 isolate is also similar to *P. kluyveri* culture CBS:188 (KY104555.1), *P. fermentans* strain *UniFGPF1* (KT029804.1), *P. fermentans* strain *UFLA CWFY24* (KM402062.1), and *P. fermentans* strain *YF12b* (EU488722.1, DQ674358.1).

P. fermentans have the ability to ferment and assimilate glucose, D-xylose, succinate, lactate, citrate, and glycerol [24]. *Candida lambica* is an anamorphic form of *P. fermentans* which can assimilate glucose and xylose but cannot assimilate arabinose, galactose, and selobiosa [26]. In addition, *Issatchenkia orientalis*, a teleomorphic form of *Candida krusei* that usually incorrectly identified as *Candida lambica*, can assimilate glucose sufficiently but cannot assimilate galactose, maltose, sucrose, lactose, raffinose, and trehalose [27].

According to Bengoa *et al*., despite *P. fermentans* and *C. lambica* can growth at a temperature up to 37°C, the optimal temperature is 25–30°C [28]. Such strain as *I. orientalis* has the unique properties, as this microorganism can grow at a higher temperature level. Miao *et al*. reported that *I. orientalist* strains optimally grows and produces a high amount of ethanol at 41°C, which indicates its thermostability [29].

CONCLUSION

Three species of indigenous yeast were isolated from napa cabbage waste. The highest cellulose-degrading enzyme activity (1.188 U/mL) displayed the S8 isolate incubated at 37°C for three days. Its average cellulose-degrading activity was 0.684 U/mL. According To the species identification, the S8 isolate showed a 100% similarity to *Pichia fermentans UniFGPF2* (KT029805.1).

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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Heavy metal content in farmed rainbow trout in relation to aquaculture area and feed pellets

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Abstract: Fish contamination by heavy metals, even at low levels, has an adverse effect on human health. Mercury (Hg), cadmium (Cd), and lead (Pb) are the most common heavy metals that contaminate sea foods. Rainbow trout is a fish species which is widely cultured in fresh water regions, e.g. in Yasuj, southwest of Iran. Heavy metal contamination was measured in three different culture areas (A, B, and C), with three different feed pellets used in Yasuj farms (I, II, and III). The sampling was conducted during February-April 2018 and the metals were measured using cold vapour atomic absorption with a Perkin Elmer 4100. The mean values of Hg, Cd, and Pb levels in the muscular tissue of the samples were 0.022, 0.105, and 1.07 mg/kg, respectively. Concentrations of Hg and Cd in edible tissues of rainbow trout were lower than the permitted values set by the WHO, the FDA, and the EC. The samples fed on mixture pellets III showed a significantly higher Hg content and a lower concentration of Cd in the muscle tissue compared to those given feed mixtures I and II ($P < 0.05$). Pearson correlation tests revealed significant correlations between the Cd and Pb concentrations and the weight of the fish samples ($r = –0.519$, $r = –0.580$). The lowest Cd concentration (0.076 mg/kg^{-1}) was found in site A located close to the spring and not polluted by sewage from urban or rural areas. The study showed a correlation between the concentration of heavy metals in the fish samples and their weight, the degree of pollution, and the feeding mixture used in the farms.

Keywords: Rainbow trout, heavy metals, mercury, cadmium, lead

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INTRODUCTION

Heavy metals in contaminated food crops, even at low concentrations, produce deleterious effects on human health [1]. Metals pollution naturally occurs in the environment; however, human activities including mining and other industries have particular effects on the ecosystem, as well as the aquatic environment [2–4]. Despite the progress in sewage effluent technologies, water contamination is still a threat in many developing countries due to sewage discharge [5]. Heavy metals have an impact on aquatic ecosystems and eventually enter the human's food chain [6]. Rainbow trout (*Oncorhynchus mykiss* L.), a native fish of North America, is known as one of the most valuable members of the Pacific trout that became the main freshwater fish species farmed in Iran [7, 8]. The first farm of this fish in Iran was established in 1959. Its production increased from 599 tons in 1978 to 140 000 tons in 2016, making Iran one of the world-leading producers of this salmon [9].

Mercury, cadmium, and lead are known as toxicants associated with fish consumption [10]. They are listed as sixth most dangerous contaminants by the International Program of Chemical Safety (IPSC) [11]. Lead poisoning can affect various systems of the body including renal, haematological, cardiovascular, gastrointestinal, and reproductive systems [12]. Renal exposure to cadmium results in its deposition in proximal tubular cells and causes renal failure due to decreased glomerular filtration rates. Also, skeletal system anomalies occur due to the secondary effects of renal dysfunction and accumulation of lead in bones [13]. Methyl mercury exposure through the consumption of contaminated fish in prenatal period leads to serious abnormalities such

as cerebral palsy, mental backwardness, neurological disorders, and infant mortality [14]. Fish, which is an important aquatic component of the human food chain, has been a subject of investigation with regard to metal pollution [15]. Therefore, numerous reports have described metal residues in types of fish species [16–20]. The accumulation of heavy metals in fish tissues is influenced by a number of factors such as feeding habits, nourishment sources, habitat, age, and size [21–23]. In this study, concentrations of heavy metals (Hg, Cd, and Pb) were assessed in fish feed mixtures and edible tissues of rainbow trout farmed in three different culture areas.

STUDY OBJECTS AND METHODS

Study area and sample collection. Between February and April 2018, rainbow trout (*Oncorhynchus mykiss* L.) samples were collected from six farms (five fishes from each farm) in three different culture areas in Yasuj, southwest of Iran (Fig. 1), the third leading producer of this trout in the world [9]. The culture areas were located very close to the spring (site A: 30.502935, 51.743184), downstream of cities and villages (site B: 30.710630, 51.514926), and downstream of a rural area (site C: 30.789658, 51.329715). Their choice was determined by the level of contamination probability. The farms used a raceway farming system, with water supplied by the spring. All the farms practiced manual feeding with commercial pellets two times a day. The fish were collected randomly from two farms in each area (10 fishes in each site). The experiments were approved by the Animal Care and Use Committee of Yasuj University of Medical Sciences (YUMS) in compliance with the 'Guidelines for the Care and Use of Animals'. At first, the samples' biological parameters were recorded including wet body weight and total length. Then, they were washed, preserved in ice-boxes, and transported to the Food Chemical Laboratory at YUMS for heavy metal (Hg, Cd, and Pb) determination. The fish were filleted, placed in polyethylene bags, and kept at −20°C prior to analysis.

Four pellets of three types (I, II, and III) of commercially manufactured feed mixtures were frequently applied in the aquaculture trout farms during the fish sampling (12 pellets) and studied to determine the content of heavy metals.

Analytical procedures. Both feeds and fillets were oven-dried at 105°C for 1 h and then cooled. To measure the level of heavy metals, the samples (dry weight) were digested in a mixture of 6 mL concentrated HNO_3 (super pure quality; Romil Ltd., Cambridge, UK) and 2 mL H_2O_2 (supra pure quality; Merck, Darmstadt, Germany) in a microwave digestion system (MARSXpress, CEM). When cooled to room temperature, the digested sample solutions were filtered and adjusted to 50 mL with ultrapure water. The levels of Hg, Cd, and Pb content were determined using cold vapour atomic absorption with a Perkin Elmer 4100 (FIMS 400 Perkin Elmer Inc., USA). The blank samples were also processed to avoid possible contamination during the analysis [22].

Human health risk assessment. The estimated daily intake (mg/kg bw/day) (EDI) of heavy metals was measured to evaluate the daily/weekly intake of heavy metals by the human body through the consumption of fish [24]. The daily intake of metals in adults was calculated as:

$$\text{EDI (mg/kg bw/day)} = (E_F \cdot E_D \cdot F_{IR} \cdot C_F \cdot C_M)/(W_{AB} \cdot T_A) \times 10^{-3} \quad (1)$$

Where E_F and E_D are the exposure frequency (365 days/year) and the exposure duration (60 years), respectively; F_{IR} is the fish ingestion rate (25.2 g/day for Iran); C_F is the conversion factor to convert fresh weight to dry weight (0.208); C_M is the metal concentration in

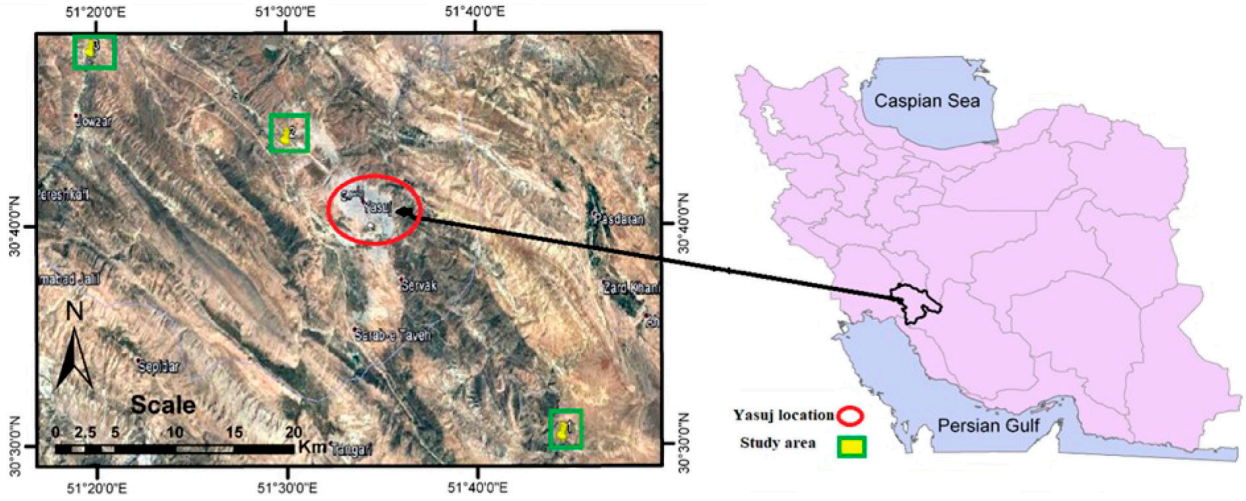

Figure 1 Location of Yasuj, southwest of Iran and the study area (site A: 30.502935, 51.743184, site B: 30.710630, 51.514926, site C: 30.789658, 51.329715)

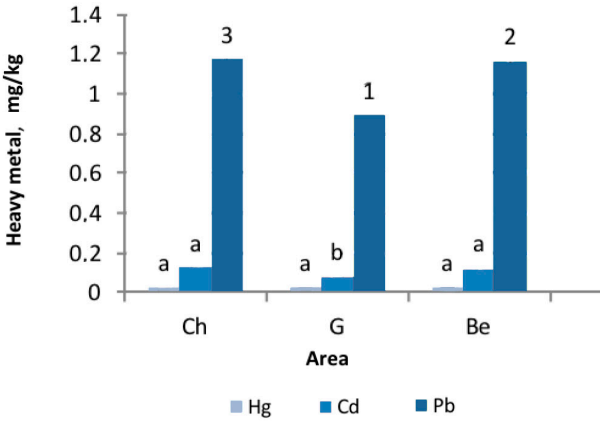

Figure 2 Heavy metal levels in muscle tissue of farmed rainbow trout from three aquaculture sites (G: site A; Be: site B; Ch: site C). All results are expressed as means ± SD for five fish in each group. a and b: groups with different letters differ significantly ($P < 0.05$)

the fish tissue (µg/g dry weight); W_{AB} is the average body weight for adults (65 kg for Iran); and T_A is the average exposure time for non-carcinogens ($E_F \cdot E_D$) [4].

The percentage of provisional tolerable weekly intake (PTWI) and target hazard quotient (THQ) were calculated for each heavy metal by the following equations:

$$PTWI = EWI/PTWI \times 100 \quad (2)$$

$$THQ = EDI/RfD \quad (3)$$

where PTWI, EWI, and RfD are provisional tolerable weekly intake (mg/kg bw/week), estimated weekly intake (mg/kg bw/week), and oral reference doses (mg/kg/day), respectively.

When the THQ is less than one, the risk of noncarcinogenic toxic effects for exposed consumer populations is presumed to be low. When it is greater than or equal to one, it is considered as a concern for consumer populations, indicating potential health risks [25].

Biomagnification factor. The biomagnification factor (BMF) is the ratio between the concentration of an element in fish and the concentration of this element in its diet. The BMF was calculated by the following equation [26]:

$$BMF = C_{fish}/C_{feed} \quad (4)$$

where C_{fish} is a heavy metal concentration in fish edible tissues and C_{feed} is a metal concentration in trout commercial pellets.

Statistical analysis. Statistical analysis was performed using the SPSS Statistics 19.0 software package. The mean and standard deviation (mean ± SD) levels of metal concentrations were reported for different areas and foods. The differences between heavy metal levels in edible tissues of fish from different farms and in different commercial foods were tested by the one-way analysis of variance (ANOVA), followed by Duncan's post hoc test. The Pearson correlation test was used to check for significant relationships between metal concentrations, length, and net weight of fish. $P < 0.05$ was considered as the level of significance.

RESULTS AND DISCUSSION

Heavy metal concentration in rainbow trout. Concentrations of heavy metals in edible tissues of rainbow trout farmed in three different areas are shown in Fig. 2.

The mean ± SD levels of Hg in the muscle tissues of rainbow trout farmed in sites A, B, and C were 0.021 ± 0.0027, 0.023 ± 0.0026, and 0.024 ± 0.0027 mg/kg^{-1}, respectively. The lower mercury level in site A, compared to the other two sites, had no significant difference ($P > 0.05$). In addition, the mean concentration of Cd in sites A, B, and C were 0.076, 0.119, and 0.120 mg/kg^{-1}, respectively. The lowest Cd concentration was found in edible tissues of the fishes farmed in locations close to the spring, not polluted by sewage from urban or rural areas. The highest Pb concentration was detected in site B (1.171 mg/kg^{-1}), followed by sites C and A (0.893 mg/kg^{-1}) ($P < 0.05$). The fishes cultured in sites B and C had significantly higher contents of cadmium and lead, compared to those farmed in site A ($P < 0.05$). The results indicated that concentrations of Hg and Cd were below the permitted values determined by [27–29]. However, the level of Pb in the muscle tissue of farmed trout exceeded the value set by the WHO (Table 1) [30].

Below the levels established by the WHO, the FDA, and the EC were heavy metal concentrations in different fish species studied in Turkey, in the Barents Sea commercial fish, the fish from Lake Chini in Malaysia, wild fresh water fish from the Khersan river in Iran, rainbow trout and freshwater fish species from Lake Pamvotis in Greece [11, 31–35]. In addition, Bat *et al.* reported that the Cd, Hg, and Pb concentrations in *Cyprinus carpio* from the Karasu Stream, Sinop and in four fish species from Sarikum Lake were within certified values allowed to consumers [36, 37].

The Pb content was higher than the WHO allowed level in the fish cultured along the river's upstream in

Table 1 Maximum permissible limit of heavy metals established by international organisations

Heavy metal, µg/g^{-1}	In this study	WHO (2007)	FDA (2001)	FAO (2007)	EC Regulation No. 1881/2006
Hg	0.022	0.5	0.05–1.0	0.5	0.5–1.0
Cd	0.105	0.5	4.0	0.5	0.5
Pb	1.070	0.5	1.7	2.0	1.0

Table 2 Heavy metal levels in edible tissue of rainbow trout from different locations according to literature data: mercury (Hg), cadmium (Cd), lead (Pb)

Fish species	Region	Unit	Heavy metals			Ref.
			Hg	Cd	Pb	
Rainbow trout	Hamadan, Iran	mg/kg dw	–	3.74 ± 4.24	14.07 ± 14.56	[38]
Large sea trout	Sinop fish market, Turkey	mg/kg^{-1} wet wt	0.15–0.42	0.012–0.044	0.08–0.23	[39]
Wild rainbow trout	Khersan river, Iran	mg/kg dw	0.023 ± 0.004	0.110 ± 0.028	1.120 ± 0.130	[33]
Rainbow trout	Gilan, Mazandaran and Chabahar, Iran.	µg/kg (ppb)	22.1 ± 0.8	36 ± 32.2	249.4 ± 88.6	[40]
Farmed rainbow trout	Chaharmahal-va-Baghtiari, Iran	µg/g dw	0.314 ± 0.195	0.097 ± 0.058	1.108 ± 0.400	[16]
Wild rainbow trout	Zayandeh-Rood river, Iran	µg/g dw	0.292 ± 0.181	0.130 ± 0.068	1.201 ± 0.373	[16]
Rainbow trout	Khorramabad, Iran	mg/kg dw	0.297 ± 0.04	0.123 ± 0.03	0.741 ± 0.02	[34]
Rainbow trout	Karakaya Dam Reservoir, Turkey	µg/kg ww	–	0.00052	0.053	[41]
Brown trout	Munzur Stream, Tunceli, Turkey	µg/kg^{-1}	0.01 ± 0.00	4.08–2.83	0.10 ± 0.00	[42]
Rainbow trout	Chaharmahal and Bakhtiari, Iran	mg/kg^{-1}	–	–	12.40	[43]
Rainbow trout	Yasuj, Iran	mg/kg^{-1}	0.022	0.105	1.07	this study

Ghadirabad, Pakistan, wild fresh water fish from the Khersan river in Iran, farmed and wild rainbow trout in the Zayandeh Rood river in Iran, and fresh water fish in North Mexico (4298 mg/kg) [16, 19, 20, 33]. These data support the findings in our study. Table 2 demonstrates the comparison of heavy metal levels in the muscle tissue of rainbow trout from different locations reported in literature.

However, lower Hg, Cd, and Pb levels in site A, compared to the other sites, indicated an important role of water supply in trout aquaculture with regard to heavy metals accumulation in fish tissues. The release of industrial wastewater and pollutants caused by human activities increased the lead content in Liza fish from the Karun River in Iran and from the coast of Cochin in India [44, 45]. It was also reported that wild carps in the downstream areas of the Ravi and the Indus rivers in Pakistan showed a high contamination by heavy metals [46, 47].

Emara *et al.* observed significant differences in the Cd and Pb accumulation in the muscle tissue of fish in two distinct farms using different water sources [48]. Based on health standards, the concentration of heavy metals such as lead was higher in *Mugil cephalus* and *Trachurus mesiteraneus* in the Gulf of Iskenderun [49]. Wagner and Boman reported a greater amount of calcium and iron in the contaminated areas, compared to non-industrial zones [41]. High levels of cadmium and nickel were recorded in fish from Kuetsjarvi Lake (Russia) due to the contamination of surrounding regions and the proximity of smelting plants. A lower concentration of heavy metals in fish was detected in the areas away from factories and contaminating sources [52]. Power plants can reduce water acidity which causes an increase in the water solubility of lead and cadmium, resulting in high accumulation of the metals in aquatic organisms [11].

Table 3 Health risk parameters for the Iranian population consuming farmed rainbow trout cultured in Yasuj compared to other studies

Health risk parameter	This study			[42]	[50]			[38]			[51]	
	Hg	Cd	Pb	Pb	Hg	Cd	Pb	Hg	Cd	Pb	Cd	Pb
EDI, mg/kg bw/day	0.17×10^{-5}	0.84×10^{-5}	0.86×10^{-4}	0.77×10^{-3}	0.008	0.039	4	0.007	0.007	0.004	0.12×10^{-4}	0.18×10^{-4}
EWI, mg/kg bw/week	0.12×10^{-4}	0.59×10^{-4}	0.6×10^{-3}	0.54×10^{-2}	–	–	–	0.049	0.048	0.027	0.87×10^{-4}	1.30×10^{-4}
PTWI, mg/kg bw/week	0.004	0.007	0.025	–	–	–	–	–	–	–	–	–
PTWI, %	0.30	0.84	2.41	–	–	–	–	–	–	–	1.25	0.52
RfD, mg/kg bw/day	0.0003	0.001	0.004	–	–	–	–	–	–	–	–	–
THQ	0.0059	0.008	0.021	0.22	0.037	0.014	0.097	–	–	–	12.46×10^{-3}	9.28×10^{-3}
TTHQ	0.0349			–	0.146				–	–	–	–

EDI: Estimated Daily Intake; EWI: Estimated Weekly Intake; PTWI: Provisional Tolerable Weekly Intake; RfD: Oral Reference Dose; THQ: Target Hazard Quotient; TTHQ: total THQ

Table 4 Heavy metal levels in commercial feed mixtures consumed by farmed rainbow trout in three different aquaculture sites

Feed mixtures	Lead (Pb)	Cadmium (Cd)	Mercury (Hg)
I	5.8 ± 0.365*	0.61 ± 0.093	0.075 ± 0.013
II	4.52 ± 0.259	0.54 ± 0.106	0.063 ± 0.018
III	4.15 ± 0.384	0.66 ± 0.076	0.071 ± 0.026

All results are expressed as means ± SD (mg/kg^{-1}) for three feed mixtures
* indicates a higher level of lead in pellet I compared to the European Commission standards

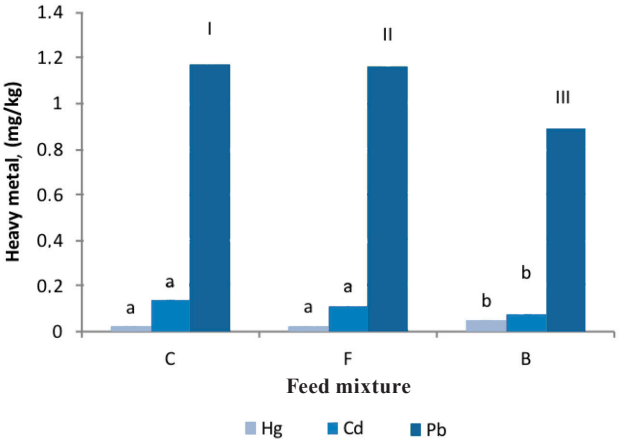

Figure 3 Heavy metal concentrations in muscle tissue of farmed rainbow trout in relation to the type of commercial feed mixture (C: pellet I; F: pellet II; B: pellet III). All results are expressed as means ± SD for six fish in each group. a and b: groups with different letters differ significantly ($P < 0.05$)

Health risk assessment. Several parameters widely used to assess human health risks include the estimated daily intake (EDI), the estimated weekly intake (EWI), the target hazard quotient (THQ), and the provisional tolerable weekly intake (PTWI). Health risk assessment values including the EDI, the EWI, the THQ, and the PTWI with regard to farmed rainbow trout are presented in Table 3. These parameters showed the following pattern in all the samples under study: Pb > Cd > Hg. We found that the EDI, the EWI, the PTWI, and the THQ obtained here were far below the recommended amounts, compared to the set values (RfD and PTWI) or other studies (Table 3). The THQ values were lower than one for all three studied metals. The findings indicated that the consumption of farmed rainbow trout in the study area does not pose a potential non-carcinogenic risk to human health.

Feeds and heavy metal concentrations. The mean ± SD contents of heavy metals in the commercial feed mixtures (I, II, and III) used in the rainbow trout farms of this region are shown in Table 4. The results indicated some differences in commercial feeds based on the content of heavy metals. Food I contained higher levels of Pb (5.8 mg/kg^{-1}) compared to the European Commission Regulations (EC) [53]. Using different raw materials in fish pellets resulted in some alteration of heavy metal contents in fish feed mixtures.

The mean concentrations of Hg, Cd, and Pb in muscle tissues of rainbow trout with regard to the type of food consumed in each of the farms are presented in Fig. 3.

We observed that various commercial mixtures could influence the accumulation of heavy metals in farmed rainbow trout. The concentrations of Hg, Cd, and Pb in the fishes that consumed pellet II were 0.05, 0.076, and 0.893 mg/kg^{-1}, respectively. Using pellet III resulted in a higher content of mercury and a lower concentration of cadmium, compared to the fishes fed on mixtures I and II ($P < 0.05$). On the whole, we concluded that the accumulation of heavy metals in fish was mainly influenced by water, food, and sediment. However, the accumulation of these elements in water and food is due to various factors including ecology, metabolism, pollution of slope water, food, and sediment, as well as other factors such as solubility, temperature, and interaction of various parameters [54, 55].

In our study, however, food intake had a significant effect on the concentration of heavy metals in rainbow trout muscle tissue: the consumption of pellet III resulted in a marked increase in mercury and a considerable reduction in cadmium and lead. Researchers have reported that there is a large association between the concentration of heavy metals in fish and its nutritional habits [22]. Mixture I had higher levels of Pb (5.8 mg/kg^{-1}) compared to the values established by the European Commission Regulations (EC) [53]. Using different raw materials to prepare feed pellets resulted in some alteration of heavy metal contents in the commercial food mixtures. The accumulation of heavy metals in fish depends on food habits, reproductive status, size, and sex [21, 56]. Deep sediments contain large amounts of heavy metals. Compared to the epipelagic organisms, benthos occupying the deepest layers of water is the largest source of heavy metals [22].

Biomagnification factor. The BMF values for all the metals were under 1 (Table 5). Actually, the concentrations of the examined metals in the commercial pellets used in this region were higher than those in the rainbow trout tissues. As Table 5 demonstrates, the BMF values for the three metals had the following pattern: Hg > Pb > Cd (Table 5). The BMF was applied to show the capability of a contaminant to bioaccumulate. When a metal BMF is less than one, it indicates that no biomagnification occurred in the bio system [26]. The current study showed that the BMF values were lower than one for all the metals (Table 5), suggesting that these metal contaminants were not biomagnified by rainbow trout from the diet. This finding was similar to the result obtained by Varol *et al.* [57]. Nevertheless, biomagnification implies inadequate

Table 5 Biomagnification factors of three metals

Aquaculture farms	Lead (Pb)	Cadmium (Cd)	Mercury (Hg)
Site A	0.15	0.11	0.28
Site B	0.25	0.22	0.36
Site C	0.37	0.22	0.38

Table 6 Biometric characteristics of farmed rainbow trout sampled from different farming sites

Feed mixtures	Length, cm	Weight, g
Site A	33.66 ± 2.33	504.66 ± 122.65
Site B	28.16 ± 2.78	262.00 ± 77.80
Site C	34.83 ± 5.07	446.33 ± 226.37

All results are expressed as means ± SD for eight fish in each group

information on the real threat from heavy metals in an aquatic food chain [58].

Physical characteristics and heavy metal levels. Table 6 shows the biometric characteristics of the sampled rainbow trouts. The relationship between metal concentrations and fish size (length and weight) is shown in Table 7. Increasing the weight and length of farmed trouts generally reduced the concentration of the three heavy metals. The Pearson correlation test revealed significant negative associations between the cadmium and lead concentrations and the weight of the fish samples ($r = –0.519$ and $r = –0.580$, respectively).

Few studies have focused on the relationship between physical characteristics and heavy metal accumulation. To the best of our knowledge, this is the first study on the relationship between the length and weight of farmed rainbow trout and the concentration of mercury, lead, and cadmium. Most of the authors have found a negative relationship between heavy metal accumulation and the size of fish (length and weight). They suggested that metabolic activity is one of the most important factors affecting the accumulation of heavy metals in marine fish [59]. Higher metabolic activity in juvenile fish leads to a higher accumulation of heavy metals [60, 61].

The correlation between the spectroscopic parameters and the concentration of heavy metals has been reported to be negative in various species of fish [61]. Heavy metal content in fish after a certain age remains almost constant [62]. In contrast to our results, Widianarko *et al*. found that in sturgeon species in the Caspian Sea, a higher accumulation of heavy metals with an increase in age, length, and weight of fish [63]. In general, there is a consensus that metals in living organisms are detoxified and depleted by a special mechanism, which is significantly dependent on the

Table 7 Relationships between heavy metal concentrations and farmed rainbow trout length and weight

Physical characteristics	Lead (Pb)	Cadmium (Cd)	Mercury (Hg)
Length	–0.383	–0.296	–0.217
Pearson correlation			
sig. (two-tailed)	0.116	0.232	0.386
Weight	–0.580*	–0.519*	–0.266
Pearson correlation			
sig. (two-tailed)	0.012	0.027	0.286

* indicates a significant correlation ($P < 0.05$)

metabolism in the particular weight [64, 65]. Therefore, the negative relationship between heavy metal concentrations and the size of fish does not necessarily mean that a certain amount of metals will accumulate in the body at the beginning of the growth, and no more metals will be subsequently absorbed [66]. It has also been suggested that the absorption of metals in low-contaminated water sources is more affected by nutrition [67]. In other words, a significant reduction in the amount of heavy metals in organs at the maturity stage is due to a decrease in the daily fish diet with age [68]. The fish at the highest nutritional level are expected to have the highest accumulation of heavy metals [69, 70].

CONCLUSION

According to the results of the study, the levels of mercury, cadmium, and lead in the muscle tissue of rainbow trout farmed in Yasuj were found to be below the permitted values. The findings showed that the health risk assessment parameters (EDI, EWI, THQ) were far below the recommended values. This indicated that the consumption of farmed rainbow trout in the study area did not have any adverse effect on human health caused by heavy metal contamination.

However, the release of urban and rural wastewater and pollutants from human activities into the rivers leads to increased levels of lead and cadmium in the fish farmed in the downstream fields of the countryside and cities. Moreover, the application of various commercial pellets containing different levels of heavy metals can affect the accumulation of these metals in farmed trout.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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A multi-criteria sensory assessment of *Cucumis melo* (L.) using fuzzy-Eckenrode and fuzzy-TOPSIS methods

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Abstract: The paper introduces a multi-criteria assessment system that can be used for sensory analysis by fuzzy-Eckenrode and fuzzy-TOPSIS methods. Respondents evaluated the sensory characteristics of *Cucumis melo* (L.), which included aroma, colour, taste, texture, and overall acceptance, after six days of storage. The product was stored under three different temperature conditions: 10°C (B1), 14°C (B2), and room temperature (27–30°C) (B3). The product was also stored at three types of packaging: unpackaged stem (A1), packaged fruit with one layer of banana stem (A2), and packaged fruit with two layers of banana stem (A3). The best result was demonstrated by the *Cucumis melo* that was stored at 14°C and packaged in a two-layered banana stem (A3B2). Both fuzzy-Eckenrode and fuzzy-TOPSIS method provided an easy, fast, and unambiguous calculation of multi-criteria sensory assessment.

Keywords: Banana stem, hedonic scale, *Cucumis melo* (L.), sensory assessment, TOPSIS, Eckenrode

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INTRODUCTION

Cucumis melo L. is a tropical and sub-tropical fruit that easily decays and rots because of its high-water content (70–95%). For the fruit to maintain its quality and freshness, it has to be handled properly during and after harvesting. A good quality fruit should be fresh, with a smooth, undamaged, and flawless skin. Compared to other cucumbers (*Cucumis*), *Cucumis melo* has a greener colour, more crunchy texture, higher water content, and sweeter taste. In addition, *Cucumis melo* can be harvested at an earlier stage.

Packaging is extremely important in post-harvest handling. It creates proper condition for the fruit to maintain its quality during the desired period. Packaging is a container or wrapper that can help to prevent or reduce damage to the packaged/wrapped object. The main functions of packaging are to keep food products from contamination, to protect them from physical damage, and to inhibit their quality degradation.

In the Province of Aceh (Indonesia), *Cucumis melo* is usually packaged in traditional manner by using banana stem, because banana leaves are cheap, easy to find, and eco-friendly. The fruit is placed in the middle part of banana stem, which are then folded into two parts (Fig. 1). Banana stem are able to protect the fruit from shocks and damage during transportation from producer to consumer. When ripe, the epidermis *of Cucumis melo* cracks, and banana stem help keep its shape and texture. Usually, *Cucumis melo* is protected with a single layer of banana stem.

According to Lukman [1], banana stem is part of banana pseudo stem [1]. Its structure is very different from that of woody plants, because it is an apparent trunk formed by tightly packed, over-leaping stem. The fibre of banana stem are strong and waterproof to both fresh and salt water. The packaging of *Cucumis melo* with a various amount of banana stem is necessary to preserve its wholeness and texture, because this fruit is easily broken when ripe. The storage temperature varies from room temperature to cold temperature, which is also expected to prolong the shelf life of *Cucumis melo.*

A quick method to find out consumer acceptance towards the food product is to perform a sensory assessment by collecting respondents' opinions on the product. This multi-criteria assessment method was completed with a weighting assessment approach,

Figure 1 *Cucumis melo* packaged in banana stem

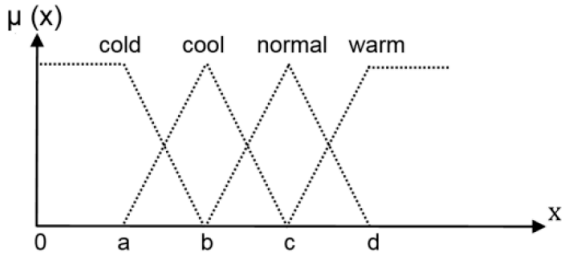

Figure 2 Membership function

which is usually used in decision making. Therefore, this article introduces a multi-criteria assessment system that performs a sensory analysis by using fuzzy-Eckenrode and the fuzzy-TOPSIS (Technique for Order Performance by Similarity to Ideal Solution) methods. According to the system, the respondents evaluated each product and rated its level of acceptance according to a multi-criteria sensory assessment, which included aroma, colour, taste, texture, and overall acceptance.

Fuzzy logic. Fuzzy logic is a development of the set theory, where each member has a degree of membership that ranges in value between 0 and 1. It means that fuzzy sets can represent interpretation of each value according to the opinion, or decision, and its probability. Rating 0 represents 'wrong', rating 1 represents 'right', and there are still other numbers between the 'right' and 'wrong' [2, 3].

In fuzzy sets, there are two attributes. The first one is linguistic attribute: it is a naming of a group which represents a certain situation or condition by using a natural language such as 'cold', 'cool', 'normal', or 'warm'. The second attribute is numeric: it is a value (number) which shows a measure of a variable, such as 10, 30, 50, etc. [4]. Membership function is a curve that defines how each point in the input room is mapped into the membership value (degree of membership between 0 and 1). If U states universal sets and A is fuzzy function sets in U, so A can be stated as sorted pair as following [2]:

$$A = \{(x, \mu_A(x)) | x \in U\} \quad (1)$$

where $\mu_A(x)$ is a membership function that gives value of degree of membership x to fuzzy set A, which is:

$$\mu_A : U \to [0,1] \quad (2)$$

In a fuzzy set, there are several membership functions of a new fuzzy set, which result from basic operation of the fuzzy set, i.e.:

$$\text{Intersection: } A \cap B = \min(\mu_A[x], \mu_B[y]) \quad (3)$$

$$\text{Union: } A \cup B = \max(\mu_A[x], \mu_B[y]) \quad (4)$$

$$\text{Complement: } \sim A = 1 - \mu_A[x] \quad (5)$$

Membership function is stated as follows:

$$\mu(x) = \begin{cases} 0; & x \le a \text{ or } x \ge c \\ (b-a)/(x-a); & a \le x \le b \\ (b-x)/(c-b); & b \le x \le c \end{cases} \quad (6)$$

In a fuzzy system, there is a linguistic variable. This is a variable that has a value in verbal form in a natural language. Each linguistic variable is related to a certain membership function. Figure 2 gives an example of membership function.

Fuzzy-Eckenrode. The Eckenrode method was initially known as a weighting multiple criteria method, which was introduced by Robert T. Eckenrode from Dunlop and Association, Inc. in 1965 and has been widely used until today [5–8]. The Eckenrode method is simpler and more efficient in determining the importance weight in a decision [9–11]. The Eckenrode weighting analysis method is one of weighing methods used in determining the degree of importance, or Weight (B), from each Criteria (K) and Sub-criteria (SK), which have been set in decision making [12]. This weight determination is perceived as very important because it affects the final total value of each chosen decision. The concept used in this weighting method is by doing a change of order to value where, for instance, first order (1) has the highest rate (value) and the fifth order (5) has the lowest rate.

Fuzzy-TOPSIS. TOPSIS belongs to the Multiple Attribute Decision Making (MADM), which was firstly introduced by Yoon, Yoon *et al.* and Hwang *et al.* [13–15]. It has been widely applied in various studies related to decision making, such as Kumar *et al.*, Han *et al.*, Tyagi, Estrella *et. al.*, Roszkowska *et al.*, Selim *et al.* [16–21]. TOPSIS can only be implemented for a criterion whose weight has been known or calculated before, because there is a step in TOPSIS which involves the process of multiplication of criterion weight and the alternative value of the criterion.

In many situations, the data available is insufficient for a real life problem, because human assessment, which is considered as preference, is unclear, and the preference cannot be estimated with exact numeric value. The verbal expression, e.g. 'low', 'medium', 'high', etc., is considered as a representation of the decision maker. Thus, fuzzy logic is necessary in making a structured decision of the preference maker.

Table 1 Attributes of multi-criteria sensory assessment of *Cucumis melo*

Attribute	Assessment consideration
Aroma (C1)	Typical, no sour smell
Colour (C2)	Yellowish-green
Taste (C3)	Sweet and not sour
Texture (C4)	Solid, not watery, no wrinkles
Overall acceptance (C5)	Yellowish-green in colour, solid, and sweet

The Fuzzy theory helps to measure the uncertainty associated with human judgement, which is subjective. Therefore, evaluation is necessary to be done in an environment. According to Ningrum *et al.* and Fadhil *et al.*, fuzzy logic can help improve failure, which happens when only Eckenrode or TOPSIS method is used [4, 22].

STUDY OBJECTS AND METHODS

This study used *Cucumis melo* (L.) which was harvested in two months after planting. The harvested *Cucumis melo* was cleaned by washing and then stored under three different conditions: without banana stem packaging (A1), with one layer of banana stem packaging (A2), and with two layers of banana stem packaging (A3). *Cucumis melo* was then stored for six days under three temperature regimes: 10°C (B1), 14°C (B2), and at room temperature (27–30°C) (B3).

Procedure of assessment. The multi-criteria sensory assessment of *Cucumis melo* included aroma, colour, taste, texture, and overall acceptance (Table 1). The attribute weight of respondents' assessment toward the multi-criteria was determined according to the hedonic scale. The hedonic scale is a preference of respondent's opinion based on likes or dislikes that are converted into number (Table 2).

The framework of this study included four steps: (1) selection of respondents and criteria, (2) determination of criterion weight of the assessment by using the fuzzy-Eckenrode method, (3) determination of the best alternative of all treatments by using fuzzy-TOPSIS, and (4) recommendation of the best acceptance from all respondents. Figure 3 shows the complete framework.

Combinations of storage conditions were as follows:
A1B1: without banana stem-packaging at 10°C;
A1B2: without banana stem-packaging at 14°C;
A1B3: without banana stem-packaging at 27–30°C;
A2B1: with one layer of banana stem-packaging at 10°C;

Table 2 Assessment of preference according to hedonic scale

Score	Preference
5	Like very much
4	Like
3	Neither like nor dislike
2	Dislike
1	Dislike very much

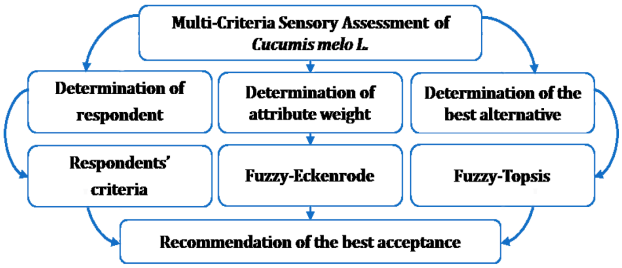

Figure 3 Research framework

A2B2: with one layer of banana stem-packaging at 14°C;
A2B3: with one layer of banana stem-packaging at 27–30°C;
A3B1: with two layers of banana stem-packaging at 10°C;
A3B2: with two layers of banana stem-packaging at 14°C;
A3B3: with two layers of banana stem-packaging at 27–30°C.

Fuzzy-Eckenrode method. According to the Eckenrode weight calculation method, the respondents were asked to make a rating (e.g. from R_1 until R_n, where n ranking, j = 1, 2, 3,…, n, ranking j = R_j) for each criterion (criterion i is notated with K_i, which is presented in a number of n criteria, i = 1, 2, 3,…, n) [11]. Table 3 shows the obtained data. Next, N_i was calculated based on P_{ij} and R_{n-j}.

$$Ni = G_{j=1}\, P_{rij}\, x\, R_{n-j},\ j = 1, 2, 3,\ldots, n. \quad (7)$$

$$\text{Total Score} = G_{i=1}\, N_i,\ i = 1, 2, 3,\ldots, n. \quad (8)$$

Then, criterion weight Bi (which are B_1, B_2, B_3,…, B_n) was calculated, where i = 1, 2, 3,…, 3, by using the following formula:

Tabel 3 Calculation of criterion weight according to the Eckenrode method

Criteria	Rank						Score	Weight
	R_1	R_2	.....	R_j	.....	R_n		
K_1	P_{11}	P_{12}	.....			P_{1n}	N_1	B_1
K_2	P_{21}	P_{22}	.....			P_{2n}	N_2	B_2
.....	.....	.....	.....			.....	.....	.....
K_i				P_{ij}				
.....								
K_n	P_{n1}	P_{n2}	.....			P_{mn}	N_n	B_n
Multiplier factor	R_{n-1}	R_{n-2}	.....	R_{n-j}		R_{n-n}	Total Score	1.00

*R*j = ranking order at j, j = 1, 2, 3,…, n
*K*i = criterion type i, i = 1, 2, 3,…, n
*P*ij = number of respondents who chose ranking j for criterion i
*R*n-j = multiplier factor j, which was obtained from the reduction of number of criteria or number of ranking (which is n) with the rank order on the column. For instance, if there are five criteria, so the multiplier factor for column of 3rd rank (if j = 3) is n–j = 5–3 = 2
*B*i = weight of criterion i.

Table 4 Scale of weighting comparison among criteria of fuzzy-Eckenrode method

Scale	Annotation	TFN membership function
˜1	Very unimportant	1, 1, 2
˜2	Less important	1, 2, 3
˜3	Neutral	2, 3, 4
˜4	Important	3, 4, 5
˜5	Very important	4, 5, 5

$$B_i = (N_i/\text{Total Score}) \quad (9)$$

To find the level of importance of each sub-criterion within a criterion, the respondents were also asked to rank each sub-criterion within a criterion. Then, by using the same procedure, the weight of each sub-criterion was calculated (B_{1i}, the weight of sub-criterion 1 in criterion i). Thus, the weighted weight (BT) from sub-criterion 1 in criterion i was obtained, which was $BT_1 = B_{1i} \cdot B_i$. Then, to find the score of each criterion, the respondents were asked to rate each sub-criterion within each criterion [23].

The assessment of each sub-criterion was calculated by using a geometric mean formula according to the assessment result from all respondents, which was multiplied with the weighted weight of each sub-criterion. Each criterion (K_1 to K_5) was calculated by summing up the total score of all sub-criteria in each criterion. To assess the weighting by the respondents, the fuzzy-Eckenrode method was applied with the value of preference, as shown in Table 4.

Fuzzy-TOPSIS method. The analysis with the fuzzy-TOPSIS method included the following tasks [24]:

To rank the fuzzy from each decision made, Dk; (k = 1, 2, 3,…, k) can be represented as triangular fuzzy number ˜Rk; (k = 1, 2, 3,…, K) with membership function μ˜R (x).

To produce an appropriate alternative, to determine the criteria of evaluation, and to organise the group of decision-maker. It was assumed that there were m alternatives, n criteria of evaluation, and decision k.

To choose the linguistic variable according to the weight of criterion importance = $(\tilde{w}_j = l_{ij}, m_{ij}, u_{ij})$ and alternative linguistic rankings on criterion $(\tilde{x}_{ij})$ in Triangular Fuzzy Number (TFN).

To do a weight aggregation of each criterion to obtain fuzzy weight aggregate $(\tilde{w}_j)$ in criterion C_j and to determine the fuzzy aggregate value from alternative A_i on each criterion C_j.

$$\tilde{x}_{ij} = \frac{1}{k}[\tilde{x}_{ij}^1 + \tilde{x}_{ij}^2 + \ldots + \tilde{x}_{ij}^k] \quad (10)$$

i = 1, 2,.., m; and j = 1, 2,..., n

$$\tilde{w}_j = \frac{1}{k}[\tilde{w}_j^1 + \tilde{w}_j^2 + \ldots + w_j^k] \quad (11)$$

j = 1, 2,..., n

To build a fuzzy decision matrix.

$$\tilde{D} = \begin{array}{c} \\ A_1 \\ A_2 \\ A_m \end{array} \begin{array}{c} \begin{array}{cccc} C_1 & C_2 & \ldots & C_n \end{array} \\ \begin{bmatrix} \tilde{x}_{11} & \tilde{x}_{12} & \ldots & \tilde{x}_{1n} \\ \tilde{x}_{21} & \tilde{x}_{22} & \ldots & \tilde{x}_{2n} \\ \tilde{x}_{m1} & \tilde{x}_{m2} & \ldots & \tilde{x}_{mn} \end{bmatrix} \end{array},$$

$$\tilde{W} = [\tilde{w}_1, \tilde{w}_2 \ \ldots \ \tilde{w}_n] \quad (12)$$

To do normalisation of the decision matrix, where:

$$\tilde{R} = [\tilde{r}_{ij}]_{mxn} \text{ i = 1, 2,..., m; and j = 1, 2,..., n} \quad (13)$$

Calculating $[\tilde{r}_{ij}]_{mxn}$ can be done with:

$$\tilde{r}_{ij} = \left(\frac{l_{ij}}{U_j^*}, \frac{m_{ij}}{U_j^*}, \frac{u_{ij}}{U_j^*}, \right) \quad (14)$$

where $U_j^* = \max u_{ij}$.

To determine the weight normalisation of the fuzzy decision matrix. Based on different importance on each criterion, the fuzzy decision of the weighted normalisation matrix can be arranged as:

$$\tilde{V} = [\tilde{v}_{ij}]_{mxn}$$

with, i = 1, 2,..., m; and j = 1, 2,..., n (15)

where:

$$\tilde{v}_{ij} = \tilde{r}_{ij} \otimes \tilde{w}_{ij}$$

with, i = 1, 2,..., m; and j = 1, 2,..., n (16)

To determine fuzzy positive ideal solution (FPIS) S^+ and fuzzy negative ideal solution (FNIS) S^-:

$$S^+ = (\tilde{v}_1^+, \tilde{v}_2^+, \ldots., \tilde{v}_n^+) \quad (17)$$

$$S^- = (\tilde{v}_1^-, \tilde{v}_2^-, \ldots., \tilde{v}_n^-) \quad (18)$$

where: $\tilde{v}_1^+ = \max \{v_{ij3}\}$ and $\tilde{v}_j^- = \min \{v_{ij1}\}$ with $\tilde{v}_j$ are TFN normalisation weight.

To calculate the interval between each alternative value and the value of FPIS (Fuzzy Positive Ideal Solution) and FNIS (fuzzy negative ideal solution).

$$d(A_1, A_2) = \sqrt{\frac{1}{3}[(l_1 - l_2)^2 + (m_1 - ml_2)^2 + + (u - u_2)^2} \quad (19)$$

$$d_1^+ = \sum_{j=1}^n d(\tilde{v}_{ij}, \tilde{v}_j^+), \ i = 1, 2, \ldots, m \quad (20)$$

$$d_1^- = \sum_{j=1}^n d(\tilde{v}_{ij}, \tilde{v}_j^-), \ i = 1, 2, \ldots, m \quad (21)$$

To calculate the closeness coefficient (*CCi*) and the ranking according to the coefficient value obtained using the following equation:

$$CC_i = \frac{d_i^-}{d_i^+ + d_i^-}, \ i = 1, 2, \ldots., m \quad (22)$$

To rate each alternative by the respondents, we used the fuzzy-TOPSIS method with preference value, as in Table 5.

Figure 4 illustrates the procedure of the analysis.

Selection of respondents. A total of 10 respondents were chosen to do a multi-criteria sensory assessment of

Table 5 Comparison scale of determination of the fuzzy-TOPSIS method alternative

Scale		TFN Linguistics
Dislike very much	(STS)	1, 1, 2
Dislike	(TS)	1, 2, 3
Neither like nor dislike	(N)	2, 3, 4
Like	(S)	3, 4, 5
Like very much	(SS)	4, 5, 5

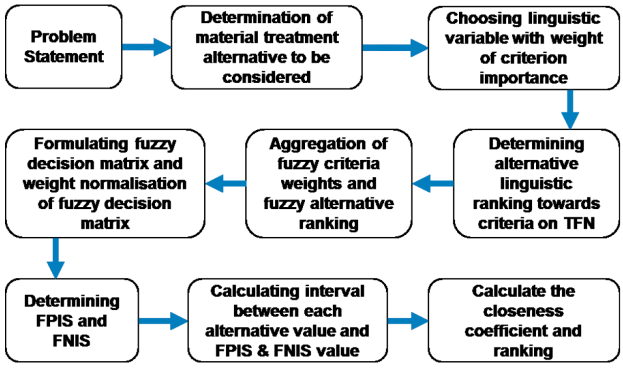

Figure 4 Steps of the fuzzy-TOPSIS method analysis

Cucumis melo. The respondents were selected according to several criteria. The potential respondents had to:

1. like *Cucumis melo*, raw or processed;
2. be experienced in sensory assessment;
3. be healthy, as flu, cough, mouth ulcers, etc. can bother the sensory assessment process;
4. be able to distinguish colours.

RESULTS AND DISCUSSION

Determination of assessment criteria weight. A hedonic scale was used to evaluate the results of determination of respondents' assessment of criteria weight towards multi-criteria which were considered in the sensory assessment. After that, they were translated into fuzzy logic functions (Table 6).

As for the data of respondents' assessment towards criteria of importance weight determination from each sensory attribute, the values of lower bound (low), middle (medium), and upper bound (upper) were arranged as summarised on Table 7. The next step was to calculate the score and the weight of each criterion. Figure 5 represents a radar diagram.

Table 6 Respondents' weighting score of criteria based on the fuzzy-Eckenrode method

No	Criteria	Order				
		1	2	3	4	5
1	C1	~4	~3	~1	~1	~1
2	C2	~3	~4	~1	~1	~1
3	C3	~3	~1	~4	~1	~1
4	C4	~2	~2	~4	~1	~1
5	C5	~5	~2	~1	~1	~1
Value (∑ criteria-order)		4	3	2	1	0

According to the respondents' assessment of the criteria with the help of the fuzzy-Eckenrode method, the order of criteria weight was obtained from the highest to the lowest: (1) overall acceptance, 0.216; (2) colour, 0.211; (3) aroma, 0.203; (4) taste, 0.191; and (5) texture 0.176.

Determination of the best alternative. The priority of the best alternative from the multi-criteria sensory assessment of *Cucumis melo* was determined by summarising all respondents' preferences. The preferences were chosen based on the mode number, i.e. the value that appears most often from each choice of material treatment. The mode number was chosen by the respondents. The next step was to arrange the matrix of the respondents' assessment on all alternatives (Table 8). The data of respondents' assessment was then transformed into TFN linguistic data, as presented in Table 9.

After that, we formulated the normalised weight matrix on each alternative. The value normalisation can be done by using Eqs. (13) and (14). Table 10 shows the results of the TFN value normalisation.

Then, we arranged the matrix of multiplication between criteria weights and normalisation value of each alternative. This process can be done by using Eqs. (15) and (16). Table 11 summarises the results of the matrix multiplication.

Table 7 TFN value of experts' weighting on criteria of the fuzzy-Eckenrode method

No	Criteria	1			2			3			4			5			Score	Weight
		l	m	u	l	m	u	l	m	u	l	m	u	l	m	u		
1	C1	4	5	5	1	2	3	1	1	2	1	1	2	1	1	2	82	0.206
2	C2	3	4	5	1	2	3	1	2	3	1	1	2	1	1	2	84	0.211
3	C3	1	2	3	1	1	2	3	4	5	1	2	3	1	1	2	76	0.191
4	C4	1	1	2	1	2	3	3	4	5	1	2	3	1	1	2	70	0.176
5	C5	3	4	5	1	2	3	1	1	2	1	2	3	1	1	2	86	0.216
Value (∑ criteria-order)		4			3			2			1			0			398	1,000

l = lower, m = middle, u = upper

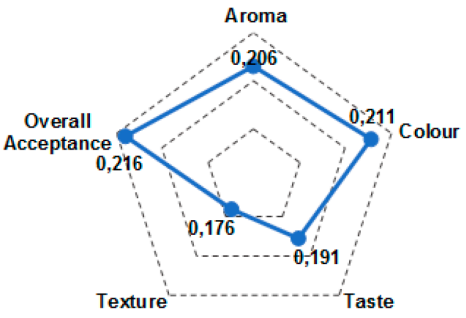

Figure 5 Radar diagram of criteria weight

The next step was to determine the positive ideal solution value (FPIS) S^+ and the negative ideal solution value (FNIS) S^-. When determining both values, the characteristic of data available should be taken into consideration. To obtain both groups of values, one can use Eqs. (17) and (18). Table 12 demonstrates FPIS and FNIS values.

After that, the interval between each alternative value and FPIS and FNIS was calculated by using Eqs. (19), (20), and (21). The results of the interval calculation between alternative value toward FPIS and FNIS can be observed from Table 13 and Table 14.

We evaluated the criteria distance value to the fuzzy positive ideal solution (FPIS) and the fuzzy negative ideal solution (FNIS) according to comparison of d+ and d–. It showed preference of product acceptance on a radar diagram (Fig. 6). For instance, the treatment of *Cucumis melo* without packaging at temperature of 10°C (A1B1) had such d+ and d– values that showed the biggest distance from the positive ideal and the negative ideal.

Table 8 Matrix of experts' assessment on alternatives

Alternatives	Criteria				
	C1	C2	C3	C4	C5
A1B1	3	2	1	1	3
A1B2	3	2	2	1	3
A1B3	2	2	2	1	1
A2B1	4	3	3	2	4
A2B2	4	4	3	2	4
A2B3	2	2	2	1	1
A3B1	5	4	5	4	5
A3B2	5	5	5	5	5
A3B3	1	1	1	1	1

The final step was to calculate the closeness coefficient (CCi) of each alternative by using Eq. (22). From the calculation result, we obtained ranking from the highest to the lowest (Fig. 7). The biggest coefficient value was the main alternative, which was suggested to be chosen or prioritised, compared to other alternatives based on respondents' preference (product acceptance).

According to the closeness coefficient (CCi), an alternative ranking can be arranged from the biggest to the lowest as follows: two-layer banana stem-packaging at 14°C (A3B2), two-layer banana stem-packaging at 10°C (A3B1), one-layer banana stem-packaging at 14°C (A2B2), one-layer banana stem-packaging at 10°C (A2B1), without banana stem packaging at 14°C (A1B2), one-layer banana stem-packaging at room temperature (A2B3), without banana stem packaging at 10°C (A1B1), without banana stem packaging at room temperature (A1B3), and two-layer banana stem-packaging at room temperature (A3B3) (Fig. 7).

The analysis with fuzzy-TOPSIS approach showed

Table 9 Matrix of respondents' assessment on alternative in TFN scale

Alternatives	Criteria				
	Aroma (0.191, 0.206, 0.211)	Colour (0.206, 0.211, 0.216)	Taste (0.176, 0.191, 0.206)	Texture (0.176, 0.176, 0.191)	Overall acceptance (0.204, 0.216, 0.216)
A1B1	(2, 3, 4)	(1, 2, 3)	(1, 1, 2)	(1, 1, 2)	(2, 3, 4)
A1B2	(2, 3, 4)	(1, 2, 3)	(1, 2, 3)	(1, 1, 2)	(2, 3, 4)
A1B3	(1, 2, 3)	(1, 2, 3)	(1, 2, 3)	(1, 1, 2)	(1, 1, 2)
A2B1	(3, 4, 5)	(2, 3, 4)	(2, 3, 4)	(1, 2, 3)	(3, 4, 5)
A2B2	(3, 4, 5)	(3, 4, 5)	(2, 3, 4)	(1, 2, 3)	(3, 4, 5)
A2B3	(1, 2, 3)	(1, 2, 3)	(1, 2, 3)	(1, 1, 2)	(1, 1, 2)
A3B1	(4, 5, 5)	(3, 4, 5)	(4, 5, 5)	(3, 4, 5)	(4, 5, 5)
A3B2	(4, 5, 5)	(4, 5, 5)	(4, 5, 5)	(4, 5, 5)	(4, 5, 5)
A3B3	(1, 1, 2)	(1, 1, 2)	(1, 1, 2)	(1, 1, 2)	(1, 1, 2)

A1B1: without banana stem-packaging at 10°C
A1B2: without banana stem-packaging at 14°C
A1B3: without banana stem-packaging at 27–30°C
A2B1: with one layer of banana stem-packaging at 10°C
A2B2: with one layer of banana stem-packaging at 14°C
A2B3: with one layer of banana stem-packaging at 27–30°C
A3B1: with two layers of banana stem-packaging at 10°C
A3B2: with two layers of banana stem-packaging at 14°C
A3B3: with two layers of banana stem-packaging at 27–30°C

Table 10 Matrix of TFN scale normalisation

Alternative	Criteria				
	Aroma (0.191, 0.206, 0.211)	Colour (0.206, 0.211, 0.216)	Taste (0.176, 0.191, 0.206)	Texture (0.176, 0.176, 0.191)	Overall acceptance (0.204, 0.216, 0.216)
A1B1	(0.40, 0.60, 0.80)	(0.20, 0.40, 0.60)	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)	(0.40, 0.60, 0.80)
A1B2	(0.40, 0.60, 0.80)	(0.20, 0.40, 0.60)	(0.20, 0.40, 0.60)	(0.20, 0.20, 0.40)	(0.40, 0.60, 0.80)
A1B3	(0.20, 0.40, 0.60)	(0.20, 0.40, 0.60)	(0.20, 0.40, 0.60)	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)
A2B1	(0.60, 0.80, 1.00)	(0.40, 0.60, 0.80)	(0.40, 0.60, 0.80)	(0.20, 0.40, 0.60)	(0.60, 0.80, 1.00)
A2B2	(0.60, 0.80, 1.00)	(0.60, 0.80, 1.00)	(0.40, 0.60, 0.80)	(0.20, 0.40, 0.60)	(0.60, 0.80, 1.00)
A2B3	(0.20, 0.40, 0.60)	(0.20, 0.40, 0.60)	(0.20, 0.40, 0.60)	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)
A3B1	(0.80, 1.00, 1.00)	(0.60, 0.80, 1.00)	(0.80, 1.00, 1.00)	(0.60, 0.80, 1.00)	(0.80, 1.00, 1.00)
A3B2	(0.80, 1.00, 1.00)	(0.80, 1.00, 1.00)	(0.80, 1.00, 1.00)	(0.80, 1.00, 1.00)	(0.80, 1.00, 1.00)
A3B3	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)	(0.20, 0.20, 0.40)

Table 11 Matrix of multiplication of criteria weights and alternative normalisation values

Alternatives	Criteria				
	Aroma (0.203, 0.204, 0.209)	Colour (0.196, 0.203, 0.204)	Taste (0.188, 0.196, 0.203)	Texture (0.188, 0.188, 0.196)	Overall acceptance (0.204, 0.209, 0.209)
A1B1	(0.08, 0.12, 0.17)	(0.04, 0.08, 0.13)	(0.04, 0.04, 0.08)	(0.04, 0.04, 0.08)	(0.08, 0.13, 0.17)
A1B2	(0.08, 0.12, 0.17)	(0.04, 0.08, 0.13)	(0.04, 0.08, 0.12)	(0.04, 0.04, 0.08)	(0.08, 0.13, 0.17)
A1B3	(0.04, 0.08, 0.13)	(0.04, 0.08, 0.13)	(0.04, 0.08, 0.12)	(0.04, 0.04, 0.08)	(0.04, 0.09, 0.13)
A2B1	(0.11, 0.16, 0.21)	(0.08, 0.13, 0.17)	(0.07, 0.11, 0.16)	(0.04, 0.07, 0.11)	(0.12, 0.17, 0.22)
A2B2	(0.11, 0.16, 0.21)	(0.12, 0.17, 0.22)	(0.07, 0.11, 0.16)	(0.04, 0.07, 0.11)	(0.12, 0.17, 0.22)
A2B3	(0.04, 0.08, 0.13)	(0.04, 0.08, 0.13)	(0.04, 0.08, 0.12)	(0.04, 0.04, 0.08)	(0.04, 0.04, 0.09)
A3B1	(0.15, 0.21, 0.21)	(0.12, 0.17, 0.22)	(0.14, 0.19, 0.21)	(0.11, 0.14, 0.19)	(0.16, 0.22, 0.22)
A3B2	(0.15, 0.21, 0.21)	(0.16, 0.21, 0.22)	(0.14, 0.19, 0.21)	(0.14, 0.18, 0.19)	(0.16, 0.22, 0.22)
A3B3	(0.04, 0.04, 0.08)	(0.04, 0.04, 0.09)	(0.04, 0.04, 0.08)	(0.04, 0.04, 0.08)	(0.04, 0.04, 0.09)

Table 12 Positive ideal solution and negative ideal solution values

Criteria	Aroma	Colour	Taste	Texture	Overall acceptance
S (+)	(0.21, 0.21, 0.21)	(0.22, 0.22, 0.22)	(0.21, 0.21, 0.21)	(0.19, 0.19, 0.19)	(0.22, 0.22, 0.22)
S (–)	(0.04, 0.04, 0.04)	(0.04, 0.04, 0.04)	(0.04, 0.04, 0.04)	(0.19, 0.19, 0.19)	(0.22, 0.21, 0.22)

Table 13 Intervals between criteria value and FPIS

FPIS (d^+)	Criteria					d+
	Aroma	Colour	Taste	Texture	Overall acceptance	
A1B1	0.096	0.136	0.156	0.143	0.095	0.626
A1B2	0.096	0.136	0.133	0.143	0.095	0.603
A1B3	0.134	0.136	0.133	0.143	0.135	0.681
A2B1	0.062	0.096	0.097	0.122	0.059	0.436
A2B2	0.062	0.060	0.097	0.122	0.059	0.400
A2B3	0.134	0.136	0.133	0.143	0.160	0.706
A3B1	0.034	0.060	0.039	0.057	0.030	0.219
A3B2	0.034	0.030	0.039	0.030	0.030	0.162
A3B3	0.158	0.161	0.156	0.143	0.160	0.778

Table 14 Interval between criteria value and FNIS

FPIS (d^-)	Criteria					d–
	Aroma	Colour	Taste	Texture	Overall acceptance	
A1B1	0.093	0.057	0.027	0.143	0.095	0.416
A1B2	0.093	0.057	0.056	0.143	0.095	0.445
A1B3	0.057	0.057	0.056	0.143	0.135	0.449
A2B1	0.131	0.094	0.090	0.122	0.059	0.496
A2B2	0.131	0.134	0.090	0.122	0.059	0.536
A2B3	0.057	0.057	0.056	0.143	0.160	0.474
A3B1	0.154	0.134	0.147	0.057	0.030	0.521
A3B2	0.154	0.158	0.147	0.030	0.030	0.518
A3B3	0.027	0.026	0.027	0.143	0.160	0.384

that the respondents preferred *Cucumis melo* stored in a two-layer banana stem packaging at 14°C (A3B2). Since the scores were fairly close between *Cucumis melo* stored in a two-layer banana stem packaging at 14°C (A3B2) and *Cucumis melo* stored in a two-layer banana stem packaging at 10°C (A3B1), both products were favored by consumers (respondents' preferences).

CONCLUSION

According to the consumer assessment of all types of the six-day storage of *Cucumis melo*, the optimal storage conditions involved packaging with two layers of banana stem at the temperature of 14°C (A3B2). The fuzzy-Eckenrode and fuzzy-TOPSIS methods were very helpful in calculating the results of the multi-criteria

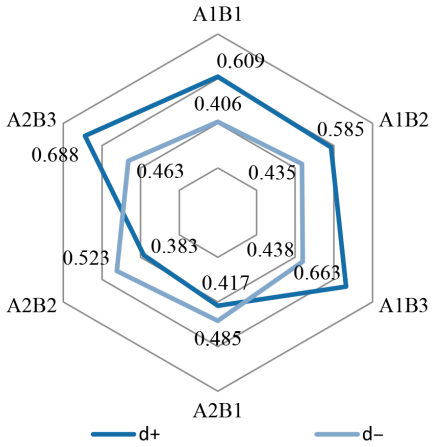

Figure 6 Evaluation of d+ and d–

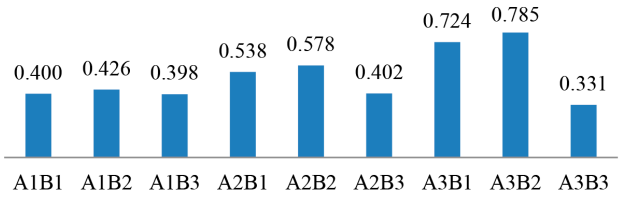

Figure 7 Alternative ranking of *Cucumis melo* product acceptance

sensory assessment through weighing. They made the process of determining consumers' acceptance easier, faster, and more certain.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Comparative evaluation of approaches to modelling kinetics of microbial thermal death as in the case of *Alicyclobacillus acidoterrestris*

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Abstract: Microbial death kinetics modelling is an integral stage of developing the food thermal sterilisation regimes. At present, a large number of models have been developed. Their properties are usually being accepted as adequate even beyond boundaries of experimental microbiological data zone. The wide range of primary models existence implies the lack of universality of each ones. This paper presents a comparative assessment of linear and nonlinear models of microbial death kinetics during the heat treatment of the Alicyclobacillus acidoterrestris spore form. The research allowed finding that single-phase primary models (as adjustable functions) are statistically acceptable for approximation of the experimental data: linear – the Bigelow' the Bigelow as modified by Arrhenius and the Whiting-Buchanan models; and nonlinear – the Weibull, the Fermi, the Kamau, the Membre and the Augustin models. The analysis of them established a high degree of variability for extrapolative characteristics and, as a result, a marked empirical character of adjustable functions, i.e. unsatisfactory convergence of results for different models. This is presumably conditioned by the particularity and, in some cases, phenomenology of the functions themselves. Consequently, there is no reason to believe that the heat treatment regimes, developed on the basis of any of these empirical models, are the most effective. This analysis is the first link in arguing the necessity to initiate the research aimed at developing a new methodology for determining the regimes of food thermal sterilisation based on analysis of the fundamental factors such as ones defined spore germination activation and their resistance to external impact.

Keywords: Microorganisms, death kinetics, survival kinetics, sterilising effect, *Alicyclobacillus acidoterrestris*, model

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INTRODUCTION

Microbiological safety is one of the key tasks in developing food production technology [1–4]. Most existing solutions are based on microbial inactivation via chemical or physical methods, as well as competitive substitution of pathogenic and opportunistic pathogenic microflora with probiotic bacteria. At the same time, thermal sterilisation remains the most common way to achieve the required level of microbiological safety.

The first stage in developing the thermal sterilisation mode is traditionally the analysis of the microbial death kinetics in the medium of the product for which this mode is developed [5]. Microbiological studies are carried out under isothermal conditions and repeated at different temperatures. Then the experimental data are approximated with a particular model [6–8]. Naturally, the adequacy of the selected model to the experimental data confirms the adequacy of ideas about the microbial death kinetics in general and, in particular, regarding extrapolation at concentrations of microorganisms down to 1 CDU/g. Obtaining correct quantitative experimental microbiological data for these concentrations is associated with geometrically increasing resource intensity and experimental error.

According to Whiting and Bushanan, all mathematical models describing the microbial response to the external negative effects can be divided into three large groups – primary, secondary and tertiary models. Thus, primary models approximate experimental data of the microbial death kinetics under isothermal conditions

as a function of processing duration. Secondary models connect primary models, approximating them as a function of additional factors, which include primarily the temperature of processing, when developing thermal sterilisation regimes. Tertiary models include the software implementation of secondary models [9]. However, we believe, it would be more logical if the adaptation of secondary models to non-isothermal sterilisation regimes within specified boundary conditions was referred to tertiary models.

The primary models of thermal inactivation of microorganisms broke into development at the turn of the XXth century. Then, basing on the analogy with the first order chemical reaction, Chick, and later Bigelow presented a model reflecting the kinetics of the microbial death resulted from external adverse factors (Bigelow model) as a linear kinetic model of the first order [10, 11]. Bigelow showed later that in general the kinetics sought for can be adequately represented in semi-logarithmic coordinates [12]. Due to the simplicity of form and further manipulations, this model has become classic:

$$\lg N = \lg N_0 - k \cdot \tau \quad (1)$$

where N is a trough concentration of microorganisms, CFU/g;

N_0 is an initial concentration of microorganisms, CFU/g;

k is the rate of microbial death kinetics (often defined as a constant), lg (CFU/g)·min^{-1} (time may be expressed in s, h, etc.);

τ is a duration of processing, min (time may be expressed in s, hour, etc.).

This model is based on the assumptions that all cells of microorganisms have the same resistance to the thermal impact in the processed product, and their death kinetics complies with statistical regularities [9, 13]. As a result, the death of each individual cell is considered from the point of view of accidental inactivation of the 'critical molecule'.

To avoid pure empiricalness, some researchers attempted to adjust the Bigelow model. They expressed the dependence of the microbial death rate on the temperature of the process similarly to the dependence of chemical kinetics on the activation energy according to the Arrhenius equation [5]:

$$k = N_0 \cdot \exp\left[-\frac{E_0}{R \cdot (T + T_{abs})}\right] \quad (2)$$

where E_0 is activation energy, J/mol;

R is the universal gas constant (8.3144598 J/mol·K);

T_{abs} is the absolute temperature of the triple point of water (273.16 K);

T is the process temperature, °C.

However, Peleg *et al.* question the adequacy of this approach in their work [14]. They argue this with the cardinal difference of the microbial death kinetics from the conventional chemical kinetics. The difference manifests itself in the absence of microflora inactivation under normal conditions and, therefore, in absence of continuity of functional dependence of microbial concentration on temperature over the entire range of its determination.

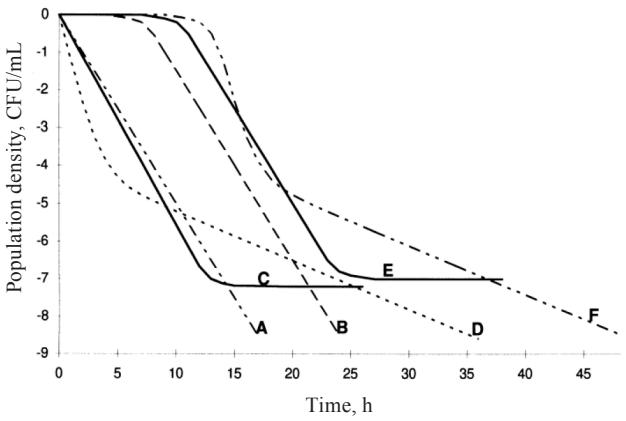


Figure 1 Dynamics of microbial death according to [15]. (A) linear kinetics; (B) linear kinetics with a lag phase; (C) and (D) nonlinear kinetics with 'tails'; (E) and (F) sigmoidal kinetics

Nowadays, there are at least four main types of kinetics of microbial death as a result of heat treatment, including linear (Fig. 1) [15].

Kinetics with a lag phase is one of the frequent deviations of microbial death kinetics [16]. This type of dependence approximates satisfactorily the Whiting-Buchanan model proposed by Whiting and Buchanan [17, 18]:

$$\lg N = \begin{cases} \lg N_0, & \tau \le \tau_{lag} \\ \lg N_0 - k \cdot (\tau - \tau_{lag}), & \tau > \tau_{lag} \end{cases} \quad (3)$$

where τ_{lag} is a lag phase duration.

Van Boekel carried out a large-scale study and analysed more than 120 curves of microbial death kinetics. He concluded that linear models described no more than 5 percent of cases. It implies that this kinetics is an exception rather than the rule [19]. Van Boekel suggested that non-linear models should be used for the most adequate description of kinetics. It should be understood that nonlinearity of models is determined primarily by their parameters, since the graphical representation resulting from approximation can have both nonlinear and linear views [16]. The simplest nonlinear model, the Weibull model, is based on the idea of statistical distribution of probability for the death of cells and/or microbial spores under the adverse external conditions as a result of their individual variability [20]:

$$\lg N = \lg N_0 - \left(\frac{\tau}{\delta}\right)^p \quad (4)$$

where δ is a coefficient.

It is notable that at $P > 1$ the model represents a convex curve, while at $P < 1$ the curve is concave. When $P = 1$, the model becomes identical to the Bigelow model. The coefficient δ becomes equivalent to the value of D in the interpretation of the same model. It probably gave Mafart a reason to adopt the coefficient δ as the duration of the process required to reduce the microbial concentration by one order [20].

Bhaduri *et al.* were the first who showed that the empirical modified Gompertz equation, the Gompertz model, can effectively describe the thermal death kinetics of *Listeria monocytogenes* [21]:

$$\lg N = \lg N_0 - C \cdot \exp\left\{-\exp\left[-b \cdot (\tau - M)\right]\right\} \quad (5)$$

where *C*, *b* and *M* are coefficients.

A little earlier, Casolari proposed a model that satisfactorily approximates the microbiological data of kinetics with 'tails', i.e. the first Casolari model [22]:

$$\lg N = \lg N_0 - \lg\left(\frac{1}{1 + b_T \cdot \tau}\right) \quad (6)$$

where b_T is a coefficient.

He linked the microbial death to the critical activity of water molecules when their energy exceeds a threshold value E_0, thus combining the probability theory and Maxwell energy distribution:

$$b_T = \left(\frac{N_a}{M_{H_2O}}\right)^2 \cdot \exp\left[-\frac{2 \cdot E_0}{R \cdot (T + T_{abs})}\right] \quad (7)$$

where N_a is Avogadro's constant ($6.022140857 \times 10^{23}$, mol^{-1});

M_{H_2O} is molar mass of water (18.01528 g/mol).

In the same work and later in [6, 23] was presented the modification of this model, i.e. the second Casolari model. It included a quadratic dependence on the processing duration:

$$\lg N = \lg N_0 + \lg\left(\frac{1}{1 + b_T \cdot \tau^2}\right) \quad (8)$$

In their turn, Daugthry *et al.* proposed an exponentially decreasing model, i.e the Daugthry model [24]. They justified its advantage over linear models due to the approximation accuracy of the experimental data on the death kinetics of *Escherichia coli* and *Staphylococcus aureus*

$$\lg N = \lg N_0 - k \cdot \tau \cdot \exp(-\lambda_d \cdot \tau) \quad (9)$$

where k is the initial rate of inactivation;

λ_d is the descending factor.

Data in [25, 26] demonstrate the expediency of a logistic function, i.e. the Fermi model, for describing the kinetics of microbial death limited by a number of stressful factors:

$$\lg N = \lg N_0 + \lg\left\{\frac{1 + \exp(-b \cdot \tau_{lag})}{1 + \exp\left[b \cdot (\tau - \tau_{lag})\right]}\right\} \quad (10)$$

where τ_{lag} is a lag phase duration.

Cole *et al.* proposed a four-factor logistic model, i.e. the Cole model [27]:

$$\lg N = \lg N_0 + \frac{\omega - \lg N_0}{1 + \exp\left[\frac{4 \cdot \sigma \cdot (\tau - \tau_{lag})}{\omega - \lg N_0}\right]} \quad (11)$$

where ω is the value of the lower asymptote of microbial death kinetics, lg (CFU/g);

σ is the maximum rate of kinetics.

The model satisfactorily described the thermal inactivation of *Salmonella typhimurium*, *Cl. botulinum*, *Salmonella enteritidis* and *E. coli*.

Membre *et al.* proposed a modified logistic function, the Membre model, to describe the thermal inactivation of *Salmonella typhimurium*. They assumed that the model could extrapolate on other microorganisms and products [28]:

$$\lg N = (1 + \lg N_0) - \exp(k \cdot \tau) \quad (12)$$

Another kinetic model, the Kamau model, is proposed by Kamau *et al.* in relation to *Listeria monocytogenes* and *Staphylococcus aureus* [29]:

$$\lg N = \lg N_0 + \lg\left[\frac{2}{1 + \exp(k_d \cdot \tau)}\right] \quad (13)$$

where k_d is a coefficient.

The complex logistic model, the Baranyi model, was developed as an alternative to the Gompertz model described by formula (5). It takes into account the advantages of the Gompertz model and levels its disadvantages. Xiong *et al.* modified the Baranyi model, which took the form [30]:

$$\lg\frac{N}{N_0} = \lg\left\{q_b + (1 - q_b) \cdot \exp\left[-\mu_m \cdot (\tau - B_T)\right]\right\} \quad (14)$$

$$B_T = \frac{r}{3} \cdot \left\{\frac{1}{2} \cdot \ln\left[\frac{(r+T)^2}{r^2 - r \cdot T + T^2}\right] + \sqrt{3} \cdot \operatorname{arctg}\left(\frac{2 \cdot T - r}{r \cdot \sqrt{3}}\right) + \sqrt{3} \cdot \operatorname{arctg}\left(\frac{1}{\sqrt{3}}\right)\right\} \quad (15)$$

where q_b is an indicator of the 'tail' of kinetics, reflecting its manifestation or absence;

μ_m is a maximum relative level of thermal inactivation;

r is a lag parameter, numerically equal to half μ_m;

B_T is a coefficient.

A distinctive feature of the Baranyi model is the possibility to derive the kinetic model of the first order on its basis.

Geeraerd *et al.* proposed the Geeraerd model as a complex approach to describing the complex kinetics of thermal microbial inactivation [23]. The model takes into account the presence or absence of the 'tail' of the function, and also a lag phase in microorganisms responding to the thermal effect:

$$\lg N = 10^{\lg N_{res}} + \left(10^{\lg N_0} - 10^{\lg N_{res}}\right) \cdot \exp(-k \cdot \tau) \cdot$$

$$\cdot\left[\frac{\exp\left(k\cdot\tau_{lag}\right)}{1+\left[\exp\left(k\cdot\tau_{lag}\right)-1\right]\cdot\exp\left(-k\cdot\tau\right)}\right] \quad (16)$$

The analysis of these models shows that in many cases logistic functions are mathematical abstractions that describe experimental microbiological data more or less satisfactorily. The Augustin model proposed in [31] and originally intended to describe the kinetics of thermal inactivation of *Listeria monocytogenes* is no exception

$$\lg N=\lg N_0-\lg\left[1+\exp\left(\frac{\lg\tau-m}{s^2}\right)\right] \quad (17)$$

where m and s are coefficients.

However, due to the commonality of this model with other logistic functions, this model may be ostensibly applicable to the description of thermal inactivation kinetics of other microorganisms.

The Fernandez model stands apart. It was proposed in [32] and based on the statistical approach to estimating the density of distribution:

$$\lg N=a^{-b}\cdot b\cdot\tau^{b-1}\cdot\exp\left[-\left(\tau^a\right)^b\right] \quad (18)$$

A new empirical nonlinear model was developed, i.e. the Chiruta model [33]:

$$\lg\frac{N}{N_0}=1-\exp\left[a+b\cdot\ln\tau+c\cdot\left(\ln\tau\right)^2\right] \quad (19)$$

where a, b and c are coefficients.

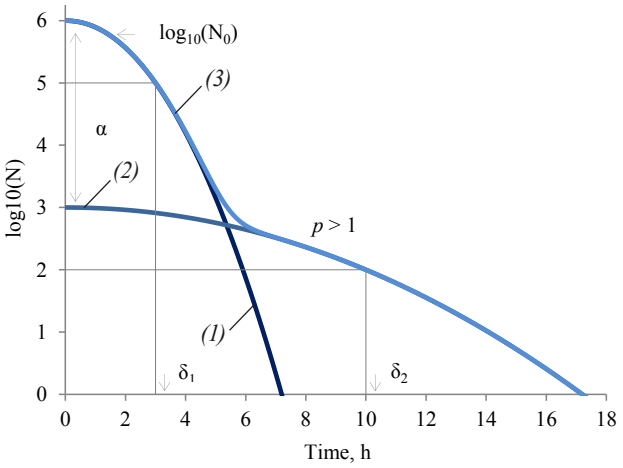
The model is a modified polynomial function with all properties characteristic of approximation functions of this class. The properties are satisfactory interpolation and sensitivity to approximated data. Extrapolation of this model is possible, but it must be carried out with caution and mandatory experimental validation.

Analysing the existing array of the experimental microbiological data, Cerf was one of the first to who suggested that the deviation of kinetics from linear (in semilogarithmic coordinates) is most likely a consequence of simultaneous presence of at least two microbial subpopulations with different resistance to external negative effects in a genetically homogeneous population [34]. The deviation may also be caused by artefacts (generated by a set of perturbation factors that are not taken into account, or are not levelled in the formulation and execution of studies). Figure 2 presents a graphical interpretation of this approach.

In relation to thermal inactivation kinetics, the result of this conclusion is the use of two-phase models, taking into account the contribution of each subpopulation to the integral response. Models of this kind were developed by Geeraerd *et al.*, Kamau *et al.*, Xiong *et al.*, Cerf, Whiting and Buchanan, and Coroller *et al.* [23, 29, 30, 34–36].

The Cerf model is described by the formula:

$$\lg N=\lg N_0+\lg\left[f\cdot\exp\left(-k_1\cdot\tau\right)+\right.$$



(1) – I subpopulation *(2)* – II subpopulation *(3)* – superposition

Figure 2 Graphical interpretation of the subpopulation approach to the microbial death kinetics during heat treatment (adapted from [36])

$$\left.+\left(1-f\right)\cdot\exp\left(-k_2\cdot\tau\right)\right] \quad (20)$$

where f is a share of the first subpopulation in the test culture;

1 and 2 are indices of coefficients belonging to the subpopulation.

The Kamau model is described by the formula:

$$\lg N=\lg N_0+\lg\left[\frac{2\cdot f}{1+\exp\left(b_1\cdot\tau\right)}+\frac{2\cdot\left(1-f\right)}{1+\exp\left(b_2\cdot\tau\right)}\right] \quad (21)$$

The Whiting-Buchanan model:

$$\lg N=\lg N_0+\lg\left\{\frac{f\cdot\left[1+\exp\left(-b_1\cdot\tau_{lag}\right)\right]}{1+\exp\left[b_1\cdot\left(\tau-\tau_{lag}\right)\right]}+\right.$$

$$\left.+\frac{\left(1-f\right)\cdot\left[1+\exp\left(-b_2\cdot\tau_{lag}\right)\right]}{1+\exp\left[b_2\cdot\left(\tau-\tau_{lag}\right)\right]}\right\} \quad (22)$$

The Coroller model:

$$\lg N=\lg N_0+\lg\left[f\cdot10^{-\left(\frac{\tau}{\delta_1}\right)^{p_1}}+\left(1-f\right)\cdot10^{-\left(\frac{\tau}{\delta_2}\right)^{p_2}}\right] \quad (23)$$

The Xiong model:

$$\lg\frac{N}{N_0}=1\begin{cases}0, & \tau\le\tau_{lag}\\ f(\tau), & \tau>\tau_{lag}\end{cases} \quad (24)$$

where

$$f(\tau)=\lg\left\{f\cdot\exp\left[-k_1\cdot\left(\tau-\tau_{lag}\right)+\left(1-f\right)\cdot\right.\right.$$

$$\left.\left.\cdot\exp\left[-k_2\cdot\left(\tau-\tau_{lag}\right)\right]\right]\right\} \quad (25)$$

The Geeraerd model:

$$\lg N=\lg N_0+\lg\left\{f\cdot\exp\left(-k_1\cdot\tau\right)+\left(1-f\right)\cdot\right.$$

$$\cdot \exp\left(-k_2 \cdot \tau\right) \cdot \frac{\exp\left(k_1 \cdot \tau\right)}{1+\left[\exp\left(k_1 \cdot \tau_{lag}\right)-1\right] \cdot \exp\left(-k_1 \cdot \tau\right)} \Bigg\} \quad (26)$$

Theoretically, there may be a significant difference in the concentration of subpopulations (10 or more times). As a result, the calculation of the share of the first subpopulation f may be complicated due to its small correction from unity. To solve this problem, Geeraerd proposed to substitute the indicator f with its more variable form:

$$\alpha = \lg\left(\frac{f}{1-f}\right) \quad (27)$$

Then:

$$f = \frac{10^{\alpha}}{1+10^{\alpha}} \quad (28)$$

A wide variety of primary models implies the lack of universality of each of them. It turns the application of such models into their adjusting to specific experimental data of microbial thermal inactivation kinetics. At the same time, it is still unclear whether the results of extrapolating these models outside the scope of determining the experimental values of input and output factors are adequate. The adequacy can be partly justified with the arguments by the authors of the models. However, as the authors of the models suggested, the arguments are also very empirical.

Accordingly, the issue of adequate applicability or non-applicability of empirical approaches to determining thermal sterilisation regimes requires comparative assessment of models and modes predicted with the help of the approaches. The convergence of their results as well as compliance with the existing trends of the development of food technologies and requirements for quality and safety of food products should be taken into consideration.

Thus, the aim of this work was to analyse the convergence of existing approaches to modelling the microbial death kinetics during heat treatment. To achieve this goal, we solved the following tasks:

– an analytical review of existing approaches to modelling the microbial death kinetics during the heat treatment;

– a comparative evaluation of (a) primary kinetic models of microbial spore death; (b) secondary kinetic models of microbial spore death; and (c) models describing rate dynamics of microbial death kinetics.

STUDY OBJECTS AND METHODS

The research focused on the following objects: the spore form of the guaiacol-positive strain of *Alicyclobacillus acidoterrestris* RNCIM V-1008 from the centre for culture collection of microorganisms, the Laboratory of Quality and Food Safety of the Russian Research Institute of Canning Technology.

The spore suspension of *A. acidoterrestris* was prepared according to [37]. For this purpose, the culture from the collection was activated by means of double or triple relocation to the liquid nutrient enrichment medium, i.e. the YSG medium (HiMedia Laboratories Pvt. Ltd., India). Subsequently, the actively growing daily culture was planted into Petri dishes with pre-prepared BAT-agar (HiMedia Laboratories Pvt. Ltd., India).

For this purpose, 0.1–0.2 cm^3 of the cell culture fluid was evenly distributed on the surface of the medium with a spatula. The platings were thermostated at 40°C for 96 h. To detect the spores, the native sample was studied with phase contrast microscopy, using the Zeiss Axioscope microscope, equipped with Canon PC 1200 camera and original AxioVision Rel.4.8 software. The culture contained light refractive shiny spores. Their amount was not less than 70% compared to the total number of cells. The spores produced on a solid nutrient medium were washed off with a phosphate buffer (0.1M aqueous solution of phosphate buffer, pH 6.98), according to [38], approximately 10–15 cm^3 solution per 75 cm^2 surface. The spores were separated from the medium by centrifuging the culture fluid at 275 g for 30 min. Washing and centrifuging were repeated several times.

The washed sediment was suspended in the medium of concentrated apple juice. The resulting suspension had a spore concentration of not less than 10^7 CFU/g. To inactivate the remaining vegetative cells, the suspension was heated at 80°C for 10 min. The concentration of spores in the suspension was determined by plating appropriate dilutions on BAT-agar within Petri dishes. The obtained suspension was used to determine the parameters of thermal stability in the concentrated apple juice (ACJ).

The capillary method was used to determine the parameters of thermal stability of the spores in the studied juice. For this purpose, the medium was contaminated by applying the spore suspension in sterile conditions. The capillaries were thin-walled glass tubes, 75 mm long, outer diameter of 3 mm. The spore suspension was injected into capillaries by 0.1 cm^3. Each capillary contained spores at a concentration of 5.31 lg (CFU/g). The filled capillaries were warmed up in the circulating thermostat series LOIP LT-311 (Russia) in the glycerine medium at 100°C and over and the aqueous medium at temperatures below 100°C.

The contaminated samples were thermostated in capillaries at 90 and 95°C for 420 s, 100°C for 300 s, and 105°C for 150 s. The trough concentration of the surviving spores was established after 0, 120, 240, 360 and 420 s for 90 and 95°C; after 0, 60, 120, 180, 240, and 300 s for 100°C, and after 0, 30, 60, 90, 120, 150 s for 105°C. The trough concentration of the surviving spores was determined according to [37] by direct inoculation method. The samples of 1 cm^3 were analysed using YSG-agars as dense nutrient media. The initial processing of the inoculation results was carried out according to [39]. All microbiological studies were carried out in four-fold repetition, rejecting statistically unreliable data.

To determine the dynamics of heating the ACJ in the least heated zone, the sterile medium was placed in a glass jar of 100 cm^3 with a twist-off lid and a thermocouple element fixed by a vertical axis of the jar, 15 mm off the inside surface of the bottom. Hermetically sealed jars with ACJ were thermostated at 90, 95, 100 and 105°C.

The temperature was measured in the least heated point at 30-second intervals for 42 min. After that, the jars were cooled in a water tank at 15°C for 10 min. The automatic multichannel thermometer CTF9008 (Ellab A/S) connected to the thermocouples was used for temperature control during the heat treatment. To reduce statistical error, each experiment was carried out in a three-fold repetition, rejecting statistically unreliable data.

Mathematical processing and modelling was carried out using a spreadsheet processor Microsoft Excel 2010 (Microsoft Corporation) with installed add-ons 'Data Analysis', 'Solution Search' and 'Parameter Selection', as well as specialised software — TableCurve 2D v.5.01 (SYSTAT Software Inc.) and Wolfram Mathematica 10.4 (Wolfram Research Inc.).

Approximation of the experimental data was carried out under the following parameters of TableCurve 2D Fitting Controls option of TableCurve 2D v.5.01: linear approximation was by Singular Value Decomposition; the level of robustness (stability) of nonlinear approximation was high (Pearson VII Lim); minimisation by natural logarithm of the square root of the sum '1 + squared remainder'.

RESULTS AND DISCUSSION

Comparative evaluation of primary kinetic models of the microbial spore death. The literary data review showed that at present a basic provision underlying any approach to determining the thermal sterilisation regimes for canned products is experimental determination of the microbial death kinetics in the analysed product with the subsequent approximation and extrapolation of the obtained model. Therefore, the initial criterion for assessing the adequacy of the particular primary model application to describe experimentally fixed kinetics is the convergence of approximating (averaging) and interpolating properties (corresponding to the numerical values in the nodes, i.e. experimental points).

In the first approximation, this criterion is numerically equivalent to the determination coefficient, i.e. the square of the correlation coefficient. However, not all models can be calculated directly. Thus, in this study, linearising transformations were carried out previously for a number of models, i.e. the Kamau model, the Membre model and the Augustin model:

– the Kamau model

$$\ln(K) = \ln\left(\frac{2 \cdot N_0}{N} - 1\right) = k \cdot \tau \qquad (29)$$

– the Membre model

$$\ln(M) = \ln\left[-\lg\left(\frac{N}{10 \cdot N_0}\right)\right] = k \cdot \tau \qquad (30)$$

– the Augustin model

$$\ln(A) = \ln\left(\frac{N_0}{N} - 1\right) = \frac{\lg\tau - m}{s^2} \qquad (31)$$

The lower threshold of the determination coefficient of 0.9 was adopted as a boundary condition determining the applicability of the primary model for approximation of the experimental data.

Thus, out of the described set of primary models, only seven models complied with the experimental data of survival kinetics of *A. acidoterrestris*. There were two linear models (the Bigelow model and the Whiting-Buchanan model) and five nonlinear models (the Weibull model, the Fermi model, the Kamau model, the Membre model and the Augustin model). In addition, in order to expand the potential of the primary models used for the Bigelow model, the activation energy of the microbial spore death was calculated in each of the temperature options according to Arrhenius. That indirectly increased the number of analysed models. At the same time, both the Bigellow model and its modification by Arrhenius in its primary form were actually identical.

The analysis of the study results showed a somewhat larger aggregate (for all temperature variants) adequacy of nonlinear models at experimental data approximation (Table 1). Thus, the determination coefficient did not fall below 0.965 for all temperature variations in nonlinear models. On the other hand, this coefficient decreased up to 0.936 and 0.956 for the Bigelow model and the Whiting-Buchanan model, respectively, in linear models. The non-linear Membre model was the only exception, comparable in aggregate adequacy to the Whiting-Buchanan model.

It is noteworthy that the activation energy of the microbial death, corresponding to the Bigellow model as modified by Arrhenius, was not constant. When the processing temperature was increasing, this value was monotonously decreasing, which presumably confirms the fact that many factors impact microbial resistance to external adverse conditions. When the temperature increases within the range of values corresponding to proteins denaturation, the number of such factors inevitably decreases. As a result, less energy is required to reach the target effect of the microbial death.

Table 1 presents characteristic indicators and determination coefficients corresponding to linear and non-linear models.

Table 1 demonstrates the heterogeneity of the approximation efficiency for any of the selected models within the temperature values in the experiment variants. It should be noted for most nonlinear models (except the Augustin model) that the approximation adequacy decreases when the linearity of experimentally determined kinetics increases and vice versa. The latter was established by the determination coefficient increase

Table 1 Characteristic indicators and determination coefficients corresponding to linear and nonlinear models

Temperature, °C	Linear models						
	Bigelow model			Bigelow model modified by Arrhenius	Whiting model		
	k, lg (CFU/g) /s	D, min	r^2	E_0, J/mol	k, lg (CFU/g)/s	τ_{lag}, s	r^2
90	1.089×10^{-3}	15.31	0.9585	57 745	1.136×10^{-3}	13.904	0.9611
95	2.964×10^{-3}	5.62	0.9876	55 183	2.985×10^{-3}	2.85×10^{-11}	0.9875
100	5.560×10^{-3}	3.00	0.9932	53 980	5.591×10^{-3}	1.226	0.9933
105	14.291×10^{-3}	1.17	0.9355	51 889	16.357×10^{-3}	13.041	0.9564
	Non-linear models						
	Weibull model			Membre model		Kamau model	
	p	δ, s	r^2	k, s^{-1}	r^2	k, s^{-1}	r^2
90	1.17	797.976	0.9722	9.14×10^{-4}	0.9748	3.802×10^{-3}	0.9764
95		340.534	0.9711	20.31×10^{-4}	0.9526	8.663×10^{-3}	0.9652
100		187.498	0.9853	35.44×10^{-4}	0.9651	15.848×10^{-3}	0.9841
105		74.419	0.9686	82.25×10^{-4}	0.9960	38.245×10^{-3}	0.9698
	Fermi model			Augustin model			
	b, s^{-1}	τ_{lag}, s	r^2	m, ln (s)	s, $[\lg(s)]^{1/2}$	r^2	
90	5.045×10^{-3}	182.908	0.9806	2.432	0.561	0.9957	
95	8.701×10^{-3}	3.30×10^{-11}	0.9649	1.965	0.496	0.9980	
100	15.834×10^{-3}	2.02×10^{-11}	0.9822	1.789	0.436	0.9975	
105	51.767×10^{-3}	39.110	0.9949	1.596	0.356	0.9881	

for linear models. It proves a low universality of the primary (in fact – adjustable) models. The consequence is an increase in the deviation of model-predicted values of the surviving microbial spore concentrations when extrapolating from the experimental data, intuitively expected on the basis of visual estimation (Figs. 3 and 4).

Thus, at 105°C, extrapolating both linear and most non-linear models towards increasing the duration showed lower heat treatment efficiency than it was intuitively assumed from the visual estimation of experimental data. It is true even for the Augustin model, approximating experimental data as formally adequately as possible. Conclusions were formed on the basis of modelling microbial death kinetics under thermal influence and its subsequent extrapolation. They were expressed in the form of the ratio of temperature and duration of treatment to achieve a given sterilising effect. Though, as a result of the above said, the conclusions will be inevitably overrated against the true state of affairs.

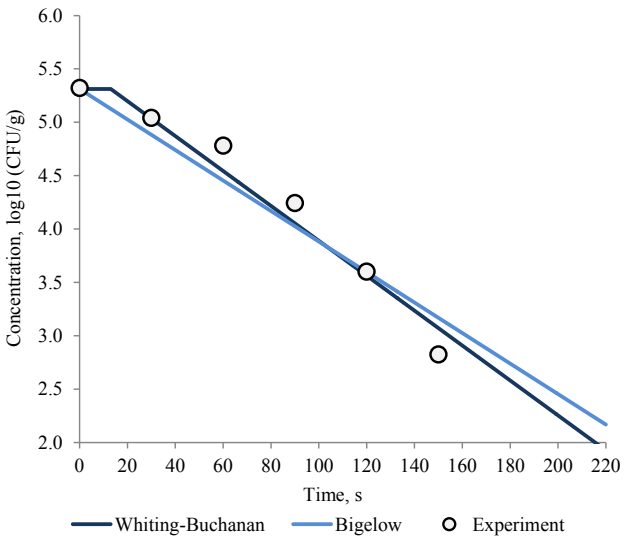


Figure 3 Approximation of experimental data of microbial death kinetics of *A. acidoterrestris* during heat treatment (t = 105°C) by using linear models

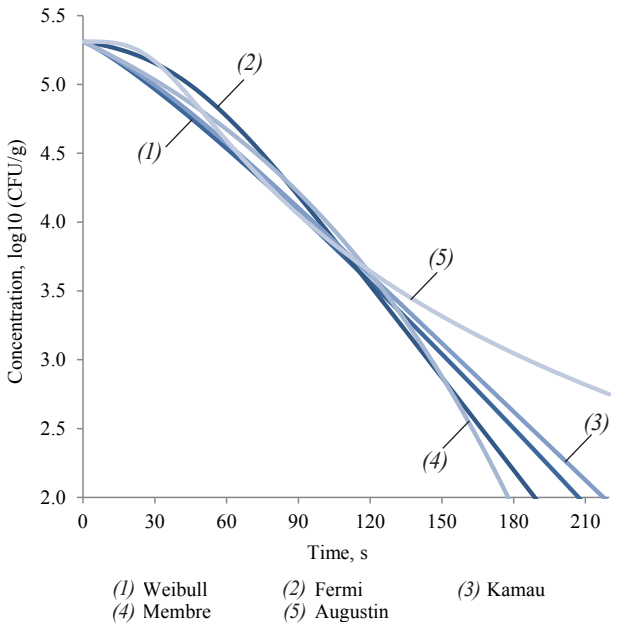


Figure 4 Approximation of the experimental data of microbial survival kinetics of *A. acidoterrestris* during heat treatment (t = 105°C) by using nonlinear models

Thus, finding the primary model satisfying all variants of experimentally established kinetics even in the field of determining variable independent factors is a non-trivial problem. This problem appears in conditions of empiricism and simplification, and in many cases phenomenology of both existing primary models and approaches to their development. This conclusion is compounded by the ambiguity of extrapolating properties of the models. However, the whole further algorithm of determining the sterilisation regimes was built on these properties.

Comparative evaluation of secondary kinetic models of the microbial spore death kinetics. The numerical values of coefficients for primary microbial death models are different when calculated as a result of approximation of experimental data at different temperatures of heat treatment. It suggests some dependence of these values on temperature. The format of primary models has a certain logic though simplified. Unlike them, the functional dependencies of these models coefficients on the processing temperature are exclusively adjustable functions. These functions approximate an array of numerical values most efficiently and extrapolate logically. They extend the value of the independent factor in any direction beyond the scope of determining experimental values.

Thus, the coefficients of linear primary models depend on temperature as follows:

– the Bigelow and Whiting-Buchanan models:

$$\lg k = -a + \frac{T}{b} \quad (32)$$

– the Bigelow model:

$$\lg D = a - \frac{T}{b} \quad (33)$$

– the Whiting-Buchanan model:

$$\lg \tau_{lag} = a + b \cdot \exp\left[-\exp\left(-\frac{T-c}{d} \right) - \frac{T-c}{d} + 1 \right] \quad (34)$$

– the Bigelow model as modified by Arrhenius:

$$\lg E_0 = -a + T \cdot b \quad (35)$$

This approach resulted in differences in functional description of secondary models: the Bigelow model and the Bigelow model as modified by Arrhenius. The coefficient k carried the function of the kinetic rate of the thermal microbial death and in the first case it depended on temperature linearly (in semilogarithmic coordinates). Conversely, in the second case, this dependence acquired nonlinearity due to the complex of activation energy and the processing temperature. The activation energy itself had a linear dependence on the processing temperature in semi-logarithmic coordinates:

$$k = N_0 \cdot \exp\left[-\frac{10^{-a+T \cdot b}}{R \cdot (T + T_{abs})} \right] \quad (36)$$

The coefficients of nonlinear primary models depended on temperature as follows:

– the Weibull model:

$$\lg \delta = a - \frac{T}{b} \quad (37)$$

– the Fermi model:

$$\lg b = -a + \frac{T}{b} \quad (38)$$

$$\lg \tau_{lag} = a + b \cdot \exp\left\{ -\frac{1}{2} \cdot \left[\frac{\ln\left(T \cdot c^{-1}\right)}{d} \right]^2 \right\} \quad (39)$$

– the Kamau model and the Membre model:

$$\lg k = -a + \frac{T}{b} \quad (40)$$

– the Augustin model:

$$\lg m = a - \frac{T}{b} \quad (41)$$

$$\lg s = -a + \frac{T}{b} \quad (42)$$

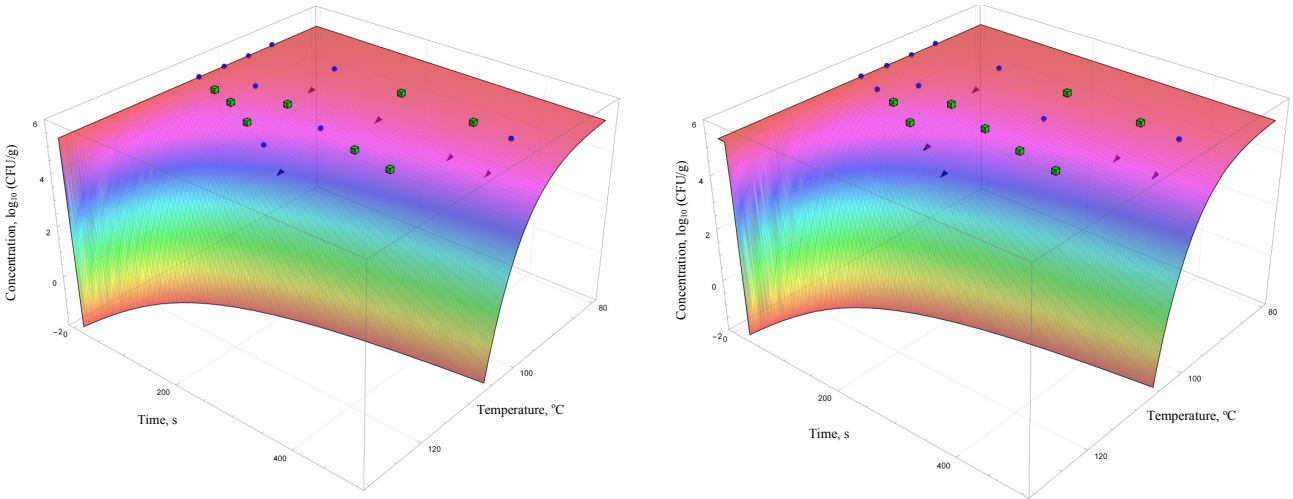
The coefficients are functionally expressed through formulae (32)–(42). In general, they allowed obtaining more informative – secondary – analogues for each of the analysed primary models (Figs. 5–8).

It is mandatory to bear in mind that primary models represent some degree of approximation of the functional idea of the microbial death dynamics as a result of thermal effect at the specified temperature.

In its turn, the functional dependence of coefficients on temperature allows determining their values in the process of inter- and/or extrapolation with a certain degree of approximation as well. Piling up with the error generated by the primary model, it determines the total degree of approximation to the experimental data in determining the temperature and processing time. This also sets some uncertainty in further extrapolation. The consequence of this conclusion presents itself in the degree to which secondary models comply with the experimental data on the basis of which these models were obtained.

The models were superimposed on the array of experimental microbiological data in Figs. 5–8. Colour variations indicated the consistency of experimental data, taking into account their errors, and the model. The green cubes show experimental data that exceeded the concentration of the surviving microbial spores calculated on the basis of the model. Black triangles show the data having values below the calculated ones. Blue spheres indicate values the same as the model within the error range of experimental data.

The analysis of the convergence of experimental data and models showed that there is a poor convergence with the kinetics at 95°C in the case of linear primary models. In addition, the secondary models – the Whiting-Buchanan model (Fig. 5b) and the Bigelow model as modified Arrhenius (Fig. 6a) – showed a



(a) (b)

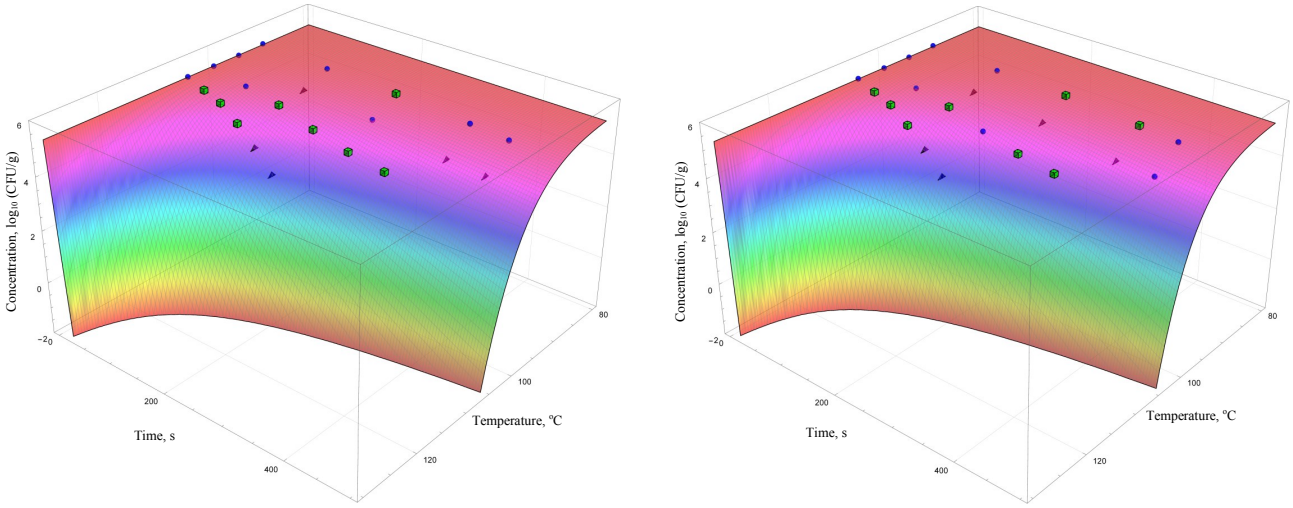
Figure 5 Secondary linear models of microbial death of *A. acidoterrestris* during heat treatment. (a) Bigelow model, (b) Whiting-Buchanan model

greater progression in the microbial death kinetics at 100°C compared to the kinetics as determined by the experimental data. However, these same models showed unsatisfactory extrapolative properties at 105°C towards overrating the concentration of the surviving microorganisms.

There was even a greater heterogeneity in the case of secondary models based on nonlinear primary models. Thus, the secondary model based on the Fermi model (Fig. 7a) has the visually worst approximation properties and, as a consequence, extrapolative characteristics. This model overestimates the calculated concentration of the surviving microorganisms for the most part in comparison with the experimental values. The Weibull model (Fig. 6b) is characterised by almost the same disadvantages as those noted for the Whiting-Buchanan model and the Bigelow model modified by Arrhenius.

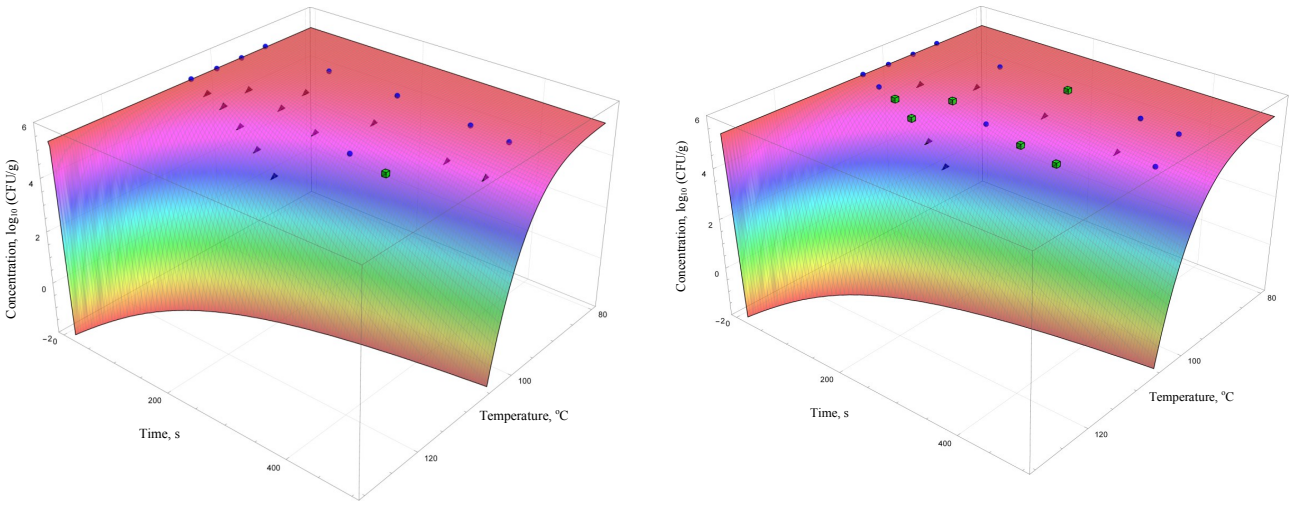
The same statement, but to a lesser extent, can be applied to the secondary models, i.e. the Kamau model (Fig. 7b) and the Membre model (Fig. 8a). Of all secondary models, the Augustin model was the most appropriate in terms of convergence with the experimental data. However, it was also characterised by overestimating the calculated values of surviving microbial concentration at the treatment temperature of 95°C, as well as overestimating the calculated values at extrapolation at the temperature of 105°C.

However, the graphical analysis of the secondary models showed that each of them was characterised



(a) (b)

Figure 6 Secondary linear model and nonlinear model of microbial death *A. acidoterrestris* during heat treatment. (a) Bigelow model modified by Arrhenius, (b) Weibull model



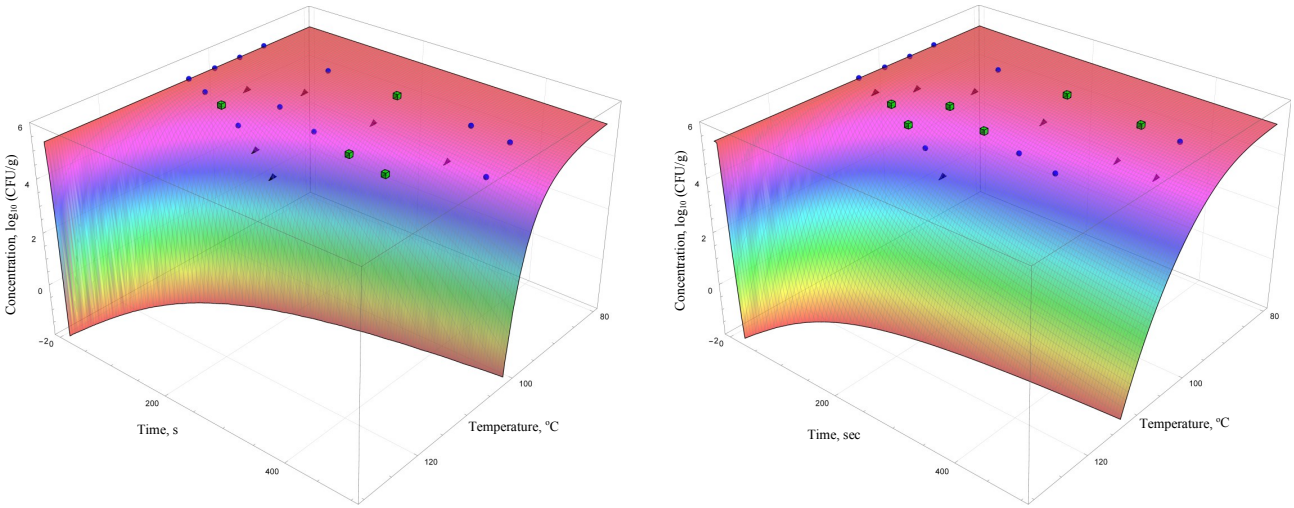
(a) (b)

Figure 7 Secondary nonlinear models of microbial death of *A. acidoterrestris* during heat treatment (a) the Fermi model, (b) Kamau model

by the continuous area in the 'duration – temperature' coordinates. It was true for this area that a trough concentration of surviving microbial spores reached the value, not exceeding a given threshold. This fitted well into the formal logic that 'a higher temperature value corresponds to a shorter processing time'. In Figs. 5–8, the trough concentration of surviving microbial spores of 10^{-2} CFU/g was randomly chosen as such threshold value. It corresponded to the sterilising effect of reducing microbial concentrations from the initial value by more than seven orders.

Comparative evaluation of rate dynamics models for microbial death kinetics. The graphical display of the secondary models was characterised by external homogeneity (if connection to the experimental data was removed). However, the key factor for the overall microbial death kinetics was the change rate indicator of concentration of the surviving microorganisms after the heat treatment. The process rate was defined as a value derived from the kinetics of the analysed index (in this case, the concentration of microorganisms).

Therefore, if there was a functional dependence reproducing the kinetics of the analysed index, the rate could be defined as the first time derivative. If the dynamics of microbial concentration increase was negative, which occurred during heat treatment, the rate value was also be negative. However, for greater convenience and clarity without levelling adequacy,



(a) (b)

Figure 8 Secondary nonlinear models of microbial death of *A. acidoterrestris* during heat treatment (a) Membre model, (b) Augustin model

we used the rate value with the opposite sign in further calculations and representations. Thus, the rate of microbial death kinetics corresponding to the secondary models could be expressed as the following:

– the Bigelow and Whiting-Buchanan model:

$$\upsilon = 10^{-a+\frac{T}{b}} \quad (43)$$

where υ is the rate of microbial death kinetics, lg (CFU/g) /s;

– the Bigelow model modified by Arrhenius:

$$\upsilon = N_0 \cdot \exp\left[-\frac{10^{a-T\cdot b}}{R\cdot\left(T+T_{abs}\right)}\right] \quad (44)$$

– the Weibull model:

$$\upsilon = p\cdot\tau^{-1}\cdot\left(\tau\cdot 10^{-a+\frac{T}{b}}\right)^{p} \quad (45)$$

– the Kamau model:

$$\upsilon = \frac{10^{-a+\frac{T}{b}}}{\left[1+\exp\left(-\tau\cdot 10^{-a+\frac{T}{b}}\right)\right]\cdot\ln(10)} \quad (46)$$

– the Fermi model:

$$\upsilon = \frac{10^{-a_b+\frac{T}{b_b}}}{\left\{1+\exp\left[10^{-a_b+\left(\frac{1}{b_b}-b_{\tau_{lag}}\right)T}\cdot\left(10^{a_{\tau_{lag}}}-\tau\cdot 10^{T\cdot b_{\tau_{lag}}}\right)\right]\right\}\cdot\ln(10)} \quad (47)$$

where a_b is the coefficient corresponding to the coefficient a in formula (38);

b_b is the coefficient corresponding to the coefficient b in formula (38);

$a_{\tau_{lag}}$ is the coefficient corresponding to the coefficient a in formula (39);

$b_{\tau_{lag}}$ is the coefficient corresponding to the coefficient b in formula (39);

– the Membre model:

$$\upsilon = 10^{-a+\frac{T}{b}}\cdot\exp\left(\tau\cdot 10^{-a+\frac{T}{b}}\right) \quad (48)$$

– the Augustin model:

$$\upsilon = \frac{10^{-2\cdot a_s+\frac{2\cdot T}{b_s}}\cdot\exp\left[10^{-2\cdot a_s+\frac{2\cdot T}{b_s}}\left(\frac{\ln(\tau)}{\ln(10)}-10^{a_m-\frac{T}{b_m}}\right)\right]}{\left\{1+\exp\left[10^{-2\cdot a_s+\frac{2\cdot T}{b_s}}\left(\frac{\ln(\tau)}{\ln(10)}-10^{a_m-\frac{T}{b_m}}\right)\right]\right\}\cdot\tau\cdot\left[\ln(10)\right]^2} \quad (49)$$

where a_m is the coefficient corresponding to the coefficient a in formula (41);

b_m is the coefficient corresponding to the coefficient b in formula (41);

a_s is the coefficient corresponding to the coefficient a in formula (42);

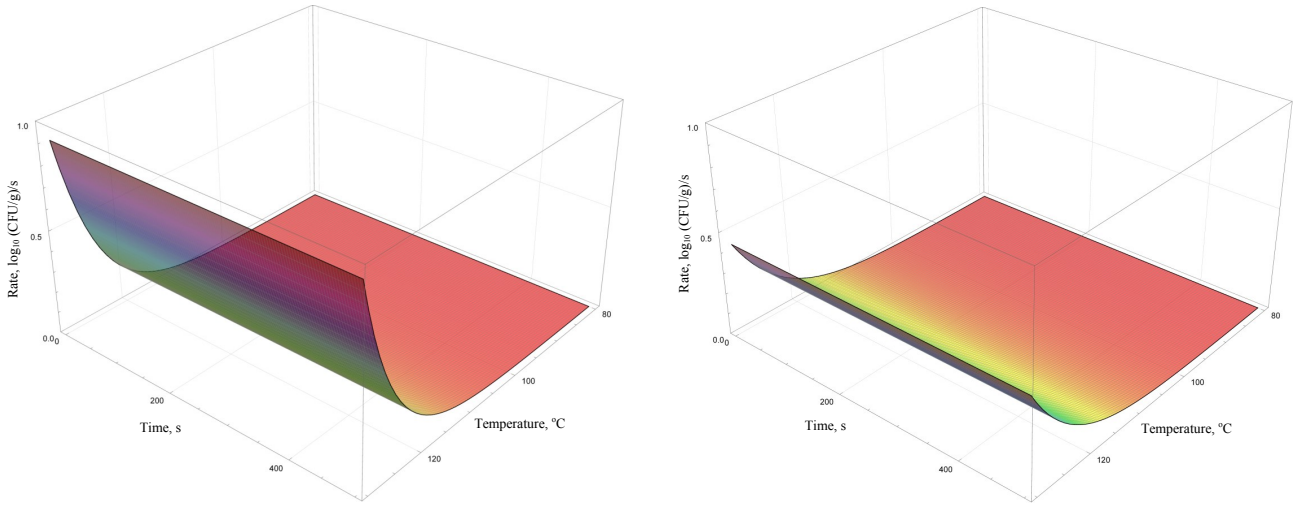
b_s is the coefficient corresponding to the coefficient b in formula (42).

Models of the rate dynamics for microbial death kinetics during the heat treatment are featured in Figs. 9–12.

Analysis of formulae (43)–(49) and their graphical representation showed that all linear models (Figs. 9 and 10a) were invariant in terms of the heat treatment duration, while nonlinear models (Figs. 10b, 11, 12) included the time component.

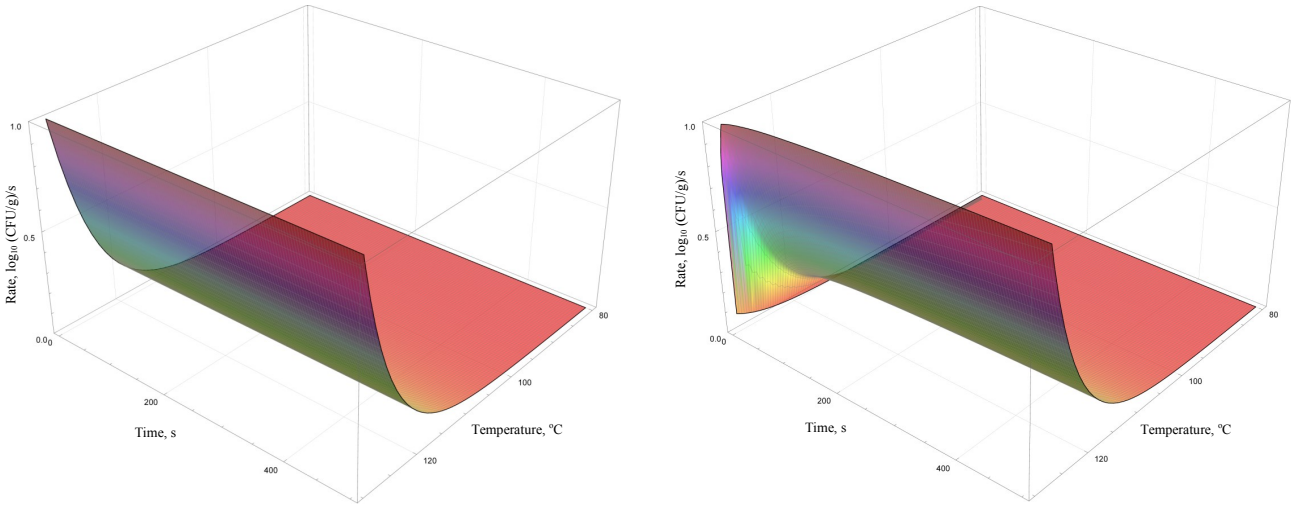
The rate growth intensity of microbial death kinetics differed visually almost twice even in externally similar linear models, such as the Bigelow model and the Bigelow model modified by Arrhenius. It occurred within graphically represented area of temperature determination of the heat treatment (80–130°C).

Some nonlinear models (the Weibull model, the Fermi model and the Kamau model) demonstrated a pronounced effect of treatment duration on the rate



(a) (b)

Figure 9 Model of the rate dynamics for microbial death kinetics of *A. acidoterrestris* during heat treatment. (a) Bigelow model, (b) the Bigelow model modified by Arrhenius



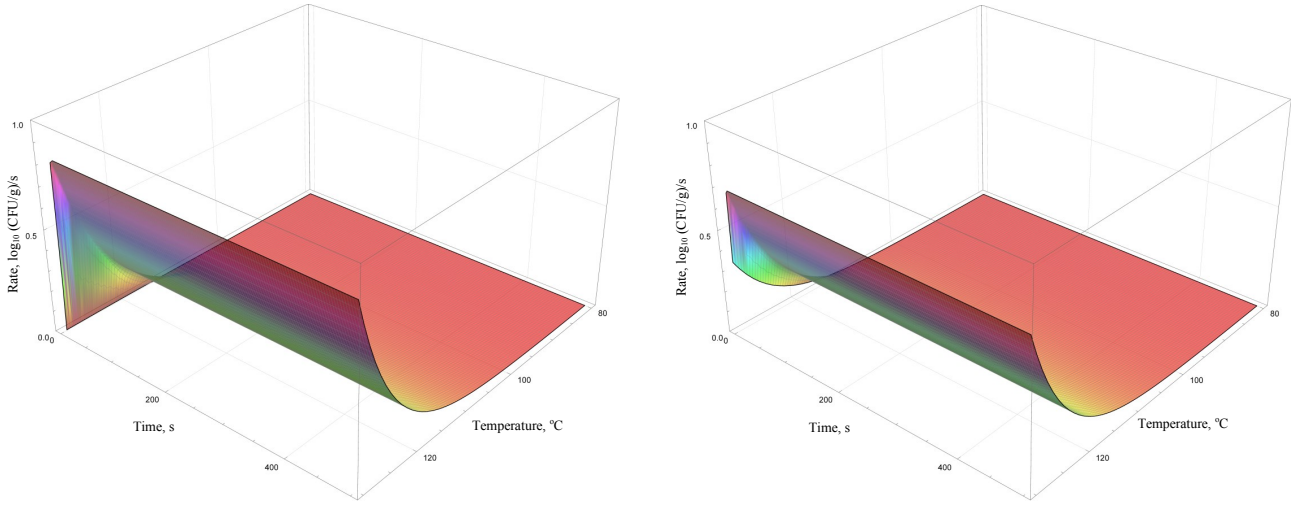
(a) (b)

Figure 10 Model of the rate dynamics for microbial death kinetics of *A. acidoterrestris* during heat treatment. (a) Whiting-Buchanan model, (b) Weibull model

of microbial death kinetics only at the initial stages of processing at temperatures over 100°C. For others (the Membre model), the rate was determined by the pronounced effect of both factors on the entire graphically represented area of determination. In the Augustin model, the rate dynamics decreased to zero by increasing both temperature and the processing duration.

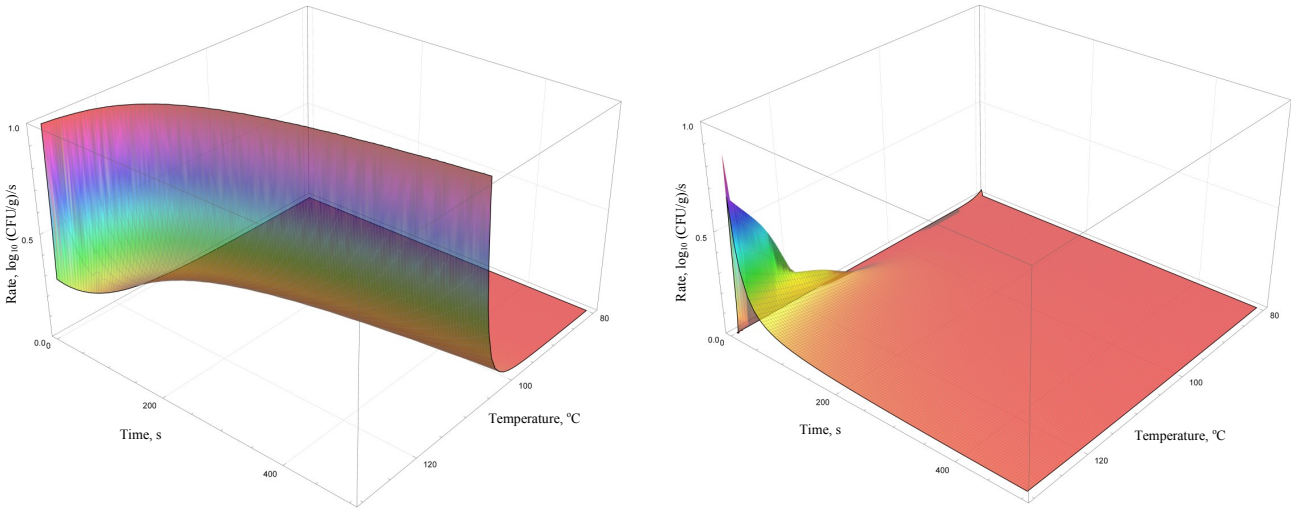
Presumably, these differences did not have any profound fundamental effect due to the initial representation of the primary models in question. The models were used for further constructions as adjustable (empirical) functions that were not bound to fundamental aspects, i.e. molecular and possibly supermolecular mechanisms, which directly determine the spore resistance to thermal treatment.

The dependence of the rate dynamics for microbial death kinetics on the processing duration for nonlinear models results in the need to determine the formal starting moment of the heat treatment. This moment should serve the starting point for measuring the real duration in order to determine the actual values of the sterilising effect for each temperature value obtained during the experimental heating. Indeed, the initial (starting) concentration of microbial spores in the product in the real conditions of thermal sterilisation was significantly (by 5–8 orders) lower than their initial



(a) (b)

Figure 11 Model of rate dynamics for microbial death kinetics of *A. acidoterrestris* during heat treatment. (a) Fermi model, (b) Kamau model



(a) (b)

Figure 12 Model of rate dynamics for microbial death kinetics of *A. acidoterrestris* during heat treatment. (a) Membre model, (b) Augustin model

concentration in microbiological experiment of heating the contaminated product. Then, in general terms, the conditions for achieving the starting microbial spore concentration and the final concentration achieved at an arbitrary time point under the known functional dependences which approximated microbiological data satisfactorily, we got the following form:

$$\begin{cases} \lg N_{st} = \lg N_0 - f\left(\tau_{st}, T\right) \\ \lg N = \lg N_0 - f\left(\tau, T\right) \end{cases} \quad (50)$$

where N_{st} is a starting microbial spores concentration in the product, CFU/g;

τ_{st} is a theoretical duration of the heat treatment to achieve the starting microbial concentration according to the selected model (starting treatment duration), s.

In this case, at an arbitrary time point, the sterilising effect n was defined as:

$$\mathrm{n} = \lg N_{st} - \lg N = f\left(\tau_{st}, T\right) - f\left(\tau, T\right) \quad (51)$$

The obvious consequence of formula (51) was the conclusion that reaching $n \geq 0$ required fulfilling the condition $\tau \geq \tau_{st}$.

To simplify the calculations in this study, the starting microbial spore concentration was assumed equal to 1 CFU/g. Then $\lg N_{st} = 0$. In this case $\mathrm{n} = -f\left(\tau, T\right)$. This conclusion could be used in the calculation of sterilising effects at each temperature value. For this, the calculated sterilisation duration for each given temperature value at the real moment of determination, adjusted to τ_{st}, must be substituted into the formula of the rate of microbial death kinetics.

The analysis of secondary models showed that for a given N_{st} value τ_{st} was a function of the process temperature. However, due to the peculiarities of formulas describing secondary models, not each of them could have an explicit form of dependence. In this regard, the ratios $\{T, \tau_{st}\}$ in the area of temperature determination from 80 to 130°C were numerically determined for each model. Then they were approximated with the following functions:

– the Bigelow model, the Weibull model, the Kamau model and the Membre model:

$$\lg\left(\tau_{st}\right) = a - b \cdot T \quad (52)$$

– the Bigelow model modified by Arrhenius:

$$\lg\left(\tau_{st}\right) = a + b \cdot \exp\left(-c \cdot T\right) \quad (53)$$

– the Whiting-Buchanan model and the Fermi model:

$$\left[\lg\left(\tau_{st}\right)\right]^2 = a + b \cdot T + c \cdot T^2 \quad (54)$$

– the Augustin model:

$$\lg\left(\tau_{st}\right) = a + b \cdot \exp\left(-\frac{T}{c}\right) \quad (55)$$

Figure 13 shows graphical representations of functional dependences of τ_{st} on temperature, corresponding to both linear and non-linear models.

The visual analysis showed a relatively high degree of variability of this dependence for nonlinear models. It indicated the pronounced variability in the dynamics of extrapolative properties for the different models studied due to the expressed empirical character of the adjustable functions. In its turn, this variability was presumably conditioned by the particularity and, in some cases, the phenomenological nature of the functions themselves, while there was no adequate connection with the fundamental mechanism of microbial spore inactivation. In addition, this variability must inevitably lead to the significant variability of the final regimes of thermal sterilisation. The regimes could be determined by

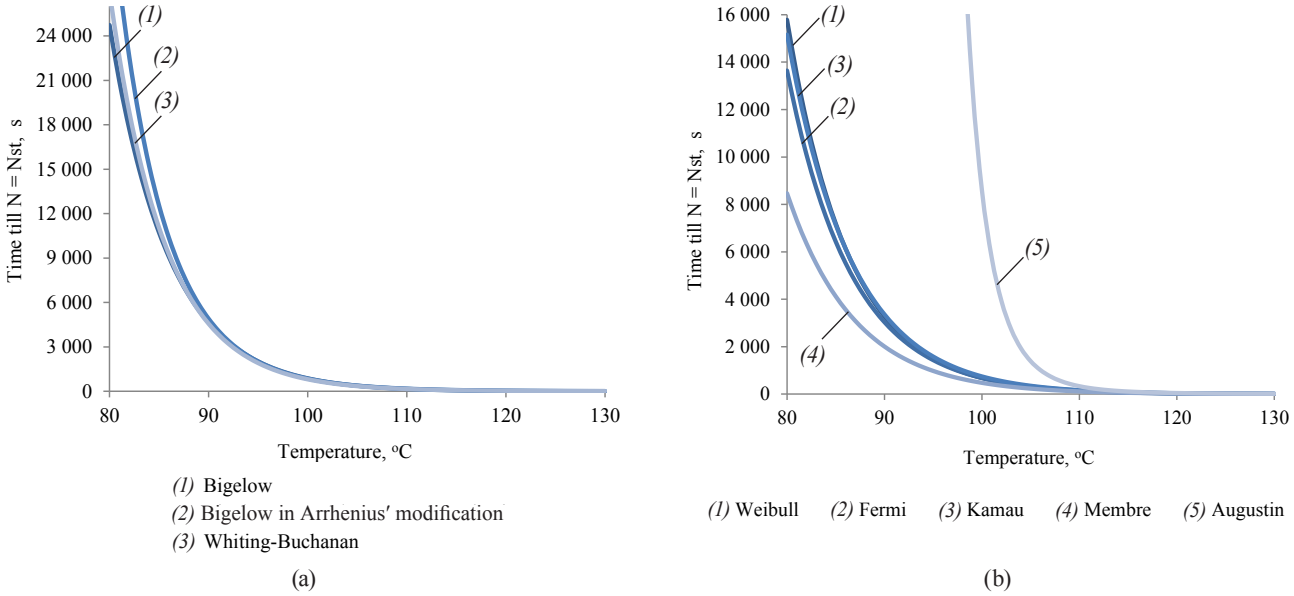


Figure 13 Dependence of starting treatment duration required for concentration of surviving *A. acidoterrestris* to reach N_{st} on sterilisation temperature for linear (a) and nonlinear (b) models

applying the analysed models to the same array of initial microbiological and thermophysical data.

CONCLUSION

According to the results of the research and subsequent analysis, the single-phase primary models (adjustable functions) are statistically acceptable (at $r^2 > 0.9$) for approximation of experimental data on the kinetics of thermal death of *Alicyclobacillus acidoterrestris* spores. The models were linear, namely the Bigelow model, the Bigelow model modified by Arrhenius, and the Whiting-Buchanan model, and nonlinear, such as the Weibull model, the Weibull model modified by Malfart *et al.*, the Fermi model, the Kamau model, the Membre model, and the Augustin model. At the same time, nonlinear models approximate experimental microbiological data on death kinetics of microbial spores during the heat treatment statistically more adequately.

The unsatisfactory convergence of extrapolation results and dynamics caused by the rate models and the temperature coefficient was shown for the first time. In other words, the expressed empirical use of adjustable functions was established analytically. This was presumably conditioned by the particularity and, in some cases, phenomenology of the functions themselves. Other causes were the lack of the criteria for the unambiguous choice of the original model and the absence of adequate connection with a fundamental mechanism of microbial spore inactivation based on the targeted blocking of the system of spore germination initialisation in combination with the conditions of the environment.

Consequently, there is no reason to believe that heat treatment regimes based on these empirical models were the most effective and provided a maximum sterilising effect at a minimum heat load. Thus, the analysis was the first link in arguing the necessity to initiate the research aimed at developing a methodology for determining the regimes of thermal sterilisation for food products including the analysis of the fundamental factors of spore germination activation and their resistance to external impact.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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DNA authentication of brewery products: basic principles and methodological approaches

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Abstract: Beer DNA authentication is the process of authentication by identification of barley malt *Hordeum vulgare* or its substitutes, as well as hops and yeast. The method is based on molecular genetic analysis of residual quantities of nucleic acids extracted from the cellular debris of the final product. The aim of the study was to analyse scientific and methodical approaches to extraction of residual quantities of beer raw materials nucleic acids and beer DNA authentication for their later application in determining brewing products authenticity. The technological level discloses the method of DNA extraction from wines, modified for extraction of nucleic acids from beer samples. The method includes the following characteristic peculiarities: stage enzymatic hydrolysis of polysaccharides and polypeptides of dissolved lyophilisate, multiple sedimentation and resursuspension of nucleoproteid complex, RNA removal followed by DNA extraction by organic solvents, and additional DNA purification by magnetic particle adsorption. This review presents the analysis of genetic targets used as molecular markers for gene identification of malting barley varieties and beer DNA authentication. We also provided the interpretation of PCR analysis of *Hordeum vulgare* varieties and samples of commercial beer. Data on SSR- and SNP-markers of *Hordeum vulgare* nuclear DNA, used for barley varieties identification and potentially suitable for beer DNA authentication, are also presented. We also analysed genetic targets used in malting barley substitute detection, as well as hops and yeast identification in beer. Data on correlation of amplified DNA targets with beer quality indicators were systematised.

Keywords: Alcoholic beverages, malting barley, *Hordeum vulgare*, DNA, authentication, identification, marker, PCR

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INTRODUCTION

Wide assortment of brewery products and their multicomponent composition refers them to the segment of difficult-to-identify goods. Their authentication is aimed at protecting consumers and manufacturers' rights [1].

One of the strategically important tasks achievable by multidisciplinary science-intensive approaches is the search for objective identification criteria with a high degree of authenticity assessment of brewery products [2].

Molecular and genetic research methods can provide the technological process of DNA authentication of beer brands, thereby expanding the complex scheme of brewery products identification, traditionally based on documentary, visual, sensory and physical and chemical analyses [3, 4].

Beer brands DNA authentication is a technological process of the authenticity verification by the gene identification of *Hordeum vulgare* barley malt, or its substitutes, as well as its key ingredients – hops and yeast, by molecular genetic analysis of residual quantities of nucleic acids extracted from the cellular debris of the products [3].

The analysis of scientific and methodological approaches points to the applicability of DNA technologies for detecting counterfeit and falsified brewery products.

RESULTS AND DISCUSSION

Extraction of DNA residues of beer raw materials. The technological level discloses a method for DNA extraction from wines [5, 6]. It was later

modified for extraction of nucleic acids from beer samples [3]. The method includes the following characteristic peculiarities: stage enzymatic hydrolysis of polysaccharides and polypeptides of dissolved lyophilisate, multiple sedimentation and resursuspension of nucleoproteid complex, RNA removal followed by DNA extraction by organic solvents, and additional DNA purification by magnetic particle adsorption.

Figure 1 demonstrates stages of DNA extraction according to the modified method. In particular, enzymatic hydrolysis of polysaccharides by α-amylase (*Bacillus licheniformis*) takes 3 h instead of 1 h, when DNA is extracted from wines [3, 5]. The time of enzymatic hydrolysis of polypeptides by proteinase K (*Tritirachium album*) is also increased up to 3 h. The sedimentation time of non-hydrolysed cellular debris by centrifugation at 8000 g is reduced to 1 min instead of 15 min when DNA is extracted from wines. At the stage of DNA extraction from the lyophilised beer powder, the sedimentation of the nucleoprotein complex is carried out by mixing the supernatant with two volumes of cold absolute ethanol instead of two volumes of cold isopropanol. At the next stage we mixed a solution of unpurified DNA with an equal volume of 70% ethanol. The maturing of the mixture at 0°C takes 3 min instead of 10 min, as with wines. During the subsequent nucleoprotein complex sedimentation, along with the stepwise addition of 10 μL of 3M sodium acetate and two volumes of cold isopropanol to the pre-transferred transparent supernatant, 3 μL of Ethachinmate linear polyacrylamide is added. After RNA removal and deproteinisation, the sedimentation of purified DNA is carried out without adding 70% ethanol (Cf. DNA extraction from wines involves in the nucleic acids sedimentation in 0.2 M NaCl and two volumes of cold ethanol, followed by washing with 70% ethanol). Later, nucleic acids precipitate, resuspended in the elution buffer, undergoes an additional purification by adsorption on magnetic particles, which is one of the key modification elements of the method for extracting residual DNA of beer raw materials [3].

The ability of magnetic particles to bind DNA reversibly and easily be deposited from the suspension in the magnetic field ensures high quality of nucleic acids purification and their preservation. Magnetic particles, as a rule, are a paramagnetic core with a highly developed surface covered with a polymer film with exposed covalent-bond carboxylic groups. Magnetic tripods, used in manual and automated modes, are made of neodymium magnets resistant to demagnetisation.

The additional purification by adsorption on magnetic particles of the modified method of extraction of nucleic acids from beer samples actually took the place of polymer polyvinylpyrrolidone widely used to reduce the inhibitory effect of polyphenols on PCR [3, 7–10].

Approaches to beer DNA authentication. Genetic targets, used as molecular markers for malting barley varieties identification, can also be analysed for commercial beer DNA authentication (Table 1) [3].

Polygalacturonase is an enzyme that performs hydrolytic cleavage of α-1,4-glycoside bonds in pectin. The DNA target was the locus of its gene (*HvPG1*) eamplified by a corresponding pair of primers constructed by Pulido *et al.* based on the analysis of expressed sequence tag (EST) deposited in GenBank (A/N: EF427919) [11]. The generated PCR products *a* and *b* of the *HvPG1* gene locus detected in the barley and beer samples were 89% and 79% identical to the previously deposited nucleotide sequence mRNA polygalacturonase *Hordeum vulgare*. Among the studied Japanese barley varieties, only the high quality 'Ryofu', recommended for brewing, generated two discrete fragments (*a*, *b*), like most American and Australian barley varieties, except for Stimling (Table 2). All the beer samples were marked only by the country of manufacture. They generated the PCR product *b* and more than half of the samples generated the additional fragment *a* (Table 2). The analysed DNA target was included in the group of DNA markers of identification and differentiation of beer samples, but did not correlate with the indicators of beer quality [3].

Hordeins are polymorphic proteins of barley grain coded by 7 *HrdA-G loci* which are localised in the short arm of the 5th *Hordeum vulgare* chromosome [12, 13]. Due to the established connection of the hordein-coding loci alleles with brewing qualities of barley grain, this block of targets is a priority for molecular and genetic analysis [14, 15]. From the three analysed loci (*HrdA*, *HrdB* and *HrdC*) only one (*HrdC*) was able to identify a single sample of beer out of 22 investigated by the presence of a specific PCR product *e* (Table 2) [3]. However, high variability of *HrdA* locus (up to 90% identity of nucleotide sequences of compared barley varieties with corresponding reference sequence (GenBank A/N: AF474373) indicates a certain potential of DNA authentication of beer on the analysed target by sequencing the amplified locus. The block of DNA targets under study also did not correlate with the indicators of beer quality [3].

Amylosis content in barley starch influences the quality of malt barley. Therefore, waxy-barley varieties may be a preferred option for their malting in brewing because starch with low amylosis content is more susceptible to enzymatic hydrolysis [18]. Molecular mechanism is embedded *in Hordeum vulgare* waxy-genes located on 7 HS chromosome. They lead to the elimination of granule-bound starch synthase (GBSS) [18, 19]. Primers selected for *Waxy*-locus amplification had the positive control status due to generation of specific PCR product in all the samples of barley and beer [3]. Their sequeneed nucleotide DNA sequences were identical to each other and showed 98% identity to the corresponding reference *Hordeum vulgare subsp. Vulgare* sequence, previously deposited to GenBank (A/N: X07931) [20].

1. LIOPHILISED BEER POWDER RESUSPENDING

Lyophilisate dissolving in 500 µL of resuspending buffer (0.1M Tris-HCl (pH 8.0), 0.1 M NaCl)

2. POLYSACCHARIDES ENZYMATIC HYDROLYSIS

Beer suspension processing with 100 µL of thermostable α-amylase (*Bacillus licheniformis*)

Incubating the produced mixture at 80°C for 3 h

3. POLYPEPTIDES ENZYMATIC HYDROLYSIS

Suspension processing with 100 µL of proteinase K (*Tritirachium album*) with 0.2% SDS

Incubating the produced mixture at 55°C for 3 h

4. NON-HYDROLYZED CELL DEBRIS SEDIMENTATION

Centrifugation at 8000 g for 1 min at 40°C

Supernatant transfer to a new tube

5. NUCLEOPROTEID COMPLEX SEDIMENTATION

Mixing the supernatant with two volumes of cold absolute ethanol and holding the mixture at 0°C (on ice) for 15 min

Centrifugation at 8000 g for 15 min at 4°C

6. NUCLEOPROTEID COMPLEX RESUSPENDING

Sediment resuspending in 300 µL of elution buffer (0.1M Tris-HCl (pH 8.0), 0.1 M EDTA)

7. NUCLEOPROTEID COMPLEX SEDIMENTATION

Mixing the crude DNA solution with an equal volume of cold 70% ethanol and holding the mixture at 0°C (on ice) for 3 min

Transfer the clear supernatant to a new tube and stepwise addition of 10 µL of 3M sodium acetate, 3 µL of linear polyacrylamide Ethachinmate and 2 volumes of cold isopropanol

Centrifugation at 8000 g for 15 min at 4°C

8. NUCLEOPROTEID COMPLEX RESUSPENDING

Sediment resuspending in 300 µL of elution buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)

9. RNA REMOVAL

Suspension Treatment with RNAse A at 55°C for 30 min

10. DEPROTEINISATION

Extraction with equal volume of neutral phenol

Recovery of the aqueous phase by centrifugation at 8000 g for 15 min (4°C)

Extraction with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)

Recovery of the aqueous phase by centrifugation at 8000 g for 15 min at 4°C

11. SEDIMENTATION OF PURIFIED DNA

Repeat stage 7 without adding 70% ethanol

12. RESUSPENDING OF SEDIMENTAL DNA

Sediment resuspending in 125 µL of elution buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)

13. ADDITIONAL STAGE OF DNA PURIFICATION BY ADSORPTION ON MAGNETIC PARTICLES

Figure 1 Stages of DNA extraction from lyophilised beer powder

Hemicelluloses are vegetable homo- and heteropolysaccharides, which are an integral part of the endosperm cell walls. The highest content of xylanes was reported to be among the main components of hemicellulose [21]. The malt barley softens as a result of the decomposition of the cell wall. Xylanase is involved in the degradation of xylanes to xylooligosarachides, whose gene locus was used as a target for primers originally designed for DNA analysis of rice samples [3]. It is noteworthy that among the 16 varieties of barley, only three varieties (Metcalfe, Nishinohoshi and Ryofu) showed a positive amplification signal (Table 2). At the same time, due to possible obtaining inconclusive data, the authors [3] presented neither the results of PCR of beer samples, nor data on amplification of the *HrdB* locus.

Barley Z proteins are the main beer protein which influence beer quality, especially foam stability [22–24]. In addition, Z4 and Z7 proteins can be used as positive and negative markers of foam stability [25]. DNA-markers of foam stability developed by Limure *et al.* were also used in by Nakamura *et al.* for barley varieties identification and beer DNA-authentication [3, 25]. Identifying and differentiating barley and beer samples procedure by the gene locus, encoding proteins Z4 and Z7 differ. In the first case PCR analysis is performed by interpreting three discrete PCR products (*h, i-a, i-b*), and in the second – by the presence or absence of a specific fragment *j*.

Based on the analysis, the authors recommended the further use of the tested primers for amplification of the analysed gene loci [3]. In addition, a negative correlation of the amplified PCR product *h* gene locus encoding Z7 protein with beer bitterness, as well as a positive correlation of PCR product *i-a* similar locus with foam stability (Table 1) were revealed.

Many enzymes, incl. α-amylase and β-amylase, are activated in the malting process [26, 27]. Their substrates are amylosis and amylopectin or products

Table 1 Genetic targets used as molecular markers for brewing barley varieties identification and beer DNA authentication

Target	PCR product	Primer sequence	Correlation (+/–)	Source
Polygalacturonase (*HvPG1*)	*a*	F: 5′-GACAGAATGGCGTTCAAGAACAT-3′	n/a	[3, 11]
	b	R: 5′-AGCAAGTTGCCTTCCAGCTTGAT-3′	n/a	
Hordein A (*HrdA*)	c	F: 5′-AGATAGCGTTTTGAAGGTCAC-3′ R: 5′-TAGACCTGCAATAATTTCCA-3′	n/a	[3, 16]
Hordein B (*HrdB*)	*d-1*	F: 5′-TCACACATAAGGTTGTGTGAC-3′	n/a	[3, 17]
	d-2	R: 5′-CAAGCTTTCCCACAACAACCA-3′	n/a	
Hordein C (*HrdC*)	*e*	F: 5′-AATTTAAACAACTAGTTTCGGGTGG-3′ R: 5′-CAAGCTTTCCCACAACAACCACCAT-3′	n/a	[3, 16]
Barley starch synthase (*waxy*)	*f*	F: 5′-CAATTCATCCGATCACTCAATCAT-3′ R: 5′- CAGGCCGACAAGGTGCTG -3′	n/a	[3, 16]
Xylanase	*g*	F: 5′-GGTACAACGTCGCGTCGG-3′ R: 5′-CGTGTACCAGACGGTCCAGATACAGC-3′	n/a	[3, 21]
Protein Z7	*h*	F: 5′-GGTCACATGACGTGTATTAATCTCC-3′	–*	[3, 24]
	i-a	R: 5′-CGTTGGTGGCAGCAGACTCGGGG-3′	+**	
	i-b		n/a	
Protein Z4	*j*	F: 5′-GAGACGTGTAGTAATCTTCG-3′ R: 5′-GCGAGCACAAATTGCACCACC-3′	–***	[3, 24]
α-amylase	*k*	F: 5′-AAGGTCTCGTGTCGATCCCAAGGAGGC-3′ R: 5′-CTAAGCCTCGTCTTCGTCCCC-3′	n/a	[3]
Barley lipoxygenase (*LOX1*)	*l*	F: 5′-GCAACGGAGGGAGTAAAACA-3′ R: 5′-CGATGGCTTGGACCAATTAC-3′	+****	[3, 34]
Barley yellow mosaic virus (*rym5*)	*m*	F: 5′-GAGTCGTCACAACGTACCTTGC-3′ R: 5′-GTGGCTGTAAATAGGCTAAGGCC-3′	n/a	[3, 34]
Barley powdery mildew (*mlo*)	*n*	F: 5′-TAGCAATCACGGTCACGTCAAC-3′	n/a	[3, 34]
	o	R: 5′-CCGCAAGGCTGCTATGAAAAGGG-3′	n/a	
Barley trypsin inhibitor (*Itr1*)	p	F: 5′-CAACTAACAGAAAGTCAGAAAGCAC-3′ R: 5′-CACAATACTGAAAATACTCTGATGC-3′	–*****	[3, 37]
Barley β-glucanase (*HvCslF6*)	*s*	F: 5′-GCCAAGACCAAGTACGAGAAGC-3′ R: 5′-TGTTCTTGGAGAAGAAGATCTCG-3′	n/a	[3, 40]

–* a negative correlation of the amplified PCR product *h* of the gene locus encoding the protein Z7 with beer bitterness
+** a positive correlation of the amplified PCR product *i-a* of the gene locus encoding the protein Z7 with foam stability
–*** a negative correlation of the amplified PCR product *j* of the gene locus encoding the protein Z4 with the detectable PCR product *h* of the gene locus encoding the protein Z7
+**** a positive correlation of the amplified DNA target with beer taste saturation
–***** a negative correlation of the detected DNA matrix with the saturation of beer taste
N/A not applicable

Table 2 Interpreted results of PCR analysis of brewing barley varieties and beer samples

Barley varieties	PCR products																		
	a	*b*	*c*	*d–1*	*d–1*	*e*	*f*	*g*	*h*	*i–a*	*i–b*	*j*	*k*	*l*	*m*	*n*	*o*	*p*	*s*
Vlamingh	+	+	–	+	+	–	+	–	–	–	+	–	+	–	–	–	–	–	–
Hamelin	+	+	–	+	+	+	+	–	+	+	+	–	+	–	–	–	–	+	–
Stimling	–	+	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	+
Bardin	+	+	–	–	+	+	+	–	–	+	–	+	+	–	–	–	–	+	+
Salute	+	+	+	–	+	–	+	–	–	–	+	–	+	–	–	–	–	–	+
Schouner	+	+	–	+	+	–	+	–	–	+	–	+	–	–	–	–	–	+	–
Maritime	+	+	–	–	+	+	+	–	–	–	+	+	+	–	–	+	–	+	+
Flag ship	+	+	–	–	+	–	+	–	–	+	–	–	–	–	–	–	–	+	+
Metkafe	+	+	–	+	+	–	+	+	+	+	+	+	–	+	+	+	–	+	+
Harushizuku	–	+	+	+	–	–	+	–	–	+	–	+	+	–	–	–	+	–	–
Houshun	+	–	+	+	–	–	+	–	–	+	–	–	–	–	+	+	–	–	–
Mikamogolden	–	–	+	+	–	–	+	–	–	–	+	+	–	–	+	–	+	–	–
Skygolden	–	+	+	+	–	+	+	–	–	–	+	+	+	–	+	–	+	–	–
Nishinohoshi	–	–	+	+	–	+	+	+	–	+	–	+	–	+	+	+	–	–	+
Nishinochikara	–	–	–	+	–	+	+	–	–	+	–	+	+	+	+	–	+	+	+
Ryofu	+	+	+	+	–	+	+	+	–	–	+	+	+	+	+	+	–	+	+
Samples of beer	PCR products																		
	a	*b*	*c*	*d–1*	*d–1*	*e*	*f*	*g*	*h*	*i–a*	*i–b*	*j*	*k*	*l*	*m*	*n*	*o*	*p*	*s*
Czechoslovakia–a	+	+	+	n/a	n/a	–	+	n/a	+	+	+	–	+	+	n/a	n/a	n/a	+	+
USA–a	–	+	+	n/a	n/a	–	+	n/a	–	+	–	–	+	+	n/a	n/a	n/a	+	+
Belgium–a	–	+	+	n/a	n/a	–	+	n/a	+	+	+	–	–	+	n/a	n/a	n/a	–	+
USA–b	+	+	+	n/a	n/a	–	+	n/a	+	+	+	+	+	+	n/a	n/a	n/a	–	+
Netherlands–a	+	+	+	n/a	n/a	+	+	n/a	–	+	+	–	+	+	n/a	n/a	n/a	+	+
Thailand–a	+	+	+	n/a	n/a	–	+	n/a	–	+	+	–	+	+	n/a	n/a	n/a	–	+
Denmark–a	+	+	+	n/a	n/a	–	+	n/a	–	+	+	–	+	–	n/a	n/a	n/a	–	+
England–a	–	+	+	n/a	n/a	–	+	n/a	–	+	+	–	+	+	n/a	n/a	n/a	–	+
Germany–a	–	+	+	n/a	n/a	–	+	n/a	–	+	–	–	+	+	n/a	n/a	n/a	–	+
Australia–a	–	+	+	n/a	n/a	–	+	n/a	–	+	–	–	+	–	n/a	n/a	n/a	+	+
Mexico–a	–	+	+	n/a	n/a	–	+	n/a	–	+	–	–	+	+	n/a	n/a	n/a	–	+
USA–c	+	+	+	n/a	n/a	–	+	n/a	+	+	+	–	+	+	n/a	n/a	n/a	+	+
Germany–b	+	+	+	n/a	n/a	–	+	n/a	+	+	+	–	+	+	n/a	n/a	n/a	–	+
England–b	–	+	+	n/a	n/a	–	+	n/a	–	+	+	–	+	+	n/a	n/a	n/a	+	+
Peru–a	–	+	+	n/a	n/a	–	+	n/a	–	+	–	–	+	+	n/a	n/a	n/a	–	+
England–c	+	+	+	n/a	n/a	–	+	n/a	+	+	–	+	–	–	n/a	n/a	n/a	+	+
Germany–c	+	+	+	n/a	n/a	–	+	n/a	+	+	–	+	–	–	n/a	n/a	n/a	+	–
Italy–a	+	+	+	n/a	n/a	–	+	n/a	+	–	–	+	+	–	n/a	n/a	n/a	+	+
Japan–a	+	+	+	n/a	n/a	–	+	n/a	+	–	–	+	+	–	n/a	n/a	n/a	+	–
Japan–b	+	+	+	n/a	n/a	–	+	n/a	+	+	–	–	+	–	n/a	n/a	n/a	+	–
Japan–c	+	+	+	n/a	n/a	–	+	n/a	–	+	–	–	+	+	n/a	n/a	n/a	–	+
Japan–d	+	+	+	n/a	n/a	–	+	n/a	+	–	–	–	+	–	n/a	n/a	n/a	+	+

+ a positive amplification signal
– a negative amplification signal
n/a not applicable

of their hydrolysis. Primers developed on the basis of nucleotide sequence of the gene locus encoding α-amylase initiated the amplification of PCR product *k* in most of the barley varieties and beer samples (Table 2) [3, 28]. It is noteworthy that the amino acid sequence of the target had 69% identity with *Mla*-locus of resistance to powdery mildew *Hordeum vulgare* (GenBank A/N: AF427791) [29]. The used set of primers was included in the group of molecular labeling systems of barley varieties, and therefore has a certain potential of practical application for beer authentication, although the authors did not mention it [3].

Lipoxygenase-deficient barley varieties with reduced or lost activity of *LOX* genes have a positive impact on quality indicators such as beer taste and foam stability [30–33]. The set of primers constructed by Nagamine *et al.* resulted in amplification of the specific PCR product *l* in a small number of studied barley varieties and in more than half of beer samples, whose sequenced nucleotide sequences had 99% identity with the reference sequence

of locus *LoxA*-gene *Hordeum vulgare* (GenBank A/N: L35931) [3, 34, 35]. The tested set of primers was recommended for further use in the amplification of the analysed gene locus for barley varieties identification and beer brands differentiation. It should also be noted that the authors [3] additionally revealed a positive correlation between the amplified DNA target and beer taste saturation (Table 1).

The selection of barley varieties with genetic resistance to viral, bacterial and fungal diseases is aimed at high-quality grain production [36]. A number of DNA markers of resistance of barley to yellow mosaic virus (*rym5*-locus) and powdery mildew (*mlo*-locus) integrated into breeding programs can also be used in molecular labelling of brewing barley varieties, which is clearly demonstrated in the work [3, 34]. The authors interpreted the PCR analysis data of barley samples taking into account the presence or absence of specific PCR products *m* (*rym*), *n* and *o* (*mlo*) recorded on the corresponding electrophoregrams. But the results of the PCR analysis of beer samples and their correlation with quality indicators were not provided [3].

Protein inhibitors of proteolytic enzymes play an important role both in formation of homeostatic reactions in plants and in the process of seed maturation and germination. Selected primers to the trypsin inhibitor (*Itr1*) gene locus led to the amplification of the specific PCR product *p* in half of the tested barley varieties and beer samples [37]. Thus, the DNA marker was concluded to be highly informative [3]. Additionally, the DNA sequences of the *Itr1*-gene locus of the material had 94% identity with the same locus of the *Hordeum vulgare subsp. vulgare gene* (GenBank A/N: (X65875) [38]. Also, in the study [3] a negative correlation of the detected DNA matrix with beer taste saturation was revealed (Table 1).

The content (1–3, 1–4) of β-D-glucan in barley grain, which determines its hardness, is much higher compared to other cereals [39]. However, for barley varieties used in brewing, a lower the content of this polysaccharide in the grain is desirable in order to achieve a more effective flow of the malting process [40]. The amplification procedure of the locus of the *HvCslF6* gene with a selected primer pair led to the production of a specific PCR product *s* in a number of American, Australian and Japanese brewing barley varieties [3, 40]. The most of the beer samples also gave a positive amplification signal (Table 2). The obtained amino acid sequence of the target had 83% identity with *Hordeum vulgare CslF6*-gene (GenBank A/N: EU267181) [41]. The used primer set was also included in the group of systems of barley varieties molecular labelling and beer DNA-authentication [3].

Microsatellites are widely used molecular markers which are suitable for identification of *Hordeum vulgare*. A wide variety of SSR-markers are being used [42–44]. Tomka *et al*. described a high potential of the five SSR-markers for brewing barley varieties identification [45].

Table 3 shows the sequence of oligonucleotide primers of the corresponding *Hordeum vulgare* SSR-markers of nuclear DNA, as well as the range of lengths of detected alleles and their number. The genetic identification procedure includes PCR method with subsequent data interpretation by horizontal or vertical gel electrophoresis and DNA fragmentary analysis of capillary gel electrophoresis. The SSR-markers, potentially suitable for beer DNA authentication, are advisable to test in the formulation of single PCR, with a set of primers of a single SSR-marker to achieve a reproducible result.

Alongside with SSR-markers, SNP-markers, used for barley varieties identification, including brewing ones, also have high identification capacity [46–48].

Table 4 shows oligonucleotide primers sequences of the corresponding SNP markers of *Hordeum vulgare* nuclear DNA, as well as the size of amplified loci of discriminated alleles [46]. The procedure of gene identification is carried out by the Amplification Refractory Mutation System (ARSM-PCR), followed by data interpretation by horizontal gel electrophoresis or by high resolution melting curves (HRM) analysis on PCR platforms in real time. It should be mentioned that we selected five SNP-markers (out of nine described by Chiapparino *et al.* [46] as potentially suitable for beer DNA authentication due to generation of relatively small allele-specific PCR products, whose size was not more than 200 bp (Table 4).

Table 3 SSR-markers of nuclear DNA *Hordeum vulgare* used for genetic identification of brewing barley varieties, potentially suitable for beer DNA authentication

No.	SSR marker	The sequence of oligonucleotide primers	Allel lenghts, bp	Number of alleles
1	*Bmac 0040*	F: 5′-AGCCCGATCAGATTTACG-3′ R: 5′-TTCTCCCTTTGGTCCTTG-3′	196-226 bp (196/200/208/214/220/226)	6
2	*Bmac 0134*	F: 5′-CCAACTGAGTCGATCTCG-3′ R: 5′-CTTCGTTGCTTCTCTACCTT-3′	140-174 bp (140/144/162/168/174)	5
3	*Bmag 0125*	F: 5′-AATTAGCGAGAACAAAATCAC-3′ R: 5′-AGATAACGATGCACCACC-3′	128-148 bp (128/132/138/144/148)	5
4	*Bmag 0211*	F: 5′-ATTCATCGATCTTGTATTAGTCC-3′ R: 5′-ACATCATGTCGATCAAAGC-3′	150-170 bp (150/154/162/170	4
5	*Bmag 0222*	F: 5′-ATGCTACTCTGGAGTGGAGTA-3′ R: 5′-GACCTTCAACTTTGCCTTATA-3′	140-178 bp (140/144/162/168/170/174/178)	7

Table 4 SSR-markers of *Hordeum vulgare* nuclear DNA used for brewing barley varieties identification, potentially suitable for beer DNA authentication

No.	Locus (position)	The sequence of oligonucleotide primers	PCR product, bp
1	MWG2062 (325 A-G)	FOP: 5′-GTTGTGTCAAGCATATCGGTTGCTCTT-3′ ROP: 5′-CAGCACGTTCGAAAACAATAGGATCC-3′	198 bp
		FIP: 5′-AAGAATTATGCCAATTATTGGCGTGTCA-3′	101 bp (A allele)
		RIP: 5′-CACACTGCATGTCATCAAACAAGCAC-3′	151 bp (G allele)
2	ABC465 (254 C-T)	FOP: 5′-CAGGTACACCTGGAAGCTCTACTCAGAG-3′ ROP: 5′-CAGCAGCCTGAATTCAACAAAACATAC-3′	236 bp
		FIP: 5′-TGGAGATGTTCTACGCTCTCAAGTACAGT-3′	130 bp (T allele)
		RIP: 5′-CTGTTGGTCAGATAACCTACCAGGATG-3′	162 bp (C allele)
3	MWG2218 (175 G-C)	FOP: 5′-CTCTCCGACATCGACCGCTTCCTCTTCG-3′ ROP: 5′-GCCGCATCATCCCTGGTGTCATCACCT-3′	215 bp
		FIP: 5′-GGGGACGTCATCCACGTCTGTCGACC-3′	127 bp (C allele)
		RIP: 5′-GTTCCCGCGGTGGGCTTTGTTTCCTC-3′	140 bp (G allele)
4	ABC156 (231 T-G)	FOP: 5′-CTTGGTCCATATAGGTCTCTCTTTTC-3′ ROP: 5′-CCTCCTGATATACTTGAGAGACTCAATA	74 bp
		FIP: 5′-TCCATATAGGTCTCTCTTTTCTTATTATG-3′	70 bp (G allele)
		RIP: 5′-TGAGAGACTCAATACTCATGAATTTCA-3′	60 bp (T allele)
5	MWG801 (344 G-A)	FOP: 5′- CAACAACCCCAATACCAGGCCAGCTCCACA-3′ ROP: 5′-AACCCTCGACTGCTCAAGGCAGAGCCGC-3′	256 bp
		FIP: 5′-GAAGCATGCTCGCACGACACCCATCC-3′	175 bp (C allele)
		RIP: 5′-CGGCAGCGGAGGGGAAGGGGAGCAGT-3′	133 bp (A allele)

FOP is a forward outer primer; ROP is a reverse outer primer; FIP is a forward inner primer; and RIP is a reverse inner primer

The detection of brewing barley substitutes in beer, which is often used as a cheap source of starch, makes it possible to evaluate the products sold for qualitative, quantitative, information and complex falsification. Table 5 demonstrates primer sequences targeting genetic targets used in the detection of brewing barley substitutes in beer, such as granule-bound starch synthase of rice, β-conglycinin of soya, and zein of maize [49, 50]. Nevertheless, other PCR systems developed for the identification of cereals in food products can also be suitable for beer DNA authentication [51].

The effect of hops and yeast on beer quality is well-known. Thus, hop has a bactericidal effect on beer as well as provides its bitterness, aroma and foam stability [52]. Yeast is used in beer fermentation and

Table 5 Genetic targets used in detecting brewing barley substitutes and identifying hops and yeast in beer

Target	PCR product	Primer sequence	Correlation (+/–)	Source
GBSS (rice)	*t*	F: 5′-GGATGAAGGCCGGAATCCTG R: 5′-CTTGCCCGGATACTTCTCCT	missing	[3, 49]
B-conglycinin	*u*	F: 5′-TTTGGCATTGCTTACTGGGAAAAAGAG R: 5′-TCTGTAGGAGTCTCTGTCGTCGTTG	missing	[3, 50]
Zein	v	F: 5′-CACATGTGTAAAGGTGAAGCGAT R: 5′-GCTCGCCGCAAGCGCTTGTTG	missing	[3, 49]
Hop-a	*w*	F: 5′-GGAACCGTTGCCTAATCCTAAGATT R: 5′-GTGTTTTCCGTATCTACGCGCTGGG	missing	[3]
Hop-b	*x*	F: 5′-AATTAGGGCATGCCATGAATATT R: 5′-TGGCATAGTTAAATTATTTCG	–* –**	[3]
Hop-c	*y*	F: 5′-AAATAAAACTTTACATGTGATA R: 5′-CTGAATTGTCGGCGT	missing	[3]
Yeast-a (*S. cerevisiae*)	*z-a*	F: 5′-GTTTTGCGCTCATTAAAACCTAGTGGGAG R: 5′-GTCATTTTTTTTAGTGGTGCTAATC	+*** –****	[3]
Yeast-b (thioredoxin)	*z-b*	F: 5′-ATGGTCACTCAATTAAAATCCGCTTCT R: 5′-CTATACGTTGGAAGCAATAGCTTGCTTG	missing	[3]

–* a negative correlation of the amplified PCR product *t* of the corresponding locus of the hop gene (Hop-b) with beer bitterness
–** a negative correlation of the amplified PCR product *t* of the corresponding hop gene locus (Hop-b) with beer astringency
+*** a positive correlation of the amplified PCR product *z-a* of the corresponding yeast gene locus with beer acidity
–*** a negative correlation of the amplified PCR product *z-a* of corresponding locus of the *S. cerevisiae* gene yeast with beer umami
N/A is not applicable

impacts its character and taste [53]. Table 5 also presents sets of primers which initiate the amplification of specific PCR products of the corresponding loci of hops and yeast genes. They also allow the identifying or differentiating of commercial beer samples [3]. In addition, a negative correlation of the amplified PCR product *t* of the corresponding locus of the hop gene (Hop-b) with beer bitterness and astringency was revealed. The amplified PCR product *z-a* of the corresponding locus of the yeast gene *S. cerevisiae* showed a positive correlation with beer acidity and a negative correlation with beer umami [3]. Taking into account the rapid development of genomic and bioinformation technologies, metagenomic analysis, which allows determining yeast species diversity in beer samples without microorganisms allocating and cultivating, is one of the promising approaches to beer DNA authentication [54, 55].

CONCLUSION

Analysis of scientific and methodical approaches to extraction of residual quantities of nucleic acids of beer raw materials and beer DNA-authentication indicates the applicability of molecular and genetic analysis in detecting counterfeit and falsified brewery products. The use of DNA technologies helps determine the authenticity and origin of the brewery industry products. Molecular labelling systems suitable for identification of *Hordeum vulgare* barley malt, or its substitutes, as well as hops and yeast, can ensure traceability of the product life cycle. Systematic data on correlation of amplified DNA targets with beer quality indicators can be of practical importance when choosing raw materials for brewery production.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Effect of pre-treatment conditions on the antiatherogenic potential of freeze-dried oyster mushrooms

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Abstract: Oyster mushroom (*Pleurotus ostreatus* L.) is a valuable food product. It possesses an antiatherogenic potential, which has to be preserved during processing. The paper features the production of oyster mushroom sublimates. It focuses on such pre-treatment conditions as grinding, disinfection, and cryostabilisation, and their effect on the antiatherogenic potential of oyster mushrooms. A set of *in vitro* experiments was performed to measure the levels of lovastatin and antioxidant, catalase, anti-inflammatory, and thrombolytic properties. Various pre-treatment conditions proved to produce different effects on the biological activity of the freeze-dried oyster mushroom product. The best results were obtained after the mushrooms were reduced to pieces of 0.5 cm, underwent UV disinfection, blanched, treated with hot air, and cryostabilised with a 1.5% apple pectin solution. The best conditions for the antioxidant properties included ozonation, UV disinfection, and cryoprotection with pectin. The critical conditions for the antioxidant properties included homogenisation, blanching, and cryostabilisation with 10% solutions of sucrose and lactose. The catalase properties did not depend on the degree of grinding and were most pronounced after ozonation. The optimal conditions for the anti-inflammatory properties included UV disinfection and cryostabilisation with lactose. Ozonation proved to be critical for anti-inflammatory properties. The optimal conditions for thrombolytic properties included ozonation and cryoprotection with a 5% sorbitol solution, while hot air disinfection proved critical. Therefore, the research provided an experimental substantiation for individual pre-treatment conditions or their combinations that turn sublimated oyster mushrooms into a valuable functional product with antiatherogenic properties.

Keywords: Oyster mushroom, freeze-drying, functional food, antiatherogenic potential, lovastatin, antioxidant properties, catalase properties, anti-inflammatory properties, thrombolytic effect

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INTRODUCTION

According to statistics, atherosclerosis and its complications remain the main cause of death worldwide [1]. The mechanisms of atherogenesis are complex and multiple. Its main causes include hyperlipidemia, oxidative stress, thrombosis, and inflammation [2].

Modern medicine is striving to find a way to curb this trend. Various therapeutic approaches are being introduced to combat atherosclerosis. However, many of them remain expensive and have various contraindications and side effects, which limits their clinical use [3]. As a result, more and more attention is given to functional food products with medicinal properties and minimal side effects. Scientists are looking for biologically active raw materials that could modify human metabolism and prevent the development and progression of atherosclerosis [4, 5].

In this regard, the oyster mushroom (*Pleurotus ostreatus* L.) is considered advantageous. Its fruit body has a high nutritional value, natural statin, and a whole complex of other biologically active substances (BAS) [6, 7]. Recent researches proved that the oyster mushroom possesses hypolipidemic, antioxidant, anti-inflammatory, and thrombolytic properties, which makes it a valuable raw material [8–11]. Thus, oyster mushrooms can help to improve the existing anti-atherogenic functional foods and develop new ones.

However, the concentration and effectiveness of biologically active compounds depend not only on cultivation conditions and age of the mushrooms, but also on the processing methods [12, 13].

Some of the existing food processing technologies make it impossible to preserve the entire complex of biologically active substances [14, 15]. Today, freeze-drying is considered the least harsh and the most reliable treatment method of BAS production. It ensures stability of thermolabile and hydrolytically unstable substances, increases shelf life, and optimises storage conditions [16–18]. However, even when all the necessary regulations for freeze-drying have been observed, the properties of the product depend on the pre-processing conditions. An appropriate use of various pre-treatment methods significantly increases the efficiency of drying, improves the quality of the product, and preserves its properties [19–21]. A careless use of pre-treatment methods can lead to a decrease in the content of certain BAS in sublimates [22–24]. Thus, each raw material requires its own freeze-drying technology based on experimental data about the effect that pre-treatment conditions produce on the specific properties of the finished sublimates.

The antiatherogenic effects of freeze-dried oyster mushrooms have already become focus of scientific studies [25]. However, there have been no studies connected with the effect of pre-treatment methods on the preservation of BAS and natural antiatherogenic potential of sublimated oyster mushrooms, which adds to the relevance of the present research.

STUDY OBJECTS AND METHODS

The present research used the following chemicals: chloroform ($CHCl_3$), hydroxyamine hydrochloride ($NH_2OH{\cdot}HCl$), reduced iron, perchloric acid ($HClO_4$), ethanol (C_2H_5OH) (Sigma-Aldrich), microbiologically pure lovastatin ($C_{24}H_{36}O_5$) (TEVA, Hungary), reagent (chromogen containing an $ABTS^{\bullet+}$ radical) (Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus), trolox 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ammonium thiocinate (NH_4NCS), ferrous chloride ($FeCl_2$), oleic acid ($C_{18}H_{34}O_2$), hydrogen peroxide (H_2O_2), monosubstituted potassium phosphate (KH_2PO_4), disubstituted sodium phosphate (sodium hydrogen phosphate 12-water, $Na_2HPO_4{\cdot}12H_2O$) (Sigma-Aldrich), dextrose ($C_6H_{12}O_6{\cdot}H_2O$), sucrose ($C_{12}H_{22}O_{11}$) (Sigma), monohydric citric acid ($C_6H_8O_7$), sodium citrate ($C_6H_5Na_3O_5$), sodium chloride (NaCl), lactose ($C_{12}H_{22}O_{11}{\cdot}H_2O$), and sorbitol ($C_6H_{14}O_6$) (Sigma-Aldrich). All the substances were purchased from Diaem (Russia).

The study featured oyster mushroom (*Pleurotus ostreatus* L.), strain NK35 (SYLVAN, Hungary). It was harvested in 2018 and cultivated under the standard mushroom production conditions in the Stavropol Region. The fruit bodies were of the same size and maturity, undamaged. During the experiments, the fresh mushrooms were stored in a refrigerator at 5–7°C. Before the experiments, the fruit bodies were thoroughly washed under running water.

The antiatherogenic potential of the freeze-dried oyster mushroom product was evaluated *in vitro* based on the concentration of lovastatin, as well as antioxidant, catalase, anti-inflammatory, and thrombolytic properties.

The first stage featured the effect of the degree of preliminary grinding on the antiatherogenic properties of the sublimates. The grinding was conducted by reducing the fruit bodies into pieces with the side sizes of 2.0–2.5 cm and 0.5–1.0 cm. The pieces were homogenised using a laboratory Sterilmixer 12 (PBI, Italy) at No. 9 high-speed mode. Whole mushrooms served as control sample. The oyster mushroom samples were spread in one layer on separate stainless steel trays. The homogenised substance was poured into the trays to form an even layer with a thickness of ≤ 0.8–1 cm. All samples were frozen in a SE-45 refrigerator (TEFCOLD, Denmark) at –40°C for 72 h and subsequently freeze-dried.

The second stage tested the effect of preliminary disinfection methods on the preservation of antiatherogenic properties in the sublimates. The mushrooms were subjected to blanching, UV disinfection, ozonation, and hot air treatment [26, 27].

Blanching is one of the most common pre-treatment methods. It reduces microbial challenge and inactivates the enzymes that reduce the quality of the freeze-dried product. According to Galoburda *et al.*, the optimal blanching temperature regime is 70–80°C, since it provides the best drying performance for mushrooms [28]. Hence, the oyster mushrooms were blanched in water at 70°C for 3 min, cooled under running water, and drained in a sieve for several minutes.

The UV disinfection of the oyster mushrooms was performed using an Azov portable ultraviolet irradiator, modification OBN-35-01 UHL 4.2 (Russia). The fruit bodies were put on plastic trays in one layer, placed at a distance of 60 cm from the irradiator and treated for 15 min.

The ozonation was performed using a universal ozoniser of air and water Ozone OViV (Ukraine). The ozonation was carried out in a ventilation hood at 22°C in a 10-litre chamber improvised from PVC film. The ozonation mode was based on [29] and the operation manual: ozonator power, 100%; gas flow rate, 2.0 dm^3/min; ozone concentration, 8 mg/dm^3; exposure time, 20 min.

The hot air treatment was performed using a TS-1/80 SPU dry-air thermostat (Smolensk Special Design-Technological Bureau of Software Management Systems, Russia). The mushrooms were placed on a wire shelf and kept in the thermostat under forced ventilation at 60°C for 60 min.

The third stage assessed the effect of various cryoprotectors on the atherogenic potential of the oyster mushroom sublimates. The experiment involved natural

substrates that are widely used in food industry: a 10% sucrose solution, a 10% lactose solution, a 5% sorbitol solution, and a 1.5% pectin solution. In all cases, the whole fruit bodies were soaked in aqueous solutions of the cryoprotectors (volume ratio = 1:20) for 30 min. The untreated oyster mushroom fruit bodies acted as control sample.

After the disinfections and cryostabilisations, the fruit bodies were placed on separate sheets, frozen, and freeze-dried.

All the samples were dried in an LS-500 freeze dryer (Prointech, Russia), which included a freeze dryer and a vacuum station. The glass lid of the drying chamber was covered with an opaque material to prevent degradation of antioxidants by photo-oxidation. The working pressure in the drying chamber was 80–90 Pa; the condenser temperature was 48–49°C. The temperature of the samples did not exceed 29–30°C during the entire drying process. The average drying time was 26–27 h.

The mushrooms were dehydrated until the residual moisture content was 6–8%. The moisture content in the dried oyster mushroom samples was measured using an MB 25 moisture content analyzer (Ohaus, China). The indicators were measured using the following automatic measurement mode: heating temperature = 75°C, measurement time = 5 min. The resulting oyster mushrooms sublimates were placed into a dry, hermetically sealed container and stored in dark at ≤ 25°C for further analysis.

The amount of lovastatin in the sublimates was estimated according to the authentic method using the hydroxam method after lovastatin had been extracted with chloroform and concentrated [30, 31]. The ground dried mushrooms were weighed into portions of 0.1–0.2 g, extracted with 5.0–10.0 cm^3 of chloroform, and filtered. The filtrate was evaporated using a RV 10 Basic V rotary vacuum evaporator (IKA, Germany). The remaining filtrate was diluted with 1.0 cm^3 of a 0.9 M alcohol alkaline solution of hydroxylamine and 5.0 cm^3 of a 5.73 mM solution of ferric (III) chloride. After that, pH was adjusted to 1.2 ± 0.2 with a 2M hydrochloric acid solution. The extinction of the resulting magenta solution was measured using an SF-102 spectrophotometer (Research and Development Centre NPO INTEROFOTOFIKA, Russia) at a wavelength of λ = 513 nm. The calculation was performed according to the calibration curve.

To assess the antioxidant activity of the sublimates, we measured the radical absorption and the degree of inhibition of lipid peroxidation (LPO). To assess the radical absorption, the dry oyster mushrooms were made into powder. Then the powder was extracted with bidistilled water in a shaker at 50–60°C for 3 h. The rotation speed was 190 rpm. After that, the material was filtered as described in [32]. The antioxidant activity of the extract was determined *in vitro* using the OxiStat test system (Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus). It was a one-stage assessment of reduction value of the resulting $ABTS^{\bullet+}$ radical by antioxidants. The scheme is described in [33] as follows: $ABTS^{\bullet+} + AO \rightarrow ABTS + AO^{\bullet+}$.

When antioxidants interacted with $ABTS^{\bullet+}$, the optical density of the solution of the cation radical fell down to 600–800 nm in proportion to the concentration and activity of the antioxidant. The optical density was measured using a spectrophotometer at a wavelength of 675 nm. The optical path length of the cuvettes was 1.0 cm.

To provide a quantitative assessment of the antioxidant activity, we used trolox, i.e. a standard antioxidant, which is a water-soluble analogue of vitamin E:

$$\% \text{ inhibition} = 100(1-\Delta A_o/\Delta A_c) \quad (1)$$

$$AA = [C_{st}]/\% \text{ standard inhibition} \times \times \% \text{ sample inhibition} \quad (2)$$

where:

AA – antioxidant activity;

ΔA_o – optical density of the experimental sample;

ΔA_c – optical density of the control sample (buffer);

C_{st} – standard concentration (trolox).

The radical absorption results were expressed in mg of trolox equivalent per gram of dry matter (mg TE/g)

To evaluate the LPO inhibition activity, 0.1 g of powdered dry mushrooms was added to 2.0 cm^3 of bidistilled water. After 24 h of maceration at room temperature, the extract was filtered and centrifuged at 1300 rpm for 10 min. The LPO inhibition activity of the obtained extract was measured in an oleic acid emulsion system according to the slightly modified procedure described in [34]. 0.1 cm^3 of the extract was added to 4.0 cm^3 of phosphate buffer (50 mM, pH 7.0), and 0.1 cm^3 of oleic acid was added to 4.0 cm^3 of ethanol (95 wt%, aqueous solution). The total volume was brought to 10.0 cm^3 with distilled water, mixed in a sealed conical tube with a screw cap, and incubated at 40°C in the dark for 7 days. The oxidation state was evaluated using iron thiocyanate at 24 h intervals. The reaction solution (100 μL) was mixed with 4.7 cm^3 of ethanol (75 wt%, aqueous solution), 0.1 cm^3 of an ammonium thiocyanate aqueous solution (30% w/v), and 0.1 cm^3 of an iron chloride (II) solution (20 mM in 3.5% (v/v) HCl). After 3 min, the absorbance was measured at a wavelength of 500 nm using a UV spectrophotometer. An increase in optical density meant an increase in the level of oleic acid oxidation. Trolox (0.95 mmol/dm^3) was used as a reference. The blank sample contained deionised water instead of the extract.

When calculating both the antiradical activity and the activity of LPO inhibition, we took into account the fact that the extracts had their own colour, which absorbed a particular wavelength in the visible spectrum.

The catalase activity of oyster mushroom sublimates was measured using a modified technique based on the Beers and Sizer spectrophotometric method [35].

This fast and accurate analysis presupposes a hydrogen peroxide dehydrogenation and determination of its loss at λ = 240 nm. The powdered oyster mushrooms were weighed into portions of 0.020–0.025 g and extracted with 5.0 cm^3 of chilled 50 mM phosphate buffer with pH = 7.0 for 15 min in the cold with periodic stirring. The extract was filtered and centrifuged at 4°C for 20 min and acceleration of 1200 g. The experimental solution contained 1.0 cm^3 of 50 mM phosphate buffer with pH=7.0 and 1.0 cm^3 of 0.1% hydrogen peroxide. Its optical density (D_1) was measured at 240 nm relative to the control solution, which contained 2.0 cm^3 of 50 mM phosphate buffer. After filtration, 0.1 cm^3 of the sublimate sample extract was introduced into the experimental sample. In addition, 0.1 cm^3 of extract was added to the control solution. The optical density of the experimental solution was determined after 30 s (D_2).

The enzymatic activity was calculated for 1 mmol of substrate (H_2O_2) split in 1 min with 1 g of sublimate sample according to the following formula:

$$A= ((D_1-D_2)V_1 \cdot n)/D_1 \cdot m \cdot V_2 \cdot t) \quad (3)$$

where:

A – enzymatic activity, mmol/g·min;

D_1 – optical density of the hydrogen peroxide solution before the extract was introduced;

D_2 – optical density of the hydrogen peroxide solution after incubation with the extract;

V_1 – total extract volume, cm^3;

n – amount of hydrogen peroxide introduced, mmol;

m – weight of oyster mushroom sublimate in the extract, g;

V_2 – volume of extract for analysis, cm^3;

t – incubation time, min.

The anti-inflammatory activity of the oyster mushroom sublimates was determined *in vitro*. It employed the method used for assessing the osmotic resistance of erythrocyte membranes [36]. The dry mushroom sublimate was made into powder, suspended in distilled water at a concentration of 5.0 mg/cm^3, and incubated at 4°C for 12 h. The suspension was centrifuged at 7000 rpm for 10 min, after which the supernatant was filtered. Blood was obtained from healthy white laboratory Wistar rats and mixed in a 1:1 ratio with Alsever solution. The latter contained equal volumes of aqueous solutions of 2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% sodium chloride. The resulting solution was centrifuged at 4000 rpm for 10 min. The precipitated cells were washed with physiological saline and centrifuged three time until the red blood cells were 10% by suspension volume in physiological saline. The extracts of oyster mushroom sublimates were separately mixed with 1.0 cm^3 of phosphate buffer, 2.0 cm^3 of hypotonic sodium chloride solution (0.42%), and 0.5 cm^3 of red blood cell suspension. The control sample contained 2.0 cm^3 of distilled water instead of the hypotonic solution. The mixes were incubated at 37°C for 20 min and centrifuged at 3000 rpm.

After that, the supernatant liquid was decanted, and the haemoglobin content was estimated using a spectrophotometer at λ = 560 nm. The percentage of resistance of red cell membranes was assessed based on the fact that the haemolysis obtained in the control sample was 100%. It was calculated by the formula:

Percentage of resistance = 100 – (optical density of the experimental sample/optical density of the control sample) × 100

To assess the thrombolytic activity of the sublimates, blood obtained from white Wistar rats was distributed into different pre-weighed sterile microcentrifuge tubes (0.5 cm^3 in each) and incubated at 37°C for 45 min. After the clot was formed, the serum was completely removed without disturbing the clot, and each tube was again weighed to calculate the weight of the clot. 100 μL of sublimate extract was added into each tube with a pre-weighed clot. All tubes were incubated at 37°C for 90 min. After incubation, the released liquid was removed, and the tubes were weighed again. The difference in weight before and after clot dissolution was expressed as a percentage [37].

The content of substances and their activity were expressed in terms of absolute dry raw materials. All quantitative parameters were triplicated. The results were recorded as arithmetic mean ± standard error of the arithmetic mean (M ± m) and subjected to statistical processing using the method of one-way ANOVA test and the Biostat software (version 4.03). The significance of the differences was measured at $P \leq 0.05$.

RESULTS AND DISCUSSION

A single-phase ANOVA was conducted to compare the quantitative values of the properties responsible for the antiatherogenic potential of the freeze-dried oyster mushroom product. It also made it possible to check whether there was any significant difference in these properties after various pre-treatment methods.

As a potential antiatherogenic product, the oyster mushrooms were checked for the concentration of lovastatin. This natural statin reduces the production of endogenous cholesterol as it inhibits the activity of hydroxyl-3-methyluracil-coenzyme reductase [38]. The experiment took into account the level of antioxidant and catalase activities that resist the accumulation of excess reactive oxygen. Together with excessive lipids in the blood, reactive oxygen is known to cause atherosclerosis [39]. We tested the abilities of freeze-dried oyster mushrooms to inhibit the inflammation and thrombosis. They are considered the key pathogenetic mechanisms of atherosclerosis as they facilitate the transformation of risk factors into morphological changes [40].

A set of experiments was performed to define the effect of disinfection, cryoprotection, and various degrees of preliminary grinding on the safety and activity of the abovementioned properties.

Table 1 Effect of preliminary grinding of oyster mushroom fruit bodies on the bioactive properties of freeze-dried product (M ± m)

№	Grinding size	Lovastatin, mg/kg	Radical absorption activity, mgTE/g	Catalase activity, mmol/g·min	Anti-inflammatory effect, %	Thrombolytic activity, %
1	Whole fruit bodies (control sample)	316.2 ± 8.3[a]	9.6 ± 0.3[a]	17.9 ± 0.6[a]	34.6 ± 0.8[a]	15.2 ± 0.4[a]
3	Pieces with side size 2.0–2.5 cm	305.6 ± 7.6[a]	9.1 ± 0.3[a]	18.7 ± 0.6[a]	35.1 ± 0.7[a]	14.1 ± 0.3[a]
4	Pieces with side size 0.5–1.0 cm	298.4 ± 6.1[a]	9.4 ± 0.4[a]	17.3 ± 0.5[a]	36.4 ± 0.9[a]	12.4 ± 0.3[b]
5	Homogeneous state	174.8 ± 4.2[b]	5.2 ± 0.2[b]	16.9 ± 0.5[a]	34.1 ± 0.8 [a]	9.9 ± 0.2[c]
6	Sources	50.0–505.0 (*Pleurotus ostreatus*) Gunde-Cimerman *et al.* [43]; 165,3–606,5 Chen *et al.* [44]	0.61–14.07 (*Pleurotus citrinopileatu*) Nattoh *et al.* [45]	14.66 (*Pleurotus Ostreatus*) Susmitha *et al.* [46]	18.66–43.50 (*Pleurotus florida*) Pandimeena *et al.* [47]; 54,33–85,12 (*Pleurotus florida*) Varghese *et al.* [36]	18.62 (*Pleurotus ostreatus*) Islam *et al.* [8]

Mean values with different letters in the same column are statistically different (P < 0.05). mgTE/g

Thickness, shape, and volume ratio of samples are known to affect the drying rate and quality of the finished product [41].

The whole dried oyster mushroom fruit bodies selected as control sample were tested for the abovementioned properties. The obtained quantitative indicators appeared to be comparable with the data for *Pleurotus* mushrooms presented in previous studies (Table 1). When the fruit bodies were ground to pieces with side sizes of 2.0–2.5 cm and 0.5–1.0 cm at the pre-treatment stage, it did not affect the concentration of lovastatin in the freeze-dried product. However, the content of lovastatin in the homogenised sublimates turned out to be 45% less than in the whole-dried samples. The technological process of homogenisation probably reduced the degree of heterogeneity of the distribution of chemicals and phases by volume. It might have changed the sensitivity of lovastatin to the conditions of the subsequent stages of lyophilisation. In addition, homogenisation is known to cause a shift in the pH of raw materials. According to Piecha, when pH of the medium increases, the lactone structures of statins can be partially or completely converted to the corresponding forms of hydroxyacids [42].

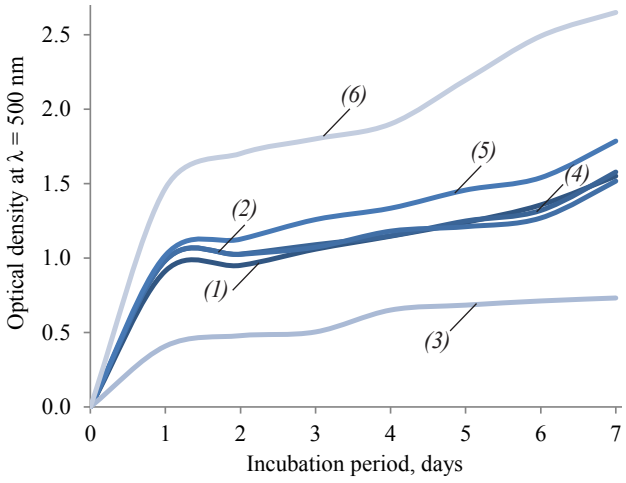
The assessment of the effect of grinding size on the preservation of antioxidant properties had a similar result. Homogenization reduced the activity of radical absorption by almost 46%. The homogenised samples also demonstrated minimal LPO inhibition activity (Fig. 1).

The data were consistent with some studies that featured the effect of homogenisation during freeze-drying of berries [19]. According to Paciulli *et al.*, the results may be explained by the fact that large tissue damage caused a loss of antioxidant substances [48].

However, the preliminary grinding of oyster mushroom affected neither catalase nor anti-inflammatory properties of its sublimates.

Pleurotus mushrooms owe their thrombolytic properties to the high level of biosynthesis of the protease enzyme complex. Proteases have an affinity for fibrin and cause its lysis [49]. The thrombolytic properties of freeze-dried oyster mushrooms depended on the degree of grinding at the pre-treatment stage: the experiment showed a statistically significant decrease as the fruit bodies were ground into smaller pieces. The samples subjected to preliminary homogenisation demonstrated the lowest thrombolytic activity. Such result might be connected with the fact that cellular disruption facilitates interaction between proteolytic enzymes and extracellular protease inhibitors.

Although pre-treatment grinding may facilitate the drying process, it proved irrational in terms of preservation of lovastatin and other antioxidant and thrombolytic substances [41].



(1) No treatment
(2) 0.5–1.0 cm pieces
(3) Trolox
(4) 2.0–2.5 cm pieces
(5) Homogeneous sample
(6) Blank sample

Figure 1 Effect of preliminary grinding on the LPO inhibition of the freeze-dried product. (Note: in Figs. 1–3, a lower optical density at 500 nm corresponds to a higher LPO inhibition)

Table 2 Effect of various methods of preliminary disinfection on the bioactive properties of freeze-dried product (M ± m)

№	Disinfection	Lovastatin, %	Radical absorption activity, mgTE/g	Catalase activity, mmol/g×min	Anti-inflammatory action, %	Thrombolytic activity, %
1	No treatment (control sample)	316.2 ± 7.9[a]	8.8 ± 0.3[a]	16.0 ± 0.4[a]	35.2 ± 0.8[a]	15.7 ± 0.4[a]
2	UV	308.4 ± 8.1[a]	10.3 ± 0.4[b]	15.6 ± 0.4[a]	65.1 ± 1.6[b]	14.8 ± 0.4[a]
3	Ozonation	217.4 ± 7.1[b]	9.4 ± 0.3[b]	21.6 ± 0.5[c]	6.3 ± 0.5[c]	23.9 ± 0.6[b]
4	Blanching	311.3 ± 8.1[a]	4.6 ± 0.2[c]	17.1 ± 0.5[a]	33.2 ± 0.8[a]	16.1 ± 0.5[a]
5	Hot air	299.4 ± 6.5[a]	8.4 ± 0.3[a]	1.6 ± 0.1[b]	36.4 ± 1.0[a]	10.6 ± 0.3[c]

Mean values with different letters in the same column are statistically different ($P < 0.05$)

Food security is as important as its nutritional and biological value. Microbiological contamination is an indicator of food security. Therefore, disinfection is a necessary pre-treatment stage. Blanching and hot air treatment had no statistically significant effect on the content of lovastatin in the finished sublimates if compared with the control samples (Table 2). UV disinfection may cause photodegradation of statins. However, it also demonstrated no significant changes in the concentration of lovastatin in the sublimates. The only difference was a slight decrease in the content of lovastain, which is consistent with the results obtained by [42], according to which lovastatin proved to be the most UV resistant statin.

The ozonation resulted in a significant loss of lovastatin. Its concentration in the sublimates decreased by 31.4% compared to the control samples, which confirmed the data published in [50], according to which oxygen makes lovastatin instable.

UV disinfection and ozonation resulted in a higher radical absorption and LPO inhibition (Fig. 2). Such results are consistent with other studies that proved a better preservation, and sometimes even an increase, of antioxidant substances in mushrooms and fruits after ozonation and UV disinfection [51–53]. According to Sudheer *et al.*, ozone can trigger the formation of such secondary metabolites as phenols and flavonoids [54].

Hot air disinfection caused no statistically significant changes in the antiradical activity and LPO inhibition (Fig. 2). These results contradicted with those described in [55]. On the one hand, the effect might be explained by the thermally induced extraction of previously bound or polymerized molecules of antioxidants, in particular, phenols. On the other hand, it may be due to the inactivation of enzymes involved in their catabolism, as demonstrated by recent studies of vegetable drying processes [56]. In addition, the obtained results might be explained by the fact that hot air treatment can trigger the formation of new compounds, e.g. Maillard reaction products, which possess good antioxidant properties [57].

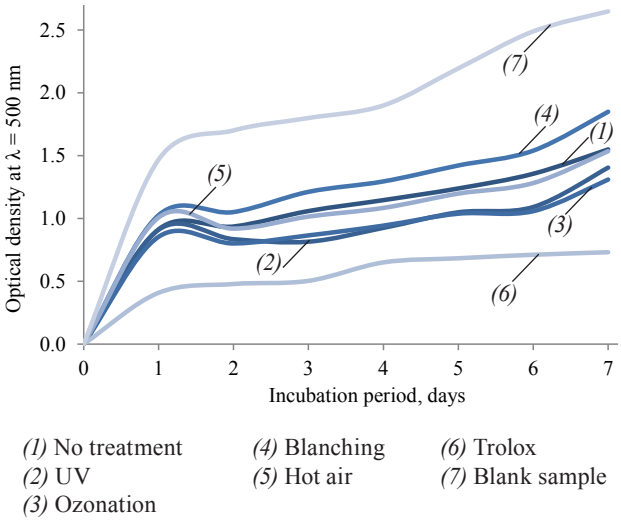
The blanching produced a significant decrease in the antiradical activity of the sublimates. Its value was 47.7% lower than that of the control sample. The results confirmed the data described in [58, 59]. According to Lam *et al.* and Radzki *et al.*, leaching and a low ability to absorb oxygen radicals resulted in a lower concentration of antioxidant substances after preliminary blanching. In addition, the blanched sublimates showed a minimal LPO inhibition [58, 59].

Various disinfection methods produced different results on the level of catalase activity of the sublimates. Hot-air treatment resulted in the lowest catalase activity. These results confirmed those described in [60], according to which a higher drying temperature reduced the residual activity of the oyster mushroom catalase enzyme.

The ozonation produced the highest catalase activity. These results confirmed those described in [61, 62], which showed an increase in the catalase activity of fruits after ozonation. The increase was explained by the fact that ozone came into contact with the biological tissue of mushrooms and caused oxidative stress, which was accompanied by activation of various antioxidant enzyme systems, e.g. catalase.

Contrary to previous assumptions [63], the blanching caused no changes in the level of catalase activity. According to Egbebi *et al.*, blanching of mushrooms inactivated catalase. In our opinion, it can be explained by the relatively short blanching time [63]. This presumption corresponds with the observations published in [64], which described catalase inactivation only in cases when blanching lasted > 10 min.

Various disinfection methods produced various effects on the anti-inflammatory activity of the product



(1) No treatment
(2) UV
(3) Ozonation
(4) Blanching
(5) Hot air
(6) Trolox
(7) Blank sample

Figure 2 Effect of various methods of preliminary disinfection on the LPO inhibition of the freeze-dried product

that had undergone a spray-freeze drying. The anti-inflammatory properties were determined using the erythrocyte membrane stabilisation test. The UV-treated samples showed the highest anti-inflammatory activity. Mushrooms owe most of their anti-inflammatory properties to polysaccharides, especially glucans [65, 66]. Thus, UV treatment served as an elicitor that increases production of extracellular polysaccharides in mushrooms. In addition, UV treatment might have produced phenolic compounds that produce a protective effect on biological membranes [67].

The ozonation resulted in the lowest anti-inflammatory activity. This effect might have been caused by ozone-induced oxidative reactions. According to Mzoughi *et al.*, ozone-induced oxidative reactions lead to the selective depolymerisation of polysaccharides, followed by a possible increase or, conversely, a decrease in their biological activity [68].

The thrombolytic properties also proved to depend on the methods of preliminary disinfection. Thus, the maximum thrombolytic properties were manifested in the ozonised samples. This result might have been caused by the ability of ozone to inactivate protease inhibitors [69]. The minimal thrombolytic properties were detected in the samples that had been treated with hot air. According to Ali *et al.*, protease inhibitors in mushrooms are thermally stable [70]. According to Rai *et al.* and de Castro *et al.*, proteases demonstrate the maximum activity at 55–60°C and may be wasted on the autohydrolysis of proteins heating [71, 72].

Freezing is an obligatory stage of freeze-drying. Freezing can damage the cell structure with ice crystals. The degree of damage depends on the size of the crystals and the heat transfer rate. It can affect the rheological and textural properties of the product, as well as redox processes in favour of oxidation. As a result, the number and the biological activity of the substances in the product may change. Therefore, a stabilising cryoprotector should be applied before freezing. Cryoprotectors maintain the quality of the frozen product by increasing the reversibility of the process and preserving BAS [73]. In this regard, we assessed various natural cryoprotectants and their effect on the anti-atherogenic properties of the freeze-dried oyster mushrooms (Table 3).

The chemical analysis demonstrated that the cryoprotectors had a different effect on the amount of lovastatin in the mushroom sublimates. The samples pretreated with a 1.5% solution of pectin showed no statistical changes in the concentration of oxidation-sensitive natural statin. However, there was a clear tendency to its increase. It confirmed the results published in [74], which proved that insoluble polysaccharides effectively inhibit oxidation processes in frozen semi-finished products.

The samples pretreated with a 5% sorbitol solution demonstrated a 47.6% decrease in lovastatin. The samples pretreated with a 10% sucrose solution and a 10% lactose solution appeared to contain no lovastatine. It was probably due to the hydrolysis of lovastatine in the aqueous medium of the cryoprotectors. Thus, freezing did not prove to be a limiting factor with respect to lovastatin concentration. Hence, cryoprotectors are not obligatory in this aspect.

1.5% pectin solution proved to be the best cryoprotector for oyster mushrooms as it ensured the maximum preservation of antiradical and LPO inhibition (Fig. 3). The results were consistent with [75], according to which pectin has a greater water absorption capacity compared with sorbitol and monosaccharides. The results may be attributed to the antioxidant properties of pectin itself, since its diffused part could enhance the antioxidant properties of the obtained dry product. According to Kopjar *et al.*, if added to bioactive substances containing phenolic compounds, pectin provides a synergistic effect on their antioxidant properties [76].

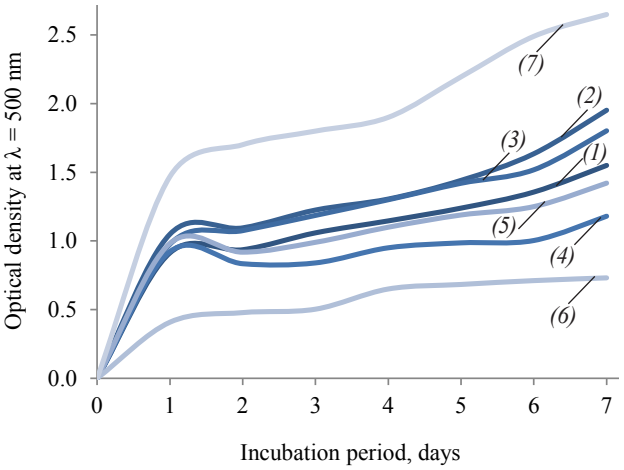
10% solutions of lactose and sucrose resulted in a significant decrease in the level of antiradical activity and LPO inhibition. On the one hand, these results may be explained by the extraction of antioxidant substances into the aqueous solutions of the cryoprotectors. On the other hand, the decrease might have been caused by the cryoprotective effect itself, since it reduces both ice crystal formation in the mushroom tissue matrix and damage to the cell structure. According to Yang *et al.*, cryoprotective effect increases extraction of antioxidant substances, e.g. phenolic compounds, from cells [77].

Cooling is known to reduce the catalase properties of certain substances [78]. However, catalase activity decreased significantly in all the experimental samples. Its value was minimal in the sublimates pretreated with a 10% lactose solution and a 5% sorbitol solution.

Table 3 Effect of pre-treatment with cryoprotectors on the bioactive properties of freeze-dried product (M ± m)

№	Cryoprotector	Lovastatin, mg/kg	Radical absorption activity, mgTE/g	Catalase activity, mmol/g·min	Anti-inflammatory action, %	Thrombolytic activity, %
1	No treatment (control sample)	310.3 ± 7.6^{a}	7.9 ± 0.3^{a}	17.1 ± 0.5^{a}	30.3 ± 0.9^{a}	14.9 ± 0.4^{a}
2	10% sucrose solution	–	5.4 ± 0.2^{b}	9.5 ± 0.4^{b}	29.4 ± 0.8^{a}	15.4 ± 0.4^{a}
3	10% lactose solution	–	5.6 ± 0.2^{b}	2.9 ± 0.1^{c}	56.4 ± 1.5^{b}	16.0 ± 0.5^{a}
4	1.5% pectin solution	339.2 ± 8.5^{a}	15.5 ± 0.5^{c}	7.0 ± 0.3^{d}	31.8 ± 1.0^{a}	14.6 ± 0.3^{a}
5	5% sorbitol solution	152.4 ± 5.9^{b}	7.5 ± 0.3^{a}	3.1 ± 0.1^{c}	28.6 ± 1.1^{a}	25.1 ± 0.6^{b}

Mean values with different letters in the same column are statistically different ($P < 0.05$)



(1) No treatment
(2) 10% sucrose solution
(3) 10% lactose solution
(4) 1.5% pectin solution
(5) 5% sorbitol solution
(6) Trolox
(7) Blank sample

Figure 3 Effect of pre-treatment with cryoprotectors on the LPO inhibition of the freeze-dried product

The maximum anti-inflammatory activity was manifested in the sublimates pretreated with a 10% lactose solution. The results confirmed those published in [79], according to which lactose proved to be a more advantageous cryoprotector than sorbitol or sucrose when used in freeze-drying of liposome preparations. It can be explained by the fact that disaccharides produce a greater stabilising effect on cell membranes during freezing than other cryoprotectors, thereby preserving polysaccharides and glycoproteins of cell membranes.

As for the thrombolytic properties, sublimates pretreated with sorbitol showed the best results. Unlike mono-, di-, and oligosaccharides, sorbitol can penetrate into cells [80]. It protects intracellular proteases and their fibrinolytic properties from possible denaturation caused by low temperature.

CONCLUSION

A set of biochemical experiments was performed to study the effect of various pre-treatment conditions on the biologically active properties that provide the antiatherogenic potential of freeze-dried oyster mushrooms. The antiatherogenic properties under study included the content of natural statin (lovastatin), as well as antioxidant, catalase, anti-inflammatory, and thrombolytic properties. The results showed that each pre-treatment method produced a different effect on the abovementioned properties of the freeze-dried product.

The experiments demonstrated that the best results for lovastatin were obtained when the raw material was ground to pieces with a side size of ≥ 0.5 cm, subjected to UV disinfection, blanched, treated with hot air, and cryoprotected with a 1.5% pectin solution.

As for the antioxidant properties, such as radical absorption and LPO inhibition, the best conditions included UV disinfection, ozonation, and cryoprotection with a 1.5% pectin solution. Homogenisation, blanching, and cryostabilisation with 10% solutions of sucrose and lactose were found critical for antioxidant properties.

The catalase activity of the product did not depend on the degree of grinding, blanching, and UV disinfection. It was maximal after ozonation. The list of critical pre-treatment conditions included hot air treatment and exposure to all the cryoprotectors except pectin.

The anti-inflammatory properties were best preserved after UV disinfection and cryoprotection with a 10% lactose solution. Ozonation appeared to be the only critical pre-treatment factor.

The best results for thrombolytic properties were obtained when the oyster mushrooms were ozonated and cryoprotected using a 5% sorbitol solution. Critical factors included homogenisation and hot air treatment.

Thus, the experiments revealed advantages of individual pre-treatment conditions and their combinations. The applied conditions can turn freeze-dried oyster mushrooms into a functional food product or ingredient. The new functional product significantly improved the properties that affect such pathogenetic factors of atherogenesis as hyperlipidemia, oxidative stress, inflammatory reaction, and thrombosis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests related to this article.

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Dietary fibres in preventative meat products

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Abstract: This paper is based on literature and our own studies of high-quality dietary fibres of various types, as well as food materials and products. It provides data on the physiological features, functional and technological properties of dietary fibre, as well as its main uses in food technology. In particular, we assessed the texture of dietary fibre, constructed rheograms for the flow of fibre-water systems, and analysed the histological structure. Our results form a scientific basis for the development of safe meat products of high quality and healthy diets. We established specific structural characteristics, properties, and rheological behaviour of various dietary fibres, as well as their advantages. We found that potato fibres demonstrated greater uniformity in texture and rheology, compared to wheat fibres. Wheat fibres had a clear phase structure (fibre/water), whereas potato fibres showed significant hydrophilic and structuring properties, attributing them to colloidal fibres. The established patterns contribute to the rational selection of dietary fibre to create products with desired properties. In particular, we developed a technology for a restructured poultry product with preventative properties using soluble and insoluble dietary fibres. The paper provides data on the product's safety indicators, nutritional and biological values, as well as functional, technological, microbiological, and other properties. We also conducted microstructural studies to analyse the uniformity of distribution of the curing mixture in the developed meat product. We concluded that using potato and wheat fibres can expand the range of meat products in line with the concepts of rational and healthy nutrition, as well as increase the product's succulence and prevent syneresis and mass loss.

Keywords: Dietary fibre, diet, rheogram, histological structure, food, poultry

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INTRODUCTION

Nutrition is a vital element of human interaction with the environment that has a decisive influence on human health, performance, and resistance to harmful effects of production and environmental factors. A regular diet of nutritious foods containing vital substances is particularly important for maintaining human health and activity in old age.

Nutrition issues are a major physiological and hygienic problem. Studies have shown a recent decrease in the consumption of meat, dairy, and fish products, as well as fresh vegetables and fruits among certain groups of Russian population. Another negative fact is a decline in energy intake from food (91%), especially due to a reduced amount of animal proteins in the diet. Moreover, certain groups consume only 55–60% of the recommended content of vitamins* [1–3].

The importance of enriching foods with various substances for health improvement purposes is specified in the Decree of the RF Government No. 1134-r of June 30, 2012 (amended on February 6, 2014) 'On approving

* MR 2.3.1.2432-08. Normy fiziologicheskikh potrebnostey v ehnergii i pishchevykh veshchestvakh dlya razlichnykh grupp naseleniya Rossiyskoy Federatsii [Methodological Guidelines 2.3.1.2432-08. The norms of physiological requirements for energy and nutrients for various population groups in the Russian Federation]. Moscow: Federal Center for Hygiene and Epidemiology of Rospotrebnadzor; 2009. 36 p.

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an action plan to implement the principles of the Russian Federation state policy in the field of healthy nutrition for the period until 2020'**.

Meat works are forced to use polysaccharide structure-forming agents, such as carob bean gum, carrageenans or alginates to improve food consistency and increase not only the output, but also economic indicators. This raises the price and adds more E numbers on the food labels, which is negatively perceived by the consumer [4].

Current trends in healthy nutrition demonstrate a need for low energy meat products with a minimum fat content, higher protein content, and special substances that improve digestion, absorption, and metabolism [5–7].

Modern scientific aspects of physiology and biochemistry encourage food experts and manufacturers to change requirements for food production. They develop new formulations and adjust the amount of nutrients and fibres in accordance with a person's physiological and professional status, as well as climatic and social living conditions.

Dietary fibres are increasingly used not only in the production of specialised foods, but also products of mass consumption. The reasons for their popularity include improved gastrointestinal motility (according to Ugolev's theory of adequate nutrition), minimum energy value, the ability to bind moisture and fat (taking into account a large amount of refined foods in the diet), structural variety, and safety of use [8, 9].

The daily intake of dietary fibre is 25–35 g. Today, nutritionists recommend increasing this amount to 40–42 g per day. Recent studies have found a decrease in daily fibre intake in many countries. It was revealed that Russian population consumes only 30–35% of the recommended amount of dietary fibre, mostly from wholemeal flour and grain. Even 30 years ago, vegetables and fruits were an integral part of Russian diet. They are immensely rich in cellulose and therefore have a balanced amount of soluble and insoluble fibres [10]. Nutritionists recommend a 3:1 ratio of those, respectively [11].

Numerous studies have proven that a deficiency in dietary fibre causes a risk of developing various diseases, including irritable bowel syndrome, hypomotor colon dyskinesia, intestinal diverticulosis, and even colon and rectal cancer. Annually, about half a million malignant tumours in the large intestine are diagnosed worldwide, 35% of which are rectal cancer. Rectal cancer comes 6th or 7th on the list of all malignant tumours [12, 13].

A connection was established between a consumption of smoked or fried foods and a risk of rectal cancer. Carcinogens (benzopyrene) that form during such types of heat treatment cause point mutations and translocations. As a result, cellular pro-oncogenes are turned into active oncogenes which contribute to the initial synthesis of oncoproteins and the transformation of a healthy cell to a cancerous cell [13]. Scientists believe that dietary fibre, when passing through the gastrointestinal tract, adsorbs water, thereby increasing the amount of faeces. As a result, faeces move faster through the intestines, reducing the risk of colon cancer [14]. In addition, a low energy value of dietary fibre and the feeling of fullness that it induces help people to control their appetite. Ballast substances contribute to the production of insulin, which affects blood sugar.

Comprehensive studies have found that dietary fibre in functional foods affects digestion processes in the gastrointestinal tract, including symbiont digestion. In particular, it improves clinical and metabolic parameters by normalising the functional activity of the intestinal microbiota. Also, it benefits the anthropometric parameters helping to reduce body weight and waist circumference. Therefore, dietary fibre can be used in the treatment and prevention of obesity [15, 16].

Thus, dietary fibre reduces the incidence of atherosclerosis, obesity, diabetes, metabolic syndrome, varicose veins, venous thrombosis of the lower extremities, etc. [17]. In addition, dietary fibre maintains a water-salt balance in the body, contributing to the prevention of gallstone disease, and is a nutritious medium for beneficial intestinal microflora [18, 19].

Until recently, ballast substances contained in vegetables and fruits were considered the main sources of dietary fibre. However, collagen, especially its fractions obtained by various methods, is just as good functionally as its plant analogues. This means that protein hydrolysates and composites can also be regarded as fibrous, anisotropic, three-dimensional food systems [20–22]. In the growing agricultural sector, there is a need for improving the production of hydrolysates and concentrates of various biopolymers (polysaccharides, proteins, etc.).

An important factor is that nanoclusters (for example, in cellulose) are highly likely to preserve various biologically active substances, which ensures their safety in the further cycle of food production [23].

A number of studies have shown that animal analogues of dietary fibres (in particular, modified collagen and ichthys collagen) can be used as a matrix base. For example, a study was conducted to determine the sorption properties of collagen fermentolisate in relation to heavy metals, using Cd^{2+} and Pb^{2+} ions. The study found that the biomodified connective tissue protein showed a similar ability to bind Pb^{2+} ions to that of cellulose, for which the sorption range was 0.10−0.23 mg/g [24]. Thus, hydrolysed forms of collagen are able to bind heavy metal ions in the digestive tract to form insoluble complexes that are excreted from the body without being absorbed. This mechanism can be used in the prevention of heavy metal salt poisoning.

Of scientific interest is also the process of joint sorption of several protein components and bioactive

substances. A systematic study of the sequential and joint sorption of several binary protein mixtures and some bioactive substances (for example, ion-exchange components of plant origin such as ascorbic acid or iodine) showed that the binding process was complicated by synergistic phenomena. Such phenomena were promoted by the strong binding of protein to individual components of various nature, which can be determined as the number of fixed ionogenic groups of the sorbent on one protein molecule. A decreased local concentration of ionogenic groups of plant-based bioactive components contributes to the transition to a synergistic mechanism of competitive sorption. Such sorption of bioactive substances on a collagen-based matrix can preserve up to 70% of organic components such as ascorbic acid or iodine that are easily destroyed by heat treatment [25].

We should note that the mechanism of such sorption has not been established yet. However, we know that all proteins have a pronounced ability of non-specific binding to SH groups, the guanidine group of arginine, and other amino acid components. It is possible that the biomodification of connective tissue contributes to the breakdown of peptide chains of collagen. As a result, the previously mentioned functional groups become more accessible for interaction with metals and biologically active substances [26].

Thus, the connective tissue modified by chemical, physical or biological methods is a highly active sorbent for heavy metals and biologically active substances. It therefore has a potential of being used as a functional additive in the production of foods, particularly meat products [24–27].

An important issue in the context of dietary fibre physiological properties is the consumer's attitude to functional components in food production. An online survey among young respondents using Google services demonstrated a positive response to functional ingredients, in particular to dietary fibre, if the necessary information is given on the packaging [28].

However, consumers are concerned about the safety of certain ingredients. According to GRAS, refined wheat, oat, corn, and other dietary fibres are safe to use.

Most studies, both in Russia and abroad, have focused on stabilising systems based on hydrocolloids and composites containing several components, for example, polysaccharide and protein composites, etc. Noteworthily, hydrocolloids can be produced by various methods: chemical, physical, biological, etc [4]. In addition, genetic modification is now used in crop breeding to accelerate the production of target products. However, it is extremely undesirable, especially in terms of consumer demand [28].

The technological aspects of using fibrous food systems or compositions require a study of their rheological properties, including viscosity, emulsifying ability, colloidal and molecular features, as well as hydration characteristics of imported and domestic

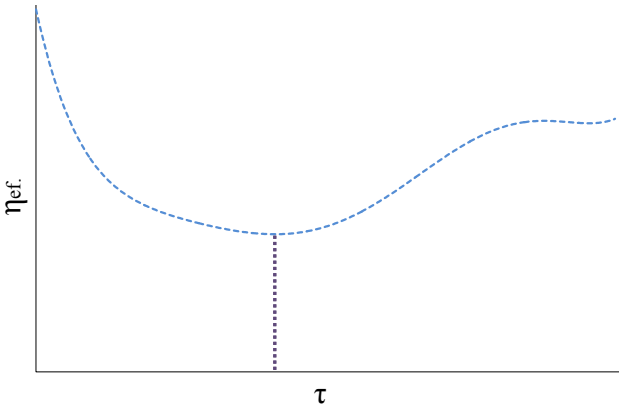


Figure 1 Rheogram for CMC solution

additives for better development of formulations and processes [29, 30].

For example, a solution of carboxymethyl cellulose (CMC) from the group of colloidal fibres is characterised by a thixotropic flow with a decrease in viscosity at constant load and a rather significant increase in effective viscosity after unloading, as shown in Fig. 1 [4].

In connection with the above, we aimed to study the rheological and microstructural properties of various types of dietary fibre that contribute to a jelly-like structure with similar mechanical and sensory properties to those of food raw materials, for their further joint use in the development of restructured poultry products.

STUDY OBJECTS AND METHODS

This study used dietary fibres of various SuperCel groups (manufactured by J. Rettenmaier & Soehne GmbH & Co. KG, Germany and supplied by Rettenmaier Rus), namely:

– insoluble: WF 200 R, WF 300 R, WF 400 R, and WF 600 R wheat fibres;
– semi-soluble: KF 200 and KF 500 potato fibres; and
– soluble: psyllium P 95.

Nutritional value indicators were determined as follows:

– moisture mass fraction: according to State Standard R 51479-99***;
– protein mass fraction: on a semi-automatic Tecator Kjeltec System 1002;
– fat mass fraction: according to State Standard 23042-2015****;
– ash mass fraction: according to State Standard R 53642-2009*****; and
– carbohydrates mass fraction: by the computational method.

The digestibility of *in vitro* proteins was examined using the Pokrovsky-Ertanov method and a modified device. The degree of dietary fibre hydration was

*** State Standard R 51479-99. Meat and meat products. Method for determination of moisture content. Moscow: Standartinform; 2010. 4 p.
**** State Standard 23042-2015. Meat and meat products. Methods of fat determination. Moscow: Standartinform; 2016. 9 p.
***** State Standard R 53642-2009. Meat and meat products. Determination of total ash. Moscow: Standartinform; 2010. 8 p.

Table 1 Technological properties of SuperCel fibres

Type of fibre	Grade	Average fibre length, μm	Average fibre thickness, μm	Degree of hydration	Water binding capacity, g water/g	Fat absorption, g fat/g	Bulk weight, g/dm^3
Insoluble: wheat	WF 200 R	250	25	1:8	8.3	6.9	72–98
	WF 300 R	350	25	1:9	9.2	7.3	58–80
	WF 400 R	500	25	1:10	10.5	11	37.5–62.5
	WF 600 R	80	20	1:5	4.2–5.5	3.7	200–240
Semi-soluble: potato	KF 200	200-350	–	1:8	15	–	250–400
	KF 500	400–1000	–	1:8	15	–	80–250
Soluble: psyllium	P95	250	–	1:25	20	–	170

determined visually. The Lipatov Jr. method was used to measure the water holding capacity of meat samples.

Structural and mechanical properties of meat products, namely shear stress and cutting work, were determined on an Instron-1140 testing machine using a Kramer shear press.

Microbiological tests and product safety studies were conducted in accordance with Technical Regulations of the Customs Union 021/2011******, 034/2013*******, and Methodological Guidelines 4.2.2747-10********.

Sensory tests were guided by ISO 11037-2013********; the yield of the finished product was measured by the weight method.

Rheological properties were studied using an RPE-1M Polymer rotary viscometer with a T1-B1 rotor-cylinder sensing system. Microstructural studies of meat samples were guided by State Standard 19496-2013********. They were conducted using an AxioImager A1 light microscope (Carl Zeiss, Germany), an AxioCam MRc 5 video camera, and an AxioVision 4.7.1.0 computer-based image analysis system.

The results were processed using standard methods of variation statistics. The differences were considered significant at a confidence interval of > 0.05.

RESULTS AND DISCUSSION

The technological properties of the dietary fibres under study are shown in Table 1.

According to sensory analysis, wheat and potato fibres showed the most rational properties for use in meat production. In addition, potato fibres had an increased hydrating and swelling ability, contributing to the formation of three-dimensional food products.

This information is relevant to selecting dietary fibre for further use in the production of various foods.

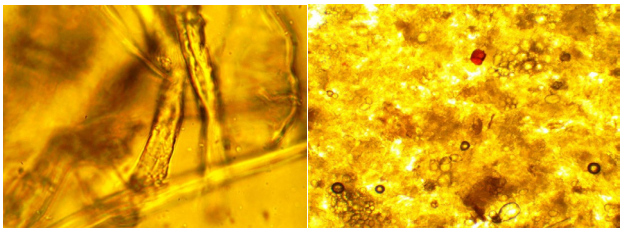
The histological structure of SuperCel wheat and potato fibres of the WF 600 and KF 500 grades, respectively, is shown in Fig. 2.

The analysis (Fig. 2) showed that wheat fibres had a three-dimensional structure characteristic of plant tissues. Under the light microscope, we could clearly see the surface of the fibres: the core, the periphery, and threads with varying degrees of deformation. Observation at different sharpening levels revealed a certain spatial network formed by the wheat fibres.

Potato fibres had a relatively uniform composition with differentiated fragments of cellular structures, round-shaped starch grains of various diameters, and optical density fluctuations over the entire structure of the preparation.

In order to optimise the processes, we then studied the rheological properties of model systems (dietary fibre-water), using the ratios recommended by the manufacturer.

The analysis of the graphs (Fig. 3) showed that WF 600 SuperCel wheat fibres had a more complex rheological behaviour at the initial stage of testing. In our opinion, this was due to the difficulty in rotor spinning at that stage caused by their complex 3D structure and, presumably, adhesion. Initially, the shear rate gradient was 2.7–5.5 s^{-1}.



(а) (б)

Figure 2 Histological structure of SuperCel dietary fibres: (a) WF 600 wheat; (b) KF 500 potato (40 magnification)

****** TR TS 021/2011. Tekhnicheskiy reglament Tamozhennogo soyuza 'O bezopasnosti pishchevoy produktsii' [TR CU 021/2011. Technical regulations of the Customs Union 'On the safety of food products']. 2011.

******* TR TS 034/2013. Tekhnicheskiy reglament Tamozhennogo soyuza 'O bezopasnosti myasa i myasnoy produktsii' [TR CU 034/2013. Technical regulations of the Customs Union 'On the safety of meat and meat products']. 2013.

******** MUK 4.2.2747-10. Metody sanitarno-parazitologicheskoy ekhspertizy myasa i myasnoy produktsii, provodili mikrobiologicheskie issledovaniya i izuchali pokazateli bezopasnosti razrabotannykh produktov pitaniya [Methodological Guidelines 4.2.2747-10. Methods of sanitary and parasitological examination of meat and meat products]. 2011.

******** State Standard ISO 11037-2013. Sensory analysis. Guidelines for sensory assessment of the colour of food products. Moscow: Standartinform; 2014. 16 p.

******** State Standard 19496-2013. Meat and meat products. The method of histological investigation. Moscow: Standartinform; 2014. 10 p.

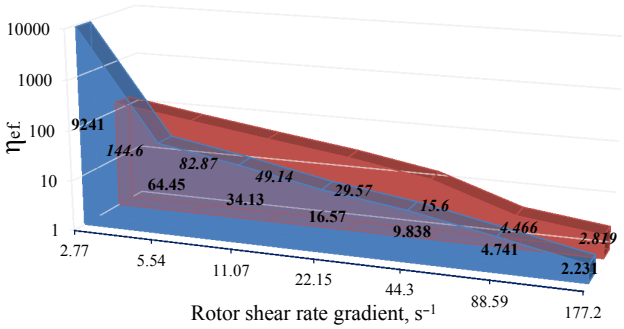


Figure 3 Rheograms for various types of dietary fibre: blue for WF 600 wheat; red for KF 500 potato

Further stages were carried out in the range from 4.2 to 177.2 s^{-1}. In those cases, KF 500 SuperCel potato fibres had a more effective viscosity, expressed in logarithmic coordinates (from 1 to 10000 Pa·s).

The rheograms above can be correlated with typical curves for viscous flow materials that are liquefied by shear. A significant part of food materials (apple pulp, puree-like products, mayonnaise, dairy products, pumping pickles, etc.) are non-Newtonian. It means that their rheological behaviour depends on the shear gradient, and the graph may feature a yield strength. Differentiated on the rheograms, which can be built in logarithmic coordinates, are Newtonian viscosity regions (low shear values), a zone of reduced viscosity as a power function (structural dispersion), and a Newtonian region of high shear [31, 32]. These data are important for predicting the course of production cycles and for food quality control.

The data shown in Fig. 3 suggest that these fibres can be attributed to colloidal structures that are mostly hydrophilic and are able to swell and bind food materials. In addition, due to increased hydration and micelle formation, they form more stable and uniform food masses that can be easily introduced into the formulations of meat products.

Based on the results, we can assume that the KF 500 SuperCel potato fibres correspond to such modified cellulose additives as methyl cellulose and carboxymethyl cellulose in terms of rheological properties and consistency. It means that they can exhibit pseudoplastic and non-thixotropic flow properties. Thus, these potato fibres can form clusters of polymer chains and 3D structures.

Consequently, these data can help us rationalise the processes of mixing, moulding, pressing, and heat treatment, as well as prevent syneresis, layering, and other processes. More stable functional and technological properties can also be used to optimise the stages of packaging and storing semi-finished and finished products (for example, convenience meat products, snacks, etc.).

In connection with the above, of great interest is the use of dietary fibres in the production of various types of meat products, for example, cooked sausages, minced products, pastes, pork products, etc. [33–37]. Taking into account consumers' desire to buy inexpensive high-quality meat products, manufacturers are developing new ways of restructuring meat.

Therefore, our further studies aimed to identify possible uses of dietary fibre in the meat technology, particularly in the development of restructured products from poultry meat.

Pieces of poultry meat, both red and white, were minced in a meat mincer with a hole diameter of 16–25 mm to be used as a meat raw material. Salt and granulated sugar were used as curing ingredients.

Meat raw materials were massaged on a vibrating massager at a rotation speed of 10 min^{-1} for 40 min. The amount of brine was 20% of the initial weight of the material. Further process stages included brining, forming, cooking at 80°C until the product reached 72 ± 2°C in the centre, and air cooling at 4 ± 2°C until the finished product was 8°C in the centre.

The composition of brines is shown in Table 2.

Sensory evaluation is one of the determining factors in assessing the quality of food products (Fig. 4).

As can be seen in Fig. 4, the test sample containing dietary fibres of the selected grades was just as good as the control product in its sensory parameters. The test

Table 2 Brine composition

Ingredients	Ingredient amount, kg per 100 L of brine	
	Control	Test
Water	89.5	84.5
Granulated sugar	0.5	0.5
Salt	10.0	10.0
SuperCel WF 600 wheat fibres	–	1.0
Fucus	–	3.5
SuperCel KF 500 potato fibres	–	0.5
Total:	100	100

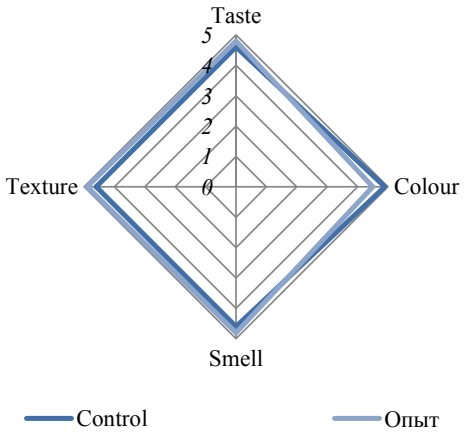


Figure 4 Sensory evaluation of restructured poultry products

Table 3 Safety indicators for restructured poultry products containing dietary fibres

Indicator	Requirements according to			Results
	Technical Regulations of the Customs Union 034	Technical Regulations of the Customs Union 021	Methodological Guidelines	
Microbiological indicators				
Coliforms	not allowed in 0.1 g			not detected
Sulphite-reducing clostridia	not allowed in 0.01 g			not detected
S. aureus	not allowed in 1 g			not detected
E. coli	not allowed in 1 g			not detected
Pathogenic, incl. salmonella	not allowed in 25 g			not detected
QMA&OAMO, CFU/g	max 1×10^3			not detected
L.monocytogenes	not allowed in 25 g			not detected
Content of toxic elements, mg/kg, max				
Lead mass fraction	0.5			< 0.001
Arsenic mass fraction	0.1			< 0.001
Cadmium mass fraction	0.05			< 0.0001
Mercury mass fraction	0.05			< 0.0001

sample's consistency received the highest rating from the panelists.

Microbiological tests and safety indicators analysis can be used to establish the degree of product safety. Safe products do not contain pathogenic or conditionally pathogenic microorganisms and do not exceed the maximum permissible concentration of toxic elements, pesticides, mycotoxins, antibiotics, hormones, and radionuclides. We found that the test sample met the requirements of the Methodological Guidelines and Technical Regulations of the Customs Union 034 and 021 (Table 3).

With regard to strength characteristics, we concluded that introducing dietary fibre into the brine for massaging the test sample contributed to an increase in shear stress and cutting work, compared to the control (Fig. 5). It improved the consistency of the finished product and its sensory characteristics (Fig. 4).

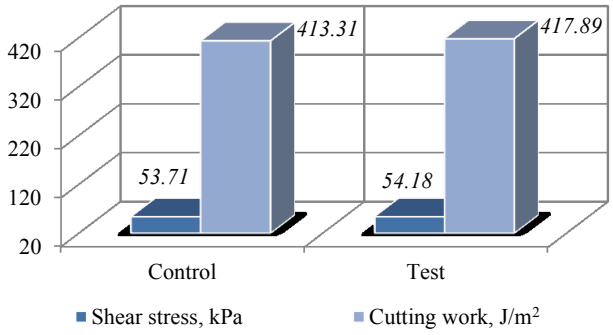


Figure 5 Structural and mechanical properties of restructured poultry products

Table 4 Functional and technological properties of restructured poultry products

Samples	Yield, %	Thermal loss, %	Moisture, %	Water-holding capacity, % to total moisture
Control	83.8	14.7 ± 0.8	72.5 ± 2.3	79.1 ± 2.1
Test	87.3	9.3 ± 0.7	74.1 ± 2.4	87.2 ± 2.0

The meat products containing dietary fibre had a lower mass loss during heat treatment and higher water-holding capacity and yield, compared to the control sample (Table 4).

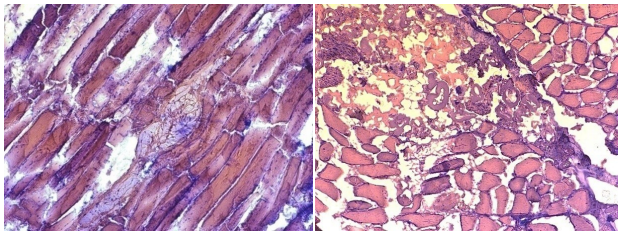
Table 5 shows the influence of dietary fibre on the indicators of biological and energy value of the meat products.

The total energy value of the test sample decreased by 8% due to a reduced fat content. We believe that the decrease in the mass fraction of fat was caused by the formation of a capsule of dietary fibre around fat droplets, which prevented the extraction of the lipid component during its determination.

The digestibility of *in vitro* proteins in the test sample was 10% lower than in the control. This was due to the presence of ballast substances in the restructured poultry product that are not digested by enzymes of the gastrointestinal tract.

Table 5 Nutritional indicators of restructured poultry products

Indicators	Control	Test
Moisture mass fraction, %	66.80 ± 2.32	68.10 ± 2.73
Protein mass fraction, %	16.77 ± 0.49	16.70 ± 0.56
Fat mass fraction, %	13.40 ± 0.37	11.30 ± 0.31
Ash mass fraction, %	3.03 ± 0.09	3.40 ± 0.10
Carbohydrates mass fraction, %	Traces	0.30 ± 0.02
Energy value, kcal/100 g of product	188.40 ± 5.24	170.00 ± 4.86
Digestibility of *in vitro* proteins, mg tyrosine/100 g protein:		
by pepsin	4.84 ± 0.09	4.63 ± 0.09
by trypsin	9.92 ± 0.29	9.83 ± 0.29
Total:	14.76 ± 0.43	14.46 ± 0.43



control test

Figure 6 Microstructure of restructured poultry products (10 × magnification)

Figure 6 shows the results of microstructural studies to assess the uniformity of distribution of the curing mixture in the finished products.

The analysis of histological preparations did not reveal any significant differences between the control and the test samples. Their microstructure showed the presence of exclusively muscle tissue with a few fragments of adipose and connective tissue that make up the muscular skeleton. We also found some components of endomysium, coarse fibrous interlayers of perimysium, and a small amount of fat cells. Noteworthily, cell membranes of muscle and connective tissue retained their integrity outside the fragmentation zone. A distinctive feature of the test sample's microstructure was a local presence of dietary fibre fragments and an increased amount of muscle tissue decomposition products.

CONCLUSION

We established specific structural characteristics, properties, and rheological behaviour of various dietary fibres. We found that potato fibres demonstrated greater uniformity in texture and rheology, compared to wheat fibres.

Wheat fibres had a clear phase structure (fibre/water), whereas potato fibres showed significant hydrophilic and structuring properties, attributing them to colloidal fibres.

Comprehensive studies revealed that a combined use of wheat and potato fibres in massage brines contributed to the production of restructured poultry products with good functional and technological properties. It also increased meat succulence and prevented liquefaction, syneresis, volume loss, etc.

Using potato and wheat fibres can help producers to expand the range of meat products in line with the concepts of rational and healthy nutrition, i.e. preventative products.

Our experimental material can become a basis for further research aiming to create combined dietary fibre complexes that can be used in the production of biologically active dietary supplements and specialised meat products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Raw poultry meatballs with soya flour: Shelf life and nutritional value

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Abstract: Poultry meat is a valuable source of protein for human consumption. It plays an important role in countries with poor ungulate meat production, including the Republic of Kazakhstan. The intake of fibre by the Kazakh population also remains low, while the intake of saturated fatty acids is excessive. Therefore, it is recommended to combine meat with plant products, e.g. soya flour. In the present research, we developed and evaluated a new meatball product containing different amounts of soya flour. The meatballs proved to be a semi-finished high-protein product. They also demonstrated a good fatty acid and mineral profile. The product with 30% of soya flour showed the best results: 27% of protein, low content of saturated fatty acid, and shelf life of 48 h. To extend the shelf life of the meatballs under refrigerator conditions, new disinfection methods should be developed.

Keywords: Soya flour, chicken, meatballs, nutritional value, predictive microbiology, shelf life

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INTRODUCTION

People living in developing countries, such as Kazakhstan, need high nutritive products. In this context, meat is considered one of the main sources of protein for consumers due to its high nutritive value. However, meat is expensive. Moreover, Kazakhstan has a specific lack of food-producing animals, and, thus, a low production of meat obtained from domestic and wild ungulates [1]. As a result, the food security of meat products there remains unsatisfactory [2]. Hence, Kazakhstan needs to develop a more competitive meat industry to improve meat production and market [3].

A relatively low production of beef in Kazakhstan is becoming an urgent problem, considering that Muslims represent a large group of Kazakh population, and they do not eat pork. Poultry meat could also improve protein intake by Kazakh people. Combining meat with products of plant origin is highly convenient for several nutritional purposes. A recent study by Shakiyeva *et al.* of the nutritional status of Kazakh people aged over 40 y.o. demonstrated a low fibre intake and excessive levels of saturated fatty acids [4]. Most plant proteins have a good fatty acid profile, which makes them preferable for human consumption. In addition, vegetables are an important source of fibre. Therefore, the nutritional composition of vegetables has several benefits for human health.

Soya is one of the plant products that could be combined with meat to formulate a new product. Although soya has lower levels of lysine or sulphur amino acids compared with meat, this food product is an important source of protein and fibre [5]. Soya also possesses isoflavones, which have been implicated as substances with important health benefits for more than a decade [6]. A recent research conducted by Ferguson *et al.* demonstrated the positive effect of moderate consumption of isoflavones on metabolic response [7]. This property makes soya beneficial for consumers suffering from obesity or insulin resistance [8]. All these aspects justify the formulation of meat products that combine ordinary or germinated soya flour with poultry meat.

The introduction of innovative flour-based functional foods into the market demonstrated a positive economic effect [9]. However, the high initial bacteria load in raw soya, raw germinated soya, and poultry meat is one of the main problems associated with this type of product. Moreover, meat is an excellent nutritional source for several types of bacteria, even taking into consideration

that this type of product requires thermal treatment. Therefore, producers of this type of food should be careful when defining the shelf life and storage conditions.

The present paper introduces meatballs containing minced poultry meat with different percentages of germinated and non-germinated soya flour. Direct and indirect methods, e.g. predictive microbiology, were employed to determine the nutritional composition, minerals, heavy metals, and the shelf life of the new product.

STUDY OBJECTS AND METHODS

Formulation and elaboration. The meatballs were made of chicken breast. The minced chicken meat was combined with different concentrations and types of germinated and non-germinated soya flour to produce four different meatball samples. Sample A contained 70% of minced chicken and 30% of dry germinated soya flour. Sample B included 85% of minced chicken and 15% of dry germinated soya flour. Sample C contained 70% of minced chicken and 30% of dry non-germinated soya flour. Sample D consisted of 85% of minced chicken and 15% of dry non-germinated soya flour. The meatballs were stored at 4–6°C during the research process.

Microbiological analysis. The microbiological analysis was performed on days 0, 3, and 7. The samples were tested for *Salmonella spp.* and *Listeria monocytogenes* and proved to contain neither. 25 g of meatballs were homogenised with 225 mL of buffered peptone water (Merck, Germany) in a stomacher (MIX2, AES-Laboratory, France) for 2 min. The total viable counts were evaluated in plate count agar (Liofilchem, Teramo, Italy) incubated at 32°C for 72 h. *Enterobacteriaceae* were counted in violet red bile glucose agar (Liofilchem) incubated at 32°C for 24 h. Coliforms were detected in violet red bile lactose agar. *Escherichia coli* were detected in Fluorocult® (Merck) incubated at 42°C for 24 h. The presence of presumptive *Staphylococcus aureus* was evaluated in Baird–Parker agar (bioMérieux, Marcy l´Étoile, France) incubated at 37°C for 48 h. The presence of *Salmonella* was detected according to ISO 6579-1:2017 [10]. The meatball homogenate prepared as described above was incubated at 37°C for 24 h. A 100-µL aliquot of the incubated peptone water was transferred to 10 mL of Rappaport–Vassiliadis (RV) enrichment broth and incubated at 42°C for 24 h. Next, one RV broth loopful was streaked on xylose-lysine-deoxycholate agar (Oxoid) and SM-ID2 (bioMérieux) and incubated at 37°C for 24 h. *Listeria monocytogenes* was determined according to ISO 11290-1:2018 [11]. Twenty five grams of meatballs were incubated in half-strength Fraser broth (Oxoid) at 30°C for 24 h. Then, 100 µL was transferred to a tube containing 10 mL of Fraser broth and incubated at 37°C for 48 h. Finally, the half- and full-strength Fraser broths were plated out on Aloa® agar (bioMérieux), and the plates were incubated at 37°C for 48 h. All analyses were performed in duplicate.

Predictive microbiology. The data obtained for the microbiological analysis were compared with the data and scenarios obtained from ComBase, www.combase.cc (University of Tasmania, Tasmania, Australia; and the USDA Agricultural Research Service, Beltsville, MA, USA), which is a free on-line modelling database for predictive microbiology. The parameters used were those obtained from the initial analysis of the meatballs. Several conditions for bacterial growth were tested to assure the results obtained for the shelf life of the product.

pH measurement. The pH level was measured using a Crison PH 25+ pH meter with a penetration electrode (Crison Instruments, Barcelona, Spain) by introducing the electrode into the meatballs. Determinations for each treated meatball were performed in triplicate every three days.

Nutritional analysis. All analyses for the proximate composition were performed using standard AOAC methods [12]. The moisture content was determined by drying samples in a laboratory drying oven (Selecta, Barcelona, Spain) until the weight became constant. The total protein was determined by the Kjeldahl method. A factor of 6.25 was used to convert total nitrogen into crude protein. The samples were digested using a Kjeltec 1007 digester (Tecator, Höganäs, Sweden) and distilled using a Kjeltec 1026 distilling unit (Tecator). The lipid content was assayed by extraction with diethyl ether/petroleum benzene (1/1, v/v) in a Soxhlet system (Soxtec HT 1043, Tecator). The ash content was assessed by incineration in a muffle furnace (Utena, Lithuania). The carbohydrate quantity and energy content were obtained by calculations.

Minerals and heavy metals. Minerals and heavy metals were analysed by the method of inductively coupled plasma-optical emission spectrometry (ICP-OES). One gram of sample and 4 mL of 69% HNO_3 (Hiperpur, Panreac, Spain) were homogenised in glass tubes and incubated at room temperature for 1 h. Afterwards, 1.5 mL of 33% (w/v) H_2O_2 (Panreac) was added, and the mixture was heated first at 120°C for 10 min in a heater block (Selecta) and then at 130°C for 3 h. After the samples were cooled down to room temperature, Milli-Q water was added until the volume reached 25 mL. The samples were analysed in an Optima 4300 DV ICP-OES (PerkinElmer, MA, USA) under the following conditions: plasma flow, 15 L/min; auxiliary flow, 0.2 L/min; nebuliser flow, 0.8 L/min; sample flow, 1.5 mL/min.

Urease activity. The urease test was conducted as follows: 10 mL of a buffered urea solution (pH = 7.0) was added to 0.2 g of finely ground soya (test sample), and 10 mL of a phosphate-buffered solution was added to 0.2 g of the same sample (blank sample). The two solutions were incubated at 30°C for 30 min under stirring. In the presence of significant urease activity, the pH of the test solution increased due to the

ammonia released from the urea. Immediately after the incubation, the pH of the solutions was determined, and the difference between the pH of the test and the blank samples was calculated as the urease activity index. The pH was measured as described in section 2.4.

RESULTS AND DISCUSSION

Table 1 shows the microbiological analysis results of the four samples.

The total bacterial count, as well as coliform and enterobacteria counts were higher for the sample with germinated soya flour compared with that with non-germinated soya flour. The values reached one logarithmic cycle or above. This result was expected, since germinated soya contains more sugars than oligosaccharides, which should be a good advantage for bacterial growth [13]. However, the *E. coli* counts were very similar in all the samples.

L. monocytogenes and *Salmonella* were not isolated from any of the analysed samples. Variations in *Salmonella* prevalence depend on the origin of the poultry meat, as shown in [14]. Despite the importance of *Salmonella* tests for poultry production, the incidence of this foodborne pathogen has decreased in recent years [15]. In the case of *L. monocytogenes*, the past century saw a substantial improvement in quality regarding the prevalence of this microorganism in food [16]. For instance, the prevalence of *L. monocytogenes* in poultry breast was reported to reach 20% in 1990s. However, it has dropped to 8.6% in the last few years [17].

According to the ComBase results obtained for *Salmonella*-positive poultry samples, a value of 3.68 log CFU/g could be reached after one week under the following conditions of storage: temperature, 7°C; pH, 6.4; a physical state for bacteria, 1; no lag phase. Comparatively higher values of *L. monocytogenes* could be reached, even at temperatures below 7°C, for the same storage time. The total aerobic count was a good indicator of the shelf life of the product, and values above 7 log CFU/g indicated a marked alteration in the meatballs. The product needs to be stored at refrigeration values. Thus, *Pseudomonads* or *Brochothrix thermosphacta* can be selected in ComBase to predict the storage stability of the meatballs, as these bacteria are frequently related to meat spoilage [18, 19].

For *Pseudomonads* and *B. thermosphacta,* values above 7.5 log CFU/g could be achieved at 48 h of storage under the following conditions: temperature, 5°C; pH, 6.4; water activity, 0.99; initial value, 5 log CFU/g; physical state for bacteria, 1; no lag phase. However, the meatballs developed in this study showed values higher than 7.5 log CFU/g (total aerobic count) after just one week of storage. The fact that a physical state of 1 implies no lag phase presupposes an extreme case that could rarely occur in real situations. In any case, a semi-manufactured product, such as the meatballs under study, could only have a maximum shelf life of 48 h at refrigeration temperature. The obtained data and the fact that the meatballs contained only raw ingredients proved that an adequate shelf life could be achieved by packaging or disinfection methods, e.g. ionising radiation, or refrigeration [20].

The values obtained for the urease activity confirmed the absence of thermal treatment in soya flour. The pH value ranged from 7.14 for the product with only 15% of germinated soya flour to 8.30 for the product with 30% of germinated soya flour. In the control samples and the samples with the cooked soya flour, the values were always ≤ 7 due to the absence of urease in the treated product. For the products with 15 and 30% of germinated soya flour, pH was 6.63 and 6.82, respectively. According to Craven and Mercuri, several commercial texturised soya proteins were used in meat products with no increase in bacterial counts relative to the control samples [21]. In the present study, the less processed soya flour caused higher bacterial counts.

Table 1 Microbial counts and pH on day 0, 3, and 7 in meatball samples with soya flour

Samples (minced meat/soya proportion)	Day	pH	TAC*	*Enterobacteriaceae*	Coliforms	*Escherichia coli*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Salmonella*
70/30 germinated soya	0	6.44	8.8×10^6	6.2×10^5	1.8×10^4	2.0×10^2	< 50	nd	nd
	3	6.89	4.3×10^7	3.2×10^6	6.4×10^5	2.6×10^2	< 50	nd	nd
	7	7.12	9.1×10^8	6.8×10^6	1.2×10^6	3.3×10^3	< 50	nd	nd
85/15 germinated soya	0	6.33	5.0×10^6	2.4×10^5	1.0×10^4	3.1×10^2	< 50	nd	nd
	3	6.78	6.3×10^7	9.2×10^5	2.4×10^5	8.6×10^2	< 50	nd	nd
	7	6.99	9.8×10^8	3.8×10^6	5.9×10^5	3.6×10^3	< 50	nd	nd
70/30 non-germinated soya	0	6.31	3.5×10^5	4.5×10^4	2.0×10^3	1.2×10^2	< 50	nd	nd
	3	6.84	4.8×10^6	2.2×10^5	2.4×10^5	4.5×10^2	< 50	nd	nd
	7	6.92	4.2×10^7	2.8×10^6	5.9×10^5	9.2×10^2	< 50	nd	nd
85/15 non-germinated soya	0	6.39	1.7×10^6	6.2×10^5	9.0×10^3	1.5×10^2	< 50	nd	nd
	3	6.90	5.2×10^7	1.5×10^6	3.3×10^4	1.8×10^2	< 50	nd	nd
	7	7.21	2.2×10^8	9.3×10^6	8.5×10^5	4.1×10^2	< 50	nd	nd

*TAC: total aerobic coun
nd: not detected

Table 2 Nutritional composition of meatball samples with soya flour

Parameter	Samples (minced meat/soya proportion)			
	70/30 germinated soya	85/15 germinated soya	70/30 non-germinated soya	85/15 non-germinated soya
Dry weight	43.45	33.40	45.31	35.03
Protein	27.18	24.37	29.49	25.69
Fat	2.85	2.52	2.17	3.20
Saturated fat	0.73	0.65	0.50	0.82
Monounsaturated fat	0.57	0.54	0.44	0.66
Polyunsaturated fat	1.55	1.33	1.23	1.72
Ash	2.23	1.79	2.53	1.66
Carbohydrate	11.19	4.79	11.12	4.48
Energy, kcal/100 g	179.13	139.04	181.97	149.48
Sodium, mg/100 g	40.38	48.97	45.18	53.57

As for nutritional properties, the idea of using soya protein is more than 30 years old [21]. Chicken breast was found to achieve a maximum protein content of 34.5%, although values of 24% are most frequently reported [22, 23]. For the products developed and studied in the present research, similar values to those described by Lonergan *et al.* were obtained in the meatballs prepared with 15% of soya flour [23]. However, an important increase in protein content was observed for the meatballs with 30% of germinated soya flour. Their protein level was ≥ 27% (Table 2).

The primary objective of the present study was to obtain a high-protein product. The research proved that could only be achieved by adding 30% of soya flour to the meatballs. Of the two samples with 30% of soya flour, the meatballs with non-geminated soya flour showed better results, with 2% more protein than in the samples that contained germinated soya flour.

The meatballs with 30% of soya flour proved to have nutritional advantages. In addition, combination of protein and fibre can promote satiety [24]. The products with 30% of soya flour had a higher dry weight values than those with 15% of soya flour. This fact could trigger a higher water intake and, hence, an increase in satiety. Satiety is an important aspect to consider both for diabetes and/or dietetic treatment of obesity. The effect of high protein intake on satiety is so strong that a remission of pre-diabetes to normal glucose tolerance was observed in patients fed with a 30% dietetic protein for 6 months [25]. As revealed above, isoflavones in soya could also help to improve insulin tolerance [7, 8].

Serdaroglu *et al.* and Ikhlas *et al.* studied the quality of low-fat beef meatballs with 10% of various legumes, excluding soya flour [26, 27]. They reported lower protein values (≤ 24%) than those obtained for the products developed in the present study. Increased amounts of legume flour were suggested to be used as extenders for meatballs [26]. The meatball samples used in the present research showed no differences in total, saturated, and unsaturated fat (Table 2). Their values were always ≤ 4% and sometimes even ≤ 3%. Judging from these fat contents, the proposed meatballs had lower energy value and fat content than beef, pork, or even some turkey parts or duck meat [22]. Likewise, the low amount of saturated fat together with the high polyunsaturated fat content can improve traditional Kazakh diet.

As stated in [28], saturated fats should provide about 7% of dietary energy. The content of saturated fats in the

Table 3 Minerals and heavy metals in meatballs with soya flour

Minerals and heavy metals, mg/kg	Samples (minced meat/soya proportion)			
	70/30 germinated soya	85/15 germinated soya	70/30 non-germinated soya	85/15 non-germinated soya
Mg	656.22	393.57	476.01	421.97
P	1872.63	1520.86	1636.46	1629.99
K	5652.08	3930.54	4987.42	4360.88
Ca	802.88	386.48	476.07	318.47
Fe	16.14	9.20	14.27	10.97
Ni	0.50	0.21	0.35	0.6
Cu	3.58	1.33	2.51	1.51
Zn	11.09	8.02	9.36	8.66
As	0.0065	0.0055	0.0056	0.0033
Se	0.0956	0.1097	0.1067	0.1255
Cd	0.0064	0.0033	0.0047	0.0031
Hg	0.0104	0.0063	0.0055	0.0046
Pb	0.0065	0.0034	0.0032	0.0013

meatballs was ≤ 1%. Therefore, 200 g of the meatballs contained 60 g of protein and about 2 g of saturated fats, i.e. 240 and 18 kcal, respectively. It complies with the general recommendations for saturated fat intake.

As for minerals and heavy metals, the four meatball samples were tested for a total of 13 elements (Table 3).

Remarkably, the meatballs with 70% and 30% of germinated soya flour demonstrated higher contents of Mg, P, K, Ca, Fe, and Zn and a double or more of Cd, Hg, and Pb than the other samples. These results were probably due to the higher mineral content found in the soya flour, as the content increased with soya flour concentration in the produced meatballs. Therefore, plant food products can be expected to have higher amounts of minerals than animal food products.

The study conducted by a Chinese research team showed high levels of As, Cu, and Zn in poultry tissues, which were mainly attributed to feed supplements [29]. The Chinese study proved that the amounts of As found in inorganic poultry meat in Lianzhou and Guangzhou pose a significant public health risk, considering the high level of bladder or lung cancer in these cities. In the products designed in this study, the As level was an order of magnitude lower than that obtained by Hu *et al.* [29]. The levels of Cd and Pb in the present study were also lower. However, we detected higher levels of Cu and Ni. In any case, the soya flour used in this study was poorly processed and did not undergo any thermal treatment, as verified by the urease test.

Soya has important anti-nutritive agents, so this product has to be treated to avoid the effect of these compounds. These anti-nutritive factors are phytic acid, phytates, and protease trypsin inhibitors. The presence of natural phytates, for instance, significantly increases the calcium requirements. In soybeans, the phytic acid content is 1.00–1.47% of dry weight, which means more than 50% of phosphorous [30]. The treatments to eliminate trypsin inhibitors from soybean flour were recently reviewed by Vagadia *et al.* [31]. The cooking of soya flour in an alkaline system at 90°C for 15 min is sufficient to inactivate the protease trypsin inhibitors.

CONCLUSION

The present research introduced, developed, and described a new poultry meat product: meatballs formulated with germinated or non-germinated soya flour. The use of 30% of soya flour resulted in a semi-finished high-protein product. The soya flour used in the formulation produced a number of other positive effects, e.g. low and well-balanced fat content and increased amounts of fibre and isoflavones. The increase in mineral content could depend on the specific plant origin of the soya flour, and additional treatments are necessary to avoid the negative effect of anti-nutritive compounds. Direct microbiological analyses and predictive microbiology showed that the mixture of minced poultry meat and soya flour produced a product with a shelf life of 48 h. In order to extend the shelf life of the product, specific packaging procedures or disinfection techniques should be applied.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this article.

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Miscanthus plants processing in fuel, energy, chemical and microbiological industries

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Abstract: The increasing shortage of fossil hydrocarbon fuel dictates the need to search for and develop alternative energy sources, including plant biomass. This paper is devoted to the study of the Miscanthus plants biomass potential and the analysis of technologies of its processing into products targeted at bioenergy, chemistry, and microbiology. Miscanthus is a promising renewable raw material to replace wood raw materials for the production of chemical, fuel, energy, and microbiological industries. Miscanthus is characterised by highly productive (up to 40 tons per one hectare of dry matter) C_4-photosynthesis. Dry Miscanthus contains 47.1–49.7% carbon, 5.38–5.92% hydrogen, and 41.4–44.6% oxygen. The mineral composition includes K, Cl, N and S, which influence the processes occurring during biomass combustion. The total amount of extractives per dry substance lies in the range of 0.3–2.2 % for different extraction reagents. Miscanthus has optimal properties as an energy source. *Miscanthus* × *giganteus* pellets showed the energy value of about 29 kJ/g. For the bioconversion of plants into bioethanol, it is advisable to carry out simultaneous saccharification and fermentation, thus reducing the duration of process steps and energy costs. Miscanthus cellulose is of high quality and can be used for the synthesis of new products. Further research will focus on the selection of rational parameters for processing miscanthus biomass into products with improved physical and chemical characteristics: bioethanol, pellets, industrial cellulose, bacterial cellulose, carbohydrate substrate.

Keywords: Miscanthus, bioethanol, cellulose, raw materials, processing

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INTRODUCTION

Currently, the task of finding alternative energy sources that are environmentally safe and economically affordable is very urgent. Of particular interest are species of herbaceous plants with a high growth rate, characterised by high values of the above-ground vegetative mass growth and having practical application as an energy source [1].

An example is the genus Miscanthus plants, a practically inexhaustible source of renewable raw materials in the field of alternative energy. This is due not only to the chemical properties of their biomass, but also to high growth rates and enormous biological productivity, among other things in a temperate climate, which together make their use in Russia promising [2].

The main advantages of miscanthus biomass in comparison with other types of perennial grasses are associated with its higher productivity, resistance to adverse environmental conditions, increased lignin content and, consequently, increased calorific capacity.

In addition, the genus Miscanthus plants can be used to produce biologically active substances. Miscanthus extracts include fatty acids, sterols and other aromatic compounds. The main structures of phenolic compounds and sterols of the bark and core of *Miscanthus* × *giganteus* include vanilla acid, para-coumaric acid, vanillin, para-hydroxybenzaldehyde, syringaldehyde, campesterol, stigmasterol, β - sitosterol, stigmast-3,5-diene-7-one, stigmast-4-ene-3-one, stigmast-6-ene-3,5-diol, 7-hydroxy-β-sitosterol and 7-oxo-β-citerol [3].

Currently in the world there is an increase in cultivation of Miscanthus driven by the characteristic

high growth rates and a high degree of its biological needs compliance with agro-climatic conditions.

The purpose of this review was to analyse the modern methods of processing Miscanthus plants for bio-ethanol production, technical and bacterial cellulose, as well as products for microbiological and biotechnological industry.

STUDY OBJECTS AND METHODS

The representatives of the genus Miscanthus (*Miscanthus Anderss* L.) graminoid family (*Poaceae*) were the materials of this research. We analysed botanical characteristics and geographical distribution of various studied plants, made chemical composition analysis, and summarised the main processing methods according to the sources of scientific literature. These resulted in the analysis of modern methods of obtaining products for fuel, energy, chemical and microbiological industries.

RESULTS AND DISCUSSION

The botanical characteristics and distribution of the genus Miscanthus plants. The Miscanthus family includes about 40 species of monocotyledonous herbaceous perennial, sustainable plants with long curved linear leaves and small buds that bloom in late summer or early autumn, and about 20 species of miscanthus proper (Fig. 1).

In the Russian Far East (Primorsky Krai, Sakhalin and the Southern Kuril Islands), there are three species of miscanthus: *Miscanthus sinensis*, *Miscanthus sacchariflorus* and *Miscanthus purpurascens*. It can grow in the climatic conditions of Central and Eastern Europe [4]. In the late 20th century there appeared new miscanthus genotypes adapted to growth in the Northern regions, including Russian territories.

Figure 1 Chinese Miscanthus 'Gracillimus'

For the countries of the European Union (EU) it is recommended to grow miscanthus in the continental climate zone and the North Mediterranean, where soil and climatic resources correspond to the requirements of the plants [5, 6].

In Europe the plants reach a height of 3–4 m, and the representatives of tropical and subtropical species may reach 5 m or more in warm and humid climatic conditions. In the Central part of Russia, according to research of scientists of the Russian Timiryazev State Agrarian University, miscanthus giant reaches the height of 2 m, in Western Siberia – 2.5 m, and 3.9 m in the middle Volga region. The stems are upright and resistant to lodging because of their considerable thickness. It reaches 6 cm in homeland regions (China, Japan, Russian Far East, USA East coast); in the Middle Volga regions plants of 1–4 years of life are 0.8–1.5 cm thick [7, 8].

Miscanthus does not impose high requirements to soil and can grow well on marginal and low density soils whose granulometric composition is dominated by sand fractions. In Ukraine it is cultivated on sod-podzolic type of soils; in the forest-steppe of Novosibirsk Priobye, Middle Volga and Moscow region – on gray forest soils [9, 10].

For optimal growth and development plants require certain thermal and water regimes. Miscanthus seed germination requires ≥ 20°C soil heating with soil moisture of 60–80% of full field moisture capacity. To resume the shoots and the active growing season on crops of previous years, the temperature of the air must be in the range of 20–25°C. As shown in the literature, physiological activity of the studied representatives of the genus is sharply reduced at temperatures below 6°C.

Optimal temperature for adequate photosynthesis is considered to be 28–32°C. In Eastern Europe this is enough to produce sufficiently high yields of biomass [11].

It is known that miscanthus belongs to the C_4 plants, characterised by: optimum temperature for photosynthesis of 30–45°C, 40–80 mg/dm^{2}h CO_2 assimilation in full sunlight, more economical water consumption as compared to C_3 plants (twice and more), high drought and heat resistance, salt tolerance. These lead to better assimilation activity and, consequently, biological productivity.

The vast majority of well-known scientific studies are devoted to the three species of the genus miscanthus: *Miscanthus giganteus*, *Miscanthus sinensis* and *Miscanthus sacchariflorus*, which are the most widespread in Russia and abroad [12].

Chinese miscanthus (*M. sinensis*) is one of the most common types of ornamental grasses, named differently in different countries. For example, 'Chinese silver grass' or 'magic grass', sometimes 'Chinese reeds'. In nature it is widespread in the Russian Far East up to the taiga zone, also in China, Korea, Japan. As an adventive species it occurs in many countries, e.g. USA, Brazil and African States (Fig. 2) [13].

M. sacchariflorus is a species growing on wet meadows, forest clearings and stony slopes in the

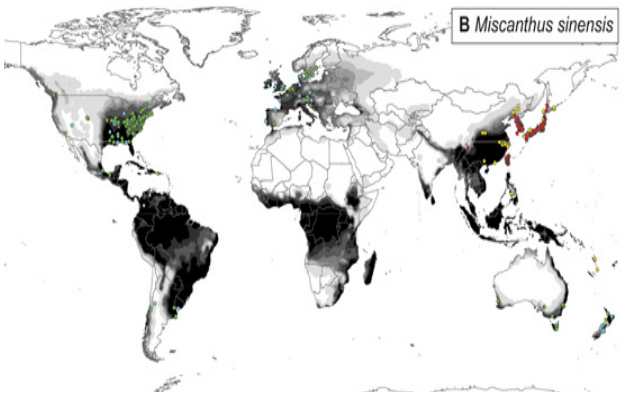

Figure 2 Distribution of Chinese Miscanthus (*M. sinensis*)

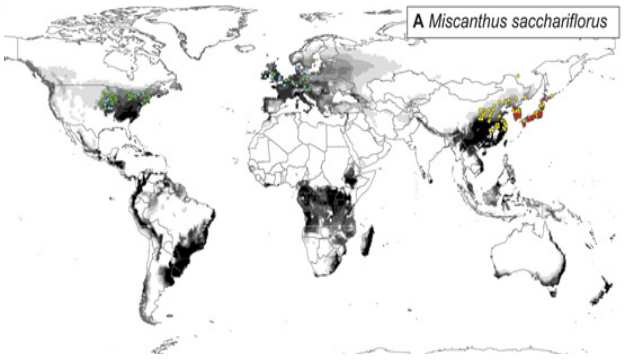

Figure 3 Distribution of *M. sacchariflorus*

Primorsky Krai, China, Korea, Japan, and also in the above-mentioned areas [14].

This also is a perennial long-stem herb up to 2 m in height. Stems are erect, thick and numerous. Leaves are rather rigid, linear, flat, long-acuminate, 7–18 mm wide, up to 60 cm long. Flowers are small, in spikelets with long, white, silky hairs. Inflorescence is 15–25 cm long, pinkish-silvery, white, fan-shaped, consisting of 8–20 spiciform branchlets with fruits. It blooms in late August–September. Flowering often occurs in July [15]. Not demanding to soil fertility.

Giant miscanthus (*Miscanthus* × *giganteus*), as described above, has a C_4 photosynthetic path and provides high productivity of plant biomass. The genome of this species includes a triple set of chromosomes that do not divide during meiosis because gametes are not viable. As a rule, seeds are formed sterile, which significantly limit reproduction of the species, this is a barrier to the establishment of new fields.

The rhizome structure of giant miscanthus grows very slowly and decreases proliferation [16, 17]. Therefore, planting material is produced by cultivation of mother plantations (Queen cells), pre-multiplying it *in vitro*, or by rhizomes by targeted separation from plants of the previous planting year. This technology is limited by insufficient amount of seedling material and the lack of landing equipment [18, 19]. In this regard, high-quality uniform planting material can be grown in special nurseries with the use of modern biotechnologies.

Chemical composition and properties of genus Miscanthus plants. Analysis of miscanthus chemical composition biomass allows planning proper use of species. The three main components of lignocellulosic materials are: cellulose, hemicellulose and lignin. Their content in each organ is not equivalent and depends on the plant's functional and physiological properties. The amount of cellulose in the stem, as a rule, is higher than that in the leaves. Lignin contains three-dimensional phenylpropyl-based polymer that provides structural rigidity and integrity, as well as prevents lingocellulose swelling [20–22].

Table 1 shows the differences in concentration of these substances in the cell wall in the three species growing on the territory of European countries, after 3–5 years of cultivation [14].

According to the data presented in the table, the types of miscanthus *giganteus* and *sacchariflorus* differ insignificantly in pulp content and hemicellulose/lignin ratio.

Table 2 shows the averages of biochemical composition of leaves, stems and a whole 4-year-old plant of Miscanthus varieties [4].

Data analysis indicates that the stem of miscanthus is the most suitable raw material for obtaining a large amount of high quality cellulose, as it has lower content of ash and lignin and a higher yield of the target product.

In the process of turning cellulose into ethanol such indicators as degree of polymerisation (n) and its crystallinity are of supreme value. The number of glucose units that make up one polymer molecule is called the degree of polymerisation.

X-ray diffraction and solid state 13 CP/MAS NMR spectroscopy are the two most commonly used methods of cellulose crystallinity determination. The crystallinity of cellulose for the species Miscanthus sinensis was measured by X-ray diffraction. Subject to the dimensions of the particles, differences in the index of cellulose crystallinity were revealed (Table 3) [23].

Table 1 Composition of miscanthus species cell walls

Species	Cellulose, %	Hemicellulose, %	Lignin, %	H:L ratio
M. × *giganteus*	50.34–52.13	24.83–25.76	12.02–12.58	2.06–2.05
M. sacchariflorus	49.06–50.18	27.41–28.11	12.10–12.13	2.26–2.30
M. sinensis	43.18–45.52	33.83–33.98	9.69–10.32	3.49–3.29

Table 2 Chemical composition of the Soranovski plant variety

The organs of the plant	Performance (in terms of dry substance, %)				
	WGF*	ash content	lignin	pentosan	cellulose by Kürschner
Whole plant	4.98 ± 0.05	5.87 ± 0.05	22.0 ± 0.5	21.0 ± 0.5	53.1 ± 0.5
Leaf	6.32 ± 0.05	9.23 ± 0.05	23.6 ± 0.5	20.3 ± 0.5	43.3 ± 0.5
Stem	2.68 ± 0.05	2.13 ± 0.05	15.0 ± 0.5	23.0 ± 0.5	55.7 ± 0.5

*WGF – wax glaze fraction

Table 3 Miscanthus cellulose crystallinity according to the diffraction of X-rays

Particle size, μm	Cellulose crystallinity, %
250–355	54.2
150–250	50.7
63–150	41.9
< 63	24.8

It is generally believed that cellulose crystalline regions are harder to decompose than amorphous domains, due to the strong intermolecular hydrogen bonds. For the studied species, the researchers found that the initial rate of cellulose hydrolysis increased with decreasing crystallinity [24, 25].

Unlike cellulose, hemicelluloses have lower degree of polymerisation, typically 50–300; they also have a branched structure and are amorphous. The predominant hemicellulose polymer for the miscanthus is the arabinoxylane, which contains a chain of 1.4-linked xylonic links. Sugar components in hemicellulose can participate in the formation of lignin-carbohydrate complexes (LCC) by covalent linkages between lignin and carbohydrates.

Despite considerable analytical studies aimed at the characterisation of the LCC, they still remain poorly defined, and their biosynthesis pathways require further study [26]. Distribution, structure and content of lignin is considered to be one of the important factors responsible for the recovery of lignocellulosic enzymatic degradation.

Considering miscanthus as fuel, the values of specific heat of combustion, mineral composition, ash content and content of volatile substances were analysed. The specific heat of combustion parameter is closely linked to the elemental composition and ash content. For *Miscanthus* × *giganteus* it ranges from 17 to 20 MJ/kg. Dry raw material contains on average 47.1–49.7% carbon, 5.38–5.92% hydrogen, and 41.4–44.6% oxygen. Mineral composition includes the following elements: K, Cl, N and S, which have an impact on the processes occurring during biomass combustion [27].

Increased content of K and Cl can reduce the melting point of ash and cause corrosion. High concentrations of N and S can lead to increased NO_x and SO_2 formation during combustion. Miscanthus mineral concentration varies depending on the type of plant, place of growth, time of harvest and even the type of fertilisation. Late harvest is the preferred fuel due to the lower content of K, Cl and N. Some studies provide the trace element composition of the miscanthus: S – 0.7–1.9 g/kg, Ca – 0.5–1.4 g/kg, Mg – 0.2–0.6 g/kg, P – 0.4–1.1 g/kg [28].

Ash content is an important parameter for fuel. The indicator represents the mass fraction of non-combustible residue (calculated as anhydrous weight) percentage, which results from mineral impurities of the fuel during its complete combustion. According to generalised data, miscanthus ash consists of 20–40% SiO_2, 20–25% K_2O, 5% P_2O_5, 5% CaO and 5% MgO. Its composition depends on the content of silt and clay in the soil. High ash content leads to the formation of slag and causes thermal process agglomeration, thereby lowering combustion efficiency of biomass plant [29].

Biomass high moisture content impedes its combustion, causing a problem of transportation. Moreover, in the process of wet fuel combustion, a large number of volatile side-products are released. Table 4 shows composition of volatile products, ash content, and molar internal energy (Ea) of miscanthus. The parameters presented are influenced by the harvest period, plant species, and climate [30].

In addition to biofuels production, the Miscanthus plants can be used for obtaining biologically active substances. The total amount of extractives based on the dry substance is redistributed in the range of from 0.3 to 2.2% with different extraction reagents. Also more than 20 hydroxycinnamic acids and their derivatives were discovered and described. The interest in these compounds is justified by the potential of plant phenols

Table 4 Energy characteristic of the genus Miscanthus plants

Species	Ash, %*	Moisture, %	Volatile matter, %*	Coke residue, %**	Ea, kJ/mol
M. × *giganteus*	2.7	4.2–4.9	73.6–73.9	19.3–19.8	76.3–76.7
M. sacchariflorus	2.2–2.3	3.8–4.1	73.4–73.6	20.3–20.4	69.0–69.3
M. sinensis	3.0–3.2	4.2–4.4	74.7–74.9	17.7–17.9	64.6–65.7

*dry matter
**dry matter ash-free basis

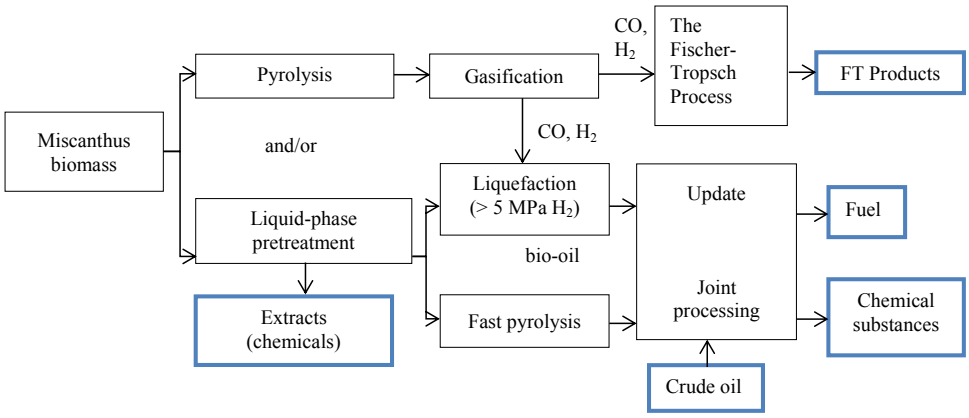

Figure 4 Simplified scheme of fuel and chemicals production

in the pharmaceutical industry. They can be used as antioxidant, antimicrobial, anti-inflammatory, anti-cancer biological active substances for manufacturing drugs to prevent HIV, thrombosis and atherosclerosis, reduce cholesterol, etc. [31, 32].

Features of processing Miscanthus raw materials for energy industry products. Miscanthus is the main energy culture, because it has the most optimal flow ratio of in/out energy content parameters [33]. Miscanthus as a lignocellulosic biomass with a low moisture content can be processed into fuel thermochemically. Figure 4 shows a simplified scheme of the two main ways of producing chemicals and fuels from thermal conversion of miscanthus biomass [34].

The first way is gasification, followed by Fischer-Tropsch synthesis, which requires large-scale installations. Large-scale installations cannot be adapted to biomass supply chain without biomass pyrolysis energy compaction before its long range transport. The second way is fast pyrolysis or biomass liquefaction, with the consequent quality biological oils increase in modified refrigerators [35].

Fast miscanthus pyrolysis was investigated in a fluidised bed reactor for production of bio-oil depending on the temperature (350–550°C), particle size (0.3–1.3 mm), feed rate and gas flow rate. The highest bio-oil yield of 69.2% was observed at the temperature of 450°C. With increasing temperature the amount of oxygenates in bio-oil gradually decreased, and the amount of water and aromatics increased. The output of the bio-oil did not depend on particle size or feed speed. The use of gaseous products as a medium for fluidisation increased the yield of bio-oil. It was also shown that partial removal of sodium and potassium increases the yield of *Miscanthus* × *giganteus* volatile substances due to the formation of semi-coke [36].

Miscanthus gasification study was carried out in a fluidised layer using olivine as the primary catalyst. It was shown that miscanthus raw material produces about 1.1 m^3/kg gas containing more than 40% of H_2 and 24% CO. Gas outlet and H_2 concentration increase with temperature while the yields of tar, semi-coke, CO, CO_2 and CH_4 decrease.

Experiments on miscanthus gasification were carried out in a circulating fluidised bed in the presence of oxygen, magnesite or olivine as a granular catalyst and kaolin as the additive to reduce agglomeration of the layer. Alkaline elements, mainly Na, K and Cl in the ash of miscanthus lead to agglomeration of the silica-rich material in the fluidised bed. The use of magnesite as an additive or as a bed material leads to a significant increase in the hydrogen fraction volume in the gaseous product. Its maximum volume fraction can reach up to 40% during the gasification of biological material with a layer of magnesite. Magnesite has also shown excellent results in resin content reduction and increase in hydrogen/carbon dioxide ratio (H_2:CO).

Thus, the analysis of scientific literature confirms the prospects of miscanthus as a source of energy. It can be briquetted or granulated. Combusted pellets from raw materials of miscanthus (*Miscanthus* × *giganteus*) demonstrated that the energy value of this product reaches 29 kJ/g. Meanwhile, low-temperature slow pyrolysis is energetically more favourable [37].

Features of processing plant biomass to bioethanol. The use of lignocellulosic biomass as a source of raw materials for the production of bioethanol has some complications, lying in its complex structure. It is established that the necessary preliminary chemical treatment of raw materials is needed. The process of raw materials bioconversion into bioethanol may include both separate hydrolysis and fermentation and simultaneous saccharification and fermentation, known, scientifically, as SHF and SSF processes, respectively.

One of the main advantages of SHF is the ability of enzyme preparations and microorganisms to operate under their optimal conditions. However, a disadvantage of stages continuous implementation is excessive length. For the purposes of optimisation, today the consistent process is faced with an alternative of SSF.

The advantage of this process is the carrying-out of saccharification and fermentation in one

reactor, shortening time process steps and reduction in energy consumption. It is also known that in the simultaneous process with introduction of bioethanol producers reducing substances begin to escape from the system, getting used for the synthesis of bioethanol. Thus, the equilibrium of the cellulose hydrolysis enzymatic reaction is continuously shifted toward the formation of reaction products (glucose), achieving saccharification intensification. However, one of the drawbacks of the simultaneous method is the difference in optimum temperatures needed for enzyme activity at saccharification stage (45–50°C) and for microorganisms cultivation (28–30°C) [9].

The main bioethanol producer in Russia is the yeast *Saccharomyces cerevisiae*, used in ethyl alcohol production both on food raw materials and hydrolysis media. In some sources these microorganisms are considered as bioethanol producers on hydrolysates of various types obtained from miscanthus raw materials.

For example, the paper by Baibakova shows the scheme of obtaining ethanol as a result of bioconversion using *Saccharomyces serevisiae* RNCIM Y-1693, isolated from the reactor of Kotlas (Arkhangelsk region) pulp and paper mill [28]. The peculiarity of the strain is its resistance to harmful impurities of hydrolysates. Optimal conditions for the strain are the temperature of 26–28°C and native active acidity of the extract of 4.5–4.7 pH. Earlier it was shown that this strain is resistant to lack of nutrients in the medium, products of its own metabolism and media obtained from cellulose-containing raw materials by enzymatic hydrolysis. The raw material was subjected to preliminary chemical treatment by alkaline delignification, after which the products of alkaline delignification were converted into a solution of monosaccharides by enzymatic hydrolysis. Further, bioethanol was synthesised on the obtained media [29].

Another paper provides information that bioethanol is also obtained by converting the strain with *Saccharomyces cerevisiae* Y-1693, but a solution of nitric acid is used for pretreatment. In this case, bioethanol yield reached 70.9 % [28, 30, 31].

The use of consortium for enzymatic hydrolysate of miscanthus cellulose based on *Pachysolen tannophilus* and *Saccharomyces cerevisiae* strains is also described. The yield of ethanol amount to 44% for *P. Tannophilus* RNCIM Y-1532 producer; to 62.5% for *S. cerevisiae* RNCIM Y-1693 of theoretically possible. With the combined use of cultures, the rate of fermentation increases by 10% compared to *S. cerevisiae* RNCIM Y-1693, but there is no increase in the proportion of ethanol yield. Joint use of strains was considered inappropriate [28, 31].

Bioconversion by enzyme preparations in combination with hydrolysis by dilute nitric acid at 90–96°C or alkaline delignification by 4% sodium hydroxide solution at 90–96°C is used for pretreatment of raw materials from miscanthus plants. Preparations 'Cellolux-A' (Sibbiopharm Ltd, Berdsk) and 'Bruzime BGX' (Polfa Tarchomin Pharmaceutical Works S. A., Poland) are used as cellulolytic enzymes.

'Cellolux-A' is positioned in the market as cellulase for non-starch polysaccharides fermentation, 'Bruzime BGX' – as hemicellulase [38]. As a result of enzymatic methods of miscanthus raw materials hydrolysis, bioethanol with a low content of ethers and fusel oils was obtained. There is no methanol in bioethanol obtained from miscanthus. However, saccharomycetes do not ferment pentoses, whose amount in hydrolysates can be significant (depending on the type of raw material and the method of hydrolysate obtaining), into ethanol. Several types of yeast are known to ferment xylose into ethanol: *Pachysolen tannophilus*, *Candida shehatae*, *Candida tropicalis*, *Pichia stipitis*, etc. To select a bioethanol producer, it is necessary to determine the specific rate of yeast biomass growth and the rate of substrate utilisation on synthetic media.

In addition to the use of wild strains, work is underway to obtain recombinant ones with increased capacity for bioconversion of raw materials. Thus, the patent CN 106701605 Huazhong Agricultural University presents a modified *Saccharomyces cerevisiae* SF4 yeast for efficient ethanol fermentation using xylose [32].

Specifics of processing plants into products for the chemical and microbiological industry. Beside the process of converting miscanthus raw materials to produce biofuels, a large amount of research is devoted to the production of cellulose fibres. Cellulose is widely used in modern industry, e.g. as a tablet excipient in pharmaceuticals, for the manufacture of fabrics, paper, plastics, explosives, etc.

The paper by Gismatulina describes obtaining cellulose from miscanthus of Soranovski variety (*Miscanthus sinensis Andersson*) by the nitrate method featuring two consecutive stages of processing the crushed material with diluted solutions of nitric acid, then sodium hydroxide [33].

The cellulose obtained by the nitrite method is characterised by high quality: the mass fraction of α-cellulose is 96.1%, the degree of polymerisation is 970, the ash content and mass fraction of lignin are 0.11 and 0.65%, respectively, the mass fraction of pentosans is 0.8%. Miscanthus cellulose is similar in quality to cotton cellulose. With these parameters, it can be successfully used for the synthesis of cellulose ethers and other valuable products.

In another paper, miscanthus samples are cellulose from the leaf and stem of miscanthus obtained separately by two methods (nitrite and combined) [34]. The nitrite method consists in cooking raw material in a dilute solution of nitric acid at atmospheric pressure, followed by treatment with a dilute solution of sodium hydroxide. Thus, cellulose obtained from the stem by the nitrite method has a better quality than that from the leaf. This is reflected in high values of α-cellulose content (94.4% vs. 91.7%) and degree of polymerisation (800 vs. 580), and also low values of noncellulosic compounds mass

fraction: ash – 0.07% vs. 1.01%, acid-insoluble lignin – 0.45% vs. 1.51%.

Celluloses obtained by the combined method demonstrate the same regularity: cellulose from the stem is characterised by higher quality than that from the leaf. The data show high value of polymerization degree – 1040 vs. 640 and low noncellulosic compounds mass fraction: ash – 0.14% vs. 0.75%, acid-insoluble lignin – 0.88% vs. 4.12%, pentosans – 6.38% vs. 8.53%. The cellulose obtained by the nitrite method may be suitable for chemical modifications, including nitration. The cellulose obtained by the combined method can be used in paper industry [31].

The use of miscanthus as a medium for bacteria cultivation can be carried out without the targeted production of simple sugars for use in the food, feed and pharmaceutical industries, as well as a substrate for bacterial growth. Some studies on bacterial celluloses production present media based on incomplete miscanthus hydrolysates.

Thus, Gladysheva describes obtaining bacterial cellulose by bioconversion of *Medusomyces gisevii* bacteria on a synthetic nutrient medium, including sucrose, black tea extract, starch hydrolysate, enzymatic miscanthus hydrolysate [35]. Cultivation was carried out in static conditions at 25–29°C for 13 days.

Gismatulina also used miscanthus raw materials to obtain a nutrient medium for the growth of bacteria producing bacterial cellulose [36, 37]. Pre-hydrolysis was carried out with 0.2% solution of nitric acid at 90–95°C for 1 h. Nitric acid treatment was carried out with 4% nitric acid solution at 90–95°C for 6 h. Washing was performed successively with 1% sodium hydroxide solution, and then 1 % nitric acid solution. The resulting pulp was pressed with a vacuum filter, washed to a neutral reaction of washing water, dried to a moisture content of 7–10 %.

The raw material for the experiments was ground to a particle size of 10–15 mm. It was established that optimal conditions of the principal and longest stage of obtaining cellulose by the combined method (alkaline treatment) are: sodium hydroxide concentration, 4%; temperature, 90–98°C; duration, 6 h. Cellulose extraction under such conditions allows obtaining the maximum yield of the target product – 35–40% with α-cellulose content of 87.0–90.3%, degree of polymerisation 950–990, residual lignin content of 2.0 to 3.0%, ash content of 0.3–0.4%, and pentosan content of 3.0 to 8.0%.

Cellulose isolated from miscanthus by the combined method is a promising substrate for enzymatic hydrolysis, with the degree of its conversion was 91–93% by weight of the substrate. High quality indicators of the substrate allow predicting the effectiveness of its use for the subsequent bacterial cellulose biosynthesis.

Also miscanthus raw materials can provide organic acids, alcohols and adsorbents. The paper describes obtaining formic acid from lignocellulose or its major components, which comprises two successive stages:
– acid-catalysed depolymerisation (polysaccharides hydrolysis, delignification);
– subsequent monomers (monosaccharides, phenolic derivatives) oxidation into formic acid. A high yield of formic acid equal to 45% was obtained [23].

Organosolv method of cooking miscanthus raw materials can also deliver ferulic, vanilla and para-cumaric acids, sterines, among which the main factions are β-sitosterol, 7-oxo-β-sitosterol, stigmasterol and campesterol. However, this method has not become widespread, as sterol derivates are oxidized during preliminary treatment with organic solvents.

CONCLUSION

Furthering lignocellulose biomass integrated processing by chemical and/or biotechnological methods into a range of competitive products and energy is a modern and fundamental area of industrial biotechnology developing in industrial countries.

The conducted botanical properties analysis of chemical composition and modern methods of processing miscanthus species biomass proved that it was a promising renewable wood-substituting raw material for products of chemical, fuel, energy, and microbiological industries. Further research will focus on the selection of rational parameters of processing miscanthus biomass into valuable products with improved physical and chemical characteristics, such as bio-ethanol, pellets, technical cellulose, bacterial cellulose, and carbohydrate-containing substrate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chemical composition of indigenous raw meats

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Abstract: Using non-traditional raw meats is a promising direction of the meat industry. They include venison and meat from yak, elk, red deer, rabbits, ostriches, upland game, and musk-ox. The study aimed to compare the biochemical and physicochemical parameters of the meat from reindeer, elk, and musk-ox living in the Yenisei North. We found that these meats had a high biological value. First, we studied the composition of amino acids, fatty acids, vitamins, and minerals in the meat from reindeer of various sex and age groups. We found that the difference between the contents of unsaturated and saturated fatty acids and their ratio in adult and young reindeer males was in favour of young males, while these parameters did not differ in the meat from reindeer females of various age groups. Nevertheless, reindeer females had a higher content of saturated acids compared to reindeer male meat. Further, we studied the composition of amino acids, fatty acids, vitamins, and minerals in Taimyr elk and musk-ox meats. According to the results, the contents of essential amino acids in Taimyr elk and musk-ox meats were 60% and 55%, respectively. In both cases, the dominant amino acids were leucine, isoleucine, lysine, and valine. The study revealed the benefits of using indigenous meats in sausage production. Finally, we showed the influence of starter cultures on the quality of dry sausages and developed formulations of venison-based sausages.

Keywords: Meat industry, ungulate meat, protein quality index, amino acids, vitamins, macroelements, microelements, indigenous animals

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INTRODUCTION

Numerous studies have shown that food production in Russia does not meet the physiological needs of Russian people, especially those in the Extreme North. The northern population, including children, has a severely unbalanced diet, which has been confirmed by extensive epidemiological studies and recent monitoring of nutritional status. Such food patterns result in an increased occurrence of nutritional diseases, lower efficiency, and reduced life expectancy, leading to unjustified social and economic losses. According to the WHO, nutrition accounts for 70% of people's health and physical development [1].

Using local raw materials and creating an indigenous metabolic type of nutrition can be the fastest and most effective way to improve the diet and eliminate micronutrient deficiencies [2–5].

Enriching foods with functional ingredients is one of the promising directions in this area. Such ingredients can regulate the amount of essential substances in the human body [6, 7].

Of no less importance is the production of combined meat products from indigenous animals with the addition of plant materials with high consumer appeal [8–11].

STUDY OBJECTS AND METHODS

The objects of the study were meat samples derived from ungulates of various sex and age groups. Nutritional value indicators were determined as follows:
– moisture mass fraction: according to State Standard 23042-86*;
– protein mass fraction: using the Kjeldahl method for nitrogen determination, State Standard 25011-81**;

* State Standard 23042-86. Meat and meat products. Methods of fat determination. Moscow: Standartinform; 2010. 5 p.

** State Standard 25011-81. Meat and meat products. Methods of protein determination. Moscow: Standartinform; 2010. 7 p.

Table 1 Amino acid composition of reindeer meat by age and sex group

Amino acid	Content, g/100 g						
	Calves		Youngsters		Adults		
	male	female	male	female	male	female	castrates
Non-essential, incl.:	30.91 ± 0.59	31.45 ± 0.45	32.37 ± 0.13	30.95 ± 0.43	31.60 ± 0.22	31.63 ± 0.44	32.33
Tryptophan	0.71 ± 0.03	0.75 ± 0.01	0.72 ± 0.01	0.79 ± 0.02	0.84 ± 0.01	0.79 ± 0.02	0.82
Isoleucine	3.87 ± 0.11	3.85 ± 0.10	3.92 ± 0.16	3.85 ± 0.08	3.94 ± 0.07	3.36 ± 0.19	3.89
Threonine	3.20 ± 0.06	3.30 ± 0.07	3.14 ± 0.05	3.19 ± 0.05	3.29 ± 0.05	3.11 ± 0.06	3.23
Valine	4.33 ± 0.06	4.20 ± 0.07	4.55 ± 0.07	4.32 ± 0.05	4.23 ± 0.07	4.46 ± 0.10	4.32
Methionine	1.35 ± 0.03	1.36 ± 0.02	1.31 ± 0.02	1.33 ± 0.02	1.36 ± 0.01	1.34 ± 0.04	1.35
Methionine + cystine	2.48 ± 0.05	2.53 ± 0.04	2.45 ± 0.04	2.49 ± 0.04	2.54 ± 0.03	2.44 ± 0.05	2.49
Leucine	6.81 ± 0.54	7.32 ± 0.16	7.96 ± 0.11	6.84 ± 0.42	7.30 ± 0.13	7.88 ± 0.17	7.99
Phenylalanine	3.47 ± 0.05	3.37 ± 0.07	3.68 ± 0.06	3.46 ± 0.04	3.35 ± 0.04	3.56 ± 0.09	3.52
Lysine	4.69 ± 0.08	4.76 ± 0.05	4.64 ± 0.06	4.68 ± 0.07	4.75 ± 0.04	4.69 ± 0.08	4.72
Essential, incl.:	24.68 ± 0.30	24.27 ± 0.38	26.07 ± 0.27	24.98 ± 0.23	24.19 ± 0.27	25.66 ± 0.37	24.82
Oxyproline	0.052 ± 0.006	0.052 ± 0.008	0.055 ± 0.01	0.052 ± 0.004	0.052 ± 0.01	0.054 ± 0.01	0.052
Serine	2.38 ± 0.03	2.33 ± 0.05	2.51 ± 0.03	2.36 ± 0.02	2.33 ± 0.03	2.49 ± 0.07	2.41
Glycine	3.75 ± 0.03	3.66 ± 0.05	4.03 ± 0.05	3.74 ± 0.03	3.66 ± 0.04	3.83 ± 0.07	3.73
Alanine	3.16 ± 0.12	3.19 ± 0.04	3.47 ± 0.05	3.12 ± 0.09	3.17 ± 0.03	3.38 ± 0.07	–
Glutamine	6.82 ± 0.07	6.63 ± 0.14	7.19 ± 0.11	6.81 ± 0.06	6.69 ± 0.10	6.98 ± 0.16	6.76
Proline	3.89 ± 0.11	3.67 ± 0.17	3.86 ± 0.16	3.84 ± 0.09	3.55 ± 0.12	4.18 ± 0.24	3.87
Arginine	4.16 ± 0.25	4.28 ± 0.11	4.46 ± 0.07	4.59 ± 0.19	4.27 ± 0.08	4.26 ± 0.14	4.25
Total:	55.59 ± 0.70	55.72 ± 0.81	58.44 ± 0.23	55.93 ± 0.53	55.79 ± 0.49	57.29 ± 0.79	57.15

– mineral composition: by mass spectrometry;
– amino acid composition: using Hitachi L-8800 and Hitachi-835 amino acid analysers;
– fat content: using a Soxhlet apparatus, State Standard 23042-86;
– fatty acid composition: by gas-liquid chromatography; and
– vitamin content: by infrared spectroscopy.

Coliform bacteria were assayed according to State Standard R 52816-2007***; Salmonella – according to State Standard R 50480-93****. The microstructural indicators of meat were obtained using a Stereo Discovery V8 microscope. The 192 × magnified images were taken using a computer video system with Zeiss lenses.

RESULTS AND DISCUSSION

The first stage of the study focused on the amino acid, vitamin, and mineral composition of raw meats. The second stage aimed to develop a sausage technology.

Table 1 shows the amino acid composition of reindeer meat.

A high content of essential amino acids in reindeer meat makes it a balanced type of raw meat.

In the body, tryptophan is converted into biologically active compounds containing an indole ring (tryptamine, serotonin, and adrenochrome) and a pyridine ring (nicotinic acid, or vitamin PP). Tryptophan is used in treatment of heart disease to control body weight, suppress appetite, alleviate migraine attacks, and reduce the harmful effects of nicotine. Phenylalanine is oxidized to tyrosine. These amino acids are substrates for the synthesis of thyroxine, adrenaline, and noradrenaline. Phenylalanine is involved in the synthesis of collagen and connective tissue. It improves memory, attention, and circulation and contributes to the formation of insulin. Sulphur-containing amino acids are vital biologically active compounds. They are substrates for the synthesis of glutathione, insulin, lipoic acid, vitamin B_1, and a number of enzymes.

The fatty acid, vitamin, and mineral compositions of reindeer meat are presented in Tables 2–4.

According Table 2, the meat of females had a higher content of saturated fatty acids than that of males. Unsaturated fatty acids were dominated by oleic acid, accounting for 65.3%.

The comparison of age and sex groups showed that the meat of female calves was richer in vitamins compared to male calves. The adult meats had a higher vitamin content compared to the young meats.

We noted that the content of macro- and microelements in reindeer meat increased with the age of the animals.

Elk meat is rich in phosphorus, potassium, and sodium. Phosphorus is part of organic compounds in phospholipids, nucleotides, and phosphoproteins. It is involved in the metabolism and maintains the acid-base

*** State Standard R 52816-2007. Food products. Methods for detection and quantity determination of coliformes. Moscow: Standartinform; 2010. 17 p.
**** State Standard R 50480-93. Food products. Method for detection of Salmonella. Moscow: Izdatel′stvo Standartov; 1993. 13 p.

Table 2 Fatty acid composition of reindeer meat by age and sex group

Fatty acid	Content, g/kg					
	Calves		Youngsters		Adults	
	male	female	male	female	male	female
Saturated, incl.:	7.65 ± 0.07	7.44 ± 0.17	8.76 ± 0.09	8.73 ± 0.10	8.90 ± 0.11	9.04 ± 0.07
Lauric	1.10 ± 0.04	1.14 ± 0.04	1.11 ± 0.03	1.1 ± 0.03	1.14 ± 0.03	1.08 ± 0.03
Myristine	0.06 ± 0.003	0.06 ± 0.004	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Palmitic	2.26 ± 0.05	2.28 ± 0.09	2.12 ± 0.03	2.18 ± 0.05	2.28 ± 0.06	2.25 ± 0.04
Stearin	5.62 ± 0.09	5.37 ± 0.19	5.42 ± 0.12	5.30 ± 0.09	5.34 ± 0.15	5.58 ± 0.07
Eicosanic	0.07 ± 0.001	0.07 ± 0.003	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Unsaturated, incl.:	7.78 ± 0.03	7.79 ± 0.05	6.46 ± 0.04	6.32 ± 0.05	6.32 ± 0.06	6.30 ± 0.03
Palmitoleic	0.81 ± 0.01	0.80 ± 0.02	0.90 ± 0.01	0.88 ± 0.03	0.80 ± 0.01	0.80 ± 0.01
Oleic	4.55 ± 0.04	4.53 ± 0.03	4.59 ± 0.02	4.59 ± 0.02	4.53 ± 0.02	4.54 ± 0.02
Linoleic	0.83 ± 0.03	0.83 ± 0.07	0.81 ± 0.03	0.70 ± 0.04	0.83 ± 0.05	0.81 ± 0.02
Linolenic	0.15 ± 0.004	0.15 ± 0.01	0.16 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
Total:	15.44 ± 0.09	15.23 ± 0.20	15.23 ± 0.12	15.04 ± 0.10	15.21 ± 0.15	15.33 ± 0.09

Table 3 Vitamin content in reindeer meat by age and sex group

Vitamin	Content						
	Calves		Youngsters		Adults		
	male	female	male	female	female	male	castrates
E, mg/kg	5.41 ± 0.14	5.52 ± 0.09	5.62 ± 0.07	5.23 ± 0.10	5.79 ± 0.10	5.50 ± 0.06	5.55 ± 0.09
B_1, mg/kg	1.16 ± 0.03	1.19 ± 0.02	1.23 ± 0.01	1.18 ± 0.02	1.24 ± 0.02	1.18 ± 0.01	1.12 ± 0.03
B_2, mg/kg	1.67 ± 0.04	1.74 ± 0.03	1.81 ± 0.02	1.71 ± 0.03	1.91 ± 0.03	1.72 ± 0.02	1.79 ± 0.04
B_3, mg/kg	5.04 ± 0.11	5.08 ± 0.08	5.17 ± 0.05	4.96 ± 0.08	5.30 ± 0.09	5.8 ± 0.05	5.12 ± 0.11
B_5, mg/kg	56.22 ± 1.27	55.02 ± 2.12	59.11 ± 0.57	55.57 ± 0.92	59.45 ± 1.10	55.57 ± 1.42	57.98 ± 1.21
B_6, mg/kg	2.25 ± 0.06	2.27 ± 0.04	2.45 ± 0.06	2.31 ± 0.04	2.38 ± 0.04	2.56 ± 0.03	2.32 ± 0.07
B_{12}, μg/kg	26.87 ± 0.70	27.31 ± 0.44	28.69 ± 0.20	26.64 ± 0.50	29.43 ± 0.45	28.09 ± 0.33	29.12 ± 0.32

Table 4 Mineral content in reindeer meat by age and sex group

Mineral	Content						
	Calves		Youngsters		Adults		
	male	female	male	male	female	male	castrates
Calcium, %	0.13 ± 0.04	0.15 ± 0.02	0.15 ± 0.04	0.12 ± 0.02	0.13 ± 0.03	0.22 ± 0.08	0.17 ± 0.05
Phosphorus, %	0.78 ± 0.03	0.79 ± 0.05	0.70 ± 0.11	0.77 ± 0.03	0.66 ± 0.09	0.73 ± 0.02	0.70 ± 0.12
Potassium, g/kg	12.95 ± 0.62	13.12 ± 0.45	11.63 ± 0.38	13.51 ± 0.43	12.71 ± 0.42	13.92 ± 0.33	12.82 ± 0.44
Sodium, g/kg	2.83 ± 0.15	2.86 ± 0.15	2.67 ± 0.15	2.75 ± 0.11	2.76 ± 0.12	2.83 ± 0.06	2.79 ± 0.11
Magnesium, g/kg	1.25 ± 0.07	1.23 ± 0.11	1.18 ± 0.13	1.25 ± 0.12	1.17 ± 0.11	1.23 ± 0.06	1.19 ± 0.11
Iron, mg/kg	183.25 ± 18.12	255.25 ± 46.61	161.67 ± 12.65	178.5 ± 13.8	187.92 ± 16.97	191.4 ± 15.8	189.21 ± 13.12
Manganese, mg/kg	2.25 ± 0.36	2.39 ± 0.23	1.87 ± 0.19	2.22 ± 0.29	2.63 ± 0.42	2.37 ± 0.30	246 ± 0.17
Copper, mg/kg	5.43 ± 0.87	5.36 ± 0.75	4.03 ± 0.47	4.91 ± 0.65	5.47 ± 0.50	5.16 ± 0.58	5.33 ± 0.41
Zinc, mg/kg	99.59 ± 10.34	99.13 ± 11.11	86.25 ± 9.51	96.73 ± 8.21	103.50 ± 8.46	109.67 ± 8.81	106.20 ± 7.23

balance in the body. Potassium and sodium are elements of the reticuloendothelial system present in the hydrated layer of bone tissue crystals. They play an important role in maintaining the osmotic pressure of the blood. Elk meat is also rich in iron, a vital part of haemoglobin. It promotes oxygen transfer from the lungs to tissues and performs a catalytic function, participating in redox reactions. In addition, elk meat has a rather high content of zinc and copper participating in many biochemical processes.

Tables 5–8 show the contents of amino acids, vitamins, and minerals, as well as the fatty acid composition of Taimyr elk meat.

We found that Taimyr elk meat had all essential amino acids in the amounts close to the standard. Arginine is a vital component of muscle tissue metabolism. It maintains the optimal nitrogen balance in the body, slows down tumour growth, and stimulates the immune system. The biochemical analysis of elk meat indicated its high biological value.

Table 5 Amino acid composition of Taimyr elk meat

Amino acid	Content, g/100 g
Non-essential, incl.:	15.29
Tryptophan	0.79
Isoleucine	3.82
Threonine	3.52
Valine	2.26
Methionine	1.43
Methionine + cystine	2.67
Leucine	3.47
Phenylalanine	1.72
Lysine	4.89
Essential, incl.:	24.57
Oxyproline	0.045
Serine	2.02
Glycine	2.22
Alanine	2.70
Glutamine	3.66
Proline	0.98
Arginine	3.66

Table 6 Fatty acid composition of Taimyr elk meat

Acid	Content, g/kg
Saturated, incl.:	32.62
Lauric	1.08
Myristine	0.73
Palmitic	25.37
Stearin	5.36
Eicosanic	0.08
Unsaturated, incl.:	51.39
Palmitooleic	6.54
Oleic	43.60
Linoleic	1.09
Linolenic	0.16
Total:	84.1

As seen from Table 6, the ratio of unsaturated to saturated fatty acids in elk meat was 1.58:1, which is indicative of the quality of intermuscular fat. Among unsaturated fatty acids, oleic acid was prevalent in all the samples, accounting for 84.9% of all unsaturated acids.

The results of the vitamin content analysis revealed that among group B vitamins, the highest concentrations were of pantothenic acid and cyanocobalamin.

The mineral composition of elk meat included a variety of minerals that are known to form chelate, ionic, and other biologically active complexes with functional properties. Elk meat contained 85.85% protein, 1.63% fat, and 4.84% ash elements. The calorie content in 100 g of meat was 358.07 kcal (1500 kJ).

We also determined the composition of amino acids, fatty acids, vitamins, and minerals in musk-ox meat. The results are presented in Tables 9–12.

According to the results, the content of essential amino acids in musk-ox meat was slightly higher than that

Table 7 Vitamin content in Taimyr elk meat

Vitamin	Content
A, mg/kg	–
E , mg/kg	4.53
B_1, mg/kg	0.91
B_2, mg/kg	1.36
B_3, mg/kg	4.08
B_5, mg/kg	46.33
B_6, mg/kg	3.62
B_{12}, µg/kg	30.22
Total:	60.83

Table 8 Mineral content in Taimyr elk meat

Mineral	Content
Calcium, g/kg	1.37
Phosphorus, g/kg	7.47
Potassium, g/kg	13.50
Sodium, g/kg	1.67
Magnesium, g/kg	0.96
Iron, mg/kg	130.00
Manganese, mg/kg	1.70
Copper, mg/kg	5.40
Zinc, mg/kg	125.00

Table 9 Amino acid composition of musk-ox meat

Amino acid	Content, g/100 g
Non-essential, incl.:	20.33 ± 1.69
Tryptophan	0.54 ± 0.11
Isoleucine	3.26 ± 0.07
Threonine	2.65 ± 0.40
Valine	3.28 ± 0.39
Methionine + cystine	1.98 ± 0.40
Methionine	1.08 ± 0.11
Leucine	4.86 ± 0.63
Phenylalanine	2.34 ± 0.32
Lysine	3.91 ± 0.56
Essential, incl.:	23.90 ± 2.11
Oxyproline	0.05 ± 0.01
Serine	2.59 ± 0.48
Glycine	2.72 ± 0.51
Alanine	3.65 ± 0.70
Glutamine	6.18 ± 0.45
Proline	2.33 ± 0.22
Arginine	2.81 ± 0.06

of non-essential acids. Essential acids accounted for 54%.

Glutamine, alanine, and arginine prevailed among non-essential amino acids.

Tyrosine is a substrate for the synthesis of thyroxine, adrenaline, and noradrenalin. Methionine is a methyl group donor for the formation of many compounds, such as adrenaline, creatine, anserine, choline, and cysteine. In the body, cysteine turns into cysteamine, which mitigates the damaging effects of ionising radiation.

Table 10 Fatty acid composition of musk-ox meat

Acid	Content, g/kg
Saturated, incl.:	33.60 ± 0.54
Myristine	1.57 ± 0.32
Palmitic	18.58 ± 0.50
Stearin	13.45 ± 0.79
Unsaturated, incl.:	55.82 ± 0.62
Palmitooleic	3.67 ± 0.27
Oleic	47.58 ± 0.24
Linoleic	3.83 ± 0.13
Linolenic	0.73 ± 0.09
Total:	89.42 ± 0.80

Table 11 Vitamin content in musk-ox meat

Vitamin	Content
D, mg/kg	1.62 ± 0.09
E, mg/kg	248.33 ± 6.12
B_1, mg/kg	37.38 ± 0.92
B_2, mg/kg	9.37 ± 0.24
B_3, mg/kg	28.38 ± 0.69
B_5, mg/kg	19.35 ± 0.47
B_6, mg/kg	16.61 ± 0.41
*B_{12}, µg/kg	124.57 ± 3.08

Many enzymes owe their biological activity in the body to cysteine present in the molecule of sulfhydryl groups.

We calculated that the ratio of unsaturated to saturated fatty acids in musk-ox meat was about 1.66 : 1.

As seen from Table 10, oleic acid was prevalent in the meat, accounting for 85% of total unsaturated acids.

Table 12 Mineral content in musk-ox meat

Mineral	Concentration
Calcium, g/kg	0.50 ± 0.10
Phosphorus, g/kg	4.40 ± 0.50
Potassium, g/kg	5.40 ± 2.32
Sodium, g/kg	2.78 ± 0.28
Magnesium, g/kg	0.60 ± 0.05
Iron, mg/kg,	96.67 ± 8.82
Manganese, mg/kg	1.07 ± 0.13
Copper, mg/kg	5.60 ± 1.19
Zinc, mg/kg	81.23 ± 9.56

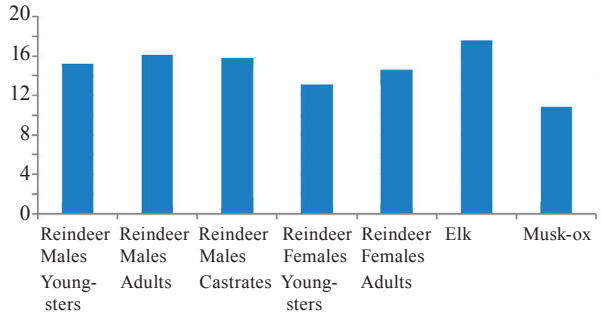

Figure 1 Protein quality index of meats, g/100 g of product

Musk-ox meat had quite a high content of fat-soluble vitamins, especially vitamin E. It was richer in vitamin B_{12} (125 µg/kg) than venison (27–29 µg/kg) or elk meat (30 µg/kg).

The analysis showed that musk-ox meat, in the same way as reindeer meat, had a high content of mineral elements such as phosphorus, potassium, and sodium. Of trace elements, musk-ox meat was rich in iron, which is

Table 13 Formulation of dry smoked and cured venison sausages

Ingredients	Sausages			
	dry smoked			dry cured
	Severnaya	Polyarnaya	Taimyrskaya	Taimyrskaya
	Unsalted raw meat, kg per 100 kg			
First grade trimmed venison	50	75	–	–
Single grade venison	–	–	75	75
Semi-fat trimmed pork	25	–	–	–
Pork breast	25	–	–	–
Back fat	–	25	25	25
Spices and materials, g (per 100 kg of unsalted raw meat)				
Edible salt	3000	3000	3000	3000
Sodium nitrite (solution)	10	10	10	10
Granulated sugar or glucose	100	100	100	100
Ground black or white pepper	150	150	100	100
Ground cardamom or nutmeg	25	–	–	–
Fresh minced garlic	–	150	300	300
Ground coriander	–	–	200	200
Madeira	250	–	–	–
Cognac	–	250	–	–
Chemical composition				
Protein, % (at least)	33	30	29	27
Fat, %	40	37	37	37
Energy value, kcal / 100g	492	453	449	441

known to be part of many protein and enzyme systems, involved in immunobiological processes. In addition, musk-ox meat had a relatively high content of zinc, which stimulates the immune system and protects the body from infections.

One of the criteria for evaluating a product's nutritional value is the protein quality index (PQI) – a ratio of tryptophan to oxyproline. This index determines the ratio of muscle and connective tissue proteins. The PQI for ungulate meats is shown in Fig. 1.

As can be seen in Fig. 1, elk meat had the highest PQI compared to the other samples. It was probably due to nutrition patterns of this animal species.

At the next stage, we used the obtained data on the indigenous meats to develop a sausage technology.

Starter cultures were used in the production of dry smoked and cured sausages. Lactic acid bacteria, which are contained in starter cultures, ferment sugar and form lactic acid. The pH of the product decreases to the required level within 24–48 h, creating optimal conditions for sausage firming, reducing microbiological contamination, and rapid uniform drying.

The quality evaluation of the products included the following indicators: microbiological, sensory (appearance, slice colour, aroma, taste, texture, and overall evaluation), and physicochemical (pH, moisture content). We found that introducing a concentrate of starter cultures into coarsely cut meat during salting improves the sensory, colour, physicochemical, structural and mechanical, microstructural, and microbiological parameters of the products, increasing their biological activity.

The ripening of venison products is based on lactic acid bacteria that gradually become dominant and inhibit the development of undesirable microflora. The introduction of starter cultures doubled or even tripled the level of lactic acid microflora in the minced meat. In addition, using starter cultures eliminates the need for cooking meat at high temperatures, thus maintaining the quality of raw meat protein.

The microstructural analysis showed that starter cultures intensified the process of muscle tissue fermentation and, consequently, the structuring of the product. Thus, bacterial preparations can accelerate and stabilise the structural changes of the minced meat.

The pH value gradually decreased from 5.8 to 5.0–5.1 during the process. The moisture content decreased from 59.79 to 30%. Salt increased by 2.55–2.60%, remaining within the maximum permissible value. Smoked sausages were dried to 32–39% and cured sausages to 25–37%.

The decrease in moisture during the production of dry smoked sausages was accompanied by a significant reduction of the bacterial content in minced meat (from 2690 to 140–150 microbial cells in 1 g of product).

Table 13 gives a few examples of venison product formulations.

CONCLUSION

We studied a number of ungulate meats, namely reindeer, elk, and musk-ox meats. According to the results, all the meats contained a whole complex of biologically active substances, including essential unsaturated fatty acids, amino acids, vitamins, and minerals. In addition, the studied raw meats had a balanced combination of vital micronutrients. Therefore, we concluded that the meat of reindeer, elk, and musk-ox can be used to replenish their deficiency in the diet of people living in the Extreme North.

We also developed the formulation of dry smoked and cured venison sausages with desirable characteristics, including shelf life and sensory attributes.

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Coconut meal: Nutraceutical importance and food industry application

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Abstract: In tropical regions coconut is the tree of great significance: it provides millions of people with food, employment, and business opportunities. The fruit is referred to as 'miracle fruit' due to its inherent rich profile of macro- and micro-nutrients for human nutrition and health. Different products, such as coconut water, milk, raw kernels, oil and desiccated coconut are commercially processed. Coconut flour is a byproduct of coconut milk and oil industry which is made from coconut meal leftover after processing. Being a rich source of dietary fibre and protein, it has found numerous applications in different functional foods. Coconut flour can be successfully incorporated into various food products, such as bakery, extruded products, snacks, and sweets. It has antidiabetic and anticancer effects, prevents cardiovascular diseases, and improves immune function. Coconut flour is also gluten-free and its nutritional composition is quite comparable to that of wheat flour. Gluten-free food products enriched with coconut flour are a healthy and viable option for the people with celiac disease. In this paper, we summarised the present use of coconut flour. There is an apparent need to convert the food processing byproducts into functional ingredients in order to implement their environment-friendly and efficient utilisation.

Statement of Novelty: The review discusses the recent research of coconut meal valorisation with the focus on technologies allowing the promotion of coconut meal and its commercial availability.

Keywords: Coconut flour, dietary fibres, celiac disease, functional food, anticancer, antidiabetic, pasta, bakery

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INTRODUCTION

Coconut (*Cocos nucifera* L.) palm is a monocotyledon belonging to *Arecaceae* or *Palmae* family. Coconut is among the ten most useful trees in the world and is referred to as 'tree of life', 'tree of heaven', 'tree of abundance' due to its multi-utilisation in nutrition, medicine, cosmetics, etc. [1]. Coconut palm can be processed into coconut water, coconut milk, coconut sugar, coconut oil, and coconut meat. Coconut consists of outer fibrous coat, or husk known as exocarp, and inner hard protective endocarp, or shell. A white albuminous part is endosperm, or coconut meat, and inner cavity is filled with clear fluid called coconut water (Fig 1).

There are two main varieties of coconut, i.e. tall and dwarf ones, depending on fruiting time. The tall varieties

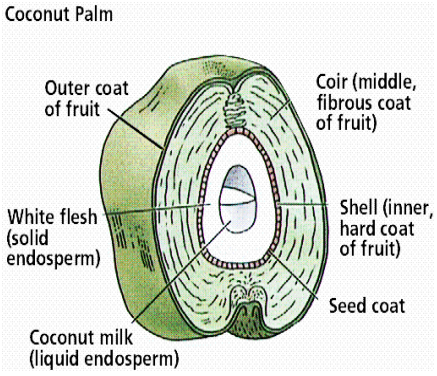

Figure 1 Internal view of Coconut [2]

grow slowly, and their fruiting occurs after 6–10 years of plantation. The dwarf varieties grow faster, and fruiting occurs within 4–5 years of plantation [3]. Indonesia, Philippine and India are the largest coconut producers in the world, with the annual production of 61, 18 and 15.86 million tons, respectively [4]. In India, Kerala's coconut production is 48.79%, followed by Karnataka (18.90%), Tamil Nadu (17.70%), and Andhra Pradesh (5.50%), which together accounts for 91% of the total production in the country [5].

Virgin coconut oil (VCO) is the main product from the coconut industry of great economic importance. VCO is extracted from the fresh, mature coconut kernel mechanically with or without the use of heat. Refining, bleaching or deodorising is avoided in VCO extraction to retain all its natural properties. The white residue, or meal, left after the extraction of VCO is called Virgin Coconut Meal (VCM). It can be milled to obtain flour rich in dietary fibre and considered as a suitable ingredient in the formulation of functional foods. Production of coconut flour has been increased recently due to its functional properties such as prevention of diseases like diabetes, cardiovascular diseases, and colon cancer. Waste generated in the fruit, vegetable and nut industry is full of valuable nutrients and needs to be reused in value added food products thus providing additional economic benefits [6, 7].

RESULTS AND DISCUSSION

Processing of coconut flour. De-oiled cakes of coconut and flour are a good source of dietary fibres. Being a rich source of dietary fibre and protein, it has found numerous applications in different functional foods. Non-starch polysaccharides (NSP) or dietary fibre are protective against gastrointestinal cancer, including esophageal one [8].

There are two methods for VCO extraction: dry and wet ones. In dry processing the fresh mature coconuts are dehusked and deshelled. After the removal of brown coat, the coconuts are grated and dried at 65–70°C in tray or vacuum drier. The dried coconuts are then subjected to cold press expeller to extract coconut oil. The white low fat residue/meal obtained is grinded to make coconut flour. In wet processing, the coconut milk is extracted from the fresh kernel which is then fermented naturally (at 35–40°C for 16–24 h) to obtain VCO from coconut curd by phase separation. The meal is milled into coconut flour [9]. The solvent extraction method is generally avoided because of health hazards and low quality meal [10]. Normally, the meal or the residue obtained after the extraction of coconut oil is used as cattle and poultry feed. Nevertheless, coconut meal has nutraceutical properties, which make it useful for human consumption, and should be incorporated in various food products [11]. The meal contains carbohydrates, protein and dietary fibre, which can also be utilised for food enrichment. In addition, another advantage of the meal is its low cost.

During food production and processing, hygiene and sanitation are prerequisites to avoid any hazards to enter in food supply chain [12]. The main concern in food utilisation is that the unhygienic processing makes copra press cake unsuitable for consumption by humans. However, in order to overcome this problem, coconut meal obtained from virgin coconut oil extraction can be processed into coconut flour [13]. Coconut flour can be marketed to the bakery, snacks, and noodles manufacturers [14]. There is an apparent need to convert the food processing by-products into functional ingredients in order to implement environment-friendly and efficient utilisation of these by-products.

Nutritional composition of coconut flour. Composition of coconut flour mainly depends upon the method employed for the extraction of coconut oil. However, varieties have slight influence on oil content of coconut and, thus, on flour. According to [15], coconut flour is quite superior to wheat flour in terms of protein, fibre, mineral, and lipid profile (Table 1).

Khan *et al.* investigated coconut flour composition made from coconut meal obtained by dry processing method [16]. The reported composition was: moisture, 6.7%; ash, 1.55%; protein, 14.3%; fat, 54.0%; fibre, 20.50%; and carbohydrates, 23.40%. Igbabul *et al.* investigated the composition of coconut flour obtained from fermented coconut slices which were deoiled by dry processing method [17]. Thus, obtained coconut flour possesses moisture of 5.27%; ash, 2.76%; protein, 12.31%; fat, 0.48%; fibre, 11.81%; and carbohydrates, 67.37%. Gunathilake *et al.* investigated the dry processed coconut flour composition as: moisture, 9.76%; ash, 0.51%; protein, 9.90%; fat, 0.87%; fibre, 0.50%; and carbohydrates, 78.46% [18]. Coconut flour produced by dry processing is rich in protein, while flour from wet processing is predominantly rich in fibre content.

Health benefits of coconut flour. Functional food is any food that imparts a positive effect on people's health and provides basic nutrition. Coconut milk residue and virgin coconut oil meal are rich sources of dietary fibre [19]. Dietary fibre is the best ingredient to be used in the development of functional foods, due to its health promoting effects, such as controlling cholesterol and blood sugar levels, increasing the fecal bulk volume, proliferation of gut microflora, decreasing intestinal

Table 1 Comparative proximate composition of wheat flour and coconut flour

Component	Wheat flour, %	Coconut flour, %
Moisture	9.76	4.20
Ash	0.51	5.96
Protein	9.90	21.65
Fibre	0.50	10.45
Fat	0.87	8.42
Carbohydrates	78.46	59.77

Table 2 Health benefits of coconut flour

Component	Health benefit	Biological action	Reference
Fiber	Antidiabetic effect	Reduces postprandial glucose levels	[20]
	Cardiovascular disease prevention	Lowers the serum cholesterol, triglycerides, and LDL cholesterol levels	[23]
	Prebiotic and immune modulator	Oligosaccharides produced by enzymatic hydrolysis of polysaccharides present in coconut meal are a potent prebiotic substance for humans	[33]
	Weight control	Gives bulkiness without supplying fats, resulting in consumption of less food and low calories	[36]
Fat	Cardiovascular diseases prevention	Coconut fats elevate the levels of high density lipoprotein (HDL) cholesterol, a good cholesterol known to lower the risk of coronary heart disease	[25]
Protein	Hypolipidemic effect	Arginine checks hypercholesterolemia by correcting the reduced concentrations of EDRF	[26]
Phenols and flavonoids	Anticancer effect	Coconut flour extracts affect the integrity of the Caco-2 cancer cells due to release of lactate dehydrogenase as found in the cytotoxicity assays	[28]

transit time, trapping carcinogenic agents, etc. Fibre can be supplemented using coconut flour to develop healthy foods low in calories and fats. Coconut flour is a potent functional ingredient used in food products with wide health benefits (Table 2).

Antidiabetic effect. Glycemic index (GI) is a therapeutic principle for diabetes mellitus. GI can be estimated by determining the increase in blood glucose concentration after the intake of a test meal for a specific period of time and compared to equivalent control meal consisting of bread or glucose. Low glycemic index food reduces the postprandial blood glucose levels. The glycemic index of coconut flour supplemented foods decreases with increase in the amount of coconut flour due to its high fibre content. This forms a scientific basis for its utilisation as a functional food for the dietary control and management of diabetes mellitus [20].

Not only the coconut dietary fibre but also the coconut kernel proteins have an antidiabetic effect. Coconut is rich in L-arginine which contributes to glucose homeostasis through nitric oxide synthase pathway leading to pancreatic beta cell regeneration [21]. It regulates blood glucose levels and improves insulin sensitivity in diabetic and non-diabetic individuals [22].

Cardiovascular diseases prevention. An increase in fibre intake is beneficial for heart health as it lowers blood pressure and serum cholesterol levels thus reducing the risk of stroke and hypertension. Coconut fire was shown to lower the serum cholesterol, triglycerides and LDL cholesterol levels thus reducing the risk of coronary heart diseases. After fat digestion, bile acids are reabsorbed in the body and then converted into cholesterol. Coconut fibres prevent the re-absorption of bile acids into enterohepatic circulation and bile acid are excreted through feces along with fibre. This justifies the need for the development of coconut flour food products as a functional food [23].

The short chain fatty acids (butyrate, acetate and propionate) produced by the fermentation of dietary fibres in large intestine inhibits the synthesis of cholesterol in the liver. Propionate is known to inhibit HMG (3-hydroxy-3-methylglutaryl) CoA synthase, a limiting enzyme for the synthesis of cholesterol from acetyl- CoA in the liver. This fermentation metabolite decreases the cholesterol synthesis by the rate of 45% [24].

Coconut fats increase the level of high density lipoprotein (HDL) cholesterol, a good cholesterol lowering the risk of coronary heart disease. Although the polyphenol content in coconut is high, it maintains normal levels of lipids in tissues by trapping the reactive oxygen species in plasma and interstitial fluid of the arterial wall resulting in the inhibition of LDL oxidation, reversal of cholesterol transport and reduction in the intestinal absorption of cholesterol [25].

Coconut proteins also exhibit hypolipidemic effect due to the high content of L-arginine (24.5%), a precursor of nitric oxide [26]. Nitric oxide is an endogenous vasodilator and also prevents the aggregation and adhesion of platelets and was identified as an endothelium derived relaxing factor (EDRF). The consumption of dietary arginine checks hypercholesterolemia by correcting the reduced concentrations of EDRF [27]. Consumption of coconut products, such as desiccated coconut and coconut flour, can therefore help improve the lipid profiles.

Anticancer effect. Colorective cancer is the cancer of digestive tract, which depends on genetic factors (over expression of enzyme cyclo-oxygenase-2), oxidative stress and diet. Antioxidants are free radical scavengers and are considered as the potent chemo-preventive components. Coconut flour contains high content of phenols and flavonoids, which have antioxidant properties (Table 1).

The study reported that coconut flour extracts affect the integrity of the Caco-2 cancer cells of the colon due to release of lactate dehydrogenase as found in the cytotoxicity assays. The extracts also increase the activity of detoxifying enzyme catalase which is responsible for the neutralisation of hydrogen peroxide [28]. Butyrate produced during the intestinal fermentation of fibres has anti-inflammatory and anticarcinogenic properties [29]. It enhances cell

Table 3 Coconut flour food products

Food product	Ingredients used	References
Bread	Refined wheat flour, coconut flour (10–30% substitution with refined wheat flour)	[18]
Plain cake	Wheat flour, coconut flour (10–30% substitution with wheat flour), egg, sugar, powder milk, baking powder and soybean oil	[41]
Cookies	Wheat flour, coconut flour fiber concentrate (10% and 20% substitution with wheat flour), sugar, fat, corn flour, salt, ammonium carbonate, sodium bicarbonate and water	[19]
Extruded ready-to-eat (RTE) snacks	VCM flour (5–20% substitution with rice flour), rice flour, sugar and cardamom powder	[16]
Cold extruded and baked RTE snacks	African breadfruit flour, cashew nut flour, defatted coconut flour (in the ratio of 8:5:2 respectively), sugar, water, salt, flavourings and wheat flour	[46]
Gluten free cookies	Rice flour, coconut flour (30% and 50% substitution with rice flour), maple syrup, butter, egg powder, ammonium bicarbonate and sodium bicarbonate	[44]
RTE Breakfast cereals	Maize flour, African yam bean seeds flour, coconut flour (10–50% incorporation in maize and African yam bean flour blends), sorghum malt extract, sugar, salt and water	[50]
Noodles	Wheat flour, coconut flour (10–30% level incorporation in wheat flour), water and salt	[15]
Biscuits	Refined wheat flour, virgin coconut meal (5–25% replacement with refined wheat flour), hydrogenated fat, sugar, baking powder and water.	[43]
Breakfast cereal	Composite flour (rice flour and soybean flour in the ratio of 50:50), coconut flour (5–20% replacement with composite flour)	[51]
Gluten free cookies	Rice cream, coconut flour, potato starch, sugar, butter, eggs, xanthum gum, salt, sodium carbonate, lemon juice, honey essence and chocolate chips	[48]
Sweet meat (ladoo)	VCM, desiccated coconut powder, whole wheat flour, sugar, cashew nut and hydrogenated fat	[53]

differentiation which prevents tumor formation in colon. Excess bile acids induces oxidative stress by the production of reactive oxygen species which causes repeated DNA damage leading to mutations and carcinogenesis in the gastrointestinal tract [30]. The secondary bile acids contribute to the risk of colorectal cancer. Dietary fibre binds with the bile acids and facilitates their effective fecal excretion [31].

Bioactive peptides also possess antioxidant properties and other health benefits. The two peptides from the coconut cake protein isolates, Pro-Gln-Phe-Tyr-Trp and Arg-Pro-Glu-Ile-Val, are the peptides exhibiting free radical scavenging activities with 4.28 and 7.65 µg/mL IC50 value respectively (IC50 is the concentration of the peptide needed to scavenge 50 percent of the radical activity) [32].

Prebiotic and immune modulator. Dietary fibres act as prebiotics and are essential for intestinal microflora. Oligosaccharides produced by enzymatic hydrolysis of polysaccharides present in coconut meal are a potent prebiotic substance for humans [33]. Prebiotics are the non-digestible oligosaccharides favouring the growth of *Bifidobacteria*, *Lactobacilli* and other microorganisms responsible for the intestinal immune responses [34]. These bacteria carry out the fermentation of the prebiotic oligosaccharides and produce short chain fatty acids. *ß*-1,4-Mannobiose is a disaccharide obtained from coconut flour which exhibits immune modulating and prebiotic effect. It increases the production of antibody IgA which prevents the growth of pathogenic bacteria by increasing the phagocytic activity. The branched structure of *ß*-1,4-Mannobiose shows similarity with the bacterial cell wall components and thus acts as a toll-like receptor agonist. Toll-like receptors activate the signaling pathways that are responsible for the host defense mechanisms [35].

Weight control. Dietary fibre is an important tool for obesity control by the nutrition and diet professionals. Consumption of dietary fibre is known to show significant changes in the gut hormones, glycemic index, gastric emptying, and satiation indices. As coconut consists of fibre, it absorbs water and provides a feeling of fullness. It gives bulkiness without supplying fats, resulting in consumption of less food and low calories and thus regulating weight. Hypoenergetic diet, when supplemented with coconut flour, lowers glucose and cholesterol levels thus managing obesity [36].

Gluten free flour. Celiac disease is genetically inherited chronic disease characterised by an immune response on the consumption of gluten. This auto immune response causes villi damage of the small intestine and thus hampers the absorption of nutrients into the body leading to gastrointestinal distress and malnutrition [37]. At present, the only treatment for gluten intolerance is strict avoidance of gluten containing foods made up of wheat, barley, and rye. The main constraint in gluten free diets is that these are quite poor in dietary fibres and carbohydrates.

On the contrary, coconut flour contains abundant amount of fibres (Table 1). Gluten free foods can be enriched with natural non-cereal gluten free fibre sources such as vegetables, fruits, nuts, legumes, or seed. Coconut flour is marketed as a gluten free product for flour replacement and as an additive to cereal products (Table 3). Thus, gluten free food products enriched with coconut flour are a healthy and viable option for the people with celiac disease.

Antinutrients. Anti-nutrients are the substances which either inhibit the absorption of nutrients or hinder in their further metabolic pathways. Major substrate cereals, legumes and millets have certain antinutrients, such as phytic acid, lathyrogens, saponins, lectins, haemagglutinins, alpha amylase inhibitors, etc. They lower the bioavailability of minerals and inhibit the protein digestion. Coconut flour can be considered as a good substitute for gluten free products and other processed due to the absence of an antinutritional factor [38].

Coconut flour. Coconut flour is successfully incorporated into bakery, extruded products and traditional sweets.

Bakery products. Cereal based cookies, bread, crackers (an integral part of human diet) accounts mainly for energy source in human nutrition therefore they are good vehicles for supplementation of nutrients [39]. Gunathilake *et al.* used coconut flour in different proportions (10, 20, and 30%) for refined wheat flour bread to enhance proteins, amino acid profile and dietary fibres [18]. Cereal proteins are not a valuable source of lysine [40]. The mixing behavior of the wheat flour and coconut flour blends was analysed. It was found that water absorption decreased while dough development time, arrival time, and stability increased with 20% substitution. The study concluded that acceptable quality of bread can be made by 20% substitution of the wheat flour with coconut flour.

Hossain *et al.* made nutritionally rich cake by incorporating coconut flour in wheat flour [41]. Substitution of wheat flour with 20% coconut flour for the preparation of plain cake resulted in increase in moisture by 0.75%, protein by 0.58%, fat by13.84%, ash by 0.91%, crude fibre by 0.9%, while the carbohydrate content decreased by 16.16%. The moisture content increased due to the presence of water holding matrix i.e. dietary fibres (cellulose, hemicelluloses, lignin, etc.), which resulted in an enhancement of cake volume.

Yalegama and Chavan made fibre supplemented cookies with coconut flour fibre extracts [13]. Fats, proteins and sugars were removed from coconut flour and the purified fiber was incorporated into cookies. 10% coconut fibre substituted cookies showed good overall acceptability. The coconut flour was found to contain 13.4 g/100g fat, 9.3 g/100g crude fibre, 3.7 g/100g moisture, and 18.8 g/100g total sugars. Cell wall polysaccharide concentrate was prepared from the coconut flour with free flowing properties and was used in the preparation of cookies. The cookies showed a slight increase in moisture content by 1% and ash content by 0.19%. The protein content decreased by 0.1% and carbohydrate increased by 3.2%. The protein content decreased and carbohydrate increased due to addition of extra sugar and fat in the cookies. The sensory properties decreased with increasing level of substitution with coconut fibre. High fibre cookies can be made by supplementation with coconut fibre.

Sivakami and Sarojini formulated biscuits using defatted coconut flour, rice flakes, and defatted soya flour [42]. The biscuits contained lower amount of fibre (2 g) which might be due to highly processed flours used for the preparation of biscuits. The carbohydrate content of biscuits was found to be in the range of 54–57 g. The biscuits provided 464 kcal of energy per 100 g.

Srivastava *et al.* prepared virgin coconut meal (VCM) incorporated refined wheat flour biscuits [43]. Sample investigations showed that incorporation of 15% VCM was most suitable for the acceptable sensory qualities of biscuits. Fibre and protein content was comparatively high as compared to the 100% wheat flour biscuits. Addition of VCM increased the hardness of the dough and decreased the adhesiveness. Addition of VCM also resulted in an increase of moisture by 1.14% (due to high water absorption capacity), fat by 14.55%, protein by 1.41%, ash content by 0.47%, and fibre by 3.40%. The 5–25% VCM resulted in decrease in palmitic and oleic acids, while lauric, myristic, caprylic and capric acids increased. Potassium content increased by 382.82 mg/100 g, calcium by 14.8 mg/100 g, iron by 4.94 mg/100 g, sodium by 32.98 mg/100 g, and zinc by 0.51 mg/100 g. The hardness of dough increased by 2.41 N on 10% replacement with VCM. Coconut meal flour is used owing to its high energy density, high food grade proteins, functional properties and other health benefits. The study found that de-oiled coconut meal flour biscuits were rich in all nutrients and can be used for the production of supplementary protein foods.

Paucean *et al.* developed gluten-free cookies from rice and coconut flour blends with pleasant flavour and taste [44]. The nutritional properties of coconut flour make it suitable for the value addition of the baked foods. 50% substitution of coconut flour with rice flour was most acceptable in sensory qualities and showed increase in moisture by 6.65%, total fat by 2.15%, proteins by 2.55% and ash by 0.38%.

Snacks. Modern lifestyle improved living standard, and changing eating habits have opened a huge market of snacks [45]. Khan *et al.* made virgin coconut meal (VCM) incorporated rice based extruded ready-to-eat snacks [16]. The extrusion temperature was maintained from 40 to 185°C for 9 zones with screw speed of 150 rpm. Incorporation of VCM in the amount of 10% in rice snacks was most acceptable. It showed a decrease in expansion ratio by 0.233, mass flow rate by 16 g/min while increase in bulk density by 0.36 g/ml, water holding capacity by 1.83 g/g, fibre content by 0.21%, and protein by 0.77%.

Okafor and Ugwu made cold extruded and baked RTE snacks from blends of breadfruit (*Treculia Africana* L.), cashewnut (*Anacardium occidentale* L.) and coconut meal [46]. Coconut flour addition showed an increase in fibre by 0.47%, fat by 1.16%, bulk density by 0.06%, pro vitamin by 2.6 mg/100g, vitamin B_2 by 0.046mg/100g, vitamin B_3 by 0.116 mg/100g while decrease in moisture

by 1.75%, protein by 1.32%, carbohydrates by 3.87%, porosity by 0.04% and lateral expansion by 2.33%. The fibre content of the extruded snacks increased with the addition of defatted coconut flour. As was proved, fibre rich foods control the blood glucose levels in diabetic people; therefore the snacks with coconut flour as one of the ingredients have potential application as diabetic food.

Mihiranie *et al.* made snack crackers with coconut flour to improve the dietary fiber, protein and mineral content [47]. Addition of coconut flour decreases the thickness and puffiness of the crackers. 20% of the defatted coconut flour can be successfully added to the wheat flour without compromising the sensory properties. It was concluded that defatted coconut flour is a potent ingredient for the formulation of healthy and low cost snack crackers.

Queiroz *et al.* prepared potato starch gluten free cookies with 10% incorporation of coconut flour [48]. This amount of coconut flour resulted in an increase in moisture by 10.5%, ash by 0.12%, protein by 1.08%, lipid by 3.46%, as well as in a decrease in carbohydrates by 15.19% and energy value by 25.1 kcal/g. The enrichment of the cookies with coconut flour improved its nutritional quality and provided superior physical and physicochemical properties to the product. The lipid and protein content was increased but the carbohydrate content was decreased.

Breakfast cereal. Ready-to-eat breakfast cereals are gaining popularity due to convenience and improved nutritional value [49]. Okafor and Usman prepared RTE breakfast cereals from blends of maize, African yam bean, defatted coconut cake and sorghum malt extract [50]. It was concluded that addition of defatted coconut flour increased the pH and water absorbing capacity of the formulation but decreased the bulk density, oil absorbing capacity and viscosity of the breakfast cereal. Increased levels of coconut flour addition showed more protein digestion. 50% substitution of coconut flour resulted in an increase in water absorbing capacity by 8.07%, in vitro protein digestibility by 15.9% and in a decrease in oil absorption capacity by 0.45%, foam capacity by 1.01%, viscosity by 11.35%, and emulsification capacity by 1.64%.

Ojali *et al.* made breakfast cereals by blending rice, soybean, and defatted coconut flour blends [51]. It was found that the protein and crude fibre content increased with the addition of soybean and defatted coconut flour, respectively, while the carbohydrate content decreased. Thus, the nutritional quality of the breakfast cereals can be enhanced by addition of soybean and defatted coconut flour. 20% coconut flour composition resulted in an increase in ash by 1%, crude fibre by 0.54%, carbohydrate by 1.76%, crude fat by 0.7% and a decrease in crude protein by 6.72%, tannin content by 0.27%, and oxalate content by 1%.

Pasta. Pasta is the primary convenience food available in different shapes and size like spaghetti, noodles, vermicelli, etc., which are consumed worldwide [52]. The nutritional and functional characteristics of noodles can be increased by their fortification with proteins and fibres. Noodle fortification can be an effective public health intervention. Gunathilake and Abeyrathne developed coconut flour incorporated wheat flour noodles to supplement wheat flour noodles with fibre and protein [15]. Wheat flour noodles were used as a medium for the incorporation of fibre and protein because in Asia 40% of the wheat flour is used for making noodles. The noodles made by addition of 20% coconut flour were not much different from 100% wheat flour noodles in sensory properties. 20% substitution showed more stable dough due to stabilisation of gluten by coconut proteins and thus better textural properties.

Sweets. Coconut burfi enjoys great popularity, especially in Southern India. It is generally made from desiccated coconut. Coconut ladoo is also a popular Indian sweet rich in fibre and protein. It generally contains sugar, wheat flour, hydrogenated fat, dry fruits and flavouring substances in desiccated coconut powder [11]. Srivastava *et al.* made Indian traditional Sweet meat (ladoo) from VCM and analysed the shelf life of the product under different conditions at the ambient temperatures of 15–35°C [11]. The main ingredients used in the formulation were VCM, sugar, and water.

The study found that the samples with potassium sorbate, an antimycotic agent, showed a shelf life of 4 months as compared to the samples without it, whose shelf life was only three weeks due to mold growth and fermenting odour. The loss of moisture from the samples and sorbic acid degradation rates were higher in polypropylene packaging as compared to the metalised polyester packaging.

Normally, coconut ladoo is made from desiccated coconut and sold by the small scale confectioners without proper protective packaging material. The study found that during storage the samples packaged in polypropylene turned hard and brittle because of the moisture loss, while the samples packaged in metalised polyester remained soft longer.

Awasthi made gluten-free coconut flour ladoo and coconut flour burfi [53]. Coconut flour is a useful product for gluten-allergic people because it is gluten-free. It can be used as a wheat flour substitute for preparing various dishes. Fat and protein contents in ladoo were 33.8% and 2.1%, respectively, while those in burfi were 46.6% and 2.91%. Table 3 shows coconut flour incorporated to food products.

CONCLUSION

Coconut meal obtained from extraction of virgin coconut oil can be used in the form of coconut flour as it is nutritious and a good source of proteins, minerals and dietary fibre. Coconut flour made from coconut meal promotes health and prevents diseases such as diabetes, obesity, colon cancer, and cardiovascular diseases. The flour can be used in the preparation of gluten-free products for individuals with celiac disease.

Utilisation of coconut meal in the form of coconut flour aims at incorporation of dietary fibres and proteins

into the gluten free food. In India, the meal obtained after the extraction of coconut oil is usually discarded or used as animal feed. If hygienically processed, the meal can be used to make flour, which can be utilised for making a variety of food products. Agro-industrial waste can be used in an efficient way for human consumption.

Extruded products such as pasta, noodles, and ready-to-eat snacks can also be made using coconut flour. Coconut flour is an underutilised product of coconut industry and its present use is very limited. There is immense need of commercial processing techniques to enhance utilisation of coconut flour from coconut meal. Coconut flour extruded products will be convenience products with nutritional and health benefits. Coconut flour is a high protein, fibre-rich and gluten-free functional food product.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Functional dairy products enriched with plant ingredients

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Abstract: Milk and dairy products are staple foods in the diet of all social groups. Plant additives are of multifunctional use in the dairy industry. Wild plants are a source of vitamins, minerals, and other biologically active substances. Due to these compounds, they improve digestion, cardiovascular activity, and emotional state. This review describes the latest trends in creating functional milk drinks enriched with plant components. They include drinks based on whole milk and cream, dairy by-products (whey, buttermilk), as well as fermented milk drinks with probiotic cultures (kefir, drinking yogurt). We found that aqueous extracts were most commonly introduced into milk raw materials. Fruits and berries were dried and added to milk raw materials in the powder form. Special attention was paid to 'hairy roots' as a promising technology for producing various functional foods. In addition to being economically viable, this technology can help us expand the range of plant materials with endangered species. Functional milk-based drinks enriched with plant extracts can improve the immune system and be used as part of supportive therapy. They are also suitable for daily use to replenish the balance of essential nutrients. These properties make their production a promising direction in the dairy industry.

Keywords: Milk drinks, plant extracts, functional ingredients, biologically active substances

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INTRODUCTION

Milk and dairy products are the most common foods in the diet of all categories of the population. The reasons for their popularity lie in the unique properties and components of milk, as well as a possibility of producing a wide variety of foods from this material. Milk is used as a basis for combined foods produced in two ways: 1) by adding plant materials to milk and dairy products and 2) by adding dairy ingredients to plant materials [1].

Combining plant and milk proteins provides a better amino acid composition compared to milk proteins. Milk protein is one of the most valuable proteins of animal origin since, unlike meat proteins, it does not contain purine bases, whose excess has a negative effect on kidney function. Its biological value is close to the value of a standard chicken egg protein. Milk protein has an optimal ratio of amino acids, which is close to the amino acid composition of human proteins. Dissolved milk proteins are readily available for digestive proteinases without prior denaturing. Milk proteins have higher digestibility (95–97%) than the proteins of meat, fish, and cereals. In addition, they are rich in essential amino acids which are often lacking in the human diet, namely lysine, tryptophane, methionine, etc. [1, 2].

The choice of dairy ingredients for functional foods can be justified by their medicinal properties widely utilised in therapeutic, preventative, and dietetic nutrition. It seems difficult to clearly distinguish between ordinary and medicinal dairy products, since even conventional dairy products can be used for dietetic and medicinal purposes due to their chemical composition. In addition, preference is usually given to fermented milk products due to their dietetic and medicinal properties. These properties result from microbiological and biochemical processes that occur during the ripening of milk curd.

The enzymatic systems of lactic acid bacteria break down milk proteins during fermentation into simpler and more easily digestible substances. Organic acids in fermented milk products affect the secretory activity of the stomach and intestines. Helping the glands of the digestive tract to secrete enzymes, they speed up digestion and improve the absorption of food. In addition, beneficial properties of fermented milk products lie in their ability to inhibit the growth of pathogenic intestinal microflora. This is especially important in view of a high incidence of intestinal dysbiosis even among healthy people [3].

Milk ingredients are often used in the production of functional dairy products. They are isolated from conventional dairy products, such as milk, cheese, whey or butter. Thanks to special treatment, they acquire desired properties, for example, texture, taste or water content. They include whey powder, lactose, protein concentrate, milk fat, protein isolate, casein, and albumin. These ingredients are used to create special products, for example, for diabetics, athletes, and children [1, 3].

The world's largest processing companies, such as Fonterra, Lactalis, Friesland Campina, Dairy Farmers of America, and Arla Foods are big investors in the production of milk ingredients. In Russia, it is still a new market. According to Streda Consulting, Russia annually imports about 110 000 tons of such products worth $200 million. These are whey, protein concentrates (used in dairy and confectionery production), and dehydrated milk fat. Belarussian products account for up to 55% of all imported ingredients and up to 25% of their cost.

Russia has a large source of whey which can be used to produce dry whey powder, whey protein concentrate, isolate, and hydrolysate. It is due to the growing production of cheese, where whey is the main by-product. In 2017, Russia produced over 603000 tons of cheese, an 8.5% growth compared to 2016. A significant amount of whey powder produced in Russia is used to meet the needs of the dairy industry. In 2017, its output reached 129 000 tons, a 15% increase since 2010 [1, 2].

Plant additives are quite widely used in the dairy industry for various functional purposes. In recent years, we have seen a clear trend towards combining plant materials with various milk additives [1]. Highly promising is the use of wild plants, edible and medicinal. Wild plants are a raw material for nutraceuticals, one of the main groups of dietary supplements. They are a source of vitamins, minerals, and other biologically active substances. Thanks to these compounds, wild plants improve digestion, cardiovascular activity, and emotional state [1, 4].

Functional properties of dairy products are normally improved by correcting their composition of fatty acids, amino acids, and minerals, as well as fortifying them with micronutrients [2, 3]. Combining milk materials with plant components allows regulating the content of vitamins, carbohydrates, minerals, and dietary fibre in the products. In addition, they give dairy products a pronounced plant taste and smell, as well as an attractive appearance. Using biologically active compounds obtained from plant materials, including medicinal plants, is a promising direction in the production of medicinal, preventative, and functional products [1, 2].

This review is devoted to the latest trends in creating functional milk drinks enriched with plant components. It describes the principles of producing various types of functional drinks, namely drinks based on whole milk and cream, drinks based on dairy by-products (whey, buttermilk), and fermented milk drinks with probiotic cultures (kefir, drinking yogurt).

STUDY OBJECTS AND METHODS

Our objects of study were scientific publications and patents of Russian and foreign authors on the production of milk drinks enriched with plant materials. Our main method was generalisation. In particular, we analysed statistical and economic data on the world-wide production of functional milk drinks, the scientific principles of using plant ingredients in milk drinks, and findings of practically-oriented studies and original research on new types of functional plants.

RESULTS AND DISCUSSION

Modern formulations and technological regulations provide for the use of various forms of medicinal plants. Quite popular are syrups and extracts from wild medicinal herbs with various preventative properties (antimicrobial, immunostimulating, antitoxic, radioprotective, and others). A study of their chemical properties showed that most plants have a unique set of substances, such as vitamins, dietary fibre, antioxidants, minerals, and organic acids [5].

Functional plants used in phytocompositions can be classified according to their pharmacological action. For example, a group of plants used in Russia to strengthen blood vessels include *Tilia Cordata*, *Comarum*, and *Aegopodium podagraria*. Plants that stimulate the cardiovascular system and prevent it from weakening include *Adonis vernalis*, *Betula pendula*, *Crataegus*, *Hypericum*, *Fragaria*, *Calendula officinalis*, *Viburnum opulus*, *Convallaria majalis*, *Melissa officinalis*, *Hippophae*, *Parmelia*, *Leonurus*, *Matricaria Chamomilla*, *Sorbus sibirica*, *Aronia melanocarpa*, and *Gnaphalium* [4, 5].

Medicinal plants with psychotropic properties fall into four groups, namely:

– sedatives: *Valeriana officinalis*, *Leonurus*, *Crataegus*, *Mentha*, *Humulus lupulus*, *Chamaenerion angustifolium*, *Polemonium caeruleum*, *Calluna vulgaris*, *Origanum vulgare*, *Cichorium*, *Melilotus officialis*, *Levisticum officinale Koch*., *Gnaphalium uliginosum*, *Thymus*, and *Bidens tripartita*;

– plants with a combined calming and tonic effect (intermediate group): *Paeonia anomala*, *Rhaponticum carthamoides*, *Acorus calamus*, *Rubus idaeus*,

Taraxacum officinale, *Pastinaca*, and *Origanum majorana*;
– stimulants: *Aralia elata*, *Oplopanax elatus*, *Rhodiola rosea*, and *Echinops*; and
– antidepressants: *Hypericum*, *Rhaponticum carthamoides*, *Aralia elata*, and *Oplopanax elatus*.

Plants with phytoncidal properties include *Calendula* officin*alis*, *Tilia*, *Arctostaphylos uva-ursi*, *Paeonia anomala*, *Matricaria Chamomilla*, *Prunus padus*, and *Rosa*.

Plants with the richest vitamin content include *Hippophae*, *Sorbus sibirica*, *Aronia melanocarpa*, *Salvia officinalis*, and *Rosa* [5].

The consumption of juices and other drinks is on the rise both in Russia and all over the world. There is a growing interest in drinks that not only quench thirst, but also have a positive effect on various systems of the body and human health in general. Depending on the intended action, functional drinks can act as general tonics, boost energy, stimulate mental activity, help to relax, prevent cholesterol metabolism disorders, etc. [1]. The concept of 'drinks for health' has become fundamental for many European manufacturers and is an effective brand that allows them to successfully compete in the market. According to market research, consumers prefer functional drinks made from natural and environmentally friendly materials and ingredients [1, 2].

Milk drinks can also be divided into the following groups: 1) drinks based on whole milk and cream; 2) drinks based on dairy by-products (whey, buttermilk); and 3) fermented milk drinks with probiotic cultures (kefir, drinking yoghurts).

A large number of studies on whole milk drinks have aimed not only to enrich the product with functional substances, but also to extend the shelf-life of the finished product. Some plants contain various compounds that can affect microbial growth, reproduction, or basic cell functions. These include phenols, polyphenols, trace elements, essential oils, and other compounds. They are mainly present in various herbs. Extracts of these plants can be used as natural food preservatives that can inhibit the growth of unwanted microorganisms. Their antimicrobial activity is determined by a high content of phenolic compounds – substances containing aromatic rings with a hydroxyl group and their functional derivatives. These include tannins, flavonoids, glycosides, phenol carboxylic acids, phenol alcohols, anthocyanins, bitter substances, and simple phenols [6, 7].

The disk diffusion method was used to establish the antimicrobial activity of aqueous extracts obtained from the following plants: *Thymus vulgaris*, *Lavandula angustifolia*, *Melissa officinalis*, *Ocimum basilicum*, *Allium schoenoprasum*, and *Petroselinum crispu*. Their antibacterial activity was tested on strains of microorganisms that cause spoilage of milk. The highest antibacterial activity was found in the aqueous extracts of *Ocimum basilicum*, *Allium schoenoprasum*, and *Petroselinum crispu* [6].

Mohamed *et al.* tested the antibacterial properties of aqueous extracts of oregano, marjoram, sage, and liquorice against *B. subtilis* and *E. coli* pathogenic microorganisms [7]. These plants are widely used in the production of functional milk drinks. The study showed that these extracts had a higher antibacterial activity against *B. subtilis* rather than *E. coli.* In addition, oregano extract exhibited the highest antibacterial activity against the studied bacteria compared to marjoram, liquorice, and sage. Also, the mass spectrometric analysis revealed some new volatile compounds in these extracts which could potentially become new antibacterial drugs to be used in the food industry.

Apart from the antibacterial effect, plant additives are able to prevent spoilage of dairy products. They do it by directly absorbing photons of light and act as internal filters that protect sensitive food components by removing radicals and preventing photodegradation and oxidation. Such properties are common for flavonoids, in particular quercetin [8].

Russian manufacturers of dairy products use dihydroquercetin, a natural antioxidant obtained from Siberian and Dahurian larch. Dihydroquercetin is included in the list of food additives as an antioxidant (State Sanitary Standard 2.3.2.1078-01*). Another functional ingredient is larch arabinogalactan – dietary fibre enriched with various contents of dihydroquercetin (5–20%). The use of dihydroquercetin in the dairy industry has scientific and practical significance. In particular, it inhibits the process of lipid oxidation, enriches the products with a natural biologically active water-soluble substance, and increases their shelf-life. Therefore, this group of natural ingredients is used in the production of functional dairy products [9].

The antioxidant properties of plant extracts not only protect the product from spoilage, but also prevent the action of free radicals in the human body, slowing down the aging process. Milk has its own antioxidant system represented by enzymes (catalase, peroxidase, peroxide dismutase, etc.) and non-enzymatic components (vitamins A, E, C, SH-compounds, metal ions Zn^{2+}, Se^{2+}, Cu^{2+}, Mn^{2+}). In addition, milk contains synergists – substances that restore antioxidants, such as citric, tartaric, and lactic acids. However, the amount of these antioxidants is not stable, depending on various factors, and their activity decreases during milk processing. Lazareva *et al.* studied various plant extracts in combination with sterilised and pasteurised milk. They found that the greatest antioxidant effect on lipid peroxidation was exhibited by sterilised milk enriched with extracts of lingonberry leaves and green tea [2].

* SanPiN 2.3.2.1078-01. Gigienicheskie trebovaniya bezopasnosti i pishchevoy tsennosti pishchevykh produktov [State Sanitary Standard 2.3.2.1078-01. Hygienic requirements for food safety and nutritional value]. Moscow: Federal Center for Sanitary Inspection of the Ministry of Health of Russia; 2019. 145 p.

Researchers in [3] recommend honeysuckle powder as an antioxidant component for milk-based drinks, due to a high content of vitamin C [3]. Honeysuckle berries are also rich in vitamin P, iodine, and biologically active substances with health beneficial and diuretic effects. *Aronia melanocarpa* is another promising raw material for various dairy products. Its fresh fruits are used as a source of vitamins for treating hypertension of stages I and II, and as an adjuvant for treating rheumatism, measles, typhus, scarlet fever, allergic reactions, etc. Its juice strengthens the walls of blood vessels [10]. Optimal conditions were developed for enriching milk with *Aronia melanocarpa*: its puree and oligofructose powder were added to milk heated to 75°C, mixed, and kept for 15 min [11].

Thyme extract is used in milk drinks due to a large content of anthocyanins and flavonoids, in addition to the above compounds. Other sources of vitamins, macro- and trace elements, essential amino acids, and dietary fibre include peanuts, walnuts, rose hips, peppermint, and thyme, as well as beetroot, carrots, and oats [12].

A high antioxidant index was also found in pomegranate, oranges, lemons, apples, pomelo, tangerines, and persimmons, which makes them good additives for milk drinks.

Whey is a widely used raw material in the dairy industry. The main types of whey products include whey powder and permeate (59%), demineralised and delactosed whey powder (10%), whey protein concentrates (12%), and lactose (19%) [20]. The composition and properties of whey are determined by the type of the basic product and its technology. Whey contains about 20% of milk proteins. In addition, whey proteins are richer in essential amino acids than milk, and their content is more balanced in terms of nutrition physiology.

The biological value of whey protein is higher than that of chicken egg protein, a gold standard among food products. According to the FAO/WHO scale, the biological value of whey proteins is 112%, whereas that of milk casein is only 78%. Whey proteins are some of the most valuable components of milk. They are rich in sulphur-containing amino acids (cystine, lysine, and tryptophan). Thus, introducing whey proteins in food products, especially of plant origin, contributes to a significant increase in their biological value due to a highly balanced composition of amino acids [4].

Of great interest is a possibility of expanding the range of whey-based drinks and regulating their biological value. Fortifying them with plant extracts rich in biologically active substances with antioxidant properties can help prevent a number of pathological conditions – stress, atherosclerosis, myocardial infarction, malignant neoplasms, and others. In addition, plant extracts increase their shelf-life [4–6].

All components of whey can be fully utilised in the production of drinks [4]. Whey drinks were fortified with black and green tea containing flavonoids – antioxidants that protect the body from premature aging and cardiovascular diseases [5]. Tea normalises blood pressure, dilates blood vessels, and improves the work of the heart. The antihypertensive (lowering pressure) effect of tea is associated with a high content of polyphenols. It was found that tea lowers the level of bad cholesterol in the blood serum, reduces the intensity of sclerotic processes in the arteries, and prevents the accumulation of fats in the blood and the liver.

Tea alkaloids that remain stable during processing include caffeine, theobromine, theophylline, adenine, xanthite, hypoxanthine, guanine, etc. The caffeine content in tea varies from 2 to 4% of dry mass. The studies confirmed the possibility of creating tonic drinks based on aromatic medicinal plants and whey. In addition to black and green tea, mate tea can be used for these purposes. Lorena *et al*. developed formulations for milk drinks with green mate extracts (*Ilex paraguariensis*), cloves (*Syzygium aromaticum*), and lemongrass (*Cymbopogon citratus*) [13].

Keldibekova *et al*. formulated a functional product based on whey and rosehip [14]. Rosehips contain up to 5.5% ascorbic acid (vitamin C), 12–18 mg% carotene (provitamin A), 0.03 mg% vitamin B_2, vitamin K, flavonoids, about 18% sugar, 4% pectin, up to 4.5% tannins, about 2% citric acid, as well as malic and other acids. Rosehip gives the drink a sedative, anti-sclerotic, and tonic effect. The sensory evaluation of the new whey-based drink and its acidity analysis showed that the most optimal amount of rosehip infusion was 15% of whey weight. The physical and chemical parameters of the whey drink meet the requirements of Federal Law No. 88**. In addition, rosehip is an excellent diuretic and choleretic agent. It can also have a sedative, anti-sclerotic, and tonic effect.

Another group of researchers developed drinks based on milk materials combined with apple pectin, rosehip blooms, lemongrass leaves, and barberry fruits [15]. These materials provided the drinks with immunomodulating, antihypertensive, anti-inflammatory, and antiseptic properties.

The current search for new strong natural antioxidants has evoked interest in xanthones, natural polyphenolic compounds. High concentrations of xanthones are present in the pericarp of mangosteen (*Garcinia mangostana* L.), an exotic fruit common in Southeast Asian countries such as Thailand, India, Sri Lanka, Myanmar, Cambodia, Vietnam, China, and others. Xanthones have a wide range of physiological effects: cardiotonic, diuretic, choleretic, psychotropic, antitumor, antifungal, etc. Multicomponent functional drinks based on whey are food systems with low aggregative stability, i.e. they are prone to sedimentation during storage. Therefore, various stabilisers (pectins, gums, seaweed products, etc.) are introduced into their formulations to ensure a uniform structure.

** Federal′nyy zakon № 88. Tekhnicheskiy reglament na moloko i molochnuyu produktsiyu [Federal Law No. 88. Technical Regulations for Milk and Dairy Products]. Moscow, 2008.

Cherevach *et al.* developed jellylike whey-based drinks enriched with mangosteen pericarp and extracts of Far Eastern plants, such as *Rosa cinnamomea*, *Aronia melanocarpa*, *Actinidia kolomikta*, *Vitis amurensis*, and *Oxycoccus quadripetalus Gilib* [16]. Their production process was made up of the following basic stages:

– preparation of milk curd whey (clarification at 90°C for 20 min, filtering, and cooling to 25°C);

– preparation of compositions of extracts from Far Eastern plants and mangosteen by dissolving gellan gum in a small amount of whey at 80°C and thorough stirring;

– preparation of plant components in the form of fruit and vegetable purees: primary treatment, cutting, cooking at 85–90°C for 20 min and rubbing through a sieve with 0.5 mm holes (for berry purees – only rubbing), pasteurisation at 70–75°C for 5 min, cooling to 25°C, mixing the ingredients by stirring;

– pasteurisation at 60–65°C for 5 min; hot filling, corking, marking, and cooling to 23–27°C followed by storage at 4 ± 2°C and relative air humidity 70 ± 2%.

The developed drinks had a significantly higher concentration of antioxidant substances compared to analogous products and met the requirements of State Standard R 52349-2005***. The drink with a rosehip extract had the highest concentration of flavonoids. One portion of this drink contains twice as many flavonoids as are recommended for daily intake. The drinks with aronia, cranberries, and grapes were also rich in flavonoids (16.5–89.6% of the daily norm). All the drinks provided 18.6–22.5% of the daily need for xanthones. These drinks should be consumed systematically in order to improve health and reduce the risk of cardiovascular diseases and common cold.

Another study aimed to formulate functional drinks based on dairy by-products and raw materials of plant origin, namely scorzonera and water caltrop [17]. All parts of water caltrop contain flavonoids, tannins, a variety of vitamins, phenolic compounds, as well as mineral salts and beneficial nitrogen compounds. The fruits contain 7.5% fat, 15% protein, and carbohydrates, including 3% sugar and 52% starch. Due to its antiviral, antimicrobial, and immunomodulatory properties, water caltrop can be used in the combined therapy for PTSD. The plant is also known to exhibit astringent, antispasmodic, sedative, choleretic, tonic, and diaphoretic properties. Scorzonera produces beneficial effects due to a variety of biologically active substances. Its roots contain saccharides (20%); pectin substances (2%); vitamins C, B_1, B_2, E, and PP; and salts of copper, potassium, iron, manganese, phosphorus, zinc, and calcium. However, its major medicinal properties are determined by a high content (about 10%) of inulin, as well as asparagine and levulin, making it suitable for diabetics. Asparagine has a positive effect on the work of the heart and activates the kidneys.

*** State Standard R 52349-2005. Foodstuffs. Functional foods. Terms and definitions. Moscow: Standartinform; 2005. 8 p.

Khramtsov *et al.* developed a formulation for milk drinks based on whey from heat-acid cheese production. They also used aqueous extracts of Japanese quince (*Chaenomeles japonica* L.), Chinese magnolia-vine (*Schisandra chinensis* L.), and common barberry (*Berberis vulgaris* L.) (pH 3.5–4) as coagulants [18]. Japanese quince contains 180 mg ascorbic acid per 100 g of product. It is also rich in organic acids, pectin, fibre, fructose, sucrose, essential oils, vitamins B, PP, A, and E, and minerals. Thanks to these components, Japanese quince can increase immunity, strengthen the conducting vessels, remove toxins and salts during intoxication, and normalise blood pressure. It is also used for treating inflammation in the oral cavity and upper respiratory tract, as well as intestinal disorders and other diseases.

The fruits of Chinese magnolia-vine contain sugar, tannins and colouring compounds, fatty acids (glycerides of linoleic, linolenic, oleic, and other acids) and organic acids (malic, citric, and tartaric). In addition, they are rich in essential oils, ascorbic acid, and vitamin E, as well as schizandrol and schizandrin – the compounds that determine basic biological properties of the plant. They improve physical and mental activity, enhance body resistance to negative factors, and stimulate the heart and blood vessels, contributing to the preservation of human health. Common barberry is valued for its content of alkaloids, carotene, tannins, ascorbic acid, tocopherol, and organic acids. Its beneficial properties are used in treating various pathologies, as well as to improve appetite. It also has laxative, antiseptic, tonic, antipyretic, and diaphoretic properties.

Fortified probiotic drinks are a new step in the development of the food industry. Fermented milk products are functional foods that contain biologically active substances with health-beneficial properties. It is generally recognised that probiotics serve as an important tool to prevent and treat dysbiosis resulting from irrational antibiotic therapy, intestinal diseases, improper nutrition, or stress. Among conventional probiotics are lactobacilli and bifidobacteria. Their beneficial effects are manifested in normalising intestinal microflora, activating the entire gastrointestinal tract, and improving calcium absorption. They also perform anti-allergenic and immunostimulating functions [19].

The greatest positive effect on human health can be achieved by using symbiotic products containing both pre- and probiotics. Prebiotics are substances that stimulate the growth and activity of microorganisms (probiotics) and improve their adhesion to the intestinal walls. Such properties are common for nonhydrolyzable oligo- and polysaccharides of plants, such as pectin, inulin, fructo-oligosaccharides, xylo-oligosaccharides, and resistant starch [19, 20].

Probiotics are widely used in the production of dairy products, but the recent focus has been on cultivating

lactic acid and bifidobacteria in dairy products with plant additives. The benefits of plant products are determined by high contents of vitamins, antioxidants, minerals, and phytoelements. Thus, current research efforts aim to develop formulations for functional dairy products enriched with plant additives with probiotic properties, improve their technology, and assess their consumer appeal.

Danilova developed a phytocomposition for a functional fermented milk product with gerodietetic properties [19]. The phytocomposition was made up of medicinal plants growing in Western Siberia. It was based on *Comarum* extract that strengthens the joints, which is especially important for older people. The phytocomposition also included extracts of *Crataegus*, which stimulates the cardiovascular system, and *Origanum vulgare*, which has a calming sedative effect on the nervous system.

Crataegus fruits contain flavonoids (up to 3%, mainly hyperin), organic acids (citric and tartaric), sugars (up to 0.29% sucrose; pentose and fructose), carotene (2–30 mg%), vitamin C (25–375 mg%), choline, essential oil, colouring agent (carotene pigment), fats, nitrogen wastes (0.8–1.5%), ash (1%), trace elements (potassium, calcium, manganese, magnesium, iron); tannins, and extractives. Also present are vitexin glycoside, hyperoside, leucocyanidins – bioside, rutin, sesculin, and purine derivatives, triterpene saponins (ursolic and oleanolic acids), soroite, and choline-like substances. *Crataegus* fruits contain a mixture of triterpenic acids (categus, ursolic, chlorogenic, oleanolic, and caffeic acid), flavone glycosides, acetylcholine, and phytosterols. *Crataegus* flowers contain caffeic and chlorogenic acids, hyperoside, choline, acetylcholine, essential oil, trimethylamine, flavone glycosides, hyperoside, and quercetin. The leaves are rich in phytoncide and the roots contain okonakintin (a quinine substitute) [19].

Crataegus primary nutrients are flavone glucosides – crystalline dyes of orange and red colour. This plant is a rich source of vitamin P. The maximum amount of flavonoids in the P-vitamin complex accumulates in the green leaves (4–5% for *Crataegus sanguinea*), remaining in the fallen leaves. An infusion of *Crataegus* fruits and flowers or a liquid fruit extract reduce the excitability of the central nervous system and have a tonic effect on the heart muscle. They increase blood circulation in the coronary vessels of the heart and brain and eliminate tachycardia and arrhythmia by normalиzing the rhythm of cardiac activity. In addition, they slightly reduce blood pressure, improve sleep and a general state of health. *Crataegus* medicines have a beneficial effect on the functioning of the heart, expanding its vessels, which is especially important for the elderly [19].

Origanum vulgare contains up to 1.2% of an essential oil (so-called 'intoxicating' oil) that has a pleasant smell and bactericidal properties. It consists of aromatic alcohol, phenols, thymol (up to 3.8–10.2%) and its carvacrol isomer, as well as bi- and geranyl acetate (up to 5%). The plant also contains free alcohols (up to 15%), sesquiterpenes (12.5%), ascorbic acid (up to 565 mg% in leaves), and flavonoids. In addition, it is a source of polyphenolic compounds (up to 12–20%), five flavonic glycosides, tarry substances (10%), coumarins (1.4%), tannins (1.9–4%), and colouring agents. The content of ascorbic acid is 565 mg% in the leaves, 58 mg% in the stems, and 166 mg% in the flowers. Phytocomponents enrich products with micronutrients – biologically active substances that increase their nutritional and biological value. They also provide products with functional properties. Further studies in using non-conventional plants as raw materials for functional products will help us replenish the deficiency of nutrients in the human body. In addition, they will give us an extra opportunity for using natural resources [19].

Potoroko *et al.* patented a formulation composed of skim milk powder, 30% cream, aqueous malt extract, fried green malt, a ginseng dietary supplement, eleutherococcus, milk thistle, echinacea, starter culture of lactic streptococci, Bifilact D and thermophilic bacteria, a stabiliser, fruit or vegetable puree, honey, fat-soluble vitamin D, and water [20]. This formulation ensures a high biological value, long shelf-life, and good sensory characteristics.

In another study, Potoroko *et al.* described the preparation of a functional kefir drink enriched with alfalfa extract [21]. After introducing alfalfa extract into milk, it was fermented at about 20°C for 10–12 h. Then the temperature was lowered to 12–16°C and the product was left at rest for 4–6 h for yeast to develop. After that, the product was cooled to 8–10°C and left for 12–24 h to ripen. Ethanol and carbon dioxide accumulated as a result of yeast development, giving the finished product a specific taste and smell. Alfalfa extract was chosen due to its composition. It contains organic and inorganic compounds, amino acids, monosugars, phenolic compounds, and trace elements characteristic of plant materials, as well as humic and other biologically active substances not commonly found in plant extracts. The extract affected the fermentation rate and intensified lactose conversion and proteolytic reactions, making the kefir drink dietetic. Most importantly, it did not contain any limiting amino acids.

Skorkina *et al.* created a formulation for biokefir based on skim milk and two plant components, hawthorn puree and stevia syrup [22]. Hawthorn puree contains substances that expand the blood vessels of the heart and improve the absorption of oxygen by the heart muscle, relieving arrhythmia. In addition, hawthorn reduces blood pressure and has a calming effect. It contains vitamins C and PP, carotene, some acids, and plenty of sugars (fructose) and pectin, which removes heavy metal salts and other harmful substances

from the body. Stevia is rich in glycosides (stevioside, rebaudioside (A, C, D, E); dulcoside, and steviolbioside) which help to improve carbohydrate metabolism and stimulate the secretion of inulin in diabetes mellitus. It also contains vitamin C, β-carotene, and minerals (zinc, selenium) with antioxidant properties. Its mild diuretic effect helps to remove metabolic products, toxins, and salts of heavy metals from the body. The syrup sweetness has a factor of 1:30. According to the study, the acidity of biokefir with natural additives increased throughout its shelf-life, but remained within the normal range.

Lyu patented a formulation for fermented milk yogurt with mild diuretic properties. It contained 200–220 parts of purple sweet potatoes, 10–12 parts of skimmed milk powder, 6–7 parts of dates, 2–4 parts of *Houttuynia cordata*, 5–6 parts of liquorice root, 8–10 parts of peppermint aqueous extract, 2–3 parts of corn fibres, 4–6 parts of algae, 3–5 parts of pomegranate peel, 6–8 parts of *Centaurea*, 0.2–0.4 part of stevioside, 10–15 parts of honey, 10–12 parts of glucose, as well as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* bacteria [23]. The product had a pleasant taste, a long shelf-life, and probiotic properties. It helped to cleanse the urinary system.

Joung *et al.* developed yogurt with extracts from two traditional Korean plants: persimmon (*Diospyros kaki* L.) and lotus (*Nelumbo nucifera* L.) [24]. The extracts were prepared by boiling in a water bath at 100°C for 9 min, with periodic stirring and further filtration of the aqueous part. The resulting product was vacuum-dried at max. 50°C. The plant additives were introduced into whole milk prior to fermentation. Then, *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* bacteria cultures were added in the amounts of 2.95 and 1.14 log CFU/mL, respectively. The plant extracts prolonged the product's shelf-life, reduced the fermentation time, improved the viability of the starter culture, structured the product, and enriched it with phenolic compounds with antibacterial, antioxidant, and immunomodulatory properties.

The authors of another study formulated fermented milk drinks enriched with ayrampo fruit extract [25]. Ayrampo aqueous extract is a rich source of natural beta-cyanine pigments and antioxidants, highly stable during heat treatment and storage.

Oh *et al.* proposed using aqueous extracts of *Cudrania tricuspidata* and *Morus alba* (commonly known as white mulberry). These extracts work as prebiotic additives that increase the rate of bacterial growth and fermentation intensity [26]. *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus* were used as probiotic microorganisms. The plant extracts enriched the drinks with monosaccharides, as well as non-chlorogenic, chlorogenic, and caffeic acids, which have a mild stimulating effect on the body.

Chiodelli *et al.* evaluated the effect of *Aloe barbadensis* and *Aloe arborescens* extracts on the properties of a dairy product fermented with *Lactobacillus* bacteria [27]. The extracts helped to structure the product, gave it a pleasant taste and smell, and enriched it with secondary metabolites, improving enzymatic processes and increasing the product's nutritional value. Aloe extracts contain enzymes, vitamins, phytoncides, aloin, nataloin, rabarberon, homonatalain, emodin (1.66%), tarry substances, and traces of essential oils. The latter have a pronounced anti-inflammatory properties, increase the secretion of digestive glands, improve appetite and digestion, and prevent the development of pathogenic flora. In addition, milk drinks enriched with aloe and *Lactobacillus rhamnosus* reduce the size of adipocytes and increase their number. They can also lower body weight and blood glucose levels, which makes them effective in fighting excess weight and treating type II diabetes [28].

The extracts obtained from the roots of *Rhodiola rosea*, *Eleutherococcus senticosus*, and *Panax ginseng* can also be effectively used to enrich fermented milk drinks. These plants are the most widely used adaptogens and natural stimulants. *Panax ginseng* is a rich source of ginsenosides. *Eleutherococcus* contains several eleutherosides which are responsible for adaptogenic activity. *Rhodiola rosea* contains salidroside, tyrosol, and rosavins, which are presumably active compounds. Molgaard *et al.* studied the properties of pasteurised milk drinks enriched with *Rhodiola rosea*, *Eleutherococcus senticosus*, and *Panax ginseng.* The content of active components was determined by HPLC after pasteurisation [29]. The results showed that eleutherosides from *Eleutherococcus* and ginsenosides from *Panax ginseng* could survive pasteurisation, while salidroside and rosavin from *Rhodiola rosea* root were destroyed. Thus, the authors warned against using this additive in heat-treated products.

In the work by Kurnakova, blueberries were used to increase the nutritional value, enhance taste, and prolong the shelf-life of the product. These effects are due to anthocyanosides, which are detrimental to *E. coli* and other pathogenic microorganisms [30]. Anthoconosides protect the cardiovascular system, prevent varicose veins, have antibacterial properties, and are beneficial for vision.

Gabriel *et al.* developed a new probiotic product called 'Rosalact'. It was made from pasteurised milk enriched with extracts of medicinal plants (rosehip, liquorice) and probiotic ABT-5 culture [31]. It was found that liquorice root extract contains carbohydrates and related compounds (glucose, fructose, sucrose, and maltose), polysaccharides (up to 34% starch, up to 30% cellulose, and pectin substances), organic acids (succinic, fumaric, citric, malic, and tartaric), essential oils, triterpenoids (glycyrrhizic acid), resins, steroids (β-sitosterol), phenolcarboxylic acids and their

derivatives (ferulic, synomal, and salicylic), coumarins (herniarin, umbelliferone, etc.), tannins (8.3–14.2%), flavonoids (liquiquirithin, isoliquirithin, liququiritozide, quercetin, kempferol, apigenin, etc.), higher aliphatic hydrocarbons and alcohols, higher fatty acids, and alkaloids. Rose hips give the dairy product a wide range of functional properties, making it suitable for daily use, as well as in supportive therapy for colds, kidney disease, cardiovascular disease, and prevention of vitamin deficiency.

In another study, liquorice root extract and sea buckthorn fruits were used to enrich milk-based drinks [32]. Milk was mixed with the plant extracts and fermented at 42°C for 5 h using ABY-3 culture bacteria (*Bifidobacterium*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii subsp. bulgaricus*). As a result, the final product had an increased content of vitamins B_1, B_2, C, E, K, P, as well as flavonoids, folic acid, carotenoids, betaine, choline, coumarins, glucose, fructose and phospholipids, macroelements and microelements (sodium, magnesium, iron, silicon, aluminium, lead, nickel, manganese, strontium, and molybdenum). In addition, the product had an extended shelf-life.

Mariola *et al.* studied the effect that phenolic compounds of rosemary, hyssop, nettle, caraway, and lemon balm extracts had on the growth of *Lactobacillus acidophilus* and *L. delbrueckii* bacteria [33]. It was shown that rosemary extract suppressed the growth and activity of the bacteria. Lemon balm extract had the maximum amount of antioxidant substances, which extended the product's shelf-life. Thus, the authors did not recommend using rosemary as a functional additive for drinks containing lactobacilli. Alternatively, they could be added at the very end of the process, after fermentation.

In view of the above, there is a clear need to fully utilise plant biodiversity and create effective and safe functional products. Russia is home to many medicinal plants that are absent in the pharmacopoeias of other countries. They include *Eleutherococcus senticosus*, *Schisandra chinensis*, *Paeonia anomala*, *Leonurus cardiaca*, *Rhodiola rosea*, *Rhaponticum carthamoides*, *Thermopsis lanceolata*, *Colchicum*, *Astragalus dasyanthus*, *Phlojodicarpus sibiricus*, *Peganum harmala*, *Hedysarum alpinum*, *Filipendula ulmaria*, *Lespedeza bicolor*, *Lespedezae hedysaroides*, *Securinega suffruticosa*, *Salsola collina*, *Sphaerophysa salsula*, and *Scutellaria baicalensis* [34, 35]. This work should involve research into using cultivated agricultural plants as a source of medicinal raw materials [36].

Many plant species, especially endemic, have disappeared or are threatened with extinction and listed in the Red Book of Russia. Although there is a high demand for them in medicine, pharmacists have to exclude them from the pharmacopoeia. These factors have created a need for further research into their reproduction and return to favourable habitats. Many of these plants are the only sources of unique substances that can be used in treating cancer, Alzheimer's, neurological and other diseases. For example, vogonosin, a flavone of *Scutellaria baicalensis* has apoptotic properties and is able to target cancer cells and destroy them without affecting the healthy ones [36]. This plant grows in the natural environment in very scarce amounts, therefore its medical substances can only be produced by cell bioengineering methods.

Over 40 years ago, scientists tried to propagate cell and tissue cultures *in vitro* and select the most productive cells and differentiated tissues. In most cases, it was impossible to isolate a sufficient amount of required metabolites from plant materials. One of the turning points was the discovery of genetic transformation using *Agrobacterium rhizogenes* soil bacterium [37].

The agrobacterial transformation of plant roots made it possible to obtain secondary metabolites for medical use: alkaloids, coumarins, phenolic compounds, and some others [38]. Plant studies in this direction are especially relevant.

The lack of secondary growth in the roots inhibits the production of a wider range of biologically active substances. It is known that the activity of secondary substances often increases in roots with secondary growth, which can contribute to a greater yield of target metabolic products. Therefore, we need to develop various methods that induce the production of secondary metabolites in hairy root cultures and their secretion into the culture medium.

One of the problems is how to preserve the roots for a long time without causing repeated subinoculation. Although there are numerous methods available today that maintain and preserve the created cultures, further research is needed to develop more advanced methods of cryopreservation and those using bioreactors.

This market segment has a huge growth potential. In Russia, hairy root cultures are still a fairly new concept. Only few scientific groups conduct fundamental and applied research using hairy roots as model objects. Moreover, there are no commercially successful Russian projects in this area. The hairy roots technology could be used in the production of functional foods, lowering costs and extending the list of biologically active plants, including endangered species [39].

CONCLUSION

In general, the state of people's health in Russia calls for more advanced research and full utilisation of local medicinal plants to obtain biologically active substances for using in the food industry. Considerable funds are currently allocated to support innovative research and development of advanced technologies in this area.

There are a number of objective and subjective reasons behind the growing production and consumption

of functional products all over the world. These include:
– changes in the structure and quality of modern nutrition: a significant decrease (2–3 times compared to 100–150 years ago) in vitamins, minerals, dietary fibre, and other vital substances;
– a real risk of chemical and biological contamination of foods with nitrates, nitrites, salts of heavy metals (mercury, tin, lead, copper, cadmium, antimony, vanadium, chromium, molybdenum, manganese, and cobalt), microscopic fungi, pathogenic microorganisms, dyes, preservatives, etc.;
– a need for certain essential nutrients, which are not formed in the body, to come with food: some macro- and microelements (selenium, magnesium, vanadium, zinc, iron, molybdenum, etc.), vitamins (E, D, A, etc.), amino acids (methionine, leucine, lysine, histidine, etc.), and polyunsaturated fatty acids (linoleic, linolenic, arachidonic, etc.); they are important for metabolic processes, the synthesis of enzymes, hormones, and vitamins, for haematopoiesis and tissue repair, etc.;
– a decrease in human motor activity and overconsumption of refined foods with various additives, leading to a 40–60% deficiency of vitamins and essential macro- and microelements in the diet;
– a growing attention to one's own health and efforts to cut down on drugs by having a balanced diet and consuming more functional foods;
– high incidence of chronic diseases (cardiovascular, endocrine, Alzheimer's, motor disease, etc.), which require functional products for medicinal and preventative purposes;
– high cholesterol levels among over 20% of the population, encouraging them to prefer functional foods to reduce the risk of cardiovascular disease;
– a growing number of obese children and adults with a high risk of heart disease, asthma, diabetes, and cancer; and
– active involvement of specialised medical associations and funds in the prevention of cardiovascular, diabetic, orthopaedic, oncological, and other diseases (their logos and recommendations, e.g. glycaemic index, are indicated on food labels); better design and quality of food packaging materials; more packages suitable for microwave ovens [36, 38, 39].

Functional products, including drinks, have a variety of positive effects on metabolic processes. They reduce glucose and cholesterol levels in the blood and help the absorption of trace elements in the large intestine. In addition, they strengthen the immune system, help to prevent cancer, and exhibit a wide range of other properties: anti-allergic, anti-inflammatory, anti-thrombotic, antimicrobial, stimulating, health-beneficial, antispasmodic, and antioxidant. Functional foods increase resistance to infectious diseases and enhance the body's ability to adapt to adverse environmental factors (weather, ionisation, oxygen deficiency, intensive workload, etc.). These adaptogens increase the sensitivity of cells to endogenous insulin, normalising the metabolism of carbohydrates, proteins, and fatty acids [39, 40].

Thus, functional milk-based drinks enriched with plant components are a promising direction in the dairy industry. They improve the immune system and can be used as part of supportive therapy. They are also suitable for daily use to replenish the balance of essential nutrients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Erratum: Optimisation of functional sausage formulation with konjac and inulin: using D-Optimal mixture design

Fahimeh Safaei, Khadijeh Abhari, Nader Karimian Khosroshahi, Hedayat Hosseini and Mojtaba Jafari

The Editorial Office of Foods and Raw Materials would like to report an error in the published paper 'Optimisation of functional sausage formulation with konjac and inulin: using D-Optimal mixture design'. Foods and Raw Materials, 2019, vol. 7, no. 1, pp. 177–184. DOI: http://doi.org/10.21603/2308-4057-2019-1-177-184.

The affiliation of Mojtaba Jafari should be changed from 'Food Sciences and Technology Department, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran' to 'Department of Food Science and Technology, Faculty of Industrial and Mechanical Engineering, Islamic Azad University, Qazvin Branch, Qazvin, Iran'.

We apologise to the author for any inconvenience caused by this mistake. The change does not affect the scientific results. The manuscript will be updated and the original will remain available on the article webpage.

The online version of the original article can be found under doi:10.21603/2308-4057-2019-1-177-184.

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Review
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Review of A. Yu. Prosekov's textbook 'Fundamentals of Food Technology'

The textbook reviewed is designed for the education and training of food industry specialists. It covers the basic aspects of physical and colloid chemistry and their application in the food industry.

Consumer properties of food products, namely elasticity, viscosity, consistency and resistance to destruction, are determined by their physical properties. As physical and colloid chemistry methods are widely used while processing food raw materials into marketable products, the knowledge of these basic disciplines appears essential for future specialists. Understanding the processes that occur in food systems during both production and storage stages allows us to predict and change the consumer properties of food products.

The textbook consists of 13 chapters covering the main processes and phenomena of physical and colloid chemistry. It involves aspects of chemical thermodynamics, kinetics of chemical reactions and rheology. The textbook also discusses surface phenomena (surface tension, adsorption, the role of surfactants in foam and emulsion formation), colloidal interactions, as well as the processes of aggregation, sedimentation and coalescence in dispersed systems. In addition, proper attention is given to nucleation, a relatively rare subject of detailed consideration in textbooks on physical and colloid chemistry.

Classic textbooks on physical and colloid chemistry consider ideal systems, while the processes occurring in non-ideal systems are complicated by a number of factors. Currently, most of the food products are multicomponent systems which are not thermodynamically stable and characterized by specific kinetic reaction determined by their heterogeneous composition. Therefore, consideration of the basic processes of physical and colloid chemistry as related to the aspect of food production is not only justified, but also promising.

What is more, students often experience difficulties in fundamental disciplines studying as they cannot see ways of practical application for the acquired data. The textbook removes this problem because readers are addressed a number of practice-oriented questions even in the introduction section. An undoubted advantage of the textbook reviewed is references to the process tasks arising in the course of food production.

As stated in the introduction section, the book is intended to understanding; hence, it contains a large number of analogies and examples allowing students to visualise the processes occurring in food systems. Each chapter presents examples of solving practical tasks, which activates mental processes and allows readers to correlate theoretical provisions with practice, as well as to see problems and find ways of their solutions. Another advantage of the textbook is optimally structured material for acquirement.

The textbook is written at a high academic and methodological level and can be recommended to students and postgraduates studying food production technology.

Andrey N. Petrov,
Dr. Sci. (Eng.), Academician of RAS,
All-Russia Scientific Research Institute of Technology of Canning

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