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Original Article

Anti-*Salmonella* activity and probiotic trends of *Kluyveromyces marxianus* S-2-05 and *Kluyveromyces lactis* S-3-05 isolated from a French cheese, Tomme d'Orchies

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Abstract

Kluyveromyces marxianus S-2-05 and *Kluyveromyces lactis* S-3-05 were recently isolated from a traditional French cheese, Tomme d'Orchies, and characterized here for their advantages using a different application perspective. First, we established their anti-*Salmonella* activity and downregulation of the virulence *sopD* gene of *Salmonella enterica* subsp. *enterica* serovar Typhimurium, mainly in the presence of *K. marxianus* S-2-05. In addition to their antagonism, these non-*Saccharomyces* yeasts were able to survive under conditions mimicking the gastrointestinal environment and to form biofilms on an abiotic device such as polystyrene. These strains also displayed highly hydrophilic cell wall surfaces properties and capacity for adhesion to intestinal Caco-2 cells, thus enhancing their potential as probiotic strains.

Keywords: Non-*Saccharomyces* yeasts; Anti-*Salmonella* antagonism; Probiotic

1. Introduction

As we near the end of the golden era of antibiotic discovery, natural antimicrobials are being viewed as options for confronting increased bacterial resistance. According to the European Center for Disease Prevention and Control (ECDC), 25,000 people die each year from infections caused by multiresistant bacteria. Such collateral damage greatly increases the cost, as well as extra healthcare services and productivity loss [1]. The combat against bacterial resistance to antibiotics includes strategies outlined in different reviews [2–4], but fecal microbiota transplantation, use of nanoparticles, essential oils and, to a lesser extent, probiotics, are also weapons that need to be considered. The ultimate goal of all these strategies is to tackle bacterial resistance. Alternatives to antibiotics must be considered in both animal agriculture and human medicine. Use of probiotics in resolving medical problems is safer and less expensive than antibiotic therapy. Probiotics are live microorganisms with beneficial claims. Their intake in adequate amounts may confer health benefits upon the host, according to FAO/WHO guidelines of 2002 [5]. Most probiotic products are issued from lactic acid bacteria (LAB) strains, mainly *Lactobacillus* and *Lactococcus* species or *Bifidobacterium* specie, but other bacteria such as *Escherichia coli* Nissle and even yeast species are also used for probiotic applications [6]. The role of probiotics as adjunctive measures led to a body of evidence supporting their role in primary prevention of *Clostridium difficile* infection (CDI), which is a leading cause of hospital-acquired bacterial

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infections and the most deadly enteric pathogen in the United States [7,8]. In a recent review, Elzouki [9] underlined how probiotics can control several gastrointestinal diseases such as antibiotic-related diarrhea, *C. difficile* toxin-induced colitis and infectious diarrhea. Elsewhere, substantial documentation highlighted antimicrobial resistance among pathogenic bacteria due to overuse of antibiotics in the poultry industry, which caused public and governmental outcries. Antibiotics that are provided to healthy animals as growth promoters at subtherapeutic doses have led to selection of bacterial resistance in *Campylobacter*, *Salmonella*, *Enterococcus* and *E. coli* [10].

The rational design of probiotics includes, in addition to their health claims, several criteria– stability during the manufacturing processes, viability during gastrointestinal transit and functionality at the desired target site [11]. Adhesion to mucus, cell surface properties, safety of the host and antagonism toward intestinal pathogens also are determinant in the selection of probiotics.

Traditionally, probiotics are associated with dairy products, especially those derived from cheese, which are remarkable for their microbial diversity. Most studies on yeast probiotics focused on *Saccharomyces boulardii* [12,13]. The recent decade has witnessed much evidence concerning non-*Saccharomyces* yeasts as probiotic candidates [14,15]. A recent report from Li et al. [16] elucidated the GRAS status of *Kluyveromyces* species. Yeasts might offer more advantages compared to bacteria, as they are insensitive to antibiotics and can be easily used for antibiotic-associated diarrhea treatment [13,17]. Yeasts are also able to produce major metabolites such as vitamins and/or antioxidants [18,19], and inhibit growth of *E. coli*, *Staphylococcus aureus*, and *Aspergillus westerdijkiae* [20,21].

Taking advantage of the GRAS status of *Kluyveromyces* species, the present work sought to further elucidate the beneficial effects of *Kluyveromyces marxianus* S-2-05 and *Kluyveromyces lactis* S-3-05, recently isolated from a French traditional cheese named “Tomme d'Orchies”. These yeasts were able to inhibit growth of the foodborne pathogen *Salmonella* spp. and downregulate the virulent *sopD* gene of *Salmonella enterica* subsp. *enterica* serovar Typhimurium.

2. Material and methods

2.1. Strains, growth conditions and inhibitory activity

K. marxianus S-2-05 and *K. lactis* S-3-03 were grown at 25 °C with shaking at 160 rpm in YEG medium (pH 6.9) containing yeast extract 5 g/L and glucose 20 g/L. *S. boulardii* Ultralevure (Biocodex, France) and *Candida albicans* ATCC 10231 were grown at 37 °C under agitation at 160 rpm in YEG medium. *S. enterica* subsp. *enterica* serovar Enteritidis SR071, *S. enterica* subsp. *enterica* serovar Paratyphi B SR425 (from the FARAH laboratory, Belgium) and *S.* ser. Typhimurium ATCC 14028 were grown at 37 °C under agitation of 160 rpm in brain-heart infusion medium (BHI) (Sigma–Aldrich, Germany). For gene expression analysis, *Salmonella* spp. were also grown in Luria broth (LB) (Sigma–Aldrich, Germany) under the above-cited conditions. The assessment of antagonism was carried as recently reported [22]. Briefly, the method consisted of flooding a Mueller-Hinton agar plate with *Salmonella* sp. at about 10^6 CFU/mL, and then adding 5 µL of yeast cultures grown for 16–18 h and diluted to about 10^8 CFU/mL. The plates were then incubated at 25 °C and inspected after 48 h for inhibition.

2.2. Expression of S. ser. Typhimurium ATCC 14028 virulence genes

The method from Guri et al. [23] was used in this study. Briefly, *K. marxianus* S-2-05, *K. lactis* S-3-03 and *S.* ser. Typhimurium ATCC 14028 were grown overnight. Then, *S.* ser. Typhimurium ATCC 14028 at about 5×10^4 CFU/mL was co-inoculated with 1×10^6 CFU/mL of each yeast and incubated for 4 h under agitation at 37 °C. *S. ser.* Typhimurium ATCC 14028 alone was used as a negative control assay, and each experiment was carried out at least in triplicate. The cultures were centrifuged (4 °C, 10 min, 5000*g*), and the resulting pellets were treated with the RNeasy Mini kit (Qiagen, Venlo, Netherlands) and the DNase I recombinant RNase-free kit (Roche Applied Sciences, Vilvoorde, Belgium) in order to extract and purify total RNA. DNase was inactivated by heating the suspension for 10 min at 75 °C, and then placed on ice. The cDNA high capacity reverse transcription kit (Life Technologies, Carlsbad, USA) was used on the purified total RNA following this program: 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C. Quantitative real-time PCR (qRT-PCR) was used to quantify the expression of virulence genes *hilA*, *ssrB2*, *sop*D and housekeeping genes *gmk*, *rpoD* and 16S rDNA of *S.* ser. Typhimurium ATCC 14028 (Table 1). The Q2 housekeeping gene with the utmost expression stability was used for ratio calculation. Primers used in this study and listed in Table 1 [23] were purchased from Eurogentec (Liège, Belgium). PCR mix was prepared with GoTaq®qPCR Master Mix (Promega, Leiden, Netherlands), and run on the ABI 7000 thermocycler (Applied Biosystems, Singapore) using the following program: initial denaturation 10 min at 95 °C; 40 cycles: 30 s at 95 °C; 30 s at 54 °C; and 30 s at 72 °C. The melting curve was obtained by decreasing the temperature from 95 °C to 60 °C with a rate at 0.1 °C/s.

The formula below [24] was used for gene expression. Ct_{CTRL} was the threshold value of each related gene of *S.* ser. Typhimurium ATCC 14028 and Ct_S was the threshold value of genes of *S.* ser. Typhimurium ATCC 14028 when grown concomitantly with the yeast strain.

$$r = \frac{2^{(Ct_{CTRL} - Ct_S)} of\ tested\ gene}{2^{(Ct_{CTRL} - Ct_S)} of\ housekeeping\ gene}$$

A ratio below −1 was considered as a downregulated gene, while a ratio above +1 was considered as upregulation.

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Table 1
Primers used for the quantitative real-time PCR.

Amplified gene	Primers		Information
hil	Forward	5′-TGT CGG AAG ATA AAG AGC AT-3′	Virulence factor – Invasion protein regulator
	Reverse	5′-AAG GAA GTA TCG CCA ATG TA-3′	
sopD	Forward	5′-ATT AAT GCC GGT AAC TTT GA-3′	Virulence factor – Secreted effector protein
	Reverse	5′-CTC TGA AAA CGG TGA ATA GC-3′	
ssrB2	Forward	5′-TGG TTT ACA CAG CAT ACC AA-3′	Virulence factor – Type III secretion system regulator (transcriptional activator)
	Reverse	5′-GGT CAA TGT AAC GCT TGT TT-3′	
gmk	Forward	5′-TTG GCA GGG AGG CGT TT-3′	Housekeeping genes
	Reverse	5′-GCG CGA AGT GCC GTA GTA AT-3′	
ropD	Forward	5′-ACA TGG GTA TTC AGG TAA TGG AAG A-3′	
	Reverse	5′-CGG TGC TGG TGG TAT TTT CA-3′	
16S rRNA	Forward	5′-GAG TTT GAT CMT GGC TCA G-3′	
	Reverse	5′-TAC GGT TAC CTT GTT ACG AC-3′	

2.3. Growth of yeasts under different physiological conditions

K. marxianus S-2-05 and *K. lactis* S-3-05 were grown overnight, streaked on YEG plates and incubated at 4, 8, 25, 30 and 37° for 7 days in order to determine the optimal growth temperature. Two other isolates on YEG plates were incubated at −20 °C and 55 °C for 7 days and at 25 °C for another period of 7 additional days. Afterwards, plates were inspected for colony growth.

To assess pH tolerance, overnight cultures were used to inoculate YEG broth (dilution: 1/100) adjusted to pH 5.0 using citrate buffer, to pH 6.0, 7.0 and 8.0 using phosphate buffer.

To assess sodium chloride (NaCl) tolerance, overnight cultures were used to inoculate YEG broth (dilution: 1/100) in the presence of different NaCl concentrations (0, 1, 2.5, 5, 8.5, 10, 15 or 30% (w/v)). Broths were incubated under agitation for 7 days at 25 °C.

2.4. Safe properties

2.4.1. Biofilm formation

Biofilm formation was performed as previously described [25]. Each strain was grown overnight and diluted to about 5×10^6 CFU/mL, placed into 96 round polystyrene well plates (Sarstedt, Germany) and incubated at 25 °C for 24 h or 48 h. Wells were washed twice with trypton-salt solution (TSS) consisting of 1 g/L trypton and 8.5 g/L NaCl, followed by addition of absolute ethanol, allowing biofilm fixation. Ethanol was eliminated and crystal violet at 0.1% (w/v) was added for 45 min before washing with TSS. Finally, glacial acetic acid at 33% (v/v) was added to dissolve the crystal violet fixed to biofilm. The resulting solution was transferred to a new 96-round-well plate and read at 595 nm to estimate the biofilm formation capacity. Negative controls were wells without yeast cells.

2.4.2. Determination of cell affinity to solvents

The microbial adhesion to solvents (MATS) was determined as previously reported [26]. Overnight cultures of *Kluyveromyces* strains were centrifuged (room temperature, 15,000×*g*, 10 min) and the resulting pellets were suspended in 100 mM phosphate buffer in order to reach a concentration of about 10^7 CFU/mL. The optical densities (ODs) were measured at 405 nm (A0), and suspensions were mixed for 90 s with hexadecane, chloroform, ethyl acetate or decane at a proportion of 1:7. The ODs of aqueous phases (A) were taken after 5 min of contact. Cell affinity for each solvent was calculated using the following formula:

$$Affinity = \left[1 - \left(\frac{A}{A0}\right)\right]$$

The affinity for chloroform and hexadecane delineates the electron donor potential, whilst the affinity for ethyl acetate and decane indicates the electron acceptor potential of the cell surface [26]. Affinity of yeasts for hexadecane can be used as a measurement of cell surface hydrophobicity. Antagonistic yeasts were assessed for their affinities to solvent, using *C. albicans* ATCC 10231 and *S. boulardii* as controls.

2.4.3. Evaluation of yeast survival in conditions mimicking the gastrointestinal tract

Survival under conditions mimicking the gastrointestinal tract was evaluated as previously reported [27,28]. The contents of the gastric and intestinal fluids are given in Table 2. Yeasts from overnight cultures were added at 2×10^7 CFU/

Table 2
Composition of artificial gastro-intestinal fluids.

Compound	Gastric fluid	Intestinal fluid
KCl	6.9 mM	6.8 mM
KH_2PO_4	0.9 mM	0.8 mM
$NaHCO_3$	24.5 mM	85 mM
NaCl	47.7 mM	38.4 mM
$MgCl_2(H_2O)_6$	0.105 mM	0.33 mM
NH_4HCO_3	1 mM	–
HCl	15 mM	8.4 mM
$CaCl_2$	0.15 mM	0.6 mM
Pepsin (porcine)	4000 U mL^{-1}	–
Pancreatin (porcine)	–	100 U mL^{-1}
Bile extract (porcine)	–	60 g L^{-1}
pH	3.0 ± 0.2	7.0 ± 0.2

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mL in gastric fluid and incubated under agitation for 2 h at 37 °C. Yeasts were then diluted (1:1) using the intestinal fluid and incubated under agitation for 2 h at 37 °C. Before treatment and after each step, samples were withdrawn, diluted in saline water (9 g/L NaCl) and then spread on YEG agar in order to determine the number of surviving cells. Experiments with the pathogen *C. albicans* ATCC 10231 and probiotic *S. boulardii* were run in the same conditions.

2.4.4. Yeast adhesion to intestinal Caco-2 cells

Adhesion to Caco-2 cells from a human adenocarcinoma intestinal cell line was performed as recently reported [28]. To this end, overnight yeast cultures were suspended in Dulbecco's Modified Eagle Medium (DMEM, Dominique Dutscher, Brumath, France) without antibiotic and fetal bovine serum (FBS). Yeasts were used at 2×10^7 CFU/mL for the controls (*C. albicans* ATCC 10231 and *S. boulardii*), and at 6×10^5 CFU/mL for probiotic candidates *K. marxianus* S-2-05 and *K. lactis* S-3-05. Each yeast suspension was added onto the monolayer culture of Caco-2 at about 1.5×10^5 cells/well, on 24-well plates. They were first washed with phosphate buffered saline (PBS) to avoid any interfering reaction, and incubated for 2 h at 37 °C in the presence of 5% CO_2. Afterwards, cells were washed twice with PBS to remove non-adherent yeasts and lysed using PBS and 0.1% Triton X100. The lysates were serial-diluted and spread on YEG agar plates to determine the adherent yeast cell percentage per Caco-2 cells.

2.4.5. Intestinal cell cytotoxicity

Yeast cultures 24-h old were diluted to 2×10^6 CFU/mL in DMEM to be assessed for the cytotoxicity assay and 100 µL of each suspension was used at a ratio of 1:4, Caco-2/yeast and the monolayer culture of Caco-2 at about 5×10^4 cells/wells on 96-well plates. Strains were first washed with DMEM without any supplementation. The plate with Caco-2 and yeast was incubated for 24 h at 37 °C in the presence of 5% CO_2. Afterwards, cells were washed twice with PBS in order to remove non-adherent yeasts, and PBS with 1 µg/µL of propidium iodide was added. Fluorescence was measured using Xenius XM (SAFAS, Monaco) with excitation/emission wavelength parameters of 575/615 nm and PMT voltage of 1000 V. Each yeast strain was tested on three independent cultures, with three assays per culture. Wells containing only Caco-2 cells were used for control of total viability.

2.5. Statistical analysis

Statistical analysis was performed with software SPSS® Statistic v20, IBM® (Armonk, NY, USA). Normality of value distributions was checked with Shaphiro–Wilk and Skewness–Kurtosis tests. Normal values were compared with one-way ANOVA and the Tukey test or Games–Howell and T2 of Tamhane for heterogeneous variances. Non-normal values were treated with Kruskal–Wallis and Conover–Iman tests.

3. Results

3.1. Kluyveromyces *yeasts inhibited foodborne* Salmonella *spp.*

K marxianus S-2-05 and *K. lactis* S-3-05 displayed inhibitory activities against *S.* ser. Paratyphi B, *S.* ser. Typhimurium ATCC 14028 and *S.* ser. Enteriditis used as target organisms (Table 3). Here, housekeeping gene *rpoD* was used for calculation of gene expression [24]. Expression of three *Salmonella* virulence genes for *K. marxianus* S-2-05 led to the following values: -10.67 ± 1.84 (*sopD*), -1.12 ± 4.82 (*ssrB2*) and 2.41 ± 0.44 (*hil*). In *K. lactis* S-3-05, data were not available for the *hil* gene, while expression of *sopD* and *ssrB2* was estimated at -9.33 ± 10.92 and *ssrB2* at -1.67 ± 10.81, respectively. These data delineate, for *K marxianus* S-2-05, significant downregulation of the *sopD* gene of *S.* ser. Typhimurium (Fig. 1).

3.2. K. marxianus *S-2-05 and* K. lactis *S-3-05 defied harsh growth conditions*

K. marxianus S-2-05 was able to grow until 8.5% NaCl, whereas *K. lactis* S-3-05 was even able to grow up until 10% NaCl. Both strains grew at pH values comprised between 5 and 8, with an optimum pH 7. Each yeast displayed viability after 7 days at −20 °C and grew between 8 and 37 °C, but both strains were killed upon 2 h treatment at 55 °C. Growth for *K. marxianus* S-2-05 was between 25 and 37 °C, while that of *K. lactis* S-3-05 was between 25 and 30 °C (data not shown).

3.3. *Novel insights into* K. marxianus *S-2-05 and* K. lactis *S-3-05 probiotic properties*

3.3.1. Biofilm formation on polystyrene

K. lactis S-3-05 and *K. marxianus* S-2-05, as well as *S. boulardii*, showed weak capacity to form biofilms on polystyrene after 24 h. The pathogenic human fungus *C. albicans* ATCC 10231 showed a different behavior in terms of biofilm

Table 3
Antagonistic activities of yeasts isolated from the "Tomme d'Orchies" cheese against *Salmonella* spp.

Target strains	Yeasts	
	K. marxianus S-2-05	*K. lactis* S-3-05
K. rhizophila CIP 53.45	13.9 mm ± 1.4; I	14.0 mm ± 1.4; I
S. ser. Enteritidis (laboratory collection)	10.4 mm ± 0.7; D	9.9 mm ± 0.9; D
S. ser. Paratyphi B (laboratory collection)	12.7 mm ± 0.6; D	9.8 mm ± 0.9; D
S. ser. Typhimurium ATCC 14028	10.6 mm ± 1.1; D	10.8 mm ± 0.6; D

Kocuria rhizophila CIP 5345 was used as target reference strain because of its sensitivity to antimicrobials. Values (±SD) are the means of at least three independent experiments. "I" corresponds to the inhibition activity and "D" to a decrease of growth density.

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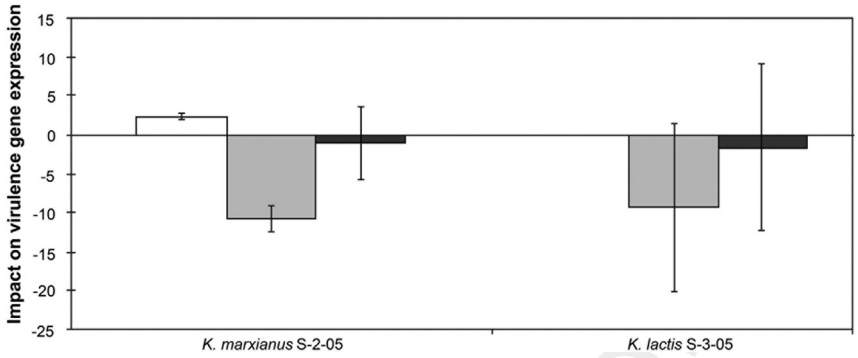

Fig. 1. Effects of *K. marxianus* S-2-05 and *K. lactis* S-3-05 on virulence gene expression of *S. enterica* subsp. enterica serovar Typhimurium ATCC 14028. Results are illustrated for *hil* (white), *sopD* (gray) and *ssrB2* (dark gray). Values (±SD) are means of at least three independent experiments.

formation on polystyrene. After 48 h, *Kluyveromyces* strains showed quite similar capacities to form biofilms, in contrast to the control strains (Fig. 2).

3.3.2. Cell surface properties

jerriBased on the MATS values (Table 4), all tested strains showed affinity to hexadecane under 10%, which delineate a strong hydrophilic property. However, *K. marxianus* S-2-05 affinity for hexadecane was estimated to be +8.89% ± 4.90 and appeared to be different from the other strains. *Kluyveromyces* strains and *S. boulardii*, showed a very strong basic property while *C. albicans* ATCC 10231 was moderately basic. On the other hand, almost neutral acidic property was reported for *K. marxianus* S-2-05 and *C. albicans* ATCC 10231, while a moderate acidic property was observed for *S. boulardii*, and a moderate repulsion of electron was observed for *K. lactis* S-3-05. *Kluyveromyces* strains used were close to the probiotic *S. boulardii* rather than *C. albicans* ATCC 10231.

3.3.3. In vitro digestion

The mimicking of passage through the entire gastrointestinal tract, with an initial concentration of 1×10^7 CFU/mL, permitted designing two significantly different groups based on the Tukey test. The highly resistant group was composed of *C. albicans* ATCC 10231, with $1.1 \times 10^7 \pm 1.6 \times 10^6$ CFU/mL after digestion, and *S. boulardii* with $9.4 \times 10^6 \pm 1.9 \times 10^6$ CFU/mL. The less resistant group was composed of *K. marxianus* S-2-05, with $4.7 \times 10^6 \pm 2.1 \times 10^6$ CFU/mL, and *K. lactis* S-3-05, with $4.5 \times 10^6 \pm 2.8 \times 10^6$ CFU/mL.

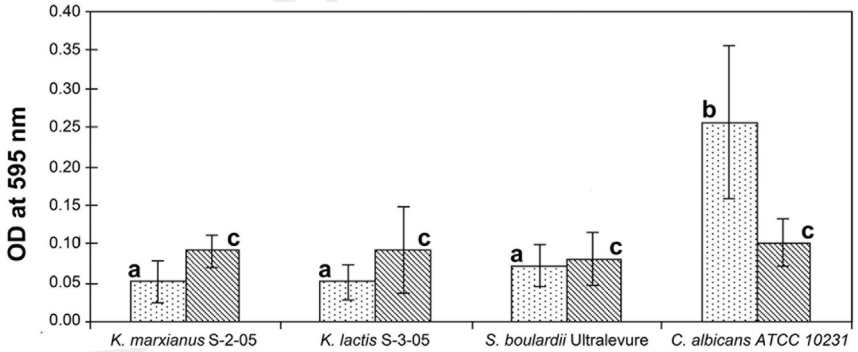

Fig. 2. Biofilm formation on polystyrene plates. Results obtained after 24 h of incubation are given in light gray and those from 48 h of incubation are depicted in dark gray. Values (±SD) are means of at least three independent experiments. Groups that were statistically similar are indicated by letters. Statistical analysis was performed using ANOVA and T2 of Tanhame for 24 h of incubation and the Kruskal–Wallis method for 48 h of incubation.

Table 4
MATS test of yeast cells cultured overnight in YEG.

Strain	Percent of adhesion to solvent			
	Hexadecane	Chloroform	Ethyl acetate	Decane
K. marxianus S-2-05	+8.89 ± 4.90 − (a)	+60.41 ± 10.46 − (a)	+21.78 ± 8.09 − (a)	+20.13 ± 7.56 − (a)
K. lactis S-3-05	−4.00 ± 5.22 − (a)/(b)	+53.92 ± 11.49 − (a)	+2.55 ± 4.90 − (b)	+17.96 ± 14.86 − (a)/(c)
S. boulardii Ultralevure	−8.94 ± 6.29 − (b)	+22.72 ± 8.48 − (b)	+11.48 ± 3.48 − (b)	−0.28 ± 4.51 − (b)
C. albicans ATCC 10231	−8.97 ± 10.99 − (b)	+4.00 ± 6.33 − (c)	+5.51 ± 3.06 − (b)	+5.12 ± 3.63 − (b)/(c)

Values (±SD) are means of at least three independent experiments. Values with the same letter are not significantly different ($P < 0.05$). Groups were made using ANOVA and Tukey test for chloroform, ethyl acetate and decane and ANOVA with Games–Howell test for hexadecane.

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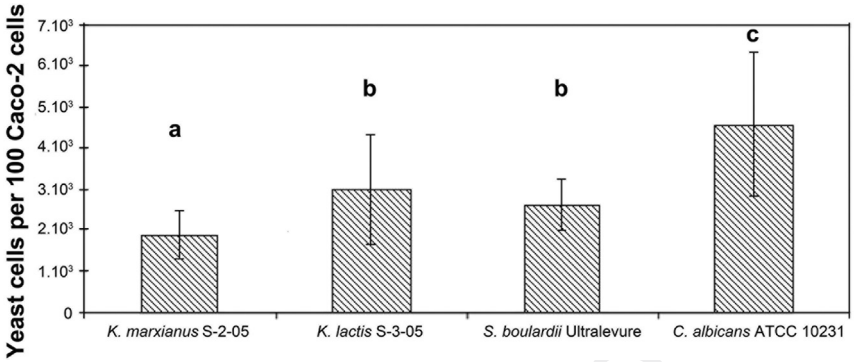

Fig. 3. Adhesion of yeast cells to Caco-2 intestinal adenocarcinoma cells, after 4 h of contact. Values (±SD) are means of at least three independent experiments. Letter corresponds to statistical groups of similarity made using ANOVA and Games–Howell analysis.

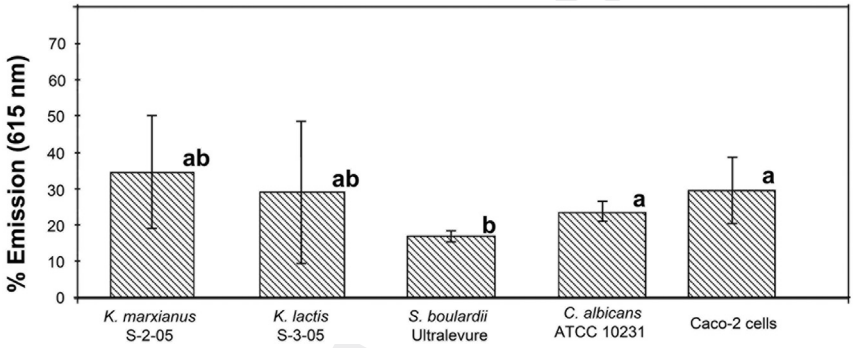

Fig. 4. Cytotoxicity of yeast cells on Caco-2 intestinal adenocarcinoma cells after 24 h of contact using a ratio of 1:4 Caco-2/yeast cells. Values (±SD) are means of at least three independent experiments. Letter corresponds to statistical groups of similarity gathered from ANOVA and Games–Howell analyses.

3.3.4. Adhesion to intestinal cells revealed no cytotoxicity for Kluyveromyces

Kluyveromyces strains adhered to Caco-2 intestinal cells upon 2 h of contact, reaching 1.9×10^3 for *K. marxianus* S-2-05 cells/100 Caco-2 cells and 3.0×10^3 for *K. lactis* S-3-05 cells/100 Caco-2. Interestingly, a similar adhesion score was reported for the probiotic strain *S. boulardii*, with 2.6×10^3 cells/100 Caco-2, and an elevated adhesion score was registered for *C. albicans* ATCC 10231, with 4.6×10^3 cells/100 Caco-2. Remarkably, the adhesion abilities of *K. lactis* S-3-05 and *S. boulardii* were shown to be statistically similar, in contrast to those observed for *K. marxianus* S-3-05 and *C. albicans* ATCC 10231, which displayed different statistical data (Fig. 3). Furthermore, these assays discarded any cytotoxicity effect associated with yeast strains on Caco-2 cells (Fig. 4). It should be noted that iodure propidium interacts only with dead cells, so the emission percentage increased consequently with the number of dead cells. Thus, the probiotic *S. boulardii* displayed an increase in cell viability rather than a decrease.

4. Discussion

Production of antimicrobial substances is of major importance when confronted with bacterial resistance to antibiotics [2–4]. Antagonism is a key criterion for selection of probiotic strains. Microorganisms expected to be used as probiotics, and especially LAB, are well known to produce a cocktail of antimicrobial substances, among them bacteriocins [29]. Nevertheless, yeasts may also be sources of antimicrobial agents [30–32]. Here we have corroborated the antagonistic properties of *K. marxianus* S-2-05 and *K. lactis* S-3-05 against diverse bacteria and the human pathogen fungus *C. albicans* [22]. These *Kluyveromyces* species, whose genetic relatedness was established [33,34], are expected to play a determinant role in biotechnology, food safety and probiotic use. Here we show that *K. marxianus* S-2-05 and *K. lactis* S-3-05 are able to inhibit *Salmonella* spp. strains. It is noteworthy that inhibition of *S.* ser. Typhimurium, by *K. marxianus* S-2-05 induced downregulation of the chromosomal *sopD* gene, which is involved in epithelial cell invasion [36]. To the best of our knowledge, this the first report showing a link between an antagonistic yeast strain and gene expression of *S.* ser. Typhimurium, a primary enteric pathogen infecting both humans and animals.

Moreover, these non-*Saccharomyces* yeasts were not toxic for intestinal Caco-2 cells after 24 h of joint incubation. *K. marxianus* strains were reported to modulate the immune response in Caco-2 [35] and to exert inhibition of the I-converting enzyme [16].

As reported by Smith et al. [15], *K. marxianus* and *Metschnikowia gruessii* were able to repair *Salmonella*-induced epithelial cell barrier disruption. On the other hand, França et al. [14] revealed the ability of *Pichia pastoris* X-33

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to inhibit *S.* ser. Typhimurium and to hamper adhesion of this pathogen to HCT-116 intestinal cells. The process of adhesion to intestinal cells can be mediated by extracellular polysaccharide (EPS) production and temperature [36,37]. It was shown that the intestinal mucus is mainly composed of MUC2 mucin, which forms an aqueous gel [38]. The acidic properties of mucin [38], coupled with the basic properties of *K. marxianus* S-2-05 and *K. lactis* S-3-05, could lead to ionic interactions and therefore to adhesion. The adhesion capacities of *K. marxianus* S-2-05 and *K. lactis* S-3-05 are similar to those of *S. boulardii*, *Kluyveromyces* strains and *C. albicans* ATCC 10231. Moreover, *S. boulardii* showed a highly hydrophilic profile, while a strong basic profile was observed for the *Kluyveromyces* antagonistic strains.

K. lactis S-3-05 and *K. marxianus* S-2-05 also possess a remarkable capacity to survive in vitro gastrointestinal conditions, which are usually used for probiotic screening. Remarkably, the potential of *K. marxianus* to defy bile salts was observed in vitro and confirmed in mice [39]. The data obtained here are encouraging, and future efforts aimed at confirming the observed effects should be performed in vivo to drive novel probiotic claims.

Conversely to *C. albicans*, which is a biofilm-forming microbe [41], *K. lactis* S-3-05 and *K. marxianus* S-2-05, but also *S. boulardii*, displayed a weak level of biofilm formation attributable to their highly hydrophilic cell surfaces. Our data differ from those reported by Brugnoni et al. [42] regarding biofilm formation by *K. marxianus* strains. Indeed, biofilm formation seems to be exerted in a strain-dependent manner.

A weak drop in cell counts was registered during in vitro gastro-intestinal digestion for *K. lactis* S-3-05, *K. marxianus* S-2-05 and *C. albicans* ATCC 10231, but not for *S. boulardii*. Nonetheless, survival of *K. lactis* S-3-05 and *K. marxianus* S-2-05 was in good agreement with that of *K. marxianus* B0399 [35].

In addition to examining anti-*Salmonella* activity and downregulation of *sopD* gene expression, this study provides additional evidence for the probiotic properties of *K. lactis* S-3-05 and *K. marxianus* S-2-05 isolated from a traditional French cheese.

Uncited references

[40].

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