DAIRY PRODUCTS RESEARCH CENTRE

MOOREPARK

END OF PROJECT REPORT 2000 DPRC No. 32

Stimulation of Propionic Acid Bacteria by Lactic Acid Bacteria in Cheese Manufacture

S. Condon and T.M. Cogan

The interactions between lactic acid bacteria and propionic acid bacteria were studied using a whey model in 32 paired combinations of commercial starter strains.
In all combinations, some degree of stimulation of propionic acid bacteria was found - probably due to peptides, but no inhibition was found.

Improving the Quality of European Hard Cheese by controlling interactions between Lactic Acid Bacteria and Propionic Acid Bacteria (LAB/PAB)

(Stimulation of Propionic Acid Bacteria by Lactic Acid Bacteria in Cheese Manufacture)

Armis No. 4397

Project Team:
Prof. S. Condon* and Prof. T.M. Cogan (Leaders)
Dr. P. Piveteau*
Dr. J. O'Callaghan*
Ms. B. Lyons*
**Microbiology Department, University College Cork*

The Dairy Products Research Centre
Moorepark, Fermoy, Co. Cork.

Teagasc acknowledges with gratitude grant aid under the EU Framework Programme (FAIR 96-CT-1024).
The project was part of a larger EU funded project FAIR 96-CT-1024 involving the following institutions:
Institut Technique Française des Fromages, 35062 Rennes Cedex, France; Entremont, 56140 Malestroit Cedex, France; Centro per lo Studio Tecnologico, Bromatologico e Microbiologico del Latte, National Recherche, 21033-Milan, Italy; Valio Ltd., 00101-Helsinki, Finland; Department of Food Science and Technology, Agricultural University of Athens, 11855-Athens, Greece; Lactalis, 35240 Retier Cedex, France; and Microbiology Department, University College, Cork.

ISBN 1 84170 1866
DPRC No. 32

Teagasc 19 Sandymount Avenue
Ballsbridge Dublin 4

EUROPEAN UNION
European Regional Development Fund

Summary and Conclusions

In the manufacture of Swiss-type cheese two successive fermentations occur. During manufacture, lactic acid bacteria (LAB), particularly ***Streptococcus thermophilus****,* ***Lactobacillus helveticus*** *and* ***Lb. delbrueckii*** *subsp.* ***lactis****, convert lactose to lactate while, during ripening, propionic acid bacteria (PAB) convert lactate to propionic acid, acetic acid and carbon dioxide (CO_2). CO_2 is responsible for eye formation and propionic acid results in the typical nutty flavour of Swiss-type cheese. There have been a few reports of interactions between a small number of LAB and PAB but the compounds involved have not been identified. A better understanding of this phenomenon is necessary to select strains of PAB for cheesemaking and improve the quality of hard cheeses. Cheese cannot be used for such a study because of its complexity and the length of time it is ripened. Hence, a simple whey-based model developed by Piveteau et al (1995) was successfully used to study the interactions between LAB and PAB. In this procedure, the LAB were grown overnight in milk and the whey was collected by centrifugation. After neutralisation and filter-sterilisation, the growth of strains of PAB in this whey and in a control whey produced from the same milk by acidification with lactic acid were compared.*

The objectives of this study were to refine the model of Piveteau et al (1995) to study the interactions between LAB and PAB and to determine the nature of the stimulant(s) produced by the LAB.

Main Conclusions and Achievements

** Thirty-two combinations of different commercial strains of PAB and LAB were evaluated in a modified whey model. None showed any inhibition and all showed some degree of stimulation but the extent of*

the stimulation depended on the particular pair of PAB and LAB used.

** An inhibitor of PAB was found in milk, which prevented the growth of PAB from low (10^5 cfu/ml) but not from high inocula (10^7 cfu/ml). The inhibitor was heat stable (to autoclaving for 15 min), of low molecular mass and could be removed by pre-growth of some but not all starter LAB in milk.*

** Growth of **P. freudenreichii** DPC 3801 in control whey was stimulated by peptone, tryptone, casein hydrolysed by the crude proteinase of **Lb. helveticus** DPC 4571 and by pre-growth of the lactobacillus in milk, but not by vitamins (riboflavin, thiamine, PABA, Ca panthothenate, biotin and nicotinic acid) or minerals ($MgSO_4$, $MnCl_2$, $CoCl_2$ and $CuSO_4$).*

** Growth of **Lb. helveticus** DPC 4571 in milk resulted in significant increases in peptide and amino acid production but the amino acids produced did not stimulate the growth of the PAB. Based on these results it was concluded that the stimulation was due to production of peptides by the LAB from casein.*

** The whey model developed by Piveteau et al (1995) to study the interactions between PAB and LAB was shown to be reproducible. Adjustment of the pH of the whey to 5.4 rather than 6.0, incubation at 24ºC rather than 30ºC and addition of 1% NaCl, to simulate cheese ripening conditions allowed growth of all the PAB tested.*

** Several chromatographic procedures, including ion-exchange, gel permeation and reverse-phase, high-pressure liquid chromatography failed to categorically identify the peptide(s) responsible for the stimulation of the PAB. In some of these chromatographic systems,*

the stimulatory activity was shown to be present in several peaks implying that different peptides were involved.

Research and Results

Improvement of the Whey Model

The original whey model to study the interactions between LAB and PAB consisted in comparing the growth of the PAB strain in control and starter whey at pH 6.0 at 30ºC. The repeatability of this model was very good. The growth rates of *P. freudenreichii* DPC 3801 in control and *Lb. helveticus* DPC 4571 starter wheys were 0.065 h^{-1} ± 0.004 and 0.087 ± 0.010 h^{-1}, respectively (n = 4). After 72 h incubation, the culture reached an A_{600} in control and starter wheys of 0.59 ± 0.043 and 0.95 ± 0.114, respectively (n = 4). Similar results were obtained with *P. freudenreichii* P23 in control and *Lb. delbrueckii* subsp. *lactis* LL51 wheys. The growth rate in control and starter whey was 0.079 ± 0.012 h^{-1} and 0.076 ± 0.018 h^{-1} respectively (n = 3). The final biomass (A_{600}) reached in control and starter whey was 1.19 ± 0.012 and 2.04 ± 0.196 respectively (n = 3). Young Swiss-type cheese contains about 1% NaCl, has a low pH (~5.3 - 5.4), and is ripened for a period ~ 24ºC to promote the growth of PAB. In addition, rennet and a mixed lactic starter flora (mainly *Str. thermophilus* and *Lb. helveticus*) are used in cheese making.

Changing the growth conditions by adding 1% NaCl (w/v), adjusting the pH to 5.4, incubating at 24ºC or adding rennet during whey production reduced the growth of *P. freudenreichii* DPC 3801 in control and starter wheys by no more than 10%. Less than 10%

variation in the growth in LH 41 whey was also observed with *P. freudenreichii* P23 on adding NaCl or rennet, or changing the temperature of incubation. Less growth was observed at lower pH, but the intensity of the stimulation was not affected. Finally, when a strain of *Str. thermophilus* was added to the strain of *Lb. helveticus* (ratio 2:1) for the production of starter whey, both PAB strains produced higher amounts of biomass.

Combining these modifications (i.e. 1% NaCl, pH 5.4, incubation at 24ºC, inclusion of *Str. thermophilus* and rennet), resulted in an increase in the intensity of the stimulation of both PAB strains, even though *P. freudenreichii* P23 reached a lower final biomass compared to the standard conditions (no salt added, pH 6.0, 30ºC). This may be due to the presence of inhibitors in the milk used for these experiments (*see below*).

Using the modified whey model has two major advantages. Firstly, the conditions are close to those encountered in a young Emmental cheese and, secondly, the intensity of stimulation (i.e. the ratio of the biomass produced in starter whey to that produced in control whey) is larger in the modified wheys than in the unmodified whey model. This may be due to the use of sub-optimal conditions of growth in the modified model.

The whey model facilitates the testing of large numbers of strains and gives good reproducibility. Thirty-two pairs of PAB/LAB were tested *(Table 1)*.

No inhibition was observed, and all pairs showed some degree of stimulation compared with the growth of the PAB in control whey. Stimulation was assessed as an increase in final biomass or an increase in growth rate. The increase in final biomass was generally

more dramatic than the increase in growth rate. For a particular PAB strain, the intensity of the stimulation varied with the LAB strain used.

		Lactobacillus							
P. freuden-reichii	**Con-trol**	**CNRZ 32**	**DPC 4571**	**LH 41**	**LH 1**	**LH 56**	**LH 77**	**LB 230**	**LL 51**
DPC 3801	**0.25**	**0.86**	**0.70**	**0.78**	**0.89**	**0.41**	**0.80**	**0.82**	**1.27**
P 23	**0.22**		**0.39**	**0.63**	**0.68**	**0.42**	**0.78**	**0.93**	**1.34**
P 1	**0.32**				**0.93**	**0.58**	**0.87**		**1.44**
P 9	**0.27**				**0.84**	**0.73**	**0.99**		**1.54**
P 20	**0.28**				**0.79**	**0.51**	**1.12**		**1.90**
P 131	**0.22**		**0.42**	**0.51**				**0.67**	**0.94**
CIP 103026	**0.29**	**1.09**							

*Table 1: Stimulation of **P. freudenreichii** by various lactobacilli, measured by final OD at 600 nm in control and lactobacillus wheys.*

Effect of supplementation of control whey on stimulation of PAB

Six vitamins (thiamine, riboflavin, biotin, nicotinic acid, pantothenic acid and p-amino benzoic acid) and four minerals ($MgSO_4$, $MnCl_2$, $CoCl_2$ and $CuSO_4$), at concentrations of 2-20 mg/L and 0.015-1.0 g/L, respectively, had no effect on biomass production by *P. freudenreichii* DPC 3801 in control whey. Some of the minerals, particularly $CoCl_2$ and $CuSO_4$, were inhibitory at concentrations >0.05 g/L.

Inhibitory effect observed in whey produced from commercial skim milk

On several occasions, growth inhibition was observed in control whey produced from commercial skim milk. The occurrence of the inhibition appeared to be related to the date of purchase of the milk. Further work showed that the same effect was obtained in both Irish and French reconstituted skim milk powders and was due to the level of inoculum of PAB used - low inocula (10^5 cfu/ml) did not grow while high inocula (10^7 cfu/ml) did. *P. freudenreichii* P23 was especially sensitive to this inhibition. The inhibitory factor was stable for at least 4 weeks at 20ºC in acid whey. Pasteurising, boiling or autoclaving acid whey did not destroy the inhibitor for *P. freudenreichii* DPC 3801. The inhibitor(s) was soluble in 2M perchloric acid and was not dialysable through a 1200 Da cut-off dialysis tubing; it was lost after dialysis through 12,000 Da dialysis tubing.

Growth of *Lb. helveticus* DPC 4571 in milk resulted in destruction of the inhibitor but incubation for at least 2 days was required to

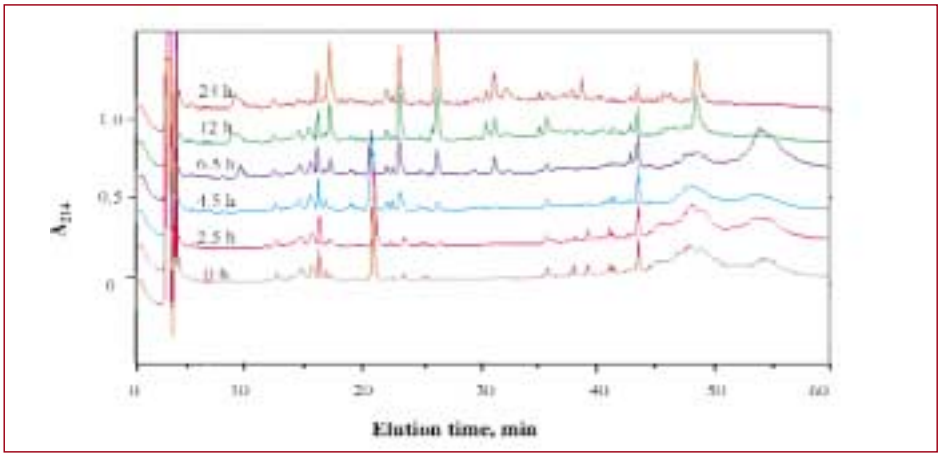


Fig. 1: Shows the utilisation and production of peptides during growth of ***Lactobacillus helveticus*** *in milk at 37ºC. The times of incubation are indicated.*

overcome the inhibition. Growth of *Lb. helveticus* LH 51 and *Str. thermophilus* NCDO 2941 also destroyed the inhibitory compound but growth of *Lb. delbrueckii* subsp. *lactis* LL1, *Str. thermophilus* NCDO 1968, *Lc. lactis* subsp. *lactis* C10 or *Lc. lactis* subsp. *cremoris* AM2 did not.

*Effect of time of incubation of **Lb. helveticus** on subsequent stimulation of PAB*

The increase in the biomass of *P. freudenreichii* DPC 3801 in *Lb. helveticus* DPC 4571 whey, corresponded with an increase in the peptide and amino acid concentration of the whey. The peptide profiles of these wheys also increased as the growth of the lactobacillus increased and some peptides present in the initial milk were used during the fermentation *(Fig. 1)*. The amino acid levels also increased as the incubation progressed but the total amount remained low (~1.6 mmol.L^{-1}). Proline and alanine were the major amino acids produced and arginine, tyrosine, serine, threonine, methioine or isoleucine were not produced. When the amino acid composition of control whey was adjusted to resemble that of starter whey, no stimulatory effect was observed. Aspartate stimulated growth but only at concentrations 50 times higher than that present in the starter whey. Moreover, dialysis of starter whey did not affect the stimulation, even though the amino acid composition decreased to 0.1 mmol.L^{-1}. Therefore, peptides, produced by the proteolytic system of *Lb. helveticus* DPC 4571 were considered to be the most probable cause of the stimulation. Several peptides which have been reported to be released from α_S- and β-casein by the cell wall associated proteinase of other strains of *Lb. helveticus*, CNRZ 303 and CP 790, had only a small effect on biomass production by *P. freudenreichii* DPC 3801 in control whey. Nitrogen in the form of yeast extract and tryptone stimulated growth at all concentrations tested (0.05 - 1% [w/v]).

Effect of casein hydrolysis on PAB stimulation

The crude proteinase of *Lb. helveticus* DPC 4571 was isolated and used to hydrolyse sodium caseinate. Samples were taken at different time intervals over a 24 h incubation period. Stimulatory activity was obtained and was highest after 24 h of hydrolysis. The peptide content of the hydrolysates was examined at each time interval by reverse phase HPLC on a C-18 Primesphere column. A comparison of the profiles before and after hydrolysis showed an increase in the size and frequency of peaks; a major peak was present at ~ 40 min which increased significantly with time.

All of these findings are strong evidence that the stimulation of ***P. freudenreichii*** *by* ***Lb. helveticus*** *is due to production of peptides.*

Fractionation of stimulatory whey

Several chromatography systems including ion-exchange (Dowex A50), gel-permeation (2 different Sephadex G-25 columns and Biogel P-2, Superdex Peptide HR and Spherogel TSK 2000SW columns) and reverse phase HPLC (Primesphere RP and Ultrasphere C-18 columns) were used in an attempt to purify the stimulant(s) produced by *Lb. helveticus* DPC 4571 in milk for *P. freudenreichii* DPC 3801 but all of them were unsuccessful. In many cases, the stimulatory activity was not confined to one area of the chromatograms but spread across several peaks.

This suggests that several peptides were involved but, despite repeated attempts, it proved difficult to isolate and identify them.

The small molecular mass of the compound(s) coupled with the presence of free amino acids in many of the fractions after chromatography made this task particularly difficult. Sometimes there was also a reduction in stimulatory activity after chromatography, which complicated the picture.

Publications

Piveteau, P., Condon, S. and Cogan, T.M. (1995). Interactions between lactic and propionic acid bacteria. *Le Lait* **75**, 331-343.

Piveteau, P. (1997). Stimulation of Propionic Acid Bacteria by Lactic Acid Bacteria. PhD Thesis, *National University of Ireland*.

Quirke, A., Piveteau, P., Cogan, T.M. and Condon, S. (1998). Effect of the level of inoculation on the growth of propionibacteria in whey. Poster presented at the *2nd International Symposium on Propionibacteria*. Cork, Ireland, 25th-27th June 1998.

Piveteau, P., Condon, S. and Cogan, T.M. (1998). Inhibition of lactose utilisation during lactate fermentation of whey. Poster presented at the *2nd International Symposium on Propionibacteria*. Cork, Ireland, 25th-27th June 1998.

Lyons, B., Piveteau, P., Cogan, T.M. and Condon, S. (1998). Purification of stimulant(s) for *Propionibacterium freudenreichii* produced by *Lactobacillus helveticus*. Poster presented at the *2nd International Symposium on Propionibacteria*. Cork, Ireland, 25th-27th June 1998.

Piveteau, P. (1999). Metabolism of lactate and sugars by dairy propionibacteria. A review. *Le Lait* **79**: 23-42.

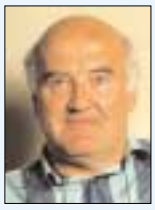
Lyons, B., Piveteau, P., Cogan, T.M. and Condon, S. (1998). Purification of stimulant(s) for *Propionibacterium freudenreichii* DPC 3801 produced by *Lactobacillus helveticus* DPC 4571. *Irish Journal of Agricultural and Food Research* **38**: 184-185.

Piveteau, P., Condon, S. and Cogan, T.M. (2000). Inability of dairy propionibacteria to grow in milk from low inocula. *Journal of Dairy Research* **67**: 65-71.

Piveteau, P., O'Callaghan, J., Lyons, B., Condon, S. and Cogan, T.M. (2001). Invited Paper to the *3rd International Symposium on Propionibacteria.* Zurich Switzerland, July 2001.

For further information, please contact:
Prof. T.M. Cogan

Prof. S. Condon



Prof. T.M. Cogan

DAIRY PRODUCTS RESEARCH CENTRE
Moorepark, Fermoy, Co. Cork, Ireland
Tel: +353 (0) 25 42222 - Fax: +353 (0) 25 42340
E-Mail: reception@moorepark.teagasc.ie