

## Characterisation of the stimulants produced by *Lactobacillus helveticus* in milk for *Propionibacterium freudenreichii*

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**Abstract** — Growth of *P. freudenreichii* DPC 3801 in whey was stimulated by peptone, tryptone, pre-growth of *Lactobacillus helveticus* DPC 4571 in milk and by sodium caseinate hydrolysed with the crude proteinase of *Lb. helveticus* DPC 4571. Addition of vitamins (riboflavin, thiamine, PABA, Ca panthothenate, biotin and nicotinic acid) and minerals ( $MgSO_4$  and  $MnCl_2$ ) to control whey did not improve the growth of *P. freudenreichii*.  $CoCl_2$  and  $CuSO_4$  inhibited growth. Growth of the lactobacillus 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. 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. Several tetra-, penta- and hexa-peptides produced by other strains of *Lb. helveticus* from  $\alpha_s$ - and  $\beta$ -casein had a small but significant effect on growth of *P. freudenreichii* DPC 3801. Based on these results it was concluded that stimulation of *P. freudenreichii* was due to production of peptides by *Lb. helveticus* from casein.

*Propionibacterium freudenreichii* / *Lactobacillus helveticus* / peptide / whey

**Résumé** — Caractérisation des stimulants produits par *Lactobacillus helveticus* dans le lait pour *Propionibacterium freudenreichii*. La croissance de *Propionibacterium freudenreichii* DPC 3801 dans du lactosérum était stimulée par la peptone, la tryptone, une pré-croissance de *Lactobacillus helveticus* DPC 4571 dans le lait et un caséinate de sodium hydrolysé par la protéase purifiée de *L. helveticus* DPC 4571. L'ajout de vitamines (riboflavine, thiamine, PABA, Ca panthothenate, biotine et acide nicotinique) et de minéraux ( $MgSO_4$  et  $MnCl_2$ ) au lactosérum témoin ne stimule pas la croissance de *P. freudenreichii*;  $CoCl_2$  et  $CuSO_4$  l'inhibent. La croissance de *L. helveticus* dans le lait entraîne une augmentation significative en peptides et acides aminés, mais les acides aminés produits ne stimulent pas la croissance des PAB.

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Différentes méthodes chromatographiques, incluant l'échange d'ions, la perméation sur gel, et la phase inverse ne permirent pas d'identifier le(s) peptide(s) responsables de la stimulation. L'activité stimulante était présente dans plusieurs pics chromatographiques indiquant que différents peptides sont impliqués. Plusieurs tetra- et hexapeptides produits par d'autres souches de *L. helveticus* à partir de caséines  $\alpha$  et  $\beta$  ont un effet stimulant faible mais significatif sur la croissance de *P. freudenreichii* DPC 3801. Sur la base de l'ensemble de ces résultats, la stimulation de *P. freudenreichii* serait due à la production par *L. helveticus* de peptides à partir des caséines.

### *Propionibacterium freudenreichii* / *Lactobacillus helveticus* / peptide / lactosérum

## 1. INTRODUCTION

In the manufacture of Swiss-type cheese two successive fermentations occur. The first one, the lactic acid fermentation (LAF) occurs during manufacture and early ripening while the second one, the propionic acid fermentation (PAF), occurs after several weeks of ripening. The LAF is carried out by lactic acid bacteria (LAB), predominantly *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lb. delbrueckii* subsp. *lactis*, which convert lactose to lactate. The PAF is carried out by (PAB), which multiply from  $\sim 10^5$  to  $\sim 10^9$  cfu·mL<sup>-1</sup> during ripening in the hot-room ( $\sim 24$  °C) and convert lactate to propionate, acetate, and CO<sub>2</sub>. This secondary fermentation is essential for the characteristics of the cheese as CO<sub>2</sub> is responsible for eye formation and propionic acid contributes to the typical nutty flavour associated with Swiss-type cheese [4, 6].

Interactions between LAB and PAB apart from the provision of lactate as a carbon source, have been previously reported for both co-cultures [9] and sequential fermentations [2, 5, 8, 10, 14]. The stimulatory effect of LAB on PAB was strain dependent [9, 10] and has been reported in experimental cheeses [8], whey [14] and minimal media [1].

The compounds involved in these interactions have not been identified although Hunter and Frazier [5] reported the stimulants to be dialysable and heat- and acid-stable. Piveteau et al. [10] noted that the

stimulants were also heat-stable and soluble in perchloric acid.

A better understanding of the interactions occurring between LAB and PAB would be useful in selection of strains of PAB to improve the quality of hard cheeses. It is difficult to study this interaction in cheese because of its complexity and the length of time required for ripening. Piveteau et al. [10] developed a simple whey-based model to study the interactions between LAB and PAB. The LAB are grown overnight in reconstituted skim milk after which a sterile whey is produced. Then growth of PAB in this whey and in a whey produced with lactic acid from the same milk are compared. The objectives of this study were to determine the nature of the stimulant(s) for PAB which are produced in milk by LAB.

## 2. MATERIALS AND METHODS

### 2.1. Organisms

*Lb. helveticus* DPC 4571 and *Propionibacterium freudenreichii* DPC 3801 from the culture collection maintained at the Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork were used in this study. *Lb. helveticus* was stored at  $-80$  °C in MRS containing 50% glycerol and was subcultured at least twice in 10% RSM before use. *P. freudenreichii* was grown in YEL broth [7] for 3 d at 30 °C. The composition of YEL was 1% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v)

$\text{KH}_2\text{PO}_4$  and 1% (w/v) D/L sodium lactate, pH 6.5.

## 2.2. Whey production

Starter whey was produced by growing *Lb. helveticus* DPC 4571 for 24 h at 37 °C in sterile (121 °C, 5 min) 10% reconstituted skim milk (RSM). The coagulated milk was centrifuged (8000 g, 10 min, 4 °C), the supernatant was adjusted to pH 6.0 with 2 N NaOH and incubated at 45 °C for 30 min to allow precipitation of colloidal Ca salts to occur. After a second centrifugation (8000 g, 30 min, 4 °C) 1% NaCl was added to the supernatant, the pH was adjusted to 5.4 and the supernatant was filtered through a Whatman No. 1 filter and subsequently filter-sterilised (0.22 µm). Modified control whey was produced by acidifying 10% RSM with 1% of 50% (w/v) DL lactic acid, adding NaCl and adjusting the pH values as described above. Sterile wheys were stored at 4 °C until required.

## 2.3. Treatment of starter whey

Perchloric acid (PCA) was added to starter whey to a final concentration of 2 N. The mixture was centrifuged (5000 g, 10 min, 4 °C) and the PCA in the supernatant precipitated by adjusting the pH to 6.0 with 2 N KOH. This mixture was centrifuged again and the supernatant whey was concentrated by freeze-drying for application to chromatography columns.

## 2.4. Dialysis of starter whey

Dialysis of neutralized PCA treated starter whey (50 mL) was performed over three days in 1200 MWCO benzoylated cellulose tubing (Sigma, Poole, UK) in 8 L of distilled water at 4 °C. The water was changed morning and evening each day. Various MWCO centrifugal concentrators were also used to estimate the size of the stimulatory material.

## 2.5. Stimulation assay

Fractions to be assessed for stimulatory activity were freeze-dried or rotary-evaporated to dryness at 60 °C and reconstituted in modified control whey to a volume equivalent to that which was put on the column. This mixture was filter sterilised, inoculated (1% v/v) with *P. freudenreichii* DPC 3801 and 300 µL aliquots dispensed into wells of microtitre plates. The plates were incubated for 96 h at 30 °C and growth was assessed as an increase in turbidity at 600 nm using a plate reader. Modified control whey was used as the control.

## 2.6. Effect of time on stimulation of PAB

*Lb. helveticus* DPC 4571 was incubated in 10% RSM at 37 °C for 24 h. Samples were collected periodically during the incubation and their pH adjusted to 3.57, the pH after 24 h incubation, with a 50% mixture of DL lactic acid. Whey was produced from each sample and their stimulatory activity assessed by measuring growth of *P. freudenreichii* DPC 3801 at 600 nm after 72 h incubation with. Peptide profiles were analysed by reverse phase HPLC on a semi-preparative reverse phase C-18 Primesphere™ wide-bore column (10 × 250 mm, Phenomenex, Macclesfield, UK) connected to a Shimadzu chromatography system (controller SCL-10A, detector SPD 10A, auto injector SIL 10A, liquid chromatograph, L6-10A).

## 2.7. Cell wall associated proteinase of *Lb. helveticus*

*Lb. helveticus* DPC 4571 was grown anaerobically in 2 L of MRS broth supplemented with 20 mmol·L<sup>-1</sup> CaCl<sub>2</sub> at 37 °C and pH 6.0. After 12 h incubation, the cells were harvested, washed twice in 50 mmol·L<sup>-1</sup> β-glycerophosphate buffer, pH 7.0, containing 20 mmol·L<sup>-1</sup> CaCl<sub>2</sub>.

The proteinase was extracted by three successive washings with 50 mmol·L<sup>-1</sup> Tris-HCl buffer (pH 7.8) in 1/20 the culture volume according to the method described by Zevaco and Gripon [14]. The crude proteinase extract was shown to be free of lactate dehydrogenase activity indicating that little or no cell lysis occurred during the extraction [12]. The crude extract was stored at -80 °C.

### 2.8. Hydrolysis of casein

Sodium caseinate (Kerry Ingredients, Tralee, Ireland) was reconstituted at 3% (w/v) in 0.05 mmol·L<sup>-1</sup> phosphate buffer, pH 7.5, containing 0.1% (w/v) sodium azide. An equal volume of the crude proteinase was added and the solution was incubated for up to 24 h at 37 °C. Samples were collected periodically and trifluoroacetic acid TFA [1.1% (v/v) final concentration] was added to stop enzyme activity. After centrifugation to remove undigested casein, the supernatants were freeze-dried and reconstituted in control whey. Samples were assayed for stimulation by the microtitre plate assay and proteolysis was estimated by HPLC on the C-18 Primesphere column.

### 2.9. Measurement of amino acids

Total amino acids were estimated by the procedure of Folkertsma and Fox [3], as modified by Baer [1]. Amino acid profiles were determined on a cation exchange column (Model 6300, Beckman Instruments Ltd., UK).

### 2.10. Fractionation of the stimulatory whey

PCA treated starter whey was applied to a Dowex 50 cation exchange column (30 × 120 mm) according to the method described by Porfirio et al. [11]. The column was washed with 200 mL volumes of

distilled water, 2 N NaOH, distilled water, and 2 N HCl and was equilibrated with 300 mL 0.2 mol·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, pH 2 and 200 mL of 0.02 mol·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, pH 2. The sample was applied at pH 2 and was washed through with 0.02 mol·L<sup>-1</sup> Na<sub>2</sub>PO<sub>4</sub>, pH 2 (acid fraction). The remaining material was eluted from the column with a 0 to 100% NH<sub>4</sub>OH (1 N) gradient. Only this material was collected and assayed. The flow rate was 1.5 mL·min<sup>-1</sup> and 10 mL fractions were collected. The absorbance of the fractions was measured at 214 nm, rotary evaporated at 60 °C and assayed for stimulatory activity.

PCA treated starter whey was also chromatographed on a Spherogel-TSK-2000SW (7.5 × 600 mm, Beckman, Fullerton, California, USA) gel-permeation column and stimulatory fractions were applied to a Superdex Peptide HR 10/30 column (10 × 300 mm, Pharmacia). The mobile phase for both columns was 30% (v/v) acetonitrile containing 0.1% TFA. The flow rates were 1 mL·min<sup>-1</sup> and 0.5 mL·min<sup>-1</sup>, respectively and 1 mL fractions were collected. The fractions were pooled according to the peak profile (A<sub>214</sub>) and were concentrated by rotary evaporation at 60 °C and assayed for activity.

Gel-permeation chromatography of the stimulatory whey was also conducted on two Sephadex G25-50 columns (Pharmacia, Uppsala, Sweden). The larger column (25 × 900 mm) was eluted with 30% (v/v) acetonitrile and the smaller one (17 × 500 mm) with distilled water. The flow rates were 0.3 mL·min<sup>-1</sup> and 3 mL fractions were collected. Their absorbances were monitored at 214 and 280 nm and pooled according to the 214 nm profile. The peaks were freeze dried, reconstituted in control whey, and assayed for stimulatory activity. A Biogel P2 column (10 × 250 mm) was used to reduce the concentration of amino acids in the fractions from the small Sephadex G-25 column. The eluate was distilled water and the flow rate was

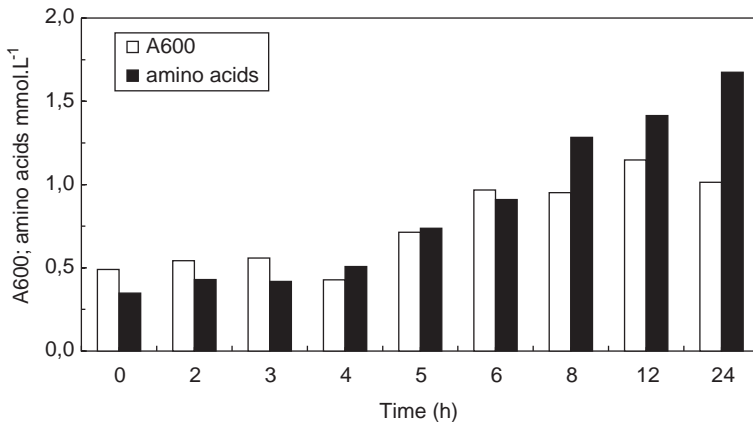
3 mL·h<sup>-1</sup>. The effluent was monitored at 214 nm and fractions were pooled according to the peaks on the chromatogram.

The peaks from the G-25 column displaying stimulatory activity were subjected to HPLC chromatography on either a semi-preparative, reverse phase Primosphere™ C-18 column (10 × 250 mm, Phenomenex) or an analytical Coulter Ultrasphere® 5µ C18 RP-HPLC column (4.6 × 250 mm, Beckman). The former was connected to a Shimadzu chromatography system (controller SCL-10A, detector SPD 10A, auto injector SIL 10A, liquid chromatograph, L6-10A) while the latter was connected to a Beckman system (solvent module 125 NMD, detector 168 NM and autosampler 508). For the Primosphere system, a gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid (TFA) in 60 min and a flow rate of 2.5 mL·min<sup>-1</sup> was used. For the Beckman system, the parameters were a gradient of 0 to 65% acetonitrile in 0.1% TFA in 80 min and a flow rate of 1 mL·min<sup>-1</sup>. A dual wavelength detector at 214 and 280 nm was used and fractions were collected every min.

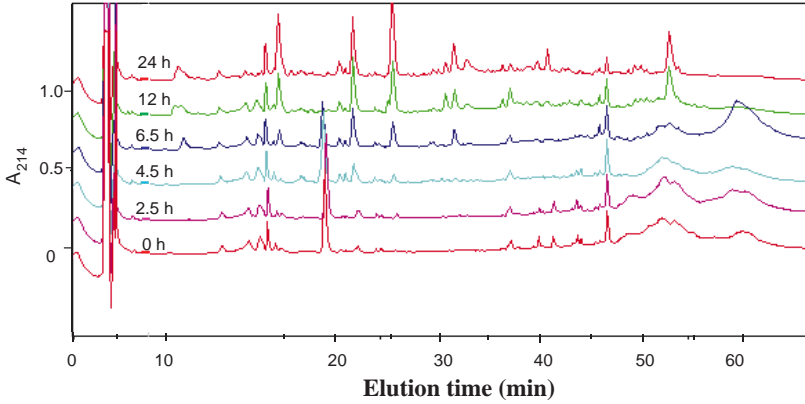
### 3. RESULTS

#### 3.1. Effect of time of incubation of LAB on stimulation of PAB

The effect of whey, produced after growth of *Lb. helveticus* DPC 4571 for different times, on the growth of *P. freudenreichii* DPC 3801 is shown in Figure 1. Stimulation was apparent even after 2 h of fermentation, reached a maximum after 12 h of fermentation and decreased at 24 h. Extensive proteolysis occurred during growth of the *Lactobacillus* as indicated by the increase in the free amino acid contents from 0.36 mmol·L<sup>-1</sup> to ~1.6 mmol·L<sup>-1</sup>. Proline and alanine were the major amino acids that accumulated and arginine, tyrosine, serine, threonine, methioine and isoleucine were not detected (data not shown). However, no evidence was obtained which suggested that the stimulants of the PAB were amino acids. When the amino acid composition of control whey was adjusted to resemble that of starter whey, produced after 24 h incubation of the lactobacillus, a stimulatory effect on the growth of *P. freudenreichii* DPC



**Figure 1.** Growth of (open bars) *P. freudenreichii* DPC 3801 in whey produced from milk fermented for various times with *Lb. helveticus* DPC 4571. The free amino acid levels of the wheys prior to inoculation with the PAB are also shown (closed bars).



**Figure 2.** Peptide profiles, determined by RP-HPLC on a C18-Primesphere column, of wheys produced from milk fermented for various times, indicated on the profiles, with *Lb. helveticus* DPC 4571. For clarity, the data is offset on the y-axis.

3801 was not observed. Dialysis of starter whey through a 1200 MW cut off membrane did not result in a loss of stimulation, even though the amino acid levels decreased to  $0.10 \text{ mmol}\cdot\text{L}^{-1}$ . Centrifugal concentrator dialysis gave an estimated molecular mass of the stimulant(s) of between 1 and  $3 \text{ kg}\cdot\text{mol}^{-1}$ , which is significantly greater than that of an amino acid.

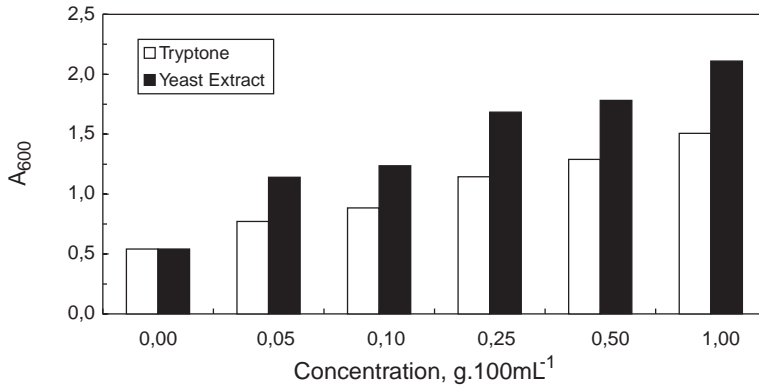
Extensive changes in the peptide composition of the whey also occurred during growth of *Lb. helveticus* DPC 4571 in the milk as shown by analysis using a column designed to separate peptides. Peptide profiles of whey analysed following increasing times of incubation of the *Lactobacillus* are shown in Figure 2. Several peaks present in the milk at inoculation disappeared during growth of the *Lactobacillus*, e.g., those with retention times of 20.5, 37.9, 39.1 and 41.1 min, while others increased, e.g., those with retention times of 23.8, 26.7, 30.2, 31.5 and 48.8 min (Fig. 2). The peak at 43.5 min increased in intensity during the 24 h incubation. The significance of the latter observation is unclear.

### 3.2. Supplementation of control whey

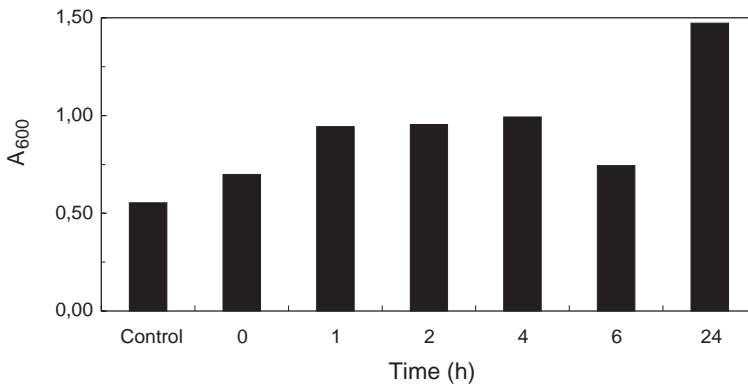
Six vitamins (thiamine, riboflavin, biotin, nicotinic acid, pantothenic acid and *p*-amino benzoic acid) at final concentrations of 2, 10 and  $20 \text{ mg}\cdot\text{L}^{-1}$  and two salts ( $\text{MgSO}_4$  and  $\text{MnCl}_2$ ) at concentrations of 0.015 to  $1 \text{ g}\cdot\text{L}^{-1}$ , had no effect on biomass production by *P. freudenreichii* DPC 3801 in control whey.  $\text{CoCl}_2$  and  $\text{CuSO}_4$  also had no stimulatory activity and were, in fact, inhibitory at concentrations  $>0.015 \text{ g}\cdot\text{L}^{-1}$ . In contrast, yeast extract and tryptone stimulated growth of *P. freudenreichii* DPC 3801 in control whey at all concentrations tested [0.05–1% (w/v)] (Fig. 3).

### 3.3. Effect of casein hydrolysates on PAB stimulation

The crude proteinase of *Lb. helveticus* DPC 4571 contained no LDH activity indicating that lysis of the cells did not occur during extraction of the enzyme and that the enzyme is associated with the cell wall. Sodium caseinate was hydrolysed for 24 h



**Figure 3.** Effect of addition of yeast extract and tryptone on the growth of *P. freudenreichii* DPC 3801 in control whey.



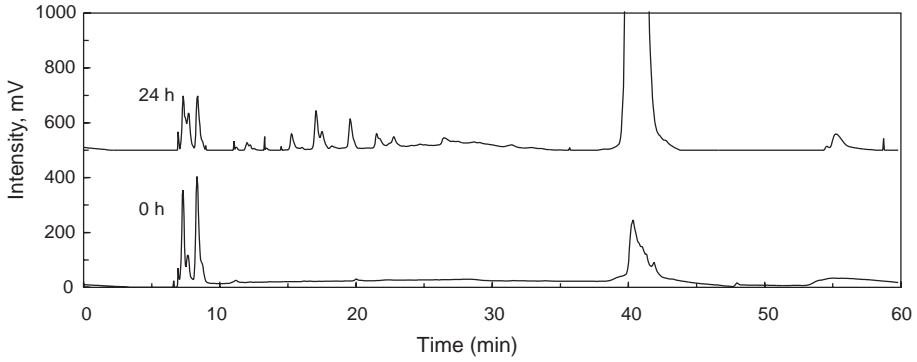
**Figure 4.** Stimulation of growth of *P. freudenreichii* DPC 3801 by hydrolysates of sodium caseinate produced during incubation with the crude proteinase of *Lb. helveticus* DPC 4571 for up to 24 h.

with the crude enzyme. The hydrolysates stimulated *P. freudenreichii* DPC3801 when added to control whey. Stimulatory activity was highest after 24 h incubation of the casein with the *Lactobacillus* proteinase (Fig. 4). The peptide composition of the hydrolysates was examined on a reverse phase HPLC on a C-18 Primesphere system and a comparison of the peptide profiles before and after hydrolysis for 24 h is shown in Figure 5. An increase in the size and frequency of peaks was apparent particularly at the beginning of the chromatogram; a major peak was present at ~40 min which increased significantly with time. A com-

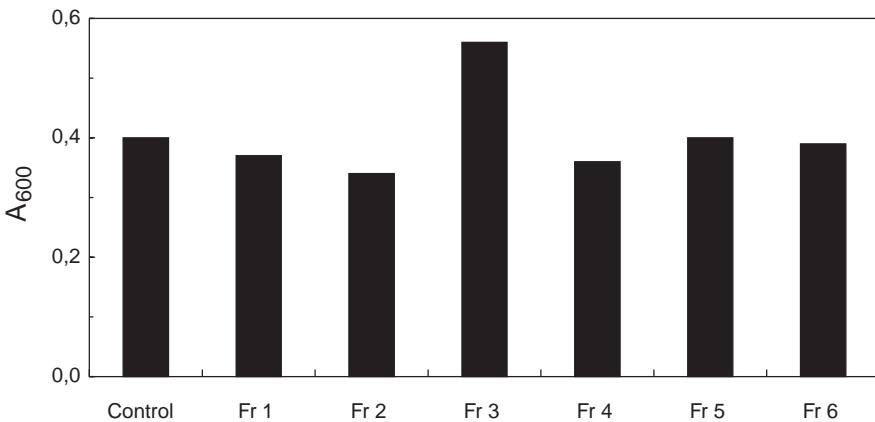
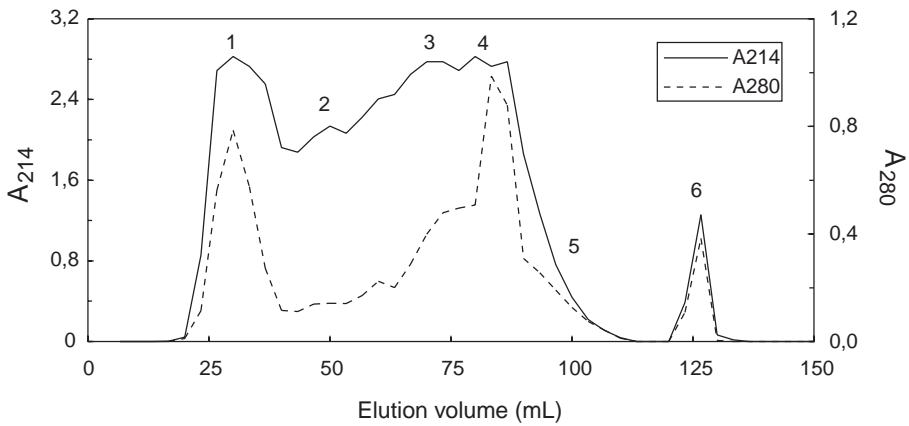
parison of the peptide profiles before and after hydrolysis for 24 h is shown in Figure 5.

### 3.4. Fractionation of stimulatory whey

The stimulant for *P. freudenreichii* DPC 3801 is soluble in 2 mol·L<sup>-1</sup> PCA [10] and this was frequently used as an initial step in purification of the stimulatory compounds. Several chromatography systems including ion-exchange, gel-permeation and reverse phase HPLC were used in an



**Figure 5.** Comparison of the peptide profiles of sodium caseinate before and after hydrolysis for 24 h with the crude proteinase of *Lactobacillus helveticus*. For clarity the 24 h data are offset by 500 mV in the plot.



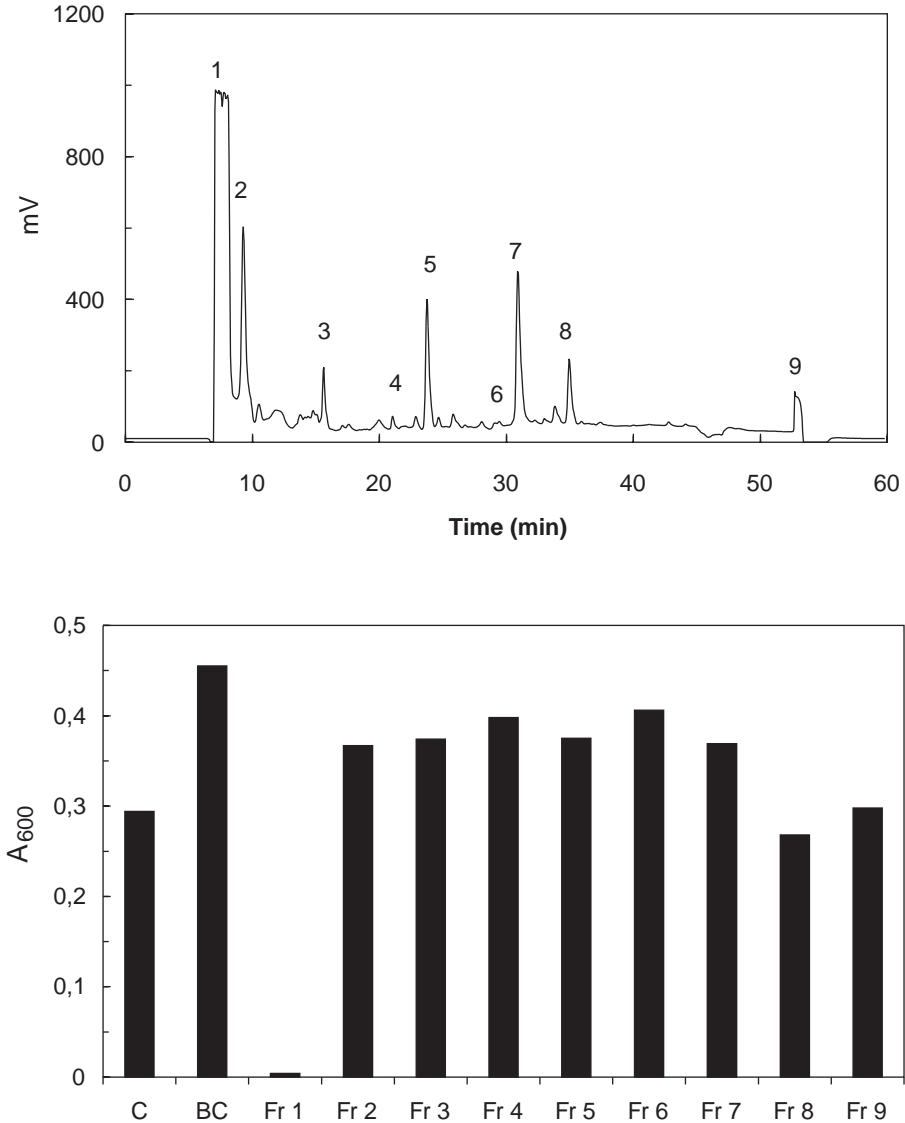
**Figure 6.** Chromatogram (top) and bioassay (bottom) of 10 mL of *Lb. helveticus* DPC 4571 when on a Sephadex G25-50 column (17 × 500 mm) eluted with distilled water at a flow rate of 20 mL·h<sup>-1</sup>.



attempt to purify the stimulants(s) but none were successful. Chromatography of the PCA extracted starter whey on a Dowex A50, weak cation exchange column, using  $\text{NH}_4\text{OH}$  as eluent or on a Spherogel-TSK-2000SW gel-permeation column did not re-

solve the activity but the stimulatory activity was spread among several peaks (data not shown).

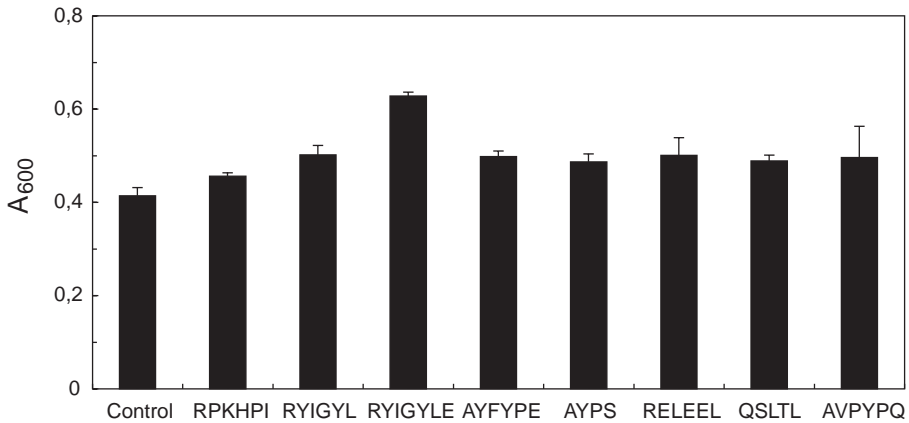
On Sephadex G-25, using water as the eluent, the material separated into 2 major peaks on the basis of  $A_{280}$  but was poorly



**Figure 7.** Chromatogram (top) and activity (bottom) of fraction 3 from the G25-50 Sephadex column after RP-HPLC on a C18-Primesphere column.

resolved on the basis of  $A_{214}$  (Fig. 6). Fractions were mixed on the basis of  $A_{214}$  and all the activity was concentrated in fraction 3. Free amino acids were also present in this fraction. Chromatography of this peak on a Biogel P2 column reduced the levels of free amino acids in the peak but did not eliminate them. Further purification of fraction 3 was conducted on a Primesphere C-18 HPLC column. Nine peptide peaks were obtained, six of which were stimulatory to

*P. freudenreichii* (Fig. 7); fraction 1 was inhibitory. The stimulation was less in all peaks than in the unfractionated material applied to the column, implying that several peptides are likely to be involved. Peak 5 was re-chromatographed on the Primesphere column and an amino acid sequence of the peptide by Edman degradation failed because of the presence of free amino acids in the sample. It was felt that peptides could be adsorbing on to the Sephadex column



**Figure 8.** Stimulation of growth of *P. freudenreichii* DPC 3801 in control whey by peptides derived from  $\alpha_s$ - and  $\beta$ -casein. The peptides and their positions are summarized in Table I.

**Table I.** Small peptides produced from  $\alpha_s$ - and  $\beta$ -casein by *Lactobacillus helveticus*.

Peptide	Casein	Fragment	Strain of <i>Lb. helveticus</i>
RPKHPI	$\alpha_s$ -	1-6	CP 790 and CNRZ 303
RYLGYL*	$\alpha_s$ -	90-95	
RYLGYLE*	$\alpha_s$ -	90-96	
AYFYPE	$\alpha_s$ -	143-148	CP 790 and CNRZ 303
AYPS	$\alpha_s$ -	158-161	CP 790
RELEEL	$\beta$ -	1-6	CNRZ 303
QSLTL	$\beta$ -	123-127	CP 790
AVPYPQ	$\beta$ -	176-182	CP 790

All were synthesised at the University of Nottingham except those marked by \* which were obtained from Sigma.

with water as the eluent. However, the use of 30% acetonitrile as eluent on a larger G-25 Sephadex column did not improve the resolution (data not shown).

Since the purification of the putative peptides appeared to be very difficult, several peptides which have been reported to be released from  $\alpha_s$ - and  $\beta$ -casein by the cell wall associated proteinase of *Lb. helveticus* CNRZ 303 and *Lb. helveticus* CP 790 [13, 15] were synthesised (Tab. I). These and two which are available commercially (Sigma) were tested for their effect on *P. freudenreichii* DPC 3801. The stimulation of growth by all of them was small but statistically significant ( $p > 0.05$ ) (Fig. 8). RYLGYLE differs from RYLYL by only one amino acid, yet growth stimulation by the former was much greater than by the latter.

#### 4. DISCUSSION

The stimulation of *P. freudenreichii* DPC 3801 became apparent in whey following a 2 h fermentation by *Lb. helveticus* DPC 4571 and reached a maximum after 24 h. The concentration of lactate in the whey at various times was constant, eliminating this nutrient as a factor in stimulation. A variety of vitamins and minerals had no effect on the growth of *P. freudenreichii* DPC 3801 in control whey. Some of the minerals, particularly  $\text{CuSO}_4$  and  $\text{CoCl}_2$  were inhibitory at higher concentrations. This indicates that the vitamin and mineral composition of control whey are satisfactory for the growth of PAB. Nitrogen, added as either yeast extract or tryptone, was stimulatory even at the lowest concentration tried (0.05% w/v) suggesting that peptides or amino acids were involved.

The increase in the biomass of *P. freudenreichii* in whey after pre-growth of *Lb. helveticus*, corresponded with an increase in the peptide and amino acid concentrations of the whey. The overall level

of amino acids in the whey was low and no stimulation of growth was observed when these levels were added to the control whey. Therefore, peptides, produced by the proteolytic system of *Lb. helveticus* DPC 4571 were considered to be the most probable cause of stimulation. An increase in stimulatory activity was obtained on addition of casein hydrolysed with the crude proteinase of *Lb. helveticus* DPC 4571 and this correlated with an increase in the concentration of peptides as measured by RP-HPLC. The data in Figure 2 showed a marked increase in the number of peptides produced by the *Lactobacillus* during growth in milk. These findings are strong evidence that the stimulation of *P. freudenreichii* by *Lb. helveticus* is due to production of peptides in the milk.

Several different chromatographic systems were used in an attempt to isolate the stimulatory compounds produced by the *Lactobacillus* in milk and most did not resolve the activity to any great extent. In many cases, the stimulatory activity was not confined to one area of the chromatogram but spread across several peaks. This suggests that several peptides may be involved which, individually, have only limited activity but when present together exert a significant influence on growth. Despite several attempts, it proved difficult to isolate them. The small molecular mass of the compound(s) coupled with the presence of free amino acids in many of the fractions after chromatography appeared to be the reason for this. Sometimes there was also a reduction in stimulatory activity after chromatography, which complicated the picture.

Support for the involvement of peptides in the stimulation of *P. freudenreichii* was obtained when several tetra-, penta- and hexa-peptides previously shown to be produced by two other strains of *Lb. helveticus* from casein [13, 15] were synthesised and shown to stimulate growth to a small but statistically significant extent. One

hepta-peptide, RYLGYLE, which is not a product of the hydrolysis of  $\alpha_s$ -casein by these strains of *Lb. helveticus*, had a marked effect on growth. The same peptide without the last amino acid had a much lower effect. From all of this data it is concluded that several peptides are involved in the stimulation of growth of *P. freundenreichii* by *Lb. helveticus*.

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