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# Hydrophilic and hydrophobic peptides produced in cheese by wild *Lactococcus lactis* strains

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**Aims:** To study the production of hydrophilic and hydrophobic peptides in cheese by 32 wild *Lactococcus lactis* strains of different RAPD patterns and to compare them with the peptides produced by lactococcal cells incubated with whole casein.
**Methods and Results:** Chromatograms of peptides from cheeses made using each strain as single starter culture were divided into five regions, and strains were classified in three groups by hierarchical cluster analysis of region areas. Thirty out of the 32 wild *L. lactis* strains produced higher levels of hydrophobic peptides in cheese than on whole casein.
**Conclusions:** Cheese was a more favourable substrate than whole casein for hydrophobic peptide formation by *L. lactis* strains.
**Significance and Impact of the Study:** New strains of lactococci should be screened for bitterness under cheese conditions, as the formation of hydrophobic peptides may be underestimated in assays with casein as substrate.

## INTRODUCTION

The water-soluble fraction of cheese is formed mainly by peptides and amino acids produced from casein breakdown by rennet and proteinases and peptidases from *Lactococcus lactis* (Fox *et al.* 1994). Bitter fractions have been associated to late running material on RP-HPLC, thought to be hydrophobic peptides, and savoury fractions with early running material (Cliffe *et al.* 1993). A relationship between the level of hydrophobic peptides and cheese bitterness has been found (Gómez *et al.* 1997).

Numerous studies in aqueous buffers have identified that different lactocepins (cell-envelope associated proteinase of *L. lactis*) produce different peptides from casein hydrolysis (Monnet *et al.* 1986, 1989; Visser *et al.* 1988, 1994; Reid *et al.* 1991a, b, 1994; Juillard *et al.* 1995). During casein degradation *in vitro* with cell-wall proteinase fractions, less bitterness was generated by *L. lactis* AM1, a strain with lactocepin III, than by *L. lactis* HP, a strain with lactocepin I (Visser *et al.* 1983a). An examination of the peptides produced in cheeses made with different starters revealed the presence of bitter peptides in cheeses made with bitter strains and with nonbitter strains, and it was concluded that bitterness depended on the amount of bitter peptides produced (Visser *et al.* 1983b).

In a previous work we carried out a classification of wild strains of *Lactococcus lactis* based on their activity on casein fractions, and on their peptide profiles after incubation of lactococcal cells with whole casein (Morales *et al.* 2001). Here, we have analysed their peptide profiles in cheese to compare them with the peptide profiles obtained on whole casein, and to relate them to the type of proteinase.

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## MATERIALS AND METHODS

### Bacterial strains

Nine wild strains of *L. lactis* subsp. *cremoris* and 23 wild strains of *L. lactis* subsp. *lactis* isolated from ewes' raw milk cheeses (Gaya *et al.* 1999), with different RAPD patterns, were used in our experiments. Five collection strains of *L. lactis* subsp. *cremoris* (AM1, AM2, HP, SK11 and Wg2) and one collection strain of *L. lactis* subsp. *lactis* (NCDO 763) were obtained from NIZO (Ede, The Netherlands). All lactococcal strains were maintained in M17 broth (Biolife Srl, Milan, Italy) at − 80 °C.

### Cheese manufacture

Small cheeses, approx. 260 g weight, were made from 2 l pasteurized whole milk (total viable counts under 100 cfu $ml^{-1}$) using each *L. lactis* strain as single starter culture. Milk at 31 °C with 0·1 g $l^{-1}$ $CaCl_2$ added was inoculated with 1% of a fresh culture in sterile milk and incubated for 20 min at this temperature. After that, 1·33 ml of a fresh 2% (w/v) dilution of Maxiren 150 rennet (Gist Brocades, Delft, The Netherlands) were added and milk was held at 31 °C for 40 min to clot. The curd was cut, heated to 38 °C and incubated for 15 min at this temperature to favour whey expulsion. The whey was discarded and the curd was pressed overnight at 20 °C. Next morning cheeses were salted for 20 min in a 150-g $l^{-1}$ NaCl solution at 20 °C, and left to ripen for 3 d at 12 °C. Afterwards they were wrapped in aluminium foil, vacuum packed and kept at − 40 °C until analysis.

### Extraction and analysis of water-soluble peptides

The water-soluble fraction of cheeses was obtained by a modification of the method described by Aston and Creamer (1986). Grated cheese (60 g) was mixed with 50 ml of distilled water, homogenized in an Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany), and centrifuged at 6000 rev $min^{-1}$ for 20 min at 4 °C. The aqueous layer was kept and the residue was homogenized again with 50 ml of water and then centrifuged at 12 000 rev $min^{-1}$ for 30 min at 4 °C. The aqueous layer of both extractions were mixed, and methanol was added in the ratio 2 : 1 aqueous layer : methanol. This mixture was kept overnight at 4 °C, and then filtered through Whatman 42 paper to eliminate the methanol-insoluble material. The extract was concentrated in a Büchi Rotavapor-R (Büchi, Switzerland) to eliminate methanol, and then freeze-dried. Lyophilizate was weighed to calculate the recovery, dissolved at 100 mg $ml^{-1}$ in water (HPLC grade), filtered (Millex HV 0·45 $\mu$m) and subjected to RP-HPLC analysis.

Hydrophobic and hydrophilic peptides were determined by RP-HPLC as described by Lau *et al*. (1991). A Beckman System Gold chromatograph (Beckman Instruments España SA, 28034 Madrid, Spain), equipped with a programmable solvent module 126, a diode array detector module 168 and an autosampler 502 was used. Detection was at 214 and 280 nm. Peaks with retention times from 14·6 min were considered to correspond to hydrophobic peptides (Gómez *et al*. 1997). Results were expressed as units of chromatogram area $mg^{-1}$ cheese dry matter.

### Statistical analysis

Cluster analysis was used to discover the natural grouping of the strains. The statistical program used was SPSS Win 5·0 (SPSS, Chicago, IL, USA). Data obtained from RP-HPLC chromatograms were subjected to hierarchical cluster analysis (HCA) using Ward's method, with the Euclidean distance as a measure of the proximity between two strains and variables previously standardized to a normal distribution. The dendrogram shows, in a 0–25 scale, the rescaled linkage distance, equivalent to 25 linkage distance/maximal distance.

Levels of hydrophobic and hydrophilic peptides recorded for cheeses made with strains from different HCA groups were subjected to one-way analysis of variance and comparison of means by Tukey's test.

## RESULTS

Collection strains showed three patterns of RP-HPLC chromatograms (data not shown), the first for *L. lactis* strains HP and Wg2, the second for strains AM2 and NCDO 763, and the third for strains AM1 and SK11. Chromatograms of peptides produced in cheese by collection strains and wild strains were subdivided into three hydrophilic regions and two hydrophobic regions, as previously described (Morales *et al*. 2001). An HCA was carried out with the levels of peptides in each of the five regions for the different strains, and the results are shown in Fig. 1. Three main groups of strains were obtained, and in each of them two collection strains were present. Group 1 contained collection strains AM1 and SK11, both with type III proteinase, together with wild strains with type III proteinase (R20, N20, C11, G17), I or I/III proteinase (A1) and II or II/III proteinase (H18, G16, F15, P21). Group 2 harboured collection strains HP, with type I proteinase, and Wg2, with type I/III proteinase, together with wild strains with type I or I/III proteinase (L20, S26), type III proteinase (D12, Q25) and type II or II/III proteinase (B6, E14, S27, A5, J16, E13, K16). Finally, collection strains AM2, with type II/III proteinase, and NCDO 763, with type II proteinase, together with wild strains with type II or II/III proteinase (A3, A4, N22, C10, P24, O23), type III proteinase (B8, M21, I19, B7, C9) and type I or I/III proteinase (A2) were included in group 3. There was no clear relationship between peptide profiles obtained from HPLC analysis of the water-soluble fraction of cheese and the type of proteinase.

Data for the strains included in the three groups of Fig. 1 were subjected to one-way analysis of variance and comparison of means (Table 1). Only two regions, R1 and R4, were significantly different between groups of collection strains, but all five regions were significantly different between groups of wild strains. The highest mean values of total hydrophilic and total hydrophobic peptides were found for collection strains and wild strains in group 3. Wild

strains showed lower mean levels of total hydrophilic peptides than collection strains in all three groups. Regarding mean levels of total hydrophobic peptides, wild strains had higher values than collection strains in groups 1 and 2, and a lower value in group 3 (Table 1).

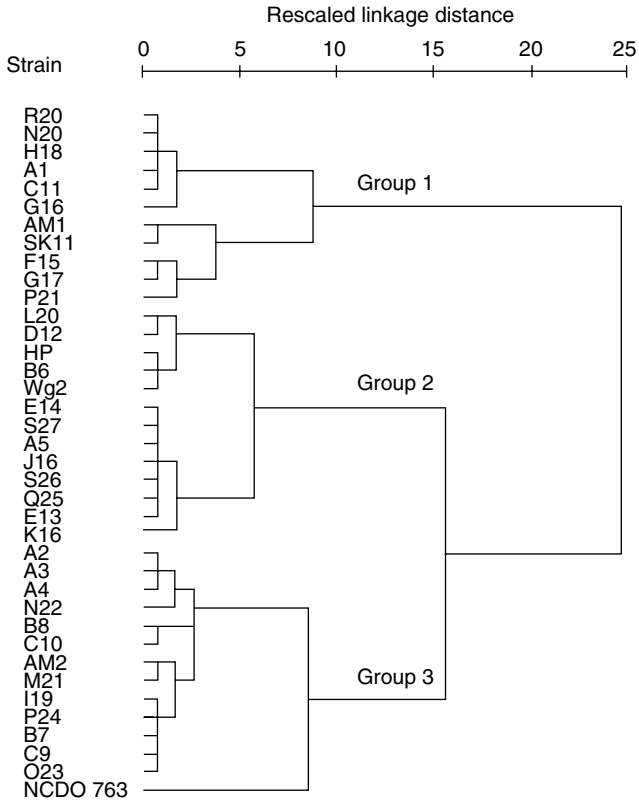


**Fig. 1** Dendrogram from the hierarchical cluster analysis of collection and wild strains of *Lactococcus lactis* with the chromatogram areas of three regions of hydrophilic peptides and two regions of hydrophobic peptides produced in cheese. Collection strains were *L. lactis* AM1, SK11, HP, Wg2, AM2 and NCDO 763

Levels of peptides produced on whole casein (Morales *et al.* 2001) were subjected to one-way analysis of variance with the strain groups defined in the present work (Fig. 1) as main effect. Collection strains and wild strains in group 3 exhibited the highest mean values of total hydrophilic and total hydrophobic peptides produced on whole casein (data not shown).

Peptide regions in cheese (Table 1) have units different from those of peptide regions from whole casein (Morales *et al.* 2001) and cannot be directly compared. For it to be feasible, peptides produced in cheese and on whole casein have been expressed as a percentage relative to peptides produced in the same substrate by *L. lactis* strain HP, chosen as reference because it has been previously described as a bitter strain (Visser *et al.* 1983a). Wild strains produced more hydrophobic peptides in cheese than on whole casein, with the only exceptions of strains N22 and M21 (Table 2). No significant correlation ($r = 0{\cdot}251$) was found between hydrophobic peptides formed by wild strains in cheese and on whole casein.

## DISCUSSION

Different wild *L. lactis* strains were grouped with collection strains in dendrograms constructed with casein degradation data, hydrophilic and hydrophobic peptides, or selected hydrophilic peptides (Morales *et al.* 2001). In the present work, the dendrogram obtained with peptides in the chromatograms from the water-soluble fraction of cheeses (Fig. 1) also showed considerable differences in strain classification when compared with the dendrogram constructed with peptides in chromatograms from assays on whole casein (Morales *et al.* 2001). Strains in group 3 of the dendrogram in Fig. 1 exhibited the highest levels of

**Table 1** Hydrophilic and hydrophobic peptides* produced in cheese by 32 wild strains and six collection strains of *Lactococcus lactis*†

| | Collection strains | | | Wild strains | | |
|---|---|---|---|---|---|---|
| Peptides | Group 1 | Group 2 | Group 3 | Group 1 | Group 2 | Group 3 |
| Hydrophilic | | | | | | |
| R1 | $24{\cdot}97^{a}$ | $41{\cdot}58^{b}$ | $40{\cdot}60^{ab}$ | $27{\cdot}90^{a}$ | $35{\cdot}11^{b}$ | $35{\cdot}97^{b}$ |
| R2 | $34{\cdot}08^{a}$ | $25{\cdot}88^{a}$ | $43{\cdot}32^{a}$ | $27{\cdot}08^{a}$ | $26{\cdot}17^{a}$ | $33{\cdot}08^{b}$ |
| R3 | $13{\cdot}57^{a}$ | $18{\cdot}33^{a}$ | $24{\cdot}45^{a}$ | $12{\cdot}64^{a}$ | $15{\cdot}56^{a}$ | $18{\cdot}71^{b}$ |
| Total | $72{\cdot}62^{a}$ | $85{\cdot}80^{a}$ | $108{\cdot}37^{a}$ | $67{\cdot}62^{a}$ | $76{\cdot}84^{b}$ | $87{\cdot}76^{c}$ |
| Hydrophobic | | | | | | |
| R4 | $18{\cdot}40^{a}$ | $30{\cdot}64^{ab}$ | $39{\cdot}70^{b}$ | $28{\cdot}39^{a}$ | $36{\cdot}27^{b}$ | $39{\cdot}26^{b}$ |
| R5 | $47{\cdot}79^{a}$ | $47{\cdot}33^{a}$ | $65{\cdot}36^{a}$ | $62{\cdot}51^{b}$ | $52{\cdot}98^{a}$ | $60{\cdot}31^{b}$ |
| Total | $66{\cdot}20^{a}$ | $77{\cdot}97^{a}$ | $105{\cdot}06^{a}$ | $90{\cdot}90^{ab}$ | $89{\cdot}25^{a}$ | $99{\cdot}57^{b}$ |

*Expressed in chromatogram area units per mg of cheese dry matter. Mean values of collection strains or wild strains with the same superscript do not differ ($P < 0{\cdot}05$).
†Groups 1, 2 and 3 of strains are those in Fig. 1.

**Table 2** Hydrophobic peptides* produced in cheese and on whole casein by 32 wild strains and six collection strains of *Lactococcus lactis*†

| Group 1 | Cheese | Casein | Group 2 | Cheese | Casein | Group 3 | Cheese | Casein |
|---|---|---|---|---|---|---|---|---|
| AM1 | 86 | 105 | HP | 100 | 100 | AM2 | 114 | 129 |
| SK11 | 79 | 100 | Wg2 | 94 | 101 | NCDO 763 | 148 | 117 |
| R20 | 121 | 82 | L20 | 98 | 27 | A2 | 120 | 72 |
| N20 | 124 | 60 | D12 | 93 | 53 | A3 | 118 | 68 |
| H18 | 121 | 71 | B6 | 94 | 82 | A4 | 128 | 82 |
| A1 | 114 | 31 | E14 | 119 | 62 | N22 | 107 | 124 |
| C11 | 107 | 47 | S27 | 121 | 46 | B8 | 143 | 87 |
| G16 | 136 | 41 | A5 | 122 | 72 | C10 | 141 | 78 |
| F15 | 91 | 44 | J16 | 123 | 49 | M21 | 125 | 131 |
| G17 | 103 | 97 | S26 | 114 | 40 | I19 | 122 | 79 |
| P21 | 102 | 43 | Q25 | 116 | 78 | P24 | 125 | 86 |
| | | | E13 | 109 | 58 | B7 | 117 | 81 |
| | | | K16 | 115 | 36 | C9 | 117 | 107 |
| | | | | | | O23 | 126 | 93 |

*Expressed as a percentage on the hydrophobic peptides produced in cheese or on whole casein, respectively, by *Lactococcus lactis* subsp. *cremoris* HP.
†Groups 1, 2 and 3 of strains are those in Fig. 1. Collection strains were *L. lactis* AM1, SK11, HP, Wg2, AM2 and NCDO 763.

total hydrophobic peptides in cheese. Therefore, cheeses made with those strains should be the most bitter, according to the relationship found between levels of hydrophobic peptides and intensity of bitter flavour (Gómez *et al*. 1997).

Collection strains used in this work were chosen because their proteolytic system was well known. However, incubation conditions may affect peptide formation and degradation. Thus, the cell-wall proteinase fraction of strain AM1 produces less bitterness on whole casein than that of HP, but the bitter score is higher for AM1 than for HP when the assay is carried out with whole cells (Visser *et al*. 1983a). The essential role of peptidases in debittering and the importance of physico-chemical conditions for peptide uptake have been proved (Visser 1993).

In the present work, 28 out of 32 wild *L. lactis* strains produced higher levels of hydrophobic peptides in cheese than strain HP, and the rest ranged from 90 to 100% the level of strain HP hydrophobic peptides (Table 2). As *L. lactis* strain HP is considered a bitter strain, it is highly probable that most of our wild strains would develop bitterness in cheese. However, if hydrophobic peptides formed in the casein assay were taken into account, five out of nine strains in group 1 and five out of 11 strains in group 2 would yield levels of hydrophobic peptides under 50% the level of strain HP and might therefore be considered nonbitter strains.

From the above results it may be concluded that peptide formation and accumulation by new strains of lactococci should be investigated under cheese conditions, because assays on casein may underestimate the potential formation of hydrophobic peptides and the risk of bitterness in cheese.

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