Autolytic phenotype of *Lactococcus lactis* strains isolated from traditional Tunisian dairy products

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Running headline: AUTOLYTIC PHENOTYPE IN DAIRY *LACTOCOCCUS LACTIS* STRAINS

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SUMMARY

Aims: To evaluate the autolytic properties of *Lactococcus lactis* strains isolated from artisan Tunisian dairy products, the peptidoglycan hydrolases content and their activity spectrum.

Methods and results: The autolytic phenotype of *Lactococcus* strains was evaluated under starvation conditions in potassium phosphate buffer. The results obtained highlighted a high degree of diversity among the analyzed strains allowing the identification of high and low autolytic *Lactococcus lactis* strains. Peptidoglycan hydrolases content was evaluated by renaturing SDS-PAGE using cells of *Micrococcus lysodeikticus* as a target for the enzymatic activity and a major activity band migrating at about 45 kD was observed. The lytic activity, evaluated in the presence of different chemicals, was retained in 8% NaCl, 15 mM $CaCl_2$ and in a range of pH between 5 and 9.5. The substrate specificity of peptidoglycan hydrolase from *Lactococcus* strains was evaluated in renaturing SDS-PAGE incorporating cells of different bacterial species. The major autolysin of *Lactococcus lactis* was active against cells of *Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus* and *Listeria monocytogenes*.

Conclusions: The autolytic activity is widely distributed in *Lactococcus lactis* and the rate of autolysis is strain-dependent. The major peptidoglycan hydrolase showed a wide spectrum of activity against several lactic acid bacteria and bacterial species involved in food-related infection.

Significance and Impact of the study: The autolytic phenotype of *Lactococcus lactis* strains isolated from Tunisian artisan dairy products has been determined and the data obtained should allow the selection of strains of technological interest in cheese ripening process.

INTRODUCTION

The autolytic activity of lactic acid bacteria (LAB) is an important factor in cheese ripening due to the release of intracellularly-located enzymes into the curd and their action on flavour development. Autolysis is a complex process involving different enzymes that are classified with the generic name of peptidoglycan hydrolases. In particular, *N*-acetylmuramidases, *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidase and endopeptidases play a key role in the cell autolysis (Chapot-Chartier 1996). Because of the importance of LAB autolysis in cheese ripening, several studies have been done to detect and characterize autolytic enzymes (Buist *et al.* 1997; Pillidge *et al.* 1998; Cibik and Chapot-Chartier 2000; Husson-Kao *et al.* 2001). To date the most characterized LAB peptidoglycan hydrolase is the major autolysin of *Lactococcus lactis* (Buist *et al.* 1995; Pillidge *et al.* 1998) and several food-grade rDNA systems have been developed to control their release into the curd with the aim of accelerating cheese ripening and/or to modify cheese flavour development (Buist *et al.* 1997; de Ruyter *et al.* 1997; Sanders *et al.* 1997). However, the use of genetically modified starters in cheese is not always accepted and an alternative approach consisting on the selection of natural starter strains having ideal autolytic properties has been recently suggested (Pillidge *et al.* 1998).

The aim of this study is to characterize *Lactococcus lactis* strains isolated from traditional Tunisian fermented milk products, on the basis of their autolytic activity evaluating also the autolysin spectrum of activity against LAB species commonly used in starter preparation.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Strains of *Lactococcus lactis* and *S. thermophilus* ATCC 20617^T were routinely maintained at 4 °C after growth at 30 °C for 12 or 24 h in M17 broth (Difco) or M17 broth implemented with glucose (GM17) at the final concentration of 1% (w/v). For longer term maintenance, stock cultures were stored in 20 % (v/v) glycerol, 80 % (v/v) M17 at - 20 °C and - 80 °C. The strains of *Lactococcus lactis* used in this work and their origin are shown in Table 1. Strains of, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T and *Lactobacillus helveticus* ATCC 15009^T, were grown in MRS broth at 37 °C, *Staphylococcus aureus* DSM 799, *Listeria monocytogenes* MACa1 and *Clostridium sporogenes* CL14 were grown at 37 °C in nutrient broth, Brain Heart Infusion broth and Reinforced Clostridial Medium respectively.

DNA extraction and PCR condition

For the PCR reaction 100 µl of an overnight culture in M17 broth were processed as previously described (Mora *et al.* 2000). All the DNA solutions obtained were stored at -20 °C. The subspecies-specific PCR assay targeted to the histidine biosynthesis operon was carried out as described by Beimfohr et al. (1997). One set of primer targeted to the *acmA* gene sequences (Buist *et al.* 1995; Pillidge *et al.* 1998) was used for the amplification of 450 bp fragment from the coding region of the gene (ACMAF 5' – GCTGTTCTTATTGCCGGAAC –3'; ACMAR 5' – GTCTTGATAGGTGGCAGCA –3') using the following PCR conditions: reactions were performed in 25 µl of volume containing 1 µl of bacterial DNA solution obtained as above, 1/10 volume of 10X reaction buffer (Amersham Pharmacia Biotech), 200 µmol l^{-1} of each deoxynucleoside triphosphate (dNTP), 2.5 mmol l^{-1} of $MgCl_2$ and 0.5 µmol l^{-1} of each primers. Temperature profile was carried out with a primary DNA denaturation step at 94 °C for 2 min followed by 35 cycles of 45 sec at 94 °C, 35 sec at 58 °C and 40 sec at 72 °C, the final extension was continued for 7 min at 72

°C. After the amplification, 5 µl of product were electrophoresed at 5 V/cm (1.5 % agarose gel, 0.2 mg ml^{-1} of ethidium bromide) in TAE buffer and photographed in UV light.

Autolysis of whole cells in buffer solution

The autolytic phenotype of *Lactococcus* strains was evaluated on harvested exponential phase cells ($O.D._{600}$ = 1-1.5) grown in M17 or GM17, washed in potassium phosphate buffer (50 mmol l^{-1}, pH 6.5) and resuspended in the same buffer or in MES buffer (2-(N-Morpholino-ethanesulfonic acid) (50 mmol l^{-1}, pH 6) to an $O.D._{600}$ of 0.6-0.8 and incubate at 30 °C. The degree of autolysis was expressed as the percentage decrease of the $O.D._{600}$ after 72 h..

Mutanolyisin activity

Mutanolysin actyvity was evaluated on *Lactococcus* cells harvested in exponential phase and resuspended in MES buffer (50 mmol l^{-1}, pH6) supplemented with 1 mmol l^{-1} $MgCl_2$ to an $O.D._{600}$ of about 0.5. A volume of 3 ml of cells suspension were equilibrated at 37 °C and the $O.D._{600}$ was evaluated. Subsequently 50 µl of mutanolysin solution (150 U ml^{-1}) (Sigma) prepared in TES buffer (N-tris-Hydroxymethyl-methyl-2-aminoethansulfonic acid) (50 mmol l^{-1}, pH7), 1 mmol l^{-1} $MgCl_2$ were added to the cell suspension and the $O.D._{600}$ were evaluated after 20 min at 37 °C. The mutanolysin activity were expressed as the percentage decrease of the $O.D._{600}$.

Cell proteins extraction

Whole-cell SDS extracts were carried out by resuspending harvested cells from 5 ml GM17 culture, previously washed in sterile water, in 100 µl of a solution containing 10 mmol l^{-1}Tris-HCl pH 8, 10 mmol l^{-1} EDTA, 10 mmol l^{-1} NaCl and 2% SDS (w/v). The obtained suspension was vigorously mixed and boiled at 100 °C for 5 min, centrifuged at 13000 *g* for 10 min and the supernatant fluid containing the cell-protein extract recovered. Protein contents were determined by the Bradford method (Bradford, 1976) and 3-4 µg of protein extraction were analyzed in renaturing SDS-PAGE.

Renaturing SDS-PAGE and detection of peptidoglycan hydrolase activity

Renaturing SDS-PAGE electrophoresis was performed as described by Buist et. al. (1995) with 12% polyacrilamide separating gel in a Mini-Protean III apparatus (Biorad Laboratories). Autoclaved cells of *Micrococcus lysodeikticus* ATCC 4698 (Sigma) were incorporated into polyacrylamide running gels at a concentration of 0.2 % (w/v). Substrate specificity of peptidoglycan hydrolases was evaluated as follows: cells of *Lactococcus lactis* subsp. *lactis* BMG6.5, *Streptococcus thermophilus* MIM-M2, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T, *Lactobacillus helveticus* ATCC 15009^T, *Sthaphylococcus aureus* DSM 799, *Listeria monocytogenes* MACa1 and *Clostridium sporogenes* CL14 were harvested from 25 ml of culture broth grown 12-18 h in the appropriate media at 30-37°C, washed and resuspended in 1 ml of sterilized distilled water and incorporated into polyacrylamide running gel. Sample preparation was carried out mixing 40 µl of whole-cell SDS extract and 30 µl of loading buffer containing 500 mmol l^{-1} Tris-HCl, pH 6.8, 10% (w/v) SDS, 25% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.5% (w/v) bromophenol blue. After electrophoresis, gels were soaked in 200 ml of sterilized, distilled water for 30 min and then in 200 ml of 25 mmol l^{-1} Tris-HCl (pH 7) containing 1% (w/v) of Triton X-100 for 12-36h. Bands of lytic activity were visualized by staining the gel with 1% (w/v) methylene blue (Sigma) in 0.01% (w/v) KOH and subsequent destaining in sterilized distilled water.
The molecular weight of the lytic bands were estimated by running in the same gel a prestained molecular weight marker (New England Biolabs; MBI-Fermentans). The effects of different chemicals on peptidoglycan hydrolases activity were evaluated incubating gel slices in renaturation buffer containing $CaCl_2$ (0.5-15 mmol l^{-1}) or NaCl (0.5-8%). The effect of pH was evaluated using renaturation solution buffered with 10 mmol l^{-1} sodium acetate (pH 5) or 10 mmol l^{-1} Tris-HCl (pH 7-9.5).

RESULTS

Subspecies specific identification of *Lactococcus lactis* strain and PCR detection of major autolysin gene *acmA*

All *Lactococcus lactis* strains listed in table 1 were identified at a subspecies level using PCR primer targeted to the histidine biosynthesis locus as described by Beimfohr et al. (1997). The results obtained showed that 26 of the 31 *Lactococcus lactis* strains isolated from traditional Tunisian dairy products were identified as *Lactococcus lactis* subsp. *lactis* while the remaining 6 strains were ascribed to the subspecies *cremoris*. With regards to the reference strains, the histidine targeted PCR approach confirmed their taxonomic position. A specific PCR assay developed for the amplification of a 450 bp fragment from the coding region of the *acmA* major lactococcal autolysin was developed. All the strains listed in table 1 showed positive PCR signals indicating the wide distribution of acmA genetic determinant in *Lactococcus lactis* strains.

Autolytic behavior of dairy *Lactococcus* strains and mutanolysin sensitivity

The extent of autolysis of *Lactococcus* strains in phosphate buffer or MES buffer after 72 h of incubation at 30 °C was evaluated using exponential phase cells grown in M17 or GM17. The incubation time of 72h was chosen because no significant variations were observed upon further incubation. The results obtained are reported in Figure 1. The autolytic behavior of *Lactococus* strains grown in presence of glucose plus lactose (GM17) and in presence of lactose (M17) was different for most of the analysed strains. The extent of autolysis ranged between 10.3 to 56.8% and 6.8 to 54% for GM17 and M17 cultures respectively. Interestingly, one of the most autolytic strain grown in GM17 (strain BMG6.5) showed the lower autolytic extent when grown in M17 without glucose. As reported in Figure 1, most of the analyzed strains showed 10 % more of autolytic extent when grown in presence of glucose and for strains BMG6.5, ATCC 11454, BMG6.8, BMG6.33, BMG6.19 and BMG6.112, the

increase of autolytic activity in GM17 ranged between 21.8 to 35.2%. The cell-wall structure and the cell osmotic sensitivity of *Lactococcus lactis* strains was carried out evaluating the mutanolysin activity for each *Lactococcus* strains harvested in exponential grow phase in M17. Despite the major autolytic activity of *Lactococcus lactis* strains being detected when cells were grown in glucose containing medium, the mutanolysin activity was evaluated in cells grown in lactose containing medium because of the dairy utilization of *Lactococcus lactis* strains. Also in this case, a high variability was detected among the analyzed strains. The extent of mutanolysin activity ranged between 9% (strain BMG6.20) and 93.7% (strain ATCC 11603) with values ranging between 20 and 35% for most of the strains (Fig. 2).

Detection and activity spectrum of peptidoglycan hydrolase activity

All *Lactococcus lactis* strains were analyzed for the detection of peptidoglycan hydrolase activity in renaturing SDS-PAGE containing cells of *Micrococcus lysodeikticus*. The autolytic profiles obtained for *Lactococcus lactis* strains, compared with those of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T and *Lactobacillus helveticus* ATCC 15009^T, were shown in Figure 3a. *Lactococcus* strains were characterized by a major autolytic band at molecular weight of about 45 kDa that was visible after few hours of renaturation (Fig. 3a), according to the reported data of Buist et al. (1995) and Pillidge et al. (1998), Moreover, secondary lytic signals, that appeared after 12 or 24 h of renaturation, were detected at about 54 kDa and three more weak bands were observed at a molecular weight ranging between 30 and 35 kDa (Fig. 4a). The peptidoglycan hydrolases activity of all *Lactococcus* strains, compared under the same conditions, showed the same electrophoretic profiles with some variations in the intensity of the bands (Fig. 3b). The main lytic bands of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. helveticus* migrated at a molecular weight of about 30-32 kDa according to experimental data previously detected by Valence and Lortal (1995) and Lortal et al.(1997) (Fig. 3a). The activity spectrum of peptidoglycan hydrolases of *Lactococcus lactis* strains were evaluated in renaturing SDS-PAGE containing cells of lactic

acid bacteria, cells of pathogenic species and cells involved in spoilage of dairy products. Substrate specificity of peptidoglycan hydrolases was evaluated on the strain BMG6.02 whole-cell SDS. The lytic pattern showed by *Lactococcus* strains against cells of *Micrococcus lysodeikticus* was maintained only when *Lactococcus lactis* subsp. *lactis* ATCC 11454 and *Lactobacillus helveticus* ATCC 15009^T cells were used as substrate while lytic activity showed against *L. delbrueckii* subsp. *lactis*, *Streptococcus thermophilus* MIM M2 and *Listeria monocytogenes* MACa1 was retained only by the main peptidoglycan hydrolase band of 45 kDa. No activity was detected on *Staphylococcus aureus* DSM 799 and *Clotridium sporogenes* CL14.

Influence of $CaCl_2$, NaCl concentration and pH on peptidoglycan hydrolases profile and activity

The activity of peptidoglycan hydrolases of *Lactococcus* strains were tested in renaturing SDS-PAGE using several concentration of $CaCl_2$ (1-15 mmol l^{-1}) and NaCl (0.5-8%; w/v) in the renaturation buffer. Surprisingly, the major autolysin, retained activity in the presence of 15 mmol l^{-1} $CaCl_2$ and in presence of 8% NaCl as shown in the Figure 4b-d. Interestingly, secondary lytic bands, at 30, 35 and 54 kDa lost activity in presence of 0.5-1% of NaCl and 1 mM of $CaCl_2$ (Fig. 4b-d). Concerning the effect of the pH, the major lytic band (45 kDa) showed activity from pH 5 to 9.5, while secondary bands at 54, 30-35 KDa were well visible on renaturing gel at pH 7. At pH5 and pH8 secondary bands showed a weak activity that was not detectable at pH 9.5. Moreover, further experiments carried out using renaturation buffers containing 10 mM $CaCl_2$, 6% NaCl at pH 5 and 8, clearly showed that the main peptidoglycan hydrolase and a secondary molecular species at about 32 kDa were still active (Fig. 4h).

DISCUSSION

In this study the autolytic phenotype of several *Lactococcus lactis* strains isolated from traditional Tunisian Dairy products was carried out. Furthermore, the identification at a subspecies level of the strains was performed with the aim of highlighting potential differences in the autolytic phenotype and peptidoglycan hydrolases content between *Lactococcus lactis* subspecies. The identification at a subspecies level, carried out using the histidine locus targeted PCR described by Beinmfohr et al. (1997), revealed that the majority of *L. lactis* strains (81%) belonged to the subspecies *lactis* and the remaining 19% to the subspecies *cremoris*. In this context, the prevalence of the subspecies *lactis* should be related to the artisan production of Tunisian dairy products, characterized by a limited utilization of starters. In fact, *L. lactis* subsp. *lactis* strains are commonly isolated from environmental sources, whereas *L. lactis* subsp. *cremoris* strains are isolated principally from dairy environments in which starters are often used (Corroler *et al.* 1998).

With regards to the autolytic phenotype of *L. lactis* strains, a high degree of diversity was highlighted but no significant differences were detected between the two subspecies. The autolysis of *L. lactis* strains under starvation condition appeared as a strain-dependent character strongly influenced both by the carbon source utilized for cells culture preparation and by the buffered solution composition. As previously reported for *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and for *Leuconostoc* strains (Moustafa and Collins, 1968; Cibik and Chapot-Chartier, 2000) the highest extent of autolysis was observed by growing strains in glucose containing medium confirming the hypothesis of a weak cell wall structure in cultures grown in presence of glucose. The use in this study of GM17 medium, containing glucose and lactose, suggests also that in the presence of both carbon sources *Lactococcus lactis* preferentially utilizes glucose. With regards to the buffer used in the starvation condition significant differences in the extent of autolysis were observed between phosphate

and MES buffer while the main autolytic strains in the two buffers were mostly the same. MES buffer allowed a higher extent of autolysis in half of the tested strains. The results obtained suggested that factors other than the carbon source in the medium could play a role in the rate of autolysis in *L. lactis*. In this context, the mutanolysin test underlined differences in the cell-wall and/or cell osmotic sensitivity to lytic enzymes among the analysed strains. The results obtained were compared with the extent of autolysis evaluated in the same condition (MES buffer) (Fig. 2) but it was not possible to extrapolate a strict correlation between the two enzymatic activities. Nevertheless, the lowest values of mutanolysin activities were detected in strains showing a weak autolytic behavior, likewise the highest extents of mutanolysin activities were detected within the group of the most autolytic strains (Fig. 2). These results were according with data reported by Meijer and coworkers (Meijer *et al.* 2001) that observed a correlation between cell stability and the cell-wall sensitivity to mutanolysin and the autolytic activity in *Lactococcus lactis* strains.

Peptidoglycan hydrolase content was almost the same in all the analysed strains with some variations in the intensity of the bands. According to Buist et al. (1995) and Riepe et al. (1997) one major lytic band and several secondary molecular species were detected in all the analyzed strains. The major lytic band at about 45 kDa should correspond to the major peptidoglycan hydrolase AcmA described by Buist et al. (1995). The disagreement in apparent molecular weight (45 kDa of the major lytic band detected instead of 42 kDa of AcmA) could be due to variance in protein electrophoretic mobility between traditional SDS-PAGE and SDS-PAGE containing cells of *Micrococcus lysodeikticus*. The major lytic band of about 45kDa was equally active on *Lactococcus lactis* cells, and on cells of other lactic acid bacteria commonly used in the preparation of dairy starters such as, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*. These data confirmed the observation of Mercier et al. (2000) on *S. thermophilus* and highlight the potential effect of lactococcal muramidase on a mixed bacterial community

during the cheese ripening. Moreover, the effect of the major peptidoglycan hydrolase on *Listeria monocytogenes* could have important effect in biopreservation processes.

The activity of peptidoglycan hydrolases of *Lactococcus lactis* strains, evaluated using renaturing buffers of several composition and different pH, revealed that the major molecular species at about 45 kDa and a secondary band were active in high salt concentration and low pH suggesting their potential role in cheese ripening condition when the pH is low and Ca ions and NaCl concentration were increasing. Moreover, the different levels of activity of the secondary peptidoglycan hydrolases bands evaluated when different renaturation conditions were carried out, could substantiate the hypothesis of the presence of more than one enzymatic activity as suggested by Pillidge et al. (1998) and that not all the bands detected by renaturing electroporesis are related to the AcmA degradation as indicated by Buist et al. (1995).

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REFERENCES

Beimfohr, C., Ludwig, W. and Schleifer, K. H. (1997) Rapid genotypic differentiation of *Lactococcus lactis* subspecies and biovar. *Systematic and Applied Microbiology* **20**, 216-221.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry* **72**, 248-254.

Buist, G., Kok, J., Leenhouts, K. J., Dabrowska, M., Venema, G. and Haandrikman, J. (1995) Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis* a muramidase needed for cell separation. *Journal of Bacteriology* **177**, 1554-1563.

Buist, G., Karsens, H., Nauta, A., van Sinderen, D., Venema, G. and J. Kok. (1997) Autolysis of *Lactococcus lactis* caused by induced overproduction of its major autolysin, AcmA. *Applied and Environmental Microbiology* **63**, 2722-2728.

Chapot-Chartier, M.-P. (1996) Les autolysins des bactèries lactiques. *Lait* **76**, 91-109

Cibik, R. and Chapot-Chartier, M.-P. (2000) Autolysis of dairy leuconostocs and detection of peptidoglycan hydrolases by renaturing SDS-PAGE. *Journal of Applied Microbiology* **89**, 862-869.

Corroler, D., Mangin, I., Desmasures, N. and Gueguen, M. (1998) An ecological study of lactococci isolated from raw milk in the Camembert Cheese Registered Designation of origin area. *Applied and Environmental Microbiology* **64**, 4729-4735.

de Ruyter, P.G.G.A., Kuipers, O.P., Meijer, W.C. and de Vos, W.M. (1997) Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. *Nature Biotechnology* **15**, 976-979.

Husson-Kao, C., Mengaud, J., Benbadis, L. and Chapot-Chartier, M.-P. (2000) MurI, a *Streptococcus thermophilus* peptidoglycan hydrolase devoid of a specific cell wall binding domain. *FEMS Letters* **187**, 69-76.

Lortal, S., Valence, F., Bizet, C. and Maubois, J.-L. (1997) Electrophoretic pattern of peptidoglycan hydrolases, a new tool for bacterial species identification: application to 10 *Lactobacillus* species. *Research in Microbiology* **148**, 461-474.

Meijer, W, van Schalkwijk, S., Kingma, F., Abee, T., Bunthof, C. and Hugenholtz, J. (2001) Role and control of starter lysis in cheese production. Proceeding of the NIZO Dairy Conference on Food Microbes 2001.

Mercier, C., Domakova, E., Tremblay, J. and Kulakauskas, S. (2000) Effects of a muramidase on a mixed bacterial community. *FEMS Microbiology Letters* **187**, 47-52.

Mora, D., Fortina, M.G., Parini, C., Daffonchio, D. and Manachini, P.L. (2000) Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationships between pediocin AcH/PA-1 producing and non-producing strains. *Microbiol.-UK* **146**, 2027-2038.

Moustafa, H.H., and Collins, E.B. (1968) Role of galctose or glucose-1-phosphate in preventiong the lysis of *Streptococcus diacetylactis*. *Journal of bacteriology* **95**, 592-602.

Pillidge, C. J., Govindasamy-Lucey, S., Gopal, P. K. and Crow, V.L. (1998) The major lactococcal cell wall autolysin AcmA does not determine the rate of autolysis of *Lactococcus lactis* subsp.*cremoris* 2250 in cheddar cheese. *International Dairy Journal* **8**, 843-850.

Riepe, H.R., Pillidge, C.J., Gopal, P.K. and McKay L.L. (1997). Characterization of the highly autolytic *Lactococcus lactis* subsp. *cremoris* strains CO and 2250. *Applied and Environmental Microbiology* **63**, 3757-3763.

Sanders, J.W., Venema, G. and Kok, J. (1997) A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*. *Applied and Environmental Microbiology* **63**, 4877-4882.

Valence, F. and Lortal, S. (1995) Zymogram and preliminary characterization of *Lactobacillus helveticus* autolysins. *Applied and Environmental Microbiology* **61**, 3391-3399.

Table 1. *Lactococcus lactis* strains, their origin, relevant characteristics and subspecies PCR identification.

Strain		subspecies PCR identification	Origin and relevant characteristic
L. lactis	ATCC 11454	*lactis*	Nisin A producer
" "	L481	*lactis*	Lactococcin 481 producer
" "	BMG6.3	*lactis*	Leben (fermented milk)
" "	BMG6.125	*lactis*	Leben (fermented milk)
" "	BMG6.01	*lactis*	Leben (fermented milk)
" "	BMG6.02	*lactis*	Leben (fermented milk)
" "	BMG6.89	*lactis*	Leben (fermented milk)
" "	BMG6.48	*lactis*	Leben (fermented milk)
" "	BMG6.4	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.12	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.43	*lactis*	Artisan Tunisian pressed cheese
" "	BMG6.7	*lactis*	Artisan Tunisian white cheese
" "	BMG6.19	*lactis*	Artisan Tunisian white cheese
" "	BMG6.14	*lactis*	Artisan Tunisian white cheese
" "	BMG6.112	*lactis*	Artisan Tunisian white cheese
" "	BMG6.121	*lactis*	Artisan Tunisian white cheese
" "	BMG6.04	*lactis*	Leben (fermenred milk)
" "	BMG6.6	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.20	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.31	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.36	*lactis*	Leben (fermented milk)
" "	BMG6.37	*lactis*	Leben (fermented milk)
" "	BMG6.21	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.25	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.33	*lactis*	Artisan Tunisian pressed cheese
" "	BMG6.5	*lactis*	Artisan Tunisian "ricotta-like" cheese
" "	BMG6.8	*lactis*	Artisan Tunisian whitre cheese
" "	ATCC 11602	*cremoris*	Containing lysogenic bacteriophage
" "	ATCC 11603	*cremoris*	
" "	MIMLac	*cremoris*	Lactococcin A producer
" "	L117	*cremoris*	
" "	BMG6.42	*cremoris*	Artisan Tunisian white cheese
" "	BMG6.18	*cremoris*	Artisan Tunisian pressed cheese
" "	BMG6.74	*cremoris*	Caillè (fermented milk)
" "	BMG6.13	*cremoris*	Caillè (fermented milk)
" "	BMG6.2	*cremoris*	Leben (fermented milk)
" "	BMG6.26	*cremoris*	Leben (fermented milk)

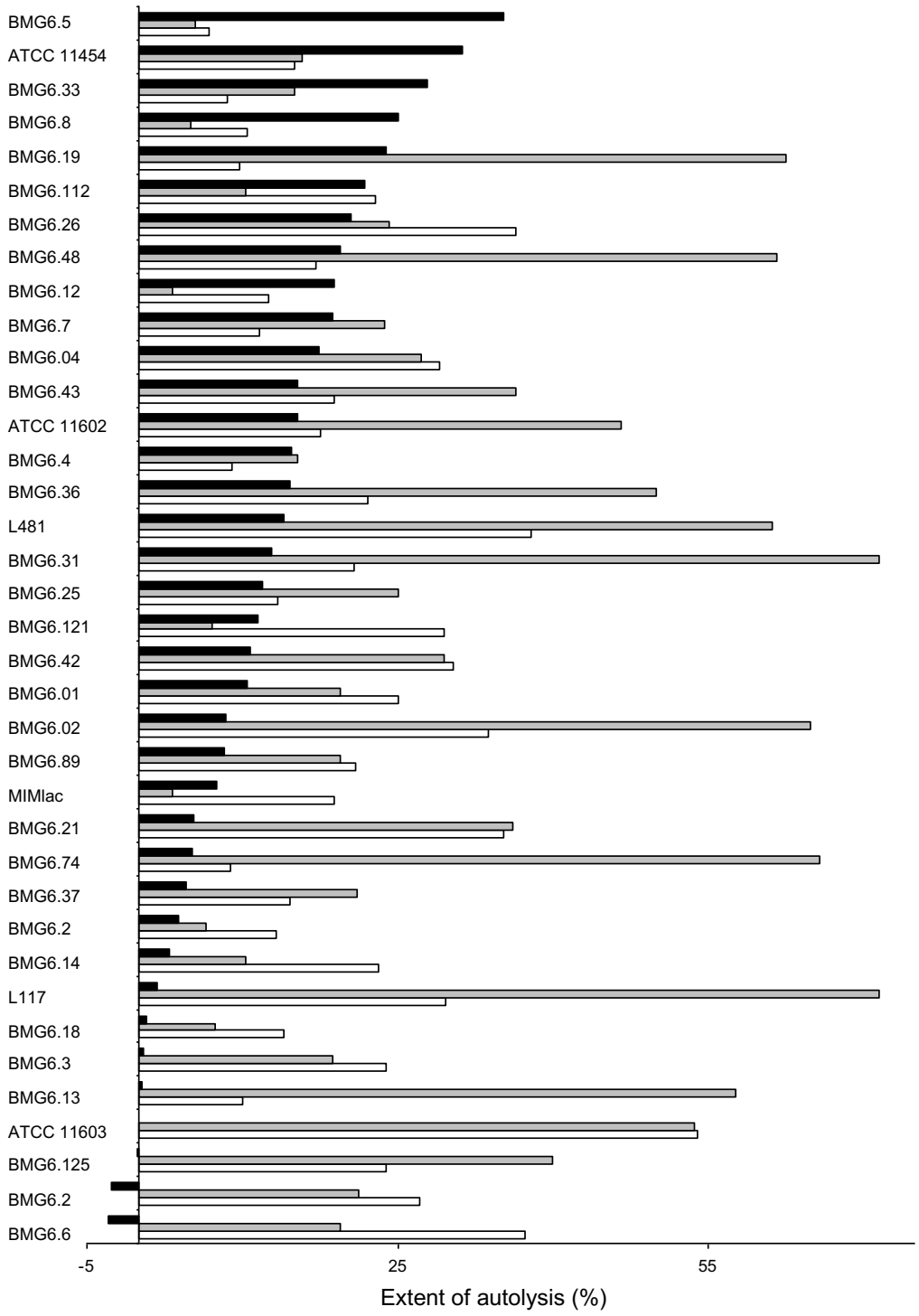

Fig. 1 Extent of autolysis of *Lactococus lactis* strains grown in M17 broth resuspended in phosphate buffer (white bars) or in MES buffer (grey bars). Differences of autolysis extent in phosphate buffer between strains grown in M17 and GM17 broth (black bars).

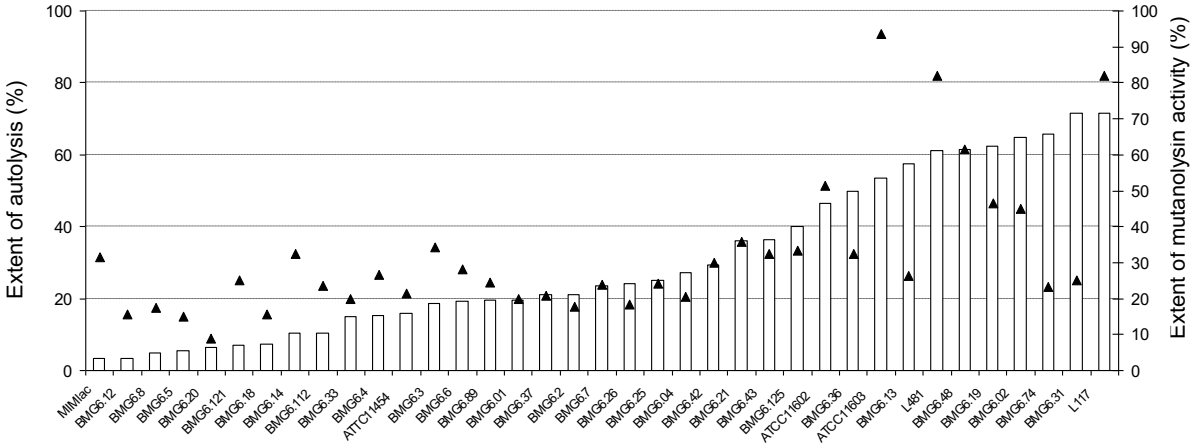

Fig. 2 Extent of autolysis of *Lactococcus lactis* strains grown in M17 broth and resuspended in MES buffer (bars) as well as the extent of mutanolysin activity in the same buffer (black triangles).

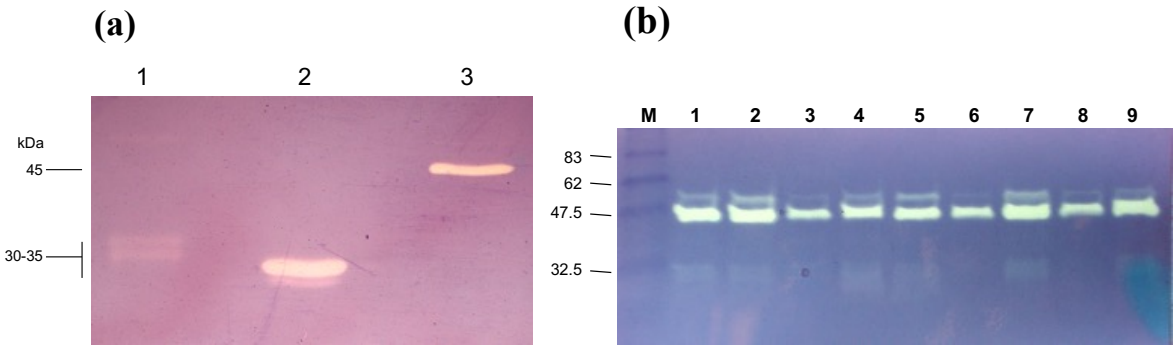

Fig. 3 (a) Peptidoglycan hydrolases profile of lactic acid bacteria evaluated by renaturing SDS-PAGE containing autoclaved cells of *Micrococcus lysodeikticus*. Lane 1, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^{T}; lane 2, *Lactobacillus helveticus* ATCC 15009^{T}; lane 3, *Lactococcus lactis* subsp. *lactis* BMG6.02. (b) Peptidoglycan hydrolases profile of *Lactococcus lactis* strains isolated from traditional Tunisian dairy products. Lane 1 to 6 *L. lactis* subsp. *lactis* strains BMG6.02, BMG6.4, BMG6.8, BMG6.14, BMG6.20, BMG6.43; lane 7 to 9 *L. lactis* subsp. *cremoris* strains BMG6.2, BMG6.13, BMG6.42.

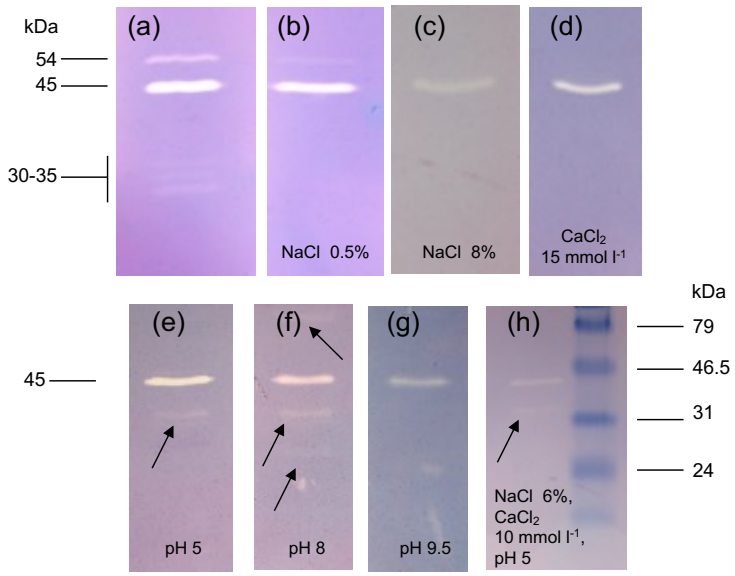

Fig. 4 Peptidoglycan hydrolases profile of *L. lactis* subsp. *lactis* BMG6.02 evaluated against autoclaved cells of *Micrococcus lysodeikticus* using several renaturation conditions. (a) 25 mmol l^{-1} Tris-HCl (pH 7), 1% of Triton X-100 with addition of: (b) NaCl 0.5 %, (c) NaCl 8%, (d) $CaCl_2$ 15 mmol l^{-1}. Peptidoglycan hydrolases profile of *L. lactis* subsp. *lactis* BMG6.02 evaluated as above described, using renaturing buffer at different pH. (e) 10 mmol l^{-1} sodium acetate pH 5; (f) 10 mmol l^{-1} Tris-HCl pH 8. (g) 10 mmol l^{-1} Tris-HCl pH 9.5. (h) Peptidoglycan hydrolases profile of *L. lactis* subsp. *lactis* BMG6.02 evaluated as above described, using renaturing buffer at pH 5 containing NaCl 6% and $CaCl_2$ 10 mmol l^{-1}. In all pictures, arrows indicate secondary lytic bands.