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Antifungal activity of non-starter lactic acid bacteria isolates from dairy products

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ABSTRACT

The antifungal activity of 81 NSLAB isolates from traditional dairy products against moulds developed on the surface of hard cheeses as well as yeasts was studied. Twenty isolates of facultatively heterofermentative and eleven of obligately heterofermentative lactobacilli from Feta cheese exhibited antifungal activities. These isolates were classified to species level by phenotypic criteria and the SDS–PAGE of whole-cell proteins. The former group also showed a broad spectrum of antibacterial activities with preference towards *Listeria monocytogenes* and other food-borne pathogens. The extracellular antimicrobial substances were sensitive to proteolytic enzymes, suggesting that the inhibitory activity was due to bacteriocin-like substances.

1. Introduction

Lactic acid bacteria (LAB) have traditionally been used as natural biopreservatives in food and animal feed, sauerkraut and silage. Their preserving effect relates mainly to the formation of organic acids and hydrogen peroxide, competition for nutrients and production of antimicrobial substances (Stiles, 1996). Biopreservation refers to extended shelf-life and enhanced safety of foods obtained by the natural or added microflora or their antimicrobial products (Schnürer & Magnusson, 2005). The preserving capacity of bacteria naturally occurring in food has gained increasing interest during the recent years, due to consumers demand for reduced use of chemical preservatives.

Moulds and yeasts are common spoilage organisms of food products, such as cheese. Benzoic acid and sodium benzonate are primarily used as antifungal agents as well as natamycin produced by *Streptomyces natalensis* (Davidson, 2001). However, moulds and yeasts are becoming resistant to antibiotics but also to sorbic and benzoic acids (Brul & Coote, 1999; Viljoen, 2001). Lactic acid bacteria may produce compounds with antifungal activity, such as proteinaceous compounds (Magnusson & Schnürer, 2001), phenyllactic acid and cyclic dipeptides (Ström, Sjögren, Broberg, & Schnürer, 2002) and hydroxylated fatty acids (Sjogren, Magnusson, Broberg, Schnürer, & Kenne, 2003). Bacteriocin-like substances and other low and medium molecular weight mass compounds produced by LAB have been reported as antifungal (Niku-Paavola, Laitila, Matilla-Sandholm, & Haikara, 1999; Okkers, Dicks, Silvester, Joubert, & Odendaal, 1999; Rouse, Harnett, Vaughan, & van Sinderen, 2008). However, studies on the effect of LAB on fungi are complicated by the sensitivity of most fungi to metabolites, lactic and acetic acids (Bonestroo, Dewit, Kusters, & Rombout, 1993).

In the present study, the antifungal effects of NSLAB isolates from Greek traditional cheeses and yoghurt were investigated. For this purpose, the fungal inhibitory spectra of 80 isolates of NSLAB against fungi and yeast strains were screened and an attempt was made to determine the nature of the antimicrobial substances produced by interesting group of isolates.

2. Materials and methods

2.1. Isolates of NSLAB

The isolates of non-starter lactic acid bacteria (NSLAB) used in this study were coming from traditional cheeses and yoghurt and belong to our collection. The eight strains of enterococci and the 10 strains of *Leuconostoc mesenteroides* had been isolated from Teleme cheese from goat milk (Litopoulou-Tzanetaki & Tzanetakis, 1992). Seventeen isolates from various cheeses characterized as *Lactobacillus paracasei* subsp. *paracasei* (Mama, Hatzikamari, Lombardi, Tzanetakis, & Litopoulou-Tzanetaki, 2002) and 15 as *Lactobacillus delbrueckii* subsp. *bulgaricus* (Xanthopoulos, Petridis, & Tzanetakis, 2001) were also used. Twenty isolates obtained from the surface growth of Feta cheese during dry-salting and 11 isolates also from Feta, characterized by simple biochemical criteria (growth at 15 and 45 °C, CO_2 production from glucose and NH_3 from arginine; Sharpe, 1979) as facultatively and obligately heterofermentative lactobacilli, respectively (unpublished data), were also studied. The strains were kept at −80 °C in either MRS broth (rods) or M17 broth (cocci) plus glycerol (70:30).

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2.2. Target strains

The isolates of NSLAB were screened against four mould and two yeast strains. The three mould strains (M1, M4 and MT1) were isolates from the surface of ripening Kasseri cheese, characterized as *Penicillium* spp. by a Key based on the morphology of sporing structures (Harrigan, 1998). *Penicillium candidum* was obtained from Wiesby (Laboratorium "Visby", Tonder aps., DK-6270 Tonder), while the yeasts *Debaryomyces hansenii* strain 29 and *Saccharomyces cerevisiae* strain 51 were isolates from the surface of Feta cheese (Tzanetakis, Hatzikamari, & Litopoulou-Tzanetaki, 1996).

2.3. Fungal inocula

Moulds were grown on malt extract agar slants at 25 °C for several days (until sporulation). The spores were collected after vigorously shaking of slants with sterile peptone water (0.2% w/v). Mycelial debris was removed from spore suspension by filtering twice through several layers of sterile damp cheese cloth (Osman, 2004). Yeast cell inocula were prepared from cultures grown in malt agar slants at 25 °C for 48 h. The growth was collected in sterile peptone water (0.2% w/v) by vigorous shaking. Inocula of both, mould spores and yeasts, (1 ml of the 10^{-1} dilution) were used for the assays.

2.4. Antifungal overlay assay

The antifungal activity of LAB was investigated with an overlay assay (Lind, Jonsson, & Schnürer, 2005; Magnusson & Schnürer, 2001). Bacteria were inoculated in two 2-cm lines on MRS agar plates and allowed to grow at 30 °C. Ten milli liter of soft (7%) malt extract agar containing 1 ml of inoculum of mould and/or yeast was then poured onto the agar plates and incubated at 30 °C. After 48 h, the zone of inhibition was measured. The degree of inhibition was calculated as the area of inhibited growth in relation to the total area of the petri dish and the scale was the following: − = no visible inhibition, (+) = weak inhibition in the soft agar above the bacterial growth, + = no fungal growth on 0.1–3% of plate area/bacterial streak, ++ = no fungal growth on 3–8% of plate area/bacterial streak, +++ = no fungal growth on >8% of plate area/bacterial streak.

2.5. Characterisation of the facultatively and obligately heterofermentative lactobacilli

2.5.1. Characterisation by phenotypic criteria

On the facultatively heterofermentative, Gram-positive, catalase-negative rods the following tests were applied: acid production from cellobiose, glucose, mannitol, melibiose, mannose, raffinose, rhamnose and ribose (Ballows, Trüper, Dworkin, Harder, & Schleifer, 1991; Dellaglio, de Roissart, Torriani, Curk, & Janssens, 1994; Fitzsimons, Cogan, Condon, & Beresford, 1999; Sharpe, 1979). The obligately heterofermentative lactobacilli were classified with the following tests: acid production from arabinose, cellobiose, galactose, mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, ribose, sucrose and xylose (Ballows et al., 1991; Sharpe, 1979). Carbohydrate fermentation was determined using sterile multiwell plates (Greiner Labortechnik, Frickenhousen, Germany) and the methodology suggested by API (BioMerieux SA, 69280 Marcy-l'Etoile, France).

2.5.2. Characterisation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins

The SDS–PAGE of whole-cell proteins was used as an additional taxonomic tool for the characterization of facultatively and obligately heterofermentative lactobacilli. Whole-cell protein analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described elsewhere (Ambadoyiannis, Hatzikamari, Litopoulou-Tzanetaki, & Tzanetakis, 2004). The protein content of the disrupted cells was determined according to Lowry, Rosebrough, Farr, and Randall (1951) and appropriate amounts were subjected to SDS–PAGE according to Laemmli (1970). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of the strains by the Pearson product-moment correlation coefficient (r) and UPGMA (Unweighted Pair Group Method using Arithmetic Averages) cluster analysis were performed with the software package GelCompar version 4.0 (Applied Maths, Kortzijk, Belgium).

Identification of the isolates was performed by comparison of their protein patterns to the fingerprints of the reference strains. Reference strains of *L. paracasei* subsp. *paracasei* (LMG 11459), *Lactobacillus paraplantarum* (LMG 16673; BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University of Gent, Gent Belgium), *Lactobacillus plantarum* (ATCC 14917; ATCC, American Type Culture Collection, Rockville, Maryland), *Lactobacillus pentosus* (NCFB 363; NCFB, National Collection of Food Bacteria, AFRC, Institute of Food Research, Reading, England), *Lactobacillus rhamnosus* (NCFB 7469), *Lactobacillus brevis* (DSM 2647), *Lactobacillus buchneri* (DSM 5987), *Lactobacillus collinoides* (DSM 20515) and *Lactobacillus fermentum* (DSM 20052; DSMZ, Deutsche Sammlung von Microorganismen and Zellculturen GmbH, Braunschweig, Germany).

2.6. Antimicrobial activity assay-inhibitory spectrum

Activated strains of facultatively and obligately heterofermentative lactobacilli grown for 16 h at 30 °C were tested for inhibitory activity by the method of Kekessy and Piquet (1970), which may detect the production of bacteriocins. Each of the indicator strains was grown in the appropriate broth until an optical density of ~0.45 at 600 nm. The producer strains were inoculated by spot inoculation on the surface of MRS agar plates. After incubation (48 h, 37 °C) the agar was detached from the edges of the Petri dish with a sterile spatula. The plate was then inverted into the lid and the new sterile surface was overlaid by the indicator strains inoculated (1% of the first dilution) into an appropriate soft agar. After incubation the zones of inhibition, whenever formed, were measured. Food-borne pathogens used for testing the antibacterial activities of the strains were: *Escherichia coli* 0:44 NCTC 9702 (NCTC, National Collection of Type Cultures, AFRC, Reading, England), *Staphylococcus aureus* NCTC 9751, *Yersinia enterocolitica* 0:9/4360 (supplied by the Pasteur Institute, Paris), *Listeria monocytogenes* Scott A and a *Bacillus cereus* strain (supplied by the School of Medicine, Aristotle University of Thessaloniki).

2.7. Sensitivity of antimicrobial substances to pH, catalase and proteolytic enzymes

The well diffusion assay (Roy, Batish, Grover, & Neelakantan, 1996) was performed on eight facultatively heterofermentative strains exhibiting both, antifungal and antimicrobial activities with an interesting tendency to inhibit preferentially *L. monocytogenes* Scott A and other food-borne pathogens. Culture extracts from the strains were obtained from MRS broth cultures after centrifugation (12,000*g*, 10 min, 4 °C, Sigma centrifuge, type 3K20). Antimicrobial substances (AMS) samples were neutralized by addition of 5 N NaOH to exclude the organic acid effect. These antibacterial crude preparations were designated as neutralized cell-free-supernatants (NCFS). Catalase (150 IU/ml) was added to neutralized samples to exclude the effect of H_2O_2. The samples were centrifuged again (12,000*g*, 10 min, 40 °C) to remove contaminants.

NCFS were treated with trypsin, pepsin, proteinase K and α-chymotrypsin (Merck, Darmstadt, Germany) to a final concentration of

Table 1
Fungal inhibition spectra[a] of facultatively and obligately heterofermentative lactobacilli strains.

Strains	Indicator fungus					
	Penicillia				Yeasts	
	M1	MT1	M4	*P. candidum*	*D. hansenii*	*S. cerevisiae*
Facultatively heterofermentative						
2032	++	–	–	++	+++	–
2035	++	–	–	++	+	–
2102	++	–	–	++	+	–
2192	++	–	–	++	++	–
2193	+	–	–	++	+	–
2026	++	–	–	++	+++	–
2096	++	–	–	++	+	–
2114	++	–	–	++	++	–
2116	+	–	–	++	+	–
2171	+	–	–	++	+	–
2176	+	–	–	++	+	–
2071	++	–	–	++	+	–
2101	++	–	–	++	+	–
2185	++	–	–	++	+	–
2194	+	–	–	++	+	–
2092	++	–	–	++	++	–
2093	++	–	–	++	++	–
2099	++	–	–	++	+	–
2111	++	–	–	++	+	–
Q20.3	+	+	–	–	++	–
Obligately heterofermentative						
R30.9	+	+	+++	–	++	–
R3.2	+++	–	–	–	+	–
R3.6	++	+	–	–	+	–
R3.8	++	–	–	–	–	–
Q30.8	++	++	–	+	+++	–
Q20.4	++	+	–	+	++	–
Q30.10	–	+	–	–	+	–
Q 30.1	++	+	–	–	+	–
W21.13	–	+	++	–	+	–
Q30.9	–	+	–	++	+++	–
L30.6	++	++	–	–	+	–

[a] – = no visible inhibition; + = no fungal growth on 0.1–3% of plate area/bacterial streak; ++ = no fungal growth on 3–8% of plate area/bacterial streak and +++ = no fungal growth on > 8% of plate area/bacterial streak.

1 mg/ml (Ahn & Stiles, 1990) in 0.5 mM Tris–0.2 mM $CaCl_2$ buffer (pH 8.5). Samples with and without enzymes were incubated at 37 °C for 1 h. Activity remained in the samples after treatment with enzymes, was detected by the well diffusion method. Antifungal activity was tested against the strain of *Penicillium* M1. Malt extract agar plates were overlaid with 5 ml of soft (0.7%) malt extract agar inoculated with spores of *Penicillium* strain M1. The well opened on the plates were filled with 50 μl extracts and remained for 2 h in the refrigerator. The plates were then incubated at 30 °C for 24 h and checked for the presence of inhibition zones.

3. Results and discussion

3.1. Antifungal activity of LAB

Varying degrees of inhibition were detected against the isolates of penicillia, *P. candidum* and the yeasts *D. hansenii and S. cerevisiae*. Mould strain M1 was the most sensitive indicator strain. None of the isolates had activity against *S. cerevisiae*; mould strain M4 was inhibited only by two obligately heterofermentative lactobacilli isolates.

Mould isolates M1 and MT1 were inhibited by one *Enterococcus hirae* strain (+++ and +, respectively), which also exhibited a weak activity against *P. candidum* and *D. hansenii*. A weak inhibitory activity was detected in four *L. bulgaricus* strains against the mould M1. This mould was subjected to various degrees (+ to +++) of inhibition by 12 strains of *L. paracasei* subsp. *paracasei*. Five isolates of the latter species exhibited inhibitory activity against the mould strain MT1, nine against *P. candidum*, and eight towards *D. hansenii*. The activity ranged from + to ++. All isolates of leuconostocs inhibited mould M1 (+ to ++) and the majority the mould strain MT1 (+). *P. candidum* and *D. hansenii* were inhibited (+) by two isolates each (data not shown).

The antifungal activities exhibited by the isolates of facultatively and obligately heterofermentative lactobacilli are presented in Table 1. All facultatively heterofermentative isolates showed activity (+ to +++) against the mould M1, *P. candidum* and *D. hansenii*. The isolates of obligately heterofermentative lactobacilli also

Table 2
Phenotypic characterization of facultatively and obligately heterofermentative lactobacilli isolates form Feta cheese.

	Facultatively heterofermentative				
	L. plantarum (five strains)	*L. paraplantarum* (six strains)	*L .pentosus* (four strains)	*L. rhamnosus* (three strains)	*L. paracasei* subsp. *paracasei* (two strains)
Acid from					
Cellobiose	+	+	+	+	+
Glucose	+	+	+	+	+
Mannitol	+	+	+	+	+
Mannose	+	+	+	+	+
Melibiose	+	+	+	2+, 1–	–
Raffinose	+	–	+	–	–
Rhamnose	–	–	1+, 3 weak	+	–
Ribose	3+, 2 weak	5+, 1–	2+, 2–	+	+

	Obligately heterofermentative		
	L. brevis (six strains)	*L. buchneri* (three strains)	*L. fermentum* (two strains)
Acid from			
Arabinose	+	+	–
Cellobiose	–	2+, 1–	1+, 1–
Galactose	+	+	+
Mannitol	–	–	–
Mannose	1+, 5–	1+, 2–	+
Melezitose	–	+	–
Melibiose	3+, 3–	+	+
Raffinose	–	1+, 2–	1+, 1–
Rhamnose	–	–	–
Ribose	+	+	+
Sucrose	1+, 5–	2+, 1–	+
Xylose	+	1+, 2–	–

exhibited activities of varying degree (+ to +++) against moulds isolates M1, M4 and MT1, *P. candidum* and *D. hansenii*.

Fungi are sensitive to fermentation products, lactic and acetic acids (Bonestroo et al., 1993). Heterofermentative LAB produce acetic acid in relatively high amounts and propionic acid in traces and their effect is often dependent on the decrease in pH caused by lactic acid (Eklund, 1989). Propionic acid reduces fungal growth especially at lower pH (Woolford, 1984) and inhibit amino acid uptake (Eklund, 1989). A number of antifungal metabolites, e.g. peptides, phenyllactic acid, proteinaceous compounds and 3-hydroxylated fatty acids have also been isolated from lactic acid bacteria (Schnürer & Magnusson, 2005).

There are several reports on antifungal activity of LAB. Okkers et al. (1999) found that *L. pentosus* exhibited fungistatic effects against *Candida albicans*. Lavermicocca et al. (2000) reported the production of antifungal compounds by a sourdough *L. plantarum* strain. The same was observed for *Lactobacillus coryneformis* Si3 (Magnusson & Schnürer, 2001) as well as isolates of *L. plantarum*, *L. coryneformis*, *Lactobacillus salivarius*, *Lactobacillus sake*, *E. hirae, and Enterococcus durans* from various sources (Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003). Ström et al. (2002) also found that *L. plantarum* MiLAB 393 produced antifungal substances, Addis, Fleet, Cox, Kolak, and Leung (2001) reported that LAB inhibited the growth at several yeasts with *L. plantarum* being active against *S. cerevisiae* and other yeast species. Durlu-Ozkaya, Karabiçak, Kayali, and Essen (2005) found LAB, such as *L. plantarum*, *L. paracasei*, subsp. *paracasei* and *Lactobacillus lactis* isolated from a Turkish traditional cheese to inhibit *S. cerevisiae*, several species of *Candida*, *Torulopsis glabrata* and *Rhodotorula rubra*, isolates from the same cheese. Several LAB isolates from sourdough bread cultures were screened for antifungal activity against a battery of moulds and the most inhibitory was *L. paracasei* subsp. *tolerans* (Hassan & Bullerman, 2008). Also, LAB with potential to eliminate fungal spoilage in foods were obtained from malted cereals (Rouse et al., 2008).

3.2. Characterization of facultatively and obligately heterofermentative lactobacilli

3.2.1. Characterization by phenotypic criteria

Our data on facultatively heterofermentative lactobacilli suggest that five biochemical patterns existed among the 20 isolates

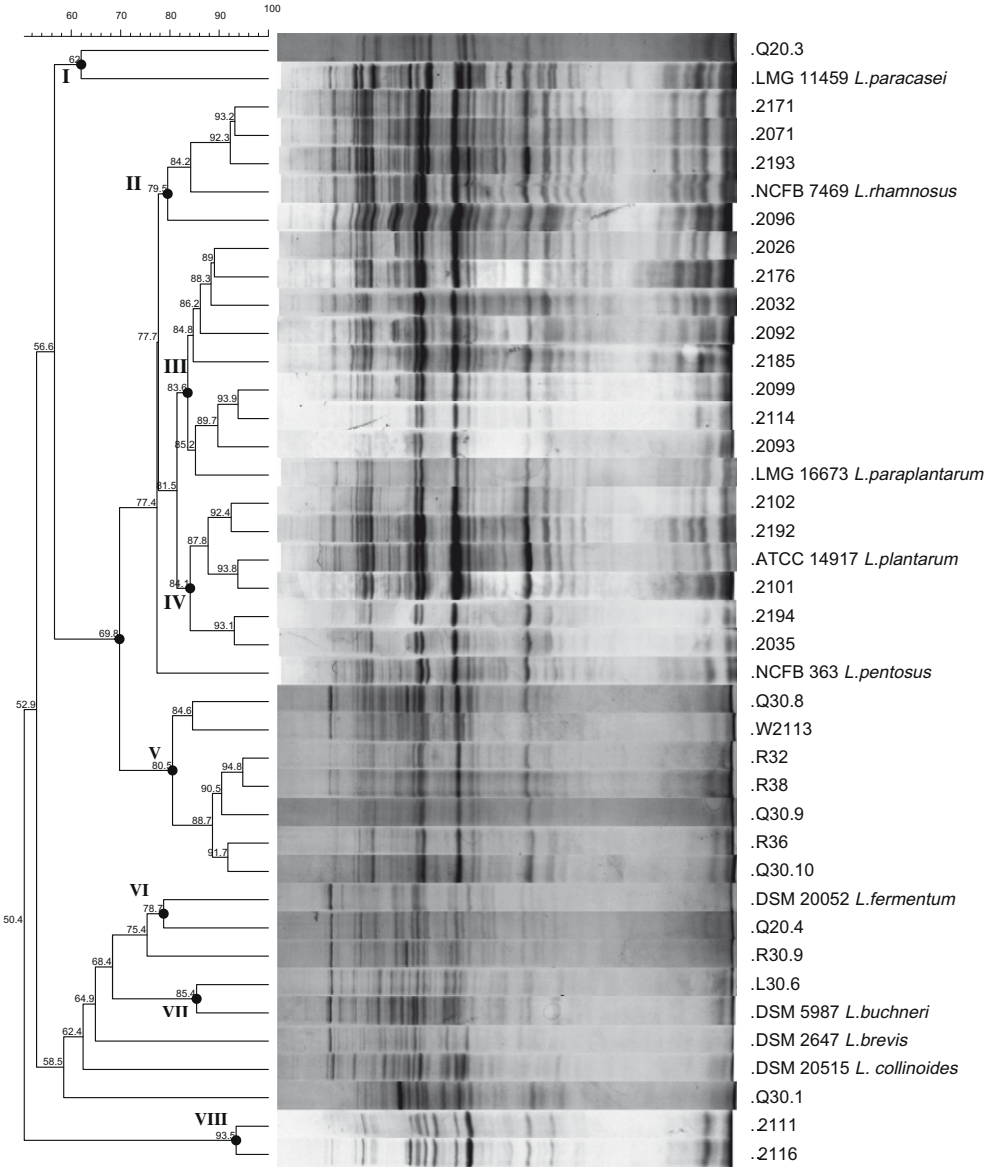

Fig. 1. Dendogram of the normalized and digitized protein patterns of lactobacilli isolates by SDS–PAGE of whole-cell proteins.

tested (Table 2). Five strains (2032, 2035, 2102, 2192 and 2193) able to form acid from cellobiose, glucose, mannitol, mannose, melibiose, raffinose and ribose were characterized as *L. plantarum* (Ballows et al., 1991; Dellaglio et al., 1994; Sharpe, 1979). Six strains (2026, 2096, 2114, 2116, 2171 and 2176) producing acid from cellobiose, glucose, mannitol, mannose, melibiose and ribose (except for strain 2171), but not from raffinose and rhamnose were considered as *L. paraplantarum* (Fitzsimons et al., 1999). Four strains (2071, 2101, 2185 and 2194) could form acid from cellobiose, glucose, mannitol, mannose, melibiose, raffinose, rhamnose and ribose (two of them) and were allocated to *L. pentosus* species; another three (2092, 2093 and 2099), able to produce acid from all the above, except for raffinose, were classified as *L. rhamnosus*; two strains (2111 and Q20.3) producing acid from cellobiose, glucose, mannitol, mannose and ribose were considered as *L. paracasei* subsp. *paracasei* (Ballows et al., 1991; Dellaglio et al., 1994; Fitzsimons et al., 1999; Sharpe, 1979).

All strains, except two (Q20.4 and Q30.10), of the obligately heterofermentative lactobacilli were able to grow at 15 °C (data not shown). Six strains (R30.9, R3.2, R3.6, R3.8, Q30.1 and Q30.8) forming acid from arabinose, galactose, melibiose (50%), ribose and xylose, but not from cellobiose, mannitol, mannose (except one), melezitose, raffinose, rhamnose and sucrose, were characterized as *L. brevis* (Table 2; Ballows et al., 1991; Dellaglio et al., 1994). Two strains (Q20.4 and Q30.10) produced acid from galactose, mannose, melibiose, ribose and sucrose and were considered as closely related to *L. fermentum*. Three strains (W21.13, Q30.9 and L30.6) could form acid from arabinose, galactose, melibiose, melezitose and ribose and were considered as *L. buchneri* (Ballows et al., 1991; Dellaglio et al., 1994; Fitzsimons et al., 1999).

3.2.2. Characterization by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell protein

According to the SDS–PAGE results (Fig. 1), the phenotypic identification was confirmed for three *L. plantarum*, three *L. paraplantarum*, one *L. paracasei* subsp. *paracasei*, one *L. buchneri* and one *L. fermentum* strain. The intragel reproducibility was ~92%. Thus, eight clusters of two to nine strains each were distinguished. Strain Q20.3 and the *L. paracasei* subsp. *paracasei* reference strain formed cluster I (at mean correlation $r = 0.62$). Cluster II (mean correlation $r = 0.80$) comprised four strains (phenotypically identified as *L. plantarum*, one; *L. paraplantarum*, two; *L. pentosus,* one) and the *L. rhamnosus* reference strain. Cluster III ($r = 0.84$) was composed of eight strains (three of each species, *paraplantarum* and *rhamnosus,* and two *L. plantarum* strains according to the phenotypic characterization) and included also the *L. paraplantarum* reference strain. Five strains (phenotypically identified, three as *L. plantarum* and two as *L. pentosus*) and the reference strain of *L. plantarum* formed Cluster IV ($r = 0.84$). Group V comprised one group of seven strains (at similarity of 80.5%) phenotypically identified as *L. buchneri.* (two strains), *L. brevis (four strains),* and *L. fermentum* (one strain), which did not group with any reference strain. One *L. fermentum* (D20.4) strain and the reference strain of *L. fermentum* composed Cluster VI ($r = 0.79$). Strain L30.6, phenotypically characterized as *L. buchneri* grouped with the *L. buchneri* reference strain ($r = 0.85$) and formed cluster VII. Strain Q30.1 (*L. brevis*) formed a unique profile and did not group any other strain. Similarly, strains 2111 and 2116, phenotypically identified as *L. paracasei* subsp. *paracasei* and *L. paraplantarum*, respectively, with a very similar overall profile, but different from the other strains formed group VIII ($r = 0.94$) and delineated separately.

3.3. Antibacterial activity of the facultatively and the obligately heterofermentative lactobacilli

None of the obligately heterofermentative lactobacilli showed inhibitory activity against any of the target bacterial strains. On the contrary, several patterns of activities were recorded for the facultatively heterofermentative lactobacilli (Table 3). *Y. enterocolitica* was not inhibited by any strain. All strains inhibited the growth of *Lactobacillus casei*, *Lactobacillus reuteri*, *Enterococcus faecalis, Pediococcus pentosaceus, L. monocytogenes* Scott A, *Listeria innocua*, *Clostridium sporogenes, S. aureus* and *E. coli*. In addition, most (95%) of the strains were antagonistic towards *B. cereus*. *L. lactis* subsp. *lactis* and *Streptococcus thermophilus* were inhibited by the 70% and 75% of the strains, respectively, and *Lactobacillus helveticus* was sensitive to the inhibitory substances formed by the 80% of the strains. It is interesting to note, that the inhibition zone was stronger for the pathogens (except for *L. innocua*), ranging from 12 to 70 mm, than LAB and clostridia (inhibition zone <18 mm). The strongest inhibitory activity was shown for *L. monocytogenes*.

LAB are well known to produce antibacterial substances against food-borne pathogens, such as *S. aureus, L. monocytogenes, Clostridium. botulinum* and others (Aslim, Yuksekbag, Sarikaya, & Beyatli, 2005; Psoni, Kotzamanidis, Yiangou, Tzanetakis, & Litopoulou-Tzanetaki, 2007; Psoni et al., 2006). In agreement with our results, Messi, Bondi, Sabia, Battini, and Manicardi (2001) found that *L. plantarum* strain 35d exhibited strong inhibitory activity towards *S. aureus* and *L. monocytogenes.* Since LAB are commonly used as starters, investigators have explored the use of bacteriocin producers as starter cultures (Sarantinopoulos et al., 2002).

It is worthwhile noting that our strains of facultatively heterofermentative lactobacilli exhibited a broad antibacterial activity and interesting fungistatic effects, as also observed by Atanossova

Table 3
Inhibition zones (cm), of facultatively heterofermentative lactobacilli isolated from the surface of Feta cheese towards selective target strains and pathogens.

Target strains	Inhibition zones by strains																			
	2032	2035	2102	2192	2193	2026	2096	2114	2116	2171	2176	2092	2093	2099	2185	2194	2071	2101	2111	Q20.3
Lactococcus lactis subsp. *lactis*	0.4	0.6	–	1.8	1.0	0.5	0.8	–	–	0.8	0.8	0.8	0.8	0.8	1.8	2.0	0.8	–	–	–
Lactobacillus casei	0.4	1.0	1.2	0.4	0.4	1.2	0.4	1.4	0.8	0.6	1.0	0.4	0.4	0.4	0.4	0.8	0.4	1.0	0.8	0.5
Lactobacillus reuteri	0.2	0.4	0.4	0.8	0.4	0.4	0.4	0.8	0.8	0.8	0.4	0.4	0.4	0.4	1.0	0.4	0.4	0.8	0.4	0.5
Lactobacillus helveticus	–	0.2	0.6	0.6	0.4	1.2	–	0.4	0.4	0.4	0.4	–	–	0.4	0.6	0.4	0.4	0.6	0.4	0.6
Enterococcus faecalis	0.6	0.8	1.0	1.4	1.2	0.8	0.8	0.8	1.0	0.6	0.6	1.0	1.0	1.0	2.0	1.4	1.0	1.4	0.6	0.2
Listeria innocua	0.4	0.4	0.6	0.8	0.8	0.6	0.4	0.2	0.4	0.4	0.4	0.2	0.4	0.2	0.6	0.8	0.2	0.4	0.2	0.2
Clostridium sporogenes	0.2	0.6	0.4	0.6	0.4	0.3	0.2	0.2	0.2	0.6	1.0	0.2	0.2	0.2	0.6	0.4	0.2	0.6	0.2	0.2
Clostridium tyrobutyricum	0.2	0.4	0.6	0.6	0.4	0.2	0.4	0.2	0.4	0.6	0.6	0.4	0.4	0.4	0.6	0.4	0.4	0.4	–	–
Pediococcus pentosaceus	0.4	0.8	0.4	0.8	0.6	0.4	0.4	0.2	0.4	0.6	1.0	0.4	0.4	0.4	0.6	0.6	0.4	0.4	0.2	0.2
Streptococcus thermophilus	0.4	0.4	–	0.6	0.6	0.4	0.4	–	–	0.6	0.8	0.4	0.4	0.4	0.8	0.6	0.4	–	–	0.4
Bacillus cereus	6.0	4.0	3.0	3.6	9.0	6.0	5.0	6.0	2.0	4.0	6.0	1.2	2.0	6.0	5.6	–	6.0	2.6	6.0	6.0
Staphylococcus aureus	1.2	1.6	2.0	4.0	3.2	2.0	3.0	1.8	2.0	2.8	2.6	3.0	3.0	2.0	2.8	2.4	3.0	3.0	1.2	2.0
Escherichia coli	1.6	2.0	2.0	2.6	3.2	1.4	2.4	1.8	2.4	3.0	1.8	3.0	2.4	2.6	3.0	3.0	2.0	2.4	2.0	2.0
Listeria monocytogenes	2.0	6.0	3.4	7.0	3.2	6.0	6.0	1.4	5.0	5.0	6.0	3.0	3.6	6.0	4.0	6.0	2.4	3.0	1.8	1.8

Table 4
Sensitivity of inhibition compounds towards Penicillium strain M1 from facultatively heterofermentative lactobacilli to catalase and pH and various proteolytic enzymes by the agar well method.

	Strains							
	2032	2035	2071	2176	2192	2114	2026	2116
Control	20[a]	6	5	5	4	4	4	6
Catalase and pH 7.0	8	5	4	5	4	4	4	4
Pepsin	+[b]	+	–	+	+	+	–	+
Trypsin	–	+	–	+	+	+	+	+
α-chymotrypsin	+	+	+	+	+	+	+	+
Proteinase K	+	+	+	+	+	+	+	+

[a] mm of inhibition zone.
[b] + or –; Effect or no effect of the proteolytic enzyme on the inhibitory substances.

et al. (2003) for the *L. paracasei* subsp. *paracasei* M_3 strain. Therefore, they may be considered interesting as biopreservatives, from this point of view.

3.4. Inhibitory metabolites by strains of facultatively heterofermentative lactobacilli

The nature of the inhibitory activity exhibited by the clear supernatant of eight selected strains, was tested against the mould M_1 by the agar well method (Table 4). Catalase treatment and neutralization of the supernatant reduced the inhibitory activity of four strains (2032, 2035, 2071 and 2116), suggesting that antimicrobial activity cannot be wholly related to the H_2O_2 or acid (Aslim et al., 2005; Mastromarino et al., 2002). For five strains (2035, 2176, 2192, 2114 and 2116) the antagonistic activity was lost after treatment with pepsin, trypsin, α-chymotrypsin, and proteinase K. The culture supernatant of strain 2032 was not affected by trypsin and the same was observed for 2071 for pepsin and trypsin; antimicrobial activity from strain 2026 was also not reduced by pepsin. These results suggest, that besides to acid formation and low pH conditions created by isolates of facultatively heterofermentative lactobacilli (*L. plantarum*, *L. paraplantarum* and *L. pentosus*) from the surface of Feta cheese, substances of proteinaceous nature were also formed. Gourama and Bullerman (1997) found that *L. casei* subsp. *pseudoplantarum,* a component of a commercially available silage inoculant, exhibited antifungal and antiaflatoxin activity against *Aspergillus flavus,* which was due to a small peptide, sensitive to treatment with trypsin and α-chymotrypsin. Okkers et al. (1999) characterized a medium length peptide from *L. pentosus* that had fungistatic effects against *C. albicans*. Magnusson and Schnürer (2001) also found a small peptide (~3 kDa) from *L. coryneformis* subsp. *coryneformis* strain Si3 with antifungal effect, totally inactivated by proteinase K. The proteinaceous compound characterized by Atanossova et al. (2003) from *L. paracasei* subsp. *paracasei* M_3 with broad antibacterial and fungistatic effects was a hydrophobic protein of approximately 43 kDa.

The overall study showed, that LAB from Greek traditional dairy products may exhibit antifungal activity against common moulds, as well as yeast species, developed on the surface of cheeses. Facultatively heterofermentative lactobacilli grown on the surface of Feta cheese (Tzanetakis et al., 1996) may produce interesting antifungal with concominant antibacterial activity substances, inhibiting strongly inhibit *L. monocytogenes* and other food-borne pathogens and may protect Feta cheese from their growth. It also seems, that the inhibitory activity is caused by proteinaceous compounds. Further investigations on their nature and mechanism of action could have a great potential for the control of spoilage fungi and food-borne pathogens. Moreover, interesting strains could be employed as novel biopreservatives of cheese.

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