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Full Length Research Paper

Characterization of *Enterococci* isolated from Moroccan dairy products

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Twenty three *Enterococcus durans* isolates collected from Moroccan dairy products were identified by species-specific PCR and their technologically relevant biochemical properties were studied. According to biochemical activities, the majority of the strains displayed weak acidification and autolysis activities in milk. In contrast, they showed high extracellular proteolytic activity. All isolates produced exopolysaccharides and most of them could metabolize citrate and tolerate a high concentration of nisin. Absence of vancomycin resistance and haemolytic activity may suggest the use of these isolates as adjunct starters in food fermentations process.

Key words: *Enterococcus durans*, Moroccan dairy products, species-specific PCR (Polymerase chain reaction), biochemical properties, adjunct starter cultures.

INTRODUCTION

Lactic acid bacteria (LAB) have gained interest as starter cultures to improve the quality and safety of fermented foods. *Enterococci* are ubiquitous LAB that occurs frequently in large numbers in dairy products and other fermented foods (Giraffa et al., 1997; Foulquié Moreno et al., 2006).

They are part of the normal intestinal microbiota (Huycke et al., 1998). *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* are the most frequently found species in dairy products (Foulquié Moreno et al., 2006). They play an important role in cheese making and contribute to the development of the sensory characteristics of many varieties of cheese (Centeno et al., 1996; Manolopoulou et al., 2003).

The positive effects of *Enterococci* on cheese are due to specific biochemical traits such as lipolytic activity, citrate utilisation and aromatic volatile compounds production. Because of these interesting metabolic traits, *enterococci* have been proposed as part of defined starter culture combinations for different European cheeses such as water-buffalo Mozzarella (Villani and Coppola, 1994), Feta (Sarantinopoulos et al. 2002a), Venaco (Casalta and Zennaro, 1997), Cebreiro (Centeno et al. 1999), and Cheddar (Gardiner et al. 1999). Generally, the presence of the added enterococcal flora throughout ripening positively affects taste, aroma, colour, structure, as well as the overall sensory profile, of the full-ripened cheeses. This seems linked to the fact that, in cheeses made with *Enterococci,* soluble nitrogen, total free amino acids, volatile free fatty acids, long-chain free fatty acids, diacetyl and acetoin contents are generally higher (Centeno et al. 1999; Oumer et al. 2001; Sarantinopoulos et al. 2002a).

According to the literature, the potentially beneficial role of *Enterococci* has been examined by including *E. faecalis* or *E. durans* strains in experimental cheese making. Litopoulou-Tzanetaki et al. (1993) and Tzanetakis et al. (1995) reported that body and texture were developed better in Feta cheese made with *E. durans* strains, and their adjunct enhanced the growth of Lactococci and Streptococci. Moreover, some

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Table 1. List and source of the *enterococci* isolated.

Isolate code	Dairy source of isolation	Medium and incubation conditions
LB2	Lben: natural fermented skimmed cow milk	MRS at 37°C
LB5	Lben: natural fermented skimmed cow milk	MRS at 37°C
LB6	Lben : natural fermented skimmed cow milk	MRS at 37°C
LB11	Lben : natural fermented skimmed cow milk	MRS at 37°C
LB13	Lben : natural fermented skimmed cow milk	MRS at 37°C
LC'1	raw goat milk	MRS at 37°C
LC1	raw goat milk	M17 at 37°C
LC2	raw goat milk	MRS at 37°C
LC'3	raw goat milk	M17 at 37 °C
L1	fresh pasteurized cow milk	MRS at 37°C
L3	fresh pasteurized cow milk	MRS at 37°C
L4	fresh pasteurized cow milk	MRS at 37°C
LF4	raw cow milk	MRS at 37°C
LV13	raw cow milk	MRS at 37°C
LV15	raw cow milk	MRS at 37°C
LV16	raw cow milk	MRS at 37°C
CLF5	raw cow milk	M17 at 37°C
R2	Raib : cow curd milk (traditional product)	MRS at 37°C
R5	Raib: cow curd milk (traditional product)	M17 at 37°C
R10	Raib: cow curd milk (traditional product)	MRS at 37°C
R11	Raib: cow curd milk (traditional product)	MRS at 37°C
S2ac	Commercial yoghurt	MRS at 37°C
S5	Commercial yoghurt	MRS at 37°C

Enterococci from dairy products have also been reported to produce bacteriocins (enterocins) having antimicrobial activity against a broad spectrum of spoilage and pathogenic organisms such as *Listeria monocytogenes, Staphylococcus aureus, Clostridium* spp., and *Bacillus* spp. (Giraffa, 1995; Ennahar and Deschamps, 2000; Sarantinopoulos et al., 2002b; De Vuyst et al., 2003; De Kwaadsteniet et al., 2005; Ghrairi et al. 2008). Specific enterococcal strains have been used as probiotic adjunct cultures in Cheddar cheese because of their ability to improve the intestine microbial balance (Gardiner et al., 1999; Giraffa, 2003). But, in spite of all this, the clinical research on *Enterococci* underlines that the safety of dairy products containing *Enterococci* is an issue that the industry must carefully address before proceeding to their application (Giraffa, 2003; Ogier and Serror, 2008).

In the present work, *E. durans* isolated from different Moroccan raw milk, traditionally prepared dairy products and commercial yoghurt, collected in various Rabat-Sale Zemour Zair areas, were identified at species level by means of species-specific PCR. Isolates were evaluated for technologically relevant biochemical activities such as acid production and proteolytic activities. Furthermore, sensitivity of the isolates to vancomycin and nisin was assayed. This study is an important step for the constitution of the National Center of LAB (starter, adjunct cultures and probiotics) in Morocco.

MATERIALS AND METHODS

Bacterial isolates and media

About 300 Gram positive and catalase negative isolates were collected from different Moroccan dairy products. Among them, 23 small size-cocci, occurring in pairs or short chains, were selected and characterised (Table 1). The following strains were used as reference in the genetic assays: *E. faecalis* DSM 20478, *E. faecium* DSM 20477 (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), and *E. durans* LMG 10746 (BCCM/LMG bacteria collection, Ghent, Belgium). The cultures were maintained as frozen stocks at -80°C in M17 broth (Biokar Diagnostics, Beauvais, France) supplemented with 15% (v/v) glycerol until use. For carrying out the experimental tests they were revitalized by overnight incubation at 37°C in M17 broth.

Identification of isolates by PCR

The 23 isolates were previously identified at genus and then at species level, by means of specific-PCR, using primers and protocol described by Ke et al. (1999) and Jackson et al. (2004) (Table 2). In this work each species-specific primer pair was used separately instead of performing a multiplex PCR as developed by Jackson et al. (2004). PCR amplifications were performed in an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany). 10 µl of PCR product was electrophoresed using a 2% 1X Tris-acetate-EDTA agarose gel at 100V, and then stained in ethidium bromide solution (0.5 µg $m1^{-1}$). DNA molecular weight marker 1 kb plus (Invitrogen, Milan, Italy) was used as standard.

Table 2. Primers used in this study for the identification of isolates.

Target	Primer	Sequence (5’-3’)	Product size (bp)	Reference
Enterococcus genus	ENT1	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	Ke et al., 1999
E. durans	DU1 DU2	CCT ACT GAT ATT AAG ACA GCG TAA TCC TAA GAT AGG TGT TTG	295	Jackson et al., 2004
E. faecalis	FL1 FL2	ACT TAT GTG ACT AAC TTA ACC TAA TGG TGA ATC TTG GTT TGG	360	Jackson et al., 2004
E. faecium	FM1 FM2	GAA AAA ACA ATA GAA GAA TTA T TGC TTT TTT GAA TTC TTC TT T A	215	Jackson et al., 2004

The PCR profiles were visualized under ultraviolet light, and digital photos of gels were taken with Kodak DC 120 Camera (Kodak Digital Science 1D LE 3.0 Software).

Technological characteristics

Acidifying activity

The acidifying activity of the selected isolates was measured by the change in pH ‘δpH’ during time. After growth in M17 broth at 37°C for 24 h, the microbial culture was inoculated at a level of 1% in reconstituted sterile skim milk (10% w/v) (Fluka, Sigma-Aldrich). The pH was measured, after 2, 4, 6 and 24 h of incubation at 37°C, by a pH-meter (pH 211 microprocessor pH meter, HANNA Instruments Inc, Italy) previously calibrated using two buffers (pH 4.0 and pH 7.0). After each run, the electrodes (HI 1413B, HANNA Instruments) were disinfected in ethanol. The acidification rate was calculated as δpH = pHf (final value) - pH0 (initial value). The experiments were carried out in duplicate.

Proteolytic activity

To determine the proteolytic activity, isolates were subcultured twice in reconstituted skim milk (10% w/v), containing yeast extract (0.3% w/v), for 24 h at 37°C and using 1% (v/v) inoculum. Final growth was performed in skim milk (10% w/v) for 24 h at 37°C (1% v/v inoculum). The proteolytic activity was determined by the quantity of free amino acids released, according to the method of Church et al. (1983). Results were expressed as glycine equivalents (mM) according to a standard curve, prepared using glycine in the range of 0 - 10 mM.

Autolytic activity

To determine the autolytic activity, the overnight cultures were centrifuged (5000 × g for 15 min at 4°C). The cell pellet was washed twice in potassium phosphate buffer (10 mM, pH 7.0) and then resuspended in potassium phosphate buffer (10 mM, pH 5.5). The cell suspension was subjected to one cycle of freezing (-20°C for 22 h) and thawing, then incubated at 45°C for 2 h. The autolytic activity was determined as the percentage decrease in the absorbance at 650 nm at different time intervals as described by Boutrou et al. (1998), which was defined as follows: (A0 - At) x 100/A0 where A0 = initial absorbance and At = absorbance measured after t hours of incubation.

Citrate metabolism

Bacteria citrate utilization, in the presence of carbohydrates, was studied on the KMK agar as described by Kempler and McKay (1980). The blue bacteria colonies and/or large blue center colonies were considered citrate positive.

Exopolysaccharides (EPS) production

The exopolysaccharides production was evaluated as reported by Mora et al. (2002). Overnight cultures were streaked on the surface of plates containing ruthenium red milk (10% skim milk powder, 1% sucrose, 0.5% yeast extract, 0.08 g 1^{-1} ruthenium red, 1.5% agar). After incubation at 37°C for 24 h, non-ropy isolates gave red colonies due to the staining of the bacterial cell wall, while ropy isolates appeared as white colonies.

Haemolytic activity

Blood haemolysis was evaluated on Columbia agar plates (Oxoid Limited, Basingstoke, England) supplemented with 5% sheep blood which were incubated at 37°C for 24 h (Maragkoudakis et al., 2009).

Nisin sensitivity

The nisin sensitivity of different selected isolates was tested and evaluated as the minimal inhibitory concentrations (MICs) values of nisin (from *Lactococcus lactis*, Biochemika, Fluka; Sigma-Aldrich) in M17 agar medium (Oxoid), using a standard agar dilution screen technique (Bouksaim et al., 1998). Plates were incubated at 37°C for 48 h.

Vancomycin sensitivity

The minimum inhibitory concentration (MIC) of vancomycin was determined according to the reference broth microdilution procedure recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006). The assay was performed in a 96 well

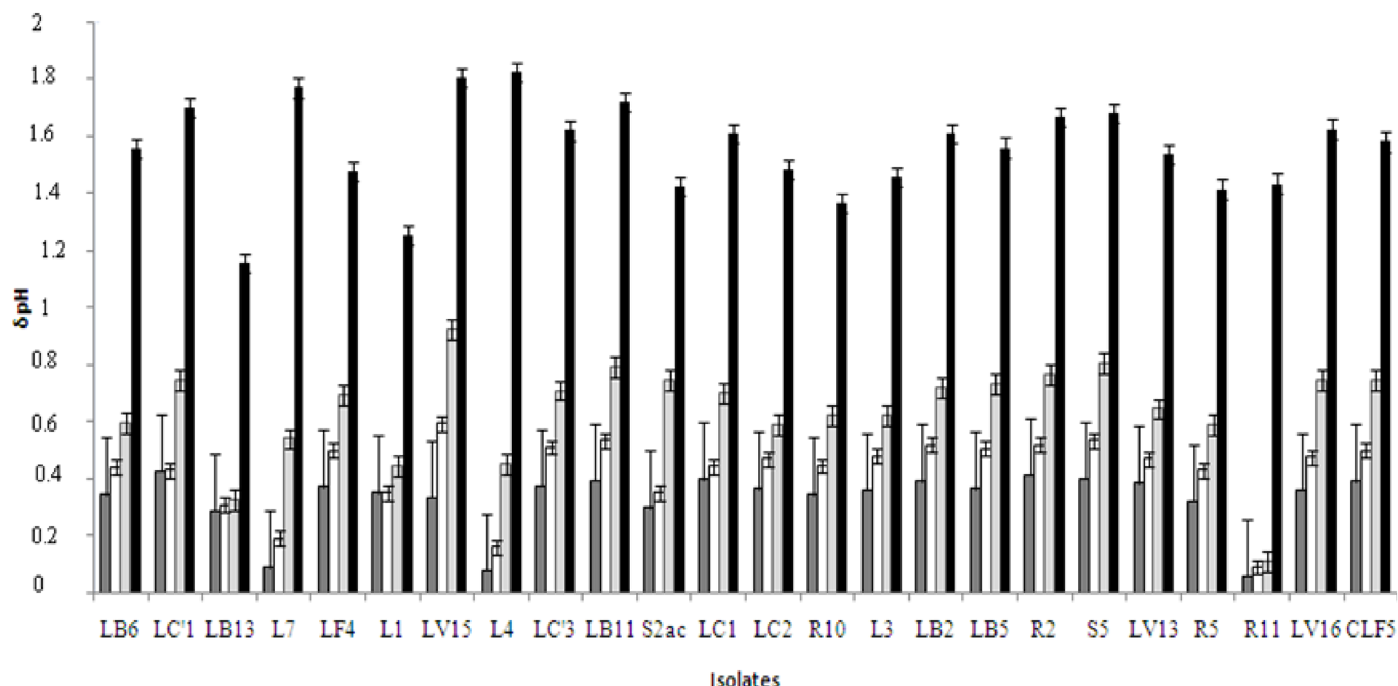

Figure 1. pH decrease in reconstituted skim milk after 2 ‘δpH2’, 4 ‘δpH4’, 6 ‘δpH6’ and 24 ‘δpH24’ h of incubation at 37°C, respectively. Values are mean ± standard deviation.

microtitre plate using cation-adjusted Mueller-Hinton broth test medium (Bection Dickinson and Company Cockeysville, MD, USA). One hundred microliters of the standardized 0.5 McFarland suspension were added to 10 ml of cation adjusted Muller-Hinton broth obtaining a concentration of 1 × 10^6 CFU $m1^{-1}$. After vortexing, the cell suspension was transferred in a sterile multi-channel solution basin. This provided a final inoculum density of approximately 5 x 10^5 CFU ml^{-1} *per* well. The concentrations of vancomycin hydrochloride (from *Streptomyces orientalis*, Biochemika, Fluka; Sigma–Aldrich) ranged from 2 to 256 µg mL^{-1}. The plates were covered with the adhesive seal and aerobically incubated at 35 ± 2°C for 24 h before visual reading of growth. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

All the 23 isolates belonged to the *E. durans* species. The results for acidifying activity in milk at 37°C are shown in Figure 1. The initial pH of the milk was 6.59. All the isolates showed a low acidifying activity after 2 (δpH2), 4 (δpH4) and 6 (δpH6) h of incubation, with values ranging from 0.06 to 0.42, 0.09 to 0.59 and from 0.11 to 0.92 pH units, respectively. In relation to their ability to reduce the pH of skim milk within 24 h (δpH24), the values of acidification activity of the 23 isolates studied ranged between 1.16 ± 0.03 and 1.83 ± 0.01 pH units. It was noted that *E. durans* L4 showed the highest acidification activity (1.83 ± 0.01 pH units) while, *E. durans* LB13 showed the lowest (1.16 ± 0.03 pH units).

After 24 h, 13 of 23 isolates lowered the pH below 5 reaching a value of 4.76. These results are in accordance with those reported by Morandi et al. (2006). They observed that most of the *E. durans* isolates tested in their study reduced the pH to 4.5 - 5 after 24 h of incubation in skim milk. Similar results are reported for *Enterococci* of dairy origin confirming the poor acidifying capacity of these bacteria in milk (Andrighetto et al., 2001; Durlu-Ozkaya et al., 2001; Sarantinopoulos et al., 2001).

Starter cultures used for cheese manufacture contain lactic acid bacteria (LAB) strains which are capable to reduce milk pH below 5.3 in 6 h at their optimum growth temperature (Beresford et al., 2001). This is essential for coagulation and for the prevention or reduction of the growth of adventitious microflora. All the *E. durans* examined in this study showed low, although variable, acidifying activity in reconstituted skim milk at the first 6 h and, therefore, they could not be used as starter organisms. However, they may be useful as adjunct cultures. These results confirm data previously reported by Giraffa (2003) indicating that *Enterococci*, in general, exhibit low milk acidifying ability.

A great variability in the ability of the isolates to hydrolyse milk proteins was observed (Table 3). Results of proteolytic activity ranged between 2.04 and 5.22 mM glycine. The *E. durans* R2 isolate showed the highest activity (5.22 ± 0.88 mM Gly), while *E. durans* CLF5 showed the lowest (2.04 ± 0.05 mM Gly). In general, the majority of the isolates exhibited high extracellular proteolytic activity. These results are not in agreement with those obtained by other authors who reported that *E.*

Table 3. Summary of the results of the biochemical tests carried out on the *E. durans* isolates.

Strains	Metabolism of citrate	EPS production	Proteolysis[a] (mM Gly)	Autolysis[a] (%)	Nisin MIC (mg ml^{-1})	Vancomycin MIC (µg ml^{-1})
LB2	+	+	2.18 ± 0.02	9.74 ± 0.91	0.8	< 2
LB5	+	+	2.64 ± 0.05	20.34 ± 0.43	0.8	< 2
LB6	+	+	2.40 ± 0.08	8.08 ± 0.33	0.8	< 2
LB11	-	+	3.10 ± 0.00	13.59 ± 0.05	1.6	< 2
LB13	-	+	2.15 ± 0.01	2.59 ± 0.75	3.2	< 2
LC'1	+	+	2.47 ± 0.00	9.30 ± 0.30	0.8	< 2
LC1	+	+	3.04 ± 0.07	18.36 ± 0.54	0.8	< 2
LC2	+	+	2.34 ± 0.01	13.04 ± 0.47	0.8	< 2
LC'3	+	+	2.33 ± 0.01	9.49 ± 0.54	0.8	< 2
L1	+	+	3.41± 0.00	11.98 ± 0.15	0.8	< 2
L3	+	+	2.55 ± 0.00	9.76 ± 0.57	0.8	< 2
L4	+	+	2.69 ± 0.05	6.94 ± 0.15	0.8	< 2
LF4	+	+	2.28 ± 0.03	13.27± 0.64	0.8	< 2
LV13	+	+	2.56 ± 0.01	6.26 ± 0.62	0.8	< 2
LV15	+	+	2.18 ± 0.10	8.24 ± 0.17	0.8	< 2
LV16	+	+	2.46 ± 0.44	11.98 ± 0.49	0.8	< 2
CLF5	+	+	2.04 ± 0.05	9.82 ± 0.11	0.8	< 2
R2	+	+	5.22 ± 0.88	15.79 ± 0.32	0.8	< 2
R5	+	+	2.26 ± 0.02	9.00 ± 0.52	0.8	< 2
R10	+	+	2.79 ± 0.02	11.96 ± 1.90	1.6	< 2
R11	+	+	2.20 ± 0.02	6.51 ± 0.53	0.8	< 2
S2ac	+	+	2.16 ± 0.02	13.30 ± 0.39	0.8	< 2
S5	+	+	2.17 ± 0.01	7.98 ± 0.05	0.8	< 2

[a] Presented values are means of duplicate determinations. ±Indicates standard deviation from the mean.

durans, in general, shows a lower proteolytic activity than *E. faecalis* strains (Morandi et al., 2006; Sarantinopoulos et al., 2001; Suzzi et al., 2000). Psoni et al. (2006) showed that 75% of the *E. durans* strains tested exhibited a low proteolytic activity (< 50 ppm Gly, that is, < 0.6 mM Gly), after 24 h of growth in milk. The proteolytic activity of our *E. durans* isolates is comparable with that reported for *E. faecalis* by Gonzáles et al. (2010) in his recent study. Moreover, other studies reported that in general *Enterococci* from food show higher proteolytic activity than veterinary isolates (Sarantinopoulos et al., 2001). A recent study by González et al. (2010) reports for 4 *E. faecalis* the same proteolytic activity values showed in our study. According to these data, proteolytic activity seems to be strain-specific rather than be related to the different *enterococci* species. The proteolytic activity of dairy LAB is essential for the bacterial growth in milk and it is involved in the liberation of interesting peptides from caseins and serum proteins (Christensen et al., 1999; Pelaez and Requena, 2005). These proteins are known to include some amino acid sequences which, upon liberation from the proteins, exert a specific biological activity on the physiology of the consumer (Meisel and Schlimme, 1996; Aimutis, 2004).

It is known that autolysis affects the release of endocellular enzymes which may have an impact on flavour and aroma during cheese ripening (Lortal and Chapot-Chartier, 2005). The *E. durans* tested in this study showed variable autolytic activities. In general, they exhibited a low autolysis rate, from 2 to 20%. Nevertheless, these values are compatible with the potential role of the strains as adjunct cultures (Franciosi et al., 2009).

The ability to metabolize citrate is another important technological feature of certain LAB species. Citrate in milk is metabolised into flavour compounds such as acetate, acetaldehyde, acetoin and diacetyl (Hugenholtz, 1993; Fox and Wallace, 1997). A high number of isolates (21 out of 23 isolates) tested in this study were able to metabolise citrate (Table 3). Therefore, if used as adjunct culture, they could be able to actively contribute to the flavour development in fermented dairy products.

Moreover, all the isolates were able to produce exo-polysaccharides (EPS) (Table 3). EPS content contributes to the viscosity and the smooth texture of yoghurt. Exo-polysaccharides stabilise the yoghurt gel and decrease its tendency to synerise (Schellhaas, 1983). Since smooth and creamy products have considerable appeal for consumers, EPS production is a relevant feature in the selection of starter strains and secondary cultures (Parente and Cogan, 2004).

Besides the possession of useful technological features, some important safety characteristics should be

assayed before using LAB strains in dairy manufacturing. The haemolytic activity constitutes a potential pathogenic trait that can be displayed by *Enterococci*. However, none of the *E. durans* tested produced haemolysin when tested on sheep blood. The absence of such activity should be a criterion for selecting strains to be used as starter or adjunct cultures in dairy products (Giraffa, 1995).

It is known that nisin is the most extensively studied bacteriocin and has been approved for use as a food preservative (Jung et al., 1992; Davies et al., 1999; Choi and Park, 2000; Chen and Hoover, 2003). All isolates of *E. durans* were able to grow in the presence of high concentration of nisin (≥ 0.8 mg $m1^{-1}$) (Table 3). These results show that the isolates of this study can be used in dairy products in the presence of nisin or nisin-producing LAB strains. Another important safety criterion to be considered when selecting strains as starter or adjunct cultures is their ability to withstand antibiotic. An increasing number of *Enterococci* resistant to different classes of antibiotic is being reported in the last years (Bertrand et al., 2000; Facklam et al., 2002; Leclercq, 2009). A major concern is the emergence of vancomycin restistant *Enterococci* (VRE). Vancomycin is considered as the last resort antibiotic to treat serious infections due to resistant Gram-positive bacteria, and given exclusively in a clinical environment, when all others fail. All the *E. durans* isolates studied had vancomycin MICs < 2 µg ml^{-1} (Table 3). According to CLSI (2006) and European Food Safety Authority (EFSA) (2008) *Enterococcus* antibiotic resistance interpretive criteria, these *E. durans* isolate had vancomycin MICs in the susceptible range (susceptible, ≤4 µg $m1^{-1}$; intermediate, 8 - 16 µg $m1^{-1}$; resistant, ≥32 µg $m1^{-1}$). The absence of vancomycin resistance can be considered a positive trait for these bacteria isolates for their use in food manufacture.

Conclusions

Among the 23 *E. durans* isolated from Moroccan dairy products studied a wide technological potential difference between isolates from the same or different sources was observed. A rapid decrease in pH during the initial steps of cheese preparation is of crucial importance in cheese manufacture, while the ability of strains to lyse and subsequently release their intracellular enzymes is a desirable trait during the ripening of cheese. In general, *enterococci* are not suitable as starter cultures because of their scarce acidifying ability, but they may be useful as good adjunct cultures. All *E. durans* characterized in this study were able to produce exo-polysaccharides (EPS), and had potential metabolic traits involved in cheese ripening and in aroma and flavour development in various fermented food. Moreover, most of the isolates were able to tolerate a high concentration of nisin, allowing their use in the presence of this food biopreservative.

As none of the *E. durans* isolated was haemolitic or showed vancomycin resistance, the possibility to safely use them in health added value dairy products could be taken into account. Further studies should be carried out on potential probiotic traits and other safety issues, as the presence of genes coding for virulence factors and antibiotic resistance, before using these isolates that colonize typical Moroccan dairy products as adjunct cultures.

For the first time in Morocco, *E. durans* isolated from different dairy products were characterized for their technological potential. The interesting technological traits showed by the isolates are important findings for the constitution of the National Center of LAB (starter, adjunct cultures and probiotics) in Morocco.

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REFERENCES

Aimutis WR (2004). Bioactive Properties of Milk Proteins with Particular Focus on Anticariogenesis. J. Nutr., 134: 989S-95S.

Andrighetto C, Knijff E, Lombardi A, Torriani S, Vancanneyt M, Kersters K, Swings J, Dellaglio F (2001). Phenotypic and genetic diversity of *enterococci* isolated from Italian cheeses. J. dairy. Res., 68: 303-316.

Beresford TP, Fitzsimons NA, Brennan NL, Cogan TM (2001). Recent advances in cheese microbiology. Int. dairy, J., 11: 259-274.

Bertrand X, Mulin B, Viel JF, Thouverez M, Talon D (2000). Common PFGE patterns in antibiotic-resistant *Enterococcus faecalis* from humans and cheeses. Food. Microbiol., 17: 543-551.

Bouksaim M, Fliss I, Merhrous J, Simard RE, Lacroix C (1998). Immunodot detection of nisin Z in milk and whey using enhanced chemiluminescence. J. Appl. Microbiol., 84: 176-184.

Boutrou R, Sepulchre A, Pitel G, Durier C, Vassal L, Gripon JC, Monnet V (1998). Lactococcal lysis and curd proteolysis: Two predictable events important for the development of cheese flavour. Int. dairy. J., 8: 609-616.

Casalta E, Zennaro R (1997). Effect of specific starters on microbiological, biochemical and sensory characteristics of Venaco, a Corsican soft cheese. Sci. Alim., 17: 79-94.

Centeno JA, Menendez S, Hermida MA, Rodriguez-Otero JL (1999). Effects of the addition of *Enterococcus faecalis* in Cebreiro cheese manufacture. Int. J. Food. Microbiol., 48: 97-111.

Centeno JA, Menendez S, Rodriguez-Otero JL (1996). Main microbial flora present as natural starters in Cebreiro raw cow's-milk cheese (northwest Spain). Int. J. Food. Microbiol., 33: 307-313.

Chen H, Hoover DG (2003). Bacteriocins and their food application. Comprehensive Rev. Food Sci. Food Safety, 2: 82-100.

Choi MH, Park YH (2000). Selective control of lactobacilli in Kimichi with nisin. Lett. Appl. Microbiol., 30: 173-177.

Christensen JE, Dudley EG, Pederson JA, Steele JL (1999). Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek, 76: 217-246.

Church FC, Swaisgood HE, Porter DH, Catignani GL (1983). Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy. Sci., 66: 1219-1227.

Clinical and Laboratory Standards Institute (CLSI) (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard—seventh edition. CLSI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.4.

Davies EA, Milne CF, Bevis HE, Potter RW, Harris JM, Williams GC,

Thomas LV, Delves-Broughton J (1999). Effective use of nisin to control lactic acid bacterial spoilage in vacuum packed bologna-type sausage. J. Food. Protect., 62: 1004-1010.

De Kwaadsteniet M, Todorov S, Knoetze H, Dicks L (2005). Characterization of a 3944 Da bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram+ and Gram- bacteria. Int. J. Food. Microbiol., 105: 433-444.

De Vuyst L, Foulquié Moreno MR, Revets H (2003). Screening for enterocins and detection of hemolysin and vancomycin resistance in *enterococci* of different origins. Int. J. Food. Microbiol., 84: 299-318.

Durlu-Ozkaya F, Xanthopoulos V, Tunail N, Litopoulou-Tzanetaki E (2001). Technologically important properties of lactic acid bacteria isolates from Beyaz cheese made from raw ewes' milk. J. Appl. Microbiol., 91: 861-870.

Ennahar S, Deschamps N (2000). Anti-Listeria effect of enterococin A, produced by cheese-isolated *Enterococcus faecium* EFM01, relative to other bacteriocins from lactic acid bacteria. J. Appl. Microbiol., 88: 449-457.

European Food Safety Authority (EFSA) (2008). Technical guidance-Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J., 732: 1-15.

Facklam RR, Carvalho MGS, Teixera LM (2002). History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of *Enterococci*. In: Gilmore MS (eds) The *Enterococci*: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington, DC, Chapter, 1: 1-54.

Foulquié Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006). The role and application of *Enterococci* in food and health. Int. J. Food. Microbiol., 106: 1-24.

Fox PF, Wallace JM (1997). Formation of flavor compounds in cheese. Adv. Appl. Microbiol., 45: 17-85.

Franciosi E, Settanni L, Cavazza A, Poznanski E (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. Int. dairy. J., 19: 3-11.

Gardiner GE, Ross RP, Wallace JM, Scanlan FP, Jagers PP, Fitzgerald GF, Collins JK, Stanton C (1999). Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of Cheddar cheese. J. Agr. Food. Chem., 47: 4907-4916.

Ghrairi T, Frere J, Berjeaud J, Manai M (2008). Purification and characterisation of bacteriocins produced by *Enterococcus faecium* from Tunisian rigouta cheese. Food. Control, 19: 162-169.

Giraffa G (1995). Enterococcal bacteriocins: their potential as anti-Listeria factors in dairy technology. Food. Microbiol., pp 291-299.

Giraffa G (2003). Functionality of *enterococci* in dairy products. Int. J. Food. Microbiol., 88: 215-222.

Giraffa G, Carminati D, Neviani E (1997). *Enterococci* isolated from dairy products: a review of risks and potential technological use. J. Food. Protect,, 60: 732-738.

González L, Sacristán N, Arenas R, Fresno JM, Tornadijo M E (2010). Enzymatic activity of lactic acid bacteria (with antimicrobial properties) isolated from a traditional Spanish cheese. Food. Microbiol., 27: 592-597.

Hugenholtz J (1993). Citrate metabolism in lactic acid bacteria. FEMS. Microbiol. Rev., 12: 165-178

Huycke MM, Sahm DF, Gilmore MS (1998). Multiple-drug resistant *enterococci*: the nature of the problem and an agenda for the future. Emerg. Infect. Dis., 4: 239-249.

Jackson CR, Fedorka-Cray PJ, Barrett JB (2004). Use of a genus -and species-specific multiplex PCR for identification of *enterococci*. J. Clin. Microbiol., 42: 3558-3565.

Jung DS, Bodyfelt FW, Daeschel MA (1992). Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. J. Dairy. Sci., 75: 387-393.

Ke D, Picard FJ, Martineau F, Ménard C, Roy PH, Oullette M, Bergeron MG (1999). Development of a PCR Assay for Rapid Detection of *Enterococci*, J. Clin. Microbiol., 37: 3497-3503.

Kempler GM, Mc Kay LL (1980). Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl. Env. Microbiol., 39: 926-927.

Leclercq R (2009). Epidemiological and resistance issues in multidrug resistant staphylococci and *enterococci*. Clin. Microbiol. Infect., 15: 224-231.

Litopoulou-Tzanetaki E, Tzanetakis N, Vafopoulou-Mastrojiannaki A (1993). Effect of the type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese. Food. Microbiol., 10: 31-41.

Lortal S, Chapot-Chartier MP (2005). Role mechanisms and control of lactic acid bacteria lysis in cheese. Int. dairy J., 15: 857-871.

Manolopoulou E, Sarantinopoulos P, Zoidou E, Aktypis A, Moschopoulou E, Kandarakis, IG, Anifantakis EM (2003). Evolution of microbial populations during traditional Feta cheese manufacture and ripening. Int. J. Food. Microbiol., 82: 153-161.

Maragkoudakis PA, Konstantinos CM, Psyrras D, Cremonese S, Fischer J, Cantor MD, Tsakalidou E (2009). Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and *Salmonella enteriditis.* Int. J. Food. Microbiol., 130: 219-226.

Meisel H, Schlimme E (1996). Bioactive peptides derived from milk proteins: Ingredients for functional foods?. Kieler Milchwirtschaftliche Forschungsberichte, 48: 343-357.

Mora D, Fortina MG, Parini C, Ricci G, Gatti M, Giraffa G, Manachini PL (2002). Genetic diversity and technological properties of *Streptococcus thermophilus* strains isolated from dairy products. J. Appl. Microbiol., 93: 278-287.

Morandi S, Brasca M, Andrighetto C, Lombardi A, Lodi R (2006). Technological and molecular characterisation of *enterococci* isolated from north-west Italian dairy products. Int. Dairy. J., 16: 867-875.

Ogier JC, Serror P (2008). Safety assessment of dairy microorganisms: The *Enterococcus* genus. Int. J. Food. Microbiol., 126: 291-301.

Oumer A, Gaya P, Fernández-Garcia E, Mariaca R, Garde S, Medina M, Nuñez M (2001). Proteolysis and formation of volatile compounds in cheese manufactured with bacteriocin-producing adjunct starter. J. dairy. Res., 68: 117–129.

Parente E, Cogan TM (2004). Starter cultures: general aspects. In: Franciosi E, Settanni L, Cavazza A, Poznanski E (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. Int. dairy J., 19: 3-11.

Pelaez C, Requena T (2005). Exploiting the potential of bacteria in the cheese ecosystem. Int. dairy J., 15: 831-844.

Psoni L, Kotzamanides C, Andrighetto C, Lombardi A, Tzanetakis N, Litopoulou-Tzanetaki E (2006). Genotypic and phenotypic heterogeneity in *Enterococcus* isolates from Batzos, a raw goat milk cheese. Int. J. Food. Microbiol., 109: 109-120.

Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea M.C, Lombardi A, Cogan TM, Kalantzopoulos G, Tsakalidou E (2001). Biochemical properties of *enterococci* relevant to their technological performance. Int. Dairy J., 11: 621-647.

Sarantinopoulos P, Kalantzopoulos G, Tsakalidou E (2002a). Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. Int. J. Food Microbiol., 76: 93-105.

Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalaki M, Kalantzopoulos G, Tsakalidou E, De Vuyst L (2002b). Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. Int. J. Food. Microbiol., 72: 125-136.

Schellhaas SM (1983). Characterization of exocellular slime produced by bacterial starter cultures used in the manufacture of fermented dairy products. In: Wouters JTM, Ayad EHE, Hugenholtz J, Smit G (2002). Microbes from raw milk for fermented dairy products. Int. dairy J., 12: 91-109.

Suzzi G, Caruso M, Gardini F, Lombardi A, Vannini L, Guerzoni ME, Andrighetto C, Lanorte MT (2000). A survey of the *enterococci* isolated from an artisanal Italian goat's cheese (semicotto caprino). J. Appl. Microbiol., 89: 267-274.

Tzanetakis N, Vafopoulou-Mastrojiannaki A, Litopoulou-Tzanetaki E (1995). The quality of white-brined cheese from goat's milk made with different starters. Food. Microbiol., 12: 55-63.

Villani F, Coppola S (1994). Selection of enterococcal strains for water-buffalo Mozzarella cheese manufacture. Ann. Microbiol. Enzim., 44: 97-105.

Wessels D, Jooste PJ, Mostert JF (1990). Technologically important characteristics of *Enterococcus* isolates from milk and dairy products. Int. J. Food Microbiol., 10: 349-352.