Technological performance of the enterocin A producer *Enterococcus faecium* MMRA as a protective adjunct culture to enhance hygienic and sensory attributes of traditional fermented milk 'Rayeb'

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Abstract

Enterococcus faecium MMRA is an enterocin A producer isolated from 'Rayeb', a Tunisian fermented milk drink. In this work, safety aspects and its behaviour in raw milk were investigated to assess its suitability as a protective adjunct culture. *E. faecium* MMRA showed interesting features such as the absence of several virulence traits, susceptibility to vancomycin and other clinically relevant antibiotics, and lack of haemolytic activity. To evaluate its performance as an adjunct culture for Rayeb, changes in the overall composition of control (non-inoculated) and experimental Rayeb (inoculated with 1 % v/v *E. faecium* MMRA) were determined throughout duplicate fermentations of raw milk using microbiological, chemical, HPLC and HSGC-MS analyses. *E. faecium* MMRA could multiply in raw milk and produced enterocin A. Interestingly, a higher content of volatile compounds including ethanol, diacetyl and 2-propanol was observed in the presence of this bacteriocin producer. Furthermore, this strain was capable of inhibiting the growth of *Listeria monocytogenes* CECT 4032 in pasteurized milk, although total killing was not achieved. Further experiments confirmed the development of resistant variants to enterocin A. On similar challenge assays, *L. monocytogenes* CECT 5672 growth was halted by the presence of the enterocin producer but viability was only slightly reduced during cold storage. According to our results, *E. faecium* MMRA meets the criteria for an autochthonous protective adjunct culture to enhance both the hygienic and the sensory attributes of Rayeb.

Keywords: Rayeb; enterocin A; anti-*listeria;* safety; protective culture; flavour.

Introduction

For centuries fermented products have played an important role in human nutrition. Traditional fermented dairy products vary considerably in composition, flavour and texture, depending on the properties of the fermenting organisms, type of milk, region of production and method of manufacture. These products have always proved very popular with consumers. Rayeb is one such popular indigenous dairy drink consumed in Tunisia, mostly in the summer as a dessert or refreshing beverage, and plays a major role in the diet of rural communities, as occurs with similar dairy products from other North African countries (Benkerroum & Tamime, 2004). Rayeb is traditionally made from the raw milk of cows, ewes or goats, placed in earthenware pots and kept undisturbed without temperature control for 24 h. It is produced through spontaneous fermentation of the milk, and is sometimes started via backslopping (inoculation of raw milk with a small quantity of the previous successful fermentation). On an industrial scale, it is produced from pasteurised cows' milk, with the addition of starter cultures and rennet.

Contamination by *Listeria monocytogenes* of traditional dairy drinks similar to Rayeb has been previously reported (El Marrakchi et al., 1993). Thus, the safety of this fermented beverage should be improved. Raw milk, in particular, is widely recognised as a source of *L. monocytogenes* contamination and a vehicle of listeriosis (Ryser, 1999). *L. monocytogenes* is the causative agent of a wide range of pathologies, ranging from gastroenteritis to meningitis and abortion with mortality rates of 20-30% and has long been recognized as one of the most important food safety issues to address (Aureli et al., 2000; Lundén et al., 2004; Cossart & Toledo-Arana, 2008). It is able to survive under severe physico-chemical conditions such as refrigeration temperatures, low pH values and high salt concentrations (Lou & Yousef, 1999), promoting persistence in foods and on food processing equipment.

Due to the increasing demand for minimally processed foods, free from chemical additives, the use of bacteriocinogenic lactic acid bacteria (LAB) in food biopreservation has gained

widespread attention (Deegan et al., 2006; Gálvez et al., 2007). Among LAB, enterococci are known to produce a number of enterocins that can effectively inactivate food spoilage micro-organisms and pathogenic bacteria such as *L. monocytogenes*, suggesting their role as 'protective' bacteria (Giraffa, 1995). Enterococci are present in milk and several dairy products, particularly those produced in Mediterranean countries (Giraffa, 2002; Franz et al., 2003). Although their presence in dairy products has been regarded as an indicator of insanitary production methods, they have a long history of safe use (Foulquié Moreno et al., 2006; Ogier & Serror, 2008). Despite their beneficial effects on the sensory characteristics and the digestibility of dairy products, interest in the use of enterococci in starter cultures has somewhat diminished due to the fact that some strains may have virulence factors and antibiotic resistance genes (Foulquié Moreno et al., 2006). Moreover, biogenic amines produced by some enteroccocal strains are also a source of concern in the food industry, due to their toxigenic potential in humans (Bover-Cid et al., 1999). With this in mind, the selection of enterococcal strains for use in food fermentations requires a careful safety assessment (Ogier & Serror, 2008).

We have previously isolated the strain *Enterococcus faecium* MMRA from traditional Tunisian Rayeb (Rehaiem et al., 2010). It synthesises the pediocin-like bacteriocin, enterocin A, a class IIa bacteriocin with strong anti-listeria activity (Aymerich et al., 1996). In the present study, we have assessed the suitability of this strain as a potential protective adjunct culture for the manufacture of the traditional Rayeb. Both safety and technological issues have been addressed.

Material and Methods

Bacterial strains and culture conditions

Enterococcus faecium MMRA, an enterocin A producer, was previously isolated from home-

made, traditional Tunisian 'Rayeb' (Rehaiem et al. 2010). It was routinely grown on M17 broth supplemented with 0·5% (w/v) lactose (LM17) (Scharlab, Barcelona, Spain) at 37 ºC for 18 h in aerobiosis. *Listeria monocytogenes* CECT 4032 and *L. monocytogenes* CECT 5672, used in challenge assays, were obtained from the Spanish Culture Collection (CECT) and propagated in Tryptone soya broth (TSB) (Difco laboratories, USA) at 37 ºC. *Listeria innocua* CECT 910 was used to detect enterocin A by the agar diffusion test, and was also grown in TSB at 37 ºC for 18 h in aerobiosis (Rehaiem et al. 2010). A spontaneous mutant of *E. faecium* MMRA, resistant to rifampicin (Rif^r) was obtained by plating 10^8 cfu of an overnight culture on LM17 plus 100 µg/mL rifampicin (Sigma Co, St. Louis, USA). This strategy has been successfully used in the past to allow differential enumeration (Rilla et al., 2003). *E. faecium* MMRA Rif^r was grown in UHT milk and incubated for 24 h at 37 °C to be used as protective adjunct starter. All the strains were stored at -80 ºC with 15% glycerol.

Haemolytic activity, antibiotic resistance and enzymatic profile

Haemolysin activity was determined by measuring zones of clearing on Columbia agar plates containing 5% (w/v) sheep blood (BioMérieux, Macy-L'Etoile, France) after 48 h of incubation at 37 °C. The susceptibility of the *E. faecium* MMRA strain to 23 commonly used antibiotics (Table 1) was performed by the disk diffusion method on Muller-Hinton agar, according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (2008) Antibiotic disks were obtained from BioMérieux. The enzymatic profile was assayed using API Zym galleries (BioMérieux) according to the manufacturer's instructions.

PCR amplification

PCR reactions to detect the presence of genes involved in the expression of the aggregation substance (*agg*), cytolysin (*cylL*), gelatinase (*gelE*), enterococcal surface protein (*esp*), and

hyaluronidase (*hyl*) were performed according to Gasson et al. (2001) with the primers listed in Table 2, using PuRe Taq Ready-to-go PCR Beads (GE Healthcare, Buckinghamshire, UK). DNA from *E. faecalis* FI9190 (Pérez-Pulido et al., 2006) and *E. faecalis* V583 (Paulsen et al., 2003) was used as a positive controls for virulence traits. Likewise, PCR reactions to detect histidine (*hdc*) and tyrosine (*tdc*) decarboxylase genes were carried out in the same way using the appropriate primers (Table 2) and conditions as previously described (Le Jeune et al., 1995; Lucas & Lonvaud-Funel, 2002; Fernández et al., 2006). DNA from *E. durans* IPLA 655 (Fernández et al., 2004) and *E. faecalis* V583 was used as a positive control for the *hdc* and *tdc* genes, respectively.

Rayeb manufacture

Fresh raw cow's milk was supplied by a collaborative farm. Two batches of 'Rayeb' were manufactured in duplicate, with each vat containing 100 mL of raw milk. A 1% (v/v) overnight culture of the rifampicin resistant *E. faecium* MMRA (adjunct culture) was added to the experimental vats, while no adjunct culture was added to the control vats. Incubation was performed at 37ºC for 24 h. Samples were taken aseptically during the fermentation process at 12 h and 24 h for further analyses as described below. Two independent trials were carried out.

Microbiological analyses

Samples of raw milk (10 mL) and Rayeb (10 g) were aseptically taken. Rayeb samples were homogenised in 90 mL of a prewarmed sterile 2% sodium citrate solution in a Stomacher Lab-Blender (Seward Medical, London, UK). Decimal dilutions of milk and homogenates were made in quarter-strength Ringer solution (Merck KGaA, Damstadt, Germany) and plated on several different types of culture media. Total aerobic viable bacteria were pour plated on PCA agar (Scharlau Microbiology, Barcelona, Spain), total lactic acid bacteria on Elliker (EK) Agar

(Scharlau Microbiology), and coliforms on Violet Red Bile Agar VRBA (Biokar Diagnostics, Beauvais, France). Enterococci were spread plated on Kenner Fecal Agar (KF Agar) (Sharlau Microbiology) supplemented with 1% triphenyl tetrazolium chroride (TTC) (Scharlau Chemie, Barcelona, Spain) and KF agar supplemented with 100 μg/mL rifampicin (Sigma) was used for *E. faecium* MMRA Rif^r counting. KF and VRBA plates were further overlaid with 10 ml of the same medium. Depending on the medium requirements, plates were incubated for 48 h at 32 °C (PCA and EK) or 37 °C (VRBA and KF). Microbiological count data were expressed as $\log_{10}$ CFU/mL and carried out in duplicate. To determine enterocin A activity, samples of Rayeb (1 g) were homogenised with 0.02 HCl (1:1, v/v) and centrifuged at 12,000 × *g* for 20 min at 4 ºC. The extracts were tested for bacteriocin activity against *L. innocua* CECT 910 by the agar diffusion test and expressed in mm to represent the zone of inhibition.

Physicochemical analyses

Acidity was measured by titration of milk and 'Rayeb' samples to pH 8.2 with 0.1 M NaOH (Panreac, Barcelona, Spain). Data were expressed as grams of lactic acid per 100 mL of sample. pH was measured with a MicropH 2001 pH meter (Crison, Barcelona, Spain). Dry matter, fat and protein content were determined according to IDF Standard 4A (1982), IDF Standard 152 (1991) and IDF Standard 20B (1993), respectively.

HPLC analysis and detection of volatile compounds.

Major sugars and organic acids were determined by HPLC as described by Fernández et al., (2007). Briefly, 25 mL of 4.5 H_2SO_4 mM were added to 5 mL of milk or 5 g of Rayeb, extracted for 1 h, and centrifuged (12,000 × g, 5 min). Supernatants (50 μL) were isocratically separated in a 300×7.8 mm HPX-87H Aminex ion-exchange column (Bio-Rad Laboratoires, Inc., Hercules, CA, USA) protected by a cation H+ Microguard cartridge (BioRad), at a flow rate of

0.7 mL/min and a temperature of 65°C. Sulfuric acid (3 mM) was used as the mobile phase. Organic acid and sugars concentrations were determined using a chromatographic system composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 and a 410 Differential Refractometer detector, connected in series, and controlled by Millennium 32 software (Waters, Milford, MA, USA). Solutions of organic acids and sugars were used as standards in the identification and quantification procedure.

Analysis of volatile compounds

Volatile compounds were determined by HSGC-MS according to Salazar et al., (2009). A G1888 headspace system (HS), connected to a Agilent 6890N gas chromatograph (GC) coupled to a 5975B inert mass selective detector (MSD) was used. Data were recorded and analysed with a ChemStation Software (Agilent Technologies, Santa Clara, CA, USA). Milk or curd samples (5 g) with cyclohexanone (0.36 mg/mL) as the internal standard and 5 g of anhydrous sodium sulphate were added into a 20 mL headspace glass vial, hermetically sealed. Samples in the HS were held for 30 min at 50 °C with stirring. Injections were made at a split ratio of 20:1, and the temperature was maintained at 220 ºC. Volatile compounds were separated on a HP-Innovax column (60 m × 0.25 mm, 0.25-μm film thickness). The chromatographic conditions were 35 °C for 5 min, a temperature increase of 5°C/min up to 100ºC and a second heating ramp of 8 ºC/min up to 240 ºC, held for 5 min. Helium was the carrier gas. Signals were recorded by the MSD by electron impact ionisation set at 70 eV operating in the scan mode. Volatile compounds were identified by comparing their mass spectra with those in the Wiley 138 library (Agilent). The peaks were quantified as the relative total ionic count abundance with respect to the IS. The concentration (μg/mL) of each volatile compound was calculated by using linear regression equations ($R^2 > 0.99$) of the corresponding standards.

TLC analysis of biogenic amines

Culture supernatants were obtained by centrifugation and their amine content determined by Thin-Layer Chromatography (TLC) as described by García-Moruno et al., (2005). Briefly, amines were converted to their fluorescent dansyl derivatives and fractionated on precoated silica gel 60 F_{254} TLC plates (Merck) in chloroform:triethylamine (4:1). The fluorescent dansyl derivative spots were visualized under UV-light (312 nm).

Challenge assays

Commercial pasteurised whole milk (Hacendado, Spain) was contaminated with overnight cultures of either *Listeria monocytogenes* CECT 4032 (aproximately 10^6 CFU/mL) or *L. monocytogenes* CECT 5672 (aproximately 10^4 CFU/ml). For each batch, one vat (100 mL) was used as control and a second vat (100 ml) was inoculated at 1% (v/v) with an overnight culture of *E. faecium* MMRA. The vats were incubated at 37 ºC for 24 h and were subsequently stored at 4 ºC for 2 days. Samples were aseptically taken at time intervals. For microbiological analysis, decimal serial dilutions in sterile Ringer solution were made and plated on *Listeria* selective Oxford agar containing Oxford selective supplement (Scharlau Microbiology) for *L. monocytogenes* enumeration, and on KF to quantify *E. faecium* MMRA. Plates were incubated for 48 h at 37 °C. Aliquots of co-cultures were also centrifugated at 12000 × g for 10 min, and the supernatants were tested for bacteriocin activity by the agar diffusion test against *L. innocua* CECT 910. Two independent challenging experiments were carried out.

Statistical analysis

It was performed using the SPSS-PC+11.0 software (SPSS, Chicago, IL, USA). Data related to microbiological counts, pH, acidity, gross composition (dry matter, fat and protein content),

carbohydrate consumption, organic acid and volatile compounds production were subjected to one-way ANOVA using two factors: 'type of Rayeb' with two categories (control and experimental) and 'incubation time' with three categories (0, 12 and 24 h). The least significant difference (LSD) test ($P<0.05$) was applied for means comparison.

Results and Discussion

The role of enterococci in food fermentations is not yet fully understood. They are ubiquitously distributed in traditionally fermented products and contribute positively to the sensory attributes. However, they may pose a risk as a potential reservoir of antibiotic resistance and virulence genes which could be transferred to human strains in the gastrointestinal tract. Therefore, it is mandatory that prior to any application in food, future starters or adjunct cultures undergo a careful screening to consider the safety and technological issues on a case-by-case basis (Ogier & Serror, 2008). On the other hand, it should be noted that food borne pathogens have been a continuous concern and can pose a serious health risk for consumers, *L. monocytogenes* being one of the common pathogens in milk and fermented milk products (WHO, 2007). Since the use of bacteriocin-producing strains can be a nice strategy to fight against undesirable bacteria (Gálvez et al., 2007), the high bacteriocinogenic potential of enterococci may play a protective role against *L. monocytogene*s in traditional fermented dairy products. Accordingly, in this work, we have focused on the enterocin A producer *E. faecium* MMRA, previously isolated from Rayeb, as a potential protective adjunct dairy culture. To our knowledge, no such studies have been done to assess the suitability of any LAB strain isolated from the traditional Tunisian Rayeb.

Preliminary safety assessment of *E. faecium* MMRA

Prior to evaluating the feasibility of the enterocin A producer *E. faecium* MMRA as a protective

adjunct culture for the elaboration of 'Rayeb', several risk factors were assessed. The presence of genes coding for five virulence factors, often found among enterococci, as well as those coding for amino acid decarboxylases involved in the synthesis of biogenic amines, was checked by PCR (Table 2). None of the potential virulence genes, including those coding for the aggregation pheromone (*agg*), hyaluronidase (*hyl*), and enterococcal surface protein gelatinase (*esp*), which are supposed to contribute to host colonization or hydrolysis of host proteins, could be amplified.

An exception was the amplification of *tdc* coding for the tyrosine decarboxylase enzyme, involved in tyramine production (Table 2). This is not surprising as tyramine is the biogenic amine most frequently produced by enterococci that have been isolated from dairy products (Bover-Cid et al., 1999; Sarantinopoulos et al., 2001). However, this biogenic amine was neither detected by TLC in overnight LM17 culture supernatants nor in Rayeb samples inoculated with *E. faecium* MMRA. It is conceivable that during the short fermentation time needed for Rayeb manufacture, the proteolytic activity of *E. faecium* MMRA and that of the indigenous microbiota is not high enough to reach the free tyrosine threshold that triggers tyramine production (Linares et al., 2009).

E. faecium MMRA was shown to be not haemolytic when grown on sheep blood agar (data not shown) and was susceptible to several β-lactams, aminoglycosides, and other broad-spectrum antibiotics (Table 1). Of note, *E. faecium* MMRA was susceptible to the glycopeptide vancomycin. This is of special interest as it is used as a last resort antibiotic against multiple antibiotic resistant enterococci (Klein, 2003; Franz et al., 2003; Ogier & Serror, 2008). The strain displayed intermediate resistance to some cephalosporins and resistance to oxacillin (Table 1). Thus, the antibiotic susceptibility profile of *E. faecium* MMRA is in agreement with previous reports concerning strains of enterococci that are commonly found in foods (Valenzuela et al., 2008; Barbosa et al., 2009; Ben Belgacem et al., 2010).

The enzymatic potential of the strain was evaluated using API ZYM strips (Table 1). Both Leucine and valine aminopeptidase, and alkalin and acid phosphatase, showed the strongest activities, while relatively weak esterase and protease activity was observed. *E. faecium* MMRA showed neither lipase nor potentially procarcinogenic activities (β-glucuronidase and β-glucosidase).

Overall, this preliminary safety assessment of *E. faecium* MMRA supports the notion that foodborne *E. faecium* poses a low risk when used in foods, since they are generally free of virulence determinants, or these determinants are found less frequently than in other enterococcal species (Gasson et al., 2001; Franz et al., 2001; Mannu et al., 2003; Abriouel et al., 2008).

Performance of *E. faecium* MMRA as an adjunct culture in Rayeb production

Preliminary assays had shown that *E. faecium* MMRA could grow up to 8.5 $\log_{10}$ CFU/ml and synthesise enterocin A in pasteurized milk, with slight acidification down to pH 6 within 24 h (our unpublished results). However, Rayeb is commonly made with raw milk, in which the autochthonous microbiota could hinder the development of any starter or adjunct culture. Therefore, two batches of Rayeb made of raw milk were manufactured to evaluate the viability and the technological performance of the enterocin A producer. To differentiate *E. faecium* MMRA from endogenous enterococci, a spontaneous rifampicin resistant mutant (MMRA Rif^r) was used to inoculate raw milk in the experimental vat (7.76 ± 0.8 $\log_{10}$ CFU/ml). A non-inoculated raw milk vat was used as control. The vats were incubated at 37 ºC for 24 h. It should be noted that the Rif^r strain displayed similar growth properties and enterocin A production to the parent strain in both LM17 and pasteurized milk (data not shown).

With regards to the physicochemical analyses, a similar pH, titratable acidity and gross composition (dry matter, fat, protein) was recorded in both control and experimental Rayeb after

24 h of incubation (P>0.05) (Table 3). Thus, the presence of *E. faecium* MMRA did not seem to disturb either the spontaneous fermentation of raw milk or the gross composition of the fermented raw milk.

Counts of the major bacterial populations, namely total viable counts, lactic acid bacteria, coliforms and enterococci, throughout Rayeb fermentation, are shown in Table 4. The adjunct strain *E. faecium* MMRA Rif^r in the experimental vat showed good growth in milk and consistently higher microbial counts on PCA and EK culture media were observed relative to the control at 12 (P<0.05) and 24 h (P<0.01) of incubation. The same applied for the enterococcal population as counted on KF agar, which reached a population 3.8 $\log_{10}$ higher (P<0.001) in the experimental Rayeb at 24 h. Based on the similar counts on KF with and without rifampicin, we presumed that *E. faecium* MMRA Rif^r was the main enterococcal strain present in the experimental vats. However, despite the fact that indigenous rifampicin resistant enterococci were below the limit of detection (<10 CFU/mL) in raw milk samples, they were detected after incubation in control Rayeb. Nonetheless, this was 6 $\log_{10}$ units lower than was observed in experimental Rayeb *(P*<0.001). Bacteriocin activity was only detected in experimental Rayeb (Table 3). This issue is relevant as bacteriocin production may be hampered in a complex food environment such as raw milk, as shown for *E. faecium* FAIR-E 198 (Sarantipoulos et al., 2002).

The total viable counts, coliforms and enterococci detected in raw milk are indicative of poor hygienic quality. Nevertheless, the decrease in pH (and the increase in acidity) likely contributed during fermentation to reduce the number of coliforms by 1.01-1.42 $\log_{10}$ CFU/mL in 24 h in both control and experimental vats. In fact, low pH is indeed a major hurdle in food preservation (Leistner 2000). However, the hygienic conditions of the fermented drink seemed to improve in the presence of *E. faecium* MMRA since a lower level of coliforms was detected, although not statistically significant (P>0.05). Considering that enterocin A is not active against Gram negatives, it is possible that a synergistic effect among different preserving compounds takes

place when the enterocin A producer is added to raw milk. Besides low pH, the higher diacetyl content detected in experimental Rayeb (see below) could have contributed largely because Gram-negative bacteria are particularly sensitive to this volatile compound (Jay, 1982).

Enhancement of the sensory attributes of Rayeb using *E. faecium* MMRA as an adjunct culture

From a technological point of view, selection of LAB in the dairy industry is mostly based on their ability to acidify and produce aromatic compounds (IDF Standard 149, 1991). Therefore, residual lactose, minor carbohydrates (glucose and galactose) and organic acids were also quantified throughout the Rayeb fermentations (Table 5). Incorporation of the strain MMRA as an adjunct culture did not seem to affect carbohydrate metabolism, as comparable values of lactose consumption (about 30%) were observed in both the control and experimental fermentations ($P>0.05$). Additionally, glucose was completely metabolized and similar levels of galactose occurred in both fermented milks ($P>0.05$). Accordingly, lactic acid production followed a similar pattern regardless of the presence of *E. faecium* MMRA ($P>0.05$), and accounted for over 90% of the carbohydrates consumed. Similar content of formic acid was also detected in the control and experimental Rayeb ($P>0.05$) (Table 5) As lactic acid production by the indigenous LAB microbiota is a critical parameter to ensure safe and successful raw milk fermentation, our results highlight the compatibility between the indigenous lactic acid bacteria and *E. faecium* MMRA.

Interestingly, citric acid, an important precursor for aroma development, was completely consumed after 24 h in both fermented milks (Table 5). Citrate fermentation gives rise to several volatile carbonyl compounds (mainly diacetyl) which greatly contribute to the organoleptic properties of the fermented products. Accordingly, diacetyl was detected in both control and experimental Rayeb but the relative abundance was notably higher in the presence of the adjunct starter strain from 12 h onwards ($P<0.001$) (Fig. 1). The inoculation of raw milk with *E. faecium*

MMRA also resulted in a higher production of ethanol (P<0.05) (Fig. 1). It is also worth noting that another alcohol, 2-propanol, was only detected on the experimental Rayeb (P<0.001) (Fig. 1). Other volatile compounds such as 2-propanone and acetoin were also detected in Rayeb whether or not the enterococcal strain was present. Therefore, *E. faecium* MMRA seemed to potentially enhance the aroma of Rayeb. Moreover, this strain can hydrolyze lactose via ß-galactosidase and exhibited high peptidase activity, mainly leucine and valine aminopeptidase (Table 1), which may further contribute to a better flavour and texture of dairy products (Arora et al., 1990). Similarly, other *E. faecium* strains isolated from dairy foods have been reported as active contributors to sensory characteristics of fermented dairy products (Andrighetto et al., 2001; Sarantinopoulos et al., 2001).

Inhibition of *L. monocytogenes* by *E. faecium* MMRA in milk

As far as we know, no published data exist on the incidence of *L. monocytogenes* in Tunisian Rayeb. However, studies in other Arab countries revealed the presence of *L. monocytogenes* in raw milk and traditional Raib (moroccan name for Rayeb) with up to 10% of samples being contaminated (El Marrakchi et al., 1993). More recently, *L. monocytogene*s has been detected in 2.61% of raw milk samples from algerian farms (Hamdi et al., 2007). Considering the protective role of enterocins in food preservation (Khan et al., 2010) and once established that *E. faecium* MMRA did not disturb Rayeb fermentation, we proceeded to carry out challenge experiments to determine if it could inhibit the growth of *L. monocytogenes* in milk (presumably through production of enterocin A), providing a natural hurdle for protecting this traditional dairy product.

Challenge experiments were performed in pasteurised milk which was incubated at 37 °C for 24 h, and subsequently kept at 4 ºC for two days to mimic storage conditions. Milk was contaminated with either *L. monocytogenes* CECT 4032 (approximately 10^6 CFU/mL) or *L.*

monocytogenes CECT 5672 (approximately 10^4 CFU/ml). These two strains were chosen on the basis of their dairy origin and their different susceptibilities to enterocin A. *L. monocytogenes* CECT 4032 was inhibited by 1.28×10^4 AU/mL while *L. monocytogenes* CECT 5672, more resistant, was inhibited by 2.56×10^4 AU/mL.

The fate of these two strains of *L. monocytogenes* was followed in the presence and absence of the adjunct *E. faecium* MMRA (Fig. 2). In both challenge assays, *E. faecium* MMRA grew during the incubation at 37 ºC and the population remained viable throughout cold storage (Fig. 2). Enterocin A was detected at 24 h in the experimental Rayeb and remained stable during cold storage for two days. As expected, no bacteriocin activity was detected in control Rayeb (Fig. 2). Cocultures of *L. monocytogenes* CECT 4032 and CECT 5276 with the enterocin A producer in milk resulted in a pH decrease from 6.65 at the inoculation time to 5.17 and 5.49, respectively, at 24 h. In both cases, the level of enterocin A detected, correlated to the growth of the producer strain.

Growth of *L. monocytogenes* CECT 4032 occurred in the absence of *E. faecium* MMRA, the viable counts reaching 10^9 CFU/mL in the first 24 h, and even increased further during cold storage (Fig. 2a) but counts were markedly reduced from $\geq 10^6$ to 10^2 CFU/mL in 24 h ($P<0.001$) and further on during the 2 days of storage in the presence of the enterocin A producer ($P<0.001$). However, total clearance of the pathogen was not achieved (Fig. 2a). Additional experiments were performed to understand why *L. monocytogenes* CECT 4032 was not completely inhibited despite their susceptibility to enterocin A. For this purpose, we randomly chose four representative colonies from the Oxford counting plates and tested their susceptibility to enterocin A by the agar diffusion test. No zones of inhibition were observed on any of them, demonstrating that the surviving *Listeria* cells had become resistant (data not shown). This is not a surprising result since natural resistance by *Listeria* strains against class IIa bacteriocins such as enterocin A has been previously reported (Ennahar et al., 2000). Susceptible strains can also

acquire resistance at a relative high frequency upon exposure to bacteriocins (Gravesen et al., 2002). Compositional changes in the cell membrane that result in modifications of the bacterial surface charge has been associated to the resistance to class IIa bacteriocins (Vadyvaloo et al. 2004), but downregulation of some genes from the mannose PTS operon also results in bacteriocin resistance (Tessema et al., 2009).

The behaviour of *L. monocytogenes* CECT 5672 was somewhat different (Fig. 2b). This strain grew rapidly in milk at 37 ºC and slow growth occurred at 4 ºC. The presence of *E. faecium* MMRA prevented *L. monocytogenes* proliferation but did not reduce viable counts during the first 24 h. Later on, upon cold storage, a reduction of CECT 5672 by 1 $\log_{10}$ unit was detected. This strain was slightly less sensitive to enterocin A than CECT 4032, which might have accounted for a higher rate of survival, as previously described for nisin resistant variants (Martínez et al., 2005). It cannot be ruled out that a higher resistance to pH could have also influenced the survival rate. However, both strains were isolated from a dairy environment where a low pH is often encountered. These results also point to the fact that the success of bacteriocin intervention strategies depends largely on the differing susceptibilities of target strains to the bacteriocins (Katla et al., 2003). Despite this, the use of bacteriocinogenic strains to inhibit *L. monocytogenes* growth in dairy products has been successful (Sulzer & Busse, 1991; Rodríguez et al., 1997; Callewaert et al., 2000; García et al., 2004; Foulquié Moreno et al., 2006).

5. Conclusions

Our results have shown that the enterocin A producer *E. faecium* MMRA isolated from Rayeb should be regarded as a potential protective adjunct culture. This strain lacks haemolytic activity, known antibiotic resistance genes and several significant virulence factors. It grew competitively in raw milk, was able to produce the bacteriocin *in situ* and suppressed the growth

of *L. monocytogenes*, thus decreasing the risk of Rayeb contamination by this foodborne pathogen. This study also provides data concerning gross composition and sugar; organic acid and volatile fractions of Rayeb. Thus, the combination of *E. faecium* MMRA with the indigenous raw milk microbiota seems to be suitable for enhancing the hygienic conditions of traditional Rayeb and could help to preserve the traditional characteristics typical of this fermented dairy product. Finally, it should be noted that as far as we know, this is the first study about the use of a bacteriocin-producing strain to control the contamination of North African fermented dairy products by *L. monocytogenes.*

Acknowledgements.

This work has been partially funded by grant BIO2007-65061 from Ministerio de Ciencia e Innovación (Spain). Amel Rehaiem is the recipient of a MAEC-AECID fellowship (Spain). We thank Ana Herrero and María Fernández (IPLA-CSIC, Spain) for their technical assistance on biogenic amine determination and for supplying the specific primers. Luis Cintas (Universidad Complutense de Madrid, Spain) and Manuel Martínez-Bueno (Universidad de Granada, Spain) are also thanked for sharing primers, strains and DNA used as positive controls, respectively. The English usage in the manuscript has been revised by Emma Meader (Institute of Food Research, UK).

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Figure legends

Fig.1. Changes in main volatile compounds during Rayeb fermentation process, (a) ethanol; (b) 2-propanol; (c). diacetyl. (White bars, control Rayeb manufactured without *E. faecium* MMRA as adjunct culture); (Grey bars, experimental Rayeb manufactured with *E. faecium* MMRA). Volatile compounds are expressed as relative abundance (peak area of compound/peak area of internal standard). Data reported are means ± standard deviations of two replicates. (**P*<0.05; ***P*<0.01; ****P*<0.001)

Fig. 2. Effect of the enterocin A-producing *E. faecium* MMRA on *L. monocytogenes* CECT 4032 (a) and *L. monocytogenes* CECT 5672 (b) viability in Rayeb. (Dark bars, *E. faecium* MMRA); (White bars, *L. monocytogenes* in the presence of *E. faecium* MMRA); (Grey bars, *L. monocytogenes* in control Rayeb); (♦,◊) Bacteriocin activity in experimental and control Rayeb (in mm). Bars are the means ± standard deviations of two independent experiments. (****P*<0.001)

Table 1
Susceptibility to antimicrobial agents (disk diffusion method) and enzymatic profile (API Zym system) of *E. faecium* MMRA

Antibiotic (μg/disk)	Susceptibility[a]	Enzyme	Reaction[b]
Amoxicillin (25)	S	Alkaline phosphatase	≥ 40
Ampicillin (30)	S	Esterase(C4)	20
Cephalothin(30)	S	Esterase lipase (C8)	20
Ceftazidim (30)	I	Lipase (C14)	0
Cefazolin (30)	I	Leucine aminopeptidase	≥ 40
Cefotaxim (30)	I	Valine aminopeptidase	≥ 40
Cefuroxim (30)	S	Cystine arylamidase	20
Ceftriaxon (30)	S	Trypsin	0
Cephalothin (30)	S	α -Chymotrypsin	0
Carbenicillin (100)	S	Acid phosphatase	≥ 40
Chloramphenicol (30)	S	Naphthol-AS-BI-phosphohydrolase	5
Gentamicin (10)	S	α -Galactosidase	0
Imipenem (10)	S	β -Galactosidase	20
Kanamycin (30)	S	β -Glucuronidase	0
Ofloxacin (5)	S	α -Glucosidase	0
Oxacillin (1)	R	N-Acetyl- β -glucosaminidase	5
Penicillin (30)	S	α -Mannosidase	0
Streptomycin (10)	S	α -Fucosidase	0
Tetracyclin (30)	S	β -Glucosidase	0
Tobramycin(10)	S		
Vancomycin (30)	S		

[a](S-I-R) (sensitive-intermediate-resistant)

[b]Enzyme activity (nM of chromophore released after 6 h of incubation at 37 °C).

Table 2
Primer sequences for PCR amplication of virulence factors genes[a] and amino decarboxylase genes[b] in *E. faecium* MMRA

Genes	Primer	Oligonucleotide sequence[c]	Expected amplicon size (pb)	PCR amplification
Aggregation substance *agg* [a]	TE3	5'-AAGAAAAAGAAGTAGACCAAC-3'	1553	–
	TE4	3'-AAACGGCAAGACAAGTAAATA-5'		
Gelatinase *gelE* [a]	TE9	5'-ACCCCGTATCATTGGTTT-3'	419	–
	TE10	3'-ACGCATTGCTTT TCCATC-5'		
Cytolysin *cyl* [a]	CylLLs	5'-GTGTTGAGGAAATGGAAGCG -3'	324	–
	CylLLs	3'-TCTCAGCCTGAA CATCTCCAC-5'		
Surface protein *esp* [a]	TE34	5'-TTGCTAATGCTAGTCCACGAC C-3'	933	–
	TE36	3'-GCGTCAACACTTGCATTGCCGAA-5'		
Hialuronidase *hyl* [a]	Hyl n1	5'-TTGCTAATGCTAGTCCACGACC-3'	276	–
	Hyl n2	3'-GCGTCAACACTTGCATTGCCGAA-5'		
	Tdc1	5'- AACTATCGTATGGATATCAAG-3'	720	+
Tyrosine decarboxylase *tdc* [b]	Tdc2	5'- TAGTCAACCATATTGAAATCTGG-3'		
	P1-rev	5'- CCRTARTCNGCNATAGCRAARTCNGTRTG -3'	924	+
	P2-for	5'- GAYATNATNGGNATNGGNYTNGAYCARG-3'		
Histidine-decarboxylase *hdc* [b]	JV16HC	5'-AGATGGTATTGTTTCTTATG-3'	367	–
	JV17HC	5'- AGACCATACACCATAACCTT-3'		

[c] Y = C or T, R = A or G,

Table 3. Gross composition of 'Rayeb' at 24 h of incubation

Incubation time (h)	Type of Rayeb	pH	[a]Titratable acidity (%)	[b]TS (%)	Fat (as %TS)	Protein (as %TS)	Bacteriocin activity
0	Raw milk	6.65 ± 0.06	0.18 ± 0.01	12.975 ± 0,67	33.35 ± 0.66	20.33 ± 0.65	-
24	C	4.24 ± 0.01	0.76 ± 0.05	18.81 ± 1.5	43.94 ± 0.948	51.67 ± 0.45	-
	E	4.25 ± 0.02	0.77 ± 0.04	20.33 ± 0.65	44.57 ± 1.322	51.23 ± 2.79	+

Data are reported as means ± standard deviations of two batches. No significant differences were detected between control and experimental Rayeb (P>0.05)

C: Rayeb made with non-inoculated raw milk (control)

E: Rayeb made with *E. faecium* MMRA Rif^r inoculated raw milk (experimental)

[a]Titratable acidity expressed in g of lactic acid per 100 mL or 100 g

[b]TS, total solids (mg per 100 g)

Table 4 Counts of the major bacterial populations (log_{10} CFU/mL) throughout the 'Rayeb' manufacturing period

Incubation time (h)	Type of Rayeb (C/E)	Total viable counts	Total lactic acid bacteria	Coliforms	Enterococci	Enterococci Rif^r
0	Raw milk	6.64 ± 0.08	6.16 ± 0.10	4.72 ± 0.43	3.84 ± 0.90	0.00 ± 0.00
12	C	8.34 ± 0.20	7.74 ± 0.06	4.90 ± 0.48	6.26 ± 0.03	3.07 ± 0.03
	E	8.97 ± 0.09*	9.04 ± 0.84*	4.45 ± 0.39	8.88 ± 0.84**	8.86 ± 0.80***
24	C	8.37 ± 0.13	7.92 ± 0.02	3.71 ± 0.57	5.63 ± 0.26	3.30±0.26
	E	9.87 ± 0.60**	9.58 ± 0.69**	3.30 ± 0.64	9.43 ± 0.55***	9.41 ± 0.65***

Data are reported as means ± standard deviations of two batches. Significant differences were detected between control and experimental Rayeb at 12 and 24 h (*P<0.05; **P<0.01; ***P<0.001)
C: Rayeb made with non-inoculated raw milk (control)
E: Rayeb made with *E. faecium* MMRA Rif^r inoculated raw milk (experimental)
[c]Rifampin resistant enterococci were determined in KF supplemented with 100 µg/ml rifampicin

Table 5 Evolution of carbohydrates and organic acids (mg/L) throughout the 'Rayeb' manufacturing period.

Incubation time (h)	Type of Rayeb (C/E)	Lactose	Glucose	Galactose	Lactic acid	Citric acid	Formic acid
0	Raw milk	38,357.7 ± 257.24	39.0 ± 0.57	60.3 ± 0.34	122.2 ± 5.09	1,259.7 ± 11.54	00.0 ± 0.00
12	C	28,434.9 ± 203.58	0.00 ± 0.00	53.64 ± 24.23	7,276.0 ± 142.92	224.3 ± 10.78	23.7 ± 0.70
	E	28,212.9 ± 80.48	0.00± 0.00	54.15 ± 31.22	7,375.6 ± 98.35	222.4 ± 15.90	25.8 ± 0.54
24	C	27,085 ± 218.17	0.00 ± 0.00	37.89 ± 15.62	8,569.2 ± 32,45	0.00 ± 0.00	58.17 ± 5.58
	E	27,351.5 ± 56.18	0.00 ± 0.00	36.99 ± 19.66	8,285.9 ± 91.44	0.00 ± 0.00	59.19 ± 5.58

Data are reported as means ± standard deviations of two batches. No significant differences were detected between control and experimental Rayeb at 12 and 24 h ($P>0.05$)

C: Rayeb made with non-inoculated raw milk (control)

E: Rayeb made with *E. faecium* MMRA Rif^r inoculated raw milk (experimental)

Fig. 1 (Rehaiem et al.)

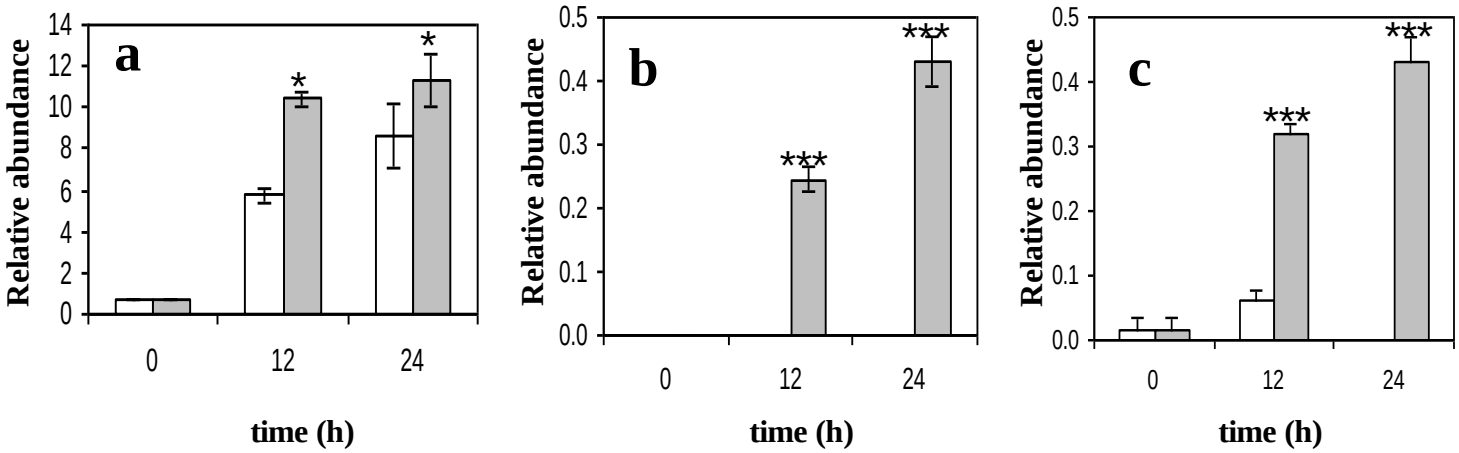

Fig. 2. Rehaeim et al.