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# Assessment of potential probiotic properties and multiple bacteriocin encoding-genes of the technological performing strain *Enterococcus faecium* MMRA

Amel Rehaiem [a,b,*], Zouhaier Ben Belgacem [a], Mohammad Reza Edalatian [c], Beatriz Martínez [d], Ana Rodríguez [d], Mohamed Manai [a], Nelson Pérez Guerra [e]

[a] *Laboratoire de Biochimie et Biologie Moléculaire, Faculté des Sciences de Tunis, Campus universitaire, Tunis, Tunisia*
[b] *Institut Supérieur de Biotechnologie de Béja, Université de Jandouba, Avenue Habib Bourguiba, BP 382, Béja 9000, Tunisia*
[c] *Department of Food Science and Technology, Ferdowsi University of Mashhad, Mashhad, Iran*
[d] *Instituto de Productos Lácteos de Asturias (IPLA-CSIC), 33300 Villaviciosa, Asturias, Spain*
[e] *Departamento de Química Analítica y Alimentaria, Facultad de Ciencias de Ourense, Universidad de Vigo, As Lagoas s/n, 32004 Ourense, Spain*

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In the present study, *Enterococcus faecium* MMRA, a safety and technological performing strain, previously isolated from a well-known fermented dairy product of Tunisia, was evaluated for certain properties relevant to probiotic including acid and bile tolerance, gastrointestinal juice resistance and adhesive properties. Acidic pH (2.0–5.0) and bile salt concentrations of 0.3% were used as stress conditions. The adhesive properties were assessed by determination of bacterial hydrophobicity. The strain MMRA showed interesting features such as high tolerance to gastrointestinal tract transit, an extremely high survival rates under stress caused by acidic pHs or bile salts, and a high adhesive potential linked to the elevated percentage of hydrophobicity. Furthermore, the genome of this strain was examined for the occurrence of known enterocin (enterococcal bacteriocins) genes by means of specific PCR assays and this study revealed the genes coding for enterocins A, B, P and X. Although the joint production of these bacteriocins has not been verified, producers of multiple enterocins could have a great technological potential as protective and/or probiotic cultures in the food industry. The results suggested that *E. faecium* MMRA should be considered as a potential probiotic which meets the criteria to enhance healthy attributes of fermented dairy product besides both of hygienic and sensory aspects.
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## 1. Introduction

Probiotics are mono- or mixed-cultures of live micro-organisms which beneficially affect the health of animals or humans when consumed in sufficient amounts, by improving the properties of the indigenous gastrointestinal flora (Howarth & Wang, 2013). Any more, research has shown that addition of probiotics to food provides numerous health benefits including reduction in the level of serum cholesterol, enhanced gastrointestinal function, improved immune system, and lower risk of colon cancer (Howarth & Wang, 2013; Vasiljevic & Shah, 2008). Most probiotic cultures are of intestinal origin and belong to the genera *Bifidobacterium* and *Lactobacillus*, while enterococcal strains are only occasionally used as potential probiotics (Mullan, 2002).

*Enterococcus* species, a group of lactic acid bacteria (LAB), are ubiquitous microorganisms which constitute a large proportion of the autochthonous microflora found in the gastrointestinal tract of humans and a variety of farm animals as well as in several different food sources, such as meats, milk and cheeses (Foulquié, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006; Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2012; Klein, 2003). Enterococci are also commonly isolated from plants, water and soils, probably as a result of dissemination from fecal sources and their natural tolerance to adverse environmental conditions (Abriouel et al., 2008). Certain enterococcal strains are associated with nosocomial infections and cause human diseases such as bacteraemia, endocarditis or urinary tract infections. These pathogenic strains often carry multiple antibiotic resistances and virulence factors such as adhesins, invasins, and haemolysin (Franz et al., 2012; Keyser 2003; Ogier & Serror, 2008).

* Corresponding author. Laboratoire de Biochimie et Biologie Moléculaire, Faculté des Sciences de Tunis, Campus Universitaire, 2092 El-Manar, Tunis, Tunisia. Tel.: +216 23 81 63 26; fax: +216 78 45 90 98.
*E-mail address:* rehaiem_amel@yahoo.fr (A. Rehaiem).

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However, like most other LAB, some enterococcal strains are applied in food fermentation processes as starter or protection cultures, feed supplements and as probiotics to treat a number of diseases in humans and slaughter animals (Foulquié et al., 2006; Franz et al., 2012). Thus, a considerable number of strains belonging to different species of the genus *Enterococcus*, in particular *Enterococcus faecium*, display many interesting biochemical and biotechnological properties such as proteolytic, lipolytic, esterolytic and other enzymatic activities, citrate utilization and bacteriocin production (Foulquié et al., 2006; Sarantinopoulos et al., 2001).

Enterococci produce a wide array of bacteriocins (enterocins), a family of ribosomally synthesized antimicrobial peptides or proteins with the potential to inhibit the growth of food-borne pathogenic and spoilage bacteria (Franz, Van Belkum, Holzapfel, Abriouel, & Gálvez, 2007). Therefore, the application of enterocins, either as food additives or through *in situ* production by an adjunct starter culture, during food fermentation, seems promising (Aymerich et al., 2000; Leroy, Foulquie, & De Vuyst, 2003). In addition, the increasing interest for the usage of probiotics has suggested some active enterococci strains as safe candidates, along with the most common currently used strains of *Lactobacillus* and *Bifidobacterium*.

When selecting a probiotic strain (FAO/WHO, 2002), a number of aspects should be considered and the theoretical basis for selection should involve functional as well as desirable technological properties. Examples of desirable characteristics for a probiotic strain include the ability to survive and retain viability at conditions (acid and bile concentrations) mimicking the harsh environment of a healthy human gastrointestinal tract (GIT) (Charteris, Kelly, Morelli, & Collins, 1998), safety criteria such as the absence of acquired antibiotic resistance genes, as well as the ability for producing antimicrobial substances (FAO/WHO, 2002; Martín et al., 2006; Ogier & Serror, 2008; Perez-Pulido et al., 2006).

In our earlier work we isolated and characterized *Ent. faecium* MMRA from traditional Tunisian fermented milk “Rayeb” (Rehaiem, Martínez, Manai, & Rodríguez, 2010). This bacterium produces the pediocin-like bacteriocin (enterocin A), a class IIa bacteriocin with strong antilisterial activity (Rehaiem et al., 2010). In addition, both safety and technological issues that have been also addressed on *E. faecium* MMRA proven that this strain meets the criteria for an autochthonous protective adjunct culture to enhance both the hygienic and the sensory attributes of “Rayeb” (Rehaiem, Martínez, Manai, & Rodríguez 2012). For these reasons, in the present study, we have assessed other probiotic properties of *E. faecium* MMRA including the multiple bacteriocin production by this strain and its suitability as a potential probiotic adjunct culture, acid and bile tolerance, gastrointestinal juice resistance and adhesive properties.

## 2. Material and methods

### 2.1. Bacterial strains, culture media and growth condition

*Ent. faecium* MMRA, an enterocin A-producing strain, was previously isolated from homemade, traditional Tunisian ‘Rayeb’ (Rehaiem et al., 2010). This strain was propagated in de Man Rogosa and Sharpe (MRS) broth.

### 2.2. PCR detection of known enterocin structural genes

PCR amplification of well-known structural genes of enterocin A (entA), enterocin B (entB), enterocin P (entP), enterocin L50 (entL50A–entL50B), enterocin AS48 (entAS48) (Du Toit, Franz, Dicks, & Holzapfel, 2000), enterocin 1071A and 1071B (ent1071A–ent1071B) (Martín et al., 2006), enterocin Q (entQ) (Citti et al., 2002), enterocins KS (entKS), enterocin X (entX) (Edalatian et al., 2012), was performed with specific bacteriocin PCR primers listed in Table 1. DNA from well-known enterocin-producing strains was used to provide positive controls. Total DNA was extracted from the bacteriocin-producing *E. faecium* MMRA grown overnight in MRS broth at 32 °C for the detection of known enterocin-encoding genes. The DNA was isolated and purified using the GenElute™ Bacterial Genomic DNA kit (Sigma–Aldrich) following the manufacturer’s recommendations. The DNA concentration was measured at 260 nm using a spectrophotometer (Digilab, Hitachi Ltd., Tokyo, Japan). PCR was performed in a volume of 50 μL containing 10 pmol of each primer, 25 μL of a 2× master mix containing DNA polymerase (Ampliqon, Skovlunde, Denmark), 100 ng of DNA from the producer strain, and molecular grade water (added up to the reaction volume) (Sigma–Aldrich). Amplifications were performed in an iCycler (Bio-Rad, Richmond, CA, USA), employing an initial denaturation cycle at 95 °C for 5 min, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (as indicated in Table 1 for the different primer pairs) and elongation (72 °C for 10 s), and a final extension step at 72 °C for 7 min. PCR products were resolved by electrophoresis in 1% agarose gels, the bands

**Table 1**
Primer sequences for PCR amplification of enterocin genes in *Enterococcus faecium* MMRA and their amplification details.

| Genes | Primer | Oligonucleotide sequence[c] | Expected amplicon size (pb) | Annealing temperature (°C) | PCR amplification | References |
|---|---|---|---|---|---|---|
| Enterocin A | EntAF | 5′-AAATATTATGGAAATGGAGTGTAT-3′ | 475 | | + | Du Toit et al., 2000 |
| | EntAR | 3′-GCACTTCCCTGGAATTGCTC-5′ | | 56 | | |
| Enterocin B | EntBF | 5′- GAAAATGATCACAGAATGCCTA -3′ | 159 | | + | Du Toit et al., 2000 |
| | EntBR | 3′- GTTGCATTTAGAGTATACATTTG-5′ | | 50 | | |
| Enterocin p | EntPF | 5′- GGTAATGGTGTTTATTGTAAT -3′ | 117 | | + | Du Toit et al., 2000 |
| | EntPR | 3′- ATGTCCCATACCTGCCAAAC-5′ | | 48 | | |
| Enterocins L50A, B | EntL50F | 5′- GGAGCAATCGCAAAATTAG-3′ | 150 | | − | Du Toit et al., 2000 |
| | EntL50R | 3′- ATTGCCCATCCTTCTCCAAT-5′ | | 55 | | |
| Enterocin AS48 | EntAS48F | 5′- GAGGAGTTTCATGATTTAAAG-3′ | 185 | | − | Du Toit et al., 2000 |
| | EntAS48R | 3′- CATATTGTTAAATTACCAAGC-5′ | | 50 | | |
| Enterocins 1071A, B | Ent1071F | 5′- GGGGAGAGTCGGTTTTTAG -3′ | 273 | | − | Martin et al., 2006 |
| | Ent1071R | 3′- ATCATATGCGGGTTGTAGCC-5′ | | 50 | | |
| Mundticine KS | EntKSF | 5′- CTACGGTAATGGAGTCTCATG-3′ | 275 | | − | Edalatian et al., 2012 |
| | EntKSR | 3′-CATCTGCATACAGGCTATACC-5′ | | 50 | | |
| Enterocin Q | EntQF | 5′- CAAGAAATTTTTTCCCATGGC-3′ | 95 | | − | Citti et al., 2002 |
| | EntQR | 3′- CTTCTTAAAAATGGTATCGCA-5′ | | 55 | | |
| Enterocin X | EntXF | 5′- GTTTCTGTAAAAGAGATGAAAC-3′ | 500 | | + | Edalatian et al., 2012 |
| | EntXR | 3′-CCTCTTAATCATTAACCATAC-5′ | | 50 | | |

stained with ethidium bromide (0.5 μg/mL), and photographed under UV light.

### 2.3. In vitro assessment of the probiotic properties

#### 2.3.1. Tolerance of Ent. faecium MMRA to acidic pH

The potentially probiotic lactic acid bacterium was grown in MRS broth at 37 °C overnight in culture tubes, then sub-cultured into the same medium during 18 h. The cultures were centrifuged at 5000 × *g* for 15 min at 4 °C, the pellets were washed in sterile phosphate-buffered saline (PBS: 10 mM sodium phosphate monobasic, 10 mM sodium phosphate dibasic, 130 mM sodium chloride, pH 7.2), and re-suspended in sterile PBS. The strain was diluted 1/100 in sterile PBS adjusted to pH 1.0, 2.0, 3.0, 4.0 and 5.0. Incubation times were 1, 2 and 4 h. Serial decimal dilutions in sterile PBS were prepared, and aliquots (0.1 mL) from the dilutions were then spotted on MRS agar plates for determining the number of surviving cells. Each experiment was repeated twice and each reading represents the mean of three observations (Guerra, Fajardo, Méndez, Cachaldora, & Pastrana, 2007).

#### 2.3.2. Preparation of simulated gastric and pancreatic juices

Preparation of simulated gastric and small intestinal juices was according to Huang & Adams (2004). Simulated gastric juice was prepared fresh by dissolving pepsin (Sigma) from porcine stomach mucosa (3 g/L) in sterile saline solution (5 g/L) and adjusting the pH to 2.0, 3.0 and 4.0 with 12 M HCl. Simulated pancreatic juices were prepared by suspending pancreatin (Sigma) from porcine pancreas (1 g/L) in sterile saline solution (5 g/L), with or without 0.3% (w/v) bile salts and adjusting the pH to 8.0 with 0.1 M NaOH.

#### 2.3.3. Upper gastrointestinal transit tolerance assay

The tolerance of washed cell suspensions of *Ent. faecium* MMRA to simulated gastric and small intestinal transit was determined according to the method of Charteris et al. (1998). An aliquot (0.2 mL) of each washed cell suspension was transferred to a 2.0 mL capacity screw-cap tube, and then mixed with 0.3 mL of NaCl (0.5%, w/v), and 1.0 mL of simulated gastric (with pH values of pH 2.0, 3.0, or 4.0) or small intestinal juices (pH 8.0). The mixture was then vortexed at maximum setting for 10 s and incubated at 37 °C. When screening gastric transit tolerance, aliquots of 0.1 mL were removed after 60, 180 and 360 min for determination of total viable count. For screening small intestinal transit tolerance, aliquots of 0.1 mL were removed after 5, 40, 180 and 300 min for the determination of total viable count. Each experiment was repeated twice and each reading represents the mean of three observations.

#### 2.3.4. Determination of total viable counts

Following exposure to juices, serial decimal dilutions of the remaining bacterial suspensions in sterile PBS were prepared, and aliquots (0.1 mL) from these dilutions were then spotted on MRS agar plates (in triplicate) for determining the number of surviving cells. Plates were incubated at 37 °C for 48 h and colonies on MRS plates were counted using a colony counter. The viable counts at 0 min ($vc_0$) were determined as follows (Huang & Adams, 2004):

$$vc_0 = \frac{vc_0(48\ h) \times DF}{Vf} \times VCS$$

Where $vc_0(48\ h)$ is the mean viable count of the cell suspensions at 0 min in the corresponding agar plates after incubating them for 48 h, DF is the dilution factor (10, $10^2$, …), Vf is the final volume (1.5 mL) obtained after mixing the volumes of washed cell suspension of *E. faecium* MMRA (0.2 mL), solution of 0.5% w/v of NaCl (0.3 mL) and simulated gastric or small intestinal juices (1.0 mL). VCS is the volume of washed cell suspension of *E. faecium* MMRA (0.2 mL).

The viable counts after exposure to juices at each sampling time ($vc_t$) were determined as follows:

$$vc_t = \frac{vc_t(48\ h) \times DF}{1.5\ mL} \times 0.2\ mL$$

Where $vc_t(48\ h)$ is the mean viable count of the cell suspensions at each sampling time in the corresponding agar plates after incubating them for 48 h.

#### 2.3.5. Cell surface hydrophobicity

The *in vitro* cell surface hydrophobicity was determined by the bacterial adherence to hydrocarbon assay modified from the method of Rosenberg, Gutnick, and Rosenberg (1980). Briefly, the test bacteria were grown in MRS broth at 37 °C under anaerobic conditions. The 18–24 h (stationary phase) test culture was harvested after centrifugation at 5000 × *g* for 10 min, washed twice and re-suspended in 50 mM $K_2HPO_4$ buffer (pH 6.5) to an optical density ($OD_{560}$) of 0.8–1.0 ($A_0$) measured spectrophotometrically. A portion of 0.6 mL of n-hexadecane was added to 3.0 mL of bacterial suspension. The mixture was blended using a vortex mixer for 120 s. The tubes were allowed to stand at 37 °C for 30 min for phase separation. The n-hexadecane phase was carefully removed and the $OD_{560}$ of the remaining lower aqueous phase (A) was measured. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the initial aqueous bacterial suspension. The percentage of cell surface hydrophobicity (% H) of the strain adhering to hexadecane was calculated using the equation:

$$\%H = \frac{A_0 - A}{A_0} \times 100$$

## 3. Results and discussion

Enterococci have different useful applications in the dairy industry. As starters or adjunct cultures, these LAB fulfil a significant role in improving flavour development and quality of cheeses. As probiotics, enterococci can be used for treatment of gut disorders in humans and animals because these strains contribute to the intestinal health of the host by the improvement of microbial balance of the gut (Franz, Holzapfel, & Stiles, 1999; Franz, Stiles, Schleifer, & Holzapfel, 2003).

One of the functional properties used to characterize probiotics is the production of antimicrobial compounds such as organic acids, hydrogen peroxide and bacteriocins (Foulquié-Moreno et al., 2006). The latter compounds have many interesting properties which suggest that bacteriocins might be used as viable alternatives to traditional antibiotics. These properties include their high potency (as determined *in vitro* and *in vivo*) against food-borne pathogenic, spoilage bacteria and antibiotic-resistant strains, their low toxicity, the availability of both broad and narrow spectrum peptides, the possibility of *in situ* production by probiotics, and the fact that these peptides can be bioengineered (Cotter, Ross, & Hill, 2013). Enterococci are among the most common human intestinal LAB, which harbour numerous useful biotechnological traits, such as the production of bacteriocins (enterocins) (Foulquié-Moreno et al., 2006; Franz et al., 2012).

The use of bacteriocin-producing *Ent. faecium* strains with probiotic properties alone or in combination with other beneficial intestinal bacteria was investigated before in different studies (Banwo, Sanni & Tan, 2013; Ben Omar et al., 2004; Sanchez et al., 2007). Based on the results obtained in these studies, in the

present work, the technological performing strain *E. faecium* MMRA (Rehaiem et al., 2012), isolated from 'Rayeb', a traditional Tunisian curdled dairy product (Rehaiem et al., 2010) was assessed for its multiple bacteriocin production and its *in vitro* probiotic criteria.

### 3.1. PCR detection of known enterocin structural genes

Purified DNA of *Ent. faecium* MMRA was subjected to PCR amplification to determine the existence of structural genes encoding the described enterocins (Table 1) (EntA, EntB, EntP, EntQ, Ent1071, EntL50A/EntL50B, EntAS48, EntKS and EntX), all known to be readily spread among enterococci (Du Toit et al., 2000; Martin et al., 2006). The PCR results (Table 1 and Fig. 1) indicated that strain MMRA carried enterocins A, B, P and X structural genes, whereas the other primers used did not yield any visible band. According to the classification scheme of Franz et al. (2007), enterocins A and P are grouped in the class IIa bacteriocins (pediocin-like bacteriocins), which have a very effective anti-listerial activity, and enterocin B is grouped in the class II.3 bacteriocins (linear nonpediocin-like bacteriocins). Casaus et al. (1997) reported that enterocin B exhibited synergistic activity with enterocin A. Enterocin X, composed of two antibacterial peptides (Xα and Xβ), is a newly found class IIb bacteriocin in *E. faecium* strains that produce enterocins A and B (Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010).

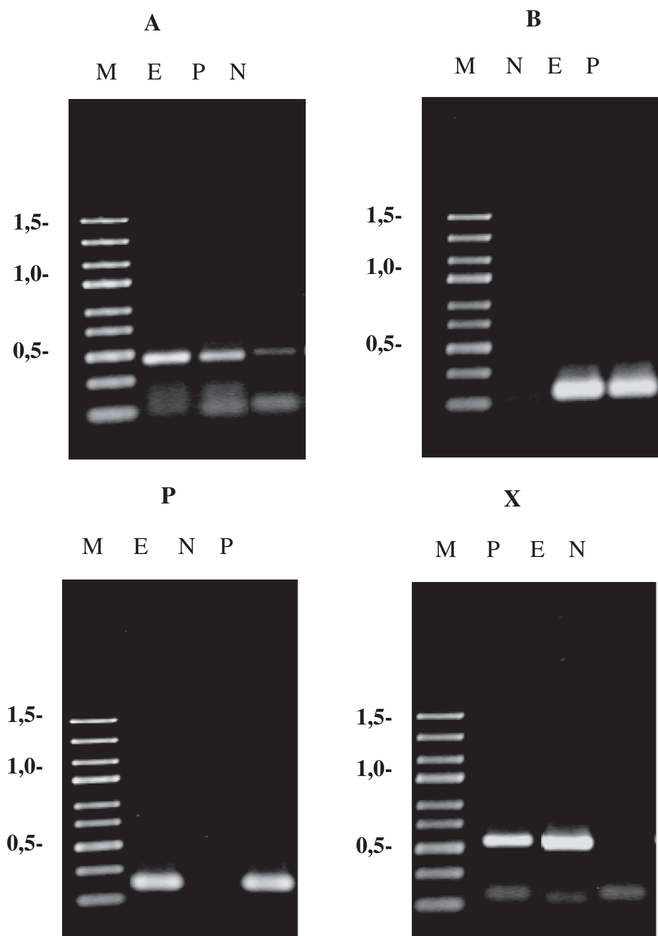


**Fig. 1.** Amplification results for genes encoding enterocin *A* (panel *A*), enterocin *B* (panel *B*), enterocin *P* (panel *P*), enterocin *X* (panel *X*). Order of strains in all panels: E, *E. faecium* MMRA. M, molecular weight marker (kpb). N, negative control: reaction without template DNA. P, positive reaction using purified DNA from a producer strain, as indicated in the text.

Production of multiple bacteriocins is not unusual and seems to be a common feature of enterococci. It has been reported that many enterococci produce multiple bacteriocins, such as *E. faecium* WHE81 (Ennahar, Asou, Takeshi, Kenji, & Ayaaki, 2001), *E. faecium* KV-B5 (Hu et al., 2010), *E. faecium* NKR-5-3A (Ishibashi et al., 2012), *E. faecium* DAC2 (Sanchez et al., 2007) and *E. faecium* JCM 5804$^T$ (Park, Itoh, & Fujisawa, 2003).

According to several studies, the multiple enterocins-producing isolates are likely more efficient and might show a broader range of inhibition in preventing the growth of undesirable bacteria than a simple bacteriocin producer (Ishibashi et al., 2012; Sanchez et al., 2007). According to Sanchez et al. (2007), the coproduction of EntA and EntP by *E. faecium* DAC2 produces a higher antagonistic activity than those of the controls *E. faecium* P13 (producer of enterocin P) and *E. faecium* T136 (producer of enterocin A). However, the occurrence of several enterocin structural genes in *Enterococcus* isolates does not always correlate with a higher bacteriocin activity in their supernatants, and not all enterocin genes must be expressed at the same time (Casaus et al., 1997).

The co-production and variability of enterocin genes in enterococcal strains detected in our study is probably facilitated by the horizontal gene transfer among enterococcal strains and by the well-known ability of enterococci to incorporate DNA material (Pangallo et al., 2004). The acquisition of multiple bacteriocins by *Ent. faecium* MMRA may not only be useful to antagonize various microbial pathogenic and spoilage bacteria, but also to confer a selective advantage and enhance intra specific competition of the strain against other competitors for the microbiological niches (Franz et al., 2012; Strompfova, Laukova, Simonova, & Marcinakova, 2008). Moreover, in populations where the dominant bacteriocinogenic strain produces multiple bacteriocins, the development of resistant bacteria could be slowed down in comparison with the populations in which the dominant producer synthesizes a single bacteriocin (Tessema, Møretrø, Kohler, Axelsson, & Naterstad, 2009).

Further investigations are also needed for the determination of synergistic mode of action of these bacteriocins. Above all, bacteriocin production is considered, from a healthy point of view, to be a key trait of strains used in probiotic formulations employed to prevent the establishment of microbial intestinal pathogens.

### 3.2. Effect of acidic pH, simulated gastric and small intestinal transit, and bile concentration on viability of E. faecium MMRA

In order to act as a probiotic in the gastrointestinal tract and to exert their beneficial effect on the host, probiotic bacterium must survive during transition through the stomach and upper part of the intestinal tract and reach the intestine in sufficiently high numbers (Bao et al., 2010; Sathyabama, Vijayabharathi, Bruntha Devi, Ranjith Kumar, & Priyadarisini, 2012). The acid and bile tolerance are two fundamental properties that indicate the ability of probiotic microorganism to survive the passage through the upper gastrointestinal tract, particularly acidic condition in the stomach and the presence of bile salts in the small intestine (Sumeri et al., 2010). Thus, to be used as a probiotic organism, the tested strain must have good viability in the harsh conditions of the gastric and intestinal transit.

Therefore, the survival of *E. faecium* MMRA strain was firstly tested under acidic conditions (at pH values of 1.0, 2.0, 3.0, 4.0 and 5.0). The number of survivors (as $\log_{10}$ CFU/mL) of the cultures during 4 h of incubation showed the highest viability at pH 5.0, 4.0, and 3.0. In contrast, the viable counts decreased substantially at pH

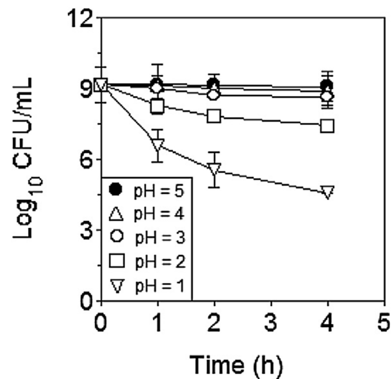


**Fig. 2.** Viability of *Ent. faecium* MMRA strain (as $\log_{10}$ CFU/mL) at different pH exposure during 4 h. CFU, colony forming units. Data are means ± standard deviations (SD) of two independent experiments with three replicates each.

2.0 and 1.0 in concomitance with the increase in the incubation time (Fig. 2).

The effect of the gastrointestinal transit commencing in the stomach is exercised by pepsin, at pH values of range 2.0, 3.0 and 4.0 (Kos, Šušković, Goreta, & Matošić, 2000). Fig. 3 shows the viability (as $\log_{10}$ CFU/mL) of *E. faecium* MMRA under simulated gastric conditions. The viability of strain MMRA was directly influenced by pH. Thus, at the end of the assay, the total declines in the initial bacterial counts were: 1.09-$\log_{10}$ (at pH 4.0), 2.44-$\log_{10}$ (at pH 3.0) and 3.64-$\log_{10}$ (at pH 2.0), which represent viability losses of 12%, 28%, 41%, respectively. According to these results, the strain MMRA could be considered as acceptably resistant to gastric transit (Guerra et al., 2007). The observed decrease in the viable counts at pH 3.0 in presence of pepsin (Fig. 3) in comparison to that at the same pH without enzyme (Fig. 2) indicates that it is better to use buffered food carriers (milk, yoghurt or milk protein-based foodstuffs) for delivery the potential probiotic strain to the gastrointestinal tract (Gardiner et al., 1999; Kos et al., 2000). This could make the potential probiotic strain MMRA less susceptible to the damaging effect of pepsin during the gastric transit and a high number of cells could reach the small intestine and the colon (Guerra et al., 2007).

However, to exert a positive effect on the health and well being of a host, probiotics need to colonize and survive in the small intestine (Havenaar, Ten Brink, & Huis In't Veld 1992), and the condition of this environment may in fact be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar, Ten Brink, & Huis In't Veld, 1992). Consequently, another important property required in a probiotic strain is

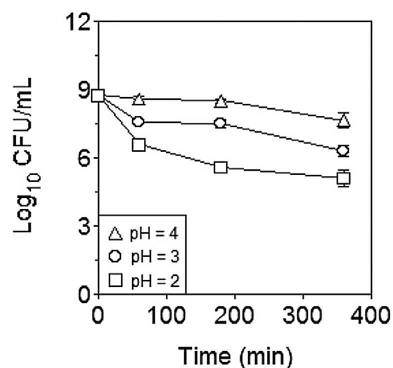


**Fig. 3.** Viability of *Ent. faecium* MMRA strain (as $\log_{10}$ CFU/mL) at simulated gastric juice exposure during 360 min. Data are means ± standard deviations (SD) of two independent experiments with three replicates each.

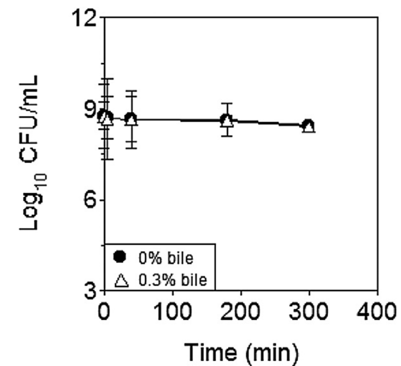


**Fig. 4.** Effect of simulated small intestinal juices on the viability of *Ent. faecium* MMRA strain (as $\log_{10}$ CFU/mL) during 300 min of small intestine transit in absence and in presence of 0.3% (w/v) bile salts. Data are means ± standard deviations (SD) of two independent experiments with three replicates each.

the bile tolerance to obtain a high survival in the small intestine (Kos et al., 2000).

Bile salts are natural detergents that facilitate digestion and absorption of hydrophobic components of the diet. Although the physiological concentration of bile salts in the small intestine varies between 0.2 and 2.0% (Gunn, 2000), it is not clear at which bile concentration a selected strain should be tolerant. However, for the bile tolerance study, a level of 0.3% is considered to be a critical and suitable concentration in selection of resistant strains and probiotic organisms for human use (Pancheniak & Soccol, 2005).

Although bile reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids (Burns et al., 2008), in the present study, the bile salts concentration used (0.3%) did not show any killing effect on the *E. faecium* MMRA strain (Fig. 4). Thus, no decrease on the viable count (as log CFU/mL) is observed up to 300 min of incubation, indicating that *E. faecium* MMRA is tolerant to these conditions. In fact, this strain showed stronger bile tolerance than those LAB (*e.g. Lactobacillus sakei* and *Lactobacillus curvatus*) studied by other researchers (Papamanoli, Tzanetakis, Litopoulou-Tzanetaki, & Kotzekidou 2003).

The high bile tolerance observed in strain MMRA and in other studied *Enterococcus faecalis* and *E. faecium* strains (Saavedra, L., Taranto, Sesma, & de Valdez 2003) is not surprising, because these bacteria are well known to be commensal of the gastrointestinal tract of human and animals, and in this ecological niche, these bacteria come in contact and interact with bile salts (Bhardwaj et al., 2010). Furthermore, bile salt resistance was probably linked to the ability of deconjugating bile salts, which could play a role in preserving the balance of the gut microflora (Begley, Hill, & Gahan, 2006).

Overall, strain MMRA showed high capacity of upper gastrointestinal transit tolerance and may be used as an alternative bacterium for future probiotic development.

### 3.3. Cell surface hydrophobicity

The ability to adhere to epithelial cells and mucosal surfaces can give information about the possibility of microorganisms to colonize and modulate the host immune system. This ability has been suggested to be an important criterion to select probiotic bacteria, since it leads to close host–microbe contact, that contributes to ensure a higher resistance to the intestinal flow of digesta, an optimal functionality and expression of health promoting physiological functions and may also contribute to competitive exclusion

(Collado, Gueimonde, Hernandez, Sanz, & Salminen, 2005; Gionchetti et al., 2000; Gueimonde & Salminen, 2006).

Several mechanisms about the adhesion of microorganisms to intestinal epithelial cells have been reported before (Savage, 1992). Hydrophobic property is generally thought to be one of the factors that may contribute to adhesion of bacterial cells to host tissues (Van Loosdrecht, Lyklema, Norde, Schraa, & Zehnder, 1987; Xiao-Hua, Jong-Man, Hyang-Mi, Shin-Young, & Jae-Myung, 2010) and the cell surface hydrophobicity is commonly accepted as one of the main factors to non-specific adhesion (Rijnaarts, Norde, Bouwer, Lyklema, & Zehnder, 1993). Thus, this property could indicate an important advantage for bacterial maintenance in the human gastrointestinal tract (Naidu, Bidlack, & Clemens, 1999).

In the present study, the potential of MMRA to adhere and colonize in the gut was measured *in vitro* by evaluating the cells surface hydrophobicity toward n-hexadecane (Schillinger, Guigas, & Holzapfel, 2005). This method was reported to be qualitatively valid to estimate the ability of a strain to adhere to epithelial cells (Kiely & Olson, 2000).

The high mean value of hydrophobicity (74.06 $\pm$ 2.06%) found for the *E. faecium* MMRA strain in the present study, may contribute to adhesion of bacterial cells to host tissues that could favour the bacterial maintenance in the human gastrointestinal tract. Although little information is available concerning the adhesion of enterococci to human as well as animal mucus (Botes, Loos, van Reenen, & Dicks, 2008; Lauková, Strompfová, & Ouwehand, 2004), the hydrophobicity of strain MMRA seems to be high compared to those found in several probiotic microorganisms belonging to the genera *Lactobacillus* or *Bifidobacterium*. For example, different studies have shown that the cell surface hydrophobicity values of some strains of lactobacilli (Lähteinen et al., 2010; Lee & Poung, 2002; Kaushik et al., 2009) and bifidobacteria (Del Re, Sgorbati, Miglioli, & Palenzona, 2000; González-Rodríguez, Ruiz, Gueimonde, Margolles, & Sánchez 2013) varied from 23 to 95% and from 20 to 70%, respectively.

In our previous studies with *Ent. faecium* MMRA (Rehaiem et al., 2012), several risk factors were assessed, prior to evaluating the feasibility of this bacterium as a protective adjunct culture for the elaboration of 'Rayeb', a traditional fermented dairy beverage from Tunisia. In these studies, we observed that the MMRA strain did not show haemolytic activity when grown on sheep blood agar, and lacked known antibiotic resistance genes and several significant virulence factors (Rehaiem et al., 2012).

Antibiotic susceptibility of potential probiotic strains is also considered as an important selection criterion for potential probiotic status, since bacteria used as probiotics may cause a variety of infections and act as reservoirs that spread antibiotic resistance genes to virulent enterococci and to other pathogenic or opportunistic bacteria (Franz et al., 2012).

The antibiotics profile of *Ent. faecium* MMRA showed that it displayed intermediate resistance to some cephalosporins and resistance to oxacillin. However, this bacterium was sensible to clinically relevant antibiotics including vancomycin, several β-lactams, aminoglycosides and other broad-spectrum antibiotics.

Thus, the antibiotic susceptibility profile of *Ent. faecium* MMRA is in agreement with previous reports concerning enterococci strains that are commonly found in foods and have safety criteria (Banwo, Sanni, & Tan, 2013; Belgacem, Abriouel, Gálvez, & Manai, 2010; Franz et al., 2012; Valenzuela et al., 2008), and mainly, with strains used as probiotics such as *Lactobacillus* ssp. and *Bifidobacterium* (Katla, Kruse, Johnsen, & Herikstad, 2001; Yazid, Shuhaimi, Kalaivaani, Rokiah, & Reezal 2000).

Another concern is the possibility to disseminate virulence genes. *Enterococcus* species with the highest virulence are often medical isolates, however, food isolates could test positive for one or more virulence determinants (Ben Omar et al., 2004; Yoon, Kim, & Hwang, 2008). Concerning the *Ent. faecium* MMRA, 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 also investigated in a previous study (Rehaiem et al., 2012). 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, were found. An exception was the amplification of tdc coding for the tyrosine decarboxylase enzyme, involved in tyramine production (Rehaiem et al., 2012). This is not surprising, as tyramine is the biogenic amine most frequently produced by enterococci that have been isolated from dairy products (Bover-Cid & Holzapfel, 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 *Ent. faecium* MMRA strain (Rehaiem et al., 2012).

Therefore, since the strain MMRA seems not to be any more resistant to antibiotics or carry transmissible virulence genes than the present-day commercial probiotic, *E. faecium* MMRA could be selected as a probiotic candidate that can be incorporate into food products without being a threat for spread of genes encoding antibiotic resistance and/or virulence traits among bacteria in the intestinal ecosystem.

Additionally, our earlier studies (Rehaiem et al., 2012) also provided data on the performance of *Ent. faecium* MMRA as an adjunct culture in "Rayeb" production. From a technological point of view, strain MMRA grew competitively in raw milk, was able to produce enterocin A *in situ* and to suppress the growth of *Listeria monocytogenes*. Moreover, the combination of *Ent. faecium* MMRA with the indigenous microbiota of raw milk seemed suitable for enhancing the hygienic conditions of traditional fermented dairy beverages, as well as, potentially enhancing their aroma, while preserving their typical traditional characteristics. All these above-described interesting features highlight *Ent. faecium* MMRA as a suitable strain for widespread use as a potential probiotic that may further contribute to a better flavour and texture of fermented products.

The role of using a probiotic as a functional food adjunct in many food products has been recently applied, and its significance for human health is continually increasing each year. The control and safety of foods that contain enterococci is a special challenge to food industry, because of the robust nature, the wide distribution and the stability in the environment of these bacteria. Despite the large number of research studies conducted on the safety of the use of enterococci in foods, as well as their utilization as probiotics in humans and slaughter animals (Banwo et al., 2013; Ben Omar et al., 2004; Franz et al., 2003; Franz et al., 2012; Ogier & Serror, 2008; Perez et al., 2006; Sanchez et al., 2007), only few *Enterococcus* strains have been well investigated with respect to their functional and safety properties. Two of the best characterized strains regarding the safety issue are *E. faecium* SF68 (NCIMB 10415 produced by Cerbios Pharma SA, Barbengo, Switzerland) and *E. faecalis* Symbioflor 1 (produced by Symbiopharm, Herborn, Germany) (Franz et al., 2012). Both strains are produced in form of pharmaceutical preparations.

While most of the species and genera (lactobacilli and bifidobacteria) are apparently safe, certain micro-organisms may be problematic, particularly the enterococci. This is explained by the fact that the evaluation, authorization of health claims and legislation regulating the safety of probiotics recognized by health authorities in individual member states of the EU (the European Food Safety Authority EFSA) (European Food Safety Authority, 2010, 2012, 2013) and around the world has been problematic and

rigorous (Ogier & Serror, 2008). For these reasons, a thorough and careful examination of enterococci, for their toxicological effects and functional properties on animal models and human volunteers, is required.

## 4. Conclusion

The results obtained in this study showed that the *Ent. faecium* MMRA strain has a great potential to be used as an effective probiotic organism in human food and/or animal feed. Thus, PCR analysis revealed the genes coding for four enterocins (A, B, P or X) in strain MMRA, whose concomitant production and synergistic activity could be likely more efficient in preventing the growth of undesirable bacteria than a single bacteriocin. In addition, the high tolerance to the passage through the gastrointestinal conditions and the high cell surface hydrophobicity suggested a high adhesiveness of *Ent. faecium* MMRA to host tissues, which can favour the colonization and survival of this bacterium in the gastrointestinal tract. Moreover, its functionality is strengthened by important features including being non-pathogen, the production of high concentrations of enterocin and lactic acid, as well as the absence of transferable antibiotic resistance genes.

However, a thorough investigation is required, mainly concerning health benefit properties and their toxicological effects (if any) on animal models and human volunteers, before the application of the *Ent. faecium* MMRA strain in the formulation of functional probiotics intended for oral or vaginal delivery.

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