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Letter to the editor

Evaluation of resistance to low pH and bile salts of human *Lactobacillus* spp. isolates

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Abstract

There are nearly 100 trillion bacteria in the intestine that together form the intestinal microbiota. They are 'good' bacteria because they help to maintain a physiological balance and are called probiotics. Probiotics must have some important characteristics: be safe for humans, be resistant to the low pH in the stomach, as well as bile salts and pancreatic juice. Indeed, their survival is the most important factor, so that they can arrive alive in the intestine and are able to form colonies, at least temporarily.

The aim of our study was the evaluation of resistance of *Lactobacillus* isolates from fecal and oral swabs compared to that found in a commercial product.

Seven strains were randomly chosen: *L. jensenii, L. gasseri, L. salivarius, L. fermentum, L. rhamnosus, L. crispatus*, and *L. delbrueckii*. We observed a large variability in the results: *L. gasseri* and *L. fermentum* were the most resistance to low pH, while only *L. gasseri* showed the best survival rate to bile salts. Interestingly, the commercial product did not show tolerance to both low pH and bile salts.

Keywords

Lactobacillus, probiotic, resistance, low pH, bile salts

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Introduction

Lactic acid bacteria (LAB) represent a large group of Gram-positive organisms that are found in humans,[1] however, *Lactobacillus* spp. are among the best characterized. In Italy, their use in food dates back some 30 years. In the past, they were used in food products such as dietary supplements, in combination with vitamins and minerals, and they were called 'organic food' with the aim of restoring the intestinal microbiota. As a result, they have become industrially important and are used in many foods, such as yogurt and fermented milk. In 2002, with the law EU Directive 2002/46/EC, the term 'probiotic bacteria' was introduced and they were permitted to be used as nutritional supplements without other components added. According to the official definition of the FAO and WHO, probiotics are 'live organisms which, when administered in adequate amounts, confer a health benefit on the host'. For bacterial species to be called 'probiotic' they must possess several characteristics: they must compete for receptors

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Table 1. Claims rejected by EFSA.

Strains	No cause: administration-effect correlation
L. casei Shirota[12]	Maintenance of immune defenses of the upper respiratory tract against pathogens
L. fermentum CECT5716[13]	
Combination *L. gasseri* PA 16/8, *B. bifidum* M20/5 and *B. longum* SP 07/3[14]	
L. rhamnosus LB21 NCIMB 40564[15]	Reduction of pathogenic microorganisms to the health of the digestive tract; reduction of *Streptococcus mutans* in the oral cavity
L. paracasei B21060[16]	Reduction of pathogenic microorganisms, maintenance of normal intestinal transit time and reduction of gastrointestinal disorders
L. plantarum 299v[17]	Reducing flatulence and bloating; protection of DNA, proteins, and lipids from oxidative damage
L. johnsonii NCC 533 (La1) (CNCM I-1225)[18]	Improvement of the immune system against gastrointestinal microorganisms; skin protection from damages caused by UV rays
Combination *L. paracasei* CNCM I-1688 and *L. salivarius* CNCM I-1794[19]	Reduction of gastro-intestinal disorders, decrease of potentially pathogenic gastro-intestinal microorganisms, increased lactose digestion, and increased production of IL-10

and adhere to cells and then colonize and stay alive in the intestine;[2] they must effectively compete with pathogenic bacteria already present through acidification and production of antimicrobial compounds;[3–5] they must affect the enzymatic modification of the receptors for bacterial toxins;[6] and they must improve immune defenses against microorganisms.[7] Moreover, in the evaluation of health claims, in accordance with EC Regulation 1924/2006, EFSA states that 'increasing the number of any group of bacteria' or 'increased levels of microflora' in itself does not confer health benefits.[8,9] However, the microorganisms that can be used in foods and food supplements must meet some important requirements such as being considered safe for humans and arriving alive in the intestine in sufficient quantity to be able to multiply thus ensuring a temporary colonization of the intestine (minimum daily dose should be 10^6–10^9 CFU). Therefore, the most important feature that a bacterial strain must have is surviving gastrointestinal transit, and then resistance to the low pH in the stomach and to bile salts released into the intestine.[8–10] Moreover, for the purposes of safety checks, the evaluation of the antibiotic resistance profile is needed, as well as the taxonomic identification to the species level by molecular techniques.[11] EFSA has recently rejected most of the claims used by food companies on food labels and supplements; one of the victims were probiotics (Table 1).[13–19] In the digestive tract the enzymatic decomposition of food takes place, there is the absorption of nutrients and water, and finally, there is the excretion of non-digestible parts. Ingested food passes into the stomach where the molecules are digested by hydrochloric acid and then pass into the small intestine. The small intestine is the first, and the longest, part of the intestine, and it is here that the completion of digestion takes place thanks to pancreatic juice and bile salts, thus allowing the absorption of nutrients into the blood. In addition to enzymes, bacteria strains belonging to the human microbiota take part in digestion. HCl and bile salts play a fundamental role and the effect of probiotic bacteria must be generated in the presence of these biological fluids; hence, the importance of surviving them. The mechanism by which probiotic bacteria could survive the stress caused by fluid remains unclear, but genomic techniques have revealed that genes and proteins are involved, and they are responsible for resistance or sensitivity. The application of genomic techniques to analyze the behavior of the strains *in vivo* would be useful to identify the key players. Therefore, it would be possible to improve the survival of probiotic strains along the gastrointestinal tract.[20]

The aim of this study was focused on the demonstration of *Lactobacillus* species' survival, isolated from human samples, in gastro-intestinal transit and therefore the resistance at low pH in the stomach and to the bile salts released into the small intestine. Moreover, the comparison with the probiotic activity claimed by a commercial product was also carried out. For this, we proceeded as proposed by Bolado-Martinez et al. in 2009.[21] The protocol includes 'the evaluation of probiotic activity' with bacterial counts and relative survival

rate after stressed growth under acidic conditions and in the presence of porcine bile salts.[21]

Materials and methods

Isolation and identification

Sixty-six wild-type strains (belonging to the Department of Biomedical and Biotechnological Sciences, University of Catania, Italy) were previously classified as *Lactobacillus* spp.

They were isolated from human samples (oral and fecal samples from volunteers) using the polyphasic approach. The samples were spread on Rogosa (Oxoid, Thermo Fisher Scientific Inc.) and on blood agar (5% sheep blood), this was done to inhibit the growth of Gram-negative bacteria and to be able to perform an initial phenotypic evaluation of the colonies. *Lactobacillus* strains were obtained from fecal samples using selective culture media (with vancomycin and bromocresol green, LAMVAB agar), thus the fecal microbiota is inhibited by vancomycin and a low pH.[22] The method of selection was based on color, size, and metabolic tests: white small/medium colonies, catalase negative, were transplanted onto MRS agar (Oxoid, Thermo Fisher Scientific Inc.) and onto LSM agar (90% Isosensitest agar [Oxoid, Thermo Fisher Scientific Inc.], 10% MRS agar w/v).[23] They were grown at 37°C in microaerobic atmosphere, for 24–48 h. The isolated lactobacilli were then cryopreserved in MRS with 10% glycerol, at −80°C.

For identification DNA was extracted by the mechanical disruption method in which zirconia beads are added to the tube containing samples and the tube is then shaken as described by Randazzo et al.[24] Briefly, each strain was inoculated into LSM broth at 37°C overnight; broths were centrifuged at 8000 r/min for 15 min, at room temperature. The supernatant was discarded and the cell pellet was suspended in 0.5 mL of TE buffer (Tris, acetic acid, EDTA). Zirconia beads (0.4 g, 0.1 mm in diameter; Biospec, UK) were then added to the cell suspensions. After the repeated bead-beating (RBB) treatment the samples were centrifuged at +4°C for 5 min at 13,000 r/min and the supernatant was collected. Extracted DNA was stored at −20°C.[24]

The strains were identified by amplification of 16S rDNA/RFLP (Restriction Fragment Length Polymorphism) as proposed by Randazzo et al.[24] RFLPs of 16S rDNA PCR products were performed by restriction enzyme digestion with Hae III, MspI I, and Alu I (MBI Fermentas™). Electrophoresis of the products on a 2% (w/v) agarose gel in 1X TBE buffer (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA; pH 8.0) containing Sybr Safe 1X (Invitrogen™) was carried out and the gel was visualized on a transilluminator Safe Imager (Invitrogen™). Strain restriction profiles were compared with those obtained from known *Lactobacillus* type strains belonging to the DSMZ catalog.[24,25] Unfortunately, due to the difficult interpretation of the gel, not all strains were identified; we thus proceeded with a multiplex PCR method as described by Song et al.[26] to discern 11 different *Lactobacillus* spp: a two-step multiplex method based on the nucleotide sequences of *16S-ITS-23S rDNA* and the flanking region of the 23S rDNA.[25,26] Lactobacilli were first grouped by multiplex PCR-G and then identified to the species level by four multiplex PCR assays. Amplifications obtained with multiplex PCR-G were 450 bp (group I *L. delbrueckii*), 300 bp (group II), 400 bp (group III), and 350 bp (group IV). After this, using species-specific primers, it was possible to discern *L. acidophilus* (ca. 210 bp) and *L. jensenii* (ca. 700 bp) with *multiplex* II-1, *L. crispatus* (ca. 522 bp) and *L. gasseri* (ca. 360 bp) with *multiplex* II-2, *L. rhamnosus* (ca. 113 bp) and *L. paracasei* (ca. 312 bp) with *multiplex* III, *L. fermentum* (ca. 192 bp), *L. salivarius* (ca. 411 bp), *L. reuteri* (ca. 303 bp), and *L. plantarum* (ca. 248 bp) with *multiplex* IV.[25,26]

Finally, we made a PCR study based on the *tuf* gene as described by Ventura et al.[27] From the length of the amplicons we discerned exactly *L. rhamnosus* (ca. 540 bp), *L. paracasei* (ca. 200 bp), and *L. casei* (ca. 350 bp). Amplifications obtained were visualized by electrophoresis (70V) on 1.5% agarose gel in 1X TAE buffer (Tris, acetic acid, EDTA), stained with SYBR Safe 1X (Invitrogen™) and observed on a transilluminator Safe Imager (Invitrogen™). DNA ladder used was 1 Kb (BioRad™).[25,27]

The methods, used for clinical isolates, were also used for the commercial strain.

For our aim we randomly chose seven wild-type strains (five oral strains and two fecal strains), one strain of *Lactobacillus* isolated from a well-known commercial product and, as internal positive control, we chose *E. coli* ATCC 35218.

Evaluation of resistance to low pH and bile salts

The strains of *Lactobacillus* spp. and the strain of *E. coli* ATCC 35218 were grown, in two different

sets, for 48 h at 37°C in 10 mL of LSM broth (pH 6.0) with cysteine (0.05% w/v). After incubation, the tubes were centrifuged at room temperature for about 20 min (Figure 1).

Then, following Bolado-Martinez et al. in 2009,[21] we performed a modified protocol that includes 'the evaluation of probiotic activity' with bacterial counts and relative survival rate after stressed growth under acidic conditions and in the presence of different concentrations of porcine bile salts (BS).

For the first set of tubes, the supernatant was removed, and the pellet resuspended in 10 mL saline. Serial ten-fold dilutions in NaCl (0.85% w/v) were done, and each dilution was spread on LSM agar (cysteine 0.05% w/v) as a control, and on LSM agar (cysteine 0.05% w/v) with porcine bile salts (0.5%, 0.25%, and 0.12% w/v) to ascertain the resistance to bile salts.

All plates were incubated for 48 h at 37°C in microaerobic atmosphere. Resistance to bile salts was expressed as the percentage of surviving cells: CFUs found on LSM agar with porcine bile salts against CFUs found on LSM agar control (Figure 1).[28]

For the second set of tubes, after centrifugation, the supernatant was discarded and the pellet was resuspended in LSM broth at pH 3.0. The cultures were incubated for 1 h at 37°C under stirring (to simulate the transit through the stomach). We then proceeded as for the first set: serial ten-fold dilutions in NaCl (0.85% w/v) were done, and each dilution was spread on LSM agar (cysteine 0.05% w/v) as a control, and on LSM agar (cysteine 0.05% w/v) with porcine bile salts (0.5%, 0.25%, and 0.12% w/v).

The plates were incubated at 37°C in a microaerobic atmosphere for 48 h, at the end of which the counts were made (Figure 1).[14,28] Ten independent experiments were performed. For each independent experiment we performed two different runs.

One run was carried out with an initial controlled concentration of the strains (10^6 CFU/mL) but plates had no visible colonies, thus swabs were taken from the plates where colonies were not visible and spread onto LSM agar. This was done to evaluate if the effect of bile salts were bactericidal or bacteriostatic. The plates were incubated for 24 h at 37°C in microaerobic atmosphere, after which visible colonies of lactobacilli were evaluated. Therefore, there is a bacteriostatic effect even at the maximum concentration of bile salts (0.5%). Because of the lack of growth, we proceeded to a second run of the experiment with LSM agar in decreasing concentrations of bile salts (0.5%, 0.25%, 0.12% w/v), but in the absence of cysteine and having an initial concentration between $\geqslant 10^7$ CFU/mL $\geqslant 10^{11}$ (Table 2).

Results

The oral strains used were *L. jensenii, L. gasseri, L. salivarius, L. crispatus*, and *L. delbrueckii*. The fecal strains used were *L. fermentum* and *L. rhamnosus*.

Thanks to the use of three different PCR methods we were able to identify the commercial strain: it was *L. paracasei* (but was marketed as *L. casei*). We repeated the *tuf* gene PCR method proposed by Ventura et al.[27] five times to be sure of the final result.

All strains were grown on LSM agar without supplements. *L. fermentum* and *L. gasseri* were particularly resistant to the treatment at pH 3.0, but all other strains demonstrated tolerability, however *L. salivarius* and *L. paracasei* were reduced significantly (Table 2). These results were comparable for both runs of 10 independent experiments.

E. coli ATCC 35218 invariably grew in all conditions considered.

Regarding the sensitivity to bile salts, the first run of the experiment was performed with bacterial cultures having an initial concentration of 10^6 CFU/mL. It was observed that all strains were sensitive to treatment BS 0.5% – Cys 0.05% w/v both pre- and post-treatment at low pH. Furthermore, the bacteriostatic action, demonstrated *in vitro* by high concentrations of bile salts, showed that the strain was still alive though not able to multiply.

Instead, when we proceeded to the second run of the experiment with decreasing concentrations of bile salts (0.5%, 0.25%, 0.12% w/v) and an initial concentration of bacteria $\geqslant 10^7$ CFU/mL, the results indicated that they were able to multiply even in the presence of the highest concentration of bile salts (pre- low pH treatment) but with a strain-dependent resistance/sensitivity (Table 2). However, after 1 h at a low pH, the bacterial concentration was drastically reduced, obtaining similar results to those of the first run of the experiment.

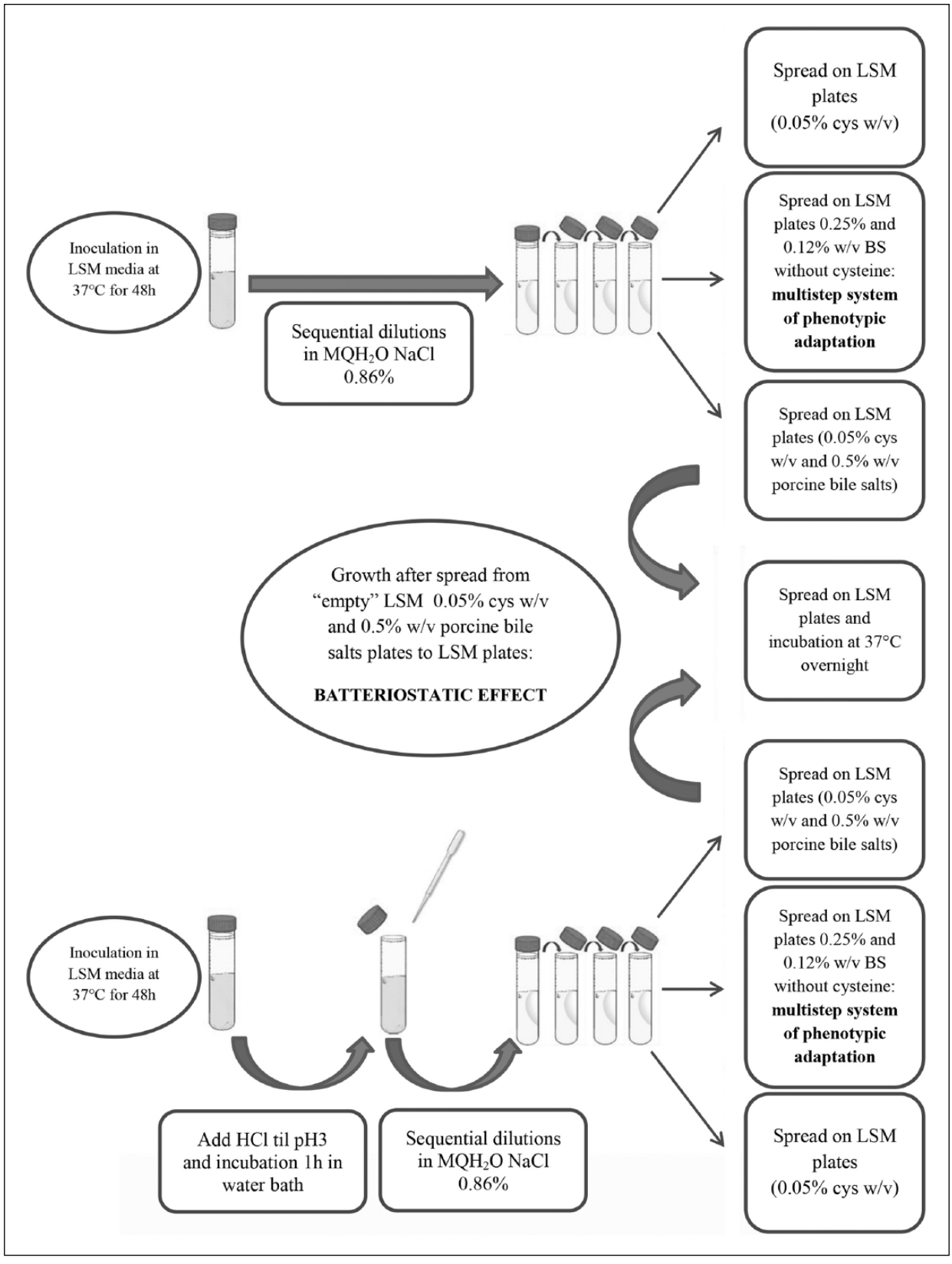

Figure 1. Tolerance evaluation procedure to low pH and bile salts.

Table 2. Survival percentage pre- and post-treatment at low pH and in the presence of bile salts (BS) at different concentrations (% w/v).

Percentage survival (%) pre-treatment at low pH					
Strain	LSM	BS 0.5% Cys 0.05%	BS 0.5%	BS 0.25%	BS 0.12%
L. jensenii	1.0×10^7	1.5	2	2,5	4
L. crispatus 58	1.0×10^8	10	20	40	80
L. delbruekii	2.0×10^7	2.4	25	35	40
L. gasseri 44	10×10^{11}	20	40	50	70
L. salivarius 26	7.0×10^{11}	14.2	28.6	40	43
L. rhamnosus 33	6.0×10^7	16	33	58	75
L. fermentum 42	8.0×10^7	18	38	50	62.5
L. paracasei trademark	1.0×10^9	1	3	4.5	5
Percentage survival (%) post-treatment at low pH					
L. jensenii	1.0×10^3	0	0.2	0.4	1
L. crispatus 58	1.0×10^3	0	10	15	30
L. delbruekii	1.0×10^3	0	20	20	20
L. gasseri 44	5.0×10^7	0	15	30	40
L. salivarius 26	1.0×10^3	0	40	55	60
L. rhamnosus 33	2.0×10^5	0	10	20	25
L. fermentum 42	3.0×10^5	0	10	15	20
L. paracasei trademark	1.4×10^3	0	0	0	0

Discussion

Intestinal microbiota plays an important role in normal gut function and maintaining host health.[2] The mechanism is by means of cross-talk with the epithelial cells.[29] Any change in the intestinal microbiota could have a negative impact on host health: intestinal disorders, allergies, obesity, and infections. Thus, it is easy to understand that when necessary it is useful to take these good bacteria to restore the gut microbiota.[8,9]

First of all, it is important that bacteria arrive alive in the intestine, but our *in vitro* experiments prove that the loss of lactobacilli, caused by two important factors (gastric acid and bile salts), is remarkable.

The method proposed by Bolado-Martìnez in 2009[21] was chosen because, unlike other similar works,[30,31] it offers a more realistic representation of the physiological conditions of the human body, simulating the series of events (resistance to bile salts following exposure to low pH) which bacteria are exposed to in the gut during digestion.[21]

Unlike Bolado-Martìnez et al.,[21] who conducted experiments on 20 strains of *L. reuteri*, we preferred to randomly choose different species of lactobacilli in order to see which had greater resistance to the conditions.

In the commercial preparation of probiotics, tablets or capsules are already used as vehicles for oral administration of these bacteria. However, a pharmaceutical vehicle must not only be able to contain bacteria and protect them from the external environment until they are needed, but it must be able to improve their adaptive capacity to adverse conditions within the body to arrive in the intestine, not only alive, but also able to multiply, at least temporarily. This is the reason why we chose to run tests even on a strain isolated from a commercial product, but our results were not positive. Therefore, we need to find a protection to allow the highest possible number of bacteria to remain viable.

Our studies are ongoing taking into account the coupling of the bacterial strain with a biological support which acts as a protection and provides nutrition during the 'journey' towards the colonization of the intestine. Meanwhile, for the most resistant strains there are ongoing *in vitro* experiments about adhesiveness properties, bacterial interference, and antimicrobial activity against bacterial pathogens thanks to bacteriocin production (data not shown).

Declaration of conflicting interests

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