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Title: Lactobacillus plantarum and Streptococcus thermophilus as starter cultures for a donkey milk fermented beverage

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Keywords: Lactobacillus plantarum; Streptococcus thermophilus; donkey milk; lysozyme; fermentation

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Abstract: Donkey milk is recently gaining attention due to its nutraceutical properties. Its low casein content does not allow caseification, so the production of a fermented milk would represent an alternative way to increase donkey milk shelf life. The aim of this study was to investigate the possibility of employing selected Streptococcus thermophilus and Lactobacillus plantarum isolates for the production of a novel donkey milk fermented beverage. Lysozyme resistance and the ability to acidify donkey milk were chosen as main selection parameters. Different fermented beverages (C1-C9) were produced, each with a specific combination of isolates, and stored at refrigerated conditions for 35 days. The pH values and viability of the isolates were weekly assessed. In addition, sensory analysis was performed. Both S. thermophilus and L. plantarum showed a high degree of resistance to lysozyme with a Minimum Bactericidal Concentration >6.4 mg/mL for 100% of S. thermophilus and 96% of L. plantarum. S. thermophilus and L. plantarum showed the ability to acidify donkey milk in 24 h at 37 °C, with an average ΔpH value of 2.91±0.16 and 1.78±0.66, respectively. Four L. plantarum and two S. thermophilus were chosen for the production of fermented milks. Those containing the association S. thermophilus/L. plantarum (C1-C4) reached a pH lower than 4.5 after 18 hours of fermentation and showed microbial loads higher than 7.00 log cfu/mL until the end of the storage period. Moreover, comparing the microbial loads of samples containing both species and those containing S. thermophilus alone (C5), we highlighted the ability of L. plantarum to stimulate S. thermophilus replication. This boosted replication of S. thermophilus allowed to reach an appropriate pH in a time frame fitting the production schedule. This was not observed for samples containing a single species (C5-C9). Thus, L. plantarum strains seem to be good candidates in the production of a novel type of fermented milk, not only for their probiotic potential, but also for the enhancing effect on S. thermophilus growth.

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02/04/2017

Dear Editor,

Please find enclosed our revised manuscript entitled " *Lactobacillus plantarum* and *Streptococcus thermophilus* as starter cultures for a donkey milk fermented beverage" to be evaluated as an original paper for “International Journal of Food Microbiology”. We appreciated a lot the constructive and helpful comments by the three reviewers. All changes required have been provided and highlighted in the text. Detailed answers to each specific question have been given in the response letter.

Thank you again for considering our work.

Please address all correspondence concerning this manuscript to my e-mail addresses: barbara.turchi@unipi.it.

Yours Sincerely,

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Reviewer #2:

Q: L130-131 - Please give a very short decription...i.e. was it an ELISA test?
A: Following the reviewer suggestion, a very short description of the principle of the lysoplate assay method was provided (lines 144-145).

Q: L138 -- please chck with L159 (Is it 1% or 0.5% S. thermophilus?)
A: At line 138 authors described the protocol employed for the determination of the ability of each isolate to ferment sterile donkey milk. Each *S. thermophilus* isolate was inoculated alone at a ratio of 1%. At line 159 authors described the protocol used to prepare fermented donkey milks. In this case, two *S. thermophilus* isolates were alway used (even in C5 sample), each of them at a ratio of 0.5% to obtain a final load in *S. thermophilus* of 1%.

Q: L156 --Why not pasteurise the milk?
A: Authors choose to not pasteurise milk to retain donkey milk nutritional characteristics as unaltered as possible.

Q: L200 "Determination of isolate susceptibility..."
A: The text has been modified accordingly (line 216).

Q:L365-L378 --- Please add any comments of the panel on the mouthfeel or body (viscosity) of the fermented milk.
Maybe also suggest on how to improve the organoleptic characteristics of the fermented donkey milk beverage.
A: In discussion section, a general comment concerning this issue has been added (line 415-421).

Q: Maybe a better justification as to why the certain pool of microroganisms chosen? Are they probiotic or just for the technological PROPERTIES?
A: For this paper, we based our selection only on lysozyme resistance, technological properties and isolates origin, without considering the probiotic potential of the isolates.

Q: References -- check all papers and update the ones that are not "in press"
A: The references have been updated. In particular, Aspri et al. (2016) and Russo et al (2016) have been modified (line 449 and line 571)

Reviewer #3:

Q: L 5 .. Francesca ...
A: The name has been corrected (line 5)

Q: L37 .. L. plantarum, ...
A: We are not sure what the suggested change is, since the addition of a comma does not seem appropriate.
Q: L55 The authors could insert the following literature about antibacterial activity of donkey milk in semihard cheese: Cosentino et al., 2015a (Cosentino, C., Paolino, R., Valentini, V., Musto, M., Ricciardi, A., adduci, F., D'adamo, C., Pecora, G., Freschi, P. (2015a) Effect of jenny milk addition on the inhibition of late blowing in semihard cheese Journal of Dairy Science, 98(8), 5133-5142.)
A: The reference has been added (line 56)

Q: L116 ... on to...
A: The text has been corrected accordingly (line 130)

Q:L119 ...Both MIC and MBC determinations were carried out in triplicate.

A: The text has been revised accordingly (line 133)

Q: L188 (1 extremely negative, ...)
A: The text has been revised accordingly (lines 202-203)

Q: L264 .....(Figure 1A-B)....
A: The text has been revised accordingly (line 286)

Q: L290 (Brumini et al., 2016; Cosentino et al., 2015b), The authors could insert the following literature about donkey milk in semihard cheese: Cosentino C., Paolino R., Musto M., and Freschi P. (2015b). Innovative use of jenny milk from sustainable rearing. (Book Chapter pp 113-132). The Sustainability of Agro-Food and Natural Resource Systems in the Mediterranean Basin. Editor Antonella Vastola School of Agricultural, Forestry, Food and Environmental Science (SAFE) University of Basilicata Potenza, Italy - Springer Open.
A: Th suggested reference has been added (line 316).

Q: L362-364 This sentence should be revised back to the information essential to the discussion.
A: A reference to the data observed in our study was added to the sentence (line 402)

Reviewer #4

The manuscript provides an interesting contribution about a promising application of selected lactic acid bacteria for production of a fermented beverage based on donkey milk.
The subject is not new, but some results are of interest. For the first time it has been highlighted a synergistic effect between S. thermophilus and L. plantarum, two species of lactic acid bacteria that are not usually used together in the formulation of dairy starter cultures. The growth of S. thermophilus was enhanced in presence of non-growing cells of L. plantarum. Unfortunately, the Authors have not deepened this aspect, assuming that this effect could be ascribed to the production of secondary metabolites.
The work is well described, but further clarifications and some additional information are required. The number of the tables can be reduced, and the figure caption must be improved to be self-explanatory (see specific comments).

SPECIFIC COMMENTS

Q: INTRODUCTION
Since the paper is referred to the use of two LAB species for preparation of a fermented donkey milk, an overview also on the main characteristics of *S .thermophilus* is required.
A: A brief overview on *S. thermophilus* characteristics has been provided (see line 87-95).

Q: MATERIALS AND METHODS
Page 6, Line 146
Based on lysozyme susceptibility profile (MIC), acidifying activity and origin of isolation, … These information were used to select the strains. It would be better to collect the data in a single table. The tables 1 and 2 may be joined. The suggestion is to remove data relating to pH changes at 2, 4, 6, and 8 hours (never used in the text).
A: Authors agree with the reviewer and provided the suggested changes (see new Table 1). In addition, the tables numbering has been changed accordingly (lines 217; 238; 258; 268; 306; 359).

Q: RESULTS
Page 9, line 201
… *for the tested* **microorganisms**.
A: The correction has been made (line 217).

Q: Pages 9, paragraph 3.3

The strains were selected based on acidifying activities evaluated in sterile donkey milk (24 h, 37°C), where the lysozyme was inactivated (page 9, line 217). The milk used for preparation of beverages was a thermised donkey milk, with a residue of lysozyme of about 0.5-1.0 mg/mL. Although the strains were chosen among the most resistant to lysozyme, the cultures made with single strain/species (i.e. from C5 to C9) showed a great difficulty to grow and acidify the thermised milk within 18 h at 37°C (Tab. 3, Fig. 1). Can the Authors provide some explanation? How is it possible that acidifying strains highly resistant to lysozyme (MIC over 6.4 mg/mL) … *were not able to perform a good acidification in thermised donkey milk* ?
The resistance to lysozyme assessed according to the MIC method might have been overestimated?
The MIC method is a microbiological assay based, in this case, on the activity of the enzyme. The analytical data of microbiological assays for determination of lysozyme were reported to be often overestimated [Pellegrino L., Tirelli A. (2000) A sensitive HPLC method to detect hen's egg white lysozyme in milk and dairy products. International Dairy Journal, 10, 435-442].
A comment would be appropriate.

A: Authors agree that the discrepancy between MIC and acidification results poses some questions.
One possible explanation could be that even though lysozyme represents the major antibacterial molecule in donkey milk, others molecules such as lactoperoxidase, lactoferrin, immunoglobulins etc contribute to its characteristic antibacterial activity (Nazzaro et al., 2010). These molecules could have acted in a synergistic way in milk and have somewhat impaired the isolates replication.
Another aspect to consider is that MIC determination was performed using lysozyme from chicken egg white, which is commercially available, but also has a slightly different aminoacidic sequence compared to donkey milk lysozyme (Godovac-Zimmermann et al., 1988).
Moreover, even though it has been shown that lysoplate assay is less accurate than other quantification methods, the difference between observed lysozyme MIC values and lysozyme concentration measured in thermised milk was such as not to raise the suspect that the low acidifying activity was due to an underestimated lysozyme content.
Authors added a comment concerning some of these aspects in the Discussion section (lines 388-400).

Q: Page 11, lines 255-264
To aid in understanding of the results that are shown in Figure 1, it is important to include more precise indications.
- line 258: … *were always higher than 7.00 log cfu/mL* **(Fig. 1A-B-C)**,
- line 259: … *containing only S. thermophilus isolates* **(Fig. 1C)**.
- line 261: … *reaching values higher than 8 log cfu/mL at T0 in samples C1-C4* **(Fig. 1C)**, …
A: The suggested changes have been made (lines 280; 283).

Q: Figure 1, Caption
In relation to the above, the caption of Figure 1 should be better specified.
E.g.:
Mean microbial counts (log cfu/mL±ds) of Streptococcus thermophilus **(1C)** *and Lactobacillus plantarum* **(1A - 1B)** *in different fermented milks, inoculated in combinations* (**fermented milks** *C1-C4) and alone* (**fermented milks** *C5-C9).*
A: Figure 1 caption has been improved as suggested (see new Fig. 1 caption).

Q: DISCUSSION
Page 14, lines 326-330
The good degree of acidification of heat-treated donkey milk, besides being related to the specific composition of donkey milk, is also allowed by the inactivation of lysozyme at 121°C (page 9, line 217). This has to be mentioned in the text.
In fact, the good acidifying activity was demonstrated in sterile donkey milk, whereas in thermised milk the behavior was completely different (page 10, line 241-243).
A: This is true. Authors added a comment concerning this aspect (line 356).

The surprising result is, if anything, the growth of *S. thermophilus* in thermised donkey milk in presence of non-proliferating cells of *L. plantarum*. Even in the absence of growth, as shown by the results, *L. plantarum* had a positive impact on *S. thermophilus*.

***Highlights (for review)**

- Donkey milk was employed for the production of fermented milks
- *S. thermophilus* and *L. plantarum* isolates were evaluated for their use as starters
- Lysozyme resistance and acidifying activity were used as selection parameters
- An enhanced growth of *S. thermophilus* in presence *L. plantarum* was observed
- All fermented milks showed a microbial load higher than 7 log cfu/mL for 35 days

Lactobacillus plantarum and *Streptococcus thermophilus* as starter cultures for a donkey milk fermented beverage

Running header: *L. plantarum* and *S. thermophilus for* a donkey milk fermented beverage

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Abstract

Donkey milk is recently gaining attention due to its nutraceutical properties. Its low casein content does not allow caseification, so the production of a fermented milk would represent an alternative way to increase donkey milk shelf life. The aim of this study was to investigate the possibility of employing selected *Streptococcus thermophilus* and *Lactobacillus plantarum* isolates for the production of a novel donkey milk fermented beverage. Lysozyme resistance and the ability to acidify donkey milk were chosen as main selection parameters. Different fermented beverages (C1-C9) were produced, each with a specific combination of isolates, and stored at refrigerated conditions for 35 days. The pH values and viability of the isolates were weekly assessed. In addition, sensory analysis was performed. Both *S. thermophilus* and *L. plantarum* showed a high degree of resistance to lysozyme with a Minimum Bactericidal Concentration >6.4 mg/mL for 100% of *S. thermophilus* and 96% of *L. plantarum*. *S. thermophilus* and *L. plantarum* showed the ability to acidify donkey milk in 24 h at 37 °C, with an average ΔpH value of 2.91±0.16 and 1.78±0.66, respectively. Four *L. plantarum* and two *S. thermophilus* were chosen for the production of fermented milks. Those containing the association *S. thermophilus/L. plantarum* (C1-C4) reached a pH lower than 4.5 after 18 hours of fermentation and showed microbial loads higher than 7.00 log cfu/mL until the end of the storage period. Moreover, comparing the microbial loads of samples containing both species and those containing *S. thermophilus* alone (C5), we highlighted the ability of *L. plantarum* to stimulate *S. thermophilus* replication. This boosted replication of *S. thermophilus* allowed to reach an appropriate pH in a time frame fitting the production schedule. This was not observed for samples containing a single species (C5-C9). Thus, *L. plantarum* strains seem to be good candidates in the production of a novel type of fermented

milk, not only for their probiotic potential, but also for the enhancing effect on *S. thermophilus* growth.

Keywords: *Lactobacillus plantarum*; *Streptococcus thermophilu~~e~~s*; donkey milk; lysozyme; fermentation

1. Introduction

Donkey milk is currently receiving an increasing attention due to its similarity to human milk and its antibacterial activity (Aspri et al.~~.~~, 2016; Cosentino et al., 2015b; Fratini et al., 2016; Murgia et al., 2016). Among the beneficial properties, it is well known that donkey milk is rich in lysozyme, which can be present in concentrations up to 3.7 mg/mL and resists to thermal treatments, such as pasteurization (Cosentino et al., 2016; Zhang et al., 2008). Since donkey milk is poor in caseins, and consequently not suitable for cheese-making, some authors proposed its employment for the production of fermented beverages, also containing probiotic strains (Chiavari et al., 2005; Perna et al., 2015; Tidona et al., 2015). If on one hand, donkey milk composition could support Lactic acid Bacteria (LAB) growth, on the other, it is known that different species have different susceptibility profiles towards lysozyme (D'Incecco et al., 2016).

Lactobacillus plantarum represents a very flexible and versatile microorganism, which can be isolated from various sources and it has the biggest genome (~ 3.3 Mb) among the LAB group (Martino et al., 2016). In recent years, the interest towards *L. plantarum* has increased, especially in relation to its probiotic potential (Bujalance et al., 2007; Huang et al., 2015; Khan and Kang, 2016; Li et al., 2015) and its possible application in different fermented foods and beverages (Blana et al., 2014; Capozzi et al., 2012; Dal Bello et al., 2007; Hütt et al., 2014; Milioni et al., 2015; Russo et al., 2016).

Recently, it has been proved that *L. plantarum* WCFS1 is able to survive to the antibacterial activity of lysozyme. This is related to the peculiar O-acetylation of peptidoglycan N-acetyl muramic acid (MurNAc), due to the activity of *oatA* gene, encoding for the MurNAc O-acetyltransferase (Bernard et al., 2011). This trait could be exploited for the employment of this LAB species in donkey milk products. However, no data are available concerning LAB resistance phenotype to high lysozyme concentrations.

L. plantarum is commonly isolated from ripened cheeses, where it could be naturally present or employed as adjunct culture (Ciocia et al., 2013; dos Santos et al., 2015). However, several studies highlighted a poor growth of this microorganism associated with a weak acidification when inoculated in sterilized cow milk (Ma et al., 2016; Xanthopoulos et al., 2000). This suggests that *L. plantarum* may take advantage of metabolites from other LAB microorganisms in cheeses.

On the other hand, *Streptococcus thermophilus* is widely used in dairy industry for the production of cheeses and fermented milks. It is commonly isolated from dairy environment, but also from plant sources (Andrighetto et al., 2002; Giraffa et al., 2001; Michaylova et al., 2007) and unlike *L. plantarum*, is well adapted to milk (Goh et al., 2011). The main parameter for the selection of *S. thermophilus* strains to be employed in milk fermentation is lactose metabolism, which should be able to provide a rapid acidification. In addition, carbohydrates metabolism, urease activity, proteolytic activity, exopolysaccharides production represent other major aspects usually considered for technological applications (Iyer et al., 2010).

The aim of the present study was to investigate the possibility of employing selected *L. plantarum* and ~~*Streptococcus*~~ *S. thermophilus* ~~and *L. plantarum*~~ isolates, alone and in association, for the production of a donkey milk fermented beverage. For this purpose, lysozyme resistance and acidifying activity in donkey milk were chosen as the main selection parameters.

2. Materials and methods

2.1 Bacterial isolates and growth conditions

Forty-seven *L. plantarum* and eight *S. thermophilus* isolates from different food sources (whey, milk, curd, cheese, and fermented meat products) and belonging to the Department of Veterinary Science (University of Pisa) collection were employed (Table 1). The type strain ATCC®14917™ (*L. plantarum*) was also included in the study and it was obtained from the American Type Culture Collection (Rockville, MD, USA). *L. plantarum* isolates were grown on MRS broth or agar (Oxoid Thermo Scientific, Milan, Italy) at 37°C in aerobiosis condition for 24 h (broth cultures) or 48 h (agar cultures). *S. thermophilus* isolates were grown on M17 broth or agar (Oxoid Thermo Scientific, Milan, Italy) with 2% lactose (Oxoid Thermo Scientific, Milan, Italy) at 37 °C in aerobiosis condition for 24 h (broth cultures) or 48 h (agar cultures).

2.2 Determination of isolates susceptibility to lysozyme

For each isolate, the resistance to lysozyme was evaluated assessing Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values. MIC values were determined by a broth microdilution method, using MRS or M17 (2% lactose) broth as diluent for *L. plantarum* and *S. thermophilus*, respectively. Microtiter plates containing serial lysozyme (Lysozyme Chicken Egg White, FS-10706, Fisher Molecular Biology, Rome, Italy) dilutions (ranging from 6.4 mg/mL to 0.000390625 mg/mL) were inoculated with 5 μL of a standardized bacterial suspension (0.5 McFarland turbidity scale) to obtain a final volume of 100 μL in each well. For each plate, a positive and a negative control were included. Microtiter plates were incubated at 37 °C in aerobiosis for 24 h and MIC values were visually determined as the lowest lysozyme concentration at which a significative inhibition of bacterial growth was observed compared to the positive control. The type strain ATCC®14917™ (*L. plantarum*) was included in each plate as internal control.

To determine lysozyme MBC values, for each isolate, a drop from the microplate wells corresponding to lysozyme concentrations equal and higher to the MIC was streaked on_to MRS or M17 (2% lactose) agar plates and incubated at 37°C for 48 h. MBC value was determined as the lowest lysozyme concentration that allowed no colonies growth on agar plates. Both MIC and MBC determinations were carried out in triplicate. ~~replicated three times.~~

2.3 Donkey milk samples: collection, determination of total microbial load, pH and lysozyme content

All bulk-tank donkey milk samples were collected from the same farm located in San Sepolcro (Arezzo, Italy), where *Romagnolo* donkeys were raised. Milk samples were collected immediately after milking and stored at refrigerated conditions during transportation to the laboratory. Determination of total mesophilic bacterial count was carried out on Plate Count Agar (PCA) (Oxoid Thermo Scientific, Milan, Italy) after 72 h incubation at 30 °C in aerobic conditions. The pH value of each milk sample was determined (pH-metro XS-instruments, pH7 Portable meter, Bormarc srl, Carpi-Modena, Italy). For all milk samples employed in the trials, lysozyme milk content was determined according to Fratini et al. (2016) by lysoplate assay, which relies on the ability of lysozyme from donkey milk whey to lyse *Micrococcus luteus* cells.

2.4 Evaluation of isolates acidifying activity in donkey milk

The isolates acidifying activity was assessed by recording pH variations of donkey milk cultures. Briefly, after revitalization, 6 mL of broth culture were centrifuged at 6,000 rpm x 10 min and pellets were resuspended in 6 mL of sterile saline solution. Isolates were then inoculated in 20 mL of sterilized donkey milk (121 °C X 5 min) at a ratio of 2% (v/v) for *L. plantarum* and 1% (v/v) for *S. thermophilus*. ~~The~~ pH was measured after ~~2, 4, 6, 8 and~~ 24 h of incubation at 37 °C. The use of sterilized milk allowed us to evaluate the ability to ferment donkey milk carbohydrates ruling out the effect of lysozyme, which in sterilized milk is expected to be denatur~~at~~ed and thus not able to affect the acidifying activity. Results are expressed in ΔpH, which was calculated as the difference between the pH of a control sample (not inoculated milk) and the pH of each inoculated sample.

2.5 Preparation of fermented donkey milk beverages

Based on lysozyme susceptibility profile (MIC), acidifying activity and origin of isolation, four *L. plantarum* and two *S. thermophilus* isolates were chosen to be used in combination, for the preparation of a donkey milk fermented beverage. In each trial, four different beverages (C1, C2, C3 and C4) were prepared using a different combination of isolates. *S. thermophilus* isolates, St2 and St5, were used in all the four combinations at a ratio of 0.5% (v/v) each in order to obtain a pH value lower than 4.5 in a suitable period of time, while a different *L. plantarum* isolate was employed in each fermented milk as follows: Lp5 was used for C1, Lp7 for C2, Lp27 for C3 and Lp43 for C4. Five additional fermented donkey milks (C5, C6, C7, C8 and C9) were prepared using St2-St5, Lp5, Lp7, Lp27 and Lp43, respectively, in order to evaluate the isolates performances when not grown in co-culture.

The fermented milks were prepared as follows: raw donkey milk was thermised at 65 °C x 5 min and dispensed in 100 mL sterile plastic containers; after revitalization, selected isolates were centrifuged (6,000 rpm x 10 min) and resuspended in sterile saline solution; the isolates were then inoculated in milk at a ratio of 2% (v/v) for *L. plantarum* and of 0.5% (v/v) for each *S. thermophilus* isolate.

Enumeration of *inocula* was performed in order to determine their bacterial cell concentrations. Inoculated milks were then incubated at 37 °C overnight. Fermented milks were then stored at refrigerated conditions for 35 days. The experiment was replicated three times in different days.

2.6 Evaluation of pH of donkey milk beverages and of microorganisms viability

For all the trials, *L. plantarum* and *S. thermophilus* cell concentrations and pH values were determined after 18 hours of fermentation (T0) and then weekly during the 35 days of storage at refrigerated condition (T7, T14, T21, T28, T35). For bacterial counts, decimal dilutions of the fermented milks were performed in sterile saline solution. *L. plantarum* was enumerated on MRS agar after incubation at 37 °C for 48 h, while *S. thermophilus* on M17 agar (added with 2% lactose) after incubation at 37 °C for 48 h.

2.7 Sensory analysis of fermented milks

Quantitative Descriptive Analysis (QDA) was carried out on the four different types of fermented beverage containing the association of *L. plantarum* and *S. thermophilus*. The analysis was performed for two trials 4 days after fermented milk production by a panel formed by six trained assessors chosen among the staff of the Department of Veterinary Sciences of Pisa University. For sensory evaluation, 40 mL of each sample were presented in a white plastic cup, codified anonymously with a three digit random number and served at room temperature following a balanced design (Macfie et al., 1989). Panellists were instructed to gently stir the sample with a spoon before assessment. Mineral water was provided for mouth cleansing between samples. Twelve attributes were evaluated using a 10 cm long unstructured scale: two related to appearance (white colour intensity, serum separation), three related to aroma (characteristic aroma intensity, acid, animal); five related to flavour (acid, sweet, salty, bitter, animal) and two related to texture (viscosity, chalkiness). Assessors were also asked to give an overall score using a 9 point structured balanced scale (1 ~~=~~extremely negative, 5 neither negative nor positive, 9 extremely positive).

2.8 Statistical analysis

All statistical analyses were performed with the software R ver. 3.3.1 (R Foundation for Statistical Computing, Vienna) and differences considered significant if associated with a p-value lower than 0.05. Data from bacterial counts were previously transformed in log cfu/mL. One way ANOVA was used to test the statistical significance of differences in bacterial counts for each fermented beverage at different sampling times, and among different milk beverages at each sampling time, and to test differences in sensory scores among different fermented beverage. Tukey HSD test was used for *post-hoc* comparisons.

3. Results

3.1 Determination of isolate~~s~~ susceptibility to lysozyme

Table 1~~2~~ shows MIC and MBC values of lysozyme for the tested ~~microrganisms~~microorganisms. A wide range of MIC values was observed, going from 0.00625 mg/mL to >6.4 mg/mL, while MBC values were less heterogeneous and ranged from 1.6 mg/mL to >6.4 mg/mL. Specifically, as concerns *L. plantarum*, 17/48 (35%) isolates showed a MIC value ≥6.4 mg/mL lysozyme; 16/48 (33%) isolates showed a MIC value ranging from 3.2 mg/mL to 0.8 mg/mL lysozyme; 6/48 (13%) isolates showed MIC values from 0.2 mg/mL to 0.1 mg/mL lysozyme and 9/48 (19%) isolates had a MIC value lower than 0.1 mg/mL lysozyme. As for *L. plantarum* MBC values, the majority of the isolates (46/48; 96%) showed a value ≥6.4 mg/mL lysozyme and only few isolates (2/48; 4%) showed a value lower than 6.4 mg/mL lysozyme, but still remarkably high (3.2 mg/mL and 1.6 mg/mL lysozyme). *S. thermophilus* isolates showed a good degree of resistance towards lysozyme as well, with 1 isolate out of 8 (12%) with a MIC value of 6.4 mg/mL and 7/8 isolates (88%) with a MIC value from 3.2 mg/mL to 1.6 mg/mL lysozyme. All *S. thermophilus* isolates had a MBC value ≥6.4 mg/mL.

3.2 Evaluation of isolates acidifying activity in donkey milk

Sterilized donkey milk samples employed in this trial showed a total mesophilic bacterial count lower than 100 cfu/mL, a null lysozyme content and a pH value of 7.40. These data were expected after the heat treatment of the milk.

Table 1~~2~~ shows the results of the evaluation of the acidifying activity at 37 °C of the tested isolates (results expressed as ΔpH after ~~2, 4, 6, 8 and~~ 24 hours from the *inoculum*).

As expected, the acidifying activity was higher in *S. thermophilus* than in *L. plantarum* isolates. Indeed, *S. thermophilus* isolates showed an average ΔpH at 24 h of 2.91±0.16 (min 2.81; max 3.31), while *L. plantarum* isolates showed an average ΔpH value at 24 h of 1.78±0.66 (min 0.15; max 2.64). Among the *L. plantarum* isolates, the reference strain ATCC 14917 showed the lowest acidifying activity (ΔpH value at 24 h of 0.15).

3.3 Preparation of fermented donkey milk beverages

Based on the results obtained, St2, St5, Lp5, Lp7, Lp27 and Lp43 were chosen to be used as a starter culture for a donkey milk fermented beverage. The mean inocula concentrations in milk samples before fermentation were 7.30 ± 0.39 log cfu/mL for Lp5, 7.24 ± 0.09 log cfu/mL for Lp7, 7.64 ± 0.09 log cfu/mL for Lp27, 7.65 ± 0.10 log cfu/mL for Lp43 and 6.30 ± 0.52 log cfu/mL for St2-St5 (Figure 1). Raw milk samples presented a mean total mesophilic bacterial count of 4.39±0.32 log cfu/mL and a mean pH of 7.47 ± 0.07, while the thermised milk samples showed a mean total mesophilic bacterial count of 2.38 ± 0.63 log cfu/mL and a mean pH of 7.42 ± 0.07. As concerns the thermised milk lysozyme content, it ranged from 0.5 mg/mL to 1 mg/mL.

After 16 hours of incubation at 37 °C (T0), all the fermented milks inoculated with a co-culture of *L. plantarum* and *S. thermophilus* (C1-C4) reached a pH significantly lower than the other samples and always below 4.5 (Table 23). Conversely, at T0, the milk samples inoculated only with *L. plantarum* isolates (C6-C9) did not reach such low pH values, with mean values ranging from 6.39 ± 0.27 (C7) to 7.13 ± 0.09 (C8). Surprisingly, when grown alone, *S. thermophilus* isolates were not able to perform a good acidification in thermised donkey milk as well.

3.4 Evaluation of pH of donkey milk beverages and microorganisms viability

Table 23 shows the pH values of fermented milks. Fermented beverages obtained with the employment of both *S. thermophilus* and *L. plantarum* isolates (C1, C2, C3, C4) maintained their pH values almost unaltered along the storage period at refrigeration condition, ranging from 4.25 at T0 (C3 and C4) to 4.12 at T35 (C1 and C3). The samples inoculated only with *S. thermophilus* or *L. plantarum* isolates (C5-C9) showed more heterogeneous trends. As mentioned before, none of them reached a pH value lower than 4.5 at T0, however C7 sample continued to acidify during the storage at refrigerated condition with a final mean pH of 4.88 ± 0.31 (T35), suggesting a peculiar ability of the isolate Lp7 to replicate at low temperatures.

As concerns microorganisms viability, it was possible to detect a general suitable load of both *S. thermophilus* and *L. plantarum* in all the fermented milks for the whole period of storage at refrigerated temperature. Indeed, final mean microbial concentrations were always higher than 7.00 log cfu/mL (Figure 1A-B-C), except for the sample C5 containing only *S. thermophilus* isolates (Figure 1A-B-C). However, while the concentration of *S. thermophilus* significantly increased after 18 hours of fermentation in all samples, reaching values higher than 8 log cfu/mL at T0 in samples C1-C4 (Figure 1C), none of the *L. plantarum* isolates was able to replicate in donkey milk in the same time frame. This is clearly shown by the fact that an equal concentration of *L. plantarum* cells was detected in milk right after inoculation and after the fermentation (Figure 1-A-B).

Comparing the growth of microorganisms inoculated in milk in combination and alone, it was possible to highlight an enhanced growth of *S. thermophilus* isolates when cultured in combination with *L. plantarum* (Figure 1C). Indeed, C5 presented *S. thermophilus* mean counts always significantly lower than those obtained for samples C1-C4, with a difference higher than 1 log cfu/mL. For samples C1-C4, high *S. thermophilus* counts were obtained starting from T0, with mean values ranging from 8.72±0.31 log cfu/mL (C3) to 8.45±0.27 log cfu/mL (C2). No significant decrease was detected from T0 to T35. As mentioned before, C5 showed a significantly different trend, with a *S. thermophilus* concentration lower than those obtained for samples C1-C4, and the lowest mean microbial load at T0 (7.16±0.22 log cfu/mL). As concerns *L. plantarum* microbial loads (Figure 1A-B), no significant differences were detected, when comparing the growth in milk of the isolates inoculated alone or in combination with *S. thermophilus*. However, in all fermented milks, *L. plantarum* isolates retained their initial concentrations until 35 days of storage at refrigerated condition, with final values ranging from 7.62±0.67 log cfu/mL (C7) to 7.31±0.31 log cfu/mL (C9).

3.5 Sensory analysis of fermented beverages

Table 34 shows the results from the sensory analysis of fermented milk beverages. Regarding QDA scores, C2 fermented milk had the highest scores in colour, aroma intensity, acid aroma, fermented milk flavour, acid flavour. Except for colour, each one of these characteristics statistically differentiated C2 samples from at least one other type of samples.

4. Discussion

The aim of the present work was to evaluate the possibility to employ *L. plantarum* and *S. thermophilus*, alone and in association for the production of a donkey milk fermented beverage. Considering the remarkable content of antimicrobial molecules in donkey milk (Brumini et al., 2016; Cosentino et al., 2015a), the first parameter that we chose to assess was the resistance to lysozyme. In this regard, previous studies that investigated LAB lysozyme susceptibility were focused on foods and beverages where lysozyme is used as additive or on probiotic strains selection, since lysozyme represents the first barrier in the oral cavity. Carini et al. (1985) highlighted the possible effect of the employment of lysozyme on hard cheeses production and their ripening, due to the inhibition of metabolite production and/or proteolytic activity by dairy species. Few years later, Neviani et al. (1988a; 1988b) investigated the lysozyme resistance of *Lactobacillus helveticus* and *S. thermophilus*, reporting a higher resistance for streptococci than lactobacilli. More recently, Ugarte et al. (2006) characterized some Non Starter LAB (NSLAB) belonging to the species *Lactobacillus casei*, *L. plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, and *Lactobacillus perolens*, observing a general resistance to 0.025 mg/mL lysozyme. Furthermore, Solieri et al. (2014) exposed strains belonging to the species *L. rhamnosus, L. casei*, *L. paracasei*, *Lactobacillus harbinensis* and *L. fermentum* to 0.1 mg/mL lysozyme and observed different behaviours, with lysozyme-sensitive strains; lysozyme-adaptive strains, which, after a decline in their survival rate, slightly increased it after 120 min of exposure; and a low number of highly lysozyme-resistant strains belonging to the species *L. rhamnosus*, *L. parcacasei* and *L. casei*. Our results highlight that *L. plantarum* isolates can show a wide range of phenotypic behaviours against lysozyme, with MIC values ranging from 0.00625 mg/mL to >6.4 mg/mL. However, the high MBC/MIC ratio observed for those isolates with the lowest MIC values indicates that *L. plantarum* isolates can tolerate lysozyme. This is not surprising and is likely due to the O-acetylation of peptidoglycan N-acetyl muramic acid (MurNAc) (Bernard et al., 2011). A remarkable resistance to lysozyme was also observed for *S. thermophilus* isolates, since all of them showed high MIC and MBC values. In this case, no reports are currently provided on the specific mechanism behind this phenotype.

As for acidifying activity, the most interesting data are those related to *L. plantarum* isolates. Since this species belongs to the NSLAB group, it is generally not tested for its ability to acidify milk. Moreover, the few available data concerning this aspect show how *L. plantarum* is only able to cause a weak acidification of skim milk. In particular, the highest ΔpH obtained by Xanthopoulos et al. (2000) was 0.92 after 24 hours of incubation at 30 °C. In addition, Georgieva et al. (2009), evaluating the technological properties of some *L. plantarum* strains, showed that they were not able to coagulate skim milk in 16 h at 37°C. More recently, Ma et al. (2016) demonstrated that six amino acids (Ile, Leu, Val, Tyr, Met, and Phe) and at least one purine (adenine or guanine) are essential nutrient requirements for the fermentation of milk by *L. plantarum*. The same authors also showed that *L. plantarum* strains were able to grow and acidify fortified raw milk, while this was not the case for control raw milk samples, suggesting an inability of *L. plantarum* to hydrolyze milk caseins. Despite this, our results prove that some *L. plantarum* isolates are able to cause a good degree of acidification of heat-treated donkey milk at 37 °C, with an average ΔpH at 24 h of 1.78±0.66. This could be related not only to the inactivation of lysozyme at 121 °C, but also to the specific composition of donkey milk, especially in terms of saccharides. Indeed, compared to mare and cow milk, donkey milk has higher levels of lactose (Guo et al., 2007; Malissiova et al., 2016). From Table 1~~2~~, it is possible to notice that those *L. plantarum* isolates showing a weak acidifying activity at 24 hours (ΔpH<0.6) also had the lowest MIC values against lysozyme, while the contrary was not always observed. As concerns S. *thermophilus*, since this species is widely used in dairy industry and well adapted to milk, it was not surprising that all the isolates showed a good acidifying activity, with an average ΔpH value at 24 h of 2.91±0.16.

Selecting the isolates with the best features in terms of lysozyme resistance and acidifying activity, different donkey milk fermented beverages were prepared. Our results are in accordance with those from other authors (Chiavari et al., 2005; Coppola et al., 2002; Nazzaro et al., 2010a; Perna et al., 2015; Tidona et al., 2015) who showed that donkey milk is a good carrier for the delivery of LAB strains, including probiotic ones, reaching loads higher than 7 log cfu/mL. However, the employment of only *Lactobacillus* strains as starter cultures could lead to the necessity of a longer fermentation, in order to obtain a pH value lower than 4.5. Indeed, Chiavari et al. (2005), Coppola et al. (2002) and Nazzaro et al. (2010a) performed a 48 hours-fermentation. Conversely, Perna et al. (2015) and Tidona et al. (2015) using *S. thermophilus* strains have managed to reach a suitable pH value in a shorter time. This is the main reason why we chose to test the association *L. plantarum*/*S. thermophilus*. Not only we evaluated the ability of different associations of isolates to acidify donkey milk and retain their concentration at refrigeration conditions for 35 days of storage, but we also looked at the behaviour of each single species in donkey milk in order to evaluate potential synergies or inhibitions. The most evident aspect that we observed was the enhanced growth of *S. thermophilus* when inoculated in donkey milk together with *L. plantarum*. To the best of our knowledge, this is the first report of such a specific interaction. The mechanisms behind this could be ascribed to the production of secondary metabolites by *L. plantarum*, which stimulate *S. thermophilus* growth. Further studies would be needed in order to understand this phenomenon in more details.

On the other hand, *L. plantarum* isolates seemed not to be positively affected by the presence of streptococci. Indeed, no significant difference was observed in their concentration before and after fermentation. Furthermore, *L. plantarum* cell concentrations were not significantly different among all the analysed samples. ~~Observing~~ Considering the data obtained for ~~*the* data on~~ acidifying activity of *L. plantarum* isolates in sterilized donkey milk and for lysozyme resistance, we expected a better acidification in fermented milks.~~,~~ It has been previously shown that lysoplate assay is less accurate than other methods for the quantification of lysozyme in milk (Pellegrino and Tirelli, 2000). However, the difference between lysozyme MIC values of chosen isolates and lysozyme concentration measured in thermised milk was such as not to raise the suspect that a slowed acidifying activity was due to an underestimated lysozyme content. I~~i~~t could be possible that 18 h of fermentation were not sufficient for *L. plantarum* isolates to cause a suitable pH decrease. Moreover, other antimicrobial molecules contained in donkey milk, such as lactoperoxidase, lactoferrin, immunoglobulins (Nazzaro et al., 2010b), together with residual lysozyme, could have slowed the isolates replication. Nonetheless, as long as the product contains a concentration of viable cells higher than 7 log cfu/mL along the entire storage period, as observed in our study, that would be suitable also for a probiotic dairy product~~s~~ (Shah, 2000).

Another aspect that needs to be taken into account are the sensory properties, as the consumer should also accept the product. In this regard, C2 samples had the highest overall score. This could be related to the higher aroma and milk flavour intensity. Conversely, the lower overall score of C3 milk beverage could be ascribed to a more bland flavour. Some authors report that a pronounced acid aroma or flavour in fermented milk negatively influences sensory scores (Mani-López et al., 2014; Muir et al., 1999). This was not the case in our study where C2 samples had high scores both in acid aroma and flavour and in overall evaluation. This could be ascribed to acidity being an essential part of the sensory characteristics of fermented milks and becoming negatively evaluated only above a certain point. In addition, also in the study by Chiavari et al. (2005), the fermented milk with a higher “pleasantness” score was also the one with a more prominent “acidified lactic” aroma.

An aspect that would generally need to be improved is the body of the product. Indeed, the recorded scores for viscosity were low for all the four fermented milks. Some authors already proposed the fortification of donkey milk with Na-caseinate, pectin, and sunflower oil in order to ameliorate this aspect (Salimei and Fantuz, 2012; Tidona et al., 2015).

None the less, ~~T~~the overall scores of fermented milk samples, and particularly of C2 milks that had only positive scores for all samples and all panellists, confirm that donkey milk fermented beverages could be well accepted among fermented milk consumers, as previously reported (Chiavari et al., 2005; Perna et al., 2015).

5. Conclusion

This work highlighted the ability of some LAB isolates to grow and acidify donkey milk, despite its remarkable content in antimicrobial molecules. We showed that the association *S. thermophilus*/*L. plantarum* could be employed for the production of a novel fermented beverage. The role of *L. plantarum* strains in this new product is crucial. Indeed, *L. plantarum* not only would contribute to the beneficial aspects of the fermented milk, but also speed up the acidification process enhancing *S. thermophilus* growth. Future investigations are needed in order to elucidate if this mechanism is something strictly related to donkey milk properties or not.

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Conflict of Interest: Turchi B., Torracca B., Fratini F., Mancini S., Galiero A., Pedonese F., Nuvoloni R., Montalbano B., Cerri D. declare that they have no conflict of interest.

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Tables

Table 1. Minimum Inhibitory Concentration (MIC) (mg/mL) and Minimum Bactericidal Concentration (MBC) (mg/mL) values of lysozyme and acidifying activity at 37 °C (ΔpH values ~~after 2, 4, 6, 8 and~~ 24 hours after the *inoculum* in donkey milk) for all bacterial isolates.

ID	Source	Origin	MIC (mg/mL)	MBC (mg/mL)	ΔpH 24 h
ATCC14917	-	-	0.2	>6.4	0.15
Lp2	ewe’s raw milk cheese	~~Siena~~ SI (Italy)	0.025	>6.4	2.34
Lp3	ewes’ raw milk cheese	~~Siena~~SI (Italy)	>6.4	>6.4	2.06
Lp4	ewe’s raw milk cheese	~~Siena~~SI (Italy)	>6.4	>6.4	1.82
Lp5	ewe’s raw milk cheese	~~Siena~~SI (Italy)	>6.4	>6.4	2.05
Lp6	ewe’s raw milk cheese	~~Siena~~SI (Italy)	>6.4	>6.4	2.25
Lp7	ewe’s raw milk cheese	~~Siena~~SI (Italy)	>6.4	>6.4	2.44
Lp8	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	2.41
Lp9	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.2	>6.4	2.38
Lp10	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	2.32
Lp11	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.8	>6.4	2.64
Lp12	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	0.8	>6.4	2.48
Lp13	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	1.6	>6.4	2.31
Lp14	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	1.6	>6.4	2.35
Lp15	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	1.6	>6.4	2.25
Lp16	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	1.6	>6.4	2.35
Lp17	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	1.6	>6.4	2.28
Lp18	ewe’s raw milk cheese	~~Massa-Carrara~~MC (Italy)	0.025	6.4	0.94
Lp19	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.1	6.4	1.37

Lp20	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.00625	6.4	0.44
Lp21	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.00625	6.4	0.46
Lp22	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	2.02
Lp23	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.00625	>6.4	0.52
Lp24	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.00625	6.4	0.51
Lp25	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.025	>6.4	2.06
Lp26	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	6.4	>6.4	1.71
Lp27	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	6.4	>6.4	1.72
Lp28	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	6.4	>6.4	1.49
Lp29	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	6.4	>6.4	1.45
Lp30	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.025	1.6	2.05
Lp31	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	1.50
Lp32	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	2.19
Lp33	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	2.15
Lp34	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	>6.4	>6.4	2.15
Lp35	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	3.2	>6.4	2.17
Lp36	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.0125	3.2	1.58
Lp37	ewe’s raw milk	~~Pisa~~PI (Italy)	0.1	6.4	2.07
Lp38	salami	~~Pisa~~PI (Italy)	3.2	>6.4	1.38
Lp39	salami	~~Pisa~~PI (Italy)	3.2	>6.4	1.02
Lp40	salami	~~Pisa~~PI (Italy)	3.2	>6.4	1.17
Lp41	salami	~~Pisa~~PI (Italy)	3.2	>6.4	0.78
Lp42	salami	~~Pisa~~PI (Italy)	1.6	>6.4	1.11
Lp43	donkey milk	~~Grosseto~~GR (Italy)	6.4	>6.4	1.47
Lp44	ewe’s pasteurized milk cheese	~~Pisa~~PI (Italy)	0.2	>6.4	2.19
Lp45	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	1.6	>6.4	2.49
Lp46	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	0.1	>6.4	2.37
Lp47	ewe’s raw milk cheese	~~Pisa~~PI (Italy)	1.6	>6.4	2.26
Lp48	ewe’s milk curd	~~Pisa~~PI (Italy)	1.6	>6.4	2.23
St1	ewe’s whey	~~Grosseto~~GR (Italy)	1.6	>6.4	2.81
St2	ewe’s whey	~~Grosseto~~GR (Italy)	3.2	>6.4	2.82
St5	ewe’s whey	~~Grosseto~~GR (Italy)	6.4	>6.4	3.31
St6	ewe’s whey	~~Grosseto~~GR (Italy)	1.6	>6.4	2.85
St7	ewe’s whey	~~Grosseto~~GR (Italy)	3.2	>6.4	2.86

St8	ewe's whey	~~Grosseto~~GR (Italy)	3.2	>6.4	2.90
St9	ewe's whey	~~Grosseto~~GR (Italy)	1.6	>6.4	2.86
St10	ewe's whey	~~Grosseto~~GR (Italy)	3.2	>6.4	2.88

Lp: *Lactobacillus~~.~~ plantarum*; St: *Streptococcus~~.~~ thermophilus*

SI: Siena; PI: Pisa; MC: Massa-Carrara; GR: Grosseto

Table 2~~3~~. Mean pH values ± ~~d~~sd of fermented milks (T0-T35) obtained with different strains as inoculums.

Samples	T0	T7	T14	T21	T28	T35
C1	4.27±0.07^{aC}	4.20±0.07^{abC}	4.16±0.06^{abC}	4.07±0.04^{bC}	4.10±0.01^{abC}	4.12±0.06a^{bB}
C2	4.36±0.1^{aC}	4.22±0.05^{abC}	4.23±0.08^{abC}	4.12±0.06^{bC}	4.13±0.03^{abC}	4.14±0.06^{bB}
C3	4.25±0.05^{C}	4.05±0.14^{C}	4.15±0.06^{C}	4.08±0.05^{C}	4.11±0.02^{C}	4.12±0.06^{B}
C4	4.25±0.03^{C}	4.11±0.09^{C}	4.17±0.06^{C}	4.11±0.04^{C}	4.12±0.03^{C}	4.14±0.06^{B}
C5	6.91±0.09^{A}	7.23±0.08^{A}	7.17±0.09^{A}	7.02±0.12^{A}	6.97±0.16^{A}	6.59±0.54^{A}
C6	6.99±0.16^{A}	7.08±0.05^{A}	7.09±0.10^{A}	6.92±0.15^{A}	6.89±0.14^{A}	6.62±0.59^{A}
C7	6.39±0.27^{aB}	6.02±0.03^{abB}	5.55±0.16^{abB}	5.29±0.18^{abB}	5.16±0.11^{bB}	4.88±0.31^{bB}
C8	7.13±0.09^{A}	7.26±0.01^{A}	7.21±0.14^{A}	7.12±0.18^{A}	7.08±0.18^{A}	6.73±0.54^{A}
C9	7.12±0.17^{A}	7.22±0.12^{A}	7.18±0.17^{A}	7.01±0.25^{A}	6.99±0.19^{A}	6.65±0.41^{A}

Different lowercase letters in the same row show statistically significant differences among pH values at different times ($p < 0.05$); different uppercase letters in the same column show statistically significant differences among different fermented milks ($p < 0.05$).

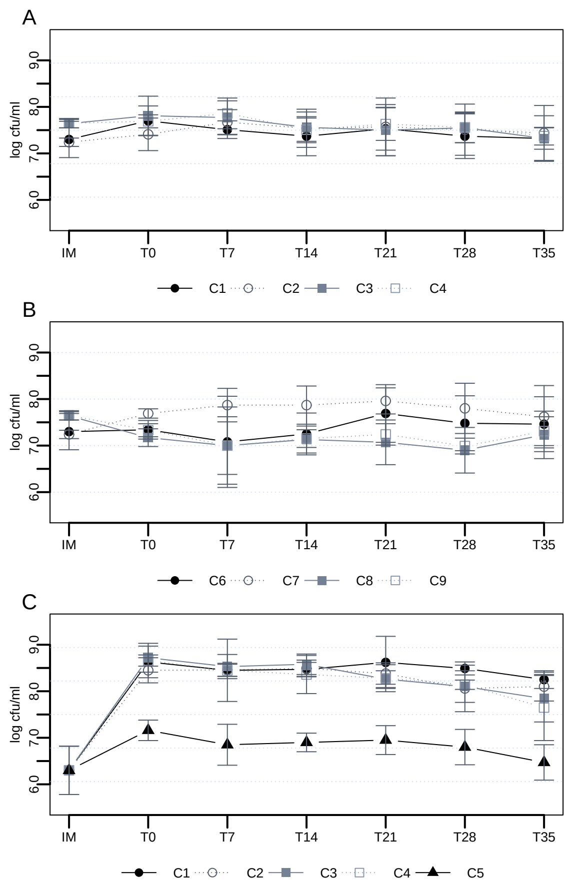
Table 3~~4~~. Sensory analysis scores (mean ± sd) of the four fermented milk beverages.

	C1	C2	C3	C4
Appearance				
Serum separation	3.13 ± 0.72^{b}	3.27 ± 1.01^{ab}	4.15 ± 0.62^{a}	3.71 ± 0.73^{ab}
Colour	7.75 ± 0.55	8.21 ± 0.60	7.74 ± 0.46	7.84 ± 0.64
Aroma				
Aroma intensity	6.72 ± 0.88^{b}	8.13 ± 0.73^{a}	6.20 ± 0.85^{b}	7.22 ± 0.95^{ab}
Acid	0.75 ± 0.37^{ab}	1.14 ± 0.59^{a}	0.61 ± 0.30^{b}	0.89 ± 0.43^{ab}
Animal	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Flavour				
Fermented milk	6.83 ± 0.25^{ab}	7.40 ± 1.00^{a}	6.53 ± 0.51^{b}	7.22 ± 0.68^{ab}
Acid	3.67 ± 0.65^{ab}	4.33 ± 0.54^{a}	3.53 ± 0.72^{b}	3.40 ± 0.65^{b}
Sweet	2.67 ± 0.69	2.70 ± 0.68	1.97 ± 0.49	2.70 ± 1.00
Bitter	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.11	0.00 ± 0.00
Animal	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Metallic	2.58 ± 1.65	2.57 ± 1.77	2.32 ± 1.67	2.02 ± 1.49
Salty	1.32 ± 0.73	1.23 ± 0.60	1.40 ± 0.48	1.03 ± 0.67
Texture				
Viscosity	0.56 ± 0.50	0.73 ± 0.74	0.77 ± 0.65	0.73 ± 0.75
Chalkiness	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.09	0.00 ± 0.00
Overall score	5.90 ± 0.57^{ab}	6.40 ± 0.52^{a}	5.50 ± 0.53^{b}	6.20 ± 0.42^{a}

Different lowercase letters in the same row show statistically significant differences among sensory scores ($p < 0.05$).

Figure


A
log cfu/ml
6.0
7.0
8.0
9.0
IM
T0
T7
T14
T21
T28
T35
C1
C2
C3
C4
B
log cfu/ml
6.0
7.0
8.0
9.0
IM
T0
T7
T14
T21
T28
T35
C6
C7
C8
C9
C
log cfu/ml
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9.0
IM
T0
T7
T14
T21
T28
T35
C1
C2
C3
C4
C5

Figure

Caption:

Fig. 1. Mean microbial counts (log cfu/mL±sds) of *Streptococcus thermophilus* (1C) and *Lactobacillus plantarum* (1A-B) in different fermented milks, inoculated in combinations (fermented milks C1-C4) and alone (fermented milks C5-C9).

Footnote:

C1: St2-St5-Lp5; C2: St2-St5-Lp7; C3: St2-St5-Lp27; C4: St2-St5-Lp43; C5: St2-St5; C6: Lp5; C7: Lp7; C8: Lp27; C9: Lp43; IM: inoculated milk; T0: after 18 hours of fermentation at 37°C; T7, T14, T21; T28, T35: 7, 14, 21, 28, 35 days at refrigerated conditions, respectively.