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Use of lactobacilli strains with probiotic potential in traditional fermented milk and their impact on quality and safety related to *Listeria monocytogenes*

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ABSTRACT

UHT bovine milk (low-fat) was inoculated with a starter culture and one adjunct strain, *Lactobacillus plantarum* 2035 or *Lb. plantarum* T571, with probiotic potential, and also with a cocktail mixture of three strains of *Listeria monocytogenes* at three different initial levels of inoculum and the milk was fermented to pH 4.5. Microbiological, physicochemical, molecular and sensory analyses were performed during storage at 4 and 12 °C. The probiotic samples displayed higher counts of lactic acid bacteria, higher acidity, lower pH and reduced counts of *L. monocytogenes*, in shorter time period than the control at 4 °C. Molecular analysis verified the presence of the probiotic strains until the end of storage at both temperatures and in adequate amounts; survival of *L. monocytogenes* strains depended on the treatment. Sensory evaluation showed that the probiotic fermented milks had desirable organoleptic characteristics, similar to the control.

1. Introduction

A variety of traditional and industrialised fermented milk products are produced worldwide with more than 400 generic names (Tamime, Wszolek, Bozanic, & Ozer, 2011). Their differentiation depends on milk type used, treatment of the milk, fermentation process and later processing of the product (Zamfir et al., 2006). Fermented dairy beverages, like drinkable yoghurt, fall in the category of fermented milk that is manufactured using mainly bovine milk with lactic fermentation (typical yoghurt culture and/or probiotic culture) (Tamime et al., 2011; Uysal-Pala, Karagul-Yuceer, Pala, & Savas, 2006). There is increasing commercial interest in drinkable yoghurt products because of their convenience, portability and ability to carry all the health and nutritional benefits of yoghurt (Allgeyer, Miller, & Lee, 2010). As demand for healthier products grows and to attract health conscious consumers, manufacturers are exploring development of novel products with high added value, such as products with new probiotic strains (Allgeyer et al., 2010; Zamfir et al., 2006). A probiotic product should contain 10^6–10^7 per mL or g of viable cells at the time of consumption, for the product to provide a therapeutic effect (Ranadheera, Vidanarachchi, Rocha, Cruz, & Ajlouni, 2017). However, despite the health promoting effects of these products, overall product properties, i.e., sensory profile, nutritional composition and variety of probiotic cultures, along with the price of such products, influences the purchasing decisions of consumers (Bayarri, Carbonell, Bariios, & Costell, 2011).

Although fermented dairy products have been characterised as safe because of low pH (4.0–4.5), low storage temperature and the possible production of bactericidal/bacteriostatic compounds produced by the fermenting microorganisms, some pathogens like *Listeria monocytogenes* (Farber & Peterkin, 1991), *Salmonella enterica* or *Escherichia coli* O157:H7 (Mufandaedza, Viljoen, Feresu, & Gadaga, 2006) have been reported to survive or grow in fermented milk and yoghurt (Farber & Peterkin, 1991; Mufandaedza et al., 2006). Among dairy products, the pathogen *L. monocytogenes* has been frequently found in soft, semi soft or hard cheeses and also in milk processing plants (Doijad et al., 2011; EFSA, 2015; Jamali, Radmehr, & Thong, 2013). A recent study concerning contamination scenarios with *L. monocytogenes* in food processing facilities showed that the pathogen is a common coloniser of food processing environments. At both farmhouse and

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large food producing facilities the risk of cross contamination with this pathogen is ever-present (Muhterem-Uyar et al., 2015). Furthermore, *L. monocytogenes*, as a ubiquitous pathogen, has been recognised as a post-processing contaminant of dairy products such as yoghurt (Tirloni, Bernardi, Colombo, & Stella, 2011).

Based on the above, the aim of the current study was to evaluate the performance of two *Lactobacillus plantarum* strains with probiotic potential, as adjunct cultures in fermented milk during storage and their effect on safety regarding *L. monocytogenes*. Hence, fermented milk was manufactured with probiotic strains 2035 and T571 (probiotic cases A and B, respectively) or without them (control) and samples were also inoculated with a cocktail mixture of three strains at three different initial inoculum levels of *L. monocytogenes*. All products were stored at 4 and 12 °C, for a total storage period of 32 and 23 days, respectively. Microbial and physicochemical analyses were performed during storage for all cases. Additionally, samples without *L. monocytogenes* were used for sensory analysis. Also, strain distribution, i.e., *L. monocytogenes* and mesophilic lactic acid bacteria (LAB), during storage was evaluated using molecular tools.

2. Materials and methods

2.1. Microbial cultures

A typical yoghurt culture *Lactobacillus delbrueckii* ssp. *bulgaricus* (ACA-DC 84) and *Streptococcus thermophilus* (ACA-DC 6) (ACA-DC culture collection of Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens) and the potential probiotic strains *Lb. plantarum* T571 (culture collection of Hellenic Agricultural Organisation-DEMETER, Institute of Technology of Agricultural Products) and *Lb. plantarum* 2035 (culture collection of Laboratory of Food Microbiology and Hygiene, Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki), were used to manufacture the probiotic fermented milk. *Lb. plantarum* 2035 and *Lb. plantarum* T571 strains had been isolated from traditional Greek dairy products and their probiotic potential was previously explored in vitro (Kotzamanidis, Kourelis, Litopoulou-Tzanetaki, Tzanetakis, & Yiangou, 2010; Pavli et al., 2016). All lactobacilli were revived from stock cultures stored at −80 °C inoculated into 10 mL of MRS broth (MRS broth, 4017292, Biolife, Milano, Italy) and incubated overnight at 30 °C. A subculture was prepared in a fresh 10 mL of MRS broth and incubated for 24 h at 30 °C. *Str. thermophilus* was revived from a stock culture stored at −80 °C, inoculated into 10 mL of M17 broth (M17 broth, BK012HA, Biokar Diagnostics, Allonne, France) and incubated overnight at 37 °C. A subculture was prepared in a fresh 10 mL of M17 broth and incubated at 37 °C for 24 h. For milk inoculation, the monocultures of the LAB strains were harvested by centrifugation at 10,000 ×g for 5 min, washed twice with ¼ strength Ringer's solution (Ringer solution Tablets, 96724-100TAB, Merck, Darmstadt, Germany) and resuspended in Ringer solution. The concentration of the monocultures in the Ringer solution was approximately 9.0 log cfu mL^{-1} for lactobacilli and 8.0 log cfu mL^{-1} for *Str. thermophilus*. All strains were then serially diluted on the decimal scale with ¼ strength Ringer solution to give a final concentration of approximately 7.0 log cfu mL^{-1} in the milk before fermentation for each case (control, probiotic). To verify inoculation level, the same dilutions were pour-plated on MRS- ISO Agar and M17 Agar (M17 Agar, 4017192, Biolife).

Three strains of *L. monocytogenes* (FMCC-B-129, FMCC-B-131, FMCC-B-133) were used as a cocktail to inoculate the milk at three different inoculum levels (3.0, 5.0, and 7.0 log cfu mL^{-1}). The strains originated from Greek food industries and were from the Food Microbiology Culture Collection (FMCC), Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens. The strains were revived from a stock culture stored at −80 °C, in 10 mL of Brain Heart Infusion broth (BHI, LabM, LAB049, Lancashire, UK) and incubated overnight at 37 °C. A subculture was prepared in a fresh 10 mL of BHI and incubated for 18 h at 37 °C. Monocultures of the strains were harvested by centrifugation at 10,000 ×g for 5 min and washed twice with ¼ strength Ringer's solution, resuspended in 10 mL of Ringer solution. The concentration of the monocultures in the Ringer solution was approximately 9.0 log cfu mL^{-1} each. To prepare the 3-strain cocktail, the strains were serially diluted on the decimal scale with ¼ strength Ringer solution and then mixed in equal volumes to give the final concentrations of the three different inocula. These final solutions containing the three strains were used to inoculate the milk at levels of 3.0, 5.0 and 7.0 log cfu mL^{-1} for all cases (control and probiotic cases A and B) before fermentation. To verify the inoculation levels, the same dilutions were spread-plated on Palcam Agar (Palcam Agar, BK145HA, Biokar Diagnostics, France).

2.2. Fermented milk production

The fermented milks were manufactured using low fat (1.5%) UHT bovine milk with initial pH 6.7. Milk was inoculated with the yoghurt starter culture (*Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus*) (control) and with the addition of *Lb. plantarum* 2035 (probiotic case A) or *Lb. plantarum* T571 (probiotic case B) as adjunct cultures. The inoculum amount of cultures added were estimated at population levels of approximately 7.0 log cfu mL^{-1} and the ratio of the adjunct culture was 1:1 compared with the starter culture, as described as section 2.1. All samples were incubated at 42 °C until the pH value of the product reached 4.5 (after approximately 5 h of incubation). After the fermentation process (42 °C, 5 h, pH 4.5), the curd was shaken to break the gels and samples were placed in 100 mL screw cap vessels and stored at 4 and 12 °C for 32 days and 23 days, respectively. The same procedure was followed for inoculation of control and probiotic samples with the pathogen. More specifically, 3.0, 5.0, and 7.0 log cfu mL^{-1} of initial inoculum level of *L. monocytogenes* (cocktail of three strains) were added to the UHT milk and then fermented. Samples from each treatment were collected at various times intervals (0, 3, 6, 10, 14, 18, 21, 25, 28 and 32 days at 4 °C, and 0, 2, 5, 7, 9, 13, 15, 16, 20 and 23 days at 12 °C) and subjected to microbiological, physicochemical and sensory analyses. All experiments were carried out using two different batches with three replicates each (2 × 3). Molecular analysis was performed for each treatment (control, probiotic cases A and B) at the beginning (day 0), middle and end of storage for both storage temperatures.

2.3. Physicochemical analysis

During production and storage, pH measurements were carried out using a pH-meter (HI2211, pH/ORPMeter, HANNA instruments, Smithfield, RI, USA) by immersing the glass electrode in the sample after microbiological analysis. Titratable acidity was measured according to IDF 81: 1997 method. In brief, 10 g of fermented milk was homogenised with distilled water, added to a 100 mL volumetric flask, filtered and then 25 mL of the solution was titrated with 0.1 N NaOH using phenolphthalein as the indicator. Results were expressed as % lactic acid.

2.4. Microbial enumeration

Microbiological analyses were carried out during storage at 4 and 12 °C. To estimate the number of viable cells, 1 mL fermented milk samples was aseptically transferred to 9 mL sterilised ¼

strength Ringer's solution and subsequent serial dilutions were prepared with the ¼ strength Ringer solution (Merck) and 0.1 or 1 mL samples of the appropriate dilutions were spread or mixed on the following media: Plate Count Agar (PCA, 4021452, Biolife) for total viable counts, incubated at 30 °C for 48–72 h, M17 Agar (M17 Agar,4017192, Biolife) for the enumeration of lactococci and *Str. thermophilus* overlaid with the same medium and incubated at 37 °C for 48 h, Rose Bengal Chloramphenicol Agar (RBC Agar, BK151HA, Biokar Diagnostics) for yeasts and moulds incubated at 25 °C for 72 h, de Man-Rogosa-Sharp medium (MRS ISO, 4017282, Biolife) for mesophilic lactic acid bacteria (LAB) overlaid with the same medium and incubated at 30 °C for 48–72 h, Violet Red Bile Glucose Agar (VRBGA, 402185, Biolife) for *Enterobacteriaceae* counts, overlaid with the same medium and incubated at 37 °C for 18–24 h, Palcam Agar with Palcam selective supplement (BS00408, Biokar Diagnostics) for the enumeration of *L. monocytogenes* incubated at 30 °C for 24 and 48 h.

To reduce the detection limit of the enumeration method for the pathogen detection to 0.48 log cfu mL^{-1}, 1 mL of the suspension was spread equally on 3 agar plates of Palcam Agar. Additionally, to ensure the presence/absence of *L. monocytogenes*, enrichment was followed according to ISO 11290-1:1996/Amnd1:2004. In more detail, for the samples with low initial inoculum levels, enrichment was applied throughout storage, while for higher inocula enrichment was applied when pathogen levels were found below 3.0 log cfu mL^{-1}.

2.5. Isolation and growth of lactic acid bacteria and Listeria cells

Fermented milks were sampled at specific time intervals depending on storage temperature. From plates that corresponded to beginning (day 0), middle and final storage time, approximately 20% of the colonies were randomly collected from the appropriate countable dilution of MRS ISO agar and Palcam agar, for mesophilic LAB and *Listeria*, respectively. *L. monocytogenes* cells were also isolated from Palcam petri dishes (3–10 isolates) from the enrichment procedure when counts were below the detection limit for the enumeration method. Purified cultures were stored at −80 °C in MRS Broth for mesophilic LAB or BHI Broth for *Listeria* supplemented with 20% (v/v) glycerol (Penta, Radimova, Praha, Czechia). Before further analysis, each isolate was grown twice in MRS broth for LAB at 30 °C for 24 h and BHI broth for *Listeria* strains at 37 °C for 24 h. Purity of the culture was always checked on MRS-ISO and BHI agar plates before use. Isolates were subsequently screened with PFGE as described in section 2.6.

In addition, a Multiplex PCR Using RAPD-Derived Primers based approach as described in previous work by Saxami et al., 2016, was used to specifically detect the presence of *Lb. plantarum* 2035 in the product during storage at both temperatures. In brief, after enumeration of mesophilic LAB in MRS ISO agar at the beginning (day 0) and at the end of storage time for 4 and 12 °C, plates corresponding to the concentration of ≥6.0 log cfu mL^{-1} were washed with 1 mL sterilised ¼ strength Ringer solution, and then cell suspensions were subjected to molecular analysis based on Multiplex PCR Using RAPD-Derived Primers as described on section 2.7.

2.6. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PGFE) was performed according to Doulgeraki, Paramithiotis, Kagkli, and Nychas (2010) to monitor for the presence of *Lb. plantarum* T571, while determination at strain level for *L. monocytogenes* was performed according to Kagkli, Iliopoulos, Stergiou Lazaridou, & Nychas (2009). For mesophilic LAB and *Listeria* isolates the restriction enzyme *Apa*I (10U) (New England Biolabs, Ipswich, MA, USA) was used according to the manufacturer's recommendations for 16 h. Restriction fragments were separated in a 1% PFGE grade agarose gel (Bio-Rad, Hercules, CA, USA) in 0.5 mM Tris-borate buffer on CHEF-DRIII (Bio-Rad) equipment with running parameters as described by Papadopoulou, Argyri, Varzakis, Tassou, and Chorianopoulos (2018). The obtained restriction profiles were compared with the PFGE fingerprints of the inoculated *L. monocytogenes* FMCC-B129, FMCC-B131, FMCC-B133 strains and *Lb. plantarum* T571 strain.

2.7. Multiplex PCR using RAPD-derived primers

To confirm the presence of *Lb. plantarum* 2035, genomic DNA from the LAB suspensions was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacture's recommendations. PCR reactions were carried out as recently described (Galanis, Kourkoutas, Tassou, & Chorianopoulos, 2015).

2.8. Sensory evaluation

The sensory analysis (samples without *L. monocytogenes*) performed in this study was in line with an earlier study for yoghurt products (Bayarri et al., 2011). In detail, quantitative descriptive analysis (QDA) was performed to quantify the descriptive attributes of the fermented milk samples. Prior to sensory assessment, 15 people (staff from the Institute of Technology of Agricultural Products of HAO-DEMETER) were trained and tested in evaluating odour perception (fermented milk attribute, acidic aroma), taste (sweetness, bitterness, acidity and saltiness), appearance (white colour) and texture (creaminess, homogeneity). For the perception of taste, three different concentration solutions of sucrose (1, 4 and 8%), quinine (0.01, 0.05 and 0.1 mM), lactic acid (0.1, 0.8 and 1.0%) and sodium chloride (0.1, 0.5 and 1.5%) were evaluated by the panel. Three sessions of 2 h each were used in which the assessors had to demonstrate an accuracy of at least 75% for the perception of the examined attributes, to be selected for the sensory evaluation panel. The final selected team for the sensory evaluation was composed of 10 people, 4 men and 6 women aged between 30 and 55 years old.

For the sensory assessment, an evaluation sheet with 10-cm intensity scale was used. Each sheet contained all the examined attributes in a hedonic scale ranging from 0 to 10. Direction of the hedonic scale was from left to right with increasing intensities, e.g., weak to strong, little to much, etc. (Cruz et al., 2013). Overall acceptance, along with total taste, total aroma, total appearance and total texture of the control and probiotic fermented milk were evaluated by the panel. Sensory evaluation was conducted in triplicate under artificial light in individual booths in a special organoleptic assessment room in the Institute of Technology of Agricultural Products. The same trained panel was used for all evaluations and were unaware of the identity of the samples tested. Fermented milk samples were served in plastic glasses codified with 3 digits. Panellists used water and unsalted crackers to clean their palates in-between samples.

2.9. Statistical analysis

Each experiment was replicated twice (two independent batches) with three samples analysed each time. Significance was established at $P < 0.05$. Physicochemical (pH and titratable acidity), microbiological and sensory results were analysed for statistical significance with analysis of variance (ANOVA). Duncan's multiple range test was used to determine significant differences among results [coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica v.5.0, (Statsoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Microbiological analysis

Population dynamics of the microbiota in the fermented milks during storage at 4 and 12 °C are shown in Fig. 1. The initial population in all cases (control and probiotic) consisted of mesophilic LAB (approximately 8.0 log cfu mL^{-1}) and lactic cocci/streptococci (approximately 7.8 log cfu mL^{-1}). Counts of mesophilic LAB for both probiotic cases remained stable (approximately 8.0 log cfu mL^{-1}) during storage of products at 4 °C with slight differences among different storage sampling days ($P < 0.05$, Supplementary material Table S1, Fig. 1). Lactic cocci/streptococci counts, fluctuated around a population level of approximately 7.5 log cfu mL^{-1} for all cases during storage ($P > 0.05$, Supplementary material Table S1, Fig. 1). Total Viable Counts (TVC) represented the dominant microbiota in each case, which for control and probiotic samples was mesophilic LAB (Fig. 1).

The microbial groups in samples stored at 12 °C, were similar to those at 4 °C, (mesophilic LAB and lactic cocci/streptococci). In more detail, probiotic case A, exhibited the highest counts in comparison with the other cases (Fig. 1). Also, the population of mesophilic LAB was 8.3 log cfu mL^{-1} during storage, while lactic cocci/streptococci were found at similar population levels as the mesophilic LAB populations. In probiotic case B, the population of mesophilic LAB during storage was approximately 7.8 log cfu mL^{-1} a similar result was observed for lactic cocci/streptococci too. In control samples, populations of mesophilic LAB and lactic cocci/streptococci were lower (approximately 7.5–7.7 log cfu mL^{-1}), during storage compared with probiotic case A. However, mesophilic LAB population was found above 7.0 log cfu mL^{-1} for all cases at the end of storage at 12 °C. No yeasts/moulds or *Enterobacteriaceae* were detected after enumeration (detection limit 1 and 0 log cfu mL^{-1} respectively) in the current study and it should also be noted, that the non-inoculated fermented milk samples remained

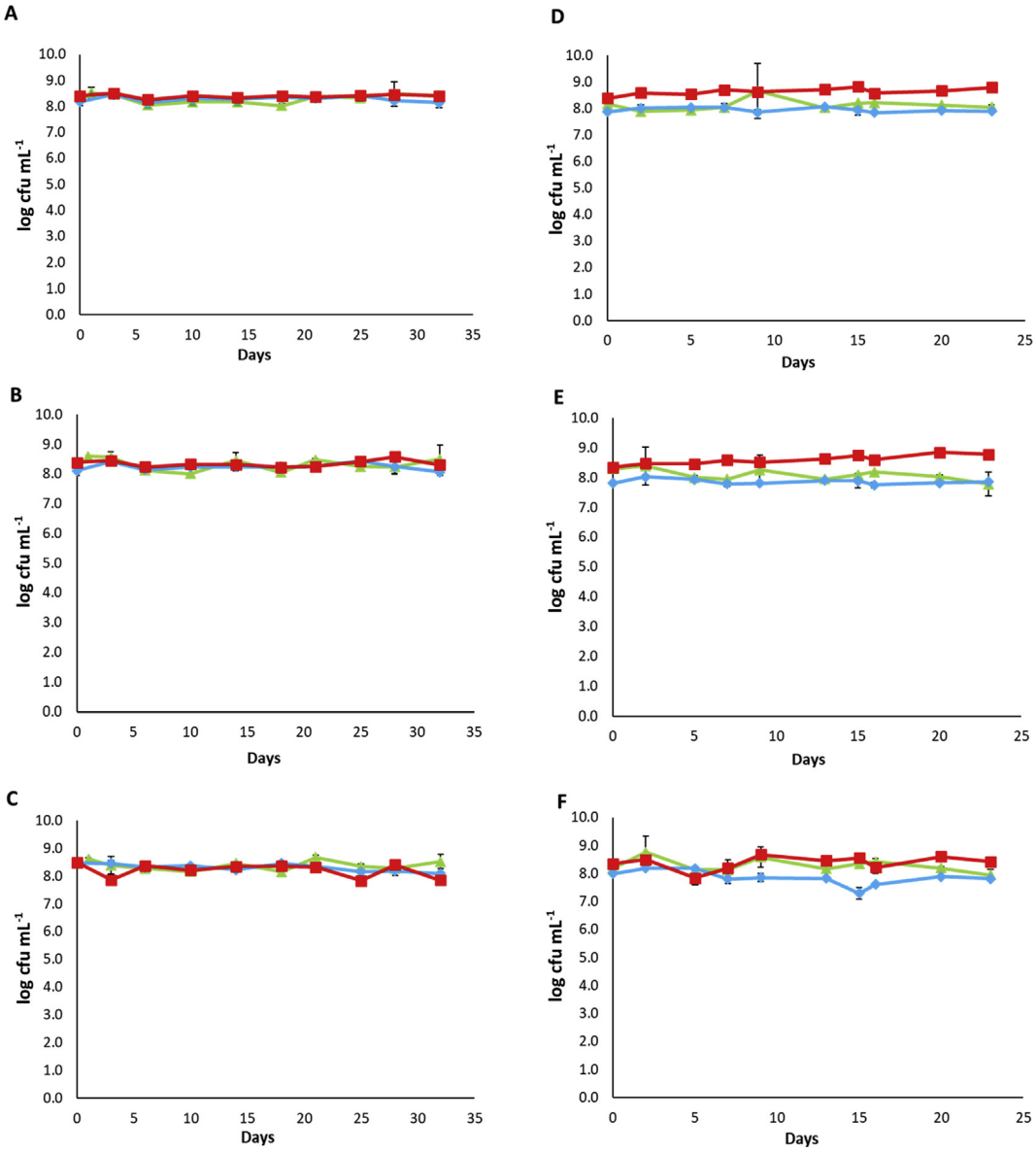

Fig. 1. Growth curves of total viable counts (A,D), mesophilic LAB (B,E) and lactic cocci/streptococci (C,F) during storage of fermented milk at 4 °C (A,B,C) and 12 °C (D,E,F): ◆, control sample; ■, *Lb. plantarum* 2035 (probiotic case A); ▲, *Lb. plantarum* T571 (probiotic case B). The bars represent the mean values ± standard deviations (two biological samples, each sample analysed three times).

Listeria free throughout manufacture and storage at both temperatures for both probiotic and control cases, since no cells were detected by the enrichment method.

The findings of this study were similar or higher as compared with literature findings, for the population of the added probiotic strains in dairy products. More specifically, the microbial evolution that was monitored during storage, was similar for the two probiotic cases, where mesophilic LAB population levels were $\geq$7.8 log cfu mL^{-1}, while in control samples, mesophilic LAB levels were found slightly lower (7.5 log cfu mL^{-1}), a result reported in other studies (Kakisu, Irigoyen, Torre, De Antoni, & Abraham, 2011; Patrignani, Lanciotti, Mathara, Guerzoni, & Holzapfel, 2006; Sidira et al., 2013). Kakisu et al. (2011) found similar results for a probiotic fermented milk, when a two-strain starter culture (probiotic strain and *Str. thermophilus*) was used, the probiotic strain had similar population levels as *Str. thermophilus* throughout storage. Sidira et al. (2013), used a probiotic strain of *Lactobacillus casei* for the production of a probiotic yoghurt along with *Lb. delbrueckii* ssp. *bulgaricus* and results showed after 30 days of cold storage, the probiotic strain had viable populations over 6.0 log cfu g^{-1} in contrast to *Lb. delbrueckii* ssp. *bulgaricus* where cell viability decreased under 6.0 log cfu g^{-1} after 14 days of cold storage.

However, contradictory results were found in other studies, dealing with fermented products with added probiotic cultures, where lower numbers of probiotic population were recorded for probiotic samples, in contrast to control samples (products with commercial starter culture) during shelf life in fermented milk or yoghurt (Allgeyer et al., 2010; Maragkoudakis et al., 2006; Ng, Yeung, & Tong, 2011; Oliveira, Sodini, Remeuf, & Corrieu, 2001). Oliveira et al. (2001), showed that added probiotic cultures of lactobacilli in fermented milk resulted in lower population levels when probiotic cultures were used as mixed cultures along with the starter culture, either than used as pure mono-cultures, possibly due to nutritional competition. However, the probiotic strains maintained adequate population numbers throughout storage, to regard the product as probiotic. Finally, Ng et al. (2011), studied differences of survival of probiotic strains of *Lb. acidophilus* during storage of yoghurt when the product was made using individual or dual starter cultures species along with the probiotic strains and concluded that the antagonistic effect was greater for the dual species, leading to lower populations of probiotic strains.

3.2. *Population dynamics of* Listeria monocytogenes

Survival of the pathogen (three-strain cocktail) inoculated at 3 different initial levels in both control and probiotic cases (A & B) and stored at 4 and 12 °C is presented in Fig. 2. The population of the pathogen at the three different inocula increased in all cases ($P < 0.05$, Supplementary material Table S2, Fig. 2) (control, probiotic A & B) during fermentation (5 h, 42 °C) approximately 0.5–1.0 log cfu mL^{-1}, depending on the case. More specifically, the highest increase (approximately 1.0 log cfu mL^{-1}) after fermentation was observed in the control and in probiotic case B for samples inoculated with the low inoculum (3.0 log cfu mL^{-1} of initial inoculum level) of the pathogen at both temperatures (Fig. 2A,D). During cold storage for the low inoculum level of pathogen, probiotic case A was found *Listeria* free after 18 days, while probiotic case B and control, remained *Listeria* positive after enrichment. At medium inoculum case (5.0 log cfu mL^{-1} of initial inoculum level), the pathogen's population decreased ($P < 0.05$, Supplementary material Table S2) for both probiotic cases (A and B) during cold storage. More specifically, for probiotic case A, *Listeria* was found only after enrichment, at day 32 of storage, while probiotic case B, *Listeria* was not detected with enrichment after 25 days of storage. In control samples, the population of the pathogen remained at 2.2 log cfu mL^{-1} ($P > 0.05$), until the end of cold storage. At high inoculum (7.0 log cfu mL^{-1} of initial inoculum level), a greater decrease was observed for probiotic case B and control (approximately 5.0 log reduction), while for probiotic case A, *Listeria* population were found approximately 1.0 log higher (Fig. 2). Regarding the population of the rest of the microbiota, population levels were comparable with the samples produced without the pathogen, for both control and probiotic fermented milk samples. Thus, mesophilic LAB were the dominant microbiota and population was estimated over 7.5 log cfu mL^{-1} during the whole storage period of the fermented milk at 4 °C. In addition, yeasts/moulds or *Enterobacteriaceae* were not detected after enumeration (detection limit 1.0 and 0 log cfu mL^{-1} respectively) in the samples of all cases.

At 12 °C and at low inoculum, control samples and probiotic case A, were found negative for the presence of *Listeria* after enrichment at the end of storage. However, probiotic case B was found *Listeria* free earlier compared with the other two cases ($P < 0.05$, Supplementary material Table S2). More specifically, probiotic case B was found negative for the presence of *Listeria* after enrichment from the 16th day of storage onwards. At medium inoculum level case, all samples were found *Listeria* free after enrichment at day 20, while for the high inoculum level (7.0 log cfu mL^{-1} of initial inoculum level), all samples were found *Listeria* positive after enrichment at the end of storage (Fig. 2). Finally, mesophilic LAB comprised the dominant microbiota (>7.0 log cfu mL^{-1}) throughout storage at 12 °C for control and probiotic fermented milk inoculated with *L. monocytogenes* as also shown for 4 °C.

Monitoring evolution of different strains of *L. monocytogenes* during shelf life of dairy products is of great importance, to examine the potential impact on food safety. To date, many studies have been conducted considering growth and survival of the pathogen on dairy products, since *L. monocytogenes* is considered to be a major food safety concern, however, to our knowledge no data are available for fermented milk. Tirloni et al. (2011) observed a reduction of *Listeria* counts after inoculation in yoghurts, though, *Listeria* was found very resistant and was always detectable till the end of shelf life of the products. Similar results were observed when yoghurt was inoculated with a high inoculum of *Listeria innocua*, where its population decreased faster at abusive temperatures than at cold storage (Belessi, Papanikolaou, Drosinos, & Skandamis, 2008). Experiments dealing with Galotyri, a soft acid curd traditional Greek cheese with low pH (4.0), showed that the pathogen was able to survive throughout storage at 4 and 12 °C, regardless the low pH and different initial inoculum levels and survival was characterised by a long “tailing” of the pathogen population (Rogga et al., 2005). In a recent study, cottage cheese, an acidic cheese product with pH value 4.5, was inoculated with *L. monocytogenes* with or without the addition of probiotic strains and it was shown that *Listeria* had a higher increase in control samples compared with the probiotic cases for both strains examined (Abadía-García et al., 2013). The findings of the current study showed that the pathogen was eliminated in a shorter time under an abuse temperature than in cold storage. This result comes in line with previous works in dairy products dealing with several storage temperatures, which have also reported the shorter inactivation time of the pathogen for abuse temperatures compared with colder temperatures (Kagkli, Iliopoulos, Stergiou, Lazaridou, & Nychas, 2009; Papadopoulou et al., 2018).

3.3. *Physicochemical analysis*

Results for pH and titratable acidity measurements are presented at Fig. 3, for 4 and 12 °C. The pH values after fermentation were similar for both probiotic and control cases ($P > 0.05$, Supplementary material Table S3) and decreased from initial pH 4.5

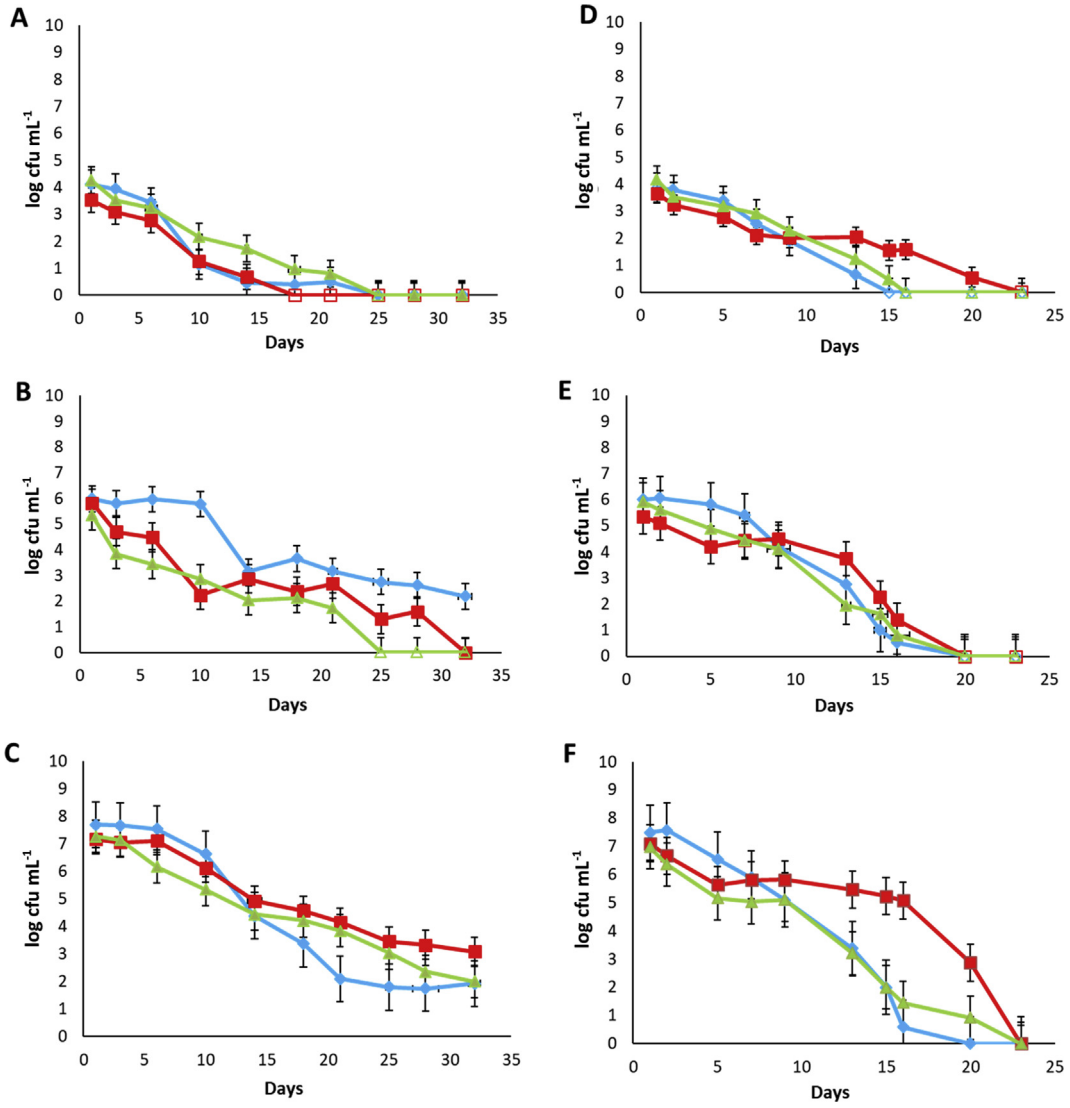

Fig. 2. Growth curves of *Listeria monocytogenes* during storage of fermented milk at A, B and C, low, medium and high inoculum, 4 °C, respectively and D, E, and F, low, medium and high inoculum, 12 °C, respectively: ◆, control sample; ■, *Lb. plantarum* 2035 (probiotic case A); ▲, *Lb. plantarum* T571 (probiotic case B). Empty markers indicate absence of *L. monocytogenes* after enrichment. Filled markers indicate presence of *L. monocytogenes* after enrichment. The bars represent the mean values ± standard deviations (two biological samples, each sample analysed three times).

to pH 4.1 during storage at 4 °C ($P < 0.05$, Supplementary material Table S3). Titratable acidity was found slightly higher ($P < 0.05$, Supplementary material Table S3) during storage at 4 °C for probiotic cases, in comparison with control samples. In contrast, during storage at 12 °C, pH and titratable acidity showed a faster change for fermented milk inoculated with probiotic strains in comparison to control samples ($P < 0.05$). In detail, pH value was 4.2 and titratable acidity was 0.9% g L^{-1} of lactic acid, for control samples by the end of storage, while values for pH and titratable acidity for both probiotic samples were approximately 4.0 and 1.05% g L^{-1} of lactic acid, respectively.

In terms of physicochemical analysis, pH decreased faster in both probiotic and control samples and reached lower levels during storage at 12 °C, in comparison with cold storage (Fig. 3). Post acidification was noticed during cold storage of the probiotic fermented milk, too, as a result of the acid-resistant nature of the strains used and residual acidification during storage. This is in line with previous work in fermented dairy products, where lower pH values were recorded for fermented milk inoculated with probiotic cultures as adjuncts, due to post acidification during storage (Kakisu et al., 2011; Mani-Lopez, Palou, & Lopez-Malo, 2014). Results of the aforementioned studies, observed pH values around 3.9–4.2 at the end of storage, while the initial pH values of the dairy products were 4.5–4.7.

3.4. Monitoring the survival of the probiotic strains

A total of 200 isolates were recovered from petri dishes with the highest countable dilution (≥6.0 log cfu mL^{-1}) for both temperatures and the presence of *Lb. plantarum* T571 in all cases was confirmed by PFGE. The results demonstrated that 100% of the isolates (beginning, middle and final storage time) recovered, displayed a PFGE profile corresponding to *Lb. plantarum* T571, for both storage temperatures; other mesophilic LAB were not detected. Additionally, the population of *Lb. plantarum* T571 was found at levels required for conferring a health benefit to the consumer (≥6.0 log cfu mL^{-1}) throughout storage. Similar results were obtained in a previous work dealing with Feta cheese and the use of

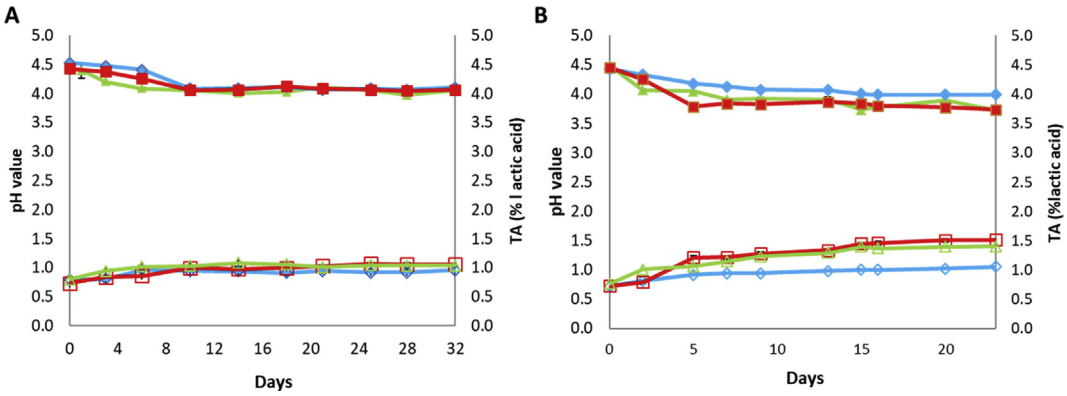

Fig. 3. Changes in fermented milk samples during storage at 4 °C (A) and 12 °C (B) in terms of pH values (primary axis): ◆, control sample; ■, *Lb. plantarum* 2035 (probiotic case A); ▲, *Lb. plantarum* T571 (probiotic case B) and in terms of titratable acidity (secondary axis) (TA % lactic acid): ◇, control sample; □, *Lb. plantarum* 2035 (probiotic case A); △, *Lb. plantarum* T571 (probiotic case B).

Lb. plantarum T571 as an adjunct co-culture (Papadopoulou et al., 2018). In the latter study, it was shown that the strain T571 was capable of surviving in high population levels during long term storage and finally overcame the commercial starter culture in Feta cheese.

In the case of *Lb. plantarum* 2035, the presence of the strain in petri dishes corresponding to the concentration of ≥6.0 log cfu mL^{-1} was verified by Multiplex PCR using the novel RAPD-Derived primers. It was found that, *Lb. plantarum* 2035 was present at desirable levels (≥6.0 log cfu mL^{-1}) in the fermented milk samples at both beginning and end of storage for both temperatures (Fig. 4).

Currently, the demand for probiotic products is growing, due to consumers preferences for food products with beneficial properties for well-being and health maintenance (Patrignani et al., 2006). The global dairy industry has been using fermented products as carriers for probiotic strains for over 30 years, thus, research into novel probiotic strains has been increasing at the international level (Maragkoudakis et al., 2006). To consider a product as probiotic, the population of the probiotic strain must exceed 6.0 log cfu per g or mL throughout storage, with bacterial species being able to be accurately identified at strain level to meet the health claims of probiotic products (Raeisi, Ouoba, Farahmand, Sutherland, & Ghoddusi, 2013). The survival of probiotic *Lb. plantarum* 2035 was confirmed with multiplex PCR using RAPD-derived primers, while

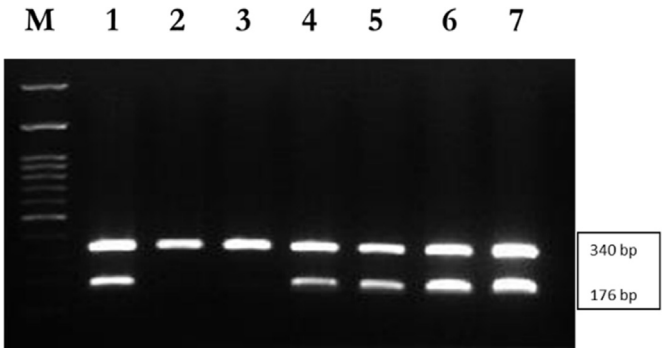

Fig. 4. Molecular identification of *Lb. plantarum* 2035 in fermented milk. After mesophilic LAB enumeration on MRS agar plates, the presence/absence of *Lb. plantarum* 2035 (probiotic case A) at levels ≥ 6 log cfu mL^{-1} in fermented milk during storage at 4 and 12 °C was confirmed by multiplex PCR using RAPD-derived primers. Lanes are: M, DNA ladder 100 bp; 1, *Lb. plantarum* 2035; 2 and 3, control sample at 4 °C and 12 °C, respectively; 4 and 5, *Lb. plantarum* 2035 at 4 °C, beginning and end of storage, respectively; 6 and 7, *Lb. plantarum* 2035 at 12 °C, beginning and end of storage, respectively.

the survival of *Lb. plantarum* T571 was determined by PFGE and both probiotics were found at desirable levels during storage at 4 and 12 °C. It is well known that, lactobacilli can grow and be viable in fermented food products with low pH values of 3.7–4.3 (Tripathi & Giri, 2014), as was evident in this study, too. Results in this work indicated that the high survival rates of both strains *Lb. plantarum* 2035 and *Lb. plantarum* T571, are in line with previous studies concerning survival of added probiotic cultures in fermented dairy products such as fermented milk (Raesi et al., 2013), yoghurts (Saxami et al., 2016; Sidira et al., 2013, 2017) and in a variety of cheeses (Angelopoulou et al., 2017; Papadopoulou & Chorianopoulos, 2016; Papadopoulou et al., 2018). In the above stated studies, strain identification was also monitored with molecular methods.

3.5. *Monitoring the distribution of* Listeria monocytogenes *strains*

A total of 560 isolates were screened with PFGE to monitor survival and distribution of the *L. monocytogenes* strains after milk inoculation and fermented milk storage at 4 and 12 °C for both control and probiotic samples. Distribution of the above strains recovered based on PFGE profiles, is presented in Fig. 5. At milk inoculation, the strains were added in a ratio of approximately 1:1:1 at each case, as presented in Fig. 5. Remarkable information was exhibited regarding the distribution of strains in control and probiotic samples (case A and B) during storage for both temperatures. At the beginning of storage at 4 °C and at low inoculum level, strain FMCC-B131 was found to be present at a level of 60% for the probiotic case B (*Lb. plantarum* T571), while for control and probiotic case A (*Lb. plantarum* 2035), the recovery percentage were 8.33% and 16.67%, respectively. However, strain FMCC-B131 was not detected at the middle and the end of storage at 4 °C for all cases, but for probiotic case B it was detected only after enrichment at the final storage day. It has to be noted that, in the cases that the isolates were recovered with the enrichment method, the presence of each strain has to be characterised as random and the appearance of the strains in the figures should be interpreted as qualitative information rather than % contribution in the samples. In general, the latter strain was found to be the most susceptible strain at 4 °C (Fig. 5). The strain FMCC-B129 was present at higher percentage at low inoculum level and in one case was found to be the only recovered strain (middle of storage, probiotic case A), while strain FMCC-B133 displayed the highest recovery percentage at high inoculum level, in all cases by the end of storage at 4 °C. At 12 °C for low and medium inoculum, the 3 strains exhibited similar percentages at the middle of storage, while at the end no strains of the

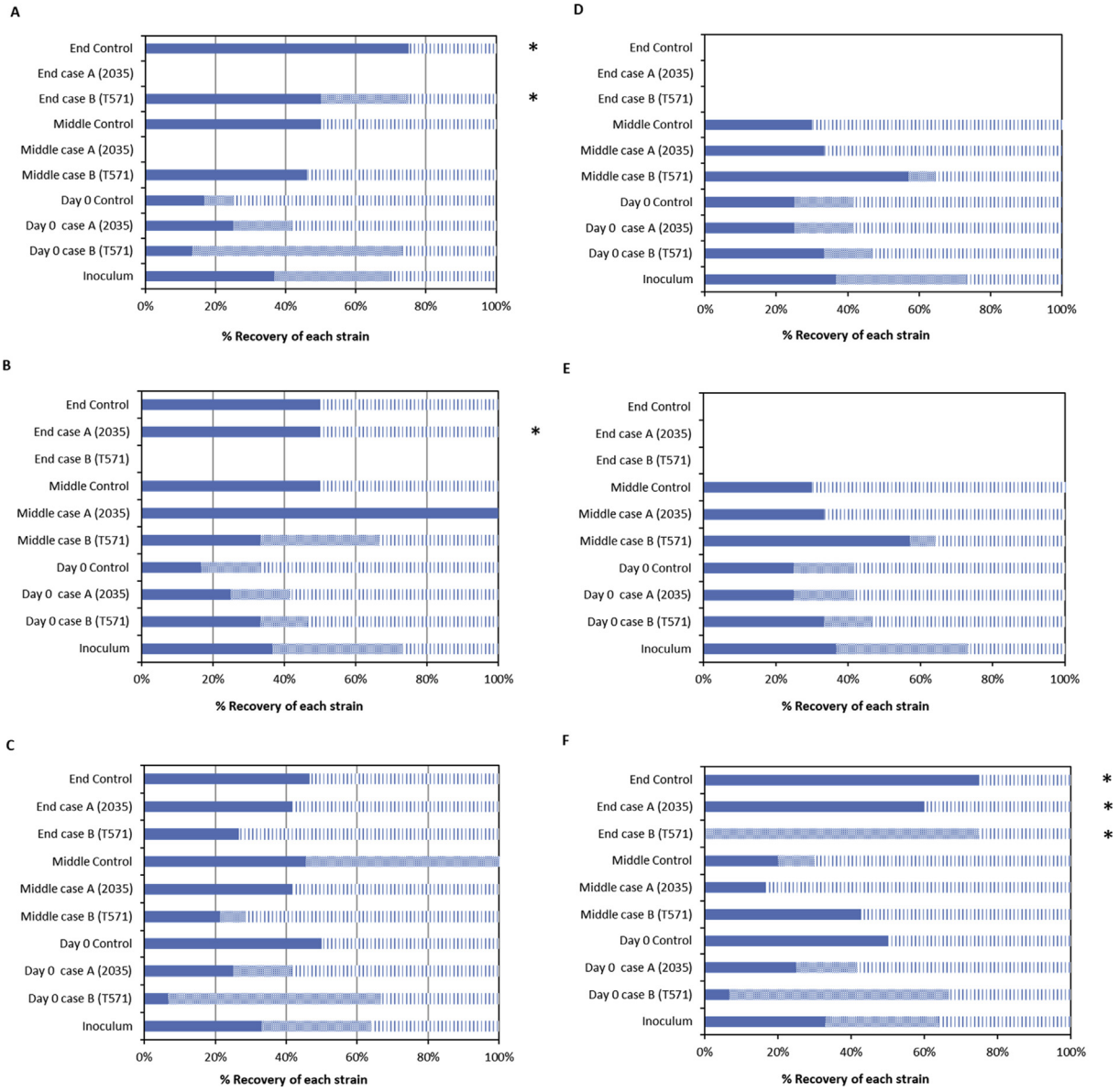

Fig. 5. Distribution of *Listeria monocytogenes* isolates (FMCC-B129: dark shade, FMCC-B131: light shade, FMCC-B133: vertical lines) recovered during storage of fermented milk samples based on PFGE profiles: A, B, and C, low, medium and high inoculum, 4 °C; D, E and F, low, medium and high inoculum, 12 °C. An asterisk indicates pathogen detected after applying the enrichment method.

pathogen where detected after enrichment. (Fig. 5). At high inoculum, strain FMCC-B129 was recovered at higher percentage in control samples and probiotic case A, while in probiotic case B, the latter strain was not recovered. In contrast, the strain FMCC-B131 displayed the highest recovery rate (75%), for the latter case.

Occurrence of *L. monocytogenes* in dairy foods has been recently studied to trace potential routes of contamination. Results demonstrated that the pathogen is widely distributed in the environment and can enter through various scenarios (contact materials, inappropriate personnel movements, food workflows, etc.) in the food processing environment and colonise food, making the risk of cross contamination always possible (Muhterem-Uyar et al., 2015; Ruckerl et al., 2014). Ruckerl et al. (2014) used PFGE and other molecular tools for typing isolates that were recovered from an Austrian cheese processing facility over a three-year period. The study revealed that different genotypes that were dominant in the food processing environment were isolated from food equipment, floors and drains during the first year, while in the second year of research, new PFGE types were introduced through raw materials. However, although a variety of *L. monocytogenes* strains can enter a dairy plant, some subtypes of the pathogen have a global distribution among food processing plants (Ruckerl et al., 2014). Other studies conducted on a variety of cheeses using PFGE for strain typing of *L. monocytogenes* showed that pathogen survival was strain dependent and was affected by storage time, different temperatures (Kagkli et al., 2009) and/or the presence/absence of probiotic strains (Papadopoulou et al., 2018).

In accordance with the above, in the current study, it was evident that each strain reacted differently in the control or probiotic samples and was depended on added probiotic cultures and storage temperature. This observation could be explained by the presence of the different probiotic bacteria which influence the survival of pathogenic strains, to one strain over the other. In addition, the pH value of the fermented milk, which was always

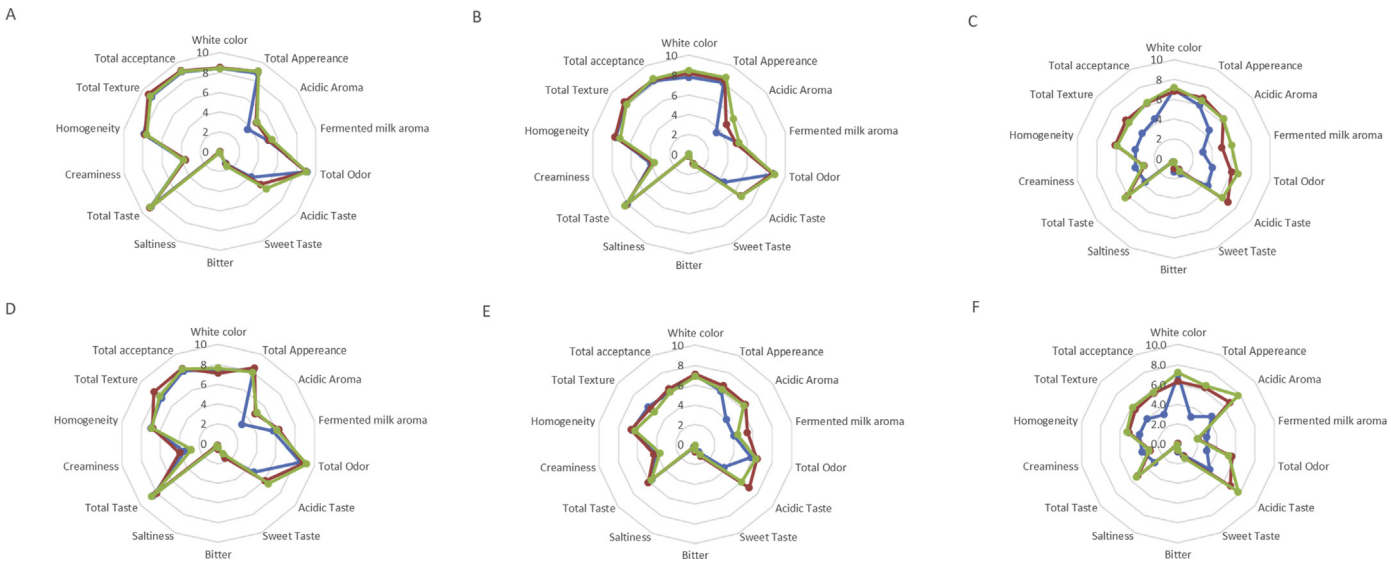

Fig. 6. Sensory evaluation of fermented milk samples, during storage at 4 °C (A, B and C: 0, 14, and 25 days, respectively) and 12 °C (D, E and F: 0, 10, and 16 days, respectively): ◆, control sample; ■, *Lb. plantarum* 2035 (probiotic case A); ▲, *Lb. plantarum* T571 (probiotic case B).

close to the tolerance limit of the pathogen, might have had an impact on the survival of the strains in each case. Finally, storage temperature affected growth and survival of the strains leading to a variability in recovery rate of each strain. To this respect, the findings of this research suggest that different storage conditions, e.g., 4 and 12 °C, addition of different probiotic strains (*Lb. plantarum* 2035 and *Lb. plantarum* T571) as adjunct cultures and variability in inoculum levels (3.0, 5.0 and 7.0 log cfu mL^{-1}), affected the diversity of the *Listeria* population, as well as its growth and survival in a real food ecosystem.

3.6. Sensory evaluation

Results of the fermented milk sensory evaluation during storage at 4 and 12 °C showed that the end of shelf life of the products depended on the case (Fig. 6). At cold storage, the shelf life of control samples was determined to be the 25th day of storage, while for probiotic cases A and B the corresponding day was the 28th. In detail, the sensory panel evaluated as better the probiotic fermented milks with probiotic strains, compared with the respective controls for taste and odour during storage at 4 °C. In addition, the texture scores and appearance scores were similar to the control ($P > 0.05$), while overall acceptance was found always higher for probiotic cases. At 12 °C, the shelf life of the products was shorter in comparison with those of stored at 4 °C. The control samples were found marginally acceptable until the 13th day of storage, while the respective day for probiotic cases was the 16th day of shelf life at 12 °C. At day 20, all samples were considered unacceptable. The sensory panel evaluated the fermented milk samples stored at 12 °C with similar or lower scores for overall acceptance in contrast to the samples stored at 4 °C.

Lb. plantarum strains 2035 and T571 were applied individually as co-starters adjuncts in fermented milk manufacture and both probiotic strains exhibited a satisfactory performance in fermented milk sensorial analysis throughout storage at both temperatures. It was evident from the results that there were no significant changes in the fermented milk typical characteristics. In detail, colour, an important attribute in the products as it is usually the first characteristic that consumers observe (Mani-Lopez et al., 2014), was not affected by the addition of the probiotic strains. This result was also noticed in other studies of fermented dairy products during fermentation with different probiotic strains, where colour remained constant (Mani-Lopez et al., 2014). In the present study, taste and odour was slightly affected, in terms of higher acidity, by the addition of the probiotic strains, a result that was not observed in other studies where no flavour differences were detected (Kakisu et al., 2011; Mani-Lopez et al., 2014).

4. Conclusions

Findings of the present study showed that the population levels of the two potential probiotic strains in a fermented milk drink throughout storage, fulfilled the international recommendations and guidelines for products manufactured with live probiotic bacteria. In respect to their contribution in product safety, the strain T571 showed better results in antagonising strains of *Listeria* by minimising their growth and survival at all inoculum levels studied, during storage at 4 °C. The pH values and titratable acidity of the new products were similar to those of control samples, as well as with respect to sensory scores. Thus, addition of strains with probiotic potential as adjunct cultures, led to qualitative products with increased added value. Although further clinical studies are required for the potential probiotic strains, this type of fermented milk can act as a suitable carrier for probiotic bacteria, while maintaining high-quality characteristics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.06.006.

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