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Microbiological shelf life at different temperatures and fate of *Listeria monocytogenes* and *Escherichia coli* inoculated in unflavored and strawberry yogurts

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ABSTRACT

Three different trials were performed on unflavored and strawberry yogurts produced in a small-scale dairy plant. In the first trial, the microbiological shelf life of the products was evaluated at 4, 8, and 20°C. At 4°C the product showed low total viable counts until the end of the trial (d 35 = 3.0 ± 0.7 and 1.5 ± 0.0 log cfu/g in unflavored and strawberry yogurt, respectively). The loads were lower in strawberry yogurt at 4°C compared with unflavored yogurt because of the antimicrobial activity exerted by potassium sorbate present in the fruit puree added. Yeasts were confirmed to be the specific spoilage agents of this product, reaching rapidly high loads with thermal abuse (5.9–7.4 log cfu/g at d 18). In the second trial, *Escherichia coli* and especially *Listeria monocytogenes* added at 2 concentrations (2 and 5 log cfu/g) showed a rapid decrease in both types thanks to the acidic conditions provided by the products, but *L. monocytogenes* was very resistant; its presence was always detected until the end of the period considered (d 68). In the third trial, no statistically significant differences were detected between wild and acid-adapted strains of *L. monocytogenes* added to the products, due to the quick adaptation that probably occurred after inoculation.

Key words: yogurt, *Listeria monocytogenes*, *Escherichia coli*, survival, acid adaptation

INTRODUCTION

Yogurt is a functional dairy product consumed worldwide thanks to its positive effect on human health. However, if the selection of raw materials is not well controlled, if good production practices are not used, especially in small-scale production, or if storage conditions are unfavorable, yogurt spoilage occurs in a short time, leading to an unacceptable product for consumers due to its yeasty, fermented odor and flavor, and a visible swelling caused by the gas produced (Fleet, 1990, 1992; Mataragas et al., 2011; Gougouli et al., 2011).

Although yeasts are mainly known as positive organisms because of their fermentative activity for the production of several foodstuffs and beverages (e.g., wine, beer, and bread), they also play a major role as specific spoilage organisms and are the main cause of microbial alterations in yogurt. The ability to grow at low temperatures and in environments with high sugar concentration (up to 50–60%) allows yeasts to replicate in yogurt (especially in fruit-based ones). Yeasts are generally more resistant than bacteria in extreme conditions, in particular thanks to low pH and the presence of preservatives (Deak, 1996); above all, yeast spoilage has recently increased as a result of mild preservation processes required for developed standards of food quality (Fleet, 1992). The growth of yeasts on food components generates different end products, determining a substantial change in physical-chemical and sensorial characteristics. In particular, their ability to ferment lactose and sucrose, which are then converted to ethanol and carbon dioxide, leading to a distinctive alcoholic flavor, is well known. Moreover, the production of several secondary end products such as higher alcohols, organic acids (prevalently succinic acid and acetic acid, the main volatile compound produced by sugar fermentation), esters, aldehydes, and ketogenic substances has been described (Suriyarachchi and Fleet, 1981; Nykänen, 1986; Fleet, 1992). The production of proteolytic and lipolytic enzymes that hydrolyze milk fat and protein is also widely documented (Fleet, 1992).

At the time of production, yogurt should contain no more than 1 yeast cell per gram, even if higher counts have been recorded immediately after production (Suriyarachchi and Fleet, 1981; Fleet and Mian, 1987); in any case, spoilage starts to be evident when loads reach 5 to 6 log cfu/g (Fleet, 1990). The main species related to spoilage of yogurt are *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Rhodotorula mucilaginosa*, *Rhodotorula rubra*, *Rhodotorula glutinis*, and *Saccharomyces cerevisiae* (Minervini et al., 2001; Viljoen, 2001; Mayoral et al., 2005). According to Italian Standard Uni 10358 (Uni Ente Italiano di

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Normazione, 1993), "a product could be defined as yoghurt if it is obtained by acid coagulation of milk without subsequent subtraction of the serum, for exclusive action of the fermentation process due to 2 specific microorganisms in combination: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. In particular, the characteristics and properties of yogurt are linked to the presence of the above microorganisms alive and vital: each of the specific thermophilic lactic acid bacteria should not be less than 6 log cfu/g and together they must be present in a total amount of not less than 7 log cfu/g in the finished product for the whole shelf-life assigned."

Although not numerous, outbreaks related to the consumption of contaminated yogurt with potential pathogenic bacteria (enterohemorrhagic *Escherichia coli, Salmonella* Typhimurium, and *Clostridium botulinum*) are reported in the literature (O'Mahony et al., 1990; Morgan et al., 1993; Evans et al., 1999). Moreover, *Listeria monocytogenes*, a ubiquitous pathogen able to adapt to stressful environments, is recognized as a postprocessing contaminant of major concern in dairy products such as yogurt.

Previous studies showed that several potential pathogenic bacteria (*Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, and *E. coli* O157:H7) intentionally added to yogurt or fermented milk before or after fermentation, thus simulating cases of ineffective heat treatment or postcontamination, were hardly able to survive or otherwise multiply (Alm, 1983; Čuk et al., 1987; Zúñiga-Estrada et al., 1995; Pazakova et al., 1997; Issa and Ryser, 2000; Benkerroum et al., 2002; Belessi et al., 2008; Hsieh et al., 2010; Osaili et al., 2013).

The aims of the current study were to evaluate the shelf life of yogurt produced in a small dairy plant in optimal refrigeration and thermal abuse conditions. In a second phase, the survival of potentially pathogenic microorganisms (*E. coli* and *L. monocytogenes*) voluntarily inoculated in the product was evaluated. The third phase of the study was conducted to evaluate the ability of adaptation of *L. monocytogenes* to the acidic environment of yogurt samples.

MATERIALS AND METHODS

Experimental Design and Raw Material

Three studies were carried out in total: in the first study, the microbiological shelf life of unflavored and strawberry yogurts was evaluated at different temperatures (4, 8, and 20°C). In a second study, the survival of *L. monocytogenes* and *E. coli* inoculated at 2 different concentrations in yogurt samples was evaluated at 4°C. In the last study, the influence of previous adaptation to yogurt environment of a *L. monocytogenes* strain was evaluated.

Unflavored and strawberry yogurt samples were obtained from a small-scale dairy producer located in the north of Italy. Briefly, milk was pasteurized at 85°C for 30 min, the blend was homogenized (136.1–170.1 atm), and milk was cooled to 42°C. Afterward, the starter cultures (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) were mixed into the milk, which was held at 42°C until the pH 4.5 was reached. Finally, the yogurt was cooled to 4°C to stop the fermentation process. The strawberry yogurt was produced from unflavored yogurt with the addition of strawberry puree (550 g/4 kg of yogurt), composed of sugar, strawberries (35%), water, pectin E440 as a gelling agent, flavoring, potassium sorbate E202 (0.13%) as a preservative, and sodium citrate E331 as an acidity regulator. The 2 types of yogurt were packaged into 125-g jars and submitted to the subsequent tests.

First Trial: Evaluation of Microbiological Shelf Life

At established sampling times (0, 3, 7, 10, 15, 18, 21, 24, 28, 31, and 35 d from production), the samples stored at 4°C were analyzed in triplicate. Samples maintained at 8 and 20°C were analyzed at the same sampling times until 18 d after production. For microbial counts, 10 g of each sample was homogenized in 90 mL of a diluent solution (0.85% NaCl and 0.1% tryptone), and serial 10-fold dilutions were prepared. Total mesophilic count excluding lactic acid bacteria was determined onto gelatin peptone bios agar (Biogenetics, Ponte San Nicolò, Italy) and subsequently incubated at 30°C for 48 h. *Lactobacillus delbrueckii* ssp. *bulgaricus* was enumerated on de Man-Rogosa-Sharpe agar (Oxoid, Basingstoke, UK) acidified at pH 5.2 according to ISO/FDIS 7889 IDF 117 standard (ISO, 2002). *Streptococcus thermophilus* was enumerated onto M17 agar (Oxoid) supplemented with lactose (5 g/L) and incubated under aerobic conditions at 45°C for 24 h (IDF, 1981). The number of *Enterobacteriaceae* was determined by ISO 21528–2:2004 (ISO, 2004b) method. *Escherichia coli* were enumerated according to ISO 16649–2:2001 (ISO, 2001) method. Coagulase-positive staphylococci were determined by ISO 6888–1:1999 (ISO, 1999) method. Yeasts and molds were enumerated according to ISO 21527–1:2008 (ISO, 2008) method. *Salmonella* spp. detection was performed by the methods ISO 6579:2002/Cor 1:2004 (ISO, 2004a). Detection of *L. monocytogenes* was performed according to the AFNOR (1998) method (AFNOR BRD 07/4–09/98).

At the same sampling times, pH was measured by a pH meter (Amel Instruments, Milano, Italy); 3 independent measurements were performed on each sample and means were calculated.

Second Trial: Inoculation Test

Escherichia coli ATCC 25922 and *L. monocytogenes* ATCC 7644 strains were stored in cryovials (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada) at −70°C until use. For each strain, a loop of the frozen culture was transferred to a test tube containing 10 mL of tryptic soy broth (**TSB**; Oxoid, Basingstoke, UK) and incubated overnight at 30°C. Both the strains were re-inoculated into cooled TSB tubes and the initial absorbance (540 nm; UV1601, Shimadzu, Chicago, IL) was measured. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in the exponential growth phase, defined as a change of absorbance of 0.05 to 0.2 at 540 nm. If necessary, the cultures were diluted to obtain the final concentrations of 4.1 and 7.1 log cfu/mL. Afterward, 0.1 mL of each preculture was inoculated into the 2 types (unflavored and strawberry) of yogurt to obtain the final concentrations of 2 and 5 log cfu/g in the products, maintained at 4°C. At settled sampling times (0, 1, 2, 3, 4, 7, 10, 13, 16, 20, 23, 26, 33, 40, 47, 54, 61, and 68 d from the inoculation), the samples were analyzed in duplicate. For microbial counts, 10 g of each sample were homogenized in 90 mL of a diluent solution (0.85% NaCl and 0.1% tryptone), and serial 10-fold dilutions were prepared. Detection and enumeration of *L. monocytogenes* were performed according to AFNOR methods (AFNOR BRD 07/4–09/98 and AFNOR BRD 07/05–09/01, respectively). *Escherichia coli* were enumerated according to the ISO 16649–2:2001 method (ISO, 2001). Detection of *E. coli* was performed inoculating 25 g of product into 225 mL of Brilliant Green Bile Lactose Broth (Oxoid), subsequently incubated at 37°C for 24 h in aerobic conditions. Afterward, 0.1 mL of the broth was plated onto Tryptone Bile X-Glucuronide Agar, incubated at 37°C for 24 h. From the plates of the Rapid'*L.mono* at d 68, colonies of *L. monocytogenes* were picked, purified in TSB, and stored in cryovials. At the same sampling times, pH was measured by a pH meter (Amel Instruments): 3 independent measurements were performed on each sample and means were calculated.

Third Trial: Adaptation Test

Listeria monocytogenes ATCC 7644 (wild) and *L. monocytogenes* isolated at d 68 as previously described, adapted to yogurt environment, were stored in cryovials, then transferred to a test tube containing 10 mL of TSB and incubated overnight at 30°C. The strains were prepared as described in the previous section. The cultures were diluted to obtain the final concentrations of 6.1 log cfu/mL. Afterward, 0.1 mL of each preculture was inoculated into the 2 types of yogurt at the final concentrations of 4 log cfu/g and stored afterward at 4°C. At settled sampling times (0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 d from the inoculation), the samples were analyzed in duplicate. For microbial counts, 10 g of each sample was homogenized in 90 mL of a diluent solution, and serial 10-fold dilutions were prepared. Detection and enumeration of *L. monocytogenes* were performed according to AFNOR methods (AFNOR BRD 07/4–09/98 and AFNOR BRD 07/05–09/01). At the same sampling times, pH was measured by a pH meter (Amel Instruments): 3 independent measurements were performed on each sample and means were calculated.

Statistical Analysis

All the analytical data were statistically compared by one-way ANOVA using SAS/STAT package version 8.0 (SAS Institute Inc., Cary, NC). During the first trial, the differences between unflavored and strawberry samples were evaluated. In the second trial, the differences between unflavored and strawberry samples were deepened considering the same starting bacterial concentration. Finally, in the third trial, the differences between wild and adapted strains were evaluated for the same product type. The differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Microbiological Shelf Life

In this study, we analyzed artisanal Italian yogurt maintained at 3 static temperatures (4, 8, and 20°C) to determine their microbiological shelf life (Figure 1). Considering *Lactobacillus delbrueckii* ssp. *bulgaricus* and *S. thermophilus* counts, the products were stable at the 3 temperatures considered. The products maintained the characteristics required by Uni Ente Italiano di Normazione (1993) for the definition of yogurt; in all samples, the count of either of the 2 microorganisms was not <6 log cfu/g and the count of the 2 combined was >7 log cfu/g. Lactic acid bacteria were the dominant microflora due to the inoculation of starter cultures, as already reported in many previous studies (Mataragas et al., 2011). This population was stable during the storage period at different temperatures: at 4°C in unflavored samples, the loads were from 9.0 log cfu/g (d 0) to 8.8 log cfu/g (d 35), whereas in straw-

berry samples, the loads were from 8.8 log cfu/g (d 0) to 8.9 log cfu/g (d 35); similar trends were detected at 8°C in unflavored (from 8.9 log cfu/g at d 0 to 8.6 log cfu/g at d 18) and in strawberry samples (from 8.6 log cfu/g at d 0 to 8.8 log cfu/g at d 18). At 20°C, the same situation was registered for unflavored samples (from 8.9 log cfu/g at d 0 to 8.6 log cfu/g at d 18); the only exception was observed in strawberry samples at 20°C, where a slight reduction was detected (from 8.6 log cfu/g at d 0 to 7.6 log cfu/g at d 18). Considering TVC at 4°C, both the types of yogurt showed low loads for the whole period of 35 d, with fluctuations between 1.5 and 2.4 log cfu/g in strawberry samples and 1.7 and 3.0 in unflavored samples, without any clear trend of increase. At 8°C, the loads in both the series were below the detection limit (2 log cfu/g) until d 3; afterward, a rapid and constant increase was seen, reaching, at d 18, loads of 5.9 ± 0.1 and 7.5 ± 0.1 log cfu/g in strawberry and unflavored yogurts, respectively. At 20°C, the loads in both the series were below the detection limits just at d 0 in unflavored samples and at d 0 and 3 in strawberry ones. Afterward, a rapid increase was observed, reaching, at d 7 in strawberry samples and at d 10 in unflavored samples, a plateau between 5.7 and 6.1 log and between 6.3 and 7.4 cfu/g, respectively. Considering the whole period, lower values were observed in strawberry samples, even if no statistically significant differences were detected.

As expected, yeasts were the limiting factor for determining the microbiological shelf life of the product analyzed, confirming to be the specific spoilage organisms for yogurt (Jakobsen and Narvhus, 1996). The ability of yeasts to grow in yogurt environment is due to their metabolic activity, such as the fermentation of lactose and sucrose and the hydrolysis of milk casein (Mataragas et al., 2011). At 4°C, the loads of strawberry samples remained below the detection limit (1 log cfu/g) for the 35 d considered, whereas unflavored samples showed significantly higher values from d 18, when a moderate increase started, reaching 3.1 ± 0.0 log cfu/g at the end of the trial. At 8 and 20°C, a rapid increase was observed from d 3, except for unflavored samples at 20°C where the increase started at d 0. Unlike our results, Viljoen et al. (2003) found that, at 5 and 10°C, yeasts increased significantly only after 10 d, reaching 5 log cfu/g at d 15 of storage. The delay in yeast growth in our samples during the first days, especially in fruit samples, was likely related to the presence of potassium sorbate in the fruit puree in a rate of 0.13%, corresponding to a final concentration in the product of 156.8 ppm; this additive is well known for its inhibitory effect against yeast flora and is widely used as antimicrobial in the food industry (Mihyar et al., 1997). Generally, the residual presence of this antimicrobial substance in strawberry yogurt seemed to affect yeasts counts. In particular, at 4°C, significantly higher values ($P < 0.05$) were obtained during the period between d 15 and 35 in unflavored samples.

At 8°C, unflavored samples showed higher values than strawberry for the whole period, reaching at the end of the trial (d 18) loads of 6.3 ± 0.3 log and 5.9 ± 0.7 cfu/g, respectively. Also at 20°C unflavored samples

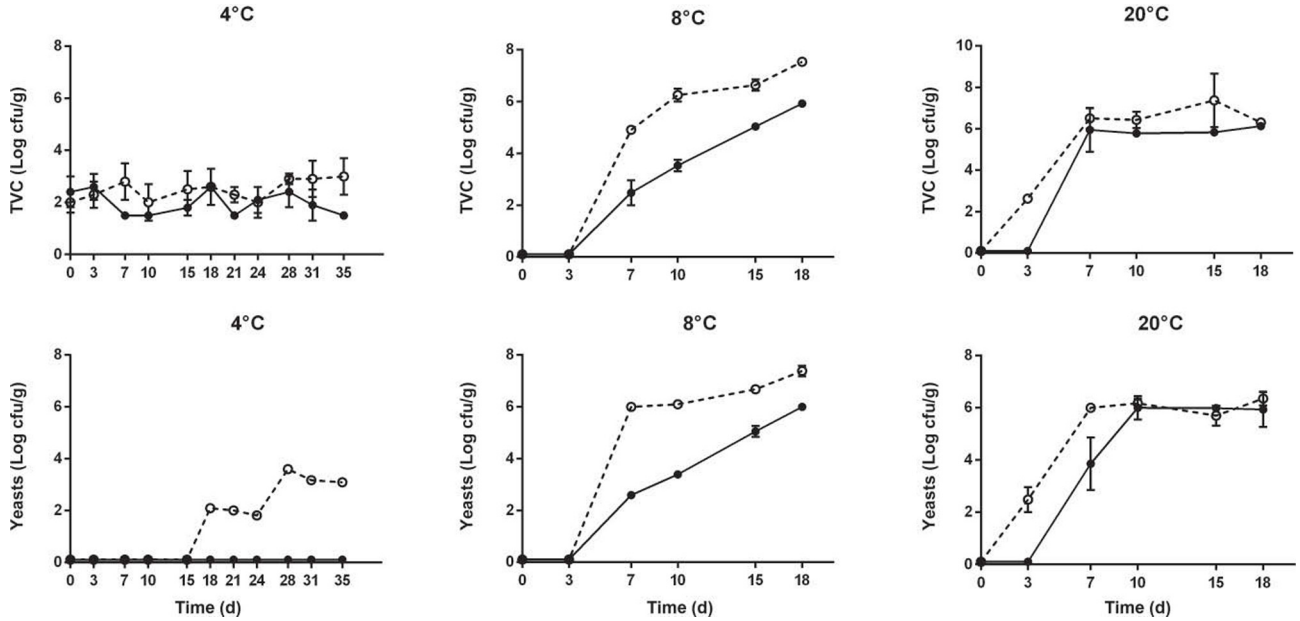

Figure 1. Trends of total viable count (TVC; means ± SD) and yeast count in unflavored (○) and strawberry (●) yogurt at 4, 8, and 20°C.

showed higher values until d 7 compared with strawberry, reaching more rapidly the plateau level between 5.7 and 6.3; however, the trends observed at 8 and 20°C were not statistically significant. The same effect was revealed also by Mihyar et al. (1997), who showed that at 5°C, yeasts in Lebanon yogurt reached 6 log cfu/g after 21 d of storage with the presence of 150 ppm of potassium sorbate, an amount very similar to that included in the puree used to obtain fruit yogurt in our study. Generally Mihyar et al. (1997) found that to ensure counts below 5 log cfu/g for 14 d at 5°C of the most sensitive yeast tested in that study (*Trichosporon cutaneum*), 100 ppm was needed, whereas 200 ppm was necessary to ensure the same level for 21 d. For all the other species tested in that study (including *Pichia farinosa* and *Debaryomices hansenii*), higher concentrations (>400 ppm) were required. Davis (1970) suggested standards for yeast counts in freshly made plain and fruit yogurts and defined as satisfactory the products with <1 organism/g and unsatisfactory those with loads >10, to ensure a sufficient shelf life in real storage conditions. A limit of 5 log cfu/g has also been determined as the threshold above which the consumers begin to perceive the sensorial alterations of yogurt (Suriyarachchi and Fleet, 1981); in our study, at 4°C the samples never exceeded this value in both types analyzed. Unflavored samples exceeded this concentration after 7 d at 8 and 20°C, whereas strawberry samples exceeded this limit after 15 or 10 d if stored at 8 or 20°C, respectively. *Escherichia coli*, *Enterobacteriaceae*, coagulase-positive staphylococci, and molds were below the detection limit and *Salmonella* spp. and *L. monocytogenes* were always absent in 25 g of product at all the sampling times.

The colonies isolated from our samples at T68 were identified as *Debaryomyces* spp.; the presence of this genus might be expected because these yeasts are typically associated with dairy products and especially with yogurt spoilage, and are recognized to possess proteolytic and lipolytic activities, even if these characteristics are strain specific (Alford and Pierce, 1961; Deak, 1996).

Constant pH values were detected in the products, without any clear trend in all the samples stored at the 3 temperatures with values ranging between 4.3 and 4.4 (4°C), 4.1 and 4.5 (8°C), and 4.0 and 4.4 (20°C). However, a slight decrease in samples maintained at 20°C was observed and was likely due to an increase in lactose and sucrose fermentation due to the high temperature, as described by other authors (Suriyarachchi and Fleet, 1981; Mataragas et al., 2011). Moreover, as already discussed by Mihyar et al. (1997) and Mataragas et al. (2011), the acidification of the substrate leads to an increase of the antimicrobial effect of potassium sorbate; as the pK_a of potassium sorbate (4.75) is higher than pH of the product, the proportion of acid in its undissociated form is higher, leading to a stronger antimicrobial effect.

Inoculation Test

The results obtained from the evaluation of the survival of *L. monocytogenes* and *E. coli* inoculated at 2 concentrations in the yogurt are shown in Figure 2. We wanted to simulate the presence of these 2 microorganisms in yogurt to reflect postprocessing contamination rather than survival during production processes. Considering the samples inoculated with *E. coli* at a concentration of 2 log cfu/g, a rapid decrease in the loads was observed in both types of product; the results were below the detection limit (1 log cfu/g) at d 10 in unflavored samples and at d 16 in strawberry samples. Afterward, all the loads were below the detection limit, with the only exception of unflavored samples at d 16 where the concentration of 1 log cfu/g was counted. However, *E. coli* was present in 25 g of product until d 26 in unflavored samples, whereas it was present in strawberry samples until d 23 (Table 1).

Considering the samples inoculated with *E. coli* at a concentration of 5 log cfu/g, a constant decrease was observed, with detectable counts until d 23 in unflavored samples and until d 26 in strawberry samples (total reduction of 5.2 and 5.4 log cfu/g, respectively). Afterward the loads were below the detection limit (1 log cfu/g). However, *E. coli* was present until d 47 in unflavored samples, whereas in strawberry samples it was present only until d 26 (Table 1). The presence of an acid resistance ability of enterohemorrhagic *E. coli* is well recognized because this microorganism can survive in acidic conditions at 4°C for prolonged periods (Lin et al., 1996). Previous studies have focused on the ability of *E. coli* O157:H7 to survive in yogurt during manufacturing and storage at different temperatures (Massa et al., 1997; Bachrouri et al., 2002; Lee and Chen, 2005; Osaili et al., 2013), but few works evaluated the acidic resistance of nonenterohemorrhagic *E. coli* (Canganella et al., 1998). Resistance to low pH is an important feature of foodborne pathogens for survival in specific foods as well as in the gastrointestinal tract. As suggested by Lin et al. (1996), weak acids are produced in many foods by the fermentation of natural microflora (as lactic acid bacteria in fermented dairy products such as yogurt), whereas acidulation is very often applied for the extension of shelf life of many fermented foods and for the inhibition of the growth of potential pathogenic bacteria. The results obtained from our study evidenced a slower decrease rate in *E. coli* inactivation compared with previous results by

Table 1. Presence in 25 g of *Listeria monocytogenes* (LM) and *Escherichia coli* (EC) in unflavored (white; W) and strawberry (S) yogurt

	Sampling time (day)																	
Item[1]	0	1	2	3	4	7	10	13	16	20	23	26	33	40	47	54	61	68
EC 2																		
W	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	−	−
W	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
S	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−
S	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−
EC 5																		
W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
S	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
S	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
LM 2																		
W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LM 5																		
W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[1]2 = starting concentration of 2 log cfu/g; 5 = starting concentration of 5 log cfu/g.

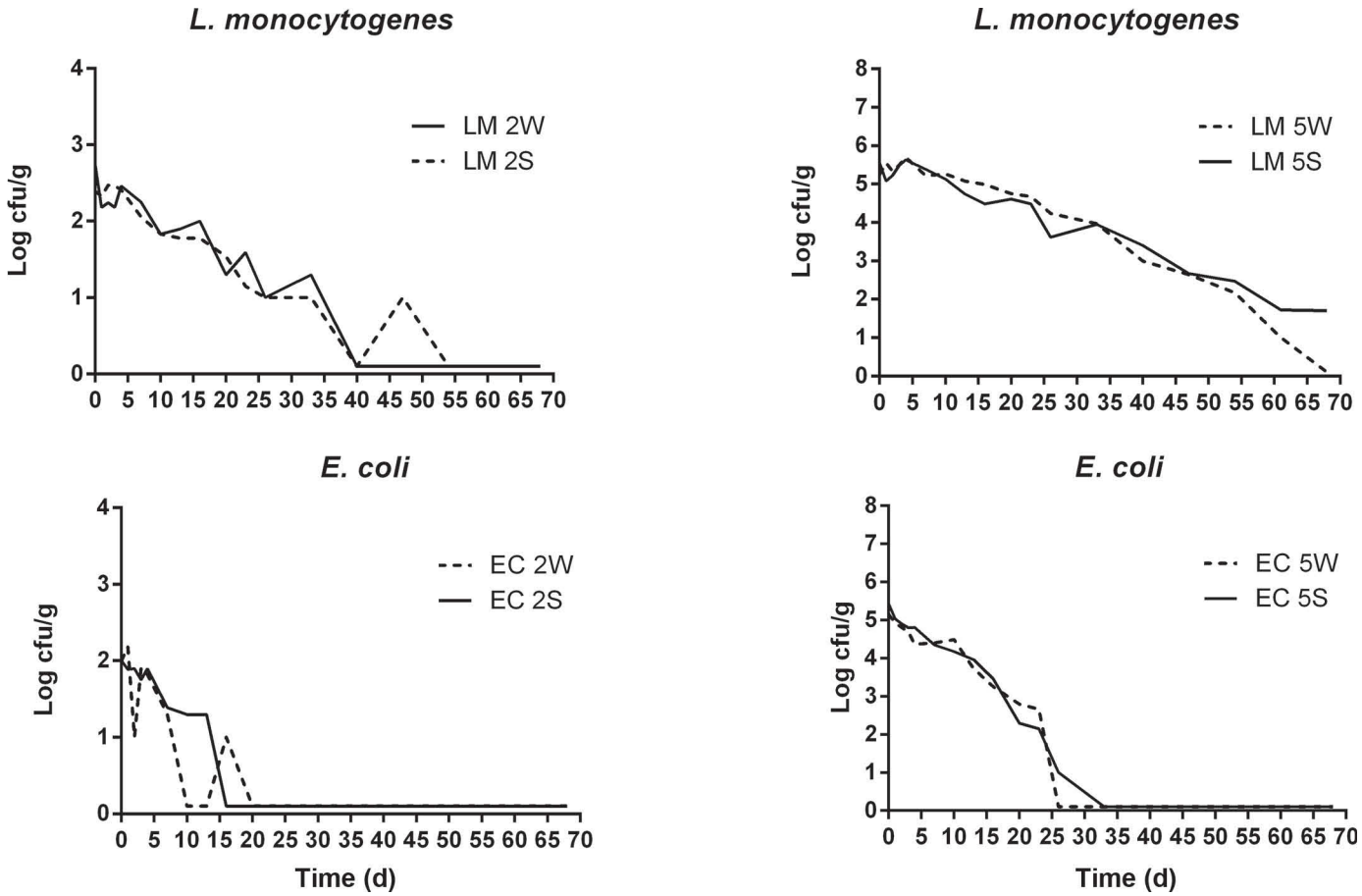

Figure 2. Trends of *Listeria monocytogenes* (LM) and *Escherichia coli* (EC) counts in unflavored and strawberry yogurt samples inoculated at 2 different concentrations. 2W = unflavored (white) yogurt inoculated at 2 log cfu/g; 2S = strawberry yogurt inoculated at 2 log cfu/g; 5W = unflavored (white) yogurt inoculated at 5 log cfu/g; 5S = strawberry yogurt inoculated at 5 log cfu/g.

Bachrouri et al. (2002) in pathogenic or non-*E. coli* strains. This could be most likely due to the different strains used in the challenge tests and in particular to the greater decrease in pH observed in that study (between 4 and 3.9), whereas in our samples a gradual increase was noticed. The pH values in samples inoculated at 2 log cfu/g ranged from 4.12 to 4.15 at d 0 and reaching 4.47 and 4.60 in strawberry and unflavored samples, respectively, at the end of the trial. A similar trend was found in samples inoculated at 5 log cfu/g, with values ranging from 4.08 to 4.21 at d 0 and final values of 4.60 and 4.54 in strawberry and unflavored samples, respectively. In any case, no statistically significant differences between unflavored and strawberry samples were observed. Our results about the presence of this microorganism (until d 47 in unflavored samples inoculated at 5 log cfu/g, but in strawberry samples only until d 26; Table 1) could be compared with those obtained by Canganella et al. (1998), who found the presence of *E. coli* after 24 d (in fruit yogurt inoculated at a moderate level between 2 and 3 log cfu/g) and after 28 d (in fruit yogurt inoculated at a high level between 4 and 6 log cfu/g) and maintained at 4°C. The greater resistance detected in our unflavored samples compared with strawberry samples could be due as already explained to the presence of potassium sorbate, even if very slight antimicrobial activity against this microorganism was revealed in previous studies (Zhao et al., 1993).

Considering the samples inoculated with *L. monocytogenes* at a concentration of 2 log cfu/g, a gradual decrease in the loads was observed with detectable counts until d 33 in both series (total reduction of 1.4 log cfu/g); afterward, the loads were below the detection limit (1 log cfu/g), with the only exception of strawberry samples at d 47 when a concentration of 1 log cfu/g was counted. However, *L. monocytogenes* was present in 25 g of product until the end of the trial (d 68; Table 1). For samples inoculated with *L. monocytogenes* at a concentration of 5 log cfu/g, a very moderate, gradual decrease was observed, with detectable counts until d 61 in unflavored samples and until the end of the trial in strawberry ones (total reduction pointed out of 4.3 and 3.9 log cfu/g, respectively); comparing the 2 types of products, strawberry samples showed significantly lower loads from d 61 until the end of the trial ($P < 0.05$).

The presence of live *L. monocytogenes* in both series for the whole period, independent of the starting concentration, was expected. As suggested by Gahan et al. (1996), the ability of *L. monocytogenes* to tolerate acid stress provides a useful model for the analysis of adaptive tolerance responses. This microorganism was recognized to exhibit an important adaptive acid tolerance response after exposure to mild acids and put in place mechanisms of defense in the presence of lethal acid pH (close to 3.5; Hill et al., 1995). The reduction rates observed in our study were in agreement with those obtained by Hsieh et al. (2010) who found reductions after 6 d of storage in retail yogurt products maintained at 4 to 7°C, between 0.7 and 1.8 log cfu/g if inoculated at a moderate dosage (4.5 log cfu/g); in our samples the reduction observed after the same storage period was between 0.8 and 1.1 if inoculated at 5 log cfu/g. According to Belessi et al. (2008), *Listeria innocua,* physiologically similar to *L. monocytogenes*, inoculated in yogurt (pH 4 to 4.3) with endogenous fungal flora, revealed a reduction of about 4 log cfu/g at 3°C in 22 d and of more than 5 log cfu/g at 5°C in 15 d. These data were inconsistent with our results, as the inactivation was evidently faster. As already reported for the samples inoculated with *E. coli*, this could be due to the different strains used in the challenge tests and after all to the higher decrease in pH observed in that study (reaching values between 4 and 4.1). In fact, a gradual increase was seen in our samples with values from 4.1 to 4.2 at d 0 to 4.5 to 4.6 in both series at the end of the trial.

Generally, members of the family *Enterobacteriaceae*, such as *E. coli*, can deal with low-pH stress in the natural nonhost environment as well as during the passage from the stomach to the intestine.

In any case, *L. monocytogenes*, as expected, generally resulted in significantly better resistance to acidic conditions present in the substrate of inoculum than *E. coli*, due to better adaptation to stressful conditions (gram-positive bacteria may encounter a wide range of low-pH environments), suggesting the possibility of an acquired increased persistence of this microorganism in this substrate. This aspect was deepened in the second inoculation trial.

Adaptation Test

Acid-adapted microorganisms (especially *Salmonella* spp., *E. coli*, and *L. monocytogenes*) are often recognized to have an increased survival in foods compared with nonadapted ones (defined also as wild). Moreover, *L. monocytogenes* cells, when are in adverse growth conditions such as strongly acidic environments, respond by increasing the synthesis of essential virulence factors; it was also demonstrated that the adaptation to hostile environments determined an increase of resistance to following stresses (O'Driscoll et al., 1996). In our study, we evaluated the survival of wild and adapted *L. monocytogenes* strains, inoculated at the same concentration around 4 log cfu/g; a gradual decrease in the loads was observed until d 28 in unflavored and strawberry

Table 2. Presence in 25 g of *Listeria monocytogenes* (LM) in wild or adapted in unflavored (white; W) and strawberry (S) yogurt

	Sampling time (day)										
Item[1]	0	7	14	21	28	35	42	49	56	63	70
LM wild											
W	+	+	+	+	+	+	+	+	−	−	−
W	+	+	+	+	+	+	+	+	−	−	−
S	+	+	+	+	+	+	+	+	+	−	−
S	+	+	+	+	+	+	+	+	+	+	+
LM adapted											
W	+	+	+	+	+	+	+	+	−	−	−
W	+	+	+	+	+	+	+	+	+	−	−
S	+	+	+	+	+	+	+	−	−	−	−
S	+	+	+	+	+	+	−	−	−	−	−

[1]Wild = strain not adapted; adapted = strain isolated from yogurt at T68 of the inoculation test.

samples inoculated with the wild strain (Figure 3). Afterward, the loads were below the detection limit (1 log cfu/g). A similar decrease was also observed in the samples inoculated with the adapted strain, with detectable counts until d 28 in unflavored samples and until d 21 in strawberry samples; afterward, the loads were below the detection limit. However, *L. monocytogenes* was present in the product until d 56 in adapted samples and until d 63 in wild ones (Table 2). Considering the whole period, no statistically significant differences were detected between the wild and adapted strains (Figure 3). This could be due to a rapid adaptation of *L. monocytogenes* at the environmental conditions characterized by low but not inactivating pH values (ranging between 4.1 and 4.3), allowing a better survival of the cells in the whole period considered. As reported by Uyttendaele et al. (2004), *L. monocytogenes* tested in different combined stress conditions and previously adapted to slight acidic environments, did not show a significantly shorter lag phase or higher specific growth rate compared with the wild strain, confirming a very rapid adaptation of the strains in a new slightly acidic substrate.

CONCLUSIONS

Yogurts stored at 4°C were very stable, whereas, under thermal abuse conditions, the growth of yeasts was the limiting factor in microbiological shelf life. Potassium sorbate in the strawberry yogurt had antimicrobial action and it is considered one of the main protective factors for fruit yogurts. The acidic environmental conditions provided by the products allowed the survival of *E. coli* and especially *L. monocytogenes* in samples at both starting inoculum concentrations. The strong acid resistance evidenced by *L. monocytogenes* resulted in the presence of live pathogens in both types of yogurt for the whole period considered, with rapid adaptation to a moderately acidic substrate. The environmental persistence of this pathogen is an important concern for artisanal dairy producers and should be considered in risk assessment, especially in small-scale facilities.

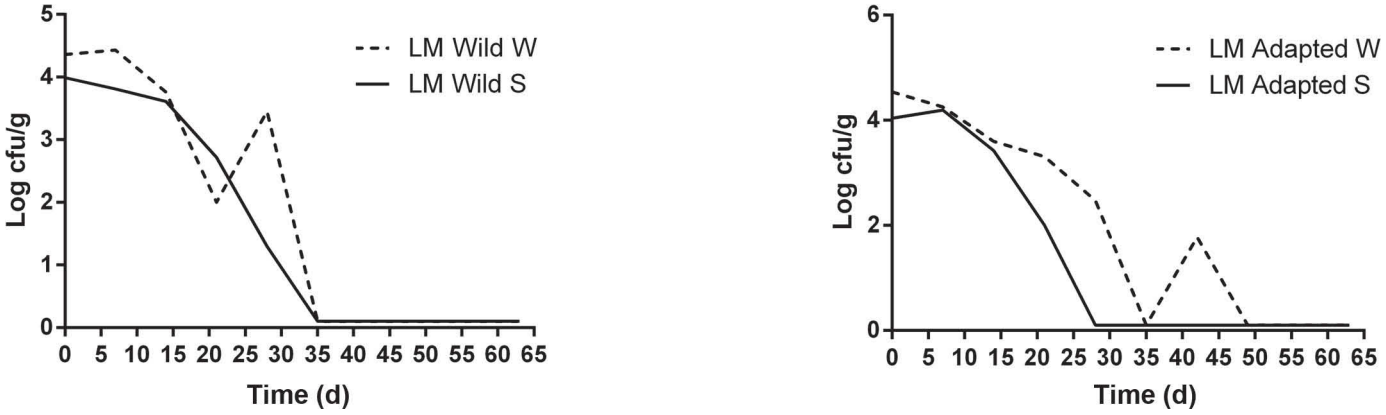

Figure 3. Counts of wild and adapted strains of *Listeria monocytogenes* (LM) inoculated in unflavored and strawberry yogurt samples. W = unflavored (white); S = strawberry.

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