Food Microbiology, 2001, **18**, 597–604 doi:10.1006/fmic.2001.0435
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ORIGINAL ARTICLE

Enterococci vs non-lactic acid microflora as hygiene indicators for sweetened yoghurt

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Coliforms are usually used as a measure of hygiene status in the processing and packaging of dairy products. However, their limited chances of survival have placed a question mark over this role in acid products. Some authors propose Enterococcus *as a group for hygienic condition inspections in process lines of fermented dairy products. The aims of this work were, first, to evaluate the viability of enterococcus and non-lactic acid microflora (coliforms,* Pseudomonas*,* Staphylococcus aureus *and yeasts) in whole set sweetened yoghurt during refrigerated storage and; second, to evaluate the occurrence of bacterial contaminants in industrial process lines of whole set sweetened yoghurt through a critical control points plan (CCP).*

Test strains were inoculated at a level of 5·5 log orders. Enterococcus remained viable for 21 days and was still detectable after up to 49 days of cold storage. The viability of Pseudomonas *was very poor (*D*-values lower than 0·69 days).* Klebsiella *spp.,* Escherichia coli *and* Citrobacter *spp. showed* D*-values of 1·61, 1·85 and 2·56 days, respectively, while two* S. aureus *strains showed* D*-values of 0·61 and 1·56 days.* Kluyveromyces marxianus *var.* lactis *strains tested in this study remained viable for at least 42 days.*

The in vitro *assays performed during this study demonstrated that enterococci could remain viable for a longer period than coliforms. However, from analysis of the industrial reality it became evident that in whole set yoghurt lines, coliforms are the most frequent contaminants. In addition, they can remain viable during the fermentation step and, in some cases, during cold storage of the product. Finally, coliform detection is cheaper and faster than enterococci counts. It can thus be concluded that enterococci have little value as hygiene indicators in the industrial processes of yoghurt. Consequently, coliforms are a suitable hygiene indicator as long as they are determined in the first days after manufacture.*

Introduction

In yoghurt production, the lactic acid produced by the starter bacteria generates a barrier effect against the proliferation of microbial contaminants. This fact, and the probable release of other antimicrobial metabolites such as bacteriocins, make yoghurt a product that is rarely implicated as an intoxication source or a disease vector. Actually, this is true only if lactic fermentation is carried out under optimal conditions. If little acidifying activity of the lactic starter is observed, spoilage or pathogen

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Received: 12 January 2001

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0740-0020/01/060597 +08 $35.00/0

micro-organisms (that are introduced into the industrial process because of poor hygiene conditions) can develop or remain viable in the product (Canganella et al. 1992).

Indicator micro-organisms are used to monitor hygiene conditions in production lines. Coliforms have been used as a measure of hygiene status in the processing and packaging of dairy products since they are killed by pasteurization. Hence, any coliform found in the product indicates contamination after pasteurization (White 1998). Application of coliform tests is not intended to detect faecal pollution but rather to measure the quality of the practices used to ensure proper processing and to minimize bacterial contamination of processed dairy products (Christen et al. 1992).

Because of its limited ability to survive in acidified products such as yoghurt, it would not seem safe to use the coliform group as a hygiene indicator for such products. Reliable information about post-production contaminants is therefore difficult to obtain. For this reason, enterococcus has been proposed for hygienic condition inspections in process lines of fermented products (Vanos 1991). The presence of enterococci in dairy products has long been considered an indication of inadequate sanitary conditions during the production and processing of milk (Giraffa et al. 1997). These bacteria are widely distributed in the environment, principally inhabiting the human and animal gastrointestinal tract, and are also found in plants, insects and soil (Deibel and Silliker 1963, Sneath 1994). Although they are not related to food intoxication, they are sometimes associated with abdominal and hospital infections because of their resistance to antibiotics (Deibel and Silliker 1963). *Enterococcus faecalis*, *Enterococcus feacium* and *Enterococcus bovis* were the main species isolated from contaminated fermented milks (Vanos 1991). In fermented dairy products the interpretation of their number will depend on each individual product and factory, according to local conditions. Thus, enterococci could be considered indicator organisms only in a broad sense (Giraffa et al. 1997). On the other hand, some authors (Gardiner et al. 1999) have proposed *E. faecium* as a potential probiotic organism.

The hazard analysis/critical control points (HACCP) system has rapidly gained regulatory status as a preventive strategy for managing hazards associated with foods. Risk assessment provides the linkage between HACCP criteria and a measure of the associated human health risks to help determine which hazards it is essential to control, reduce or eliminate. Also, it is possible to verify that CCP and assigned critical limits effectively result in risk reduction (Hathaway 1997, Lammerding 1997, van Schothorst 1997).

Taking into account that coliforms have traditionally been used to evaluate sanitary conditions in spite of their poor survival in acidified products and that enterococcus has also been suggested as a possible indicator of hygiene, the aims of this work were twofold. First, to evaluate the viability of enterococci and non-lactic acid microflora (coliforms, *Pseudomonas*, *Staphylococcus aureus* and yeasts) in yoghurts during refrigerated storage and, second, to evaluate the occurrence of bacterial contaminants in industrial process lines of yoghurt by means of a CCP plan.

Materials and Methods

Strains, starter and culture conditions

Enterococcus spp. (strains 47, 127 and 158) were cultured (37°C, 24 h) in M17 broth (Biokar, Beauvais, France); *S. aureus* (strains 6 and 50), *Escherichia coli* 26, *Klebsiella* spp. 58, *Citrobacter* spp. 21, *Pseudomonas* spp. 156, *Pseudomonas fluorescens* 259 and *Pseudomonas aureuginosa* 195 were cultured in Nutrient broth (Britania, Buenos Aires, Argentina) (24 h, 37°C) and *Kluyveromyces marxianus* var. *lactis* (strains M11 and M21) was cultured in Yeast extract broth (Britania, Buenos Aires, Argentina) (25°C, 24 h). All the strains belong to the Programa de Lactología Industrial collection.

For industrial yoghurt manufactures, a commercial lyophilized mixed culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (30/70), identified as IA, was used as direct vat starter (DVS). The starter was used according to the manufacturer's instructions: 500 ml of heat-treated milk (20 min –

90°C) were inoculated (2% v/v) and kept 1 hour at 30°C; then it was inoculated (2% v/v) in the fermentators (batch production) (37°C, 4 h).

Yoghurt samples

Yoghurt samples were provided by a factory near Santa Fe City (Argentina). Whole set sweetened yoghurt was manufactured with whole milk (3·2% v/v fat content), sucrose (13% w/v), skimmed powder milk, stabilizer, natural colour and flavour. The protein, carbohydrate and fat contents were 3% w/v, 16·5% w/v and 3% v/v, respectively. Yoghurts were aseptically packaged.

Microbial viability assays

Samples (cups of 200 g) were taken from ordinary production runs for microbial viability assays at the end of the production line. An overnight culture of each contaminant microorganism was used to inoculate the cups of whole set yoghurt. Initial numbers ranged from 10^5 to 10^6 cfu ml^{-1}. The containers were then hermetically sealed. All samples were stored at 6°C. Periodically, samples were taken for microbiological analysis, and for pH (pHmeter ORION SA 720) and acidity (percentage lactic acid, measured by titration with a N/9 NaOH solution upto pH 8·4) measurements. The experiments were carried out in triplicate.

Colony counts

Yoghurt samples were decimally diluted in sterile peptone water (0·1% w/v) and 0·1-ml aliquot dilutions plated over different media in duplicate. The following culture media and incubation conditions were used: KF Streptococcus agar (Merck, Darmstadt, Germany) for 48 h at 37°C (enterococcus); VRB agar (Merck) for 24 h at 30°C (*Escherichia coli*, *Klebsiella* and *Citrobacter*); nutrient agar (Merck) for 48 h at 37°C (*Pseudomonas* and *S. aureus*), YGC agar (Britania, Buenos Aires, Argentina) for 72 h at 25°C (moulds and yeasts) and Skim Milk agar (Plate Count Agar, Britania) added to 10% w/v of reconstituted 10% w/v Skim Milk, Merck) for 72 h at both 30°C (total mesophilic counts for milk samples; IDF 1991) and 37°C (total lactic acid bacteria counts and *Strep. thermophilus*/*L. delbrueckii* ssp. *bulgaricus* differential counts for yoghurt samples; Vinderola and Reinheimer 1999).

Critical control points in industrial manufacture of whole set yoghurt

On the basis of the flow chart for whole set sweetened yoghurt manufactures (Fig. 1), a hazard analysis chart (Aru 1993, Byrne and Bishop 1998) was carried out for the description of CCP (Table 1). In CCP, besides pH measurements, the following microbial counts were performed: lactic acid bacteria, enterococci, coliforms, and moulds and yeasts. Values for these parameters were determined for 10 industrial manufactures of whole set sweetened yogurt carried out on different days.

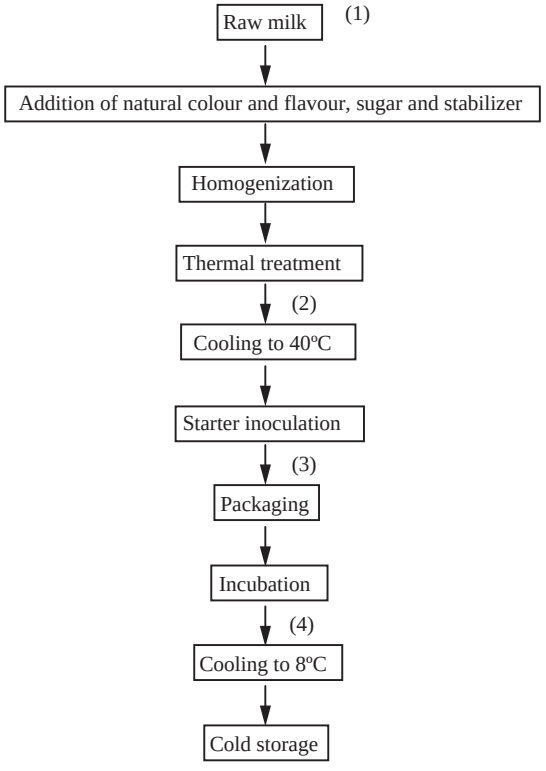

Figure 1. Flow chart for manufacture of whole set sweetened yoghurt. Critical control points are indicated (1)–(4).

Table 1. Hazard analysis critical control points (CCP) description for whole set sweetened yoghurt

CCP/process step	Hazard/concern	Control point	Critical limit
Raw milk receiving	Microbiological	Temperature	7°C or less
	Chemical drug residues	Acidity	$<0\cdot16$% lactic acid
		β-lactam screening	No positives
Thermal treatment	Microbiological	Temperature	95°C
		Time	20 min
Starter inoculation and filling	Microbiological	Asepsis	
		Proper concentrations	2% (v/v)
Incubation	Microbiological	Temperature	42°C
		Acidity	$0\cdot90$% lactic acid

Statistical analysis

Cell counts, pH and acidity values were compared statistically to detect significant differences using the test for homogeneity of variances (Miller et al. 1992) and Duncan and Student's tests (Miller and Miller 1993). *D*-values were determined at 6°C from linear regression of death kinetics using Origin 4.10 (Microcal Software, Inc., Massachusetts, USA).

Results

The viability of enterococcus strains in whole set sweetened yoghurt during refrigerated storage at 6°C is shown in Fig. 2. The initial numbers of enterococci remained almost constant during the first 20 days of cold storage, showing a clear fall after this period. In this sense, enterococcus strain 158 was the most sensitive strain. The other bacteria tested (coliforms, *Pseudomonas* spp. and *S. aureus*) were more sensitive to the acidic conditions of the yoghurt environment (Fig. 3). The initial number of coliforms (*E. coli* strain 26, *Klebsiella* strain 58 and *Citrobacter* strain 21) showed a minimum viability loss of six log orders in 9 days, as did *Staphylococcus* strain 6. Neither *Pseudomonas* strains nor *Staphylococcus* strain 50 were detected after 2–5 days. The decimal reduction time ($D_{6°C}$ values) for these organisms are shown in Table 2. The lowest $D_{6°C}$ values (ranging from 0·46 to 0·69 days) were obtained for *Pseudomonas* strains, while for *S. aureus* the values found depended on the strain considered

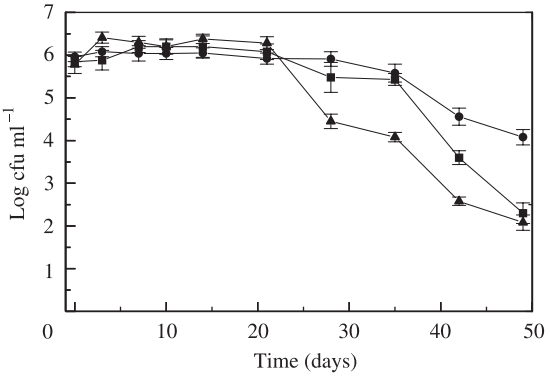

Figure 2. Mean (s.d.) cell viability of enterococcus spp., strains 47 (■), 127 (●), and 158 (▲), in whole set sweetened yoghurt during storage at 6°C. Experiment performed in triplicate.

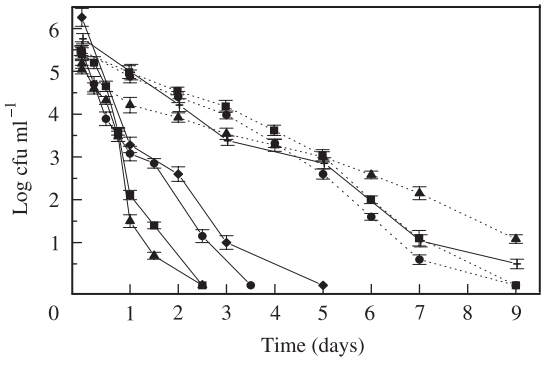

Figure 3. Mean (s.d.) cell viability of *Escherichia coli* strain 26 (■), *Klebsiella* strain 58 (●) and *Citrobacter* strain 21 (▲) (dotted lines), and *Pseudomonas* spp. strain 156 (■), *Pseudomonas fluorescens* strain 259 (●), *Pseudomonas aeruginosa* strain 195 (▲) and *Staphylococcus aureus* strains 50 (◆) and 6 (+) (solid lines), in whole set sweetened yoghurt during storage at 6°C. Experiment performed in triplicate.

(0·61 and 1·56 days). Finally, coliform bacteria showed $D_{6°C}$ values ranging from 1·61 to 2·56 days.

The viability of *Kluyveromyces marxianus* var. *lactis* during cold storage is shown in Fig. 4. During the first 25 days, *K. marxianus* var. *lactis* strains M11 and M21 maintained almost the initial numbers of viable cells. After this period, the cell concentration fell less than one log order until 42 days.

Table 2. Decimal reduction times ($D_{6°C}$ values) for coliforms, *Staphylococcus aureus* and *Pseudomonas* in whole set sweetened yoghurt stored at 6°C

Micro-organism	*D* (days)
Pseudomonas aeruginosa 195	0·46
Pseudomonas fluorescens 259	0·59
Pseudomonas sp. 156	0·69
S. aureus 50	0·61
S. aureus 6	1·56
Klebsiella 58	1·61
Escherichia coli 26	1·85
Citrobacter sp. 21	2·56

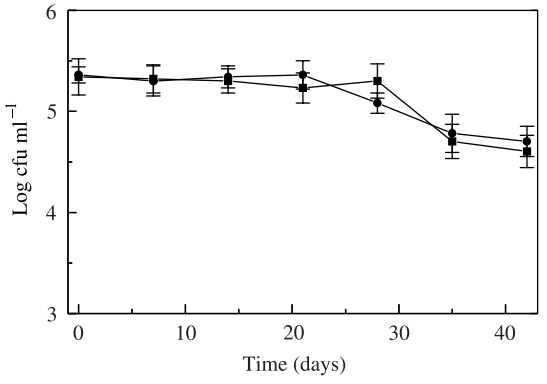

Figure 4. Mean (s.d.) cell viability of *Kluyveromyces marxianus* var. *lactis*, strains M11 (■) and M21 (●), in whole set sweetened yoghurt during storage at 6°C. Experiment performed in triplicate.

Table 3 shows changes in pH and microbial counts for nine typical industrial manufactures of whole set sweetened yoghurt. The mean (s.d.) initial pH and acidity of raw milk were 6·72 (0·11) and 0·18 (0·059)% lactic acid, respectively. Thermal treatment reduced the total bacterial counts (cfu ml^{-1}) of raw milk samples by approximately five log orders. After starter inoculation and fermentation, total lactic acid bacteria counts reached mean (s.d.) values of 5·21 (0·13) and 8·73 (0·32) log orders (cfu ml^{-1}), respectively. This last count value did not change significantly ($P<0·05$) for at least 20 days. The initial mean (s.d.) content of the starter lactic acid microflora was 8·46 (0·28) log orders (cfu ml^{-1}) for *L. delbrueckii* ssp. *bulgaricus* and 8·48 (0·32) log orders (cfu ml^{-1}) for *Strep. thermophilus*. After 49 days, whole set yoghurts kept at 6°C showed a viable cell diminution ranging from 2·2 to 3·8 log orders (cfu ml^{-1}) for *L. delbrueckii* ssp. *bulgaricus* and *c.* 1·2 log orders (cfu ml^{-1}) for *Strep. thermophilus* (data not shown). Coliforms, enterococci, yeasts and moulds were not detected in the milk after heat treatment, but a reduced contamination (mean 0·6 and 1·3 log orders (cfu ml^{-1}) after starter inoculation and fermentation, respectively) of coliform bacteria was detected in the cups. However, after 24 h of refrigerated storage, no viable coliforms were detected in the yoghurt samples. The mean (s.d.) pH (4·51 (0·08)) and acidity (0·92 (0·036)% lactic acid) values of yoghurt samples did not show significant ($P<0·05$) changes during cold storage.

Table 4 shows a particular case among the ten industrial runs studied. In this case,

Table 3. Mean (s.d.) pH and cell counts for nine industrial manufactures of whole set sweetened yoghurt

CCP	pH	Cell count (log orders cfu ml^{-1})				
		Total	Enterococci	Coliforms	Yeasts	Moulds
Raw milk	6·7 (0·1)	5·9 (0·3)	4·0 (0·3)	3·5 (0·2)	4·9 (0·4)	2·2 (0·3)
Heat treatment	6·6 (0·1)	1·1 (0·2)	n.d.	n.d.	n.d.	n.d
Starter inoculation/filling	6·5 (0·3)	5·2 (0·1)	n.d.	0·6 (0·3)	n.d.	n.d.
Fermentation	4·5 (0·1)	8·7 (0·3)	n.d.	1·3 (0·3)	n.d.	n.d.
Cold storage after 1 day	4·5 (0·3)	8·2 (0·4)	n.d.	n.d.	n.d.	n.d.

CCP, Critical control points plan. n.d., Not detected (<1 cfu ml^{-1}).

Table 4. pH and cell counts values for one industrial manufacture of whole set sweetened yoghurt

CCP	pH	Cell count (log orders cfu ml^{-1})				
		Total	Enterococci	Coliforms	Yeasts	Moulds
Raw milk	6·86	5·7	4·2	3·8	3·0	1·8
Heat treatment	6·69	1·2		n.d.	n.d.	n.d.
Starter inoculation/filling	6·67	3·7	n.d.	0·5	1·6	1·4
Fermentation	4·70	8·6	n.d.	1·4	n.d.	n.d.
Cold storage (days)						
1	4·68	8·5	n.d.	1·1	n.d.	n.d.
3	4·72	8·3	n.d.	1·2	n.d.	n.d.
5	4·71	8·5	n.d.	1·0	n.d.	n.d.
10	4·69	8·3	n.d.	1·1	n.d.	n.d.
14	4·66	8·0	n.d.	0·2	n.d.	n.d.
16	4·67	8·3	n.d.	n.d.	n.d.	n.d.
18	4·62	8·1	n.d.	n.d.	n.d.	n.d.

CCP, Critical control points plan. n.d., Not detected (<1 cfu ml^{-1}).

coliforms, moulds and yeasts were detected in the cups (after starter inoculation) at a level ranging from 0·5 to 1·6 log orders (cfu ml^{-1}). The coliform counts remained almost constant up to day 10, but were no longer detected at day 16.

Discussion

Coliform bacteria were traditionally suggested as hygiene indicators for fermented milks (Christen et al. 1992, Abd El Ghani et al. 1998). However, this role might become less important because of the high sensitivity to the acidic environment in yoghurt. Thus, the detection of coliforms in acidified products may not offer information about the presence of other post-processing contaminants. Vanos (1991) has therefore postulated that enterococci are a more reliable index of hygiene since they can survive in adverse environmental conditions (Mosel and Moreno García 1985, Giraffa et al. 1997).

In this study, although the enterococcus strains used to contaminate the yoghurt samples did not increase their numbers at 6°C, they remained constant for 21 days. After this period, the population experienced a significant loss in cell viability but cells were still detectable at 49 days of cold storage. These results showed good cell viability for enterococci at low pH values, although cell growth at pH values near 4, as had been previously reported (Deibel and Silliker 1963), was not observed.

Some authors have reported that the survival of enterobacteria in yoghurt was very limited. The survival ability of *Salmonella typhimurium* in cold stored yoghurt was not higher than 3–6 days, while *E. coli* survived for 10 days (Marth 1985). According to another study (Vanos 1991) *Enterobacter aerogenes* and *E. coli* survived for only 4 days at 7°C when added to yoghurt. Marth (1985) reported that *S. typhimurium* was completely inhibited by mixed cultures of *Strep. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. A similar result was observed for pathogenic strains of *E. coli* when inoculated together with a lactic acid starter culture, while coliform bacteria isolated from dairy products survived for up to 14 days at 5°C depending on initial counts (Sadovski et al. 1980). These authors also stated that enterobacteria can survive better when they are added during the fermentation process than when the contamination occurs after it, and that their survival is related to the magnitude of the initial contamination and storage temperature. In this work, initial numbers of 5·5 log orders (cfu ml^{-1}) of *E. coli* and *Klebsiella* spp. remained viable for only 7 days in whole set sweetened yoghurt at 6°C (*D*-values of 1·85 and 1·61 days, respectively), while *Citrobacter* spp. survived for 9 days (*D*-value of 2·56 days) after inoculation into the product.

The viability of *S. aureus* in dairy products has previously been reported (Marth 1985) as

being very limited (ranging from 2 to 4 days) when inoculated at levels of approximately five log orders (cfu ml^{-1}). In this work, *S. aureus* was detected until days 3 and 7 of cold storage (*D*-values of 1·56 and 0·6 days), depending on the strain.

The most common micro-organisms isolated from refrigerated dairy products (e.g. *Pseudomonas*, *Alcaligenes*, *Achromobacter* and *Flavobacterium*) could not be detected in cold stored yoghurts (Sadovski et al. 1980). These authors reported that *Pseudomonas* had very low survival in the acidic conditions of the samples as they were no longer detected after 36–60 h of storage depending on the strain. These micro-organisms only tolerate environments with pH values ranging from 5·6 to 8 (Brown and Corlett 1980).

Yeasts are the most important spoilage micro-organisms in yoghurt because they can develop in the acidic conditions of the product (Rasic and Kurmann 1978). They are the most acid-tolerant organisms found in foods (Baird Parker 1980) and may cause organoleptic changes and packaging deformations because of gas formation. The minimum quantity of lactic acid necessary for yeast inhibition is not found in yoghurts obtained in a normal fermentative process (Brown and Corlett 1980). In studies on the survival of undesirable micro-organisms in yoghurts, yeasts were found to be viable for upto 32 days of refrigerated storage (Canganella et al. 1992). *Kluyveromyces marxianus* var. *lactis* strains tested in this work remained (initial counts of 5·3 log orders) viable in whole sweetened set yoghurt for at least 42 days.

In this work, enterococci and non-lactic acid microflora (coliforms, moulds and yeasts) were monitored in 10 industrial manufacturers of whole set sweetened yoghurt by means of a CCP plan. It was found that the thermal treatment of milk killed these microbial groups completely. Hence, the presence of coliforms indicates post-processing contamination, because these organisms are unable to survive the heat treatments applied during yoghurt manufacture (Abd El Ghani et al. 1998). This step is the only one at which pH is not yet a limiting factor for the growth of eventual microbial contaminations (Vanos 1991, ICMSF 1991). For yoghurt set processes, pot filling is the following CCP since, after the starter inoculation, milk is pumped through pipelines upto the filler. In all cases coliform bacteria were detected, but in one process run they were accompanied by moulds and yeasts (both at low numbers). Enterococci were never detected. Only the coliforms were capable of surviving the fermentation step, slightly increasing in number. However, 24 h later no coliforms were detected in the final product for nine industrial manufactures. In only one of 10 cases did the coliform bacteria remain viable for 15 days of cold storage, though at low numbers (approximately one log order).

The *in vitro* assays performed in this study demonstrated that enterococci could remain viable for more than 40 days in whole set sweetened yoghurt, while coliforms showed a faster loss of viability. Contrary to this, Abd El Ghani et al. (1998) found a higher cell viability of coliforms than enterococci in experimental yoghurts during refrigerated storage for 15 days. However, from the analysis of the industrial reality it was seen that in whole set yoghurt lines, coliforms are the most frequent contaminants. In addition, they can remain viable during the fermentation step and, in some cases, throughout cold storage of the product. Finally, coliform detection is cheaper and faster than enterococci counts and so coliforms are suitable hygiene indicators as long as they are determined in the first days after production. It can be concluded that enterococci have little value as hygiene indicators in the industrial processes of sweetened yoghurt.

Acknowledgements

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica of Argentina (Project PICT 97 N° 09-00000-00747).

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