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A combination of Direct Viable Count and Fluorescence *in situ* Hybridization (DVC-FISH) for Specific Enumeration of Viable *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus.*

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DVC-FISH to detect viable LAB

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Abstract

Aims: We have developed a DVC-FISH procedure for quickly and easily discriminating between viable and non viable cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains, the traditional yogurt bacteria.

Methods and Results: Direct Viable Count method (DVC) has been modified and adapted for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* analysis by testing different times of incubation and concentrations of DNA-gyrase inhibitors. DVC procedure has been combined with fluorescent *in situ* hybridization (FISH) for the specific detection of viable cells of both bacteria with specific rRNA oligonucleotide probes (DVC-FISH). Out of the four antibiotics tested (novobiocin, nalidixic acid, pipemidic acid and ciprofloxacin), novobiocin was the most effective for DVC method and the optimum incubation time was 7 hours for both bacteria. The number of viable cells was obtained by enumeration of specific hybridizated cells which were elongated at least twice their original length for *Lactobacillus* and twice their original size for *Streptococcus*.

Conclusions: This technique was successfully applied to detect viable cells in inoculated feces.

Significance and Impact of the Study: Results showed that this DVC-FISH procedure is a quick and culture-independent useful method to specifically detect viable *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in different samples, being applied for the first time to Lactic Acid Bacteria (LAB).

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus*, FISH, Direct Viable Count, rapid method.

Abbreviation key: FISH = fluorescent *in situ* hybridization, DVC = direct viable count, LAB = Lactic Acid Bacteria

Introduction

Probiotics have been defined as living organisms that, when included as a part of the diet, confer some favourable effect on the host (Fuller 1991). Fermented milks have the property of containing these live bacteria mainly belonging to *Lactobacillus* and *Bifidobacterium* genera. It has been suggested that, in order to exert an action as probiotics, the lactic acid bacteria (LAB) should arrive alive and in a certain number to the intestine. This way, it is possible to appreciate their effects and to reach adhesion, implantation or multiplication in the intestinal tract (Bouhnik 1993).

Plate counting is the classical method for the determination of the LAB viability. However, it has several disadvantages such as being time-consuming and the underestimation of the number of viable bacteria due to the irregular distribution of microorganisms in the sample and the loss of culturability under stress conditions.

Therefore, there is a clear need for culture-independent methods to quantify viable LAB in microbial communities like fecal samples (Del Campo *et al.* 2005). Ribosomal RNA probe hybridization (FISH) has become widely adopted for detection and enumeration of specific bacterial groups in mixed populations (Amann *et al.* 1995). This approach has been successfully used to quantify *Lactococcus* and *Leuconostoc* in fermented foods (Ampe *et al.* 1999), *Lactococcus, Enterococcus* and *Streptococcus* in milk and LAB in fecal samples after ingestion (Ouwehand *et al.* 2004). The rRNA content varies among cell types depending on growth rate and physiological state (Moter and Göbel, 2000) and some authors suggest that it is not enough to determine the viability of a cell (Uyttendaele *et al.* 1997; Keer and Birch, 2003). DVC method discriminates among viable and non-viable cells by direct microscopy (Kogure *et al.* 1979). Viable bacteria incubated in presence of nutrients and a gyrase inhibitor (antibiotic) are able to elongate and increase their cellular metabolic activity being their division inhibited by the presence of antibiotic. Viable cells can then be easily discriminated from nonviable cells, due to differences in their respective sizes. The combination of DVC

incubation which increases intracellular rRNA levels, with FISH performed on rRNA-targeted sequences could prove useful in detecting and identifying viable cells in mixed microbial communities (Kalmbach *et al*. 1997). A DVC-FISH combination has been applied to detect *Escherichia coli* in water (Armisen and Servais, 2004) and other pathogens such as *Helicobacter pylori* in water (Piqueres *et al*. 2006).

Lactobacillus delbruecki subsp. *bulgaricus* and *Streptococcus thermophilus* are used as starters in the manufacture of fermented dairy products, such as yogurt. The detection on feces of viable LAB from the ingestion of food products has allowed to demonstrate on several occasions the survival of these bacteria to the gastrointestinal tract (Brigidi *et al.* 2003; Collado *et al.* 2006). The aim of this study was to develop a rapid DVC-FISH procedure for the *in situ* specific enumeration of viable LAB cells from yogurt (*L. delbruecki* subsp. *bulgaricus* and *S. thermophilus*) in fecal samples. Several antibiotics with different combinations of concentrations and incubation times were tested to establish the optimal conditions to elongate the cells of lactobacilli or to increase the diameter of streptococci. 23S rRNA probes were designed to hybridize with *Lactobacillus delbruecki* subsp. *bulgaricus* and *Streptococcus thermophilus* in FISH analysis and the specificity of these probes were tested. This improved procedure could be an effective culture-independent method to determine the presence of viable LAB in fecal samples in future survival studies after yogurt ingestion.

Materials and Methods

Bacterial strains and Culture conditions

L. delbrueckii subsp. *bulgaricus* and *S. thermophilus* strains (CECT 4005T and CECT 801) were used for all the assays. Bacteria were grown in MRS agar (Difco Laboratories, Detroit, USA) under anaerobiosis conditions **(**Anaerogen Compact system**;** Oxoid AN010C). A 48 h plate culture of these bacteria was inoculated into two flasks containing MRS broth under

anaerobiosis conditions at 37ºC. After 48 h of incubation, the culture was tested for DVC incubation.

DVC-assays

For each bacterium, 1 ml aliquots from the broth culture were added to twenty different flasks containing 25 ml of MRS broth (Difco Laboratories, Detroit, USA) with different concentrations of one of the antimicrobial agents shown in Table 2. The inhibitors of DNA gyrase nalidix acid, novobiocin, ciprofloxacin and pipemidic acid (Sigma Chemical Co., St. Louis, Mo) were tested for the DVC assay at different concentrations (0.5, 1 , 5, 10, and 100 μg ml^{-1}). The antibiotic stock solutions were appropriately diluted in distilled water and added to 25 ml of MRS broth flasks. A flask without antibiotic was also inoculated and used as a control. Flasks were incubated at 37ºC under microaerobic conditions for 24 h. All assays were performed in duplicate.

An aliquot of each flask was sampled immediately after inoculation and after 3, 7, 9 and 24 h of incubation and fixed for FISH and Scanning Electron Microscopy (SEM) analysis.

Probes

FISH analysis for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* was performed with two specific and complementary 23S rRNA oligonucleotide probes (LDE23 probe: 5′-GCGTGTTCCRTCCTTAAGC-3′ and STH23 probe 5′-CATGCCTTCGCTTACGCT-3′) designed in this study. Probes sequences were confirmed to match exclusively with *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* by the gapped Probe Match at RDP II (Michigan State University) and by a BLAST (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/blast/]) search. LDE23 and STH23 probe specificity was evaluated by whole-cell hybridization with different species of *Lactobacillus, Streptococcus*

and *Enterococcus* (Table 1). Probes were synthesized and labelled by Tib Molbiol (Berlin, Germany) with CY3.

FISH analysis

Samples were fixed with paraformaldehyde (PFA) 4% as described by Amann *et al*. (1995) and subsequently hybridized with 20% formamide at 46ºC for 2 h with LDE23 and STH23 rRNA probes as previously described by Moreno *et al*. (2003).

Hybridized samples were examined with an Olympus microscope BX50 equipped with a 100W mercury high-pressure bulb and set filters U-MWB, U-MWIB and U-MWIG. Colour micrographs were taken with an Olympus DP 10 digital camera (Olympus Optical Co., Hamburg, Germany).

In accordance with other authors (Kogure *et al*. 1979; Servis, *et al*. 1995; Moter and Göbel, 2000; Regnault *et al*. 2000; Moreno *et al*. 2011), cells were estimated as viable when their elongation was at least twice their original length for lactobacilli and at least twice their original diameter for streptococci.

Viability of bacteria was also tested by spreading 100 µl of different dilutions from each MRS flask sample on MRS agar plates which were incubated under anaerobic conditions at 37ºC for 48 h. Counts were carried out in duplicate.

Scanning electron microscopy (SEM)

For SEM, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* cells were fixed by adding 0.1 M sodium phosphate buffer (PBS) (pH 7.2), containing 2.5% glutaraldehyde (Sigma Chemical Co.) and 2% osmium tetroxide (Sigma) at 4ºC for 8 h. The fixed cells were washed in 0.1 M PBS. This suspension was carefully dispensed onto the surface of 25 mm (pore size: 0.2 µm) Poretics polycarbonate membranes (Sigma) and immersed in a liquid nitrogen bath.

Membranes were coated with gold and examined by using a JEOL JSM-5410 (JEOL, Ltd., Tokyo, Japan) scanning electron microscopy operating at 20 kV.

Detection of viable *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in inoculated feces samples

A total of five fecal samples were taken from people who had not eaten yogurt or other fermented products and had not been given antibiotics. Samples diluted (1:10) in PBS 1x (130 mM sodium chloride, 10 mM sodium phosphate (pH 7.2)) were used to inoculate *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* at different concentrations (ranging from 10^2 to 10^7 cfu g^{-1}) in order to evaluate the detection limit. They were processed using the *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* DVC-FISH method. A volume of 1 ml of each inoculated sample was introduced in different flasks containing 25 ml of MRS broth (Difco Laboratories, Detroit, USA) supplemented with 10 μg ml^{-1} novobiocine and processed as described above for the DVC-FISH detection. Two additional flasks were also inoculated with MRS broth not containing any antibiotics as a negative control. All flasks were incubated at 37ºC in anaerobiosis for 7 h. After that period, 1 ml was taken from each flask for analysis by FISH and two aliquots of 100 μl were also plated in MRS and M17 to detect *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* by culturable methods. MRS and M17 plates were incubated for 48 h at 37ºC and 42ºC, respectively.

Results

DVC-FISH assays

STH23 and LDE23 rRNA-targeted oligonucleotide probes were designed. In order to determine the hybridization specificity of the probes, other reference strains were tested with these probes and no hybridization occurred (Table 1).

179 In the optimal conditions for the DVC procedure, viable bacteria continue to metabolize
180 nutrients and they become elongated or bigger in size (in the case of the morphology of
181 cocaceas) but do not replicate. As the optimal bacterial response (antibiotic and its
182 concentration and incubation times) depends on the species of bacteria, effectiveness and
183 optimal concentrations were tested for each antibiotic: ciprofloxacin, nalidix acid, novobiocin
184 and pipemidic acid (Tables 2 and 3). As the optimal incubation time for cell elongation
185 depends on their growth rate (Barcina *et al.* 1995), different incubation periods were tested for
186 each antibiotic concentration.
187 For both bacteria, when pipemidic acid, ciprofloxacin and nalidix acid were used for the DVC
188 technique, no important increase in cell length or size was observed for any of the
189 concentrations tested. Therefore, this procedure was ruled out.
190 For *L. delbrueckii* subsp. *bulgaricus,* the maximum cell elongation was obtained after
191 incubation with novobiocin at a concentration of 10 μg ml^{-1} for 7 h and 24 h (Table 2). These
192 conditions made possible to differentiate between live elongated cells and dead cells after
193 probe hybridization. For *S. thermophilus,* the maximum diameter of cells was also obtained
194 after incubation with novobiocin at a concentration of 10 μg ml^{-1} for 7 h (Table 3). Lactobacilli
195 and streptococci were considered as viable when their length and their diameter were at least
196 twice the original ones, respectively (Figure 1). Replicate experiments yielded similar results
197 for every antibiotic and concentration tested.
198 Scanning electron microscopic observation of *L. delbrueckii* subsp. *bulgaricus* cell suspensions
199 after DVC treatment revealed changes in cell length from 9 μm before treatment to 59 μm after
200 treatment (Figure 2). In the case of *S. thermophilus,* cell diameter increased from 0.8 μm before
201 treatment to 2.3 μm after treatment (Figure 3).
202
203 **Determination of viable *L. delbrue*ckii subsp. *bulgaricus* and *Streptococcus thermophilus***
204 **on inoculated fecal samples**

In order to assess the suitability of the developed DVC-FISH protocol, it was applied in inoculated feces and results were compared with those obtained by traditional detection methods.

None of the culture media used (MRS and M17) was found to be selective enough for detection and quantification of the studied bacteria due to the massive growth of fecal background microbiota.

When the effectiveness of the developed DVC-FISH technique was tested in fecal samples inoculated with yogurt bacteria using novobiocine as antibiotic, the elongation and the increase in size of viable *L. delbrue*ckii subsp. *bulgaricus* and *S. thermophilus,* respectively, were similar to the values obtained in the *in vitro* assay with pure culture (Figure 4).

The detection limit of the DVC-FISH method was also evaluated for the five trials of artificially inoculated feces. For this purpose, the observation of at least one viable cell from the two bacteria inoculated in the prepared dilutions was required. In the five replicates, analysis yielded a detection limit for the DVC-FISH technique of 10^3 cells.g^{-1} of material tested of viable *L. delbrue*ckii subsp. *bulgaricus* and *S. thermophilus* (Figure 5), considering as viable cells those elongating or increasing at least twice their original size or diameter, respectively.

Discussion

Villarino *et al*. (2000) considered death as an irreversible state where no growth, cell elongation or protein synthesis may occur. DVC-FISH, which requires active or reactivable cellular machinery would be adequate for monitoring bacterial viability. In this study, four antibiotics at different concentrations were evaluated for the optimization of the DVC procedure, according to previous reports (Jous and LeBaron 1997; Guyard *et al.* 1999; Besnard *et al.* 2000; Regnault *et al.* 2000; Moreno *et al*. 2007).

For the DVC procedure, bacteria must be incubated in a broth media with an optimal concentration of antibiotics which inhibits cell replication but allows other synthetic pathways to continue (Buchrieser and Kaspar 1993).

For *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus,* the maximum cell elongation or increase in diameter was obtained after incubation with novobiocin at a concentration of 10 $\mu g.ml^{-1}$ and after 7 h and 24 h. The intensity of the fluorescent signal of the hybridized cells after the DVC treatment was stronger than before the treatment for both bacteria. This effect could be due to an increase of intracellular rRNA levels, which has been verified by others authors (Baudart *et al.* 2002).

No reduction in the number of bacteria after 7 and 24 hours of incubation with novobiocine was detected. It could be concluded that a 7 h incubation period with 10 $\mu g\ ml^{-1}$ of this antibiotic is the most suitable when applying DVC technique for *L. delbrue*ckii subsp. *bulgaricus* and *S. thermophilus*. These results are in line with other authors that described the effectiveness of novobiocin against Gram-positive bacteria (Buchrieser and Kaspar, 1993; Regnault *et al*. 2000; Bersnard *et al.* 2000).

Colony counts in plating media has traditionally been the method for enumerating viable cells. However, sometimes microorganisms may be viable but not able to form colonies on a synthetic medium.

Plate culture media used was not an effective method for detecting *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in artificially inoculated feces due to the huge growth in background microbiota, which masked the potential growth of these bacteria when it happened. In other feasibility studies comparing both methods, the total number of live cells by DVC-FISH was one-fold higher than by the plating method (Auty *et al*. 2001; Piqueres *et al*. 2006).

The presence of endogenous *Lactobacillus* and *Streptococcus* in the gastrointestinal tract and in human feces makes difficult to differentiate them from the ones ingested. Therefore, it is hard to unequivocally demonstrate the survival of ingested lactobacilli and streptococci through the

256 gastrointestinal tract using traditional methods such as culture media. To improve the
257 efficiency of detection and enumeration methods, specific rRNA probe hybridization without
258 culturing has become widely adopted for detection of bacterial groups in fecal samples
259 (Marteau *et al.* 2001). Therefore, probes in this study were designed to be specific for *L.*
260 *delbrueckii* subsp. *bulgaricus* and *S. thermophilus,* avoiding this way the hybridization with
261 other bacteria present in feces.
262 It has been reported that the sensitivity of the FISH technique can be affected by some
263 environmental factors, such as contamination (Bouvier and del Giorgio 2003). However, in the
264 present work, background fluorescent signals due to non-specific probe attachment did not
265 interfere with the FISH specific signal.
266 Some hybridizations were performed in feces without inoculation of *L. delbrueckii* subsp.
267 *bulgaricus* and *S. thermophilus*, checking that no non-specific hybridization occurred with the
268 fecal microbiota. It was found that the DVC-FISH method for detecting viable cells of *L.*
269 *delbrueckii* subsp. *bulgaricus* and *S. thermophilus* was valid for its use in this complex matrix.
270 Background noise did not interfere in its detection and it was possible to differentiate between
271 viable and nonviable bacteria.
272 Some studies have used the DVC-FISH method for the detection of viable cells of *Escherichia*
273 *coli* (Villarino *et al*. 2000), for direct enumeration of viable cells from the family
274 *Enterobacteriaceae* in freshwater and drinking water after membrane filtration (Baudart *et al*.
275 2002) and for the detection of viable cells of pathogens such as *Helicobacter pylori* in complex
276 matrices (Piqueres *et al*. 2006). Until now, there have not been viability studies of LAB using
277 the DVC-FISH method. DVC-FISH arises as a novel, rapid and suitable method for the
278 enumeration of total traditional yogurt LAB in feces, although it is certain that it is easier to
279 discriminate between viable and non viable cells by the length in the case of lactobacilli, as the
280 measure of the diameter of the streptococci is more complex. Moreover, this technique
281 provides a reduction in the detection time compared to other detection techniques such as

plating in selective culture media and can be applied the same day as the samples are collected. Furthermore, it gives more information about the physiological state of bacteria. Therefore, DVC-FISH combination can be considered as a useful tool for rapid and specific *in vitro* detection of the presence of viable *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.

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410 **Table 1** Strains used to test the probe specificity.

Species or subspecies	Strain	Hybridization with LDE23	Hybridization with STH23
L. delbrueckii subsp. *bulgaricus*	CECT 4005T	+	-
L.delbrueckii subsp. *delbrueckii*	CECT 286	-	-
*L.*paracasei	CECT 4022	-	-
L.brevis	CECT 4121	-	-
L.acidophilus	CECT 903	-	-
L.casei	CECT 475	-	-
L.rhamnosus	CECT 278	-	-
L.salivarius	CECT 4063	-	-
S. thermophilus	CECT 986	-	+
S. intermedius	CECT 803	-	-
Enterococcus faecalis	CECT 407	-	-
Enterococcus faecium	CECT 4102	-	-

411 CECT: Colección Española de Cultivos Tipo, Spain.

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415 **Table 2** Length of *L. delbrueckii* subsp. *bulgaricus* using several antibiotics, concentrations and after different

416 times of DVC treatment.

417

Antibiotic	Conc. μg ml^{-1}	Length (μm) 0h		Length (μm) 3h		Length (μm) 7h		Length (μm) 24h	
		min	max	min	max	min	max	min	max
Nalidix acid	0.5	4	9	4	9	4	9	4	9
	1	4	9	4	9	4	9	4	9
	5	4	9	4	9	4	9	4	9
	10	4	9	4	9	4	9	4	9
	100	4	9	4	9	4	9	4	9
Novobiocin	0.5	4	9	4	9	4	9	4	9
	1	4	9	4	9	4	9	4	9
	5	4	9	4	9	4	23	4	22
	10	4	9	4	9	4	**59**	4	**54**
	100	4	9	4	9	4	**50**	4	**52**
Ciprofloxacin	0.5	4	9	4	9	4	9	4	9
	1	4	9	4	9	4	9	4	9
	5	4	9	4	9	4	9	4	9
	10	4	9	4	9	4	12	4	9
	100	4	9	4	9	4	29	4	15
Pipemidic acid	0.5	4	9	4	9	4	9	4	9
	1	4	9	4	9	4	9	4	9
	5	4	9	4	9	4	9	4	9
	10	4	9	4	9	4	9	4	9
	100	4	9	4	9	4	9	4	9

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Table 3 Diameter of *S. thermophilus* using several antibiotics, concentrations and after different times of DVC treatment.

Antibiotic	Conc. µg ml^{-1}	Length (µm) 0h		Length (µm) 3h		Length (µm) 7h		Length (µm) 24h	
		min	**max**	**min**	**max**	**min**	**max**	**min**	**max**
Nalidix acid	0.5	0.8	1	0.8	1	0.8	1	0.8	1
	1	0.8	1	0.8	1	0.8	1	0.8	1
	5	0.8	1	0.8	1	0.8	1	0.8	1
	10	0.8	1	0.8	1	0.8	1	0.8	1
	100	0.8	1	0.8	1	0.8	1	0.8	1
Novobiocin	0.5	0.8	1	0.8	1	0.8	1	0.8	1
	1	0.8	1	0.8	1	0.8	1	0.8	1
	5	0.8	1	0.8	1	0.8	1	0.8	1
	10	0.8	1	0.8	1	0.8	**2.3**	0.8	**2.2**
	100	0.8	1	0.8	**2.2**	0.8	**2.2**	0.8	**2.2**
Ciprofloxacin	0.5	0.8	1	0.8	1	0.8	1	0.8	1
	1	0.8	1	0.8	1	0.8	1	0.8	1
	5	0.8	1	0.8	1	0.8	1	0.8	1
	10	0.8	1	0.8	1	0.8	1	0.8	1
	100	0.8	1	0.8	1.2	0.8	1.2	0.8	1
Pipemidic acid	0.5	0.8	1	0.8	1	0.8	1	0.8	1
	1	0.8	1	0.8	1	0.8	1	0.8	1
	5	0.8	1	0.8	1	0.8	1	0.8	1
	10	0.8	1	0.8	1	0.8	1	0.8	1
	100	0.8	1	0.8	1	0.8	1	0.8	1

Legend to Figures

Figure 1. FISH micrographs showing hybridization of live and dead cells of *L. delbrueckii* subsp. *bulgaricus* (A) and *S. thermophilus* (B) after 7 h incubation with novobiocin (10 µg ml^{-1}) by application of probe LDE23 and STH23 respectively.

Figure 2. Electron microscopic image of *L. delbrueckii* subsp. *bulgaricus* cells after 7h incubation with novobiocin (10 µg ml^{-1}) (bar, 20 µm).

Figure 3. Electron microscopic image of *S. thermophilus* cells after 7h incubation with novobiocin (10 µg ml^{-1}) (bar, 10 µm).

433 Figure 4. Detection by FISH of live and dead cells of *L. delbrueckii* subsp. *bulgaricus* (A)
434 and *S. thermophilus* (B) in artificially inoculated feces after 7 h incubation with novobiocin
435 (10 μg ml^{-1}).
436 Figure 5. Detection of viable cells of *L. delbrueckii* subsp. *bulgaricus* (A) and *S. thermophilus*
437 (B) by DVC-FISH in artificially inoculated feces at a concentration of 10^3 cells g^{-1}.
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