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Fluorescence *in situ* hybridization method using a peptide nucleic acid probe for identification of *Lactobacillus* spp. in milk samples

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ABSTRACT

Lactobacillus species constitute one of the dominant and beneficial bacteria in our body and are used in developed countries as a microbial adjuvant. Identification of these probiotic bacteria is traditionally performed by culture-based techniques. However, such methods are very time-consuming and can give inaccurate results, especially when *Lactobacillus* is present in mixed bacterial complex communities. Our study aimed to accurately identify *Lactobacillus* spp. using a novel Peptide Nucleic Acid (PNA) Fluorescence *In Situ* Hybridization (FISH) probe. The probe (Lac663) was tested on 36 strains belonging to different *Lactobacillus* species and on 20 strains of other bacterial species. The sensitivity and specificity of the method were 100% (95% confidence interval (CI), 88.0 to 100.0%) and 95.0% (95% CI, 73.1 to 99.7%), respectively. Additionally, we tested the applicability of the method on milk samples added with *Lactobacillus* strains at probiotic range concentrations and other taxonomically related bacteria, as well as pathogenic bacteria. The Lac663 probe bound exclusively to *Lactobacillus* strains and the described PNA-FISH method was capable of directly quantifying *Lactobacillus* spp. in concentrations at which these potential probiotic bacteria are considered to have an effective benefit on human health.

1. Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Gaurner et al., 2008). The addition of probiotics to enhance the nutritive value and potential health benefits of foods is now a matter of great interest. Among the most used organisms are those belonging to the genus *Lactobacillus*, which are believed to have beneficial effects on human health (Matsumoto et al., 2006; Saxelin et al., 2005). However, there is no consensus regarding the minimum number of probiotic bacteria that need to be consumed to produce a beneficial effect (Farnworth, 2008; Gaurner et al., 2008). The Fermented Milks and Lactic Acid Bacteria Beverages Association of Japan has set a minimum of 10^7 bacteria/g or ml (Ishibashi and Shimamura, 1993). Considering that each dose should not have more than 100 ml, the concentration should not be lower than 10^7 CFU/ml (FAO/WHO, 2006; Lourens-Hattingh and Viljoen, 2002).

The *Lactobacillus* genus is a heterogeneous group with more than 100 species and subspecies, many of them used as probiotics, silage inoculants and as starters in fermented food in industrial technology. Nevertheless, a wide range of important strains remain to be discovered and characterized which may be used in food and feed biotechnology applications (Giraffa et al., 2010; Matsumoto et al., 2006). Currently, there is a great need to improve the experimental detection of *Lactobacillus* species, thus the design of screening methods for these microorganisms is still under development (Satokari et al., 2003).

In food microbiology, the choice of an appropriate technique to study microbial communities depends on the aims of the research, the complexity of the community and the required resolution and sensitivity level. Identification is traditionally performed by culture-based techniques, but molecular methods are able to detect non-cultivable microorganisms, providing a more comprehensive picture of the total community (Bernardeau et al., 2008). One of the most disseminated methods for bacterial community analysis is based on the extraction of the total community DNA, followed by PCR amplification of the nucleotide sequence of interest (Spiegelman et al., 2005). Nevertheless, the presence of numerous usual compounds such as polysaccharides, carbohydrates, proteins or even salts may hamper DNA extraction and affect PCR efficiency during the amplification stages (Mothershed and Whitney, 2006).

Fluorescence *In Situ* Hybridization (FISH) is another technique used for bacterial identification, which combines the simplicity of microscopy observation and the specificity of DNA/rRNA hybridization. This methodology is based on the hybridization of labeled DNA probes to taxon-specific regions of the bacterial ribosomes (16S and

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23S rRNA sequences), that are usually detected by fluorescence microscopy. In addition, the FISH procedure can be accomplished in a few hours, allowing fast *in situ* analysis (Amann and Fuchs, 2008; Justé et al., 2008). After Langer-Safer et al. (1982) developed FISH, this technique has been used to detect and quantify the presence or absence of specific DNA/rRNA sequences. FISH fluorescent probes show a high degree of specificity to complementary sequences and therefore have been applied in numerous fields of research. In the specific context of food technology, FISH has been also applied for the detection of specific lactic acid bacteria (LAB) in natural whey cultures for the production of hard cooked cheeses, a matrix which is very similar to fermented milks (Bottari et al., 2010; Matte-Taillez et al., 2001), in wine (Sohier and Lonvaud-Funel, 1998) and for probiotic bifidobacteria quantification in fermented food (Kaufmann et al., 1997; Lahtinen et al., 2005; Shah and Lankaputhra, 1997; Tabasco et al., 2007). Moreover, FISH is well established as an advantageous method for cultivation-independent detection of microorganisms in many different sample types.

However, DNA probes frequently showed low fluorescent responses in hybridized samples due to numerous factors, such as, low ribosome content of cells, difficult cell wall permeabilization and the presence of rRNA secondary and tertiary structures (Justé et al., 2008). Consequently, peptide nucleic acid (PNA) probes have emerged as an alternative to DNA probes as a more efficient molecular method for rapid microbial detection (Stender et al., 2002). PNAs recognize and bind to their complementary nucleic acid sequences with higher thermal stability and specificity than the corresponding deoxyribooligonucleotides. PNA probes targeting specific 16S and 23S rRNA sequences of bacteria with clinical, industrial and environmental relevance have been successfully described by several researchers (Almeida et al., 2009, 2010; Guimaraes et al., 2007; Matte-Taillez et al., 2001).

Our main goal consisted in the design, characterization and evaluation of a new fluorescently labeled PNA probe for the specific detection of the *Lactobacillus* genus by FISH. To validate our probe, we determined its specificity and sensitivity against a great variety of *Lactobacillus* strains and other related bacterial strains. Subsequently, the PNA FISH method was evaluated on fresh milk samples to which lactobacilli were supplemented in concentrations found in several products after probiotic fermentation.

2. Materials and methods

2.1. Culture of bacterial strains

The bacterial strains used in this study are listed in Table 1. All strains were maintained on Brain Heart Infusion agar (BHI; Oxoid, United Kingdom) or de Man, Rogosa and Sharpe agar (MRS; Sigma, Portugal) at 37 °C (or 30 °C in the case of *L. pentosus* strains) and streaked onto fresh plates every 24 h. Plates were incubated at 37 °C or 30 °C under anaerobic conditions (AnaeroGen Atmosphere Generation system; Oxoid, United Kingdom) for 20–24 h prior to FISH experiments.

2.2. PNA probe design

To identify *Lactobacillus* genus potential targets for the probe design, we used the Primrose program (http://www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html; Ashelford et al., 2002) coupled with the 16S rRNA databases from the Ribosomal Database Project II (version 10.0; http://rdp.cme.msu.edu/; Cole et al., 2009). The complementarity to a lower number of non-target and to the higher number of target sequences were the main reasons for the PNA probe design.

The selected sequence was synthesized (Panagene, Daejeon, South Korea) and the oligonucleotide N terminus was attached to an Alexa Fluor 488 molecule via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker (PNA Probe: Lac663, Alexa Fluor 488-OO-ACATGGAG TTCCACT; HPLC purified > 90%).

Table 1
Bacterial strains used in PNA-FISH assays in the present study. The PNA probe efficiency was tested in triplicate experiments for each strain, with the following qualitative evaluation: (−) absent hybridization; (+) poor hybridization; (++) moderate hybridization; (+++) good hybridization; and (++++) optimal hybridization. The table shows the average value from the three experiments for each strain.

Code	Species	Strain	PNA probe efficiency
L1	*Lactobacillus (L.) pentosus*	CECT4023	++++
L2	*L. casei*	CECT5275	++++
L3	*L. rhamnosus*	CECT288	++++
L4	*L. coryniformis sub. torquens*	CECT4129	++++
L5	*L. paracasei*	CECT227	++++
L6	*L. acidophilus*	ATCC4356	++++
L7	*L. agilis*	CCUG 31450	++++
L8	*L. animalis*	ATCC35046	+++
L9	*L. bifermentans*	ATCC35409	+++
L10	*L. brevis*	ATCC14869	++++
L11	*L. buchneri*	ATCC4005	+++
L12	*L. fermentum*	ATCC11739	+++
L13	*L. crispatus*	ATCC33820	++++
L14	*L. curvatus sub. Curvatus*	ATCC25601	++++
L15	*L. delbrueckii sub. delbrueckii*	ATCC9649	+++
L16	*L. delbrueckii sub. Lactis*	ATCC12315	+++
L17	*L. farciminis*	DSM20182	++++
L18	*L. fructivorans*	ATCC8288	+++
L19	*L. gallinarum*	CCUG31412	++++
L20	*L. gasseri*	ATCC9857	++++
L21	*L. graminis*	DSM20719	++
L22	*L. hamster*	ATCC43851T	+++
L23	*L. helveticus*	ATCC15009	++++
L24	*L. hilgardii*	NCFB962	+++
L25	*L. intestinalis*	ATCC49335	+++
L26	*L. johnsonii*	ATCC11506	++++
L27	*L. murinus*	ATCC35020	++++
L28	*L. parabuchneri*	ATCC12936	++++
L29	*L. paracasei sub. paracasei*	CCUG27320	+++
L30	*L. plantarum*	NCIMB8827	+++
L31	*L. reuteri*	NCFB2656	+++
L32	*L. rhamnosus*	ATCC7469	++++
L33	*L. ruminis*	ATCC27781	++++
L34	*L. sakei sub. carnosus*	CCUG8045	++
L35	*L. salivarius*	DEVRIESE94/438	+++
L36	*L. plantarum*	NCCB46043	+++
E1	*Lactococcus lactis* 53	−	−/++
E2	*Streptococcus thermophilus* A	−	−
E3	*Streptococcus thermophilus* B	−	−/+++
E4	*Leuconostoc mesenteroides*	−	−/+
E5	*Bacillus subtilis*	DSM 7–10	−
E6	*Enterococcus faecium*	CECT410	−
E7	*Enterococcus faecalis*	CECT184	−
E8	*Bacillus cereus*	−	−
E9	*Enterobacter aerogenes*	CECT684	−
E10	*Salmonella enterica*	−	−
E11	*Escherichia coli O157:H7*	NCTC12900	−
E12	*Staphylococcus aureus*	CECT976	−
E13	*Staphylococcus aureus*	CECT86	−
E14	*Shigella flexneri*	ATCC12022	−
E15	*Listeria monocytogenes*	−	−
E16	*Klebsiella pneumoniae* subsp. *ozaenae*	ATCC11296	−
E17	*Salmonella typhi*	−	−
E18	*Listeria seeligeri*	CECT917	−
E19	*Escherichia coli*	CECT434	−
E20	*Listeria monocytogenes*	CECT5873	−

2.3. Theoretical assessment of probe specificity and sensitivity

In order to compare the performance of the PNA probe developed in the current study with that of other probes previously described (Table 2), their theoretical specificities and sensitivities were calculated according to Almeida et al. (2010), using updated databases available at the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu/) through Primrose software and then confirmed by a BLAST search at

the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Only *Lactobacillus* strains with at least 1200 base pairs and good quality sequences were included. Briefly, theoretical sensitivity was calculated as $Ls/(TLs)\times 100$, where *Ls* stands for the number of *Lactobacillus* strains detected by the probe and *TLs* for the total number of *Lactobacillus* strains present in the RDP II (http://rdp.cme.msu.edu/probematch/, last accession date, March 2012). Theoretical specificity was calculated as $nLs/(TnL)\times 100$, where *nLs* stands for the number of non-*Lactobacillus* strains that did not react with the probe and *TnL* for total of non-*Lactobacillus* strains examined.

2.4. FISH protocol development

Both hybridization procedures (in glass slides and in suspension) are able to detect and quantify potential probiotic lactobacilli. Moreover, glass slide hybridization is the more commonly used technique in analytical laboratories (Amann and Fuchs, 2008). While hybridization in suspension is usually used to avoid autofluorescence background in complex matrix samples (Almeida et al., 2009) and it is also the hybridization technique applied in flow cytometry (Amann et al., 1990; Amann and Fuchs, 2008).

2.4.1. Hybridization procedure on glass slides and in suspension

Protocols were developed both for hybridization on slides and in suspension. Even though the FISH protocols on slides and in suspension herein used are based on the previous work by Almeida et al. (2009, 2010), optimal fixation and hybridization conditions are crucial for a specific FISH method development. Therefore, these procedures were optimized considering the following modifications. Hybridization was done at 60 °C for 90 min and washing (60 °C for 30 min) was prepared less than 24 h before use. The glass slides were allowed to air dry before microscopy visualization, while suspension samples were stored at 4 °C in the dark for a maximum of 24 h before microscopy visualization.

2.4.2. Hybridization on milk samples

All strains were grown on MRS or BHI plates, for 24–48 h (see Table 1). Afterwards, strains were suspended in distilled water and then homogenized by vortexing for 1 min. Then, 1 ml of cell suspension was pelleted by centrifugation at 10,000 ×*g* for 5 min, resuspended in 500 μl of 4% (wt/vol) paraformaldehyde (Fisher Scientific, United Kingdom) and fixed for 1 h. The fixed cells were washed in sterile water by centrifugation at 10,000 ×*g* for 5 min, resuspended in 500 μl of 50% (vol/vol) ethanol, and incubated for 30 min at −20 °C. Following this, a 100 μl aliquot was pelleted by centrifugation, washed with sterile water and finally resuspended in 100 μl of hybridization solution with 200 nM PNA probe. Next, the samples were incubated at identical hybridization time and temperature ranges as the ones referred above (see Section 2.4.1). Subsequent to the hybridization step, the sample was centrifuged again at 10,000 ×*g* for 5 min, resuspended in 500 μl of wash solution and then incubated for 30 min at the same temperature of the hybridization step. The washed suspension was pelleted by centrifugation and resuspended in 500 μl of sterile water. The final step consisted of filtering 200 μl of cell suspension through a 0.2 μm cellulose nitrate membrane (Whatman, United Kingdom) or alternatively by spreading 20 μl of the suspension on a microscope slide, allowing then the membrane or slide to air dry. After hybridization samples were stored at 4 °C in the dark for a maximum of 24 h before microscopy visualization.

2.5. Microscopic visualization

Before the microscopic evaluation, one drop of non-fluorescent immersion oil (Merck, Portugal) was added to either slides or filters and covered with coverslips. Microscopy visualization was performed using an Olympus BX51 (Olympus Portugal SA, Porto, Portugal) epifluorescence microscope equipped with one filter sensitive to the Alexa Fluor 488 molecule attached to the PNA probe (BP 470–490), and an Olympus DP 72 camera. Other filters present in the microscope that are not capable of detecting the probe fluorescent signal were used to confirm the absence of autofluorescence (FT 500, LP 516). In each experimental assay, a negative control was performed simultaneously in which all the steps described above were carried out, but where no probe was added in the hybridization step. All images were acquired using Olympus CellB software using a magnification of ×1000.

2.6. Experimental assessment of probe specificity and sensitivity

After the hybridization optimization, the specificity and sensitivity of the PNA probe were tested using 36 representative strains from *Lactobacillus* genus and 20 representative strains from related genera belonging to the Lactobacillales order (Kandler and Weiss, 1986; Hammes and Vogel, 1995) and common pathogens in food industry (see Table 1). Specifically, strains from the following genera were included: *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Streptococcus*, *Staphylococcus*, *Shigella*, *Listeria*, *Salmonella*, *Escherichia*, *Enterobacter*, *Bacillus* and *Klebsiella*. All experiments were performed in triplicate at identical conditions and the experimental specificity and sensitivity were determined.

2.7. PNA FISH validation against fresh milk samples

For the direct detection of bacterial cells in milk, we selected a well-known probiotic and an uncommon probiotic *Lactobacillus*

Table 2
Theoretical specificity and sensitivity obtained for probes previously developed for the detection of *Lactobacillus* spp.

Probe	Type	Sequence (5′ 3′)	No. of *Lactobacillus* strains detected[a]	No. of non-*Lactobacillus* strains detected[a]	Specificity (%)[a]	Sensibility (%)[a]	Reference or source
Eub338[b]	DNA	GCTGCCTCCCGTAGGAGT	11,842	904,088	5.99	98.22	Amann et al., 1990
Lab158[c]	DNA	GGTATTAGCA(C/T)CTGTTTCCA	11,198	6,203	99.36	92.88	Harmsen et al., 1999
LGC354A[d]	DNA	TGGAAGATTCCCTACTGC	11,852	11,585	98.80	98.30	Meier et al., 1999
LAB759[e]	DNA	CTACCCATRCTTTCGAGCC	10,371	2,823	99.72	80.17	Zijnge et al., 2010
LbpV3[f]	DNA	CCGTCAATACCTGAACAG	831	4	99.99	6.89	Ercolini et al., 2003
Name not available[g]	PNA	GAATCTTCCACAATGG	11,873	14,126	98.53	98.47	Burton et al., 2003
Lact663	PNA	ACATGGAGTTCCACT	11,035	3,248	99.66	91.52	This work

[a] Calculated through ProbeMatch/ (last accession, January 2012) with the following data set options: Strain—Both; Source—Both; Size—>1200 bp; Quality—Both.
[b] DNA probe for Eubacteria being unspecific for *Lactobacillus* spp. but useful for comparative value.
[c] DNA probe also detects members of *Enterococcus*, *Pediococcus*, *Weissella*, *Vagococcus*, *Leuconostoc* and *Oenococcus* spp. used in Lebeer et al. (2011).
[d] DNA probe for mainly members of *Lactobacillales* and *Bacillales*, such as *Lactobacillus* spp., used in Olsen et al. (2008).
[e] DNA probe also detects members of *Ruminococcaceae* sp. and *Pediococcus* sp. used in Quevedo et al. (2011); the R symbol of the DNA probe sequence may be adenosine or guanosine, therefore Quevedo et al. (2011) used in fact two DNA probes to detect *Lactobacillus* spp.
[f] DNA probe only hybridizes with certain strains *Lactobacillus plantarum*.
[g] PNA probe for the detection of *Lactobacillus*-related genera.

strain, more precisely, *L. casei* CECT 4023 (also known as ATCC 393; Sidira et al., 2010) and *L. pentosus* CECT 5275 (Lafarge et al., 2004; Zhu et al., 2007), respectively. These two strains were selected to demonstrate Lac663 capability for potential probiotic lactobacilli detection in complex matrix samples, such as milk samples. An inoculum of either *Lactobacillus pentosus* CECT 4023 or *L. casei* CECT 5275 was prepared in phosphate buffered saline (PBS) and, using a calibration curve of CFU vs O.D. (at a wavelength of 600 nm), fresh milk (also known as raw milk) samples were inoculated with microbial concentrations ranging from 1×10^2 to 1×10^8 CFU/ml. Also, we performed a spiked-milk inoculation with a mixture of *Lactobacillus casei* CECT 5275 and two non-*Lactobacillus* strains: *Lactococcus lactis* (E1) and *Streptococcus thermophilus* B (E3; Table 1). This spiked-milk sample was inoculated with a final concentration of 1×10^8 CFU/ml of each bacteria. Taking into account the previously reported detection limit of 2×10^5 CFU/ml for *Cronobacter* in powdered infant formula (Almeida et al., 2009), a simple and direct detection approach, after a ten-fold dilution (to avoid the interference of milk protein autofluorescence), was considered adequate to reach the desired detection limit. Therefore, all samples were diluted 1:10 and 1 ml aliquots of each dilution were concentrated by centrifugation as described above. The fresh milk was previously pasteurized at 66 °C for 30 min (before FISH procedure) to reduce the naturally occurring microbial load from the initial lactobacilli or related bacterial strain inoculation in the milk samples. The pasteurization step was performed in our laboratory to ensure an efficient procedure and to maintain the same bacterial concentration from the initial inoculation, avoiding further proliferation of the bacteria in fresh milk samples through temperature inactivation. Hybridization was performed in suspension or on glass slides, then samples were visualized by epifluorescence microscope and concentration was determined by counting a total of 15 fields (1000×) for each sample. Also, the samples were plated on MRS agar for CFU counts.

3. Results and discussion

3.1. Theoretical assessment of probe specificity and sensitivity

The sequence of the selected PNA probe is the shorter from all probes in Table 2, and it hybridizes between positions 663 and 677 of the *Lactobacillus* sp. strain MDL2 16S rRNA sequence (Genebank ID: HM753265.1), consequently it was denominated as Lac663. According to the consulted database of the RDP II Project, the Lac663 probe is fully complementary to 11,837 matches that correspond to target sequences. Since the *Lactobacillus* genus reveals 12,936 target sequences, the Lac663 probe theoretical sensitivity was found to be 91.52% (see Table 2). Considering a total of 1,018,924 non-*Lactobacillus* sequences evaluated (isolates with good quality and sequence size > 1200 bp) from which 1,015,376 did not react with Lac663 probe, a theoretical specificity of 99.66% was determined.

These sensitivity and specificity equations allowed us to compare our Lac663 PNA probe with other probes that had previously been developed to detect and enumerate *Lactobacillus* spp. strains (Table 2). Lac663 theoretical performance was quite similar to what had previously been reported for the other probes mentioned in Table 2, except for Eub338 and LbpV3, which were designed for the detection of Eubacteria (Eub338) and *Lactobacillus plantarum* (LbpV3) and, as such, are not specific for the *Lactobacillus* genus. Although probes Lab158, LGC354A and the probe described by Burton et al. (2003) detected approximately 1 to 8% more *Lactobacillus* strains in comparison with our probe, Lac633 was found to be the probe with the lowest number of false positive hits (Table 2). In fact, the Lac663 probe does not cross-react with 3,617, 8,781 and 11,332 non-*Lactobacillus* strains that are detected with the Lab158, LGC354A and Burton et al. (2003) probes, respectively. Moreover, 5 of the 22 non-*Lactobacillus* genera detected by the Burton et al. (2003) PNA probe belong to the Carnobacteriaceae family and consequently to the LAB group (König and Fröhlich, 2009), more precisely, the following genera: *Dolosigranulum*; *Atopostipes*; *Alloiococcus*; *Alkalibacterium*; and *Marinilactibacillus*. This family of gram-positive, lactic acid-producing bacteria is used in several food applications, including probiotic milk and its products (Afzal et al., 2010; Bourdichon et al., 2012). A total of 51.95% of bacterial species from the Carnobacteriaceae family are detected by the Burton et al. (2003) PNA probe in contrast with only 0.34% detected by our probe. This demonstrates that the Lac663 probe shows a better specificity and applicability for analysis of fresh milk samples, which can be contaminated by a huge variety of bacteria. From Table 2 it can be concluded that only the LAB759 probe was more specific than the currently developed Lac663 probe. However, the LAB759 probe shows a clearly lower sensitivity percentage (80.17%) compared to our probe (91.52%). It is also important to note that our probe has the shortest oligonucleotide sequence from all the probes included in Table 2, more precisely 1 and 3 nucleotides less than the other PNA probe and the shorter DNA probe (LGC354A), respectively. This implies that the Lac663 probe should penetrate better through the cell wall and that 1 base mismatch can be more easily discriminated. Also, because it's a PNA based probe, it has higher resistance against enzymatic activity (such as DNases or proteases) from the bacterial cell, enabling superior FISH efficiency as previously discussed by Cerqueira et al. (2008).

In conclusion, our *in silico* analysis indicates that the Lac663 probe is able to induce hybridization more efficiently to *Lactobacillus* strains in complex samples than the other probes reported so far.

3.2. FISH protocol development

From the different temperatures and time periods tested, the best hybridization conditions were found to be 60 °C for 90 min. Hybridization conditions revealed strong signal-to-noise ratio from 58 °C to 62 °C, with incubation times ranging from 60 to 120 min. As such, our FISH protocol can be performed within 3 h, which constitutes a significant improvement as compared to previously described similar DNA probes by Lebeer et al. (2011) (Lab158 DNA probe with overnight hybridization of 12 h). Also, as previously mentioned, all samples were visualized with other available filters and no autofluorescence was observed (see examples in Fig. 1). Specifically, the absence of autofluorescence in L14 and L34 pictures on red filter confirms the specificity of the Lac663 probe.

3.3. Experimental assessment of probe specificity and sensitivity

As expected by the *in silico* analysis, the Lac663 probe hybridized with all *Lactobacillus* collection strains whereas no hybridization was observed for the other species used, except for *L. lactis* 53, *S. thermophilus B* and *L. mesenteroides*, which showed some cross-hybridization with the probe if a washing step of 15 min was used. To clarify these results we analyzed *in silico* 16S rRNA gene sequences from these three bacterial strains and we found only one mismatch at positions 670 for *L. lactis* (T) NCDO607T 16S rRNA gene sequence (RDPII ID: S000439498) and *S. thermophilus* (T) ATCC19258 16S rRNA gene sequence (RDPII ID: S000400852), more exactly a single medium-strength T/C mismatch. However, extending the washing step to 30 min and using fresh washing solution allowed the removal of the Lac663 probe poorly bound from all non-*Lactobacillus* strains with this single medium-strength T/C mismatch in our bacteria culture collection. *L. mesenteroides* (T) NCFB529 (RDPII ID: S000003774) revealed a total common oligonucleotide sequence identical to *Lactobacillus* spp., which was already predicted in the theoretical evaluation and this actually occurs with all the probes developed for detection of *Lactobacillus* spp. described in Table 2. Despite of the cross-hybridization for this species, its coccus morphology allows differentiation from *Lactobacillus* spp., which have a rod-shaped morphology. Importantly, Lac663 probe

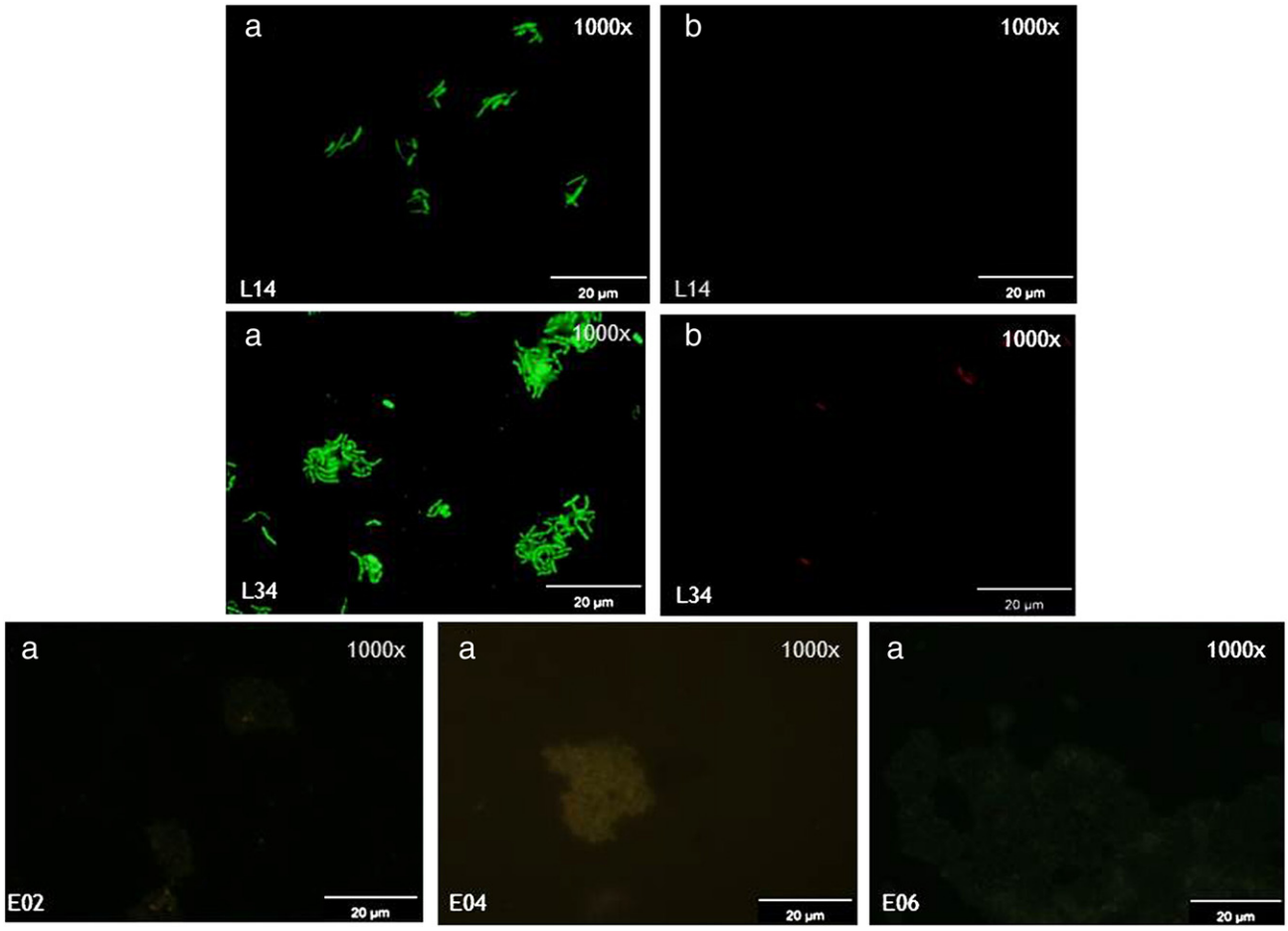

Fig. 1. Fluorescence microscopy pictures of *Lactobacillus* spp. and other related bacteria by specific PNA probe (Lac663) associated with Alexa Fluor 488 fluorochrome. All images were obtained using the same exposure time. (a) Green filter (BP 470–490). (b) Red filter (FT 500, LP 516). *Code:* L14, *L. curvatus sub. curvatus* ATCC25601; L34, *L. sakei sub. carnosus* CCUG8045; E02, *S. thermophilus* A; E04, *L. mesenteroides*; E06, *E. faecium*.

showed an absence of hybridization with several bacterial species from the Bacilli class, such as *S. thermophilus A*, *Enterococcus faecium* CECT 410, *Enterococcus faecalis* CECT 184, *Bacillus subtilis* DSM 7–10 and *Bacillus cereus*. In addition, the probe also did not hybridize with several pathogenic bacteria which may contaminate industrial food such as *Salmonella* spp., *Escherichia coli*, *Shigella* spp. and *Listeria monocytogenes*. Therefore, these results support the advantages previously mentioned about the reliable application of PNA probes in FISH methodology and corroborates the theoretical prediction. Based on this test, experimental sensitivity of 100% (95% confidence interval (CI), 88.0 to 100.0%) and specificity of 95.0% (95% CI, 73.1 to 99.7%) were obtained.

3.4. PNA FISH validation against fresh milk samples

After the optimization of the Lac663 FISH protocol the method was adapted for the detection and quantification of *Lactobacillus* spp. in a milk sample enriched with probiotic strains. According to Ishibashi and Shimamura (1993), we have defined a desired detection limit of 1×10^7 CFU/ml. As expected, after hybridization with the Lac663 probe, microscopic visualization showed that *Lactobacillus* species could be detected up to the concentration of 1×10^7 CFU/ml (Fig. 2 and Table 3). The concentration of inoculated bacteria in the fresh milk samples was determined by conventional plating techniques (as CFU/ml) and by PNA FISH counts (as cell/ml; Table 3). As expected, PNA FISH counts were higher than CFU, which most probably was due to the presence of non-cultivable cells. Furthermore, we performed a spiked-milk experiment with a mixture of *L. casei* CECT 5275 and the two previously mentioned non-*Lactobacillus* strains with one single medium G/T mismatch, more exactly, *L. lactis* (E1) and *S. thermophilus* B (E3; Table 1). The aim of this experiment was to confirm that Lac663 probe only hybridized to the *Lactobacillus* genus using a washing step of 30 min. No unspecific hybridization was observed in the spiked milk sample hybridization (*data not shown*), thus confirming Lac663 probe applicability in the detection and quantification of *Lactobacillus* species in complex samples.

Epifluorescence microscopy has become a widely used technique for direct estimation of bacteria in several industrial samples. In fact, many authors demonstrated the efficiency of FISH methodology in lactobacilli analysis (Bernardeau et al., 2001; Lebeer et al., 2011; Matte-Taillez et al., 2001); however none of them achieved the simplicity and specificity that our method offers. For example, Bernardeau et al. (2001) successfully performed a quantitative analysis of lactobacilli in probiotic feed, but lactobacilli were counted by an unspecific fluorochrome (DAPI—4′, 6-diaminidino-2-phenylindole–2HCL). Matte-Taillez et al. (2001) also used DNA and PNA FISH methodology for the detection and identification of lactic acid bacteria on milk samples. However, these authors had to apply multiple PNA probes for lactobacilli identification, which makes the analysis more expensive and complex.

Regarding the traditional culture method for enumeration of *Lactobacillus* spp., it takes at least 48 h, even with a more advanced Petrifilm™ AC system used by Champagne et al. (2009). Similar results were obtained by Jackson et al. (2002), who used two selective media for the detection and enumeration of lactobacilli followed by a PCR for lactobacilli confirmation.

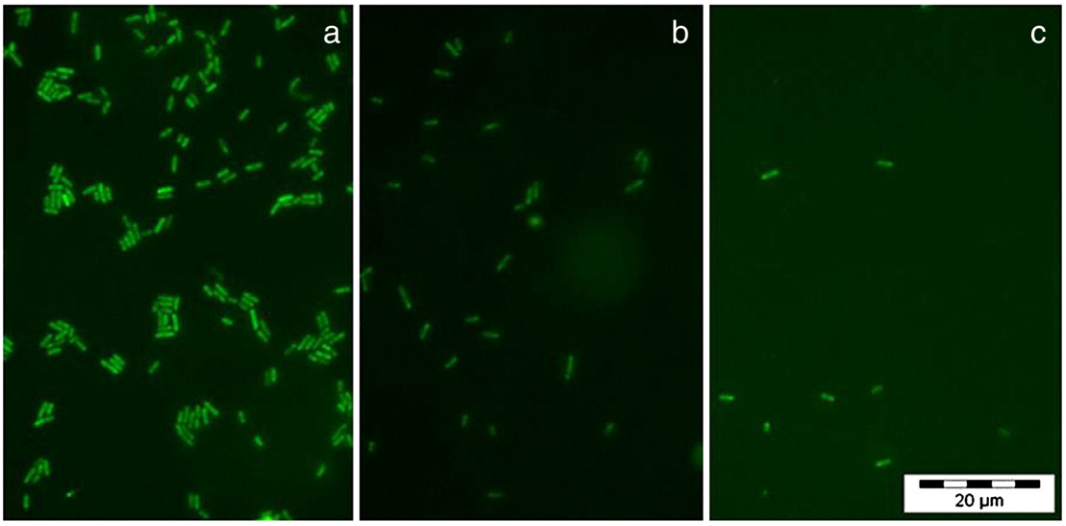

Fig. 2. Examples of fluorescence microscopy pictures of *Lactobacillus pentosus* CECT 4023 from fresh milk samples by specific PNA probe (Lac663) associated with Alexa Fluor 488 fluorochrome. Total cell counting determined for fresh milk samples with different initial concentrations, such as 10^9 CFU/ml (a), 10^8 CFU/ml (b) and 10^7 CFU/ml (c).

As regards to molecular methods other than FISH, Collado and Hernández (2007) used an Amplified Ribosomal DNA Restriction Analysis (ARDRA) and a Randomly Amplified Polymorphic DNA (RAPD) method that allowed discrimination of lactobacilli from other bacteria present on milk samples, but both techniques involved a much more complex protocol and were not capable of enumerating lactobacilli in the samples. Quantitative Real-Time PCR (qPCR) methods, which allow a quantitative detection, have been developed by Haarman and Knol (2006). qPCR methods can be performed at the same time as the FISH methodology; however they might also suffer from the presence of inhibitory substances in food samples, such as the other PCR techniques.

In summary, the *Lactobacillus* genus is present in probiotic milk and other-related products, such as cheese and yogurts. Quality assurance of the health or technological benefits of these products requires a fast detection and quantification of these bacteria.

The Lac663 probe was found to be a specific and sensitive PNA probe for *Lactobacillus spp.* that together with FISH methodology may be a reliable and fast (approximately 3 h) alternative technique for potential probiotic lactobacilli detection and/or quantification in complex matrices, such as the probiotic milk samples. Using this method, only milk samples with an effective number of probiotic bacteria (at least 10^7 CFU/ml) will show appropriate results. However, if a lower detection limit is desired, a destabilizing solution may be applied to the milk samples followed by a filtration step, to concentrate the samples.

Further studies are necessary to determine if the Lac663 probe validated here for milk samples, is also useful for other applications such as the detection and enumeration of *Lactobacillus spp.* in feed probiotic samples. It might also be combined with other PNA probes in multiplex assays, thus allowing the simultaneous detection and quantification of other bacterial species.

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Table 3
Detection level of *Lactobacillus spp.* species in milk samples by PNA-FISH assay as compared to the conventional culture plates. All experiments were done in triplicate and results represent the average ± standard deviation.

Concentration of cells (CFU/ml)	*L. pentosus* CECT4023		*L. casei* CECT5275	
	Culturable cells (CFU/ml)	FISH count (cells/ml)	Culturable cells (CFU/ml)	FISH count (cells/ml)
1×10^8	$7.00\times10^7\pm1.96$	$2.14\times10^8\pm0.24$	$4.50\times10^7\pm0.83$	$2.59\times10^8\pm0.09$
1×10^7	$6.54\times10^6\pm2.73$	$6.97\times10^7\pm1.87$	$3.76\times10^6\pm0.20$	$8.32\times10^7\pm2.29$
1×10^6	$7.43\times10^5\pm1.84$	–	$4.48\times10^5\pm1.75$	–
1×10^5	$7.56\times10^4\pm1.79$	–	$4.89\times10^4\pm2.45$	–
1×10^4	$1.08\times10^4\pm0.61$	–	$5.34\times10^3\pm2.59$	–
1×10^3	$6.99\times10^2\pm0.74$	–	$9.44\times10^2\pm6.52$	–

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