Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 6, No. 1, 69-76, 2016

Isolation and Identification of *L. plantarum* from Iranian Fermented Cucumbers by Conventional Culture and PCR Methods

Z. Nilchian [a], E. Rahimi [b*], S. H. Razavi [c], M. Momeni Shahraki [d]

[a] *Ph. D. Student of the Department of Food Science and Technology, School of Agriculture, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.*
[b] *Assistant Professor of the Department of Food Science and Technology, School of Agriculture, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.*
[c] *Associate Professor of the Department of Food Science and Engineering, Faculty of Biosystem Engineering University of Tehran, Iran.*
[d] *Member of the Biotechnology Research Center, ShahreKord Branch, Islamic Azad University, ShahreKord, Iran.*

Received: 19 December 2013 Accepted: 30 April 2014

ABSTRACT: The species of *Lactobacillus* are widely present in many natural environments that are important in fermented processes. Among these species some belong to the lactic acid bacteria group that are useful in fermented food. One of the species of lactobacilli group such as *L. plantarum* provides health benefits, but their identification is one of the main issue. In order to proceed with this project, conventional culture method and PCR primers have been employed to identify *L. plantarum*. According to the conventional culture method, colonies were Gram-positive and catalase-negative in the form of coccoid-rod, short, long, thin and fine. Under the PCR conditions, *L. plantarum* and *L. brevis* strains were detected in only one sample at 25^0C. In chemical technique, *L. plantarum* was separated from *L. brevis* in four cultures medium with 4-7% NaCl where *L. brevis* became inactive. The isolated *L. plantarum* that was used in the fermented cucumbers prevented the growth and development of pathogenic organisms.

Keywords: *Biochemical Methods, Iranian Fermented Cucumbers, L. plantarum, PCR.*

Introduction

Lactic acid bacteria (LAB) strains have been widely used as probiotics for human and animals (Tsai *et al*., 2010). The species of *Lactobacillus* are widely present in many natural environments that are important in fermented processes. Among these are the lactic acid bacteria group that is useful in fermented food products (Ercolini *et al*., 2006). LAB species, such as *Lactobacillus plantarum*, is a major LAB species which might provide health benefits, including improvement of gastrointestinal function, immune modulation and cholesterol-lowering functions for hosts (De Roos & Katan, 2000). *L. plantarum* is a homofermentative LAB which is predominantly found in fermented foods. *L. plantarum* completes the final stage of natural fruit and vegetables fermentation due to its acid tolerance that is higher than other lactic acid bacteria (Torriani *et al*., 2001). The identification of lactobacilli (*L. plantarum*) is one of the main issues of applied microbiology and is also one of the rapid and reliable methods in natural ecosystems (Ercolini *et al*., 2006). For the identification of LAB cells, including *Lactobacillus spp.* and *Bifidobacterium spp.,* many studies have been based on DNA probes and PCR primers (Tsai *et al*., 2010). These primers and probes obtained from the

*Corresponding Author: ebrahimrahimi55@yahoo.com

sequences of recA gene (Torriani *et al*., 2001), tuf gene (Ventura *et al*., 2003), 16S and 23S rRNA genes (Moura *et al*., 2007). On the other hand, real-time PCR methods were used for the simultaneous identification and quantification of specific LAB cells in cultures or fermented food samples. The growth and fermentative activity of *L. plantarum* greatly affects the quality of the final product in cucumber and cabbage fermentation (Lu *et al*., 2003). Therefore, the objective of this study is to isolate and evaluate the *L. plantarum* in microbial control of cucumbers fermentation.

Materials and Methods

- *Fermented cucumber samples*

In this study, the samples of fermented cucumber were supplied from four different centers in Iran where these products were prepared with native cucumbers that were placed in glass jars with added fresh brine (7-9% NaCl). The chemical characteristics of these samples are shown in Table 1.

- *Microbial analysis by conventional culture method*

In this experiment the samples were prepared by adding 2-3 g mixture of fermented cucumbers to 250 ml of sterile fresh MRS broth (Merck, Darmstadt, Germany) and then incubated at different temperatures (25, 30, 35 °C). When the pH reached below 4.2, the process was stopped. The Pour plate technique was performed using 0.1ml liquid samples in molten media (MRS agar) and surface spreading technique was performed by spreading 0.1ml liquid samples on the surface of the media (MRS agar). In all the cases, duplicate plates were prepared and were incubated at 25, 30, 35 ˚C for 13h. Colonies were randomly selected and purified by re-plating on MRS agar plates. The purified colonies were primarily identified by Gram staining and catalase tests. Only Gram-positive and catalase-negative strains are selected and stored in MRS broth with 60% (v/v) glycerol at -80 ˚C (Modified of Paramithiotis *et al*., 2010).

- *Genomic DNA preparation*

For LAB cells, the total chromosomal DNA was prepared from 100 µl dilute cell cultures (500 µl with sterile water) that remained for one night. This bacteria suspension was boiled at 100 °C for 10 min to release the DNA and then was cooled at 4 ^{0}C for 10 min. After using centrifugal force with 3000 rpm in 3 min, 1 µl supernatant was used as the DNA source for PCR amplification (Tsai *et al*., 2010).

- *PCR primers*

The DNA sequence coding for 16S, 23S rRNA of LAB was selected for the design of *L. brevis* and *L. plantarum* specific oligonucleotides. The less homologous sequences of these genes were used as a design for oligonucleotide primers and probes of *L. brevis* and *L. plantarum* (Table 2) (Tsai *et al*., 2010).

Table 1. Chemical characteristics of fermented cucumbers

samples	pH	titratable acidity (% acetic acid)	NaCl (g)	shelf-life
number1	3.95	0.48	4.05	35 days
number2	4.01	0.42	4.26	30 days
number3	4.05	0.37	4.38	20 days
number4	3.65	0.66	3.12	1 year

Table 2. Primers used for the detection of Lactobacillus spp.

Group of species	Primers	Sequences(5′-3′)	Location within gene	Product size (bp)
L. plantarum	LPL-1	GAAACCTACACACTCGTCGA	21-40(ITS)	598
	LPL-2	CCTGAACTGAGAGAATTTG	619-599(23S rDNA)	
L. brevis	23SP10	GGCCTATAGCTCAGCTGGT		730
	23SP11	CCTTTCCACGGTACTG		

- *PCR amplification*

20 µl of mixed sample (840 µl diluter, 100 µl PCR buffer, 40 µl $MgCl_2$, 20 µl dNTP), 1 µl PCR primer sets and Taq enzyme were used. 22.2 µl PCR mixture and 1-5 µl DNA were heated at 95°C for 5 min by a thermal cycler that contained 35 PCR cycles. For each PCR cycle, denaturation, annealing, and extension were carried out at 94 °C for 1 min and at 58 °C for 1min and 20s and at 72°C for 1min and 20s respectively. Final extension was carried out at 72°C for 7min. To detect the amplified product, 14 µl of the PCR product was examined by electrophoresis through 1% agarose gel in 20ml of 1×TBE buffer (10×TBE: 27 g/250ml Tris, 13.91 g/250ml Buric acid, 1.86 g/250 ml EDTA) (Modified of Tsai et al., 2010).

- *Survey antimicrobial activity of L. plantarum*

In this survey, the industrial fermentation of cucumbers were carried out with 10^6-10^8 cfu/ml isolated *L. plantarum* strain from Iranian fermented cucumbers in fresh brine (5-7% NaCl). In order to detect the viable cells in inoculated samples, the total plate count method was employed (Li *et al*., 2010). When the fermentation was completed, 0.5 ml of brine was added to Listeria Enrichment Broth to survey the *Listeria monocytogenes* and then this media was incubated at 37°C for 72 h, then 0.1 ml of media broth was spread on the surface of Listeria Selective Agar and incubated at 37°C for 24–48 h. The determination of *S. aureus* was carried out by spreading 0.1 ml of brine on Baird-Parker selective agar (Merck, Darmstadt, Germany) and incubated at 37°C for 24-48 h. Vibrio spp. was determined using 0.1 ml brine that was spread on the surface of TCBS agar (Thiosulfate-Citrate-Bile-Salt-sucrose Agar) and incubated at 25°C for 24-48 h (Modified of Paramithiotis *et al*., 2010; Panagou *et al*., 2008).

Results and Discussion

- *Microbial analysis by conventional culture methods*

Microbiological examination of the fermented cucumbers revealed that *Lactobacillus* species could tolerate pH of below 4.2, and according to (Sánchez *et al*., 2000), they are quite tolerant in acid media and some can grow in pH of about 3.8. After cultivation on MRSA, the 10^5 cfu/ml cells obtained by surface spreading technique revealed that the colonies on 15 plates were white, flat, globular with few yeast like colonies after 13h incubation at 25, 30 and 35 °C (Figure 1). The colonies were Gram-positive and catalase-negative on pour plating technique and surface spreading technique. In morphological survey, colonies were in the shape of coccoid-rod, short, long, thin and fine in chain form and in accordance with the taxonomic criteria (Axelsson, 1998), they might be related to the *Lactobacillus* genus while few of them were in the shape of oval capsules (Figure 2). Lactobacillus colonies were purified and isolated by re-plating on MRS agar plates (Figure 3). After isolation, LAB colonies of each plate were cultivated on MRS broth

medium for 24 h at 25, 30 and 35˚C. The production of gas was observed in all medium. In this aspect, obligately homofermentative lactobacilli can convert glucose almost exclusively to lactic acid, and facultatively and obligately heterofermentative lactobacilli can convert hexoses to lactic acid, acetic acid, ethanol and CO2 at different proportions (Kandler & Weiss, 1986).

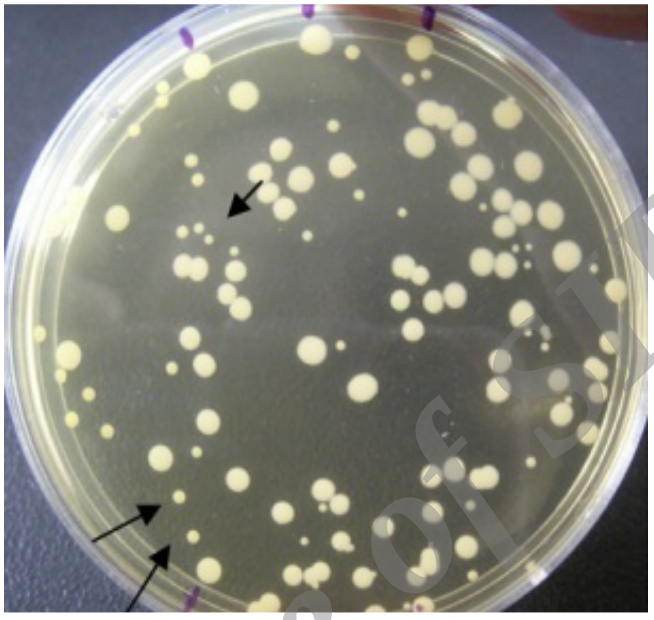

Fig.1. Lactobacillus colonies

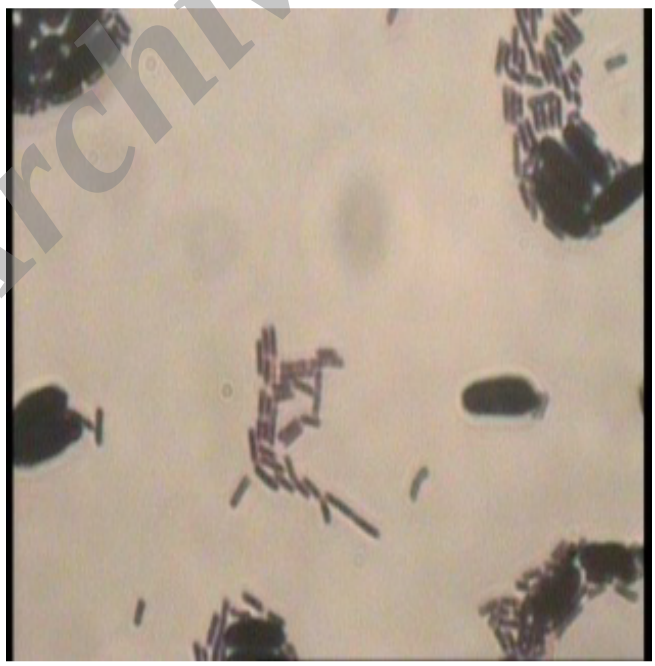

Fig. 2. Morphology shape

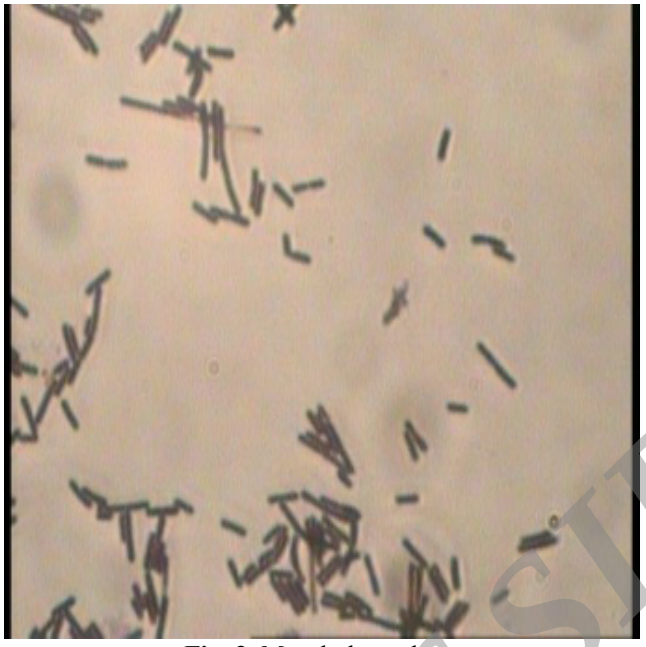

Fig. 3. Morphology shape

- *Multiplex PCR assay*

Based on the described methods, a multiplex PCR was performed with the *recA* gene of LPL-1/LPL-2 and 23SP10/23SP11 primers that were used as PCR primers for specific detection of *L. plantarum* and *L. brevis,* respectively. In this method, the DNA amplification of *L. plantarum* and *L. brevis* strains didn't produce an expected product and identities of the two fragments were not confirmed by sequencing. According to (Sánchez *et al*., 2000), *L. plantarum* is facultatively heterofermentative and arginine-negative lactobacilli (Subgroup A) and *L. brevis* is obligately heterofermentative and arginine-negative lactobacilli (Subgroup D). But the multiplex PCR protocol can produce the DNA amplification of *L. plantarum, L. pentosus,* and *L. paraplantarum* strains that the *recA* sequences of this facultatively heterofermentative lactobacilli belong to the same 16S rRNA phylogenetic group. Therefore, the clear distinction is obtained by short gene sequences for the closely related species (Torriani *et al*., 2001).

- *Detection limit of the PCR primers*

According to the described procedures, a separate PCR was performed by primers for specific detection of *L. plantarum* and *L. brevis*. Under described PCR conditions, only in the 4th sample (surface spreading technique at 25°C) these strains were obtained from the PCR products with molecular weight equal to 598 bp and 730 bp, respectively. The sequence analysis of 16S, 23S and 16S-23S ITS rRNA genes might be used to design genus or species-specific oligonucleotide primers and probes for rapid identification and detection of bacteria species including LAB (Moura *et al*., 2007). However, the detection of *L. plantarum* is difficult because it is genotypically and phenotypically very similar to *L. pentosus* and *Lactobacillus paraplantarum* (Curk *et al*., 1996). *L. plantarum* and *L. pentosus* 16S rRNA sequences have almost the same identity (Collins *et al*., 1991). In this respect, the use of ITS sequence could show that two strains of *L. plantarum* is related to the species, i. e., *L. pentosus* but LPL-1/LPL-2 primers would

not show a false positive result (Tsai *et al*., 2010). The specificity of these PCR primers of *L. plantarum* was similar to the designed primer of recA gene as (Torriani *et al*., 2001) reported. These primers of recA gene were able to distinguish *L. plantarum* from *L. pentosus* and *L. paraplantarum* strains (Torriani *et al*., 2001).

- *Separation of L. plantarum from L. brevis*

L. plantarum is homofermentative and arginine-negative lactobacilli and *L. brevis* is obligately heterofermentative and arginine-negative lactobacilli and both of them are mesophilic bacteria (Sánchez *et al*., 2000). *L. brevis* colonies appear in the shape of thin, short and long rods and in the pairs and short chains (Kandler & Weiss, 1986). In all of the samples, the shape of colonies on MRSA was irregular, white and rough and some of them had a raised centre (Sánchez *et al*., 2000). In these strains, the recognition of colonies and separation of *L. plantarum* from *L. brevis* are difficult, therefore, their resistance against chemical ingredient such as NaCl was evaluated. According to the results of (Sánchez *et al*., 2000), *L. plantarum* and *L. pentosus* are more salt-tolerant than other strains and they can grow in 8% (w/v) NaCl. Therefore, the samples were cultivated in MRS broth with 2, 3, 4, 5, 6 and7% NaCl and incubated for 24h at 25°C. In this survey, the growth and production of gas were observed only in the media with 2% NaCl. Based on the described methods, PCR was carried out for six samples (2-7% NaCl) where in the four medium with 4-7% NaCl, *L. brevis* became inactive and *L. plantarum* remained active.

- *Antimicrobial activity of L. plantarum*

After the fermentation was completed, all of the samples were examined for *Listeria monocytogenes, S. aureus* and *Vibrio spp*. on 10 plates with different medium. All of the fermented cucumbers with inoculation of 10^6-10^8 cfu/ml *L. plantarum*, the levels of *Listeria monocytogenes, S. aureus and Vibrio spp* were undetected on the surface of the plate and there were no growth of pathogenic bacteria. *Listeria monocytogenes* is a pathogenic organism in a non-acidified, refrigerated pickle products and in cucumber juice fermentation (Romick, 1994). In some of the fermented vegetables like green table olives, the product can support the survival of *Listeria spp*. despite its low pH and high salt concentration (Caggia *et al*., 2004) and also *Listeria* can find some advantage for survival in cold-fermented olives (Abriouel *et al*., 2011), but in this assay *Listeria spp.* was not observed. Abriouel *et al*. (2011) could detect the potentially pathogenic bacteria such as *Vibrio spp.* only at the beginning of fermentation where the low pH and anaerobic conditions of olive fermentation do not favor the survival of this contaminant. In salty fermented cucumber, a large number of antagonistic lactic acid bacteria could act against pathogenic bacteria (Singh & Ramesh, 2008). Todorov *et al*. (2007) showed the activity of *L. plantarum* AMA-K bacteriocin against Gram-negative bacteria where the number of *Listeria innocua F* cells decreased to undetectable levels after 24 h (Todorov *et al*., 2007). Tamang *et al.* (2009) isolated LAB strains with antagonistic activities from ethnic fermented vegetable and bamboo shoot products (Tamang *et al*., 2009). Therefore, isolated *L. plantarum IB2 (BFE948)* could show a bacteriocin activity against *S. aureus S1*.

LAB can produce many antibacterial substances like organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds, bacteriocins, antibiotics, fatty acid, phenyllactic acid that have positive influence on the shelf-life of the fermented product (Valerio *et al*., 2008). Therefore, growth of LAB seems to inhibit the growth and development of the rest of the microorganisms (Paramithiotis *et al*., 2010).

Conclusion

In one out of the four Iranian fermented cucumbers, two strains of *L. plantarum* and *L. brevis* were observed that were purified and isolated with different values of NaCl and identified by PCR method. In our results, the growth of LAB (*L. plantarum)* could inhibit the growth and development of the pathogenic organisms in fermented cucumbers. In future, *L. plantarum* strain might be employed to control the fermentation of products.

References

Abriouel, H., Benomar, N., Lucas, R. & Gálvez, A. (2011). Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. International Journal of Food Microbiology 144, 487-496.

Axelsson, L. (1998). Lactic acid bacteria: classification and physiology; in: Salminen S, von Wright A. (Eds). Lactic Acid Bacteria. Microbiology and Functional Aspects. 2 Edition, Marcel Dekker. New York. Basel. Hong Kong, 1-72.

Caggia, C., Randazzo, C. L., Di Salvo, M., Romeo, F. & Giudici, P. (2004). Occurrence of *Listeria monocytogenes* in green table olives. Journal of Food Protection 67, 2189-2194.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A. & et al. (1991). Phylogenetic analysis of the genus Lactobacillus and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiology Letters 77, 5-12.

Curk, M. C., Hubert, J. C. & Bringel, F. (1996). *Lactobacillus paraplantarum sp.* Nov., a new species related to *Lactobacillus plantarum.* International Journal of System *Appl. Environ. Microbiol* Bacteriology 46, 595-598.

De Roos, N. & Katan, M. (2000). Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. American Journal of Clinical Nutrition 71, 405-411.

Ercolini, D., Villani, F., Aponte, M. & Mauriello, G. (2006). Fluorescence in situ hybridisation detection of Lactobacillus plantarum group on olives to be used in natural fermentations. International Journal of Food Microbiology 112, 291-296.

Kandler, O. & Weiss, N. (1986). Bergey's Manual of Systematic Bacteriology, Willians and Wilkins, Baltimore MD, pp, 1208-1234.

Li, L., Feng, L., Yi, J., Hua, C., Chen, F., Liao, X., Wang, Z. & Hu, X. (2010). High hydrostatic pressure inactivation of total aerobic bacteria, lactic acid bacteria, yeasts in sour Chinese cabbage. International Journal of Food Microbiology 142, 180-184.

Lu, Z., Breidt Jr, F., Fleming, H. P., Altermann, E. & Klaenhammer, T. R. (2003). Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, ΦJL-1, from a cucumber fermentation. International Journal of Food Microbiology 84, 225-235.

Moura, P., Simões, F., Gírio, F., Loureiro-Dias, M. C. & Esteves, M. P. (2007). PCR monitoring of *Lactobacillus* and *Bifidobacterium* dynamics in fermentations by piglet intestinal microbiota. Journal of Basic Microbiology 47, 148-57.

Panagou, E. Z, Schillinger, U., Franz, C. M. A. P. & Nychas, G. J. E. (2008). Microbiological and biochemical profile of cv. Conservolea green olives processed by the Spanish-method. Food Microbiology 23, 199-204.

Paramithiotis, S., Hondrodimou, O. L. & Drosinos, E. H. (2010). Development of the microbial community during spontaneous cauliflower fermentation. Food Research International 43, 1098-1103.

Romick, T. L. (1994). Biocontrol of Listeria monocytogenes, a psychrotrophic pathogen model, in low salt, non-acidified, refrigerated vegetable products, PhD Diss, NC State University, Raleigh, NC.

Sánchez, I., Palop, L. & Ballesteros, C. (2000). Biochemical characterization of lactic acid bacteria isolated from spontaneous fermentation of 'Almagro' eggplants. International Journal of Food Microbiology 59, 9-17.

Singh, A. K. & Ramesh, A. (2008). Succession of dominant and antagonistic lactic acid bacteria in fermented cucumber: Insights from a PCR-based approach. Food Microbiology 25, 278-287.

Tamang, J. P., Tamang, B., Schillinger, U., Guigas, C. & Holzapfel, W. H. (2009). Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas. International Journal of Food Microbiology 135, 28-33.

Todorov, S. D., Nyati, H., Meincken, M. & Dicks, L. M. T. (2007). Partial characterization of bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K isolated from naturally fermented milk from Zimbabwe. Food Control 18, 656-664.

Torriani, S., Felix, G. E. & Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Applied and Environmental Microbiology 67, 3450-3454.

Tsai, C. C., Lai, C. H., Yu, B. & Tsen, H. Y. (2010). Use of PCR primers and probes based on the 23S rRNA and internal transcription spacer (ITS) gene sequence for the detection and enumerization of *Lactobacillus acidophilus* and *Lactobacillus plantarum* in feed supplements. Anaerobe 16, 270-277.

Valerio, F., De Bellis, P., Lonigro, S. L., Visconti, A. & Lavermicocca, P. (2008). Use of Lactobacillus plantarum fermentation products in bread-making to prevent Bacillus subtilis ropy spoilage. International Journal of Food Microbiology 122, 328-332.

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T. R. & Zink, R. (2003). Analysis, characterization, and loci of the tuf genes in lactobacillus and bifidobacterium species and their direct application for species identification. Applied and Environmental Microbiology *Appl. Environ. Microbiol Appl. Environ. Microbiol* 69, 6908-6922.