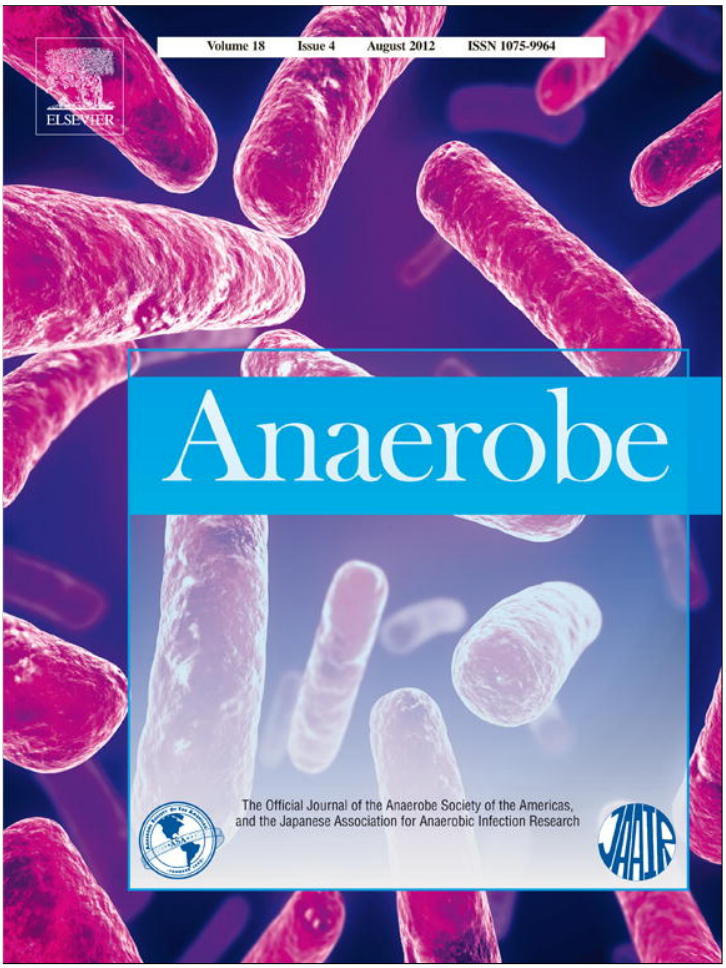

Anaerobe 18 (2012) 417–420

Contents lists available at SciVerse ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular biology, genetics and biotechnology

Distinct adhesion of probiotic strain *Lactobacillus casei* ATCC 393 to rat intestinal mucosa

Georgia Saxami [a], Petros Ypsilantis [b], Marianthi Sidira [a], Constantinos Simopoulos [b], Yiannis Kourkoutas [a], Alex Galanis [a,*]

[a] *Applied Microbiology and Molecular Biotechnology Research Group, Department of Molecular Biology and Genetics, Democritus University of Thrace, Dragana, Alexandroupolis GR-68100, Greece*
[b] *Laboratory of Experimental Surgery and Surgical Research, School of Medicine, Democritus University of Thrace, Alexandroupolis GR-68100, Greece*

ARTICLE INFO

Article history:
Received 8 February 2012
Received in revised form
2 April 2012
Accepted 9 April 2012
Available online 17 April 2012

Keywords:
Probiotics
Adhesion
Lactobacillus casei ATCC 393
Rats

ABSTRACT

Adhesion to the intestine represents a critical parameter for probiotic action. In this study, the adhesion ability of *Lactobacillus casei* ATCC 393 to the gastrointestinal tract of Wistar rats was examined after single and daily administration of fermented milk containing either free or immobilized cells on apple pieces. The adhesion of the probiotic cells at the large intestine (cecum and colon) was recorded at levels ≥6 logCFU/g (suggested minimum levels for conferring a probiotic effect) following daily administration for 7 days by combining microbiological and strain-specific multiplex PCR analysis. Single dose administration resulted in slightly reduced counts (5 logCFU/g), while they were lower at the small intestine (duodenum, jejunum, ileum) (≤3 logCFU/g), indicating that adhesion was a targeted process. Of note, the levels of *L. casei* ATCC 393 were enhanced in the cecal and colon fluids both at single and daily administration of immobilized cells (6 and 7 logCFU/g, respectively). The adhesion of the GI tract was transient and thus daily consumption of probiotic products containing the specific strain is suggested as an important prerequisite for retaining its levels at an effective concentration.

1. Introduction

The development of novel functional foods containing probiotic microorganisms, such as bifidobacteria and lactic acid bacteria (LAB) represents a highly growing area of the food industry and attracts special interest from the field of nutrition, due to their beneficial properties to human health [1]. Intake of probiotics has been linked with the prevention or treatment of various pathological conditions including, rotaviral gastroenteritis, antibiotic-associated diarrhea and intestinal inflammatory disorders such as chronic pouchitis, ulcerative colitis, Crohn's disease and irritable bowel syndrome (IBS) [2,3]. However, to deliver their health benefits, probiotics must be present in food products above a threshold level (≥6 logCFU/g) at the time of consumption, to compensate for the loss of cells during the passage through the upper and lower parts of the gastrointestinal (GI) tract [4,5].

Among LAB, *Lactobacillus casei* ATCC 393 strain has been extensively incorporated into food products due to its excellent technological properties [6,7]. Moreover, it has been demonstrated that *L. casei* ATCC 393 displayed beneficial effects for the removal of cholesterol [8] and activity against cancer cell proliferation [9]. In addition, our group has showed recently that *L. casei* ATCC 393 survived the passage through the GI tract and caused significant reduction of staphylococci, enterobacteria, coliforms and streptococci counts, in a rat model, indicating potential regulation of the intestinal microbiota [10]. Interestingly, the probiotic bacteria were detected at feces at levels ≥6 logCFU/g, by 12 h after single dose administration of fermented milk [10].

Although studies on the survival of administered probiotic bacteria by analyzing fecal samples offer an indication that the strains survived the harsh conditions of the intestine, they do not reveal the number of cells that remain attached to the intestinal tract. Thus, the main objective of this study was to investigate adhesion of probiotic *L. casei* ATCC 393 to the gastrointestinal epithelia.

Adhesion of probiotics to the intestinal mucosa is an important prerequisite for colonization of the gastrointestinal tract. It has been studied mainly using *in vitro* model systems, such as the Caco-2 and HT-29 human epithelial cell lines [11–13]. However, as each *in vitro* model describes a different part of the GI tract, it is difficult

* Corresponding author. Tel.: +30 25510 30634; fax: +30 25510 30624.
E-mail address: agalanis@mbg.duth.gr (A. Galanis).

1075-9964/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2012.04.002

to extrapolate *in vitro* results to the *in vivo* situation. The use of biopsies from the intestinal mucosa is considered the most accurate means of determining adhesion [11,14]. Hence, in our study the adhesion properties of *L. casei* ATCC 393 were examined *in vivo*, using a rat model system.

2. Materials and methods

2.1. Bacterial strain and culture conditions

L. casei ATCC 393 (DSMZ, Germany) was grown at 37 °C for 72 h on MRS broth. Pressed wet weight cells (≈0.5–1.0 g dry weight) were prepared and used directly for cell immobilization or in probiotic-fermented milk production.

2.2. Cell immobilization and fermented milk production

Cell immobilization on apple pieces and fermented milk production was carried out as described previously [7]. In brief, pressed wet cells of *L. casei* ATCC 393 were re-diluted in sterilized MRS broth, the proper amount of apple pieces was added and the mixture was allowed overnight at 37 °C without agitation. When the immobilization was completed, the fermented liquid was decanted and the immobilized cells were washed twice with sterilized ¼ Ringer's solution. The level of immobilized cells equaled to ≈9 logCFU/g was determined after homogenization of apple pieces with ¼ Ringer's solution, serial diluting and plating on acidified MRS agar (Fluka) at 37 °C for 48 h anaerobically. Probiotic fermented milk was produced by inoculating pasteurized milk with either free or immobilized cells to a concentration of approximately 9 logCFU/g and the mixture was allowed stationary overnight at 37 °C [7]. The final pH in both cases was ≈3.8. At the time of administration, both products contained ≈9 logCFU/g *L. casei* ATCC 393 cells. The number of cells was verified daily by serial diluting and plating on acidified MRS agar as described above. For the negative control experiments, only commercial pelleted diet was followed.

2.3. Animals

A total of 72 Wistar rats, 3–4 months of age, weighing 250–300 g, were used. They were housed in polycarbonate cages, 1 rat per cage, at room temperature, on a 12 h light: 12 h dark cycle and were provided with tap water and commercial pelleted diet (Mucedola) free of lactobacilli, consisted of soybean, soybean oil, wheat, maize, fish and lucerne meal, whey powder, mineral dicalcium phosphate, corn gluten feed, halzenut skins, calcium carbonate, yeasts, magnesium oxide and sodium chloride, *ad libitum* in sterile cages.

2.4. Study design

The study was divided in two experiments: At experiment 1, 12 rats were randomly assigned into 2 groups of 6 animals each and were administered a single dose of 1 g/rat of fermented milk containing free (group F1-24) or immobilized (≈1 g of apple) (group I1–24) *L. casei* ATCC 393 cells by intragastric gavage using a blunt-ended needle. Similarly, at experiment 2, 12 rats were randomly assigned into 2 groups of 6 animals each and fermented milk containing ≈9 logCFU/g of either free (group F2–24) or immobilized (group I2–24) *L. casei* ATCC 393 cells was administered orally at a dose of 1 g/rat/day for 7 days. In both cases, an additional group (N1–24 and N2–24, respectively) of 6 animals administrated no probiotic fermented milk was used as negative control. 24 h post administration, intestinal content and tissue samples from distinctive parts of the small (duodenum, jejunum, ileum) and the large (cecum and colon) intestine, were collected and subjected to microbiological and molecular analysis. The experiment procedures were repeated and samples were collected 48 h post administration (groups F1–48, I1–48, F2–48, I2–48, N1–48, N2–48, respectively). Samples from all animals were tested and each analysis was repeated twice. Prior to collection, the animals were subjected to sevoflurane anesthesia. The experimental protocols were approved by the Animal Care and Use Committee of the local Veterinary Service since they were in compliance with Directive 86/609/EEC.

2.5. Microbiological analysis and enumeration of lactobacilli

The small and large intestines were removed aseptically and 3-cm long individual sections were cut longitudinally. After collection of the intestinal fluids, the tissue samples were washed twice with sterile buffered peptone water and vortex mixed to break down bacterial clumps and to remove loosely attached bacteria, as it is well described in previous studies [15,16]. Then, the tissues were homogenized in a tissue grinder with 5 ml sterilized buffered peptone water [16]. Both intestinal and tissue samples were subjected to serial dilutions and lactobacilli [Gram (+), catalase (−)] counts were determined on acidified MRS agar (Fluka) at 37 °C for 48 h anaerobically [10].

Noticeably, analysis of lactobacilli was also carried out in the commercial pellets used to feed the rats. Lactobacilli [Gram (+), catalase (−)] counts were determined after blending of representative 10 g portions of duplicate samples with 90 ml of sterilized ¼ Ringer's solution, serial diluting and plating on acidified MRS agar (Fluka) at 37 °C for 48 h anaerobically. As expected, no colonies were observed.

2.6. Identification of L. casei ATCC 393 by multiplex PCR

To confirm the presence of *L. casei* ATCC 393 in the intestinal samples, after growth of the cultures on MRS agar, the plates corresponding to all dilutions were washed with 1 mL sterilized ¼ Ringer's solution and then the cell suspension was subjected to molecular analysis based on multiplex PCR methodology, as described before [17]. Briefly, genomic DNA from lactobacilli suspensions prepared after microbiological analysis was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Multiplex PCR reactions were carried out in a total volume of 50 μL, containing 5 units Taq DNA polymerase (HyTest Ltd), 400 μM each dNTPs (Promega), 1.5 mM $MgCl_2$ (HyTest Ltd) and 100 ng template DNA. The specific primers of *L. casei* ATCC 393 consisted of GGCGACCAAGGCAGCG (10 pmol), CTTCGGTTTCATCTTCC (50 pmol) and GGCCAACTTTTTCCATA (50 pmol). Additionally, a set of primers consisted of AGCAGTAGGGAATCTTCCA (10 pmol) and ATTYCACCGCTACACATG (10 pmol) was used as positive control [18]. Amplification was carried out in a Thermal Cycler (Mastercycler, Eppendorf) under the following conditions: 94 °C (2 min), followed by 25 cycles of 94 °C (15 s), 51 °C (15 s), 72 °C (30 s), followed by a final extension step at 72 °C (1 min). The PCR products were separated on 1.5% w/v agarose gels, visualized under UV illumination and photographed with a digital camera (Gel Doc EQ System, Biorad).

3. Results and discussion

Adhesion of the intestine is crucial for providing the beneficial effects of probiotics, since it may influence interaction with the host and the other bacteria present, affect the local microbial composition and/or stimulate the host's immune system. Although many

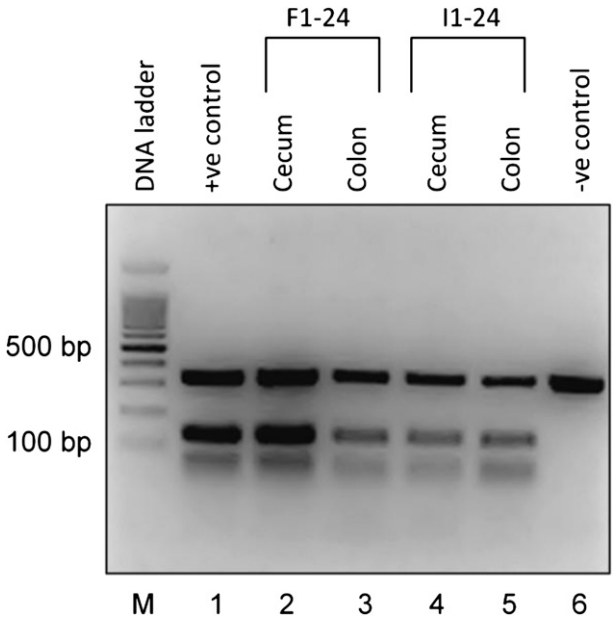

Fig. 1. Identification of *L. casei* ATCC 393 by multiplex PCR. Tissue samples from cecum and colon were collected 24 h after single dose administration of fermented milk containing either free (lanes 2, 3), or immobilized cells (lanes 4, 5). The samples were first subjected to microbiological analysis and then the presence of *L. casei* ATCC 393 at levels ≥5 logCFU/g was confirmed by multiplex PCR. Pure culture of *L. casei* ATCC 393 served as a positive control (lane 1). PCR products of 67 bp and 144 bp are unique for *L. casei* ATCC 393, whereas the PCR product of 340 bp is universal for lactobacilli. Lane 6 that corresponds to no probiotic fermented milk served as a negative control.

in vitro studies have demonstrated the adherence of *Lactobacillus* to the epithelial surface, a limited number of *in vivo* adhesion studies have been performed [15,19,20].

In the present study, we investigated *in vivo* adhesion of *L. casei* ATCC 393 to rat intestinal mucosa. Probiotic fermented milk produced by either free or immobilized *L. casei* ATCC 393 cells was administered orally at a single dose or daily for 7 days in Wistar rats and the effects of the delivery vehicle used and the frequency of consumption on the adhesion ability of the probiotic bacteria were studied.

At 24 h post single dose administration, microbiological and multiplex PCR analysis demonstrated that in both groups (F1–24 and I1–24), *L. casei* ATCC 393 was detected at levels of ≥5 logCFU/g at the cecum and colon (Fig. 1), levels were reduced to 3 logCFU/g at the jejunum and ileum, and were undetected at the duodenum (Table 1). The probiotic strain was undetected at the GI tract 48 h post administration in both free (F1–48) (Fig. 2A) and immobilized (I1-48) (Fig. 2B) groups. Similarly, daily administration indicated adhesion of the large intestine by both free and immobilized *L. casei* ATCC 393 at levels ≥6 logCFU/g, at 24 h post administration (Table 2). The levels of the probiotic strain were lower at the duodenum, jejunum and ileum (Table 2). Yet again, the probiotic strain was not found in the GI tract at 48 h post administration (groups F2–48, I2–48) (data not shown). Of note, the levels of *L. casei* ATCC 393 were enhanced in the cecal and colon fluids following single and daily administration of immobilized cells (≥6 and ≥7 logCFU/g, respectively) (Tables 1, 2). Remarkably,

Table 1
Levels of *L. casei* ATCC 393 recovered from intestinal content and tissue samples of the GI tract of rats 24 h after single dose administration of fermented milk containing either free (Group F1–24) or immobilized (Group I1–24) probiotic bacteria.

Specimens	Group F1–24 (logCFU/g)	Group I1–24 (logCFU/g)
Duodenal fluid	ND	ND
Jejunal fluid	3	3
Ileal fluid	3	3
Cecal fluid	5	6
Colon fluid	5	6
Duodenum	ND	ND
Jejunum	3	3
Ileum	3	3
Cecum	5	5
Colon	5	5

ND: not detected.

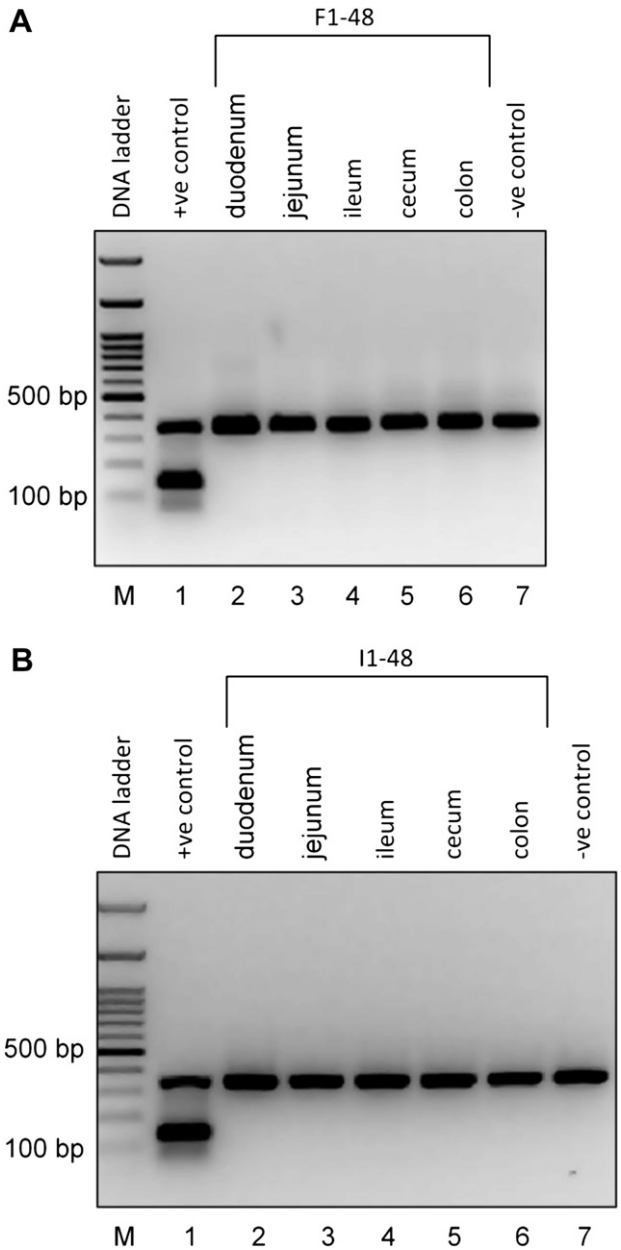

Fig. 2. Investigation of the presence of *L. casei* ATCC 393 on the small (duodenum, jejunum, ileum), and the large intestine (cecum and colon). Tissue samples were collected 48 h after single dose administration of fermented milk containing (A) free *L. casei* ATCC 393 cells (B) immobilized *L. casei* ATCC 393 cells on apple pieces and subjected to microbiological and strain-specific multiplex PCR analysis. Pure culture of *L. casei* ATCC 393 served as a positive control (lane 1). PCR products of 67 bp and 144 bp are unique for *L. casei* ATCC 393, whereas the PCR product of 340 bp is universal for lactobacilli. Lane 7 that corresponds to no probiotic fermented milk served as a negative control.

Table 2
Levels of *L. casei* ATCC 393 recovered 24 h post administration from intestinal content and tissue samples of the GI tract of rats orally dosed with fermented milk containing either free (Group F2-24) or immobilized (Group I2–24) probiotic bacteria daily for a 7-day period.

Specimens	Group F2–24 (logCFU/g)	Group I2–24 (logCFU/g)
Duodenal fluid	2	2
Jejunal fluid	3	3
Ileal fluid	3	3
Cecal fluid	6	7
Colon fluid	6	7
Duodenum	2	2
Jejunum	3	3
Ileum	3	3
Cecum	6	6
Colon	6	6

L. casei ATCC 393 was not detected in the intestinal fluids and tissues in the groups of rats (N1–24, N2–24, N1–48, N2–48) used as negative controls (data not shown).

For many probiotics, the aim is to achieve at least transient adhesion, in which case they grow or at least metabolize in the intestine. Here, we showed that *L. casei* ATCC 393 was able to survive transit through the stomach and small intestine and adhere to the large intestine at levels compatible with a physiological effect ($\geq$6 logCFU/g). However, the probiotic strain was not detected at 48 h post administration, demonstrating that the adhesion of the GI tract was only transient. These results extend our previous findings reporting that the levels of *L. casei* ATCC 393 at fecal samples were $\geq$6 logCFU/g 24 h post administration, reduced to 4 logCFU/g at 36 h, while no probiotic bacteria were detected by 48 h [10]. Apparently, daily consumption of probiotic products containing the specific strain is a prerequisite for retaining its levels at an effective concentration, information that could be valuable in food industry.

Lower levels of *L. casei* ATCC 393 were detected in tissue samples from the duodenum, jejunum and ileum, indicating that adhesion of the GI tract is a distinctive process and as previous studies demonstrated it may differ even among high related strains [15,21]. Indeed, it has been established that the mechanism of action of each probiotic is based on distinct molecular and cellular parameters, such as the rate of cell growth and division [22], or the presence of unique genes and gene products that offer improved adaptation in the host [23,24]. Accordingly, it has been demonstrated, that the adhesion ability of *L. casei* ATCC 393 to the human epithelial cell line Caco-2 enhanced when the probiotic bacteria heterologously expressed the collagen-binding protein gene *cnb* originally derived from *Lactobacillus reuteri* Pg4 [25]. Apparently, the ability of *L. casei* ATCC 393 to adhere to intestinal epithelial cells is relatively poor [26,27]. This also may explain the fact that in our study no differences concerning adhesion ability were observed among free and immobilized cells, although it has been clearly documented that cell immobilization enhances the viability of cultures [6,7] and both the delivery vehicle and the food matrix may influence surface properties and adhesion abilities of lactobacilli [11,14]. Although, further research is required, our findings may be highly instructive in elucidating the mechanisms underlying the physiological effects exhibited by probiotic strain *L. casei* ATCC 393.

In conclusion, this study demonstrated adhesion of the gastrointestinal epithelia by *L. casei* ATCC 393 after dietary probiotic treatment of Wistar rats. Distinct adhesion of the large intestine at levels that might be compatible with a probiotic effect was documented by microbiological and multiplex PCR analysis.

References

[1] Saxelin M. Probiotic formulations and applications, the current probiotics market, and changes in the marketplace: a European perspective. Clin Infect Dis 2008;2:S76–9.
[2] Sullivan A, Nord CE. Probiotics and gastrointestinal diseases. J Int Med 2005; 257:78–92.
[3] Marteau PR, de Vrese M, Cellier CJ, Schrezenmeir J. Protection of the gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 2001;73(Suppl.): 430S–6S.
[4] Boylston TD, Vinderola CG, Ghoddusi HB, Reinheimer JA. Incorporation of bifidobacteria into cheeses: challenges and rewards. Int Dairy J 2004;19: 315–87.
[5] Tamime AY, Saarela M, Korslund Sondergaard A, Mistry VV, Shah NP. Production and maintenance of viability of probiotic micro-organisms in dairy products. London: Tamime AY: Probiotic Dairy Products, Blackwell Publishing Ltd; 2005. pp. 39–72.
[6] Kourkoutas Y, Bosnea L, Taboukos S, Baras C, Lambrou D, Kanellaki M. Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces. J Dairy Sci 2006;89:1431–51.
[7] Kourkoutas Y, Ksolis V, Kallis M, Bezirtzoglou E, Kanellaki M. *Lactobacillus casei* immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. Process Biochem 2005;40:411–6.
[8] Lye HS, Rusul G, Liong MT. Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. J Dairy Sci 2010;93:1383–92.
[9] Choi SS, Kim Y, Han KS, You S, Oh S, Kim SH. Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress *in vitro*. Lett Appl Microb 2006; 42:452–8.
[10] Sidira M, Galanis A, Ypsilantis P, Karapetsas A, Progaki Z, Simopoulos C, et al. Effect of probiotic-fermented milk administration on gastrointestinal survival of *Lactobacillus casei* ATCC 393 and modulation of intestinal microbial flora. J Mol Microb Biotech 2010;19:224–30.
[11] Ouwehand AC, Salminen S. *In vitro* adhesion assays for probiotics and their *in vivo* relevance: a review. Microb Ecol Health Dis 2003;15:175–84.
[12] Tuomola EM, Salminen SJ. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int J Food Microb 1998;41:45–51.
[13] Vizoso Pinto MG, Schuster T, Briviba K, Watzl B, Holzapfel WH, Franz CM. Adhesive and chemokine stimulatory properties of potentially probiotic *Lactobacillus* strains. J Food Prot 2007;70:125–34.
[14] Deepika G, Charalampopoulos D. Surface and adhesion properties of lactobacilli. Adv Appl Microb 2010;70:127–52.
[15] Wang B, Li J, Li Q, Zhang H, Li N. Isolation of adhesive strains and evaluation of the colonization and immune response by *Lactobacillus plantarum* L2 in the rat gastrointestinal tract. Int J Food Microb 2009;132:59–66.
[16] Wang B, Huang Q, Zhang W, Li N, Li J. *Lactobacillus plantarum* prevents bacterial translocation in rats following ischemia and reperfusion injury. Dig Dis Sci 2011;56:3187–94.
[17] Karapetsas A, Vavoulidis E, Galanis A, Sandaltzopoulos R, Kourkoutas Y. Rapid detection and identification of probiotic *Lactobacillus casei* ATCC 393 by multiplex PCR. J Mol Microb Biotech 2010;18:156–61.
[18] Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* Species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microb 2001;67:2578–85.
[19] Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Appl Environ Microb 2004;70:1176–81.
[20] Mare L, Wolfaardt GM, Dicks LMT. Adhesion of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 to the intestinal tract of piglets, as recorded with fluorescent in situ hybridization (FISH), and production of plantaricin 423 by cells colonized to the ileum. J Appl Microb 2006;100:838–45.
[21] Ibnou-Zekri N, Blum S, Schiffrin EJ, von der Weid T. Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties *in vitro*. Infect Immun 2003;71:428–36.
[22] Lee YK, Ho PS, Low CS, Arvilommi H, Salminen S. Permanent colonization by *Lactobacillus casei* is hindered by the low rate of cell division in mouse gut. Appl Environ Microb 2004;70:670–4.
[23] Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, et al. Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. Proc Natl Acad Sci USA 2009;106: 17193–8.
[24] Lebeer S, Verhoeven TL, Francius G, Schoofs G, Lambrichts I, Dufrêne Y, et al. Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. Appl Environ Microb 2009;75:3554–63.
[25] Hsueh HY, Yueh PY, Yu B, Zhao X, Liu JR. Expression of *Lactobacillus reuteri* Pg4 collagen-binding protein gene in *Lactobacillus casei* ATCC 393 increases its adhesion ability to Caco-2 cells. J Agric Food Chem 2010;58:12182–91.
[26] Edelman S, Westerlund-Wikström B, Leskelä S, Kettunen H, Rautonen N, Apajalahti J, et al. *In vitro* adhesion specificity of indigenous lactobacilli within the avian intestinal tract. Appl Environ Microb 2002;68:5155–9.
[27] Liu JR, Yu B, Liu FH, Cheng KJ, Zhao X. Expression of rumen microbial fibrolytic enzyme genes in probiotic *Lactobacillus reuteri*. Appl Environ Microb 2005;71: 6769–75.