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Effect of Probiotic-Fermented Milk Administration on Gastrointestinal Survival of *Lactobacillus casei* ATCC 393 and Modulation of Intestinal Microbial Flora

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Key Words
Fermented milk · *Lactobacillus casei* · Probiotics · Wistar rats · Multiplex PCR

Abstract
The aim of the present study was to assess the survival of free and immobilized *Lactobacillus casei* ATCC 393 on apple pieces, contained in probiotic-fermented milk, after gastrointestinal (GI) transit and to investigate the potential regulation of intestinal microbial flora in a rat model. In in vitro GI stress tolerance tests, immobilized *L. casei* ATCC 393 exhibited significantly higher survival rates compared to free cells. At a second stage, probiotic-fermented milk produced by either free or immobilized cells was administered orally at a single dose or daily for 9 days in Wistar rats. By 12 h after single-dose administration, both free and immobilized cells were detected by microbiological and molecular analysis at levels ≥6 logCFU/g of feces. Moreover, daily administration led to significant reduction of staphylococci, enterobacteria, coliforms and streptococci counts. In conclusion, *L. casei* ATCC 393 contained in fermented milk survived GI transit and modulated intestinal microbiota.

Introduction

Nowadays there is an upsurge of interest in developing novel foods containing probiotic microorganisms, such as bifidobacteria and lactic acid bacteria (LAB). Such functional foods demonstrate a great potential in promoting human health. Maintenance of the intestinal microbial homeostasis, prevention of pathogenic infections, stabilization of the gastrointestinal (GI) barrier function and production of anti-carcinogenic and anti-mutagenic compounds [Boirivant and Strober, 2007; Choi et al., 2006; Saulnier et al., 2009] are included among the beneficial effects of probiotic-based foods, mainly yogurt and other dairy products. Therefore, a probiotic-rich diet is linked with the prevention and potential treatment of several severe digestive disorders, such as inflammatory bowel disease [Boirivant and Strober, 2007], colorectal cancer [Fotiadis et al., 2008], etc.

To deliver the health benefits, probiotics need to contain an adequate amount of live bacteria (at least 10^6–10^7 CFU/g) [Boylston et al., 2004; Oliveira et al., 2002], able to survive the acidic conditions of the upper GI tract and proliferate in the intestine, a requirement that is not always fulfilled [Boylston et al., 2004; Vinderola et al., 2002]. It is established that cell immobilization enhances the viability of cultures [Kourkoutas et al., 2005, 2006a;

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Mattila-Sandholm et al., 2002]. Many efforts have focused on immobilization of LAB in various supports such as starch [Mattila-Sandholm et al., 2002], wheat grains [Bosnea et al., 2009] and casein [Dimitrellou et al., 2008, 2009], aiming in stabilization of cells and formulation of new types of foods fortified with encapsulated health-promoting bacteria that are only released upon reaching the human gut. Likewise, fruit pieces [Kopsahelis et al., 2007; Kourkoutas et al., 2005, 2006a] were previously used as immobilization supports of LAB and the immobilized biocatalysts were tested in fermented milk [Kourkoutas et al., 2005] and cheese production [Kourkoutas et al., 2006a]. Enhanced cell survival, improved quality characteristics and elevated resistance to contamination were evident. As fruit pieces contain cellulose that is not digested, immobilized probiotic cultures on fruit pieces may act advantageously in cell survival and proliferation in the colon, enhancing thus the beneficial effects.

Among LAB, *Lactobacillus casei* ATCC 393 strain has been extensively incorporated into food products [Kourkoutas et al., 2005, 2006a; Li et al., 2009]. Recently, a new method for rapid detection and identification of the above strain based on multiplex PCR was demonstrated [Karapetsas et al., 2010]. The presented methodology proved an efficient tool for accurate, convenient and reliable identification of the *L. casei* ATCC 393 in food products.

Although there are several publications concerning in vitro and in vivo evaluation of many LAB strains as probiotics [Guo et al., 2009; Minelli et al., 2004; Schillinger et al., 2005], there is only a limited number of preliminary studies available in literature concerning the beneficial effects of *L. casei* ATCC 393, focusing mainly in activity against cancer cell proliferation [Choi et al., 2006] and osteoporosis [Lee et al., 2005, 2006]. Hence, the aim of the present study was to assess the probiotic properties of free and immobilized *L. casei* ATCC 393 on apple pieces, contained in probiotic-fermented milk, by investigating their survival after transit through the GI tract and their role as potential regulators of the intestinal microbial flora.

Results

In vitro Assessment of L. casei *ATCC 393 Survival*

The effects of GI stress tolerance tests on the survival of free and immobilized *L. casei* ATCC 393 are summarized in tables 1 and 2. Although acidic conditions resulted in a significant reduction of *L. casei* ATCC 393 levels in both free and immobilized cells, counts of immobilized cells were significantly higher after 120 min at pH 2.0 and after 30, 60, 90 and 120 min at pH 1.5 compared to free cells. Likewise, cell immobilization resulted in significantly higher survival rates in pancreatic juices supplemented with 0.45% bile salts after 240 min and in bile salts after 120 min.

Table 1. Effect of acidic conditions on the survival of immobilized and free *L. casei* ATCC 393

pH	Time min	Immobilized *L. casei* logCFU/g	Free *L. casei* logCFU/g
4.0	0	9.30 ± 0.2	9.16 ± 0.1
	30	8.74 ± 0.2	9.03 ± 0.1
	60	8.53 ± 0.3	8.30 ± 0.3
	90	7.56 ± 0.2*	7.69 ± 0.3*
	120	7.17 ± 0.1*	7.39 ± 0.2*
3.0	0	9.30 ± 0.2	9.16 ± 0.1
	30	7.60 ± 0.3*	7.77 ± 0.2*
	60	7.28 ± 0.2*	7.54 ± 0.2*
	90	7.23 ± 0.2*	7.38 ± 0.2*
	120	7.11 ± 0.1*	7.24 ± 0.1*
2.0	0	9.51 ± 0.2	9.05 ± 0.2
	30	8.14 ± 0.1*	8.00 ± 0.3
	60	7.56 ± 0.3*	6.30 ± 0.2*
	90	5.39 ± 0.2*	5.21 ± 0.2*
	120	5.13 ± 0.1*	3.98 ± 0.2*, #
1.5	0	9.51 ± 0.2	9.05 ± 0.2
	30	6.47 ± 0.2*	4.17 ± 0.1*, #
	60	4.74 ± 0.2*	3.04 ± 0.3*, #
	90	4.71 ± 0.2*	2.84 ± 0.2*, #
	120	4.36 ± 0.2*	2.30 ± 0.2*, #

* $p < 0.05$ vs. time 0; # $p < 0.05$ vs. immobilized.

In vivo Tests

The general health and appearance of the rats fed with the probiotic-fermented milk products was good and weight gain was regular for both the treated and untreated animals. No intestinal disturbance such as diarrhea was observed.

Experiment 1: Effect of Single-Dose Administration of Probiotic-Fermented Milk on GI Survival of *L. casei* ATCC 393

In both groups of rats administered a single dose of fermented milk containing free (group F-1) or immobilized *L. casei* ATCC 393 (group I-1), the above strain was detected at levels of ≥6 logCFU/g of feces at 12 and 24 h post-administration, while it was undetectable at 0 h (fig. 1).

Table 2. Effect of simulated gastric transit, pancreatic juice and bile salts on the survival of immobilized and free *L. casei* ATCC 393

Gastrointestinal stress tolerance tests	Time min	Immobilized *L. casei*, logCFU/g	Free *L. casei* logCFU/g
Simulated gastric transit tolerance	0	9.30 ± 0.2	9.16 ± 0.1
	1	8.11 ± 0.1*	7.90 ± 0.2*
	90	8.17 ± 0.1*	7.85 ± 0.2*
	180	8.17 ± 0.1*	7.54 ± 0.2*
Tolerance to pancreatic juices	0	9.30 ± 0.2	9.16 ± 0.1
	1	8.55 ± 0.2	7.70 ± 0.2*
	240	8.59 ± 0.2	7.90 ± 0.2*
Tolerance to pancreatic juices supplemented with 0.45% bile salts	0	9.30 ± 0.2	9.16 ± 0.1
	1	5.75 ± 0.2*	4.74 ± 0.2*
	240	5.71 ± 0.2*	3.25 ± 0.2*, #
Tolerance to bile salts	0	9.30 ± 0.2	9.16 ± 0.1
	120	6.23 ± 0.2*	3.66 ± 0.3*, #

* $p < 0.05$ vs. time 0; # $p < 0.05$ vs. immobilized.

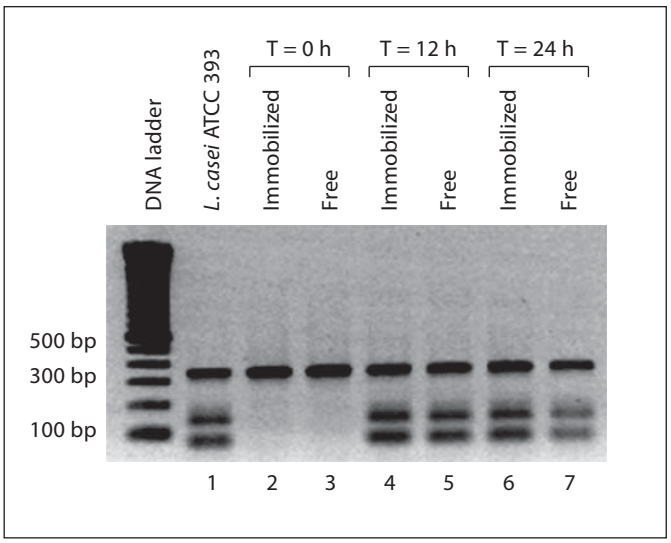

Fig. 1. Molecular identification of *L. casei* ATCC 393 at levels ≥6 logCFU/g in rat feces using multiplex PCR assay at 0 h (lanes 2, 3), 12 h (lanes 4, 5) and 24 h (lanes 6, 7) after single-dose administration of fermented probiotic milk products containing free or immobilized cells. Pure culture of *L. casei* ATCC 393 served as a positive control (lane 1). PCR products of 67 and 144 bp are unique for *L. casei* ATCC 393. PCR product of 340 bp is universal for lactobacilli.

Levels were reduced to 4 logCFU/g at 36 h, while *L. casei* ATCC 393 was not detected at 48 h (data not shown).

Identification of *L. casei* ATCC 393 after growth on MRS agar plates was carried out by multiplex PCR assay using specific primers that generated PCR products of 67 and 144 bp [Karapetsas et al., 2010]. A set of universal for lactobacilli primers generating a PCR product of 340 bp was used as positive control [Walter et al., 2001].

Experiment 2: Effect of Daily Administration of Probiotic-Fermented Milk on Intestinal Microbial Flora

At all time-points (1–9 days) after administration of fermented milk containing free (group F-2) or immobilized (group I-2) cells, *L. casei* ATCC 393 was detected at levels ≥6 logCFU/g in rat feces (data not shown). Microbiological analysis of feces showed that administration of milk with apple pieces (group MAP-2) or acidified milk (group AM-2) resulted in no significant reduction of microbial counts in all cases (fig. 2). In contrast, there were fluctuations in total aerobic counts in group I-2. Staphylococci counts were significantly reduced in both I-2 and F-2 groups by days 1 and 2, respectively. Coliforms and enterobacteria counts were significantly reduced in group I-2 by day 7, while in group F-2 at day 9. A decrease in streptococci counts was noted at days 1, 2, 7 and 9 in group I-2 and only at day 9 in group F-2. No differences were observed in lactobacilli counts (fig. 2).

Discussion

L. casei ATCC 393 has been proposed for the production of dairy products due to its excellent technological properties [Bosnea et al., 2009; Kourkoutas et al., 2005, 2006a], while cell immobilization on apple pieces has shown significantly increased survival rates over free cells during food manufacture and storage [Kourkoutas et al., 2005, 2006a]. However, the probiotic properties of the above strain have not been documented. Thus, the scope of the present study was to assess survival of *L. casei* ATCC 393 during GI transit, which constitutes an essential requirement for probiotic characterization. The strategy adopted involved initially in vitro GI stress tolerance tests and subsequently in vivo assays to study GI survival of free and immobilized cells contained in probiotic-fermented milk using Wistar rats, since they have been proposed as animal model in many similar studies [de Waard et al., 2002; Liong et al., 2006; Minelli et al., 2004; Montesi et al., 2005]. Finally, possible regulation of fecal microbial flora was investigated.

The pH of the gastric juice is considered among the main factors determining the survival of probiotic bacteria upon passage through the stomach to the intestine. In

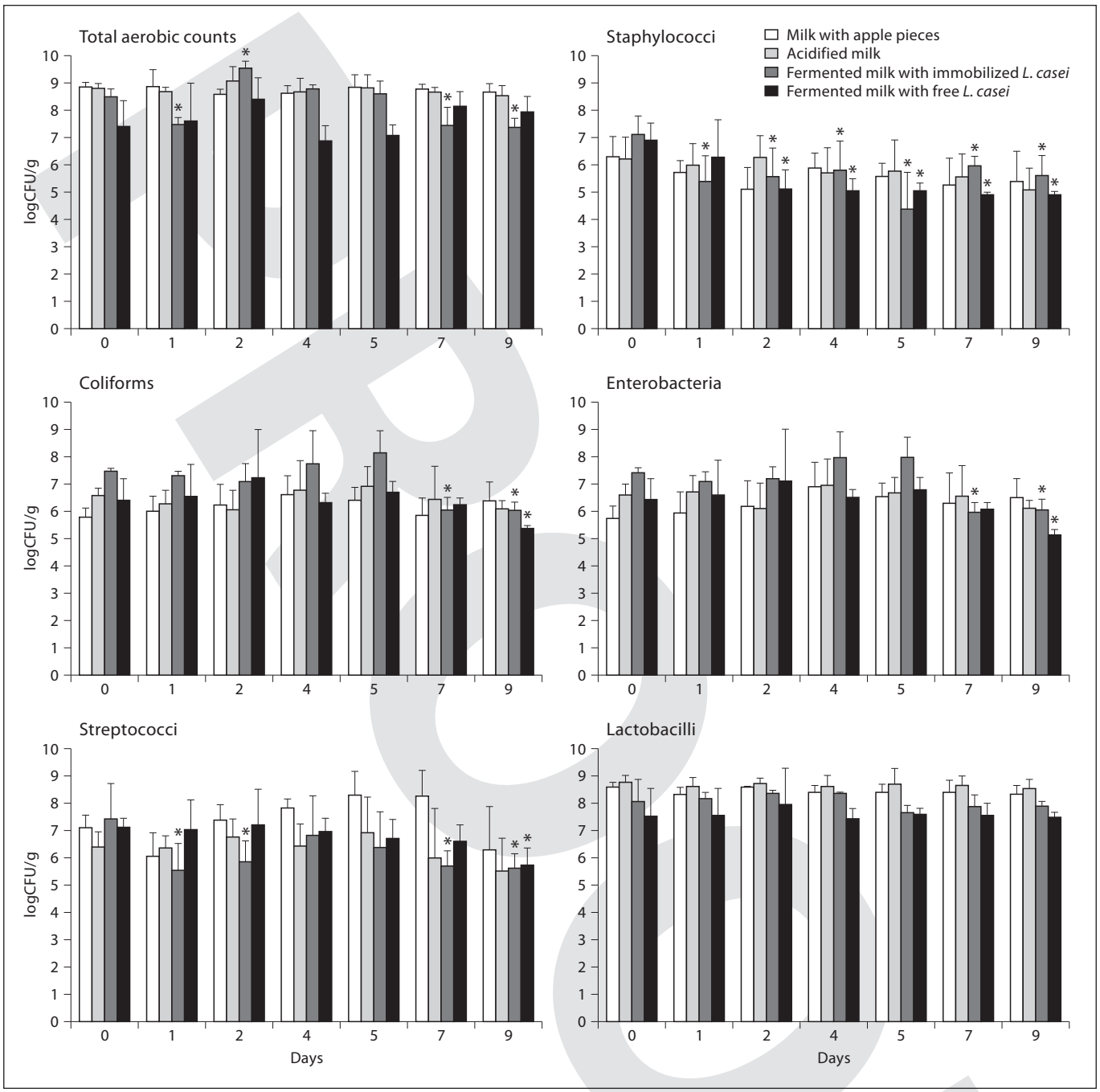

Fig. 2. Effect of daily administration of milk with apple pieces, acidified milk and probiotic-fermented milk containing free or immobilized *L. casei* ATCC 393 on intestinal microbial flora (*$p < 0.05$ vs. day 0).

addition to the buffering capacity of the food, which is a major factor affecting pH, and the rate of gastric emptying, a potential protective environment may significantly influence cell survival in the GI transit. Acid tolerance is essential not only for withstanding gastric stresses, but it is also a prerequisite for the production of acid probiotic food products [Kourkoutas et al., 2005, 2006a].

To accurately predict tolerance to upper GI transit, either free or immobilized cells of *L. casei* ATCC 393 were exposed to in vitro conditions simulating acidic environ-

ment, gastric and pancreatic juices, and bile salts. The significantly improved resistance of immobilized cells to GI stress suggested that immobilization enhances cell survival [Kourkoutas et al., 2005, 2006a; Mattila-Sandholm et al., 2002]. Similarly, microencapsulation of *Bifidobacterium lactis* in traditional African fermented beverages resulted in significantly higher survival rates in the presence of simulated gastric juice and considerably higher viability over the shelf life compared to free cells [McMaster et al., 2005]. Likewise, significant improvement of survival of probiotic bacteria in GI transit and gastric acidity, due to the protective action of milk components, has also been previously reported [Charteris et al., 1998; Gardiner et al., 1999; Schillinger et al., 2005].

During the in vivo experiments, both free and immobilized *L. casei* ATCC 393 were detected in rat feces at levels required for conferring a probiotic effect (at least 6 logCFU/g) [Minelli et al., 2004] shortly after a single-dose administration (12 h), documenting that the above strain survived passage through the stomach and the intestine. These findings could be of vital importance in the clinical setting in order to treat enteropathogenic-derived diseases. However, our study demonstrated that daily consumption of probiotic products emerged as a prerequisite for retaining the levels of the strain at an effective concentration. Although there are several studies investigating the in vivo GI survival of probiotic strains [Djouzi et al., 1997; Huang et al., 2003; Montesi et al., 2005], references reporting determination of the levels of the administrated strain in animal feces are scarce in the literature.

The impact of *L. casei* ATCC 393 on modulating fecal microbial flora was also evaluated. The significantly reduced counts of staphylococci, enterobacteria, coliforms and streptococci in rat feces after oral administration of free or immobilized cells contained in probiotic-fermented milk revealed regulation of intestinal content microbiota, as administration of milk with apple pieces and acidified milk had no significant effect. Hence, it is not unreasonable to suggest the development of a microbial association, due to *L. casei* ATCC 393 cells, leading to repression of facultative pathogens, although pathogenesis has not been considered for all strains of the above microbial species. Suppression of staphylococci growth has been described in various food products by mixed LAB cultures [Dimitrellou et al., 2009; Kourkoutas et al., 2006b], while reduction of pathogenic bacteria counts in feces [de Waard et al., 2002; O'Mahony et al., 2001] and intestines of rats [de Waard et al., 2002; Liong et al., 2006; Montesi et al., 2005], due to the action of LAB and bifidobacteria has been previously reported. Further research using deliberately spiked animals with pathogens will give more insight into the role of *L. casei* ATCC 393 as endogenous protecting shield.

In conclusion, although cell immobilization resulted in enhanced survival in in vitro GI stress tolerance tests, both free and immobilized *L. casei* ATCC 393 survived passage through the GI tract and were detected in rat feces at the essential concentration for providing the health benefits after 12 h of probiotic treatment. In addition, the significant reduction of staphylococci, enterobacteria, coliforms and streptococci counts due to probiotic-fermented milk administration was observed, indicating potential protection against pathogens. However, more research is still needed to better understand the mechanisms of action of probiotics so that their activity in the balance of intestinal ecosystem can be appreciated. More evidence on the beneficial effects is still required to consolidate their role not only in GI disorders, but in many infectious diseases.

Experimental Procedures

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.

Cell Immobilization and Fermented Milk Production

Cell immobilization on apple pieces and fermented milk production was carried out as described previously [Kourkoutas et al., 2005]. In brief, pressed wet cells of *L. casei* ATCC 393 were rediluted in sterilized MRS broth, the proper amount of apple pieces was added and the mixture was allowed to stand 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 solution. The level of immobilized cells equaled to ≈9 logCFU/g and was determined after homogenization of apple pieces with ¼ Ringer solution, serial diluting and plating on acidified MRS agar (Fluka, 69964) at 37°C for 48 h anaerobically (Anaerobic Jar, Anerocult C, Merck).

Probiotic-fermented milk was produced by inoculating pasteurized milk with either free or immobilized cells up to a concentration of approximately 9 logCFU/g and the mixture was allowed to stand overnight at 37°C [Kourkoutas et al., 2005]. The final pH in both cases was ≈3.8.

For the control experiments, milk with apple pieces (pH: 6.7) and acidified milk (pH: 3.8) with addition of lactic acid were prepared.

In vitro GI Stress Tolerance Tests

Resistance to Acidic Conditions

Resistance was tested by inoculating tubes containing 9 ml MRS broth adjusted to pH 4.0, 3.0, 2.0 or 1.5 using 1 M HCl [Minelli et al., 2004] with 1 ml of liquid cell culture of *L. casei* ATCC

393 grown for at least 12 h or 1 g of immobilized cells (≈9 logCFU/ml). Survival was evaluated by plate count on MRS agar, after 30, 60, 90 and 120 min of incubation in acidic MRS broth at 37°C.

Resistance to Simulated Gastric Juices

Resistance was tested by inoculating tubes containing 9 ml simulated gastric juices [prepared fresh daily by suspending pepsin (Sigma-Aldrich, Poole, UK) (3 g/l) in sterile saline (0.5% w/v)] adjusted to pH 2.0 using 1 M HCl [Charteris et al., 1998] with 1 ml of liquid cell culture of *L. casei* ATCC 393 or 1 g of immobilized cells, as described above. Survival was evaluated by plate count on MRS agar, after 1, 90 and 180 min of incubation in simulated gastric juices at 37°C.

Resistance to Pancreatic Juices

Resistance was tested by inoculating tubes containing 9 ml simulated pancreatic juices [prepared fresh daily by suspending pancreatin USP (Sigma-Aldrich) (3 g/l) in sterile saline (0.5% w/v)] adjusted to pH 8.0 using 0.1 M NaOH at 37°C [Charteris et al., 1998] and simulated pancreatic juices supplemented with 0.45% bile salts with 1 ml of liquid cell culture of *L. casei* ATCC 393 or 1 g of immobilized cells, as described above. Survival was evaluated by plate count on MRS agar, after 1, 90 and 180 min of incubation in simulated gastric juices at 37°C.

Resistance to Bile Salts

Resistance was tested by inoculating tubes containing 9 ml MRS broth supplemented with 1% (w/v) bile salts (Sigma-Aldrich) with 1 ml of liquid cell culture of *L. casei* ATCC 393 or 1 g of immobilized cells, as described above. Survival was evaluated by plate count on MRS agar, after 120 min of incubation in MRS broth supplemented with bile salts at 37°C.

In vivo Assessments

Animals

36 Wistar rats, 3–4 months of age, weighing 250–300 g, were used. They were housed in polycarbonate cages, 1 rat per cage, at 20–22°C room temperature, on a 12 h light:12 h dark cycle and were provided with commercial pelleted diet and tap water ad libitum. The facilities were in accordance with Directive 86/609/EEC.

Study Design

Experiment 1: Single-Dose Administration of Probiotic-Fermented Milk

12 were randomly assigned into two groups of 6 animals each and were administered a single dose of 1 g/rat of fermented milk containing immobilized (≈1 g of apple) (group I-1) or free (group F-1) *L. casei* ATCC 393 by intragastric gavage using a blunt-ended needle. Feces were collected at 12, 24, 36, and 48 h post-administration and subjected to microbiological and molecular analysis in order to monitor *L. casei* ATCC 393 counts.

Experiment 2: Daily Administration of Probiotic-Fermented Milk

24 rats were randomly assigned into four groups of 6 animals each. Fermented milk containing ≈ 9 logCFU/g of either immobilized (group I-2) or free (group F-2) *L. casei* ATCC 393 was administered orally at a dose of 1 g/rat/day for 9 days. For comparison reasons, milk with apple pieces (group MAP-2) or acidified milk (group AM-2) was also administered orally as described above. Feces were collected daily and subjected to (a) molecular and microbiological analysis to monitor *L. casei* ATCC 393 counts and (b) microbiological analysis to assess microbial counts. Day 0 feces served as controls. After each collection, rats were placed in sterile cages.

The experimental protocols were approved by the Animal Care and Use Committee of the local Veterinary Service (permission No. T/1533/13-5-2009) since they were in compliance with Directive 86/609/EEC.

Microbiological Analysis

Rat feces were homogenized in sterilized buffered peptone water and subjected to serial dilutions. The following tests on microbiological analysis were performed: (i) total aerobic counts on plate count agar (Fluka, Buchs, Switzerland) at 30°C for 48 h, (ii) staphylococci on Baird Parker egg yolk tellurite medium (Fluka) at 37°C for 48 h and confirmed by a positive coagulase test, (iii) coliforms on violet red bile agar (Fluka) after incubation at 30°C for 24 h, (iv) enterobacteria on violet red bile glucose agar (Fluka) at 37°C for 24 h, (v) streptococci on bile esculin azide agar after incubation at 37°C for 24 h (Fluka) and (vi) lactobacilli [Gram (+), catalase (–)] on acidified MRS agar (Fluka) at 37°C for 48 h anaerobically (Anaerobic Jar, Anerocult C, Merck). All incubations were further extended up to 120 h, but no extra colonies were observed. Gram staining and catalase tests were performed for LAB confirmation. Results are presented as log of mean colony-forming units on solid media culture plates containing between 30 and 300 colonies per gram of feces. For determination of *L. casei* ATCC 393 levels in fecal samples, after growth of the cultures on MRS agar, the plates were washed with 1 ml sterilized ¼ Ringer solution and then the cell suspension was subjected to molecular analysis.

Molecular Identification of L. casei *ATCC 393*

Molecular identification of *L. casei* ATCC 393 strain in fecal samples was carried out as described recently [Karapetsas et al., 2010]. Briefly, genomic DNA from lactobacilli suspensions prepared after microbiological analysis as described above, 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 for *L. casei* ATCC 393 primers that recognize the *hsp60* gene consisted of GGCGACCAAGGCAGCG (10 pmol), CTTCGGTTTCATCTTCC (50 pmol) and GGCCAACTTTTTCCATA (50 pmol) [Karapetsas et al., 2010]. Additionally, a set of primers that recognize the *16S rRNA* gene of all *Lactobacillus* strains was used as positive control. It consisted of AGCAGTAGGGAATCTTCCA (10 pmol) and ATTYCACCGCTACACATG (10 pmol) [Walter et al., 2001]. 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).

Statistical Analysis

Data were expressed as means ± SD and were subjected to repeated measures analysis of variance (ANOVA). Differences between means were evaluated using the Student's t test. A probability of less than 5% ($p < 0.05$) was considered to be statistically significant. Data were computed using the Microsoft Excel 2007 software.

Disclosure Statement

The authors have declared no conflict of interest.

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