

Safety and persistence of orally administered human *Lactobacillus* sp. strains in healthy adults

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Abstract

The aim of the study was to evaluate the safety and persistence of selected *Lactobacillus* strains in the gastrointestinal tract (GIT) of healthy adult volunteers after oral consumption of high doses of lactobacilli to identify potential candidates for probiotic and biotechnological applications. In the first phase of the study, nine individuals consumed capsules containing *Lactobacillus gasseri* 177 and E16B7, *Lactobacillus acidophilus* 821-3, *Lactobacillus paracasei* 317 and *Lactobacillus fermentum* 338-1-1 (each daily dose 1×10^{10} cfu) for 5 consecutive days. Data on gut health, blood parameters, and liver and kidney function were collected. The persistence of *Lactobacillus* strains was assessed by culturing combined with arbitrarily primed polymerase chain reaction (AP-PCR) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) on days 0, 5, 8, 10 and 20 from faecal samples. All strains survived gastrointestinal passage and were detected on the 5th day. *L. acidophilus* 821-3 was detected in four volunteers on the 8th day (4.3 to $7.0 \log_{10}$ cfu/g) and in two on the 10th day (8.3 and $3.9 \log_{10}$ cfu/g, respectively). In the second phase of the study, five additional volunteers consumed *L. acidophilus* 821-3 (daily 1×10^{10} cfu) for 5 consecutive days. The strain was subsequently detected in faeces of all individuals using real-time PCR on the 10th day (range 4.6-6.7; median $6.0 \log_{10}$ cell/g) in both phases of the study for at least 5 days after discontinuation of consumption. The administration of high doses of different *Lactobacillus* strains did not result in any severe adverse effects in GIT and/or abnormal values of blood indices. Thus, the strain *L. acidophilus* 821-3 is a promising candidate for probiotic and biotechnological applications. Further studies will be performed to confirm the strain persistence and safety in a larger number of individuals.

Keywords: probiotic, survival, colonisation, cultivation, real-time PCR

1. Introduction

Lactobacilli are microorganisms that form part of the human microbiota, having an important role in the first line of defence against opportunistic and invasive pathogens (Lidbeck and Nord, 1993; Stecher and Hardt, 2008). Some *Lactobacillus* strains have been used as probiotics in food products and dietary supplements for decades (Reid *et al.*, 2008). Probiotics are defined by the FAO/WHO (2001) as live microorganisms which, when administered in adequate numbers, confer a health benefit on the host. The health-

promoting effects of some *Lactobacillus* strains have been associated with high antimicrobial activity against pathogens, antioxidative properties and immunostimulatory potential (Annuk *et al.*, 2003; Hutt *et al.*, 2006; Mikelsaar and Zilmer, 2009; Saarela *et al.*, 2000; Strahinic *et al.*, 2007; Timmerman *et al.*, 2007; Verdenelli *et al.*, 2009).

During recent years, different genetically-engineered, e.g. second generation, probiotics ('designer probiotics') have been developed for the application of lactobacilli as vehicles for delivery of both active and passive immunity (Detmer

and Glenting, 2006; Seegers, 2002). The transformation of *Lactobacillus* strains for delivery of antibodies has thus been proposed both for prophylaxis and treatment of selected infections (Krüger *et al.*, 2002; Pant *et al.*, 2006).

However, before application as a probiotic and/or biological delivery system, the safety and persistence of high doses of the selected *Lactobacillus* strains in the gastrointestinal tract (GIT) of healthy adult volunteers has to be assessed. Lactobacilli have generally been regarded as safe, but there are several theoretical concerns in the safety of putative probiotics such as the development of infection (such as bacteremia or endocarditis), toxic or metabolic effects on the GIT, the transfer of antibiotic resistance to the gastrointestinal microbiota (Snydman, 2008), and adverse effects mediated by immunomodulation. In addition, the putative probiotic strains should be accurately identified (Huys *et al.*, 2006; Vankerckhoven *et al.* 2008). Antibiotic resistance pattern, tolerance to acid, bile and pancreatic juice, absence of haemolytic activity, and safety should also be proven in animal models (FAO/WHO, 2002; Frias *et al.*, 2009; Pavan *et al.*, 2003; Vesterlund *et al.*, 2007).

Prior to this study, five *Lactobacillus* strains had been isolated from the gastrointestinal tract of healthy Estonian children (Mikelsaar *et al.*, 2002; Sepp *et al.*, 1997) and selected based on the antibiotic resistance pattern, auto-aggregation ability, tolerance to acid, bile and pancreatic juice and absence of haemolytic activity (Köll *et al.*, 2010). According to their properties, these five strains were considered as potential probiotics and candidates for biotechnological applications such as delivery of therapeutic molecules in the gastrointestinal tract. To further investigate the suitability of the strains for this purpose, the present study was conducted to assess the safety, survival, and persistence of the selected *Lactobacillus* strains in the gastrointestinal tract in healthy volunteers upon oral consumption.

2. Material and methods

Lactobacillus strains

Strains used for randomly amplified polymorphic DNA-polymerase PCR (RAPD PCR) and the colonisation studies are listed in Table 1. For the colonisation study, five *Lactobacillus* strains previously isolated from the gastrointestinal tract of healthy Estonian children (Mikelsaar *et al.*, 2002; Sepp *et al.*, 1997) and subsequently characterised (Köll *et al.*, 2010) were used. The strains were re-identified by sequencing the 16S RNA gene as *Lactobacillus gasseri* 177, *Lactobacillus acidophilus* 821-3, *Lactobacillus gasseri* E16B7, *Lactobacillus paracasei* 317 and *Lactobacillus fermentum* 338-1-1 (Köll *et al.*, 2010). The safety of these strains has previously been confirmed using an animal model (Köll *et al.*, 2010).

Table 1. Bacterial strains used in this study.

Species	Strain ¹	Origin	Source of reference ²
<i>L. acidophilus</i>	821-3	Human intestine	Köll <i>et al.</i> , 2010
	JCM 1021	Rat intestine	JCM
	JCM 1023	Rat intestine	JCM
	JCM 1028	Human intestine	JCM
	JCM 1229	Human intestine	JCM
	JCM 2124	Vaginal tract	JCM
	JCM 5342	Human	JCM
	JCM 11047	Human intestine	JCM
	JCM 20315	Unknown	JCM
	La5	Dairy culture	Chr Hansen, Denmark
	ATCC 4356 ^T	Human	ATCC
<i>L. gasseri</i>	177	Human intestine	Köll <i>et al.</i> , 2010
	E16B7	Human intestine	Köll <i>et al.</i> , 2010
	TU171	Human intestine	Tartu University
	TU172	Human intestine	Tartu University
	ATCC 9857	Vaginal tract	ATCC
<i>L. paracasei</i>	317	Human intestine	Köll <i>et al.</i> , 2010
	TU194	Human intestine	Tartu University
	TU199	Human intestine	Tartu University
<i>L. plantarum</i>	TU191	Human intestine	Tartu University
	TU192	Human intestine	Tartu University
	TU193	Human intestine	Tartu University
	ATCC 14917	Pickled cabbage	ATCC
<i>L. fermentum</i>	338-1-1	Human intestine	Köll <i>et al.</i> , 2010
	TU153	Human intestine	Tartu University
	TU154	Human intestine	Tartu University
	ATCC 14931	Fermented beets	ATCC
<i>L. delbrueckii</i>	CRL 945	Sour grain mash	ATCC

¹ The strains used in the colonisation study are highlighted in bold and the other strains were used for the development of the real time PCR.

² ATCC = American Type Culture Collection; JCM = Japanese Collection of Micro-organisms.

Preparation of capsules

Lactobacilli were grown in de Man-Rogosa-Sharpe (MRS) medium (Oxoid, Basingstoke, UK) at 37 °C under micro-aerobic conditions, lyophilised and encapsulated (10¹⁰ cfu per capsule). The viability of the encapsulated lactobacilli was checked by cultivation before use in the human trials.

Study population

Inclusion criteria for the subjects were: a desire to participate, age 20-68 years, and no known health problems. Participants were asked to maintain their normal diet but refrain from consuming probiotic products. Exclusion criteria included a history of gastrointestinal disease, food allergy and acute infection, use of any antimicrobial

agent within the preceding month or use of any regular concomitant medication including non-steroidal anti-inflammatory drugs and antioxidant vitamins, pregnancy and breastfeeding. Fifteen healthy volunteers (six men, nine women, mean age 36 years) volunteered to participate in the study (interventional, single-arm, open trial). All participants signed a written informed consent and were informed that they could withdraw from the study at any time. The Ethical Committee of Tartu University approved the study protocol. The trial was registered in Current Controlled Trials (ISRCTN30946841).

Study protocol

The study was divided into two phases, the first one aiming to identify the best colonising *Lactobacillus* strain among five candidates and the second phase aimed to repeat the colonisation study with the best persisting strain. The first phase of the colonisation study included a conventional plating method and molecular methods (arbitrarily primed polymerase chain reaction (AP-PCR), PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and real-time PCR). In the second phase, the faecal samples were analysed by real-time PCR only.

First phase

The first phase involved ten volunteers (5/5; men/women). They all received capsules containing 5 freeze-dried putative probiotic strains *L. gasseri* 177, *L. gasseri* E16B7, *L. acidophilus* 821-3, *L. fermentum* 338-1-1 and *L. paracasei* 317 in a daily dose of 10^{10} cfu each for 5 consecutive days. Faecal samples were collected at days 0, 5, 8, 10 and 20.

The volunteers completed a self-reporting questionnaire containing questions on welfare, nutritional habits, and habitual gastrointestinal symptoms (abdominal pain, flatulence, bloating and stool frequency) during the trial (Svedlund *et al.*, 1988). The impact of the consumption of the five selected strains on the volunteers' health was also evaluated. Blood samples were collected at recruitment and at the end of the trial (day 20) to confirm absence of infection/inflammation: white blood cell counts (WBC), high-sensitive C-reactive protein (hs-CRP), and function indices of liver and kidney (aspartate aminotransaminase (ASAT), alanine aminotransaminase (ALAT), albumin and serum creatinine) were measured.

Second phase

The second phase included five new healthy adult volunteers (1/4; man/women). They consumed capsules containing freeze-dried *L. acidophilus* 821-3 in a daily dose of 10^{10} cfu for 5 consecutive days. Inflammatory indices (WBC, hs-CRP) were tested before the start of the second phase to exclude infection. The other blood analyses listed

above were not measured in the second phase due to the absence of abnormal values of blood indices in the first phase using the same strain. Faecal samples were collected and questionnaires were completed as described above.

Collection of faecal samples

Approximately 2 g of voided stool was collected in sterile plastic containers at the beginning on day 0 and after end of administration on the 5th, 8th, 10th, and 20th day. The faecal samples were initially stored 1 day maximum in a freezer at -20 °C or delivered to the laboratory immediately, where they were subsequently frozen at -80 °C. Samples from both phases of the study were sent to the Department of Laboratory Medicine at Karolinska Institute on dry ice for real-time PCR analysis.

Microbiological analyses of faeces

The counts of total faecal lactobacilli, isolates of indigenous lactobacilli, and the consumed *Lactobacillus* strains during the trial were evaluated in the faecal samples by a conventional cultivation method. Serial dilutions (10^{-2} - 10^{-9}) of the weighed faecal samples were prepared with sterile saline and 0.05 ml aliquots were seeded onto MRS and Rogosa agar medium (Songisepp *et al.*, 2005). The plates were incubated at 37 °C for 3 days microaerobically in a 10% CO₂ environment (incubator IG 150, Jouan, France).

Provisional identification of lactobacilli isolates was based on a gram-positive rod-shaped nonsporulating cell morphology and negative catalase reaction. Further identification included biochemical characteristics and/or API 50 CHL (BioMérieux, Marcy-l'Étoile, France). A total of 727 isolates were provisionally identified as lactobacilli and were further analysed for fermentation type. The ability of isolates to grow in MRS broth for 24 h in a 10% CO₂ environment at 15 °C and 37 °C and to produce gas in MRS agar with 1% glucose was also assessed. The fermentation of glucose without gas production, growth at 37 °C and no growth at 15 °C identifies obligate homofermentative lactobacilli; growth both at 15 °C and 37 °C without gas production is characteristic of facultative heterofermentative lactobacilli, whereas gas production at 37 °C and variable growth at 15 °C are characteristic of obligate heterofermentative lactobacilli. The count of *Lactobacillus* species was expressed as log₁₀ colony forming units per gram of faeces (log₁₀ cfu/g). The detection level of lactobacilli was 3.0 log₁₀ cfu/g faeces.

AP-PCR typing

The putative *Lactobacillus* isolates were typed by AP-PCR. Pure cultures were cultivated on MRS agar microaerobically 24 h at 37 °C in 10% CO₂. Genomic DNA was extracted with the QIAamp DNA Mini Kit 50 (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

AP-PCR typing was performed using three primers: ERIC1R (5'-ATGTAAGCTCCT GGGGATTCAC-3'), ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG -3') and Hayford primer (5'-ACGCGCCCT-3') (DNA Technology A/S, Aarhus, Denmark) as described previously (Alander *et al.*, 1999; Hayford *et al.*, 1999; Zhong *et al.*, 1998). The PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide (0.1 µl/ml) in tris/ acetic acid/EDTA (TAE) electrophoresis buffer (Bio-Rad Laboratories, Hercules, CA, USA) at constant voltage of 100 V. A 1 kb ladder (Fermentas, Vilnius, Lithuania) was used as a marker. The banding patterns of isolates were visualised with UV light and compared with that of the consumed *Lactobacillus* strains.

PCR-DGGE

PCR amplification

The DNA of faecal samples was extracted using QIAamp DNA Stool Mini Kit (Qiagen GmbH). The amount of DNA was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide. PCR to investigate the *Lactobacillus*-specific GIT microbiota was performed using the 16S rDNA primers Univ-515r-GC (5'-CGCCGGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGATCGTATTACCGCGGCTGGCA-3') and Lab-0159f (5'-GGAAACAGRTGCTAATACCG-3'). Nested PCR was performed with these primers on previously generated products from amplification with 7f (AGAGTTTGATCTACTGGCTCAG) and Lab677-r (5'-CACCGCTACACATGGAG-3') (Heilig *et al.*, 2002).

DGGE analysis of PCR products

The DGGE analysis of PCR amplicons was performed using a Dcode™ System apparatus (Bio-Rad). Polyacrylamide gels (8%[wt/vol] acrylamide-bisacrylamide [37.5:1]) in 0.5× TAE buffer with a denaturing gradient were prepared with a gradient mixer and Econopump (Bio-Rad). Gradients from 30 to 60% urea-formamide were employed for the separation of the products amplified with primers specific for *Lactobacillus* spp. Gels were analysed according to Pearson correlation, using BioNumerics 2.5 (Applied Maths, St. Martens Latem, Belgium) software (Heilig *et al.*, 2002).

Real-Time PCR

Total DNA from faeces was extracted using the QIAamp DNA Stool Minikit (Qiagen GmbH) with some modifications in the protocol. Faecal samples weighing 200 mg each were resuspended in 1:10 volume of cold 1× PBS and homogenised using a mixer mill (Retsch MM301; Retsch GmbH, Haan, Germany) at 20 Hertz for 3 min. The suspension was centrifuged for 30 s at 100g to remove debris. Supernatants were collected and centrifuged for 5

min at 1,6000g. Pellets were washed once with 1 volume of 1× PBS and once with 1 volume of 10 mM Tris pH8, 10 mM EDTA. Faecal samples were then resuspended in 10 mM Tris pH8, 10 mM EDTA, 10 mg/ml lysozyme, 400 u/ml mutanolysin and 40 µg/ml RNase, and incubated for 15 min at 37 °C. After the incubation, the faecal suspensions were transferred to screw-cap tubes containing 300 mg of zirconium beads (0.1 mm) and 1.4 ml of ASL buffer (QIAamp DNA Stool Mini Kit), and were beaten in a Fast Prep at 6.5 ms⁻¹ for 3×45 sec with cooling on ice between runs. After that, samples were centrifuged at 16,000g for 1 min to pellet stool particles and DNA was extracted from supernatants using the QIAamp DNA Stool Mini kit following the manufacturers' instructions.

Real-time PCR protocols for detection and quantification of *Lactobacilli*

The RAPD technique using primer 1254 (5'-CCGCAGCCAA-3') (Torriani *et al.*, 1999) was initially applied to detect strain-specific sequences of *L. acidophilus* 821-3. The RAPD band patterns of 11 different *L. acidophilus* strains and other 17 *Lactobacillus* strains (Table 1) were compared and a specific band of 250 bp was obtained for *L. acidophilus* 821-3. The sequence of the 250 bp RAPD fragment showed homology with an ORF that encodes a phage major capsid protein of *L. gasseri* ATCC 33323 (GenBank accession number YP_815279) and *L. reuteri* ATCC 55730 (GenBank accession number ABO43800). In order to obtain a longer sequence to design strain-specific primers for the real-time PCR, inverse PCR was performed to determine the sequence of the surrounding region of this 250 bp fragment. As a result, the sequence of a 3.3 kb region containing the ORFs that encoded putative phage proteins was obtained (GenBank accession number HM598408).

Strain-specific primers and a Taqman-MGB (Taqman-minor groove binding) probe (Kutyavin *et al.*, 2000) were designed within the 3.3 kb region using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The pair of primers q821-3D_Fw (5'-CTTCACGGGAGGAATACTACAC-3') / q821-3D_Rv (5'-GATAACGAATAATAAAGGAAAGCTGCTT-3') was selected based on its specificity. The specificity of these primers were tested in PCR and real-time PCR using as negative controls, DNA from 28 lactobacilli strains (Table 1) and four DNA samples extracted from the faeces of 4 volunteers at day 0 of the first phase of the study. The designed Taqman-MGB probe q821-3_P (5'-CGTTACGGTCAAATA-3') was located in the ORF that encoded a protein that showed homology with a putative phage head-tail adaptor of *L. gasseri* ATCC 33323 (GenBank accession number YP_815280).

The primers and the Taqman-MGB probes used to determine total counts of lactobacilli and *L. acidophilus*

were described previously (Haarman and Knol, 2006). For quantification of total lactobacilli the primers F_allact_IS (5'-TGGATGCCTTGGCACTAGGA-3') and R_allact_IS (5'-AAATCTCCGGATCAAAGCTTACTTAT-3') and the probe P_allact_IS (5'-TATTAGTTCCGTCCTTCATC-3') were used. For quantification of total *L. acidophilus*, the primers F_acid_IS (5'-GAAAGAGCCCAAACCAAGT GATT-3') and R_acid_IS (5'-CTTCCCAGATAAATCAA CTATCGCTTA-3') and the probe P_acid_IS (5'-TACC ACTTTGCAGTCCTACA-3') were used.

All the probes were labelled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher NFQ-MGB (Applied Biosystems). The PCR reactions were performed in a total volume of 20 µl containing 0.5 U of AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 0.2 U of AmpErase UNG, 2 µl of 10× Taqman Buffer A, 200 µM of each dNTP, 2 µl of purified DNA and optimised concentrations of primers, probe, and MgCl₂. The amplification and detection program contained: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curves were made using ten fold dilutions of plasmid pGEM-FT DNA (Zhao *et al.*, 2009) containing the corresponding amplicon ranging from 10⁶ to 10 copies. Each sample was analysed in duplicates. The reproducibility of each assay was determined calculating the coefficients of variation (CV) from duplicates of three independent runs testing DNA isolated from faeces spiked with a bacterial monoculture.

For the quantification of the strain *L. acidophilus* 821-3, the optimal concentrations of primers, probe, and magnesium were 300 nM forward primer, 900 nM reverse primer, 200 nM probe, and 5 mM MgCl₂. Under these conditions, the detection limit of the assay was 20 copies per reaction, corresponding to 5×10⁴ cell/g faeces. Regarding the reproducibility, the CV was 0.53%. In the case of the assays for the quantification of total lactobacilli and total *L. acidophilus* the optimal conditions were 900 nM forward primer, 900 nM reverse primer, 200 nM probe, and 5 mM MgCl₂. The detection limit and CV were 10 copies per reaction (5×10³ cell/g faeces) and 0.99% for total lactobacilli and 20 copies per reaction (5×10⁴ cell/g faeces) and 0.91% for total *L. acidophilus*.

To validate the real-time PCR method for detection of *L. acidophilus* 821-3, the faecal samples from the first phase of colonisation study were analysed and the collected data was compared to the results obtained with other molecular methods.

Statistical analysis

Statistical analysis was performed using R 2.10.1 (A Language and Environment, <http://www.r-project.org>). A statistical evaluation of the significance of the differences

in the numbers of lactobacilli obtained at different sampling times was performed by using the Wilcoxon signed rank test. All measurements of clinical data were given as means and standard deviations. For evaluation of the changes in clinical parameters at different time points, the paired t-test or Wilcoxon signed rank test were applied according to the data distribution. The Fisher exact test was used to determine the between-group differences in gastrointestinal symptoms. Differences were considered statistically significant if the *P*-value was <0.05.

3. Results

First phase of colonisation study: identification of the best coloniser

In the first phase of the study, ten volunteers consumed capsules containing a mixture of five *Lactobacillus* strains in order to select the best colonising strain. One person was excluded at the start of the study due to signs of infection in the blood analyses.

All consumed strains survived the gastrointestinal passage and were detected by conventional cultivation of faecal samples and confirmed by molecular methods (AP-PCR and PCR-DGGE) on the 5th day of consumption. Nonetheless, there were differences in persistence among the selected strains of human origin. Only two (*L. acidophilus* 821-3 and *L. paracasei* 317) strains persisted for up to five days after discontinuing the administration, albeit with diminishing counts. The different detection methods showed results mostly concordant in prevalence of *L. acidophilus* 821-3 and *L. paracasei* 317 (Figure 1A and 1B). The best temporal colonisers were *L. acidophilus* 821-3 and *L. paracasei* 317 that were found in 7 out of 9 subjects on the 5th day and 4 and 3 (respectively) out of 9 volunteers even on the 8th day (Figure 1 and 2). The counts of the two strains detected on the 10th day were quite variable. The strain *L. acidophilus* 821-3 was detected in two volunteers (8.3 log₁₀ cfu cell/g and 3.9 log₁₀ cfu cell/g respectively) and *L. paracasei* 317 was detected in one volunteer (4.3 log₁₀ cfu cell/g) on that day (Figure 2D). The volunteers colonised by *L. acidophilus* 821-3 and *L. paracasei* 317 on the 10th day were also colonised by these two strains on the 5th and 8th day.

At baseline, the total number of faecal indigenous lactobacilli varied between 4.3 and 6.3 log₁₀ cfu/g of faeces. The counts of indigenous lactobacilli were significantly increased on the last day of administration (day 5, median 6.6 log₁₀ cfu/g) in comparison to baseline values (day 0) (median 5.0 log₁₀ cfu/g). The total count returned to baseline values 3 days after stopping the administration of lactobacilli (day 8) (median 5.3 log₁₀ cfu/g).

We observed an increase in the intensity of some bands as well as new bands in the DGGE profiles in faecal samples

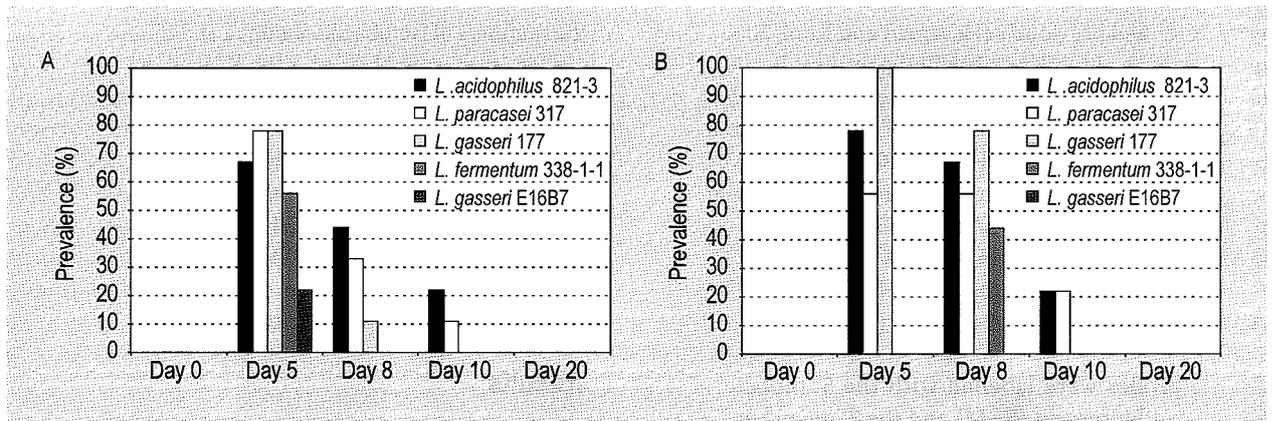


Figure 1. Prevalence of recovered *Lactobacillus* strains at five sampling times using (A) conventional plating method with typing AP-PCR and (B) by PCR-DGGE. The prevalence is defined as the percent of individuals in whom the strain was detected in faeces. None of the strains was detected on day 0 and 20.

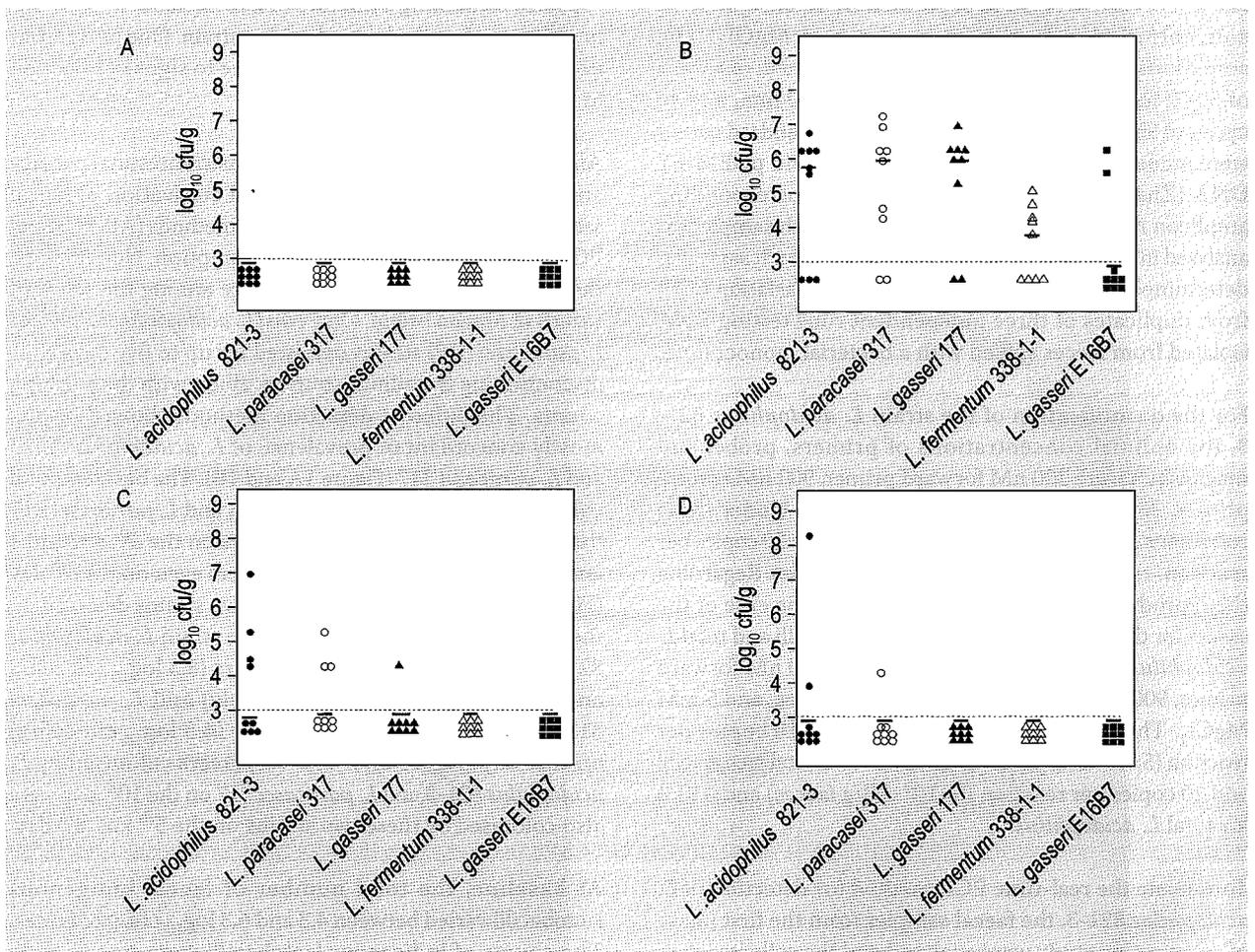


Figure 2. Counts of recovered lactobacilli using a conventional plating method with typing AP-PCR. (A) Before the administration (day 0), (B) on the last day of administration (day 5), (C) 3 days after consumption (day 8) and (D) 5 days after the last consumption (day 10) (range, median) in the first phase of study. Limit of detection, 3.0 \log_{10} cfu/g faeces (dashed line). Each dot represents the \log_{10} cfu/g faeces of lactobacilli for each individual. The median is indicated by a dash. None of the strains was detected on day 20 (not shown).

collected on the 5th day of administration of lactobacilli indicating an increased complexity of the *Lactobacillus* community after application of the mixture of strains (data not shown).

All haematological and functional indices of liver and kidney remained normal in all participants who completed the trial (Table 2). According to the semi-assisted questionnaire, the study subjects tolerated the consumption of lactobacilli well though some individual differences were noted. Mild abdominal symptoms (abdominal pain, flatulence, or abdominal bloating) were reported by three persons at the beginning of the study. Two participants had complaints of abdominal pain accompanied with flatulence and bloating on day 3; a single participant marked all three listed abdominal symptoms on day 6 (one day after the cessation of consumption). These mild symptoms seemed not to be related to the temporary colonisation of the volunteers by these strains. There was no difference in the presence of the *Lactobacillus* strains on day 5 between individuals with and without abdominal complaints. Furthermore, although the three participants were still colonised with *L. acidophilus* 821-3 on day 8, 4 other participants colonised on the same day did not experience any symptoms (Table 3).

Given that *L. acidophilus* 821-3 was the best temporary coloniser in the first phase of the study, a real-time protocol for detection of this strain in faeces was developed. In terms of prevalence, the real-time-PCR showed a good correlation with conventional cultivation combined with AP-PCR or PCR-DGGE (Table 3). However, real-time PCR was more sensitive and detected slightly more positive individuals than a combination of conventional methods and AP-PCR (Table 3). Thus, the absence of detection of *L. acidophilus* 821-3 and *L. paracasei* 317 in two individuals out of nine on day 5 using cultivation (Figure 2B) is probably due to the

lower sensitivity of the method. Furthermore, the counts of *L. acidophilus* 821-3 isolates measured with real-time PCR were significantly higher (up to 2 log₁₀ cfu higher) compared to the results obtained by conventional cultivation on the last day of consumption (day 5) (median 8.0 log₁₀ cell/g vs. 5.8 log₁₀ cfu/g, $P=0.004$) and on the 8th day (5.4 log₁₀ cell/g vs. <3.0 log₁₀ cfu/g, $P=0.022$, respectively). The counts of *L. acidophilus* 821-3 were decreased at the 10th day as detected by both methods (Table 3).

Although real-time PCR method seemed to be highly specific, an amplicon was detected in one of the subjects on day 0 (before consuming the capsules containing the strain) and on day 20 of the same volunteer (Table 3). The sequence of this amplicon presented 100% homology with the target sequence which is localised within a putative open reading frame that is homologous to ORFs found in other lactobacilli, so it would be possible to find this sequence in other intestinal strains. Nevertheless, these 'false' positive results did not critically affect the data; therefore, the developed method could be used to detect this strain in faeces in the second phase of the colonisation study.

Second phase of colonisation study: temporal colonisation with *Lactobacillus acidophilus* 821-3

In the second phase of the study, the best temporary colonising strain, *L. acidophilus* 821-3, was consumed as a single preparation. At the starting point, the five volunteers selected for the second phase did not show any symptoms/indices of inflammation. The count of total lactobacilli, *L. acidophilus* as a species and the strain *L. acidophilus* 821-3 were determined by real-time PCR (Figure 3). *L. acidophilus* 821-3 was detected in all five subjects on day 5 (median 6.8 log₁₀ cell/g), on day 8 (median 6.0 log₁₀ cell/g), and on day 10 (median 6.0 log₁₀ cell/g) (Figure 3A).

Table 2. Haematological and biochemical indices of subjects in the first phase of colonisation study (mean ± SD).

Indices ¹	At beginning	At end	Reference values ²	P values
WBC (×10 ⁹ /l)	6.0±1.3	6.1±0.8	3.5-8.8×10 ⁹ /l	0.734
Eosinophils (×10 ⁹ /l)	0.18±0.09	0.17±0.07	0.0-0.5×10 ⁹ /l	0.674
Basophils (×10 ⁹ /l)	0.09±0.12	0.05±0.04	0-0.1×10 ⁹ /l	0.586
Monocytes (×10 ⁹ /l)	0.42±0.15	0.45±0.13	0.1-0.8×10 ⁹ /l	0.441
Neutrophils (×10 ⁹ /l)	3.27±1.10	3.27±0.41	2.0-7.0×10 ⁹ /l	1.0
Lymphocytes (×10 ⁹ /l)	2.08±0.40	2.20±0.64	1.0-3.0×10 ⁹ /l	0.910
hs-CRP (mg/l)	0.7±0.4	0.8±0.5	<5 mg/l	0.344
ASAT (U/l)	24.9±7.0	25.6±6.4	F <32 U/l; M <38 U/l	0.518
ALAT (U/l)	21.4±7.5	25.4±12.9	F <31 U/l; M <41 U/l	0.201
Albumin (g/l)	43.1±2.1	44.3±2.7	34-48 g/l	0.075
Serum creatinine (µmol)	76.7±15.9	77.6±10.7	F <81 µmol; M <107 µmol	0.529

¹ WBC = white blood cells; hs-CRP = high sensitive C-reactive protein; ASAT = alanine aminotransaminase; ALAT = aspartate aminotransaminase.

² F = female; M = male; Anonymous (2004); Heil *et al.* (2008); Lewis (2001).

Table 3. Detection of *Lactobacillus acidophilus* 821-3 by three different molecular methods in the first phase colonisation study. Data are expressed in log₁₀ cfu/g faeces for AP-PCR and log₁₀ cell/g for real-time PCR.

Subject	Prefeeding period ^c			Feeding period ^c			Postfeeding period ^c								
	Day 0			Day 5			Day 8			Day 10			Day 20		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
P1 ^a	-	-	-	6.8	+	8.7	5.3	+	7.1	-	+	6.0	-	-	-
P2 ^b	-	-	-	5.6	-	6.7	4.5	+	5.1	-	-	-	-	-	-
P3 ^a	-	-	-	6.3	+	8.2	7.0	+	8.2	8.3	+	7.1	-	-	-
P4	-	-	-	6.3	+	7.0	-	-	-	3.9	-	-	-	-	-
P5	-	-	5.2	5.8	+	8.1	4.3	+	7.8	-	-	-	-	-	5.5
P6	-	-	-	-	+	7.3	-	-	5.1	-	-	-	-	-	-
P7	-	-	-	-	+	7.8	-	+	-	-	-	5.1	-	-	-
P8	-	-	-	6.3	+	8.5	-	+	5.4	-	-	-	-	-	-
P9	-	-	-	-	-	8.0	-	-	7.3	-	-	5.0	-	-	-
Prevalence	0/9	0/9	1/9	6/9	7/9	9/9	4/9	6/9	7/9	2/9	2/9	4/9	0/9	0/9	1/9
Median	0	0	0	5.8		8.0	0		5.4	0		0	0		0

^a Experienced mild symptoms on day 3.

^b Experienced mild symptoms on day 6.

^c A = cultivation with typing AP-PCR; B = PCR-DGGE; C = real-time PCR; - = under detection limit, + = presence of a specific pattern by PCR-DGGE.

It was still found in one subject 15 days after cessation of consumption (day 20) (5.1 log₁₀ cell/g). Compared to the first phase, it persisted in a higher proportion of individuals five days after discontinuation of consumption (4 out of 9 positive individuals in the first phase vs. 5 out of 5 in the second phase according to real time PCR detection). Taken together, the results from both phases showed that

the *L. acidophilus* 821-3 strain was detectable in 2/14 persons by real-time PCR even 15 days after cessation of consumption. The values of total counts of *L. acidophilus* (Figure 3B) and lactobacilli (data not shown) did not change significantly during the second phase of the study. None of the individuals showed any symptoms/indices of inflammation following consumption of *L. acidophilus*

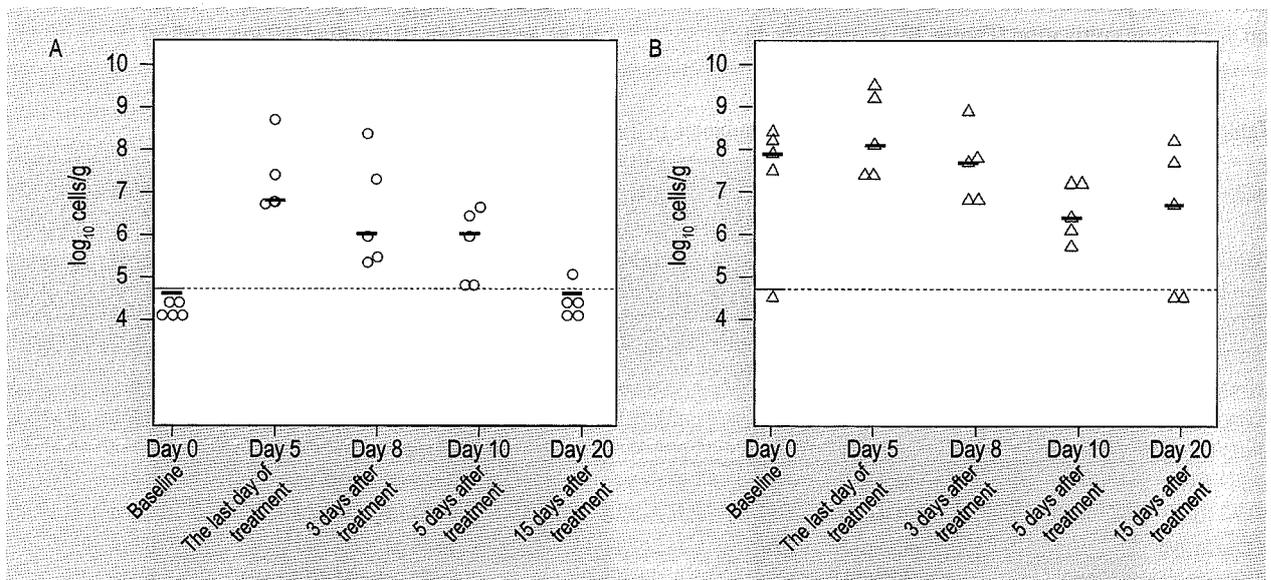


Figure 3. Counts of (A) *Lactobacillus acidophilus* 821-3 and (B) *L. acidophilus* detected by real time PCR in the second phase of the colonisation study. Limit of detection, 4.7 log₁₀ cell/g faeces (dashed line). Each dot represents the log₁₀ cell/g faeces of lactobacilli for each individual. The median is indicated by a dash.

821-3, neither reported any symptoms during feeding of *L. acidophilus* 821-3.

4. Discussion and conclusion

During previous years, a biotechnological approach, e.g. transformation of *Lactobacillus* strains for delivery of therapeutics, has been proposed for prophylaxis and treatment of selected infections (Detmer and Glenting, 2006; Krüger *et al.*, 2002). Before being used as delivery system for therapeutics or as probiotics, however, the safety of consumption of high doses of the lactobacilli and persistence of the strains in the gastrointestinal tract has to be assessed in healthy volunteers (Snydman, 2008).

In order to select a safe and good colonising strain of lactobacilli, a two phase study with human volunteers was performed. In the first phase, the volunteers were orally given five strains of human origin previously been chosen among several candidates due to their resistance to acid and bile *in vitro*, antibiotic resistance profile, and safety in an animal model (Köll *et al.*, 2010). The results indicate that *L. acidophilus* 821-3 was the best strain in temporally colonising the gastrointestinal tract when given in a mixture. The colonising ability of *L. acidophilus* 821-3 was confirmed in the second phase when the strain was administered alone. Even with the low numbers of volunteers involved, there was a trend towards a longer persistence of *L. acidophilus* 821-3 when fed as a single strain compared to when fed as part of a mixture. These results suggest that in the first phase, competition with co-administered lactobacilli might have reduced the colonisation by *L. acidophilus* 821-3. However, clinical trials with larger number of individuals will be necessary to confirm these results.

The *L. acidophilus* 821-3 strain was shown to persist for at least five days after discontinuation of consumption which is good in comparison to other published studies. In the dose-response studies of Saxelin *et al.* (1995), the authors showed that the well-known probiotic *Lactobacillus* GG, administered orally in high doses (capsules 1.2×10^{10} cfu/day), could be detected only for a short period (up to three days) after cessation of consumption. Similar to our results, Jacobsen *et al.* (1999) re-isolated the selected *Lactobacillus rhamnosus* strains from faeces 5 days after cessation of the 18-day consumption period of a *Lactobacillus* strains mixture. However, one study shows poor survival and persistence of the particular probiotic *L. acidophilus* NCFB 1748 after consumption of triple-strain yoghurt for 10 days. Namely, according to RAPD PCR results the survival of the *L. acidophilus* was detected in 3 of the 14 subjects, while persistence in 1 of the 14 subjects 4 days after cessation of the triple-strain yoghurt (Mättö *et al.*, 2006).

The mechanism underlying the difference of persistence of lactobacilli in the GIT has not been elucidated yet. It may

be influenced by the origin of lactobacilli and particular strain properties, either of human characteristics or food matrices (Walter, 2008). In our study, the latter did not influence the results as we used capsulated strains.

An increase of total lactobacilli counts after consumption of either a capsulated probiotic and/or a fermented by probiotic goat milk yoghurt has been shown previously (Kullisaar *et al.*, 2003; Songisepp *et al.*, 2005). Recently, Dommels *et al.* (2009) described that consumption of a low-fat probiotic spread containing *L. rhamnosus* GG or *L. reuteri* DSM 17938, increased the total counts of lactobacilli. Similarly, in our study, the consumption of capsulated, putatively probiotic lactobacilli significantly increased the indigenous lactobacilli counts and even the species diversity of lactobacilli. However, the increase in indigenous lactobacilli was not observed when feeding *L. acidophilus* as a single strain suggesting that this property might be dependant of the strain or the dose used.

During the current human colonisation study, the FAO/WHO recommendations to assess the safety of consumption of lactobacilli were followed. Several previous volunteer trials have applied different indices (haematological values, tests for liver and renal function) for recording adverse effects and tolerance following consumption of probiotics (Vankerckhoven *et al.*, 2008; Vlieger *et al.*, 2009). We selected several inflammatory (WBC, hs-CRP) and biochemical indices (ALAT, ASAT, albumin, creatinine) to exclude infection and detrimental effects on liver and kidney function as the most important clinical indices for health status. Although 30% of the volunteers in the first phase of our study reported mild abdominal symptoms, the feeding of high doses of the mixture of selected *Lactobacillus* strains did not induce any severe adverse effects in the GIT and did not cause any change in blood and biochemical indices. In the second phase of the study, no symptoms were observed and it could not be ruled out that the mild symptoms observed in the first trial were caused by the co-feeding with other lactobacilli. In spite of the low number of participants in each phase of the study (9 in the first phase and 5 in the second phase), the results of the safety assessments from each phase, taken together with the *in vitro* and animal studies reported before (Köll *et al.*, 2010), suggest that the tested strains can be considered as potentially safe for human consumption. Nevertheless, further safety studies with larger amounts of human volunteers would be needed to confirm this assumption.

In previous studies, specific and sensitive quantitative PCR methods for detection of probiotic bacteria from faeces have also been applied (Ahlroos and Tynkkynen, 2009; Bartosch *et al.*, 2005; Brigidi *et al.*, 2000). Similar to these methods, the real-time PCR assay developed to detect the strain *L. acidophilus* 821-3 showed a high specificity, sensitivity and reproducibility. When real-time PCR assay was compared

to conventional cultivation followed by AP-PCR, the counts of the strain *L. acidophilus* 821-3 determined by real-time PCR were significantly higher. These data are in good accordance with previous studies on the topic (Ahlroos and Tynkkynen, 2009; Dommels *et al.*, 2009). Real-time PCR methods quantify bacterial loads inferred from the number of copies of a particular DNA target sequence. Thus, this method does not differentiate between dead and live bacteria which leads to an overestimation of the number of viable cells. Furthermore, in relation to conventional cultivation methods, not all the colonies may originate from a single viable cell, which leads to an underestimation of the number of viable cells. In order to obtain a more accurate measurement of viable cells using quantitative PCR, Cenciarini-Borde *et al.* (2009) have suggested treating of bacteria with propidium or ethidium monoazide prior to the DNA isolation for selective suppression of amplification of DNA released from dead cells.

In conclusion, a combined approach using culture-based and molecular methods is suitable to detect the gastrointestinal survival and persistence after discontinuation of the consumption of the probiotic candidate *L. acidophilus* 821-3. This strain can be considered as a good temporal coloniser if administered in high doses either as a single strain or in a mixture with other lactobacilli. The consumption of high doses of different strains of *Lactobacillus* species, including *L. acidophilus* 821-3, did not result in any severe gastrointestinal adverse effects and/or abnormal values of blood indices in human volunteers. Thus, the *L. acidophilus* 821-3 strain is a promising candidate for probiotic and biotechnological application and further studies will be performed to confirm the persistence and safety in a larger number of individuals.

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