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Safety of a probiotic cheese containing *Lactobacillus plantarum* Tensia according to a variety of health indices in different age groups

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ABSTRACT

Safety of the probiotic *Lactobacillus plantarum* strain Tensia (DSM 21380) was tested in vitro, in semihard Edam-type cheese, in an animal model and after consumption of the probiotic cheese in double-blind randomized placebo-controlled human intervention studies with different age groups. The susceptibility of *L. plantarum* Tensia to 8 antibiotics, and the presence of tetracycline (*tet* M, S, O, K, L) genes and class 1 integron was assessed by applying epsilometer-test and PCR-based methods. Production of biogenic amines by the probiotic strain in decarboxylation medium containing 1% of L-histidine, L-glutamine, L-ornithine, L-arginine, or L-lysine and in cheese was tested by gas chromatography. The biosafety of *L. plantarum* Tensia was evaluated on National Institutes of Health-line mice fed cheese containing Tensia at a concentration of 9.6 log cfu/g for 30 consecutive days. In human intervention trials in adults and the elderly, the effects of different doses of Edam-type cheese and the probiotic bacterium on BW, gut functionality indices, and host metabolism were evaluated. The strain *L. plantarum* Tensia was susceptible to all tested antibiotics and did not possess the tetracycline resistance-determining genes *tet*(L), *tet*(S) and *tet*(O), nor did it contain the integron (*Int1*) gene. However, the strain was *tet*(K) and *tet*(M) positive. *Lactobacillus plantarum* Tensia did not produce potentially harmful biogenic amines, such as histamine or cadaverine. The amount of tyramine produced in the cheese environment during ripening and after 15 wk of storage was below the clinically significant content. In the animal model, no translocation of the administered strain or other microbes into the blood or organs of mice was detected. No harmful effect was observed on body mass index, inflammatory markers, or serum lipidograms during human intervention trials with different age groups at a daily dose of 10.3 or 8.17 log cfu/serving for 3 wk. No negative effect on gastrointestinal welfare was observed, but the consumption of 100 g/d for 3 wk caused hard stools from the second week of the trial. The content of total lactobacilli increased in feces, and the presence of the ingested probiotic strain was confirmed after the consumption of cheese. Thus, *L. plantarum* strain Tensia is suitable for generally recognized as safe (GRAS) and qualified presumption of safety (QPS) criteria because it did not have any undesirable characteristics. The regular semihard Edam-type cheese (fat content of 26%) with the probiotic additive at a daily dose of 50 g or in excess (100 g) and with a probiotic daily dose of 10 log cfu for 3 wk was safe.

Key words: Edam-type cheese, *Lactobacillus plantarum*, probiotic, safety

INTRODUCTION

To date, the close link between intestinal microbiota, nutrition, and human metabolism has increased interest in functional food applications (e.g., pre- and probiotic products) for health promotion in different age groups. A variety of probiotic dairy products, including cheeses with particular functional properties, is available on the market worldwide (Gomes et al., 1995; Gardiner et al., 1998; Songisepp et al., 2004; Ross et al., 2005; Ibrahim et al., 2010). Probiotics are defined as live microorganisms, which, when consumed in appropriate amounts in the food, confer a health benefit on the host (FAO/WHO, 2002). Their identity, safety, and health claims have attracted a large amount of attention from different public and regulatory organizations.

The putative probiotic strain should be accurately characterized and identified (Vankerckhoven et al., 2008), with its functional properties and the ability for temporal colonization (bile, gastric acid tolerance, and adhesive properties) confirmed in vitro (Saarela et

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al., 2000; Kõll et al., 2010). Recent recommendations include the absence of hemolytic activity and transferable antibiotic resistance of the selected *Lactobacillus* strain, whereas the safety should be proven in animal models (FAO/WHO, 2002; Vesterlund et al., 2007; Kõll et al., 2010). Next, pilot clinical trials on healthy volunteers are needed, to exclude probiotic administration having adverse effects on gut health and biochemical and cellular indices of the blood, reflecting the proper functions of human organs (Reid, 2005; Mercenier et al., 2008; Rijkers et al., 2010). Further, only after these procedures should the expression of the functional properties of the strain—either by improving some physiological functions (e.g., antimicrobial, metabolic, immunogenic, antioxidative) of the host or by reducing the risk of some diseases after consumption of the probiotic product—be tested in large groups of volunteers. Several studies have found an inverse relationship between the intake of low-fat dairy products and the incidence of cardiovascular diseases (**CVD**), preclinical atherosclerosis, and cardiovascular risk factors in middle-age and older age persons (Djoussé et al., 2006; Engberink et al., 2009; Levitan et al., 2009; Toledo et al., 2009). However, few safety assessments have been directed toward the control of biomarkers of host basic metabolism, particularly carbohydrates, lipids, and AA turnover, after administration of a dairy probiotic. Furthermore, it has not been elucidated how the addition of a probiotic strain to a full-fat dairy product affects gut functionality indices of the host.

Here, we report on clinical probiotic food intervention trials in adults and the elderly in which the tolerability and safety of a cheese containing a probiotic *Lactobacillus plantarum* strain was tested according to multiple health markers. In particular, a regular Edam-type cheese containing the probiotic *L. plantarum* strain Tensia (DSM 21380) was clinically evaluated in healthy Estonian individuals of 2 different age groups (adults and the elderly). The effects of different doses of cheese and probiotic bacteria on gut health indices; BW; functionality markers of the liver and kidneys; and hematological (hemoglobin, erythrocytes, and lymphocytes), metabolic (plasma glucose, glycohemoglobin, and lipids), and inflammatory indices [white blood cell count, high-sensitivity C-reactive protein (**hs-CRP**)] were evaluated.

MATERIALS AND METHODS

Probiotic Strain

A novel probiotic *Lactobacillus* strain was previously isolated from a fecal sample of a healthy child (Mikelsaar et al., 2002; Annuk et al., 2003). The molecular identification of the strain as *L. plantarum* was confirmed by internal-transcribed spacer PCR and 16S rRNA sequencing. The functional properties and health effects of the strain have been described elsewhere (Songisepp et al., 2009). The strain *L. plantarum* Tensia was deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) under the registration number DSM 21380 on April 16, 2008.

Hemolytic Activity

A single line of lactobacilli culture [grown in de Man, Rogosa, Sharpe broth (**MRS**; Oxoid, Basingstoke, UK) for 48 h] was streaked onto blood agar plates containing either human or sheep blood. Hemolysis of *L. plantarum* Tensia was evaluated after 24 and 48 h of incubation in aerobic, microaerobic (10% CO_2), and anaerobic (90% N_2, 5% CO_2, 5% H) environments. One *Staphylococcus aureus* strain (ATCC 25923) and 1 *Streptococcus pyogenes* strain (ATCC 19615) were used as positive controls.

Antibacterial Susceptibility Testing

The susceptibility to kanamycin, ampicillin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, and quinupristin/dalfopristin was assessed according to European Union Commission recommendations for probiotic safety (EFSA, 2008). The MIC of antibiotics to *L. plantarum* Tensia was determined by applying an epsilometer-test strip (AB Biodisk, Piscataway, NJ; Mayrhofer et al., 2008) on an inoculated Lactic Acid Bacteria Susceptibility Test Medium containing Iso-Sensitest agar (90%, vol/vol) and MRS agar (10%, vol/vol), pH 6.7 (both from Oxoid; Klare et al., 2005). The results were read after 24 h of incubation at 35°C in a microaerobic environment (CampyPak Plus, BD, Franklin Lakes, NJ).

Presence of tet and Int1 Genes Analyzed by PCR

Total DNA of *L. plantarum* Tensia was extracted using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Polymerase chain reactions were carried out in a 50-μL volume containing 10× PCR buffer, 2.5 mM $MgCl_2$, 2.5 mM deoxynucleotide 5′-triphosphates, 20 pmol of each primer (Table 1), 2.5 U of Taq polymerase (Fermentas, Vilnius, Lithuania), and 2 μg of template DNA. For detection of the possible presence of *tet* genes encoding ribosomal protection proteins, the degenerate primers DI and DII were used. If positive, additional PCR assays were performed with primers specific for the *tet*(M),

Table 1. Primers for PCR detection of the *tet* and *Int* genes[1]

Primer pair	Gene(s) targeted	Sequence
DI	RPP	5′-GAYACNCCNGGNCAYRTNGAYTT-3′
DII	RPP	5′-GCCCARWANGGRTTNGGNGGNACYTC-3′
DI	*tet*(M)	5′-GAYACNCCNGGNCAYRTNGAYTT-3′
TetrM-R	*tet*(M)	5′-CACCGAGCAGGGATTTCTCCAC-3′
TetO-f	*tet*(O)	5′-AATGAAGATTCCGACAATTT-3′
TetO-r	*tet*(O)	5′-CTCATGCGTTGTAGTATTCCA-3′
TetS-f	*tet*(S)	5′-ATCAAGATATTAAGGAC-3′
TetS-r	*tet*(S)	5′-TTCTCTATGTGGTAATC-3′
TetK-f	*tet*(K)	5′-TTATGGTGGTTGTAGCTAGAAA-3′
TetK-r	*tet*(K)	5′- AAAGGGTTAGAAACTCTTGAAA-3′
TetL-f	*tet*(L)	5′-GTMGTTGCGCGCTATATTCC-3′
TetL-R	*tet*(L)	5′-GTGAAMGRWGCCCACCTAA-3′
5′CS	*Int1*	5′-GGCATCCAAGCAGCAAG-3′
3′CS	*Int1*	5′-AAGCAGACTTGACCTGA-3′

[1]DI and DII = degenerate primers; RPP = ribosomal protection protein; CS = conserved segment; *tet* = tetracycline; *Int* = integron.

tet(O), and *tet*(S) genes. Next, *L. plantarum* Tensia was tested for the presence of the tetracycline efflux genes *tet*(K) and *tet*(L) (Gevers et al., 2003a,b).

For detection of the possible presence of integron, a class 1 integrase-specific fragment of the *Int1* gene was used (Lévesque et al., 1995). Polymerase chain reaction amplicons for *tet* genes were performed in an Eppendorf PCR System (Eppendorf AG, Hamburg, Germany) with the following programs: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 1 min, annealing temperature [ribosomal protection protein gene at 45°C; *tet*(M), *tet*(K), *tet*(L), *tet*(S), and *tet*(O) at 55°C] for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. Amplification specifications for integrase *Int1* were as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. Amplicons were electrophoresed on 1.5% agarose gel, and a 1-kb ladder (Fermentas) was used as a molecular size marker.

Production of Biogenic Amines

In Decarboxylation Medium. *Lactobacillus plantarum* Tensia strain DSM 21379 and *Escherichia coli* ATCC 700336 were used as positive controls. A 0.5-mL (9-log cfu/mL) amount of the microbial suspension was further seeded into 4.5 mL of the decarboxylase broth (Difco Decarboxylase Base Moeller, BD) containing 1% of L-histidine, L-glutamine, L-ornithine, L-arginine, or L-lysine and incubated at 37°C for 4 d. A 200-μL volume of reaction medium was derivatized for gas chromatography analysis by a modification of the method of Nakovich (2003). For detection of biogenic amines (**BA**), gas chromatography analyses were carried out using an HP 6890 Series GC System with an HP-5 19091J-413 capillary column (30 m × 0.32 mm; 0.25 μm i.d.; Agilent, Santa Clara, CA). The column temperature program was 160°C and was held for 1 min and increased at 20°C/min to 280°C for 15 min; the flame-ionization detector temperature was 300°C.

In Milk. Fat-free milk was inoculated with 10^7 cfu/mL of the Tensia strain and incubated at 37°C for 40 d. Samples were taken at the beginning of the trial and on d 12 and 40 from the inoculation.

Cheese Containing L. plantarum Tensia

Gas chromatography profiles of propyl chloroformate amines extracted from cheese samples were detected. The cheese samples were extracted as follows: 20 mL of 50% methanol solution was added to 10 g of cheese and incubated at 45°C for 1 h, cooled to 30°C, and centrifuged. A 200-μL volume of the upper layer was derivatized for gas chromatography and analyzed by a modification of the method of Nakovich (2003).

Cheese Preparation

The probiotic cheese containing *L. plantarum* Tensia was developed at the Dairy Cooperative E-Piim, (Põltsamaa, Estonia). Shortly thereafter, semihard Edam-type cheeses were prepared from cow's milk with 0.8 to 1% of C92 precultured cheese starter (CSK Food Enrichment, Leeuwarden, the Netherlands). Two different cheeses were prepared: regular Edam-type cheese as the control, and Edam-type cheese containing the *L. plantarum* strain Tensia as an adjunct starter. The total counts of live *L. plantarum* Tensia per gram of cheese are presented in Table 2. Before renneting, *L. plantarum* Tensia was added to the pasteurized milk together with the starter. The milk was renneted (25 min

Table 2. Designs of intervention studies with healthy adults and the elderly

	DBPC[1] intervention study		Open-label intervention study
Variable	Study 1	Study 2	Study 3
Study acronym	TE 1	ELD	TE 5
ISRCTN[2]	ISRCTN38739209	ISRCTN45791894	ISRCTN42449576
Duration of the trial (wk)	8	8	3
Duration of the test period (wk)	3	3	3
Duration of washout (wk)	2	2	—
Duration of the control period (wk)	3	3	—
Probiotic count in the cheese log (cfu/g)	8.7	6	8.3
Daily dose of probiotic ($\log_{10}$ cfu)	10.4	8.17	10.3
Daily dose of cheese (g)	50	50	100
Cheese age (wk)	12 to 15	13 to 16	12 to 15
Fat intake with cheese (g/d)	13	13	26
Saturated fat intake with cheese (g/d)	8	8	16
Cholesterol intake with cheese (mg/d)	45	45	89

[1]DBPC = double-blind placebo-controlled.

[2]ISRCTN = International Standard Randomized Controlled Trial Number.

at 32°C) in the presence of $CaCl_2$ (E-509) and KNO_3 (E-252; Chy-Max, Chr. Hansen, Hørsholm, Denmark). The curds were cut (10 to 15 min), heated (38 to 42°C), dried (25 min), pressed (70 min, 37 to 41°C), drained (1 h), salted at 12°C for 24 h (pH 4.5 to 4.9, salt concentration >1,151 g/mL), drained and dried (30 min to 24 h), and coated with plastic. The ripening of the cheese lasted 4 wk at 10 to 12°C at a relative air humidity of 80 to 85%.

The nutrient value of 100 g of cheese was as follows: lipids, 26 g; proteins, 26.4 g; carbohydrates and fiber, 0 g; saturated fatty acids, 16 g; vitamin A, 0.23 mg; vitamin B_2, 0.37 mg, and vitamin C, 3 mg. The energy provided by consuming 100 g of the cheese was 350 kcal or 1,488 kJ.

In Vivo Animal Trial

The biosafety of cheese containing *L. plantarum* Tensia was evaluated in a mouse model according to guidelines suggested by the Federation of European Laboratory Animal Science Association (Nicklas et al., 2002). The animal trial protocol was approved by the Ethics Committee on Animal Experiments of the Ministry of Agriculture of Estonia (protocol number 67/09.11.06).

Altogether, 20 National Institutes of Health mice (Harlan Laboratories Inc., Blackthorn, UK) were involved. Throughout the study, the mice were given a commercial diet (R-70; Lactamin, Kimstad, Sweden) and tap water ad libitum and a 50-g amount of cheese was added per cage daily. The average consumption of cheese per mouse per day was calculated after the leftover cheese was weighed. Ten mice belonged to the test group fed the probiotic cheese containing *L. plantarum* Tensia at a concentration of 9.6 $\log_{10}$ cfu/g for 30 consecutive days. A control group was fed the same amount of regular cheese without added lactobacilli.

Changes in behavior, coat texture, physical activity, and general health of the animals as well as changes in BW and related changes in food and water consumption were observed daily according to the Organisation for Economic Co-operation and Development Guidance Document (OECD, 2000). Feces for counts of lactobacilli were sampled on d 0, 3, 10, 15, 20, and 30. For histological analysis, tissue sections of the liver, spleen, kidneys, and lungs of killed mice were fixed in 10% of formaldehyde and embedded in paraffin. The samples were stained with hematoxylin and eosin and by using the van Gieson method. Alterative and inflammatory changes in tissues were evaluated.

Mice were killed by cervical dislocation on d 30, and samples were collected for microbiological analyses. Heart blood (10 μL) and homogenized tissue of the liver and spleen were tested for possible translocation of gut microbiota and *L. plantarum* Tensia onto blood and MRS agar, and for counts of lactobacilli, samples of the small intestine and large intestine were plated onto MRS agar (Oxoid). After 48 h of incubation in an aerobic (blood agar plates) and a microaerobic environment (MRS plates), colonies were enumerated. The *Lactobacillus* spp. were identified according to gram staining, colony and cell morphology, a negative catalase reaction, and carbohydrate fermentation patterns.

Design of Human Volunteer Trials

The present study was conducted according to guidelines laid down in the Declaration of Helsinki. All trials were carried out in accordance with good clinical practice (GCP) and approved by the Ethics Review Committee on Human Research of the University of

Tartu, Estonia (protocol nos. 158/10 from March 26, 2007; 177/T-13 from December 12, 2008; and 190/10 from 2010). All participants provided written informed consent at the enrollment of the study and were given the possibility of withdrawing from the study at any time.

Three different trials were conducted. Study 1 (**TE 1**), a double-blind placebo-controlled (**DBPC**) crossover study [International Standard Randomized Controlled Trial Number (**ISRCTN**) ISRCTN38739209], was performed to investigate the tolerability and safety of semihard Edam-type cheese containing *L. plantarum* strain Tensia on healthy adults.

Within 1 mo before the beginning of the study, participants were asked to continue their normal diet except to avoid probiotic products (e.g., food supplements, cheese, yogurt, kefir). The trial began with participants consuming 50 g of the test cheese [i.e., cheese containing *L. plantarum* Tensia (verum)] daily for 3 wk. The trial was begun with the test cheese because otherwise the control cheese could have influenced the evaluation of tolerability of the study product under investigation. After a 2-wk washout period, volunteers were crossed over to another 3 wk of consuming the control cheese (i.e., cheese without *L. plantarum* Tensia; Table 2, Figure 1).

Study 2 (**ELD**), a second DBPC crossover trial (ISRCTN45791894), was performed to investigate the safety of semihard Edam-type cheese containing *L. plantarum* strain Tensia on health markers in healthy elderly participants. Within 1 mo before the beginning of the study, participants were asked to continue their normal diet except to avoid probiotic products (e.g., food supplements, yogurt, cheese, kefir). The trial began with 3 wk of participants consuming 50 g of the test cheese [i.e., cheese containing *L. plantarum* Tensia (verum)] daily. The trial was begun with the test cheese because otherwise the control cheese could have influenced the evaluation of tolerability of the study product under investigation. After a 2-wk washout period, volunteers were crossed over to another 3 wk of control cheese (i.e., cheese without *L. plantarum* Tensia) consumption (Table 2, Figure 1).

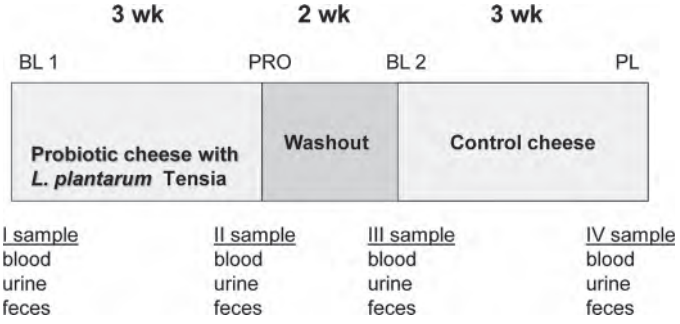

Figure 1. Design of the double-blind placebo-controlled human intervention studies with healthy volunteers (study 1) and the elderly (study 2). BL 1 = baseline 1, at recruitment; PRO = after the probiotic treatment; BL 2 = baseline 2, after washout; PL = after the control treatment.

Study 3 (**TE 5**), an open-label intervention study 3 (ISRCTN42449576), was performed to investigate the safety and dose-response effects of semihard Edam-type cheese containing *L. plantarum* strain Tensia on health markers in healthy adults. After a 3-wk run-in period, in which participants were asked to continue their normal diet except to avoid probiotic products (e.g., food supplements, yogurt, kefir), the participants consumed cheese containing a high dose (100 g) of *L. plantarum* Tensia daily for 3 wk.

Study Population

The participants were randomly chosen from free-living healthy adults aged 18 to 65 yr. The healthy elderly (>65 yr of age) were selected from the registry of family doctors and orthopedists of Tartu University Clinics (Tartu, Estonia) before orthopedic surgery. The demographic and baseline characteristics of participants are described in Table 3.

The overall inclusion criteria were a desire to participate, a suitable age, no known health problems, no health conditions that required medication, and no previous (at least 2 mo) antimicrobial treatment. Subjects following special dietary routines; having an unstable cardiopulmonary system; with a history of diabetes and malignancy, food allergy, acute infection, chronic renal or hepatic failure, or gut surgery; with the presence of an acute illness 4 wk before the study; having used any medication during the last 2 mo; or with a history of alcohol abuse, pregnancy, or breastfeeding were excluded from participation. For exclusion of participants with undiagnosed diabetes, glucose and glycohemoglobin were detected in blood sera.

Participants in the trials habitually consumed a Western diet. Diets were typically rich in potatoes, vegetables, meat, and eggs, but also characterized by a high content of fiber (rye bread and oat, wheat, or rice porridge) and high-fat dairy products, vegetable seed oils, margarine, and nonalcoholic beverages (Adlercreutz, 1990; Štšepetova et al., 2011).

Questionnaire

In all trials, a self-reported questionnaire was administered, which contained questions about participants' welfare and any adverse gastrointestinal symptoms (abdominal pain, flatulence, bloating, stool frequency, and

Table 3. Demographic and baseline characteristics of participants (mean ± SD)

	DBPC[1] intervention study		Open-label intervention study
Variable	Study 1	Study 2	Study 3
Study acronym	TE 1	ELD	TE 5
ISRCTN[2]	ISRCTN38739209	ISRCTN45791894	ISRCTN42449576
Study population	Adults	Elderly	Adults
No. of enrolled participants	13	21	29
No. of participants completing the study	12	18	26
No. of dropouts	1	3	3
Sex (male/female)	5/8	1/17	8/18
Age (yr)	29.1 ± 7.7	69.8 ± 5.5	37.2 ± 10.7
BMI[3] (kg/m^2)	24.1 ± 3.6	27.3 ± 4.2	25.7 ± 4.6
Total cholesterol (mmol/L)	4.6 ± 0.9	5.8 ± 0.8	5.6 ± 1.5
HDL cholesterol[4] (mmol/L)	1.7 ± 0.5	1.7 ± 0.3	1.7 ± 0.5
LDL cholesterol[5] (mmol/L)	2.7 ± 0.8	3.9 ± 0.8	3.6 ± 1.5
Triglycerides (mmol/L)	1.0 ± 0.6	1.1 ± 0.6	1.2 ± 0.9
HbA1c[6]	5.6 ± 0.4	5.7 ± 0.2	5.6 ± 0.2

[1]DBPC = double-blind placebo-controlled.
[2]ISRCTN = International Standard Randomized Controlled Trial Number.
[3]BMI = body mass index.
[4]HDL = high-density lipoprotein.
[5]LDL = low-density lipoprotein.
[6]HbA1c = glycohemoglobin.

stool consistency). Questionnaires were completed once a week during the trial (Svedlund et al., 1988)

Clinical Investigations

The subjects were clinically investigated, and plasma samples were collected after an overnight fast and after abstinence from any medications, tobacco, alcohol, tea, or coffee. Each participant was evaluated for anthropometrical indices. Body mass index (**BMI**) was calculated as the BW (kg) divided by the height squared (m^2). Overweight was classified as a BMI $\geq$25.0 and normal weight was classified as a BMI $<$25.0 according to the World Health Organization Global Database on Body Mass Index (http://apps.who.int/bmi/index.jsp?introPage=intro_3.html). In TE 5, the hip and waist circumference (cm) was measured and the waist-to-hip ratio was calculated.

In the DBPC trials (TE 1 and ELD), fasting blood samples were collected at recruitment, after administration of the cheese containing *L. plantarum* Tensia, after a washout period, after administration of the control cheese, and at the end of the trial. In the open-label trials (TE 5), blood samples were collected at recruitment and after the intervention.

Hematological indices (hemoglobin, erythrocytes, leukocytes, lymphocytes), inflammatory indices (white blood cell count and hs-CRP), metabolic markers [plasma glucose, glycohemoglobin, and lipids, namely, total cholesterol, low-density lipoprotein (**LDL**) cholesterol, high-density lipoprotein (**HDL**) cholesterol, and triglycerides], and functionality markers [liver and kidney functions: aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), albumin, serum creatinine, and immunoglobulin (IgE, IgA, IgM, IgG) levels] were determined by standard laboratory methods using certified assays in the local clinical laboratory (United Laboratories of Tartu University Clinics). Intervals for routine laboratory tests proposed by the Nordic Reference Interval Project (http://www.furst.no/norip/) were used as a reference.

Microbiological Analyses of Feces

Quantitative analysis of fecal cultivable lactobacilli was performed using conventional serial dilution and cultivation methods on MRS media. Samples of feces were serially diluted (10^{-2} to 10^{-9}) in PBS (pH 7.2) in an anaerobic glove box (Concept 400, Biotrace International PLC, Bridgend, UK) with a gas mixture consisting of 5% CO_2, 5% H_2, and 90% N_2, and then cultivated on freshly prepared MRS agar media (Oxoid). The bacteria were quantified by serial dilutions. The microbial counts were expressed as log colony-forming units per gram of feces. The detection limit of microorganisms was 3.0 log cfu/g. Lactobacilli were identified according to their biochemical profile by API CHL 50 medium (bioMérieux SA, Marcy L'Étoile, France).

Survival of the *L. plantarum* Tensia strain in the human gastrointestinal tract was confirmed by randomly amplified polymorphic DNA-PCR in TE 1 as follows. Genomic DNA of putative Tensia fecal isolates was

extracted from 24-h-old cultures and cultivated microaerobically on MRS agar with a QIAamp DNA Mini Kit 50 (Qiagen GmbH) according to the manufacturer's instructions.

Randomly amplified polymorphic DNA-PCR typing was done with primer M13 (GAGGGTGGCGGTTCT; DNA Technology A/S, Risskov, Denmark). A 50-μL volume of the reaction mixture consisted of 10× PCR buffer (Fermentas), 5 mM $MgCl_2$ (Fermentas), 200 μ*M* deoxynucleoside triphosphate mixture [2′-deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), 2′-deoxythymidine triphosphate (dTTP), and 2′-deoxycytidine 5′-triphosphate (dCTP); Fermentas], 2.0 μmol/L of each primer, 1.25 U of Taq DNA Polymerase (Fermentas), and 100 ng of extracted DNA. The PCR mixture was subjected to thermal cycling (Cycler 200r, Eppendorf AG) with 40 cycles of denaturation and polymerase activation at 95°C for 5 min, annealing at 42°C for 2 s, and extension at 72°C for 10 min, with a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in a horizontal 2% agarose gel containing 0.1 μL/mL of ethidium bromide in a Tris-acetic acid-EDTA buffer (40 m*M* Tris, 20 m*M* boric acid, 1 m*M* EDTA, pH 8.3; Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 120 V. A 1-kb ladder (GeneRuler; Fermentas) was used as the base pair size marker. The banding patterns of the isolates were visualized with UV light and compared with those of the *L. plantarum* Tensia.

Statistical Analysis

The sample size was calculated for crossover studies based on a predicted change in cholesterol of 0.1 U in the treatment period. Assuming a standard deviation of 0.09 U, a sample size of 8 subjects provided sufficient power (80%) to detect a significance level of $P < 0.05$ in a paired Student's *t*-test calculation. Statistical analysis was performed with R software version 2.9.0 (A Language and Environment, R Project for Statistical Computing; http://www.r-project.org). Clinical and biochemical data were expressed as means ± standard deviations. Intestinal lactobacilli counts were expressed as log colony-forming units per gram of feces. Baseline and intervention data were compared by paired *t*-test or Wilcoxon rank-sum test, according to distribution of data.

All data were given as means and standard deviations. Differences were considered statistically significant if the value was $P < 0.05$. The χ^2-test was used to determine the between-group differences in categorical variables (gastrointestinal symptoms). Study power was calculated with the program PS: Power and Sample Size Calculation (version 3.0; http://biostat.mc.vanderbilt.edu/PowerSampleSize).

RESULTS

Hemolytic Activity

Lactobacillus plantarum Tensia did not cause the lysis of erythrocytes of human and sheep blood in either of the environments, whereas complete lysis (β-hemolysis) was caused by the strains of *Strep. pyogenes* and *Staph. aureus* that were used as positive controls.

Antibacterial Susceptibility

The *L. plantarum* Tensia strain was susceptible to all the tested antibiotics. The MIC values corresponded to epidemiological cutoff (**ECOFF**) values for differentiation of wild-type lactobacilli isolates and cultures intended for probiotic or nutritional use (Klare et al., 2005): kanamycin 32 μg/mL (ECOFF 64 μg/mL), ampicillin 0.25 μg/mL (ECOFF 2 μg/mL), gentamicin 1.5 μg/mL (ECOFF 8 μg/mL), streptomycin 16 μg/mL (ECOFF 64 μg/mL), erythromycin 0.19 μg/mL (ECOFF 32.5 μg/mL), clindamycin 0.032 μg/mL (ECOFF 0.5 μg/mL), tetracycline 8 μg/mL (ECOFF 32 μg/mL), chloramphenicol 2 μg/mL (ECOFF 8 μg/mL), and quinupristin/dalfopristin 1 μg/mL (ECOFF 1 μg/mL).

Class I Integron and Tetracycline-Resistance Genes

Lactobacillus plantarum Tensia did not possess the tetracycline-resistance-determining genes *tet*(L), *tet*(S), or *tet*(O), although the *tet*(K) and *tet*(M) genes were found. The strain did not contain the integron *Int1* gene.

Production of BA

In Decarboxylation Medium. After 4 d at 37°C, *L. plantarum* Tensia produced traces of cadaverine in vitro from lysine (0.3 μg/mL) and produced putrescine from ornithine (0.5 μg/mL). The strain did not decarboxylate arginine or glutamine. Histamine was not produced from histidine. The gram-positive control strain *L. plantarum* DSM 21379 produced putrescine from ornithine (1.9 μg/mL). The control strain *E. coli* ATCC 700336 was able to produce significant amounts of cadaverine from lysine (240 μg/mL), putrescine from arginine and ornithine (18.4 and 1,599.3 μg/mL, respectively), and histamine from histidine (105.1 μg/mL).

In Milk. After incubating the Tensia strain in milk at 37°C, tyramine was detected at a concentration of 6.9 μg/mL on d 12. This concentration decreased to 1.5 μg/mL by d 40. No other BA were detected.

In Cheese. The formation of most BA, including cadaverine and histamine, in cheese during ripening and storage was not established. Only tyramine and putrescine were found in 3 batches of cheese containing Tensia in detectable amounts (0.69, 2.65, and 5.49 mg/kg of tyramine and 1.32, 7.46, and 7.29 mg/kg of putrescine, respectively) at the end of ripening (wk 4). The corresponding figures for tyramine and putrescine in control cheese (regular Edam-type cheese) were 2.31, 5.64, and 0.1 mg/kg of tyramine and 1.82, 1.84, and 0.1 mg/kg of putrescine, respectively.

In Vivo Animal Trial

The average consumption rate of cheese per mouse was approximately 4.4 g/d. Oral administration of *L. plantarum* Tensia with cheese did not influence the activity or general health status of the treated mice over 30 d. The mean BW of the mice in the test group was 25.6 ± 1.5 g at the beginning and 32.9 ± 1.6 g at the end of the trial. Respective values for the controls were 26.9 ± 1.3 g and 32.8 ± 1.7 g. Average daily BW gain for both groups (test group and control group) was 1.5 g. The BW gained at the end of the trial was 6.1 and 6.2 g, respectively. Thus, all mice showed an increase in BW.

No statistically significant increase was found in the total count of lactobacilli from the small intestine (range/median: 3.0–7.1/5.9 log cfu/g in the control group vs. 0–5.7/4.7 log cfu/g in the test group) and from the large intestine when compared with the control group (4.4–7.3/6.6 log cfu/g in the control group vs. 0–7.0/6.7 log cfu/g in the test group).

No translocation of the administered strain or other microbes into the blood or organs was detected. The heart blood, liver, kidney, and lung samples obtained at autopsy were sterile in all mice. No pathological shifts and no micro abscesses, granulomas, or inflammation were found by morphological and histological evaluation of the spleen, liver, ileum, and colon of mice.

In Vivo Human Trial Clinical Investigations

Questionnaires. In adults (TE 1), no complaints were reported regarding abdominal pain or bloating throughout the 3-wk treatment period [verum (i.e., cheese containing *L. plantarum* Tensia) vs. control (i.e., regular) cheese; $P = 1.0$], and no complaints were reported regarding flatulence in wk 1 and 3 (verum vs. control; $P = 1.0$). However, in wk 2 of the treatment, 25% of participants reported flatulence in the verum period (verum vs. control; $P = 0.22$). No significant changes in stool frequency or consistency were reported.

In adults with a high dose of probiotic cheese (TE 5), 8% of participants reported abdominal pain throughout the 3-wk treatment period; flatulence and bloating were reported by 27% of participants throughout the 3-wk treatment period. Approximately 62% of participants reported hard stools in wk 2 and 3 of the trial in comparison with the run-in period ($P = 0.023$).

In the study with the elderly (ELD), participants reported no significant abdominal pain (23%, verum vs. control; $P = 1.0$), flatulence (50%, verum vs. control; $P = 1.0$), or bloating (20%, verum vs. control; $P = 1.0$) throughout the trial in both treatment periods (probiotic and control). No significant changes in stool frequency or consistency were reported.

Abdominal Obesity and BMI. No significant changes were detected in BMI (TE 1: baseline 24.1 ± 3.6 to 24.2 ± 3.6 kg/m^2, $P = 0.584$; ELD: 27.6 ± 4.1 vs. 27.5 ± 4.2 kg/m^2, $P = 0.723$; TE 5: 25.7 ± 4.6 vs. 25.7 ± 4.7 kg/m^2, $P = 0.319$) following the 3-wk consumption of probiotic cheese. The additional markers of abdominal obesity, namely, waist circumference (cm) and waist-to-hip ratio, in adults consuming the highest dose of 100 g/d for 3 wk (TE 5) also did not increase (baseline 0.808 ± 0.075 vs. end of trial 0.806 ± 0.073; $P = 0.351$)

Blood Serum Glucose and Lipids. In TE 1, no changes in the level of blood serum glucose were detected (probiotic period: 4.5 ± 0.7 vs. 4.6 ± 0.5 mmol/L, $P = 0.922$; control period: 4.6 ± 0.6 vs. 4.7 ± 0.5 mmol/L, $P = 0.289$; Table 4).

In the elderly (ELD), a significant increase of 0.3 U was found in blood serum glucose content (from 5.1 ± 0.5 to 5.4 ± 0.5 mmol/L, $P = 0.036$) after probiotic cheese consumption (Table 4). However, the increase did not exceed the normal reference values by the Nordic Reference Interval Project (M/F >18 yr: 3.3 to 5.5 mmol/L). On the other hand, in adult volunteers (TE 5), a statistically significant reduction ($P = 0.0005$) in blood serum glucose (from 5.3 ± 0.4 to 5.0 ± 0.5 mmol/L) was registered after 3 wk of probiotic cheese consumption at the high dose (Table 4). No correlations between the changes in glucose contents with age, sex, or gastrointestinal health status and BMI were established. Blood serum lipid (total, HDL, and LDL cholesterol) levels remained unchanged in all groups consuming probiotic cheese in comparison with the recruitment values (Table 4).

Inflammation Markers. After the 3-wk probiotic treatment, the values of systemic inflammation markers (hs-CRP and white blood cell count) stayed within the normal range in both adults and the elderly (Table 5).

Table 4. Effect of 3 wk of consumption of 50 g [studies 1 (TE 1) and 2 (ELD)] or 100 g [study 3 (TE 5)] of probiotic cheese containing *Lactobacillus plantarum* Tensia on blood serum glucose and lipid values of adult volunteers (TE 1, TE 5) and the elderly (ELD; mean ± SD)[1]

	Test period			Control period			
Variable[2]	BL1	PRO	*P*-value, PRO vs. BL1	BL2	PL	*P*-value, BL2 vs. PL	Reference interval by NORIP[3]
TE 1 (n = 12)							
Total cholesterol (mmol/L)	4.6 ± 0.9	4.6 ± 1.1	0.828	4.2 ± 0.6	4.5 ± 0.9	0.102	30 to ≤ 50 yr: 3.3 to 6.9.mmol/L
HDL cholesterol (mmol/L)	1.7 ± 0.5	1.7 ± 0.3	0.628	1.6 ± 0.4	1.7 ± 0.4	0.433	≥18 yr: 1.2 mmol/L
LDL cholesterol (mmol/L)	2.7 ± 0.8	2.8 ± 1.1	0.296	2.6 ± 0.7	2.6 ± 0.7	0.827	30 to ≤50 yr: 1.4 to 4.7.mmol/L
TG (mmol/L)	1.0 ± 0.6	1.0 ± 0.5	0.978	0.9 ± 0.4	1.2 ± 0.7	0.140	≥18 yr: 0.45 to 2.6.mmol/L
Glycose (mmol/L)	4.5 ± 0.7	4.6 ± 0.5	0.922	4.6 ± 0.6	4.7 ± 0.5	0.289	≥18 yr: 3.3 to 5.5 mmol/L
ELD (n = 18)							
Total cholesterol (mmol/L)	5.7 ± 0.8	5.6 ± 0.8	0.343	5.9 ± 0.9	5.7 ± 0.8	0.198	>50 yr: 3.9 to 7.8 mmol/L
HDL cholesterol (mmol/L)	1.7 ± 0.4	1.6 ± 0.4	0.411	1.7 ± 0.4	1.7 ± 0.5	0.514	≥18 yr: 1.2 mmol/L
LDL cholesterol (mmol/L)	3.9 ± 0.8	3.8 ± 0.7	0.557	4.1 ± 0.9	3.8 ± 0.7	0.052	≥50 yr: 2.0 to 5.3 mmol/L
TG (mmol/L)	1.1 ± 0.6	1.1 ± 0.6	0.380	1.2 ± 0.5	1.1 ± 0.5	0.394	≥18 yr: 0.45 to 2.6.mmol/L
Glycose (mmol/L)	5.1 ± 0.5	5.4 ± 0.5	0.036	5.3 ± 0.5	5.4 ± 0.4	0.144	≥18 yr: 3.3 to 5.5 mmol/L
TE 5 (n = 26)							
Total cholesterol (mmol/L)	5.6 ± 1.5	5.5 ± 1.4	0.151	ND[4]	ND	—	30 to ≤50 yr: 3.3 to 6.9 mmol/L
HDL cholesterol (mmol/L)	1.7 ± 0.5	1.7 ± 0.4	0.187	ND	ND	—	≥18 yr: 1.2 mmol/L
LDL cholesterol (mmol/L)	3.6 ± 1.5	3.7 ± 1.4	0.761	ND	ND	—	30 to ≤50 yr: 1.4 to 4.7 mmol/L
TG (mmol/L)	1.2 ± 0.9	1.0 ± 0.5	0.183	ND	ND	—	≥18 yr: 0.45 to 2.6 mmol/L
Glycose (mmol/L)	5.3 ± 0.4	5.0 ± 0.5	0.0005	ND	ND	—	≥18 yr: 3.3 to 5.5 mmol/L

[1]BL1 = baseline 1, at recruitment; PRO = after the probiotic treatment; BL 2 = baseline 2, after washout; PL = after the control treatment.

[2]HDL = high-density lipoprotein; LDL = low-density lipoprotein; TG = triglycerides.

[3]Reference intervals for both sexes; NORIP = Nordic Reference Interval Project.

[4]ND = not determined.

In both age groups, no changes in the contents of IgA, IgM, and IgG antibodies were detected. Most important, no change was detected in the values of the essential allergy marker IgE or in kidney and liver markers (serum creatinine, albumin, ALT, or AST; Table 6). Even consumption of the higher dose of probiotic cheese had no negative effect on the aforementioned indices (Table 6).

Intestinal Lactobacilli. The total counts of intestinal cultivable lactobacilli increased significantly during the period of probiotic cheese consumption for both adults (TE 1; 5.5 ± 1.1 vs. 6.7 ± 1.0 log cfu/g, $P = 0.047$) and the elderly (ELD; mean value 5.2 ± 2.7 vs. 6.9 ± 1.4 log cfu/g, $P = 0.017$; Figure 2). No significant changes were seen with the consumption of control cheese.

The cheese strain administered, *L. plantarum* Tensia, was detectable in the fecal samples of 10 of 12 adult participants (TE 1) after probiotic consumption, at counts of 6.0 ± 1.5 log cfu (range: 3.2 to 8.6; median: 6.3), and after a 2-wk washout period, was detectable in the fecal samples of 2 subjects, at counts of 4.2 ± 0.2 log cfu (range: 4.0 to 4.3; median: 4.2).

DISCUSSION

We found that the addition of a particular probiotic strain to cheese did not affect the gut functionality indices or metabolism of the host. In this study, we applied the *L. plantarum* strain Tensia, originating from a healthy child (Mikelsaar et al., 2002). The human origin of the strain is a prerequisite for its harmless status and suitability for oral application. Generally, *L. plantarum* is a common indigenous component of human intestinal microbiota (Mikelsaar et al., 2002, 2010; de Vries et al., 2006). Moreover, *L. plantarum* as a species is considered by the European Food Safety Authority suitable for the qualified presumption of safety (QPS) approach (EFSA, 2007).

In characterizing a probiotic strain, the absence of antibiotic-resistance genes is of utmost importance. Mobile genetic elements containing genes determining resistance to erythromycin, tetracycline, and vancomycin have been found among lactobacilli (Tannock, et al., 1994; Gevers et al., 2003a,b; Mathur and Singh, 2005). The prevalence of tet genes in *Lactobacillus* isolates has been reported earlier (Klare et al., 2005; Gevers et al., 2003a,b). In *L. plantarum* Tensia, the *tet*(K) and *tet*(M) genes, although present, were not expressed. Gevers et al. (2003a,b) showed, in *Lactobacillus* isolates with the chromosomal *tet* gene, significantly lower tetracycline MIC values than in the case of plasmid-encoded tetracycline resistance (32 to 48 μg/mL and >192 g/mL, respectively). Thus, because we did not find phenotypic

Table 5. Effect of 3 wk of consumption of 50 g [studies 1 (TE 1) and 2 (ELD)] or 100 g [study 3 (TE 5)] of probiotic cheese containing *Lactobacillus plantarum* Tensia on the values of systemic inflammation markers [high-sensitivity C-reactive protein (hs-CRP) and white blood cell counts] of adult volunteers (TE 1, TE 5) and the elderly (ELD; mean ± SD)[1]

Variable	Test period			Control period			
	BL1	PRO	*P*-value, PRO vs. BL1	BL2	PL	*P*-value, BL2 vs. PL	Reference interval by NORIP[2]
TE 1 (n = 12)							
Leucocytes (total count × 10^9/L)	5.2 ± 0.8	5.6 ± 1.3	0.641	5.1 ± 1.1	5.5 ± 1.1	0.141	F: 3.5 to 8.8 × 10^9/L M: 3.5 to 8.8 × 10^9/L
hs-CRP (mg/L)	1.1 ± 0.6	1.0 ± 0.3	0.281	1.4 ± 0.9	1.6 ± 1.3	0.944	F/M: <5 mg/L
ELD (n = 18)							
Leucocytes (total count × 10^9/L)	5.1 ± 1.3	4.9 ± 1.3	0.330	5.0 ± 1.0	5.0 ± 1.6	0.33	F: 3.5 to 8.8 × 10^9/L M: 3.5 to 8.8 × 10^9/L
hs-CRP (mg/L)	1.6 ± 1.7	1.8 ± 1.7	0.130	1.8 ± 1.9	1.6 ± 1.5	/0.818	F/M: <5 mg/L
TE 5 (n = 26)							
Leucocytes (total count × 10^9/L)	5.1 ± 1.0	5.5 ± 1.3	0.110				F: 3.5 to 8.8 × 10^9/L M: 3.5 to 8.8 × 10^9/L
hs-CRP (mg/L)	1.1 ± 1.1	1.1 ± 1.0	0.966				F/M: <5 mg/L

[1]BL1 = baseline 1, at recruitment; PRO = after the probiotic treatment; BL 2 = baseline 2, after washout; PL = after the control treatment.
[2]Reference intervals for both sexes; NORIP = Nordic Reference Interval Project; F = female; M = male.

Table 6. Effect of 3 wk of consumption of 50 g [studies 1(TE 1) and 2 (ELD)] or 100 g [study 3 (TE 5)] of probiotic cheese containing *Lactobacillus plantarum* Tensia on metabolic markers of adult volunteers (TE 1, TE 5) and the elderly (ELD; mean ± SD)[1]

	Probiotic cheese		Control cheese		
Variable[2]	BL1	PRO	BL2	PL	*P*-value
TE 1					
AST (U/L)	20.2 ± 5.4	ND[3]	ND	18.3 ± 5.4	0.129
ALT (U/L)	17.7 ± 5.6	ND	ND	16.2 ± 4.3 4	0.247
Albumin (g/L)	45.9 ± 2.6	ND	ND	45.2 ± 1.8	0.283
Serum creatinine (μmol/L)	73.8 ± 12.0	ND	ND	70.9 ± 10.3	0.122
IgE (kU/L)	31.7 ± 21.1	ND	ND	28.9 ± 19.9	0.174
IgA (g/L)	1.7 ± 0.7	ND	ND	1.7 ± 0.6	0.29
IgM (g/L)	1.4 ± 0.7	ND	ND	1.4 ± 0.7	0.966
IgG (g/L)	11.2 ± 1.6	ND	ND	11.1 ± 1.5	0.671
ELD					
AST (U/L)	21.4 ± 4.2	ND	ND	22.1 ± 4.4	0.334
ALT (U/L)	20.2 ± 8.7	ND	ND	22.8 ± 9.4	0.050
Albumin (g/L)	42.0 ± 2.3	ND	ND	41.7 ± 2.8	0.548
Serum creatinine (μmol/L)	65.4 ± 9.7	ND	ND	64.6 ± 10.9	0.779
IgE (kU/L)	53.9 ± 85.8	ND	ND	42.8 ± 63.6	0.064
IgA (g/L)	2.4 ± 1.3	ND	ND	2.4 ± 1.2	0.664
IgM (g/L)	1.2 ± 0.6	ND	ND	1.2 ± 0.6	0.205
IgG (g/L)	10.3 ± 1.5	ND	ND	10.8 ± 1.5	0.098
TE 5					
AST (U/L)	21.9 ± 6.3	22.8 ± 6.0	ND		0.319
ALT (U/L)	21.2 ± 9.4	22.0 ± 11.3	ND		0.508
Albumin (g/L)	44.5 ± 2.0	43.9 ± 2.0	ND		0.390
Serum creatinine (μmol/L)	69.8 ± 14.6	67.5 ± 12.7	ND		0.128

[1]BL1 = baseline 1, at recruitment; PRO = after the probiotic treatment; BL 2 = baseline 2, after washout; PL = after the control treatment.

[2]AST = aspartate aminotransferase; ALT = alanine aminotransferase.

[3]ND = not determined.

tetracycline resistance in *L. plantarum* Tensia, it can be predicted as nontransferable. However, more studies are needed to verify this prediction.

Probiotics, as viable microbes with the ability to survive and be metabolically active in the gastrointestinal tract, have led to several areas of concern (Hibberd and Davidson, 2008). First is the potential of the probiotic to translocate and cause invasive infection (Salminen et al., 2004; Cannon et al., 2005). The absence of translocation and the absence of histologically detected pathologies in the NIH mouse experimental model proved the safety of the strain *L. plantarum* Tensia. Moreover, interaction with the gastrointestinal mucosa and the effect on the composition of the intestinal microbiota may also have serious consequences. Feeding the *Lactobacillus* strain of human origin for a period of 30 d did not result in an increase in the total number of lactobacilli in the mouse gut. This could be due to the human origin of the strain but also to strain specificity. It has been shown that feeding a human strain of *Lactobacillus* to mice does not always result in an increase in total counts of lactobacilli (Truusalu et al., 2004).

In our human study, the content of total lactobacilli increased in feces, and the presence of the ingested probiotic strain was confirmed after the consumption of cheese. Mutual interactions take place between a probiotic strain and the indigenous microbiota of the host in the small intestine. The consumption of probiotics may influence the metabolism and population of the indigenous microbiota.

It has been shown that ingestion of a certain probiotic may increase the total number of indigenous lactobacilli (Sepp et al., 1993; Goossens et al., 2003; Wind et al., 2010). The increase in *Bifidobacterium* and *Lactobacillus* spp. levels in the gut are correlated with numerous health markers. Microbiota changes attributable to probiotic intake include increased numbers of related phylotypes, a decrease in pathogens and their toxins, stabilization of the bacterial communities when perturbed (e.g., with antibiotics), and the promotion of a more rapid recovery from a perturbation (Floch et al., 2011).

A relatively high amount (10.3 log cfu/serving for 3 wk) of the probiotic strain in the cheese was consumed by adult volunteers. For the elderly, who are a more vulnerable population group, the daily dose was lowered: 8.17 log cfu daily for 3 wk. No adverse side effects (flatulence, bloating, abdominal pain, stool frequency) were detected with 50 g of cheese consumption in adults. The consumption of cheese in a double dose caused

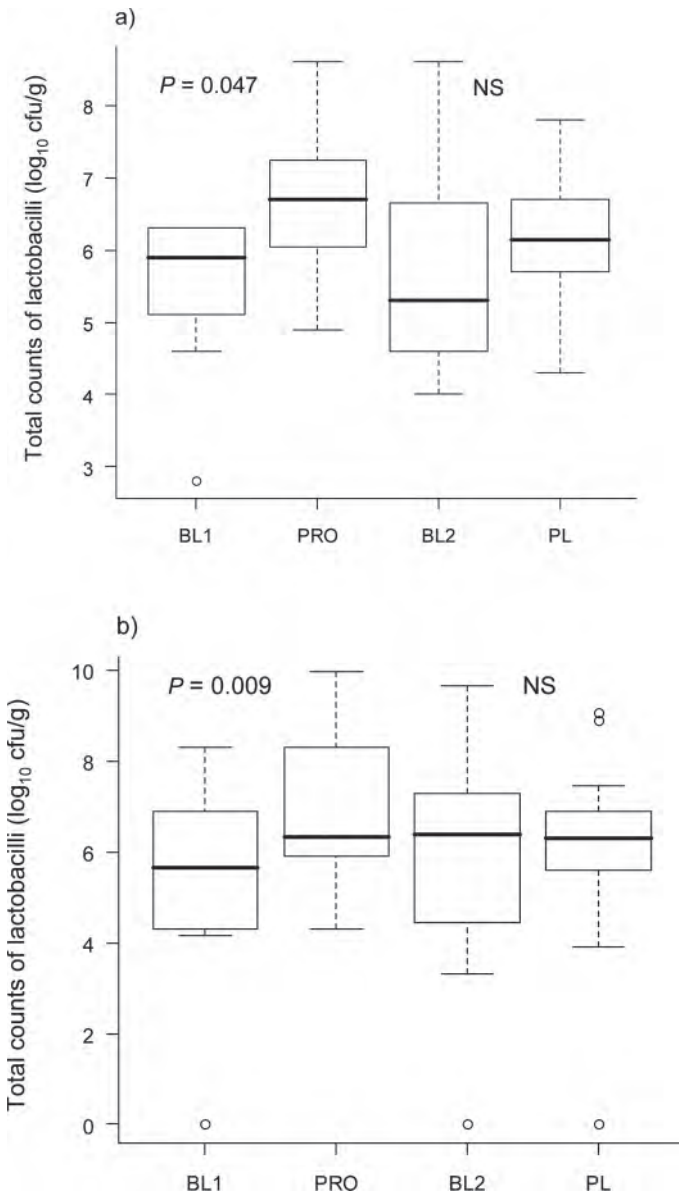

Figure 2. Changes in counts of cultivable fecal lactobacilli during the studies: a) study 1 (TE 1); b) study 2 (ELD). BL 1 = baseline 1, at recruitment; PRO = after the probiotic treatment; BL 2 = baseline 2, after washout; PL = after the control treatment. The transverse line within the box indicates the median value; the bars extending from each box represent the 25th and 75th percentiles; and the open circles represent outliers.

single cases abdominal pain, flatulence, bloating, or their combination. Because the daily dose of probiotic was similar in both studies with adult volunteers, such indigestion could have been caused by the consumption of cheese in excess, not by the probiotic strain. In the elderly, one-fourth of the participants complained about abdominal pain or bloating, and one-half of the participants reported flatulence throughout the entire trial. Because abdominal pain (12.5%), bloating (41.6%), and flatulence (63.3%) were also reported during the run-in period, these kinds of complaints seem to be symptoms of an age-related decline in physiological functions. Cheese is rich in saturated fats and proteins and lacks fiber; therefore, excessive consumption of cheese could result in constipation. We found that only the consumption of 100 g/d for 3 wk caused hard stools during the second week of the trial.

Lactobacilli could exhibit certain disadvantageous metabolic activities with regard to consumer safety, particularly if BA accumulate in the fermented products (Bernardeau et al., 2008). Biogenic amines have been implicated in several outbreaks of food poisoning and are the initiators of hypertensive crises, hypertension or hypotension, and dietary-induced migraines in certain patients. Nonstarter lactic acid bacteria (including lactobacilli) are associated with cheese-related outbreaks of histamine poisoning (Novella-Rodríguez et al., 2002). Aged cheeses, such as Cheddar, Emmenthaler, and Stilton, typically contain moderate to high concentrations of BA, including tyramine. The amount of clinically significant tyramine per serving is considered >6 mg (McCabe-Sellers et al., 2006). The cheese used in the present study was Edam-type cheese because this variety is the most common in Estonia and has a ripening period that is relatively short in comparison with other cheese varieties (e.g., Cheddar, Parmesan). Therefore, the possibility of the appearance of very high quantities of BA into this cheese variety is unlikely. However, the incorporation of relatively high viable counts of a human strain of *L. plantarum* into a protein-rich food matrix can trigger unwanted metabolic activity of the strain. Furthermore, *L. plantarum* has been identified as a producer of BA in Edam-type cheese (Bunková et al., 2010). Therefore, the possible appearance of BA in cheese containing *L. plantarum* Tensia was assessed in the present work. We demonstrated that the strain *L. plantarum* Tensia does not produce potentially harmful BA, such as histamine or cadaverine. The amount of tyramine produced in the cheese during ripening and 15 wk of storage was below the clinically significant content.

Damage to liver function is caused by liver cell damage, resulting in the leakage of enzymes into the blood circulation, indicating injury by various substances, including food. On the other hand, Higashikawa et al. (2010) reported a remarkable improvement in liver function in healthy adults, according to the serum enzymes (including AST and ALT), with intake of cultured yogurt containing a *L. plantarum* strain, and Ranganathan et al. (2010) reported a lowering of the creatinine level in patients with chronic kidney disease when given a probiotic supplement containing *L. acidophilus* and *Bifidobacterium longum*. In our trials with

adult and elderly volunteers, no changes were registered in the values of kidney and liver function markers (serum creatinine, albumin, ALT, and AST) because of probiotic cheese consumption.

No adverse effects on inflammation markers in the blood (WBC count and IgG, IgM, and IgE levels) were detected. In addition, the hs-CRP values were stable in the long-lasting trials. This is a very important finding because C-reactive protein is a marker of inflammation strongly correlated with CVD (Danesh et al., 1998; Moss and Freed, 2003).

Milk fat is considered one of the major sources of dietary cholesterol. The consumption of full-fat varieties of cheese allegedly increases the blood cholesterol level. In our study, the effect of fat on host plasma lipids by participants consuming, for 3 plus 3 wk, the semihard Edam-type cheese (fat content 26%) with and without the probiotic additive was considered negligible. No negative effect on levels of total cholesterol or cholesterol fractions (HDL cholesterol, LDL cholesterol, triglycerides) was observed. Some reports indicate that unlike other full-fat dairy products, such as whole milk or butter, consumption of cheese confers lesser plasma lipid levels, increasing the effect at comparable intakes of total fat and saturated fat (Biong et al., 2004; Tholstrup et al., 2004; Nestel et al., 2005). Some authors suggest that probiotic cultures could beneficially influence the absorption of dietary fat and cholesterol in the small intestine (Agerholm-Larsen et al., 2000; Raff et al., 2008).

Abdominal obesity has been associated with an increased risk of type 2 diabetes, dyslipidemia, hypertension, and especially CVD (de Koning et al., 2007). In contrast, the Hoorn study, with an elderly Dutch population, showed a significant inverse association between consumption of high-fat dairy products and BMI, waist circumference, and blood triglyceride indices and a positive correlation with HDL cholesterol (Snijder et al., 2007). In the present study, no BW gain was detected in the elderly consuming 50 g of cheese even though they were figured as being a less physically active population subgroup, or in adults consuming 50 or 100 g of cheese daily for 3 wk.

The statistically significant ($P = 0.0005$) decrease in blood serum glucose in adults because of a high dose of cheese consumption is probably because cheese has a low glycemic index but is otherwise a nutrient-dense food that helps postpone the feeling of hunger.

CONCLUSIONS

The present work demonstrates the safety of consuming a regular semihard Edam-type cheese (fat content of 26%) with and without the probiotic additive *L. plantarum* Tensia at a daily dose of 50 g and in excess (100 g), and with a daily dose of probiotic of 10 log cfu for 3 wk, with no harmful effect on gut health, BMI, inflammatory markers, or serum lipidograms.

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