

# Impact of probiotic *Lactobacillus plantarum* TENSIA in different dairy products on anthropometric and blood biochemical indices of healthy adults

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## RESEARCH ARTICLE

### Abstract

The blood pressure-lowering effect of dairy products holds the potential to decrease the risk of cardiovascular disease (CVD). An open question is if the successful expression of functional properties of the probiotic strain depends on host biomarkers and/or food matrix properties. The probiotic *Lactobacillus plantarum* strain TENSIA® (DSM 21380) is a novel microorganism with antimicrobial and antihypertensive functional properties. The aim of this study was to characterise the functional properties of the probiotic *L. plantarum* TENSIA and compare its effects on host anthropometric, clinical, and blood biomarkers when consumed with cheese or yoghurt. This study involved two double-blinded randomised placebo-controlled exploratory trials (ISRCTN15061552 and ISRCTN79645828) of healthy adults over a three-week period. The three-week consumption of probiotic *L. plantarum* TENSIA in a daily dose of  $1 \times 10^{10}$  cfu in probiotic cheese or a daily dose of  $6 \times 10^9$  cfu in yoghurt with different content of carbohydrates, proteins, and lipids did not significantly change the body mass index (BMI), plasma glucose and lipid levels, or inflammatory markers in the blood. Reduced lowered systolic and diastolic blood pressure values were detected, regardless of food matrix or baseline values for blood pressure and BMI. In conclusion, our study showed that three-week consumption of the probiotic *L. plantarum* TENSIA either in cheese or yoghurt lowered diastolic and systolic blood pressure regardless of food matrix and baseline values of blood pressure and BMI, confirming the impact of the functional properties of the probiotic strain in decreasing CVD risk.

**Keywords:** *Lactobacillus plantarum* TENSIA, food matrix, functional property, host biomarker

### 1. Introduction

A substantial body of evidence has suggested that dietary approaches are becoming more relevant for prevention of metabolic syndrome, including hypercholesterolaemia, glucose tolerance, and elevated blood pressure (BP) (Appel, 2009; Shirani *et al.*, 2013). A high consumption of milk and dairy products has shown a lowering effect on cardiovascular disease risk, according to a systematic review and meta-analysis (Elwood *et al.*, 2008). Moreover, the consumption of dairy products has been associated with differences in the prevalence of metabolic syndrome, obesity, and high BP in special populations (Alvarez-Leon *et al.*, 2006; Beydoun *et al.*, 2008). In contrast, however, among a representative sample of British adults, total dairy intake

and specific dairy subgroups (high- or low-fat content, fermented dairy products) were not related to BP after adjustment for confounding factors (Heraclides *et al.*, 2012). Apparent differences in genetic variants of loci that contain genes known or suspected to be involved in regulation of BP may be the reason (Ehret *et al.*, 2011).

Some reports indicate that consuming a high-fat diet (including cheese) may promote obesity with low-grade inflammation (Cani and Delzenne, 2009), which contributes to the development of atherosclerosis (Libby, 2002). One of the possibilities for compensating for these effects may be functional foods containing pre- and probiotics (Nakamura and Omaye, 2012). For instance, yoghurts and cheeses enriched with different prebiotic substances and beneficial

probiotic bacteria are used worldwide to influence human health (Kumar *et al.*, 2012). In addition, in animal models, a decrease in fat storage with probiotic bacteria has been demonstrated (Aronsson *et al.*, 2010; Beydoun *et al.*, 2008).

Probiotic bacteria have the potential for temporal colonisation of the gut when consumed, supporting the competitive exclusion of pathogens directly or influencing the immune system; furthermore, they might modulate the composition and functional activity of microbiota in beneficial ways. Studies in humans and experimental animals have shown that not only can functional food change the metabolic profile of microbiota but it also can improve host metabolism, according to biomarkers of health that have been examined (McNulty *et al.*, 2011).

What remains unexamined is if the impact of probiotics on health indices depends on the delivery form of the probiotic or on individual variation of anthropometric, clinical, and laboratory data. Some evidence suggests that food matrices play an important role in the beneficial health effects of probiotics on the host, leading to recommendations that research focus on how the food matrix and dietary content interact with the most efficient probiotic strains (Isolauri, 2007; Isolauri *et al.*, 2004).

Cheese offers a food-based delivery vehicle for probiotic cultures and biogenic substances, such as conjugated linoleic acid and bioactive peptides, while in yoghurts the conversion of lactose into lactic acid lowers the pH and consequently favours the precipitation of milk proteins. The probiotic strain *Lactobacillus plantarum* TENSIA (DSM 21380) is a novel microorganism with antimicrobial and antihypertensive properties (Mikelsaar *et al.*, 2012; Songisepp *et al.*, 2012a). The technology for its incorporation into cheese (Rätsep *et al.*, 2009; Songisepp *et al.*, 2012b) and yoghurt has been described and offers the possibility of comparing the effect of the two dairy products with different structures and lipid contents on indices of health in the general adult population.

The aim of this report was to assess some functional properties of *L. plantarum* TENSIA, such as angiotensin-1-converting enzyme (ACE) inhibitory activity and production of nitric oxide (NO) *in vitro*. Furthermore, we aimed to evaluate the impact of the probiotic strain *L. plantarum* TENSIA on anthropometric, clinical, and biochemical indices in healthy adults in two double-blinded randomised, placebo-controlled exploratory trials (ISRCTN15061552 and ISRCTN79645828) with dairy products of different food structure and content.

## 2. Material and methods

A novel probiotic strain *L. plantarum* TENSIA<sup>®</sup> was previously isolated from the gastrointestinal tract of a healthy Estonian child (Mikelsaar *et al.*, 2002). The strain *L. plantarum* TENSIA has been deposited in DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) under registration number DSM 21380. Molecular identification of the strain as *L. plantarum* was confirmed by internally transcribed spacer polymerase chain reaction and 16S rRNA sequencing (Songisepp *et al.*, 2012a). The functional properties and health effects of the strain have been patented (Songisepp *et al.*, 2012a).

### Possible mechanisms of action of *Lactobacillus plantarum* TENSIA *in vitro*

*L. plantarum* TENSIA was inoculated (2%, v/v) to 50 ml of pasteurised cow milk. Inoculation was carried out under sterile conditions, and the milk was kept for fermentation at 37 °C for 24 h. The experiment was repeated twice in parallel. To evaluate the ACE inhibitory activity of the milk during fermentation, the whey fraction was used, having been obtained as follows. The milk pH was adjusted to 3.4 by addition of 50% lactic acid. The milk acidified with the lactic acid and the milk fermented by *L. plantarum* TENSIA both were centrifuged at 6,000×g for 10 min; 10 N NaOH was added to the supernatants to increase the pH to 8.3, and the supernatant was then centrifuged at 6,000×g for 10 min. The milk hydrolysates were centrifuged at 6,000×g for 10 min. The supernatant was ultrafiltered through a 10-kDa cut-off filter (Millipore, Billerica, MA, USA) and in a second step of fractionation through a 3-kDa cut-off filter by centrifugation (4,000×g for 40 min at 15 °C). The final supernatant used for the study does not contain other compounds influencing ACE inhibition (Praveesh *et al.*, 2011).

The molar concentration of peptides in the supernatant was determined using a quantitative ninhydrin (Sigma-Aldrich, St. Louis, MO, USA) assay, a rapid and sensitive method for the quantitative determination of free amino groups. The technique involves the reaction of the free amine with ninhydrin under carefully controlled conditions and the determination of the resulting chromophore concentration in solution at 570 nm (Sarin *et al.*, 1981). Leucine (Sigma-Aldrich) was used as the standard for creating a standard curve.

The K-Assay<sup>®</sup> ACE Inhibition Screening Kit (Kamiya Biomedical Company, Seattle, WA, USA) was used for measurement of ACE inhibitory activity. The substrate for ACE was 3-hydroxybutyryl-Gly-Gly-Gly (3HB-GGG), and the amount of cleaved 3-hydroxybutyric acid (3HB) from 3HB-GGG was measured using the enzymatic method.

The inhibition activity was calculated with the following equation:

$$\text{ACE inhibitory activity (\%)} = \left[ \frac{(A_{\text{blank1}} - A_{\text{sample}})}{(A_{\text{blank1}} - A_{\text{blank2}})} \right] \times 100$$

where blank1 is positive control (without ACE inhibition) and blank2 is the reagent blank. Each sample was assayed in triplicate. For determination of  $IC_{50}$  (the concentration of an inhibitor required to inhibit 50% of the ACE activity), an inhibition curve was prepared with the sample concentration on the  $x$  axis and ACE inhibitory activity on the  $y$  axis.

Measurement of nitrogen mono-oxide production by *L. plantarum* TENSIA was carried out with 24-h old intact cells in 500  $\mu$ l of MRS broth (Oxoid, Basingstoke, UK) or in 10 ml of MRS broth containing 3 or 30 mg of  $NaNO_3$  with Apollo 4000 free radical analyser (WPI, Berlin, Germany) and electrodes of type. ISO-NOP electrode signals were registered for 5-7 min and the mean signal strength calculated. Each experimental point was measured in four independent parallels, and each parallel was measured twice. NO concentration was calculated according to standard curve correlation with the strength of the electrode signal.

#### Possible mechanisms of action of *Lactobacillus plantarum* TENSIA in vivo

The probiotic cheese Sūdamejuust Harmony™, containing *L. plantarum* TENSIA, was developed by E-Piim Production (Järva-Jaani, Estonia). The cheese production has been described in detail elsewhere (Songisepp *et al.*, 2012a). In brief, semi-hard Edam-type cheeses (fat content 26%) (control and probiotic) were prepared from cow milk with 0.8-1% of the pre-cultured cheese starter C92 (CSK Food Enrichment, Leeuwarden, the Netherlands). In probiotic cheese, the *L. plantarum* strain TENSIA was an adjunct starter.

The probiotic yoghurt was developed from adjusted and pasteurised (92-95 °C, 5 min) cow milk (fat content 4.7%) using *L. plantarum* TENSIA ( $2 \times 10^{11}$  cfu/g) as an adjunct starter (inoculation dose 1 g/t). In brief, the pasteurised milk was cooled to 35-43 °C before mixing with commercial starter cultures (Chr. Hansen, Hørsholm, Denmark) and the probiotic strain. The milk was fermented to pH 4.2-4.5 and cooled to 23-27 °C. The yoghurt was sweetened with 5% of sugar, packaged in plastic cups, and cooled to 2-6 °C. Control was similar yoghurt without the probiotic adjunct.

The human trials were performed according to the guidelines of the Declaration of Helsinki and consisted of two double-blind placebo-controlled cross-over trials. Both trials were approved by the Ethics Review Committee on Human Research of the University of Tartu, Estonia

(protocol numbers 184/T-10 from 2009; 190T-11 from 2010). All participants signed the written informed consent at enrolment and were given the option to withdraw from the study at any time.

Study TE4 (ISRCTN15061552) was performed to investigate the effect of semi-hard Edam-type cheese comprising *L. plantarum* TENSIA on health biomarkers in healthy adults ( $n=82$ ). Within one month prior to the study, the participants were asked to continue their normal diet but to avoid probiotic products (e.g. food supplements, cheese, yoghurts, kefir, etc.). The trial started with three-week consumption of probiotic cheese, i.e. cheese containing *L. plantarum* TENSIA, or control cheese (50 g daily).

Study JOG2 (ISRCTN79645828) was performed to investigate the effect of yoghurt containing *L. plantarum* TENSIA on health biomarkers in healthy adults ( $n=43$ ). Within one month prior to the study, participants were asked to continue their normal diet but to avoid probiotic products (e.g. food supplements, yoghurts, cheese, kefir, etc.). The trial started with three-week consumption of test yoghurt, i.e. yoghurt containing *L. plantarum* TENSIA, or control yoghurt (150 g daily).

In both trials, after a two-week washout period, volunteers were crossed over to another three-week period of consumption of the probiotic cheese or yoghurt, or control cheese and yoghurt. Participants underwent clinical examination, and plasma samples were collected after an overnight fast and abstinence from any medications, tobacco, alcohol, and tea or coffee. Each participant was evaluated for anthropometric indices. Body mass index (BMI) was calculated as the weight (kg) divided by squared height ( $m^2$ ) and used to classify participants as being in the normal weight range (18.5-24.9  $kg/m^2$ ), overweight ( $\geq 25.0$ -29.9  $kg/m^2$ ), or obese ( $\geq 30.0$   $kg/m^2$ ) (WHO, 2006).

Samples of fasting blood were collected four times: at recruitment, after administration of either the *L. plantarum* TENSIA-containing product or control product, after the washout period, and after the administration of the control or probiotic product at the end of the trial. Haematological indices (haemoglobin, erythrocytes, leukocytes, and lymphocytes; data not shown), inflammatory indices (white blood cell count, high-sensitivity C-reactive protein), and metabolic indices (plasma glucose and lipids: total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglycerides) were determined using standard laboratory methods with certified assays in the local clinical laboratory (United Laboratories of Tartu University Clinics, Tartu, Estonia). Intervals for routine laboratory tests proposed by the Nordic Reference Interval Project (NORIP, [www.furst.no/norip](http://www.furst.no/norip)) were used as a reference.



BP measurements were done using a mercury sphygmomanometer (Riester No 1002 DIPLOMAT presameter, Rudolf Riester GmbH, Jungingen, Germany). Participants were instructed to avoid any strenuous exercise and stimulants (alcohol, caffeine) for at least 24 h prior to any BP measurement and refrain from cold exposure, food, and fluid intake as well as smoking for at least 1 h prior to any BP measurement.

All measurements were done at the same individual time of day per person and within 07:00 and 13:00 for all participants by a properly trained nurse of the Tartu University Clinics. The BP of the volunteers was measured according to European Society of Hypertension (ESH) and the European Society of Cardiology guideline (Mancia *et al.*, 2007) suggestions, i.e. each person was allowed to sit for ca. 10-15 min in a quiet temperature-controlled room (22 °C) before beginning the BP measurements. Peripheral BP was measured in both arms twice (2-3 min between two measurements), and the mean values were calculated.

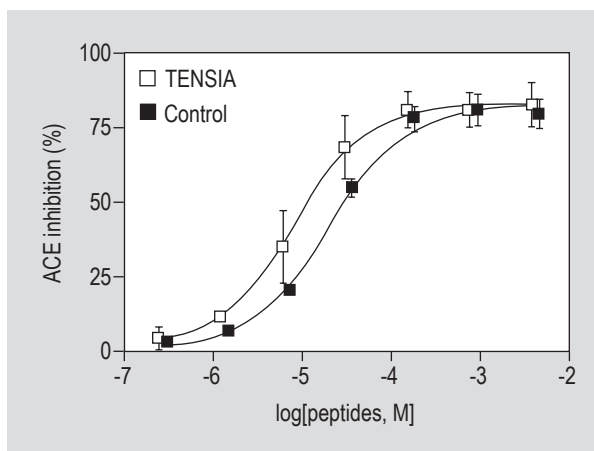
### Statistical analysis

Statistical analysis was performed by using R 2.10.1 ([www.r-project.org](http://www.r-project.org)) and GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). All data were expressed as mean and standard deviation. Baseline and intervention data were compared by paired t-tests or the Wilcoxon rank sum test according to the distribution of data. Furthermore, sex and the frequency of BMI category, systolic BP category, and number of smokers were compared between trials by the Chi-square test. The presence of correlation between variables was explored using Spearman's correlation. Differences were considered statistically significant if the *P* values <0.05.

## 3. Results

### Functional properties of *Lactobacillus plantarum* TENSIA

In the *in vitro* analysis, the ACE inhibition curves of TENSIA and control milk with cleaved 3HB as the product differed significantly ( $P < 0.05$ ) (Figure 1). Between the two types of milk screened for ACE inhibitory activity, the control milk (acidified with lactic acid) showed a maximum inhibition percentage of 82.8% while milk fermented with TENSIA showed a maximum ACE inhibition of 83.6%. Overall, ACE inhibitory activity was significantly higher ( $P < 0.05$ ) for milk fermented with TENSIA, as indicated by the lower  $IC_{50}$  of  $2.7 \pm 0.9 \mu\text{M}$  compared to that for the control milk at  $6.6 \pm 0.3 \mu\text{M}$ . *L. plantarum* TENSIA could produce NO in both MRS and skim milk and use  $\text{NaNO}_3$  as a substrate for NO production. The amount of produced NO was related to substrate concentration (Table 1).



**Figure 1.** Angiotensin-1-converting enzyme (ACE) inhibition of milk fermented with *Lactobacillus plantarum* TENSIA compared to acidified control milk.

**Table 1.** Production of nitric oxide ( $\mu\text{M}$ ) by *Lactobacillus plantarum* TENSIA *in vitro* in two different growth media in absence or presence of nitrate.

Growth environment	MRS broth	Skim milk
Medium + 3 mg $\text{NaNO}_3$	$4.5 \pm 0.9$	$3.4 \pm 0.7$
Medium + 30 mg $\text{NaNO}_3$	$11.0 \pm 2.2$	$3.6 \pm 0.7$
Control (medium with TENSIA)	$2.6 \pm 0.8$	$1.2 \pm 0.2$
Negative control (medium without TENSIA)	$0.0 \pm 0.0$	$0.0 \pm 0.0$

### Results of clinical trials

We assessed the effect of consumption of probiotic *L. plantarum* TENSIA cheese and yoghurt products separately in two different trials. The characteristics of probiotic cheese and yoghurt are given in Table 2.

In trial TE4, the number of volunteers was almost twice that of trial JOG2, although the sex, age, and smoker distribution did not differ (Table 3). However, they did differ significantly in BMI values ( $25.4$  vs.  $23.8 \text{ kg/m}^2$ ,  $P = 0.009$ ), with cheese consumers having a higher BMI. In addition, the distribution of participants according to BMI category ( $< 25$  and  $\geq 25 \text{ kg/m}^2$ ) differed between the two trials. Most of the yoghurt consumers fell into the  $\text{BMI} < 25 \text{ kg/m}^2$  group while the cheese consumers were equally distributed between the higher and lower values of  $25 \text{ kg/m}^2$  ( $P = 0.029$ ).

Both systolic and diastolic baseline BP values of participants in the cheese and yoghurt trials differed significantly ( $129.8$  vs.  $119.1 \text{ mmHg}$ ,  $P = 0.001$ ; and  $83.6$  vs.  $77.0 \text{ mmHg}$ ,  $P = 0.001$ , respectively) (Table 3). The prevalence of individuals with different systolic BP values was evenly distributed in the cheese trial whereas in the yoghurt trial, the optimal and

**Table 2. Nutritional value of cheese and yoghurt for 100 g of product and daily intake.**

Variable	Cheese	Yoghurt	Daily intake with cheese/yoghurt
Energy (kcal/kJ)	350/1,488	99.1/415	175/148.6 kcal
Protein (g)	26.4	3.4	13.2/5.1
Carbohydrates (g)	0	10.7	0/16.1
Fat (g)	26	4.7	13/7.1
Daily dose of product (g)	50	150	
Content of <i>Lactobacillus plantarum</i> TENSIA			
Log cfu/100 g of products	2×10 <sup>10</sup>	4×10 <sup>9</sup>	10 <sup>10</sup> cfu/6×10 <sup>9</sup> cfu

**Table 3. Mean baseline characteristics of participants of both trials.**

Indices <sup>1</sup>	Probiotic cheese trial <sup>2</sup> ISRCTN15061552 (n=82)	Probiotic yoghurt trial <sup>2</sup> ISRCTN79645828 (n=43)	P-value
Male	33 (40%)	11 (26%)	0.152
Female	49 (60%)	32 (74%)	
Age (y)	37.7±11.1	34.2±11.5	0.085
Body weight (kg)	76.5±13.1	69.5±14.1	0.003
BMI (kg/m <sup>2</sup> )	25.4±4.0	23.8±4.2	0.009
BMI category <sup>3</sup>			
BMI<25 kg/m <sup>2</sup>	22.3±1.8 (n=41, 50%)	21.8±1.6 (n=31, 72%)	0.160 (0.029) <sup>5</sup>
BMI≥25 kg/m <sup>2</sup>	28.4±3.2 (n=41, 50%)	29.1±4.2 (n=12, 28%)	0.890
Systolic BP (mm Hg)	129.8±11.6	119.1±12.1	<0.001
Systolic BP category <sup>4</sup>			
Optimal (<120) and normal 120-129	121.1±6.4 (n=43, 52%)	115.0±9.0 (n=35, 81%)	0.002 (0.003) <sup>5</sup>
High normal 130-139 and grade 1 hypertension 140-159	139.3±7.9 (n=39, 48%)	137.1±6.0 (n=8, 19%)	0.620
Diastolic BP (mm Hg)	83.6±8.4	77.0±7.5	<0.001
Smokers	15 (18%)	6 (14%)	0.715

<sup>1</sup> BMI = body mass index; BP = blood pressure.

<sup>2</sup> ISRCTN, *International Standard Randomised Controlled Trial Number*.

<sup>3</sup> World Health Organization classification (WHO, 2006).

<sup>4</sup> Classification systolic blood pressure levels (Mancia *et al.*, 2007).

<sup>5</sup> Comparison of category values (BMI or systolic BP) in both trials using Chi-square test.

normal BP values prevailed (optimal with normal and high-normal BP with grade 1 hypertension, 43/39 vs. 31/12,  $P=0.003$ , respectively). BMI baseline values were related to systolic and diastolic BP in both trials (probiotic cheese trial,  $r=0.240$ ,  $P=0.030$  and  $r=0.276$ ,  $P=0.012$ , respectively; probiotic yoghurt trial  $r=0.366$ ,  $P=0.016$  and  $r=0.303$ ,  $P=0.048$ , respectively).

The effect of consumption of either probiotic cheese or yoghurt over three weeks did not influence BMI. Inflammatory markers also did not change after consumption of either probiotic food product or following consumption of placebo (Table 4).

The clearest shifts were found in diastolic BP values, which significantly decreased in the probiotic period in both

trials (cheese trial,  $83.4±8.5$  vs.  $81.0±8.3$  mm Hg,  $P=0.0004$ ; yoghurt trial  $76.7±7.6$  vs.  $74.3±8.3$  mm Hg,  $P=0.002$ ). After the control cheese period, a lower decrease in diastolic BP values was also found ( $82.8±8.9$  vs.  $81.4±8.1$  mm Hg,  $P=0.046$ ). Systolic BP values decreased significantly only after consumption of probiotic cheese ( $131.0±8.1$  vs.  $127.9±10.9$  mm Hg,  $P=0.0006$ ). BP reduction was greater among persons with lower BMI (BMI<25 kg/m<sup>2</sup>) in both trials while among participants with higher BMI (≥25 kg/m<sup>2</sup>), the BP reduction was seen only in the cheese trial (Table 5).

We evaluated the relationships between changes in systolic BP with diastolic BP, BMI, glucose, cholesterol, LDL-cholesterol, HDL-cholesterol, and triglyceride values during the probiotic period in both trials. Changes in BMI were

**Table 4. Body mass index and inflammatory markers of volunteers consuming either cheese or yoghurt containing *Lactobacillus plantarum* TENSIA (mean ± standard deviation).<sup>1</sup>**

Variable	Product	Probiotic period			Placebo period		
		BL1	PRO	P-values BL1 vs PRO	BL2	PL	P-values BL2 vs PL
BMI (kg/m <sup>2</sup> )	cheese	25.4±4.0	25.3±4.0	0.441	25.4±4.0	25.3±3.9	0.454
	yoghurt	23.8±4.1	23.8±4.1	0.749	23.8±4.1	23.7±4.2	0.032
WBC (×10 <sup>9</sup> /l)	cheese	5.7±1.3	5.7±1.3	0.254	5.7±1.3	5.6±1.3	0.643
	yoghurt	5.2±1.0	5.1±1.0	0.285	5.2±1.1	5.3±1.3	0.877
hs-CRP (mg/l)	cheese	1.0±1.2	1.0±1.1	0.685	1.0±1.1	1.1±1.2	0.972
	yoghurt	0.7±0.8	0.8±1.0	0.610	0.8±1.1	0.7±0.6	0.460

<sup>1</sup> BL1 = baseline 1, at the recruitment; PRO = after probiotic treatment; BL2 = baseline 2, after washout; PL = after control treatment; BMI = body mass index; WBC = white blood cells; hs-CRP = high-sensitivity C-reactive protein.

**Table 5. Blood pressure values in volunteers consuming either cheese or yoghurt containing *Lactobacillus plantarum* TENSIA (mean ± standard deviation).<sup>1</sup>**

Variable	Product	Probiotic period			Placebo period		
		BL1	PRO	P values BL1 vs PRO	BL2	PL	P values BL2 vs PL
SBP (mm Hg)	cheese	131.0±8.1	127.9±10.9	0.0006	128.7±12.4	126.2±11.2	0.057
	yoghurt	118.9±13.0	116.7±14.0	0.055	117.3±12.7	116.5±14.1	0.352
DBP (mm Hg)	cheese	83.4±8.5	81.0±8.3	0.0004	82.8±8.9	81.4±8.1	0.046
	yoghurt	76.7±7.6	74.3±8.3	0.002	75.5±8.1	74.5±8.1	0.109
SBP [BMI<25]	cheese	130.5±7.0	126.1±10.4	0.001	126.7±13.2	124.7±10.3	0.213
	yoghurt	116.1±12.5	114.1±13.8	0.077	114.2±12.3	113.6±13.6	0.688
SBP [BMI≥25]	cheese	131.5±9.2	129.7±11.1	0.064	130.8±11.3	127.8±12.0	0.048
	yoghurt	126.1±12.0	123.4±12.6	0.298	125.4±10.4	124.0±12.9	0.505
DBP [BMI<25]	cheese	82.1±8.0	79.5±8.2	0.009	80.7±8.8	79.7±7.7	0.311
	yoghurt	75.9±7.8	73.0±8.2	0.002	74.3±7.9	73.2±8.4	0.262
DBP [BMI≥25]	cheese	84.6±8.9	82.7±8.3	0.017	84.8±8.5	83.1±8.3	0.055
	yoghurt	78.6±7.1	77.6±8.1	0.319	78.7±7.8	77.8±6.7	0.333

<sup>1</sup> BL1 = baseline 1, at the recruitment; PRO = after probiotic treatment; BL2 = baseline 2, after washout; PL = after control treatment; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure.

significantly related to changes in systolic BP in the cheese trial ( $r=0.276$ ,  $P=0.013$ ), and reduced cholesterol levels were associated with changes in systolic BP ( $r=0.311$ ,  $P=0.045$ ) in the yoghurt trial. A negative correlation between reduced systolic BP and increased blood glucose was found among yoghurt consumers ( $r=-0.353$ ,  $P=0.022$ ) (Table 6).

### Impact of probiotic food on biomarkers

The two probiotic products differed in their structure and protein, carbohydrate, and fat content (Table 2). The biggest difference was detected for carbohydrate (16 times in favour of yoghurt) and protein and fat (2.6 times and 2.0 times in favour of cheese, respectively) daily intake. At the same

time, the intake of the probiotic strain TENSIA was quite similar. The energy load from cheese was higher than from yoghurt (175 kcal and 148.6 kcal, respectively).

The biochemical blood indices of the two groups of cheese and yoghurt consumers were quite stable. Remarkably, lowered values of blood LDL were found in consumers of both probiotic and control yoghurt (Table 7). No differences were detected comparing the erythrocytes, platelets, and lymphocyte values (data not shown) or in the content of glucose and total cholesterol, HDL-cholesterol, and triglycerides either at baseline or after probiotic consumption (Table 7). Thus, the highly different (16×) content of carbohydrates did not influence blood glucose

**Table 6. Correlation between changes in systolic blood pressure with diastolic blood pressure (DBP), body mass index (BMI), and blood indices during the probiotic period.**

Indices	Probiotic cheese trial (n=82)	Probiotic yoghurt trial (n=43)
DBP	r=0.490, P=0.001	r=0.411, P=0.006
BMI	r=0.276, P=0.013	r=0.100, P=0.522
Glucose	r=-0.058, P=0.610	r=-0.353, P=0.022
Cholesterol	r=0.023, P=0.841	r=0.311, P=0.045
LDL-cholesterol <sup>1</sup>	r=-0.051, P=0.655	r=0.175, P=0.267
HDL-cholesterol <sup>1</sup>	r=0.015, P=0.897	r=0.064, P=0.687
Triglycerides	r=-0.0172, P=0.88	r=-0.007, P=0.964

<sup>1</sup> LDL = low-density lipoprotein; HDL = high-density lipoprotein.

values with either low (cheese trial) or higher carbohydrate content (yoghurt trial).

#### 4. Discussion

The functional properties of the probiotic strain *L. plantarum* TENSIA exhibiting *in vitro* ACE inhibitory activity and NO production are the putative mechanisms responsible for lowering BP. We found an increased ACE inhibition in milk fermented with *L. plantarum* TENSIA *in vitro*, which indicated a possible mechanism for lowering systolic and diastolic BP. According to earlier studies, milk bioactive peptides may act as ACE inhibitors, thus inhibiting the renin-angiotensin system with consequent vasodilatation (Jauhainen *et al.*, 2005; Turpeinen *et al.*, 2012). Recently, Gupta *et al.* (2013) found ACE inhibitory

activity in cheddar cheese. The strongest ACE inhibitors known to be produced by lactobacilli are rich of proline (i.e. Ile-Pro-Pro and Val-Pro-Pro), which makes them resistant to digestive peptidases. Moreover, besides single amino acids, also di- and tripeptides are absorbed in the intestine and thus the short peptides generated by lactobacilli may reach the host's bloodstream (De Leo *et al.*, 2009).

NO is a signalling molecule regulating a variety of biological functions. In the gastrointestinal tract it is synthesised by and sensed as a signal in the endothelial cells of local blood vessels, in tissue macrophages as well as in intestinal epithelial cells. Lactobacilli are suggested to induce NO synthase activity in host cells (Korhonen *et al.*, 2001, 2002; Hu *et al.*, 2013) and some of them are capable of producing NO by themselves (Xu and Verstraete, 2001). Gaseous signalling molecules like NO, CO or H<sub>2</sub>S easily penetrate biological barriers and the signalling distance for NO is mainly limited by its short half-life, which allows covering distances up to a few hundred µm (Arzumanian *et al.*, 2003). However, it has recently been recognised that nitrite formation from NO is not an irreversible process, and via nitrite conversion NO signalling may reach systemic range (Lundberg *et al.*, 2008). For instance, NO and nitrite formation has been suggested to be the key mechanism of how oral microbiota reduce host's blood pressure (Kapil *et al.* 2013).

Our results show production of NO by *L. plantarum* TENSIA in all growth media used, with a higher production in nitrate-rich environments, suggesting that nitrate is the preferred source for NO generation. NO synthesis was higher and depended more on nitrate concentration in MRS broth than in skim milk-based growth media implying

**Table 7. Biochemical indices in volunteers consuming either cheese or yoghurt comprising *Lactobacillus plantarum* TENSIA (mean ± standard deviation).<sup>1</sup>**

Indices	Product	Probiotic period			Placebo period		
		BL1	PRO	P-values BL1 vs PRO	BL2	PL	P-values BL2 vs PL
Glucose (mmol/l)	cheese	5.3±0.6	5.3±0.6	0.281	5.2±0.5	5.3±0.6	0.399
	yoghurt	5.0±0.4	5.0±0.6	0.856	5.0±0.5	5.1±0.5	0.445
Chol (mmol/l)	cheese	5.3±1.0	5.4±1.0	0.812	5.3±1.0	5.3±1.0	0.623
	yoghurt	5.1±1.0	5.0±1.1	0.294	5.0±1.0	5.0±1.1	0.418
LDL (mmol/l)	cheese	3.6±1.0	3.5±0.9	0.213	3.5±1.0	3.4±0.9	0.239
	yoghurt	3.2±0.9	3.0±1.0	0.006	3.1±1.0	3.0±0.9	0.015
HDL (mmol/l)	cheese	1.6±0.4	1.6±0.4	0.946	1.6±0.4	1.7±0.4	0.348
	yoghurt	1.7±0.4	1.7±0.4	0.308	1.7±0.4	1.8±0.4	0.309
TG, mmol/l	cheese	1.1±0.5	1.1±0.5	0.825	1.1±0.5	1.0±0.5	0.177
	yoghurt	1.0±0.6	1.0±0.6	0.202	1.0±0.6	1.0±0.6	0.274

<sup>1</sup> BL1 = baseline 1, at the recruitment; PRO = after probiotic treatment; BL2 = baseline 2, after washout; PL = after control treatment; Chol = total cholesterol; HDL = high-density lipoprotein; LDL = low-density lipoprotein; TG = triglycerides.



that the NO generation is not only regulated by substrate availability, but a more complex interaction between the bacteria and its environment. Xu and Verstraete (2001), who found only nitrate-derived NO production in lactobacilli, carried the experiments out in MRS broth only. Considering other previous reports (Iarullina *et al.*, 2006; Morita *et al.*, 1997) and differences between MRS broth and skim milk media observed by us, it cannot be excluded that non-nitrate sources, such as arginine, can under certain conditions also be used by TENSIA for NO generation. Nevertheless, although the mechanism needs additional clarification, NO production by TENSIA in gastrointestinal tract would be plausible. This could lead to increased levels of NO and/or nitrite in the intestinal tissue and a potential reason for decreased blood pressure. Of course, there also remains the possibility that TENSIA is inducing NO generation in host cells independently of its own NO production capability and the latter has no significant effect on blood pressure.

In two double-blind, placebo-controlled cross-over trials, we compared the effect of the probiotic strain TENSIA in different food carriers (cheese, yoghurt) on anthropometric, BP, and blood biomarkers of health in healthy adults. The main effect of the consumption of probiotic cheese and yoghurt was a decreased diastolic BP, while consuming probiotic cheese was linked to a significant reduction in systolic BP. Usually, both the amount and type of carbohydrate affect BP, and partial substitution of carbohydrate with either protein or monounsaturated fat lowers BP (Appel *et al.*, 2005). A few trials have tested the effects of periodic consumption of sugars, proteins, and fat in different probiotic products. The reduction in BP with milk products is well documented (Engberink *et al.*, 2009). Namely, dietary changes can lower BP and delay hypertension in non-hypertensive individuals (Appel, 2009). Some intervention studies have also shown a BP-lowering effect of milk products and dairy proteins (Agerholm-Larsen *et al.*, 2000; Hata *et al.*, 1996; Jauhiainen *et al.*, 2005; Seppo *et al.*, 2003; Sharafedinov *et al.*, 2013; Tuomilehto *et al.*, 2004). The result of the present comparative study is in accordance with those of a previous study finding that the most notable shifts with TENSIA were in diastolic BP values (Mikelsaar *et al.*, 2012). In obese patients on a hypocaloric diet and antihypertensive treatment, the extent of change of morning diastolic BP was closely associated with colonisation of molecularly detected probiotic *L. plantarum* TENSIA (Sharafedinov *et al.*, 2013).

In our study, the BP-lowering effect among cheese consumers was also found in the placebo period, possibly the effect of starter and nonstarter lactobacilli of the Edam-type cheese. Milk-derived biologically active peptides and other substances affecting BP are present in cheese, including Edam, and produced by starter microbes, adventitious microflora, or rennet used during cheese production

(Bütikofer *et al.*, 2008; Gupta *et al.*, 2013; Korhonen and Pihlanto, 2006; Sieber *et al.*, 2010). Similarly, the absence of clear differences in systolic BP reduction between probiotic and ordinary milk products may arise from the fact that milk compounds were present equally in probiotic and control products.

The consumption of either probiotic cheese or yoghurt for three weeks in this study did not depend on or influence BMI values or the level of plasma glucose, lipid profile, or inflammatory markers of blood despite the different carbohydrate, protein, and lipid content. Although, a low reduction of BMI was detected during the placebo intake in probiotic yoghurt trial. It may be due to the placebo effect or some carry-over effect. A previous study in healthy volunteers involving probiotic cheese containing TENSIA did not show changes in BMI values (Songisepp *et al.*, 2012a), either. However, in patients with metabolic syndrome, probiotic cheese with TENSIA caused a significant reduction in body water accompanied by decreased BMI (Sharafedinov *et al.*, 2013).

In our intervention study with healthy adults, we identified a difference at baseline in BMI indices and BP values. This result showed that the diastolic BP-lowering effect with both dairy products containing *L. plantarum* TENSIA was relevant even in the general population with a large range of baseline values of BP. Recently, the BMI for the healthy population has been lowered to <24 kg/m<sup>2</sup> or less. According to the National Heart, Lung and Blood Institute, heart disease, diabetes, and high BP are all linked to being overweight (NIH, 2004). Thus, in the general population, adults consider themselves healthy, but there is a large range of normality both for BMI and BP. It is important to know if functional food containing probiotics can positively influence borderline levels of prehypertension or reduce BP values. Evidence of a link between cheese consumption and vascular disease gives a lowered overall estimate of risk from cheese (Ciccarone *et al.*, 2003; Lockheart *et al.*, 2007). In a wide-ranging review of studies of cheese consumption, Tholstrup found 'no convincing evidence of harm' and wrote on the 'neutral' effect of cheese on coronary heart disease (Tholstrup, 2006). No risk was demonstrated by Elwood *et al.* (2007) reporting the results of a meta-analysis of cross-sectional studies of milk and/or dairy consumption and metabolic syndrome.

The only effect of probiotic *L. plantarum* TENSIA on blood biomarkers was found in yoghurt consumers in whom LDL-cholesterol was significantly lowered in both the probiotic and placebo treatment periods, possibly because of a carryover effect of probiotic yoghurt. A meta-analysis of 13 probiotic studies indicated that a diet rich in probiotics decreases total cholesterol and LDL-cholesterol concentration in plasma for participants with



high, borderline-high, and normal cholesterol levels (Guo *et al.*, 2011).

The limitations of the study were a possibly too short washout period after probiotic product consumption in both trials and a possible resulting carry-over effect that could have induced the BP reduction in the placebo period. In conclusion, our study showed that three-week consumption of probiotic *L. plantarum* TENSIA either in cheese or yoghurt lowered diastolic BP regardless of food matrix or different baseline values of BP and BMI, confirming the impact of functional properties of the probiotic strain in decreasing cardiovascular disease risk.

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