Microbial Host Interactions

Intestinal *Lactobacillus* sp. is associated with some cellular and metabolic characteristics of blood in elderly people

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ABSTRACT

The higher counts or particular groups (Firmicutes/Bacteroidetes) of intestinal microbiota are related to host metabolic reactions, supporting a balance of human ecosystem. We further explored whether intestinal lactobacilli were associated with some principal cellular and metabolic markers of blood in 38 healthy >65-year-old persons. The questionnaire, routine clinical and laboratory data of blood indices as much as the oxidized low-density lipoprotein (ox-LDL) and baseline diene conjugates in low-density lipoprotein (BDC-LDL) of blood sera were explored. The PCR-based intestinal *Lactobacillus* sp. composition and counts of cultivable lactobacilli (LAB) were tested. The facultative heterofermentative lactobacilli (*Lactobacillus casei* and *Lactobacillus paracasei*) were the most frequent (89 and 97%, respectively) species found, while *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus reuteri* were present in almost half of the elderly persons. The number of species simultaneously colonizing the individuals ranged from 1 to 7 (median 4). In elderly consuming probiotics the LAB counts were significantly higher than in these not consuming (median 7.8, range 4.2–10.8 vs. median 6.3, range 3.3–9.7 log cfu/g; $p = 0.005$), adjusted (OR = 1.71, CI95 1.04–2.82; $p = 0.035$) for age and body mass index (BMI). The colonization by *L. acidophilus* was negatively related ($r = -0.367$, $p = 0.0275$) to *L. reuteri*, staying significant after adjusting for age, sex and BMI (OR = 0.16, CI95 0.04–0.73; $p = 0.018$). However, the blood glucose concentration showed a tendency for a negative correlation for colonization with *Lactobacillus fermentum* ($r = -0.309$, $p = 0.062$) adjusted for BMI (Adj. $R^2 = 0.181$; $p = 0.013$) but not for age and sex. The higher white blood cells (WBC) count was positively related ($r = 0.434$, $p = 0.007$) to presence of *Lactobacillus reuteri* adjusted for age, sex and BMI (Adj. $R^2 = 0.193$, $p = 0.027$). The lower values of ox-LDL were predicted by higher counts of cultivable lactobacilli adjusted by sex, age and BMI ($r = -0.389$, $p = 0.016$; Adj. $R^2 = 0.184$ $p = 0.029$). In conclusion, the pilot study of elderly persons shows that the intestinal lactobacilli are tightly associated with WBC count, blood glucose and content of ox-LDL which all serve as risk markers in pathogenesis of inflammation, metabolic syndrome and cardiovascular disease (CVD).

1. Introduction

Gut microbiota forms an essential part of the complex ecosystem of the host and is involved in nutrition and health. Sequencing 16S rRNA genes of human stool samples accompanied with biochemical analyses have shown that some dominant bacterial divisions are associated with host nutrient uptake, metabolism, harvesting of energy and increased body weight. In obese people, a higher proportion of gram-positive *Firmicutes* as compared to gram-negative *Bacteroidetes* was found in gut [1,2]. However, which particular groups of microbes from the *Firmicutes* division found in the gut could mainly exert the collective metabolic influence on human health has not been fully explored among different age groups.

Elderly persons (>65 years) are the fastest growing subpopulation in the world [3]. During aging, mainly degenerative alterations in gut morphology and physiology occur. The disturbances in some metabolic pathways and decreased energy expenditure commonly result in excessive weight and obesity, driving elderly people to several medical complications − such as cardiovascular disease (CVD), hypertension, diabetes and osteoarthritis [4,5].

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A wide variety of host, microbiological, dietary and environmental factors affect bacterial colonization of the intestinal tract. In the elderly, the reduction in number and species diversity of anaerobes like bacteroides and bifidobacteria, and the increased abundance of aerobes as enterobacteria has been shown [6–8]. Nevertheless, there have been contradictory data concerning prevalence and numbers of *Lactobacillus* sp. during aging, accentuated with high inter-individual variation in species composition [9–11].

It has not been assessed if the higher counts or particular species of lactobacilli are related to host metabolic and defence reactions, performing a balanced human ecosystem.

The purpose of the pilot study was to evaluate the relations between molecularly assessed intestinal *Lactobacillus* sp. composition, the counts of fecal cultivable lactobacilli and some clinical (BMI and blood pressure) and laboratory blood indices (cellular-erythrocytes, white blood cells, platelets; metabolic-glucose, cholesterol; oxidative stress-related markers − ox-LDL and BDC-LDL) in healthy elderly people.

2. Materials and methods

2.1. Subjects

A total of 42 elderly persons from an area in Southern Estonia were recruited into the pilot study. The healthy elderly were selected from the registry of family doctors and by orthopedists of Tartu University Hospital scheduling the subjects for elective orthopedic surgery. The inclusion criteria for volunteers were: the age 65 years or above, considered themselves generally healthy and wished to participate in the study (confirmed by written informed consent). Subjects following special dietary routines, having an unstable cardiopulmonary system, with a history of diabetes and malignancy, chronic renal or hepatic failure, gut surgery, the presence of an acute illness four weeks prior to the study, on anti-hypertensive medication, with recent use of corticosteroids, non-steroid anti-inflammatory drugs, antibiotics during the last two months, or with a history of alcohol abuse were excluded from participation in this study.

2.2. Methods

The data on general health, education, welfare, nutritional habits and habitual gastrointestinal symptoms such as abdominal pain, flatulence, bloating, and stool frequency and on smoking, medications and probiotic consumption habits during the last 6 months were collected by a modified self-reported questionnaire [12]. Clinical investigations were performed, and samples for blood biochemistry and microbiological analyses of feces were collected. In two drop-outs the blood sample was clotted, and in two other persons the fecal samples were not obtained. These 4 persons did not differ in questionnaire data and BMI from these 38 (15 M and 23 F) who were included. The study was approved by the Ethics Committee of Tartu University Medical Faculty (No 139/16 20.06.2005).

2.3. Clinical investigation

All subjects completed a comprehensive medical evaluation including medical history and physical examination to evaluate the function of the cardiovascular system and determine the state of skin and mucosa. Each participant was clinically checked for anthropometrical indices: the body mass index (BMI) was calculated as the weight (kg) divided by height squared (m^2). The blood pressure (mmHg) was measured manually after the subjects remained in the supine position for 15 min and recorded to nearest 2 mmHg [13,14].

2.4. Blood samples

Blood samples were obtained in the early morning after 8 h of fasting. Samples (6 ml) were drawn from antecubital vein with vacutainer into heparinised tubes and immediately stored (on ice) at 4 °C. The plasma was separated from the cells by centrifugation at 3000 rpm for 10 min and the plasma samples were stored at −80 °C until analysis.

Hematological-clinical indices (hemoglobin, red blood cells, white blood cell count and platelets) as well as routine biochemical-clinical indices like plasma glucose and serum total cholesterol were determined by standard laboratory methods using certified assays in the local clinical laboratory of Tartu University Hospital.

2.5. Oxidative stress indices of blood

The different epitopes of LDL particles were measured by testing both the level of oxidation of apolipoprotein B (ox-LDL assay) and baseline diene conjugates in LDL lipids as a direct measure of *in vivo* LDL particle oxidation (BDC-LDL assay).

Oxidized low-density lipoprotein (ox-LDL) was measured in plasma, which had been stored at −70 °C as previously described [15].

Ox-LDL levels were measured using an enzyme-linked immunosorbent assay kit (Mercodia, AB, Uppsala, Sweden). This kit uses monoclonal antibody 4E6 [16] directed against a conformational epitope in the apo B-100 moiety of LDL that is generated as a consequence of substitution of at least 60 lysine residues of apo B-100 with aldehydes. The number of substituted lysines corresponds to the minimum number required for scavenger-mediated uptake of oxidized LDL (or apo B-100 aldehyde-modified form of ox-LDL). Substituting aldehydes can be produced by peroxidation of lipids of LDL, resulting in the generation of ox-LDL. Additionally, aldehydes that are released by endothelial cells under oxidative stress or by activated platelets may also induce the oxidative modification of apo B-100 in the absence of lipid peroxidation of LDL. For ox-LDL, within-assay variation coefficient (CV) was 6.3% and between-assay CV 4.7% [17].

For the measurement of baseline diene conjugates in low-density lipoproteins (BDC-LDL) the serum was separated from blood cells by centrifugation at $3000 \times g$ for 15 min [18]. To precipitate the LDL, 1 mg/1 ml of EDTA was added and precipitation reagents were allowed to equilibrate to room temperature. The blood sample (0.5 ml) was added to 3.5 ml of the heparin-citrate buffer. The precipitation buffer consisted of 0.064 mol/l trisodium citrate adjusted to pH 5.05 with 5 mol/l HCl, and contained 50.000 IU/l heparin. After mixing with a Vortex mixer, the suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were sedimented by centrifugation at $1000 \times g$ for 10 min. The pellet was resuspended in 0.5 ml of 0.1 mol/l Na-phosphate buffer, pH 8.0, and containing 0.9% of NaCl. Lipids were extracted from LDL samples (100 μl) by chloroform−methanol (2:1), dried under nitrogen, then re-dissolved in cyclohexane, and analysed spectrophotometrically at 234 nm for diene conjugates. Absorbance units (difference $A_{234}-A_{300}$) were converted to molar units using the molar extinction coefficient $2.95 \times 10^4 \, M^{-1} \, cm^{-1}$. For BDC-LDL, the CV for within-assay precise determinations of samples precipitated from the same serum was 4.4%, and the CV for the between-assay precision over a period of 3 months was 4.5%.

2.6. Molecular investigation of intestinal Lactobacillus sp in fecal samples

Approximately 2 g of fresh stool samples were obtained and put into sterile plastic cups. The samples were collected and delivered

within 1 h after defecation to the laboratory where the samples were stored at −20 °C before processing.

Bacterial DNA from fecal samples was extracted using a QIAamp DNA stool mini kit (QIAgen, Hilden, Germany) with some modifications. The amount of 0.22 g of feces were resuspended in 200 μl of TE buffer (10 mM Tris, 10 mM EDTA pH 8, 20 mg/ml lysozyme, 200 u/ml mutanolysin) and incubated for 1 h at 37 °C. A total of 0.3 g of 0.1 mm zirconia/silica beads and 1.4 ml of ASL solution from the stool mini kit was added to fecal samples. The tubes were then agitated for 3 min at a speed of 5000 rpm in a mini-beadbeater (BiospecProducts). The protocol was then continued as described by the manufacturer. The amount of DNA was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide.

The *Lactobacillus* species-specific qualitative PCR was carried out by primers listed in Table 1 targeted on the 16S-23S rRNA intergenic spacer region [19–21]. A reaction mixture (50 μl) consisted of 10X reaction buffer, a 200 μM concentration of each deoxynucleoside triphosphate, 10 pmol of each primers, 5 μl of bacterial DNA (extracted from fecal samples) and 1.5 U of HotStar Taq Plus DNA polymerase (QIAgen, Hilden, Germany). The amplification program was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 30 s at the appropriate annealing temperature (Table 1), and 72 °C for 30 s. A cycle of 72 °C for 10 min concluded the program. Amplification products were detected by agarose gel electrophoresis on 2% agarose gel, ethidium bromide staining, and UV transillumination. The size of PCR products was compared with the aforementioned *Lactobacillus* reference strains (*Lactobacillus acidophilus* ATCC 4356, *Lactobacillus delbrueckii spp. delbrueckii* ATCC 9649, *Lactobacillus helveticus* ATCC 8018, *Lactobacillus paracasei* DSM 20020, *Lactobacillus casei ssp. casei* NCDC150, *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus plantarum* DSM 9843, *Lactobacillus fermentum* ATCC 14931, *L. reuteri* DSM 20016). The number of different *Lactobacillus* species colonizing the individual and their prevalence in elderly was assessed.

2.7. Estimation of cultivable lactobacilli

For bacteriological analyses the weighed fecal samples were serially diluted (10^{-2}–10^{-9}) in pre-reduced phosphate buffer (pH 7.2) inside the anaerobic glove box (Sheldon Manufacturing Inc., USA, with a gas mixture: 5% CO_2; 5% H_2, 90% N_2). A quantitative analysis of the gut bacteria was performed using duplicate samples of 0.05 ml of each dilution on the de Man–Rogosa–Sharpe (MRS) agar (Oxoid) for microaerobic lactobacilli and streptococci. The inoculated MRS agar plates were incubated in a microaerobic atmosphere (CO_2–incubator"Jouan" IG 150, France), with gas mixture (10% CO_2) at 37 °C for 48 h [22].

The colony counts of the different fecal dilutions on MRS media were recorded. All colonies of different morphology grown from the highest dilutions on MRS agar were isolated and the lactobacilli were identified according to gram-positive rod-shaped morphology and the negative catalase test [23,24].

The composition of the gut microbiota was expressed as counts (CFU/g expressed in $\log_{10}$) and prevalence (%) of lactobacilli. The detection level of the micro-organisms was 3 $\log_{10}$ CFU/g.

2.8. Statistical analysis

Statistical analysis was performed by using the computer program Sigma Stat for Windows 2.0 (Jandel Corporation, USA). The Student's *t*-test or Mann–Whitney *U* test (nonparametric distribution) was applied to compare the differences in the clinical and microbiological indices of the probiotic consumers and non-consumers. The χ^2-test or Fisher exact test was used to determine the between-group differences in categorical variables. Spearman correlations were used to determine the relationships between variables. Linear multiple regression analysis with an additive model adjusted for age, sex and BMI was used to detect the association between variables 1 and 2 of Table 3. Logistic regression analyses were performed to compare the binary variables and/or continuous variables. Results are given with odds ratios (OR) with 95% CI. The models were adjusted for BMI, sex and age. Differences were considered statistically significant if the value was $p < 0.05$.

3. Results

3.1. Questionnaire data

According to self-reported questionnaires, the participants did not complain about expressed habitual gastrointestinal symptoms (abdominal pain, flatulence, bloating, and stool frequency; $p > 0.05$; data not shown). Only three current smokers were registered and 16 persons were habitual consumers of probiotic functional food. The female elderly were more frequent consumers of probiotic food than male (OR = 5.2 CI95 1.15–23.54; $p = 0.0324$).

Table 1
List of primers used in this study for detection of *Lactobacillus* sp according to literature [19–21].

Target	Primers	Sequence (5′–3′)	Annealing temp (°C)
L. acidophilus	F_acid_IS	GAAAGAGCCCAAACCAAGTGATT	59
	R_acid_IS	CTTCCCAGATAATTCAACTATCGCTTA	59
L. casei	R_casei_IS	CTTCCTGCGGGTACTGAGATGT	59
	F_casei_IS	CTATAAGTAAGCTTTGATCCGGAGATTT	59
L. delbrueckii	R_delb_IS	CGAACTCTCTCGGTCTT	58
	F_delb_IS	CACTTGTACGTTGAAAACTGAATATCTTAA	58
L. fermentum	R_ferm_IS	ACTTAACCTTACTGATCGTAGATCAGTCA	58
	F_ferm_IS	AACCGAGAACACCGCGTTAT	58
L. paracasei	F_paca_IS	ACATCAGTGTATTGCTTGTCAGTGAATAC	60
	R_paca_IS	CCTGCGGGTACTGAGATGTTTC	60
L. plantarum	F_plan_IS	TGGATCACCTCCTTTCTAAGGAAT	60
	R_plan_IS	TGTTCTCGGTTTCATTATGAAAAAATA	60
L. reuteri	F_reut_IS	ACCGAGAACACCGCGTTATTT	59
	R_reut_IS	CATAACTTAACCTAAACAATCAAAGATTGTCTCT	59
L. rhamnosus	F_rham_IS	CGGCTGGATCACCTCCTTT	59
	R_rham_IS	GCTTGAGGGTAATCCCCTCAA	59
L. helveticus	F_helv_IS	CTCTTCTCGGTCGCCTTG	56
	R_helv_IS	GAAGTGATGGAGAGTAGAGATTA	56

3.2. Clinical and blood indices

No substantial shifts were found in the functioning of cardiovascular system and the state of skin and mucosa (data not shown). The values of fasting plasma glucose, Hgb and the counts of blood cells (erythrocytes, white blood cells and platelets) responded to the reference values (Table 2). As compared to reference data the increased values of systolic (34%) and diastolic (16%) blood pressure, overweight (37%) and obesity (24%) were registered in several subjects while increased cholesterol values were found in 76% of the elderly persons.

3.3. Lactobacillus species composition of elderly

The overall prevalence of the 8 species out of 9 in the fecal samples of the elderly is depicted in Fig. 1. We could not detect the specific banding pattern of *L. rhamnosus* in any elderly person. The facultative heterofermentative lactobacilli *L. casei* and *L. paracasei* were the most frequent (89% and 97%, respectively) species found, while *L. acidophilus*, *L. plantarum* and *L. reuteri* were present in almost half of the elderly persons (47%, 58% and 53%, respectively). According to PCR products the number of species simultaneously colonizing the individuals was 1–7 (median 4).

The colonization by *L. acidophilus* was negatively related ($r = -0.367$, $p = 0.0275$) to *L. reuteri* adjusted for age, sex and BMI (OR = 0.16, CI95 0.04–0.73; $p = 0.018$) applying logistic regression analysis.

3.4. Quantitative composition of fecal microbiota

Lactobacilli were present in all investigated elderly persons, their median count was 6.8 (range 3.3–10.8 log cfu/g). The counts of lactobacilli were similar (median 7.3, range 3.7–9.7 vs. median 6.8, range 3.3–10.8 log cfu/g, $p > 0.05$) if the persons were divided according to the reference values of BMI (<24.9 and ≥ 25 kg/m^2)

Table 2
Clinical and biochemical data of healthy elderly persons.

Characteristics	Total $n = 38$	Reference values[*]
Age (years)	72 ± 5.0	
Body mass index (kg/m^2)	27.1 ± 4.0	
Normal (N %)	15 (39%)	Normal 18.5–24.9^3 kg/m^2
Overweight (N %)	14 (37%)	Overweight >25
Obesity (N %)	9 (24%)	Obese >30
Systolic blood pressure (mmHg)	134 ± 16.1	<130 mmHg
Over normal value 130 (N %)	13 (34%)	
Diastolic blood pressure (mmHg)	79 ± 8.0	<85 mmHg
Over normal value 85 (N %)	6 (16%)	
Fasting plasma glucose (mmol/l)	5.2 ± 0.6	3.1–6.4 mmol/l
Erythrocytes (×10^{12}/l)	4.6 ± 0.4	Men 4.5–5.5; women 3.8–4.8
Hgb (g/l)	139 ± 11	Men 130–175; women 120–160
WBC (×10^9/l)	5.7 ± 1.4	4–10 × 10^9/l
Platelets (×10^9/l)	227.4 ± 41	150–400 × 10^9/l
Total cholesterol (mmol/l)	5.8 ± 1.1	<5.0 mmol/l
Over normal value 5.0 (N %)	29 (76%)	
Ox-LDL (U/l)	168.3 ± 45.4	145 ± 54.2 U/l
BDC-LDL (μmol/l)	48.2 ± 12.0	45.8 ± 12.1 μmol/l

References: [13,14,17,39]. Abbreviations: Hgb – hemoglobin, WBC – white blood cells; ox-LDL – oxidized low-density lipoproteins; BDC-LDL – baseline diene conjugates in low-density lipoproteins.
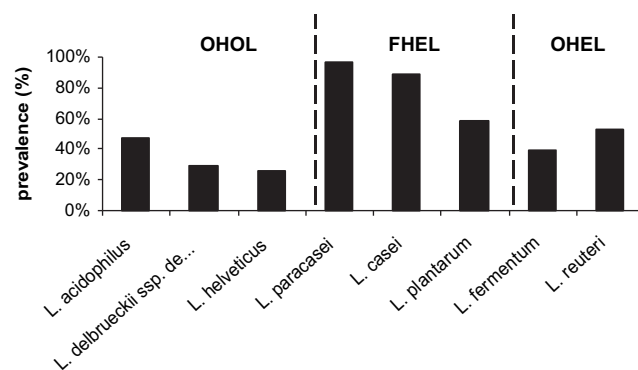

Fig. 1. Prevalence of intestinal *Lactobacillus* sp., assessed by PCR products in fecal samples of healthy elderly persons. Abbreviations: OHOL – obligate homofermentative lactobacilli; FHEL – facultative heterofermentative lactobacilli; OHEL – obligate heterofermentative lactobacilli.

and no correlations between these indices were found. Also, the counts of lactobacilli were not predicted from the *Lactobacillus* species composition and the number of species simultaneously colonizing the individuals (data not shown).

In elderly consuming probiotics the lactobacilli counts were significantly higher than in these not consuming (median 7.8, range 4.2–10.8 vs. median 6.3, range 3.3–9.7 log cfu/g, $p = 0.005$). This difference stayed significant if adjusted for age and BMI (OR = 1.71, CI95 1.04–2.82; $p = 0.035$).

3.5. Comparisons of cellular and metabolic markers with Lactobacillus species composition and counts in the elderly

A positive correlation ($r = 0.402$; $p = 0.014$) Table 3 was found between BMI and fasting blood glucose content adjusted for age and sex applying linear regression analysis (Adj. $R^2 = 0.084$, $p = 0.022$).

Also the values of ox-LDL were positively correlated to cholesterol values ($r = 0.414$, $p = 0.011$) adjusted for age, sex and BMI (Adj. $R^2 = 0.217$, $p = 0.018$). The counts and colonization with different species of lactobacilli were not directly associated with BMI of elderly. However, the blood glucose concentration showed a tendency for a negative correlation for colonization with *L. fermentum* ($r = -0.309$, $p = 0.062$) adjusted for BMI (Adj. $R^2 = 0.181$, $p = 0.013$) yet the sex and age were the confounding factors.

The increased WBC count was related ($r = 0.434$, $p = 0.007$) to presence of *L. reuteri* (Adj. $R^2 = 0.193$, $p = 0.027$) adjusted for age, sex and BMI. The values of ox-LDL were negatively related to lactobacilli counts ($r = -0.389$, $p = 0.016$) which association stayed significant according to linear regression analysis adjusted for age, sex and BMI (Adj. $R^2 = 0.184$, $p = 0.029$).

4. Discussion

In our study, the association between some principal cellular (WBC count), metabolic (concentration of plasma glucose) and oxidative stress (ox-LDL) indices of blood with intestinal *Lactobacillus* sp. was assessed, demonstrating the systemic defense potential of *Lactobacillus* sp. in host microbial ecology.

The excessive weight, increased values of blood glucose and WBC counts together with oxidative stress indices are used widely as predictors for inflammation, metabolic syndrome and cardiovascular disease (CVD) [4,25,26]. At the same time it is well-known

Table 3
Spearman rank order correlation and linear multiple regression analysis between counts and species of lactobacilli, BMI, blood glucose, cholesterol and ox-LDL indices.

Variable 1	Variable 2	Correlation coefficients r and p- values	Linear multiple regression analysis adjusted for age, sex and/or BMI (Adj. R^2, p values)
Body mass index (kg/m^2)	Plasma glucose (mmol/l)	$r = 0.402$, $p = 0.014$	Adj. $R^2 = 0.084$, $p = 0.022$
Plasma glucose (mmol/l)	Colonization with *L. fermentum*	$r = -0.309$, $p = 0.062$	Adj. $R^2 = 0.181$, $p = 0.013$ for BMI Not for age and sex
Ox-LDL (U/l)	Total cholesterol (mmol/l)	$r = 0.414$, $p = 0.011$	Adj. $R^2 = 0.217$, $p = 0.018$
Count of lactobacilli (log cfu/g)	Ox-LDL (U/l)	$r = -0.389$, $p = 0.016$	Adj. $R^2 = 0.184$, $p = 0.029$
WBC ($\times 10^9$/l)	Colonization with *L. reuteri*	$r = 0.434$, $p = 0.007$	Adj. $R^2 = 0.193$, $p = 0.027$

that over 1000 species of bacteria reside in the colons of healthy adults, actively involved in human metabolism [27,28].

We found by the species-specific PCR that the number of *Lactobacillus* species, colonizing the gastrointestinal tract of elderly persons varied largely (from 1 to 7), and it was expressed with various individual combinations. The facultative heterofermentative species like *L. casei/L. paracasei* prevailed, followed by *L. plantarum*, similar to some other reports [10,29]. Homofermentative *L. acidophilus* and obligately heterofermentative *L. fermentum* were found in nearly half of the elderly, resembling the Italian and Greece culture-based approach [30,31]. The limitation of our study could be the set of applied primers not allowing to detect *Lactobacillus ruminis* [11].

In our elderly subjects the general health was assured using strict exclusion clinical criteria confirmed by the laboratory blood indices that generally responded to the widely accepted reference values. However, 61% of the subjects had excessive weight and respectively a half suffered from expressed osteoarthritis, scheduled for corrective operation. It has been shown that the prevalence of osteoarthritis is 68% in women and 58% in men >65 years of age and the high BMI is clearly associated with an increased risk of osteoarthritis [4]. The etiology and pathogenesis of osteoarthritis (whether of inflammatory origin or due to the degeneration of cartilage) are still an issue of debate [32,33]. According to normal WBC values there was found no systemic inflammation in the elderly. However, surprisingly, in elderly persons the prevalence of intestinal *L. reuteri* was positively related to white blood cell counts and the former species negatively to the *L. acidophilus*. Though no fermented food with probiotic *L. reuteri* has been marketed in Estonia, *L. reuteri* was found in 53% of investigated fecal samples. It is a warning sign to individuals with excessive weight and predisposition for inflammation, as the introduction of a probiotic *L. reuteri* can increase the inflammatory state and the risk for CVD. Recently the leukocyte count has been used as a predictor of cardiovascular events and mortality [26].

Beside molecular studies we have applied bacteriological methods for the quantitative determination of *Lactobacillus* sp. The reasons were as follows: First, in enumeration of *Lactobacillus* sp. we aimed to avoid the registration of DNA of dead bacteria, seemingly not involved in the host and microbiota integrated metabolism. Second, the Lab158 probe hybridizes both to *Lactobacillus* and *Enterococcus* sp and therefore did not afford their clear distinction [11].

The consumption of probiotic functional food revealed by a questionnaire was correlated with higher counts of cultivable lactobacilli. This is in agreement with some other interventional probiotic studies [15,29,34]. Though there was no association between the counts of lactobacilli and the increased BMI, still, the tendency for lower content of blood glucose was predicted by colonization with *L. fermentum* adjusted for BMI but not for age and sex. In turn, the positive correlation, as anticipated, between BMI and fasting blood glucose was found. In elderly, due to large inter-individual differences in physical fitness, the BMI have not

been considered as full predictor of health status or adverse outcomes [35].

Obligately heterofermentative lactobacilli, incl. *L. fermentum* play an important role in carbohydrate breakdown and fermentation in the intestine: beside lactic and acetic acids they are also the producers of succinate [36]. It can be speculated that colonization by *L. fermentum* decreases the uptake of both glucose (hexoses) and fructose (pentoses) in the intestine due to its most effective phosphoketolase pathway of carbohydrate fermentation [37].

Further, to the best of our knowledge, this is the first study showing the inverse association between the counts of cultivable intestinal lactobacilli and oxidized LDL particles in elderly individuals. The effect of probiotic *L. rhamnosus* GG intervention on global serum lipidomic profiles of healthy adults was recently published [38]. Besides, in our study, as anticipated, a close connection between total cholesterol and ox-LDL was revealed. The increased level of ox-LDL (oxidized apolipoprotein B-100) is suggested for an important marker of systemic inflammation and oxidative stress (oxS) driven responses [25,39]. According to a large population-based study the ox-LDL is correlated with an increased incidence of metabolic syndromes overall, as well as obesity, hyperglycemia and hyper-triglyceridemia already in people who are currently young and healthy [40]. Also the relative risk of death and coronary events increase with higher levels of ox-LDL affecting the endothelium of blood vessels as well as arterial elasticity [41,42].

How can the high count of metabolically active lactobacilli operate against oxidativestress-caused damage in the host? First, some lactic acid bacteria, particularly *L. fermentum* are involved in cholesterol metabolism [43]. In our study, more than a third (39%) of the elderly was colonized with this species. Second, lactobacilli possess several anti-oxidative mechanisms: catalase, glutathione-system-related compounds, and Mn-SOD [44–46]. Recently, the role of extra-cellular antioxidant enzymes for the attenuation of proliferation caused by reactive oxygen species in human smooth muscle cells was shown [47]. In addition, different species and particular strains of lactobacilli can create anti-oxidative peptides [48]. It is also possible that lactobacilli can influence host metabolic pathways to modulate the metabolism of lipoproteins, leading to decreased amounts of ox-LDL. Previously, we have shown an improvement of blood lipoprotein metabolism (ox-LDL, lag phase of LDL and BDC-LDL) after consumption of the antimicrobial and anti-oxidative probiotic *L. fermentum* ME-3 [15]. Nowadays, the role of antioxidants as a possible "third great wave" in cardiovascular secondary prevention has been widely discussed [49].

In conclusion, the pilot study of elderly persons showed for the first time that the intestinal lactobacilli are tightly bound to WBC count, blood glucose and content of oxidized lipoprotein which all serve as risk markers in pathogenesis of inflammation, metabolic syndrome and CVD (Fig. 2). The application of particular species of probiotic lactobacilli could be a challenge in terms of the health care of elderly people concerning the complex control of their
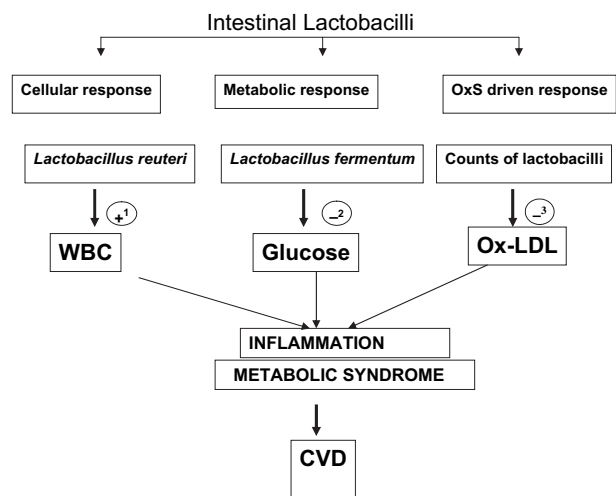
Fig. 2. Some associations between intestinal *Lactobacillus* sp. with cellular, metabolic and oxidative stress driven responses in elderly persons. [1]The higher white blood cells count was predicted by colonization with *L. reuteri* ($p = 0.007$) adjusted for age, sex and BMI (Adj. $R^2 = 0.193$, $p = 0.027$). [2]The higher plasma glucose content was negatively predicted by colonization with *L. fermentum* ($r = -0.309$, $p = 0.062$) adjusted for BMI (Adj. $R^2 = 0.181$; $p = 0.013$) but not for age and sex. [3]Values of ox-LDL were negatively related to lactobacilli counts ($r = -0.389$, $p = 0.016$ by Spearman correlation analysis) adjusted for age, sex and BMI (Adj. $R^2 = 0.184$, $p = 0.029$).

metabolic and systemic defence reactions, including oxidative stress-related ones.

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Disclosures

The authors have declared that they have no interests which might be perceived as posing a conflict or bias.

Authors' contributions

MM and HK participated in the design and coordination of the study; JS applied the molecular methods, HK has investigated the patients and collected the clinical data, ES and KL analyzed the quantitative composition of microbiota, KZ analyzed the oxidative stress related markers, PH tested the relations between the clinical laboratory and microbiological data; MM, JS, PH, ES and MZ participated in the writing of the manuscript. All authors read and approved the final manuscript.

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