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Diversity and metabolic impact of intestinal *Lactobacillus* species in healthy adults and the elderly

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Abstract

The present study aimed at assessing the counts and species distribution of intestinal lactobacilli and exploring if the data are associated with BMI and blood glucose level in healthy adults and elderly persons. The BMI ($P<0.01$), the level of fasting blood glucose ($P<0.001$) and the total counts of lactobacilli ($P<0.01$ by bacteriology; $P<0.001$ by real-time PCR) were higher in the elderly. The number of species in adults was lower ($P<0.05$), who were more often colonised with *Lactobacillus acidophilus* ($P=0.031$) and *L. helveticus* ($P<0.001$). In contrast, *L. plantarum* ($P=0.035$), *L. paracasei* ($P<0.001$) and *L. reuteri* ($P=0.031$) were more prevalent in the elderly. *L. rhamnosus* was detected in adults ($P<0.001$), but not in any elderly person. BMI was associated with counts of lactobacilli, adjusted for age and sex ($P=0.008$). The higher BMI in both groups of persons was associated with the presence of obligate homofermentative lactobacilli and *L. sakei*, both adjusted for age and sex. Plasma glucose values were positively correlated with BMI and negatively correlated with colonisation with *L. paracasei* ($P=0.0238$) in adults and on the borderline with *L. fermentum* ($P=0.052$) in the elderly. Thus, the species-specific PCR analysis of *Lactobacillus* sp. combined with viable plating data indicates substantial age-related structural differences in the intestinal lactobacilli communities. The higher counts of intestinal *Lactobacillus* sp. are associated with higher BMI and blood glucose content, while their specific fermentative groups and species of lactobacilli appear at different glucose levels both in adults and in the elderly.

Key words: Metabolic impact: *Lactobacillus* species: Faecal microbiota: Adults: Elderly

A host and its microbiota form a tightly linked microbial ecosystem. This has several health consequences that expose differentially during the lifespan. The changes in the bacterial colonisation of the gut and metabolic activities in the colonic ecosystem occur during ageing[1,2]. In the elderly, the country-specific increased prevalence and the number of *Lactobacillus* species during ageing have been assessed[2–4].

Lactobacillus sp. belongs to the members of lactic acid bacteria[5–7] and is phylogenetically included in the division of Firmicutes[8]. The genus *Lactobacillus* comprises more than ninety validly described species and some subspecies, while approximately 30% of them have been isolated from faecal sources[9] (www.bacterio.cict.fr). The role of lactobacilli has received much attention, especially due to their putative health-promoting properties. *Lactobacillus* sp. has been shown to contribute to digestion, stimulation of immunity and inhibition of pathogens[7,10–13]. Recently, some studies have reported an association between the presence of intestinal lactobacilli and an impact on metabolism and energy uptake in the host[14].

However, there are a few comparative studies of *Lactobacillus* species diversity among different ages in adults and elderly groups, either by applying bacteriological methods[15,16] or by using denaturing gradient gel electrophoresis with *Lactobacillus* genus-specific primers[17–19]. The classical division of lactobacilli has been based on their fermentative characteristics: obligate homofermentative lactobacilli (OHOL); facultative heterofermentative lactobacilli (FHEL); obligate heterofermentative lactobacilli (OHEL)[20]. During fermentation of polysaccharides in the colon, the lactobacilli strains of particular species may use different pathways, providing the host with energy differentially. Recent work by the Gordon team has shown that Gram-positive

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bacteria of the Firmicutes phylum rather than those of Bacteroidetes expressed specific enzymatic activities in obese individuals[21]. Whether this process is influenced by differences in age, sex and increased body weight has not been elucidated yet.

The aim of the present study was to assess the counts and species distribution of intestinal lactobacilli and to explore if the data of intestinal lactobacilli are associated with BMI and blood glucose level in healthy adults and elderly persons.

Materials and methods

Sampling and processing of samples

The study group comprised twenty-four healthy adults (nine males and fifteen females; age: median 27·0 years (quartiles 23–31·5)) and thirty-seven elderly persons (sixteen males and twenty-one females; age: median 73 years (quartiles 68–75·2)), $P < 0.001$ (Table 1). The present study was conducted according to the guidelines laid down in the Declaration of Helsinki. The study was approved by the Ethics Committee of the Medical Faculty of the University of Tartu (no. 139/16 20.06.2005). The baseline values of healthy adults have been included from the study assessing the impact of a probiotic product (ISRCTN38739209, also approved by the aforementioned Ethics Committee (approval no. 158/10 26.03.2007)). The inclusion criteria for adults were as follows: considering themselves healthy, no gastrointestinal disorders and no recent history of antibiotic treatment.

The healthy elderly were selected from the registry of family doctors and orthopaedists of the Tartu University Hospital, Estonia, before performing elective orthopaedic surgery. The inclusion criteria for volunteers were as follows: age 65 years or above, considering themselves generally healthy and had not had any recent antibiotic treatment.

Both adults and the elderly wished to participate in the study, which was 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 4 weeks

before the study, on anti-hypertensive medication, with recent use of corticosteroids, non-steroid anti-inflammatory drugs, antibiotics during the last 2 months or with a history of alcohol abuse were excluded from participation in the present study.

Subjects in both groups consumed habitually a Western-type diet, typically rich in potatoes, vegetables, meat, eggs but characterised also with a high content of fibre (rye bread, oat/wheat/rice porridge) and dairy products, vegetable seed oils, margarine and non-alcoholic beverages[22,23].

Participants' body weight was measured in light clothing to the nearest 0·1 kg using a calibrated scale. Height was measured without shoes to the nearest 0·1 cm using a vertical ruler. BMI was calculated as weight (kg) divided by height squared (m^2)[24].

Blood samples were obtained in the early morning after 8 h of fasting. Samples were drawn from the antecubital vein with a vacutainer into heparinised tubes and immediately stored (on ice) at 4°C. Plasma glucose (mmol/l) was determined by standard laboratory methods using certified assays in the local clinical laboratory of the Tartu University Hospital. The reference values for human BMI (kg/m^2) and glucose in blood (mmol/l) are presented in Table 1.

Approximately 2 g of fresh stool samples were placed into sterile containers. The samples collected at home or at the hospital were kept in a domestic refrigerator at 4°C for not more than 2 h before transportation to the laboratory, where the containers were stored frozen at −70°C until use.

Counts of lactobacilli in faeces by the bacteriological method

For bacteriological analyses, the weighed samples of faeces were serially diluted $(10^{-2}-10^{-9})$ in pre-reduced phosphate buffer (pH 7·2) inside an anaerobic glove box (Sheldon Manufacturing, Inc., Cornelius, OR, USA), with a gas mixture (5 % CO_2, 5 % H_2 and 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 agar (Oxoid, Basingstoke Hampshire, UK) for micro-aerobic lactobacilli. The de Man–Rogosa–Sharpe agar was incubated in a micro-aerobic atmosphere

Table 1. Clinical data of adults and the elderly persons (>65 years)

(Mean values and standard deviations)

Indices	Adults (n 24)		Elderly (n 37)		P	Reference values[24]
	Mean	SD	Mean	SD		
Age (years)	29·2	8·2	72·5	5·0	<0·001	>65 Years old (elderly)
BMI (kg/m²)	24·5	4·1	27·2	4·2	0·012	Normal: 18·5–24·9 Overweight: >25 Obese: >30
Fasting plasma glucose (mmol/l)	4·5	0·50	5·2	0·60	<0·001	3·1–6·4

(CO_2-incubator 'Jouan' IG 150, Saint-Herblain, France), with a gas mixture (10 % CO_2) at 37°C for 48 h[25]. The colony counts of the different faecal dilutions on de Man–Rogosa–Sharpe media were recorded. All colonies of different morphology grown from the highest dilutions on de Man–Rogosa–Sharpe agar were isolated, and the lactobacilli were identified according to Gram-positive rod-shaped morphology and the negative catalase test[20,26].

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

Molecular identification of Lactobacillus sp.

DNA extraction. Bacterial DNA from the faecal samples was extracted using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) with some modifications. In 200 μl of 10 mM-Tris, 10 mM-EDTA buffer (pH 8; lysozyme (20 mg/ml) and mutanolysin (200 units/ml)), 0·22 g of faeces were resuspended and incubated for 1 h at 37°C. Then, 0·3 g of 0·1 mm zirconia/silica beads and 1·4 ml of ASL solution (Qiagen) from the stool mini kit were added to the faecal samples. The tubes were then agitated for 3 min at a speed of 5000 rpm in a mini-bead beater (Biospec Products, Inc., Bartlesville, OK, USA). The protocol was then continued as described by the manufacturer (Qiagen).

Quantitative analysis of total lactobacilli by real-time-PCR. In order to establish a quantitative assay, we cloned plasmids containing the amplified region of target bacteria using the pGEM-T vector system (Promega, Madison, WI, USA). The PCR amplicon for *L. paracasei* was individually inserted into a separate plasmid vector; the recombinant vector was transformed in chemically competent *Escherichia coli*. Plasmids were purified with MaxiPrep (Qiagen). The purified plasmids were quantified by spectrophotometry (Quibit™; Invitrogen, Carlsbad, CA, USA) of multiple dilutions[27]. Quantification of target DNA was achieved by using serial tenfold dilutions from 10^2 to 10^9 plasmid copies of the previously quantified plasmid standards. Plasmid standards and samples were run in triplicate, and the average values were used for the calculation of the bacterial load.

Real-time PCR (RT-PCR) was performed with the ABI PRISM 7500 HT Sequence Detection System (Applied Biosystems, Bedford, MA, USA) using optical-grade ninety-six-well plates. The PCR was performed on a total volume of 25 μl using SYBR Green PCR Master mix (Applied Biosystems). Each reaction included 150 ng of template DNA, 12·5 μl of SYBR Green PCR Master mix (Applied Biosystems) and 2 μM of each primer[5,28] (Table 2). The conditions were set as follows: 2 min at 50°C and 10 min at 95°C, followed by forty cycles consisting of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 1 min. Data analysis was conducted with Sequence Detection Software version 1.6.3, supplied by Applied Biosystems.

Reference strains. The reference strains used in the study were composed of three different fermentation groups[29] of *Lactobacillus*: OHOL *L. acidophilus* ATCC 4356, *L. delbrueckii* spp. *delbrueckii* ATCC 9649, *L. salivarius* DSM 20555, *L. crispatus* DSM 20584, *L. johnsonii* DSM 10533, *L. gasseri* DSM 20077, *L. helveticus* DSM 20075; FHEL *L. paracasei* DSM 20020, *L. ruminis* DSM 20403, *L. curvatus* ATCC 2560, *L. casei* NCDC 150, *L. rhamnosus* ATCC 53103, *L. plantarum* DSM 9843, *L. sakei* DSM 20100; OHEL *L. fermentum* ATCC 14931, *L. reuteri* DSM 20016, *L. brevis* DSM 20054, *L. buchneri* DSM 5987.

Species-specific PCR analysis of Lactobacillus *sp. in faecal samples.* The *Lactobacillus* species-specific qualitative PCR was carried out by primers listed in Table 2, targeted on the 16S–23S ribosomal RNA intergenic spacer region[18,30–32]. The primer pair for the *L. ruminis* subgroup is specific for *L. ruminis*, *L. animalis*, *L. mali*, *L. salivarius* and *L. satsumensis*[33]. A reaction mixture (50 μl) consisted of 10 × reaction buffer, a 200 μM concentration of each deoxynucleoside triphosphate, 1 μM of each primer, 100 ng of bacterial DNA (extracted from the faecal samples) and 1·5 U of HotStar Taq Plus DNA polymerase (Qiagen). The amplification programme consisted of pre-denaturation at 94°C for 5 min, followed by thirty-five cycles of 94°C for 30 s, 30 s at the appropriate annealing temperature (Table 2), and finally 72°C for 30 s. A cycle of 72°C for 10 min concluded the programme. Amplification products were detected by agarose gel electrophoresis on 2 % agarose gel, ethidium bromide staining and UV transillumination.

The size of the PCR products was compared with that of the aforementioned *Lactobacillus* reference strains.

Statistical analyses

The statistical analysis was performed using the SIGMA-STAT 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA) and the SPSS 11.0 (SPSS, Inc., Chicago, IL, USA) statistical software packages. Data are presented as means and standard deviations or ranges and medians. Bacterial count data were logarithmically transformed; the prevalence of the species was expressed as a percentage.

According to the data of the descriptive statistics, Student's *t* test and the Mann–Whitney rank-sum test were applied to compare the differences in clinical and microbiological indices. Logistic regression analyses were performed to compare the binary variables and/or continuous variables. The Spearman rank correlation test and the multiple linear regression models were used to test the associations between microbiological and clinical or biochemical indices in both groups. The models were adjusted for BMI, age and sex. All differences were considered statistically significant if $P < 0.05$.

Table 2. List of primers used for the detection of *Lactobacillus* spp.[18,30–32]

Group	Target	Primers	Sequence (5′–3′)	Annealing temperature (°C)	Amplicon length (bp)
OHOL	*L. acidophilus*	F_acid_IS R_acid_IS	GAAAGAGCCCAAACCAAGTGATT CTTCCCAGATAATTCAACTATCGCTTA	59	85
	L. helveticus	F_helv_IS R_helv_IS	CTCTTCTCGGTCGCCTTG GAAGTGATGGAGAGTAGAGATTA	56	160
	L. delbrueckii	R_delb_IS F_delb_IS	CGAACTCTCTCGGTCTT CACTTGTACGTTGAAAACTGAATATCTTAA	58	94
	L. salivarius	R_sal_IS F_sal_IS	GTCGTAACAAGGTAGCCGTAGGA TAAACAAAGTATTCGATAAATGTACAGGTT	61	97
	L. crispatus	R_cri_IS F_cri_IS	GTAATGACGTTAGGAAAGGG ACTACCAGGGTATCTAATGG	57	85
	L. johnsonii	R_joh_IS F_joh_IS	GAGCTTGCCTAGTAGATTTTA ACTACCAGGGTATCTAATCC	58	80
	L. gasseri	R_gas_IS F_gas_IS	AGCGACCGAGAAGAGAGAGA TGCTATCGCTTCAAGTGCTT	57	360
	L. ruminis subgroup	F_rum_sub R_rum_sub	CACCGAATGCTTGCAYTCACC GCCGCGGGTCCATCCAAAA	57	182
OHEL	*L. fermentum*	R_ferm_IS F_ferm_IS	ACTTAACCTTACTGATCGTAGATCAGTCA AACCGAGAACACCGCGTTAT	58	88
	L. reuteri	F_reut_IS R_reut_IS	ACCGAGAACACCGCGTTATT CATAACTTAACCTAAACAATCAAAGATTGTCT	59	93
	L. brevis	F_bre_IS R_bre_IS	ATTTTGTTTGAAAGGTGGCTTCGG ACCCTTGAACAGTTACTCTCAAAGG	55	289
	L. buchneri	R_buc_IS F_buc_IS	GGACCAATGGAGCAACTGAA AGATTACTGACGCATTGGTTACCA	55	72
FHEL	*L. casei*	R_casei_IS F_casei_IS	CTTCCTGCGGGTACTGAGATGT CTATAAGTAAGCTTTGATCCGGAGATTT	59	132
	L. paracasei	F_paca_IS R_paca_IS	ACATCAGTGTATTGCTTGTCAGTGAATAC CCTGCGGGTACTGAGATGTTTC	60	80
	L. plantarum	F_plan_IS R_plan_IS	TGGATCACCTCCTTTCTAAGGAAT TGTTCTCGGTTTCATTATGAAAAAATA	60	144
	L. rhamnosus	F_rham_IS R_rham_IS	CGGCTGGATCACCTCCTTT GCTTGAGGGTAATCCCCTCAA	59	97
	L. curvatus	R_cur_IS F_cur_IS	TTGGTACTATTTAATTCTTAG GCTGGATCACCTCCTTTC	58	305
	L. sakei	R_sak_IS F_sak_IS	GAGCTAATCCCCCATAATGAAACTAT GATAAGCGTGAGGTCGATGGTT	57	78
	Lactobacillus group	Lac-F Lac-R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	58	341

OHOL, obligate homofermentative lactobacilli; OHEL, obligate heterofermentative lactobacilli; FHEL, facultative heterofermentative lactobacilli.

Results

Clinical indices

Clinical indices of both groups are presented in Table 1. BMI was significantly higher in the elderly ($P=0.012$). Similarly, the level of fasting blood glucose was higher in the elderly ($P<0.001$).

Quantification of lactobacilli in faeces by the culture method and real-time PCR

The culture method and RT-PCR were used to determine the total number of lactobacilli in the faeces of healthy adults and the elderly.

Lactobacilli were cultured from twenty-three (96%) out of the twenty-four adults and from thirty-six (97%) out of the thirty-seven elderly persons. In both groups, the viable counts were below the detection limit. According to the RT-PCR results, lactobacilli were found in all faecal samples of adults and the elderly. Both methods showed that in the elderly, the total counts of lactobacilli were significantly higher (3·6–10·8, median 6·4; mean 6·8 (SD 2·2) $\log_{10}$ CFU/g of faeces $v.$ 2·6–8·6, median 5·9; mean 5·3 (SD 1·8) $\log_{10}$ CFU/g of faeces; $P=0.008$ by the culture method and 7·0–9·5, median 7·9; mean 8·0 (SD 0·7) $\log_{10}$ CFU/g of faeces $v.$ 5·3–8·8, median 7·2; mean 7·2 (SD 0·7) $\log_{10}$ CFU/g of faeces; $P=0.0002$ by RT-PCR) (Fig. 1).

The values obtained for lactobacilli by RT-PCR were higher than those obtained by the culture method (mean 7·69 (SD 0·80)/$\log_{10}$ CFU/g of faeces $v.$ mean 6·19 (SD 2·1)/$\log_{10}$ CFU/g of faeces; $P=0.007$).

Identification of lactobacilli by species-specific PCR and their relationship with a person's age

Species-specific PCR analysis was performed with all samples, even with samples that were negative in bacteriological analysis. According to the PCR method, the number of species in adults was lower than in the elderly: 4–12, median 8, adults $v.$ 5–11, median 6, elderly ($P=0.042$). There was no correlation detected between the number
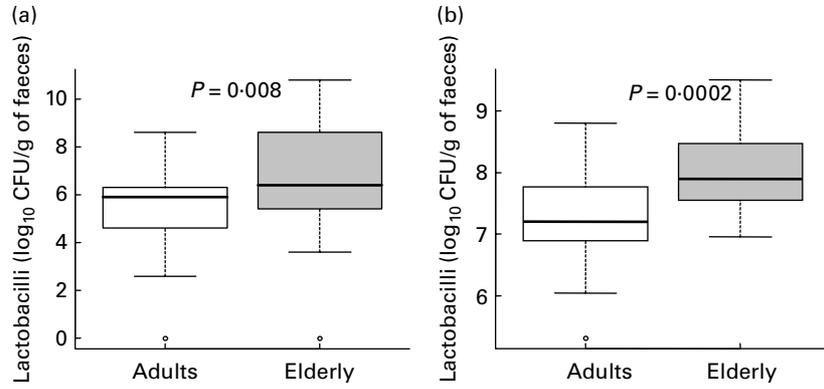
Fig. 1. Total counts of lactobacilli in adults and the elderly by (a) bacteriological analysis and (b) real-time-PCR. CFU, colony-forming units.

of species and the count of lactobacilli estimated, neither by the culture method nor by RT-PCR. The most prevalent *Lactobacillus* species detected both from adults and the elderly were *L. casei* of the FHEL group and *L. ruminis* of the OHOL group ($\geq 70\%$; Fig. 2). The prevalence of *L. crispatus* was the lowest (8%).

The age-related significant differences were found for six species of *Lactobacillus*. Concerning the OHOL metabolic pattern, the adults, in comparison with the elderly, were more often colonised with *L. acidophilus* (19/24, 79% *v.* 18/37, 47%; $P = 0.031$) and *L. helveticus* (16/24, 66% *v.* 9/37, 24%; $P = 0.001$). At the same time, *L. johnsonii* was detected only in the elderly (5/37, 13.5%) (Fig. 2), and the age range of detection was older than 66, median 68 years old.

In the FHEL group, *L. plantarum* (22/37, 59% *v.* 7/24, 29%; $P = 0.035$) and *L. paracasei* (36/37, 97% *v.* 14/24, 58%; $P = 0.0002$) were more prevalent in the elderly than in adults. Surprisingly, *L. rhamnosus* was detected in seven (29%) adults ($P < 0.001$), but not in any elderly person. The age range for the detection of *L. rhamnosus* was up to 48, median 31 years old.

The species difference was found also in the OHEL group where the higher prevalence of *L. reuteri* (19/37, 51% *v.* 5/24, 21%; $P = 0.031$) and on the borderline also with *L. buchneri* (11/37, 29% *v.* 2/24, 8%; $P = 0.059$) was observed in elderly persons (Fig. 2).

Comparison of lactobacilli counts and species with BMI, blood glucose and sex

A positive correlation between BMI and age was found (r 0.301; $P = 0.0185$), though sex was a confounding factor (Table 3). A positive correlation was found between BMI and fasting blood glucose content (r 0.463; $P < 0.0001$), adjusted for age and sex. The lower blood glucose level was predicted by the presence of intestinal *L. paracasei* (r^2 0.281; adjusted r^2 0.213; $P = 0.031$), adjusted for age in the adult group. A negative correlation of borderline significance was found between the level of blood glucose and colonisation by *L. fermentum* (r −0.321; $P = 0.052$) in the elderly group. Multiple regression analysis revealed that BMI was associated with counts of cultivable lactobacilli adjusted for age
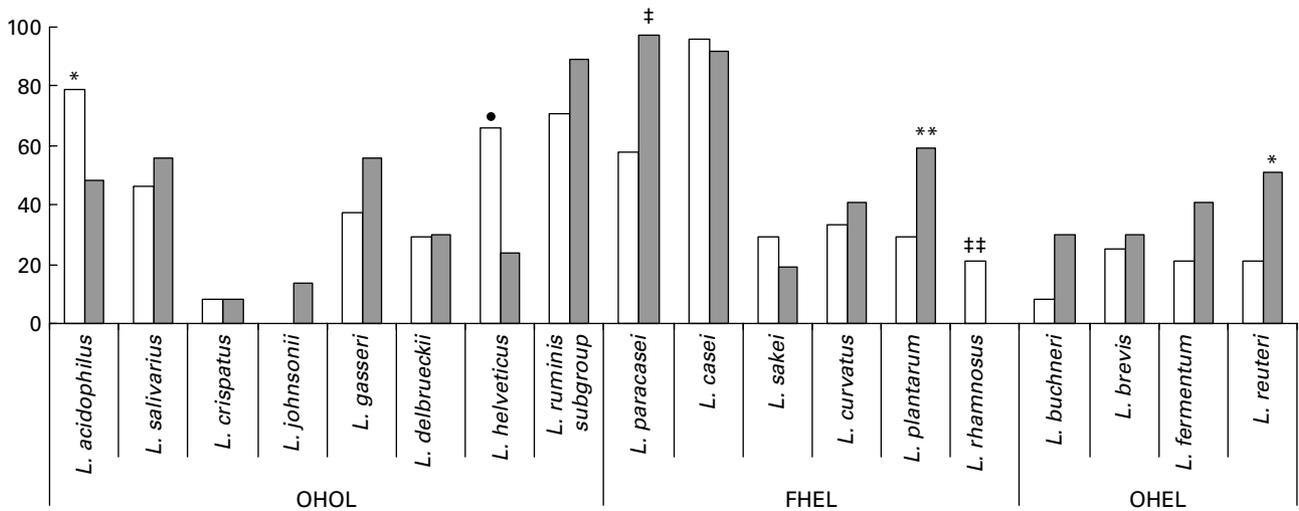

Fig. 2. *Lactobacillus* sp. diversity in adults (□) and the elderly (▨). OHOL, obligate homofermentative lactobacilli; FHEL, facultative heterofermentative lactobacilli; OHEL, obligate heterofermentative lactobacilli. *$P = 0.031$; •$P = 0.001$; ‡$P = 0.0002$; **$P = 0.035$; ‡‡$P = 0.007$.

Table 3. Spearman's rank-order correlation, linear and multiple linear regression analyses between counts and species of lactobacilli, BMI, age and sex

| Variable 1 | Variable 2 | Linear multiple regression analysis | | | | |
		Correlation coefficients (r)	P	r^2	Adjusted r^2	P
BMI (kg/m^2)	Age (years)	0·301	0·0185			NS
BMI (kg/m^2)	Fasting plasma glucose (mmol/l)	0·463	0·0001	0·182*	0·139*	0·009*
Fasting plasma glucose (mmol/l)	Presence of *Lactobacillus paracasei*	−0·460	0·0238	0·281†	0·213†	0·031†
Fasting plasma glucose (mmol/l)	Presence of *Lactobacillus fermentum*	−0·321	0·052			NS
BMI (kg/m^2)	Counts of lactobacilli (bacteriology; log$_{10}$ CFU/g of faeces)	0·309	0·00279	0·187*	0·144*	0·008*
BMI (kg/m^2)	Number of OHOL species	0·598	0·00213			NS
BMI (kg/m^2)	Presence of *Lactobacillus sakei*	0·394	0·002	0·282*	0·244*	0·001*

CFU, colony-forming units; OHOL, obligate homofermentative lactobacilli.
* Age, sex.
† Age (adult group).

and sex (r^2 0·187; adjusted r^2 0·144; $P=0·008$). The higher BMI in both groups of persons was directly predicted by the presence of OHOL and facultative heterofermentative *L. sakei* species, both adjusted for age and sex (Table 3).

Discussion

In the present study, the species-specific PCR analysis of *Lactobacillus* sp. combined with viable plating data indicates substantial age-related structural differences in the intestinal lactobacilli community of healthy adults and the elderly. In the elderly, the lactobacilli counts were higher, and their species composition was richer than those of younger persons. For the first time, it has been established that the values of BMI and glucose content are associated with counts, particular fermentation groups and species of intestinal *Lactobacillus* sp.

The present results confirm the previous culture-based studies, indicating higher viable counts of lactobacilli in healthy elderly people as compared with adults[2,34]. Lactobacilli generally account for less than 1% of the bacterial community of faeces; however, the composition of the lower parts of the gastrointestinal tract is still not well elaborated[35]. In the present study, lactobacilli were detected in 90% of the faecal samples by the culture method and in all samples by RT-PCR. In the elderly, the higher counts of intestinal lactobacilli were assessed by both methodological approaches. At the same time, previous data based on viable counts have shown that lactobacilli were present in the gastrointestinal tract of only 70–73% of humans who consumed a Western-type diet[36,37], while in a vegetarian diet, a higher prevalence of lactobacilli has been assessed[38]. In Estonia, the Western-type diet is characterised by the frequent use of fibre-rich rye bread, porridges and dairy products.

In the present study, we have had a unique possibility to compare both the cultivable and molecularly assessed counts and the PCR species composition of intestinal lactobacilli in adults and the elderly with the bacteriological data obtained previously in 1967–8 in Estonia, reviewed

later[39]. The high prevalence and high counts of lactobacilli were characteristics of adults and the elderly both in the late 1960s and 35 years later. Astonishingly, also the decreased colonisation of the elderly by homofermentative *L. acidophilus* strains coincided in both periods, irrespective of the different identification methods: physiological/biochemical properties as compared with molecular assessment. At the same time, in infants and children, the colonisation by lactobacilli has significantly decreased during 5 years of industrial development and changes in lifestyle of the Estonian community[40]. It is probable that the microbiota that was formed at a young age did not change easily by growing old.

All eighteen lactobacilli species identified in the present study were previously described as members of the faecal microbiota of humans, though some were only recently detected by molecular techniques using specific PCR primers such as the *L. gasseri* and *L. ruminis* group[13,41,42]. In the present study, *L. acidophilus*, *L. salivarius*, *L. paracasei*, *L. casei*, *L. plantarum*, *L. brevis* and *L. fermentum* were the most common species in both age groups, which is in agreement with previous studies[7,43,44]. Reuter[42] observed the predominance of *L. reuteri* among the indigenous intestinal lactobacilli, while some other authors reported this species as only a minor component of the OHEL group[17,28] or completely absent in humans[7]. In our investigations, altogether 39% of the human subjects harboured *L. reuteri*.

Besides, the composition of intestinal lactobacilli varies widely between persons and location in the gastrointestinal tract. However, the present results showed that age groups differed in their species diversity, whereas *L. johnsonii. L. plantarum*, *L. paracasei*, *L. buchneri* and *L. reuteri* were more prevalent in the elderly over 66 years of age. At the same time, *L. acidophilus* and *L. helveticus* of the OHOL group serve as more frequent colonisers of healthy adults, younger than 28 years. During ageing, the typical shifts in the *Lactobacillus* sp. population may have been caused by some degenerative shifts in the gastrointestinal function such as reduction in transit time and digestive secretions[45]. Besides, the lowered reactivity of the

immune system also emerges in the elderly[46–48]. It is possible that these changes could explain the characteristic pattern of the faecal lactobacilli associated with advancing age.

A striking difference concerned the *L. rhamnosus* species, which was found only in the faecal samples of adults (21%), but not in the elderly. Walter[13] has considered *L. rhamnosus* as one of the dominant *Lactobacillus*, though only 26% of healthy individuals and 6% of elderly harboured this species according to some other authors[4,41]. In our elderly group, the complete absence of *L. rhamnosus* is difficult to explain, considering the aggressive marketing of Gefilus, one of the *L. rhamnosus* GG products in Estonia. We hypothesise that in the infancy of the studied elderly people (with birth years from 1920 to 1940) when the resident *Lactobacillus* sp. biota of the gastrointestinal tract were formed, diet and environment did not favour the spread of this species. Furthermore, during the next years of life, even the intensive spread of this probiotic strain did not help to colonise the seniors with previously well-formed microbiota.

The present results confirm that each person of both age groups had an individual set of combinations of different lactobacilli species, which is in agreement with previous, studies[26,41,49]. The individual specificity, on the one hand, and the temporal modulation of intestinal microbiota by diet, lifestyle and stress, on the other hand, complicate the understanding of the relationships between colonic microbiota signatures and host functions throughout the lifespan[17,50,51].

The elderly are more prone to the metabolic syndrome, characterised by a higher blood glucose level and BMI[52,53]. In our previous study of the elderly, we could not find any significant link between lactobacilli counts and BMI[54]. In a larger number of individuals (adult and elderly persons), we detected a significant relationship between BMI and counts of cultivable lactobacilli, adjusted for age and sex. Why was it detected only when using bacteriological estimation of counts of lactobacilli? Discrepancies between RT-PCR and cultivable counts of lactobacilli have been found previously and were related to the multiplicity of 16S ribosomal RNA gene copies, the presence of non-viable, or viable but not cultivable, bacterial cells and to free DNA[55,56]. Additionally, due to the complex taxonomy of lactobacilli, the primers designed for lactobacilli based on 16S ribosomal RNA may also detect other related genera such as *Pediococcus* spp. and *Weissella* spp.[28].

How could the increased number of lactobacilli, particularly those of the OHOL fermentation group, influence health parameters such as the blood glucose level and BMI? The intestinal lactobacilli support intestinal epithelial cell proliferation through the production of SCFA that provide energy for the host[57]. The main fermentation end product of the OHOL group is lactic acid. The lactobacilli of the FHEL and OHEL groups, including

L. paracasei and *L. fermentum*, produce not only lactic acid, but also acetic acid and succinate[12]. It can be speculated that intestinal colonisation by some species of the latter groups decreases the content of both glucose (hexoses) and fructose (pentoses) due to its most effective phosphoketolase pathway of carbohydrate fermentation[29]. Thus, the complex metabolic activities between the host and different groups of microbiota shape the nutrient environment of the gut[58] and correspondingly the blood glucose level.

Lactobacilli also possess the ability to produce polyamines due to decarboxylation of amino acids[59,60]. The high amounts of polyamines could facilitate the absorption of glucose, increasing the number of glucose carriers in the membrane of enterocytes[61]. In Finnish elderly people, a positive correlation between the counts of administered *L. acidophilus* NCFM (OHOL fermentation type) and the values of polyamine spermine was detected[62]. This may be the reason for an increased BMI tightly bound to the glucose level of blood in persons colonised prevalently with several species of the OHOL group in the present study of both adults and the elderly.

The question arises whether the reduction of *L. acidophilus* and *L. gasseri* could influence the state of health of the elderly? Both *L. acidophilus* and *L. gasseri* harbour genes for formyl-CoA transferase and oxalyl-CoA decarboxylase that are obligate enzymes for oxalate degradation in the gastrointestinal tract. The genes for these enzymes are absent in *L. johnsonii* and *L. plantarum*[63]. It is possible that in the elderly, the higher prevalence of *L. johnsonii* and *L. plantarum* species can increase oxalate concentration in urine. This could support the development of kidney stone disease, common in the elderly[64].

According to the present study, *L. sakei* was found in a quarter of the investigated persons, yet it was unexpectedly associated with higher BMI. The ability of this species to metabolise complex carbohydrates into monosaccharides[65] may support their absorption by host cells. Some studies have indicated that *L. sakei* is one of the predominant food-associated *Lactobacillus* species that occurs in human faeces[5,28,66]. *L. sakei* has been isolated from meat, sausages and sauerkrauts, also used for the production of fermented meat products[67]. At the same time, it is one of the major spoilage organisms for vacuum-packed meat products[68].

Tempting for future applications is our finding on the decreased blood glucose level in adults colonised with *L. paracasei* of the FHEL group. This finding is supported by data showing that administration of the close species of *L. casei* to diabetic mice reduced the glucose level of blood[69]. Likewise, a diet enriched with another *Lactobacillus* species of the FHEL group (e.g. *L. rhamnosus* GG) has resulted in an improved glucose tolerance test as well as in reduced blood glycated Hb values in experimental rats and pregnant women[70,71].

Altogether, this clearly hints at the different impact of particular *Lactobacillus* species of various fermentative groups on the metabolism of adults and the elderly having to be considered in the individual probiotic administration for health's sake.

Conclusion

There are substantial age-related (adults and the elderly) structural differences in the intestinal lactobacilli communities. The higher counts of intestinal lactic acid bacteria are associated with higher BMI and blood glucose content, while colonisation with different fermentative groups of lactobacilli is bound to the glucose levels both in adults and in the elderly. In developing blood glucose- and BMI-lowering species of lactobacilli as probiotics, their age- and metabolism-related differences should be taken into account.

References

1. Hebuterne X (2003) Gut changes attributed to ageing: effects on intestinal microflora. *Gastroenterology* **6**, 49–54.
2. Tiihonen K, Tynkkynen S, Ouwehand A, *et al.* (2008) The effect of ageing with and without non-steroidal anti-inflamatory drugs on gastrointestinal microbiology and immunology. *Br J Nutr* **100**, 130–137.
3. Mitsuoka T (1992) Intestinal flora and ageing. *Nutr Rev* **50**, 438–446.
4. Silvi S, Verdanelli MC, Orpianesi C, *et al.* (2003) EU Project Crownalife: functional foods, gut microflora and healthy aging. Isolation and identification of *Lactobacillus* and *Bifidobacterium* strains from fecal samples of elderly subjects for a possible probiotic use in functional foods. *J Food Eng* **56**, 195–200.
5. Heilig HG, Zoetendal EG, Vaughan EE, *et al.* (2002) Molecular diversity of *Lactobacillus* ssp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl Environ Microbiol* **68**, 114–123.
6. Tannock GW (2004) A special fondness for lactobacilli. *Appl Environ Microbiol* **70**, 3189–3194.
7. Vaughan EE, Heilig HG, Ben-Amor K, *et al.* (2005) Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. *FEMS Microbiol Rev* **29**, 477–490.
8. Garrity GM, Bell JA, Lilburn TG, *et al.* (2004) *Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology. Release 5.0*, 2nd ed., pp. 401. New York: Springer-Verlag.
9. Sghir A, Gramet G, Saua A, *et al.* (2000) Quantification of bacterial groups within human faecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol* **66**, 2263–2266.
10. Hirano J, Yoshida T, Sugiyama T, *et al.* (2003) The effect of *L. rhamnosus* on enterohemorrhagic *E. coli* infection of human intestinal cells *in vitro*. *Microbiol Immunol* **47**, 405–409.
11. Valeur N, Engel P, Carbajal N, *et al.* (2004) Colonization and immunomodulation by *L. reuteri* ATCC 55730 in the human gastrointestinal tract. *Appl Environ Microbiol* **70**, 1176–1181.
12. Annuk H, Shchepetova J, Kullisaar T, *et al.* (2003) Characterization of intestinal lactobacilli as putative probiotic candidates. *J Appl Microbiol* **94**, 403–412.
13. Walter J (2008) Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Appl Environ Microbiol* **74**, 4985–4996.
14. Cani PD & Delzenne MN (2009) The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* **15**, 1546–1558.
15. Woodmansey EJ, McMurdo ME, Macfarlane GT, *et al.* (2004) Comparison of compositions and metabolic activities of fecal microbiota in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Appl Environ Microbiol* **70**, 6113–6122.
16. Vassos D, Maipa V, Voidarous C, *et al.* (2007) Development of human lactic acid gastrointestinal microbiota in a Greek rural population. *Cent Eur J Biol* **3**, 55–60.
17. Nielsen DS, Møller PL, Rosenfeldt V, *et al.* (2003) Case study of the distribution of mucosa-associated *Bifidobacterium* species, *Lactobacillus* species, and other Lactic acid bacteria in the human colon. *Appl Environ Microbiol* **69**, 7545–7548.
18. Song Y-L, Kato N, Lui C-X, *et al.* (2000) Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assay using group- and species-specific primers derived from the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiol Lett* **187**, 167–173.
19. Cagno R, Rizello CG, Garliardi F, *et al.* (2009) Imbalance of the faecal microbiota and volatile organic compounds between treated and untreated coeliac children. *Appl Environ Microbiol* **75**, 3963–3971.
20. Kandler O & Weiss N (1986) Regular non-sporing gram-positive rods. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1209–1234 [PH Sneath, NS Mair and ME Sharpe, editors]. Baltimore, MD: Williams and Wilkins Baltimore.
21. Bäckhed F, Ding H, Wang T, *et al.* (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* **101**, 15718–15723.
22. Adlercreutz H (1990) Western diet and western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest Suppl* **201**, 3–23.
23. Costacou T, Bamia C, Ferrari P, *et al.* (2003) Tracing the Mediterranean diet through principal components and cluster analyses in the Greek population. *Eur J Clin Nutr* **57**, 1378–1385.
24. Wenzel R (2007) Health care – associated infections. Major issues in early years of 21st century. *Clin Infect Dis* **45**, 85–88.

25. Sepp E, Julge K, et al. (2005) Intestinal microbiota and imunoglobulin E responses in 5-year-old Estonian children. Clin Exp Allergy 35, 1141–1146.

26. Mikelsaar M, Annuk H, Stsepetova J, et al. (2002) Intestinal Lactobacilli of Estonian and Swedish Children. Microb Ecol Health Dis 14, 75–80.

27. Bartosch S, Fite A, MacFarlane GT, et al. (2004) Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 70, 3575–3581.

28. Walter J, Hertel C, Tannok GW, et al. (2001) Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weisella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67, 2578–2585.

29. Axelsson L (1998) Lactic acid bacteria: classification and physiology. In Lactic Acid Bacteria, pp. 1–72 [S Salminen and A von Wright, editors]. New York: Marcel Dekker.

30. Tilsala-Timisjärvi A & Alatossava T (1997) Development of oligonucleotide primers from the 16–23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int J Food Microbiol 35, 49–56.

31. Walter J, Tannock GW, Tilsala-Timisjarvi A, et al. (2000) Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl Environ Microbiol 66, 297–303.

32. Haarman M & Knol J (2006) Quantitative real-time PCR analysis of fecal Lactobacillus species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 72, 2359–2365.

33. Matsuda K, Tsuij H, Matsumoto K, et al. (2009) Establishment of an analytical system for the human fecal microbiota, based on reverse transcription-quantitative PCR targeting of multicopy rRNA molecules. Appl Environ Microbiol 75, 1961–1969.

34. Mitsuoka T (1992) The lactic acid bacteria in health and disease. In The Human Gastrointestinal Tract, vol. 1, pp. 69–114 [BJB Wood, editor]. London: Elsevier Applied Science.

35. Marteau P, Pochart P, Dore J, et al. (2001) Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol 67, 4939–4942.

36. Tannock GW (1991) The microecology of lactobacilli inhabiting the gastrointestinal tract. Adv Microbiol Ecol 11, 147–171.

37. Finegold SM, Sutter VL & Mathisen GE (1983) Normal indigenous intestinal flora. In Human Intestinal Microflora in Health and Disease, pp. 3–31 [DJ Hentges, editor]. New York: Academic Press.

38. Finegold SM, Sutter VL, Sugihara PT, et al. (1977) Fecal microbial flora in seventh day adventist populations and control subjects. Am J Clin Nutr 30, 1781–1792.

39. Mikelsaar M (1993) Evaluation of the gastrointestinal microbial ecosystem in health and disease. PhD Thesis, University of Tartu.

40. Sepp E, Voor T & Julge K (2006) Is intestinal microbiota bound up with changing lifestyle? In Modern Multidisciplinary Applied Microbiology, pp. 708–712 [Mendez-Vilas, editor]. Weinhein: Wiley-VCN Verlag.

41. Ahrne S, Nobaek S, Jeppsson B, et al. (1998) The normal Lactobacillus flora of healthy human rectal and oral mucosa. J Appl Microbiol 85, 88–94.

42. Reuter G (2001) The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol 2, 42–53.

43. Mikelsaar M & Lencner A (1982) Mucosal lactoflora of gastrointestinal tract. In Mikrobielle Umwelt und/in antimikrobielle Massnahmen. Bd. 6. Mikroökologie des Magen-Darmkanals des Menschen (Microbial Environment and/or in Antimicrobial Action. vol. 6 Micro-ecology of the Gastrointestinal Tract of Man), pp. 89–96 [H Bernhard and M Knoke, editors]. Leipzig: Barth-Verlag.

44. Conway P (1989) Lactobacilli: fact and fiction. In The Regulatory and Protective Role of the Normal Microflora, pp. 263–283 [R Grubb, T Midtvedt and E Norin, editors]. Stockholm: Stockton Press.

45. Mariat D, Firmesse O, Levenez F, et al. (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol 9, 123.

46. Timiras PS (1996) Physiology of aging: standards for age-related functional competence. In Comprehensive Human Physiology, vol. 2, pp. 2391–2405 [R Greger and U Windhorst, editors]. Heidelberg: Springer-Verlag.

47. Gill HS, Darragh AJ, Cross M, et al. (2001) Optimizing immunity and gut function in the elderly. J Nutr Health Aging 5, 60–91.

48. Tiihonen K, Ouwehand AC, Rautonen N, et al. (2010) Human intestinal microbiota and healthy ageing. Ageing Res Rev 9, 107–116.

49. Delgado S, Suárez A, Mayo B, et al. (2007) Identification of dominant cultivatable lactobacilli species from the faeces of Spanish healthy adults. Int Microbiol 10, 141–145.

50. Lay C, Rigottier-Gois L, Holmstrm K, et al. (2005) Colonic microbiota signatures across five northern European countries. Appl Environ Microbiol 71, 4153–4155.

51. Guarner F (2006) Enteric flora in health and disease. Digestion 73, 5–12.

52. Lichtenstein AH (1999) Optimal nutrition for the mature adult in health and disease. In Lifestyle Nutrition, pp. 106–126 [JT Dwyer and JM Rippe, editors]. Malden, MA: Blackwell Science.

53. Hashizume K, Suzuki S, Masahiro H, et al. (2006) Metabolic syndrome and age-related dementia: endocrinological aspects of adaptation to aging. Mech Ageing Dev 12, 507–510.

54. Mikelsaar M, Stsepetova J, Hütt P, et al. (2010) Intestinal Lactobacillus sp. is associated with some cellular and metabolic characteristics of blood in elderly people. Anaerobe 16, 240–246.

55. Farelly V, Rainly F, Stackebrandt E, et al. (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol 61, 2798–2801.

56. Apajalahti J, Kettunen A, Nurminen PH, et al. (2003) Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. Appl Environ Microbiol 69, 5731–5735.

57. Shanahan F (2002) The host–microbe interface within the gut. Best Pract Res Clin Gastroenterol 16, 915–931.

58. Falk PG, Hooper LV, Midvedt T, et al. (1998) Creating and maintaining the gastrointestinal ecosystem, what we know and need to know from gnotobiology. Microbiol Mol Biol Rev 62, 1157–1170.

59. Bover-Cid S & Holzapfel WH (1999) Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol 53, 33–41.

60. Morene-Arribas MV, Polo MC, Jorganes F, et al. (2003) Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. Int J Food Microbiol 84, 117–123.

61. Johnson LR, Brockway PD, Madsen K, et al. (1995) Polyamines alter intestinal glucose transport. Am J Physiol 31, G416–G423.

J. Štšepetova *et al.*

62. Ouwehand AC, Tiihonen K, Saarinen M, *et al.* (2009) Influence of a combination of *Lactobacillus acidophilus* NCFM and lactitol on healthy elderly: intestinal and immune parameters. *Br J Nutr* **101**, 367–375.

63. Azcarate-Peril MA, Bruno-Barcena JM, Hassan HM, *et al.* (2006) Transcriptional and functional analysis of oxalyl-coenzyme A (CoA) decarboxylase and formyl-CoA transferase genes from *Lactobacillus acidophilus*. *Appl Environ Microbiol* **72**, 1891–1899.

64. Grases F, Costa-Bauza A, Prieto RM, *et al.* (2006) Renal lithiasis and nutrition. *Nutr J* **5**, 23.

65. van Geel-Schutten GH, Flesh F, Ten Brick B, *et al.* (1998) Screening and characterization of *Lactobacillus* strains producing large amount of polysaccharides. *Appl Microbiol Biotechnol* **50**, 697–703.

66. Dal Bello F, Hammes WP, Hertel C, *et al.* (2003) Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. *Microb Ecol* **45**, 455–463.

67. Hammes WP, Bantleon A, Min S, *et al.* (1990) Lactic acid bacteria in meat fermentation. *FEMS Microbiol Rev* **87**, 165–174.

68. Hammes WP & Hertel C (1998) New development in meat starter cultures. *Meat Sci* **49**, S125–S138.

69. Matzuzaki T, Nagata Y, Kado S, *et al.* (1997) Effect of oral administration of *Lactobacillus casei* on alloxan-induced diabetes in mice. *APMIS* **105**, 637–642.

70. Yadav H, Jain S, Sinha PR, *et al.* (2007) Antidiabetic effect of probiotic Dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high-fructose fed rats. *Nutrition* **23**, 62–68.

71. Laitinen K, Poussa T, Isolauri E, *et al.* (2009) Probiotics and dietary counselling contribute to glucose regulation during and after pregnancy: a randomised controlled trial. *Br J Nutr* **101**, 1679–1687.