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Advances in Microbiology, Infectious Diseases and Public Health

Gianfranco Donelli *Editor*

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Gianfranco Donelli
Editor

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Editor
Gianfranco Donelli
Microbial Biofilm Laboratory
Fondazione Santa Lucia IRCCS
Rome, Italy

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Biodiversity of Intestinal Lactic Acid Bacteria in the Healthy Population

Marika Mikelsaar, Epp Sepp, Jelena Štšepetova, Epp Songisepp, and Reet Mändar

Abstract

The complex ecosystem of the gastrointestinal tract involves tight interrelations among host cells, diet, and billions of microbes, both beneficial and opportunistic pathogens. In spite of advanced genomic, metagenomic, and metabonomic approaches, knowledge is still quite limited regarding the biodiversity of beneficial microbiota, including *Lactobacillus* spp., and its impact on the main biomarkers of general health. In this paper, *Lactobacillus* biodiversity is demonstrated through its taxonomy, function, and host-microbial interactions. Its prevalence, composition, abundance, intertwined metabolic properties, and relation to host age, genotype, and socioeconomic factors are reviewed based on the literature and original research experience. The species richness, e.g., the biodiversity of gut microbiota, provides the host with a variety of metabolically active species and strains that predict their response for different health conditions and extrinsic interventions. Metabolically active and safe *Lactobacillus* species and specific strains with particular functional properties increase the biodiversity of the whole intestinal microbiota. The elaborated principles for effective application of probiotics are discussed, aimed at regulating the composition of microbiota simultaneously with blood and urine biomarkers at the borderline of normality. This approach targets the impact of probiotic strains to maintenance of health with anti-infectious, cardiovascular, and metabolic support.

Keywords

Lactobacilli • Taxonomy • Metabolite • Species diversity • Functional properties • Probiotic elaboration • Health maintenance • Human

M. Mikelsaar (✉), E. Sepp, J. Štšepetova, and R. Mändar
Institute of Biomedicine and Translational Medicine, University of Tartu, Europe, Estonia
e-mail: marika.mikelsaar@ut.ee; epp.sepp@ut.ee; jelena.stsepetova@ut.ee; reet.mandar@ut.ee

E. Songisepp
Bio-Competence Centre of Healthy Dairy Products, Europe, Estonia
e-mail: epp.songisepp@tptak.ee

1 Intestinal Microbiome and Its Biodiversity

The intestinal microbiota is a dynamic complex of microbes comprising bacteria, archaea, protozoa, fungi, and different viruses. To date, this microbiota consists of 10^{14} viable microbes belonging to over 1000 species, among which anaerobic bacteria predominate (Holdeman et al. 1976; Finegold et al. 1977; Mikelsaar and Mändar 1993; Bochkov et al. 1998; Zoetendal et al. 1998; van der Waaij et al. 2005; Reid et al. 2006; Sun and Chang 2014; Ghaisas et al. 2015). The understanding of its impact on human health and well-being and possible ways to regulate it following disruptions are not yet well elaborated. New molecular methods and large-scale European and US human microbiome projects (Qin et al. 2010, 2012) have led to an explosive increase in the number of culture-independent metagenomic studies, illustrating a large ecological diversity, particularly in the gastrointestinal (GI) tract. Genomics and other -omics technologies are playing an important role in helping to maintain personal health. The revolution in DNA sequencing technologies has made it possible to sequence the microbiome and metabolome of healthy individuals and patients with diagnosed disease to tailor treatment for specific individuals (Wu 2016).

The first criteria to be evaluated were obtained by culture-based quantitative studies of microbiota of different biotopes followed by culture-independent sequencing studies of metagenomes. The large range of different microbial species (nearly 1000 for a person) was identified as having a large diversity at the ribosomal RNA (rRNA) level, confirming different biological properties of microbiota (Lozupone et al. 2012).

Three robust clusters, e.g., enterotypes of microbiota, were detected by Sanger analysis of metagenomes of different populations (Danish, French, Italian, and Spanish individuals) and including previously published pyrosequencing datasets of Japanese and US volunteers (Arumugam et al. 2011). Their abundance, relative proportions, and number of species were set as criteria for "core microbiota." Microbial abundance has been roughly characterized with a predominance of *Firmicutes* (approx. 28–40 %), followed by 20–38 % *Bacteroidetes*, and *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobiota* as minor constituents (3–10 %) (Eckburg et al. 2005; Roager et al. 2014). However, newer data from developing countries have expanded the set of phyla differentially colonizing young children from different cultures and economic conditions (Yatsunenko et al. 2012).

In contrast to the modest levels of diversification of phyla in the mammalian gut, quite high variation exists at the level of species and strains (Chow et al. 2010). The culture-independent 16S rRNA and metagenomic sequencing studies have shown a high variability of composition of healthy gut microbiota, particularly for viruses and the predominant phyla *Firmicutes* and *Bacteroidetes*, expressed in species diversity between individuals (Eckburg et al. 2005; Reyes et al. 2010; Qin et al. 2010).

The biodiversity of microbiota is largely characterized by composition as well as function. Complex studies of biological properties of cultured phenotypes of microbiota with characterized genome profiles offer the possibility of revealing functioning genes in the whole DNA community of the biotope and further modeling the metabolic network of the host in a particular region or in general. Combining targeted sequencing with mRNA-, protein-, and metabolite-level analyses has helped to measure these community properties (Lozupone et al. 2012). Surprisingly, whereas plasma and urine metabolomes of human vegans differ markedly from those of omnivores, the gut microbiota is similar (Wu et al. 2016). Thus, the gut microbiota provides an individual person by chance with a variety of metabolically active species and strains, but the variability of functional maps is considered to be smaller than the genomic variability (Huttenhower et al. 2009).

Undoubtedly, the large metabolic potential of the gut microbiota influences the production of diet-dependent gut microbial metabolites, complicating the estimation of healthy versus

dysbiotic microbiota (Sonnenburg and Sonnenburg 2014). These findings have underscored the need to consider the structurally and functionally diverse microbiome when evaluating nutritional needs, physiological variations in health biomarkers, and the impact of westernization. The rapidly progressing knowledge about the balance of microbial groups in the gut of individuals has supported their impact on health and conversely the damaging role of imbalance in the generation of various metabolic diseases. The very large bacterial divisions of predominating phyla (*Firmicutes* vs. *Bacteroidetes*) involve different genera, species, and strains with different effects on host biomarkers and health.

The individual pattern of the microbiome does not, however, anticipate the presence of a universal well-balanced host–microbial symbiosis. Individual stability is granted by different mechanisms despite temporary or sometimes even long-lasting imbalance due to various exogenous and endogenous influences. A wide variety of host genetic, environmental, and dietary factors affect bacterial colonization of the GI tract, but the symbiosis occurs with several diverse functions of microbiota including the traditional decomposition of different nutrients, maturation of intestinal cells, morphology and gut physiology, stimulation of the immune system, systemic effects on blood lipids, and inhibition of harmful bacteria (Dubos and Schaedler 1962; McFarland 2000).

The complex immune-mediated signaling processes, together with different chemical interactions, comprise a series of multidirectional interactive metabolic axes between microbe and host (Nicholson et al. 2012). The higher diversity and abundance of particular microbial groups seemingly serve as more effective factors in the various metabolic connections of different organs, secretions, and metabolites that respond to perturbations of homeostasis (Clemente et al. 2012). Although the abundant species do not always engage in the molecular functions that are important for the host, some marker genes and functional modules of bacteria significantly correlate with host age and health biomarkers such as body mass index (BMI) (Arumugam et al. 2011). Moreover, the understanding of the stability of intestinal microbiota is tightly connected with the phenotypic flexibility of host metabolism (van Ommen et al. 2014). These changes certainly also should be reflected in health biomarkers.

In 2000s many studies started to explain the relationship between individual genotype, stage of life and environment with epigenetic processes (Kanherkar et al. 2014; Shenderov and Midtvedt 2014; Remely et al. 2014). Epigenomic processes regulate when and in which manner certain genes of both host and its microbiota are turned on or off by the covalent attachment of various chemical groups to DNA, RNA, chromatin, histones during the transcriptional and in post-translational period to aminoacids and even proteins. Methylation, but also acetylation or ubiquitylation, lead to different molecular outcomes which can persist even during several cell generations and result in inactivation of the X-chromosome, genomic imprinting, or different types of cancer (Sagl et al. 2007; Paul et al. 2015). Several environmental factors are capable of eliciting positive or negative epigenetic modifications with lasting effects on development, metabolism and health. These can impact the body so profoundly as to permanently alter the epigenetic profile of an individual.

Microbiota and its metabolites influence epigenomic reprogramming. There are various molecules of microbial origin that are in complex interplay with host metabolism and physiology. For instance, *Faecalibacterium prausnitzii* and *Eubacterium rectale/Roseburia* spp. (*Firmicutes* phylum), can regulate the gene expression of storage of lipids in fat cells by histone modifications due to production of butyrate. On the other hand, the methylation of genes for receptors of fat cells can silence their epigenomic programming. Lipopolysaccharide (LPS) of gram negative bacteria is another well proved microbial factor for epigenetic regulation of immune and intestinal cells (Bierne et al. 2012; Kumar et al. 2014).

Epigenomic impact may serve as a central factor for altered homeostasis of the host in the majority of modern-world diseases

(atherosclerosis, obesity, cancer, atopy and asthma, type II diabetes). Epigenomic programming of the genome and the post-translational modification of cell products such as proteins are closely associated with embryogenesis and postnatal development for adaptation to different environmental signals. Certainly, food and microbiota with their bioactive molecules can serve as the most important environmental epiprogramming factors, possibly increasing the risk of chronic inflammatory and metabolic diseases (Kau et al. 2011; McKay and Mathers 2011). There is a clear necessity to identify more microbiota groups involved in epigenetic programming and elaborate the possibilities for epigenetic reprogramming with nutra- and microbial epigenetic-based functional foods and for use in personalized medicine.

In addition to epigenetic influences, the outcome of genetically well-defined microbiota can depend on phenotypic fluctuations at the single cell level. Recently, for genetically identical microbial cells that reside in the same microenvironment, molecular mechanisms for phenotypic variation have been outlined. The main drivers of phenotypic heterogeneity are stochastic gene expression, aged cultures, or interactions between phenotypic subpopulations in clonal groups. These modulators can provide microbial groups with new modified functionality to persist in fluctuating environments (Ackermann 2015).

In this review, we address a specific group of intestinal bacteria, *Lactobacillus* of the *Firmicutes* phylum, that are tightly involved in host-microbiota interactions. Lactic acid bacteria (LAB), mainly *Lactobacillus* (*Firmicutes*) and *Bifidobacterium* (*Actinobacteria*), are believed to benefit the host through anti-inflammatory, antitumorigenic, and pathogen exclusion properties (Vaughan et al. 2005; Marteau 2013).

Our aim is to describe *Lactobacillus* spp. biodiversity by community composition, abundance, relative proportions of biotypes in particular microbiota, and individual metabolic variety, with the resulting rich functional diversity of species and strains. Some mechanisms behind these characteristics are considered, including age, genotype, and environmental factors. The possible metabolic interactions among *Lactobacillus* species and with different species of various phyla are described and their beneficial vs. harmful impact on host health predicted. In different health states and perturbances, the metabolic activity of lactobacilli and its impact on intestinal microbiota and host metabolism can surely enlarge our understanding of the role of certain groups of microbiota and their interplay with host structure and physiology. Understanding interrelations with the other more numerous predominating microbiota can open possibilities for *Lactobacillus* spp. application as natural beneficial bacteria (probiotics) for personal correction of imbalances of intestinal microbiota and consequently for the maintenance and regulation of health.

2 Lactic Acid Bacteria in Humans: Origin, Divisions, and Characteristics

LAB are phylogenetically included in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* (Heilig et al. 2002; Tannock 2004; Vaughan et al. 2005). The family *Lactobacillaceae* contains the genera *Lactobacillus*, *Pediococcus*, *Paralactobacillus*, and *Sharpea*, which are phylogenetically intermixed (Felis and Dellaglio 2007; Haakensen et al. 2011).

Lactobacilli are Gram-positive, non-pathogenic microorganisms characterized by the production of lactic acid as the main end-product of carbohydrate metabolism. Among the other genera of LAB, the genus *Lactobacillus* (Kandler and Weiss 1986; Garrity and Lilburn 2005; Felis and Dellaglio 2007) comprises more than 154 validly described species and 19 subspecies (http://www.bacterio.net/lactobacillus.html). *Lactobacillus* spp. forms a large, heterogeneous group consisting of non-sporulating, anaerobic or microaerophilic, catalase-negative, fermentative organisms with complex nutritional requirements. In humans, these bacteria persist in oral cavities, the GI tract, and genital tracts (Kandler and Weiss 1986; Axelsson 1998;

Hayashi et al. 2005; Felis and Dellaglio 2007). Although 20 species of lactobacilli have been tightly associated with the human GI tract, new species of bacteria still are being defined (Walter et al. 2010; Oki et al. 2012; Rajilic-Stojanovic and de Vos 2014). In the small intestine, *Lactobacillus* spp. represents one of the predominant groups (Reuter 2001; Hayashi et al. 2005; Ahmed et al. 2007). Approximately 30 % of species have been isolated from fecal sources, however. At the same time, the lactobacilli group has been the focus of several studies for their prevalence, numbers, and properties in specific biotopes of host.

Moreover, a wide variety of different *Lactobacillus* strains is present on plant material and fermented food, soil, and sewage. Altogether, consumed food complicates the determination of the true inhabitants of the human organism and assessment of their role in host function. Whether the health-promoting capacities of *Lactobacillus* spp. are mainly predicted by their host and biotope-specific origin still lacks evidence-based confirmation. Yet, for health promotion, the application of *Lactobacillus* spp. strains with defined functional properties largely depends on this specificity.

2.1 Phenotypic Properties

Lactobacillus spp. are catalase-negative bacteria, generally oxygen tolerant, aciduric or acidophilic, and obligately carbohydrate fermenters with at least 50 % of the carbohydrate end-product being lactate (Hammes and Vogel 1995; Hammes and Hertel 2006). For a long time, the identification of *Lactobacillus* spp. was performed with application of different methods in phenotypic studies relying on detection of metabolites, enzymes, and/or chemical composition.

Metabolites According to the type of sugar fermentation, lactobacilli can be subdivided into three groups: a genus of homo-fermenters (OHOL), or *Thermobacterium*, and the genera of facultative hetero-fermenters (FHEL), the *Streptobacterium*, and obligate heterofermenters (OHEL), the *Betabacterium* (Table 1).

Group I – obligately homofermentative (OHOL) lactobacilli can convert hexoses into lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway while the pentoses and gluconate are not fermented because OHOL lactobacilli lack phosphoketolase; Group II – facultatively heterofermentative lactobacilli (FHEL) degrade hexoses to lactic acid by the EMP pathway and also can degrade pentoses to lactic acid and acetic acids and ethanol; the gluconate is often fermented. Group III – obligately heterofermentative (OHEL) hexoses are fermented to lactic acid, carbon dioxide, and ethanol (or acetic acid using an alternative electron acceptor), formate, and succinate. Pentoses are converted to lactic and acetic acids (Kandler and Weiss 1986; Pot et al. 1994b; Hammes and Vogel 1995; Axelsson 1998; Songisepp et al. 2005; Štšepetova et al. 2011a)

A special kit, the API 50 CHL system, for identification of lactobacilli based on their phenotypic properties, particularly on the fermentation patterns of carbohydrates, has been developed by bioMérieux, France. The kit is mainly useful for identification to the species level. Its precision can be greatly improved by computerized application of Bayes's theorem (Cox and Thomsen 1990). The great advantage with cultivation is that isolates can be recovered and further studied for their ability to use different substances and for other physiological parameters, including their antibiotic susceptibility pattern. In addition to group and species specificity, the biochemical profile can be strain-specific, to some extent depending on the number of tests in any particular kit. The fermentation profile of carbohydrates of three particular strains – *L. acidophilus* 821 (OHOL group), *L. plantarum* Tensia DSM 21380 (FHEL group), and *L. fermentum* ME-3 DSM 14241 (OHEL group) – according to the API 50 CHL kit results is presented in Table 2.

Table 1 Human *Lactobacillus* spp. fermentative properties

Indices	Group I Obligately homo-fermentative (OHOL)	Group II Facultatively hetero-fermentative (FHEL)	Group III Obligately hetero-fermentative (OHEL)
Growth at 45 °C	+	+	+/−
Growth at 15 °C	−(+)[a]	+(−)[b]	+(−)[b]
Hexose fermentation	+	+	+
Pentose fermentation	−	+	+
Fructose-diphosphate (FDP) aldolase	+	+	−
Phosphoketolase (PK)	−	+[c]	+
Gas from glucose	−	−	+
Gas from gluconate	−	+	+
NH_3 from arginine	−(+)[a]	−	+(−)[b]
Metabolites	(D-, L-, DL)	(D-, L-, DL)	(DL)
	Lactic acid	Lactic acid	Lactic acid
		Acetic acid	Acetic acid
		Ethanol	Succinic acid
			Formic acid
			Ethanol
Species of *Lactobacillus*	*L. delbrueckii*	*L. casei*	*L. brevis*
	L. acidophilus	*L. curvatus*	*L. buchneri*
	L. helveticus	*L. paracasei*	*L. fermentum*
	L. salivarius	*L. plantarum*	*L. reuteri*
	L. gasseri	*L. sakei*	*L. oris*
	L. johnsonii	*L. rhamnosus*	*L. mucosae*
	L. ruminis		
	L. crispatus		

Adapted from Bottazzi (1983), Kandler and Weiss (1986), Axelsson et al. (1993), Pot et al. (1994a), Hammes and Vogel (1995), Songisepp et al. (2005), Štšepetova et al. (2011a)
Legend: [a]mostly negative
[b]mostly positive, with a few exceptions
[c]inducible by pentose

Enzymes The enzyme profile of *Lactobacillus* spp. can be detected by the API ZYM (bioMérieux, France) www.biomerieux.fr/test kit (Table 3).

Table 3 depicts some LAB strains that can be characterized by both alpha- and beta-glycosidases and -galactosidases. Alpha-glucosidase breaks down starch and disaccharides to glucose and is close to maltase, a similar enzyme that cleaves maltose. For health, the glucosidases may pose a problem by excess produced glucose. Beta-glucosidase is an enzyme located on the brush border of the small intestine that acts on β1- > 4 bonds linking two glucose or glucose-substituted molecules (i.e., the disaccharide cellobiose). It is one of the cellulases, enzymes involved in the decomposition of cellulose and related polysaccharides; more specifically, it is an exocellulase with specificity for a variety of glycoside substrates and catalyzes the hydrolysis of terminal non-reducing residues in beta-D-glucosides with release of glucose (Cox et al. 2000).

The high content of alpha-galactosidase in *L. casei* and *L. fermentum* ME-3 (up to 93 % nmol/min/mg protein, at pH 6.5) is quite exceptional, enabling them to hydrolyze glycosides of different biologically active substances such as flavonoids and isoflavones (Uskova et al. 2010).

Table 2 API 50 CHL system (bioMérieux) profile of fermentation of carbohydrates by OHOL, FHEL, and OHEL group strains, respectively: *L. acidophilus* 821–3, *L. plantarum*, Tensia DSM 21380, and *L. fermentum* ME-3 DSM 14241

L. acidophilus 821–3 HUMB 0036 (Rööp et al. 2014)	Galactose	Cellobiose	D-mannose	
	D-glucose	Maltose	Esculine	
	D-fructose	Lactose	Salicine	
	N-acetyl-glycosamine	Saccharose	D-raffinose	
			Amidon	
			Gentiobiose	
L. plantarum Tensia DSM 21380	D-fructose	Maltose	α-methyl-D-mannoside	Mannitol
	Ribose	Saccharose	α-methyl-D-glycoside	Sorbitol
	Galactose	Lactose	Amygdaline	
	D-glucose	D-turanose	Arbutine	
	N-acglycosamine	Trehalose	Esculine	
		Cellobiose	Salicine	
			Gentibiose	
			Glyconate	
			Melezitose	
			Melibiose	
			D-mannose	
			Starch	
L. fermentum ME-3 DSM 14241	D-fructose	Maltose	D-mannose	Mannitol
	Ribose	Lactose	Esculine	Sorbitol
	Galactose	Saccharose	Melibiose	
	D-glucose		Glyconate	
			D-raffinose	

Source: Mikelsaar et al. (2006); Mikelsaar and Zilmer (2009); Songisepp et al. (2012b); Rööp et al. (2014)

The presence of beta-galactosidases is quite common for different *Lactobacillus* spp. (*L. casei*, *L. bulgaricus*) and streptococci (*Streptococcus thermophilus*) involved in lactose fermentation in the production of yogurt.

Chemical Composition Recently, the identification of bacteria by their chemical composition has gained increasing importance. Applications of modern laboratory research methods such as matrix-assisted laser desorption/ionization time-of-flight (e.g., MALDI-TOF) mass spectrometry (MS) has taken the lead in microbiology laboratories during the last decade. This method is rapid, accurate, and cost-effective and measures highly abundant proteins of microorganisms. The characteristic patterns of these proteins are used to reliably and accurately identify a particular microorganism by matching the respective pattern with an extensive database. Most studies regarding identification of microorganisms by MALDI-TOF MS are based on the Bruker system, which has been commercially developed mainly for clinical application (https://www.bruker.com).

Regarding lactobacilli, in most studies, human oral, fecal, vaginal, or non-human animal strains have been investigated (Callaway et al. 2013; Anderson et al. 2014; Dec et al. 2014; Zhang et al. 2014). In these studies, comparison of MALDI-TOF MS with 16S rDNA sequencing has given highly concordant results. Thus, MALDI-TOF MS analysis seems to be a reliable and fast tool to identify lactobacilli to the species level. Though 16S rDNA sequencing yielded more precise species identification, accuracy can be supposedly improved by extending reference databases (Anderson et al. 2014).

In a study with MALDI-TOF MS analysis and 16S rDNA analysis, both methods were used to

Table 3 API ZYM (bioMérieux, France) profile of strains *L. plantarum* Tensia DSM 21380 and *L. fermentum* ME-3 DSM 14241

Strain	Positive reaction
L. plantarum Tensia DSM 21380	Leucine arylamidase
	Valine arylamidase
	Cystine arylamidase
	Acid phosphatase
	Naphthol-AS-BI-phosphohydrolase
	β-galactosidase
	α-glucosidase
	β-glucosidase
L. fermentum ME-3 DSM 14241	Alkaline phosphatase
	Esterase (C4)
	Esterase (C8)
	Leucine arylamidase
	Valine arylamidase
	Cystine arylamidase
	Acid phosphatase
	Naphthol-AS-BI-phosphohydrolase
	α-galactosidase
	β-galactosidase

Source: Songisepp et al. (2012b)

analyze 77 vaginal and 21 oral *Lactobacillus* isolates. The concordance of both methods was at 96 % with only five samples discordantly identified (Anderson et al. 2014). In addition to protein profile analysis, this method enables analysis and comparison of bacterial lipid profiles and has also been used with lactobacilli (Calvano et al. 2011). The method additionally allows definition of several seldom described *Lactobacillus* species in fecal samples of healthy humans such as *L. acidipiscis*, *L. agilis*, *L. amylovorus*, *L. antri*, *L. coryniformis*, *L. equi*, *L. fructivorans*, *L. fuchuensis*, *L. gastricus*, *L. ingluviei*, *L. jensenii*, *L. kalixensis*, *L. kefiri*, *L. malefermentans*, *L. murinus*, *L. oligofermentans*, *L. parabuchneri*, *L. parakefiri*, *L. paralimentarius*, *L. pentosus*, *L. saerimneri*, *L. suebicus*, *L. zeae*, *L. ultunensis*, *L. vaginalis*, and *L. vitulinus*.

2.2 Phylogenetic Division

The taxonomy of LAB is quite complicated. Lactobacilli are characterized by a low G + C content (32–53 %), although the upper limit of DNA G + C content reaches 58.5–59.2 mol % for *Lactobacillus nasuensis* (Cai et al. 2012). To date, the genus *Lactobacillus* contains over 150 species with wide phenotypic and genotypic variation (Kant et al. 2011; Salvetti et al. 2012).

Different methods for genotyping have changed the understanding of phylogenetic classification of *Lactobacillus* spp. Based on DNA–DNA hybridization, lactobacilli were grouped into eight major groups: *L. buchneri*, *L. delbrueckii*, *L. casei*, *L. plantarum*, *L. reuteri*, *L. sakei*, *L. salivarius*, and *L. brevis* (Felis and Dellaglio 2007). Previous studies on the basis of 16S rRNA gene sequences have split *Lactobacillus* spp. into three clusters: *L. acidophilus*, *L. casei*/*Pediococcus*, and *Leuconostoc* (Schleifer and Ludwig 1995; Vandamme et al. 1996; Kwon et al. 2004; Martinez et al. 2014). According to the most recent taxonomic updates, with a combination of different methods based on 16S rRNA gene sequence similarity, the *Lactobacillus* spp. are categorized into 15 groups: *L. delbrueckii*, *L. salivarius*, *L. reuteri*, *L. buchneri*, *L. alimentaris*, *L. brevis*, *L. collinoides*, *L. fructivorans*, *L. plantarum*, *L. sakei*, *L. casei*, *L. coryniformis*, *L. perolens*, *L. vaccinostercus*, and *L. manihotivorans* (Fig. 1) (Collins et al. 1991; Felis and Dellaglio 2007; Salvetti et al. 2012; Mattarelli et al. 2014).

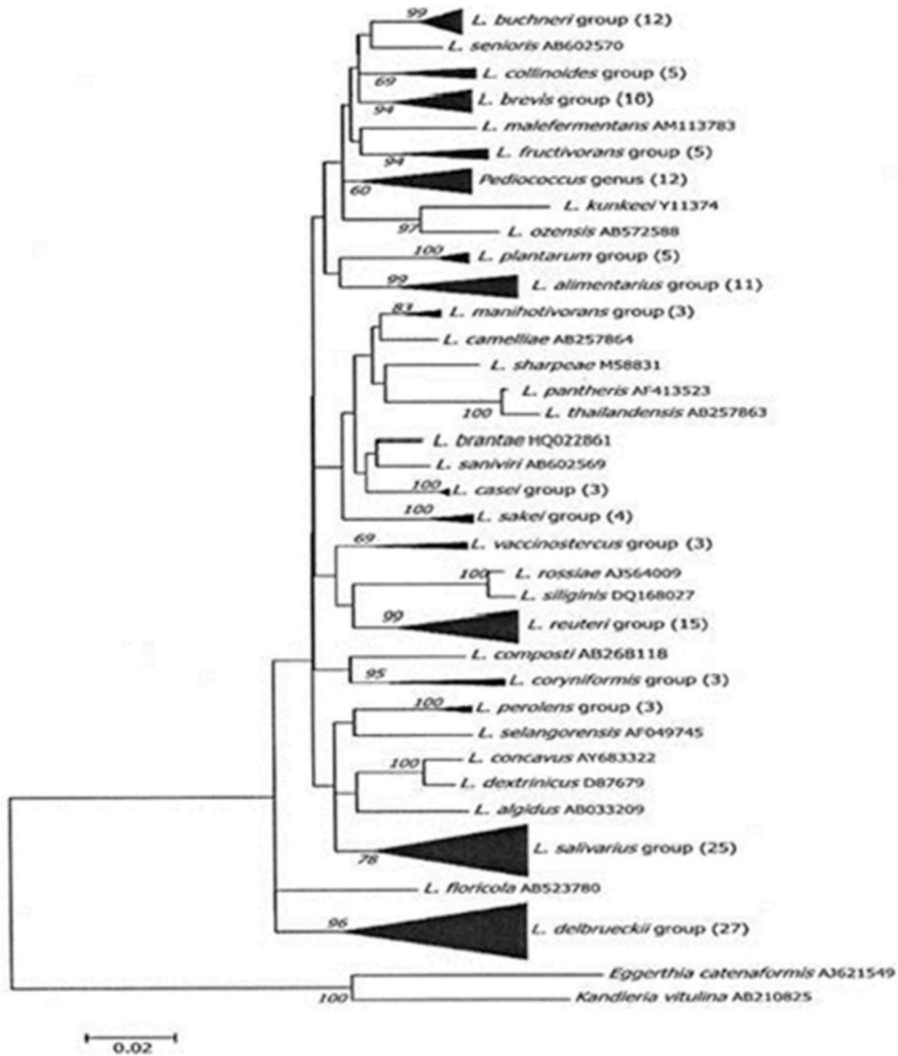


Fig. 1 Phylogenetic tree illustrating the relationship of *Lactobacillus* and *Pediococcus* spp. based on 16S rRNA gene sequence similarity (Adapted from Salvetti et al. 2012)

Application of new sequencing technology has expanded our knowledge about the full genomes of *Lactobacillus* spp. and provided unprecedented insight into microbial diversity. Presently, more than 25 completed *Lactobacillus* genomes have become available within the different databases, with many projects ongoing (Altermann et al. 2005; Nelson et al. 2010; Kant et al. 2011; Wang et al. 2011; Chen et al. 2015; Tareb et al. 2015). The comparative study of 10 complete genomes of *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, and *Oenococcus* has established that all of these LAB share a common ancestor with the bacilli and that their gene complement results from a combination of extensive gene loss and horizontal gene transfer during evolution (Makarova et al. 2006).

Detailed comparative analysis of the 20 *Lactobacillus* genomes has demonstrated that the *Lactobacillus* pan-genome consists of approximately 1400 protein-encoding genes with genomes sharing a total of 383 sets of orthologous genes that define the *Lactobacillus* core genome. This

information allowed classification of all genomes into the NCFM (*L. johnsonii*, *L. gasseri*, *L. crispatus*, *L. helveticus*, *L. acidophilus*), GG (*L. casei*, *L. rhamnosus and L. sakei*) and WCFS (*L. salivarius*, *L. reuteri*, *L. plantarum*, *L. fermentum*, *L. brevis*) groups comprising different species. Into the latter also belong the species of our probiotic strains *Lactobacillus fermentum* ME-3 (DSM 14241), *L. plantarum* Tensia (DSM 21380) and *L. plantarum* Inducia (DSM 21379). Of note, the group-specific genes present in genome of one species and absent in all species (ORFans) appear to be of value in defining the different genomic groups and providing insight into the origin and function of the species (Kant et al. 2011).

The functional prediction of the *Lactobacillus* core genome has identified 26 % of genes belonging to 'translation, ribosomal structure, and biogenesis', while 10 % of the genes belong to 'replication, recombination, and repair', 7 % to 'transcription', 6 % to 'carbohydrate transport and metabolism', and 14 % to 'unknown general function prediction'. Less than 5 % of the proteins encoded by the lactobacilli core genome were predicted to be secreted, indicating that many secreted proteins are encoded by strain-specific genes (Kant et al. 2011).

Within the last decade, *Lactobacillus* spp. identification has been revised due to the development of a wide variety of molecular techniques. The PCR-DGGE of 16S rRNA (or DNA) and 16S-23S ITS-region rRNA are widely used for selective monitoring of LAB and bifidobacteria populations (Bello et al. 2001; Heilig et al. 2002; Murray et al. 2005; Vaughan et al. 2005; Štšepetova et al. 2011a). Specific nucleotide probes targeting rDNA have been designed for different species of *Lactobacillus* that occur in the human intestine. Fluorescent in situ hybridization has been applied to morphologically intact cells and thus provides a quantitative measure of the target organism without the limitation of culture-dependent methods (Amann et al. 1995, 2001). Unfortunately, the probe panel for lactobacilli is still incomplete and lacks specific probes for intestinal species. Although lactobacilli are phylogenetically heterogeneous, two group-specific probes (Lab 158) have been designed that also cover related genera such as *Enterococcus*, *Streptococcus*, *Vagococcus*, and *Oenococcus* (Harmsen et al. 1999). The value of real-time PCR has been demonstrated in several studies. Real-time PCR can be used to quantify *Lactobacillus* spp. and strains from various samples, including feces (Requena et al. 2002; Malinen et al. 2003; Matsuki et al. 2004; Rinttila et al. 2004; Maruo et al. 2006), dairy products, and other food (Kao et al. 2007). Recently, RT-PCR also has been applied for strain-specific quantification in probiotic products (Ahlroos and Tynkkynen 2009; Kullisaar et al. 2010a; Štšepetova et al. 2011b; Sharafedtinov et al. 2013; Mikelsaar et al. 2015).

2.3 Intertwined Metabolism of *Lactobacillus* spp. with Other Intestinal Microbiota

In the microbial ecosystem of the gut, the intertwined metabolism between the host and the microbiota components has played an important role in health. In addition, the whole microbial ecosystem is developed with tight interrelations among their components, including different groups of bacteria. We have attempted to associate the metabolites of the three fermentative groups of *Lactobacillus* spp. with the other important metabolites detected in gut but produced by other groups of bacteria. The major function of the metabolism of the human gut microbiota is to aid in the harvest of nutrients and energy from the varied human diet. Carbohydrates and proteins are broken down by primary fermenters (Fig. 2), yielding gases, hydrogen, carbon dioxide, short-chain fatty acids (SCFAs; e.g., acetic, propionic, butyric), branched fatty acids (isobutyrate, isovalerate, 2-methylbutyrate), organic acids (formate, lactate, and succinate), ethanol, ammonia, amines, phenols, and indoles. Usually, organic acids do not accumulate because they are rapidly further metabolized by other bacterial species to SCFAs. These fermentation and hydrolyzation products

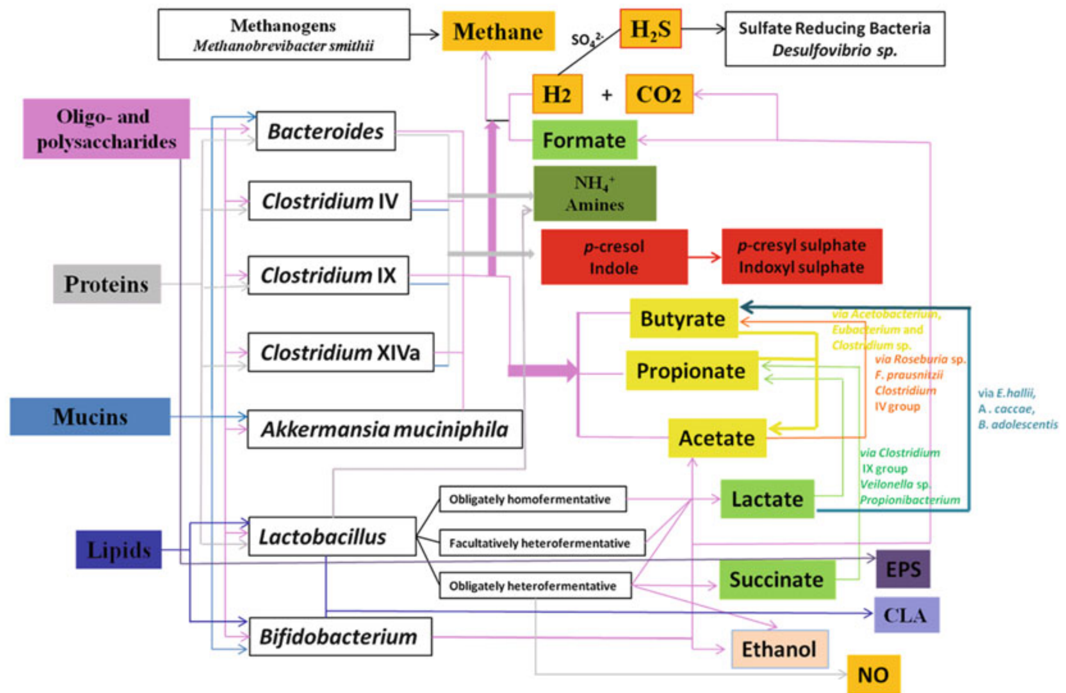


Fig. 2 Schematic view of the metabolism of *Lactobacillus* sp. intertwined with some other intestinal groups of bacteria (Modified from Sarbini and Rastall 2011)
Legend: SCFA: Butyrate, propionate, acetate created from poly- and oligosaccharides and amino acids all groups of bacteria (*Bacteroides, Clostridium* IV, IX, XIVa groups, *Akkermansia muciniphila*, *Lactobacillus, Bifidobacterium sp.*), main butyrate producres: *Clostridium* IV group (*Faecalibacterium prausnitzii*), *Clostridium* XIVa group (*Eubacterium rectale*, *E. halii*, *Roseburia* sp.); main propionate producers: *Bacteroides;* main acetate producer: Clostridium XIVa group (*Blautia hydrogeotrophyca*). Organic acids: formic, lactate and succinate created from poly- and oligosaccharides *Firmicutes: Bacillus, Enterococcus, Eubacterium*, *Lactobacillus* sp., *Lactococcus, Leucocostoc, Pediococcus, Staphylococcus, Streptococcus, Weissella, Actinobacteria*: *Bifidobacterium* sp.; *Enterobacteria.* Lactate-utilizers: *Propionibacterium, Eubacterium, Veilonella, A. caccae, B. adolescentis, C. catus, M. eldsenii*, *Desulfomicrobium* sp. Succinate-utilizers to propionate: *Clostridium* XIVa group (*Roseburtia* sp., *F. prausnitzii*), *Clostridium* IV group. Amines, $NH4^+$ *p*-cresyl sulphate, and indoxyl sulphate created from proteins and peptides by *Firmicutes* (*Clostridium, Lactobacillus*), *Bifidobacterium* sp., *Bacteroides.* Gases: CO_2 created form polysaccharides by OHEL lactobacilli and anaerobic bacteria. H_2 created by *Clostridium* XIVa group, *Lactobacillus* and Clostridium XIVa group (*Blautia hydrogeotrophyca).* H_2-consuming microbes include methanogens by creation of CH_4 (*Methanobrevibacter smithii*), acetogens by creation of acetate and sulphate-reducing bacteria by creation of H_2S. *Desulfovibrio* sp. reduced sulfate. NO created by *Lactobacillus* and *Bifidobacterium* sp. Mucin: genera *Clostridium* XIVa group *(C.clostridiiforme, C. malenominatum)*, *Bacteroides (B. thetaiotaomicron, B. uniformis)*, *Bifidobacterium (B.longum, B.bifidum)* and *Akkermania muciniphila* are able degradate mucin. Conjugated linoleic acid: *Lactobacillus* sp. can produce CLA from lipids (free linoleic acid). EPS created from monosaccharides by *Lactobacillus* sp.

are sources of carbon and energy for community members. Dietary components that are not absorbed in the proximal intestine reach the distal gut, where they are metabolized through processes that involve a wide range of different bacteria in addition to lactobacilli. Furthermore, an unexpected important role of proteins (putatively enzymes) has been demonstrated based on expression of genes involved in carbohydrate metabolism and energy generation (Biagi et al. 2010), possibly indicating a large gene repertoire of different lactobacilli strains. In contrast, Goel et al. (2015) showed that central metabolism in *Lactococcus lactis* appears to be scarcely regulated at the level of gene expression, e.g., ribosomal proteins, but rather more so

at the interacting metabolic level. The regulation of metabolic pathways is still not well understood and involves trophic interactions among members of microbial community that could be explained by epigenetic influences.

2.3.1 Promotional Functions of *Lactobacillus* spp. on Beneficial Metabolites of Microbiota

SCFAs. The SCFAs produced by intestinal bacteria, including *Lactobacillus* and *Bifidobacteria* spp., are further diversified by different bacteria acting as crucial modulators of the gut ecosystem. The main fermentation products of *Lactobacillus* spp. – acetic, lactic, and succinic acids (Fig. 2) – reach additional end-products, including formic, caproic, propionic, butyric, and valeric acids and ethanol (Corsetti et al. 1998; Zalán et al. 2011). In general terms, acetate appears to contribute 50–60 %, propionate 20–25 %, and butyrate 15–20 % of total SCFA depending on dietary variables (Topping 1996). The total beneficial effect of SCFA is the reduction in pH, which diminishes the bioavailability of alkaline cytotoxic compounds and inhibits growth of pH-sensitive organisms. Moreover, a number of specific health-supporting properties have been identified for the major SCFAs (Topping 1996).

Acetate promotes the relaxation of resistance vessels in the colonic vasculature, which changes the maintenance of the blood flow to the liver as well as the colon and increases the absorption of calcium and magnesium. Acetate can reduce the concentration of serum free fatty acids (butyric and linoleic acids), which is important in lowering tissue glucose use. Acetate is also the primary substrate for cholesterol synthesis. Bacteria (*Roseburia* spp., *Faecalibacterium prauznitzii*, *Clostridium* IV group) isolated from the human intestine can use acetate to produce butyrate in the colon (Duncan et al. 2002).

Butyrate enhances some properties of propionate and appears to be the preferred metabolic fuel for colonocytes possessing antineoplastic properties, thus contributing directly to energy production (Roediger and Millard 1995; Gillet et al. 1998; Rizkalla et al. 2000; Liong and Shah 2005b). The major groups of bacteria characterized by levels of butyrate production include the *Bacteroidetes* phylum, *Clostridium leptum*, *Roseburia* species, *Faecalibacterium prausnitzii*, and *Coprococcus* species (Guilloteau et al. 2010). The presence of butyrate may enhance the growth of *Lactobacillus* spp. and play a crucial role in colon physiology and metabolism (Roy et al. 2006; Hijova and Chmelarova 2007). In contrast, metabolites of lactobacilli such as lactate serve as the starting point for many bacteria to produce butyrate (Belenguer et al. 2011).

Gibson et al. (1995) have shown that oligofructose and inulin, which are naturally occurring indigestible carbohydrates, selectively stimulate the growth of species of *Bifidobacterium*, a producer of butyrate considered beneficial to health. At the same time, some other microbes such as bacteroides, clostridia, anaerobic cocci, and fusobacteria are decreased.

Propionate affects colonic muscular contraction, relaxation of resistance vessels, stimulation of colonic electrolyte transport and colonic epithelial proliferation, and insulin resistance. Long-term dietary supplementation with propionate decreases blood glucose in rats and humans. Another possible effect of propionate is the reduction of plasma cholesterol levels (Chen et al. 1984; Hara et al. 1999; Chambers et al. 2014). One of the determinants of the actions of propionate on serum lipids is the ratio of propionate to acetate (Cheng and Lai 2000). Propionate is subsequently metabolized by hepatocytes while acetate either remains in the liver or is released systemically to the peripheral venous system (Pomare et al. 1985).

Both butyrate and propionate may be degraded into the two smaller acetate molecules by sulfate- or nitrate-reducing acetogenic bacteria such as *Acetobacterium*, *Eubacterium*, and *Clostridium* spp. (Westermann et al. 1989). However, an increased proportion of butyrate-producing or -consuming species such as *F. prausnitzii* and *Roseburia* species can reverse this process (Duncan et al. 2002). Such interactions can involve the mutualistic production of SCFAs, with acetate produced by

B. thetaiotaomicron acting as a substrate for butyrate generation by *E. rectale* (Mahowald et al. 2009).

Lactate is not a major bacterial fermentation product, but it may be used by other bacteria in the environment. Bacteria in the GI tract that produce lactate include *Bacteroidetes*, bifidobacteria, LAB, and *Eubacterium*. However, it does not usually accumulate to a substantial extent in the colon (Duncan et al. 2002; Pessione 2012). Lactate modulates key functions of the main players in the innate response, such as myeloid and epithelial cells (Blad et al. 2012). The receptor GPR81 is specific for lactate and expressed primarily in adipocytes, having an antilipolytic effect and mediating macrophage-dependent anti-inflammatory effects (Liu et al. 2009; Hoque et al. 2014; Garrote et al. 2015). Beyond the signaling capacity through GPR81, lactate can also modulate histone deacetyl activity (Latham et al. 2012). A high concentration of lactate in the extracellular milieu has an effect on modulation of cell metabolism (Garrote et al. 2015). Some studies have shown that after co-incubation of both dl-lactate and human intestinal butyrate-producing bacteria such as *Eubacterium hallii*, *Anaerostipes caccae*, and *Bifidobacterium adolescentis*, a significant amount of lactate is converted to butyrate (Duncan et al. 2004). The high concentrations of lactate and butyrate are in agreement with the presence of *Streptococcus* spp. and *Clostridium* cluster XIVa spp., respectively (Zoetendal et al. 2012). In addition, lactate can be metabolized by propionate-forming bacteria (*Coprococcus catus*, *Megasphaera eldsenii*) or by sulfate-reducing bacteria such as *Desulfomicrobium* spp. (Louis et al. 2014).

During a randomized double-blind synbiotic cross-over intervention study with feeding probiotics (*L. fermentum* ME-3, *L. paracasei* 8700:2, *B. longum* 46) together with prebiotic (oligofructose or inulin), the counts of bifidobacteria and its metabolite butyrate increased. At the same time, the other microbes such as bacteroides, clostridia, and fusobacteria decreased (Gibson et al. 1995; Saulnier et al. 2007). In addition, the counts of lactobacilli in the intestine increased (Mikelsaar et al. 2008), accompanied by a documented increase in butyrate. The antioxidative effect of blood sera was simultaneously identified (Hutt et al. 2009).

Succinic acid is a dicarboxylic acid detected in considerable amounts in the FHEL and OHEL groups of lactobacilli as a product of the fermentation of sugars. Among microbiota, the lactobacilli, bifidobacteria, bacteroides, and *Clostridium* IV group with *Faecalibacterium prausnitzii* are involved in the metabolism of succinate and further to butyrate and propionate via the *Clostridium* IX group (Flint et al. 2012). In addition, succinate can donate electrons to the electron transport chain, leading to fumarate and ubiquinone, playing an important role in antioxidative processes. For instance, the production of succinate by a probiotic strain *L. fermentum* ME-3 (DSM14241) seems to be one mechanism of its antioxidative capacity (Mikelsaar and Zilmer 2009; Mikelsaar et al. 2012a, b). Succinic acid is a final product of the oxidation of putrescine in the small bowel of animals and may serve as a source of instantly metabolizable energy (Bardoczs et al. 1998). Increased SCFA concentrations may increase the solubility of certain minerals such as calcium and enhance the absorption and expression of calcium-binding proteins (Scholz-Ahrens et al. 2007).

The pH and peptide supply have predicted alterations in bacterial populations and SCFA ratios within microbial communities in the human colon (Leitch et al. 2007; Walker et al. 2008). However, per a recent publication on the flexibility of human metabolism, even the ribosomal protein levels and enzyme activities changed somewhat with increasing microbial growth rates, whereas the central metabolism was more regulated at the metabolic levels (van Ommen et al. 2014).

Gases

The three most abundant gas metabolites of *Lactobacillus* spp. include CO_2, intra-colonic hydrogen gas (H_2), and nitrogen mono-oxide (NO). CO_2 is a natural product of the OHEL group in carbohydrate metabolism. Excess CO_2 can create problems in the GI tract from probiotic bacteria

of this fermentation group such as *L. fermentum*, *L. brevis*, and *L. reuteri*. Special precaution is needed for children below 6 months to avoid flatulence.

Intra-colonic hydrogen gas (H_2) production has been shown by *Clostridium* XIV group, but it also can be released from acetate produced by lactobacilli and acetogens (*Blautia hydrogenotrophica*). One function of microbes during fermentation is to maintain redox balance while maximizing energy production. Many species have branched fermentation pathways that allow the disposal of reducing equivalents. The production of hydrogen is an energetically efficient way to yield higher levels of ATP (Rey et al. 2013). Hydrogen buildup inhibits reoxidation of pyridine nucleotides and forces primary fermenters to accumulate reduced compounds (e.g., butyrate, ethanol) that are key to the energy-extracting capacity of primary fermenters in microbial food webs and contribute to more efficient and complete oxidation of substrates (Wolin and Miller 1983; Stams and Plugge 2009). In the human gut, H_2-consuming microbes include methanogens, acetogens, and sulfate-reducing bacteria that in turn produce methane, acetate, and hydrogen sulfide (H_2S), respectively. They also can use H_2 or organic compounds (lactate, formate) for reduction of sulfate or their oxidized sulfur compounds to generate hydrogen sulfide. Sulfate-reducing bacteria have been found in fecal microbiota of healthy adults (Stewart et al. 2006) and in the distal mucosa and also associated with both pro- and anti-inflammatory signaling (Levine et al. 1998; Loubinoux et al. 2002; Levine and Kroemer 2008; McIntosh et al. 2009; Rajilic-Stojanovic et al. 2011). Hydrogen sulfide is produced in the gut by sulfide-reducing bacteria (main genus *Desulfovibrio*) via the reduction of diet-derived sulfate and the metabolism of sulfur amino acids and taurine (Magee et al. 2000; Scanlan et al. 2009). *Desulfovibrio* spp. can use lactate as a co-substrate for growth and sulfide formation (Marquet et al. 2009); thus, they are putatively interconnected with *Lactobacillus* spp. Sulfide is toxic to colonocytes and inhibits butyrate oxidation, which results in the breakdown of the colonocyte barrier (Roediger and Babidge 1997). Hydrogen sulfide is also genotoxic to non-transformed human cell lines in the colonic lumen, and the mechanism of DNA damage is proposed to involve creation of reactive oxygen species (ROS) (Louis et al. 2014).

Nitric oxide (NO) is a signaling molecule that regulates many biological functions. Formation of NO has been demonstrated in a wide variety of cells, including vascular endothelial, neuronal, polymorphonuclear PMN, bronchial epithelial cells, and hepatocytes. Excessive NO production, in particular by activated macrophages, has a cytotoxic or cytostatic effect, inhibiting the growth of a diverse array of infectious agents. NO production *in vitro* has been demonstrated also by lactic acid bacteria, e.g., lactobacilli and bifidobacteria (Xu and Verstraete 2001; Korhonen et al. 2001; Korhonen 2002; Sobko et al. 2005; Hutt et al. 2015). Lactobacilli produce NO from nitrate by reducing it to nitrite for further decomposition to NO either enzymatically or non-enzymatically. In addition, the sequential reduction of nitrate and nitrite by different anaerobes of gut microbiota has been shown. NO produced by LAB protects mucosa from damage and excessive permeability (Korhonen et al. 2001).

Some other strains of intestinal microbiota such as *E. coli* and *S. aureus* can counteract this process by rapid NO consumption. To date, it has been demonstrated that lactobacilli can also induce NO synthetase activity in host cells (Korhonen et al. 2001; Korhonen 2002; Hu et al. 2013). Both oxygen (oxidative stress and hypoxia) and NO are important factors in cardio vascular diseases such as atherosclerosis and hypertension. ROS production intricately balances with that of NO, and both ROS and NO affect mitochondrial function and structure, which are crucial for maintaining a stable heartbeat. ROS and reactive nitrogen species (RNS) can modulate cardiac NO signaling, causing many downstream effects. This important topic in cardiology will require further studies while

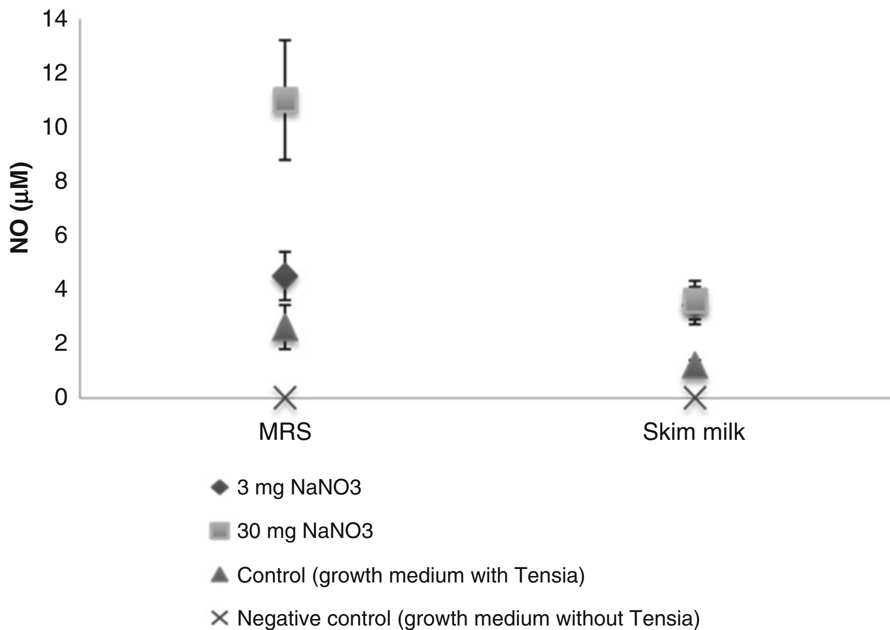


Fig. 3 Detection of NO (µM) produced by *Lactobacillus plantarum* Tensia in the presence of different contents of sodium nitrate in Man, Rogosa, Sharpe (MRS) media and skim milk (Hutt et al. 2015) Legend: The amount of NO was in close correlation with the amount of $NaNO_3$ added to the MRS culture media and skim milk for *Lactobacillus plantarum* (Tensia DSM 21380). Thus, nitrate was the preferred source for NO generation

the role of oral microbiota has been recently raised (Koren et al. 2011; Erdmann 2013; Kapil et al. 2014). Previously, specific mixtures of amino acids (arginine, cysteine) have been tried for increasing endothelial NO synthetase expression (Nisoli et al. 2008). We suggest that non-nitrate sources such as some probiotic bacteria (*Lactobacillus plantarum* TENSIA DSM21380) can also be involved in *in vitro* NO production (Hutt et al. 2015) and possibly in induction of NO synthetase activity in host cells (Hu et al. 2013).

We have measured the NO production of lactobacilli using the Apollo 4000 free radical analyzer (WPI, Berlin, Germany) and electrodes of type ISO-NOP electrode signals (Fig. 3). The NO concentration was calculated according to standard curve correlation with the strength of the electrode signal.

Mucin

Additionally, the next most important catabolic activity of the bacteria of the intestinal microbiota is degradation of mucus glycoproteins and cell membrane glycolipids (termed mucins). Mucins are composed of a peptide core rich in serine and threonine residues that is modified by oligosaccharides linked via O- or N- glycosidic bonds. The oligosaccharides are composed of one or more four primary sugars (N-acetylglucosamine, N-acetylgalactosamine, galactose, and fucose) and are terminated by sialic acids or sulfate groups (Allen 1981). Degradation of mucin is regarded as a pathogenicity factor because loss of the protective mucus layer may expose GI tract cells to pathogens (Ruseler-van Embden et al. 1995; Derrien et al. 2004). However, only 1 % of colonic microbiota can degrade host mucin-using enzymes (e.g., glycosidases and sulfatases) that can degrade the oligosaccharide chains (Hoskins and Boulding 1981). However, these findings show the role of mucin as a carbon and energy source for intestinal microbiota. Isolates belonging to the genera *Enterobacteria*, *Eubacteria*, *Ruminococcus*, *Bacteroides* (*B. thetaiotaomicron*, *B. uniformis*), *Bifidobacterium* (*B. longum*,

B. bifidum), *Clostridium* (*C. clostridiiforme*, *C. malenominatum*), and *Akkermansia muciniphila* can degrade mucin (Salyers et al. 1977; Derrien et al. 2004; Macfarlane et al. 2005). By measuring the release of reducing sugar monomers from the mucin polymer, it was shown that only mixed cultures of fecal bacteria could degrade mucin by more than 90 % whereas pure cultures *of B. fragilis*, *B. longum*, and *Clostridium perfringens* showed only partial degradation (Willis et al. 1996). Recently, it was demonstrated that the ability to degrade mucin is associated with the presence of two genes coding for the extracellular glycosidases *afcA* and *engBF*, found in *B. bifidum* and *B. longum* (Ruas-Madiedo et al. 2008).

According to the literature, *Lactobacillus* spp. cannot degrade mucin (Ruseler-van Embden et al. 1995; Macfarlane et al. 2005; Subramani et al. 2010). Supplementation with the multistrain probiotic product VSL#3, which contains LAB strains (*B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*, *S. thermophilus*) did not affect the expression levels of MUC1, MUC2, MUC3, and MUC4 genes in a mouse model of colitis (Gaudier et al. 2005) or the expression of MUC5AC in a rat model of gastric ulcer (Dharmani et al. 2013). In contrast, administration of VSL#3 to healthy Wistar rats resulted in the upregulation of MUC2 and MUC3 as well as MUC31 gene levels (Caballero-Franco et al. 2007). However, *Lactobacillus*-specific genes include mucus-binding proteins that are involved in cell adhesion and several transport systems for carbohydrates and amino acids (Klaenhammer et al. 2008). Thus, the elucidation of the metabolism of mucins by *Lactobacillus* spp. needs more research.

Cholesterol

An important property of *Lactobacillus* spp. is the ability to reduce cholesterol through a combination of two or more mechanisms that include assimilation of cholesterol during growth, binding of cholesterol to the cellular membrane, and deconjugation of bile salts (Brashears et al. 1998; Liong and Shah 2005a). Some lactobacilli can produce proteins with a cholesterol-lowering effect (Kim et al. 2008), which is strain specific. The cell-free supernatant of the *L. acidophilus* strain contains a protein (NPC1L1) that influences cholesterol absorption and is a promising target for cholesterol-lowering mechanisms (Miura and Saku 2008; Lee et al. 2010). Administration *L. reuteri* to mice reduces serum total cholesterol by 20 % and increases the ratio of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol by 17 % (Taranto et al. 2003). The *ccpA* gene encodes the catabolite control protein to play an important role in the cholesterol-reducing activity of lactobacilli. Cholesterol removal by *L. delbrueckii* results from binding of free bile acids to their cell membranes through exocellular polysaccharides (EPS). LAB strains that produce more EPS bind the greatest amount of bile acids (Pigeon et al. 2002; Tok and Aslim 2010).

The *L. plantarum* PH04 strain has been reported to be able to produce bile salt hydrolase (BSH) *in vitro*. Full genome sequencing of the Estonian probiotic strains *L. plantarum* Tensia, *L. plantarum* Inducia, and *L. fermentum* ME-3 has demonstrated the presence of BSH genes in the genome. Detection of bile acid hydrolases has been associated with improvement in the blood lipid profile in volunteers with borderline high content of low LDL cholesterol (Mikelsaar et al. 2014, 2015).

The single amino acids and di- and tripeptides of milk generated by lactobacilli (Jauhiainen et al. 2005; Turpeinen et al. 2012) can reach the host bloodstream and act as angiotensin-1–converting enzyme (ACE) inhibitory compounds. ACE inhibits the renin–angiotensin system with consequent vasodilatation. Some Estonian *L. plantarum* strains (Inducia and Tensia) produce peptides with inhibitory activity against ACE, polyamine spermidine, and NO and have been used for regulation of blood and urine biomarkers (Štšepetova et al. 2011a; Songisepp et al. 2012a; Hutt et al. 2015). In dairy food intervention and clinical trials, the lactobacilli reduce the risk of obesity and high blood pressure

(Jauhiainen et al. 2012; Xu et al. 2015). Thus, the described blood pressure–lowering effect of fermentation by probiotics as a special functional property of dairy products holds the potential to decrease the risk of cardiovascular disease.

Conjugated linoleic acid (CLA) is a group of positional (e.g., 7:9, 9:11, 10:12, and 11:13) and geometric isomers of linoleic acid (C18, *cis*-9: *cis*-12) that exert health benefits including anti-atherogenic, antidiabetic, anti-inflammatory, and anticarcinogenic properties (Maggiora et al. 2004). Many papers have reported that *Lactobacillus* spp. (*L. acidophilus*, *L. brevis*, *L. paracasei* subsp. *paracasei*, *L. pentosus*, *L. plantarum*, *L. rhamnosus*, *L. casei*) could be able to convert free linoleic acid to the conjugated form (Lin et al. 2002; Alonso et al. 2003; Sieber et al. 2004; Zhao et al. 2011).

Exopolysaccharides

Exopolysaccharides (EPS) are long-chain polysaccharides produced extracellularly by *Lactobacillus* spp. (Fig. 2). The utility of various EPS depends on monosaccharide composition, types of linkages present, degree of branching, and molecular weight. Different researchers have demonstrated that species such as *L. lactis*, *L. reuteri*, *L. sanfranciscensis*, *L. johnsonii*, *L. bulgaricus*, *L. kefir*, *L. kefiranofaciens*, *L. parakefir*, *L. delbrueckii* subsp. *bulgaricus*, *L. mucosae*, and *L. helveticus* possess high potential for synthesis of EPS (Frengova et al. 2002; Buchholz and Seibel 2003; Korakli et al. 2003; Kralj et al. 2005; Vinderola et al. 2006). EPS derived from *Lactobacillus* spp. have beneficial physiological effects on human health, such as antitumor activity, immunomodulating bioactivity, and anticarcinogenecity (Doleyres et al. 2005; Patel et al. 2012b). EPS also have a role in initial adhesion, biofilm formation, cellular recognition, and pathogenicity (Patel et al. 2012b). Additionally, the exopolysaccharide-synthesizing *Lactobacillus mucosae* DPC 6426 strain has shown cholesterol-lowering properties in an animal model of lipid-driven atherosclerosis (Ryan et al. 2015).

2.3.2 Suppressive Functions of *Lactobacillus* spp. on Detrimental Metabolites

The metabolites produced by intestinal bacteria involve not only their several beneficial components but also some toxic compounds such as ammonia, amines, indoxyl, and p-cresol sulfate created from proteins (amino acids) (Fig. 2). Concerning protein metabolism, ammonia generated by bacteria in the colon and absorbed into the portal blood is converted into urea in the liver and excreted in the urine (Eklou-Lawson et al. 2009). Bacteria species producing ammonia are Gram-negative anaerobes and *Clostridium*, *Peptostreptococcus*, and *Fusobacterium* species (Vince and Burridge 1980; Smith and Macfarlane 1996). Gram-positive non-sporing anaerobes, the streptococci and micrococci, form modest amounts, and lactobacilli and yeasts form very little ammonia (Vince and Burridge 1980). Bacteria assimilate ammonia to produce bacterial protein during carbohydrate fermentation, so the concentration of ammonia in the colon at any one time depends on the balance between amino acid deamination and bacterial protein synthesis. The consumption of the probiotic *L. acidophilus* LC1 may influence the bacterial production of toxic metabolic end-products, particularly ammonia, in the human colon (Cummings and Macfarlane 2002; De Preter et al. 2004; Geboes et al. 2005; Wutzke et al. 2010). Fermentation of prebiotics containing resistant starch and other non-digestible carbohydrates, such as lactose and lactulose, may also repress the formation and inhibit the activity of enzymes responsible for ammonia release. In the human colon, these substrate effects may decrease the amount of ammonia available to exert a toxic effect on the host (Bianchi et al. 1993; Ito et al. 1993).

Phenolic compounds

Phenolic compounds are formed following bacterial degradation of aromatic amino acids such as p-cresole and phenylpropionate from tyrosine, phenylacetate from phenylalanine, and indole propionate and indole acetate from tryptophan.

Intestinal bacteria involved in these processes include the genera *Clostridium*, *Bacteroides*, *Enterobacteriaceae*, *Bifidobacterium*, and *Lactobacillus* (Aragozzini et al. 1979; Yokoyama and Carlson 1981; Smith and Macfarlane 1996; Blaut and Clavel 2007; Evenepoel et al. 2009). Phenolic compounds are absorbed in the colon, detoxified by the liver, and excreted in urine as p-cresol with the remainder being made up of phenol and 4-ethylphenol (Tamm and Villako 1971; Evenepoel et al. 2009). Phenols are not found in the urine of germ-free animals (Bakke and Midtvedt 1970), and in humans, their urinary excretion rate can be related to protein intake (Cummings et al. 1979). In the colon, the increased carbohydrate fermentation decreases urinary phenol excretion, indicating that the amino acids are required for bacterial growth. The presence of fermentable carbohydrate (starch) may decrease the net production of phenolic compounds (Cummings et al. 1979; Smith and Macfarlane 1996). Indoxyl sulfate and p-cresyl sulfate are potentially important, therapeutically modifiable toxins in patients with chronic kidney disease. Both are protein-bound uremic retention solutes that are generated from colonic bacterial fermentation of dietary protein and have been associated with cardiovascular disease, kidney disease progression, and overall mortality in the chronic kidney disease population (Rossi et al. 2014).

A tight interplay has been shown between *Lactobacillus* spp. and the harmful phenolic, p-cresole metabolites of different anaerobes and enterobacteria. The probiotic intervention study in a cohort of hemodialysis patients has illustrated a 30 % decrease in serum indoxyl sulfate in conjunction with a decline in fecal enterobacteria (*E. coli*) ($p < 0.05$), which has one of the highest observed enzymatic activities for indoxyl sulfate production (Hida et al. 1996). In a study of oral administration of probiotic *L. gasseri* G2055SR at a high dose, the decrease in p-cresol was also important because p-cresol as a tyrosine metabolite is considered to be a promoter of skin and liver carcinogenesis in mice (Fujiwara et al. 2001). It seems that *L. gasseri* LG 2055SR ingestion may reduce the toxicological risk of tyrosine metabolites in the human GI tract (Fujiwara et al. 2001; Sanders et al. 2009). The *Bacteroides fragilis* species have a high enzymatic activity for p-cresol production (Ling et al. 1994). The combination of the decrease in p-cresyl sulfate–producing bacteria (*Bacteroidaceae*) and the increase in p-cresyl sulfate- and indoxyl sulfate–suppressing bacteria (*Bifidobacteria* and *Lactobacillus*) supports the proposed mechanistic rationale for the significant reduction in fecal p-cresol and indole (Rossi et al. 2014). De Preter et al. conducted a randomized placebo-controlled crossover study with coadministered *Lactobacillus casei* Shirota (dose 2×10^9) and oligofructose-enriched inulin, which demonstrated a significant reduction in urinary p-cresyl sulfate (De Preter et al. 2007).

Amines

Amines are produced by a long list of intestinal bacteria following hydrolysis and decarboxylation of amino acids. The amines include agmatine, methylamine, pyrrolidine, butylamine, taurine; the polyamines include putrescine spermine and spermidine; and the biogenic amines (BAs) include histamine, cadaverine, and tyramine (Drasar and Hill 1974). Normally, amines produced by colonic bacteria are detoxified by monoamine and diamine oxidases in the gut mucosa and liver (Hughes et al. 2000). Species belonging to the genera *Clostridium*, *Bacteroides*, *Enterococcus Bifidobacterium*, and *Lactobacillus* form amines in substantial quantities (Allison et al. 1989; Dudkowska et al. 2003).

In organisms, the main source for polyamines is food intake, cellular synthesis, and in the lower parts of the intestine, the intestinal microbiota (Milovic 2001). Polyamines can be formed by bacteria from arginine by the mitochondrial enzyme arginase to produce ornithine. Ornithine is then decarboxylated to putrescine by ornithine decarboxylase, or from agmatine via agmatine deiminase (AgDI) (Ladero et al. 2011). Furthermore, other polyamines are produced: spermidine from putrescine using spermidine synthetase or spermine from spermidine by spermine synthetase. These pathways has been

reported for some *P. aeruginosa*, enterococci, and lactic acid bacteria strains (Moinard et al. 2004; Makarova et al. 2006; Larque et al. 2007; Lavizzari et al. 2010). The amount of polyamines decreases with age (Matsumoto and Kurihara 2007), and it may be that polyamines are linked to senescence. Both spermidine and spermine can be converted back to putrescine, and polyamine oxidases can convert spermine and spermidine into the acetylated forms (Minois et al. 2011). The full repertoire of biological effect of polyamines is not fully known; however, many studies have addressed the toxicological effects of polyamines as a potential deterioration marker or quality indicator of food (Shinki et al. 1991; Moinard et al. 2004). These compounds are present in all human cells (Takahashi and Kakehi 2009) and involved in many physiological functions, including immunity, stress resistance, cell growth, proliferation, and the synthesis of proteins and nucleic acids (Rhee et al. 2007; Mandal et al. 2013). All of these mechanisms comprise chronic low-grade inflammation, a major risk factor for aging and related diseases. In sufficient amounts, the polyamines are important in maintaining the healthy structure and function of the intestinal mucosa (Milovic 2001). Some species of lactobacilli of the human gut microbiota producing polyamines have special roles in the suppression of low chronic inflammation, supporting the recovery of damaged tissues. Human milk is a source of spermine and spermidine that is important for child growth. There seems to be a fine balance between beneficial and deleterious effects of polyamines that is not solved in infection and cancer development. Moreover, the larger amounts of putrescine can potentiate the effects of histamine by inhibiting the detoxifying enzymes diamine oxidase and hydroxymethyl transferase (Eerola et al. 1997; Guerrini et al. 2002; Minois et al. 2011).

The production of polyamines by lactobacilli can be tested using *in vitro* and experimental settings. Moreover, in 13 different species of intestinal *Lactobacillus* and *E. faecalis*, the presence of genes and their ability to produce bio- and polyamines in the decarboxylation media supplemented with ornithine, agmatine, histidine, and tyrosine has been demonstrated (Nakovich 2003; Štšepetova et al. 2014a). Both *L. buchneri* and *L. gasseri* contain the histamine-encoding gene *hdcA*; additionally, *L. gasseri* contains the tyramine-encoding gene *tdc*. An AgDI cluster has been found in *L. brevis*, *L. buchneri*, *L. fermentum*, *L. rhamnosus*, *L. plantarum*, *L. acidophilus*, and *L. gasseri*. All of the species produce bio- and polyamines such as histamine, tyramine, and putrescine in correlation with the genes present (Štšepetova et al. 2014a).

The *LytR* gene, associated with the genetic organization of the AgDI cluster, has been found in both *L. plantarum* Inducia and *L. plantarum* Tensia using full genome sequencing. The presence of a similar gene has been demonstrated in dairy putrescine producer strains of *Lactococcus lactis* subs. *lactis* (Ladero et al. 2011).

In our studies, for elaboration of different functional properties of specific probiotic *Lactobacillus* strains, the contents were compared of a putatively beneficial polyamine *in vitro*, in food products/supplements, and as a particular biomarker of human blood or urine after consumption (Songisepp et al. 2005; Štšepetova et al. 2011a, 2012a). We illustrated this by depicting correlations between putrescine production *in vitro*, probiotic cheese comprising *L. plantarum* Inducia and Tensia, and excreted urine samples of volunteers consuming the probiotic cheeses. Surprisingly, the same proportions – comparatively higher values of putrescine – were present also in industrially produced cheese with *L. plantarum* Inducia, and a higher content of acetylated putrescine was identified in the urine of humans consuming the Inducia cheese for 3 weeks. The values *in vitro*, in cheese, and in volunteers were proportionally lower in the case of Tensia. Even in probiotic cheese with the *L. plantarum* strain Inducia, the obtained higher level did not reach the safe reference value of putrescine for lactobacilli after 3 weeks ripening (Fig. 4) (Karvicoka and Kohajdova 2005).

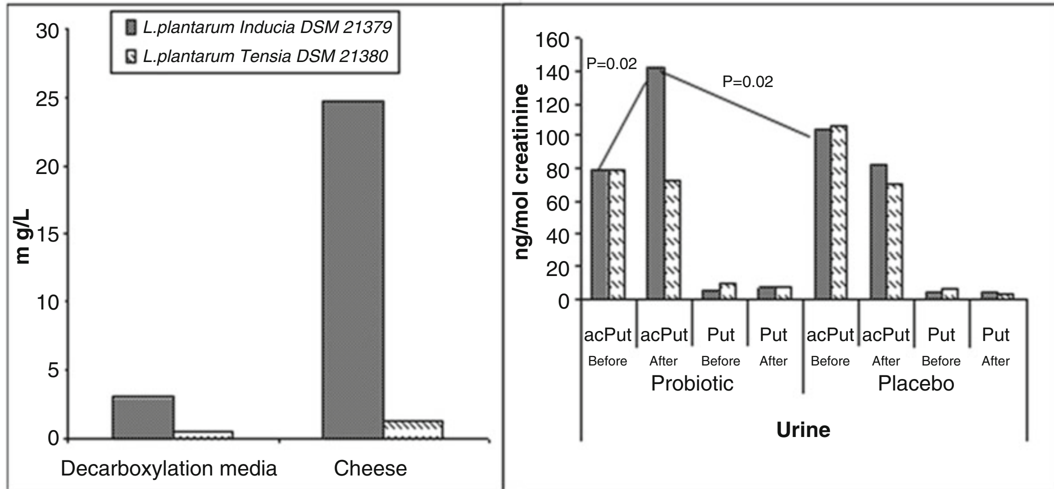


Fig. 4 Detection of putrescine (Put) and acetyl-putrescine (AcPut) in decarboxylation media, probiotic cheeses and urine of persons consuming probiotic cheeses during 3 weeks comprising *L. plantarum* Inducia DSM 21379, *L. plantarum* Tensia DSM 21380 and control cheese
Legend: Gas chromatographic (GC) analysis was performed with a Hewlett-Packard HP model 6890 (Hewlett Packard, USA). For detection of poly amines by GC analyze the decarboxylation medium cultivated with *L. plantarum* Inducia or *L. plantarum* Tensia or the supernatant of the probiotic cheeses was derivatized according modified method of Nakovich (Nakovich 2003). Polyamine concentrations in urine were expressed as ng/mol of creatinine (Štšepetova 2011a). In decarboxylation medium *L. plantarum* Inducia produced 1.9 μg/mL putrescine from ornithine while *L. plantarum* Tensia only 0.5 μg/mL, e.g. nearly in four times lower value. The similar proportions of putrescine and acetylated putrescine were detectable in cheese comprising Inducia and Tensia and in urine of volunteers consuming the probiotic cheeses

A positive association between consumption of probiotic *L. acidophilus* NCFM and the content of gut spermidine has been described (Ouwehand et al. 2009). As well, it has been reported that administration of the probiotic strain *B. animalis* LKM512 to mice for 6 months is correlated with changes in gut microbiota, increasing the amount of polyamines in feces and longevity (Matsumoto et al. 2011). In our studies, it has been shown that administration of cheese containing probiotic *L. plantarum* Tensia to obese persons for 3 weeks was associated with a reduction in BMI because of the lower water content of the body. The lactobacilli content was positively correlated with urinary putrescine content and the presence of Tensia (Sharafedtinov et al. 2013).

Thus, the presence of genes of the *Lactobacillus* strain mainly affects the yield of putrescine regardless of the variable environment with different constituents (decarboxylation media, cheese, host organism). It seems that metabolic outcome depending on the genotype of the host, the microbial genetic metabolic profile, and epigenetic influence has great flexibility in homeostasis.

Concerning biogenic amines (BAs), the lactobacilli could exhibit certain disadvantageous metabolic activities with regard to consumer safety, particularly if BAs accumulate in the fermented products (Bernardeau et al. 2008). BAs have been implicated in several outbreaks of food poisoning and are the initiators of hypertensive crises, hypertension, or hypotension, and dietary-induced migraines in certain patients. The probiotic *Lactobacillus plantarum* Tensia did not produce potentially harmful BAs, such as histamine or cadaverine, in decarboxylation media and milk according to gas chromatographic analysis. Also, the amount of tyramine produced in the cheese environment during ripening and after 15 weeks of storage was below the clinically significant content (Songisepp et al. 2012a).

Hydrogen sulfide is produced in the gut by sulfide-reducing bacteria (main genus *Desulfovibrio*) via the reduction of diet-derived sulfate and the metabolism of sulfur amino acids and taurine (Magee et al. 2000; Scanlan et al. 2006). Sulfate-reducing bacteria have been found in fecal microbiota of healthy adults (Stewart et al. 2006) and in the distal mucosa and also have been associated with both pro- and anti-inflammatory signaling (Levine et al. 1998; Loubinoux et al. 2002; Wallace et al. 2009; Rajilic-Stojanovic et al. 2011). *Desulfovibrio* spp. can use lactate as a co-substrate for growth and sulfide formation (Marquet et al. 2009); thus, they are putatively interconnected with *Lactobacillus* sp. sulfide is toxic to colonocytes and inhibits butyrate oxidation, which results in the breakdown of the colonocyte barrier (Roediger and Babidge 1997). Hydrogen sulfide is also genotoxic to non-transformed human cell lines in the colonic lumen, and the mechanism of DNA damage is proposed to involve ROS (Louis et al. 2014).

The diversity of intestinal LAB of the *Firmicutes* phylum therefore has been proven with a variety of metabolic compounds produced differentially according to their taxonomic division. The promotional role of metabolites of the three fermentative groups of *Lactobacillus* spp. is intertwined with other beneficial compounds like acetate, propionate, butyrate, and NO produced by the other groups of intestinal bacteria. On the other hand, the suppressive impact of *Lactobacillus* spp. for reduction of detrimental bacterial metabolites such as ammonia, indole, para-cresol, sulfides, H_2, ROS, or RNS and their producers is surely conferring balance on the intestinal microbial ecosystem.

3 Intestinal *Lactobacillus* Species Composition of Different Age Groups

3.1 Infants and Young Children

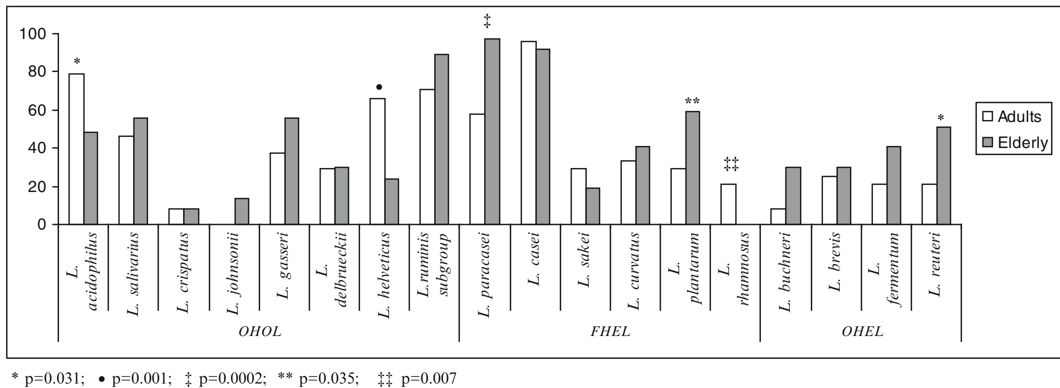
The characterization of intestinal lactobacilli by phenotypic properties and genotyping has been performed with 70 lactobacilli strains of fecal samples of healthy children (1–24 months). The species identification by the API 50 CHL divided the *Lactobacillus* strains into six species, and the concordance with molecular typing by intergenic/internal transcribed spacer polymerase chain reaction (ITS-PCR) was 89 %. The degree of antimicrobial activity of lactobacilli isolated from children against *E. coli*, *S. aureus*, *E. faecalis*, and *S. sonnei* divided the strains into three groups, which largely coincided with the fermentation types of lactobacilli: OHEL > FHEL > OHOL. Within the different fermentation types, *L. plantarum* strains expressed the strongest antagonistic activity among FHEL. *L. fermentum* strains had less antagonistic activity compared to *L. brevis* and *L. buchneri* (Annuk et al. 2003). In the majority of children, lactobacilli of different fermentation types were presented in fecal samples

Chervonsky (2012) has emphasized the production of effector molecules (antimicrobial substances and specific immunoglobulin A antibodies) as important in the control of pathogens and commensals. Tracking these indices could be helpful in the search for probiotic strains with pathogen-suppressive properties.

In the literature, the intestinal lactobacilli in breastfed infants aged 15–60 days with infantile colic (30 cases) and healthy infants (26 cases) have been evaluated. *Lactobacillus brevis* (4.3×10^8 CFU/g) and *L. lactis* ssp. *lactis* (2.5×10^7 CFU/g) were found only in colicky infants while *L. acidophilus* (2.4×10^7 CFU/g) was found only in healthy infants. *Lactobacillus brevis* and *L. lactis* ssp. *lactis* might be involved in the pathogenesis of infantile colic, increasing abdominal distension (Savino et al. 2005). One reason for the observed differences may be the heterofermentive metabolism with gas production by the latter lactobacilli that could be involved in the pathogenesis of this common disorder (Vassos et al. 2008).

3.2 Adults

In our laboratory the *Lactobacillus* species distribution was assessed in 24 adults (mean age 29.2)



* p=0.031; • p=0.001; ‡ p=0.0002; ** p=0.035; ‡‡ p=0.007

Fig. 5 Intestinal *Lactobacillus* spp. diversity in adults (n = 24) and elderly persons (n = 37) (Štšepetova et al. 2011b)
Legend: statistically significant differences were detected in prevalence (%) of *L. acidophilus* and *L. helveticus* being more prevalent in adults. *L. paracasei, L. plantarum and L. reuteri* were more prevalent in elderly

while the lactobacilli were isolated from 97 % of adults (Fig. 5). However, using molecular techniques (RT-qPCR method) the lactobacilli were present in all investigated persons.

Some correlations between the species distribution and host blood biomarkers were found. The lower blood glucose level was associated with presence of *L. paracasei* adjusted for adults age group. The higher BMI in both groups of persons was directly predicted by the presence of OHOL and facultative heterofermentative *L. sakei* species (Štšepetova et al. 2011). According literature there was found a good agreement as positive correlation between the presence of *L. sakei* and also *L. reuteri* with BMI in a study of obese individuals (Million et al. 2013).

3.3 Elderly People

Elderly people (>65 years) are the fastest growing subpopulation in the world, needing more medical and social attention. Recent studies indicate shifts in the composition of the intestinal microbiota of the elderly host, which may lead to detrimental health effects. Increased numbers of facultative anaerobes have been reported in conjunction with a decrease in beneficial microbes such as the anaerobic lactobacilli and bifidobacteria, amongst other anaerobes (Tiihonen et al. 2010). These changes, along with a general reduction in species diversity in most bacterial groups and changes to diet and digestive physiology such as prolonged intestinal transit time, may result in increased putrefaction, collection of deteriorative metabolites in the colon, and resulting greater susceptibility to disease.

On the other hand, aging increases the viable count of *Lactobacillus*, with substantial changes in species prevalence (Štšepetova et al. 2011b). In elderly individuals, two types of *Lactobacillus* spp. colonization have been detected (Mikelsaar 1969; Mikelsaar et al. 2010). In some persons the counts of lactobacilli are significantly increased compared to other elderly persons, and the other elderly people were characterized by a lower abundance, similar to that of younger adults. This grouping could be detected both with culture methods and with qPCR with LAB-specific primers in different groups of elderly. We have tried to associate this finding with health biomarkers. In elderly people, the high counts of lactobacilli are in close correlation with lower values of one important oxidative stress marker, serum oxidized LDL (Mikelsaar

et al. 2010). Thus, the higher counts of lactobacilli of some elderly (shown in Fig. 6a) also are reflected in biomarkers of blood as being correlated with lower levels of atherogenic oxidized LDL (Mikelsaar et al. 2010). By molecular detection (Fig. 6b) the infants were colonized less with lactobacilli in comparison to children and elderly persons ($p < 0.001$, both respectively) (Štšepetova et al. 2014b).

Furthermore, it was possible to demonstrate (Fig. 7) that high levels of intestinal *Lactobacillus* spp. (Štšepetova et al. 2011b) were positively associated with increased concentrations of fecal SCFAs, lactate, and essential amino acids in fecal waters by ^{1}H-NMR –based metabonomics (Le Roy et al. 2015).

Nicholson et al. (2012) proposed that the majority of metabolites in human plasma are microbe-derived, which fits well with our finding.

It seems that the unique phylotypes and variable list and number of genera and species should be addressed for discovering the functional impact of different loads of *Lactobacillus* spp. This can be addressed in food trials, e.g., functional food interventions.

That the biodiversity of intestinal microbiota increases in parallel with aging (Tiihonen et al. 2010) can be confirmed in the example of *Lactobacillus* spp. in adults and elderly persons in our studies. The number of different species detected by species-specific PCR was lower in adults than elderly (6, 5–11, median vs. 4–12, 8; $p = 0.042$). Still, the most prevalent species in both age groups were *L. casei* and *L. ruminis* (Štšepetova et al. 2011b). This finding may have some important health impact considering the differences in the metabolic profile of these strains. The six species of lactobacilli show age-related differences: adults vs. elderly are more often colonized with *L. acidophilus* (79 % vs. 47 %; $p = 0.031$) and *L. helveticus* (66 % vs. 24 %). At the same time, *L. johnsonii* was detected only in elderly people (13.5 %; median age 68 y). In adults vs. elderly *L. plantarum* (59 % vs. 29 %; $p = 0.035$) and *L. paracasei* (97 % vs. 58 %, $p = 0.0002$) both are present in higher proportions.

Similarly, the bacterial diversity of microbiota increases with age in the three studied populations of the United States, American Indians, and Malawians in adults and elderly (Yatsunenko et al. 2012). Nevertheless, in US adults, the fecal microbiota was at least diverse compared with that found in developing countries, favoring the former to some modern lifestyle–derived diseases. The decreased taxonomic diversity of individuals in Western cultures raises concern about the maintenance of important microbial symbionts in the broader population that could provide health benefits and whether global trends in diet can result in the permanent loss/extinction of bacterial species.

The solution can be seen in maintaining culture collections from individuals from the developing world, or the agrarian cultures specifically may help to preserve potentially important components of the microbiota (Lozupone et al. 2012).

Conversely, the persistence of a specific strain that is moderately resistant to antibiotics can be found in early life, as illustrated by our studies of antibiotic-resistant commensal *Escherichia coli* in different age groups (Sepp et al. 2009). In antibiotic-naive children, a significantly higher frequency of integron-bearing strains and high minimum inhibitory concentration values was present compared to healthy elderly persons (53 % versus 17 %; $p < 0.01$). Recently, the same tendency was shown for lactobacilli from young children compared to adults (personal communication by Siiri Kõljalg, University of Tartu). This surprising finding could be explained by weaker colonization resistance of intestinal microbiota of young children against exogenous strains from mothers and environment. It would be interesting to test in healthy adults born in the 1960s (~55 years old in 2015) whether, because of the fitness cost, the resistant population would be suppressed or if largely resistant *E. coli* strains would appear that resemble those of young children in the 1990s. Furthermore, during the development of commensal intestinal microbiota, the strains without resistance probably have advantages in microbial competition in the intestinal micro-ecosystem

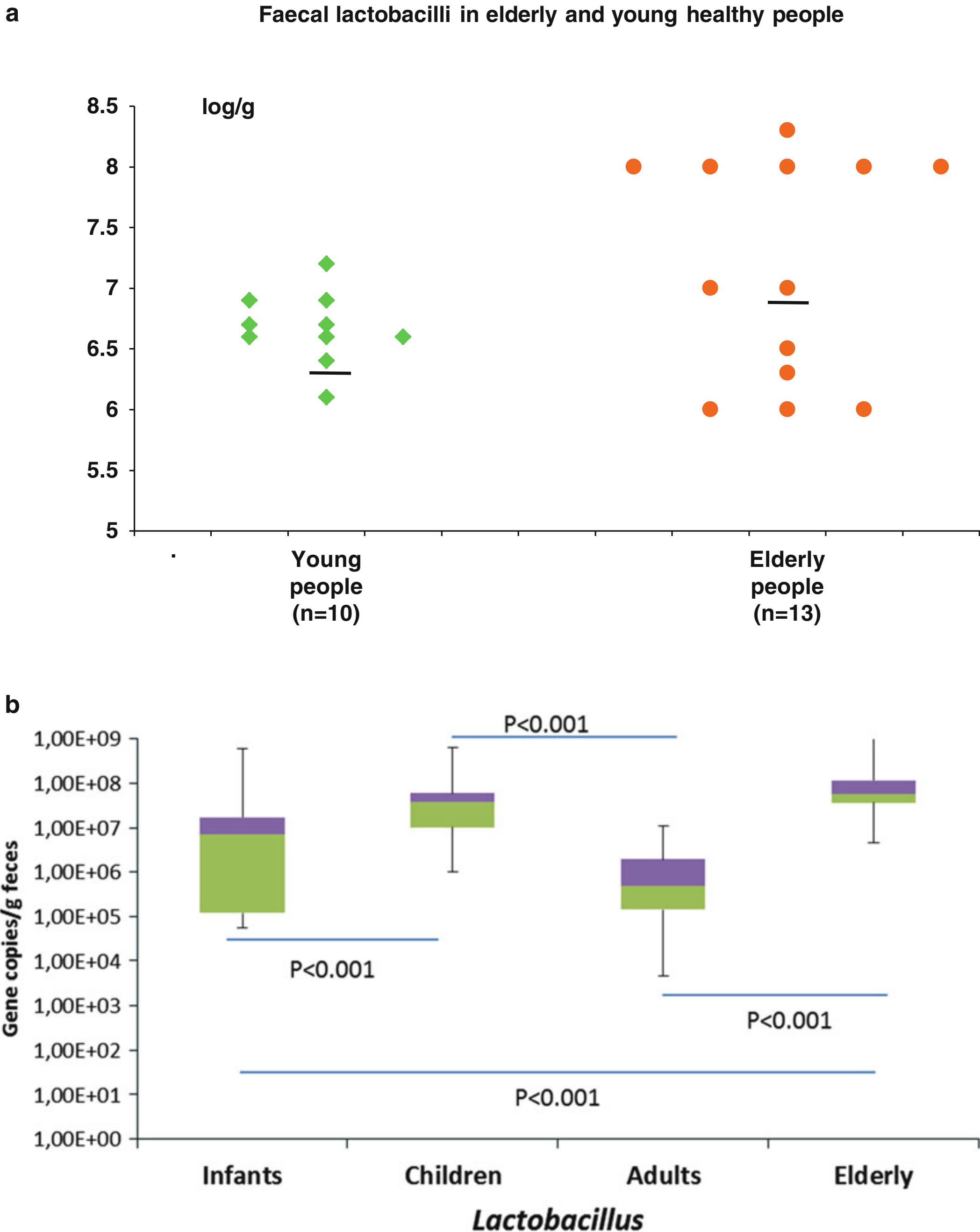


Fig. 6 (**a**) Intestinal *Lactobacillus* spp. counts in elderly persons (>65 years) as compared to adults detected by culture on MRS media (log CFU/g). (**b**) Gene copy number of total *Lactobacillus* spp. per gram of feces determined by real-time PCR in elderly people as compared to infants, children, and adults. Dot plots indicate max–min, median, and 1st and 3rd quartiles (Mikelsaar and Mändar 1993; Mikelsaar et al. 2010; Štšepetova et al. 2014b)
Legend: The differences in counts (gene copies/g feces) of lactobacilli found between all study groups. Significantly higher amounts were observed in elderly persons in comparison to adults ($p < 0.001$) with culture methods and molecular assessment

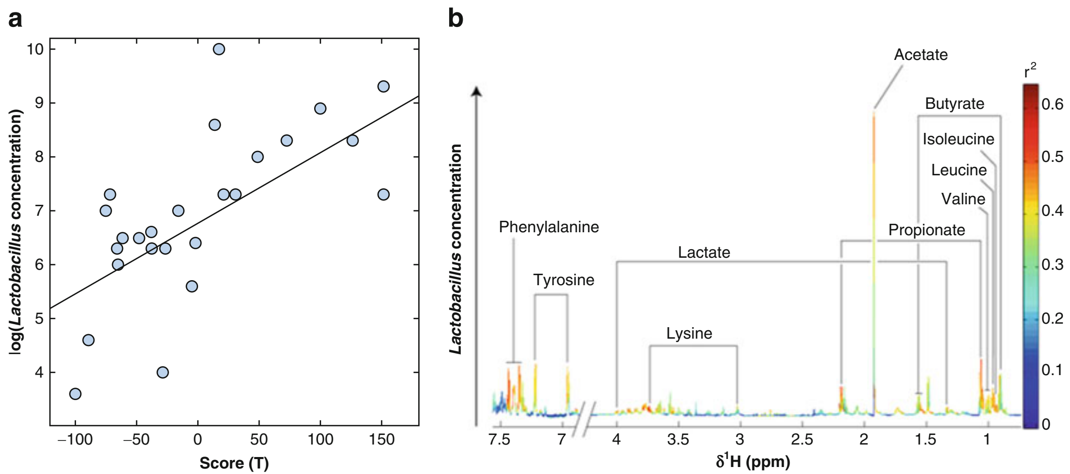


Fig. 7 High total count of *Lactobacillus* spp. is associated with higher fecal SCFAs, lactic acid, and amino acid levels. O-PLS score projection and loading plot of all individuals (Le Roy et al. 2015)
Legend: (**a**) O-PLS scores projection of 49 individuals according to the decimal logarithm of total *Lactobacillus* counts. This model is derived from 1H-NMR spectra of faecal waters using the log of total *Lactobacillus* counts as a response predictor. (**b**) Metabolic contribution of SCFAs, organic acids and amino acids to the same model (loadings plot). Metabolites pointing upwards are positively correlated with high *Lactobacillus* levels

and therefore the resistant ones may be absent in older people (Karami 2007).

3.4 Lactobacilli in Children of Different Geographic Areas

The environmental impact on putative differences in the diversity of intestinal bacteria, including *Lactobacillus* spp. of the *Firmicutes* phylum, has not been completely established. In the mid-1990s, we compared the lactoflora formation among residents of two socio-economically developed countries. Several differential tendencies were found: the prevalence of lactobacilli was different in Estonia (post-socialistic country with low income) and Sweden (well-industrialized country) during the first year of life (Fig. 8). The prevalence of lactobacilli was higher in Estonians than in their Swedish counterparts while the $p < 0.05$ significance value was reached at age 1 month and 1 year.

The same tendency could be seen for counts ($\log_{10}$ CFU/g) of lactobacilli in the two different countries (Table 4).

At the beginning of the twenty-first century, however, the abundance of intestinal lactobacilli of young children did not differ between Estonia and Sweden, in prevalence or in counts. This shift seemingly reflects the higher degree of industrialization and changes in lifestyle achieved during 10 years of independence of Estonia from the Soviet system. The increased income of the general population and improvement in food hygiene could have reduced the differences in the abundance of intestinal lactobacilli between the Estonian and Swedish infants (Voor 2005; Sepp et al. 2006).

Concerning the biodiversity of species of *Lactobacillus*, a similar trend for the higher variety was detected in Estonian as compared to Swedish infants in cross-sectional and prospective studies of healthy infants at the age of 1–2 years (born 1995–1996). In Estonians, ten species compared to only five different species of Swedish infants were detected. The study relied on cultivation, API 50 CHL, and ITS-PCR identification of lactobacilli. In both groups of children, the three main biochemically divergent groups were still present but individually, particular species

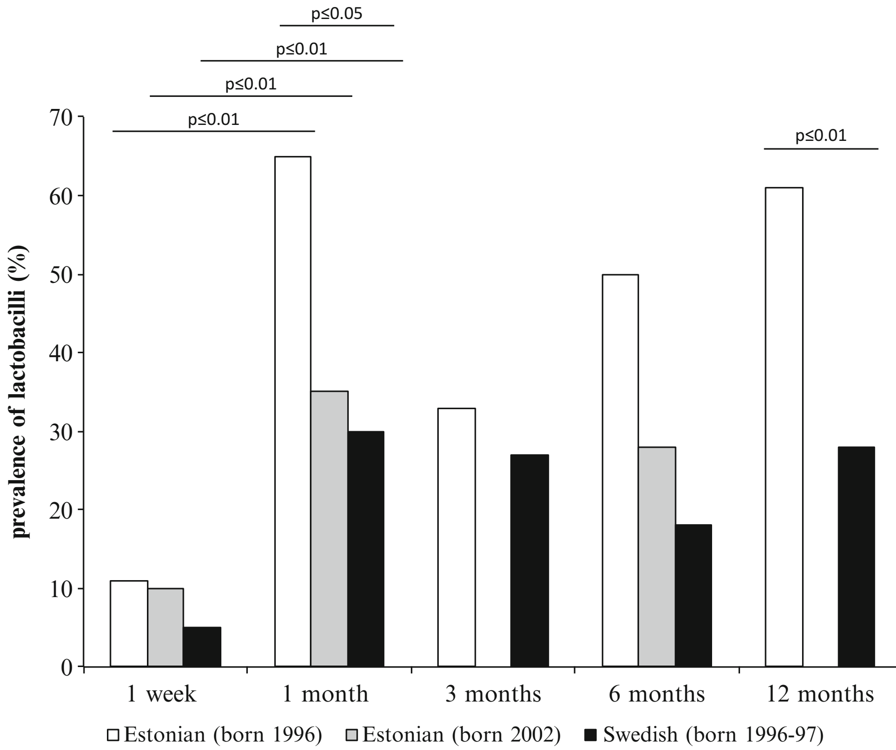


Fig. 8 Prevalence (%) of lactobacilli in Swedish children and Estonian children born in different years (1996 versus 2002) Legend: Colonization (%) with lactobacilli in Estonian and Swedish children during their first year of life (1 week, 1 month, 3, 6, and 12 months)

Table 4 The counts of intestinal lactobacilli in Estonian and Swedish children

	1 week		1 month		1 year	
	$\log_{10}$ CFU/g		$\log_{10}$ CFU/g		Log_{10} CFU/g	
	Estonian	Swedish	Estonian	Swedish	Estonian	Swedish
Lactobacilli	n = 20	n = 20	n = 20	n = 20	n = 27	n = 29
Median	<3[a]	<3	7.7[a]	<3	4.2[b]	<3[b]
Range	<3–10	<3–8.6	<3–10.8	<3–10.8	<3–10.3	<3–8.8

Source: Sepp et al. (1997, 2000)
Legend: [a]p = 0.01
[b]p = 0.001
p significance values comparing Estonians and Swedes at different ages

had different prevalences. In Estonian infants, *L. plantarum* prevailed whereas in Swedes, *L. paracasei* was the most prevalent species (Mikelsaar et al. 2002).

The difference between Estonian and Swedish children in number of detected species was also apparent at the 6th month of age, as confirmed by MALDI-TOF/Bruker identification methods

(https://www.bruker.com). In addition, the Swedish children (65 %) were more often colonized by *Lactobacillus rhamnosus* than Estonian (22 %) children ($p < 0.01$). Thus, the prevalence of *Lactobacillus* and its particular species is related to geographical location with its industrialization characteristics, birth years, and age (Bjorksten et al. 1999, 2001; Sepp et al. 2000; Mikelsaar et al. 2002; Penders et al. 2010).

3.5 Main Factors Driving the Individual Variety of Intestinal *Lactobacillus* sp.

3.5.1 Human Genotype in Determination of Individual Variety of Microbes

The individual variety in terms of abundance and functions of predominant persistent microbiota has gained large attention. To date, the specialized seminal colonizers (microbiota "core" of operational taxonomic units (OTU) by HITChip) have been found independent of delivery mode and lactation stage (Turnbaugh et al. 2007). Correlations among OTUs, metabolites, and OTUs-metabolites revealed that metabolic profiles were associated with early microbial ecological dynamics, maturation of milk components, and host physiology. One possibility to find dependence of the individual specificity of microbiota from host genotype is the use of monozygotic twin model.

We provide our own and literature data to illustrate the results of similarity e.g. selectivity of microbiota of twins obtained nearly 30 years back: first on culture-dependent level, further some 15 years later on PCR specific molecular detection level and up to date on metagenomics and metabolomics levels. At the beginning of eighties we have demonstrated that the quantitative composition of fecal cultivable microbiota of 12 pairs of healthy adult monozygotic twins had the same degree of similarity as that of the paired samples of a single healthy person (Mikelsaar et al. 1984). Additionally, in the Estonian twin pairs also the significant correlation ($r = 0.711$; $n = 29$; $p < 0.001$) between the very specific and individually stable composition of bacterial metabolites *e.g.*, excretion of urinary phenols, was assessed (Siigur et al. 1991; Mikelsaar and Mändar 1993). Later, with advanced molecular methods the similarity indices of microbiota of genotypically identical pairs were demonstrated (Zoetendal et al. 1998 2001, 2006; Vaughan et al. 2005).

The study group of Gordon's laboratory in USA confirmed the previous findings showing that intrapersonal variation of fecal microbiomes and metatranscriptomes of healthy twin pairs during repeated sampling for 16 weeks was not significantly different from interpersonal variation but occurred completely different from unrelated pairs (McNulty et al. 2011). Later several studies of twins have confirmed the concordance of microbiome and its functions (Tims et al. 2012; Bondia-Pons et al. 2014). Concluding the historic survey, we state that the comparison of microbiota in twin co-pairs indicates that factors related to the host genotype have one of the most important effects on determining the intestinal bacterial composition that are also reflected in host metabolism. However, never mind the relative stability of microbiota, the phenotypic flexibility of host metabolism can deem the host genetic makeup and environmental relations (van Ommen et al. 2014).

3.6 Ante-, Intra- and Postnatal Factors for Individual Persistent Colonization

The proved stability of the human gut microbiota suggests that in an individual most strains can be long-term residents. Faith and coauthors (Faith et al. 2013) have shown that nearly 60 % of individual strains of intestinal tract are remaining over the course of 5 years. Particularly stable components were members of *Bacteroidetes* and *Actinobacteria*, inversely correlated within the first days of life (Jost et al. 2012). Still, the

unsolved problem is the detection of the time point of selective colonization of the neonate with specific individual strains. The data from the literature have identified the mode of delivery, mother's diet, hygiene, microbiota, and standard of living as driving factors for individual variety of intestinal *Lactobacillus* spp.

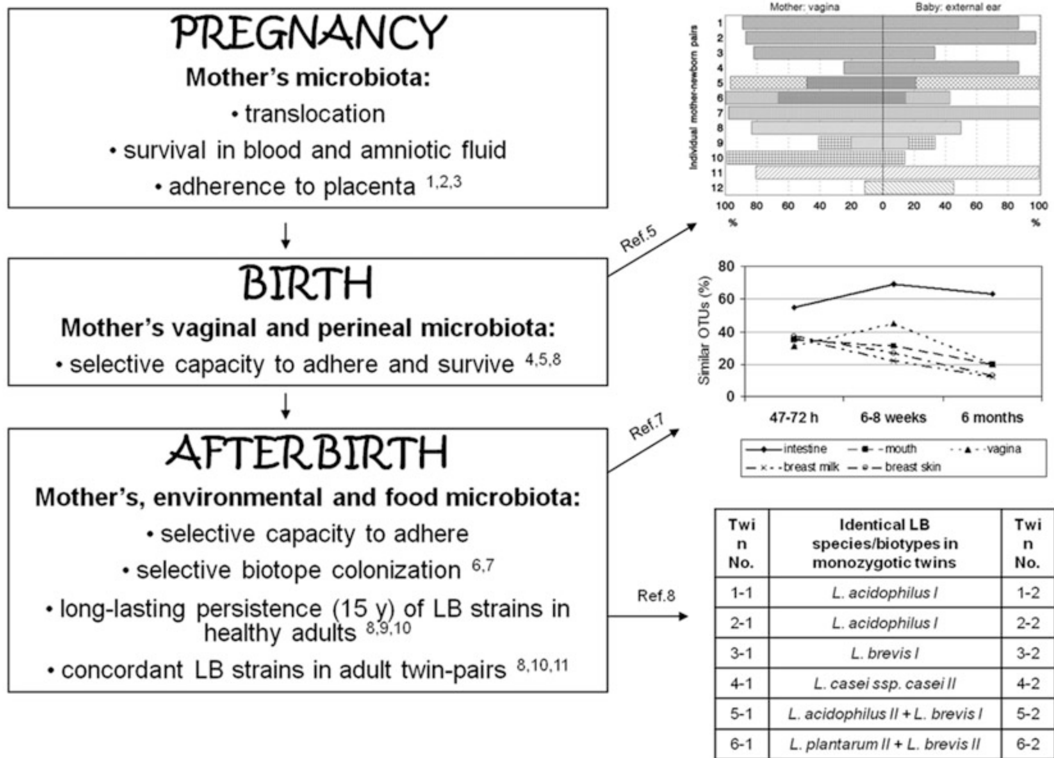
For centuries, the fetus was thought to be in a sterile *in utero* environment with the normal lactic acid bacteria of the genital tract of healthy women offering protection. The specific microbes of intestinal or vaginal microbiota of mothers located on skin and perinea have been considered the main predictors of microbiota formation and changes from newborn age to infancy (Mandar and Mikelsaar 1996). During the twenty-first century, advances in understanding early host–microbe interactions have, however, indicated that the early microbial programming begins during the fetal period and is substantially modulated by mode of birth, perinatal antibiotics, and breastfeeding. Unexpectedly, the traces of microbes were detectable in the feto-placental unit and infant meconium from healthy, term pregnancies (Satokari et al. 2009; Steel et al. 2005; Rautava et al. 2012; Mshvildadze et al. 2010). The presence of bacterial DNA – most often belonging to the common gut bacteria *Lactobacillus* spp. and *Bifidobacterium* spp. – was detected in all placentas and 43 % of amniotic fluid samples obtained after 29 sterile elective caesarean section deliveries at term without signs of infection, rupture of membranes, or onset of labor (Rautava et al. 2005).

These discoveries bring us back to the idea of antenatal development of individually specific lactoflora during pregnancy (Mikelsaar and Mändar 1993). In 1986, the hypothesis on antenatal development of individually specific lactoflora was experimentally set: a particular strain of *L. fermentum*^{ResStr}, administered to mice together with food in the last days of gestation, could colonize the offspring. This strain vanished quickly from the mother's feces but could be found in the offspring during four generations (Mikelsaar et al. 1977; Mikelsaar 1986; Mikelsaar and Mändar 1993). For human babies, some other authors reported the very first data on early controlled colonization through mothers with specific strains, e.g. *Escherichia coli* Nissle (Borderon et al. 1981; Lodinova-Zadnikova et al. 1992; Schulze and Sonnenborn 1995; Lodinova-Zadnikova and Sonnenborn 1997). Finnish scientists have confirmed the persistent colonization of human colonic mucosa by a probiotic strain LGG after oral consumption (Alander et al. 1999). Later in the 2000s, (Schultz et al. 2004) tried to apply the antenatal colonization for prevention of atopy by a *Lactobacillus rhamnosus* GG probiotic strain. In six women, the LGG was consumed in late pregnancy and stopped after delivery. At birth, the strain was present in fecal samples of 5/6 infants, and some of them remained colonized even for 12 months, yet none of the mothers was colonized after 1 month.

Individual lactoflora means the persistence of individually different lactobacilli strains in relatively stable quantities in a particular biotope of the host. Several of our human studies provide supportive evidence. Among monozygotic twins, for example, in six pairs out of ten, we found strains of the same species and biotype: *L. acidophilus* I, *L. casei* ssp. *casei* II and *L. brevis* I. From two pairs even the similar combinations of lactobacilli strains were isolated (Mikelsaar and Lencner 1982). Moreover, in 12 pairs of twins the biochemical activity of microbiota (amounts of urinary phenols) was in high concordance ($r = 0.711$; $n = 29$; $p < 0.001$) (Mikelsaar et al. 1998).

In a survey over a long 15 years period (years 1965, 1971 and 1979) of nine healthy adult persons, in addition to the stability of the number of lactobacilli in feces, the stable persistence of *Lactobacillus* spp. composition was also identified (Mikelsaar and Lencner 1982; Mikelsaar et al. 2004). Particularly, one or two *Lactobacillus* species occurred repeatedly, and in four persons, we could isolate the same species and biotype even during three estimations.

Similarly, the biological isolation of healthy persons during special training or



Twin No.	Identical LB species/biotypes in monozygotic twins	Twin No.
1-1	*L. acidophilus I*	1-2
2-1	*L. acidophilus I*	2-2
3-1	*L. brevis I*	3-2
4-1	*L. casei ssp. casei II*	4-2
5-1	*L. acidophilus II + L. brevis I*	5-2
6-1	*L. plantarum II + L. brevis II*	6-2

Fig. 9 Hypothesis on formation of individual lactoflora: persistence of individually different lactobacilli strains/biotypes with relatively stable quantities in the particular biotope of the host
Legend: During **PREGNANCY** the antenatal selective contamination of the fetus by the mother's lactoflora, possibly via translocation (1, 2), the ability of these strains to survive the bacteriocide impact of amniotic fluid, their selective capacity to adhere to placenta (3). At **BIRTH** the selective contamination of the fetus by the mother's microbiota, including lactobacilli, the ability of these strains to survive the bactericide impact of amniotic fluid; their selective capacity to adhere to newborn's ear skin (4, 5). **AFTER BIRTH** postnatally colonize particular mucosal surfaces (6, 7), enabling persistence of specific *Lactobacillus* spp. strains/biotypes detected in adults during 15 years survey (8, 9, 10) and monozygotic twins (10, 11). This could be possible by induction of tolerance toward themselves
Source: *1* (Fernandes et al. 2013), *2* (Moles et al. 2013), *3* (Aagaard et al. 2014), *4* (Mikelsaar et al. 1989), *5* (Mandar and Mikelsaar 1996), *6* (Gosalbes et al. 2013), *7* (Drell In publication), *8* (Mikelsaar and Lencner 1982), *9* (Mikelsaar et al. 1984), *10* (Mikelsaar and Mändar 1993), *11* (Mikelsaar et al. 1998)

space flights of astronauts caused some shifts to opportunistic microorganisms, but the close physical contact and flight stress could not eliminate the individual specificity of their lactoflora in terms of bacterial species (Lentsner et al. 1981). In the chapters about lactobacilli in human ecosystems we have postulated that the selective colonization of a biotope by lactobacilli and its composition (counts and prevalence) are determined during early infancy and kept during life, are a function of the host as well as of the microorganism (Mikelsaar and Mändar 1993).

In our laboratory, we have compared the transfer of maternal microbiota of the perineum (fecal, vaginal, skin origin) to the newborn, investigating the anterior ear of the newborn just after birth (Fig. 9). This area can preserve the amniotic fluid and is not contaminated with the hands of the obstetrician. The high concordance between the microbiota abundance of maternal perineal and newborn ear samples was

detected in the first prospective study. The most common microorganisms were lactobacilli, epidermic staphylococci and non-haemolytic streptococci but not all mother‘s perineal microbes colonized the newborn (Mikelsaar and Lencner 1982; Mikelsaar et al. 2004). However, after douching with chlorhexidine solution (0.005 %) the vaginal lactobacilli could not be found in newborn’s ear samples (Mandar and Mikelsaar 1996).

The transmission of maternal microbiota to the newborn also has been demonstrated by Tannock (Tannock 1990) by applying advanced plasmid profiling studies to transmitted bacteria and their putative original strains. Gosalbes et al. (2013) showed that the meconium microbiota have an intrauterine origin, participating in gut colonization and having large consequences for child health. That group used high-throughput pyrosequencing of the 16S rRNA gene for comparison of the meconium bacteria in 20 term Spanish newborns, fecal samples of seven pregnant women some days before delivery, and infant samples spanning the first 7 months of life. Some meconium strains even remained in the gut until 7 months of age and could lay the basis for gut colonization. The meconium microbiota resembled that of fecal samples from young infants yet differed from those in adult feces, vagina, and skin. The mechanism could involve the bacterial translocation from gut to lymphatic nodes and blood in pregnant women (Fernandes et al. 2013; Moles et al. 2013). A unique placental microbiome niche, composed of nonpathogenic commensal microbiota, was described recently (Aagaard et al. 2014).

In our recent prospective study (Fig. 9) we investigated the similarity (%) of mother- baby microbiomes in seven mothers and their nine babies (including two pairs of twins) at three time points – 2–3 days, 6–8 weeks and 6 months after delivery, applying next generation sequencing (Drell et al. 2016, submitted). We revealed that though the microbiomes were individually different there was present the similarity between mother and her baby. It is important to note that the similarity of baby’s intestinal tract microbiome with mother’s different microbiomes was moderate some days after birth but the intestinal similarity increased significantly in the course of time whereas the rate of other similarities (mouth, breast skin and vagina) decreased. Thus, the gradual formation of individual intestinal microbiota tends to be highly biotope-specific. Different confounding variables including environment, diet, and host genotype intervene into this process.

The persistence of the first colonizers of the neonate can be illustrated by the spread of particular *Lactobacillus rhamnosus* GG probiotic strain. In Estonian infants, up to the age of 6 months (born 1996–1998), the rare colonization with species *L. rhamnosus* (21 %) was found (Mikelsaar et al. 2002). These prevalence values did not differ in adults: In Estonian adults born at least in the 1970s and investigated in the 2000s with a mean age of 27 years, the intestinal *L. rhamnosus* species was still present in only 20 % of adults (Štšepetova et al. 2011b). Similarly, in our recent probiotic kefir trial with *L. fermentum* ME-3 (Mikelsaar et al. 2015) in adults (mean age 49.6 years, born ~ 1960s), out of 116 samples at baseline levels, only 16 (13.8 %) were positive for *L. rhamnosus*. The reason lies in the fact that in adults born before the start (at 1999 in Estonia) of marketing of the probiotic strain *Lactobacillus rhamnosus* GG (Valio OY), the species *L. rhamnosus* could not be a frequent intestinal colonizer. These data support our hypothesis that the random colonization with commensal species at infancy determines the resident microbiota later in life. Furthermore, the dependence of LGG colonization on spread of the strain in the community can be illustrated in the Finnish population. In Finland the marketing of LGG (Gefilus™, Valio OY) started from the first years of 1990s. Consequently, from 1992–1993, no young children harbored LGG, a particular strain belonging to the species *L. rhamnosus* (Kaila et al. 1998). Hence, in a

study of Finnish children (born in 1995–2003), the prevalence of probiotic *L. rhamnosus* GG showed a substantial increase. In blood sera of 94 % of infants at age 12 months, the prevalence of *L. rhamnosus* detected by immunoglobulin G antibody–reactive protein bands (58 and 65 kDa) was the highest (Talja et al. 2014). The important and interesting issue of selective colonization of the host gut by different *Lactobacillus* species still needs new experimental and clinical data, especially in frame of possible tolerance induction to first colonizers of newborn.

4 Beneficial Potential of *Lactobacillus* spp. on Host Physiological Functions

An imbalance of microbiota, expressed as a disturbance of host physiological functions, e.g., dysbiosis, is characterized by low species and gene numbers of beneficial intestinal microbiota and subsequently a reduced number of interacting balancing metabolites. This state is clearly the opposite of a "diversity of balanced" microbiota. Korpela et al. (Korpela et al. 2016) recently confirmed that the depletion of some components of microbiota caused by antibiotic use in early life has disrupted normal gut microbiota development, which is associated with development of allergy later in life.

In host defense, for reconstruction of the balanced microbiota, the beneficial bacteria of the gut microbial ecosystem have been considered to:

1. maintain and promote perturbed colonization resistance for fighting pathogens;
2. maintain human metabolic functions by absorbed bacterial metabolites; and
3. modulate innate resistance and attenuate chronic low-grade inflammation.

The increase in *Bifidobacterium* and *Lactobacillus* spp. abundance in the gut usually correlates well with numerous beneficial effects such as a reduction in the risk of enteric infections, dysbiosis, and metabolic and immune-mediated diseases (Floch 2011).

Using the stepwise collected and registered health impacts by beneficial bacteria of microbial ecosystem, the probiotic approach was developed. The regulations of the Food and Agriculture Organisation and World Health Organization (FAO/WHO 2002) have defined the principles and recommendations for a claim of a probiotic. A probiotic was defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The expert commission of the International Scientific Association for Probiotics and Prebiotics has retained the FAO/WHO definition in principle (Hill et al. 2014).

The origin of the strain (biotope/host specificity), its colonizing ability, and its safety and efficacy serve as the basic criteria for defining suitable candidates for probiotics.

Currently, according to well-designed clinical trials, systematic reviews, and meta-analysis, the efficacy of probiotics has been addressed as a general class (Hill et al. 2014). The main contribution of probiotics is to a healthy gut microbiota or/and nutrition benefit. The support of probiotics for a healthy immune system has also been acknowledged by several authors as a core benefit while considering the specificity of strains with differential immune pro- or anti-inflammatory action (Hill et al. 2014).

Concerning the general class of the probiotic bacteria with core functions, we illustrate several mechanisms of action. By these mechanisms, such as balancing intestinal microbiota, modulating the immune system, and exerting metabolic influences (dark pink, Fig. 10), several probiotics of different genera and species may help to maintain the colonization resistance of the gut against invasion of pathogens, ensure or activate the immune system, and modulate the epithelial barrier function and host metabolism with its products, thus participating in creating healthy intestinal microbiota and improving digestion and regulation of peristalsis (Mikelsaar et al. 2011b; Lozupone et al. 2012; Patel and Denning 2013; Sanders et al. 2013).

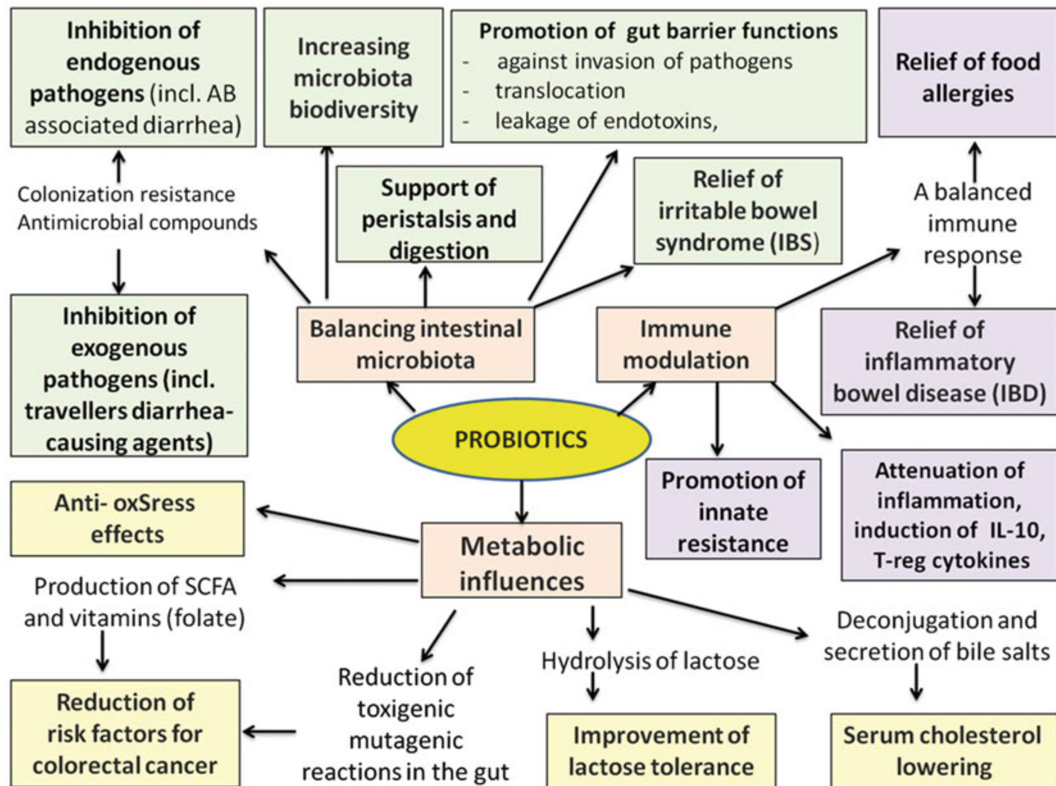


Fig. 10 Mechanisms of action of probiotics
Source: Neish et al. (2000); Hooper et al. (2001); Teitelbaum and Walker (2002); Mack and Lebel (2004); Sartor (2004); Kumar et al. (2007); Lin et al. (2008); Khailova et al. (2009); Patel et al. (2012a); Patel and Denning (2013)

At the cellular level (Fig. 10), probiotics have a number of important activities:

1. Attenuation of NF-κB activation, a major proinflammatory pathway (Jones et al. 2012): Recent data suggest that bacterial products, in the absence of viable organisms, may have similar effects on signaling pathways (Li et al. 2009) and a barrier function (Patel et al. 2012a). These bacterial products can be characterized as postbiotics or metabiotics that express biologic activity inside the host (Shenderov and Midtvedt 2014). The principal metabiotics are SCFAs but also other substances like polyamines (putrescine, spermidine, spermine) (Larque et al. 2007). Metabiotics are beneficial in promoting a healthy GI tract by creating an environment that is most favorable to probiotics, through nourishing enterocytes, reinforcing mucosal barrier function, maintaining or supporting epithelial integrity, or signaling the immune system to limit inflammatory responses both in the gut and through influencing T cells throughout the body.
2. Upregulation of cytoprotective genes (Hooper et al. 2001; Lin et al. 2008).
3. Prevention of apoptosis and cell death (Lin et al. 2008; Khailova et al. 2010).
4. Immunological mechanisms: stimulation of specific antibody-secreting cell response (Kaila et al. 1992), enhancement of pathogen phagocytosis (Schiffrin et al. 1997), and modification of cytokine production (Miettinen et al. 1996; Truusalu et al. 2010).
5. Induction of the expression of tight junction proteins necessary for barrier function (Khailova et al. 2009; Patel et al. 2012a).
6. Generation of reactive species that are important in cell signaling (Kumar et al. 2007; Lin et al. 2009; Jones et al. 2012).

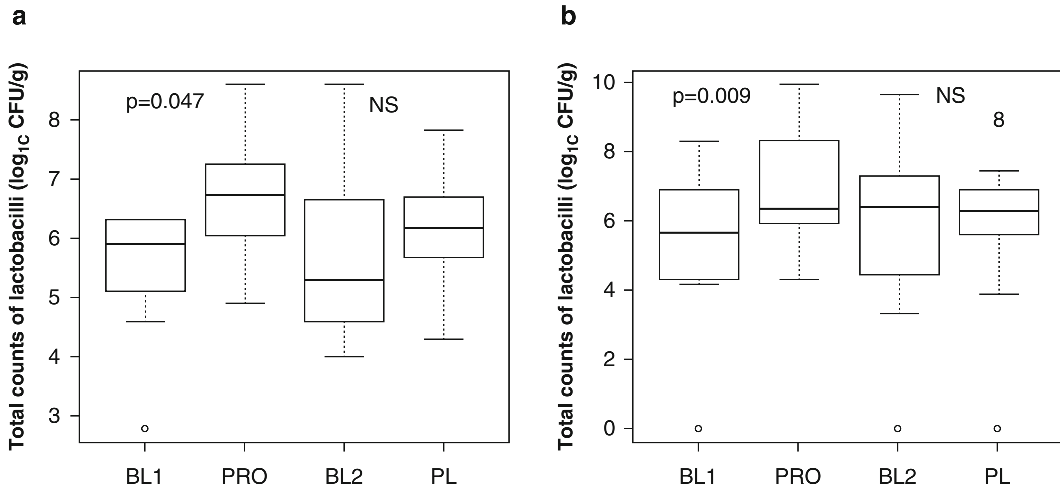


Fig. 11 Increase in total fecal lactobacilli during studies of the application of probiotic *L. plantarum* Tensia to (**a**) healthy adults; (**b**) elderly persons (Songisepp et al. 2012a)
Legend: BL 1 = baseline 1, at recruitment; PRO = after the probiotic treatment; BL 2 = baseline 2, after washout; PL = after the control treatment. The transverse line within the box indicates the median value; the bars extending from each box represent the 25th and 75th percentiles; and the open circles represent outliers

Probiotics comprise special compounds or metabolites of beneficial bacteria of the gut that can express specific health-maintaining or -improving properties. With laboratory studies, experimental studies, and translational research, however, several benefits of probiotics arise from a particular strain-specific action (Gibson 1998; Mikelsaar et al. 2011b; Million and Raoult 2013). The specific functions of probiotics addressed for particular health-improving physiological properties have gained increasing attention in the last century. Selection of *Lactobacillus* spp. strains with the potential to modulate host biomarkers is reviewed in paragraph 5.2.

4.1 Host Specificity (Mice, Humans) of Probiotics for Abundance of Intestinal Indigenous Lactobacilli

For understanding the impact of probiotic bacteria on the composition of gut microbiota, it is important to rely on their colonization ability and the possibility of increasing the abundance of lactobacilli. The question arises of whether the choice should be a strain of human origin or some environmental good antagonist. In various experimental settings (oral dose 0.5×10^8 CFU for 15 days), *Lactobacillus fermentum* ME-3 (DSM14241) of human origin did not increase counts of total lactobacilli in the distal ileum (Truusalu et al. 2010) or large intestine of mice (Truusalu et al. 2004). In contrast, the use of the same strain for 3 weeks with a daily dose 10×10^{10} CFU with fermented goat milk or a daily dose 2×10^9 CFU with a capsule caused a significant increase in intestinal lactobacilli in healthy volunteers (Songisepp et al. 2005).

A similar host distinction was seen with another intestinal species, *Lactobacillus plantarum*. The semihard Edam-type cheese comprising strain Tensia (DSM 21380) originated from another Estonian child and was fed for 30 consecutive days (dose 50 g, total 9.6 $\log_{10}$ CFU) to experimental mice. No statistically significant increase was found in the total count of cultivable lactobacilli either in the small or large intestine of mice when compared with the control group. Conversely, a significant increase was found in fecal lactobacilli counts (Fig. 11) in both groups of healthy adults and elderly individuals (DBPC crossover studies) after consuming a similar probiotic cheese with Tensia in a daily dose of 50 g, total 10.6 and 8.2 $\log_{10}$ CFU, respectively (Songisepp et al. 2012a).

Table 5 Prevalence of *L. fermentum* species in fecal samples after consumption of the probiotic strain *L. fermentum* - ME-3 in different doses and formulations

Trial characteristics	Dose per day and duration	Baseline *Lactobacillus* species CFU log/g mean ± SD	After consumption *Lactobacillus* species CFU log/g mean ± SD	Baseline prevalence % *L. fermentum*	After consumption % *L. fermentum*
Fermented goat milk trial	11.0 log_{10}CFU	6.03 ± 2.4	8.0 ± 7.5	4/16	16/16*
	3 weeks			25 %	100 %
Capsule trial	9.4 log_{10}CFU	7.0 ± 7.5	7.9 ± 8.0	2/12	4/12
	3 weeks			16.7 %	33.3 %

Legend: *In the goat milk trial, the presence of the *L. fermentum* ME-3 strain in feces of all participants after consumption was assessed by AP-PCR typing (Songisepp et al. 2005). In both trials, a large standard deviation (SD) was seen because of the minimal values of lactobacilli counts at baseline for some persons

Different tested *Lactobacillus* strains originating from the intestinal tract of Estonian children (Mikelsaar et al. 2002) (http://eemb.ut.ee) increased the abundance of human lactobacilli of feces in contrast to the experimental mice studies. Thus, settlement of the microbial ecosystem of the gut is largely granted by the host specificity for the beneficial *Lactobacillus* strain applied.

In our experiments, the amount of probiotic and the formulation applied did not seem to have selective value for increasing lactobacilli counts if daily microbial dose reached at least 10^9 CFU. In the literature, probiotic effects have been described as having a dosage threshold. The minimum effective dose that influences the intestinal environment and provides beneficial effects for human health is considered to be $>10^9$ live microbial cells per day. However, some authors state that the minimum dose depends on the particular strain and the type of foodstuffs (Reid et al. 2006; Williams 2010; Champagne et al. 2011). Thus, in case of two probiotic strains of human origin (*L. fermentum* ME-3 and *L. plantarum* Tensia), their ingestion caused a significant increase in the total count of fecal LAB of healthy volunteers in comparison with the initial count. The increase was almost the same in spite of the probiotic formulation or daily dose.

Furthermore, when the human origin strain *L. fermentum* ME-3 was applied for volunteers, the prevalence of *L. fermentum* as a species was increased from 25 to 100 % in the goat milk trial. In the capsule trial, however, the increase was more mild (Table 5) and accompanied by a smaller improvement in antioxidative biomarkers such as total antioxidative activity (TAA) and total antioxidative status (TAS), as was seen in the fermented goat milk study (Songisepp et al. 2005). Together with the increased prevalence of *L. fermentum*, some other lactobacilli species such as *L. brevis*, *L. buchneri*, and *L. acidophilus* were found in the GI tracts of the volunteers. Thus, the consumption of human-origin probiotic caused an increase in the diversity of *Lactobacillus* spp., without dominance of the consumed probiotic.

The results of our study of the response for both probiotic bacteria in adults measuring the counts of lactobacilli with RT-qPCR were not anticipated, however. No change in the intestinal abundance of intestinal lactobacilli was detected either in healthy adults consuming kefir containing *L. fermentum* ME-3 (8 weeks, daily dose 200 ml, total 8×10^9 CFU/day) or in overweight or adipose persons using cheese containing *L. plantarum* Tensia (3 weeks, daily dose 50 g, total 6×10^{10} CFU/day). Still, in both trials, after consumption, the probiotic bacteria were molecularly confirmed in 62 % and 64 % volunteers, respectively (Sharafedtinov et al. 2013; Mikelsaar et al. 2015). The absence of increase of lactobacilli counts may lie in the methods of estimation: the DNA of dead bacteria is present both at the start and completion of the study. The other possibility may be that the small intestine could be a location for propagation of the introduced strain yet the strain remains elusive for testing in clinical trials. A similar negative result for an increase in lactobacilli counts by RT-qPCR in volunteers after probiotic introduction was described by McNulty

et al. (McNulty et al. 2011). In their elegant translational study, they showed an improved metabolism with some other groups of intestinal microbes, but exceptionally not for the species of the administered probiotic.

Consequently, in clinical trials, to avoid efficacy failures due to registering dead bacteria, the metabolomic approach can reveal an increase in low-molecular-weight compounds such as amino acids, sugars, lipids, and some other organic compounds of microbial origin induced by probiotic consumption.

4.2 Interaction Between Probiotic *Lactobacillus* spp. with Some Other Groups of Intestinal Indigenous Microbiota

Mutual interactions take place between a probiotic strain and the host's indigenous microbiota in the gut. The suppression of normal microbiota has been feared for a long time. However, it has now been accepted that ingestion of a certain probiotic causes beneficial changes in the fecal flora by increasing the total number of a particular genus of probiotic such as the lactobacilli, but also of some other symbionts, such as bifidobacteria or enterococci (Sepp et al. 2013; Alander et al. 1999; Brigidi et al. 2001; Cesana 2001).

More than 20 years ago, in a study with newborns, the probiotic *Lactobacillus rhamnosus* GG, in addition to introduced lactobacilli, also boosted the abundance of some other groups of microbiota (Sepp et al. 1993). LGG was administered as a freeze-dried powder during the first 2 weeks of life. We compared the balance of microbiota of meconium and the fecal samples of neonates at 3–4, 6–7, and 28–31 days of age. In meconium, the counts of coliforms and bifidobacteria outnumbered those of bacteroides, but lactobacilli were seldom present (11 %). Yet, at 3–4 days after administration of *L. rhamnosus* GG, the prevalence of lactobacilli increased to 87 %. It is worth mentioning that in the GG group, the total counts of lactobacilli outnumbered those of the control group and that at the same time, the newborns were also more intensively colonized with bifidobacteria. Thus, the danger of profound microbiota distortion by probiotic administration to early newborns was not realized. The large quantities of administered probiotic did not outcompete the indigenous microbiota of the newborns but in a proportional manner instead increased the populations of some beneficial bacteria, including lactobacilli and bifidobacteria. Other authors later described similar trends (Goossens et al. 2003; Wind et al. 2010).

Recently, the popular probiotic *Lactobacillus rhamnosus* GG was confirmed to work as a 'facilitator' that modifies and promotes the activity of the other gut bacteria (Eloe-Fadrosh et al. 2015). A metagenomics analysis of 300,000 sequences from bacterial rRNA genes showed that beneficial bacteria substantially modified the resident microbiota within nondiseased individuals. Increased expression of *Bacteroides*, *Eubacteria*, *Faecalibacterium*, *Bifidobacterium*, and *Streptococcus* strains was identified. Concerning the immunological impact of probiotics, a study of a Finnish–Spanish–Dutch population (Ganguli et al. 2015) supported a beneficial role of probiotic administration for immune responses of the fetal gut.

Thus, beneficial strains of lactobacilli can support correction of an impaired balance of the intestinal microbiome, enriching the intentionally consumed bacteria and some other members of the microbiota. This effect is seemingly the result of the metabolic and immunologic interactions taking place between the strain used and the affected group of indigenous microbiota in the appropriate host. The metabolic outcome, depending on host genotype, the microbial genetic and phenotypic metabolite profile, and epigenetic influence from the environment, has great flexibility in homeostasis that is not fully understood.

5 Potential of *Lactobacillus* spp. Probiotic Strains for Health

5.1 Elaboration of a Lactobacillus Strain into a Probiotic Product

Development of a microbial strain into a probiotic product is a stepwise process in which host and

biotope specificity and several other issues must be checked. The putative probiotic strain should be accurately phenotypically characterized and genetically identified (Vankerckhoven et al. 2008). The specific functional properties and the ability for temporal colonization (bile, gastric acid tolerance, and adhesive properties) should be confirmed (Saarela et al. 2000; Koll et al. 2010). Recent recommendations include an absence of hemolytic activity and transferable antibiotic resistance of the selected *Lactobacillus* strain, whereas the safety should be proven in animal models (FAO/WHO 2002; Vesterlund et al. 2007; Koll et al. 2010).

Next, pilot clinical trials on healthy volunteers are needed to exclude adverse effects on gut health and biochemical and cellular indices of the blood (Reid and Hammond 2005; Wells and Mercenier 2008; Rijkers et al. 2010). Furthermore, only after improving some physiological functions (e.g., antimicrobial, metabolic, immunogenic, antioxidative) of the host or by reducing the risk of some diseases after consumption of the probiotic product can the expression of the functional properties of the strain be tested in large groups of volunteers.

The genera of *Lactobacillus* and *Bifidobacterium* are "generally regarded as safe" (GRAS status according to the US Food and Drug Administration) due to their long history of safe use in fermented foods and their presence in the normal intestinal and urogenital microbiota of humans. Several lactobacilli species, including *L. plantarum* and *L. fermentum*, have received a Qualified Presumption of Safety (known as QPS) status from the European Food Safety Authority (EFSA). Still, in rare cases, some lactobacilli species could cause clinical conditions like bacteremia and endocarditis (Snydman 2008). Therefore, screening of new probiotics should include a proper safety assessment, starting from antibiotic susceptibility estimations.

Antibiotic Susceptibility

In a study of Estonian and Swedish 1–2-year-old children, 60 intestinal lactobacilli were tested for susceptibility to different antimicrobials (Mandar et al. 2001). Most of the strains appeared to be resistant to two or three antibiotics out of nine (Fig. 12). No differences between the Estonian and Swedish isolates were found.

The most important feature for safety assurance of putative probiotics is the absence of transmissible antibiotic-resistance plasmids containing transferable antibiotic-resistance

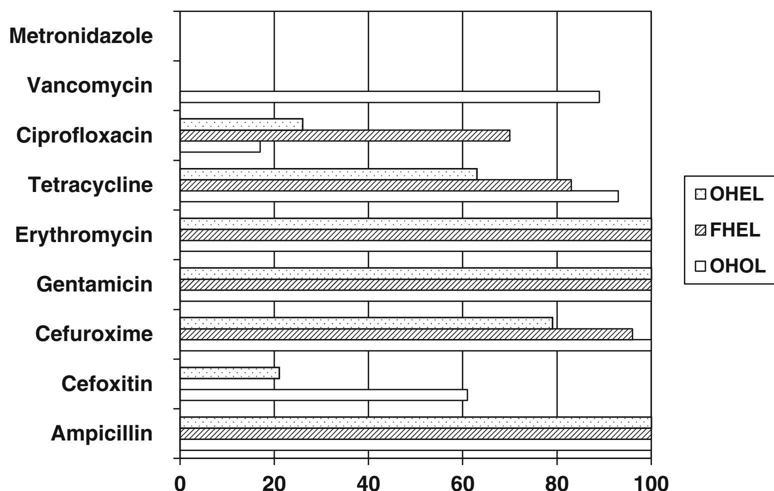


Fig. 12 Antibiotic susceptibility of lactobacilli by fermentation group
Legend: Prevalence (%) of susceptible strains is presented. The breakpoints (MIC/ml) were determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines for Gram-positive microorganisms as follows: ciprofloxacin (4 μg/ml); erythromycin (8 μg/ml); ampicillin, gentamicin, and tetracycline (16 μg/ml); cefoxitin, cefuroxime, vancomycin, and metronidazole (32 μg/ml)

SURVIVORS

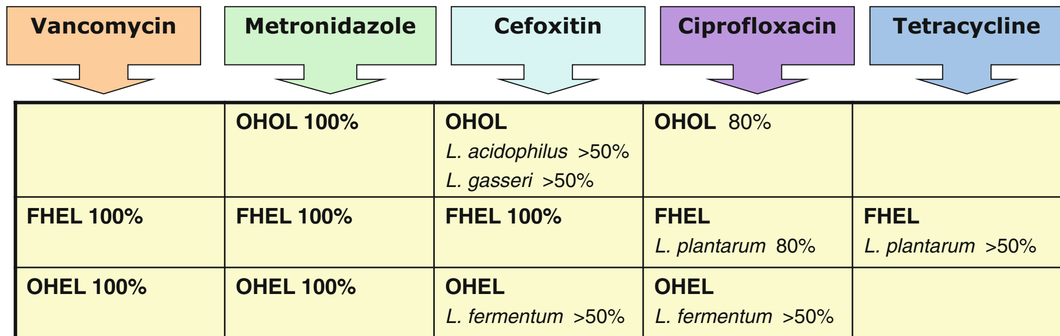


Fig. 13 Schematic view of proportions of different *Lactobacillus* species (Mikelsaar 2011) surviving after antimicrobial treatment with therapeutic doses for selective decontamination (Solomkin et al. 2010). The circles and antibiotic names are matched by color

genes (Mathur and Singh 2005). The comparison of a wild strain's susceptibility profile with the applicable *Lactobacillus* strain of the same species is the clue for confirming their natural resistance and absence of transferable resistance (Vankerckhoven et al. 2008).

In a previously reported complex safety study (Koll et al. 2010), we screened a pre-selected six lactobacilli strains for several antibiotics, including inhibitors of cell wall, protein, and nucleic acid synthesis. Plasmid-encoded erythromycin, tetracycline, and chloramphenicol resistance was reported in lactobacilli (Gueimonde et al. 2013). However, we observed no resistance to erythromycin and chloramphenicol, and only one *L. buchneri* strain was resistant to tetracycline; it was excluded from further experiments. In the case of *L. fermentum* species, a natural resistance to trimethoprim plus sulfamethoxazole, metronidazole, fluoroquinolones, and cefoxitin was interpreted as a high natural resistance to these antibiotics.

In critically ill patients, the prophylactic selective decontamination of the digestive tract simultaneously involves applying three to four different antimicrobials (vancomycin, cefoxitin, ciprofloxacin, and some new tetracyclines; also the combination of metronidazole with cephalosporins or fluoroquinolones) for the suppression of potentially pathogenic microorganisms (Solomkin et al. 2010). We have shown (Fig. 12) that *Lactobacillus* species strains are all non-susceptible to metronidazole while specific antibiotic susceptibility pattern is characteristic for different species of lactobacilli of the three fermentation groups. The simultaneous colonization of humans with several (4–12) *Lactobacillus* species expressing variable intrinsic resistance (Fig. 13) to the aforementioned antimicrobials (Štšepetova et al. 2011b) offers the possibility of preserving the colonization of the intestine with particular naturally resistant species of indigenous lactobacilli during antimicrobial treatment (Mikelsaar 2011).

In addition, in the gut, the most abundant bacteria, such as the *Firmicutes* and the *Bacteroidetes*, by production of large-scale bacteriocins seem to ensure some antibiotic activity and participate in permanent intestinal host defense against the proliferation of harmful bacteria (Drissi et al. 2015). Moreover, several probiotic products with *Lactobacillus* spp. strains that are intrinsically resistant to the above-mentioned antimicrobials can be used for balance of intestinal microbiota during antibacterial therapy.

Furthermore, we illustrate schematically (Fig. 14) the complex safety studies on the six putative *Lactobacillus* spp. strains from the

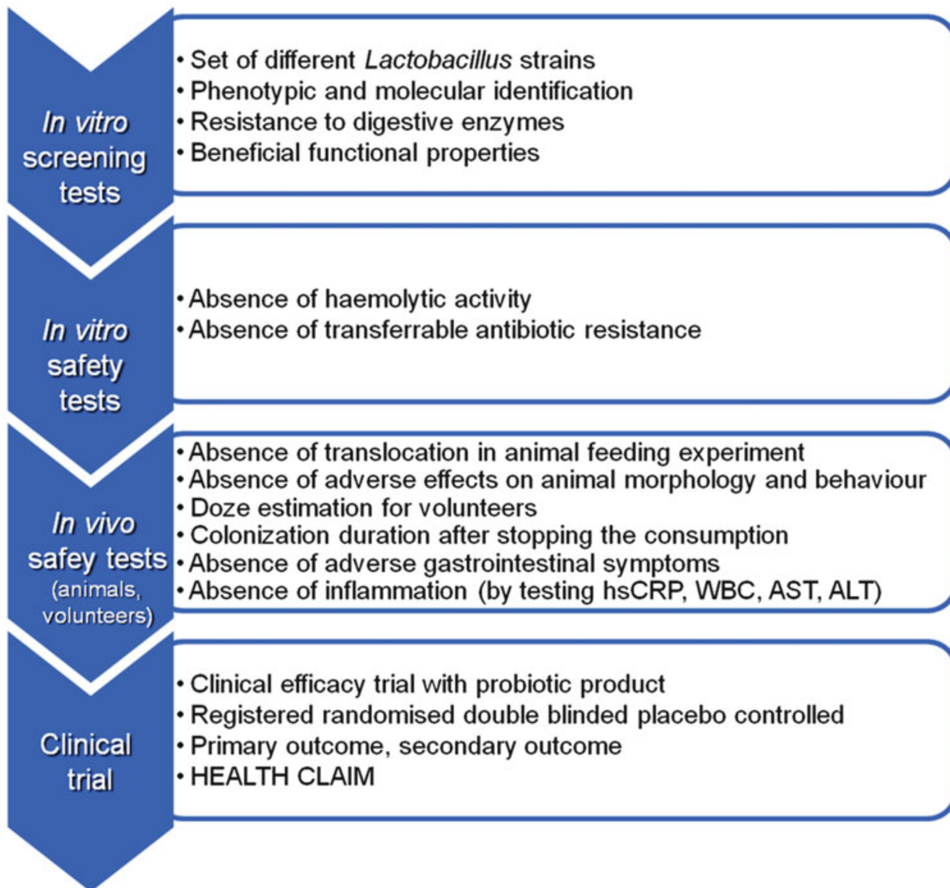


Fig. 14 Development of a safe and efficacious probiotic product
Legend: The step-by-step testing was performed including *in vitro* assays, experimental animal studies, a volunteer safety trial, and a registered clinical efficacy trial, including ethics committee approval, randomization and double blinding of participants for placebo and treatment, defining primary and secondary outcomes for efficacy confirmation, and drawing the preliminary health claim for EFSA acceptance

Human Microbiota Biobank (Acronym: HUMB registration number: 977) (Koll et al. 2010). No strain caused the lysis of erythrocytes of human blood, and in an animal trial, no translocation into blood and organs of mice was detected in the case of five strains. Still, one of the two *L. paracasei* strains translocated into the spleen of one mouse and was excluded from further development.

Next, five of these strains of different species were fed for 5 days (each daily dose 1×10^{10} CFU) to nine volunteers. The regulatory guidelines prescribe the dose delivered at a level of 1×10^{9} CFU per serving; thus, a tenfold larger dose (5×10^{10} CFU) of the putative probiotic product was tested for safety reasons. The persistence of *Lactobacillus* strains was assessed by culturing combined with arbitrarily primed PCR (AP-PCR) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) from fecal samples on consecutive days. Only two strains persisted for 10 days in detectable numbers. In the second phase of the study, five additional volunteers consumed the selected strain *L. acidophilus* 821–3 (daily 1×10^{10} CFU) for five consecutive days. Using RT-qPCR, the

strain was subsequently found in the feces of all individuals on day 10 (range 4.6–6.7; median 6.0 $\log_{10}$ cell/g) (Hutt et al. 2011). Thus, the colonizing ability of human-origin strains can be evaluated with a defined dose. More important, the administration of high doses of different *Lactobacillus* strains did not result in any severe adverse effects in the GI tract and/or abnormal values of blood indices. No specific functional property that could be biotechnologically applicable for human health improvement has been detected, however, among the five promising colonizers of humans with applying the scheme without detection of special functional properties of the best strain. Thus, without doubt, the colonizing properties and safety traits are critically necessary for application of every probiotic strain. To achieve particular health effects, though, the probiotic strain should have some specific beneficial properties that can influence the host physiology, metabolism, or immunology and that are expressed in specific host biomarkers.

5.2 Selection of Lactobacillus spp. Strains for Functional Properties

For application of probiotics, their functional properties, usually checked by improved values of human health biomarkers, must be characterized (FAO/WHO 2002). It should be kept in mind that high heterogeneity in metabolic properties exists among species and strains. Although not all strains even of human origin possess beneficial properties, numerous strains should be screened to obtain an ideal probiotic for special host function.

Recently, a functional score was suggested by Guidone et al. (Guidone et al. 2014) to simplify the simultaneous screening of several microbial isolates from different ecological niches for the potential of probiotic applications. However, the usual colonizing and safety aspects were unfortunately intertwined with particular functional properties that should be addressed in specific health claims.

The elucidation of functional properties for health maintenance and fortification requires an understanding of the risk for a borderline healthy individual for particular pathogenesis of the disease. This understanding enables testing for the potential to correct shifts in human blood, urine, and feces health biomarkers using the functional properties of lactobacilli. These properties can be found by molecular assessment of the genetic profile of the strain (whole genome profile), followed by testing the expression of specific genes and by conducting phenotypic/physiological experiments using *in vitro* tests, animal models, population surveys, and clinical trials (Sanders and Huis in't Veld 1999). For instance, to reveal the potential for reduction of cholesterol content in blood sera by a probiotic strain, the presence of bile salt hydrolase (BSH) has to be tested either in genome or/and *in vitro* experiments and the impact assessed by clinical trials (Mikelsaar et al. 2011a; Songisepp et al. 2012b; Mikelsaar et al. 2015). The specific functional impact of particular strains has been confirmed. Some *Lactobacillus* probiotic strains, e.g., *Lactobacillus acidophilus*, *L. casei*, and *L. rhamnosus*, each induce differential gene-regulatory transcriptional networks and pathways in the human mucosa (van Baarlen et al. 2011).

Milk contains a list of proteins, including some similar to the opioids, blood pressure–-lowering peptides and antithrombotic and cognitive function–improving compounds, normally in low quantities (Meisel and Bockelmann 1999). Certainly, human and animal milk serves as a most suitable environment for producing similar compounds by lactobacilli.

We have elaborated some functional properties among our collected LAB strains to be applied for different physiological states of the host. The special *Lactobacillus* strains and their functional properties with their impact on the host are depicted in Fig. 15. These strains can be used for the three main physiological axes of the host organism: defense against infection, metabolic impact, and immune modulation, all bound to a beneficial effect of LAB.

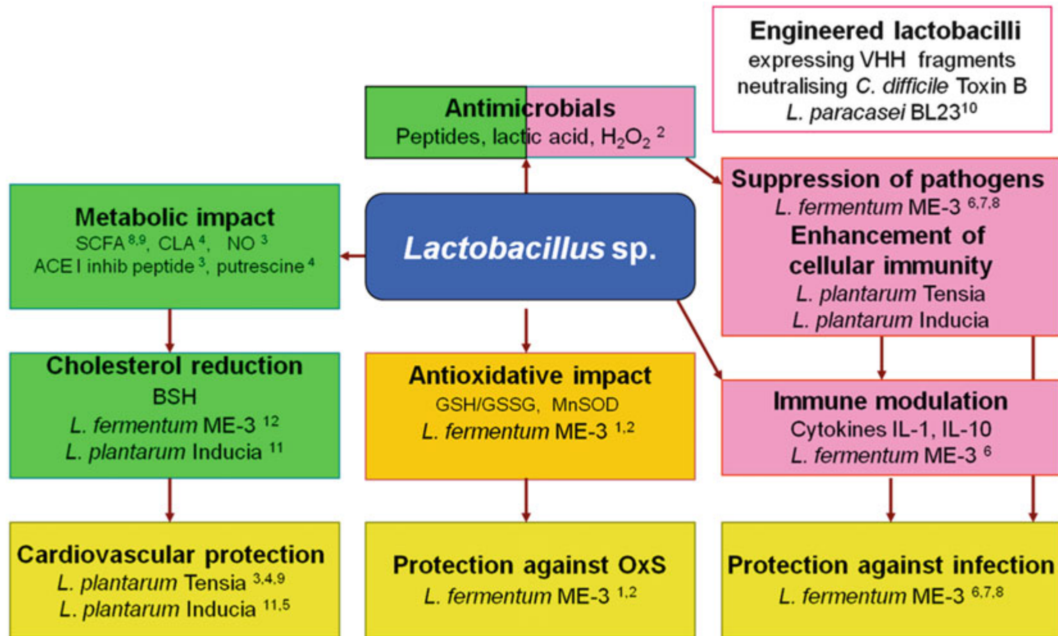


Fig. 15 LAB produced bioactive compounds with potential impact for health
Legend: *SCFA* short chain fatty acids, *CLA* conjugated linoleic acid, *NO* nitric oxide, *BSH* bile salt hydrolase, *ACE I inhib* angiotensin I-converting enzyme inhibitory peptides, *OxS* oxidative stress, *GSH/GSSG* ratio of reduced and oxidized glutathione, *MnSOD* manganese-dependent superoxide dismutase, H_2O_2 hydrogen peroxide, *IL* interleukin
References: *1* (Kullisaar et al. 2002), *2* (Kullisaar et al. 2010a), *3* (Hutt et al. 2015), *4* (Songisepp et al. 2012a), *5* (Mikelsaar et al. 2014), *6* (Truusalu et al. 2010), *7* (Truusalu et al. 2008), *8* (Annuk et al. 2003), *9* (Štšepetova et al. 2011a), *10* (Andersen et al. 2015), *11* (Mikelsaar et al. 2014), *12* (Mikelsaar et al. 2015)

5.3 Role of Environment in Expression of Functional Properties of Probiotics

The environment has recently been intensively studied for its role in the expression of different functional properties of bacteria. The differences in aeration, nutrition, and metabolic co-metabolites may promote or suppress the genetically defined properties of *Lactobacillus* spp. and some other genera by epigenetic reprogramming (Shenderov 2012; Kumar et al. 2014; Remely et al. 2014).

The lactobacilli bioactive compounds suppressing pathogens, affecting antioxidativity of host tissues, granting immune protection, and affecting metabolic impact on blood sera indices are tightly bound with different environmental influences. Below, we describe the results of testing some of these properties in different environmental conditions.

5.3.1 Suppression of Pathogens

One of the most frequent health claims for probiotics concerns the putative reduction and prevention of infectious disease in the GI tract. Although probiotics are targeted to healthy populations, the effects on prevention and alleviating the infectious diseases have to be tested in models of specific infection. The effect of probiotic strains depends on their ability to survive during passage through the stomach, as well as on their ability to persist and compete with pathogens in the GI tract.

L. fermentum ME-3 can suppress mainly Gram-negative bacteria but to some extent also enterococci and *Staphylococcus aureus*. In different environmental conditions (MRS plates cultivated in microaerobic and anaerobic milieu), the production of lactic acid by strain ME-3 correlates well with its antagonistic activity. In her PhD dissertation, Heidi Annuk (2002) showed that the *in vitro* antagonistic activity

resulting from a pH drop and organic acid (lactic, acetic, and succinic acids) and ethanol production was differentially quite characteristic for particular fermentative groups of lactobacilli (homo-, facultatively heterofermentative, and obligately heterofermentative) further identified by ITS-PCR.

The strain ME-3 produces some cationic peptides, has a suitable lectin profile for competitive adhesion to the epithelium (Annuk et al. 2001), and some immunogenic properties, as assessed in animal experiments (Truusalu et al. 2008; 2010). In addition, we recently found using a ROS analyzer (APOLLO 4000) that the ratio of H_2O_2:NO signals was 13.7, produced by strain ME-3 in microaerobic MRS medium, achieving the first rank among about 30 tested strains of *Lactobacillus* species. This result shows that strain ME-3 can manage with compounds both to suppress antagonists and/or initiate signaling using several pathways.

Reactive species (ROS, RNS, H_2O_2, OH, O*) are produced by body tissues and fluids. Principal ROS are superoxide radical, hydroxyl radical, lipid peroxyl radical, and non-radical hydrogen peroxide (the latter is produced from superoxide by superoxide dismutase (SOD)). The RNS are NO and non-radical peroxynitrite. Recent data on microbiota and gut epithelia cell signaling have demonstrated that enteric commensal bacteria rapidly generate reactive species, including ROS. Although the induced generation of ROS via stimulation of formyl peptide receptors is a cardinal feature of the cellular response of phagocytes to pathogenic or commensal bacteria, evidence is accumulating that ROS are also similarly elicited in other cell types, including intestinal epithelia. Additionally, ROS serve as critical second messengers in multiple signal-transduction pathways stimulated by proinflammatory cytokines and growth factors. This physiologically generated ROS participates in cellular signaling via the rapid and transient oxidative inactivation of a defined class of sensor proteins bearing oxidant-sensitive thiol groups. These proteins include tyrosine phosphatases that serve as regulators of MAP kinase pathways and cytoskeletal dynamics, as well as components involved in control of ubiquitination-mediated NF-κB activation. Microbial-elicited ROS mediate increased cellular proliferation and motility and modulate innate immune signaling. Certainly, for any upregulating physiological mechanism, there can be hypothesized a counterbalancing pathway, in this case antioxidative measures. These results demonstrate how enteric microbiota influence regulatory networks of the mammalian intestinal epithelia (Jones et al. 2012). The oxidative and antioxidative potential of *Lactobacillus* spp. will be discussed further in association with the review of our specific trials.

Enteric pathogens cause pathological lesions in different atmospheric conditions of the host GI tract, leading to diarrheal disease. *Salmonella* spp. and *Clostridium difficile* trigger inflammation in the ileum and colon while *Shigella* spp. clearly prefers the colonic mucosa (Huang and DuPont 2005; Pegues et al. 2005). In addition, the colon has been considered the main reservoir of *Escherichia coli* strains that cause urinary tract infections (Franz and Horl 1999). Thus, it has been postulated that the antagonistic activity of probiotic *Lactobacillus* strains and *Bifidobacterium* spp. depends on the environmental growth conditions, e.g., aerobic/anaerobic conditions (Jacobsen et al. 1999; Annuk et al. 2003).

Therefore, it is important when working with probiotic bacteria to check their functional properties in conditions where they are expected to provide the health-improving effect, e.g., antagonistic suppression of pathogens. In laboratories of the Department of Microbiology of University of Tartu (UT), some original and commercial lactobacilli strains with high antagonistic activity against enteric pathogens (*Salmonella enteritidis*, *Shigella* spp., *Helicobacter pylori*, *Clostridium difficile*), based on the production of high amounts of SCFAs, ethyl alcohol, and antimicrobial peptides, have been tested (Annuk et al. 2003; Naaber et al. 2004; Rätsep et al. 2014). The content and types of organic acids produced during the fermentation process depend on the species of lactobacilli, culture composition, and growth conditions (Lindgren

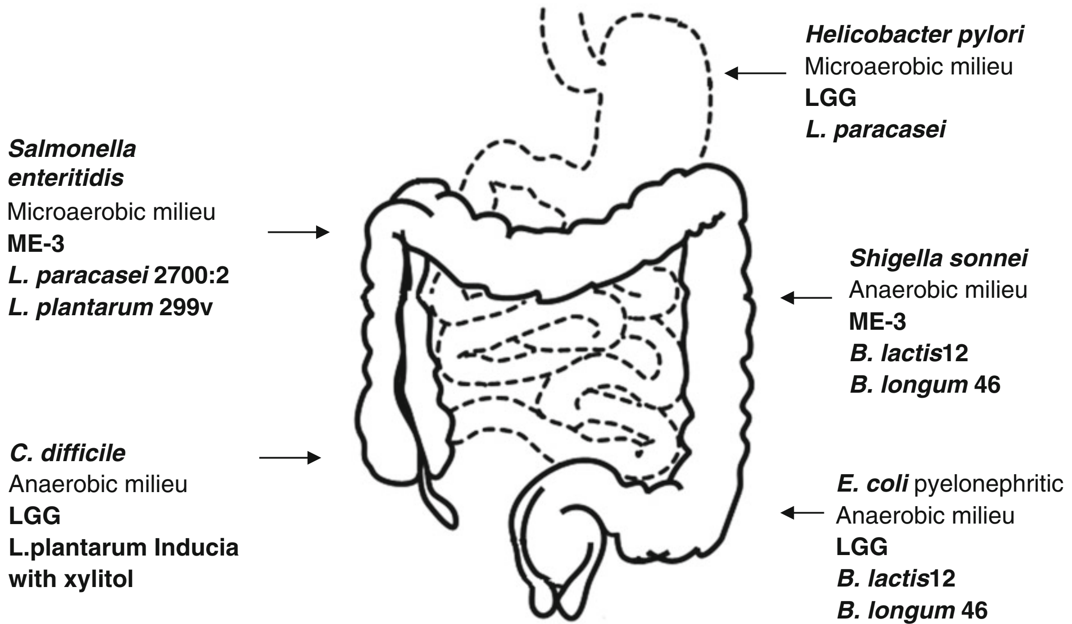


Fig. 16 Probiotic antagonistic activity against target pathogens according to the level of aeration (microaerobic, anaerobic) in the gut (Hutt et al. 2006; Mikelsaar et al. 2014)
Legend: High antagonistic activity, decrease by 5.9–6.5 $\log_{10}$ CFU ml^{-1}. Probiotic bacteria *L. rhamnosus* GG, *L. fermentum* ME-3, *L. paracasei* 2700:2, *Bifidobacterium lactis* B12, and *Bifidobacterium longum* 46. Pathogens: *Escherichia coli* ATCC 700336, *Escherichia coli* ATCC 700414, *Salmonella enterica* subspecies *enterica* ATCC 13076, *Shigella sonnei* ATCC 2593, *Clostridium difficile* VPI 10463 (ATCC 43255), and *Helicobacter pylori* NCTC 11637

and Dobrogosz 1990). In an anaerobic environment, much more ethanol and succinic acids are produced. There is a positive correlation between the production of lactic acid and the inhibitory activity of lactobacilli after cultivation under microaerobic conditions; unexpectedly, the amount of acetic acid and the inhibitory activity of lactobacilli cultured under anaerobic conditions are negatively correlated, however. Bacterial growth inhibition due to lactic acid can be explained by efficient leakage of hydrogen ions across the cell membrane, causing acidification of the cytoplasm and dissipation of the pH gradient (Blom and Mortvedt 1991).

Probiotic potential differs concerning suppression of recurrent cystitis- or pyelonephritis-causing *E. coli* strains, which both usually reside in the anaerobic environment of the colon (Fig. 16). The pyelonephritic *E. coli* strains are highly suppressed by three probiotics: *L. rhamnosus* GG and both bifidobacteria (*Bifidobacterium lactis* B12 and *Bifidobacterium longum* 46). However, the tested probiotics seemed to possess only intermediate potency for outcompeting cystitis-causing *E. coli* from the large intestine. Reid et al. (2003) reported that some strains from the aforementioned species – *L. rhamnosus* GR-1 and *Lact. fermentum* (reassigned for *L. reuteri*) RC-14 – are useful for preventing and treating urogenital infections in women. This finding confirms once more the specificity of action of a particular probiotic strain.

5.3.2 Antioxidativity against Ox-stress

Antioxidativity against ox-stress. An antimicrobial strain of *L. fermentum* ME-3 (DSM 14241) (Mikelsaar et al. 2002; Kullisaar et al. 2003; Kullisaar et al. 2010a) can reduce GSSG for GSH and express antioxidative Mn–SOD and capture oxidative superradicals (Kullisaar et al. 2010a), as has been elaborated in different

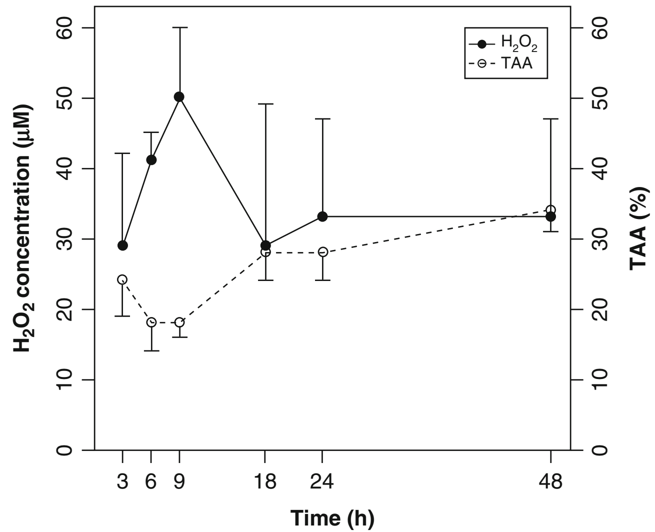


Fig. 17 H_2O_2 production (μM) and total antioxidative activity (TAA, %) in *Lactobacillus fermentum* ME-3 (Kullisaar et al. 2010b)
Legend: Growth tested in MRS broth for 48 h at 37 °C in microaerobic conditions. Hydrogen peroxide (H_2O_2 μM/L) and TAA % were expressed differentially during different growth periods of the bacteria

conditions. An *in vitro* experiment showed that H_2O_2 as a typical oxidative compound was produced by the probiotic strain *L. fermentum* ME-3 during the exponential growth phase and that the total antioxidative activity was somewhat delayed to the stationary growth phase (Fig. 17). These two opposing oxidative and antioxidative parameters showed a significant negative correlation ($p < 0.05$), predicting their interaction also in the host.

Microaerobic H_2O_2-producing lactobacilli, in contrast to strictly anaerobic bacteria, are not killed by H_2O_2 or O_2 and thus can colonize aerobic pockets in close contact with the intestinal epithelium (Berstad et al. 2015). In these conditions, the antioxidative activity of ME-3 seemingly preserves bacteria for an excess of oxygen to achieve better survival.

In the alimentary tract, the redox potential, which is positive in the proximal sections of the small intestine, drops to negative in the large bowel. Through the length of the gut, the microbes can use several electron acceptors beside O_2, including nitrate, nitrite, thiocyanate, sulfate, and trimethylaminoxide. The redox system thus promotes different microbiota in the small and large intestines. It also helps in regulating intestinal permeability, immune defense, gene expression, wound healing, and stem cell proliferation (Remely et al. 2014).

The particular *Lactobacillus fermentum* strain therefore has the potential to kill pathogens invading luminal cavities and mucosal surfaces because of its oxidative properties. On the other hand, the same strain can express high antioxidative potential according to the growth cycle, seemingly depending on the changes in redox potential and exhaustion of nutrients in the environment.

Recently, the concept of oxidative stress (oxS) has been advanced as "a disruption of redox signaling and control" (Jones et al. 2012). It emphasizes the impact of oxidative degradation of lipids (lipid hydroperoxide; LPO) during oxS and an impaired redox ratio of glutathione. In LPO, the free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. The most notable initiators in living cells are ROS such as OH·and HO_2, which combine with a hydrogen atom to make water and a fatty acid radical (Jones 2006). Thus, consumption of the multivalent probiotic *L. fermentum* ME-3, which produces Mn–SOD and GSH, contributes to the reduction of LPO in the epithelia of the GI tract (Truusalu et al. 2004; Kullisaar et al. 2010b) and in hepatocytes and prevents them from entering the circulation. This effect may lead to an improvement of the systemic picture of oxS in the host.

The expression of genes important for probiotic-linked mechanisms (*quorum sensing*) has been revealed by whole genome transcriptional profiling using varying growth conditions (pH, bile, carbohydrates) and food matrices (Klaenhammer et al. 2008). It is anticipated that probiotic strains could tolerate the highly acidic conditions present in the stomach and the variable concentrations of intestinal juices along the GI tract. Confirmation can been obtained when, after oral administration, the survival of the probiotic strain inside the gut is detected by fecal recovery of the strain and the imbalance of intestinal microflora is corrected (Songisepp et al. 2005).

Immune enhancement by functional properties of lactobacilli can be demonstrated with animal experiments. In experimental animal models of infections, some probiotic strains of lactobacilli (*L. fermentum* ME-3, *L. plantarum* Inducia) cause the enlargement of Peyer's patches and increase the number of lymphocytes and mono- and polymorphonuclears with induction of inflammatory (IFN-γ, TNF-α) and anti-inflammatory cytokine (IL-10) in the gut mucosa and organs (Truusalu et al. 2010; Truusalu 2013). This capacity serves as an important functional property of the special strain that is targeted to control of infection and correction of dysbiosis after antibiotic treatment.

In *Salmonella*-challenged mice, we have tested the ability of *L. fermentum* ME-3 to kill salmonella by ROS production and conversely to express antioxidative potential for influencing the course of infection. In this model, the phagocytes produce ROS, which are important for killing the pathogen. The excessive ROS damage the collateral intestinal epithelial cells, and the superoxide compound is responsible for generating granulomatous lesions and limiting the spread of infection (Umezawa 1995). In our work, administration of antimicrobial and antioxidative probiotic *L. fermentum* ME-3 to infected mice reduced the high number of *Salmonella* Typhimurium in ileum mucosa and reduced LPO, and no typhoid nodules were detected in the probiotic group with ME-3. This result could be explained by the improved antioxidative status of ileal mucosa thanks to the neutralization of the produced superoxides by the strain's SOD (Kullisaar et al. 2003; Truusalu et al. 2004).

The intertwined oxidative and antioxidative effects on formation of granulomas were demonstrated in experimental mice challenged with *S*. Typhimurium and treated with ofloxacin and the probiotic *L. fermentum* ME-3. In gut mucosa of *Salmonella*-challenged mice, the high ratio of GSSG/GSH (oxidized glutathione/reduced glutathione) was decreased (31 %) with application of the probiotic ME-3 strain, with remarkable antioxidative activity (TAA 48.2 ± 2 %). In liver, the number of typhoid granulomas was subsequently lowered (from 80 % in *S*. Typhimurium to 15 %) after application of ofloxacin and ME-3. Moreover, a decrease in TNF-alpha and increase in the anti-inflammatory cytokine IL-10 in the ileal mucosa and liver were detected (Truusalu et al. 2008, 2010).

5.4 Principles of Clinical Trials for Probiotic Efficacy

Probiotics are targeted toward improvement and/or maintenance of host physiological reactions or reduction in disease risk in the healthy population. To evaluate the potential of probiotic strains with defined functional properties, clinical trials for suppression of some pathogenetic factors of particular diseases need to be conducted.

In atherosclerosis, common risk markers like LDL cholesterol, HDL cholesterol, fasting triglycerides, and plasma homocysteine with additional oxS- and inflammation-related indices (oxLDL, 8-isoprostanes, and high-sensitivity C-reactive protein (hs-CRP)) are the main players. OxS indices (oxLDL, urine 8-isoprostanes, etc.) together with increased inflammatory markers (white blood cells and hs-CRP) are characteristic for patients with atherosclerotic lesions of the vascular system (Stocker and Keaney 2004). All of these markers are considered to be age- and diet-related (Mensink et al. 2003; Tiihonen et al. 2010).

For maintaining cardiovascular health, the assessed functional properties of *L. fermentum* ME-3 with different formulations were tested in

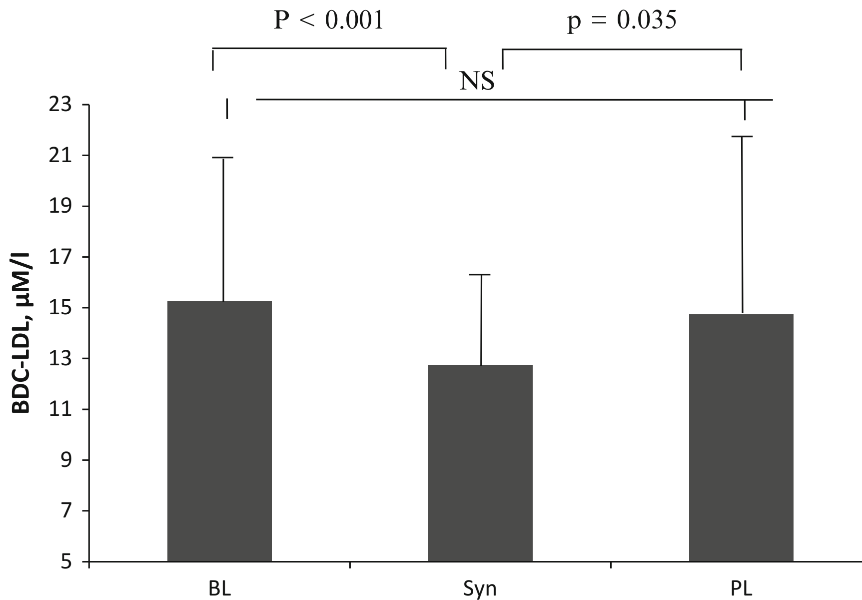


Fig. 18 Decrease of level of baseline diene conjugates of low density lipoproteins (BDC-LDL) during the synbiotic consumption compared to the placebo period in trial of *Helicobacter pylori* colonised persons (Mikelsaar et al. 2008)
Legend: *BL* baseline, *Syn* end of synbiotic consumption, *PL* end of placebo period, *NS* not significant. The BDC-LDL values decreased significantly in blood of volunteers at the end of synbiotic treatment period compared to baseline values (mean 15.2 *vs* 12.7 μM/l, $p < 0.001$). There was also a significant reduction (13 %) seen at the end of synbiotic period as compared to the end of placebo period (mean 12.7 *vs* 14.6 μM/l, $p = 0.035$)

healthy population subgroups for improvements in human blood and urine antioxidative biomarkers such as TAA, TAS, ox-LDL, baseline diene conjugate level of LDL (BDC-LDL), and urine diene conjugates (8-isoprostanes) (Kullisaar et al. 2003; Mikelsaar and Zilmer 2009).

Administering a food product (fermented goat milk) containing *L. fermentum* ME-3 to humans enhanced the systemic antioxidative activity of blood sera in a trial of volunteers (Kullisaar et al. 2003).

Furthermore, in a placebo-controlled cross-over synbiotic trial (Saulnier et al. 2007; Hutt et al. 2009; Mikelsaar and Zilmer 2009), we tested the blood biomarkers of healthy adults persistently colonized with *Helicobacter pylori*. A decreased local antioxidativity of gastric mucosa has been shown in *H. pylori* infection (Beil et al. 2000; Jung et al. 2001). The consumption of a synbiotic product containing enterocoated capsulated antimicrobial and antioxidative strains (*L. fermentum* ME-3; *L. paracasei* 8700:2; *B. longum* 46, 3×10^9 CFU, with 6.6 g Raftilose P95 twice a day for 3 weeks) increased blood TAS. In addition, the BDC-LDL values decreased significantly in the blood of volunteers to the end of the synbiotic administration and compared to the values to the end of the placebo period (Fig. 18). A quite similar distribution of *H. pylori*–positive ($n = 28$) and *H. pylori*–negative ($n = 25$) subjects, however, did not change after treatment.

Thus, the synbiotic treatment, though improving oxS indices, could not eradicate the persistent *H. pylori* infection. Still, we postulate that the risk for development and progression of atherosclerosis due to the chronic *H. pylori* infection could be reduced after 3 weeks of synbiotic consumption. Clinical studies have shown that BDC-LDL is closely linked to atherosclerosis and serves as a well-known oxS-related atherosclerosis risk factor. As an indicator, BDC-LDL clearly exceeds the sensitivity and specificity of common markers, successfully revealing mild

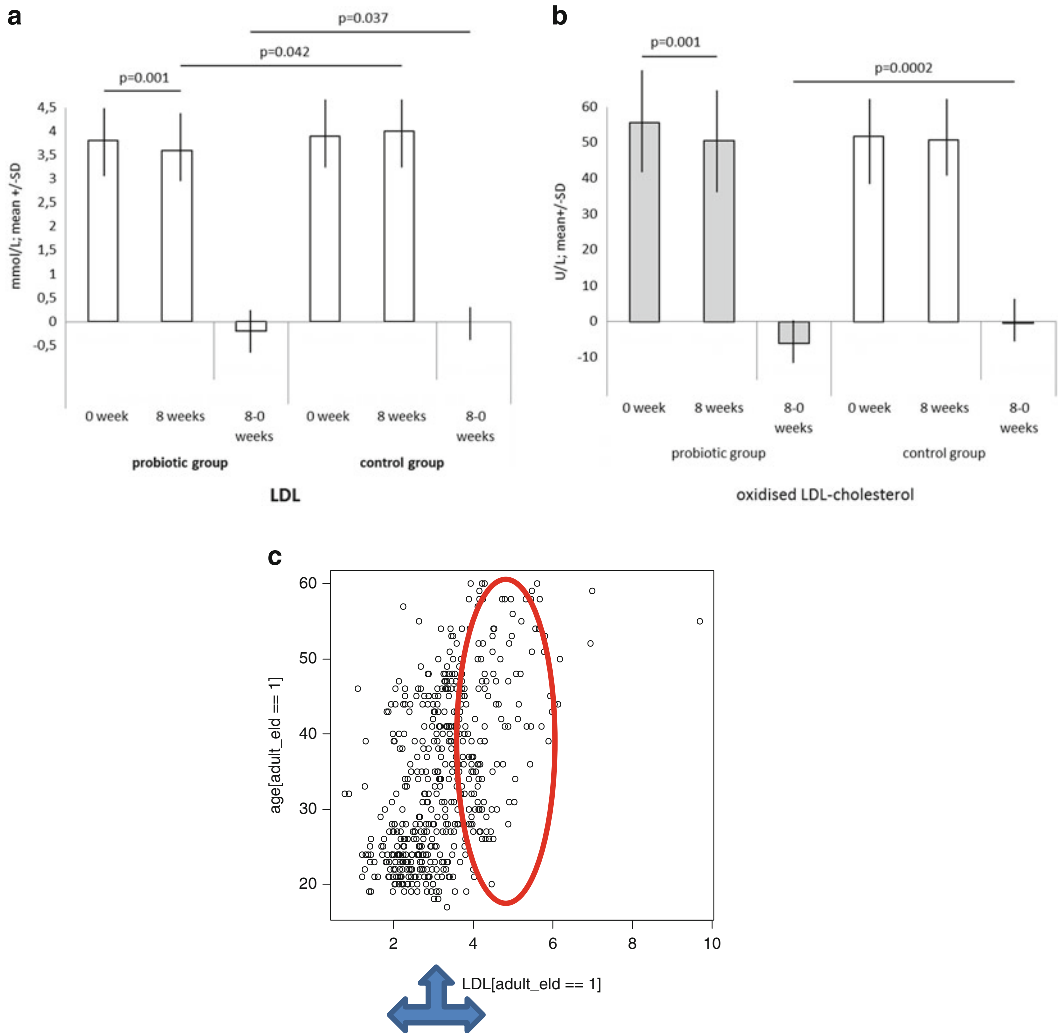


Fig. 19 (**a**) (**b**) (**c**) Reduction of borderline high >4.0 mmol/l content of (**a**) blood LDL- cholesterol and (**b**) oxidized LDL cholesterol after 8 weeks consumption of kephir comprising *L. fermentum* ME-3
Legend: The extent of changes (mean ± SD) of (**a**) LDL and (**b**) ox-LDL from the adjusted baseline to 8 weeks, for individuals in the (*left*) probiotic and (*right*) control groups were compared (Mikelsaar et al. 2015). (**c**) Distribution of LDL biomarker of blood in healthy adults, the *circle* depicts the *borderline* values of biomarkers in healthy population (Nordic Reference Interval Project; NORIP)

oxidation of LDL (Ahotupa and Asankari 1999; Brizzi et al. 2004). The BDC-LDL method distinctly improves possibilities for diagnosis, follow-up of treatment, and basic research into cardiovascular diseases.

In our recent study with *L. fermentum* ME-3, we showed that the people with biomarkers at borderline-to-normal reference values (e.g., blood LDL, Fig. 19) can be selected from the whole population and tested for the efficacy of a probiotic product (Mikelsaar et al. 2015). Apparently, the individual response to diet can vary largely, and an application aimed for personalized nutrition needs to take into consideration both the microbiota temporal signatures and the health biomarkers of the particular person.

Significantly different reductive values of LDL cholesterol ($p < 0.037$) and oxidized LDL cholesterol markers ($p < 0.0002$) were found

between the reduction in the test and control groups (Fig. 19). The results show a clear impact of probiotic food on healthy persons with biomarkers at borderline-to-normal values. This response is in consonance with the claim for maintenance of physiological health status.

Thus, we have proved the validity of the *in vitro*-assessed functional properties of some lactobacilli strains in host experimental animals and healthy volunteers.

5.5 Culture Collection of *Lactobacillus* spp.

To provide the Estonian population with beneficial bacteria of Estonian origin and for larger scientific purposes, the culture collection of microbiota was founded. Biobank was established in 1994 when the first microbiota samples and lactobacilli strains of Estonian and Swedish children were collected in the context of the first comparative studies for environmental impact in allergy development together with the universities of Linköping, Sweden, and Tartu, Estonia. From that time, the same study subjects have been repeatedly observed and sampled to continue the investigation. This unique cohort enables us to reveal changes in microbiota in connection with the changes in age, health, and environment.

The Human Microbiota Biobank (HUMB, http://eemb.ut.ee) is situated at the Department of Microbiology, University of Tartu, Estonia. The biobank was registered in 2010 at the World Data Centre for Microorganisms as the WFCC Estonian Human Microbiota Biobank (collection No. 977, acronym HUMB). HUMB is a member of ECCO (European Culture Collections' Organisation).

During the following years, the biobank was supplemented with numerous strains and microbiota samples in the context of several (including international) research projects. Indigenous bacteria of human microbiota – lactobacilli and bifidobacteria – form almost a quarter of the collection, but the collection also contains several opportunistic pathogens. Hundreds of new strains and microbiota samples from the GI, urogenital and respiratory tracts and the mouth and skin from healthy and diseased newborns, children, adults, and elderly people are added to the biobank each year. To date, the biobank contains more than 13,000 bacterial strains representing 74 genera and 201 species.

Probiotic development is an important research field in microbial ecology. In collaboration with the Bio-Competence Centre of Healthy Dairy Products, several GI probiotics have been developed: *Lactobacillus fermentum* ME-3 (DSM 14241; strain and patent belong to the University of Tartu; the strain is on the market as the dairy product brand Hellus and food supplement Reg'Activ™), *L. plantarum* TENSIA® (DSM 21380), and *L. plantarum* INDUCIA® (DSM 21379) (the two latter strains and their patents belong to Bio-Competence Centre of Healthy Dairy Products). In collaboration with the Estonian Competence Centre of Health Technologies, the search for vaginal probiotics is ongoing. In collaboration with the Institute of Dentistry of University of Tartu, a set of oral bacteria has been collected that has the potential for the development of oral probiotics against caries and periodontitis.

In addition to practical applications, the set of human microbiota is expected to provide new theoretical perspectives for determining if the health-promoting capacities of different strains of *Lactobacillus* spp. are mainly predicted by their host and biotope-specific origin, genetic and phenotypic profile, or some other epigenetic influences like age, geographic origin, environment, and type of nutrition.

6 Summary

Pinpointing beneficial LAB with important metabolic functions resulting from special pathways and released compounds seems worthwhile for studies on maintenance and modification of human ecosystems. The intestinal *Lactobacillus* spp. of the *Firmicutes* phylum represents a large group of Gram-positive bacteria. In this paper its biodiversity is demonstrated with data on taxonomy, function, and host-microbial interactions.

Its prevalence, composition, abundance, metabolic properties, and relation to host age, genotype, and socioeconomic factors have been reviewed based on the literature and the research experience of the Department of Microbiology at the University of Tartu for almost half a century.

An attempt has been made to associate schematically the metabolites of the three fermentative groups of *Lactobacillus* spp. with some metabolites produced by other groups of intestinal bacteria. Several functions of lactobacilli are well demonstrated in the reviewed *in vitro* studies and human trials. The promotional role of lactobacilli can induce the increase of beneficial compounds like acetate, propionate, butyrate, and NO. On the other hand, the suppressive impact of *Lactobacillus* spp. for the reduction of detrimental bacterial metabolites such as ammonia, indole, para-cresol, sulfides, H_2, ROS, and RNS has also been demonstrated. As a result, the intertwined metabolism can induce the simultaneous increase in the abundance of groups of intestinal microbiota other than the applied probiotic *Lactobacillus* strain and also increase the biodiversity of different *Lactobacillus* species.

In gut microbial ecology, several experimental trials have failed to demonstrate the increase in abundance of total lactobacilli after human *Lactobacillus* strain application. We have confirmed that different *Lactobacillus* strains of Estonian children did not increase the abundance of total lactobacilli in experimental rodents. However, in human studies, a tight association has been demonstrated between host specificity and achieved effects after application of human lactobacilli strains of different species. This link may rely on the specific nutrition and genetic differences in receptors for host colonization. However, this result was detected only for cultivable metabolically active lactobacilli and with daily doses at least over 10^9 CFU.

The large collection of *Lactobacillus* spp. strains (http://eemb.ut.ee) founded in 1994 originated from the first comparative studies of their environmental influence for allergy development together with the universities of Linköping, Sweden, and Tartu, Estonia. As noted, to date, this biobank contains more than 13,000 bacterial strains representing 74 genera and 201 species supplemented during several experimental, population, and clinical studies. This collection has served as the basis of several elaborated probiotic strains and for new putative candidates. For the selection of the potential for probiotic applications, FAO/EFSA has set strict regulations. There is no denying the need for precise molecular identification, safety traits, and colonizing properties as being of the utmost importance for application of every probiotic strain; however, to gain particular health effects, the probiotic strain also should have specific functional properties expressed in the host. For this purpose, physiological experiments using *in vitro* tests, animal models, population surveys, and clinical trials are decisive. It has been postulated that human metabolic status basically depends on the host genotype, microbiota metabolic profile, and epigenetic influence of the environment and normal diet, yielding great flexibility in homeostasis. In contrast, the environment, particularly the unbalanced homeostasis of the host either after infection or with metabolic disorders, can deliver epigenetic signals to the introduced probiotic strain to select the specific beneficial properties necessary for the current situation. For instance, the double functional efficacy of *Lactobacillus fermentum* ME-3 is characterized by the ability to suppress intestinal pathogens during enteric infections because of its oxidative properties, but the same strain can express highly antioxidative effects in the maintenance of cardiovascular health.

More advanced knowledge of the biodiversity of lactobacilli may open the path for evidence-based specification of the abundance and species distribution of intestinal microbiota that results in regulation of blood and urine biomarkers to reduce the risk of metabolic and immune-mediated diseases. The theory-based possibilities for the biotechnological applications of lactobacilli, together with directed and supportive personalized nutrition for large populations, appear to be part of the future of maintaining health.

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Clostridium difficile in Food and Animals: A Comprehensive Review

C. Rodriguez, B. Taminiau, J. Van Broeck, M. Delmée, and G. Daube

Abstract

Zoonoses are infections or diseases that can be transmitted between animals and humans through direct contact, close proximity or the environment. *Clostridium difficile* is ubiquitous in the environment, and the bacterium is able to colonise the intestinal tract of both animals and humans. Since domestic and food animals frequently test positive for toxigenic *C. difficile*, even without showing any signs of disease, it seems plausible that *C. difficile* could be zoonotic. Therefore, animals could play an essential role as carriers of the bacterium. In addition, the presence of the spores in different meats, fish, fruits and vegetables suggests a risk of foodborne transmission. This review summarises the current available data on *C. difficile* in animals and foods, from when the bacterium was first described up to the present.

Keywords

Clostridium difficile • Epidemiology • Animals • Food • Transmission

1 Introduction

Clostridium difficile is a spore-forming anaerobic bacterium recognised as the leading cause of antibiotic-associated diarrhoea in hospitalised patients. However, in recent years *C. difficile* infection (CDI) is increasingly common in the community, in younger patients without a previous history of hospitalisation or antibiotic treatment (Gupta and Khanna 2014). Studies worldwide have reported the presence of the bacterium in animals and foods (Songer and Anderson 2006; Hoover and Rodriguez-Palacios 2013; Rodriguez-Palacios et al. 2013) with a prevalence that varies according to the methodology used, the geographical area, the age and the animal species studied. While *C. difficile* is

C. Rodriguez (✉), B. Taminiau, and G. Daube
Department of Food Science, University of Liège-Faculty of Veterinary Medicine, Avenue de Cureghem 10, bât 43bis Sart-Tilman, 4000 Liège, Belgium
e-mail: c.rodriguez@ulg.ac.be

J. Van Broeck and M. Delmée
Belgian Reference Centre for Clostridium difficile (NRC), Pôle de microbiologie médicale, Université Catholique de Louvain, Brussels, Belgium

well known as enteric pathogen in some food producing, wild and companion animal species (Donaldson and Palmer 1999; Songer and Uzal 2005), there are several reports describing the presence of the bacterium in the intestinal contents of apparently healthy animals (Rodriguez et al. 2012; Hawken et al. 2013). Moreover, data recently published suggests that besides the nosocomial transmission, animals are an important source of human CDI, whether through environmental contamination, direct or indirect contact, or food contamination, including carcass and meat contamination at slaughter – or in the case of vegetables and other fruits, by the use of organic fertilizer or contaminated water (Rupnik and Songer 2010; Hoover and Rodriguez-Palacios 2013; Rodriguez-Palacios et al. 2013).

The European Food Safety Authority (EFSA) defines zoonoses as infections or diseases that can be transmitted directly or indirectly between animals and humans (through direct contact or close proximity with infected animals, or through the environment). As noted before (Rodriguez-Palacios et al. 2013), the relevance of the presence of *C. difficile* in some environments, animals and foods is little understood. This review describes the current knowledge regarding *C. difficile* in animals, foods, and the environment, as well as the prevalence among animals with and without signs of disease. The available data about animals and foods as vectors of CDI in humans has also been reviewed.

2 The Evolutionary History of *C. difficile* Detection in Animals and the Natural Environment

C. difficile was first reported in animals in 1960 (McBee 1960). The bacterium was isolated from a sample of a Weddell seal's large intestine contents, obtained during the course of a brief biological survey in the Ross Sea area of Antarctica. In 1974, a doctoral thesis described for the first time the presence of *C. difficile* in hay, soil, sand, and mud from the bank of the river, and in stools from diverse animals such as donkeys, horses, cows and camels, in Pakistan (Hafiz 1974). In an experimental study conducted in 1979 to reproduce neonatal diarrhoea in young gnotobiotic hares, the authors concluded that *C. difficile* was the causal agent of neonatal diarrhoea and that other strains of *Clostridium* enhanced its pathogenic effect (Dabard et al. 1979). CDI in pigs was first confirmed in 1980 when gnotobiotic pigs were accidentally exposed to *C. difficile* and accordingly suffered dehydration and excreted mucoid faeces containing specks of blood (Nagy and Bilkei 2003). In 1981 *C. difficile* was isolated from a goat (Hunter et al. 1981) and in 1982 the bacterium was obtained from rectal samples of healthy cattle in Nigeria of different breeds aged 6 months and above (Princewell and Agba 1982). Borriello et al. (1983) were the first to report the carriage of *C. difficile* in household pets and their immediate environment, including dogs, cats, ducks, geese, chicken, ring-necked parakeets, rabbits, goats, hedgehogs and guinea pigs. However, most of the recovered isolates were identified as non-cytotoxigenic. In the same year, *C. difficile* was recovered from pigs (Jones and Hunter 1983) and identified as the causative agent of antibiotic-associated colitis in a Kodiak bear (Orchard et al. 1983). Interest in the study of *C. difficile* in animals continued to increase during this period. From 1984 to 1987 three new studies described the bacterium as causal agent of enteric disease and diarrhoea in hares, European and cottontail rabbits (Carman and Evans 1984), horses (Ehrich et al. 1984) and foals (Jones et al. 1987). These findings raised the first concerns that domestic animals might be vectors of *C. difficile* among humans (Weber et al. 1988). From 1978 onwards, several studies focused on the isolation procedures and characterisation of *C. difficile* from healthy and diarrhoeic animals, including not only domestic animals such as foals (Jones 1989), cats, dogs (Weber et al. 1989; Riley et al. 1991; Martirossian et al. 1992) and captive ostriches (Frazier et al. 1993), but also wild animals such as cotton-top tamarinds (Snook et al. 1989). In 1995, *C. difficile* toxins were detected in the

small intestine and cecum of three juveniles and one adult rabbit with clinical signs of anorexia, decreased faecal output, nasal exudate and laboured breathing before death (Perkins et al. 1995). A later study in 1996 also reported the presence of *C. difficile* in animals (dogs, cats, horses, sheep and poultry) and in the environment: in soils, in river, sea and lake waters, and in swimming pool and tap waters (al Saif and Brazier 1996). Waters et al. (1998) described an outbreak of *C. difficile* in suckling piglets, and in 1999, Rieu-Lesme and Fonty isolated the bacterium from the ruminal reservoir of newborn lambs (Rieu-Lesme and Fonty 1999).

Besides clinical reports of CDI in exotic animals, such as Asian elephants (Bojesen et al. 2006) and ocelots (Silva et al. 2013a), *C. difficile* has been also isolated from faecal samples of captive white-tailed deer (*Odocoileus virginianus*) in confinement facilities in Ohio, USA, with a prevalence of 36.7 % (French et al. 2010). Furthermore, different studies have investigated the presence of the bacterium in wild animals, including wild passerine birds (Bandelj et al. 2011) and barn swallows (Bandelj et al. 2014); zoo animals (chimpanzees, dwarf goats, Iberian ibexes and plains zebras) (Álvarez-Pérez et al. 2014); sea otters (Miller et al. 2010); free-living South America coatis (Silva et al. 2014); small and medium-size wild mammals (raccoons, shrews, deer and house mice, rats, voles, opossum and groundhogs) (Jardine et al. 2013); black and Norway rats (Firth et al. 2014; Himsworth et al. 2014); feral pigs (Thakur et al. 2011) and Iberian free-range pigs (Álvarez-Pérez et al. 2013).

In the natural environment, *C. difficile* has recently been described in soils of studfarms and farms with mature horses in Sweden (Båverud et al. 2003), in homestead soils and household-stored water in Zimbabwe (Simango 2006), in tropical soils in Costa Rica (del Mar Gamboa et al. 2005) and in Slovenian rivers (Zidaric et al. 2010). In a study conducted in marine environments in the South of Italy, toxigenic *C. difficile* was also detected in seawater and zooplankton (Pasquale et al. 2011).

3 *Clostridium difficile* in Household Pets: Dogs and Cats

Rodriguez-Palacios et al. (2013) refer to the importance of household pets as common transmission routes for human infections of *C. difficile*: in modern lifestyles dogs and cats are considered family members and have access to all parts of the house, including beds, sofas, kitchens and dining rooms. Children under 16 years old often have close contact with their pets, as dogs often licked their faces and both cats and dogs usually sleep in the child's bed. In a study conducted in Canada, it was reported that very few of these children (2.9–4.4 %) recognised the need for washing their hands after contact with pets (Stull et al. 2013). A further study evaluating *C. difficile* in dogs and in the household environment indicated that 10 % of dogs were colonised by the bacterium and 31 % of households were contaminated with its spores, suggesting that exposure to this pathogen may be common (Weese et al. 2010a). In this environment, children, elderly and immune-compromised people could be more at risk of being colonised and developing CDI. In the same study, molecular characterisation of the isolates revealed that household and dog strains were different, concluding that there are sources of household *C. difficile* contamination other than dogs (Weese et al. 2010a). In any case, all dog isolates were indistinguishable from those circulating in human hospitals in the same geographical area (Rodriguez-Palacios et al. 2013). Therefore, the potential transmission of *C. difficile* between pets and humans is currently unclear.

Conversely, it has been reported that pets owned by an immune-compromised person or dogs living with a human receiving antimicrobial treatment were at greater risk of being colonised, presumably because the owner is at greater risk of developing the disease and in turn becoming a source of infection for the pet (Rodriguez-Palacios et al. 2013; Weese 2011). *C. difficile* has been detected in very high rates in healthy

dogs that visit human hospitals (58 %) (Lefebvre et al. 2006a). The risk seems to be particularly high when they accepted treats during the visit or licked patients (Lefebvre et al. 2009). However, it is not yet clear whether the contamination comes from patients or the hospital environment (Weese and Fulford 2011). Lefebvre et al. (2006b) reported the first human epidemic strain PCR-ribotype 027 in a healthy 4-year-old toy poodle that visited patients in healthcare settings in Ontario on a weekly basis. In 2009, Lefebvre and Weese (2009) reported the acquisition of toxigenic *C. difficile* by a therapy dog on its paws during a visit to an acute care facility. In this visit, the dog had been encouraged to 'shake paws' with patients. With these findings authors demonstrated that transient contamination of pet therapy animals (without colonisation) could be a source of pathogen transmission.

Regarding *C. difficile* as a cause of disease in pets, it seems that infection is more commonly community-associated rather than acquired at veterinary hospitals or after antimicrobial therapy (Weese 2011). However, the prevalence and causes of infections acquired in veterinary practices is largely unknown. A previous study identified administration of antimicrobials prior to admission, or administration of immunosuppressive drugs during hospitalisation, as risk factors for veterinary hospital-associated colonisation (Clooten et al. 2008). Murphy et al. (2010) described an important proportion of veterinary hospitals (58 %) with positive environmental swabs for *C. difficile*. While signs of disease could range from mild self-limiting diarrhoea to chronic or fatal diarrhoea (Berry and Levett 1986), the relevance of the bacterium in small veterinary clinics is still uncertain (Weese 2011; Busch et al. 2014). Different other studies have associated the presence of *C. difficile* in faeces with diarrhoea in dogs and cats (Weese et al. 2001a; 2001b; Weese and Armstrong 2003; Koene et al. 2012; Wetterwik et al. 2013). However, dogs can also be healthy carriers of *C. difficile* strains belonging to human epidemic PCR-ribotypes (Schneeberg et al. 2012; Silva et al. 2013b; Spigaglia et al. 2015), with a high colonisation in the first period of live (Perrin et al. 1993; Álvarez-Pérez et al. 2015).

Regarding CDI in cats, little information is available. It seems that colonisation rates are relatively low in the general population (0–21 %), but slightly higher among cats in veterinary hospitals (9.4–31 %) (Marks et al. 2011). The same *C. difficile* strains were recovered from cats and floor drains in the same veterinary hospital, suggesting the clinical environment was a possible source of contamination (Madewell et al. 1999).

Pet nutrition has been identified as a possible source of *C. difficile*, via pet treats (as bully sticks for dogs) and other raw or processed foods (Freeman et al. 2013; Rodriguez-Palacios et al. 2013). In a study conducted in France, *C. difficile* was not detected in any feline raw foods ($n = 20$) purchased from 20 Paris stores (Bouttier et al. 2010). However, a further study conducted in Ontario reported the presence of toxigenic *C. difficile* in turkey-based pet food. In the same study the authors recommended disinfecting food and water bowls daily with a 10 % bleach solution to reduce the potential burden of bacteria. Furthermore, it was proposed owners should not feed pets with raw diets in households with young children or immunosuppressed or elderly individuals (Weese et al. 2005).

4 Clostridium difficile in Horses

C. difficile toxins were associated with equine diarrhoea for the first time in 1984, in a study of horses in Potomac River area. In this study, Ehrich et al. (1984) concluded that toxins appeared not to be primary determinants of diarrhoea but they may have contributed to the disease. Currently, *C. difficile* is considered one of the most important causes of diarrhoea and enterocolitis in foals and horses (Arroyo et al. 2006; Weese et al. 2006; Uzal et al. 2012; Diab et al. 2013b). The prevalence of *C. difficile* in foals and adult horses with gastrointestinal disease varies considerably among studies, ranging between 5 % and 63 % (Diab et al. 2013b).

In newborn foals, *C. difficile* has been associated with spontaneous watery or bloody diarrhoea immediately after birth, depression, dehydration, toxaemia and finally death (Diab et al. 2013a). While in some cases the disease can occur without a history of antibiotic therapy or hospitalisation (Diab et al. 2013b), the major risk factors for the development of CDI in horses are antimicrobial treatment, hospitalisation, pre- or post-surgical feed withdrawal or changes in diet. The antimicrobials that have been most frequently associated with *C. difficile* diarrhoea in horses are erythromycin, clindamycin, rifampicin and gentamicin (Diab et al. 2013b).

Like other species, horses can carry *C. difficile* without showing signs of disease. In healthy foals the reported prevalence can vary between 0 and 29 % depending on different factors such the type of the study, the diagnostic test used and the method of sample collection (Diab et al. 2013b). A colonisation rate of up to 44 % has been reported in non-diarrhoeic foals under antibiotic treatment (Båverud et al. 2003). Mare-foal pairs can harbour *C. difficile* subclinically and potentially serve as reservoirs for cross-colonisation (Magdesian and Leutenegger 2011). In hospitalised horses without clinical signs of *C. difficile* disease, the observed prevalence ranged from 4.8 to 11 % (Medina-Torres et al. 2011; Rodriguez et al. 2014a), possibly under the influence of stresses that alter the intestinal flora (such as change of diet, transportation to the hospital, hospitalisation, and surgical or medical treatments) (Båverud 2004). Some studies have suggested a transient shedding of *C. difficile* in adult horses (Schoster et al. 2012) but also in other animal species including cattle (Rodriguez-Palacios et al. 2011b) and humans (Ozaki et al. 2004).

A recent study has evaluated the effect of probiotics on foals developing diarrhoea within 6 months of birth. The authors concluded that there was no benefit observable of administering a 3-week course of probiotics. Furthermore, a significantly higher incidence of diarrhoea in foals receiving probiotics than in control groups suggested a negative impact of probiotics (Schoster et al. 2015), although in vitro inhibition of *C. difficile* and *C. perfringens* by commercial probiotic strains has also been reported (Schoster et al. 2013).

5 *C. difficile* in Food-Producing Animals

In the twenty-first century the possibility of human exposure to *C. difficile* spores via environments and foods contaminated with feces of colonised animals has aroused considerable interest. Furthermore, besides the concern for zoonotic transmission, *C. difficile* is also a costly disease on companion animals and livestock production. There are no financial loss estimates for the treatment of household pets, but veterinary services and medical treatment for a case of acute diarrhoea without further complications costs between 100 and 200 euros in Europe. In production animals, *C. difficile* losses and treatment costs have also not been estimated, but *C. difficile* can produce mortality in breeding, weight loss, and delayed weight gain in animals (Rodriguez-Palacios et al. 2013; Squire and Riley 2013).

5.1 Food-Producing Animals: Swine

C. difficile has been widely described in both healthy pigs and pigs with diarrhoea (Table 1). In neonatal piglets (<15 days old), *C. difficile* has been proposed as the most common cause of diarrhoea (Songer and Anderson 2006) with a mortality rate of up to 50 % in suckling piglets (Songer 2000). Previous studies reported spore or toxin detection ranging between 23 and 93 % in faeces of diarrhoeic piglets and between 1.4 and 96 % in piglets with normal faeces (Table 1). The presence of *C. difficile* toxins in the colon of neonatal swine has been associated with: profuse non-haemorrhagic yellow pasty-to-watery diarrhoea, colitis, typhocoloitis, severe mesocolonic edema, other microscopic lesions such as erosive or ulcerative colonic lesions, infiltration of neutrophils in the lamina propia, and exudation of fibrin into the lumen, resulting

Table 1 Presence of *C. difficile* in piglets and adult pigs at farms, slaughterhouses and clinics

Area	Country/State	Year[a] From 2000	Age or situation	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Europe	Slovenia	08	1–10 days	D and ND	133/257 (51.8)	T (100)	–	Pirs et al. (2008)
		09	<10 days	D (77.7 of litters)	247/485 (50.9)	T (99.6)	066 (68.3)	Avbersek et al. (2009)
	Spain	09	1–7 days	D (49.7)	58/254 (22.8)	T (100)	–	Alvarez-Perez et al. (2009)
				ND (50.3)	76/257 (29.6)	T (90.8)		
			1–2 months	D (6)	0/12 (0)	–		
				ND (94)	0/187 (0)			
	Belgium	11	<15 days	ND (100)	18/23 (78.3)	T (100)	078 (66.7)	Rodriguez et al. (2012)
							002 (16.7)	
			At slaughter (5–6 months)	ND (100)	0/194 (0)	–	–	
		11–12	At slaughter (5–6 months)	ND (100)	1/100 (1)	T (100)	078 (100)	Rodriguez et al. (2013)
	Sweden	12	Neonatal	D and ND	45/67 (67)	T (100)	046 (100)	Norén et al. (2014)
	Germany	12	0–1 days	D (70.5)	19/31 (61)	T (100)	078 (55)	Schneeberg et al. (2013a)
				ND (29.5)	11/13 (85)		126 (20)	
			2–14 days	D (77.8)	78/84 (93)			
				ND (22.2)	23/24 (96)			
			15–77 days	D (71.4)	11/35 (31)			
				ND (28.6)	5/14 (36)			
	Austria	08	At slaughter	ND (100)	2/61 (3.3)	T (100)	–	Indra et al. (2009)
	The Netherlands	09	At slaughter	ND (100)	14/50 (28)	T (100)	015 (35.7)	Hopman et al. (2011b)
		09–10	At slaughter	ND (100)	0/100 (0)	–	–	Koene et al. (2012)
			In clinics	D (100)	9/25 (36)	T (100)	078 (77.8)	
							023 (11.1)	
							005 (11.1)	
		09–10	At slaughter	ND (100)	58/677 (8.6)	–	078 (31)	Keessen et al. (2011b)
							014 (15.5)	
							013 (12.1)	
	Switzerland	10	At slaughter	ND (100)	0/165 (0)	–	–	Hoffer et al. (2010)

USA	Texas	04–07	At farrowing	–	175/702 (24.9)	T (97.2)	078 (26.2)	Norman et al. (2011)
			Nursery		14/274 (5.1)			
			Breeding		26/604 (4.3)			
			Growth or finishing (at farm)		37/1370 (2.7)			
		06–07	Suckling	ND (100)	61/122 (50)	T93	–	Norman et al. (2009)
			Nursery		10/119 (8.4)			
			Growth or finishing (at farm)		15/382 (3.9)			
	Midwest	06	Neonatal	D (1000)	241/513 (47)	T [578 isolates]	–	Baker et al. (2010)
	Michigan	08	At farm	–	1/56	T (100)	–	McNamara et al. (2011)
	Ohio	08	16–20 weeks (at farm)	–	1/150 (0.67)	T (100)	–	Rodriguez-Palacios et al. (2014)
	Ohio	10	At farrowing	ND (100)	183/251 (73)	T (83.6)	–	Thakur et al. (2010)
	North Carolina		Sows after farrowing		32/68 (47)	T (90.6)		
	North Carolina	08–10	At farrowing (conventional farms)	ND (100)	120/350 (34.3)	T (97.8)	–	Susick et al. (2012)
			At farrowing (antimicrobial-free farms)		56/241 (23)			
			Nursing (conventional farms)		34/651 (5.2)			
			Nursing (antimicrobial-free farms)		7/491 (1.4)			
			Conventional sows in the farrowing barns		24/70 (34.3)			
			Antimicrobial-free sows in the farrowing barns		2/39 (5.1 %)			
			Finishing at conventional farms		2/579 (0.3)			

(continued)

Table 1 (continued)

Area	Country/ State	Year[a] From 2000	Age or situation	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Canada	Ontario	10	On day 2	–	90/121 (74)	T (100)	078 (94)	Weese et al. (2010c)
			On day 7		66/117 (56)			
			On day 30		45/113 (40)			
			On day 44		23/101 (23)			
			On day 62		2/54 (3.7)			
			Sows prior to farrowing		4/10 (40)	T (100)	078 (12)	
	Ontario	13	1 day	–	28/30 (93)	T (100)	078	Hawken et al. (2013)
			Market age (188 days)		1/26 (3.8)	T (100)	078 (100)	
	Various provinces	11	At slaughter	–	30/436 (6.9)	T (93.3)	078 (67)	Weese et al. (2011)
Australia	Western Australia	09	Neonatal	D (94.1)	103/174 (59.19)	T (100)	273 (100)	Squire et al. (2013)
				ND (5.9)	11/11 (100)			
	Five states	12–13	<7 days	D (12 farms with idiopathic diarrhoea) ND (9 farms without idiopathic diarrhoea)	154/229 (67.2)	T (87)	014 (23)	Knight et al. (2015b)
	Georgia	11	Before slaughter	–	55/345 (15.9)	–	–	Thitaram et al. (2011)
Japan	Kanto-Tokai	12	Finishing at farm (13–27 weeks)	–	2/250 (0.8)	T (50)	–	Asai et al. (2013)

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(–) Data not available or not applicable

in 'volcano lesions' (Lizer 2010). Scrotal edema, dyspnoea, mild abdominal distension, hydrothorax, ascites, anorexia and dehydration are other extra-intestinal symptoms probably caused by systemic sepsis (Squire and Riley 2013). However, an absence of diarrhoea does not discount possible *C. difficile* colonisation (Yaeger et al. 2007). Why some colonised piglets with toxigenic strains of *C. difficile* do not develop any signs of disease remains unclear and may be explained by the variability in colostrum intake and colostrum antibody concentration (Squire and Riley 2013). Similarly, the presence of *C. difficil*e-negative piglets has been described in litters where most of the members carried the bacterium. The reason why these piglets were negative despite being constantly exposed to the bacterium is also unknown (Weese et al. 2010c). The prevalence of the bacterium decreases with age, varying from 0 to 23 % at finishing in the farm or at slaughter (Table 1). Furthermore, outbreaks in adult pigs have only been reported in periparturient sows (Kiss and Bilkei 2005). It appears that sows are more likely to be colonised by *C. difficile* before or after farrowing (Thakur et al. 2010; Weese et al. 2010c; Susick et al. 2012), which may be due to environmental stress or the administration of antibiotics (Kiss and Bilkei 2005). While it seems sows would pose an obvious contamination source for piglets during farrowing, one study describes the predominance of different PCR-ribotypes in each group, suggesting that external sources other than sows could be responsible for CDI in piglets (Weese et al. 2010c; Hopman et al. 2011a). Widespread aerial dissemination of *C. difficile* on a pig farm was demonstrated and associated with personnel activity. Furthermore, possible aerial dispersal of the bacterium between farrowing pens was revealed by the detection of spores in the hallway following relocation of piglets (Keessen et al. 2011a). On pig farms, vermin such as house mice, drain flies, lesser houseflies and yellow mealworms were found positive for *C. difficile* and proposed as vectors for bacteria transmission (Burt et al. 2012). Despite the progress made in these studies, the sources of *C. difficile* in pig farms and aspects of the infection cycle still remain unclear. Several procedures, like surface disinfection and the use of gloves, have been proposed to reduce disease-associated mortality in piggeries (Squire and Riley 2013).

5.2 Food-Producing Animals: Cattle

As in the case of swine, the reported prevalence of *C. difficile* in cattle can vary wildly from one study to another depending on the geographical location studied, with percentages as diverse as 0 % in farms in North America and 60 % in Iran (Doosti and Mokhtari-Farsani 2014; McNamara et al. 2011) (Table 2). Furthermore, the pathogenicity of *C. difficile* in cattle is not fully understood. The bacterium and its toxins have been associated with diarrhoea in calves and dairy cows (Table 2). Using post-mortem analysis of calves infected with *C. difficile*, it has been showed that the bacterium was more frequently encountered in the cecum, where histologic lesions were also more severe (Rodriguez-Palacios et al. 2007b).

A higher prevalence (up to 56 %) has been reported in apparently healthy calves aged less than three months old (Table 2). One experimental study investigated the infection of neonatal calves by oral inoculation (in the colostrum) of toxigenic *C. difficile* spores. Results showed faecal shedding but did not detect toxins or the induction of enteric disease, and suggested that simple exposure to *C. difficile* could not cause disease in calves (Rodriguez-Palacios et al. 2007b). Colostrum can also play a protective role, providing passive immunity in neonatal calves. A natural protective effect of this first milk when ingested by calves immediately after birth is plausible (Rodriguez-Palacios et al. 2007b) and merits further investigation. In the literature, many studies have investigated hyperimmune bovine colostrum (obtained by repeated immunisation of pregnant cows) as an effective treatment for CDI in human patients (Steele et al. 2013). However, with or without signs of enteric disease, a decrease in the prevalence rate of *C. difficile* is observed in adult

Table 2 Presence of *C. difficile* in calves, dairy cattle and beef cattle at farms and slaughterhouses

Area	Country/ State	Year[a] From 2000	Age	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Europe	Slovenia	08	>21 days	D (100)	1/56 (1.8)	T (100)	033 (100)	Pirs et al. (2008)
		09	<12 weeks	D (76.1)	4/42 (9.5)	T (100)	077 (50)	Avbersek et al. (2009)
							038 (25)	
							002 (25)	
	Belgium	10	14 days (at arrival)	D (60)	5/50 (10)	T (95)	126 (36.8)	Zidaric et al. (2012)
			18 days		8/50 (16)		078 (31.6)	
			25 days		6/50 (12)		045 (10.5)	
			32 days		1/50 (2)		033 (7.9)	
			46 days		1/50 (2)		012 (7.9)	
			194 days (just before slaughter)		6/50 (0)			
		11	<3 months	ND (100)	4/18 (22.2)	T (100)	078 (75)	Rodriguez et al. (2012)
							015 (25)	
			At slaughter (11–52 months)	ND (100)	14/202 (6.9)	T (71.4)	002 (7.1)	
							014 (7.1)	
							081 (7.1)	
		11–12	At slaughter (15–56 months)	ND (100)	10/101 (9.9)	T (80)	078 (54.5)	Rodriguez et al. (2013)
							029 (18.2)	
	The Netherlands	09–10	Veal calves	ND (100)	6/100 (6)	T (100)	012 (83.3)	Koene et al. (2012)
							033 (16.6)	
			Dairy cows	D (100)	0/5 (0)	–	–	
				ND (100)	1/100 (1)	T (100)	012 (100)	
	Austria	08	At slaughter	ND (100)	3/67 (4.5)	T (3)	–	Indra et al. (2009)
	Switzerland	10	At slaughter	ND (100)	1/204 (4.2)	T (100)	078 (100)	Hoffer et al. (2010)
		10	Cows	–	1/63 (1.6)	T (100)	137 (100)	Romano et al. (2012)
			Calves		6/47 (12.7)	T (83.3)	033 (16.7)	
							003 (16.7)	
							066 (16.7)	
							070 (16.7)	

	Germany	10–12	Calves	D (100)	176/999 (17.6)	–	033 (57)	Schneeberg et al. (2013b)
							078 (17)	
							045 (9)	
		11–12	Dairy cattle	–	25/29 (86.2)	T (17)	–	Schmid et al. (2013)
			Beef cattle		4/29 (13.8)			
Asia	Iran	13	3–25 days	–	90/150 (60)	T (41)	–	Doosti and Mokhtari-Farsani (2014)
USA	Georgia	11	Dairy cattle	ND (100)	32/1325 (2.4)	–	–	Thitaram et al. (2011)
			Beef cattle		188/2965 (6.3)			
	Ohio	08	Dairy cow	–	2/330 (0.61)	T (100)	027 (50)	Rodriguez-Palacios et al. (2014)
			Beef cow					
		07	On arrival	ND (100)	24/186 (12.9)	T (92.8)	078	Rodriguez-Palacios et al. (2011b)
			Week 1		0/176 (0)			
			Week 4		3/176 (1.7)			
			Week 12		0/168 (0)			
			Week 20		5/168 (3.6)			
			Prior to slaughter		2/167 (1.2)			
			At slaughter (intestinal contents)		2/168 (1/2)			
		11	At harvest (meat processing plants)	ND (100)	17/944 (1.8)	T (0.4)	078 (5.9)	Rodriguez-Palacios et al. (2011a)
	Michigan	08	At farm	–	0/50 (0)	–	–	McNamara et al. (2011)
	South-western	08	1–6 weeks	D (82.7)	64/253 (25.3)	T (23)	–	Hammitt et al. (2008)
				ND (17.3)	7/53 (13.2)	T (30.2)		
	Pennsylvania	12	<2 weeks	ND (100)	8/200 (4)	T (7.1)	–	Houser et al. (2012)
			4–6 weeks		18/200 (9)			
			8–10 weeks		6/200 (3)			
			12–18 weeks		10/200 (5)			
			20–22 weeks		18/200 (9)			

(continued)

Table 2 (continued)

Area	Country/ State	Year[a] From 2000	Age	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Canada	Ontario	04	<1 month	D (51.8)	11/144 (7.6)	T (100)	078 (25.8)	Arroyo et al. (2005)
				ND (48.2)	20/134 (14.8)		017 (29)	
				014 (12.9)				
				027 (12.9)				
				033 (9.7)				
	Ontario	08–09	2–10 days (48 h after arrival)	–	56/174 (32)	T (98.7)	078 (67)	Costa et al. (2011)
			1 week		88/172 (51.1)			
			17 weeks		4/183 (2)			
			21 weeks		4/156 (2)			
	Alberta	09	At feedlot on arrival	–	18/539 (3.3)	T (100)	078 (100)	Costa et al. (2012)
			Mid-feeding period		18/335 (5.4)			
Australia	Western Australia	07–08	Adult cattle at slaughterhouse (intestinal contents)	ND (100)	0/158 (0)	T (98.6)	127 (50.2)	Knight et al. (2013)
							033 (19.6)	
							16 (7.7)	
	New South Wales	08–09	Adult cattle at slaughterhouse (faeces)		5/280 (1.8)		126 (5.7)	
	Queensland						3 (1.4)	
	South Australia						103 (1.4)	
							002 (1)	
	Victoria						137 (0.5)	
	Western Australia						7 (3.3)	
	Victoria	12	Calves aged < 7 days at slaughterhouse		203/360 (56)			
	Queensland		Calves 2–6 months at slaughterhouse		1/26 (3.8)			

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature (–) Data not available or not applicable

animals (Table 2). While the reason for this age effect is still unknown, a probable explanation is that the bacterium is better able to colonise and proliferate in the intestinal tract of younger animals, where the gut microbiota is less developed (Rodriguez-Palacios et al. 2006).

5.3 Food-Producing Animals: Poultry

A wide variety of zoonotic diseases can be transmitted by poultry. However, few studies have focused on the study of *C. difficile* in these animals. The limited data available shows that the situation is similar to other species, with prevalence decreasing with increasing age (ranging from 100 % in faecal samples of 14-day-old birds to 0.29 % in mature farm animals), and with bacterial colonisation observable with or without development of disease (Table 3).

Only one outbreak of *C. difficile* has been described in newly hatched ostriches (Cooper et al. 2013). In this outbreak, more than 90 % of birds died within three days of the onset of diarrhoea. At necropsy, the colon and rectum were dilated and diffusely haemorrhagic. Microscopic examination also revealed necrotizing typhilitis and colitis in all the birds. After this report, 300 additional birds from a subsequent hatching were also affected by an epidemic of necrotic enteritis. Identical symptoms were observed which may suggest that CDI is a common and important problem in captive ostrich chicks (Frazier et al. 1993).

In rural communities in Zimbabwe, chickens were identified as major reservoirs of *C. difficile*. Water probably acted as a source of the bacterium for these chickens, as spores were detected in well water and household-stored water. Sources of water contamination may be faeces of domestic animals or humans, although this was not investigated in the study. In addition, soils were also heavily contaminated with *C. difficile* by chicken faeces. The free movement of chickens between neighbouring homesteads highlights the importance of these colonised animals as vectors for widespread distribution of *C. difficile* in rural communities (Simango 2006).

5.4 Food-Producing Animals: Sheep and Goats

Other production animals such as lambs, sheep and goats have been also described as carriers of the bacterium, with a prevalence varying between 0.6 and 10.1 % (Table 3). As in other animal species, the rate of *C. difficile* detection seems to decrease with age.

On average, a lower prevalence has been reported in sheep and lambs than in swine. This may be associated with the greater use of antimicrobials in production of pigs than in sheep (Knight and Riley 2013). However, as stated before, the few studies available in the literature studying the effect of antibiotics did not find a direct relation between the use of antimicrobials and *C. difficile* colonisation or infection (Romano et al. 2012; Susick et al. 2012). While the presence of *C. difficile* in apparently healthy sheep and goats in farms and at slaughter could play a role in animal-to-animal, environmental or zoonotic transmission, there are no reports identifying the bacterium as responsible for outbreaks of enteropathogen in these animal species.

6 Clostridium difficile in Foods

Recent studies have described the presence of *C. difficile* spores in a variety of food products of both animal and plant origin. These findings highlight the potential risk of infection associated with consuming foods, particularly if they are not cooked prior to eating (Lund and Peck 2015).

6.1 Prevalence and Food Products Concerned

The contamination by *C. difficile* spores has been detected in different types of food products,

Table 3 Presence of *C. difficile* in other food-producing animals

Animal species (Origin)	Area	Country	Year[a] From 2000	Age	With (D) Without (ND) diarrhoea (%)	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b]	Study
Poultry	Europe	Slovenia	07–08	14 weeks (flock 1)	–	5 /7 (71.4)	T (100)	023 (6.8)	Zidaric et al. (2008)
				1 day (flock 2)		0/8 (0)	–		
				15 days (flock 2)		24/24 (100)	T (96.3)		
				18 weeks (flock 2)		9/22 (40.9)	T (90)		
		The Netherlands	09–10	Clinics	D (100)	2/21 (9.5)	T (57.1)	014 (28.6)	Koene et al. (2012)
				At slaughter	ND (100)	5/100 (5)		010 (28.6)	
		Austria	08	Broiler chicken at slaughter	ND (100)	3/59 (5)	T (66.7)	001 (33.3)	Indra et al. (2009)
								446 (33.3)	
	Africa	Zimbabwe	06	Chicken faeces (home leads rural community)	–	20/115 (17.4)	T (55)	–	Simango (2006)
			08	Broiler chickens at market places	–	29/100 (29)	T (89.7)	–	Simango and Mwakurudza (2008)
	USA	Ohio	08	At farm	–	1/340 (0.29)	T (0)	–	Rodriguez-Palacios et al. (2014)
		Texas	09	Broiler chicken 42 days-old at barns	–	6/300 (2.3)	T (100)	078	Harvey et al. (2011a)
Sheep	Europe	The Netherlands	09–10	Clinics	D (100)	2/11 (18.2)	T (100)	015 (50)	Koene et al. (2012)
								097 (50)	
		Slovenia	09–11	Adult sheep >1 year at farms	D (12.4)	0/27 (0)	T (100)	056 (16.7)	Avberšek et al. (2014)
								061 (16.7)	
				Lambs between 1 day and 4 months	D (7.6)	6/78 (7.7)			
	Australia	South Australia	11–12	Sheep at slaughterhouse	ND (100)	1/156 (0.6)	T (93.3)	056 (40)	Knight and Riley (2013)
		New South Wales						101 (40)	
		Victoria		Lambs at slaughterhouse or farm	–	14/215 (6.5)		m	
		Western Australia							
	USA	Michigan	08	At farm	–	0/57 (0)	–	–	McNamara et al. (2011)

Goats	Europe	Slovenia	09–11	Adult goats >1 year at farms	ND (100)	0/10 (0)	T (90)	045 (40)	Avberšek et al. (2014)
								010 (10)	
				Goats between 1 day and 4 months		10/99 (10.1)		014 (10)	
								020 (10)	
		Switzerland	10	At farm	–	3/40 (7.5)	T (100)	001 (66.7)	Romano et al. (2012)
								066 (33.3)	
	USA	Michigan	08	At farm	–	0/14 (0)	–	–	McNamara et al. (2011)

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(−) Data not available or not applicable

including seafood, vegetables and meats, with a prevalence ranging between 2.9 and 66.7 % (Tables 4 and 5). Considering that *C. difficile* is present in healthy food-producing animals at slaughter, it is not surprising that its spores have also been found in meats (Table 4). The mean prevalence of *C. difficile* spores in these products ranges between 0 and 15 %. While early studies conducted in North America reported a much higher contamination rate than elsewhere (Rupnik and Songer 2010), recent studies show the situation to be similar to other countries (Table 4). Rodriguez-Palacios et al. (2009), noting an increased recovery of the bacterium from ground beef and chops in winter in Canada, suggested a seasonal component in *C. difficile* contamination in meats, and also hypothesised a possible epidemiological connection between the prevalence of *C. difficile* in food animals, some foods and humans (Rodriguez-Palacios et al. 2013).

If the initial contamination of food products with *C. difficile* is low, the preservation method used may play a fundamental role in the spores' survival. One of the key features of *C. difficile* in foods is if the pathogen grows or resides in the dormant state, especially if there are anaerobic conditions and the cool chain is not respected. *C. difficile* has been reported in vacuum-packaged meat in France (Bouttier et al. 2010) and in New Zealand, where the bacterium was isolated from chilled vacuum-packed meats in which 'blown pack' spoilage had been observed (Broda et al. 1996). The impact of *C. difficile* survival in these storage conditions clearly demands further study.

There has also been interest with respect to thermal inactivation of *C. difficile* spores by thermal treatment. Rodriguez-Palacios and Lejeune (2011) reported that cooking food at a minimum of 96 °C for 15 min produced an inhibitory effect on *C. difficile* spores. However, minimally-processed fruits and vegetables are treated below these temperatures and therefore could be potential vectors of human infection (Rodriguez-Palacios et al. 2013). The contamination source of these fruits and vegetables could be the use of organic fertilizer containing *C. difficile* spores, or irrigation or washing with contaminated water.

6.2 Routes of Food Contamination

As stated before, *C. difficile* is present in the intestinal contents of apparently healthy food-producing animals, suggesting carcasses and meats could be contaminated during the slaughter process. A few studies have addressed the contamination of carcasses at slaughter. In pigs, *C. difficile* was detected in a total of 3 out of 20 carcasses (15 %) sampled at post-bleed and a further 3 out of 20 (15 %) at post-evisceration in a processing facility in Canada (Hawken et al. 2013). A further study reported a prevalence of 2.2 % and 2.5 % in antimicrobial-free pigs at post-evisceration and post-chill respectively (Susick et al. 2012). Harvey et al. (2011b) detected 3 positive samples from a total of 10 sponge swabs collected from carcass hide, post-excision hides and ears from pigs in a processing plant in Texas. In Belgium, the prevalence reported in carcasses from slaughter pigs was 7 % (7/100) (Rodriguez et al. 2013).

C. difficile has also been described in cattle carcasses. In Belgium, the observed prevalence in cattle carcasses reached up to 7.9 % (8/101) (Rodriguez et al. 2013). In a study conducted in Pennsylvania, Houser et al. (2012) detected the *tpi* housekeeping gene in 4 out of 100 cattle carcass swabs by PCR, but *C. difficile* was not isolated using culture techniques. The same data has been reported in an Australian study of cattle carcasses sampled in the processing area of the slaughter line where none of the samples taken (n = 151) were positive for *C. difficile* (Knight et al. 2013). Rodriguez-Palacios et al. (2011b) reported 0 positive carcasses from a total of 168 samples analysed.. In a further study conducted in the USA, samples were collected from pig hides, pre-evisceration carcasses, post-intervention carcasses and ground beef. The bacterium was detected in hides with a prevalence of 3.2 %. However, none of the carcass or meat samples tested positive, evidencing a low

Table 4 Presence of *C. difficile* in meats (in processing plants or the retail trade) and other foods (at farms or markets)

Area	Country/State	Year[a]	Sample Type	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b] (%)	Study
Asia	Iran (Isfahan)	2014	Chopped beef	1/35 (2.8)	T (100)	–	Esfandiari et al. (2014a)
			Ground beef	1/46 (2.1)			
			Chopped mutton	2/55 (3.6)			
			Ground mutton	4/64 (6.2)			
		2012	Beef meat samples	3/54 (5.6)	T (100)	–	Esfandiari et al. (2014b)
			Beef hamburger	4/56 (7.1)			
	Iran (Isfahan/ Khuzestan)	2012	Buffalo meat	6/67 (9)	T (92.3)	078 (53.8)	Rahimi et al. (2014)
			Goat meat	3/92 (3.3)			
			Beef meat	2/121 (1.7)			
			Cow meat	1/106 (0.9)			
			Sheep meat	1/150 (0.7)			
			Camel meat	0/124 (0)			
USA	Connecticut	2015	Ground beef	0/100 (0)	T (0)	–	Mooyottu et al. (2015)
			Ground pork	2/100 (2)			
			Chicken wings	0/100 (0)			
	Pennsylvania	2011–2012	Beef meat samples	5/72 (6.9)	T (100)	027 (40)/078 (40)	Varshney et al. (2014)
			Pork meat samples	9/78 (11.5)	T (66.7)	078 (44)	
			Turkey meat samples	11/76 (14.5)	T (81.8)	027 (9.1)/078 (18.2)	
			Chicken meat samples	6/77 (7.8)	T (33.3)	–	
		2012	Pork sausages	2/102 (2)	T (100)	078 (100)	Curry et al. (2012)
		2012	Ground veal products	4/50 (8)	T (100)	–	Houser et al. (2012)
	Nine different states	2009–2011	Retail meat products	0/1755 (0)	–	–	Limbago et al. (2012)
	Nebraska	2013	Ground beef	0/956 (0)	–	–	Kalchayanand et al. (2013)
	Texas	2004–2009	Ground pork and turkey	23/243 (9.5)	T (100)	078 and variants (95.7)	Harvey et al. (2011b)
			Pork chorizo and trim				
	Arizona	2007	Ground beef uncooked	13/26 (50)	T (100)	078 (2.2)/027 (4.4)	Songer et al. (2009)
			Summer sausage (cooked)	1/7 (14.3)			
			Ground pork (uncooked)	3/7 (42.9)			

(continued)

Table 4 (continued)

Area	Country/State	Year[a]	Sample Type	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b] (%)	Study
			Braunschweiger (cooked)	10/16 (62.5)			
			Chorizo (uncooked)	3/10 (30)			
			Pork sausage (uncooked)	3/13 (23.1)			
			Ground turkey (uncooked)	4/9 (44.4)			
		2010	Poultry meats	4/32 (12.5)	T (100)	078 and variants (100)	Harvey et al. (2011a)
Africa	Abidjan	2009–2010	Cooked kidney beef	19/172 (11)	–	–	Kouassi et al. (2014)
			Cooked flesh beef	30/223 (13.4)			
Europe	Belgium	2012	Ground and burger beef	3/133 (2.3)	T (100)	078 (33.3)/014 (66.7)	Rodriguez et al. (2014b)
			Ground and sausage pork	5/107 (4.7)	T (80)	078 (20)/014 (40)	
	The Netherlands	2008–2009	Beef meat	0/145 (0)	–	–	de Boer et al. (2011)
			Pork meat	0/63 (0)	–	–	
			Calf meat	0/19 (0)	–	–	
			Lamb meat	1/16 (6.3)	T (100)	045 (100)	
			Chicken meat	7/257 (2.7)	T (2.7)	001/003/087/071	
	Switzerland	2010	Minced meat products	0/46 (0)	–	–	Hoffer et al. (2010)
	France	2007–2008	Ground beef	2/105	T (100)	012 (100)	Bouttier et al. (2010)
			Pork sausage	0/59			
	Austria	2007–2008	Ground meat	3/100	T (66.7)	053 (33.3)	Jöbstl et al. (2010)
		2008	Beef meat	0/51 (0)	–	–	Indra et al. (2009)
			Pork meat	0/27 (0)			
			Chicken meat	0/6 (0)			
	Sweden	2008	Ground meat	2/82 (2.4)	T (100)	–	Von Abercron et al. (2009)

Canada	Manitoba	2007	Ground beef	2/24 (8.3)	T (100)	–	Visser et al. (2012)
			Ground pork	1/24 (4.2)			
			Ground pork				Metcalf et al. (2010a)
			Chopped pork				
	Ontario	2008–2009	Chicken thighs	10/11 (9)	T (100)	078 (100)^{2}	Weese et al. (2010b)
			Chicken wings	13/72 (18)			
			Chicken legs	3/20 (15)			
	Four provinces	2008	Ground beef	14/115 (12)	T (100)	078 (71.4)/027 (7.1)	Lefebvre and Weese (2009)
			Ground pork	14/115 (12)	T (100)	078 (85.7)/027 (7.1)	
	Various provinces	2006	Ground beef	22/149 (14.8)	T (89.3)	027 (30.8)/077 (23.1)/014 (15.4)	Rodriguez-Palacios et al. (2009)
			Veal chops	6/65 (9.2)			
	Ontario and Quebec	2005	Beef and veal ground meat	12/60 (20)	T (91.7)		Rodriguez-Palacios et al. (2007a)
Central America	Costa Rica	2013	Beef meat	1/67 (1.5)	T (100)	–	Quesada-Gómez et al. (2013)
			Pork meat	2/66 (3)			
			Poultry meat	1/67 (1.5)			

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(–) Data not available or not applicable

Table 5 Presence of *C. difficile* in other foods sampling from farms, wholesalers or markets

Food Type	Area	Country/ State	Year[a]	Sample Type	Prevalence (%)	T (% of toxigenic strains)	Main PCR-ribotypes[b] (%)	Study
Seasoned ingredients	Asia	Iran	2015	Defrosted onions	0/14 (0)	–	–	Esfandiari et al. (2014b)
				Textured soy proteins	0/14 (0)			
				17 seasoning	0/17 (0)			
Seafood	USA	Texas	2012	Fresh mussel	3/67 (4.5)	T (100)	078 (66.7)	Norman et al. (2014)
				Frozen salmon/ shrimp				
	Europe	Italy	2010–2011	*Mytilus galloprovincialis*	16/33 (48.5)	T (43.7)	014/020/078/045/ 012/	Pasquale et al. (2012)
				Tapes philippinarum	10/19 (52.6)	T (80)	002/001/003/106	
				Venus verrucosum	0/1 (0)	–	–	
			2009	*Mytilus galloprovincialis*	2/3 (66.7)	T (50)	066 (50)/010 (50)	Pasquale et al. (2011)
				Tapes philippinarum	1/1 (100)	T (0)	010 (100)	
				Callista chione	1/2 (50)	T (100)	005 (100)	
	Canada	Ontario	2010	Frozen scallop and shrimp	5/119 (4.8)	T (80)	078 (80)	Metcalf et al. (2011)
				Fresh perch and salmon				
				Cooked shrimp				
Ready-to-eat/raw vegetables	Europe	France	2013	Heard of lettuce	3/104 (2.9)	T (100)	001 (33.3)	Eckert et al. (2013)
				Lambs lettuce salad			014/020/077 (33.3)	
				Peat sprouts			015 (33.3)	
	Canada	Ontario	2008	Ginger	5/111 (4.5)	T (100)	078 (60)	Metcalf et al. (2010b)
				Carrot				
				Eddoes				
Milk	Europe	Austria	2008	Bactofugates	0/50 (0)	–	–	Jöbstl et al. (2010)

[a]Year when the study was conducted or year when the study was published
[b]Main PCR-ribotypes found with standard Cardiff nomenclature
(–) Data not available or not applicable

contamination of the production chain (Kalchayanand et al. 2013).

Regarding the environmental shedding of *C. difficile* in processing facilities, little data is available. In seven hamburger processing plants in Iran, *C. difficile* was detected in 3.5 % (2/56) of swabs taken from the environment. The authors suggested that this environmental contamination might be due to biofilm formation which could facilitate the attachment of spores (Esfandiari et al. 2014b). In contrast, in a further study conducted in three sausage-manufacturing plants, sponge swabs collected from equipment and facilities yielded no *C. difficile* isolates (Harvey et al. 2011b), while meat samples tested positive for the bacterium, indicating meat contamination with *C. difficile* from the intestinal contents of food animals.

The hands of food handers, especially of those who produce ready-to-eat food, are well-known vectors of foodborne pathogens, in most cases due to poor hygiene. However the impact of contamination of *C. difficile* by humans who handle foods without washing their hands has not yet been evaluated. In a previous study investigating the *C. difficile* contamination of foods prepared in-house at a Belgian nursing hom, only 1 out of 188 food samples tested positive for *C. difficile*. This positive sample was recovered from a meal composed of carrot salad, mustard sauce and pork sausage. However, as they were analysed together, contamination could have originated from any of the ingredients or as a result of manipulation (Rodriguez et al. 2015).

7 The Threat of Zoonotic and Foodborne Transmission

The literature of the last decade has presented several hypotheses about *C. difficile* transmission (Bauer and Kuijper 2015). Weese et al. (2002) reported a risk of zoonotic transmission of some animal diseases, including *C. difficile*, especially in small veterinary hospitals. Goorhuis et al. (2008) described PCR-ribotype 078 as frequently encountered in human CDI and in pigs with diarrhoea in The Netherlands. A further study reported that this ribotype was the most prevalent type in pig, cattle and horse species worldwide, and also reported an increase in its prevalence in humans in different countries (Rupnik et al. 2008). Other studies conducted in 2008 (Jhung et al. 2008) and in 2009 (Debast et al. 2009) showed a high degree of similarity between pig and animal *C. difficile* PCR-ribotype 078 toxinotype V strains, suggesting a common origin. Recently, Janezic et al. (2014) showed that the most prevalent *C. difficile* types in humans are also prevalent in different animals from different geographic areas, evidencing the potential for global dissemination of some strains.

In the twenty-first century, the development of different typing methods has allowed genome analysis and the comparison of animal, food and human strains (Griffiths et al. 2010). The first study investigating the phylogeny of *C. difficile* by multilocus sequence typing (MLST) analysis reported that differences between phylogenetic lineages do not correlate with the type of host (human or animal) (Pons 2004). Lemée et al. (2004) studied the genetic relationships and population structures of 72 *C. difficile* isolates from various hosts and geographic sources, including human, dog, horse, cow and rabbit stools. Results obtained in the study showed that animal isolates did not constitute a distinct lineage from human isolates. In subsequent works, the same study group (Lemée et al. 2005; Lemée and Pons 2010) observed that animal isolates were intermixed with human isolates. In the recent years, clade 5 has been largely studied as it contains *C. difficile* PCR-ribotype 078 (Knight et al. 2015a). This type was classically associated with animals, especially pigs (Álvarez-Pérez et al. 2013). However, lately it has been also reported in hospitals (Indra et al. 2015). At present, clade 5 seems to be highly heterogeneous and divergent from the rest of population (Janezic and Rupnik 2015).

Marsh et al. (2010) used multiple-locus variable number tandem repeat analysis (MLVA) to show that toxinotype V (REA group BK) human

and animal isolates were highly related but differentiated. In another study conducted in the Netherlands (Koene et al. 2012), faecal samples from healthy and diarrhoeic animals were compared with human strains isolated from patients with diarrhoea and hospitalised patients. MLVA analysis showed a genotypic correlation between animal and human PCR-ribotype 078, but a distinction between human and animal PCR-ribotypes 012 and 014.

Whole genome sequencing (WGS) has recently been used to study the epidemiology of CDI and the genetics of *C. difficile* (Knight et al. 2015a). One such study investigated the evolutionary relatedness of *C. difficile* PCR-ribotype 078 isolated from humans and pigs (in farms) (Knetsch et al. 2014). Results revealed that farmers and pigs were colonised with identical or nearly identical *C. difficile* clones (with zero or less than two single nucleotide polymorphism differences). These results supported the hypothesis of interspecies transmission between animals and humans; however, the existence of a common contamination source (in the environment) was also possible.

It seems that *C. difficile* occurs as a low-level contaminant in meats and other food products. Therefore foodborne transmission may be responsible for only a small proportion of human CDI cases (Curry et al. 2012). However, other authors have reported no molecular relationship between clinical human and meat isolates and, therefore, that sources other than meat are responsible for CDI (Esfandiari et al. 2014a). At present, the human infectious dose for *C. difficile* is not known (Hoover and Rodriguez-Palacios 2013) and the risk posed by the presence of its spores in meat and other foods is still not clarified. Among healthy people with normal intestinal flora, the ingestion of low quantities of spores may not have major repercussions. However, the consumption of these contaminated foods by vulnerable populations with gastrointestinal perturbations could lead to *C. difficile* colonisation and infection, or can contribute to the asymptomatic *C. difficile* carriage and transmission in the community.

8 Conclusions and Perspectives

Eighty years after its discovery, *C. difficile* continues to be the focus of attention in hospitals and an important topic for many research groups worldwide. Comparisons of strains have revealed that in some regions animals and humans are colonised with identical *C. difficile* clones or these strains cluster in the same lineage. Therefore, it is suggested that *C. difficile* should be considered as a zoonotic pathogen and that animals play an important role as reservoirs of the bacterium.

While many questions remain unanswered, next generation typing techniques must be applied in the future to study the relatedness of strains of human and animal origins. In this context, it will be interesting to assess the presence of *C. difficile* in close related human and animal populations, like pets and their owners or farmers in close contact with their animals. The analysis of the isolates by WGS analysis will definitively confirm the absence of host tropism of certain strains and the zoonotic transmission of the bacterium.

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Russian Kefir Grains Microbial Composition and Its Changes during Production Process

I.B. Kotova, T.A. Cherdyntseva, and A.I. Netrusov

Abstract

By combining DGGE-PCR method, classical microbiological analysis and light- and electron microscopic observations, it was found that the composition of microbial communities of central Russia regions kefir grains, starter and kefir drink include bacteria of the genera *Lactobacillus*, *Leuconostoc* and *Lactococcus,* and yeast anamorphs of the genera *Saccharomyces*, *Kazachstania* and *Gibellulopsis*. Fifteen prokaryotic and four eukaryotic pure cultures of microorganisms were isolated and identified from kefir grains. It has been shown that members of the genus *Lactobacillus* prevailed in kefir grains, whereas strains *Leuconostoc pseudomesenteroides* and *Lactococcus lactis* dominated in the final product – kefir drink. Yeasts contained in kefir grains in small amounts have reached a significant number of cells in the process of development of this dairy product. The possibility of reverse cell aggregation has been attempted in a mixed cultivation of all isolated pure cultures, but full formation kefir grains is not yet observed after 1.5 years of observation and reinoculations.

Keywords

Kefir grains • Starter • Kefir drink • Composition of microbial communities • Lactic acid bacteria • Yeasts

1 Introduction

Kefir drink is a lactic and acetic acid/alcohol-containing fermented milk. Kefir is a useful and nutritious product with unique organoleptic qualities, having a positive effect on the human wellness (Otles and Cagindi 2003; Leite et al. 2013). It is produced using a complex, naturally established microbial community of numerous microbial genera and species, which are assembled in an aggregated state, called kefir grains. Numerous research efforts to identify the precise structural and functional organization of

I.B. Kotova, T.A. Cherdyntseva, and A.I. Netrusov (✉)
Microbiology Department, Moscow Lomonosov State University, Moscow 119992, Russia
e-mail: anetrusov@mail.ru

the community have not been successful. Therefore, kefir, being essentially a natural probiotic, can not be classified as probiotic products on formal criteria. In this regard, identification of the members of kefir microbial community and the definition of their relationship is the actual problem. According to modern concepts, prevailing kefir grains microbiota includes yeast, lactic acid and acetic acid bacteria (LAB and AAB) of several groups (Angulo et al. 1993; Sarkar 2008; Leite et al. 2013). There are mesophilic cocci from genera *Lactococcus* and *Leuconostoc*, mesophilic and thermophilic rods of the genus *Lactobacillus*, acetic acid bacteria, as well as fermenting and non-fermenting lactose yeast from genera *Kluyveromyces, Dekkera* and *Saccharomyces* could be found in various kefir grains (Simova et al. 2002; Wang et al. 2008; Jianzhong et al. 2009; Garofalo et al. 2015). According to various observations (Wang et al. 2008; Dobson et al. 2011), dominant strains of bacteria in kefir grains' microbial community may change during kefir drink preparation. Also, the number of yeast cells increases during the process of kefir drink manufacture. Typically, kefir obtaining process includes kefir grains propagation in sterile milk (separate process), kefir grains growth and fermentation in starter preparation, removal of grains from starter and kefir drink obtaining with starter, prepared on the previous stage.

Kefir grains are elastic, slimy, cauliflower-like structures, varying from white to yellow in color, with irregular lobes, usually 0.5–2 cm in diameter (Leite et al. 2013). Kefir grains have been used for centuries in the Caucasus region as storage for the preparation of starter cultures and final kefir drink (Kabak and Dobson 2011). Kefir drink is prepared by simultaneous lactic acid/alcoholic fermentation of milk, usually bovine, but it could be supplemented by goat, sheep, buffalo, and camel or mare milk too. It has a distinct flavor and viscosity due to a mixture of lactic and acetic acids, acetaldehyde, ethanol, acetioin, diacetyl, exopolysaccharides (EPS), CO_2 and other minor bioactive compounds, produced in microbial fermentation processes as bacteriocins, amino acids, peptides, vitamins B_{12}, B_1, K and folic acid (Kabak and Dobson 2011; Leite et al. 2013; Garofalo et al. 2015).

Several studies were conducted to determine the microbial composition of kefir grains that are used to produce this healthy drink in various places throughout the world: Portugal (Pintado et al. 1996), Ireland (Garbers et al. 2004), South Africa (Witthuhn et al. 2004), Belgium (Ninane et al. 2007), Taiwan (Chen et al. 2008), Tibet, China (Jianzhong et al. 2009), Brazil, Canada and USA (Miguel et al. 2010), Turkey (Kesmen and Kacmaz 2011), Italy (Garofalo et al. 2015). Culture-depended methods have revealed that dominant LAB in kefir grains are *Lactobacillus* spp. and *Lactococcus lactis*, but bacteria of *Leuconostoc* genus are present in low numbers (Chen et al. 2008). Some studies have highlighted the importance of combining both culture-dependent and culture-independent methods (i.e. DGGE-PCR) in order to have a more complete and precise picture of the microbiota thriving in kefir grains ecosystems (Chen et al. 2008; Leite et al. 2012; Garofalo et al. 2015).

The use of preservatives and stabilizers is becoming subject to public concern and ever-tighter legislative control. Thus, manufacturers need procedures suitable to control the fermentation process, stabilizing microbial loads and extending kefir shelf-life without altering its traditional taste and flavor. An in-depth characterization of traditional kefir microbiota as well as the development of autochthonous starter cultures to be used by dairy factories seems to be essential pre-requisites to reach these goals and favor the production of kefir at industrial scale.

Furthermore, the study of structural and functional organization of the kefir grains has also a fundamental value, because kefir grain is a complex, naturally evolved, constantly aggregated, self-preserving and self-reproducing microbial community, which is considered by many

scientists (Nielsen et al. 2014), to be a new entity, and not only the sum of its components. Determination of kefir grains' microbial profile and exploring microorganisms of which they are composed can provide us with initial data for studying of cell-to-cell and population-to-population interactions which lead to microorganisms' close integration in such stable microbial ensembles in the form of 3D biofilm.

To the best of this authors' knowledge, there are no literary reports on the microbial composition of the kefir grains isolated and propagated from Russia. Though Caucasus region is considered to be the area of kefir grains origin, kefir grains then have spread all over Russia as well as other countries long time ago, in this respect they are still the same kind of grains. On the other hand in each region, depending on physical-chemical conditions of use and on production process flow, a "local selection" of kefir grains took place and the beverages taste differently. Consequently kefir grains from central Russia regions are most suitable for "Russian kefir type" production (in two stages), specifically under Russian conditions and in order to achieve final product's taste which is typical for those regions.

Therefore, comparative analysis of the microbial composition of kefir grain associations, starter, kefir drink, and the study of the properties of isolated pure cultures of bacteria and yeast will help to elucidate the role of different groups of microorganisms in the formation of the starter and the finished product (kefir drink). Knowing what species of microorganisms are transferred from kefir grains to the finished kefir beverage will help to establish a standard for the microbial composition of the final product. The aims of this study were to identify the microbial composition of Russian kefir grains' microbial community and to detect the microorganisms which are directly involved at different stages of kefir production, e.g. are transferred from kefir grains to starter and to the final product (ready-made kefir drink) using culture-dependent and -independent techniques, combining the results of scanning electron microscopy, viable counts on selective culture media for bacteria and yeasts and by DGGE-PCR analysis.

2 Materials and Methods

2.1 Biological Material

Constantly kept alive by regular re-inoculations, kefir grains, used for handicrafts (home-made) preparation of kefir drinks in the central Russia area, were used in this study. Several different samples of kefir grains were collected in Moscow region and then 12 samples were selected for the further studies. From those 12 samples, five were selected to make kefir drink and these products were tested for organoleptic properties with a panel of experts. The three best-quality kefir drinks were selected and from one of them, kefir grains were applied for this study as a model for development of microbial composition study algorithm and its standardization for large-scale production.

2.2 Preparing of Starter and Kefir Drink

For preparing the starter, 1 g of wet, washed kefir grains was transferred to 300 ml of sterile skimmed cow milk and incubated at 20 °C for 24 h. Then, kefir grains were removed from the starter, washed intensively by sterile tap water and used for further propagation in a fresh portion of sterile milk. For the preparing of kefir drink, 15 ml of starter were added to 200 ml of sterile skimmed cow milk and further incubated at 20 °C for 24 h (Fig. 1). Samples of kefir grains, starter and kefir made of them were studied with microscopic examination, DGGE and inoculation to different kinds of media.

2.3 The Making of Fixed Stained Cells' Preparations

To study morphological characteristics of microorganisms speciments, fixed by flame and stained with methylene blue were prepared. They were examined under light microscope Biolam Co. (St.-Petersburg, Russia) with 90X objective.

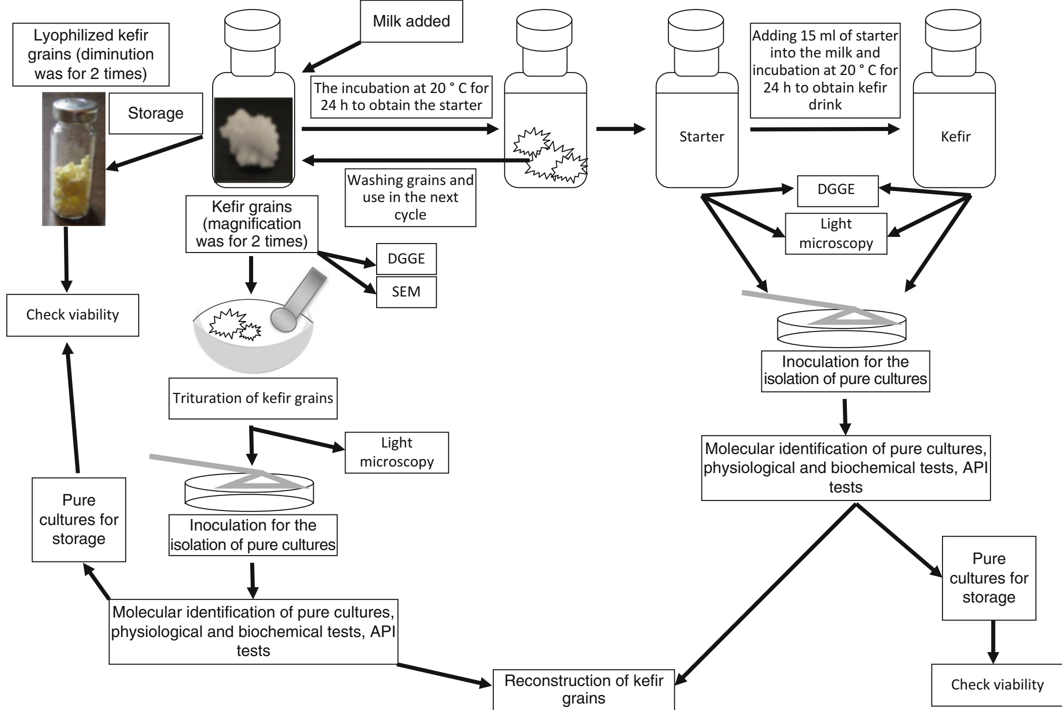

Fig. 1 Scheme of the preparation of "Russian kefir" and of the investigations

2.4 Scanning Electron Microscopy (SEM)

For kefir grains architecture visualization, community morphological variety determination and for specific morphotypes detection the kefir grain slices were fixated in 2.5 % glutaraldehyde solution and in 1.0 % osmium tetroxide, dehydrated with ascending concentrations of ethanol and with absolute acetone, then finally were sputter coated with gold particles after Solontsov and Iudina (1996). Samples were observed under a scanning electron microscope AMRAY 1830 I (USA).

2.5 Molecular Biological Analysis of Prokaryotic Component of Samples

To detect microorganisms of community that cannot be cultivated, kefir grains, samples of starter and kefir drink were analyzed with DGGE-PCR method followed by subsequent sequencing.

2.5.1 DNA Isolation

The isolation of DNA from kefir grains, samples of starter and kefir drink was done according to a technique described by Boulygina et al. (2002). The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively using spectrophotometer. Concentrations of the isolated DNA by using this method were 30–50 mkg/ml of the sample.

2.5.2 DGGE Analysis

Before carrying out DGGE analysis, a site rich G + C nucleotides was added to obtained amplicons for what reamplification was done with primer, containing G + C-clamp (515 F-GCclamp, Table 1). Obtained PCR amplicons were separated on the basis of their melting characteristics at 8 % (by volume of polyacrylamide gel) with concentration gradient of urea-formamide from 30 to 70 % [100 % corresponds

Table 1 The primers used in this study

A group of microorganisms	Gene	Procedure	Designation	Sequence (5′–3′)
Prokaryotes	16S rRNA	Amplification, DGGE	515 F-GCclamp	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGTGBCAGCMGCCGCGGTAAB
	16S rRNA	Amplification, molecular biological identification of pure cultures	8-27f	AGAGTTTGATCMTGGCTCAG
			1492r	TACGGYTACCTTGTTACGACTT
	16S rRNA	Sequencing, molecular biological identification of pure cultures	27f	AGAGTTTGATCMTGGCTCAG
			519r	GWATTACCGCGGCKGCTG
			530f	GTGCCAGCMGCCGCGG
			1114f	GCAACGAGCGCAACCC
			1492r	TACGGYTACCTTGTTACGACTT
			357f	CTCCTACGGGAGGCAGCAG
Eukaryotes (fungi)	D1/D2 domain of 28S (LSU) rDNA region	Amplification, molecular biological identification of pure cultures	ITS1f	CTTGGTCATTTAGAGGAAGTA
			NL4	GGTCCGTGTTTCAAGACGG
Eukaryotes (fungi)	D1/D2 domain of 28S (LSU) rDNA region	Sequencing, molecular biological identification of pure cultures	NL4	GGTCCGTGTTTCAAGACGG

Sanger et al. (1977), Lane (1991), Boulygina et al. (2002), Glushakova et al. (2014)

to 7 M urea and 40 % (w/v) formamide]. The electrophoresis was conducted with a constant voltage of 70 V and temperature 60 °C for 20 h using TV400-DGGE (SCIE-PLAS, England). After the electrophoresis, the gels were washed with redistilled water, stained with SYBR® Gold and bands were visualized by a transilluminator at 470 nm wavelength. All visible bands were excised, placed in vials containing 20 mkl of distilled water and left in the refrigerator overnight to elute DNA from gels. 1 μl of each eluate were used as a template for the second PCR with appropriate primers. Reamplificated products were purified by electrophoresis in 1.5 % agarose gel, from which DNA gel bands were cut and purified using a DNA purification kit (Cytokine Co., Moscow, Russia).

2.6 Media and Isolation of Pure Cultures

To isolate of cultivated pure strains of bacteria and yeast cultures and for the further study of their properties we used the media, allowing the accumulation of lactic, acetic acid bacteria and yeasts: MRS broth and agar (Merck, Germany); M-17 agar medium (Fluka, Switzerland) supplemented with 10 % lactose; PDA medium (Scharlau, Spain); 7 °B wort and wort agar (1.5 %, WA); liquid 7 °B wort with 7 % ethanol and wort agar (1.5 %) with ethanol (WAE); Saburo medium and glucose-peptone medium (all – from SSC of Applied Microbiology, Obolensk, Russia). All media were sterilized in an autoclave at 0.5 atm. To prevent fungal growth, a sterile solution of cycloheximide was added in MRS and M-17 with 10 % lactose media to a final concentration of 200 mg/L. To inhibit the growth of bacteria, a sterile solution of chlortetracycline was added in the wort, WA and Saburo medium to a final concentration 100 mg/L.

Active kefir grains look like swollen whitish granules with rubberlike texture, which can be elastically crumpled, and then restored to form, when attempting to grind them in a porcelain mortar with pest. Cutting with a scalpel did not give the desired degree of homogenization. Therefore, to grind 1 g of the grains in a sterile porcelain mortar, a finely dispersed abrasive material (sterile zeolite powder) was added, which resulted in a sufficient homogeneity of the sample. In the process of grinding, 10 ml of sterile tap water was added to sample. Microscopic control of the resulting suspension verified the preservation of the integrity of the majority of cells by this treatment. Further 10-fold dilutions of suspension of kefir grains, starter and kefir drink were prepared to degree 10^{-10}. 0.1 ml of each of 10^{-1}–10^{-10} dilutions were plated (in duplicate) with a spatula onto Petri dishes with agar media (MRS, M-17 and WA). Inoculated plates were incubated at 30 °C under aerobic conditions and in anaerobic boxes using Genbox packages (BioMerieux, France; www.apiweb.biomerieux.com). After 3–5 days of growth, the plates were viewed, description of the colonies and microscopic examination were carried out, and re-inoculation of cells from individual colonies was performed into appropriate liquid medium in serum vials with rubber stoppers (MRS for bacteria, wort and glucose-peptone medium for yeasts). After their cultivation at 30 °C and at developing of turbidity, light microscopy was carried out, and examination for purity was performed by Koch method on solid media of MRS, M-17 with 10 % lactose, WAE (for bacteria) and WA, WAE, PDA, Saburo media and glucose-peptone medium (for yeasts). After confirmation of the purity, the cultures were again grown in liquid media and microscopically checked.

2.7 Identification of Pure Cultures of Microorganisms

The taxonomic positions of isolated cultures were further identified by complete sequencing of 16S rRNA gene and by phylogenetic analysis of nucleotide sequences. The isolation of DNA from a biomass of bacteria was done according to a technique described by Boulygina et al (2002). The quantity of the isolated DNA for the use of this method was 30–50 mkg/probe. The primary

analysis of 16S rRNA gene sequence of the studied strains was performed using the BLAST software package (Camacho et al. 2009).

The genetic identification of isolated strains of yeasts was carried out on the basis of the analysis of the nucleotide sequences of D1/D2 domain of 28S (LSU) rDNA region (Table 1, Glushakova et al. 2014). The species identification was performed according to the databases of CBS (www.cbs.knaw.nl) and Genebank NCBI (www.ncbi.nlm.nih.gov).

Isolated pure cultures were tested for the ability to use different sources of carbon and energy with the API-test (BioMerieux, France). The bacterial cells were grown in serum vials with a liquid MRS medium with glucose for 1–2 days, then they were separated from the medium by centrifugation in serum vials for 10–15 min at 5000 *g* and washed three times in sterile saline solution followed by centrifugation, then suspended in 10 ml of liquid MRS without substrate but with dye. The vials were shaken until homogeneity of cell suspension and aliquots were applied for API-test. For carrying out the API-test with yeast, their pure cultures were grown for 24 h on agar glucose-peptone medium to obtain isolated colonies. Then, several colonies were transferred by loop into 2 ml of sterile saline solution to obtain a suspension of the desired turbidity. 100 μl of the suspension was introduced into the vial with the API C medium and homogenized with a pipette, avoiding the formation of bubbles. The implementation of API-tests was performed in accordance with the manufacturer's instructions using the API 50CH kit for identification of lactic acid bacteria and API 20CAUX – for identification of yeasts and other fungi. Lactic bacteria-inoculated API strips were incubated for 48 h and for yeasts and fungi – for 72 h, at 30 °C for both. The interpretation of results was performed using the manufacturer's software (www.apiweb.biomerieux.com).

The Gram-status of isolated bacteria was determined using a rapid test with 3 % KOH solution. The presence or absence of mobility and endospores in isolated cultures were determined by microscopy after staining. The test for the presence of oxidase was performed using a moistened oxidase discs (HiMedia, India). To test for catalase activity, a few drops of 3 % hydrogen peroxide were added to the colonies on an agar medium in a Petri dish.

To study the sensitivity of microorganisms to the presence of oxygen in the environment, along with changes in pH and temperature, the cells were grown at temperatures of 20, 30 and 37 °C, in the pH range from 3.0 to 8.0, in both aerobic and anaerobic conditions.

2.8 Long Storage of Kefir Grains and of Isolated Pure Cultures of Microorganisms

Periodic inoculations were used for the keeping of active kefir grains, isolates from them and pure cultures of bacteria. The grains were separated from fermented milk by a sieve, washed with sterile tap water, transferred into a new sterile container, filled with portions of sterile milk and placed in a refrigerator at 4 °C every 1–3 weeks. The bacterial cultures were reinoculated every 3–6 months to liquid anaerobic MRS medium in vials and sealed with rubber stoppers and aluminum caps. Yeasts were grown on the beveled WA and glucose-peptone agar media for 6–7 days at 30 °C, then covered with sterile vaseline oil and kept in a refrigerator at 4 °C for more than 6 months.

To test the ability of kefir grains and isolated microorganisms to maintain their properties during long-term preservation, to create a pool of standard inoculums and to avoid frequent re-inoculations lyophilization in the skimmed milk with 5 % glucose + 5 % lactose (protective medium) was performed (Rapoport et al. 2009). The washed young small kefir grains were placed in sterile Petri dishes and covered with two layers of sterile gauze fixed by adhesive tape. Isolated bacteria and yeasts grown at 30 °C, respectively, in a liquid MRS medium for 3–5 days and in glucose-peptone medium for 6–7 days in serum vials were concentrated by centrifugation at 5000 *g* for 15–20 min. The supernatant was removed by syringe, and the cells were

resuspended in 1 ml of a protective medium. Prepared samples were frozen at −20 °C and placed for the lyophilization in Free Zone freeze-drier (Labconco, USA) at T = −51 °C and P = 49 kPa for 24 h. After drying, kefir grains and pure cultures were placed in sterile serum vials, which were closed by rubber stoppers with aluminum caps and then were stored at 4 °C. After 5–7 days of storage the samples were checked for viability. 0.5 ml of the appropriate sterile liquid medium was added to the vial, mixed well and the entire volume of the slurry was transferred in the serum vial with the same medium. After incubation of bacteria and yeasts at 30 °C for 2–7 days, the developing of significant turbidity samples were inoculated on appropriate agar medium in Petri dishes. The clot formation in the milk served as testimony to the vitality of kefir grains. Freeze-dried grains (0.1 g) were placed in sterile jars and filled with 100 ml of sterile milk (Lianozovsky milk factory, Moscow, Russia, 3.2 % fat content) at room temperature. The necks of the jars were covered with four layers of sterile gauze and fixed with circular rubber bands. The jars were left to stand at room temperature for 1–3 days, stirred daily, watched for changes in cultural characteristics and performed microscopy. The living kefir grains had to curdle milk, swell, ascend and form a "cap". The active grains were thoroughly rinsed with sterile tap water and used for the preparation of starter.

2.9 The Mixed Cultivation of the Isolated Microorganisms

To establish potentiality of forming artificial kefir grains with defined composition using microorganisms with prescribed properties and to observe the stages of aggregate forming we conducted mixed cultivation of all the pure isolated cultures. Defined and standard microbiota composition is one of the probiotic product characteristics which define organoleptic properties of final beverage. To observe the possible aggregation of isolated pure cultures of microorganisms, cells for kefir grains "reconstruction", previously grown on a suitable liquid media (for bacteria – in MRS, for yeasts – in glucose-peptone medium) at 30 °C for 3–7 days. Then 1 ml of each pure culture of microorganisms was added to sterile 100 ml jars with 50 ml of sterile liquid MRS medium or sterile skimmed milk. The jar necks were covered by four layers of sterile gauze and food film, fixed by circular rubber bands, and then they were incubated at room temperature (about 20 °C), the development of mixed cultures was observed daily and thoroughly stirred. At intervals of 3–5 days, liquid mounts were made, fixed, stained with safranin and examined by light microscopy. These types of experiments were performed for 1.5 years.

3 Results

3.1 Process Stages of "Russian Kefir" Production and Morphological Variety of Kefir Grains, Starter and Kefir Drink Community

After 3 days, kefir grains turned the milk into curds, which swelled, surfaced and formed a "cap". While on the bottom of the jars they have not been observed, "eyes" (large CO_2 gas bubbles) can be seen in the thick clot. Microscopy of the liquid phase of samples showed an abundance of thin rods and small cocci, as well as a small number of large coccoid or oval cells appeared singularly, in pairs or in short chains. After the discount of starter and the washing of kefir grains with sterile tap water, both large and small white grains sized from 0.5 up to 2 cm in diameter were found (Fig. 1). Electron micrographs showed that the surfaces of the kefir grains were smooth, bumpy and had a gelatinous matrix substance, which covered the cell clusters above in the form of a thin polysaccharide film (Fig. 2a, c). Microbial cells are clearly seen from the surface in case of damage (after cutting with a scalpel). Cell-free cavities were observed inside the kefir grains (Fig. 2b). Rare bands (perhaps it was the long thread of polysaccharide films) crossed these cavities. Rod-shaped

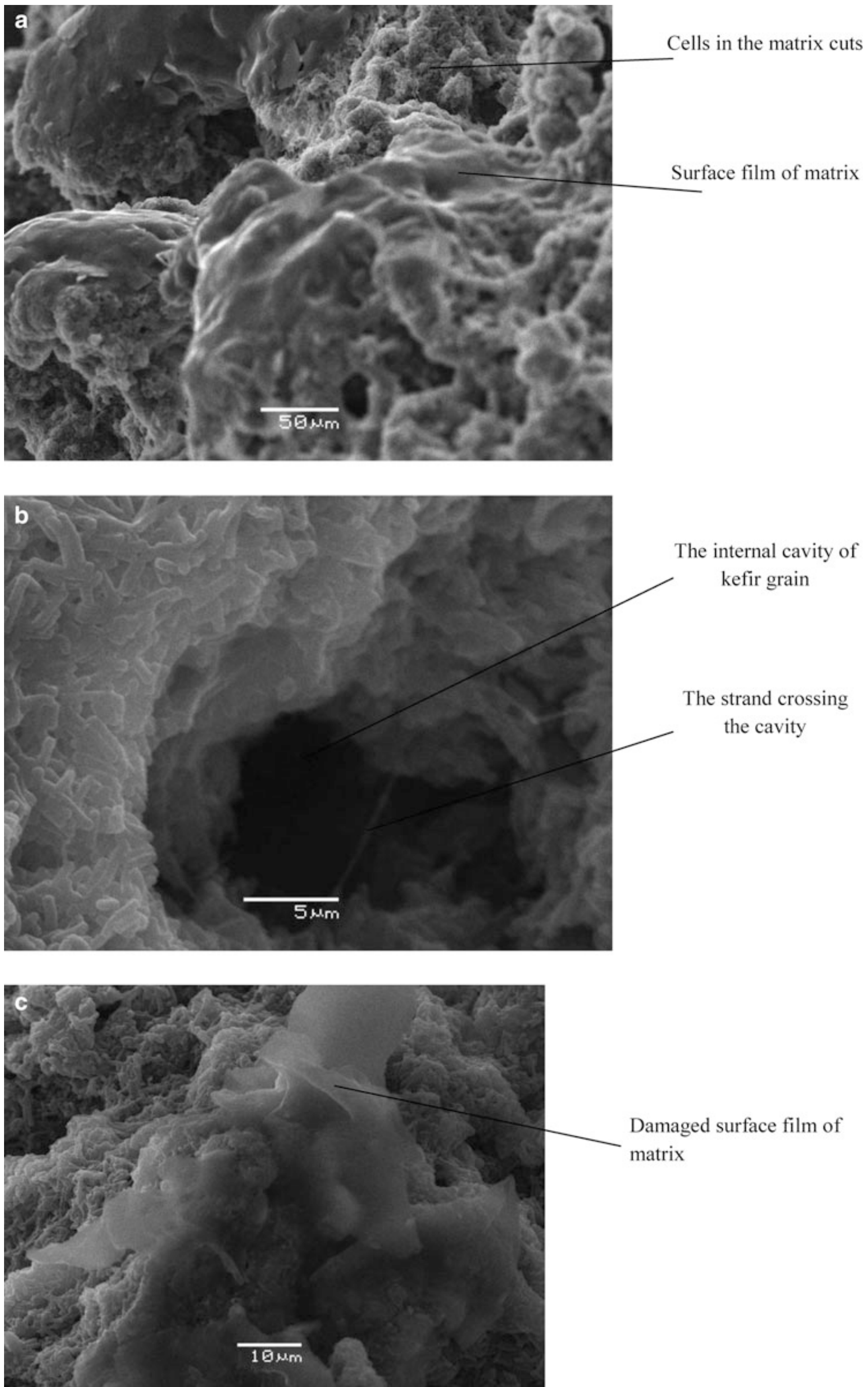

Fig. 2 Architecture of kefir grain microbial community: surface (**a**), the inner cavity (**b**) and the matrix (**c**) of kefir grain (SEM)

cells of different sizes were predominant in the community of kefir grains. Small coccoid and oval cells were presented in a significantly less amount, and yeast-like large oval and round cells were found in the amount of 1–3 in the field of view (Fig. 3a, c). Clusters of globules (possible precipitated proteins) were seen in some micrographs (Fig. 3a, b).

The starter and kefir drink obtained in repeated cycles (Fig. 1) had stable organoleptic properties and characteristic microscopic picture. The starter had a sharp, sourish taste and sour cream consistency with many "eyes", formation of which was not observed. A large number of small cocci and thin non-sporulating rods, a large number of dark-colored large diplococci and single typical yeast cells were observed in wet mount preparations. The kefir drink had a balanced taste and a thick sour cream consistency with many "eyes", also without which a layer formed. Small cocci and thin rods prevailed in the field of view, dark-colored medium-sized cocci in short chains and typical yeast cells were observed.

3.2 The Detecting of the Prokaryotic Microorganisms Composition Differences at Kefir Grains, Starter and Kefir Drink Communities

The comparison of the DGGE data for kefir grains, starter and kefir drink (Fig. 4) showed that many of the strains had approximately equal strength (except bands 7, 8 and 20) in grains, but in starter and kefir drink only six (bands 9–12, 15 and 17) and seven (bands 1–6 and 19) strains were dominant respectively. Also, three distinct bands (13, 14, and 16) with lower intensity were present in the starter sample. In the kefir drink, sample "blurred" bands in gel were also visible in these areas. In other parts of the gels of starter and kefir drink samples, bands or areas of very low intensity were observed, which could not be amplified. All well distinguishable bands (24 specimens) were sequenced consecutively (Fig. 4 and Table 2).

In kefir grains samples, seven bands could clearly be seen (Fig. 4), represented by strains of the species *Lactococcus lactis* subsp. *cremoris* and *lactis*, *Lactobacillus kefiri* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum* (Table 2). Three prokaryotic strains (bands 7, 8 and 20) were dominant in this community: (1) a microorganism that was most closely cognate *Lactococcus lactis* subsp. *cremoris* strain MFPB22D06-03 (98 % similarity of 16S rRNA sequences), (2) a strain that was most closely related to *Lactobacillus kefiri* (98 % similarity of 16S rRNA sequences) and (3) *Lactobacillus kefiranofaciens* subsp. *kefirgranum* (99 % similarity of 16S rRNA sequences). The last microorganism, apparently, was presented in the form of three different strains (one was dominant and the other two were minor). Two strains of *Lactococcus lactis* subsp. *lactis* also had insignificant number of cells in kefir grains.

Ten well visible bands (Fig. 4 and Table 2), attributed to members of the genus *Lactococcus,* were present in starter samples. These belong to different strains of the two subspecies of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*.

In the kefir drink samples, seven visible bands (Fig. 4 and Table 2) were attributed to representatives of the two subspecies of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*.

Lactobacilli were not detected as distinct bands in starter and kefir drink. Probably, they are presented in quantities not detectable by this method. Thus, lactococci have profited in starter and kefir drink compared with a kefir grains sample and lactobacilli became a small group. Strain composition of kefir drink and starter seem to be similar, but the number of distinct bands in the kefir drink decreased in comparison with the starter. Thus, the same microorganisms (bands 9–12, 15, 17 and 1–6), which were not dominant in grains, prevailed in starter and kefir drink. Two organisms were most similar to various strains of *Lactococcus lactis* subsp. *cremoris*, and three others to three different strains of *Lactococcus lactis* subsp. *lactis*. Not all strains present in kefir grains were developed in fermented milk products. So, lactobacilli were not revealed in

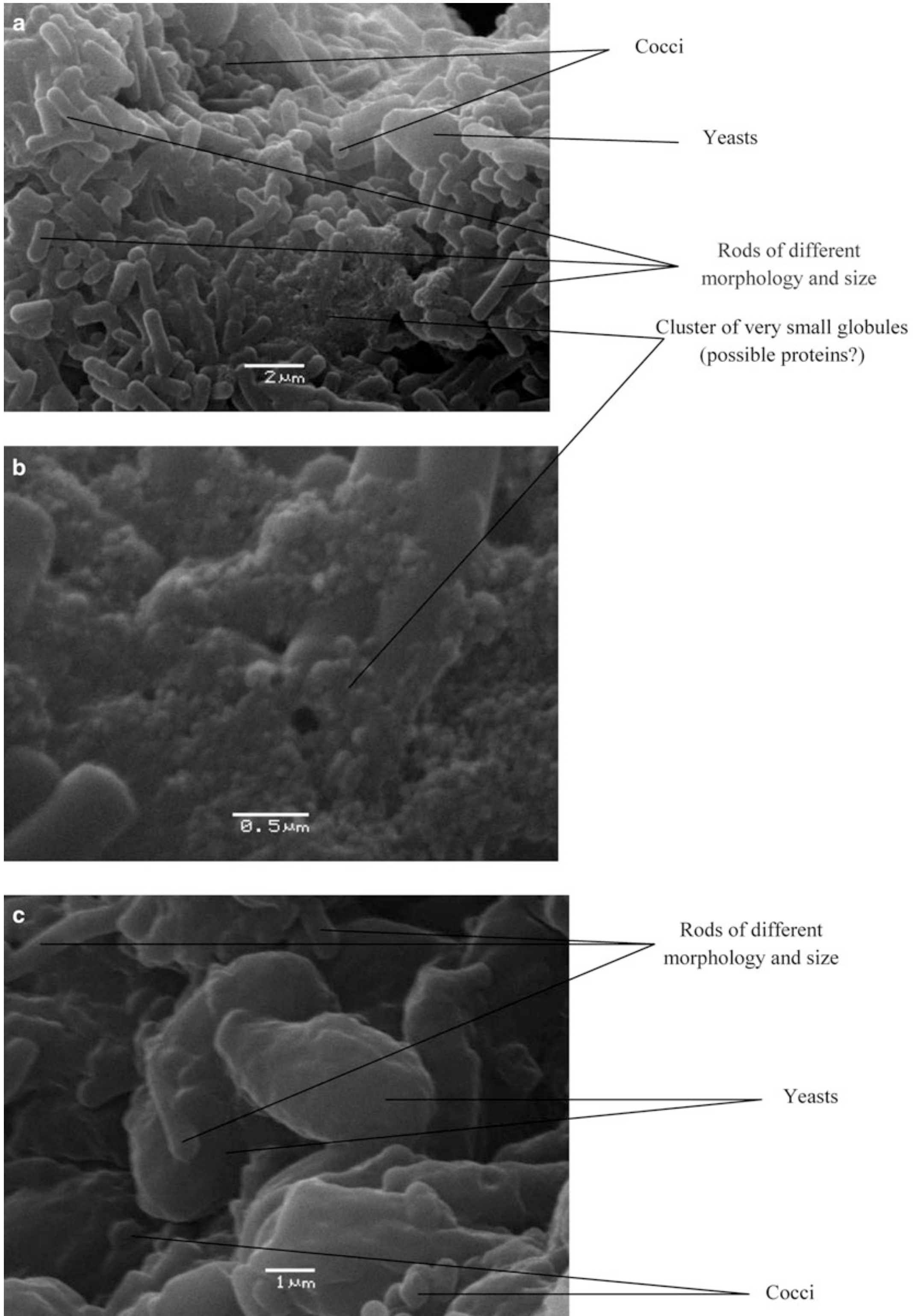

Fig. 3 Morphological diversity and spatial arrangement of cells in the microbial community of kefir grains. Different morphotypes of cells are presented, their close physical contact and location relative to each other can be seen (SEM)

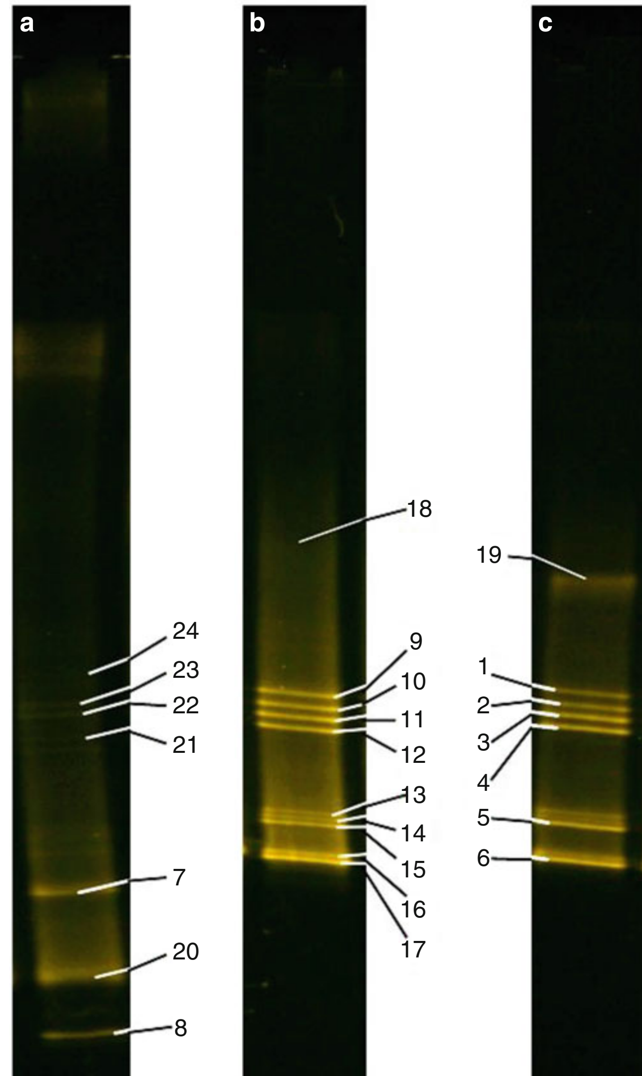

Fig. 4 DGGE gels photos of samples of kefir grains (**a**), starter (**b**) and kefir drink (**c**). Band's numbers correspond to that in Table 2

them, although they were in significant numbers in kefir grains. At the same time, it could be noticed that cells in grains initially were in a "concentrated" state, i.e. kefir grains sample contained microorganisms in a significantly higher number than in samples of dairy products (starter and kefir drink). Moreover, closely related strains of lactobacilli gave a single band on the DGGE gel. However, even taking into account the above remarks, DGGE results indicated change in the dominant groups of microorganisms under development in milk and in the process of fermentation. Two bacterial subspecies of the species *Lactococcus lactis* were detected in kefir drink and in starter. However, some strains were the same as prevailed in the starter, and the other part was replaced by other strains. At the same time, it could be argued that the lactococci are those microorganisms that pass from kefir grains in milk and then are able, through the starter, to get to the final product in significant amounts. Observed in microscopic starter and kefir drink preparations lactobacilli, apparently, is a minor group, the number of which is below the level of sensitivity of this analytical method (DGGE).

Table 2 Comparative analysis of microbial communities composition in the kefir grains, starter and kefir drink, determined by two methods with correspondent band numbers on DGGE gels (band's numbers correspond to that in Fig. 4)

Microorganisms detected in the communities					
Kefir grains		Starter		Kefir drink	
DGGE	Pure cultures (GenBank number in parentheses)	DGGE	Pure cultures (GenBank number in parentheses)	DGGE	Pure cultures (GenBank number in parentheses)
Lactococcus lactis subsp. *cremoris* (one strain – the band number 7)	Nf	*Lactococcus lactis* subsp. *cremoris* (four strains – №№ of bands 9, 13, 14 and 15)	Nf	*Lactococcus lactis* subsp. *cremoris* (three strains – №№ of bands 1, 2 and 5)	*Lactococcus lactis* subsp. *cremoris* K-24 (**KF234767**)
Lactococcus lactis subsp. *lactis* (two strains – №№ of bands 21 and 22)	*Lactococcus lactis* subsp. *lactis* KG-8 (**KF263161**)	*Lactococcus lactis* subsp. *lactis* (six strains – №№ of bands 10, 11, 12, 16, 17 and 18)	Nf	*Lactococcus lactis* subsp. *lactis* (three strains – №№ of bands 3, 4 and 6)	Nf
Nf	Nf	Nf	Nf	*Lactococcus lactis* (one strain – the band number 19)	Nf
Lactobacillus kefiri (one strain – the band number 8)	*Lactobacillus kefiri* KG-17 **(KF263157)**	Nf	Nf	Nf	*Lactobacillus kefiri* K-22 **(KF234766)**
Lactobacillus kefiranofaciens subsp. *kefirgranum* (three strains – №№ of bands 20, 23 and 24)	Nf	Nf	Nf	Nf	Nf
Nf	*Lactobacillus casei* KG-3 (**KF263159**), KG-5 (**KF263160**) and KG-9 (**KF263156**)	Nf	*Lactobacillus casei* S-18.2 (**KF263164**)	Nf	nf
Nf	Nf	Nf	*Lactobacillus paracasei* S-18.1 (**KF263163**)	Nf	Nf
Nf	Nf	Nf	*Leuconostoc pseudomesenteroides* S-2 (**KF263165**), S-7 (**KF263162**) and S-14.2 (**KF263158**)	Nf	*Leuconostoc pseudomesenteroides* K-26 (**KF234768**), K-28.1 (**KF234769**) and K-28.2 (**KF263154**)

(continued)

Table 2 (continued)

Microorganisms detected in the communities					
Nf	*Saccharomyces cerevisiae* var. *cerevisiae* KG-21.1 (**KJ162151**)	Nf	*Saccharomyces cerevisiae* var. *cerevisiae* KG-21.1 (**KJ162151**)	Nf	*Saccharomyces cerevisiae* var. *cerevisiae* KG-21.1 (**KJ162151**)
Nf	*Saccharomyces cerevisiae* var. *cerevisiae* KG-20.1 (**KJ162152**)	Nf	*Saccharomyces cerevisiae* var. *cerevisiae* KG-20.1 (**KJ162152**)	Nf	*Saccharomyces cerevisiae* var. *cerevisiae* KG-20.1 (**KJ162152**)
Nf	*Kazachstania unispora* KG-25.1 (**KJ162150**)	Nf	*Kazachstania unispora* KG-25.1 (**KJ162150**)	Nf	*Kazachstania unispora* KG-25.1 (**KJ162150**)
Nf	Nf	Nf	Nf	Nf	*Gibellulopsis piscis* K-30 (**KJ162153**)

Notes: *Nf* not found, numbers in bold in parentheses correspondent to GenBank number accession numbers of submitted sequences of 16S rRNA genes of pure cultures isolated in this study

3.3 Determination of Microbial Composition of Kefir Grains, Starter and Kefir Drink Communities Using Cultivation (Classical Microbiological Analysis)

Classical microbiological analyses were performed in parallel by plate inoculation of samples suspension's dilutions on agar media. Analysis of platings of kefir grains samples showed that the total number amenable to cultivation in these media bacteria and yeasts did not exceed 10^9 cells per 1 g of wet biomass. More morphotypes of colonies grew on all media under anaerobic conditions than in the presence of oxygen. At the same time it should be noted that identical morphological colonies may be formed by various strains of microorganisms, and the same cells on different media and under different conditions can form morphologically different colonies. At least 8 colonies morphotypes for kefir grains were recorded on all media in total. By comparison of results of samples of kefir grains, starter and kefir drink it was found that no more than five morphotypes of colonies existed. It was also noted that some isolates did not grow at the second passage to liquid or solid media. At the same time, the growth of some small colonies over or under large colonies was observed. In this case, the separation of the isolates was difficult because the cells of both colonies existed as co-culture, but could not grow alone in general. At the same time, part of the binary cultures managed to divide into two pure cultures or one of these components to isolate from them. Altogether we isolated fifteen bacterial pure cultures and ten yeast pure cultures (in accordance with its morphological features) from thirty-four primary isolates after checking for purity and after periodic passages.

3.4 Identification of Pure Culture Isolates and Their Properties Determination

A combination of molecular, morphological, physiological and biochemical tests were used to determine the taxonomic position of isolated pure cultures. The complete sequence of 16S rRNA genes was performed for pure cultures of bacteria and determination of the nucleotide sequences of D1/D2 domain 28S (LSU) rDNA region – for yeasts. The species names of isolated bacteria and yeasts were obtained by comparing the similarity of their nucleotide sequences corresponding to genes with sequences available

in the database of the NCBI GenBank (www.ncbi.nlm.nih.gov), with a high degree of similarity (98–100 %). The sequences of all isolated pure cultures were deposited in the NCBI GenBank (Table 2). All isolated pure cultures were tested for the ability to use a variety of carbon and energy sources with API-test (BioMerieux, France). The auxiliary rapid identification of microorganisms was also performed. API-testing confirmed that the isolated bacterial cultures belong to the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc,* however, in some cases, the taxonomic position of bacteria determined using API tests did not coincide with the results of molecular identification. The API-testing program observed identification as "doubtful", if the generic names did not match, and from "questionable" to "very good" if the species and subspecies names did not match. The API testing of yeast cultures confirmed the affiliation of nine isolates to the species of *Saccharomyces cerevisiae,* with identification observations from "acceptable" to "good". This does not contradict on the whole with the molecular identification, since a species *Kazachstania unispora* was previously called *Saccharomyces unispora*. Only for one isolate, *Gibellulopsis piscis,* API identification was deemed "unacceptable", which was explained by the absence of mold fungus in the database API 20 C AUX for yeasts. Like any other method, API-testing has its limitations and assumptions and is intended for identification of microorganisms, which are recorded in the database of API. Since the sequencing of the nucleotide basepairs is a more accurate method of identification, the results of molecular testing were used as the basis. To clarify the properties of pure cultures of bacteria, standard Bergey's tests were performed by (Bergey's Manual of Systematic Bacteriology 2007). Their results were used to formulate the characteristics of the isolated microorganisms (Tables 3 and 4). Prokaryotic microorganisms isolated from kefir grains, starter and kefir drink had a number of common features: they were Gram-positive; immobile; catalase- and oxidase-negative; asporogenous; belonged to aerotolerant anaerobes with a high resistance to atmospheric oxygen; and were able to grow well under aerobic conditions. All of them were chemo-organo-heterotrophic, need in complex media, rich in organic matter, and carried out different types of lactic acid fermentation using a significant amount of sugars in D-form.

Four lactobacilli strains of two species and one strain of lactococci were isolated from kefir grains (Tables 3 and 4). Three members of the *Lactobacillus casei* specie had differed colonial and cell morphology and the spectrum of substrates used, but had a similar attitude to the physico-chemical parameters of cultivation. Another strain of *Lb. casei*, differed in its properties from the strains isolated from the kefir grains, as well as the culture of *Lb. paracasei* and three different strains of *Leuconostoc pseudomesenteroides* specie (Tables 3 and 4), differed in morphological characteristics and substrates preferences, but grew in the same range of temperature and pH, succeeded in isolating from starter. Isolated from kefir drink, three leuconostoc representatives were different from each other in morphology of cells, colonies, and the set of growth substrates used (Tables 3 and 4). They also differed from strains obtained from the starter. The strains of *Lactobacillus kefiri*, isolated from kefir grains and finished kefir drink, were characterized by different morphological features and substrate preferences. The culture of *Lactococcus lactis* subsp. *cremoris* was also isolated from the kefir drink, whereas the microorganism of other subspecie (*lactis*) was isolated from kefir grains. Thus, fifteen pure cultures of lactic acid bacteria that are members of three genera (*Leuconostoc*, *Lactococcus* and *Lactobacillus*) were isolated from kefir grains and the corresponding starter and kefir drink. All isolated leuconostocs belong to the same specie of *Leuc. pseudomesenteroides* and, apparently, they are different strains since they have differences in morphology of cells and colonies and in the spectra of the used substrates. The lactococci were represented by two subspecies (*lactis* and *cremoris*) of one specie – *Lactococcus lactis*. Members of the species *Lb. kefiri* (two strains), *Lb. casei* (four strains) and

Table 3 Pure cultures of prokaryotes isolated from kefir grains, starter and kefir drink and their physiological-biochemical properties

Isolation source	Bacterial strain (GenBank accession number)	Description of colonies	Microscopic picture	Some physiological-biochemical properties			
				Temperature optimum for growth, °C	pH-optimum for growth	Type of lactic acid fermentation	Other properties
Kefir grains	*Lactobacillus casei* KG-3 (**KF263159**)	White round colonies, d = 2 mm, smooth, shiny, with homogeneous soft texture and uncurled edge	Thin medium size rods arranged one by one, in the form of “fence” and in short chains	30–40	5.5–6.5 Can grow up to 3.0	Homo-	–
	Lactobacillus casei KG-5 (**KF263160**)	White round colonies, d = 3 mm, convex, smooth, shiny, with homogeneous soft texture and uncurled edge	Straight thin rods, forming long chains	30–40	5.5–6.5 Can grow up to 3.0	Homo-	–
	Lactococcus lactis subsp. *lactis* KG-8 (**KF263161**)	Gray round colonies, d = 2–3 mm, smooth, curved, shiny, with homogeneous soft texture and uncurled edge	Oval, thick medium size cells arranged singly, in pairs, and in long-chains	30 Can grow up to 40 °C and up to 10 °C. No growth at 45 °C	5.5–6.5	Homo-	Cells do not form capsules
	Lactobacillus casei KG-9 (**KF263156**)	Gray round colonies, d = 2 mm, flat, smooth, shiny, with homogeneous soft texture and uncurled edge	Medium size rods containing granules, single, in pairs or in short chains	30–40	5.5–6.5 Can grow up to 3.0	Homo-	–
	Lactobacillus kefiri KG-17 (**KF263157**)	Gray round colonies, d = 1.5 mm, convex, smooth, shiny, with fine grain soft texture and wavy edge	Long pleomorphic rods in long chains	30–40	5.5–6.5 Can grow up to 3.0	Homo-	–
Starter	*Leuconostoc pseudomesenteroides* S-2 (**KF263165**)	White round colonies, d = 2 mm, smooth, convex, shiny, with homogeneous soft texture and uncurled edge	Oval, thick cells, single, in pairs and in short chains	37	5.5–6.5	Hetero-	In the presence of oxygen they form a large amount of slime polysaccharide (dextran) from sucrose
	Leuconostoc pseudomesenteroides S-7 (**KF263162**)	White round colonies, d = 2 mm, smooth, convex, shiny, with homogeneous soft texture and uncurled edge	Oval thick cells, forming long chains	37	5.5–6.5	Hetero-	In the presence of oxygen they form a large amount of slime polysaccharide (dextran) from sucrose
	Leuconostoc pseudomesenteroides S-14.2 (**KF263158**)	Gray round colonies, d = 3 mm, smooth, convex,	Petty oval, swollen cells, forming forks and chains	37	5.5–6.5	Hetero-	In the presence of oxygen they form a large amount of

		shiny, with homogeneous soft texture and uncurled edge					slime polysaccharide (dextran) from sucrose
	Lactobacillus paracasei S-18.1 (**KF263163**)	Opalescent gray round colonies, d = 3 mm, convex, smooth, shiny, with homogeneous soft texture and uncurled edge	Thick average length rods connected in chains	37	5.5–6.5 Can grow up to 3.0	Homo-	–
	Lactobacillus casei S-18.2 (**KF263164**)	Opalescent gray round colonies, d = 3 mm, convex, smooth, shiny, with homogeneous soft texture and uncurled edge	Thick short rods in chains	37	5.5–6.5 Can grow up to 3.0	Homo-	–
Kefir drink	*Lactobacillus kefiri* K-22 (**KF234766**)	White round colonies, d = 1.5 mm, convex, smooth, shiny, with homogeneous soft texture and uncurled edge	The rods of various lengths connected by two or in short chains	30–40	5.5–6.5 Can grow up to 3.8	Homo-	–
	Lactococcus lactis subsp. *cremoris* K-24 (**KF234767**)	Colorless round colonies, d = 2 mm, smooth, convex with a raised center, shiny, with homogeneous soft texture and uncurled edge	Large oval cells, forming long chains	30 Do not grow at 40 °C	5.5–6.5	Homo-	Cells do not form capsules. Do not grow with the addition of 4 % NaCl
	Leuconostoc pseudomesenteroides K-26 (**KF234768**)	Colorless transparent round colonies, d = 1.5 mm, smooth, convex, shiny, with homogeneous soft texture and uncurled edge	Large oval cells in long chains	37	5.5–6.5	Hetero-	In the presence of oxygen the cells form significant amount of mucous polysaccharide (dextran) from sucrose
	Leuconostoc pseudomesenteroides K-28.1 (**KF234769**)	White round colonies, d = 1.5 mm, smooth, convex, shiny, with homogeneous soft texture and uncurled edge	Large oval cells, single, in pairs or in long chains	37	5.5–6.5	Hetero-	In the presence of oxygen the cells form significant amount of mucous polysaccharide (dextran) from sucrose
	Leuconostoc pseudomesenteroides K-28.2 (**KF263154**)	Transparent round colonies, d = 1.5 mm, smooth, convex, shiny, with homogeneous soft texture and uncurled edge	Cocci and ovals, forming short chains	37	5.5–6.5	Hetero-	In the presence of oxygen the cells form significant amount of mucous polysaccharide (dextran) from sucrose

Table 4 Spectra of the substrates used by isolated cultures

Substrate	Strains						
	Lactobacillus casei **KG-3**	*Lactobacillus casei* **KG-5**	*Lactococcus lactis* subsp. *lactis* **KG-8**	*Lactobacillus casei* **KG-9**	*Lactobacillus kefiri* **KG-17**	*Leuconostoc pseudomesenteroides* **S-2**	*Leuconostoc pseudomesenteroides* **S-7**
L-arabinose	−	−	−	−	−	−	−
D-ribose	+	+	+	+	+	+	+
D-galactose	+	+	+	+	−	+	+
D-glucose	+	+	+	+	−	+	+
D-fructose	+	+	+	+	−	+	+
D-mannose	+	+	+	+	−	+	+
D-mannitol	+	+	−	+	−	−	+
Methyl – α-D-glucopyranoside	+	+	+	+	−	+	+
N-acetyl-glucosamine	+	+	+	+	−	+	+
Amygdalin	−	±	−	−	−	−	−
Salicin	+	+	+	+	−	+	+
D-cellobiose	+	+	+	+	−	+	+
D-maltose	±	±	+	−	−	+	+
D-lactose	+	±	+	−	−	+	+
D-sucrose	+	+	+	+	−	+	+
D-trehalose	+	+	+	+	−	+	+
Inulin	+	+	−	+	−	−	+
D-melezitose	−	−	−	−	−	−	+
Amidon (starch)	−	−	+	−	−	±	−
Glycogen	−	−	−	−	−	−	−
Xylitol	−	−	−	−	−	−	−
Gentibiose	+	+	+	+	−	±	+
D-turanose	+	+	−	+	−	−	+
D-tagatose	+	+	−	+	−	−	+

Notes: + used, − not used, ± the result was unclear or variable
None of the strain did not utilize glycerol, erythritol, D-arabinose, D- and L-xylose, D-adonitol, L-sorbose, L-rhamnose,

Leuconostoc pseudomesenteroides **S-14-2**	*Lactobacillus paracasei* **S-18-1**	*Lactobacillus casei* **S-18-2**	*Lactobacillus kefiri* **K-22**	*Lactococcus lactis* subsp. *cremoris* **K-24**	*Leuconostoc pseudomesenteroides* **K-26**	*Leuconostoc pseudomesenteroides* **K-28-1**	*Leuconostoc pseudomesenteroides* **K-28-2**
−	−	−	+	−	−	−	−
+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+
−	+	+	+	+	+	+	+
+	−	+	+	−	+	+	+
+	+	+	+	+	+	+	+
−	−	−	−	±	−	−	−
+	−	−	+	+	−	−	+
+	−	−	±	±	−	−	+
+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+
+	+	+	+	−	+	+	+
+	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−
−	+	+	−	−	+	+	−
±	−	−	+	±	−	−	+
−	±	−	−	−	−	−	−
−	−	−	−	−	−	−	+
+	−	−	+	+	−	−	−
±	+	+	+	−	+	+	+
−	+	+	−	−	+	+	−

Inositol, D-sorbitol, D-melibiose, D-raffinose, D- and L-fucose, D- and L-arabitol

Table 5 Pure cultures of eukaryotic microorganisms isolated from kefir grains, starter and kefir drink

Isolation source	Microorganism (GenBank number)	Description of colonies	Microscopic picture	Some properties	
				The growth temperature	Attitude to oxygen
Kefir grains, starter and kefir drink	*Saccharomyces cerevisiae* var. *cerevisiae* KG-21.1 (**KJ162151**)	Round white colonies, d = 4–5 mm, smooth, conical, matte, with homogeneous soft texture and toothed edge	Large oval grainy budding cells with vacuoles. Form ellipsoidal ascospores with a smooth wall	Under aerobic conditions, the cells grow well at 20–25, slow at above 30 and is absent at 37 °C	Facultative anaerobes
	Saccharomyces cerevisiae var. *cerevisiae* KG-20.1 (**KJ162152**)	Round white colonies, d = 8–9 mm, wrinkled, flat, matte, with fine grain soft texture and wavy edge	Large oval grainy curved budding cells. Form ellipsoidal ascospores with a smooth wall	Under aerobic conditions, the cells grow well at 20–25, slow at above 30 and is absent at 37 °C	Facultative anaerobes
	Kazachstania unispora KG-25.1 (**KJ162150**) (previously – *Saccharomyces unisporus*)	Round brown colonies, d = 1.5 mm, smooth, convex, matte, with fine grain soft texture and wavy edge	Large oval grainy curved cells. Form ascospores	Under aerobic conditions, the cells grow well at 25, slow at above 37 and is absent at 45 °C	Facultative anaerobes
Kefir drink	*Gibellulopsis piscis* K-30 (**KJ162153**)	Round white, slow-growing, immersed in agar colonies, d = 3 mm (on the ninth day), with concentric circles on the surface, convex, matte, with filamentous edge	Large curved oval budding cells and hyphae. In the early stages of development of culture is represented by yeast anamorphs	The cells grow well at 20–25 °C under aerobic conditions	Microaerophilic

Notes: + used, − not used
None of the strain did not use xylitol, Inositol, D-sorbitol, D-lactose, D-trehalose

Substrates used:											
D-glucose	L-arabinose	D-xylose	D-galactose	D-cellobiose	D-maltose	D-sucrose	D-melezitose	D-raffinose	glycerol	N-acetyl-glucosamine	Methyl-α-D-glucopiranoside
+	–	–	+	–	–	+	–	+	–	–	–
+	–	–	+	–	+	+	–	+	–	–	–
+	–	–	+	–	–	–	–	+	–	–	–
+	+	+	+	+	+	+	+	+	+	+	+

Lb. paracasei (one strain) were found among lactobacilli, and the strains of the same species had morphological peculiarities and different substrate preferences.

Ten pure cultures of eukaryotic microorganisms classified in four strains were isolated from kefir grains, starter and kefir drink. It was found that three pure cultures of each strain (*Saccharomyces cerevisiae* KG-20.1, *S. cerevisiae* KG-21.1, *Kazachstania unispora* KG-25.1), obtained from three sources, were identical, i.e. these strains are "transferred" from kefir grains in starter at first and then, finally, to a kefir drink (Table 5). Two strains of the specie *Saccharomyces cerevisiae* were different in morphological features and spectrum of substrates used. The strain *Gibellulopsis piscis* was able to isolate only from kefir drink. At the early stages of development of this culture, yeast anamorphs was presented, and then formed the typical fungal mycelium, generating a limpid vegetative hyphae and erected conidiophores. The conidiophores were slightly differing from the vegetative hyphae, bearing one-two nodes with one-three subulate phialides. The conidia which formed were ellipsoidal or short cylindrical, in most cases, simple. Chlamydospores, single or in chains, with or without vitreous cells, abundantly formed on their ends or intercalarily after 10 days of incubation. Thus, the three pure cultures of yeasts that are members of two genera were isolated from kefir grains and from the corresponding starter and kefir drink. The fourth pure culture was a mold fungus (*Gibellulopsis piscis* K-30) obtained only from kefir drink (Table 5).

3.5 Differences of Microbial Compositions of Kefir Grains, Starter and Kefir Drink Communities

Comparative analysis of the species composition of communities of kefir grains, starter and kefir beverage (Table 2) showed that not all strains of cultured microorganisms present in the grains, were able to develop well in milk and dairy products as planktonic cells. Thus, none of the strains of *Lactobacillus kefiranofaciens* subsp. *kefirgranum*, identified by using DGGE-PCR method, were possible to isolate using standard inoculations. At the same time, the number of strains which were not detected by DGGE analysis (probably due to the small number of cells) was obtained as pure cultures after plating (members of the *Leuconostoc pseudomesenteroides* specie). It should be noted that not only the strains, that are numerically dominant in the community, were easier to isolate, but also those minor groups that are more suited to the culture conditions in media used in this study. Therefore, according to the microbiological analysis, we can definitely state about changing the composition of the microbial community in the row "kefir grains – starter – kefir drink" towards increasing coccoid cultures, but also argue that they are numerically dominated in associations of these dairy products.

Microbiological analysis allowed the revealing of the eukaryotic component of these communities in the form of "passing" from grains to the kefir drink of three yeast strains, as well as isolated, only from kefir drink, a mold fungus strain *Gibellulopsis piscis* K-30 (Domsch et al. 2007; Kurtzman et al. 2011), which was not present in the plating of kefir grain and starter samples (see Table 3). Using the DGGE analysis, data and direct microscopic observations, one may judge not only the changing of the component's composition (Table 2), but also the change of the dominant strains in associations of kefir grains and dairy products (starter and kefir beverage). Thus, according to the results of SEM, rod-shaped microorganisms, numerically dominated in grains, was confirmed by the intensity of the amplicon bands of lactobacilli members on DGGE gels. In dairy products, according to DGGE data, there was a significant increase in the number of coccoid cells, and that was also evidenced by microscopic observations (light microscopy) and does not contradict with microbiological analysis. Thus, using several methods, we found that in the consecution of "kefir grains → starter → kefir drink" succession of microbial communities occurs, which is

expressed in changes in species composition, ratios of microbial cultures and in changes of the dominant species. Lactobacilli were outnumbered by lactococci in the row of kefir grains to kefir drink and coccoid cells are dominating in the finishing product.

3.6 An Attempt of Kefir Grains Formation

There was recorded acidification and significant clouding of MRS medium, acidification and converting of milk to curds after five days upon pure mixed cultures inoculation. The formation of a thick dense sediment and turbidity reduction was observed in the MRS medium on the fourteenth day. The precipitate was "broke" into very small angular aggregates after vigorous stirring. When shaking milk, it was noted that the lower portion of the culture fluid had a significantly greater density. Microscopy showed the propensity of cells in both media to the formation of clusters and microaggregates containing different morphotypes of cells. The sediment of small grains, representing aggregates of rods and cocci of various sizes and large oval cells, covered with mucus, was located on the bottom of flasks in both media after 1.5 months of mixed pure cultures cultivation with regular re-inoculations. The aggregates in the milk appeared larger and friable, but contained less mucus than cellular aggregates in a liquid MRS medium. Thus, isolated pure cultures were able to form aggregates, resembling young kefir grains, however, the formation of high-grade artificial "kefir grains" were not yet observed even after 1.5 years of cultivation with regular re-inoculations in two media (skimmed milk and MRS).

3.7 Long-Time Storage of Isolated Pure Cultures of Microorganisms and Kefir Grains

It was shown that pure cultures of lactic acid bacteria isolated in this study were well stored at 4 °C in the liquid MRS medium in serum vials, if they were subcultured into fresh medium every 0.5–2 months. Yeast cells can maintain their properties when stored in the WA and glucose-peptone agar medium, under mineral oil, at 4 °C for 6 months. The checking of the viability of freeze dried cultures of the isolated microorganisms, kept in a refrigerator at 4 °C, showed that their titer in all tested cases was not less than 10^5 cells per 1 mg of solid. Thus, lyophylization allowed the keeping of the culture without losing their properties for more than 1.5 years. The "revival" of lyophilized kefir grains samples (Fig. 1) was performed at 2, 6, 12 and 18 months after lyophilization. It was found that the kefir grains do not lose the ability to produce good kefir starter after 18 months of storage in a lyophilized state. Storage experiments with lyophilized kefir grains will continue for more than 3 further years.

4 Discussion

It was known from ancient times that the products, obtained from milk by using kefir grains, have beneficial health effects, particularly for intestinal disorders and increasing lifespan. Now, kefir grains are widely used in industry to produce dairy products that suppress pathogenic microbiota (Rodrigues et al. 2005; Londero et al. 2011). Studies of kefir grains, whose compositions vary, are conducted in many countries (Garrote et al. 2001; Wang et al. 2008; Jianzhong et al. 2009). For example, with Argentine grains, researchers found 24 strains of yeasts and 23 bacterial strains. The microbial communities of three kinds of Tibetan kefir grains were similar in bacterial composition on 78–84 %, and in the composition of the yeasts – on 80–92 %. In general, lactic acid bacteria (lactobacilli and lactococci), isolated from kefir grains, can include several strains of the same species (Simova et al. 2002; Golowczyc et al. 2008).

The DGGE-PCR method was widely used, throughout the world, to study the composition of kefir grains. Molecular methods allow

indicating the microorganisms in community which cannot be detected by inoculation and growth: uncultivated, syntrophic, the ones that do not grow on the chosen media, the ones which initial amount in community was small, microorganisms with low growth rate. However, this method cannot be considered universal, as was shown by various researchers (da Cruz Pedrozo et al. 2010; Chen et al. 2011). If the composition of kefir grains includes a large number of microbial species, then there is no clear separation of electrophoretic bands during DGGE procedure. In addition, amplicons of closely related strains cannot be separated by this method. Species with minor amount of cells are difficult to determine by DDDE. Therefore it is necessary to apply several methods that could complement each other. In the case of kefir grains, minor species which were not determined by DGGE-PCR, can grow well in milk, accumulate there and then be readily isolated as pure cultures. There will not be a complete correspondence between the list of identified species determined and isolated by different methods.

In our study, 15 pure cultures of bacteria and four strains of microbial eukaryotes were isolated from kefir grains and related dairy products and further identified to the species level. It should be noted that not all members of the microbial associations, that are present in grains and dairy products according to DGGE, were obtained as pure cultures, as well as several isolated pure cultures were not detected by the molecular analysis (Table 2). This partial discrepancy of results, due to the lack of a single universal method, which has no restrictions, for composition determination of complex multicomponent communities, has been noted by many authors (Garbers et al. 2004; Wang et al. 2008; Chen et al. 2008; da Cruz Pedrozo et al. 2010; 2011; Chen et al. 2011; Nielsen et al. 2014). It is noticed that DGGE-analysis cannot identify all closely related and/or minor strains in microbial associations, containing dozens and hundreds of members. Microbiological methods for the study of communities' composition also are not without drawbacks, because there is no universal media that supports the growth of all, without exception, strains of the community. Culture conditions do not always repeat the conditions of natural propagation of microorganisms in associations, many cells can be in the uncultivated state, or will be able to give a stable growth only in co-cultures with partner (s) microorganisms. This should be considered when the results of molecular and classical microbiological analyses are compared. Therefore, in our study we applied a range of methods, combining DGGE-analysis, classical microbiological methods of cultivation and isolation of cultures, direct observations of microorganisms and their associations in the light and electron microscopes. This allowed us to most completely characterize the composition of the kefir grains' microbial community and to track microorganisms that are "passing" into the final product.

According to our data, the kefir grains' microbial community consists of more than a dozen strains of prokaryotes having approximately equal numbers, with the exception of three strains – one representative of each *Lb. kefiri*, *Lb. kefiranofaciens* and *Lactococcus lactis* subsp. *cremoris* species. The analysis of kefir grains microphotographs confirms the dominance of rod-shaped cells (Fig. 3a). High numbers of lactobacilli cells in kefir grains of different origin were noted by other authors (Garbers et al. 2004; Golowczyc et al. 2008; da Cruz Pedrozo et al. 2010). However, the range of species may vary considerably depending on the origin of the kefir grains themselves. The presence of large quanities of cells in the kefir grains of the strains *Lb. kefiri* and *Lb. kefiranofaciens*, was noted in a number of papers (Garrote et al. 2001; Golowczyc et al. 2008; Chen et al. 2008; Jianzhong et al. 2009; da Cruz Pedrozo et al. 2010). We have isolated and described a culture of *Lb. kefiri*, but *Lb. kefiranofaciens* strains, presented in the grains and detected by molecular methods, could not be obtained in pure cultures. Based on the description of its properties, available from literature (Jianzhong et al. 2009; Lemieux et al. 2009), it can be assumed that due to the active synthesis of exopolysaccharide kefiran

and tendency to grow in close associations, it develops poorly without partners. In addition to the DGGE-detected lactobacilli strains, members of the *Lb. casei* specie, obviously having a small number of cells in the kefir grains, were isolated in pure cultures. The amount of strains in the microbial community of starter and kefir drink was reduced, and quite different members of the association, than in the kefir grains, become the dominant microorganisms. So, the role of lactic acid cocci (strains of the genera *Lactococcus* and *Leuconostoc*) increases in dairy products according to the results of molecular and microbiological analyses, and DGGE detects only the presence of intense bands of different lactococci strains. At the same time, rod-shaped and oval cells in small amounts can be seen at microscopic preparations. On the contrary, the members of minor species of *Leuconostoc preudomesenteroides*, *Lb. casei* and *Lb. paracasei* were isolated by plating because, apparently, they can propagate better at suitable cultivation conditions in media used in this study. According to other researchers' data, lactobacilli are mandatory members of starter and kefir drink, but the presence and concentration of lactococci, leuconostocs, acetic acid bacteria and yeasts can vary and depends on the origin of kefir grains, from which they are produced (Garrote et al. 2001; Simova et al. 2002; Garbers et al. 2004). For example, as it was also observed in our study, changes of species composition and quantitative relationships of partners with a predominance of lactic acid cocci in the final product are shown for kefir grains from a different origin (Simova et al. 2002; Dobson et al. 2011). According to our data, lactococci are those microorganisms that are most easily transferred from kefir grains to milk and further able to enter through the starter in the final product in significant quantities. This property of lactococci is probably due to the formation of nizin-like bacteriocins which can suppress the development of Gram-positive bacilli in the same environmental niche.

In our study, neither molecular (DGGE technique) nor microbiological analysis of kefir grains and dairy products obtained from it, have shown the presence of members of acetic acid bacteria (AAB) in microbial associations, in contrast to other authors' findings (Sarkar 2008; Leite et al. 2012). In the scientific literature, opinions about its presence in kefir grains and in the final product differ: some researchers believe AAB are small in number, but represent an integral part of these communities, others think that they are not obligatory and their availability depends on the individual samples of kefir grains. For example, Garrote et al (2001) revealed that *Lactococcus lactis* subsp. *lactis*, *Lactobacillus kefir*, *Lactobacillus plantarum* and *Acetobacter* species are present in all types of Argentine kefir grains, but strains of *Leuconostoc mesenteroides*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Lactobacillus parakefir* were detected only in some grains. According to Garbers et al (2004), AAB were absent in some grains, which was also the case for the samples tested in our study. The presence of members of the genera *Acetobacter*, *Gluconobacter* and even *Bacillus* was observed in the composition of kefir grains from Brazil, Canada and the USA, along with lactic acid bacteria (da Cruz Pedrozo et al. 2010, 2011). This fact again confirms the variations in the kefir grains microbial compositions of different origin, which was reflected in the mild sour flavor of our kefir drink. Kefir prepared with grains containing AAB should have a more acidic taste and lower pH in general. AAB could develop in kefir drink after yeasts components activity, producing ethanol, which, in turn, would be converted to acetic acid by AAB in the presence of oxygen. So, it would need certain conditions during kefir manufacturing to develop AAB in this beverage.

From the data of literature (Simova et al. 2002; Wang et al. 2008; Jianzhong et al. 2009; Golowczyc et al. 2009) it is known that the composition of kefir grains includes yeasts (usually a lactose-negative yeast from genera *Saccharomyces*, *Kluyveromyces*, *Kazachstania*, *Pichia*, *Candida*, *Torulopsis*), and as minor components – even representatives of fungal genera *Oidium*, *Penicillium*,

Verticillium and others. In some samples of kefir grains, researchers observed the presence of yeasts from genera *Zygosaccharomyces*, *Yarrowia, Dekkera* and *Cryptococcus* (Witthuhn et al. 2005; da Cruz Pedrozo et al. 2011; Garofalo et al. 2015). We have also allocated ten isolates of yeasts from the grains studied, that after identification were members of three species of typical yeasts (from *Saccharomyces* and *Kazachstania* genera) and one species of mold fungi having a yeast anamorphs at the early development stages (*Gibellulopsis piscis*). SEM and light microscopy data demonstrated that the typical yeast cells presented in kefir grains were only in a small amount, and for isolation in pure culture pre-enrichment are required, whereas in milk and dairy products they developed best and can be obtained through simple plating technique by Koch method without previous enrichment. Mutualistic relationships of yeast cells with lactic acid bacteria promote their accumulation in milk (Spenser and Spenser 1997; Ivanova et al. 2013). All yeasts isolated in this study are lactose-negative, and they are responsible for part of the fermenting process occurring in milk kefir by using glucose and galactose with production of ethanol and CO_2. Therefore, it is possible to postulate that their growth in kefir grains may occur at the expense of the galactose and glucose possibly released by lactose hydrolysis catalysed by β-galactosidase enzymes produced by LAB occurring in kefir grain microbiota. Among the species belonging to the *Kazachstania* genus, *K. unispora* was also found, even if it was less represented. *K. exigua* and *K. unispora* were previously detected in some kefir grains worldwide (Jianzhong et al. 2009; Leite et al. 2012). Many yeasts, especially *Saccharomyces cerevisiae*, are more resistant to both the active acidity and a high content of lactic acid in medium. A significant number of lactic acid bacteria, in turn, have a high resistance to yeast fermentation product – ethyl alcohol, frequently superior to that of the yeasts itself. The bactericidal effect of ethyl alcohol on living microorganisms generally increases with increasing the acidity of the medium.

The accumulation of alcohol and lactic acid by LAB and yeasts at a joint development in the rich media does not allow the development therein of foreign microorganisms. Most LAB for their development need vitamins and amino acids that yeasts can synthesize and excrete. LAB in the presence of yeasts can grow in the media in which they themselves do not grow. Coming into close physical contact with the yeasts, sometimes attaching themselves to their cells, LAB obtained a favorable environment. Inhibition of their growth rate by ethanol delays aging of the population and increases the lifespan of lactic acid bacteria in the community. At the same time, the growth of fungi cells in kefir grains association are being suppressed, and only if they enter into the milk with a significant change in physical and chemical conditions, cells of the mold fungi can reach concentrations, allowing them to be recognized by platings. The selected strain of *Gibellulopsis piscis* was isolated from the kefir drink, but it was absent in the platings of kefir grains and corresponding starter samples. There is information that in some kefir grains the presence of yeasts was not detected at all (Garbers et al. 2004).

Thus, the analysis of the literature and our own results suggests that the structure of the microbial community of kefir grains depends on the source of its origin and on the individual features of development.

Rod-shaped bacteria from *Lactobacillus* genus predominate in the investigated kefir grains, but lactic acid cocci dominate in numbers in kefir drink. Cells from the surface layers of aggregate transferred from kefir grains to milk when producing starter, because media and incubation conditions (free state, rich in milk protein and lactose medium, presence of air) are the most comfortable for them, and they are able to multiply rapidly in the milk. Those functional alternates – different strains of one species, metabolic capabilities of which are most adaptable to these physico-chemical conditions, in this case receive the advantage in development. The microbial community of one-day kefir drink is

almost similar in species composition of the starter's community, except for the replacement of species *Lb. paracasei* and *Lb. casei* by the specie of *Lb. kefiri*. This is probably due to further change of culture conditions during the kefir drink preparation using starter (lowering the pH, the formation of alcohol). Based on the analysis of our results, we can conclude that in the chain of "kefir grains → starter → kefir beverage" there is the succession of microbial community which exists, expressed in a change in its composition and proportions of the components. This is also confirmed by other authors' data (Simova et al. 2002; Witthuhn et al. 2005; Dobson et al. 2011). The description of the pure cultures properties (Bergey's Manual of Systematic Bacteriology 2007; Kurtzman et al. 2011) isolated from kefir grains, allows for defining their possible roles in the life of this complex microbial association. Lactobacilli and lactococci convert lactose into glucose and galactose and further to lactic acid with reducing pH; yeasts produce ethanol and synthesize vitamins. In the studied community, isolated yeasts cultures cannot convert lactose into metabolizing sugars, therefore they relay on the lactic bacteria for the source of carbon and energy. On the other hand, yeast can consume oxygen fast, providing low-oxygen microenvironments for the lactic bacteria developments. In acidic medium, partial acid-caused denaturation of milk protein occurs. The species of bacteria capable of producing large amounts of exopolysaccharides (*Lb. kefiranofaciens*, *Leuc. pseudomesenteroides*) may participate in the formation of kefir grains matrix, as well as creating the viscosity of the final product – kefir. The role of *G. piscis* fungus in the community still remains unclear. They can grow only on hydrolyzed lactose of the milk after β-galactosidase enzymatic action of LAB and D-glucose and D-galactose evolving in the medium. On the other hand, having a microaerophilic nature, *G. piscis* can rapidly consume oxygen from the environment, oxidizing glucose and galactose, provided by lactose-utilizing LAB presented in the same batch, making the niche for anaerobic bacteria to develop more quickly.

We were able to trace the initial stages of the formation of aggregates in the experiments on the "reverse assembly" of artificial kefir grains involving pure cultures isolated by us. Despite the possible incompleteness of the species composition of mixed pure cultures, the cells have demonstrated the ability to implement the close contact and the formation of mucus matrix, but full formation kefir grains is not yet observed within 1.5 years of experiments with repeated re-inoculations. Although confirmation of the ability of some microorganisms isolated from kefir grains to create aggregates may be found in the scientific literature (Spenser and Spenser 1997; Golowczyc et al. 2009; Jianzhong et al. 2009; Wang et al. 2012). Attempts to restore the kefir grains from the isolated pure cultures of bacteria and yeasts from artemisian grains will be continued with the hope to construct "artificial" kefir grains, which will have the same qualities as the original native ones.

We can conclude, that the studies of microbial compositions of kefir grains of various regions of world must be continued, as many aspects of metabolism of this complex microbial community remain obscure. More complex media should be developed which will allow the isolation of minor components of the microbial communities which are not propagated in the commonly used media, probable, due to lack of the nutritional components which are supplied by the partner's organisms in the close environmental niche of the kefir grains.

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