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Original Research

Enterococcus as probiotics: what is the advantage?

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Introduction

Enterococcus is a genus of lactic acid bacteria which normally colonize the intestines of mammals including humans [1]. Enterococci are among the first bacterial colonizers after birth and are able to proliferate in both the large and the small intestine. *Enterococcus faecium* and *Enterococcus faecalis* are the most common species of enterococcus found in human feces.

However, enterococci can also cause serious infection including sepsis, pneumonia, ophthalmitis, nephritis and osteomyelitis, mainly as complications of various chronic conditions associated with intestinal dysbiosis such as cancer, AIDS or chronic renal failure [2]. Most clinical strains of enterococci belong to the two species *E. faecium* and *E. faecalis*, with vancomycin-resistant enterococcal strains (VRE) being the most common cause of lethal infection. Several virulence genes have been discovered in enterococci, allowing the identification of potentially hazardous strains [3]. At the same time, enterococci have a long history, being used as starters for making fermented food products from meat, milk or vegetables [4]. Many enterococcal strains (SF68, M74, LX, etc.) from that group of probiotic strains have been used for a long time as clinically effective probiotics.

The current paper summarizes the probiotic effects of one *Enterococcus faecium* strain—*E. faecium L3*. This strain was originally isolated from starter culture used in the Russian food industry. The strain was fully characterized microbiologically and genetically and tested for the absence of virulence genes, safety in laboratory animals and it immunomodulatory features. *E. faecium* L3 has been used in Russia in several probiotic products such as Laminolakt and Bakfir for the last 20 years. The benefits of the strain have been shown in several randomized clinical studies, including some where it was used for the treatment of gastrointestinal diseases such as chronic gastritis, gastric ulcers, irritable bowel syndrome, pancreatitis and chronic hepatitis [5–7]. In addition to other clinical effects such as anti-cancer properties, a significant cholesterol-lowering activity was also shown [8].

Microbiology and physiology of the strain *E. faecium* L3

General information

The strain *E. faecium* L3 was originally isolated from starter culture used in the dairy industry in the Soviet Union to produce fermented milk products (cream and yogurt) distributed in Leningrad. 16S RNA sequencing of this strain showed it belong to the genus Enterococcus, species Faecium. Probiotic strain *Enterococcus faecium* L3 was deposited in the collection of the All-Russia Research Institute for Agricultural Microbiology and in the international collection of Laboratorium voor Microbiologie, University of Ghent (LMG P-27496). The genome of strain *E. faecium* L3 was completely sequenced (GenBank No. SUB167269): it is 2,629,318 base pairs in size and contains 2,717 genes.

E. faecium L3 exhibited strong resistance to gastrointestinal stress conditions as it could withstand acid stress at pH 1.5, 2 and 3. The bacterium also survived at a bile salt concentration of 0.45%, with better tolerance observed towards pepsin and trypsin. *E. faecium* L3 produced lactic acid as a major metabolic product, followed by butyric acid. The strain also demonstrated high heat tolerance, being able to survive at 50°C for an hour and at 80°C for 10 minutes without significant loss of viability.

The strain is able to ferment a broad range of sugars, proteins, lipids and other substances due to the production of numerous enzymes including alkaline phosphatase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase (**Tables 1 and 2**).

N	Substrate	24-Hour result
1	VP (pyruvate)	+
2	HIP (hippurate)	+
3	ESC (aesculin)	+
4	PYRA (pyrrolidonyl-2-naphthylamide)	+
5	α-GAL 6 Br 2 naphtyl αD-galactopyranoside	–
6	β-GUR naphtol ASBI βD glucuronate	–
7	β-GAL 2-naphthyl β D galactopyranoside	–
8	PAL (2 naphtylphophate)	+
9	LAP (L-leucine 2 naphthylamide)	+
10	ADH (arginine)	+
11	RIB (ribose)	+
12	ARA (L-arabinose)	–
13	MAN (mannitol)	+
14	SOR (sorbitol)	+
15	LAC (lactose)	+
16	TRE (trehalose)	+
17	INU (inulin)	–
18	RAF (raffinose)	–
19	AMD (starch)	+
20	GLYG (glycogen)	–
21	β-HEM	–

Table 1 Metabolism of *Enterococcus faecium* L3: phenotypic profile of the strain (carbohydrates fermentation, enzymatic activity, etc.)

Antagonistic activity

Original testing of the strain for antagonistic activity against different pathogenic bacteria demonstrated unusually high antagonism compared with other lactic acid bacteria (LAB) used as probiotics. Comparison with other enterococcal strains including well-known enterococcal probiotic strains such as *Enterococcus faecium* SF68 and *E. faecium* LX included in the probiotics Bifiform or Linex, demonstrated that the strain *E. faecium* L3 had superior antagonistic activity against pathogenic Group A and Group B streptococcus (**Figures 1 and 2**), gram-negative pathogens and pathogenic fungi (**Tables 3 and 4**) [9,10]. The strain *E. faecium* L3 demonstrated antimicrobial activity against gram-positive pathogens, gram-negative bacteria, pathogenic fungi (**Tables 3 and 4**) and some viruses including herpes simplex and influenza [11,12].

Data presented in Tables 3 and 4 show the superior activity of the strain against patho-

N	Enzyme	Production by the strain *E. faecium* L3	Related genes in *E. faecium* L3 genome prepared by RAST	Open reading frame start
1	Alkaline phosphatase	+++++	Alkaline phosphatase synthesis transcriptional regulatory protein PhoP	1746762
2	Esterase (C 4)	+++	Tributyrin esterase Protein probably involved in xylan degradation; possible xylan esterase	1781178
3	Esterase lipase (C 8)	++	Lipase/acylhydrolase family protein)	369488
4	Lipase (C 14)	ND	Lipase putative	1971388
5	Leucine arylamidase	+++	N-acetylmuramoyl-L-alanine amidase, family 4	1235781
6	Valine arylamidase	ND	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	2256280
7	Cystine arylamidase	++++	Cysteinyl-tRNA synthetase (EC 6.1.1.16)	723137
8	Trypsin	ND	ND	ND
9	α-Chymotrypsine	-ND	ND	ND
10	Acid phosphatase	+++++	Acid phosphatase	290681
11	Naphthol-AS-BI-phosphohydrolase	+++++	Deoxyguanosinetriphosphate triphosphohydrolase (EC 3.1.5.1)	1575781
12	α-Galactosidase	ND	Alpha-galactosidase (EC 3.2.1.22)	1973617
13	β-Galactosidase	+	Beta-galactosidase	1975859
14	β-Glucuronidase	-ND	D-Galacturonate and D-glucuronate utilization	567245
15	α-Glucosidase	-ND	Alpha-glucosidase (EC 3.2.1.20)	2144668
16	β-Glucosidase	-ND	Beta-glucosidase (EC 3.2.1.21)	2488171
17	N-acetyl-β- glucosaminidase	ND	Endo-beta-N-acetylglucosaminidase	1022392
18	α-Mannosidase	-ND	Alpha-mannosidase (EC 3.2.1.24)	594352
19	α-Fucosidase	-ND	L-rhamnose utilization isu; L-fucose utilization	

Table 2 Enzymatic activity of *Enterococcus faecium* L3 and appropriate genes located on the chromosome

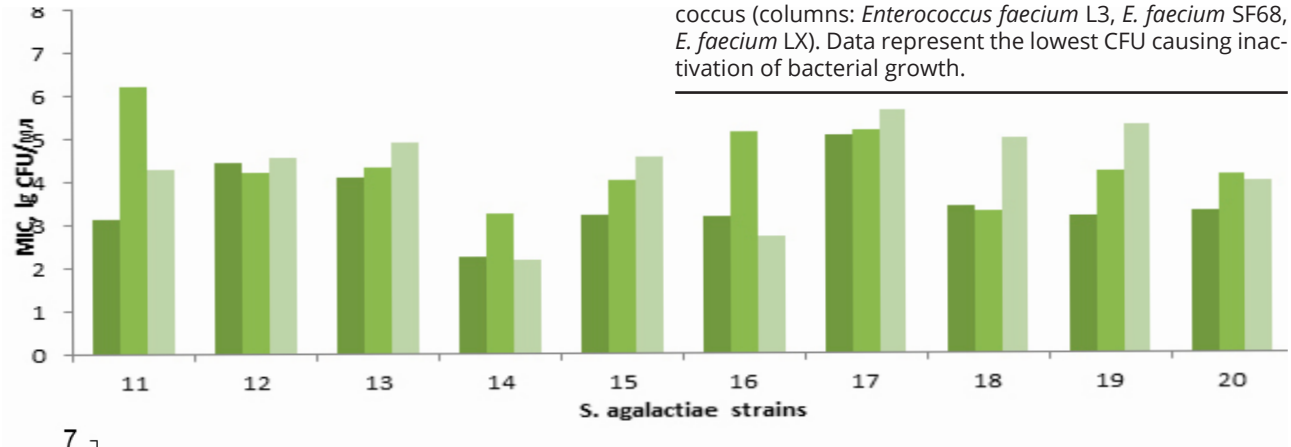


Figure 1 Antagonistic activity of three different enterococcal probiotics against individual strains of Group B streptococcus (columns: *Enterococcus faecium* L3, *E. faecium* SF68, *E. faecium* LX). Data represent the lowest CFU causing inactivation of bacterial growth.

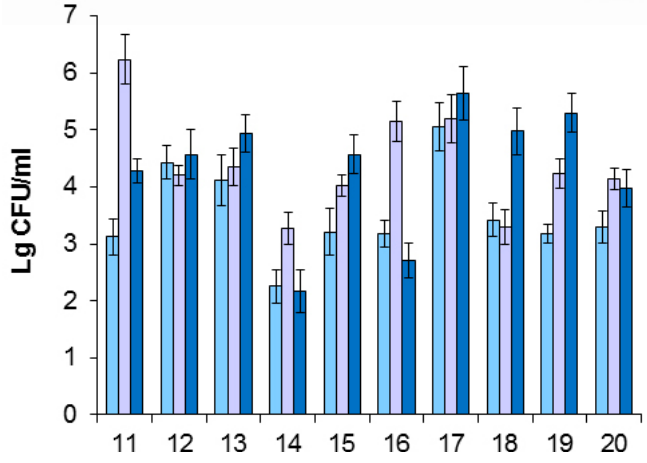


Figure 2 Antagonistic activity of enterococcal probiotic against individual strains of Group A and Group B streptococcus (columns: *Enterococcus faecium* L3, *E. faecium* SF68, *E. faecium* LX). Data represent the lowest CFU causing inactivation of bacterial growth.

	Gram-negative indicator bacteria	*E. coli* ATCC 25923	*E. coli* CS35P	*E. coli* M15	*P. aeruginosa* ATCC 27853	*Klebsiella pneumoniae*
PROBIOTICS	*E. faecium* L3	2.34±0.16	2.34±0.16	2.34±0.16	0.88±0.06	2.34±0.16
	E. faecium SF68	2.89±0.19	1.89±0.19	2.89±0.19	1.89±0.19	2.89±0.15
	E. faecium M74	3.51±0.07	2.04±0.12	4.04±0.20	2.50±0.18	2.04±0.12
	L. plantarum 8P-A3	1.33±0.08	1.66±	2.33±0.13	0.54±0.12	2.33±0.14
	L. acidophilus EP 317/402	1.76±0.05	0.76±0.04	1.76±0.08	1.57±0.27	3.76±0.19
	L. acidophilus Д № D75 и76	2.26±0.09	1.26±0.06	2.26±0.09	1.26±0.06	2.26±0.11
	L. fermentum Z*	2.51±0.07	2.51±0.07	2.51±0.07	2.51±0.13	3.51±0.21
	L. fermentum 62*	3.77±0.18	2.77±0.13	2.77±0.13	4.77±0.22	5.77±0.26
	Lactobacillus sp. 64*	5.04±0.21	4.04±0.18	5.04±0.21	5.04±0.21	6.04±0.31

Table 3 Antagonistic activity of different enterococcal and lactobacillus probiotics against indicator gram-negative pathogens. Probiotics with superior antagonistic activity are shown in cells with grey shading. Values in the cells represent the minimum inhibitory concentration (<2.5 lg) of probiotic stopping growth of the indicator bacteria

genic gram-negative bacteria and fungi compared with other enterococcal probiotic strains.

The antimicrobial activity of most LAB including various probiotics is due to different factors including antimicrobial metabolites such as lactic acids inhibiting the growth of acid-sensitive bacteria and viruses, enzymes depleting other bacteria of essential metabolites, and specific antimicrobial products such as bacteriocins. Strain *E. faecium* L3, an industrial probiotic, is a strong acid producer. After 24 hours of cultivation in MRS broth, the pH of the medium usually falls to about 5.0; however, in addition to lactic acid, the culture medium contains substances which significantly impede the growth of other bacteria (**Figures 3 and 4**).

Moreover, the antimicrobial activity of the supernatant from *E. faecium* L3 continued after boiling or neutralization of pH, but was suppressed after protease treatment, suggesting the protein nature of this antimicrobial activity.

Many enterococcal strains are able to produce small proteins belonging to the bacteriocin family. Knowledge of the sequence of the various bacteriocin genes of enterococci allowed us to identify four bacteriocin genes *entA*, *entB*, *entXa* and *entXb* in *E. faecium* L3 encoding for bacteriocins EntA, EntB, EnxA and EnxB. The last two bacteriocins usually function synergistically and so are called EnxAB. The presence of the genes was determined by PCR and ge-

Fungi strains	*C. albicans* ATCC 885-653	*Cryptococcus neoformans*
Probiotics	**6.31±0.39**	**5.31±0.29**
E. faecium SF68	>7	5.89±
E. faecium M74	>7	6±0.31
E. faecium 1	>7	>7
L. plantarum 8P-A3	3.61±0.18	3.77±0.17
L. acidophilus EP 317/402	6.61±0.28	5.61±0.31
L. acidophilus Д № 75 и 76	>7	>7
L. fermentum Z*	5.17±0.31	4.17±0.24
Lactobacillus sp. 64*	>7	>7
Lactobacillus sp. 62*	>7.2	6.2±0.30

Table 4 Antagonistic activity of different enterococcal and lactobacillus probiotics against indicator fungi pathogens. Values in the cells represent the minimum inhibitory concentration (<2.5 lg) of probiotic stopping growth of the indicator bacteria

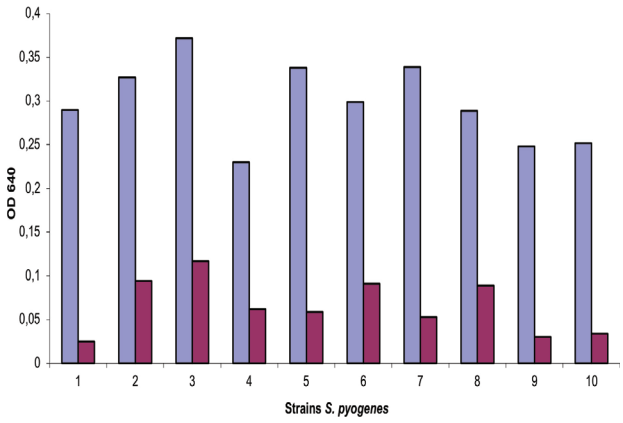


Figure 3 Antagonistic activity of *Enterococcus faecium* L3 supernatant against growing cultures of different strains of Group A streptococci. Blue columns: optical density of the strain, growing in Todd-Hewitt medium at 37°C for 8 hours. Red columns: the same strain with the addition of 5% of *E. faecium* L3 supernatant.

nomic sequencing. Reverse transcription later confirmed their expression. All these bacteriocins belong to class II antimicrobial peptides [20], many of which are processed into the active form outside the bacterial cell and regulated by other bacterial peptides called pheromones.

Gene *entA* encodes the bacteriocin EntA that is regulated by the operon which includes the immunity protein genes e*ntI* and *entF* (encoding for the pheromone), genes *entK* and *entR* (which encode a two-component regulatory system with sensory histidine kinase and regulator protein) and the genes *entT* and *entD* (responsible for secretion of enterocin A). This complex regulatory unit is quite variable in different enterococcal strains with the entA gene and is typical of the strains with a high level of antimicrobial activity [13–15].

Pheromone regulation of the expression of antimicrobial activity of the strain *E. faecium* L3 had been proved by experiments with synthetic pheromones kindly provided by Alexander Kolobov of the Institute of Highly Pure Biopreparations (St. Petersburg). Testing of three different molecules showed that one of them (Pher-2) corresponded to the putative form of the pheromone in the strain *E. faecium* L3 (**Figure 5**).

All structures had a cyclic sequence linked by the two cysteine molecules. Independently of size and amino acid sequence, they showed stronger antagonistic activity against Listeria monocytogenes than the original strain without pheromone induction (**Figure 6**). Apparently, the cyclic area of the pheromone structure is essential for induction of activity. However, the actual activity of the pheromone was different, with the original structure (Pher-2) being the most potent inducer (**Table 3**).

Taken together, these data suggest that the broad antagonistic activity of the strain *E. faecium* L3 is a complex feature that depends on the function of several extracellular peptides, which activity is regulated by the pheromones [16].

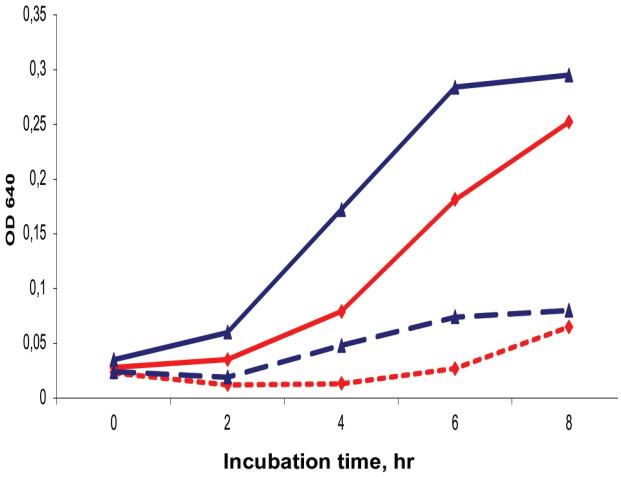


Figure 4 Antimicrobial activity of the strain *Enterococcus faecium* L3 supernatants against growing cultures of Group A and Group B streptococci. Solid blue line: time curve growth of the indicator GBS strain. Solid red line: time curve growth of the indicator GAS strain.

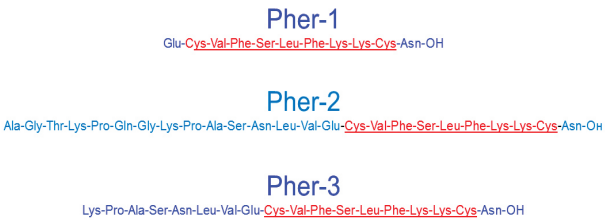


Figure 5 Structures of the synthetic pheromone inducers (Pher1, Pher2, Pher3) of antagonistic activity of bacteriocins from *Enterococcus faecium* L3. Pher2 corresponds to the original sequence of the pheromone in the strain Enterococcus faecium L3. The cyclic part of the pheromones is underlined.

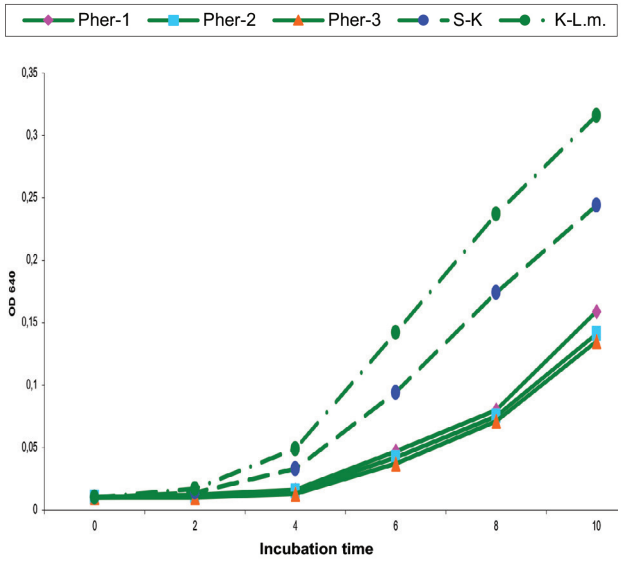


Figure 6 Pheromone-induced inhibition of the growth of Listeria monocytogenes.

Genetic profile of *E. faecium* L3

The strain *E. faecium* L3 belongs to the species Enterococcus which in certain conditions can cause severe infection. Consequently, enterococci as potential pathogens are under strict surveillance by epidemiologists worldwide. However, *E. faecium* strains differ substantially regarding their ability to cause infection.

The genomic content of enterococci varies significantly due to the presence in their genome of several antibiotic resistance determinants (vancomycin resistance is one of the most important) and genes affiliated with pathogenicity (virulence factors). Epidemiological studies show that approximately one-third of Enterococcus isolates are vancomycin resistant and cause an estimated 1,300 deaths each year [17,18]. *E. faecium* strains which are resistant to vancomycin usually belong to MLST clonal complex 17 [17]. Several enterococcal genes have been discovered and deemed virulence factor genes [20]. In many cases, especially when the genes encode for the adherence proteins, which are needed by all bacteria colonizing the human gut, the real role of this category of genes in pathogenicity is not yet understood. However, molecular epidemiological analysis allows potentially hazardous enterococci to be distinguished from the avirulent strains beneficial to health. Accordingly, probiotic enterococcal strains should be free from vancomycin resistance markers as well as the known virulence factor genes.

PCR analysis employing primers for different virulence genes revealed that the *E. faecium* L3 strain is free from the enterococcal virulence genes asa1, efa, esp, fsr, hyl, van, gel, spr and IS16 region tested by PCR and hybridization (**Figures 7 and 8**).

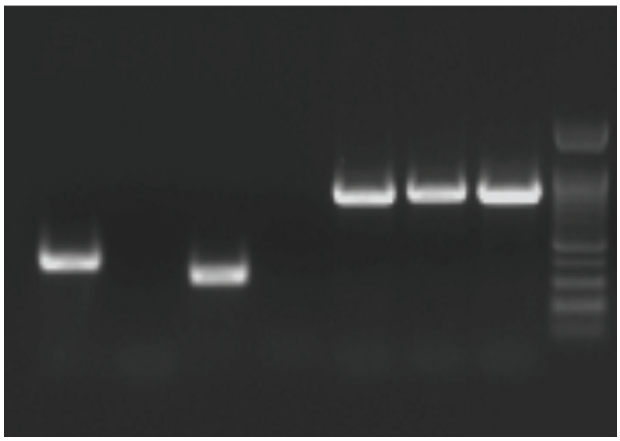


Figure 7 PCR with different enterococcal DNA employing primers corresponding to the van and hyl regions. 1: DNA *E. faecium* 1120, primers Van; 2: DNA *E. faecium* L3, primers Van; 3: DNA *E. faecium* 1120, primers Hyl; 4: DNA *E. faecium* L3, primers Hyl; 5: DNA *E. faecium* 1120, primers UniB (+PCR control); 6: DNA *E. faecalis* CCUG 52538, primers UniB (+PCR control); 7: DNA *E. faecium* L3, primers UniB (+PCR control); 8: molecular weight marker 100–1000, 1500 bp.

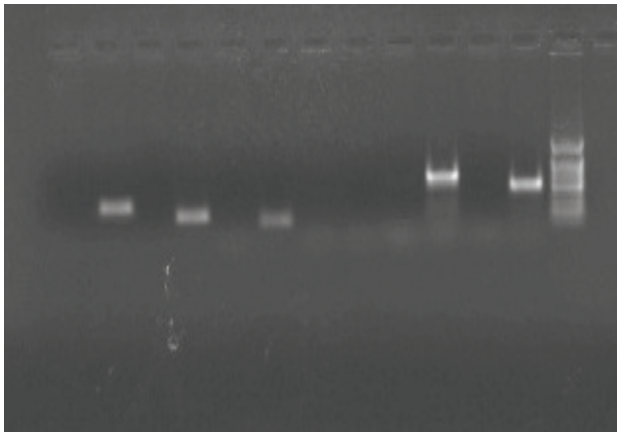


Figure 8 Electrophoresis of DNA of several *Enterococcus spp*. strains using primers corresponding to some enterococcal virulence-related genes. 1: DNA *E. faecium* L3 gelE (expected size 419 bp); 2: DNA *E. faecalis* CCUG 52538 (SMI Ekkr319), gelE+; 3: DNA *E. faecium* L3 sprE (expected size 233 bp); 4: DNA *E. faecalis* CCUG 52538 (SMI Ekkr319), sprE+; 5: DNA *E. faecium* L3 fsrB (expected size 316 bp); 6: DNA *E. faecalis* CCUG 52538 (SMI Ekkr319), fsrB+; 7: DNA *E. faecium* LAT E-253 esp (expected size 933 bp); 8: DNA *E. faecalis* CCUG 52538 (SMI Ekkr319), esp; 9: DNA *E. faecium* L3 efaA (expected size 735 bp); 10: DNA *E. faecalis* CCUG 52538 (SMI Ekkr319), efaA+; 11: DNA *E. faecium* L3 asa1 (expected size 529 bp); 12: DNA *E. faecalis* CCUG 52538 (SMI Ekkr319), asa1+; 13: molecular weight marker 100–1000, 1500 bp

Genome analysis

The genome of the strain *Enterococcus faecium* strain L3 was sequenced by Roche 454 Life Science. Contigs were sampled using the program Newbler Assembler and deposited in the GenBank sequence database (GenBank Nos. SUB167269, JRGX00000000). The draft genome sequence is composed of 74 contigs for a total of 2,643,001 bp, with 2,646 coding genes [21]. Analysis of the genomic sequence of the strain E. faecium L3 showed that the strain belongs to ST-619, which is quite rare, and is not affiliated to CC-17 or any virulent strains.

In order to proceed with the genomic analysis, we utilized the data obtained by optical mapping of the strain by OpGen Technologies. This approach is based on digestion of the genomic DNA by endonuclease NcoI. Subsequent electrophoresis allowed the contigs obtained by DNA sequencing to be allocated to one chromosome by employing the program MapSolver. The result of this work and the following analysis of DNA sequence is presented in **Figure 10**. Genomic analysis of *E. faecium* L3 revealed that the chromosome was 2.56 Mb in size, making it one of the smallest genomes sequenced so far.

Genomic analysis of *E. faecium* L3 revealed that the genome was 2.6 Mb in size, making it one of the smallest genomes sequenced so far.

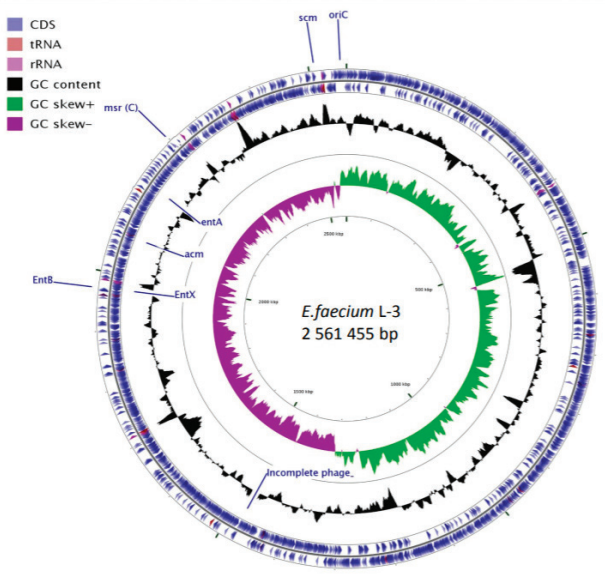
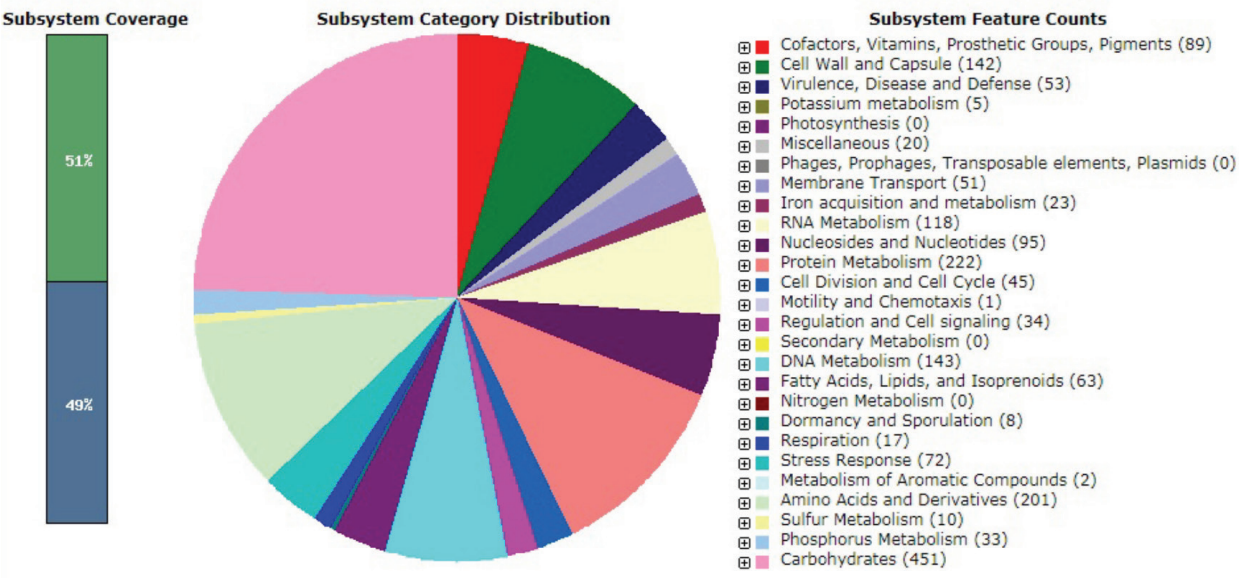


Figure 9 Circular map of the *E. faecium* L-3 chromosome generated with the program CGView. Coding sequences are designated with arrows. Diagram in black color represents the deviation of GC content from the average. Pink and Green diagrams demonstrates the value of the deviation from the average GC skew of the entire sequence-fluctuations of GC content in the leading and the lagging strands of DNA. Acm and Scm are putative collagen binding adhesins, entA, entB, lcb and entX represents the location of bacteriocin genes. putative CRISPr_1 corresponds to the location of the putative locus CRISPR.

Figure 10 Genes identified in the Enterococcus faecium L3 genome after RAST analysis.



Among the 2,623 open reading frames discovered using the program RAST, 451 genes are involved in the metabolism of carbohydrates, 222 in the metabolism of proteins, 261 in the metabolism of DNA and RNA, 142 in cell wall synthesis, and 89 in metabolism of the vitamins and cofactors.

The presence of genes encoding for biochemical pathways responsible for the synthesis of vitamins B1, B2, B6, and folic and lipoic acids, corroborates the previously discovered vitamin-producing activity of the strain.

The presence of various enzymatic activities determined by the biochemical studies was nicely confirmed by the finding of the appropriate genes on the bacterial genome (**Table 2**).

In addition to four bacteriocin genes discovered by PCR analysis, genomic study revealed a putative bacteriocin gene—lcbE linked to enterocin A operon. Genes responsible for the synthesis of biogenic amines, toxins or well-established virulence factors such as asa1, efa, esp, fsr, hyl, van, gel, spr and IS16, were not discovered in the strain genome. Interestingly, genome sequence analysis revealed the presence of the large plasmid DNA (110 kb) which was also free from the virulence factors but carried gene clusters responsible for lactose and mannose utilization. These additional genes seem to be advantageous for lactic acid bacteria cultivated on milk as a major energy source.

The stability of the plasmid in the genome is supported by a toxin–antitoxin system which eliminates the clones without the plasmid.

Analysis of the genome employing the program CRISPRFinder (PMC1933234) revealed the putative CRISPr locus.

The presence of CRISPr, which is considered an anti-viral bacterial genetic structure, corresponds to the technological stability of the strain *E. faecium* L3 being free from bacteriophage-induced loss of biomass.

Conclusion

Enterococcal probiotics and their use in the food and drug industry is a sensitive topic in microbiology and biotechnology. Underlying the clinical importance of these species, enterococci are natural inhabitants of the human gut and for centuries have been used in the production of traditional fermented milk, vegetable and meat products [4, 20-22–].

In addition, evidence demonstrating the health benefits of some enterococcal strains has led to the sale of enterococci-containing probiotics in most developed countries including Germany, Switzerland, Italy and Russia [20,23]. Enterococcal probiotics have a long history of safe clinical use. Accordingly, there is an urgent need for clear guidelines to distinguish potentially hazardous strains from strains appropriate for human consumption. The present paper is planned as the first in a series on the features and physiological impact of the strain *E. faecium L3*. The data presented show that the strain possesses a unique set of genetic tools to kill pathogens in the human organism and provide it with necessary metabolites and vitamins. It is also important to note that the strain is free from virulence factors, toxins and biogenic amines which are major obstacles to using *E. faecium* strains as probiotics [24].

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