Colonisation potential of plantaricin-producing *Lactobacillus plantarum* SF9C and S-layer-carrying *Lactobacillus brevis* SF9B among gut microbiota

Katarina Butorac
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Martina Banic
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Jasna Novak
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Andreja Leboš Pavunc
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Ksenija Uroic
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Nada Orsolic
Sveuciliste u Zagrebu Prirodoslovno-matematicki fakultet
Marina Kukolj
Sveuciliste u Zagrebu Prirodoslovno-matematicki fakultet
Slobodanka Radovic
IGA Technology Service
Simone Scalabrin
IGA Technology Service
Jurica Zucko
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Antonio Starčevic
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Jagoda Suskovic
Sveuciliste u Zagrebu Prehrambeno-Biotehnoloski Fakultet
Blaženka Kos (bkos@pbf.hr)
Faculty of Food Technology and Biotechnology, University of Zagreb https://orcid.org/0000-0003-1711-316X

Research

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Abstract

Background: The influence of an S-layer-carrying strain *Lactobacillus brevis* SF9B and a plantaricin-producing strain *Lactobacillus plantarum* SF9C on the gut microbiota composition was evaluated in the rats. Considering the probiotic potential of *Lb. brevis* SF9B, this study aimed to examine the antibacterial activity of *Lb. plantarum* SF9C and potential for their *in vivo* colonisation, which could be the basis for the investigation of their synergistic functionality.

Results: A plantaricin-encoding cluster was identified in *Lb. plantarum* SF9C, a strain which efficiently inhibited the growth of *Listeria monocytogenes* ATCC®19111™ and *Staphylococcus aureus* 3048. Contrary to the plantaricin-producing SF9C strain, the S-layer-carrying SF9B strain excluded *Escherichia coli* 3014 and *Salmonella enterica* serovar *Typhimurium* FP1 from adhesion to Caco-2 cells. Finally, DGGE analysis of the V2-V3 region of the 16S rRNA gene confirmed the transit of two selected lactobacilli through the gastrointestinal tract (GIT). Microbiome profiling via the Illumina MiSeq platform revealed the prevalence of *Lactobacillus* spp. in the gut microbiota of rats suggesting their colonisation potential in GIT.

Conclusion: The combined application of *Lb. plantarum* SF9C and *Lb. brevis* SF9B could influence the intestinal microbiota composition, which is reflected through the increased abundance of *Lactobacillus* genus, but also through altered abundances of other bacterial genera, either in the model of healthy or aberrant microbiota of rats. The obtained results contributed to the functional aspects of SF9C and SF9B strains which could be incorporated in the probiotic-containing functional foods and therefore have a beneficial influence on the gut microbiota composition.

Background

Lactobacillus strains are omnipresent in different ecological niches. The representative members dominate the microbiota of the sauerkraut and are under constant competition with other strains for nutrients and space (Collins *et al*., 2018). The antibacterial activity of *Lactobacillus* strains is an important factor for the pathogen elimination in the complex microbial communities. *Lactobacillus* strains able to produce bacteriocins may achieve a competitive advantage in the surrounding environment which represents an attractive approach in the terms of food biopreservation (Collins *et al*., 2018). Their applications are even expanding to the health point since bacteriocin production is recognised as an important probiotic trait and bacteriocins have even been proposed as alternatives to antibiotics (Chikindas *et al*., 2017; Mills *et al*., 2017). Bacteriocinogenic activity may contribute to the functionality of probiotics through direct inhibition of the pathogens. Moreover, bacteriocins help the survival of the producing strain and may act as quorum-sensing molecules in the intestinal environment. Previously, we monitored lactic acid bacteria (LAB) population during spontaneous fermentation of the *Brassica oleracea* var. *capitata* cultivar Varaždinski (Banić *et al*., 2018; Beganović *et al*., 2014; Beganović *et al*., 2011). At the onset of the spontaneous fermentation, LAB diversity was present, including *Leuconostoc mesenteroides* strains, while a restricted number of *Lactobacillus* species, mainly *Lactobacillus plantarum,* dominated in the latter stages (Beganović *et al*., 2014). *Lb. brevis* SF9B was isolated from the respective spontaneous fermentation. This strain showed desirable functional and technological properties largely influenced by S-layer proteins (Slps) which were detected by SDS-PAGE (Beganović *et al*., 2014) and identified by 2D electrophoresis followed by LC-MS analysis. Slps have a functional role in conveying increased survival of the respective strain in simulated GIT conditions and during freeze-drying. Moreover, the results indicate a prominent role of Slps in adhesion of SF9B strain to mucin, extracellular matrix (ECM) proteins, and particularly to Caco-2 cells (Banić *et al*., 2018). Besides SF9B, an

autochthonous isolate SF9C was isolated at the final stage of spontaneous fermentation. This strain was identified as *Lb. plantarum,* which is a prevalent species in sauerkraut fermentation, probably due to its competitiveness among autochthonous microbiota. Previous analysis of untreated supernatant of SF9C culture by a turbidimetric method revealed antibacterial activity towards some common pathogens (Beganović *et al*., 2014). Therefore, the aim of this study was to evaluate the competitive advantage potential of bacteriocin-producing *Lb. plantarum* SF9C and S-layer-carrying *Lb. brevis* SF9B against pathogens by *in vitro* and *in vivo* investigations. A possible bacteriocinogenic activity of SF9C strain against Gram-positive pathogens *Listeria monocytogenes* ATCC®19111™ and *Staphylococcus aureus* 3048 was tested. The stimulation of bacteriocinogenic activity in SF9C strain was performed by its coculturing with common food pathogens. To observe whether this strain has a broader spectrum of the antibacterial activity, pathogen competition and competitive exclusion of Gram-negative *Escherichia coli* 3014 and *Salmonella* Typhimurium FP1 was also evaluated. Since preclinical evidence indicate that probiotic *Lactobacillus* strains may positively influence gut microbiota composition in different disorders followed by microbiota disturbance (Distrutti *et al.,* 2014; Chen *et al*., 2016), the aim of this study was also to assess colonisation potential and the capacity of SF9C and SF9B strain to induce microbiome alterations *in vivo*, after joint application, either in healthy or $AlCl_3$-exposed rats as a model of disturbed microbiota. PCR-DGGE and sequence analysis of faeces content were employed to investigate if *Lb. brevis* SF9B and *Lb. plantarum* SF9C have the potential for *in vivo* colonisation and the shift of the microbiota in the intestinal tract (IT) of rats.

Results

Plantaricin-related genes andwhole genome sequences (WGS) of *Lb. plantarum* SF9C

Plantaricin-related genes, *plnA*, *plnE* and *plnJ,* were identified using PCR amplification, suggesting that SF9C genome could harbour a *pln* locus. Rapid Annotations using Subsystems Technology (RAST) of sequences obtained by Illumina MiSeq platform, and tblastn v.2.2.27 comparison of the assembled contigs with the sequences deposited in NCBI employed for WGS, identified SF9C as *Lb. plantarum*. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession RHLZ0000000. The version described in this paper is version RHLZ 01000000. The genome sequence is composed of 3.26 million bp (Mb) and is divided into 14 contigs. The size of *Lb. plantarum* SF9C genome of 3.2 Mb is similar to that of the other members of the species. The number of coding sequences is 3,229 and the number of RNAs is 68. According to the comparative genomic studies the estimated number of predicted protein-coding genes in *Lactobacillus* strains ranges from 1,700 to around 3,000 (Van Pijkeren and O´Tolle, 2013). The G+C content of the *Lb. plantarum* SF9C genome is 44.4%, which is similar to the other *Lb. plantarum* strains, e.g. *Lb. plantarum* WCFS1 (44.5%) and *Lb. plantarum* ATCC 14917 (44.5%) (Anukam *et al*., 2013). Subsystem category distribution of major Protein Encoding Genes (PEGs) for *Lb. plantarum* SF9C as annotated by RAST is shown in Fig. 1. The pie chart is depicting the percentage distribution of 27 most abundant subsystem categories in strain SF9C. While the most of the PEGs were related to universal cell functions such as DNA replication, transcription, translation, ribosomal structure and biogenesis, protein turnover and chaperones, and transport and metabolism of carbohydrates and nucleotides, certain PEGs were associated to the specific categories of cellular defence mechanisms and secondary metabolites biosynthesis, transport, and catabolism which may be responsible for the antimicrobial phenotype of SF9C strain.

Given that SF9B and SF9C originate from the same microenvironment, their whole genomes were compared and the cluster dendrogram that reflects the diversity among strains was constructed. Single Nucleotide Polymorphism (SNP) hierarchical clustering based on WGS revealed that S-layer-carrying *Lb. brevis* SF9B is grouped with another S-layer-expressing *Lb.brevis* (Fig. 2). Given that the phylogenetic distance between the strains was small, sauerkraut

isolate *Lb. plantarum* SF9C was grouped with SF15C strain, another isolated *Lb. plantarum* strain from the same fermentation batch (Fig. 2).

Next, WGS data were exploited to identify potential genomic triggers that may be responsible for the antibacterial phenotype. The assembled contigs were compared with so far identified bacteriocins in the NCBI using the tblastn v2.2.27. Through functional annotation and analysis of the high-coverage contigs obtained through Illumina sequencing, plantaricin production was predicted for *Lb. plantarum* SF9C. The genes involved in the bacteriocinogenic activity and their functions, which were also detected in the genomes of the other *Lb. plantarum* strains, are listed in Supplementary Table 1. The genome sequence of *Lb. plantarum* SF9C contains a cluster for biosynthesis of a putative plantaricin. *In silico* BAGEL4 analysis identified one area of interest (AOI) located at a contig 13. The pln locus of SF9C contains genes encoding their cognate immunity proteins, whose location is just downstream of the bacteriocin genes, as well as ABC transporters, probably involved in the export of peptides with a double glycine leader (Fig. 3). Finally, homology-based three-dimensional (3D) structures of SF9C two-peptide plantaricins were predicted by the SWISS-MODEL. Properties of the chosen amino acid (aa) residues that form helix of each of the two plantaricins, PlnJK and PlnEF, were calculated by HeliQuest web server. Hydrophobic residues are shown in yellow, serine and threonine in purple, basic residues in dark blue, acidic residues in red, asparagine and glutamine in pink, alanine and glycine in grey, histidine in light blue and proline in green circles (Fig. 4).

Antimicrobial activity of *Lb. plantarum* SF9C after the cocultivation with pathogens

Preliminary results regarding the antibacterial activity of *Lb. plantarum* SF9C and *Lb. brevis* SF9B clearly demonstrated the difference in the spectrum of antibacterial activity among two naturally coexisting strains (Table 1.). Interestingly, while grown cultures of both strains, SF9C and SF9B, demonstrated antibacterial activity against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048, CFS of the strain SF9B failed in the inhibition of the respective pathogens as evaluated by agar well-diffusion method (Table 1).

The antibacterial activity of the CFS of SF9C strain was partially inactivated after the treatment with proteinase K and after its exposure to high temperature of 100 °C during 30 minutes (Table 1). These findings confirm the presence of a substance with proteinaceous nature in CFS. Furthermore, the combined CFSs of both strains, SF9C and SF9B, in equal ratio, showed decreased antibacterial activity against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 which was expected since the CFS of S-layer-carrying SF9B strain did not demonstrate antibacterial activity against these two pathogens, and therefore negatively influenced on cumulative effect of both CFSs.

Similarly, the evaluation of the antibacterial activity of SF9C and SF9B against closely related LAB strains, showed that SF9C compared to SF9B, was more effective in the inhibition of the examined LAB strains, with the strongest effect observed against *Enterococcus*, moderate against *Lactococcus* and the weakest against *Lactobacillus* strains by agar well-diffusion method (data not shown). Antibacterial activity of the grown SF9C culture was additionally confirmed by agar spot test against pathogens (Table 1) and by cocultivation with the same pathogenic strains determined as bacteriocin-sensitive (Fig. 5). To assess the possibility to enhance the bacteriocin activity of *Lb. plantarum* SF9C, this strain was cocultivated with *S. aureus* 3048 and *L. monocytogenes* ATCC®19111™, respectively. The log CFU/ml values of the bacteriocin-sensitive strains, *S. aureus* 3048 and *L. monocytogenes* ATCC®19111™ were reduced to non-detectable levels after 48 and 24 h, respectively, during the cocultivation with *Lb. plantarum* SF9C (Fig. 5). Additionally, the antibacterial effect of *Lb. plantarum* SF9C, obtained by agar spot test, was significantly higher after growth in coculture with bacteriocin-sensitive test microorganisms: with *S. aureus* 3048 after 8, 10 and 22 hours of incubation, whereas with *L. monocytogenes* ATCC®19111™ after 22,

24 and 48 hours of incubation, showing that longer time of incubation is needed with *L. monocytogenes* ATCC®19111™ than with *S. aureus* 3048 for the enhancing of plantaricin activity (Fig. 5). The obtained results indicate that the plantaricin activity of *Lb. plantarum* SF9C was enhanced by the presence of the bacteriocin-sensitive bacterial cells.

Inhibition of pathogen adherence to Caco-2 cells by *Lb. brevis* SF9B

The pathogen competition and exclusion assays by S-layer-carrying *Lb. brevis* SF9B and *Lb. plantarum* SF9C, on Caco-2 human intestinal cells were performed. *Lb. brevis* SF9B inhibited the adhesion of *S.* Typhimurium FP1 and especially *E. coli* 3014 (Table 2). Previous investigation revealed that SF9B strain had completely lost the adhesion ability to Caco-2 cell lines after the removal of the S-layer (Banić *et al.* 2018).

Lb. plantarum SF9C strain failed in adhesion to Caco-2 cells and therefore did not affect the exclusion of these Gram-negative pathogens (data not shown). On the contrary, strain SF9B inhibited *S.* Typhimurium FP1 adherence to the significant levels in competitive exclusion assay. In exclusion assay, Caco-2 cells exposed to *Lb. brevis* SF9B before *S.* Typhimurium FP1 had significantly fewer *Salmonella* adhered to them (4.708±0.014 $\log_{10}$ CFU/ml) than Caco-2 cells exposed to *S.* Typhimurium FP1 alone (6.825 ± 0.099 $\log_{10}$ CFU/ml). The competition assay revealed that Caco-2 cells incubated with *Lb. brevis* SF9B had significantly fewer *Salmonella* cells adhered (5.613 ± 0.135 $\log_{10}$ CFU/ml) compared to Caco-2 cells infected with *Salmonella* alone. The inhibition effect was even stronger against *E. coli* 3014, where the high efficacy of preincubation for inhibiting an invasion of Caco-2 cells by *E. coli* 3014 was evident, with values of 2.209 $\Delta\log_{10}$CFU/ml when the pathogen was added subsequently (exclusion), or of 2.117 $\Delta\log_{10}$ CFU/ml when *Lb. brevis* SF9B cells and pathogen were added simultaneously (competition) (Table 2).

Influence of *Lb. brevis* SF9B and *Lb. plantarum* SF9C on gut microbiome composition

The colonisation capacity of examined *Lactobacillus* strains and the gut microbiome composition after their transit through GIT was monitored *in vivo* on experimental animals. Since the aberrant microbiota differs from the microbiota of healthy subjects by the prevalence of undesirable species, the $AlCl_3$-exposed rats was chosen as an animal model for microbiome dysbiosis because previous research showed that toxic metals such as aluminium (Al) have a direct negative impact on the gut microbiota in human and animals. Additionally, Lactobacillaceae, particularly *Lb. plantarum*, *Lb. rhamnosus* and *Lb. brevis*, were able to bind and remove toxic metals (Yu *et al.* 2016). A recent study by Tian *et al.* (2017) also suggests the potential of the *Lb. plantarum* strain to alleviate the aluminium-induced brain injuries in mice. Therefore, the potential of *Lb. plantarum* SF9C and *Lb. brevis* SF9B to compete among microbiota of $AlCl_3$-exposed rats was investigated. The acetylcholinesterase (AChE) activity was assessed in the brain tissue homogenates to monitor the possible influence of $AlCl_3$ treatment in the rats. The AChE activity and histopathological and immunohistochemical analyses of the brain have shown that the number of plaques and the AChE activity were significantly higher in the brains of the AT group, compared to that of the control group ($P < 0.05$) (personal communication by Ledinski *et al.* 2017). According to the result, the neuropathological changes were observed in the $AlCl_3$-exposed rats. Diffuse plaques, also called benign plaques, occurred much earlier than the neuritic plaques in the cerebellum. In the treatments, cerebellum of the AT rats was negative on the AT8 marker, but positive on 4G8 and Iba1 marker (Supplementary Fig. 1). The value of AChE in the control group was lower (Min-Max value 1.2 to 1.58 moles of substrate hydrolyzed/min/mg protein) compared to the AT group (Min-Max value from 1.38 to 2.4 moles of substrate hydrolyzed/min/mg protein). Further, the analysis of faecal microbiota of rats by Illumina MiSeq sequencing revealed that across all, control or $AlCl_3$-exposed groups

(calculated as mean values from all the experiments), the dominant phyla were *Firmicutes* and *Bacteroidetes*, which respectively made up 63% (62.35 ± 5.40%) and 22% (21.76 ± 6.20%) of total abundance, with lower contributions from *Actinobacteria* (1.65 ± 0.73%) and *Proteobacteria* (1.84 ± 0.58%) (Fig. 6a). The *Firmicutes* and *Bacteroidetes* phyla accounted for more than 85% of total sequences, similar to previous findings in the gut microbiota of rats. However, phylum- through genus-wide differences in bacterial abundance were observed among the two groups. In the microbiome of $AlCl_3$-exposed rats the abundance of *Firmicutes* and *Actinobacteria* decreased, while the abundance of *Bacteroidetes* increased compared to the control group. At the class level, the most abundant for all groups were *Bacilli*, *Clostridia* and *Bacteroidia* (Fig. 6b). *Bifidobacterium* was also consistently detected through the samples (Fig. 6a). Since our main goal was to evaluate the survival and colonisation potential of the two *Lactobacillus* strains in the model of healthy, but also in experimental animals with disturbed microbiota, the focus was on the evaluation of *Lactobacillus* abundance. The abundance in *Lactobacillus* sp. was observed in all treated rat groups, implying good adaptation of SF9B and SF9C to the GIT, especially since these two strains are not of an intestinal, but sauerkraut origin. The gut microbiome analysis revealed taxonomic differences in gut microbiota composition influenced by *Lactobacillus* treatments. The culture-independent PCR-DGGE approach was applied to verify the presence of lactobacilli in the gut microbiota of faecal samples among rats before and the treatment with SF9B nad SF9C strains (Fig. 7). The cultivation on selective agar plates revealed the presence of presumptive *Lactobacillus* in the faeces of the control group at 5.6×10^7 CFU/ml and $AlCl_3$ rats at 1.99×10^8 CFU/ml, respectively the 10^{th} day after *Lactobacillus* treatment, which is in correlation with the results obtained by the Illumina MiSeq sequencing. DGGE analysis was employed to verify which *Lactobacillus* strains are potentially responsible for the observed higher *Lactobacillus* spp. abundance levels in the microbiota of *Lactobacillus*-treated rats. DGGE of DNA fragments obtained by PCR amplification of the V2-V3 region of the 16S rRNA gene implied the presence of both *Lactobacillus* strains in the faeces of treated rats since their DNA fragment coincided with the 16S DNA fragment generated from the pure culture of *Lb. brevis* SF9B and *Lb. plantarum* SF9C (Fig. 7). The inoculation of the healthy rats with *Lactobacillus* strains led to the appearance of a new 16S DNA fragment in the DGGE profile of the sample performed from the healthy rat the 3^{rd} day after *Lactobacillus* treatment, which corresponded to *Lactobacillus reuteri*. Interestingly, the results of microbiota analysis, at the species level, have shown the presence of *Lb. reuteri* and *Lb. brevis* as well. Furthermore, in a DGGE profile of the healthy rat, an intensive band was consistently detected, assigned after the sequencing and BLAST search as *Lb. animalis*, while the 3^{rd} day after *Lactobacillus* treatment, a faint band appeared corresponding to *Lactobacillus intestinalis* strain (Fig. 7).

Discussion

Functional genomics in probiotic research has facilitated the characterisation of candidate *Lactobacillus* strains. Bacteriocin production is a desirable trait of probiotic strains (Hegarthy *et al*., 2017). Herein, *Lb. plantarum* SF9C genome sequence was determined using a WGS assembly approach, with a focus on the characterisation of the plantaricin locus. The whole genome sequencing confirmed the presence of the plantaricin (*pln*) loci in SF9C strain. *plnE* and *plnF* genes that encode for bacteriocin precursor peptide and the *plnA* which encodes induction factor and individual gene *plnJ* were also detected by PCR. Plantaricin EF (PlnEF) and plantaricin JK (PlnJK) have already been described in certain *Lb. plantarum* strains as two-peptide bacteriocins. These compounds are biosynthesised as prepeptides and are cleaved off during the transport to the cell surface to become active peptides whose activity is dependent on the complementary action of the two peptides PlnE/PlnF i.e. PlnJ/PlnK (Diep *et al.,* 2009). Predicted 3D structures of two SF9C plantaricins by SWISS homology modelling showed sequence similarity with the structures of PlnJK and PlnEF plantaricins, obtained by Rogne *et al.* (2009) and Fimland *et al.* (2008), respectively.

Bacteriocin activity together with competition for limited nutrients, competitive exclusion, and the stimulation of mucosal immunity could enhance intestinal health (Dobson *et al.*, 2012). Here the antibacterial activity of the *Lb. plantarum* SF9C against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 was explored. *Lb. plantarum* SF9C drastically decreased the pH value (3.86 ± 0.04) after overnight growth due to the lactic acid production (2.25 ± 0.24% v/v) which creates unfavourable local microenvironment for pathogenic bacteria. The mechanisms of the antibacterial activity of SF9C strain are multifactorial and include the inhibition by produced lactic acid, but also by the activity of potential SF9C-produced plantaricin, especially since the inhibition was alleviated after CFS proteinase K and boiling treatment. *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 possess several mechanisms to combat the challenges posed by acidic environments and therefore can tolerate low pH values. This claim was supported by the finding that the *Lb. plantarum* SF9C strain demonstrates antibacterial activity against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 contrary to *Lb. brevis* SF9B, which failed in the inhibition of respective pathogens, even though it is an effective lactic acid producer. Additionally, *L. monocytogenes* and *S. aureus* were deliberately chosen since these foodborne Gram-positive pathogens contaminate the wide range of fermented foods, although the pH value in these food matrices is low due to the metabolic activity of a spontaneously present population of LAB. Therefore, it was hypothesized that the potential plantaricin is involved in the antibacterial activity towards *L. monocytogenes* and *S. aureus.* Since one strategy to achieve the expression of otherwise silenced bacteriocins is the induction of their biosynthesis by growth in cocultures (Chanos and Mygind, 2016; Maldonado-Barragán *et al.*, 2013; Kos *et al.*, 2011), the potential to enhance plantaricin antibacterial activity by cocultivation of *Lb. plantarum* SF9C with *S. aureus* 3048 and *L. monocytogenes* ATCC®19111™ was studied. Antibacterial activity was initially detected after 10 hrs of incubation in the early exponential phase of the pathogen growth. The highest antibacterial activity was marked after 24 hrs in the late exponential phase of *L. monocytogenes* ATCC®19111™ and after 48 hrs for *S. aureus* 3048. This is supported by the results of Maldonado-Barragán *et al.* (2013) who suggested that the induction of bacteriocin production by means of coculturing with specific bacterial strains is a common feature among *Lb. plantarum* species. Since *L. monocytogenes* tolerates a broad pH range, it can be speculated that the SF9C plantaricin activity was potentially enhanced in the presence of *L. monocytogenes* ATCC®19111™ and the obtained antilisterial effect of SF9C could be assigned to the potential plantaricin activity. This is in agreement with the feature of *Lactobacillus* bacteriocins which are mostly active towards Gram-positive bacteria.

Since SF9C did not prevent pathogen exclusion on the Caco-2 cells, the potential of S-layer-carrying *Lb. brevis* SF9B, to exclude enteric pathogens was tested. Slps may act as mediators of bacterial adhesion and as such may contribute to the antibacterial activity against the pathogens with whom the S-layer-carrying strain competes for the same adhesion sites (Uroić *et al.*, 2016; Hynönen *et al.*, 2014; Taverniti *et al.*, 2013). In our previous paper, SF9B strain exhibited the strongest coaggregation with *E. coli* 3014 and *S.* Typhimurium FP1 and the removal of Slps negatively affected its coaggregation ability. The results of this study revealed that *Lb. plantarum* SF9C did not compete and exclude the pathogens from the Caco-2 cells, while S-layer-carrying SF9B strain demonstrated significant levels ($P < 0.01$) of exclusion capacity against both *E. coli* 3014 and *S.* Typhimurium FP1, respectively. Additionally, the competitive capacity of SF9B strain was also obtained against both Gram-negative pathogens, but more effective against *E. coli* 3014. Nevertheless, *Lb. brevis* SF9B strain may prevent and exclude *E. coli* 3014 and *S.* Typhimurium FP1, exhibited considerable coaggregation capacity (Banić *et al.*, 2018), even after only 1-hour incubation on Caco-2 cells in the respective experiment. The coaggregation enables lactobacilli to manipulate a microenvironment around the pathogenic bacteria and to inhibit their growth in the gut by secreting antimicrobial substances at their very close proximity. The results suggest that *Lb. brevis* SF9B competed more efficiently against *E. coli* 3014 than *S.* Typhimurium FP1 since the mechanisms of competition and exclusion differ and are highly

specific for each pathogen. Combining plantaricin producer *Lb. plantarum* SF9C with S-layer-carrying *Lb. brevis* SF9B offers an effective strategy to suppress *L. monocytogenes* ATCC®19111™, *S. aureus* 3048, *E. coli* 3014 and *S.* Typhimurium FP1 since the joint application of these potential probiotic strains could result in a broader spectrum of antibacterial activity. One drawback of the direct application of bacteriocins to a food product is that the loss of activity occurs over time due to enzymatic degradation and interactions with food components such as proteins and lipids (Hartmann *et al.,* 2011). Here it was suggested the potential application of the plantaricin-producing cells of *Lb. plantarum* SF9C, which can act synergistically with *Lb. brevis* SF9B, to eliminate common pathogens. *Lb. brevis* SF9B is a non-producing bacteriocin strain, but whose genome contains *plnI* gene, encoding the bacteriocin immunity protein (Banić *et al.,* 2018). Both strains, SF9B and SF9C have shown tolerance to harsh conditions of GIT because their relative survival rate displayed only a 2-log and 3-log decrease in CFU/ml, respectively, under the conditions mimicking the GIT (Banić *et al.,* 2018; Beganović *et al*., 2014). The cooperation of coexisting *Lactobacillus* strains can be also exploited to control bacterial infection for the reestablishment of the disturbed gut microbiota associated with certain diseases (Dicks *et al.,* 2018). Therefore, the potential of plantaricin-producing SF9C and S-layer-carrying SF9B strain to compete among healthy or disturbed gut microbiota was examined after their application to the healthy and $AlCl_3$-exposed rats. $AlCl_3$-exposure can cause a variety of adverse physiological effects in humans and animals, including disturbance of gut microbiota (Chen *et al*., 2016; personal communication Ledinski *et al*., 2017). After *Lactobacillus* treatment of rats, the changes in intestinal microbiota composition were observed, not only in the abundance of *Lactobacillus* genus but also in the abundance of other bacterial genera. According to microbiome analysis, *Blautia* genus was not detected in healthy rats but was identified in the $AlCl_3$-exposed rats in which its ratio decreased 3rd and 10th day after the *Lactobacillus* application. The ratio of *Bacteroides* and *Phascolarctobacterium* genera before the *Lactobacillus* treatment was higher in the $AlCl_3$-exposed rats compared to healthy reats, but 3rd day after the *Lactobacillus* administration the ratio of these genera was reduced in $AlCl_3$-exposed rats and increased in healthy rats, compared to ratio of these genera before *Lactobacillus* application. Furthermore, the abundance of the *Bifidobacterium* genus remained unchanged, before and after the *Lactobacillus* treatment, in both the healthy and the $AlCl_3$-exposed rats. *Clostridium* and *Adlercruetzia* genera were evenly present in both groups, before the *Lactobacillus* application, while the 3rd and 10th day after *Lactobacillus* application the ratio of *Adlercruetzia* genus decreased in both groups of rats and the ratio of *Clostridium* genus decreased only in $AlCl_3$-exposed rats. An abundant prevalence of *Lactobacillus* spp. was observed in the microbiota of the *Lactobacillus* treated rats, even 10th day after the *Lactobacillus* application, compared to the microbiota of the healthy rats. This increased abundance of the *Lactobacillus* genus possibly reflects an adaptation of *Lb. plantarum* SF9C and *Lb. brevis* SF9B in GIT as evaluated by PCR-DGGE. However, besides allochthone lactobacilli SF9B and SF9C, PCR-DGGE indicated a presence of other commensal lactobacilli, suggesting the possible impact of applied *Lactobacillus* strains on the competitive ability of autochthonous strains. The obtained results emphasise the influence of applied *Lactobacillus* strains on rat microbiota composition, which will be valuable for further experiments on more experimental animals, in order to investigate interactions of specific treats of *Lactobacillus* strains, such as Slps and bacteriocin production, with faecal microbiota composition. Further studies are needed to better understand the probiotic effects of these two strains on a healthy and disturbed gut microbiome composition and function, and the possible impacts on other parameters important in alleviating $AlCl_3$-induced toxicity in host.

Conclusion

The results of this research supported an enhanced functionality potential of the joined application of SF9C and SF9B strains *in vivo*. The cooperation between two strains could result in a facilitated adhesion of *Lb. plantarum* SF9C due to the competitive pathogen exclusion by coexisting *Lb. brevis* SF9B. Simultaneously, SF9B could benefit from the improved colonisation due to plantaricin production by *Lb. plantarum* SF9C, resulting in a broader spectrum of antibacterial activity of the coculture against the pathogens. The plantaricin- and S-layer-expressing *Lactobacillus* strains could be a promising probiotic candidates for an application in functional food and for the treatment of different disorders linked with a dysbiosis of gut microbiota, which require further investigation.

Materials And Methods

Bacterial strains, culture media and cultivation conditions

Bacterial strains and cultivation conditions, used in this study, are listed in Table 3.

S-layer-carrying *Lb. brevis* SF9B was previously characterised by Banić *et al.* (2018). Strains are deposited in the Culture Collection of the Laboratory of Antibiotic, Enzyme, Probiotic and Starter Culture Technologies, Faculty of Food Technology and Biotechnology, University of Zagreb (CIM-FFTB) and are maintained as frozen stocks at -80 °C in appropriate medium supplemented with 15% (v/v) glycerol.

Human cell line, culture medium and cultivation conditions

Enterocyte-like Caco-2 cells were donated by the Ruđer Bošković Institute, Zagreb, Croatia. Caco-2 cells were grown as monolayer cultures in RPMI 1640 medium (GIBCO, USA), supplemented with 15% of the fetal bovine serum (GIBCO, USA) and 4500 mg/l of glucose. Cells were grown up to confluence at 37 °C and 5% of CO_2 in T-flasks, trypsinised and seeded into 24-multiwell plates. Prior to experiments, cells reached sub-confluence.

DNA isolation and PCR analysis

Total genomic DNA, both for PCR analysis of the bacteriocin genes or WGS, was extracted according to the method of Leenhouts *et al.* (1990) with minor modifications. The purity and concentration of the extracted DNA were then determined by using a BioSpec-Nano spectrophotometer (Shimadzu, Kyoto, Japan) and the extracted DNA was stored at -20 °C. PCR screening for the prevalence of bacteriocin structural genes was performed with primers listed in Ben Omar *et al.* (2008). Therefore, amplification of DNA fragments was performed in 50 µl reaction mixtures containing 25 µl of Emerald Amp MAX HS PCR Mastermix Premix (TaKaRa, Ohtsu, Japan), 200 nmol/l of each oligonucleotide primer, 300 ng of DNA template and EmeraldAmp dH_20. A negative control, which contained all reagents except the DNA template, was used to detect contamination or non-specific amplification. The amplification was carried out in an Eppendorf Mastercycler personal thermal cycler (Eppendorf, Germany) using the conditions described by the Ben Omar *et al.* (2008). PCR-amplified products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualised on a MiniBIS Pro transilluminator (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) at 254 nm and images were captured by the GelCapture software version 7.1 (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Whole genome sequencing and identification of genesencodingbacteriocins

Genomic DNA was prepared according to Frece *et al.* (2009). Genome sequencing was done using a paired-end approach as essentially described in Banić *et al.* (2018). Briefly, the Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used to construct a library. The library was processed with the Illumina cBot and

sequenced on the MiSeq2500 (Illumina, San Diego, CA) pair-end with 300 cycles per read. Contigs were classified as belonging to *Lb. plantarum* when obtaining the best blastn v2.2.27 hit (Altschul *et al*., 1990) in the NCBI nt database. RAST server, which identifies protein-encoding, rRNA and tRNA genes, assigns functions to the genes, and predicts which subsystems are represented in the genome (Aziz *et al*., 2008), was used for the annotation, and the distribution and categorization of all sequenced genes. The assembled contigs were compared with so far identified bacteriocins in the NCBI using the tblastn v2.2.27. To further supplement the annotation, BAGEL4 sofware was used to predict genes related to bacteriocin synthesis (van Heel *et al*., 2018). The input file was the genome sequence of *Lb. plantarum* SF9C in a fasta file. Conserved genes associated with the bacteriocin synthesis were retrieved using the RAST server (Aziz *et al*., 2008). Additionally, WGS were pairwise aligned with "run-mummer3" to detect alignments and SNPs. The plot was computed with R package hclust, based on SNP frequency. Further, the 3D structure homology modelling was done using the SWISS-MODEL server (https://swissmodel.expasy.org/) based on the alignment of the amino acid sequences of the core peptides, generated from BAGEL4 software. Additionally, helix properties of each two plantaricin were calculated using heliQuest web server (Gautier *et al*., 2008).

In vitro assays

Testing of antimicrobial activity

The antimicrobial activity of the overnight grown culture of *Lb. plantarum* SF9C and *Lb. brevis* SF9B strains against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 was tested by agar spot test and well-diffusion method. The agar spot test was performed according to Leboš Pavunc *et al.* (2013). The ratio of the inhibition diameter (ID) to the spot culture diameter (CD) was calculated to determine the effective inhibition ratio (EIR) of SF9C strain: ((ID-CD)/CD). Furthermore, the agar well-diffusion method, previously described by Kos *et al.* (2008) was applied for the analysis of antimicrobial activity of the cell free supernatant (CFS). CFS was recovered by centrifugation, filtered through a 0.22 µm sterile filter (Millipore Corporation, Billerica, MA, USA) and concentrated up to 5-fold in an Amicon cell concentrator (Amicon, Beverly, MA, USA) equipped with a selective (10 kDa) membrane. The proteinaceous nature of potential inhibitory compounds in CFS was examined by treatment with Proteinase K (Invitrogen, Carlsbad, CA, USA) at a concentration of 1 mg/ml during 2 h at 37 ^{o}C and by heating the samples at 100 ˚C/30 min, according to Elayaraja *et al*. (2014). Statistical analysis was carried out using ANOVA and the results are reported as mean values of three individual experiments ± standard deviation. One-way analysis of variance (ANOVA) and Tukey tests were performed using VassarStats software to determine significant group differences and means were considered as statistically significant if $P < 0.05$.

Evaluation of the antibacterial activity after cocultivation with the targeted pathogens

The influence of cocultivation of *Lb. plantarum* SF9C with *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 on bacteriocin activity of SF9C strain was performed according to Kos *et al.* (2008) with slight modifications. The number of viable cells was determined by spot-plate method using the corresponding selective media for each strain: MRS for lactobacilli; Baird-Parker (Oxoid, Hampshire, UK) for *S. aureus* and ChromoBio (Biolab Diagnostic Laboratory, Budapest, Hungary) for *L. monocytogenes*, in 2 hour intervals during the first 10 hours, and after 22, 24 and 48 h of the incubation. Plates were incubated for 24 hours at 37 °C and the number of viable cells was expressed as log CFU/ml. Also, during the experiment, the antibacterial activity of SF9C strain, in monoculture and coculture, was tested by agar spot test as described above. Experiments were conducted in triplicate and values

were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey tests were performed for statistical analysis.

Pathogen competition and exclusion assay by *Lb. brevis* SF9B and *Lb. plantarum* SF9C on Caco-2 cell line

For exclusion and competition assay experiments, Caco-2 cells were routinely grown in 24-well culture plates until confluent differentiated monolayers were obtained. Cellular monolayers were carefully rinsed three times with PBS (pH 7.4) before addition of the bacterial cells. Two separate protocols were followed to assess the ability of viable lactobacilli strains to inhibit *E. coli* 3014, *S.* Typhimurium FP1 adhesion to Caco-2 cells. For both assays, *Lactobacillus* strains and pathogens were routinely cultivated; the cells were harvested and prepared in PBS (pH 7.4) to reach A_{620} = 1 (approximately 1 x 10^9 CFU/ml). The competition assay was performed according to the procedure described by Uroić *et al.* (2016) with few modifications. Lactobacilli and pathogens were co-incubated with Caco-2 monolayer for 1 h. For exclusion assays, *Lactobacillus* strains were cultured with Caco-2 monolayer for 1 h. Following 1 h incubation, Caco-2 monolayers were gently washed three times with PBS (pH 7.4); pathogens were added and incubated for another 1 h. A 1.0 ml aliquots of the monospecies cultures of pathogenic bacteria together with 1.0 ml of EMEM per well were used as the controls in both assays. In all the above treatments, post-incubation removal of the non-adhered bacterial cells was executed by removing the bacterial suspension and washing the Caco-2 monolayers three times with PBS (pH 7.4). The Caco-2 cells were then lysed by addition of 0.25% (v/v) Triton X-100 (AppliChem, Darmstadt, Germany) solution at 37 °C for 10 min in order to collect the adherent bacterial cells, and the total numbers of viable adhering *Lactobacillus*, *E. coli* and *S.* Typhimurium were determined by spot-plate method on MRS, Rapid (Biorad, Dubai, United Arab Emirates) and XLD (Biolife, Milano, Italy) agar plates, respectively. The efficiency of pathogen exclusion of *Lactobacillus* strains was assayed in three biologically independent experiments each with three replicates.

In vivo animal trial

Preparation of *Lb. brevis* SF9B and *Lb. plantarum* SF9C strains and administration to rats

Bacterial cultures *Lb. brevis* SF9B and *Lb. plantarum* SF9C were grown in 5 ml of MRS broth at 37 °C under anaerobic conditions until the OD value reached 1.0 at 620 nm. The as-prepared cultures were mixed in 1:1 (v/v) ratio and inoculated (4%) in 50 ml of MRS broth. After overnight incubation at optimal conditions, the cells were harvested by centrifugation at 5000 g for 10 min, suspended in saline solution and the presence of both strains was microscopically examined. The bacteria suspensions were prepared daily to ensure viability and the CFU was controlled to maintain strictly the number of CFU administered by a rat as it is described in the next chapter.

Experimental animals

Three-months-old male highly inbred Y59 strain rats, weighing 200 to 250 g, (http://www.informatics.jax.org/external/festing/rat/docs/Y59.shtml), obtained from our breeding within the Department of Animal Physiology, Faculty of Science, University of Zagreb, were used in this study. The animals were maintained under a 12/12-h light-dark cycle with free access to food and water and standard housing conditions (room temperature around 25 °C and 60% humidity). They were fed a standard laboratory diet (4 RF 21, Mucedola, Settimo Milanese, Italy) and tap water *ad libitum*. Maintenance and care of all experimental animals were carried out according to the guidelines in force in the Republic of Croatia (Law on the Welfare of Animals, NN135/06 and NN37/13) and in accordance with EU Directive 2010/63/EU for animal experiments (OJEU, 2010) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-

123. The experimental procedure was approved by the Bioethics Committee of the Faculty of Science, University of Zagreb, Croatia (No. HR-POK-012).

Rat study design and sample collection

Male rats belonging to the Y59 inbred strain were randomly divided into 2 equally sized trial groups and housed three per cage in stainless-steel cages, under the same controlled conditions. The rats were treated daily for five consecutive days with a single dose ($3x10^9$ CFU/ml) of *Lb. brevis* SF9B and *Lb. plantarum* SF9C strains suspended in saline solution, starting 24 h after the last treatment as follows: (a) first trial group represented a model of induced aluminium toxicity which was established by intraperitoneally injecting $AlCl_3$ (10 mg/kg) and D-galactose (60 mg/kg) as described by Ulusoy *et al*. (2015) and (b) second group served as healthy (control) group and was injected comparatively with saline solution in the same manner. No side effects were reported following *Lactobacillus* administration. In order to evaluate the AchE activity, which requires a brain sample, rats had to be sacrificed. Before the sacrifice rats were anesthetized using a mixture of ketamine (Narketan®10, Vetoquinol AG, Belp Bern, Switzerland) at dose of 75 mg/kg with xylazine (Xylapana® Vetoquinol Biowet Sp., Gorzow, R. Poland) at dose of 10 mg/kg. The intestinal mucosal content from each sacrificed rat was scraped and specimens were kept frozen at -80 °C until the analysis. The brain was removed and frozen at -80 °C or kept in buffered formaldehyde until the analysis. The brain tissue homogenates were used to assess acetylcholinesterase (AChE) activity by colorimetric method. AchE activity is expressed in mol/min/g tissue. The brain samples are prepared according to standard paraffin procedure. Changes related to early-stage Alzheimer's disease were also (un)confirmed by immunohistochemistry used primary antibodies Purified-β-Amyloid, 17-24 Antibody (4G8) diluted 1:2000 (BioLegend, San Diego, CA), Phospho-PHF-Tau (pSer202 + Thr205) Monoclonal Antibody (AT8) diluted 1:500 (Thermo Fisher Scientific, Waltham, MA, USA) and Iba1 diluted 1:250 (Wako Pure Chemical Industries, Japan). Photomicrographs were recorded using a digital camera (AxioCam ERc5s, Zeiss) and processed by a computer program morphometric image analysis (AxioCam ERc5s-ZEN2). The faecal samples were collected from the cages before starting the treatment and on the 3rd and 10th day following the last *Lactobacillus* administration in triplicates. Faecal samples were stored at -80 °C until analysis as described in the next chapter.

Bacterial 16S rRNA sequencing and processing using QIIME

Faecal samples were collected from rats at the end of the study and used to purify the total genomic DNA using a commercial DNA extraction kit Maxwell DNA Tissue Kit with automated extraction platform, Maxwell® 16 Research System instrument (Promega, USA). The final equimolar pool was sequenced on the Illumina MiSeq platform. PCR reactions and 16S sequencing were performed at the Molecular Research LP (MRDNA, Shallowater, Texas USA). The MiSeq instrument (Illumina) was used for sequencing the 16S amplicons following the manufacturer's instructions at MRDNA described by Garcia-Mazcorro *et al.* (2018) with slight modifications. Raw 16S data were obtained from Illumina's basespace as FASTQ files and analysed using the QIIME 2 pipeline using the procedure as described in the moving pictures tutorial (https://docs.qiime2.org/2018.11/tutorials/moving-pictures/).

PCR –DGGE analysis

PCR-DGGE analysis was performed according to Leboš Pavunc *et al.* (2012) with slight modifications in order to check the presence of the *Lb. plantarum* SF9B and *Lb. brevis* SF9C in faeces of *Lactobacillus* fed rats. DNA was extracted directly from faecal samples of healthy rats for culture-independent PCR-DGGE analysis, as well as from the bacterial colonies, isolated on MRS agar plates for culture-dependent PCR-DGGE analysis, from faeces of

healthy rats sampled before feeding (control), and 3rd and 10th day after application of *Lactobacillus* SF9B and SF9C strains. In both cases, DNA was isolated using Maxwell DNA Cell Kit with automated extraction platform, Maxwell® 16 Research System instrument (Promega, USA). The V2-V3 region of the 16S ribosomal DNA gene of bacteria in the faeces contents or from pure cultures of lactobacilli was amplified with primers HDA1-GC and HDA2. To identify the lactobacilli, recovered from rat faeces, the V2-V3 region of the 16S rRNA gene of the strains was amplified. The amplicons were sequenced using ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems). A search of sequences deposited in the GenBank DNA database was conducted by using the BLAST algorithm. The identities of the isolates were determined based on the highest score.

Statistical analysis

All the experiments were repeated three times and the results were expressed as means of three independent trials ± standard deviation (SD). Statistical significance was appraised by one-way analysis of variance. Pairwise differences between the means of groups were determined by the Tukey HSD test for post-analysis of variance pairwise comparisons (http://vassarstats.net/test). Statistical differences between groups were considered significant when P values were less than 0.05.

Declarations

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Author contributions

All authors performed the analysis, prepared the manuscript, and contributed to editing and critical reviewing.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Laboratory of Antibiotic, Enzyme, Probiotic and Starter Culture Technologies, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, Zagreb, Croatia [2] Laboratory for Biology and Microbial Genetics, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, Zagreb, Croatia [3] Department of Animal Physiology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, Zagreb, Croatia [4] IGA Technology Services srl, via Jacopo Linussio 51, Udine, Italy [5] Laboratory for Bioinformatics, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, Zagreb, Croatia

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Tables

Table 1 Comparison of the antimicrobial activity of Grown Culture (GC) and Cell Free Supernatants (CFS-A - untreated; CFS-B - treated with proteinase K; CFS-C - treated at 100 ºC for 30 min) of two *Lactobacillus* strains, SF9C and SF9B, separately and combined, against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048, evaluated by agar spot test for GC and agar well-diffusion method for CFSs

Lactobacillus strains	Diameter of the inhibition zone (cm)							
	L. monocytogenes ATCC®19111™				*S. aureus* 3048			
	GC	CFS-A	CFS-B	CFS -C	GC	CFS-A	CFS-B	CFS-C
Lb. plantarum SF9C	3,50 (±0,10)^{az}	1,92 (±0,03)^{ax}	1,10 (±0,00)^{t}	1,68 (±0,03)^{w}	2,85 (±0,13)^{ay}	1,57 (±0,06)^{av}	1,17 (±0,03)^{u}	1,52 (±0,03)^{v}
Lb. brevis SF9B	1,93 (±0,40)^{bz}	0,00 (±0,00)^{cx}	n.d.	n.d.	1,35 (±0,05)^{by}	0,00 (±0,00)^{cx}	n.d.	n.d.
Combined SF9C+SF9B	2,57 (±0,06)^{bz}	1,72 (±0,03)^{by}	n.d.	n.d.	1,50 (±0,10)^{bx}	0,98 (±0,03)^{bw}	n.d.	n.d.

n.d.- not determined; ^{abc} Different symbol means statistically significant difference ($P < 0.05$) within the same column. ^{tuvwxyz} Different symbol means statistically significant difference ($P < 0.05$) within the same row between the treatments. Statistical analysis was carried out using ANOVA and the results are reported as mean values ± standard deviation of three independent experiments

Table 2 Competition and exclusion assay of *E. coli* 3014 and *S.* Typhimurium FP1 on Caco-2 cells by *L. brevis* SF9B

Strain	*S.* Typhimurium FP1 (log CFU/ml)		*E. coli* 3014 (log CFU/ml)	
	Competition assay	Exclusion assay	Competition assay	Exclusion assay
Control	6.825 ± 0.099	6.825 ± 0.099	8.517 ± 0.157	8.517 ± 0.157
Lb. brevis SF9B	5.613 ± 0.135*	4.708 ± 0.014**	6.393 ± 0.101**	6.308 ± 0.194**

Statistical analysis was carried out using ANOVA and the results are reported as mean values of three separate experiments ± standard deviation. Asterisks indicate significant differences of adhered *E. coli* 3014 and *S.* Typhimurium FP1, without (control) and with the addition of the *Lb. brevis* SF9B strain compared to control at different levels: $^{**}P < 0.01$, $^{*}P < 0.05$

Table 3 Bacterial strains used in this study

Bacterial strain	Cultivation conditions	Reference
Lb. brevis SF9B	MRS, 37 °C, microaerophilic	Banić *et al.* (2018)
Lb. plantarum SF9C	MRS, 37 °C, microaerophilic	This study
E. coli 3014	BHI broth, 37 °C, aerobic	CIM-FFTB*
S. Typhimurium FP1	BHI broth, 37 °C, aerobic	CIM-FFTB
L. monocytogenes ATCC®19111™	BHI broth, 37 °C, aerobic	ATCC**
S. aureus 3048	BHI broth, 37 °C, aerobic	CIM-FFTB

*CIM-FFTB - Culture collection of the Laboratory of Antibiotic, Enzyme, Probiotic and Starter Culture Technologies, Faculty of Food Technology and Biotechnology, University of Zagreb; **ATCC - American Type Culture Collection

Figures

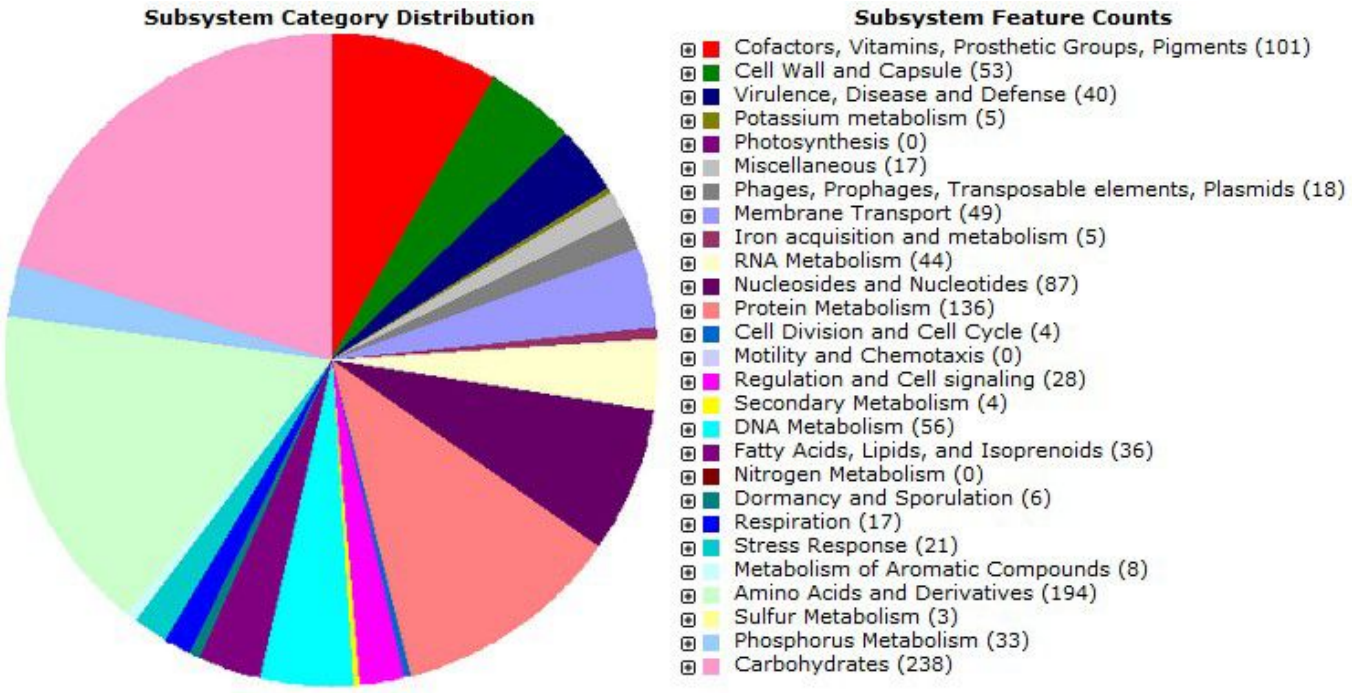

Figure 1

Distribution of Lb. plantarum SF9C subsystem gene functions. The complete genome sequence Lb. plantarum SF9C of was annotated using the Rapid Annotation System Technology (RAST) server. The pie chart showed the count of each subsystem feature and the subsystem coverage

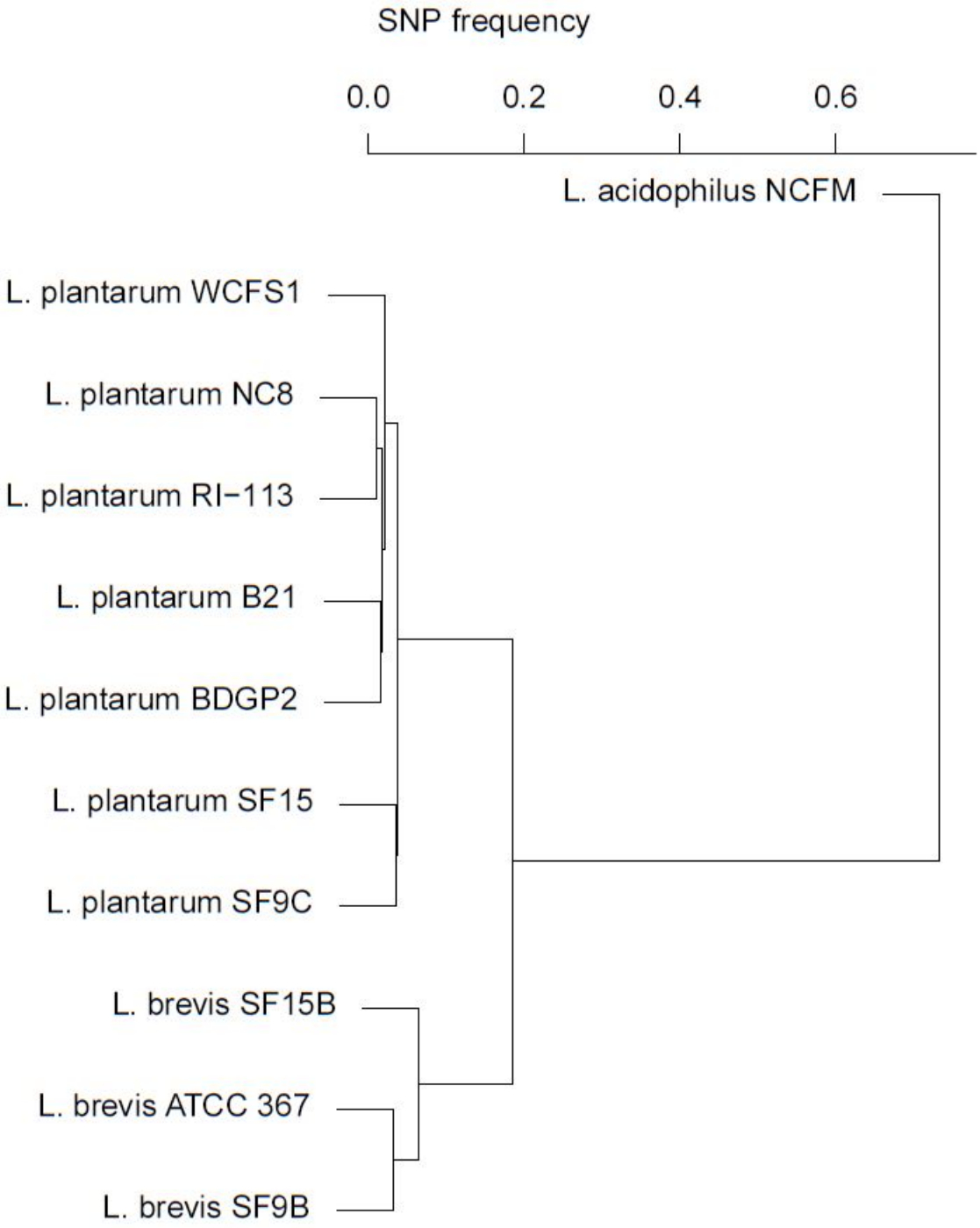

Figure 2

Single Nucleotide Polymorphism (SNP) based hierarchical clustering. Clustering of multiple Lactobacillus genomes based on SNP frequency, plotted on the y axis. SNP frequency is "number of bases divided by bases aligned"

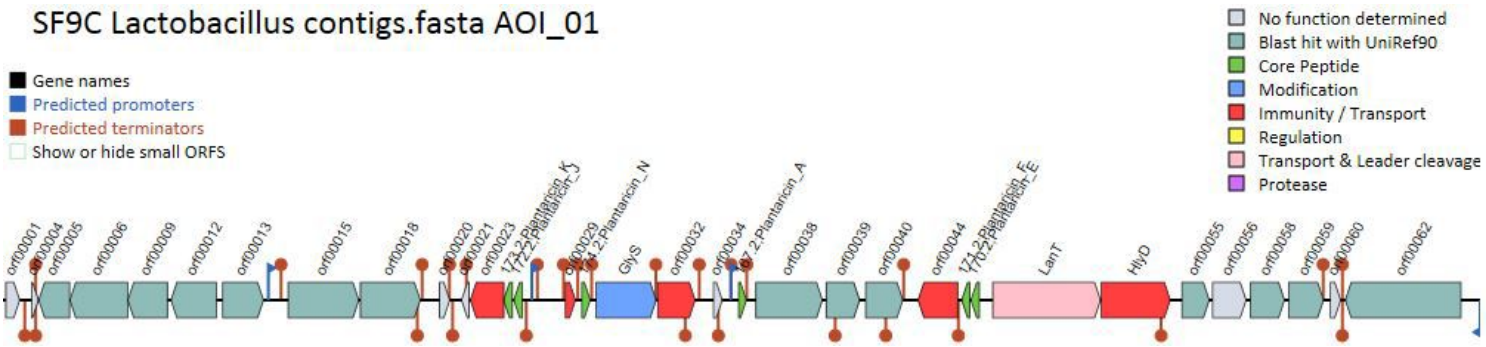

Figure 3

Genetic map of the plantaricin gene cluster in Lb. plantarum SF9C strain

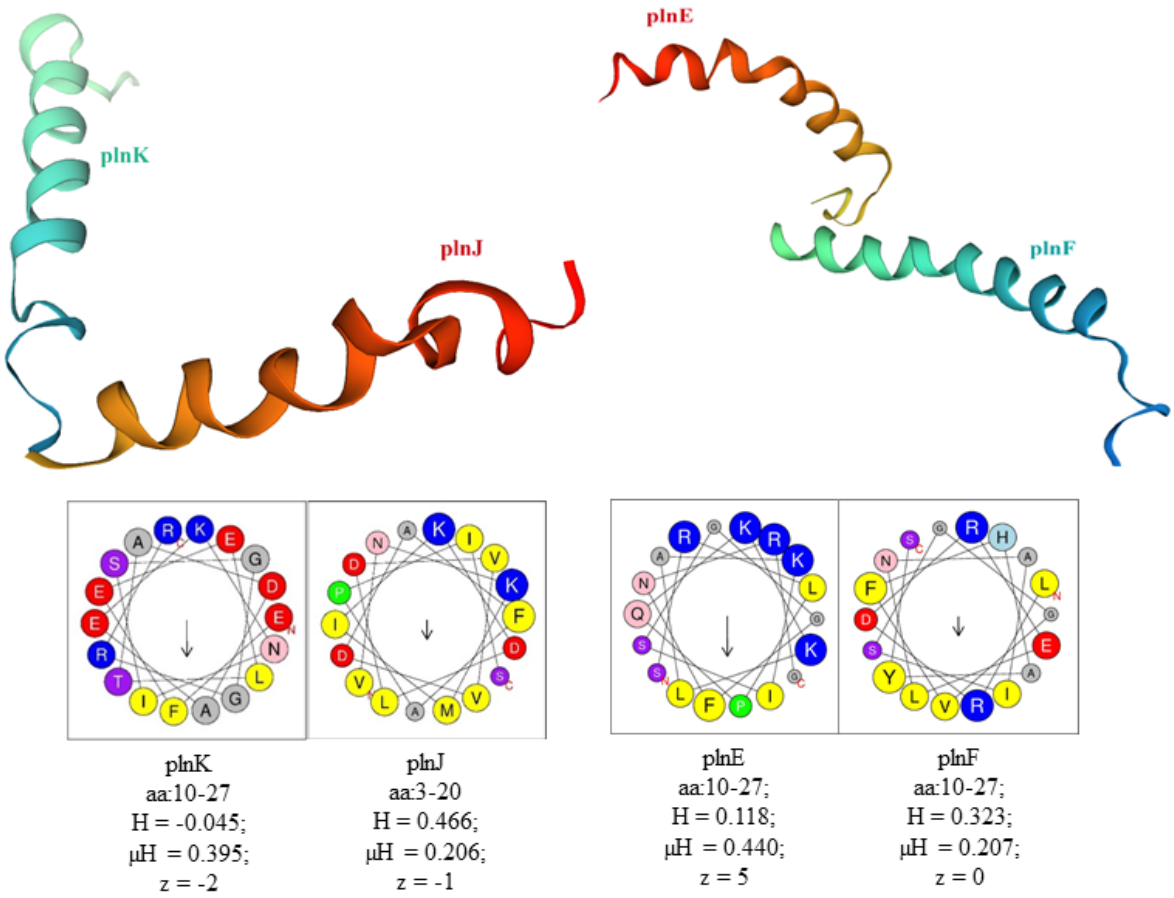

Figure 4

Homology-based 3D structures of SF9C two-peptide plantaricins predicted by the SWISS-MODEL Homology Modelling and helical wheel projections analysed by HeliQuest of the peptides PlnJ/K and PlnE/F. H and μH are the mean hydrophobicity and the hydrophobic moment calculated by HeliQuest, respectively. The net charge (z) was calculated at pH = 7.4, under the assumption that histidine is neutral and that the N-terminal amino group and the

C-terminal carboxyl group of the sequence are uncharged. aa is the amino acid residues. The one letter code for amino acids is used

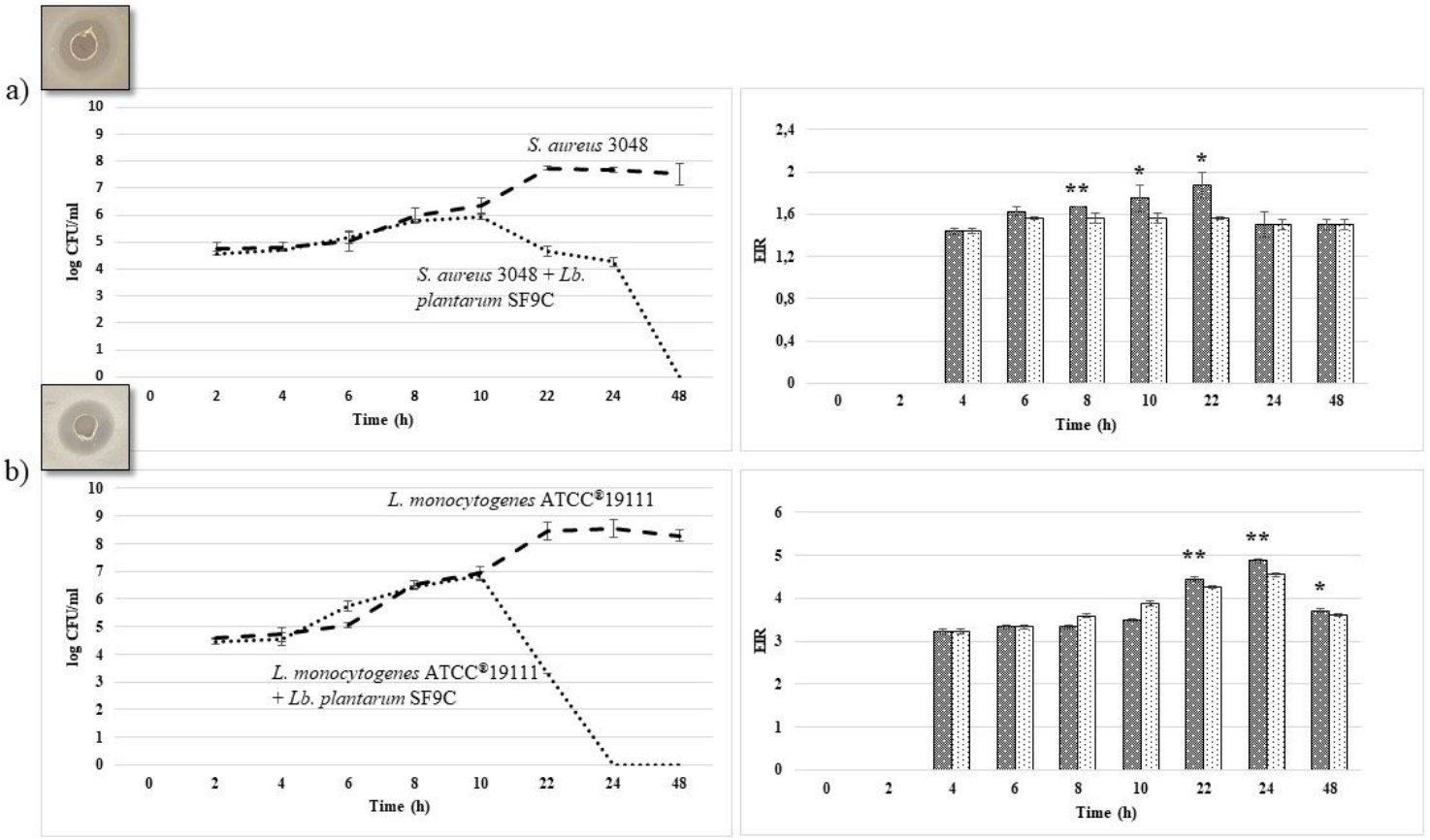

Figure 5

The growth curves of the test microorganisms: a) S. aureus 3048 and B) L. monocytogenes ATCC ®19111™ without (▬ ▬) or with (•••) Lb. plantarum SF9C cocultivation. Growth of Lb. plantarum SF9C was not influenced by incubation with test microorganisms (data not shown). The right side of the figure shows the bars representing EIR of test microorganisms, resulting from the antimicrobial activity of the Lb. plantarum SF9C after the growth in coculture with: A) S. aureus 3048 and b) L. monocytogenes ATCC®19111™ (▩), respectively, and after the growth of SF9C alone (⁘), obtained by agar spot test. Each value shown is the mean ± SD. Asterisks indicate statistically significant differences of EIR of test microorganisms obtained by the Lb. plantarum SF9C after the growth in coculture with test microorganisms and alone, at the same incubation time: *P < 0.05, **P < 0.01

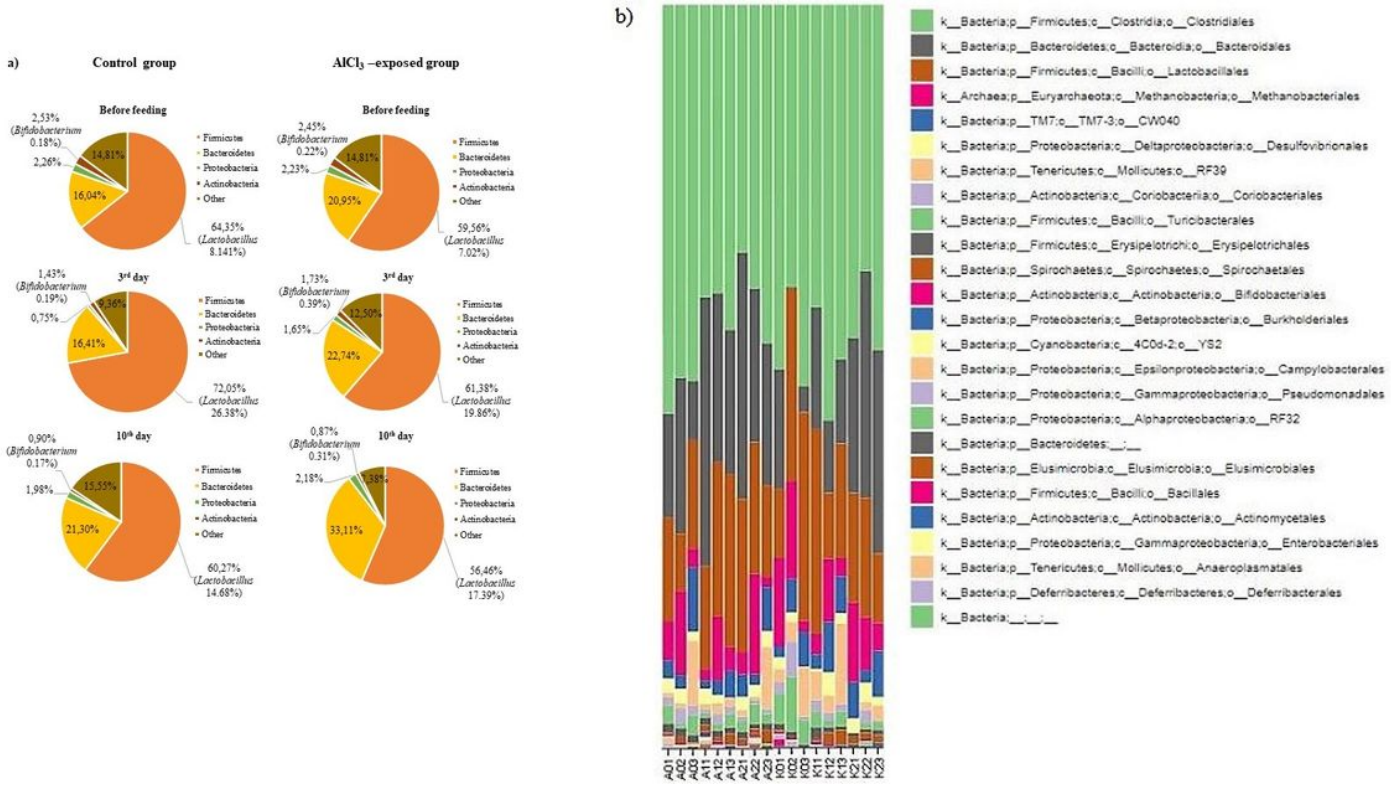

Figure 6

a) The four most abundant phyla detected in the faecal microbiota of control and AlCl3-exposed rats, both fed with Lb. plantarum SF9C and Lb. brevis SF9B b) The distribution of the bacterial classes in the faeces of control (K) and AlCl3-exposed rats (A), before application (0), and 3rd day (1), and 10th day (2) after application of SF9B and SF9C strains

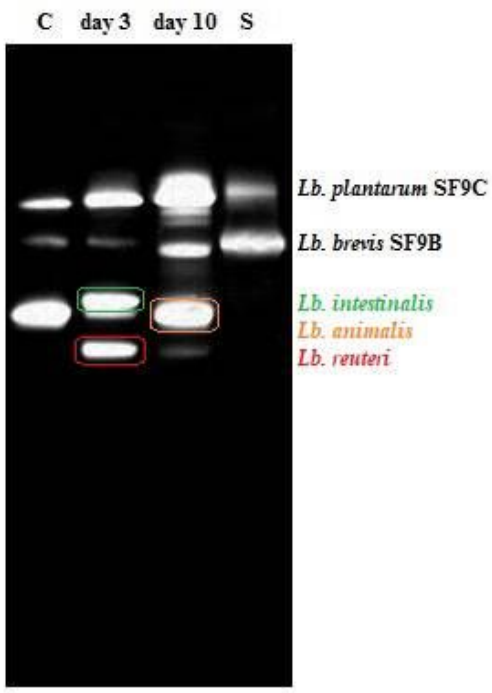

Figure 7

PCR-DGGE analysis of 16S DNA fragments generated with the universal bacterial primers HDA1 and HDA2 from the pooled DNA samples of the Lactobacillus species, isolated on MRS agar from faecal samples of rats fed with Lb. plantarum SF9C and Lb. brevis SF9B. Lanes: C - rats before application of SF9C and SF9B strains; day 3 - 3th day after application of SF9C and SF9B strains; day 10 - 10th day after application of SF9C and SF9B strains, S- the ladder of sequences from the pure cultures of SF9C and SF9B strains, respectively. Bands indicated by the symbols were excised and after amplification subjected to sequencing

Supplementary Files

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- Graphicalabstract.jpg
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- SupplementaryFigure1.jpg