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

Katarina Butorac

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski Fakultet

Martina Banic

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski Fakultet

Jasna Novak

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski Fakultet

Andreja Leboš Pavunc

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski Fakultet

Ksenija Uroic

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski 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-Biotehnoski Fakultet

Antonio Starčević

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski Fakultet

Jagoda Suskovic

Sveuciliste u Zagrebu Prehrambeno-Biotehnoski Fakultet

Blaženka Kos (✉ bkos@pbf.hr)

Faculty of Food Technology and Biotechnology, University of Zagreb <https://orcid.org/0000-0003-1711-316X>

Research

Keywords: antibacterial activity, gut colonization, *Lactobacillus*, microbiota, plantaricin, S-layer

Posted Date: February 12th, 2020

DOI: <https://doi.org/10.21203/rs.2.23400/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published on May 19th, 2020. See the published version at <https://doi.org/10.1186/s12934-020-01365-6>.

Abstract

Background: The influence of an S-layer carrying *Lactobacillus brevis* SF9B and plantaricin-producing *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 antimicrobial activity of *Lb. plantarum* SF9C and potential for their *in vivo* colonization, which could be the basis for the investigation of their synergistic functionality.

Results: We identified plantaricin encoding cluster in *Lb. plantarum* SF9C, a strain which efficiently inhibited *Listeria monocytogenes* ATCC®19111™ and *Staphylococcus aureus* 3048.

Contrary to a plantaricin-producing SF9C strain S-layer carrying SF9B strain excluded *Escherichia coli* 3014 and *Salmonella enterica* serovar *Typhimurium* FP1 from Caco-2 cells. DGGE analysis of the V2-V3 region of the 16S rRNA gene confirmed the transit of the two selected lactobacilli through the gastrointestinal tract. Microbiome profiling via the Illumina MiSeq platform revealed the prevalence of *Lactobacillus* spp. in the gut microbiota of rats suggesting their colonization potential in GIT.

Conclusion: The combined application of the two strains could influence intestinal microbiota composition, which is reflected through the increased abundance of *Lactobacillus* genus, but also along with the abundances of other bacterial genera, either in the model of health or aberrant microbiota. Obtained results contribute to the functional aspect of SF9C and SF9B strains which could be incorporated in the probiotic-containing functional foods to beneficially influence gut microbiota composition.

Background

Lactobacillus strains are omnipresent in different ecological niches. The representative members dominate the microbiota of the sauerkraut and are under the constant competition from 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. Some *Lactobacillus* strains are able to produce bacteriocins. Bacteriocin synthesis is shown to be one means by which strain producer can gain a competitive advantage and therefore is attractive in the terms of food biopreservation (Collins et al., 2018). Their applications are even expanding to the health 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 functionality of probiotics firstly through direct inhibition of the pathogens; secondly, as colonizing peptides, bacteriocins help the survival of the producing strain and finally, 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., 2011a). At the onset of the spontaneous fermentation, a LAB diversity is present, including *Leuconostoc mesenteroides* strains, while

a restricted number of *Lactobacillus* species dominates in the latter stages, consisting mainly of *Lactobacillus plantarum* (Beganović et al., 2014). *Lactobacillus brevis* SF9B was isolated from the respective spontaneous fermentation. This strain showed desirable functional and technological properties and expresses the S-layer proteins (Slps), which have a functional role in conveying the in vitro survival of SF9B in the intestinal tract (IT) stress conditions (Banić et al., 2018). Besides SF9B, autochthonous isolate SF9C, was isolated in 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 (Beganović et al., 2014). Therefore, the aim was to evaluate the competitive advantage potential of this strain, targeting on its possibility to produce bacteriocin. SF9C strain and S-layer carrying *Lb. brevis* SF9B can act synergistically and the use of substrates occurs by their combined metabolic activity. Next, a possible bacteriocinogenic activity of SF9C strain against Gram-positive pathogens *Listeria monocytogenes* ATCC®19111™ and *Staphylococcus aureus* 3048 was tested. To observe whether this strain has a broader spectrum of antimicrobial activity, antagonistic activity against Gram-negative *Escherichia coli* 3014 and *Salmonella* Typhimurium FP1 was evaluated. Apart from evaluating the antimicrobial activity, the induction of plantaricin production in SF9C producing strain was performed by coculturing with common food pathogens. Since the importance of the gut microbiota, both health and disease, is recognized, with preclinical evidence indicating that probiotic *Lactobacillus* strains may provide a means to ameliorate different disorders (Distrutti et al., 2014; Chen et al., 2016), the goal was to assess colonization potential and the capacity of the two *Lactobacillus* strains, SF9C and SF9B 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 sequencing analysis of faeces content was employed to demonstrate if *Lb. brevis* SF9B and *Lb. plantarum* SF9C have the potential for in vivo colonization and to influence the microbiota of rat's intestinal tract (IT).

Results

Plantaricin-related genes and WGS of bacteriocinogenic strain SF9C

Plantaricin-related genes *plnA*, *plnEF*, *plnJ* were identified using PCR amplification, suggesting that SF9C could harbour a *pln* locus in its genome. RAST annotation 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 the genome of *Lb. plantarum* SF9C is similar to that of other members of this species, which is average of 3.2 Mb. The number of coding sequences is 3,229, and the number of RNAs is 68. According to the comparative genomic studies that estimated the 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) of *Lb. plantarum* SF9C as annotated by RAST is shown in Figure 1. The pie chart is depicting the percentage distribution of 27 most abundant subsystem categories in strain SF9C. Next, WGS data were exploited to identify potential genomic triggers that may be responsible for the antimicrobial phenotype. The assembled contigs were compared with so far identified bacteriocins in the NCBI using the tblastn v2.2.27. The genes involved in the bacteriogenic activity and their function, after annotation of sequenced genes with RAST server, are listed in the Supplementary Table 1. Functional annotation confirmed the presence of other genes included in bacteriocin biosynthesis, also present in sequenced genomes of other *Lb. plantarum* strains (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 the 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 (Figure 2).

Induction of antimicrobial activity in *Lb. plantarum* SF9C through 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 antimicrobial activity among two naturally coexisting strains (data not shown). Interestingly, while SF9C strain demonstrated antibacterial activity against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048, strain SF9B failed in inhibition of respective pathogens by agar well-diffusion method (data not shown). Similar, 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 particular strains, with the strongest effect observed against *Enterococcus*, moderate against *Lactococcus* and the weakest against *Lactobacillus* strains by agar well-diffusion method. The results were confirmed by agar spot-test (data not shown). To assess the possibility of the potential induction of bacteriocinogenic activity, *Lb. plantarum* SF9C was cocultivated with *S. aureus* 3048 and *L. monocytogenes* ATCC®19111™, respectively. The cocultivation of *Lb. plantarum* SF9C significantly decreased *S. aureus* 3048 counts below the detection limit after 2 days (Figure 3A). The antimicrobial effect was even more pronounced when *L. monocytogenes* ATCC®19111™ was used as a cocultured strain (Figure 3B). The log CFU/ml values were significantly reduced after 24 hs to non-detectable levels when cocultivated the *L. monocytogenes* ATCC 19111 together with *Lb. plantarum* SF9C. The antilisterial activity of *Lb. plantarum* SF9C was determined by reduction of the CFU/ml. Moreover, effective inhibition ratio (EIR) values after the cocultivation of *Lb. plantarum* SF9C either with *L. monocytogenes* ATCC®19111™ or *S. aureus* 3048, were higher compared to the EIR values obtained after the growth of *Lb. plantarum* SF9C in monoculture. This could imply the possibility to induce plantaricin biosynthesis in the presence of 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, respectively 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).

By contrast, *Lb. plantarum* SF9C strain did not adhere to Caco-2 cells and did not affect the adhesion of these Gram-negative pathogens (data not shown). 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 ($5.708 \pm 0.014 \log_{10}$ CFU) than Caco-2 cells exposed to *S. Typhimurium* FP1 alone ($6.825 \pm 0.099 \log_{10}$ CFU). Interestingly, after removal of the S-layer proteins, as a strains specific feature, *Lb. brevis* SF9B completely lost the adhesion ability to Caco-2 cell line as it is shown previously by Banić *et al.* (2018), and did not influence *S. Typhimurium* FP1 and *E. coli* 3014 adhesion (data not shown). In the competition assay as well as exclusion assays, Caco-2 cells incubated with *Lb. brevis* SF9B had significantly fewer *Salmonella* cells adhered ($5.613 \pm 0.135 \log_{10}$ CFU and $4.708 \pm 0.014 \log_{10}$ CFU, respectively) compared to Caco-2 cells infected with *Salmonella* alone ($6.825 \pm 0.099 \log_{10}$ CFU). 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 when the pathogen was added subsequently (exclusion), or of $2.117 \Delta \log_{10}$ CFU 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

Acetylcholinesterase (AChE) activity was assessed in the brain tissue homogenates to monitor the possible influence of AlCl_3 treatment in the rats. In our previous paper (Ledinski *et al.* 2017), the AChE activity and histopathological and immunohistochemical analyses of the brain, number of plaques and AChE activity was significantly higher in the brains of the AT group, compared to that of the control group ($P < 0.05$). According to the result, the neuropathological changes were observed in the Al-exposed rat group (Figure 4.). Diffuse plaques, also called benign plaques, occurred much earlier than the neuritic plaques in the cerebellum. In the treatments cerebellum of the AT rats were negative on AT8 marker, but positive on 4G8 and Iba1 marker (Figure 4.). 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 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 revealed that across all, control or Al-exposed groups (calculated as mean values from all the experiments), the dominant phyla were *Firmicutes* and *Bacteroidetes*, which respectively made up 63% ($62.35 \pm 5.40\%$) and 22% ($21.76 \pm 6.20\%$) of total abundance, with lower contributions from *Actinobacteria* ($1.65 \pm 0.73\%$) and *Proteobacteria* ($1.84 \pm 0.58\%$) (Figure 5A). The *Firmicutes* and *Bacteroidetes* phyla accounted for more than 85% of total sequences, similarly 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 rats exposed to aluminium, the abundance of *Firmicutes* and *Actinobacteria* decreased, while the abundance of *Bacteroidetes* increased compared to the control group. In a bacterial class-level, the most abundant classes for all groups were *Bacilli*,

Clostridia and *Bacteroidia* (Figure 5B). *Bifidobacterium* was also consistently detected over the samples (Fig. 5A). Since our main goal was to evaluate the survival and colonisation potential of the two *Lactobacillus* strains in the model of healthy, but also of 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 well adaptation of SF9B and SF9C to the IT, especially since these two strains are not of an intestinal, but sauerkraut origin. Rat's gut microbiome analysis revealed taxonomic differences of gut microbiota composition influenced by *Lactobacillus* treatments. The culture-independent PCR-DGGE approach was applied to verify the differences in the gut microbiota composition of faecal samples among groups of rats (data not shown). Culture on selective media revealed the presence of presumptive *Lactobacillus* in the faeces of the control group at 5.6×10^7 CFU/ml and AT rat at 1.99×10^8 CFU/ml, respectively on day 10 after *Lactobacillus* treatment. 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 (Figure 6). 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 after the 3rd day of *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 as. Furthermore, in a DGGE profile of the healthy rat, an intensive band was consistently detected, which after the sequencing and BLAST search was assigned to *Lb. animalis*, while in the 3rd day after *Lactobacillus* treatment, a faint band corresponding to *Lactobacillus intestinalis* was observed (Figure 6.).

Discussion

Functional genomics in probiotic research has facilitated the characterization of probiotic *Lactobacillus* strains. These findings also contribute to the application of the probiotics in the development of the functional foods. Here *Lb. plantarum* SF9C genome sequence was determined by using a WGS assembly approach, with a focus on the characterisation of the plantaricin locus. Whole genome sequence confirmed the presence of a plantaricin (pln) loci in SF9C. 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 and plantaricin JK have already been described in certain *Lb. plantarum* strains. These compounds are translated as prepeptides and afterwards are cleaved off during the cell transport to produce active peptides whose activity is dependent on the complementary action of the two peptides PlnE/PlnF i.e. PlnJ/PlnK (Diep et al., 2009). Bacteriocin activity together with a competition for limited nutrients, competitive exclusion, and the stimulation of mucosal immunity could enhance intestinal health (Dobson et al., 2012). Here the antagonizing activity of the *Lb. plantarum* SF9C against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 was explored. *Lb. plantarum* SF9C decreases drastically pH (3.86 ± 0.04) after overnight growth due to the lactic acid production ($2.25 \pm$

0.24% v/v) which creates unfavourable local microenvironment for pathogenic bacteria. The mechanisms of the antibacterial activity of SF9C strain is multifactorial, including inhibition by produced lactic acid, but also the activity of potential SF9C - plantaricin, especially since the inhibition was alleviated after CFS neutralisation and proteinase K treatment. *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 possess a number of mechanisms that enable to combat the challenges posed by acidic environments and therefore can tolerate the low pH values. This is supported by finding that *Lb. plantarum* SF9C strain demonstrated antibacterial activity against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 while *Lb. brevis* SF9B contrariwise, although an effective lactic acid producer, failed in the inhibition of the respective pathogens. Additionally, *L. monocytogenes* and *S. aureus* were target-selected since these foodborne gram-positive pathogens are contaminating 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. We hypothesize that the potential plantaricin is involved in the antibacterial activity towards *L. monocytogenes* or *S. aureus*. Since one strategy to achieve expression of otherwise silenced bacteriocins is induction of their biosynthesis by growth in cocultures (Chanos and Mygind, 2016; Maldonado-Barragán et al., 2013; Kos et al., 2011) we studied the potential to induce plantaricin biosynthesis by cocultivation of *Lb. plantarum* SF9C with *S. aureus* 3048 and *L. monocytogenes* ATCC®19111™. Antimicrobial activity was initially detected after 10 hs of incubation in the early exponential phase of the pathogen growth. The highest antimicrobial activity was marked after 24 hs in the late exponential phase of *L. monocytogenes* ATCC®19111™ and after 48 hs 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 in *Lb. plantarum* species. One can speculate that the SF9C plantaricin activity is potentially enhanced in the presence of *L. monocytogenes* ATCC®19111™. *L. monocytogenes* tolerates a broad pH range, the antilisterial potential of SF9C could be assigned to the potential plantaricin synthesis. Contrariwise, SF9C did not exhibit inhibitory activity against enteric Gram-negative *E. coli* 3014 and *S. Typhimurium* FP1. 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. S-layer proteins (Slps) may act as a mediators of bacterial adhesion and as such may contribute to the antimicrobial activity against the pathogens with whom the 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 GHCl treatment negatively affected the coaggregation ability of SF9B strain. Moreover, the removal of Slps utterly eradicated binding of S-layered SF9B strain in the adhesion experiment (Banić et al., 2018). The results of this study revealed that S-layer depleted *Lb. brevis* SF9B did not compete and exclude the pathogens from the Caco-2 cells. S-layer expressing SF9B strain demonstrated significant levels ($P < 0.01$) of exclusion capacity against both *E. coli* 3014 and *S. Typhimurium* FP1, respectively. Conversely, SF9B strain ability to compete with pathogens for Caco-2 cell binding sites was less prominent, though significant ($P < 0.01$), with *E. coli* 3014, whereas it was not

significant ($P \geq 0.01$) with *S. Typhimurium* FP1 in comparison to pathogen adhesion to Caco-2 cells without addition of SF9B strain. *Lb. brevis* SF9B strain may possibly better prevent the adhesion of *S. Typhimurium* FP1 within in vivo conditions owing to considerable coaggregation capacity (Banić et al., 2018), alongside with longer in vivo coincubation period compared to a 1-hour incubation tested 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. In addition, the pathogen inhibition ability of *Lb. brevis* SF9B did not noticeably correlate with its adhesive capacity, which is in accordance with Uroić et al. (2016). The results clearly suggest that *Lb. brevis* SF9B competed more efficiently against *E. coli* 3014 than *S. Typhimurium* FP1, it might be possible that the mechanisms of competition and exclusion differ and are highly specific for each pathogen. Combining plantaricin producer *Lb. plantarum* SF9C with S-layers expressing *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 antimicrobial activity. One drawback of the direct application of bacteriocins to food product is that the loss of activity occurs over time because of enzymatic degradation and interactions with food components such as proteins and lipids (Hartmann et al., 2011), here we suggest the potential application of the plantaricin producing cells *Lb. plantarum* SF9C which can act synergistically with *Lb. brevis* SF9B, to eliminate common pathogens. To our knowledge *Lb. brevis* SF9B is a non-producing bacteriocin strain, whose genome contains *plnI* encoding the bacteriocin immunity protein (Banić et al., 2018). This cooperation of coexisting *Lactobacillus* strains can be also exploited to control bacterial infection for the reestablishment of the disturbed microbiota associated with certain diseases (Dicks et al., 2018). Further, in an attempt to demonstrate the potential of coculture to compete among healthy or disturbed gut microbiota, or even colonize the rat's IT, plantaricin-producing SF9C and S-layer expressing SF9B were orally applied to a healthy and AI-treated rats. AI exposure can cause a variety of adverse physiological effects in humans and animals (Chen et al., 2016; Ledinski et al., 2017). In this study, we aim to outline its adverse effects on the gut microbiome. The changes in intestinal microbiota composition were observed, not only in the abundance of *Lactobacillus* genus, but also in abundance of other bacterial genera. According to microbiome analysis, *Blautia* genus was not detected in the healthy rat group but was identified in AT rat group in which its ratio decreased as revealed 3 and 10 days after the *Lactobacillus* treatment. The ratio of *Bacteroides* and *Phascolarctobacterium* genera before the *Lactobacillus* treatment was higher than in AT rat group compared to healthy groups, but 3 days after the *Lactobacillus* administration the ratio of this genera was reduced in AT rat group. Furthermore, the abundance of *Bifidobacterium* genus remains unchanged, before and after *Lactobacillus* treatment, in both healthy and AT rat group. *Clostridium* and *Adlercruetzia* genera were evenly present in both groups, before application of two *Lactobacillus* strains, while after the 3rd and 10th day the ratio of *Adlercruetzia* genus was decreased in both groups of rats and the ratio of *Clostridium* genus was decreased only in AT rat group. An abundant prevalence of *Lactobacillus* spp. was observed in the microbiota of the *Lactobacillus* treated rats, even 10 days after inoculation of the rats, compared to the microbiota of the control group. This increased abundance of *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 strains on the competitive ability of autochthonous strains. Further studies are needed to better understand the probiotic effects of these two strains on a healthy and disturbed gut microbiome structure and function, and the possible impacts on other parameters important in alleviating AI-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. At the same, SF9B could benefit from the improved colonization due to plantaricin production by *Lb. plantarum* SF9C, resulting in a broader spectrum of antimicrobial activity of the coculture against the pathogens. The plantaricin and S-layer expressing *Lactobacillus* duo could be a promising probiotic candidate, which needs further investigation for an application in functional food or targeted application for the different disorders closely linked with a dysbiosis of gut microbiota.

Materials And Methods

Bacterial strains, culture media and cultivation conditions

Bacterial strains and cultivation conditions, used in this study, are listed in Table 1.

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 Rudjer Bošković Institute, Zagreb, Croatia. Caco-2 cells were grown as monolayer cultures in RPMI 1640 medium (GIBCO, USA), supplemented with 15% of the foetal bovine serum (GIBCO, USA) and 4500 mg/L of glucose. Cells were grown up to confluence at 37 °C and 5% of CO₂ 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 gene 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 by Ben Omar *et al.* (2008). Therefore, amplification of DNA fragments was performed in 50 µL reaction mixtures containing 25 µL of Esmerald Amp MAX HS PCR Mastermix Premix (TaKaRa, Ohtsu, Japan), 200 nmol/L of each oligonucleotide primer, 300 ng of DNA template and EsmeraldAmp dH₂O. 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 genes encoding bacteriocins

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 were done. 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 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).

***In vitro* assays**

Testing of antimicrobial activity

Antimicrobial activity of *Lb. plantarum* SF9C and *Lb. brevis* SF9B against *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 was tested by agar-spot test. 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 in the analysis of antimicrobial activity. Antibacterial activity of the overnight grown culture and cell-free supernatant (CFS) was tested. 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 thereof with

Proteinase K (Invitrogen, Carlsbad, CA, USA) at a concentration of 1 mg/mL during 2 h at 37 °C. To eliminate the inhibitory effect of the produced lactic acid, clear MRS broth was treated with lactic acid (Kemika, Zagreb, Croatia) at the same concentration and pH value present in the overnight culture of SF9B strain. Statistical analysis was carried out using ANOVA and the results are reported as mean values of three individual experiments $\pm$ 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 $P < 0.05$.

Induction of antibacterial activity

In order to induce bacteriocins synthesis of *Lb. plantarum* SF9C, cocultivation with *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048 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*, during every 2 hours for 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 antimicrobial activity of SF9C strain, in monoculture and coculture, was tested by agar spot method as described above. Experiments were conducted in triplicate and values were expressed as the mean $\pm$ 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 1×10^9 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 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, resuspended 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 four weeks as follows: (a) first trial group represented a model of induced aluminium toxicity (AT) 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 with saline solution in the same manner. The rats from each group were further assigned to three groups based on the *Lactobacillus* treatment. AT model group was divided into: AT1 group (n=3, no *Lactobacillus* treatment), AT2 group (n=3, orally-cannulated every third day during the four weeks of

treatment with a single dose (3×10^9 CFU) of *Lactobacillus* strains resuspended in saline solution) and AT3 group (n=3, orally-cannulated for five consecutive days with a single dose (3×10^9 CFU/mL) of *Lb. brevis* SF9B and *Lb. plantarum* SF9C strains resuspended in saline solution, starting 24 h after the last treatment). The control group of rats was also divided into three groups (C1-C3) in the same manner and treatments as the AT model. No side effects were reported following *Lactobacillus* administration. 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 samples were collected from the cages within two hours in after the animals were transferred into clean cages: (a) before the starting treatment (AT1-AT3 and C1-C3 groups); (b) 24 hours after the last treatment (AT1-AT3 and C1-C3 groups) and (c) on third and seventh day following the last probiotic administration (AT3 and C3 group) in triplicates. Faeces samples were stored at -80°C until analysis as described in the next chapter.

Bacterial 16S rRNA sequencing and processing using QIIME

Faeces 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 $\pm$ 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

Funding

This work has been supported by Croatian Science Foundation through the projects IP-2014-09-7009 and IP-2019-04-2237. Authors also acknowledge financial support of Zagreb University, Croatia. The authors declare that there is no conflict of interest.

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

References

1. Altschul SF, Gish W, Miller W, Myers E W, Lipman D.J. Basic local alignment search tool. J. Mol. Biol. 1990, 215(3), 403-404. doi:10.1016/S0022-2836(05)80360-2
2. Anukam KC, Macklaim JM, Gloor GB, Reid G, Boekhorst J, Renckens B. et al. Genome Sequence of *Lactobacillus pentosus* KCA1: Vaginal Isolate from a Healthy Premenopausal Woman. PLOS One 2013, 8 (3) |e59239. <https://doi.org/10.1371/journal.pone.0059239>
3. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 2008, 9(75). <https://doi.org/10.1186/1471-2164-9-75>.
4. Banić M, Uroić K, Leboš Pavunc A, Novak J, Zorić K, Durgo K, et al. Characterization of S layer proteins of potential probiotic starter culture *Lactobacillus brevis* SF9B isolated from sauerkraut. LWT-Food Sci. Technol. 2018, 93, 257-267. <https://doi.org/10.1016/j.lwt.2018.03.054>
5. Beganović J, Kos B, Leboš Pavunc A, Uroić K, Jokić M, Šušković J. Traditionally produced sauerkraut as source of autochthonous functional starter cultures. Microbiol. Res. 2014, 169 (7-8), 623-632. <https://doi.org/10.1016/j.micres.2013.09.015>
6. Beganović J, Leboš Pavunc A, Gjuračić K, Špoljarec M, Šušković J, Kos B. Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. J. Food Sci. 2011a, 76(2), M124-M129. <https://doi.org/10.1111/j.1750-3841.2010.02030.x>
7. Beganović J, Frece J, Kos B, Leboš Pavunc A, Habjanič K, Šušković J. Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. Anton. Leeuw. 2011b, 100, 43-53. <https://doi.org/10.1007/s10482-011-9563-4>
8. Ben Omar N, Abriouel H, Keleke S, Sánchez Valenzuela A, Martínez-Cañamero M, Lucas López et al. Bacteriocin-producing *Lactobacillus* strains isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operons. Int. J. Food Microbiol. 2008, 127(1-2), 18-25. <https://doi.org/10.1016/j.ijfoodmicro.2008.05.037>

9. Chen P, Miah MR, Aschner M. (2016) Metals and neurodegeneration. *F1000 Res* 5: 366. <https://doi.org/10.12688/f1000research.7431.1>
10. Chikindas ML, Weeks R, Drider D, Chistyakov V.A, Dicks LMT. Functions and emerging applications of bacteriocins. *Curr Opin Biotech.* 2017, 49, 23–28. <https://doi.org/10.1016/j.copbio.2017.07.011>
11. Chanos P. and Mygind T. Co-culture-inducible bacteriocin production in lactic acid bacteria. *Appl. Microbiol. Biot.* 2016, 100(10), 4297–4308. <https://doi.org/10.1007/s00253-016-7486-8>
12. Collins FWJ, Mesa-Pereira B, O'Connor PM, Rea MC, Hill C, Ross RP. Reincarnation of Bacteriocins from the *Lactobacillus* Pangenomic Graveyard. *Front. Microbiol.* 2018, 9: 1298. <https://doi.org/10.3389/fmicb.2018.01298>
13. Dicks LMT, Dreyer L, Smith C, van Staden AD. A Review: The Fate of Bacteriocins in the Human Gastro-Intestinal Tract: Do They Cross the Gut–Blood Barrier? *Front. Microbiol.*, 2018. 9: 2297. <https://doi.org/10.3389/fmicb.2018.02297>
14. Diep DB, Straume D, Kjos M, Torres C, Nes I F. An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. *Peptides*, 2009. 30, 1562-74. <https://doi.org/10.1016/j.peptides.2009.05.014>
15. Distrutti E, O'Reilly JA, McDonald C, Ciprian S, Renga B, Lynch MA. et al. Modulation of intestinal microbiota by the probiotic VSL#3 resets brain gene expression and ameliorates the age-related deficit in LTP. *PLoS One*, 2014. 9(9): e106503. <https://doi.org/10.1371/journal.pone.0106503>
16. Dobson A, Cotter PD, Ross RP, Hill C. Bacteriocin Production: a Probiotic Trait? *Appl. Environ. Microb.* 2012. 1-6. <https://doi.org/10.1128/AEM.05576-11>
17. Frece J, Kos B, Svetec IK, Zgaga Z, Beganović J, Leboš A, Šušković J. Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice. *J. Dairy Res.* 2009, 76:98e104.
18. Garcia-Mazcorro JF, Lage NN, Mertens-Talcott S, Talcott S, Chew B, Dowd SE. et al. Effect of dark sweet cherry powder consumption on the gut microbiota, short-chain fatty acids, and biomarkers of gut health in obese db/db mice. *PeerJ.* 2018, 6: e4195. <https://doi.org/10.7717/peerj.4195>
19. Hartmann HA, Wilke T, Erdmann R. Efficacy of bacteriocin-containing cell-free culture supernatants from lactic acid bacteria to control *Listeria monocytogenes* in food. *Int. J. Food Microbiol.*, 2011. 146, 192–199. <https://doi.org/10.7717/peerj.4195>
20. Hynönen U, Kant R, Lähtinen T, Pietilä TE, Beganović J, Smidt H. et al. Functional characterization of probiotic surface layer protein-carrying *Lactobacillus amylovorus* strains. *BMC Microbiol.* 2014, 14:199. <https://doi.org/10.1186/1471-2180-14-199>
21. Kos B, Beganović J, Jurašić L, Švađumović M, Leboš Pavunc A, Habjanič K. et al. Coculture-inducible bacteriocin biosynthesis of different probiotic strains by dairy starter culture *Lactococcus lactis*. *Mljekarstvo* 2011, 61(4), 273-282.
22. Kos B, Šušković J, Beganović J, Gjuračić K, Frece J, Iannaccone C. et al. Characterization of the three selected probiotic strains for the application in food industry. *World J. Microbiol. Biotechn.* 2008, 24, 699-707. <https://doi.org/10.1007/s11274-007-9528-y>

23. Leboš Pavunc A, Kos B, Beganović J, Uroić K, Bučan D, Šušković J. Antibiotic susceptibility and antimicrobial activity of autochthonous starter cultures as safety parameters for fresh cheese production. *Mljekarstvo*, 2013, 63(4), 185-194.
24. Ledinski M, Oršolić N, Kukolj M, Odeh D, Mojzeš A, Uroić K, Gačina, L. Analysis of intestine microbiome in the Alzheimer's disease rat model. *Annual Meeting of the Croatian Immunological Society with EFIS on Tour Zagreb*, 2017 40.
25. Leenhouts K, Kok J, Venema G. Stability of Integrated Plasmids in the Chromosom *Lactococcus lactis*. *Appl. Environ. Microb.* 1990, 56(9), 2726-2735.
26. Maldonado-Barragán A, Caballero-Guerrero B, Lucena-Padrós H, Ruiz-Barba JL. Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. *Food Microbiol.* 2013, 33: 40e47. doi:10.1016/j.fm.2012.08.009
27. Mills S, Ross RP, Hill C. Bacteriocins and bacteriophage; a narrow-minded approach to food and gut microbiology. *FEMS Microbiol. Rev.* 2017. 022(41), S129-S153. [https://doi: 10.1093/femsre/fux022](https://doi.org/10.1093/femsre/fux022)
28. OJEU. (2010). Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. *Official Journal of the European Union* 276: 33-79.
29. Taverniti V, Stuknyte M, Minuzzo M, Arioli S, De Noni I, Scabiosi C. et al. S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. *Appl. Environ. Microb.* 2013. 79, 1221-1231. [https://doi: 10.1128/AEM.03056-12](https://doi.org/10.1128/AEM.03056-12)
30. Ulusoy HB, Sonmez MF, Kilic E, Caliskan K, Bkaraca B, Kara M. et al. Intraperitoneal administration of low dose aluminium in the rat: how good is it to produce a model for Alzheimer disease. *Arch. Ital. Biol.* 2015, 153, 266-278. doi: 10.12871/00039829201543.
31. Uroić K, Novak J, Hynönen U, Pietilä TE, Leboš Pavunc A, Kant R, Šušković J. The role of S-layer in adhesive and immunomodulating properties of probiotic starter culture *Lactobacillus brevis* D6 isolated from artisanal smoked fresh cheese. *LWT-Food Sci. Technol.* 2016, 69, 625-632. <https://doi.org/10.1016/j.lwt.2016.02.013>
32. van Heel AJ, de Jong A, Song C, Viel JH, Kok J, Kuipers OP. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Res.* 2018, 2, 46(W1):W278-W281. <https://doi.org/10.1093/nar/gky383>
33. Van Pijkeren JP and O'Tolle PW. Comparative and Functional Genomics of the Genus *Lactobacillus*. In: Ljungh, Å. and Wadström, T. (eds.) *Lactobacillus* Molecular Biology: From Genomics to Probiotics. 2013. Caister Academic Press, Norfolk, UK, pp. 59-82. <https://doi.org/10.1002/elsc.200990012>

Tables

Table 1. Bacterial strains used in this study.

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

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

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

Strain	<i>S. Typhimurium</i> FP1 (log CFU/ml)		<i>E. coli</i> 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
<i>Lb. brevis</i> SF9B*	5.613 ± 0.135 ^b	4.708 ± 0.014 ^a	6.393 ± 0.101 ^a	6.308 ± 0.194 ^a

* SF9B strain completely lost the adhesion ability to Caco-2 cell lines (Banić et al., 2018) after the removal of the S-layer, and therefore probably did not impair *S. Typhimurium* FP1 and *E. coli* 3014 adhesions (the values were the same as in control).

**a means statistically significant difference (P < 0.01) of adhered *E. coli* 3014 and *S. Typhimurium* FP1, without (control) and with the addition of the *Lb. brevis* SF9B strain compared to control. ^b no statistically significant difference (P < 0.01) compared to control.

Figures

Figure 1

Distribution and categorization of sequenced genes in *Lb. plantarum* SF9C strain.

Figure 2

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

Figure 3

Antimicrobial effect of *Lb. plantarum* SF9C against *S. aureus* 3048 (A) and *L. monocytogenes* ATCC®19111™ (B) (···) (log CFU/mL). Growth curves of the strains *Lb. plantarum* SF9C (■) and the respective test-microorganism (■ ■). Bars represent effective inhibition ratio (EIR) determined after co-cultivation of *Lb. plantarum* SF9C strain with *L. monocytogenes* ATCC®19111™ and *S. aureus* 3048, respectively (▨) and during the growth of *Lb. plantarum* SF9C strain in a monoculture (⋯).

Figure 4

Photomicrograph of sagittal section in a rat cerebellum; a control group (C) and AlCl₃ treated group (AT). Morphological profile of the rat Purkinje cells (stained with Bielschowsky silver staining), diffuse plaques (4G8, scale bar 10 x = 100 µm) and expression of microglia cells markers Iba1 (scale bare 10 x = 100 µm; scale bar 40 x = 20 µm) in the molecular layer (ML) and granular layer (GL) of the cerebellum.

Figure 5

A) The four most abundant phyla detected in the faecal microbiota of control and Al exposed rats, both fed with Lb. plantarum SF9C and Lb. brevis SF9B. B) The distribution of the bacterial classes in the faeces of control and Al exposed rats consuming SF9B and SF9C Lactobacillus strains. Sample

designations: first ordered number indicates the day of faeces sampling (0-before; 1-3rd day; 2-10th day after the Lactobacillus administration, respectively); second ordered number indicates rat labels (A(AP)-AI exposed rats; K(KP)-control group).

Figure 6

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarytableS1.docx](#)
- [Graphicalabstract.jpg](#)