Evaluation of the Probiotic Properties of Lacticaseibacillus casei 431® Isolated from Food for Special Medical Purposes§

Running head: Probiotic activity of L. casei 341® in FSMPs

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SUMMARY

Research background. Increasing awareness of the importance of nutrition in health promotion and disease prevention has driven to the development of foods for special medical purposes (FSMPs). In this research, the probiotic strain Lacticaseibacillus paracasei subsp. paracasei (Lacticaseibacillus casei 431®) was incorporated into FSMPs to develop an innovative product. The aim was to investigate the influence of FSMPs matrix on the specific probiotic properties of L. casei 431® in vitro.

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Experimental approach. A series of in vitro experiments were performed as part of the probiotic approach. After evaluation of antibiotic susceptibility profiles, functional properties such as survival under simulated gastrointestinal tract (GIT) conditions, bile salt deconjugation activities, cholesterol assimilation, antagonistic activity against spoilage bacteria, and adhesion to Caco-2 cell line monolayers and extracellular matrix proteins were investigated.

Results and conclusions. L. casei 431® strain, both the lyophilised one and the one isolated from FSMPs matrix, effectively survived the adverse simulated gastrointestinal conditions without significant effect of the food matrix. The effect of FSMPs matrix on the deconjugation activity of bile salts of L. casei 431® was minimal, however, cholesterol assimilation was increased by 16.4 %. L. casei 431® showed antibacterial activity against related lactic acid bacteria whether it was implemented in FSMPs or not. Conversely, the probiotic strain isolated from FSMPs matrix showed significantly higher inhibitory activity against 6 potential pathogens than the lyophilised culture. The autoaggregation ability of L. casei 431® cells was not affected by FSMPs matrix. The adhesion of L. casei 431® bacterial cells to the extracellular matrix proteins was reduced after treatment with proteinase K, with the highest adhesion observed to laminin. Adhesion of L. casei 431® reduced E. coli 3014 binding by 1.81 log units and S. Typhimurium FP1 binding by 1.85 log units to Caco-2 cell lines, suggesting the potential for competitive exclusion of these pathogens.

Novelty and scientific contribution. The results support the positive influence of FSMPs matrix on the specific probiotic properties of L. casei 431®, such as antibacterial activity, bile salts deconjugation and cholesterol assimilation, while the incorporation of this probiotic strain brought additional functional value to the FSMPs. The achieved synergistic effect of the join application of L. casei 431® and innovative FSMPs matrix contributed to the development of the novel formulation of an improved functional food product with added value.

Keywords: Lactcaseibacillus paracasei ssp. paracasei; food for special medical purposes; functional food; probiotics

INTRODUCTION
Nowadays, global trends are placing increasing importance on functional nutrition as a basis for human well-being and health management. Increasing consumer awareness of the relationship between nutrition and health and the production of state-of-the-art food with positive health effects or probiotic food supplements opens up opportunities for both consumers and the food and pharmaceutical industries. Likewise, nutrition science points out the importance of nutrition in disease
prevention, which, along with the avoidance of harmful modern lifestyle habits, can reduce the risks of metabolic syndrome which consists of a group of risk factors: elevated blood pressure or blood sugar concentration, elevated cholesterol levels and increased abdominal fat (1). Metabolic syndrome is associated with increased incidence of cardiovascular disease, diabetes, obesity and cancer. This has stimulated the intensification of global research activities in the field of nutrigenomics, the development of personalised foods, and foods intended to prevent the occurrence or alleviate the consequences of acute or chronic diseases. Food for special medical purposes (FSMPs) is intended for the complete or partial nutrition of patients with a disturbed ability to metabolise food or for patients who have special medical requirements (2).

Another aspect of nutrition as a health-promoting concept is functional food based on the addition of probiotics that promote cognitive response, improve immune system, and overall well-being (3). The molecular mechanisms responsible for probiotic effects can be associated with a range of microbial metabolites and molecules exposed at the cell surface, such as surface (S)-layer proteins, exopolysaccharides, bacteriocins, which are also mediated by enzymatic activities of bioactive peptides produced by probiotic proteolytic enzymes (4-6). According to the European legislation, probiotic preparations are categorised as functional food ingredients or as food supplements (7). According to the US Food and Drug Administration, probiotics are not only used as health-promoting food supplements (nutraceuticals), but also as live biotherapeutic products (LBPs) (8). The quality assurance of probiotics as LBPs on the European market is established by the European Pharmacopoeia Commission (9). Probiotics are used to improve digestive health and strengthen the immune system. The International Scientific Association for Probiotics and Prebiotics (ISAPP) has supported and reaffirmed the FAO/WHO definition of a probiotic as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (10).

The most commonly recognised probiotic strains come from generally regarded as safe (GRAS) lactococci genera. Traditionally associated with fermented food, LAB favorably influence the intestinal microbiota balance (11,12). Many of them are distributed in various probiotic products such as functional foods, infant formulations, foods for special dietary and medical purposes, as well as dietary supplements (nutraceuticals) in the form of capsules, tablets, powders or solutions in lyophilised and microencapsulated form (13-15). Lacticaseibacillus paracasei strains have been documented in numerous studies as bacteria with strong probiotic activity (16). These are Gram-positive, non-sporogenic, facultatively heterofermentative bacteria that are indogenous to the intestinal microbiota. The beneficial effects of Lacticaseibacillus paracasei strains have been demonstrated in patients with various digestive disorders, infectious diseases, obesity and depression in clinical studies (17).
Various methods for determining the content, purity, and identity of microorganisms in probiotic products have been described in the scientific literature, but the influence of the product matrix on the functionality of the probiotic culture is not widely known (18).

Therefore, the aim of this work was to develop novel FSMPs product with the addition of the probiotic *Lacticaseibacillus paracasei* (*Lacticaseibacillus casei* 431®) strain. This new product would be intended for malnourished patients when, due to medical reasons, it is not possible to utilize the nutritional needs from a normal diet. Probiotic strain in the product may also provide added functional value of this final preparation.

The impact of the food matrix on the functional properties of *L. casei* 431® was tested through a series of *in vitro* experiments as part of the proposed probiotic concept, which include sensitivity or resistance to antibiotics according to EFSA (19), survival under simulated gastrointestinal tract (GIT) conditions, deconjugation activity towards bile salts, cholesterol assimilation, antagonistic capacity including potential bacteriocin activity and ability of adhesion on the Caco-2 cell line monolayer and extracellular matrix proteins.

**MATERIALS AND METHODS**

*Food for special medical purposes with Lacticaseibacillus paracasei subsp. paracasei strain L. casei 431®*

Probiotic culture *L. casei* 431® (Chr. Hansen Holding A/S, Hoersholm, Denmark) was tested as pure lyophilised culture and after isolation from the samples of FSMPs. The pure lyophilised culture contains freeze-dried cells of *Lacticaseibacillus paracasei* subsp. *paracasei*, as well as sucrose, maltodextrin and sodium ascorbate.

The food matrix of FSMPs that was produced in the development laboratories of Belupod d.d. (Koprivnica, Croatia) and in the pilot plant of Podravka d.d. (Koprivnica, Croatia). FSMPs contains maltodextrin, sunflower oil, milk proteins, sucrose, dietary fiber (inulin), medium-chain triglycerides (MCT) from coconut oil, minerals (calcium lactate, sodium chloride, iron lactate, zinc sulfate, manganese sulfate, copper sulfate, chromium (III) chloride, potassium iodide, sodium molybdate, sodium selenate, sodium fluoride, sodium selenite, copper gluconate), vitamins (L-ascorbic acid, DL-alpha tocopheryl acetate, nicotinamide, calcium D-pantothenate, retinyl acetate, cholecalciferol, pyridoxine hydrochloride, thiamine hydrochloride, phytomenadione, riboflavin, folic acid, D-biotin, cyanocobalamin, sodium L-ascorbate), emulsifier (soy lecithin), glucose syrup powder, anti-caking agent (silicon dioxide), acidity regulators (citric acid, dipotassium phosphate, dimagnesium phosphate, disodium phosphate, magnesium hydrogen carbonate), sweetener (acesulfame K),
thickeners (xanthan gum, carrageenan), antioxidant (sodium ascorbate), aroma, choline bitartrate and 0.25 % (m/m) (2.0·10⁷ CFU/g) of L. paracasei culture (Chr. Hansen Holding A/S, Hoersholm, Denmark).

The survival of the probiotic strain in FSMPs matrix during 24 months of storage at room temperature was determined by the agar plate counting method using MRS agar (Merck, Darmstadt, Germany). Plates were incubated anaerobically at 37 °C for 48 h.

**Sequencing of the 16S rRNA gene**

The taxonomic identification of the strain after recovery from FSMPs was confirmed by sequencing the 16S rRNA gene (Macrogen, Amsterdam, Netherlands) using an automatic four-capillary device ABI 3730xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA), which operates on the principle of the Sanger dideoxy method, in which DNA synthesis is stopped by the incorporation of 2', 3'-dideoxynucleotides (ddNTPs). The obtained results were compared with known sequences in the NCBI (The National Center for Biotechnology Information) database using the BLASTn (Nucleotide Basic Local Alignment Search Tool) algorithm available at the link [http://www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/) (20).

**Survival of the strain in simulated GIT juices**

The cumulative effect of simulated GIT juices on the survival of strain L. casei 431® was investigated (21). L. casei 431® cells and FSMPs matrix were exposed to simulated gastric juice for 2 h, then centrifuged to remove the gastric juice after which the cells or the matrix were subjected to the 4-hours incubation in simulated small intestinal juice. The changes in the total viable count were monitored periodically every hour during the treatment.

**Quantitative determination of bile salt hydrolase activity and cholesterol assimilation**

Bile salt hydrolase (BSH) activity was determined by quantifying the cholic acid released from the conjugated bile salt sodium taurocholate (Difco, Detroit, MI, USA) by the solvent extraction method, whereas the capability of L. casei 431® to assimilate cholesterol (AppliChem, Darmstadt, Germany) added to MRS broth (0.2 mg/mL) with 3 mg/mL Oxgall (Difco, Detroit, MI, USA) was evaluated by the modified o-phthalaldehyde method in the supernatant, cell extract, and in the washing buffer where precipitated cholesterol from the supernatant was redissolved (14).
Antibiotic susceptibility testing

Susceptibility to antibiotics was examined by E-test strips (M.I.C. Evaluator™, Oxoid, Ltd, Basingstoke, UK) according to Leboš Pavunc et al. (22) and obtained results were interpreted according to EFSA (19).

Antimicrobial activity

Antimicrobial activity was tested with an agar-well diffusion assay, an agar spot test (13), and with a microplate growth inhibition assay of cell-free supernatants of *L. casei* 431® (15). Samples for agar spot test were 10-fold dilutions of lyophilised pure cultures of *L. casei* 431® and *L. casei* 431® from FSMPs matrix that were inoculated on the surface of the MRS agar plates. Samples for the agar-well diffusion assay and microplate growth inhibition assay of cell-free supernatants of *L. casei* 431® were obtained after cultivation of a lyophilised pure culture of the *L. casei* 431® strain and from FSMPs matrix in MRS broth. The antagonistic activity of the *L. casei* 431® strain evaluated by the agar-well diffusion assay, was expressed as the diameter of the inhibition zones.

Aggregation assays

Auto- and co-aggregation assays during 5 h of incubation in phosphate buffered saline (PBS; pH=7.4; Gibco, Paisley, Scotland, UK) were performed using a microplate reader INFINITE® 200 PRO (Tecan, Männendorf, Switzerland) (21,23). The autoaggregation and coaggregation were monitored over 5 h in PBS (pH=7.4) and the obtained results were expressed as a percentage of aggregated cells.

Adhesion on Caco-2 cells and extracellular matrix proteins

Adhesion properties of *L. casei* 431® were tested according to Novak et al. (12). Caco-2 cells were first seeded on a 96-well microtiter plate in a final volume of 100 μL DMEM/F12 medium (Dulbecco's modified medium/Ham's F-12; Capricorn Scientific GmbH, Ebsdorfergrund, Germany) enriched with 10 % fetal bovine serum (FCS; Thermo Fisher Scientific, Rochester, NY, USA) and L-glutamine (Sigma-Aldrich, Schnelldorf, Germany). After that, Caco-2 cells were inoculated with a bacterial suspension at the multiplicity of infection (MOI) 50, 10 or 2 and then stained in suspension with Incucyte Rapid Red dye (Sartorius, Schloß Holte-Stokenbrock, Germany). After fixation and washing with PBS, the Caco-2 cells were stained with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Thermo Fisher Scientific, Waltham, MA, USA) and the detection of adhered *L. casei* 431® was performed on the EVOS FLc Cell Imager device (Thermo Fisher Scientific, Waltham, MA, USA).
Binding of *L. casei* 431® to human extracellular matrix proteins, (fibronectin, BD BioCoat, Erembodegem, Belgium; type I collagen, Biocoat, Leicestershire, UK; and laminin, BD BioCoat, Erembodegem, Belgium), with and without proteinase K (Invitrogen™, Waltham, MA, USA) treatment, was also examined (14).

**Competitive exclusion assay on the Caco-2 cell line**

To assess the ability of the *L. casei* 431® strain to exclude enteropathogens *Salmonella enterica* Typhimurium FP1 and *Escherichia coli* 3014, exclusion and adhesion assay was performed on the Caco-2 cell monolayers (24, 25). Viable adhering *L. casei* 431®, *E. coli* and *S. Typhimurium* (CFU/ml) were determined by the spot-plate method on MRS, Rapid (Biorad, Dubai, United Arab Emirates) and XLD (Biolife, Milan, Italy) agar plates, respectively (24,25).

**Statistical analyses**

Results were expressed as means of three independent trials±standard deviation. Statistical significance was appraised by one-way analysis of variance. Pairwise differences between group means were determined by the Tukey HSD test for post-analysis of variance pairwise comparisons (Statistics Kingdom) (26). Statistical differences between treatments were considered significant when *p* values were less than 0.05 (*p*<0.05).

**RESULTS AND DISCUSSION**

As a first criterion for achieving a positive effect on health, probiotic preparations should contain live cells of the probiotic strain in concentrations higher than 10⁶ CFU/g of the product (27). Accordingly, an important technological parameter is monitoring the survival of probiotic bacteria during the preparation and storage of the product within the declared expiration date. Therefore, the survival of *L. casei* 431® in the product was tested during 24 months of storage at room temperature. The initial number of viable cells of *L. casei* 431® in FSMPs matrix was 2.0·10⁷ CFU/g, which did not change remarkably after 24 months of storage at room temperature and was above the required number of live probiotic cells. In order to validate the probiotic culture applied into FSMPs, phenotypic and genotypic characterisation of *L. casei* 431® was undertaken. *L. casei* 431® was recovered from a sample of FSMPs on MRS agar. After culturing 20 single colonies on MRS agar overnight, the isolated bacteria were identified and confirmed as *L. casei* 431® by 16S rRNA gene sequencing.

In order to investigate the influence of the food matrix on the *in vitro* functionality of *L. casei* 431®, a series of experiments were conducted to compare the specific probiotic properties of the...
lyophilised culture and the isolate from FSMPs matrix. According to the EFSA Scientific Opinion (28), bacterial strains added to food must be sensitive to the relevant spectrum of antibiotics of human and veterinary importance. According to EFSA (19), the minimum inhibitory concentrations (MICs) of 4, 1, 32, 64, 4, 4, 64 and 4 μg/mL are proposed for ampicillin, erythromycin, gentamicin, kanamycin, clindamycin, chloramphenicol, streptomycin, and tetracycline respectively for Lacticaseibacillus paracasei. According to the results, L. casei 431® is susceptible to all tested antibiotics, with certain antibiotics inhibiting the growth even at concentrations lower than the proposed cut-offs (0.19 for ampicillin, 32 for streptomycin, 0.94 for erythromycin, 0.047 for clindamycin and 0.25 μg/mL for tetracycline) with no differences in antibiotic susceptibility profiles (MIC values) between the lyophilised and the isolate from FSMPs matrix (data not shown).

The potential of L. casei 431®, both the lyophilised culture and the cultures grown from the isolates from FSMPs matrix to survive under simulated GIT conditions was tested, as well as the influence of food matrices on its survival under these conditions. Survival of the tested strains was monitored at one-hour intervals for 2 h in simulated gastric juice and after direct transit to simulated small intestinal juice during a 4-hour incubation (Fig. 1).

Both cultures successfully survived the adverse conditions. The survival rate of the pure lyophilised culture compared to the isolate from FSMPs matrix differed by only about half a log unit. There were no significant difference in survival among lyophilised cultures and isolate from FSMPs, when L. casei 431® cells are exposed to simultaneous stress conditions of GIT. González-Vázquez et al. (29) also exposed two Lacticaseibacillus casei strains, Shirota and J57, to unfavourable GIT conditions. The viability of Lb. casei J57 did not change remarkably during 4-hour exposure to simulated gastric juice conditions, while the survival rate of the strain Lb. casei Shirota dropped drastically after only 1 h of exposure (below 40 %) and no living cells were present after 2 h. The strains were also exposed for 4 h to different bile salts. Both strains showed the highest hydrolase activity towards taurocholic acid and moderate activity towards Oxgall bile salts compared to the others, and their survival rate under adverse GIT conditions was at least 50 %.

Since Oxgall is produced from a large amount of fresh bile by rapid evaporation of water and has a complex composition with a negative synergistic effect of specific compounds on bacterial survival, the very high viable cell counts of L. casei 431®, observed even after 4 of exposure to simulated conditions of the small intestine, are a promising result (Fig. 1). The high survival rate under simulated gastric juice conditions might also be associated with cross-resistance mechanisms involving modifications of the peptidoglycan structure in both the core peptide and/or the sugar
moieties. Adaptation of LAB to acid stress involves a mechanism to maintain cytoplasmic pH, and many of these systems also provide cross-protection when cells are exposed to other stressors such as high concentrations of bile salts (30,31).

A high concentration of bile salts is the main obstacle to the colonization of probiotic strains in the ileum terminalis since bile salts express antimicrobial activity against Gram-positive microorganisms. One of the mechanisms of resistance to bile salts is established through the activity of BSH through deconjugation of bile salts (32). Therefore, the growth of L. casei 431®, as a pure lyophilised culture and isolate from FSMPs matrix, in the presence of bile salts and the deconjugation activity of the cells towards bile salts were tested using a qualitative method (data not shown). The sodium salt of taurocholic acid was used as it is the most abundant bile salt in human bile. An increase in BSH levels is also associated with a greater ability to reduce the level of cholesterol in blood, which is considered a desirable function of probiotics (32). Therefore, spectrophotometric determination of precipitated, assimilated and dissolved cholesterol was carried out after 24 h cultivation of L. casei 431®, as a pure lyophilised culture and as an isolate from FSMPs isolates, in the presence of bile salts (data not shown).

Analysis of the growth of L. casei 431® strains in the MRS medium containing 2 mg/mL sodium taurocholate, revealed that deconjugation activity was evident in both the pure lyophilised culture and the isolate from FSMPs (13 ± 0.04 % and 14.5 ± 0.6 %, respectively). Although the value for the strain isolated from FSMPs matrix is slightly higher, the influence of the food matrix on this activity and the ability of the strain to grow (apsorbance at A620 was 0.38 ± 0.04 for the pure lyophilised culture and 0.42 ± 0.05 for the isolate from FSMPs) in the presence of bile salts cannot be asserted. After 24 h of growth in the presence of cholesterol and bile salts, cells of L. casei 431® from a pure lyophilised culture removed 54.77 % of the cholesterol from the MRS medium and assimilated 32.85 % of the cholesterol. In contrast, L. casei 431® cells isolated from FSMPs matrix assimilated 49.25 % of the cholesterol, while 45.85 % of the cholesterol was deposited in the solution during the cell growth. These cells removed 96.52 % of the cholesterol from the MRS medium. Since L. casei 431® cells incorporated into FSMPs matrix assimilated 49.25 % of the cholesterol compared to 32.85 % of the pure lyophilised culture, it is reasonable to assume that a food matrix contributed to increased cholesterol assimilation by 16.4 %. Lactobacilli are thought to remove cholesterol due to deconjugation of bile salts by BSH (32). Deconjugation of bile salts leads to a drop in pH value, which destabilises cholesterol micelles, resulting in precipitation of cholesterol by free bile acids. Bacterial cells remove cholesterol from the medium by adsorption or absorption by incorporating cholesterol into the cell membrane (33). According to the recent literature data, the administration of functional food containing probiotic bacteria was effective in cholesterol assimilation under simulated GIT conditions (34). Nutrient-rich food matrices such as FSMPs might be associated with a higher capacity.
of \textit{L. casei} 431\textsuperscript{®} from FSMPs matrix to assimilate cholesterol compared to MRS medium. In this context, Chaiyasut \textit{et al.} (35) reported that the \textit{L. paracasei} HII01 strain significantly reduced the level of total cholesterol, triglycerides, and tumour necrosis factor and lipopolysaccharide in patients with hypercholesterolaemia while increasing levels of high-density lipoprotein (HDL) cholesterol, total antioxidant capacity, and propionic and lactic acid. Thus, the addition of strain \textit{L. paracasei} HII01 improved the blood lipid profile and reduced oxidative stress (35). The capacity of certain probiotic strains to lower cholesterol levels may have an impact on the prevention or incidence of hypercholesterolaemia, which is one of the major causes of cardiovascular disease (36). Albano \textit{et al.} (37) showed that among 58 LAB, the strains \textit{Lacticaseibacillus paracasei} subsp. \textit{paracasei} SE160 and VC213 reduced cholesterol concentration in the medium by 55 and 45 \%, respectively, and were subsequently tested for their capacity to lower cholesterol levels in cheese production. All strains were present in cheese in counts higher than $10^7$ CFU/g, and the highest reduction in cholesterol levels in cheese (up to 23 \%) was achieved with \textit{L. paracasei} ssp. \textit{paracasei} VC213. Overall, the implementation of probiotic lactobacilli with cholesterol-lowering capacity contributes to the development of novel functional foods, FSMPs and other nutraceuticals or fermented dairy products with reduced cholesterol content and important functional properties. The application of these strains extends to the development of nutritional supplement formulations intended for the prevention of dyslipidemia, coronary disease, and metabolic syndrome.

As mentioned above, the BSH activity of probiotic lactobacilli can generally enhance antimicrobial activity in the human GIT. When implemented as probiotic-fortified food, it would be optimal if the designed food matrix would strengthen the antipathogenic capacity of the applied probiotic strain. Therefore, the antimicrobial capacity of the two different formulations of \textit{L. casei} 431\textsuperscript{®} was evaluated. The antimicrobial activity of \textit{L. casei} 431\textsuperscript{®} cells propagated from a pure lyophilised preparation and of a isolate from FSMPs isolate against microorganisms that are potential food contaminants and causative agents of various infections was examined using the agar spot test (Table 1), and agar-well diffusion assay (Table 2). The antimicrobial activity of the cell-free supernatants was investigated using the microplate growth inhibition assay (Fig. 2).

\begin{table}[h]
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\caption{Table 1}
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According to the results, the antimicrobial activity of \textit{L. casei} 431\textsuperscript{®} was not significantly impacted whether the strain was derived from FSMPs or not, except for the antimicrobial activity against the related bacterium \textit{Leuconostoc (Lm) mesenteroides} LMG 7954 (Table 1). Isolate from FSMPs moderately inhibited whereas the cells from pure lyophilised culture weakly inhibited related LAB strains.
When testing antimicrobial activity against common food contaminants, cells from FSMPs matrix showed significantly higher inhibitory effect, suggesting that the matrix enhances the inhibitory effect of *L. casei* 431® probably by increasing the concentration of antimicrobial metabolites produced as a nutritionally rich medium in comparison to the MRS medium (Table 2).

**Fig. 2**

When testing the antibacterial activity of *L. casei* 431® culture supernatants, cells from FSMPs matrix showed a significantly stronger inhibitory effect against the tested microorganisms compared to cells recovered from lyophilised culture (Fig. 2). *L. casei* 431® cells from FSMPs matrix generally showed higher inhibition percentages of 12 %, while the inhibition percentages determined with related LAB strains were 10 % higher on average, with the exception of 24 % determined for *Lactococcus* (*Lc*) *lactis* subsp. *lactis* LMG 9450 (Fig. 2). It can be speculated that the antimicrobial activity was achieved primarily through the synthesis of a number of inhibitory metabolites in situ, including organic acids, hydrogen peroxide, ethanol, diacetyl, acetaldehyde, as well as bacteriocins that mainly inhibit Gram-positive microorganisms (38). Many studies have shown different mechanisms of antimicrobial action of lactobacilli using in vitro tests (19,39-41). Islam et al. (42) demonstrated the strong antibacterial activity of *Lacticaseibacillus paracasei* ssp. *paracasei*-1 isolated from traditional yoghurt. Radulović et al. (43) showed that three autochthonous traditional cheese isolates of *Lacticaseibacillus paracasei* strongly inhibited *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*. In general, testing the antimicrobial activity of strains cultured in liquid media is advantageous due to the facilitated diffusion of antimicrobial metabolites such as lactic acid, bacteriocins, etc. Given that bacteriocins are primarily active against closely related species, and that the cell-free supernatant of *L. casei* 431® showed strong activity against related LAB, it can be assumed that the antimicrobial activity is also bacteriocin-based. This needs to be further investigated, and as a first step, the antimicrobial activity of the neutralised supernatant should be tested to eliminate the inhibitory effect of the lactic acid produced (Fig. 2a).

In addition to antimicrobial activity, adhesion is also reported as an important criterion for probiotic strains. Adhesion to intestinal epithelial cells is an important prerequisite for the colonization of probiotic strains, because it reduces their removal by intestinal peristalsis and gives them a competitive advantage over other bacteria for a longer transit in the GIT ecosystem. Aggregation is a necessary prerequisite for the adhesion of probiotic strains to the intestinal epithelial cells (7). It is the ability of bacterial cells to settle during growth, with autoaggregation involving cells of the same bacterial strain while genetically distinct cells coaggregate. The autoaggregation of *L. casei* 431® cells,
as a pure lyophilised culture and as an isolate from FSMPs matrix, is presented in Fig. 3. Autoaggregation of pure lyophilised L. casei 431® culture was approximately 10% higher than the autoaggregation of L. casei 431® isolate from FSMPs matrix during the first three h of incubation but without statistical significance. In the latest, 5th hour of the experiment, the percentage of autoaggregation of the pure culture was lower (45.13%) compared to the isolate from FSMPs matrix which amounted to 53.90% (Fig. 3). In lactobacilli, two types of proteins are responsible for aggregation, namely soluble proteins and surface proteins (25, 44). The smallest protein, 2 kDa in size, which mediates autoaggregation, was found in Lactobacillus gasseri 2459. The protein of 200 kDa molecular weight directly involved in aggregation was found in Lactobacillus paracasei subsp. paracasei BGSJ2-8 (45).

Fig. 3

The factors that promote the coaggregation of lactobacilli differ from each other in terms of molecular weight and primary structure. Considering coaggregation, those probiotic bacteria that successfully coaggregate and also exhibit strong antimicrobial activity can create a barrier that prevents the colonization of pathogens. Additionally, coaggregation with other LAB, that tolerate low pH values, promotes the colonization of bifidobacteria in the intestinal system. Here, the coaggregation of L. casei 431® with representatives of LAB, Lactobacillus helveticus M92 and Enterococcus faecium L3 as well as with Salmonella enterica serovar Typhimurium FP1 and E. coli 3014 was tested (Table 3).

Table 3

The coaggregation with related LAB was higher compared to the test-microorganisms, where the coaggregation with L. helveticus M92 was approx. 10% higher than with E. faecium L3 (Table 3). In all coaggregation pairs, the coaggregation percentage was higher with L. casei 431® isolated from FSMPs matrix, which indicates the influence of the food matrix on the ability of coaggregation. Autoaggregation and coaggregation prevent colonization of pathogens on surfaces, because these processes are involved in the second phase of biofilm formation. The ability to form multicellular aggregates has been observed in numerous bacterial species, including lactobacilli. Autoaggregation and coaggregation are very important phenomena in several ecological niches. Autoaggregation of lactobacilli is necessary for their adhesion to epithelial cells and mucosal surfaces, and it is a desirable property of probiotic bacteria. In the same way, the coaggregation between lactobacilli and pathogenic microorganisms contributes to the creation of a barrier that prevents pathogen adhesion to the epithelium and subsequent access to tissues, thus creating an important host defense mechanism against infections in the urogenital and GIT (46). Numerous studies have confirmed the beneficial
effect of probiotic strains in biofilm formation, including increased resistance to temperature or low pH value \((47)\). Gómez et al. \((48)\) confirmed the potential of probiotic LAB’s biofilms to control the formation of \(L.\ monocytogenes\), \(S.\ Typhimurium\) and \(E.\ coli\) O157 biofilms. Falagas and Makris \((49)\) proposed the use of probiotic LAB strains for the creation of protective biofilms and their implementation in daily cleaning products to reduce the occurrence of pathogenic microorganisms.

The successful retention of probiotic strains in the large intestine, where they exert a beneficial effect on the host's health, depends on their adhesion to epithelial cells, cells of the intestinal and gastric mucosa, and to the extracellular matrix (ECM) consisting of various proteins, such as laminin, fibronectin and collagen. Probiotic bacteria express adhesins on the cell surface that mediate their adhesion to components of the ECM. Through the surface-exposed adhesins, pathogens also interact with ECM proteins, limiting peristalsis and enabling tissue invasion and infection. An example is a \(Staphylococcus\ aureus\) collagen-binding protein Can, known as a major virulence factor \((50)\). Therefore, probiotic bacteria capable of adhering to ECM can outcompete pathogens to adhere to the host surface, and thus prevent their invasion deep into the tissue. Next, the potential of \(L.\ casei\) 431\(^\circ\) to adhere to human collagen, fibronectin and laminin was evaluated \((\text{Fig. 4})\).

The adhesion capacity of \(L.\ casei\) 431\(^\circ\) to specific ECM proteins decreased in all cases after proteinase K treatment. This implies that the mediators of adhesion to the ECM are of a protein nature. Among the three ECM proteins, the highest cell adhesion was recorded for laminin. One can hypothesise that specific protein structures of \(L.\ casei\) 431\(^\circ\) are involved. The host-probiotic cell interaction mediated by surface proteins is the basis for the creation of a barrier that inhibits pathogen adhesion to the intestinal mucosa, thereby preventing pathogen colonization, which ultimately affects pathogen elimination from the intestinal environment. By sharing common host receptors, \(Lactobacillus\) strains could enable the exclusion of pathogens from the gastrointestinal or urogenital tract by direct competition for binding sites of epithelial cell proteins \((51)\). In this context, the autoaggregation-promoting protein AggLb, located on the surface of bacterial cells, is involved in the interaction of \(Lacticaseibacillus paracasei\) subsp. \(paracasei\) with collagen and fibronectin \((44,52)\). Deletion of the \(aggLb\) gene causes loss of the ability to form cell aggregates, while overexpression increases autoaggregation, hydrophobicity, and collagen binding potential. PCR screening with sets of primers constructed on the basis of the \(aggLb\) gene of \(Lacticaseibacillus paracasei\) subsp. \(paracasei\) BGNJ1-64 enabled the detection of the same type of \(aggLb\) gene in five different \(Lactobacillus\) strains that have the ability to aggregate. Heterologous expression of the \(aggLb\) gene confirmed the key role of the AggLb protein in cell aggregation and specific binding to collagen, which
indicates that AggLb can mediate effective host colonization and prevention of pathogen colonization (44).

One of the principles for selecting a potential probiotic strain is its ability to adhere to the host's GIT mucosa. Adhesion is a multi-step process that involves the mutual contact of the bacterial cell membrane with other surfaces, and is initially based on non-specific physical interactions between these two surfaces. Adhesion to the intestinal mucosa prevents the washout of probiotic cells and enables temporary colonization, immune modulation, and competitive exclusion of pathogens. The ability to adhere to the intestinal epithelium is mainly investigated by in vitro experiments using human intestinal cultures of cell lines (53). So, following the evaluation of autoaggregation potential and capacity to bind to ECM proteins, the ability of L. casei 431® in competitive exclusion of S. enterica serovar Typhimurium FP1 and E. coli 3014, using Caco-2 cell line was investigated. In vitro adhesion testing was performed using fluorescent staining of living cells, adhered to the Caco-2 cell line monolayer. Adhesion was tested at MOI 2, 10, and 50 during 1, 4, and 12 h of incubation (Fig. 5).

Fig. 5

The results show that the number of adhered cells increased with the larger incubation time, with optimal results obtained at MOI 10. Previous researches have shown high percentages of adhesion of Lacticaseibacillus paracasei strains to the Caco-2 cell line (39,54). L. paracasei subsp. paracasei KNI9 inhibited the adhesion of Y. enterocolitica subsp. enterocolitica to Caco-2 cells. AggLb of L. paracasei subsp. paracasei is not only involved in interaction with ECM components but also in the competitive exclusion of pathogens by L. paracasei subsp. paracasei strains (44). Additionally, the exopolysaccharide produced by Lacticaseibacillus paracasei subsp. paracasei strain was also shown to be involved in adhesion to intestinal epithelial cells, thereby reducing the adhesion of the E. coli to Caco-2 cells (40).

In case of testing the ability of the competitive exclusion of pathogens with the probiotic strain L. casei 431®, the results showed that the adhesion of L. casei 431® to the Caco-2 cell line reduces the attachment of E. coli 3014 by 1.81 log CFU/mL and the attachment of S. Typhimurium FP1 cells by 1.85 log CFU/mL (p<0.05), which is in accordance with the results obtained by Jankowska et al. (41) where Lacticaseibacillus paracasei IBB2588 strain inhibited the binding of the Salmonella enterica to the Caco-2 cell line. Further research of L. casei 431® can also be focused on the anticancer effect as additional probiotic property, which was obtained by L. paracasei K5, a strain with adhesion ability, but also with antiproliferative and apoptotic activity to Caco-2 colon cancer cells via modulation of expression of specific Bcl-2 family proteins (55).
CONCLUSIONS

Sequencing of the 16S rRNA gene confirmed the presence of the *L. casei* 431® strain in FSMPs matrix. Survival under adverse GIT conditions, ability to aggregate, adhesion to proteins of extracellular matrix and Caco-2 cells along with antagonistic activity indicated the competitive exclusion of *S. Typhimurium* FP1 and *E. coli* 3014 by *L. casei* 431®. Additionally, FPMPs matrix positively influenced cholesterol assimilation by *L. casei* 431®. According to the results, the incorporation of this probiotic strain in the FSMP matrix improved its probiotic activities and functional value of the new FPMPs preparation. Therefore, by adding the probiotic strain to innovative FSMPs matrix, the synergistic effect was obtained which contributed to the enhanced functionality of the developed product.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

Andreja Leboš Pavunc contributed to the design of the work, performing the analysis, data analysis, and interpretation, and drafting the article. Lenkica Penava contributed to performing the analysis, data analysis and interpretation and drafting the article. Jasna Novak contributed to the interpretation of the results and the critical revision of the manuscript. Nina Čuljak, Martina Banić, and Katarina Butorac performed the analysis, data analysis, interpretation data and drafting the work. Marijana Ceciliger, Jelena Miličević, and Danijela Čukelj contributed to the preparation of the samples,
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analysis and drafting the the article. Jagoda Šušković and Blaženka Kos contributed to the design of the work and critical revision. All authors contributed to the final approval of the version to be published.

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Fig. 1. The cumulative effect of simulated gastric juice (pH=2.0; incubation time 2 h) and simulated small intestine juice (pH=8.0; incubation time 4 h) on the survival of *L. casei* 431®, as pure lyophilised culture and isolates from food for special medical purposes (FSMPs). The results are expressed as mean values of three independent experiments, and error bars represent standard deviation. There were no statistically significant differences among the compared results (p<0.05)
Table 1. Antibacterial activity of L. casei 431® as pure lyophilised culture and as isolate from food for special medical purposes (FSMPs) against lactic acid bacteria as related Gram-positive microorganisms, tested by the agar spot test

<table>
<thead>
<tr>
<th>Lactic acid bacteria</th>
<th>Effective inhibition ratio (EIR*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactiplantibacillus plantarum</strong> LMG 9206</td>
<td>(0.5±0.1) ^a</td>
</tr>
<tr>
<td><strong>L. casei 431®</strong> FSMPs</td>
<td>(0.67±0.09) ^a</td>
</tr>
<tr>
<td><strong>Lactococcus lactis</strong> subsp. lactis LMG 9450</td>
<td>(0.43±0.11) ^a</td>
</tr>
<tr>
<td><strong>Enterococcus faecium</strong> ATCC 9430</td>
<td>(0.58±0.03) ^a</td>
</tr>
<tr>
<td><strong>Leuconostoc mesenteroides</strong> LMG 7954</td>
<td>(0.27±0.05) ^b</td>
</tr>
</tbody>
</table>

*EIR = (ID-CD)/CD; CD = diameter of the spot with grown colonies; ID = inhibition diameter; EIR<0.5 weak inhibition; 0.5< EIR<1.5 medium inhibition; EIR>1.5 strong inhibition.
The results are expressed as the mean value±standard deviation of three separate experiments. Different letters in superscript indicate statistically significant differences among data across the same column (Tukey’s HSD test, p<0.05, N=3)

Table 2. Antibacterial activity of L. casei 431® as pure lyophilised culture and as isolate from food for special medical purposes (FSMPs) against potentially pathogens as test-microorganisms, tested by the agar-well diffusion assay, and expressed as the diameter of the zone of inhibition

<table>
<thead>
<tr>
<th>Test-microorganisms</th>
<th>Inhibition zone/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. casei 431®</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong> 3048</td>
<td>(3±0.05) ^b</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong> K-144</td>
<td>(10±0.09) ^b</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong> 3014</td>
<td>(11±1.1) ^b</td>
</tr>
<tr>
<td><strong>Salmonella Typhimurium</strong> FP1</td>
<td>(9.33±1) ^b</td>
</tr>
<tr>
<td><strong>Bacillus cereus</strong> TM2</td>
<td>(12.33±0.09) ^b</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong> ATCC6633</td>
<td>(9±1) ^b</td>
</tr>
</tbody>
</table>

The results are expressed as the mean value±standard deviation of three separate experiments. Different letters in superscript indicate statistically significant differences among data across the same column (Tukey’s HSD test, p<0.05, N=3)
Fig. 2. The antibacterial activity of cell-free supernatants of *L. casei* 431®, as pure lyophilised culture and isolate from food for special medical purposes (FSMPs), after 24 h of incubation tested by the microplate growth inhibition assay against: a) lactic acid bacteria strains *L. plantarum* LMG 9206; *Lc. lactis* subsp. *lactis* LMG 9450; *E. faecium* ATCC 9430 and *Ln. mesenteroides* LMG 7954. b) test-microorganisms *S. aureus* 3048; *S. aureus* K-144; *E. coli* 3014; *S. enterica* serovar Typhimurium FP1; *B. cereus* TM2; *B. subtilis* ATCC 6633. Data show the average of three independent replicates. *Significantly different (p<0.05) from the pure lyophilised culture.

Fig. 3. Autoaggregation of *L. casei* 431® strains grown from pure lyophilised culture and isolate from food for special medical purposes (FSMPs). The results are expressed as mean values of three independent experiments, and error bars represent standard deviation. There were no statistically
significant differences between the autoaggregation properties of lyophilised culture or L. casei 431® isolate from FSMP (p<0.05)

Table 3. Coaggregation ability of L. casei 431® strains grown from lyophiliised culture and isolate from food for special medical purposes (FSMPs), after 5 h of incubation at room temperature with lactic acid bacteria and potential pathogens as test-microorganisms

<table>
<thead>
<tr>
<th>Test-microorganisms</th>
<th>Coaggregation/%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. casei 431®</td>
</tr>
<tr>
<td>Lactobacillus helveticus M92</td>
<td>(43.18±0.6)b</td>
</tr>
<tr>
<td>Enterococcus faecium L3</td>
<td>(35.13±0.8)b</td>
</tr>
<tr>
<td>Salmonella Typhimurium FP1</td>
<td>(18.43±0.9)b</td>
</tr>
<tr>
<td>Escherichia coli 3014</td>
<td>(23.68±1.1)b</td>
</tr>
</tbody>
</table>

The results are expressed as the mean value±standard deviation of three separate experiments. Different letters in superscript indicate statistically significant differences among data across the same column (Tukey’s HSD test, p<0.05, N=3)

Fig. 4. Effects of proteinase K treatment on the binding of L. casei 431® cells to immobilised extracellular matrix proteins: fibronectin, collagen and laminin. The results are the mean value of three independent experiments, and error bars represent standard deviations. *Data comparisons (intact cells versus proteinase K-treated cells) considered statistically significantly different (p<0.05) are indicated with an asterisk
Fig. 5. Fluorescence microscopy of adhesion of *L. casei* 431® cells to the Caco-2 monolayer: a) only bacterial cells *L. casei* 431® (green fluorescence) and b) double fluorescent layers of Caco-2 cell nuclei (red fluorescence) and bacterial cells *L. casei* 431® (green fluorescence) are shown for different inoculation densities (MOI 2, MOI 10, and MOI 50) after 1, 4, and 12 h of incubation at 20× magnification.