Probiotic bacteria of wild boar origin intended for piglets – An *in vitro* study

IVETA KOSTOVOVA[1] , KATERINA KAVANOVA[1] , MONIKA MORAVKOVA[1] ,
JAN GEBAUER[2], LENKA LEVA[2], MONIKA VICENOVA[2] , VLADIMIR BABAK[1] ,
MARTIN FALDYNA[2] , MAGDALENA CRHANOVA[1] [*]

[1]Department of Microbiology and Antimicrobial Resistance, Veterinary Research Institute, Brno,
  Czech Republic
[2]Department of Infectious Diseases and Preventive Medicine, Veterinary Research Institute, Brno,
  Czech Republic
*Corresponding author: magdalena.crhanova@vri.cz

Abstract: Using probiotics represents a potential solution to post-weaning diarrheal diseases in piglets on commercial farms. The gastrointestinal tract of wild boars serves as a promising reservoir of novel lactic acid bacteria with suitable probiotic characteristics. In this study, we isolated eight bacterial strains from the intestinal content of wild boars identified as representatives of the species *Bifidobacterium apri*, *Lactobacillus amylovorus*, and *Ligilactobacillus salivarius*. These isolates underwent *in vitro* analysis and characterisation to assess their biological safety and probiotic properties. Analysis of their full genome sequences revealed the absence of horizontally transferrable genes for antibiotic resistance. However, seven out of eight isolates harboured genes encoding various types of bacteriocins in their genomes, and bacteriocin production was further confirmed by mass spectrometry analysis. Most of the tested strains demonstrated the ability to inhibit the growth of selected pathogenic bacteria, produce exopolysaccharides, and stimulate the expression of interleukin-10 in porcine macrophages. These characteristics deem the isolates characterised in this study as potential candidates for use as probiotics for piglets during the post-weaning period.

Keywords: antibiotic susceptibility; antimicrobial activity; bacteriocins; exopolysaccharides; interleukin-10

Weaning is a critical stage in porcine production that presents significant challenges for farmers and can lead to substantial economic losses. During this stage, the piglets are exposed to various stressors, including separation from the sow and littermates, transportation, handling, transition from milk to solid pelleted feed, and housing with piglets from other litters (Hwang et al. 2016; Shin et al. 2019). These changes collectively influence the health of piglets resulting in greater susceptibil-ity to pathogen infection manifested in diarrhoea, transient anorexia, reduced feed conversion efficiency, loss of weight, and, in extreme cases, death.

Antibiotic feed additives were commonly used preventively to mitigate losses associated with weaning. However, the increasing risk of spreading antimicrobial resistance among pathogenic bacteria has reduced the use of antimicrobial drugs in farm animals (Tang et al. 2022). In addition, the prophylactic use of antibiotics and their

use as growth promoters have been banned in the European Union (EU) since 2006. In 2022, the EU implemented legislation restricting the prophylactic and metaphylactic use of antimicrobials in animals to only exceptional cases or instances of high-risk spread of infectious diseases, intended to reduce the overall proportion of antimicrobial use in animals (EP 2019; ECDC et al. 2021).

Therefore, there is a pressing need for an alternative to antibiotics to prevent post-weaning diarrhoea in piglets (McEwen and Collignon 2018). One promising solution is represented by probiotics, defined as microorganisms (bacteria or yeasts) that positively affect the host when administered in sufficient quantities. Lactic acid bacteria (LAB), primarily lactobacilli, and bifidobacteria are the most commonly used probiotics (Shin et al. 2019). The beneficial effects of lactic acid bacteria on the host organism include enhanced immune function, inhibition of the adhesion of pathogens to the epithelial surface, and improved digestion connected to their ability to produce lactic acid and metabolites such as antioxidants, organic acids, and antimicrobial compounds (Azad et al. 2018). This modulation improves intestinal microbial balance (Li et al. 2020; Zhou et al. 2020).

The compounds produced by LAB that inhibit pathogen growth include hydrogen peroxide, diacetyl, organic acids, and bacteriocins. Bacteriocins are antimicrobial peptides that inhibit or kill pathogenic bacteria in the host gut and alter the gut microbiome composition in animal models (Anjana and Tiwari 2022). The production of bacteriocins is, therefore, one of the most crucial parameters for selecting probiotic strains as an alternative to antibiotics. Additionally, the production of exopolysaccharides (EPs) by LAB is another important parameter for probiotic selection. EPs produced by LAB are reported to have antimicrobial, immunomodulatory, anti-inflammatory, antioxidant, anti-tumour, anti-viral, anti-diabetic, anti-ulcer, and cholesterol-lowering properties in humans (Angelin and Kavitha 2020).

Various sources including cereals, fruits and vegetables, and dairy-based products such as milk are typically used to isolate and select potential probiotic strains. However, in addition to feed and food sources, the gastrointestinal tract (GIT) of healthy individuals can also serve as a feasible isolation source (Guo et al. 2010). In contrast to domestic pigs, which are frequently exposed to antibiotics, the GIT of wild boars presents a promising reservoir of potentially probiotic strains. Wild boars inhabit forest areas and must adapt to natural resources, exhibiting strong adaptability to their ecological conditions. Unlike domestic pigs, wild boars do not rely on veterinary antimicrobials for survival, making them a promising reservoir of health-promoting LAB (Li et al. 2020).

Between 2019 and 2021, we successfully isolated more than 60 strains of LAB and bifidobacteria from the GIT of wild boars hunted in the Czech Republic. Our initial analysis focused on ensuring the safety of these isolates, which involved various measures such as antimicrobial susceptibility testing, evaluation of haemolysin production and wholegenome sequencing to detect horizontally transmissible genes associated with antibiotic resistance. Subsequently, we selected eight isolates considered "safe" and investigated their potential for EP production. Ultimately, we tested these eight isolates to determine their capability to inhibit the growth of pathogenic bacterial strains (enteropathogenic *Escherichia coli*, *Salmonella* Typhimurium, and *Yersinia enterocolitica*). Those isolates were identified as *Bifidobacterium apri* (*B. apri*) (115B), *Lactobacillus amylovorus* (*L. amylovorus*) (M597AA, M597B, M624A, M668A, M696A), and *Ligilactobacillus salivarius* (*L. salivarius*) (M494A, M698A).

This study aimed to assess *in vitro* the impact of these eight selected strains on two crucial parameters for selecting efficient probiotics: the production of antibacterial substances and the modulation of the immune system. A combination of molecular techniques was employed to detect the presence of genes for antibacterial peptides and confirm their expression at the protein level. A model of macrophages derived from monocytes was also utilised to evaluate the modulation of immune cells. These cells were stimulated with pro-inflammatory lipopolysaccharide to mimic an inflammatory response.

MATERIAL AND METHODS

Isolation of bacterial strains and growth conditions

All strains used in this study were isolated from the digestive tract of wild boars from various locations in the Czech Republic. Samples from the small and large intestines were collected during wild boar

hunts conducted in 2018 and 2019. A total of 42 digestive tract samples from wild boars were collected and immediately placed in coolers to maintain their integrity. The samples were cultured on retrieval on Rogosa agar (Oxoid, Basingstoke, UK). Cultivation was carried out simultaneously under anaerobic conditions (using anaerobic jars with palladium catalysts maintaining an atmosphere of 10% CO_2/ 10% H_2/80% N_2) and microaerophilic conditions at 37 °C. Subsequently, isolates were sub-cultured on de Mann Rogosa Sharpe agar (MRS; Oxoid) under anaerobic conditions at 37 °C for 48 hours. More than 60 strains of *Lactobacillus* and *Bifidobacterium* were isolated from the intestines of wild boars during the study.

DNA isolation and whole-genome sequencing

All strains underwent genomic DNA extraction using a Quick-DNA™ Faecal/Soil Microbe Microprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The extracted DNA was utilised for library construction using the Nextera Library preparation kit. Paired-end sequencing was conducted using the NextSeq platform, employing a NextSeq 500/550 High Output Kit v2.5 from Illumina (Illumina Inc., San Diego, CA, USA). The generated read sequences underwent trimming using Trim Galore v0.6.7 (accessed on 1 December 2020), and low-quality reads were eliminated using Cutadapt v0.6.6. Following the removal of low-quality reads, MultiQC v1.9 was employed to evaluate the quality of the remaining reads. The trimmed reads were subsequently subjected to *de novo* genome assembly using Unicycler v0.4.9b, which utilised SPAdes v3.14.1.

Strain identification

The individual isolates were identified using sequencing analysis of the 16S rRNA gene using the primers 16S27f (AGAGTTTGATCMTGGCTCAG) and 16S1492r (TACGGYTACCTT-GTTACGACTT) (Lagace et al. 2004). Subsequently, the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The resulting amplicons were sequenced in both the forward and reverse directions using a Mix2Seq Kit by Eurofins Genomics (Luxembourg City, Luxembourg). The isolated bacterial strains were identified based on sequence similarity with reference sequences in the GenBank and EzBioCloud databases accessed on 1 October 2020.

The final identification of the isolates relied on the average nucleotide identity (ANI) of all orthologous genes shared between the genome of the type strain and the genome of the particular isolate. ANI calculation was performed using the bioinformatics tool FastANI. An isolate was considered to belong to a particular species if the ANI value between the type strain genome and the genome of the isolate exceeded 95%. The genome sequences of the following type strains were used: *Bifidobacterium apri* DSM 100238, *Ligilactobacillus salivarius* DSM 20555, and *Lactobacillus amylovorus* DSM 20531.

Analysis of antibiotic resistance and bacteriocin genes

The presence of horizontally acquired antibiotic resistance genes was detected using whole-genome sequencing data. Bacterial genomes were analysed using Abricate v1.0.1 software with the following databases: Comprehensive Antibiotic Resistance Database (CARD), ResFinder, Argannot, Megares, and NCBI AMRFinderPlus. All databases were updated on 7 February 2022 (Moravkova et al. 2022).

The genes potentially responsible for bacteriocin production were identified using the web-server BAGEL4.

Antimicrobial susceptibility

Antimicrobial susceptibility testing was conducted using broth microdilution methods in accordance with ISO10932:2010 standards and the interpretation criteria suggested by EFSA FEEDAP Panel guidance (EFSA et al. 2018). The microplates were incubated for 48 h at 37 °C in an anaerobic atmosphere. The minimal inhibitory concentration (MIC) was visually read as the lowest concentration of the antimicrobial substance that inhibited bacterial growth. The following antimicrobials were tested: ampicillin (0.125–16 mg/l), streptomycin (2–256 mg/l), tetracycline (0.5–64 mg/l), erythromycin (0.063–8 mg/l), clindamycin (0.063–8 mg/l), chloramphenicol (0.25–32 mg/l), kanamycin (0.5–2050 mg/l), gentamicin (0.125–512 mg/l), vancomycin (0.25–32 mg/l) and ciprofloxacin

(0.125–128 mg/l). All tested antimicrobials were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quality control strains (*Lactobacillus plantarum* ATCC14917 and *Lactobacillus paracasei* ATCC334) were used to ensure the accuracy of susceptibility testing (Moravkova et al. 2022). The evaluation of susceptibility was based on microbiological cut-off values established by the EFSA for the *Lactobacillus acidophilus* group (used for *L. amylovorus* strains), *Lactobacillus* facultative heterofermentative (used for *Ligilactobacillus salivarius*) and *Bifidobacterium* (used for *Bifidobacterium apri*).

β-haemolysin production

To cultivate the bacterial isolates, MRS agar medium supplemented with cysteine at a concentration of 0.3 g/l (MRS+C) was utilised and incubated for 48 hours. The bacterial cultures were then mixed with a physiological solution to prepare a suspension with a 1.2 McFarland turbidity. Columbia agar with 5% sheep blood (Oxoid, Basingstoke, UK) was employed to observe the production of β-haemolysin. Five microliters of each bacterial suspension were spotted in triplicate on the surface of the Columbia agar plate. The plates were then incubated for 48 h at 37 °C under anaerobic conditions.

Antimicrobial activity

The antimicrobial activity of all bacterial isolates was assessed against pathogenic bacteria known to cause diarrhoeal infections, including three isolates of *Escherichia coli* (EC 971, EC973, EC974) producing enterotoxins, one isolate of *Salmonella* Typhimurium (STM 970) and one isolate of *Yersinia enterocolitica* (YE M108/15), all originating from the gastrointestinal tract of pigs. The agar spot test described by Monteiro et al. (2019) was performed with some modifications. Five µl of *L. salivarius* suspension in physiological solution (McFarland turbidity 1.3) was spotted on 15 ml of MRS agar plated in a Petri dish. After 24 h of incubation at 37 °C under anaerobic conditions, 10 ml of tryptic soy agar (TSA, HiMedia, Brno, Czech Republic) was overlaid onto the MRS agar containing the grown culture of a particular tested isolate. The TSA medium was allowed to solidify at room temperature; thereafter suspensions

of pathogenic bacteria (McFarland turbidity 0.5) were spread with a swab. The plates were then incubated at 37 °C for 24 h under aerobic conditions. The indication of antimicrobial activity was the formation of a clear halo around a grown probiotic culture spot. The diameter of the growth inhibition halo was measured and expressed in millimetres.

Production of exopolysaccharides

The ability to produce exopolysaccharides (EPs) was examined by picking the colonies growing on the surface of MRS agar plates with a sterile bacteriological loop and observing the formation of a filament when the loop was lifted (Ruas-Madiedo and de los Reyes-Gavilan 2005).

Preparation of supernatants for *in vitro* assays

All selected isolates were cultivated on MRS+C agar for 48 h at 37 °C. Subsequently, one bacteriological loop of the culture was transferred into 1 ml of MRS+C medium and incubated for 24 h at 37 °C. Following this, 500 µl of the suspension was transferred into 50 ml of MRS+C medium. All cultivation steps were conducted under anaerobic conditions. Once the bacterial cultures reached the beginning of the stationary phase, the bacterial cells were collected by centrifugation at 2 700 RCF for 15 minutes. The resulting supernatant was adjusted to pH 7 with 5 M NaOH. Cells were washed three times in 9.5 mM (PO_4) Dulbecco's Phosphate Buffered Saline with Calcium and Magnesium (DPBS; Lonza, Basel, Switzerland) and then resuspended in DPBS to a concentration of 4–5×10^8 CFU/ml.

Mass spectrometry analysis of secreted proteins

Supernatants obtained from bacterial cultures (as described in the previous paragraph) were collected and a protein concentration was estimated by UV280 measurement using a DeNovix DS-11 FX spectrophotometer (DeNovix, Wilmington, DE, USA) with bovine serum albumin as a calibrator. Ten µg of total protein was utilised for mass spectrometry (MS) sample preparation with the

filter-aided sample preparation (FASP) method (Wisniewski et al. 2009). Each sample underwent six washes with 8 M urea in Vivacon 500 centrifugal tubes (Sartorius Stedim, Göttingen, Germany) equipped with a 10 000 MWCO membrane filter. Dithiothreitol (10 mM, Sigma-Aldrich) and iodoacetamide (50 mM, Serva, Heidelberg, Germany) in 25 mM TEAB (triethylammonium bicarbonate; Sigma-Aldrich) buffer were used for reduction and alkylation, respectively. The proteins were then digested with trypsin (Thermo Fischer Scientific, Waltham, MA, USA) at a 1 : 50 ratio, initially for one hour at 37 °C followed by overnight digestion at 25 °C. Following centrifugation, the eluate containing digested peptides was evaporated using a DNA120 SpeedVac (Thermo Fischer Scientific), and the peptide pellet was resuspended in 0.1% aqueous formic acid (Sigma-Aldrich) serving as the mobile phase for liquid chromatography (UltiMate 3000 RSLCnano; Thermo Fischer Scientific). Peptides were separated and eluted using a 2-hour gradient with increasing concentrations of acetonitrile (0.1% formic acid in 80% acetonitrile; Sigma-Aldrich, St. Louis, MO, USA) at a flow rate of 300 nl/min. Separation of the peptides was performed on a 25 cm column (Acclaim PepMap RSLC C18, 2 μm, 100 Å, 75 μm I.D.; Thermo Fischer Scientific) with the uHPLC system connected to an EASY-spray ion source and a Q Exactive mass spectrometer (Thermo Fischer Scientific). A survey scan over the m/z range 390–1 700 was conducted to identify protonated peptides with charge states of at least 2, which were subsequently selected for data-dependent MS/MS analysis and fragmented by HCD dissociation. Ten fragment mass spectra following each full scan were recorded. The measured spectra were then searched using Proteome Discoverer (v2.4; Thermo Fischer Scientific) with Sequest HT as a searching algorithm. Uniprot unreviewed databases for the *Bifidobacteriales* taxon (from 2022/08) and *Lactobacillales* taxon (from 2022/08) were employed in Sequest HT. Peptides with a false discovery rate of less than 0.01 were considered well-identified. The quantity of identified proteins was expressed by the intensity of the chromatographic peak detected by the mass spectrometer. The summed abundances of the connected peptide groups provided the quantification of each identified protein. Relative abundance was calculated after normalization for the total peptide amount in each sample and scaled to a value of 100, representing the median abundance.

Isolation of monocyte-derived macrophages

The preparation of monocyte-derived macrophages (MDMs) followed previously described methods (Kavanova et al. 2017). CD14+ porcine monocytes were isolated from heparinised peripheral blood obtained from five-month-old pigs. Mononuclear cells were isolated using Histopaque-1077 (Sigma-Aldrich) gradient. Monocytes were further enriched to a purity of > 95% using positive magnetic bead selection (QuadroMACS™ cell separator; Miltenyi Biotec, Bergisch Gladbach, Germany) with a monoclonal antibody directed against CD14 (clone MIL2, AbD Serotec, 1 μl per 10^8 cells) and goat anti-mouse IgG microbeads along with LS separation columns (MACS).

After isolation, the cells were washed with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, New York, USA), centrifuged at 1 100 × *g* at 20 °C for 10 min, and resuspended with DMEM supplemented with 10% Fetal Bovine Serum Superb (FBS; Diagnovum, Tillburg, The Netherlands) and 1% antibiotics (Antibiotic Antimycotic Solution 100 × : 10 000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml; Sigma-Aldrich). MDM were then plated into 24-well culture plates (Biotech) at a concentration of 5 × 10^5 cells in one ml per well or, in the case of the viability/cytotoxicity assay, into black Nunc-Immuno™ MicroWell™ 96-well polystyrene plates (Sigma-Aldrich) at a concentration of 1 × 10^5 cells at 0.2 ml per well. These cells were then incubated for 5 days at 37 °C, 5% CO_2.

After 5 days of transformation into MDM, the DMEM medium was removed and the cells were washed in DPBS. Alternatively, the medium was enriched with either 10% of supernatants or washed bacteria at a ratio of 1 : 10. The cells were either left unstimulated or stimulated with LPS (1 μg/ml; lipopolysaccharides from *E. coli* O111:B4; Sigma-Aldrich) for 6 hours.

The analysis of mRNA expression based on reverse transcription qPCR was subsequently performed.

Gene expression analysis based on reverse transcription quantitative real-time PCR (RT-qPCR)

The mRNA expression of the anti-inflammatory cytokine interleukin-10 (IL-10) in MDM was determined using RT-qPCR. Following treatments, MDMs were stabilised in TRI Reagent RT

(Molecular Research Center, Cincinnati, OH, USA) and stored at –70 °C until RNA isolation. The RNA phase was obtained from the mixture with bromanisole by separation in a refrigerated centrifuge. Total RNA was isolated using a NucleoSpin RNA Mini Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions, resulting in a final volume of 36 μl RNeasy free water. The purity and integrity of RNA were assessed spectrophotometrically by measuring absorbance ratios at 230, 260, and 280 nm and by agarose gel electrophoresis. Reverse transcription was performed using M-MLV reverse transcriptase (200 IU/μl, Invitrogen) and oligo(dT) RT primer (Generi Biotech, Hradec Kralove, Czech Republic) at 37 °C for 1.5 hours.

Duplicates of 3 μl qPCR reaction were dispensed using a Nanodrop II liquid dispenser (Innovadyne Technologies, Rohnert Park, CA, USA) and qPCR was performed using a LightCycler 480 instrument (Roche, Basel, Switzerland). The reaction conditions included denaturation at 95 °C for 15 min followed by amplification in 45 cycles of 95 °C for 15 s and 58 °C for 30 s and elongation at 72 °C for 30 s according to the manufacturer's recommendations. Quantification cycle data with a variation of less than 0.5 were further analysed. Each reaction contained 10 pmol of each primer pair (Generi Biotech), 1.5 μl

of QuantiTect SYBR Green PCR MasterMix (Qiagen), and 0.5 μl of 4 × diluted cDNA. Analysis of the melting temperature confirmed the specificity of amplicons using LightCycler 480 1.5.0.39 software (Roche Applied Science, https://www.roche.com). Gene-specific primers for IL-10 were adapted from Kyrova et al. (2012). The reference housekeeping gene (REF) TBP1 (Nygard et al. 2007) was determined using a variability test (Andersen et al. 2004) among the MDM samples tested. Assuming a primer efficiency ≥1.9, normalised gene expression based on quantification cycle (Cq) values was calculated as $2^{-(CqGENE - CqREF)}$ (Livak and Schmittgen 2001; Bustin et al. 2009). REF served as a qPCR positive control. The data obtained were logarithmised and further analysed using two-factor analysis of variance in STATISTICA v13.2 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

De novo assembly

De novo genome assembly was carried out on the genomes of one *Bifidobacterium apri*, five *Lactobacillus amylovorus*, and two *Ligilactobacillus salivarius* strains. The number of assembled con-

Table 1. The genome assembly and ANI results-based strain identification

Isolate name	NCBI accession	Number of contigs	Contig size (bp)	L50 (contigs)	N50 (bp)	GC (%)	ANI (%)	Type strain with the highest similarity
115B	SAMN35847917	59	2 396 385	8	78 681	59.34	99.98	*Bifidobacterium apri* DSM 100238
M597AA	SAMN31135173	126	2 098 617	12	56 554	37.78	98.73	*Lactobacillus amylovorus* DSM 20531
M597B	SAMN31135174	122	2 095 652	13	56 022	37.78	98.65	*Lactobacillus amylovorus* DSM 20531
M624A	SAMN31135175	112	1 995 845	12	57 998	37.84	98.60	*Lactobacillus amylovorus* DSM 20531
M668A	SAMN31135176	60	1 965 416	4	172 888	37.94	96.95	*Lactobacillus amylovorus* DSM 20531
M696A	SAMN31135177	126	1 950 594	16	36 990	37.95	98.76	*Lactobacillus amylovorus* DSM 20531
M494A	SAMN35847937	109	2 072 636	15	41 742	32.53	98.51	*Ligilactobacillus salivarius* DSM 20555
M698A	SAMN35847938	93	2 059 459	12	56 073	32.72	98.45	*Ligilactobacillus salivarius* DSM 20555

ANI = average nucleotide identity; GC = the percentage of either guanine (G) or cytosine (C) bases in DNA molecule; L50 = the count of the smallest number of contigs whose length sum makes up half of genome size; N50 = the minimum contig length required to cover 50 percent of the assembled genome sequence

Table 2. Distribution of minimal inhibition concentration for selected antimicrobials

Antibiotics range (mg/l)	Strain	No.	< 0.63	< 0.125	0.125	0.25	0.5	1	2	4	8	16	32	64	128	> 128	256	512	1 024
Ampicillin (≤ 0.125–≥ 16)	*B. apri*	1	1																
	L. amylovorus	5					4	1											
	L. salivarius	2						2											
Streptomycin (≤ 2–≥ 256)	*B. apri*	1													1				
	L. amylovorus	5								1	4								
	L. salivarius	2															2		
Tetracycline (≤ 0.5–≥ 64)	*B. apri*	1							1										
	L. amylovorus	5						2	2	2	1								
	L. salivarius	2									1	1							
Erythromycin (≤ 0.063–≥ 8)	*B. apri*	1			1														
	L. amylovorus	5			1	4													
	L. salivarius	2						2											
Clindamycin (≤ 0.063–≥ 8)	*B. apri*	1			1														
	L. amylovorus	5				1	1	2			1								
	L. salivarius	2		1	1														
Vancomycin (≤ 0.25–≥ 32)	*B. apri*	1					1		1										
	L. amylovorus	5					2	3											
	L. salivarius	2									2								
Chloramphenicol (≤ 0.25–≥ 32)	*B. apri*	1									2								
	L. amylovorus	5								3	2								
	L. salivarius	2									2								
Kanamycin (≤ 16–≥ 2 050)	*B. apri*	1																	1
	L. amylovorus	5										2	1	1	1				
	L. salivarius	2															1	1	
Gentamicin (≤ 0.125–≥ 512)	*B. apri*	1									1								
	L. amylovorus	5					1		3										
	L. salivarius	2											2						
Ciprofloxacin (≤ 0.125–≥ 128)	*B. apri*	1												1					
	L. amylovorus	5												1		2			
	L. salivarius	2						1		1						2			

The gray zones represent values higher than the cut-off values for the *Lactobacillus acidophilus* group (used for *Lactobacillus amylovorus* strains), for *Lactobacillus* facultative heterofermentative (used for *Ligilactobacillus salivarius*), and for *Bifidobacterium* (used for *Bifidobacterium apri*) according to the guidance on the characterisation of micro-organisms used as feed additives or production organisms (EFSA et al. 2018). The cut-off value of ciprofloxacin and kanamycin in the case of *Bifidobacterium* is not known. *B. apri* = *Bifidobacterium apri*; *L. amylovorus* = *Lactobacillus amylovorus*; *L. salivarius* = *Ligilactobacillus salivarius*; MIC = minimal inhibitory concentratio

tigs varied from 59 to 126, with L50 and N50 values ranging from 36 990 to 172 888 bp and 4 to 16 contigs, respectively. The genome sizes ranged from 1.9 to 2.3 Mbp with an average GC content of 37–38% in the *L. amylovorus* and *L. salivarius* strains and 59% in the *B. apri* strain (Table 1). ANI values were calculated against three type strain genomes: *B. apri* DSM 100238, *L. amylovorus* DSM 20531, and *Ligilactobacillus salivarius* DSM 20555. The ANI values of all used genomes and their respective type genomes exceeded the recommended 95% threshold for species delineation. Antimicrobial susceptibility, presence of antibiotic resistance genes and β-haemolysis.

The antimicrobial susceptibility and absence of horizontally transmissible antibiotic resistance genes in the genomes of all used *L. amylovorus* strains have been published in our previous article (Moravkova et al. 2022). These data are provided in Table 2 for the convenience of the reader. The MIC values of ten different antibiotics were obtained using the same method as for the *L. amylovorus* strains to assess the antibiotic susceptibility of *B. apri* and two *L. salivarius* strains.

B. apri exhibited MIC values above the established cut-off values for streptomycin (MIC 256 mg/ml, cut-off 128 mg/ml) and gentamicin (MIC 256 mg/l, cut-off 64 mg/l).

Resistance to streptomycin, tetracycline, chloramphenicol, kanamycin, and gentamycin was

observed in the *L. salivarius* isolates (Table 2). Even though the mentioned isolates showed resistance to the antimicrobials used, whole-genome sequencing analyses using four curated databases (CARD, ResFinder, Argannot, and Megares) did not reveal any horizontally transferred resistance genes in the case of the genera *Lactobacillus* and *Ligilactobacillus*. Genome analysis of isolate 115B (*B. apri*) revealed only the presence of the genes *rpoB* and *ileS*, which are not transferable via mobile genetic elements. β-haemolysis activity was not detected in any isolate (data not shown).

Antimicrobial activity

Antimicrobial activity against enteropathogenic *E. coli*, *S.* Typhimurium and *Y. enterocolitica* strains was detected in *L. amylovorus* and *L. salivarius* strains, but not in the *B. apri* strain (Table 3). A high level of antimicrobial activity against all five tested pathogens, as evidenced by large inhibition zones, was observed in M494A (*L. salivarius*). *L. salivarius* strain M668A exhibited a significant antimicrobial activity against four tested pathogens, but not against *E. coli* 974. Similarly, *L. salivarius* strain M698 showed a high level of antimicrobial activity against *S.* Typhimurium and *Y. enterocolitica*, but a weaker antimicrobial activity against two of the three tested *E. coli* strains. Among the *L. amylovorus* representa-

Table 3. Antimicrobial activity against pathogenic strains, production of exopolysaccharides, and presence of genes for bacteriocin production in selected strains

Strain	Identification	Antimicrobial activity					EPs	Genes for bacteriocin production
		E. coli 971	*E. coli* 973	*E. coli* 974	*S.* Typhimurium	*Y. enterocolitica*		
115B	*B. apri*	−	−	−	−	−	+	−
M597AA	*L. amylovorus*	+	−	−	+	+	+	helveticin J, enterolysin A, lanthipeptides class I and IV or class I
M597B	*L. amylovorus*	+	++	−	++	+	−	
M624A	*L. amylovorus*	++	+++	−	+++	−	+	
M696A	*L. amylovorus*	++	+	−	+	+++	+	
M668A	*L. amylovorus*	+	+	+	+	-	−	
M494A	*L. salivarius*	+	+++	++	+++	+++	−	salivaricin P, enterolysin A
M698A	*L. salivarius*	++	+	−	+++	+++	−	

− = no zone of strain growth inhibition; + = the diameter of the growth inhibition was 1 to 3 mm; ++ = the diameter of the growth inhibition was 3 to 5 mm; +++ = the diameter of the growth inhibition was more than 5 mm; *B. apri* = *Bifidobacterium apri*; EPs = exopolysaccharides; *E. coli* = *Escherichia coli*; *L. amylovorus* = *Lactobacillus amylovorus*; *L. salivarius* = *Ligilactobacillus salivarius*; *S.* Typhimurium = *Salmonella* Typhimurium; *Y. enterocolitica* = *Yersinia enterocolitica*
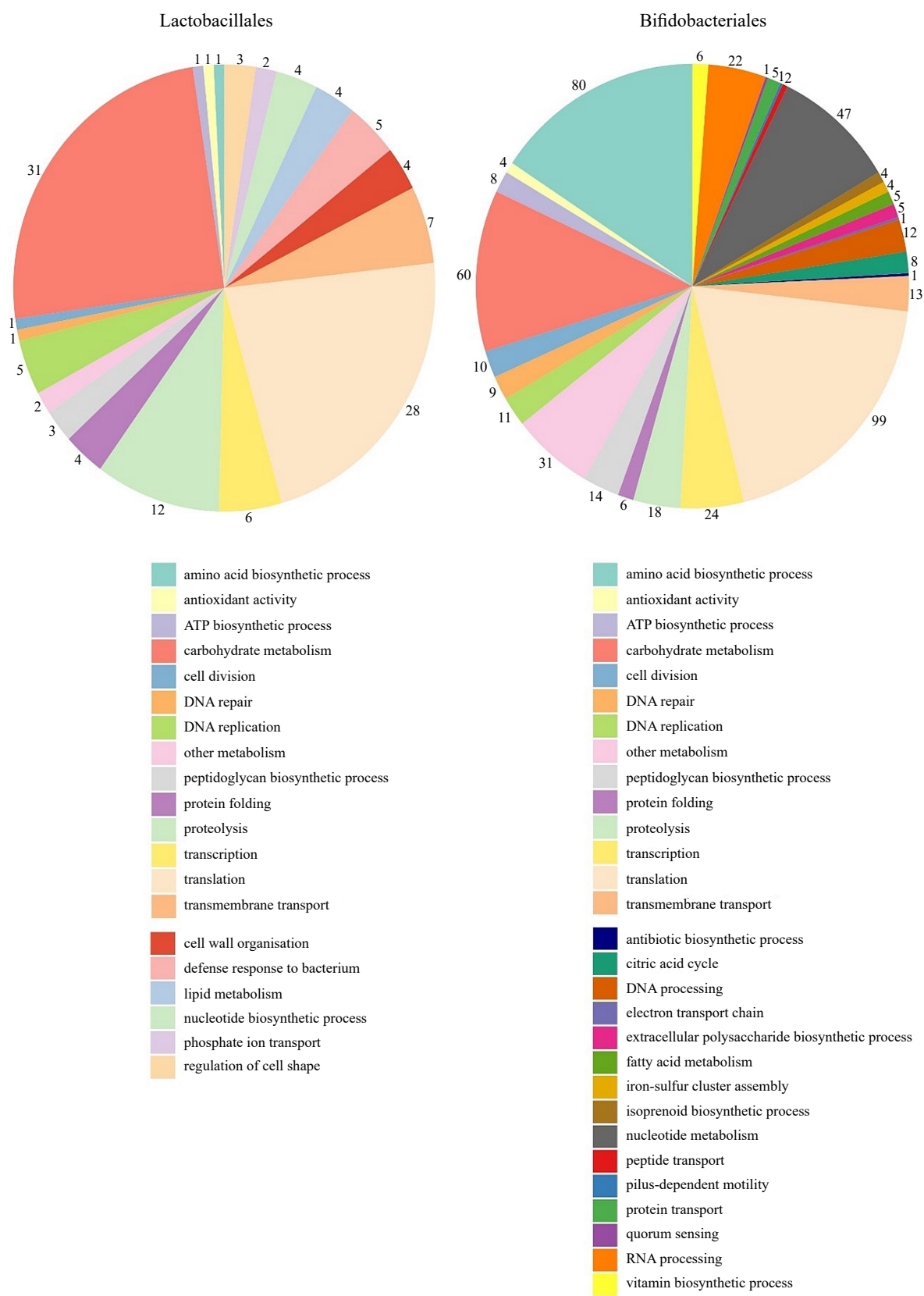
Figure 1. Proteins identified using mass spectrometry from bacterial supernatants and their associated biological processes according to gene ontology terms. Not all identified proteins are annotated for biological processes in a database (see above)
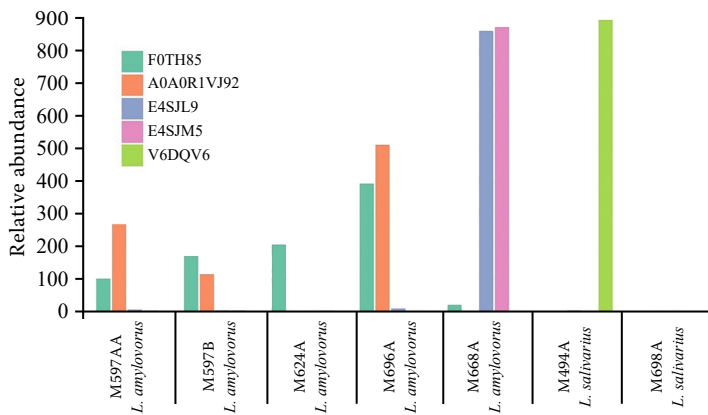
Figure 2. Relative abundance of proteins identified in supernatants of the order *Lactobacillales* involved in a "defense response to bacterium" biological process

A0A0R1VJ92 = Bacteriocin helveticin (*L. kitasatonis*); E4SJL9 = Bacteriocin helveticin (*L. amylovorus*); E4SJM5 = Bacteriocin helveticin J (*L. amylovorus*); F0TH85 = Bacteriocin immunity protein (*L. amylovorus*); *L. amylovorus* = *Lactobacillus amylovorus*; *L. salivarius* = *Ligilactobacillus salivarius*; V6DQV6 = nonfunctional salivaricin B (*L. salivarius*)
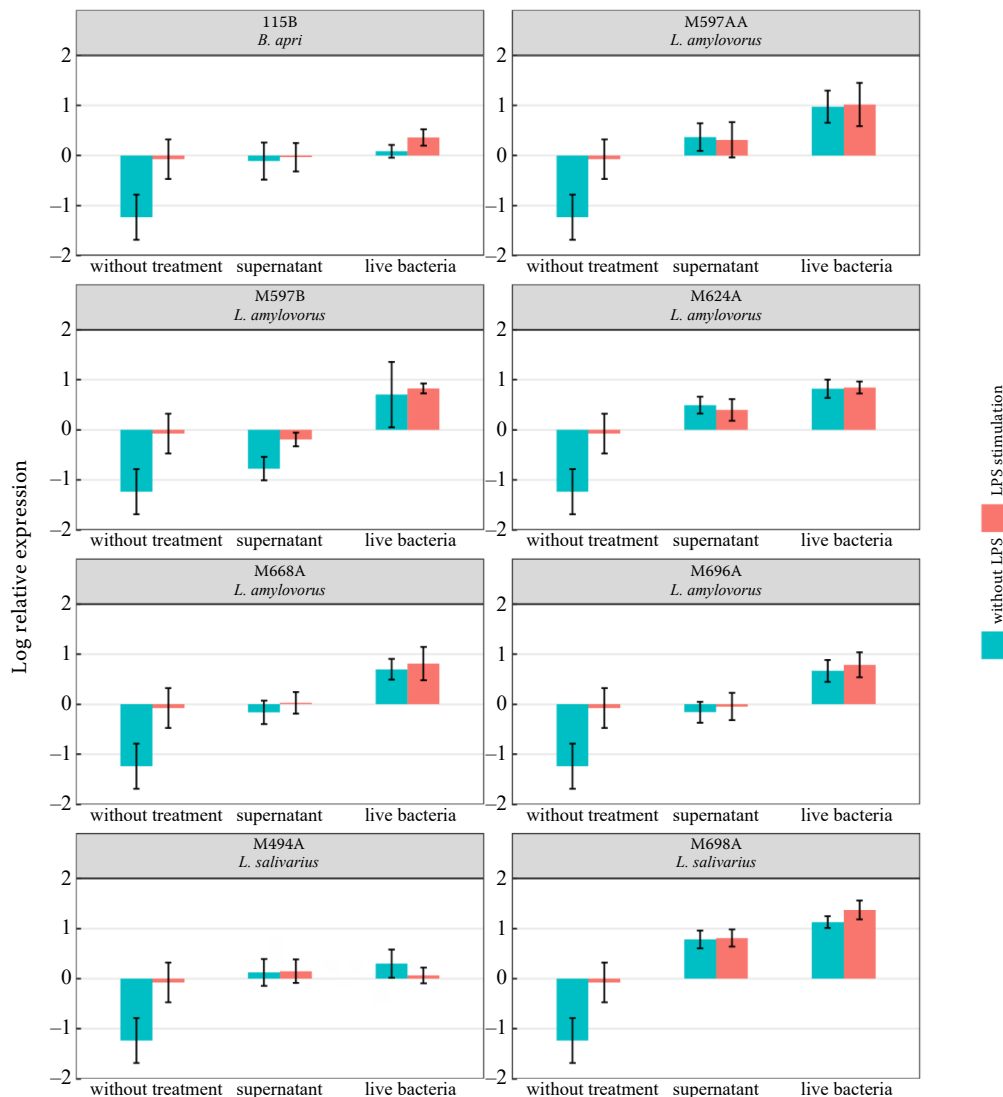

Figure 3. Log mRNA relative expression for interleukin-10 by monocyte-derived macrophages after 6-hour treatment with or without supernatants of live bacteria and stimulation with LPS or left without LPS stimulation. The data were logarithmised and the geometric mean was calculated. The error bars represent the standard deviation of the geometric mean. A two-factor analysis of variance (ANOVA) was conducted to analyse the data

B. apri = Bifidobacterium apri; *L. amylovorus = Lactobacillus amylovorus*; *L. salivarius = Ligilactobacillus salivarius*; LPS = lipopolysaccharides

tives, a high antimicrobial impact was observed in the M696A isolate, inhibiting all tested pathogens except for *E. coli* 974, while M624A exhibited strong antimicrobial activity against *S.* Typhimurium, *E. coli* 971 and *E. coli* 973. Conversely, *L. amylovorus* strains M597AA and 597B showed only weak or no antimicrobial activity, particularly against *E. coli* strains. No antimicrobial activity was detected against any of the five tested pathogens in the case of isolate 115B, belonging to *B. apri*.

Exopolysaccharide production

The production of EPs was detected visually when some isolates formed a "ropy" culture on Petri plates (Table 3). Among the examined isolates, three out of four *L. amylovorus* strains (M597AA, M624A, and M696A) as well as the *B. apri* strain (115B) were found to produce EPs.

Bacteriocin production

The potential for bacteriocin production was evaluated using genome sequence analysis using a BLAST search in the BAGEL4 database. This approach confirmed the presence of genetic elements responsible for encoding two types of bacteriocins within the genomes of *L. salivarius* (enterolysin A and salivaricin P) and *L. amylovorus* (enterolysin A and helveticin J) (Table 3).

The presence of bacteriocins was also determined at the protein level in the supernatants obtained from all isolates studied. Out of 711 well-identified proteins (with false discovery rate (FDR) < 0.01 with at least 2 unique peptides per protein) belonging to the order *Bifidobacteriales* (*B. apri*), 511 proteins were successfully annotated. In supernatants originating from *Lactobacillales* strains (the genera *Lactobacillus* and *Ligilactobacillus*), 291 proteins were identified with 125 of them having known functions. In comparison with *Lactobacillales*, *Bifidobacteriales* contain a wide range of proteins with different functions such as "antibiotic biosynthetic process", "extracellular polysaccharide biosynthetic process", "quorum sensing" and "vitamin biosynthetic process". However, none of the detected proteins from *Bifidobacteriales* supernatants participate in a "defense response to bacterium" according to gene ontology (GO)

terms. Five "defense response to bacterium" proteins were identified in supernatants derived from *Lactobacillales* cultures (Figure 1).

These bacteriocins were helveticin (E4SJL9 and A0A0R1VJ92), helveticin J (E4SJM5) and bacteriocin immunity protein (F0TH85) for *Lactobacillus amylovorus* strains and nonfunctional salivaricin B (V6DQV6) for *Ligilactobacillus salivarius* strains. Their relative abundances in each isolate of the order *Lactobacillales* are shown in Figure 2. Protein sequence identity was 36% and 37% between helveticin J (E4SJM5) and both helveticin proteins (E4SJL9 and A0A0R1VJ92, respectively). The bacteriocin helveticin had a different amino acid sequence in isolates M597AA, M597B, and M696A as compared to helveticin found in the M668A isolate. Since the latter protein belonged to *L. amylovorus*, the other helveticin protein originated from a *Lactobacillus kitasatonis* strain. The sequence identity of these two proteins was 66%.

Anti-inflammatory properties of the strains

Analysis of mRNA expression revealed that macrophages responded to LPS stimulation with an increase in interleukin-10 (IL-10) expression (Figure 3). Macrophages exhibited a slight increase in IL-10 mRNA expression when treated with 10% of the supernatants and a significantly higher expression when treated with washed live bacterial cells across all tested probiotic strains. Furthermore, treatment with live bacterial cells, and to a lesser extent with supernatants, increased the IL-10 mRNA expression in macrophages, even following an LPS stimulation.

DISCUSSION

Probiotics are live microorganisms that have beneficial effects when consumed in adequate quantities. There are several ways in which probiotics can positively influence gut well-being. These include enhancement of the epithelial barrier, inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of antimicrobial substances, and modulation of the immune system (for a review see Yousefi et al. 2019).

The bacterial strains isolated from the gut content of wild boars and tested in this study were

identified as *L. amylovorus*, *L. salivarius* and *B. apri*, *L. amylovorus* and *L. salivarius* have previously been reported to have been isolated from the GIT of domestic pigs and wild boars (Bravo et al. 2019; Shen et al. 2022), whereas *B. apri* is a newly discovered species isolated from the GIT of wild boars (Pechar et al. 2017). The safety and probiotic potential, including the production of antimicrobial compounds or bacteriocins, as well as the anti-inflammatory properties of these isolates were studied using an *in vitro* approach.

Although *B. apri* showed a MIC above the established cut-off values for streptomycin (MIC 256 mg/ml, cut-off 128 mg/ml) and gentamicin (MIC 256 mg/l, cut-off 64 mg/l), it is worth noting that *Bifidobacterium* strains are generally known for their high MICs for these two antibiotics. It has been suggested that *Bifidobacterium* resistance to both antibiotics is intrinsic (Gueimonde et al. 2013; Kim et al. 2018). In addition, the genome of *B. apri* contains two genes corresponding to the intrinsic resistance to rifamycin (*rpoB* and *ileS*) (Lokesh et al. 2018) and mupirocin (Gueimonde et al. 2013). It can, therefore, be assumed that *B. apri* is a safe probiotic capable of surviving antibiotic treatment and preventing gut dysbiosis during antibiotic therapy.

Although both *L. amylovorus* and *L. salivarius* isolates exhibited phenotypic resistance to the tested antibiotics, subsequent WGS analysis did not reveal any horizontally transferred resistance genes. The susceptibility of *L. amylovorus* isolates from wild boar GIT to antibiotics was discussed in our previous study (Moravkova et al. 2022). Conversely, *in vitro* susceptibility testing of *L. salivarius* to relevant antibiotic agents has been documented in only a few studies, each with a limited number of strains. For instance, Yeo et al. (2016) analysed susceptibility to seven antibiotics in five *L. salivarius* strains isolated from pig faeces. They observed an increased MIC to streptomycin, gentamicin, and vancomycin in a range of 128–512 mg/l, 64–128 mg/l and > 512 mg/l, respectively. A broader investigation by Dec et al. (2020) explored the phenotypic and genotypic antimicrobial resistance profiles of 16 faecal strains of *L. salivarius* from domesticated pigeons. Similarly to our findings, they reported increased resistance to streptomycin and kanamycin, with MICs ranging from 32 mg/l to 256 mg/l and 256 mg/l to 512 mg/l, respectively. Additionally, they demonstrated the absence of genes associated with resistance to aminoglycosides. These results suggest that antibiotic resistance in *L. salivarius* is probably due to chromosomal mutation or other mechanisms and that *L. salivarius* strains present a low risk for the horizontal spread of genes potentially involved in antibiotic resistance. Intrinsic resistance in *Lactobacillus* spp. has been documented against vancomycin, aminoglycosides, ciprofloxacin, and trimethoprim (Campedelli et al. 2018). However, lactobacilli are generally susceptible to penicillins, β-lactams, chloramphenicol, tetracycline, linezolid, and quinupristin/dalfopristin (Abriouel et al. 2015). Similarly, intrinsic resistance in *Bifidobacterium* has been noted against muciprocin. *Bifidobacteria* are usually susceptible to macrolides, chloramphenicol, β-lactams, and vancomycin (Gueimonde et al. 2013). Since none of the investigated isolates carried transferable genetic elements for antibiotic resistance in their genomes, while at the same time not exhibiting β-haemolytic activity, these strains are considered safe from the perspective of biosecurity.

In addition to biological safety, other probiotic characteristics are essential in selecting probiotic bacteria. For instance, the production of exopolysaccharides (EPs) in probiotic bacteria can contribute to their survival, colonization, immunomodulatory effects, and promoting healthy gut microbiota in the host (Angelin and Kavitha 2020). In our study, three out of four tested *L. amylovorus* strains, as well as the *B. apri* strain, showed production of EPs consistent with findings in the literature in which EPs production was observed in lactobacilli and bifidobacteria (Chen et al. 2014; Yuan et al. 2021).

Additionally, the ability of probiotic bacteria to exhibit antimicrobial activity is another important characteristic. The mechanisms of antimicrobial activity in bacteria are numerous. Apart from the application of the principle of competitive exclusion or acidification of the gut environment, an important mechanism of probiotic bacteria is the production of bacteriocins – antimicrobial peptides – produced as a defense mechanism by bacteria to outcompete others in their environment. The strains of *L. amylovorus* and *L. salivarius* tested in our study were able to inhibit the growth of enteropathogenic *E. coli*, *S.* Typhimurium and *Y. enterocolitica*, which are common pathogens of pigs (Weber et al. 2015; Chlebicz and Slizewska 2018). The antibacterial activity of both *L. amylovorus* and *L. salivarius* has been repeatedly dem-

onstrated *in vitro* against *E. coli* and *Salmonella* strains (Adetoye et al. 2018; Shen et al. 2022).

In our study, however, *B. apri* did not exhibit any antimicrobial activity, which contrasts with existing literature describing the antibacterial effects of bifidobacteria against specific gram-negative and gram-positive pathogens (Lim and Shin 2020). Additionally, our search using the BAGEL4 database for bacteriocin production did not reveal any genes responsible for antimicrobial activity in the genome of the *Bifidobacterium apri* isolate. Similarly, although the supernatant derived from *B. apri*, belonging to *Bifidobacteriales*, contained more identified proteins as compared to *Lactobacillales* cultures (711 vs 291), none of them were associated with the "defense response to bacterium" according to known GO terms. Conversely, the proteins identified in the supernatant of *Bifidobacteriales* exhibited a broader range of functions than those in *Lactobacillales*. Among the proteins identified in supernatants of *Bifidobacteriales* were those identified as responsible for an "antibiotic biosynthetic process", "extracellular polysaccharide biosynthetic process", "quorum sensing" or "vitamin biosynthetic process", all of which could potentially contribute to the probiotic effect on the host digestive system (Pompei et al. 2007; Sabater et al. 2020; Salman et al. 2023).

On the other hand, several genes encoding bacteriocin proteins were detected in genomic sequences and confirmed to be present at a protein level in the bacterial supernatants of *L. amylovorus* and *L. salivarius* cultures. Three helveticin proteins with different amino acid sequences were found in supernatants derived from *L. amylovorus* strains. Among them, helveticin J, defined as a heat-labile bacteriocin belonging to class III of bacteriocins (Simons et al. 2020; Luo et al. 2023), has been previously identified in *L. amylovorus* strains (Collins et al. 2017; Park et al. 2023). In the case of supernatants from *L. salivarius* cultures, only one of the two tested strains produced an antibacterial protein – the nonfunctional salivaricin B (V6DQV6). Although we identified the salivaricin gene in the *L. salivarius* genome, it is not currently present in the Uniprot protein database. Based on the amino acid sequence, it shares 100% sequence identity with "Blp family class II bacteriocin". However, analysis of *L. salivarius* strain genome sequences revealed the presence of genes encoding salivaricin P, a two-peptide bacteriocin commonly found in *L. salivarius* strains isolated from the intestines of pigs (Messaoudi et al. 2013). The presence of the gene for the bacteriocin enterolysin A was also detected in the genomes of *L. amylovorus* isolates in this study. Enterolysin A has been primarily described in enterococci (Khan et al. 2013) and *L. mucosae* (Jia et al. 2020).

Another known mechanism of how probiotic bacteria can positively influence the function of the intestine is immunomodulation. Macrophages represent innate immunity cells. One population is redistributed around specific places in the body as resident macrophages. The second population – as an inflammatory – is recruited by a chemoattractant into inflamed tissue. Macrophages can recognise corpuscular as well as soluble factors by membrane-associated pattern-recognising receptors. Both populations of macrophages play important roles in the regulation of intestinal homeostasis using the production of cytokines. One of the most important is interleukin-10 (Kole and Maloy 2014; Nguyen et al. 2021). Production of IL-10 in the small intestine is highly probably controlled by a food antigen (Kim et al. 2016). In the large intestine, however, IL-10 production is driven by microbiota (Ueda et al. 2010).

The results of our study showed that all the selected probiotic bacteria can induce the expression of IL-10 mRNA. Response to whole bacteria was significantly higher than to supernatant. Several studies demonstrated that probiotic bacteria can induce expression of mRNA for the production of the IL-10 protein. They have emphasised the role of lipoteichoic acid and its structure (Lebeer et al. 2012; Lu et al. 2022). Moreover, Engevik et al. (2021) compared the ability of cell surface components and metabolites to induce the production of IL-10, the former being more potent. These authors suggested that colonic macrophages (in contrast to colonic dendritic cells) constitutively produce IL-10 and the production is responsive for unresponsiveness to LPS stimulation. The role of commensal bacteria in these phenomena was proven by the fact that germ-free mice produced less IL-10 and had a higher response to LPS exposure. In line with this finding, our study also showed that selected probiotic bacteria can increase IL-10 production even after LPS stimulation, and cell surface structures were more potent than supernatants containing bacterial metabolites.

In this study, we isolated and selected eight potential probiotic strains originating from the di-

gestive tract of wild boars. These isolates were identified as *Bifidobacterium apri*, *Lactobacillus amylovorus* and *Ligilactobacillus salivarius*. Our primary objective was to analyse these isolates *in vitro* for their biosecurity, antimicrobial activity, production of extracellular components, and their ability to modulate immune responses in host cells.

None of the selected isolates were found to carry transferable antibiotic resistance genes or exhibit β-haemolytic activity, suggesting their biological safety. Furthermore, all strains tested in this study exhibited the combination of probiotic properties *in vitro*.

Specifically, the isolates were capable of inhibiting the growth of selected pathogens, producing exopolysaccharides and bacteriocins, and stimulating the production of the anti-inflammatory cytokine interleukin-10 in porcine macrophages. These characteristics make the aforementioned strains, originating from the gastrointestinal tract of wild boars, promising candidates for use as probiotics in piglets.

Acknowledgement

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Abriouel H, Casado Munoz MDC, Lavilla Lerma L, Perez Montoro B, Bockelmann W, Pichner R, Kabisch J, Cho GS, Franz CMAP, Galvez A, Benomar N. New insights in antibiotic resistance of Lactobacillus species from fermented foods. Food Res Int. 2015 Dec;78:465-81.

Adetoye A, Pinloche E, Adeniyi BA, Ayeni FA. Characterization and anti-salmonella activities of lactic acid bacteria isolated from cattle faeces. BMC Microbiol. 2018 Aug 30; 18(1):96.

Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004 Aug 1;64(15): 5245-50.

Angelin J, Kavitha M. Exopolysaccharides from probiotic bacteria and their health potential. Int J Biol Macromol. 2020 Nov 1;162:853-65.

Anjana, Tiwari SK. Bacteriocin-producing probiotic lactic acid bacteria in controlling dysbiosis of the gut microbiota. Front Cell Infect Microbiol. 2022 May 16;12:851140.

Azad MAK, Sarker M, Li T, Yin J. Probiotic species in the modulation of gut microbiota: An overview. Biomed Res Int. 2018 May 8;2018:9478630.

Bravo M, Combes T, Martinez FO, Cerrato R, Rey J, Garcia-Jimenez W, Fernandez-Llario P, Risco D, Gutierrez-Merino J. Lactobacilli isolated from wild boar (Sus scrofa) antagonize Mycobacterium bovis Bacille Calmette-Guerin (BCG) in a species-dependent manner. Front Microbiol. 2019 Jul 30;10:1663.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4): 611-22.

Campedelli I, Mathur H, Salvetti E, Clarke S, Rea MC, Torriani S, Ross RP, Hill C, O'Toole PW. Genus-wide assessment of antibiotic resistance in Lactobacillus spp. Appl Environ Microbiol. 2018 Dec 13;85(1):e01738-18.

Chen XY, Woodward A, Zijlstra RT, Ganzle MG. Exopolysaccharides synthesized by Lactobacillus reuteri protect against enterotoxigenic Escherichia coli in piglets. Appl Environ Microbiol. 2014 Sep;80(18):5752-60.

Chlebicz A, Slizewska K. Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: A review. Int J Environ Res Public Health. 2018 Apr 26;15(5):863.

Collins FWJ, O'Connor PM, O'Sullivan O, Gomez-Sala B, Rea MC, Hill C, Ross RP. Bacteriocin gene-trait matching across the complete Lactobacillus pan-genome. Sci Rep. 2017 Jun 14;7(1):3481.

Dec M, Stepien-Pysniak D, Nowaczek A, Puchalski A, Urban-Chmiel R. Phenotypic and genotypic antimicrobial resistance profiles of fecal lactobacilli from domesticated pigeons in Poland. Anaerobe. 2020 Oct;65:102251.

ECDC – European Centre for Disease Prevention and Control; European Food Safety Authority (EFSA); European Medicines Agency (EMA). Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA: JIACRA III 2016–2018. EFSA J. 2021 Jun 30;19(6): e06712.

EFSA – European Food Safety Authority, Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Karenlampi S, Aguilera J, Anguita M, Brozzi R, Galobart J. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA J. 2018 Mar 28;16(3):e05206.

Engevik MA, Ruan W, Esparza M, Fultz R, Shi Z, Engevik KA, Engevik AC, Ihekweazu FD, Visuthranukul C, Venable S, Schady DA, Versalovic J. Immunomodulation of dendritic cells by Lactobacillus reuteri surface components and metabolites. Physiol Rep. 2021 Jan;9(2): e14719.

EP – European Parliament and the Council of the European Union. Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC. Official Journal of the European Union; 2019.b.

Gueimonde M, Sanchez B, G de Los Reyes-Gavilan C, Margolles A. Antibiotic resistance in probiotic bacteria. Front Microbiol. 2013 Jul 18;4:202.

Guo XH, Kim JM, Nam HM, Park SY, Kim JM. Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. Anaerobe. 2010 Aug;16(4):321-6.

Hwang HS, Lee JK, Eom TK, Son SH, Hong JK, Kim KH, Rhim SJ. Behavioral characteristics of weaned piglets mixed in different groups. Asian-Australas J Anim Sci. 2016 Jul;29(7):1060-4.

Jia Y, Yang B, Ross P, Stanton C, Zhang H, Zhao J, Chen W. Comparative genomics analysis of Lactobacillus mucosae from different niches. Genes (Basel). 2020 Jan 14;11(1):95.

Kavanova L, Matiaskova K, Leva L, Stepanova H, Nedbalcova K, Matiasovic J, Faldyna M, Salat J. Concurrent infection with porcine reproductive and respiratory syndrome virus and Haemophilus parasuis in two types of porcine macrophages: Apoptosis, production of ROS and formation of multinucleated giant cells. Vet Res. 2017 May 4;48(1):28.

Khan H, Flint SH, Yu PL. Determination of the mode of action of enterolysin A, produced by Enterococcus faecalis B9510. J Appl Microbiol. 2013 Aug;115(2):484-94.

Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, Lee JY, Lee M, Surh CD. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science. 2016 Feb 19;351(6275):858-63.

Kim MJ, Ku S, Kim SY, Lee HH, Jin H, Kang S, Li R, Johnston TV, Park MS, Ji GE. Safety evaluations of Bifidobacterium bifidum BGN4 and Bifidobacterium longum BORI. Int J Mol Sci. 2018 May 9;19(5):1422.

Kole A, Maloy KJ. Control of intestinal inflammation by interleukin-10. Curr Top Microbiol Immunol. 2014;380: 19-38.

Kyrova K, Stepanova H, Rychlik I, Faldyna M, Volf J. SPI-1 encoded genes of Salmonella Typhimurium influence differential polarization of porcine alveolar macrophages in vitro. BMC Vet Res. 2012 Jul 20;8:115.

Lagace L, Pitre M, Jacques M, Roy D. Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. Appl Environ Microbiol. 2004 Apr;70(4): 2052-60.

Lebeer S, Claes IJ, Vanderleyden J. Anti-inflammatory potential of probiotics: Lipoteichoic acid makes a difference. Trends Microbiol. 2012 Jan;20(1):5-10.

Lim HJ, Shin HS. Antimicrobial and immunomodulatory effects of Bifidobacterium strains: A review. J Microbiol Biotechnol. 2020 Dec 28;30(12):1793-800.

Li M, Wang Y, Cui H, Li Y, Sun Y, Qiu HJ. Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. Front Vet Sci. 2020 Feb 11;7:49.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001 Dec;25(4): 402-8.

Lokesh D, Parkesh R, Kammara R. Bifidobacterium adolescentis is intrinsically resistant to antitubercular drugs. Sci Rep. 2018 Aug 9;8(1):11897.

Lu Q, Guo Y, Yang G, Cui L, Wu Z, Zeng X, Pan D, Cai Z. Structure and anti-inflammation potential of lipoteichoic acids isolated from Lactobacillus strains. Foods. 2022 May 30;11(11):1610.

Luo R, Liu C, Li Y, Liu Q, Su X, Peng Q, Lei X, Li W, Menghe B, Bao Q, Liu W. Comparative genomics analysis of habitat adaptation by Lactobacillus kefiranofaciens. Foods. 2023 Apr 10;12(8):1606.

McEwen SA, Collignon PJ. Antimicrobial resistance: A one health perspective. Microbiol Spectr. 2018 Mar;6(2).

Messaoudi S, Manai M, Kergourlay G, Prevost H, Connil N, Chobert JM, Dousset X. Lactobacillus salivarius: Bacteriocin and probiotic activity. Food Microbiol. 2013 Dec; 36(2):296-304.

Monteiro CRAV, do Carmo MS, Melo BO, Alves MS, Dos Santos CI, Monteiro SG, Bomfim MRQ, Fernandes ES, Monteiro-Neto V. In vitro antimicrobial activity and probiotic potential of Bifidobacterium and Lactobacillus

against species of Clostridium. Nutrients. 2019 Feb 21; 11(2):448.

Moravkova M, Kostovova I, Kavanova K, Pechar R, Stanek S, Brychta A, Zeman M, Kubasova T. Antibiotic susceptibility, resistance gene determinants and corresponding genomic regions in Lactobacillus amylovorus isolates derived from wild boars and domestic pig**s**. Microorganisms. 2022 Dec 30;11(1):103.

Nguyen HD, Aljamaei HM, Stadnyk AW. The production and function of endogenous interleukin-10 in intestinal epithelial cells and gut homeostasis. Cell Mol Gastroenterol Hepatol. 2021;12(4):1343-52.

Nygard AB, Jorgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol Biol. 2007 Aug 15; 8:67.

Park S, Kim JA, Jang HJ, Kim DH, Kim Y. Complete genome sequence of functional probiotic candidate Lactobacillus amylovorus CACC736. J Anim Sci Technol. 2023 Mar; 65(2):473-7.

Pechar R, Killer J, Salmonova H, Geigerova M, Svejstil R, Svec P, Sedlacek I, Rada V, Benada O. Bifidobacterium apri sp. nov., a thermophilic actinobacterium isolated from the digestive tract of wild pigs (Sus scrofa). Int J Syst Evol Microbiol. 2017 Jul;67(7):2349-56.

Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M. Folate production by bifidobacteria as a potential probiotic property. Appl Environ Microbiol. 2007 Jan; 73(1):179-85.

Ruas-Madiedo P, de los Reyes-Gavilan CG. Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J Dairy Sci. 2005 Mar;88(3):843-56.

Sabater C, Molinero-Garcia N, Castro-Bravo N, Diez-Echave P, Hidalgo-Garcia L, Delgado S, Sanchez B, Galvez J, Margolles A, Ruas-Madiedo P. Exopolysaccharide producing Bifidobacterium animalis subsp. lactis strains modify the intestinal microbiota and the plasmatic cytokine levels of BALB/c Mice according to the type of polymer synthesized. Front Microbiol. 2020 Nov 26;11: 601233.

Salman MK, Abuqwider J, Mauriello G. Anti-quorum sensing activity of probiotics: The mechanism and role in food and gut health. Microorganisms. 2023 Mar 20;11(3):793.

Shen J, Zhang J, Zhao Y, Lin Z, Ji L, Ma X. Tibetan pig-derived probiotic Lactobacillus amylovorus SLZX20-1 improved intestinal function via producing enzymes and regulating intestinal microflora. Front Nutr. 2022 Mar 29; 9:846991.

Shin D, Chang SY, Bogere P, Won K, Choi JY, Choi YJ, Lee HK, Hur J, Park BY, Kim Y, Heo J. Beneficial roles of probiotics on the modulation of gut microbiota and immune response in pigs. PLoS One. 2019 Aug 28;14(8):e0220843.

Simons A, Alhanout K, Duval RE. Bacteriocins, antimicrobial peptides from bacterial origin: Overview of their biology and their impact against multidrug-resistant bacteria. Microorganisms. 2020 Apr 27;8(5):639.

Tang X, Xiong K, Fang R, Li M. Weaning stress and intestinal health of piglets: A review. Front Immunol. 2022 Nov 24;13:1042778.

Ueda Y, Kayama H, Jeon SG, Kusu T, Isaka Y, Rakugi H, Yamamoto M, Takeda K. Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. Int Immunol. 2010 Dec;22(12): 953-62.

Weber N, Nielsen JP, Jakobsen AS, Pedersen LL, Hansen CF, Pedersen KS. Occurrence of diarrhoea and intestinal pathogens in non-medicated nursery pigs. Acta Vet Scand. 2015 Sep 30;57:64.

Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009 May;6(5):359-62.

Yeo S, Lee S, Park H, Shin H, Holzapfel W, Huh CS. Development of putative probiotics as feed additives: Validation in a porcine-specific gastrointestinal tract model. Appl Microbiol Biotechnol. 2016 Dec;100(23):10043-54.

Yousefi B, Eslami M, Ghasemian A, Kokhaei P, Salek Farrokhi A, Darabi N. Probiotics importance and their immunomodulatory properties. J Cell Physiol. 2019 Jun; 234(6):8008-18.

Yuan L, Chu B, Chen S, Li Y, Liu N, Zhu Y, Zhou D. Exopolysaccharides from Bifidobacterium animalis ameliorate Escherichia coli-induced IPEC-J2 cell damage via inhibiting apoptosis and restoring autophagy. Microorganisms. 2021 Nov 16;9(11):2363.

Zhou B, Albarracin L, Indo Y, Arce L, Masumizu Y, Tomokiyo M, Islam MA, Garcia-Castillo V, Ikeda-Ohtsubo W, Nochi T, Morita H, Takahashi H, Kurata S, Villena J, Kitazawa H. Selection of immunobiotic ligilactobacillus salivarius strains from the intestinal tract of wakame-fed pigs: Functional and genomic studies. Microorganisms. 2020 Oct 26;8(11):1659.