Probiotics and Antimicrobial Proteins (2021) 13:208–217
https://doi.org/10.1007/s12602-020-09687-y

Compatibility, Cytotoxicity, and Gastrointestinal Tenacity of Bacteriocin-Producing Bacteria Selected for a Consortium Probiotic Formulation to Be Used in Livestock Feed

Mégane Eveno[1,2] · Patricia Savard[2] · Yanath Belguesmia[1] · Laurent Bazinet[2] · Frédérique Gancel[1] · Djamel Drider[1,3] · Ismail Fliss[2]

Published online: 25 July 2020

Abstract

Bacteriocin-producing *Escherichia coli* ICVB442, *E. coli* ICVB443, *Enterococcus faecalis* ICVB497, *E. faecalis* ICVB501, and *Pediococcus pentosaceus* ICVB491 strains were examined for their pathogenic risks and compatibility and hence suitability as consortium probiotic bacteria. Except for *E. coli* ICVB442, all were inclined to form biofilm. All were gelatinase-negative, sensitive to most of the antibiotics tested and not cytotoxic to porcine intestinal epithelial cells (IPEC-1) when tested at a multiplicity of infection (MOI) of 1. *P. pentosaceus* ICVB491 stood apart by inhibiting the other four strains. Both *E. coli* strains and *E. faecalis* ICVB497 strain were β-hemolytic. Survival in the TIM-1 dynamic model of the human digestive system was 139% for the tested *E. coli* ICVB443 strain, 46% for *P. pentosaceus* ICVB491, and 32% for the preferred *E. faecalis* ICVB501 strain. These three potential probiotics, which are bacteriocin-producing strains, will be considered for simultaneous use as consortium with synergistic interactions in vivo on animal model.

Keywords Gut microbiota · Probiotic · Bacteriocin · Biocompatibility · TIM-1

Introduction

Antibiotics have been used widely and massively as growth promoters and therapeutic agents to stop infectious diseases in livestock [1]. Indeed, so-called growth promotion is a sparing effect on the animal immune system, which would otherwise be a metabolic burden on livestock performance. This practice has been profitable for farmers, allowing production with huge economies of scale and minimal animal death due to pathogenic bacteria. The price paid for this boon has been the development of a reservoir of bacterial resistance to antibiotics, now called the "resistome," which includes multi-resistant strains that are transmissible throughout the production chain [2]. Even more troubling, residues of antibiotics can end up in human foods, especially meat and its derivatives. Consequently, the use of antibiotics as growth promoters has been prohibited in the European Union (under directive 2001/82/CE) and in other countries [3]. Total replacement of antibiotics in livestock production has bolstered the development of new strategies of pathogen control, including the use of probiotics [4]. According to the United Nations Food and Agriculture Organization, probiotics are "live microorganisms, which when administered in adequate amounts, confer a health benefit to the host" [5]. The key criteria for selecting a microbial strain as a probiotic are safety, survival under gastrointestinal conditions, and ability to inhibit pathogens [6]. Most probiotics approved so far are species of lactic acid bacteria [3, 7, 8]. However, other bacteria have been evaluated, such as *Escherichia coli* Nissle 1917, a potent inhibitor of *Salmonella* spp. as well as other enteric pathogens and now used in the first approved probiotic formulation [9]. Studies of potential pathogenic properties are required in order to qualify strains of *E. coli* or *Enterococcus* spp. as probiotic candidates. These non-GRAS species then have to be evaluated for bacteriocin production and survival in the gastrointestinal tract, in particular

✉ Djamel Drider
djamel.drider@univ-lille.fr

[1] UMR Transfrontalière BioEcoAgro N° 1158, Univ. Lille, INRAE, Univ. Liège, UPJV, YNCREA, Univ. Artois, Univ. Littoral Côte d'Opale, ICV – Institut Charles Viollette, F-59000 Lille, France

[2] Institute of Nutrition and Functional Foods (INAF), Dairy Research Center (STELA), Department of Food Sciences, Pavillon Paul-Comtois, 2425, rue de l'Agriculture, Université Laval, Québec G1V 0A6, Canada

[3] UMR-T INRAE 1154, Institut Charles Viollette, Cité Scientifique, Polytech-Lille, Bureau C315, , Avenue Paul Langevin, 59655 Villeneuve d'Ascq, France

in the presence of bile salts and at low pH [10–12]. Their responses to unabsorbed dietary starch, non-caloric polysaccharides such as fiber and oligosaccharides (major sources of carbon and energy for some probiotics) and mucin (an inexhaustible source of oligosaccharides and glycoproteins in the gastrointestinal tract) also have to be evaluated [13, 14]. Strains of non-GRAS genera such *Entercoccus* sometimes meet these requirements [15]. Notwithstanding non-GRAS status and even potential antibiotic resistance, *Enterococcus*-based probiotic formulations have been developed and marketed. Examples are Symbioflor 1 (SymbioPharm, Herborn, Germany) and *E. faecium* SF68® or NCIMB 10415 (Cerbios-Pharma SA, Barbengo, Switzerland) [16], which are currently used for preventing or treating diarrhea in pigs [17]. Probiotic strains of *E. coli* produce bacteriocins called colicins [18], which were in fact the first bacteriocins to be characterized [19]. Of note, the antimicrobial spectrum of colicins includes pathogenic vancomycin-resistant enterococci [20], and pathogenic strains of *E. coli* [21, 22]. They bind to specific receptors on targeted cells and form voltage-dependent channels into the inner membrane [23].

Pediococcus has received GRAS status, and strains of *P. pentosaceus* and *P. acidilactici* offer ideal probiotic profiles and are already being used as probiotic supplements in animal feed [8]. As for enterococci, one of notable advantages of pediococci is the production of bacteriocins that tolerate gastrointestinal conditions [24]. These bacteriocins are pH-stable and temperature-stable [25–28]. They bear the N-terminal YGNGV(L) sequence that typifies class IIa bacteriocins and they act through membrane permeabilization or by binding to specific receptors [29, 30].

Probiotic formulations usually contain a single well-characterized strain. However, recent studies show benefits of probiotics based on multiple strains [31, 32]. To develop new therapeutic options for infections by Gram-negative or Gram-positive bacilli in farming and veterinary medicine, we recently isolated strains of *E. coli*, *E. faecalis*, and *P. pentosaceus* from livestock (swine) and claimed their bacteriocin-producing features [33].

In this study, we gained more insights and characterize these strains for their potential health risk, mutual compatibility, and survival under gastrointestinal conditions (TIM-1 dynamic model), allowing to evaluate their suitability as probiotic strain as standalone or mixed in a consortium.

Material and Methods

Bacterial Strains and Growth Conditions

Escherichia coli ICVB442 and ICVB443, *E. faecalis* ICVB497 and ICVB501, and *P. pentosaceus* ICVB491 strains were isolated and characterized for their probiotic characters, mainly their antimicrobial activity against Gram-positive and Gram-negative pathogens in a previous report focused on drops of animals living in captivity at the zoological garden of Lille (France) [33]. *E. coli* ICVB442 and ICVB443 as well as *E. faecalis* ICVB497 and ICVB501 strains were grown in brain heart infusion (BHI) medium (Sigma Aldrich, St Louis, MO, USA) at 37 °C for 24 h. *P. pentosaceus* ICVB491 strain was grown at 37 °C for 24 h in de Man, Rogosa, and Sharpe (MRS) [34] broth (Sigma Aldrich). In addition, the probiotic *Lactobacillus rhamnosus* GG (ATCC 53103) was used as control strain to assess biofilm formation, whereas *E. faecalis* Symbioflor®1 probiotic strain, *Clostridium perfringens* DSM 756 pathogenic strain, *E. faecalis* ATCC 700802, and *E. coli* ATCC 25922 reference strains were also used as controls for cytotoxicity assays. *Staphylococcus aureus* ATCC43300 and *C. perfringens* DSM 756 were used as control strains for hemolysis, antibiotics resistance, and assessment of gelatinase activity.

Evaluation of Pathogenic Properties of the Bacteriocin-Producing Strains

Resistance to Antibiotics

Overnight broth culture of each strain was diluted in peptone water (5 μL in 5 mL). The *P. pentosaceus* ICVB491 suspension was spread on MRS agar, whereas *E. coli* ICVB442, ICVB443, and *E. faecalis* ICVB497, ICVB501 strain suspensions were spread on Mueller-Hinton agar (Sigma Aldrich). Antibiotic discs (Becton-Dickenson, Mississauga, Canada) were deposited on the agar surface, and the plates were incubated overnight at 37 °C. *E. coli* was tested with gentamycin (10 μg), ampicillin (10 μg), ciprofloxacin (5 μg), cefoxitin (30 μg), nalidixic acid (30 μg), ceftazidime (30 μg), or cefotaxime (30 μg). *P. pentosaceus* was tested with penicillin (10 μg), gentamycin (10 μg), erythromycin (15 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), and vancomycin (30 μg). *E. faecalis* was tested with ciprofloxacin (5 μg), ampicillin (10 μg), gentamycin (10 μg), vancomycin (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). Antibiotic susceptibility was interpreted in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations [35], based on the diameter of the zone of inhibition formed around each disc.

Hemolytic Activity

Hemolytic activity was evaluated on Mueller-Hinton agar into which defibrinated sheep blood (5% v/v, Nutri-Bact, Terrebonne QC, Canada) was blended just before solidification (at 41 °C). Then, 18 h cultures at 37 °C in BHI broth (for

E. coli ICVB442, ICVB443 and *E. faecalis* ICVB497, ICVB501 strains) or in MRS broth (for *P. Pentosaceus* ICVB 491) were used. The broth cultures were streaked onto the blood agar plates and incubated for 36 h at 37 °C. Based on the zone of clearing, hemolytic activity was described as β-hemolysis (complete) or α-hemolysis (partial).

Gelatinase Production

Gelatinase activity was determined using Petri plates containing nutrient medium agar supplemented with 5% (w/v) gelatin (BDH Chemicals, Ltd., Poole, UK). The agar surface was inoculated by streaking the tested strain grown in its usual culture broth. After incubation for 2 to 5 days at 37 °C, Frazier reagent ($HgCl_2$ 12 g, HCl 20 mL, distilled water 20 mL) was added to reveal the hydrolysis. Formation of a halo around a colony indicated gelatinase activity.

Biofilm Formation Ability

Biofilm formation was quantified according to a method published previously [36]. *E. coli* ICVB442, ICVB443 and *E. faecalis* ICVB497, ICVB501 strains were grown overnight in 3 mL of BHI broth, whereas *P. pentosaceus* ICVB491 and *Lb. rhamnosus* GG (ATCC 53103) were grown in MRS broth. Polystyrene micro-assay plates were loaded with broth (230 μL per well) and 20 μL of overnight culture were added in triplicate. Wells containing medium only were negative controls, whereas those containing *Lb. rhamnosus* GG (ATCC 53103) were positive controls. After incubation for 24 h at 37 °C, the culture (or broth) was poured off, and the wells were washed three times with 300 μL of sterile ultrapure water. Methanol (250 μL) was added to fix adherent bacteria for 15 min. The wells were then emptied, air-dried, stained for 5 min with 250 μL of crystal violet, and the assay plate was placed under running tap water to remove excess stain. After air-drying, 250 μL of 33% (v/v) glacial acetic acid (Sigma Aldrich) were added to each well to resolubilize the dye bound to adherent cells. The $OD_{570\ nm}$ was determined for each well using a PowerWave XS2 automated 96-well assay plate reader (Bio-Tek Instruments, Winooski, VT, USA). The cutoff OD (OD_c) was defined as the mean $OD_{570\ nm}$ of the negative control values plus three standard deviations. Strains were classified on the following basis: $OD_{570\ nm} \leq OD_c$ = non-adherent, $OD_c < OD_{570\ nm} \leq 2 \times OD_c$ = weakly adherent and $2 \times ODc < OD_{570\ nm}$ = strongly adherent. The OD_c was determined for BHI and MRS broths.

Cytotoxicity Assay on the Porcine Intestinal Epithelial Cell Line IPEC-1

The cytotoxicity of bacteriocin-producing strains was determined using a porcine intestinal epithelial cell line-1 called IPEC-1 (DSMZ, Braunschweig, Germany) grown to confluence in the wells of micro-assay plates. The assay is based on measurement of lactic dehydrogenase activity released into the culture medium by lysed cells. Each strain was tested at a concentration 10^5 cfu/mL or 10^7 cfu/mL for multiplicity of infection (MOI) equal to 1 or 100. Cultures were centrifuged (8228*g*, 10 min, 4 °C), washed twice with 4 mL of phosphate-buffered saline (NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 1.8 mmol/L), and resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum and no antibiotics. The IPEC-1 cells were washed twice to remove the culture medium completely then covered with 100 μL of bacterial suspension. DMEM was used as a negative control. The plates were then incubated for 24 h at 37 °C and 5% (v/v) CO_2. Probiotic Symbioflor®1, *C. perfringens* DSM 756, *Enterococcus faecalis* ATCC 700802, and *Escherichia coli* ATCC 25922 were also used as controls. After incubation, 50 μL from each well were transferred to another micro-assay plate for testing with the lactate dehydrogenase substrate, dye, and cofactor (Sigma Aldrich) added as solutions at a 1:1:1 (v/v/v) ratio (100 μL total). The plate was returned to the incubator for 20–30 min. The absorbance in each well was then measured at 490 nm and subtracted from the absorbance at 690 nm. Epithelial cell viability was calculated from the absorbance relative to the negative control.

Mutual Compatibility of the Bacteriocin-Producing Strains

A cross-inhibition assay was performed using the deep-well diffusion method [37]. Overnight culture at 37 °C in 3 mL of BHI (*E. coli* ICVB442; ICVB443 and *E. faecalis* ICVB497; ICVB501) or MRS (*P. pentosaceus* ICVB491) was centrifuged (8334*g*, 8 min, 4 °C). Half of the supernatant was adjusted to pH 7.0. Both portions were tested in wells in BHI or MRS agar seeded with one of the five strains [38]. Plates were incubated overnight at 37 °C. The presence of an inhibition zone indicated inhibitory activity.

The compatibility assay was performed by re-suspending the centrifugal pellet in 200 μL of supernatant of another strain in micro-assay wells. BHI or MRS broth was used as a positive control and uninoculated broth was used as a negative control. The OD_{600} was measured every 15 min during 36 h of incubation at 37 °C. The plate was agitated gently before each measurement. The T_{lag} (time elapsed upon the initial 0.01 increase in optical density) and μ_{max} (specific growth rate at the inflection point) were calculated from the OD_{600} graph. Peak optical density (OD_{max}) was noted.

Survival Under Gastrointestinal Conditions

The tenacity of each strain in situ was evaluated using the TIM-1 dynamic model of the human digestive system (TNO

Nutrition and Food Research Institute, Zeist, Netherlands), which consists of four flexible compartments connected in series mimicking the stomach, duodenum, jejunum, and ileum. Computer-controlled pumps produce peristaltic movement using external water pressure. Two independent sensors connected to the stomach and small intestine compartments allow temperature control (set at 37 °C). A sensor mounted in each compartment allows monitoring and control of pH. The gastric pH was started at 6.5 and decreased gradually to 3.5 at 30 min, 1.8 at 60 min, and 1.7 from 120 min to the end of digestion at 300 min. The duodenal pH was kept at 6.0, the jejunal pH at 6.5, and the ileal pH at 7.2. Hollow-fiber modules connected to the jejunal and ileal compartments allow dialysis against small intestinal electrolyte solution to simulate absorption. Gastric secretions were composed of 150 U/mg lipase and 3200 U/mg pepsin in gastric electrolyte solution (NaCl 6.2 g/L, KCl 2.2 g/L, $CaCl_2$ 0.3 g/L, $NaHCO_3$ 1.5 g/L). Porcine bile extract and 8xUSP pancreatin dissolved in small intestine electrolyte solution (NaCl 5.0 g/L, KCl 0.60 g/L, $CaCl_2$ 0.30 g/L) were injected into the duodenal compartment. The jejunal and ileal compartments were filled initially with small intestine electrolyte. A test protocol described previously [39] was used with minor modifications. Briefly, 310 mL of skim milk inoculated with 3 mL of culture were added to the gastric compartment. Aliquots of 500 μL were removed therefrom at 0, 30, 60, 90, and 120 min, after 30, 60, 90, 120, and 150 min from the duodenal compartment, and after 60, 120, 180, and 240 min from jejunal and ileal compartments. The effluent was collected, weighed, and aliquoted at 60, 120, 180, 240, and 300 min. The semifluid mass that passed from the stomach to the duodenum intestine at 300 min (chyme) was also weighed.

Viable counts were determined from serial dilutions (to 10^{-6}) in sterile 1% (v/v) peptone water (Thermo Fisher Scientific Inc.). *E. faecalis* ICVB501 was counted on bile esculin agar (selective for group D streptococci), *E. coli* ICVB443 on eosin methylene blue agar (selective for coliforms), and *P. pentosaceus* ICVB491 on MRS agar. Plates were spread in duplicate and incubated at 37 °C for 24 h. Counts were expressed as decimal logarithm (log_{10}) colony forming units (cfu) per mL of skim milk.

Statistical Analyses

The IPEC-1 viability test was performed in triplicate. The mutual compatibility experiment was performed twice, independently for each strain. Data were analyzed using SAS software (SAS Institution, Cary, NC, USA) and expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) with the Tukey multiple comparisons test was used to identify significant differences between treatments ($P < 0.05$).

Results

Antibiotic Susceptibility Was Strain-Dependent

Based on CLSI interpretation [35], *E. coli* ICVB442, ICVB443, and *E. faecalis* ICVB497, ICVB501 were sensitive to all of the antibiotics tested. However, *E. faecalis* showed intermediate resistance to trimethoprim-sulfamethoxazole. *P. pentosaceus* ICVB491 was also sensitive, except to ciprofloxacin, trimethoprim-sulfamethoxazole, and vancomycin, to which pediococci are generally resistant [40, 41].

Hemolysis and Gelatinase

E. coli ICVB442, ICVB443, and *E. faecalis* ICVB497 colonies produced clear zones on blood agar, indicating β-hemolysis. Neither *E. faecalis* ICVB501 nor *P. pentosaceus* ICVB491 displayed this undesirable trait (results not shown). All five strains were gelatinase-negative.

Biofilm Formation

The control optical density was 0.145 in BHI broth and 0.149 in MRS broth. *E. coli* ICVB442 was non-adherent (OD less than half the OD_c), in other words, little or no tendency to form biofilm [42]. *E. coli* ICVB443 (OD = 0.511), *E. faecalis* ICVB497 (0.680), and *P. pentosaceus* ICVB491 (0.794) were strongly adherent (OD more than twice the OD_c). *E. faecalis* ICVB497 (0.373) was weakly adherent [42]. The OD obtained for *Lb. rhamnosus* GG (used as a strong positive control) was 0.658.

Cytotoxicity at Low Concentrations

Cytotoxicity is deemed significant when the IPEC-1 cell layer viability is around 50%, moderate at 60–80%, low at 80–99%, and negative at 100% [43]. As shown in Fig. 1 A and B, *C. perfringens* was highly cytotoxic at both MOI. Symbioflor® 1, *E. faecalis* (ICVB497, ICVB501), and *P. pentosaceus* ICVB491 strains displayed low cytotoxicity at both MOI as no significant difference ($P > 0.05$) was noticed compared with the untreated IPEC-1 cells, used as controls (Fig. 1A, B). The cytotoxicity of *E. faecalis* ATCC 700802 was low at a MOI of 1 and moderate at 100. *E. coli* ATCC 25922 and *E. coli* ICVB442 showed low cytotoxicity at 1 and high at 100 with percentage of IPEC-1 cells viability significantly (less than 50%) lower ($P < 0.05$) than the control (Fig. 1B). *E. coli* ICVB443 was non-toxic at 1 but highly toxic at 100.

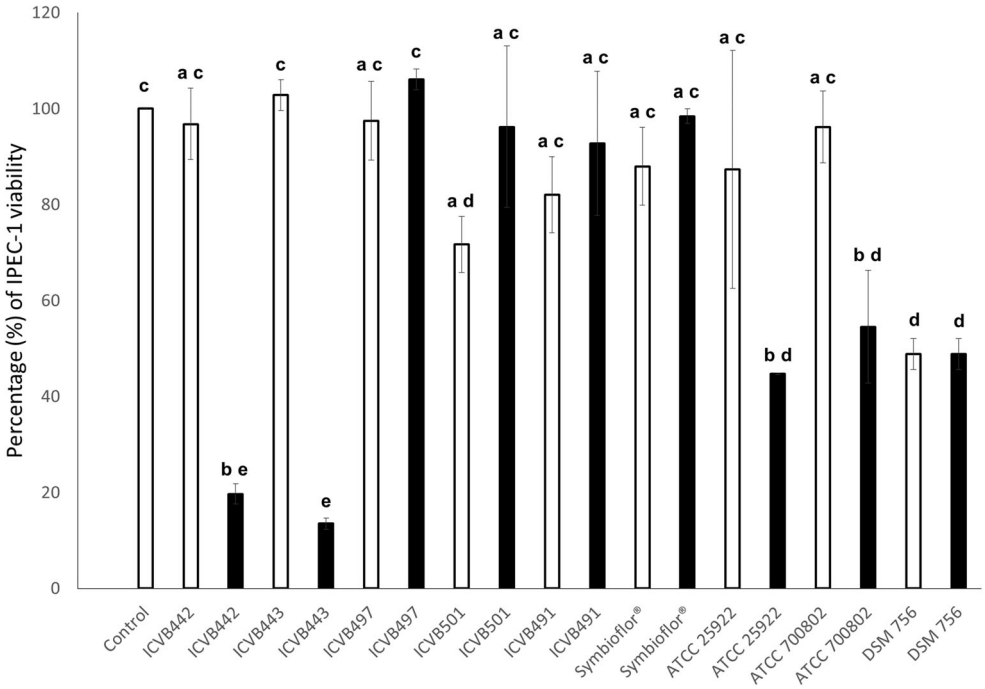

Fig. 1 Viability of IPEC-1 cells, expressed in percentage, in the presence of *E. coli* ICVB442, *E. coli* ICVB443, *E. faecalis* ICVB497, *E. faecalis* ICVB501, *P. pentosaceus* ICVB491, *E. faecalis* Symbioflor® 1, *E. coli* ATCC 25922, *E. faecalis* ATCC 700802, and *C. perfringens* DSM 756 with a 1:1 MOI (multiplicity of infection) bacterial cell/IPEC-1 cell of 1 (□); and MOI of 100 (■). Values are mean ± standard deviation; different letters indicate significant difference ($P < 0.05$)

Compatibility of the Probiotic Candidate Strains

P. pentosaceus ICVB491 culture supernatant inhibited the growth of the four other bacteriocin-producing strains (Fig. 2A). No *E. facealis* or *E. coli* strain reached an OD_{max} of even 0.2 in its presence. Both *E. faecalis* strain culture supernatants had a limited effect on the other strains as no significant difference was noticed compared with the untreated control in most cases. Figure 2B shows that each strain, and particularly *P. pentosaceus*, produced something that slowed the growth rate (μ_{max}) of the other strains significantly ($P < 0.05$). The *P. pentosaceus* ICVB491 supernatant also increased the lag time (T_{lag}) of all strains significantly reaching two- to tenfolds the control (Fig. 2C). Supernatant from *E. faecalis* ICVB497 and ICVB501 culture increased the T_{lag} of other strains except *E. coli* ICVB442, whereas *E. coli* ICVB443 supernatant had only a slight effect on the growth of the other strains.

Tolerance of Gastrointestinal Conditions

Based on hemolysis, biofilm formation, and cytotoxicity, two strains (*E. coli* ICVB442 and *E. faecalis* ICVB49) were excluded from the gastrointestinal simulation experiment. *E. coli* ICVB443, *E. faecalis* ICVB501, and *P. pentosaceus* ICVB491 were retained for survival test in the TIM-1 model. Figure 3a shows the distribution of the skim milk among the compartments during the 5-h digestive cycle. The viable counts are shown in Fig. 3b, c, d. The initial counts of *E. coli* ICVB443, *E. faecalis* ICVB501, and *P. pentosaceus* ICVB491 in the gastric compartment were, respectively, 9.13 ± 0.01, 9.19 ± 0.08, and 9.20 ± 0.10 $\log_{10}$ cfu/mL. However, after remaining stable for 1 h, the *E. coli* count fell to 7.79 $\log_{10}$ cfu/mL while *E. faecalis* dropped to the 5.51 $\log_{10}$ cfu/mL range and *P. pentosaceus* to the 4 $\log_{10}$ cfu/mL range. In the duodenal compartment, *E. coli* and *P. pentosaceus* viable counts decreased by 1 log after 2 h. Viable *E. faecalis* increased by nearly 1 log during the first hour, then dropped to 8 $\log_{10}$ cfu/mL and remained at that level. The viable counts of the 3 strains remained relatively stable during the passage through the jejunum and the ileum until the end of the simulated digestion. Survival of the proposed probiotic bacteria in the TIM-1 was 139% for *E. coli* ICVB443, 32% for *E. faecalis* ICVB501, and 46% for *P. pentosaceus* ICVB491.

Discussion

The use of probiotics to replace antibiotics is now widespread [4, 44, 45]. Prior to filing any claim about the probiotic value of a bacterial strain, its potential pathogenic risk and its tenacity under conditions as close as possible to those normally prevailing in the gastrointestinal tract must be evaluated [3, 8]. Strains that meet all safety, efficacy, and viability criteria can now be used in consortium formulations, which are being proposed as the next generation of probiotics [7].

In the present study, five bacterial strains were evaluated as probiotic candidates: *E. coli* ICVB442 and ICVB443, *E. faecalis* ICVB497 and ICVB501, and *P. pentosaceus* ICVB491. While the latter species is not a surprising choice, the two other might still raise a few brows. All were tested for

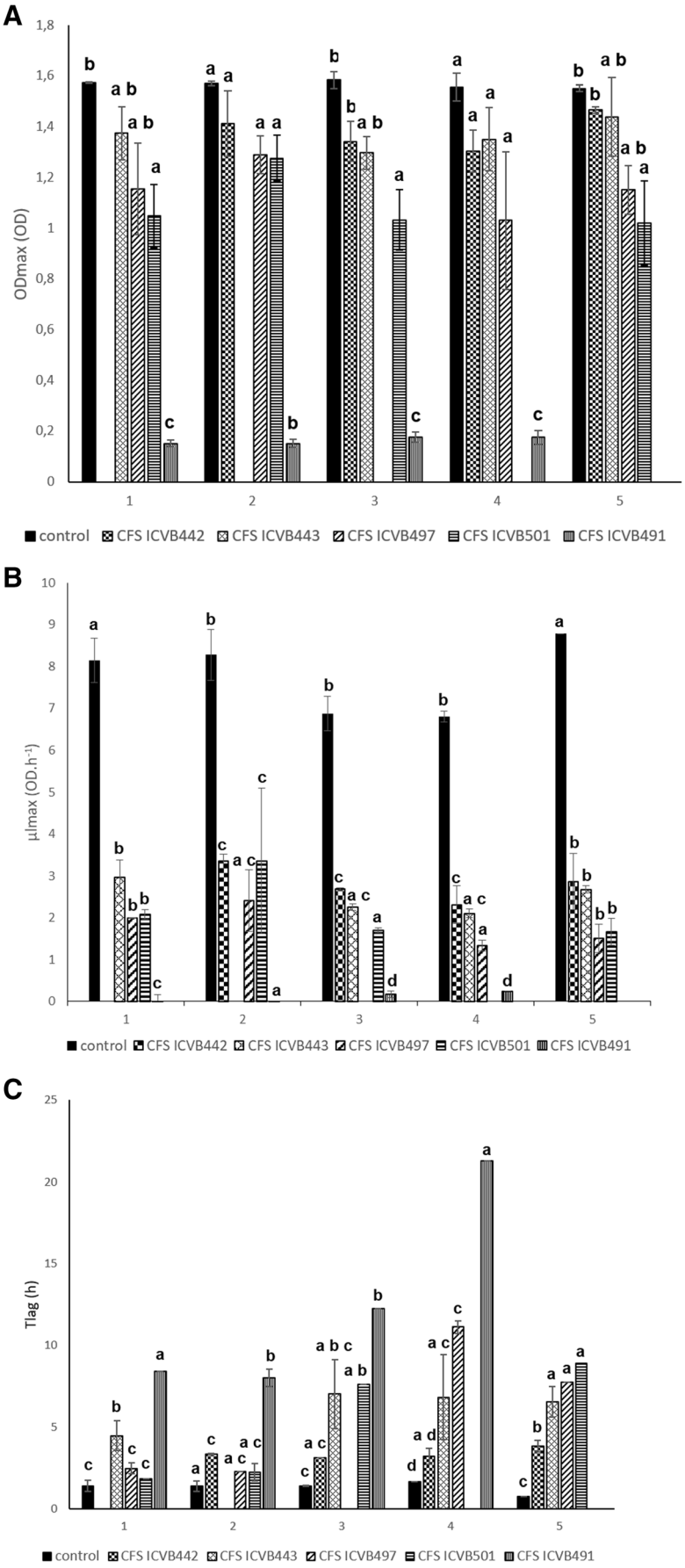

Fig. 2 Effect of culture supernatant of each bacteriocin-producing strain on the growth of the others: on *E. coli* ICVB442 (1) and ICVB443 (2); *E. faecalis* ICVB497 (3) and ICVB501(4); and *P. pentosaceus* ICVB491 (5). Left to right for each strain: control, CFS of *E. coli* ICVB442, of *E. coli* ICVB443, of *E. faecalis* ICVB497, of *E. faecalis* ICVB501, and of *P. pentosaceus* ICVB491. **A** On OD_{max}. **B** On μ_{max}. **C** On T_{lag}. Values are mean ± standard deviation; different letters indicate significant difference ($P < 0.05$)

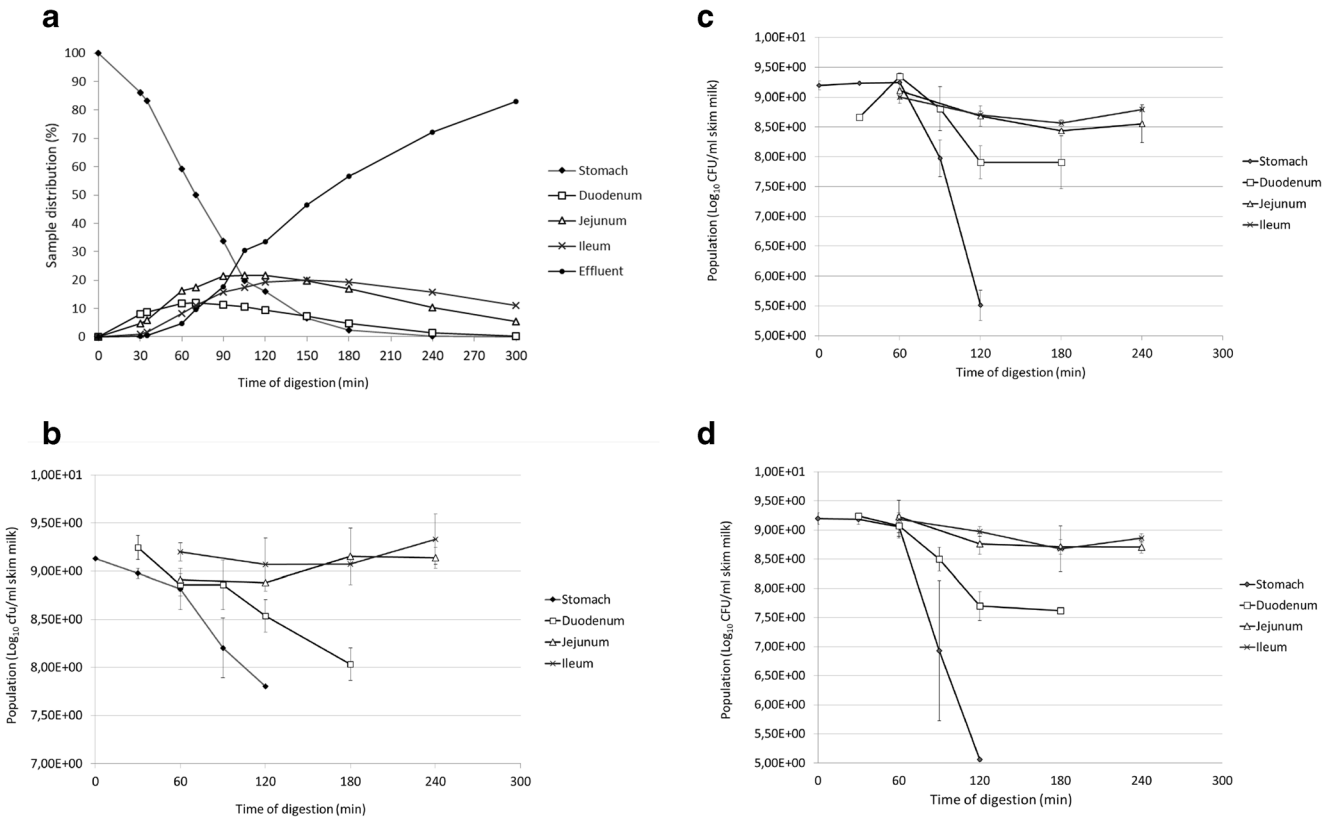

Fig. 3 Distribution of components in the TIM-1 compartments during a 5-h digestion. **a** Carrier medium (skim milk). **b** Viable *E. coli* ICVB443. **c** Viable *E. faecalis* ICVB501. **d** Viable *P. pentosaceus* ICVB491

antibiotic susceptibility, hemolytic and gelatinase activities, mutual compatibility, and cytotoxicity to a porcine intestinal epithelial cell line. None of these five strains exhibited a gelatinase activity, a property considered as a detrimental virulence factor for pathogenic *E. coli* and *E. faecalis* strains. This protease hydrolyse gelatin, casein, and hemoglobin, as well as other active compounds, can compromise the integrity of soft tissues [46, 47].

E. coli ICVB442 and *E. coli* ICVB443 strains were not resistant to the antibiotics tested. However, they were hemolytic and had a cytotoxic effect on IPEC-1 cells, albeit only at high multiplicity of infection (Fig. 1). *E. coli* ICVB443 was more inclined to form biofilm. Both *E. faecalis* ICVB497 and ICVB501 strain formed biofilm, ICVB497 somewhat more. Of note, these promising in vitro results need to be confirmed in vivo as a biofilm formation capability can help a probiotic strain to defy harsh conditions in the gastrointestinal tract, and possibly block colonization of the intestinal mucosa by pathogens [48].

Remarkably, the probiotic grade is attributed for some *Enterococcus* strains based taking into consideration their global safety profile and the absence of virulence factors. Neither *E. faecalis* ICVB497 nor ICVB501 strain displayed any antibiotic resistance, and noticeably both of them were devoid of gelatinase activity. However, *E. faecalis* ICVB497 has a β-hemolytic activity, which practically rules out its possible use as a probiotic. Notwithstanding some controversial aspects attributed to *Enterococcus*, some strains have benefitted of probiotic grade [49]. In fact, they have been tested for human use [50, 51], for example, to limit diarrheas and inflammatory bowel diseases as well as in livestock [2, 52].

Compatibility assays showed that both *E. coli* ICVB442, ICVB443 strains and both *E. faecalis* ICVB497, ICVB501 strains could function in the same probiotic formulation (Fig. 2). However, *P. pentosaceus* ICVB491 inhibited the other strains, likely because of the acidity of its culture supernatant (based on agar diffusion tests, results not shown), but possibly also by producing an inhibitory substance such as a bacteriocin [33].

Survival during gastrointestinal transit, a key factor for selecting new probiotic strains [53, 54], has been tested successfully in static models [53] and dynamic models [54]. The dynamic model designed 25 years ago by Minekus et al. [54] has proven its effectiveness for evaluating survival of beneficial microorganisms [55, 56] and the persistence of bacteriocins [39, 57], drugs [58–60], and other substances [61–63] in human and animal digestive tracts.

Of the three strains deemed worthy of testing in the TIM-1 in this study, *E. coli* ICVB443, which was retained as its non-toxic at 1 MOI, was the most robust, its final viability value reaching 139% of the initial load into the system (Fig. 3),

which is consistent with previous similar studies [64]. The *P. pentosaceus* ICVB491 strain has interestingly revealed comparable features with *P. acidilactici* UL5 tested by Kheadr et al. [39], using a skim milk culture in a TIM-1 model. *Pediococcus* strains usually survive better than other lactic acid bacteria in the TIM-1 model [65].

Finally, at 32%, the survival of *E. faecalis* ICVB501 was the lowest measured in the present study, which has been observed elsewhere for enterococci under such conditions [66, 67].

Conclusion

In summary, *E. coli* (ICVB442 and ICVB443), *E. faecalis* (ICVB497 and ICVB501), and *P. pentosaceus* ICVB491 were tested for their suitability as parts of a consortium-type probiotic formulation. Pathogenicity and cytotoxicity tests including their antibiotic resistance, hemolysis, biofilm forming ability, and their compatibility assays enabled us to select three strains that presented suitable attributes for probiotic use. These strains are *P. pentosaceus* ICVB491, which belongs to species possessing GRAS status, *E. coli* ICVB443, and *E. faecalis* ICVB501 that exhibited worthy features that deserve to be tested under a dynamic model. These strains will be the focus of future research regarding the use of these colicin-producing, enterocin-producing, and pediocin-producing commensal strains, for simultaneous use as a novel consortium probiotic supplement to promote the health of piglets.

Funding Information Mégane Eveno received a Ph.D. scholar fellowship from l'Université de Lille (France) and Université Laval (Québec, Canada) through the Natural Sciences and Engineering Research Council of Canada (NSERC) METABIOLAC Industrial Research Chair awarded to Professor Ismail Fliss and the NSERC Industrial Research Chair on Electro-Membrane processes for the ecoefficiency improvement of bio-food production lines (Grant IRCPJ 492889-15 to Laurent Bazinet). Research at Université de Lille was supported by a CPER/FEDER Alibiotech grant (2016-2021) awarded by the Région des Hauts de France.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Wierup M (2000) The control of microbial diseases in animals: alternatives to the use of antibiotics. Int J Antimicrob Agents 14: 315–319. https://doi.org/10.1016/s0924-8579(00)00143-6
2. Liao SF, Nyachoti M (2017) Using probiotics to improve swine gut health and nutrient utilization. Anim Nutr 3:331–343. https://doi.org/10.1016/j.aninu.2017.06.007
3. Yang F, Hou C, Zeng X, Qiao S (2015) The use of lactic acid bacteria as a probiotic in swine diets. Pathogens 4:34–45. https://doi.org/10.3390/pathogens4010034
4. Bogere P, Choi YJ, Heo J (2019) Probiotics as alternatives to antibiotics in treating post-weaning diarrhoea in pigs: review paper. South African Journal of Animal Science 49:403–416. https://doi.org/10.4314/sajas.v49i3.1
5. FAO/WHO (2002) Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. Guidelines for the Evaluation of Probiotics in Food https://wwwwhoint/foodsafety/fs_management/en/probiotic_guidelinespdf Accessed 30 Apr 2020
6. Baccouri O, Boukerb AM, Farhat LB, Zébré A, Zimmermann K, Domann E, Cambronel M, Barreau M, Maillot O, Rincé I, Muller C, Marzouki MN, Feuilloley M, Abidi F, Connil N (2019) Probiotic potential and safety evaluation of *Enterococcus faecalis* OB14 and OB15, isolated from traditional tunisian testouri cheese and rigouta, using physiological and genomic analysis. Front Microbiol 10:881. https://doi.org/10.3389/fmicb.2019.00881
7. Guo X-H, Kim J-M, Nam H-M, Park SY, Kim JM (2010) Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. Anaerobe 16: 321–326. https://doi.org/10.1016/j.anaerobe.2010.03.006
8. Porto MCW, Kuniyoshi TM, Azevedo POS, Vitolo M, Oliveira RPS (2017) *Pediococcus* spp.: an important genus of lactic acid bacteria and pediocin producers. Biotechnol Adv 35:361–374. https://doi.org/10.1016/j.biotechadv.2017.03.004
9. Sonnenborn U (2016) *Escherichia coli* strain Nissle 1917-from bench to bedside and back: history of a special *Escherichia coli* strain with probiotic properties. FEMS Microbiol Lett 363:https://doi.org/10.1093/femsle/fnw212
10. Barnes AMT, Dale JL, Chen Y, Manias DA, Greenwood Quaintance KE, Karau MK, Kashyap PC, Patel R, Wells CL, Dunny GM (2016) Enterococcus faecalis readily colonizes the entire gastrointestinal tract and forms biofilms in a germ-free mouse model. Virulence 8:282–296. https://doi.org/10.1080/21505594.2016.1208890
11. De Paepe M, Gaboriau-Routhiau V, Rainteau D et al (2011) Trade-off between bile resistance and nutritional competence drives *Escherichia coli* diversification in the mouse gut. PLoS Genet 7: e1002107. https://doi.org/10.1371/journal.pgen.1002107
12. Foster JW (2004) Escherichia coli acid resistance: tales of an amateur acidophile. Nat Rev Microbiol 2:898–907. https://doi.org/10.1038/nrmicro1021
13. Conway T, Cohen PS (2015) Commensal and pathogenic *Escherichia coli* metabolism in the gut. Microbiol Spectr 3. https://doi.org/10.1128/microbiolspec.MBP-0006-2014
14. Macfarlane S, Woodmansey EJ, Macfarlane GT (2005) Colonization of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. Appl Environ Microbiol 71:7483–7492. https://doi.org/10.1128/AEM.71.11.7483-7492.2005
15. Vimont A, Fernandez B, Hammami R, Ababsa A, Daba H, Fliss I (2017) Bacteriocin-producing *Enterococcus faecium* LCW 44: a high potential probiotic candidate from raw camel milk. Front Microbiol 8:865. https://doi.org/10.3389/fmicb.2017.00865
16. Hanchi H, Hammami R, Kourda R, Hamida JB, Fliss I (2014) Bacteriocinogenic properties and *in vitro* probiotic potential of enterococci from Tunisian dairy products. Arch Microbiol 196:331–344. https://doi.org/10.1007/s00203-014-0978-y
17. Ben Braïek O, Smaoui S (2019) *Enterococci*: between emerging pathogens and potential probiotics. Biomed Res Int 2019:5938210–5938213. https://doi.org/10.1155/2019/5938210
18. Gillor O, Kirkup BC, Riley MA (2004) Colicins and microcins: the next generation antimicrobials. Adv Appl Microbiol 54:129–146. https://doi.org/10.1016/S0065-2164(04)54005-4
19. Gratia A, Fredericq P (1946) Diversité des souches antibiotiques de *Bacterium coli* et étendue variable de leur champ d'action. C R Soc Biol (Paris) 140:1032–1033

20. Qiu X-Q, Zhang J, Wang H, Wu GY (2005) A novel engineered peptide, a narrow-spectrum antibiotic, is effective against vancomycin-resistant *Enterococcus faecalis*. Antimicrob Agents Chemother 49:1184–1189. https://doi.org/10.1128/AAC.49.3.1184-1189.2005
21. Setia A, Bhandari SK, House JD et al (2009) Development and *in vitro* evaluation of an *Escherichia coli* probiotic able to inhibit the growth of pathogenic *Escherichia coli* K88. J Anim Sci 87:2005–2012. https://doi.org/10.2527/jas.2008-1400
22. Walker D, Behrens HM, Six A et al (2017) The therapeutic potential of bacteriocins as protein antibiotics. Emerg Top Life Sci 1:65–74. https://doi.org/10.1042/ETLS20160016
23. Cascales E, Buchanan SK, Duché D et al (2007) Colicin biology. Microbiol Mol Biol Rev 71:158–229. https://doi.org/10.1128/MMBR.00036-06
24. Ben Said L, Gaudreau H, Dallaire L, Tessier M, Fliss I (2019) Bioprotective culture: a new generation of food additives for the preservation of food quality and safety. Ind Biotechnol 15:138–147. https://doi.org/10.1089/ind.2019.29175.lbs
25. Henderson JT, Chopko AL, van Wassenaar PD (1992) Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. Arch Biochem Biophys 295:5–12. https://doi.org/10.1016/0003-9861(92)90480-k
26. Papagianni M, Anastasiadou S (2009) Pediocins: the bacteriocins of pediococci. Sources, production, properties and applications. Microb Cell Factories 8:3. https://doi.org/10.1186/1475-2859-8-3
27. Gálvez A, Abriouel H, López RL, Ben Omar N (2007) Bacteriocin-based strategies for food biopreservation. Int J Food Microbiol 120:51–70. https://doi.org/10.1016/j.ijfoodmicro.2007.06.001
28. Franz CMAP, van Belkum MJ, Holzapfel WH, Abriouel H, Gálvez A (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 31:293–310. https://doi.org/10.1111/j.1574-6976.2007.00064.x
29. Drider D, Fimland G, Héchard Y et al (2006) The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70:564–582. https://doi.org/10.1128/MMBR.00016-05
30. Rodríguez JM, Martínez MI, Kok J (2002) Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. Crit Rev Food Sci Nutr 42:91–121. https://doi.org/10.1080/10408690290825475
31. Belguesmia Y, Alard J, Mendil R, Ravallec R, Grangette C, Drider D, Cudennec B (2019) In vitro probiotic properties of selected lactobacilli and multi-strain consortium on immune function, gut barrier strengthening and gut hormone secretion. J Funct Foods 57:382–391. https://doi.org/10.1016/j.jff.2019.04.028
32. McFarland LV (2020) Efficacy of single-strain probiotics versus multi-strain mixtures: systematic review of strain and disease specificity. Digest Dis Sci. https://doi.org/10.1007/s10620-020-06244-z
33. Eveno M, Salouhi A, Belguesmia Y, Bazinet L, Gancel F, Fliss I, Drider D (2020) Biodiversity and phylogenetic relationships of novel bacteriocinogenic strains isolated from animal's droppings at the zoological garden of Lille, France. Probiotics Antimicrob Proteins https://doi.org/10.1007/s12602-020-09657-4
34. de Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x
35. CLSI (2020) M100Ed30 | performance standards for antimicrobial susceptibility testing, 30th Edition. In: Clinical & Laboratory Standards Institute. https://clsi.org/standards/products/microbiology/documents/m100/. Accessed 30 Apr 2020
36. Stepanović S, Cirković I, Ranin L, Svabić-Vlahović M (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 38:428–432. https://doi.org/10.1111/j.1472-765X.2004.01513.x
37. Demers-Mathieu V, St-Gelais D, Audy J, et al (2013) Biocompatibility between new probiotic strains isolated and commercial mesophilic or thermophilic strains. Colloque STELA/Symposium FIL-IDF Canada, at Québec, Canada 13-16 May 2013. https://www.researchgate.net/publication/269333822_BIOCOMPATIBILITY_BETWEEN_NEW_PROBIOTIC_STRAINS_ISOLATED_AND_COMMERCIAL_MESOPHILIC_OR_THERMOPHILIC_STRAINS. Accessed 30 April 2020
38. Schillinger U, Lücke FK (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 55:1901–1906
39. Kheadr E, Zihler A, Dabour N, Lacroix C, le Blay G, Fliss I (2010) Study of the physicochemical and biological stability of pediocin PA-1 in the upper gastrointestinal tract conditions using a dynamic *in vitro* model. J Appl Microbiol 109:54–64. https://doi.org/10.1111/j.1365-2672.2009.04644.x
40. Haakensen M, Vickers DM, Ziola B (2009) Susceptibility of *Pediococcus* isolates to antimicrobial compounds in relation to hop-resistance and beer-spoilage. BMC Microbiol 9:190. https://doi.org/10.1186/1471-2180-9-190
41. Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Müller-Bertling S, Witte W, Goossens H (2007) Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. J Antimicrob Chemother 59:900–912. https://doi.org/10.1093/jac/dkm035
42. Oh S-W, Chen P-C, Kang D-H (2007) Biofilm formation by *Enterobacter sakazakii* grown in artificial broth and infant milk formula on plastic surface. Journal of Rapid Methods & Automation in Microbiology 15:311–319. https://doi.org/10.1111/j.1745-4581.2007.00103.x
43. López-García J, Lehocký M, Humpolíček P, Sáha P (2014) HaCaT keratinocytes response on antimicrobial atelocollagen substrates: extent of cytotoxicity, cell viability and proliferation. J Funct Biomater 5:43–57. https://doi.org/10.3390/jfb5020043
44. Fijan S (2016) Antimicrobial effect of probiotics against common pathogens. In Rao V and Rao LG (ed) Human nutrition and health, IntechOpen. London, UK. https://www.intechopen.com/books/probiotics-and-prebiotics-in-human-nutrition-and-health/antimicrobial-effect-of-probiotics-against-common-pathogens. Accessed 30 April 2020
45. Vieco-Saiz N, Belguesmia Y, Raspoet R, Auclair E, Gancel F, Kempf I, Drider D (2019) Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front Microbiol 10:57. https://doi.org/10.3389/fmicb.2019.00057
46. Hancock LE, Perego M (2004) The *Enterococcus faecalis fsr* two-component system controls biofilm development through production of gelatinase. J Bact 186(17):5629–5639. https://doi.org/10.1128/JB.186.17.5629-5639.2004
47. Shruthi N et al (2012) Phenotypic study of virulence factors in *Escherichia coli* isolated from antenatal cases, catheterized patients, and faecal flora. J Clin Diagn Res 6(10):1699–1703. https://doi.org/10.7860/JCDR/2012/4669.2634
48. Borges S, Teixeira P (2014) Pediococcus pentosaceus SB83 as a potential probiotic incorporated in a liquid system for vaginal delivery. Benef Microbes 5:421–426. https://doi.org/10.3920/BM2013.0084
49. Suvorov A (2020) What is wrong with enterococcal probiotics? Probiotics Antimicrob Proteins 12:1–4. https://doi.org/10.1007/s12602-020-09633-y
50. Franz CMAP, Huch M, Abriouel H, Holzapfel W, Gálvez A (2011) Enterococci as probiotics and their implications in food safety. Int J Food Microbiol 151:125–140. https://doi.org/10.1016/j.ijfoodmicro.2011.08.014
51. Shanahan F (2001) Probiotics in inflamatory bowel disease. Gut 48:609. https://doi.org/10.1136/gut.48.5.609

52. Amer M, Nadeem M, Nazir SUR, Fakhar M, Abid F, Ain QU, Asif E (2018) Probiotics and their use in inflammatory bowel disease. Altern Ther Health Med 24:16–23
53. Marteau P, Minekus M, Havenaar R, Huis in't Veld JH (1997) Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. J Dairy Sci 80: 1031–1037. https://doi.org/10.3168/jds.S0022-0302(97)76027-2
54. Minekus M, Marteau P, Havenaar R, Huis in't Veld JHH (1995) Multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. Alternatives to laboratory animals : ATLA 23(2):197–209
55. Khalf M, Dabour N, Kheadr E, Fliss I (2010) Viability of probiotic bacteria in maple sap products under storage and gastrointestinal conditions. Bioresour Technol 101:7966–7972. https://doi.org/10.1016/j.biortech.2010.05.053
56. Venema K, Verhoeven J, Verbruggen S, Espinosa L, Courau S (2019) Probiotic survival during a multi-layered tablet development as tested in a dynamic, computer-controlled in vitro model of the stomach and small intestine (TIM-1). Lett Appl Microbiol 69:325–332. https://doi.org/10.1111/lam.13211
57. Naimi S, Zirah S, Hammami R, Fernandez B, Rebuffat S, Fliss I (2018) Fate and biological activity of the antimicrobial lasso peptide microcin J25 under gastrointestinal tract conditions. Front Microbiol 9:1764. https://doi.org/10.3389/fmicb.2018.01764
58. Blanquet S, Zeijdner E, Beyssac E, Meunier JP, Denis S, Havenaar R, Alric M (2004) A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions. Pharm Res 21:585–591. https://doi.org/10.1023/b:pham.0000022404.70478.4b
59. Brouwers J, Anneveld B, Goudappel G-J, Duchateau G, Annaert P, Augustijns P, Zeijdner E (2011) Food-dependent disintegration of immediate release fosamprenavir tablets: in vitro evaluation using magnetic resonance imaging and a dynamic gastrointestinal system. Eur J Pharm Biopharm 77:313–319. https://doi.org/10.1016/j.ejpb.2010.10.009
60. Dickinson PA, Abu Rmaileh R, Ashworth L, Barker RA, Burke WM, Patterson CM, Stainforth N, Yasin M (2012) An investigation into the utility of a multi-compartmental, dynamic, system of the upper gastrointestinal tract to support formulation development and establish bioequivalence of poorly soluble drugs. AAPS J 14:196–205. https://doi.org/10.1208/s12248-012-9333-x
61. Chen L, Hébrard G, Beyssac E et al (2010) In vitro study of the release properties of soy-zein protein microspheres with a dynamic artificial digestive system. J Agric Food Chem 58:9861–9867. https://doi.org/10.1021/jf101918w
62. Nimalaratne C, Savard P, Gauthier SF, Schieber A, Wu J (2015) Bioaccessibility and digestive stability of carotenoids in cooked eggs studied using a dynamic in vitro gastrointestinal model. J Agric Food Chem 63:2956–2962. https://doi.org/10.1021/jf505615w
63. Torres-Escribano S, Denis S, Blanquet-Diot S, Calatayud M, Barrios L, Vélez D, Alric M, Montoro R (2011) Comparison of a static and a dynamic *in vitro* model to estimate the bioaccessibility of As, Cd, Pb and Hg from food reference materials *Fucus* sp. (IAEA-140/TM) and Lobster hepatopancreas (TORT-2). Sci Total Environ 409:604–611. https://doi.org/10.1016/j.scitotenv.2010.10.021
64. Gänzle MG, Hertel C, van der Vossen JM, Hammes WP (1999) Effect of bacteriocin-producing lactobacilli on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine. Int J Food Microbiol 48:21–35. https://doi.org/10.1016/s0168-1605(99)00025-2
65. Fernandez B, Hammami R, Savard P, Jean J, Fliss I (2014) Pediococcus acidilactici UL5 and Lactococcus lactis ATCC 11454 are able to survive and express their bacteriocin genes under simulated gastrointestinal conditions. J Appl Microbiol 116:677–688. https://doi.org/10.1111/jam.12391
66. Nueno-Palop C, Narbad A (2011) Probiotic assessment of *Enterococcus faecalis* CP58 isolated from human gut. Int J Food Microbiol 145:390–394. https://doi.org/10.1016/j.ijfoodmicro.2010.12.029
67. Cebrián R, Baños A, Valdivia E, Pérez-Pulido R, Martínez-Bueno M, Maqueda M (2012) Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis* UGRA10, a new AS-48-producer strain. Food Microbiol 30:59–67. https://doi.org/10.1016/j.fm.2011.12.002