

Safety assessment and characterisation of *Ligilactobacillus salivarius* PS21603 as potential feed additive for swine

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Abstract

The present study aimed to characterise *in vitro* properties of the strain *Ligilactobacillus salivarius* PS21603 and evaluate *in vivo* piglets' tolerance for its use as feed additive in swine. The ability of *L. salivarius* PS21603 of inhibiting enteropathogens' growth *in vitro* was evaluated using a co-culture assay. Low pH tolerance, bile tolerance, and resistance to osmotic changes were evaluated. The antibiotic susceptibility profile of *L. salivarius* PS21603 was assessed through broth microdilution method. Whole genome sequencing (WGS) was performed to exclude the presence of antibiotic resistance genes. *L. salivarius* PS21603 showed a high antimicrobial activity *in vitro*, reducing in a mean of 6.16 log cfu/ml eight different enterotoxigenic *Escherichia coli* strains. Moreover, *L. salivarius* PS21603 showed resistance to osmotic changes and was able to survive to a pH above 3.5 during 24 h and up to pH 2 at least during 2 h. In addition, WGS revealed that *L. salivarius* PS21603 did not harbour any resistance genes and thus there was no risk of transmissibility. Finally, an *in vivo* 28-days safety and tolerance study was performed. For that, 384 healthy piglets (28±2 days old and 7.5±1.5 kg, at weaning) were divided into three treatment groups receiving a different dose of *L. salivarius* PS21603: T1, 10⁹ cfu/day; T2, 10⁷ cfu/day; T3, control. Piglet's health status was daily controlled. Individual bodyweight and feed intake per pen were weekly recorded to determine performance parameters. Blood samples were collected in 16 piglets from each treatment group on days 0 and 28 for determination of cytokine profiles. *L. salivarius* PS21603 was safe and well tolerated by piglets, there were no differences in performance nor cytokine profile between treatment groups. In conclusion, *L. salivarius* PS21603 is a potential candidate for a probiotic prevention strategy against pig diarrhoea.

Keywords: feed additive, *Escherichia coli*, diarrhoea, pig, *Ligilactobacillus salivarius*

1. Introduction

The pig gut microbiota is a complex ecosystem harbouring several microorganisms that coexist in a state of balance. Nevertheless, the disturbance of the composition in the population of microorganisms and the loss of its diversity can predispose towards gastrointestinal disorders and infection (Dou *et al.*, 2017; Gresse *et al.*, 2017). Gastrointestinal infections in piglets are a major health concern for the swine industry accounting for nearly 17% of deaths of piglets born in Europe (Lallès *et al.*, 2007). These infections affect piglets during the first months of life and are characterised by diarrheic processes and reduced growth rates caused mainly by enterotoxigenic *Escherichia coli* (ETEC) strains (Luppi *et al.*, 2016).

Antibiotics and the mineral zinc oxide (ZnO) have been widely used to prevent and treat the gastrointestinal infections in piglets (Heo *et al.*, 2013; Jensen *et al.*, 2018; Rhouma *et al.*, 2017; Verstegen and Williams, 2002). However, widespread use of antibiotics has produced rising rates of antibiotic resistant bacteria, which are a major concern for both human and animal health in our times (Tang *et al.*, 2017). Moreover, the European Medicines Agency has concluded that benefits of using ZnO do not outweigh its risk for the environment, and the possible acceleration in the antibiotic resistance genes spread. As a result, many jurisdictions have enforced restrictions to the prophylactic use of antibiotics (Dibner and Richards, 2005), and the use of ZnO as pharmacological additive (1000-3,000 mg/kg) is banned in Europe from January

2022 (Bonetti *et al.*, 2021; EFSA, 2014). In view of these restrictions, safe alternatives to the use of antibiotics and mineral additives are needed to keep the swine industry profitable, while maintaining animal health and welfare.

Probiotics administered as feed additives are a safe and efficient alternative for fighting gut infections in piglets, maintaining the microbiota's state of balance and minimising the need for antibiotics (Gresse *et al.*, 2017). According to the widely recognised FAO/WHO definition (2001), revised by Hill *et al.* (2014), probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Lactobacilli are one of the best known bacteria used as probiotics for disease prevention (Dowarah *et al.*, 2017; König *et al.*, 2021). Certain of these bacteria act protecting the host from pathogenic bacteria by inhibiting their growth and/or restricting their adherence to intestinal epithelium (Fouhse *et al.*, 2016). Some species, such as *Limosilactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus gasseri* or *Limosilactobacillus fermentum* have shown promising effects in diarrhoea prevention in piglets (Dowarah *et al.*, 2017). Nevertheless, each strain could present unique properties, so the characterisation of novel strains is of great interest. Thus, the present study aimed to characterise *in vitro* properties and evaluate *in vivo* piglets' tolerance of the strain *Ligilactobacillus salivarius* PS21603 for its use as feed additive in swine.

2. Material and methods

In vitro characterisation of *Ligilactobacillus salivarius* PS21603

The strain *Ligilactobacillus salivarius* PS21603 from Probisearch' culture collection was included in the present study. This strain was isolated in preliminary assays from the gastrointestinal tract of a healthy wild boar and is deposited at the Spanish Type Culture Collection under the code CECT 9673.

The ability of the strain *L. salivarius* PS21603 of inhibiting enteropathogens' growth was evaluated using a co-culture assay as described previously (Coman *et al.*, 2014). Seven ETEC strains (AI32, AI33, AII12, AIII21, AIII31, B24, and B42) selected from cases of pig diarrhoea (Supplementary Material and Methods S1) and one ETEC collection strain (CECT 5010) were used as indicator strains. Altogether, the indicator strains harboured seven different virulence factors (Stx2e, K88, 987P, LTI, K99, STaP, and STb). *L. salivarius* PS21603 strain was cultured in De Man, Rogosa and Sharpe (MRS) broth and ETEC indicator strains in Brain Heart Infusion (BHI; Oxoid, Basingstoke, UK) broth and were incubated in aerobic conditions at 37 °C for 24 h. Moreover, the ability of the indicator strains of growing in MRS was verified. The co-culture assay was performed by

inoculating 1 ml of the *L. salivarius* PS21603 culture and 1 ml indicator culture (~8 log 10 cfu/ml of each strain) in 20 ml of MRS. Subsequently, the mixture was incubated in aerobic conditions at 37±1 °C for 17 h. Each co-culture was prepared in triplicate. Positive controls were prepared by inoculating the same medium either with the strain *L. salivarius* PS21603 or with the indicator strain on their own. Bacterial counts were performed on MRS agar plates for the strain *L. salivarius* PS21603 and BHI agar plates for the indicator strains after inoculation (initial time) and after 17 h of incubation (final time).

To evaluate the ability of growing in acid environment of *L. salivarius* PS21603, 200 µl of an overnight bacterial culture (37 °C, MRS broth) was inoculated in 20 ml of MRS broth adjusted to different pH, from 2 to 4, using HCl, and incubated in 96-wells microplates. MRS broth was included as control. Every 30 min OD₆₀₀ was registered for 24 h using a Biochrom Anthos Zenyth 200rt (Biochrom, Cambridge, UK). The experiment was performed in triplicate.

Tolerance of *L. salivarius* PS21603 to bile was assessed by examining the survival of this strain in presence of different concentrations of bile. For that, 200 µl of an overnight bacterial culture (37 °C, MRS broth) was inoculated in 20 ml of MRS broth adjusted to different bile concentration, from 0.1 to 0.5% (w/v) using bile extract porcine (Sigma-Aldrich, St. Louis, MO, USA) and incubated in aerobic conditions at 37 °C for 24 h. Bacterial counts of the strain *L. salivarius* PS21603 were performed on MRS agar plates at the initial and final point of the incubation. The experiment was performed in triplicate.

To study the resistance of *L. salivarius* PS21603 to osmotic changes, 20 ml MRS broth tubes containing 1, 2, 3, 4, and 5% (w/v) NaCl (Sigma-Aldrich) were inoculated with 200 µl of an overnight bacterial culture (37 °C, MRS broth) and dispensed in 96-wells microplates. MRS broth without NaCl was included as control. Every 30 min OD₆₀₀ was registered for 24 h using a Biochrom Anthos Zenyth 200rt (Biochrom). The experiment was performed in triplicate.

Sugar fermentation and enzymatic activity profiles of *L. salivarius* PS21603 were determined using API 50 CH and API ZYM kits, respectively, according to manufacturer's instructions (API system, BioMerieux, Marcy-l'Étoile, France).

Antibiotic susceptibility of *Ligilactobacillus salivarius* PS21603

Antibiotic resistance/susceptibility pattern was determined through the evaluation of the minimum inhibitory concentration (MIC) of each antibiotic and its subsequent comparison with the microbial cut-off values established by the European Food Safety Authority (EFSA, 2017a) for

the different bacterial species. The antibiotics included in the assay were: gentamicin (Gm), kanamycin (Km), streptomycin (Sm), tetracycline (Tc), erythromycin (Em), clindamycin (Cl), chloramphenicol (Cm), ampicillin (Am), and vancomycin (Va). To determine the MIC, the VetMIC™ Lact-1 assay (Swedish National Veterinary Institute, Uppsala, Sweden) was performed following the manufacturer's instructions. The assay was performed in triplicate. The MIC value was defined as the lowest concentration at which no growth was visualised.

Whole genome sequencing was performed using MiSeq Illumina system (Illumina, Inc., San Diego, CA, USA) with paired-end sequencing strategies, and *de novo* assembly was performed using SPAdes Genome Assembler software (Bankevich *et al.*, 2012). Antimicrobial resistance gene detection was carried out in the Comprehensive Antibiotic Resistance Database (CARD) and ResFinder database. ResFinder identifies acquired antimicrobial resistance genes in total or partial sequenced isolates of bacteria. The analysis was carried out under criteria: selected % ID threshold 90%; selected minimum length 60%.

***In vivo* safety and tolerance study of *Ligilactobacillus salivarius* PS21603**

An *in vivo* 28-days safety and tolerance study was performed in Topigs × Pietrain breed weaned healthy piglets (28±2 days old and 7.5±1.5 kg, at weaning) in a commercial farm adapted to perform *in vivo* assays. Environmental conditions (temperature and ventilation rate) were automatically controlled during the study period. All feeds and water were provided *ad libitum* to the piglets using one feeder and one drinker per pen placed inside the pen. Basal diets were calculated to be iso-nutritive and meet or exceed the nutrient requirements recommended for piglets. No antibiotics were included in the feed during the study. The animal experiment was conducted according to the protocol approved by the Government of Castilla y León (Spain) in accordance with the protection of animals used for scientific purposes, included in the Directive 2010/63/EU (EC, 2010).

Any piglet showing signs of illness, injury or being in poor condition was excluded from the selection process. At day 0 of the study (weaning), 384 healthy piglets were randomly allocated in pens (4 females and 4 males per pen) and divided in three treatment groups: T1, Basal diet + *L. salivarius* PS21603 10⁹ cfu/kg of feed (10⁹ cfu/day); T2, Basal diet + *L. salivarius* PS21603 10⁷ cfu/kg of feed (10⁷ cfu/day); T3, Basal diet (control group). The health status of piglets was daily evaluated by the farm veterinarian, based on the presence of clinical signs. Individual bodyweight and feed intake per pen were recorded on days 0, 7, 14 and 28 post-weaning. Average daily gain (ADG), average daily

feed intake (ADFI) and feed conversion ratio (FC) were calculated by week.

Blood samples were collected by jugular venepuncture into 10 ml vacutainer tubes on days 0 and 28 post-weaning in 16 piglets from each treatment. Blood samples were immediately centrifuged, and serum was collected and frozen at -18 °C until analysis. Multiplex immunoassay for the quantification of cytokines at day 0 and 28 in serum samples was performed in duplicate using ProcartaPlex Porcine Cytokine & Chemokine Panel 1 (Affymetrix, eBioscience, Vienna, Austria) according to the manufacturer's instructions. This multiplex immunoassay uses Luminex® xMAP technology for the quantification of 9 cytokines related to inflammation: interferon (IFN)-α, IFN-γ, interleukin (IL)-12p40, tumour necrosis factor (TNF)-α, IL-1β, IL8, IL-4, IL-6, and IL-10. The cytokine levels were determined according to standard curves using xPONENT® 3.1 software (Luminex Corporation, 's-Hertogenbosch, the Netherlands).

Statistical analysis

Statistical analyses were performed using SAS v9.0 software (SAS Institute, Inc., Cary, NC, USA) and R software (3.4.0, R-project, <http://www.R-project.org>). Changes between initial and final concentration of each strain included in the co-culture assay were evaluated using paired samples *t*-Test. Regarding the *in vivo* study, the pen was considered the experimental unit for growth performance data and the individual animal was considered the experimental unit for immunological data. Continuous variables were tested for normality using PROC UNIVARIATE. Normality was determined by evaluating data distribution using density histograms and confirming normality with Shapiro-Wilk test. Growth performance data (mean body weight per pen, feed intake per pen and feed conversion ratio) were analysed by ANOVA (PROC GLM). The model included treatment as fixed effect and pen as random effect. Initial body weight (mean body per pen) was included in the growth performance model as a covariate. One-way analysis of variance (ANOVA) or Kruskal-Wallis test were used to analyse haematological data, according to data distribution. For binary variables, the experimental unit was the individual animal. Percentage of mortality and diarrhoea was analysed using the chi-square test (PROC FREQ) as binary variables. Results were considered statistically significant with *P*-value lower than 0.05.

3. Results

Ligilactobacillus salivarius PS21603 inhibition of enterotoxigenic *Escherichia coli* strains

At the beginning of co-culture experiments, initial concentrations of *L. salivarius* PS21603 and *E. coli* strains did not differ (7.90 (0.31) vs 7.77 (0.31) ($P>0.05$). The strain *L. salivarius* PS21603 reduced a mean ($\pm$, standard deviation) of 6.16 (1.83) log cfu/ml the *E. coli* counts ($P<0.0001$) of eight enterotoxigenic strains after 17 h of incubation (Table 1), whereas *E. coli* strains used as control in MRS did not reduce the counts, increasing 0.32 (0.27) log cfu/ml after 17 h of incubation ($P>0.05$).

Functional probiotic properties of *Ligilactobacillus salivarius* PS21603

L. salivarius PS21603 was able to grow at pH above 3.5, albeit at a reduced rate (Figure 1). At a pH of 3, there was almost no growth and below 3 growth was completely inhibited. However, *L. salivarius* PS21603 was able to survive for at least 2 h at the lower pH tested (pH 2) (results not shown). No growth of *L. salivarius* PS21603 in the presence of bile salt was observed after 24 h of incubation. The strain *L. salivarius* PS21603 showed capacity to survive up to 4% (w/v) NaCl (Figure 2).

The carbohydrate fermentation and enzymatic activity profiles are presented in Table 2 and 3, respectively. *L. salivarius* PS21603 was able to ferment 13 of the 49 types of carbohydrates tested and harboured 5 of the 19 enzymes tested. The presence of the carcinogenic enzyme, β -glucuronidase was not detected.

Table 1. Counts reduction of eight *Escherichia coli* strains after 17 h co-culture with *Ligilactobacillus salivarius* PS21603.

Indicator strain	<i>E. coli</i> log cfu/ml ¹		P-value ²
	Initial load	Change	
<i>E. coli</i> CECT 501	8.02 (0.06)	-8.02 (0.06)	<0.0001
<i>E. coli</i> AI32	8.04 (0.08)	-8.04 (0.08)	<0.0001
<i>E. coli</i> AI33	7.77 (0.15)	-5.12 (1.02)	0.013
<i>E. coli</i> AII12	7.70 (0.20)	-7.70 (0.20)	<0.0001
<i>E. coli</i> AIII21	7.43 (0.76)	-3.88 (0.98)	0.021
<i>E. coli</i> AIII31	7.88 (0.11)	-4.32 (0.56)	0.005
<i>E. coli</i> B24	7.65 (0.05)	-5.62 (1.82)	0.033
<i>E. coli</i> B42	7.67 (0.06)	-6.56 (1.89)	0.027

¹ Values expressed as mean ($\pm$ standard deviation).

² Paired samples *t*-test.

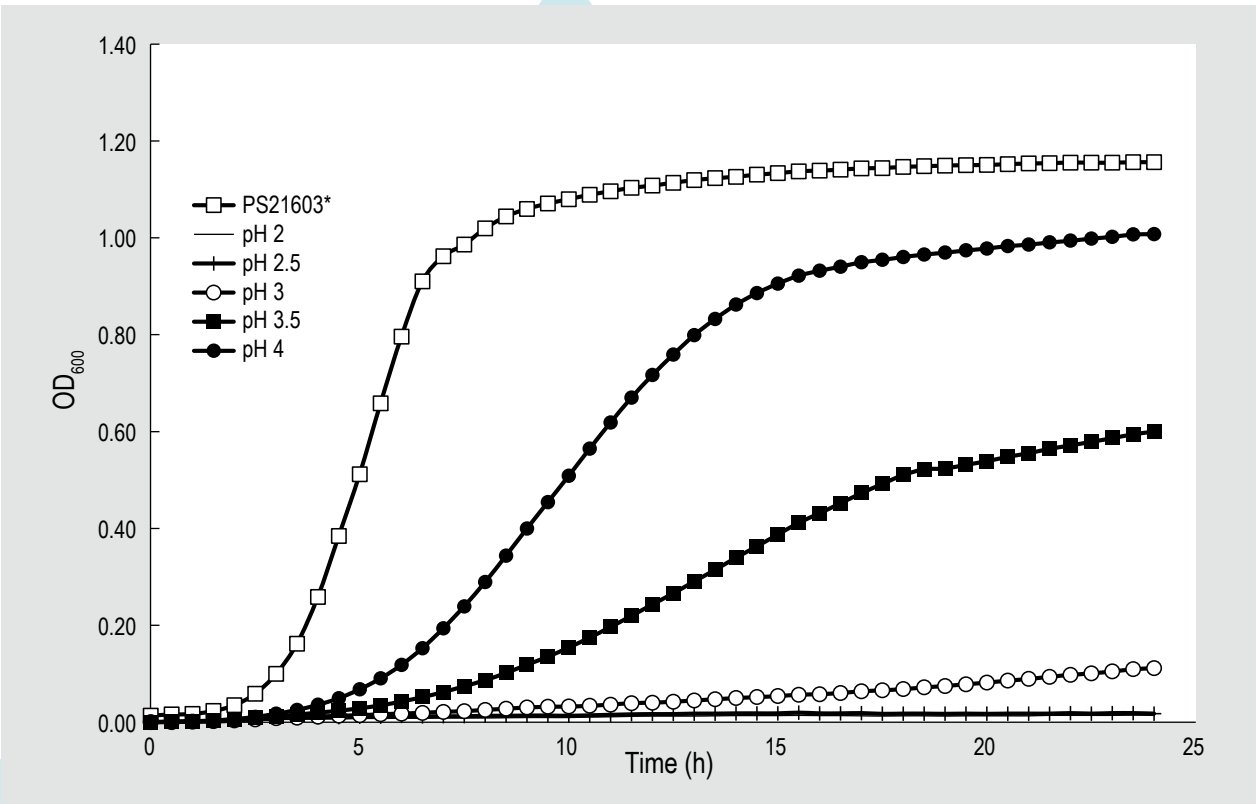


Figure 1. Growth curve of *Ligilactobacillus salivarius* PS21603 at different pH levels. White squares line represents the growth curve of *L. salivarius* PS21603 without pH adjustment of the growth media.

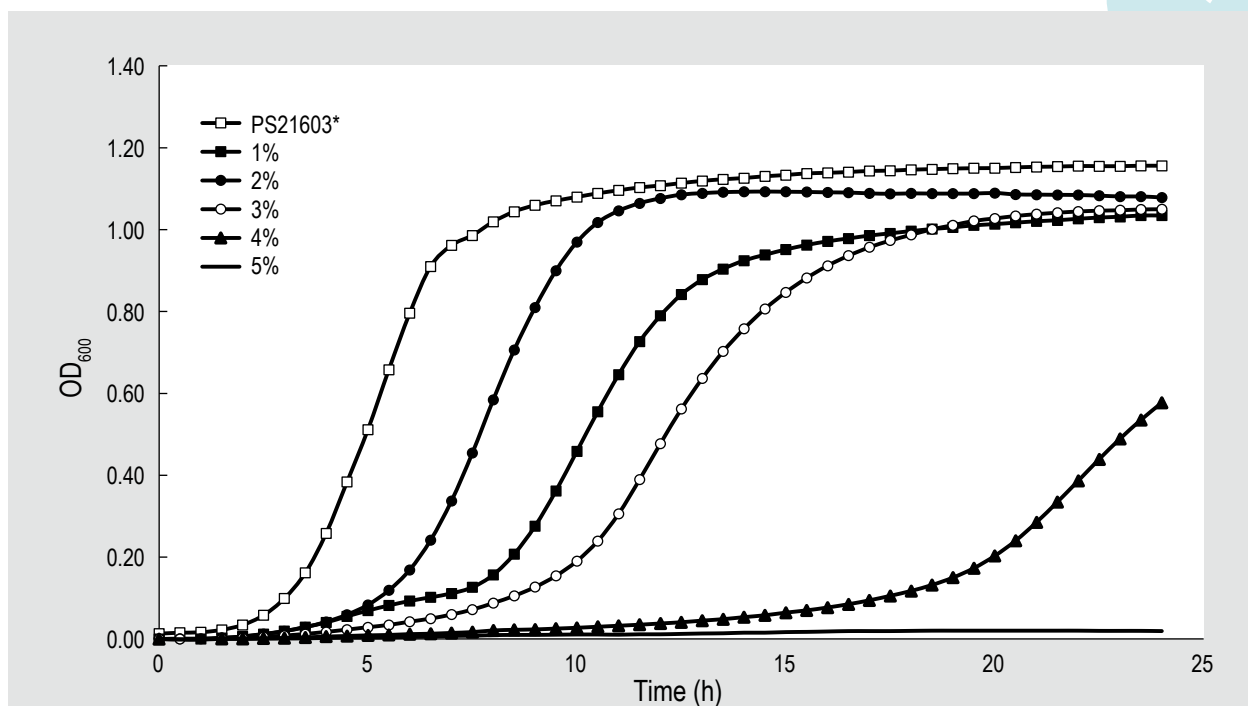


Figure 2. Growth curve of *Ligilactobacillus salivarius* PS21603 at different NaCl concentrations. White squares line represents the growth curve of *L. salivarius* PS21603 without NaCl adjustment of the growth media.

Table 2. Carbohydrate fermentation profile of *Ligilactobacillus salivarius* PS21603 determined using API 50 CH test kit.

Carbohydrate	Utilisation	Carbohydrate	Utilisation	Carbohydrate	Utilisation
Glycerol	–	D-mannitol	+	D-raffinose	–
Erythritol	–	D-sorbitol	–	Amidon	–
D-arabinose	–	Methyl- α -D-mannopyranoside	–	Glycogen	–
L-arabinose	–	Methyl- α -D-glucopyranoside	+	Xylitol	–
D-ribose	–	N-acetylglucosamine	–	Gentiobiose	–
D-xylose	–	Amygdalin	–	D-turanose	–
L-xylose	–	Arbutin	–	D-lyxose	–
D-adonitol	–	Esculin ferric citrate	–	D-tagatose	–
Methyl- β -D-xylopyranoside	+	Salicin	–	D-fucose	–
D-galactose	+	D-cellobiose	+	L-fucose	–
D-glucose	+	D-maltose	+	D-arabitol	–
D-fructose	+	D-lactose	+	L-arabitol	–
D-mannose	–	D-melibiose	+	Potassium gluconate	–
L-sorbose	–	D-saccharose	+	Potassium 2-ketogluconate	–
L-rhamnose	–	D-trehalose	–	Potassium 5-ketogluconate	–
Dulcitol	–	Inulin	–		
Inositol	+	D-melezitose	+		

Table 3. Enzymatic profile of *Ligilactobacillus salivarius* PS21603 determined using API ZYM test kit.

Enzymes	Presence	Enzymes	Presence
Esterase (C-4)	–	Phosphohydrolase	+
Esterase lipase (C-8)	–	α -Galactosidase	–
Lipase (C-14)	–	β -Galactosidase	–
Leucine aminopeptidase	+	β -Glucuronidase	–
Valine aminopeptidase	+	α -Glucosidase	–
Cystine aminopeptidase	+	β -Glucosidase	–
Trypsin	–	N-acetyl- β -glucosaminidase	–
Chymotrypsin	–	α -Mannosidase	–
Alkaline phosphatase	–	α -Fucosidase	–
Acid phosphatase	+		

Antibiotic susceptibility of *Ligilactobacillus salivarius* PS21603

The strain *L. salivarius* PS21603 showed phenotypical sensitivity to gentamicin (MIC = 4 μ g/ml), streptomycin (MIC = 64 μ g/ml), tetracycline (MIC = 0.5 μ g/ml), erythromycin (MIC = 0.12 μ g/ml), clindamycin (MIC = 0.12 μ g/ml), chloramphenicol (MIC = 1 μ g/ml), and ampicillin (MIC = 1 μ g/ml). Only phenotypical resistance to kanamycin was detected (MIC = 128 μ g/ml).

The sequence of the bacterial genome of *L. salivarius* PS21603 and its plasmid of ~186 kb was obtained and analysed for antimicrobial resistance (AMR) gene detection. There were no AMR genes based on CARD, and no acquired AMR genes were found in the genome and neither in the plasmid using ResFinder database to the following antibiotic families: aminoglycoside, beta-lactam, colistin, fluoroquinolone, fosfomycin, fusidic acid, glycopeptide, MLS – macrolide, lincosamide and streptogramin B, nitroimidazole, oxazolidinone, phenicol, and rifampicin.

In vivo safety and tolerance experiment with *Ligilactobacillus salivarius* PS21603

There were no cases of diarrhoea in any of the piglets in the present study. Faeces consistency was only slightly affected in four pens (two in T1, one in T2 and one in T3), in which a softer consistency was registered with no differences ($P>0.05$) between treatment groups. During the study period three piglets died (mortality = 1%), two due to meningitis (one in T1 and one in T2) and a runt piglet (in T1). The mean ADG of all piglets at the end of the intervention was 0.272 kg/d. There was no difference ($P>0.05$) in ADG, ADFI or FC (Supplementary Table S1) between T1, T2, and T3. No differences ($P>0.05$) in the cytokines profile analysed (IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-12, INF- α , INF- γ and TNF- α) were observed between T1, T2 and T3 (Supplementary Table S2).

4. Discussion

Lactic acid bacteria have received considerable attention as biotherapeutic agents due to their benefits for the host's health and its safety presumption, as they are classified in the Quality Presumption for Safety (QPS) list established by EFSA (2019) for their use as feed additives. Since probiotic properties are strain dependent (Gresse *et al.*, 2017), *in vitro* studies are needed to determine the functional and antimicrobial properties of each strain and *in vivo* studies are needed to ensure safety for the target animal (tolerance studies) as well as efficacy to demonstrate compliance with the requirements of Regulation (EC) No 1831/2003 (EC, 2003) to be authorised as feed additives. For that reason, the objectives of the present study were to characterise *in vitro* properties of the QPS strain *L. salivarius* PS21603 and evaluate *in vivo* piglets' tolerance for its use as potential probiotic prevention strategy against pig diarrhoea.

Gastrointestinal infections, as post-weaning diarrhoea, are one of the most common causes of disease and mortality in piglets worldwide (Dubreuil *et al.*, 2016). Stressors, as nutritional, psychosocial factors, or environmental changes, during the first weeks of life of piglets cause gastrointestinal dysbiosis (Bonetti *et al.*, 2021; Fohse *et al.*, 2016), which dramatically increase the risk of colonisation by pathogens, mainly *E. coli*, triggering diarrhoea and reduced growth rate (Gresse *et al.*, 2017). ETEC strains are frequently the primary and unique infectious agents and cause diarrhoea through a group of different enterotoxins (Dubreuil *et al.*, 2016). For that reason, seven *E. coli* strains isolated from pig diarrhoea and harbouring different virulence-associated genes were included in this study to evaluate the capacity of the strain *L. salivarius* PS21603 of inhibiting the enteropathogens' growth *in vitro*. Moreover, the collection strain *E. coli* CECT 501 was included in the *in vitro* antimicrobial assays, so that the selected *E. coli* strains covered seven virulence-associated genes. As described in the 17-h co-culture of the current study, the presence of

L. salivarius PS21603 induced a mean reduction of 6.16 log cfu/ml in eight different ETEC strains. According to these results, other authors have reported antagonistic activities against *E. coli* of some strains of *L. salivarius* (Lähteinen *et al.*, 2010; Yeo *et al.*, 2016). Nevertheless, it is broadly agreed that antimicrobial activity of different lactobacilli differs among species and strains (Lo Verso *et al.*, 2018; Salehizadeh *et al.*, 2020; Tuyarum *et al.*, 2021). For example in a previous study, only 13 out of 31 *L. salivarius* strains isolated from chickens were selected as probiotic candidates based on their antagonism towards the indicator strains, including *E. coli* (Dec *et al.*, 2014). Consequently, the antimicrobial activity of each *L. salivarius* strain needs to be studied in a case-by case basis and the *in vitro* results of this study highlight the utility of the *L. salivarius* PS21603 strain in enhancing the resistance to *E. coli* infection.

Acid and bile tolerance are important attributes of probiotic strains since they have to survive the transit through the gastrointestinal tract. The ability to overcome high concentration of NaCl is a desirable feature for potential commercial strains not only for the NaCl osmolarity of the animal gastrointestinal tract but also for resisting the technological processing (Mohd Adnan and Tan, 2007). The strain *L. salivarius* PS21603 of the present study showed acid and osmotic tolerance for 24 h, however, it did not survive to bile salt. Considering that gastrointestinal transit of piglets was reported to be lower (1.4–6 h) (Davis *et al.*, 2001), it could be expected a survival of *L. salivarius* PS21603, after continued feed administration, under physiological conditions. Other authors have reported tolerance to acidic conditions of different *L. salivarius* strains isolated from pig faeces (Yeo *et al.*, 2016), however, variable degrees of tolerance to acidic condition by lactobacilli strains, including *L. salivarius*, have been reported in other studies (Salehizadeh *et al.*, 2020; Tuyarum *et al.*, 2021). Similarly, variable results regarding tolerance to bile salt and osmo-tolerance have been reported in different lactobacilli strains (Kobierecka *et al.*, 2017). Therefore, an *in vitro* characterisation needs to be performed strain-by-strain to determine its potential use as feed additive. According to the results of the present study, it is expected that *L. salivarius* PS21603 will survive during its passage through the stomach in an *in vivo* model after continued feed administration, maintaining a number of viable cells enough to exert their effect on the pig's intestine although more studies are needed.

Previous authors have reported that sugar metabolism pattern of *L. salivarius* strains differ substantially (Kobierecka *et al.*, 2017), therefore it has to be evaluated in each strain. *L. salivarius* PS21603 had the ability to metabolise several nondigestible sugars such as mannitol, methyl-beta-D-xylopyranoside, cellobiose, and melibiose, which are used as prebiotics. Regarding the enzymatic activity, β -glucuronidase and N-acetyl- β -glucosaminidase have been associated with the induction of toxins, intestinal

diseases, and carcinogenesis in humans (Dabek *et al.*, 2008). It is recommended that bacteria to be selected as probiotic strains should not have these enzymes. *L. salivarius* PS21603 was not found to have neither β -glucuronidase nor N-acetyl- β -glucosaminidase enzymatic activity, which supports the safety of the strain.

Probiotic strains intended for use as viable cells in feed additives should not spread AMR against antibiotics considered relevant to their use in humans and animals, and thereby be safe in the host (EFSA, 2017a). The phenotypic testing of AMR based on MIC, revealed phenotypical resistance of the strain *L. salivarius* PS21603 to kanamycin. Subsequently, a search of the WGS for the presence of known AMR genes was performed to determine if there was a risk of transmissibility of AMR genes. Despite *L. salivarius* PS21603 was phenotypically resistant to kanamycin, an aminoglycoside, no AMR genes were found in this strain based on CARD and ResFinder databases that could be linked to the phenotype. Thus, there is no evidence of transmissibility of kanamycin resistance by *L. salivarius* PS21603, confirming its safety according to the criteria established by EFSA. This is in accordance with previous research, which indicates that lactobacilli, as any other anaerobic microorganisms, tend to be intrinsically resistant to aminoglycosides (EFSA, 2017b). Resistance to these antibiotic group in some lactobacilli strains has been described by several authors (Charteris *et al.*, 1998; Danielsen and Wind, 2003; Gueimonde *et al.*, 2013; Jaimee and Halami, 2016; Korhonen *et al.*, 2007; Zhou *et al.*, 2005), suggesting the existence of an intrinsic resistance. In anaerobic bacteria, the lack of oxidative metabolism and a low membrane potential imply a low rate of electron transport and a decrease in the internalization rate of big antibiotic molecules. These membrane characteristics are responsible for the resistance of some lactobacilli strains to the aminoglycosides (Elkins and Mullis, 2004; Jana and Deb, 2006; Ramirez and Tolmasky, 2010).

Regarding the *in vivo* safety and tolerance experiment, there were no cases of diarrhoea and no differences in weight gain were observed between the different treatment groups. Similarly, there was no systemic effect in the immune system of the piglets as reflected in their cytokine profile. The changes observed corresponded to the maturation of the immune system of the piglets and no changes were associated to the administered strain. Safety assessment for specific *L. salivarius* strains are scarce in the scientific literature (Chaves *et al.*, 2017) but no adverse effects have been reported in previous studies supplementing piglets with *L. salivarius* (Sun *et al.*, 2020; Yang *et al.*, 2020). The findings of the present study support that *L. salivarius* PS21603 is safe and well tolerated by piglets. All animals included in our *in vivo* experiment were exceptional in terms of general health and no gastrointestinal infections were detected. In recent studies, it has been described

that supplementation with *L. salivarius* can lead to an increase of average daily gain, decline of diarrhoea and downregulation of pro-inflammatory mediators in piglets with gastrointestinal infections or lipopolysaccharides-challenged showing the potential of *L. salivarius* species as feed additive for the swine industry. Nevertheless, the extent of probiotic activity is strain dependent and further *in vivo* efficacy trials are warranted to explore the effect of *L. salivarius* PS21603 as modulator of gut microbiota and its use as feed additive for the prevention of diarrhoea caused by ETEC in piglets.

5. Conclusions

Considering the analysis performed, the strain *L. salivarius* PS21603 has a high inhibitory activity of enterotoxigenic *E. coli* strains *in vitro*, has potential functional probiotic properties, and is safe and well tolerated for direct intake in piglets. Therefore, *L. salivarius* PS21603 is a potential promising candidate strain for use as feed additive in the swine industry for the prevention of diarrhoea caused by enterotoxigenic *E. coli* strains. Additional studies are warranted to confirm its *in vivo* efficacy.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2022.0020>.

Table S1. Growth performance parameters (ADG, ADFI, FC) in three treatment groups of piglets during 28 days post-weaning.

Table S2. Blood cytokines profile at day 0 (weaning) and day 28 post-weaning in the three treatment groups.

Materials and methods S1. Characterisation and selection of *Escherichia coli* as indicator strains.

Figure S1. Multiplex PCR for the detection of virulence associated genes of 21 *Escherichia coli* isolates. CECT 501 and CECT 685 were included as positive controls.

Figure S2. RAPD patterns and dendrogram (based on Dice similarity coefficient) of *E. coli* isolates from pig rectal swabs generated with OPL5 primer.

Conflict of interest

Authors Inmaculada Cuevas-Gómez, Javier de Andrés, Nivia Cárdenas, Irene Espinosa-Martos and Esther Jiménez are employees of ProbiSearch, SLU Spain, the funder of this research. Javier de Andrés, Irene Espinosa-Martos and Esther Jiménez are co-authors of the patent of *Ligilactobacillus salivarius* PS21603, the strain tested in this research.

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