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Autochthonous lactic acid bacteria isolated from pig faeces in Thailand show probiotic properties and antibacterial activity against enteric pathogenic bacteria

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ABSTRACT

Lactic acid bacteria (LAB) play an important role in pig health and performance that arises from their beneficial impacts on the balance of gastrointestinal microbes, ability to fight enteric pathogens, and capacity to support the immune system. The aim of this study was to evaluate the functional and safety aspects of five previously isolated autochthonous LAB strains, (*Lactobacillus plantarum* 22F, 25F and 31F, *Pediococcus acidilactici* 72N and *Pediococcus pentosaceus* 77F) from pig faeces as potential probiotics for a pig feed supplement. The functional and safety properties of the strains were assessed by *in vitro* tests. The functional properties tested were their abilities in tolerating low pH values under simulated gastric conditions, their cell surface properties (hydrophobicity, auto- and co-aggregation), antibacterial activity against the common enteric pathogenic bacteria in pigs (such as pathogenic *Escherichia coli, Salmonella* Choleraesuis and *Streptococcus suis*), and diacetyl production. The safety of the strains was analyzed based on the absent of haemolysis on blood and bile salt hydrolase activity. Although all strains demonstrated diacetyl production, good survivability and antibacterial activities, *L. plantarum* 22F and 25F showed the best performance with the strongest antibacterial actions against the indicator pathogens. Of the strains, only *P. pentosaceus* 77F exhibited haemolysis or bile salt hydrolase activity. Furthermore, a principal component analysis revealed that *L. plantarum* 22F possessed superior functional and safety aspects compared to the other four autochthonous strains and to reference strains *L. plantarum* JCM 1149 and *P. acidilactici* DSM 20284. Further *in vivo* studies using oral administration of the strains are justified to assess their effectiveness as feed supplements for pigs.

1. Introduction

FAO/WHO define probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [1]. The largest and best characterized source of probiotics are members of the lactic acid bacteria (LAB). Nevertheless, before they can be termed probiotics, these beneficial microbes are required to be isolated, characterized, identified and assessed for their probiotic properties [2,3]. Functional and safety properties such as ability to survive, colonize, and exert health benefit without potential harm towards the host are amongst the most important criteria in selection of probiotic strains [4]; therefore, detailed *in vitro* studies are required to verify these probiotics properties before *in vivo* application [5].

As a result of the excessive use of antibiotics and development of antimicrobial resistance in the pig industry, the use of probiotics has generated substantial interest and has evolved as an alternative to the use of antibiotics in pig production. Although, there are many commercial probiotic products available for veterinary use, most of them have been inaccurately labeled, especially concerning the identity of the probiotic strain, shelf-life, viable number of microorganisms, characterization of the probiotic strains, and not having been derived from the same host species, as required by probiotic safety criteria [2,6,7]. Additionally, previous studies revealed that host-species specificity is a key for promoting pig health. Feeding probiotics isolated from pigs can result in improvement in growth, and provided protective effects against diarrhoea from the common enteric pathogens: these

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include pathogenic *Escherichia coli* and *Salmonella* spp. in neonatal and weaning pigs. The probiotics act by inhibition of pathogen adherence, reduction of bacterial translocation and production of inhibitory molecules against the pathogens, but the same effects are not observed when feeding probiotics of non-porcine origin [8,9].

Even though there have been many studies published on probiotics, the discovery of new strains with promising probiotic properties is always desirable, as probiotics are strain-specific and possess different beneficial properties and actions [3]. The previously isolated five autochthonous LAB strains from healthy pig faeces in Thailand are potentially suitable as probiotics for pig feed supplements since they are well adapted to the pig's GI tract, and safer than LAB from other sources according to species-specificity criteria [10]. These LAB strains were previously identified by 16S rDNA sequencing analysis, tested for antimicrobial susceptibility using Minimum Inhibitory Concentration (MIC) following European Food Safety Authority (EFSA) criteria [11], and investigated for antiviral activity against porcine epidemic diarrhoea virus (PEDV) [12]. The objective of the current study was to access functional and safety properties of the five autochthonous LAB strains using *in vitro* evaluation, including antibacterial activity against important enteric pathogens of pigs to select the most promising strains as probiotics for further *in vivo* study as pig feed supplements.

2. Materials and methods

2.1. Strains and culture conditions

Lactobacillus plantarum (22F, 25F and 31F), *Pediococcus pentosaceus* 77F, and *Pedicoccus acidilactici* 72N were previously isolated from faeces of antibiotic-free pigs in Thailand. These were evaluated using EFSA criteria for antimicrobial susceptibility by determining MIC, tested for antiviral activity against PEDV, and were proposed as probiotic candidates for pig feed supplement [12]. *L. plantarum* JCM 1149, a commercial type strain with probiotic properties [13] and *P. acidilactici* DSM 20284 were used as reference strains. The strains were stored in MRS broth (Oxoid, Basingstoke, England) supplemented with 15% v/v glycerol at −80 °C. Five strains of enterotoxigenic *Escherichia coli* (ETEC), five strains of enterohaemorrhagic *Escherichia coli* (EHEC), five strains of *Salmonella* Choleraesuis, *Salmonella* Typhimurium ATCC 13311, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 were used for co-aggregation with enteric pathogens and antibacterial activity assays. These strains were periodically reactivated in tryptic soy broth (TSB) (Sigma-Aldrich, Munich, Germany), and stored in TSB supplemented with 15% v/v glycerol at −80 °C.

2.2. Evaluation of functional probiotic properties

2.2.1. Resistance to lysozyme

Overnight LAB cultures were harvested, washed twice in PBS and resuspended in Ringer's solution (8.5 g/l NaCl, 0.4 g/l KCl, 0.34 g/l hydrated $CaCl_2$). Ten µl of bacterial suspensions (10^8 CFU/ml) were inoculated in simulated saliva [sterile electrolyte solution (SES: 0.22 g/l $CaCl_2$, 6.2 g/l NaCl, 2.2 g/l KCl, 1.2 g/l $NaHCO_3$) supplemented with 100 mg/l of lysozyme (Sigma-Aldrich, Sydney, Australia)]. Survival percentage of viable cell counts was calculated after 30 and 120 min of incubation compared with viable cell counts at 0 min. Assays were performed in triplicate [14].

2.2.2. Resistance to 0.4% phenol

Two percent of overnight LAB cultures (10^8 CFU/ml) were inoculated in 10 ml MRS broth (Oxoid, Wesel, Germany) with or without the presence of 0.4% phenol, and were incubated at 37 °C for 24 h. The number of surviving bacteria (CFU/ml) were determined at time 0 and 24 h. Survival rate (%) was calculated as: (viable cell count at 24 h/viable cell counts at 0 h) x 100 [15].

2.2.3. Survival of LAB under low pH and simulated gastric juice

Overnight LAB cultures were harvested, washed and resuspended in sterile saline (0.85% NaCl, w/v). One ml of bacterial suspensions (10^8 CFU/ml) was mixed in either simulated gastric juice [2X SES supplemented with 0.6% (w/v) porcine pepsin (Sigma-Aldrich, Sydney, Australia)] or MRS adjusted to pH 2 or to pH 3 using 1 M HCl. Bacterial suspensions were incubated at 37 °C with agitation and collected for cell counts in MRS agar at 0, 1, 2, and 3 h. Assays were performed in triplicate [14,16]. Resistance percentages were calculated as described by Feng et al. [17].

2.2.4. Survival of LAB in different concentrations of bile

One ml of the LAB suspensions (10^8 CFU/ml) was inoculated into 9 ml of MRS broth supplemented with either 0.3%, 0.5% or 1% (w/v) bile salts (Oxgall, Sigma-Aldrich, Sydney, Australia). The suspensions were further incubated at 37 °C for 24 h under an anaerobic atmosphere. The assay was performed in triplicate trials [5]. Resistance percentage was calculated as above [17].

2.2.5. Cell surface properties

2.2.5.1. Cell surface hydrophobicity. Microbial adhesion to hydrocarbons (MATH) was used to evaluate cell surface hydrophobicity. Overnight LAB cultures were harvested, washed twice with PBS, and resuspended in PBS to optical density (OD) 0.6 at 600 nm. 1 ml of either xylene or toluene was added to 3 ml of bacterial suspensions, and mixed for 90 s by vortex and incubated for 30 min. In two phase systems, the aqueous phase was collected to measure OD at 600 nm. The assay was performed in triplicate. The surface hydrophobicity was calculated as a percentage by: H% = $[(OD_{600\ \text{before mixing}} - OD_{600\ \text{after mixing}})/(OD_{600\ \text{before mixing}})]$ x 100 [18].

2.2.5.2. Auto-aggregation. The overnight LAB cultures were harvested, washed twice, and resuspended in PBS to an OD of 0.6 at 600 nm ($A_{0\ h}$). 3 ml of each bacterial suspension was vortexed for at least 10 s and incubated at 37 °C. 1 ml of the supernatant was measured for absorbance at 600 nm ($A_{\text{final h}}$) at 1 h, 2 h, 3 h, and 4 h. The assay was performed in triplicate. Auto-aggregation (%) was calculated as followed: $(1 - A_{\text{final h}}/A_{0\ h})$ x 100 [19].

2.2.5.3. Co-aggregation. The overnight LAB cultures and pathogenic strains were harvested, washed twice, and resuspended in PBS to an OD of 0.6 at 600 nm. Equal volumes (2 ml) of LAB strain and pathogenic strain were mixed and vortexed for 10 s. After incubation at 37 °C for 4 h, the supernatants were measured for absorbance at 600 nm. The assay was performed in triplicate. The co-aggregation (%) was calculated as followed: 100 x $[(OD_{LAB} + OD_{pathogen}) - 2(OD_{mix})]/(OD_{LAB} + OD_{pathogen})$ [18].

2.2.6. Screening for antibacterial activity

2.2.6.1. Antibacterial activity by cell-free supernatants. An agar well diffusion assay modified from Lin et al. [20] was used to evaluate the antibacterial activity of the cultures. Cell-free supernatants (CFS) of the LAB strains were tested against indicator pathogenic strains including *E. coli* ATCC 25922, *S. aureus* ATCC 25923, five strains of ETEC, five strains of EHEC, five strains of *S.* Choleraesuis, *S.* Typhimurium ATCC 13311, and *S. suis* type II. CFS were obtained by centrifugation (7000 rpm, 5 min) of overnight LAB cultures and filtering this through a 0.22 µm sterile filter (Millipore, Bedford, Massachusetts). Eighty µl of the CFS and neutralized CFS (adjusted to pH 7 with 1N NaOH) were used to fill duplicate 6 mm diameter wells made in Nutrient Agar that had been spread with 100 µl of 10^7 CFU/ml of the indicator pathogen. The plates then were incubated overnight at 37 °C, and the diameters of the inhibition zone were measured and interpreted as shown in Table 3.

2.2.6.2. Antibacterial activity by live cells. A single colony of each LAB strain was cultured in MRS broth to an OD of 0.2 at 600 nm, and 50 µl of