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ORIGINAL ARTICLE

Selection of a candidate probiotic strain of *Pediococcus pentosaceus* from the faecal microbiota of horses by *in vitro* testing and health claims in a mouse model of *Salmonella* infection

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Abstract

Aims: The aim of this study was to verify the suitable use of candidate 'probiotics' selected by *in vitro* tests and the importance of *in vivo* assays to nominate micro-organisms as probiotics and alternative prophylactic treatments for *Salmonella* Typhimurium infection.
Methods and Results: Thirty-three lactic acid bacteria (LAB) isolated from foal's faeces were assessed based on the main desirable functional *in vitro* criteria. Based on these results, *Pediococcus pentosaceus* strain 40 was chosen to evaluate its putative probiotic features in a mouse model of *Salmonella* infection. Daily intragastric doses of *Ped. pentosaceus* 40 for 10 days before and 10 days after *Salmonella* challenge (10^6 CFU of *Salm.* Typhimurium per mouse) led to a significant aggravation in mouse health by increasing weight loss, worsening clinical symptoms and anticipating the time and the number of deaths by *Salmonella*. *Pediococcus pentosaceus* modulated cell-mediated immune responses by up-regulation of the gene expression of the proinflammatory cytokines IFN-γ and TNF-α in the small intestine.
Conclusion: The usual criteria were used for *in vitro* screening of a large number of LAB for desirable probiotic functional properties. However, the best candidate probiotic strain identified, *Ped. pentosaceus* #40, aggravated the experimental disease in mice.
Significance and Impact of the Study: These findings emphasize the need for prophylactic or therapeutic effectiveness to be demonstrated in *in vivo* models to make precise health claims.

Introduction

Probiotics—live micro-organisms that when administered in adequate amounts, confer a health benefit to the host (FAO/WHO 2002; Hill *et al.* 2014)—have been receiving special attention from farmers, who seek alternatives to the use of traditional antibiotics as growth promoters. Indeed, the injudicious prophylactic use of antibiotics has been banned by governmental regulatory agencies—in the European Union by Regulation EC 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (European Union 2003) and in the USA by U.S. FDA CVM GFI #209 and #213 2012 (U.S. Food and Drug Administration 2012)—because of the danger of selection of multidrug-resistant bacteria.

It has already been shown that immunomodulation by several probiotics kept the host immune system primed to respond faster and more effectively to microbial infection (Fagundes *et al.* 2011). Numerous studies have proposed the use of probiotics to improve gut health in the treatment of inflammatory bowel diseases (Heselmans *et al.* 2005; Chaves *et al.* 2011) and in the prevention of antibiotic-induced diarrhoea (Song *et al.* 2011). One of the foremost allegations of these studies regards the ability of probiotics to protect against several infectious agents, especially against enteropathogens (Cremonini *et al.* 2002; Maragkoudakis *et al.* 2010). The protective effects of certain probiotic strains against specific pathogens are undeniable; however, the scientific basis of the means by which probiotics confer protection must be well established. A great number of protective mechanisms have been proposed in protective studies such as stabilization of the gut mucosal barrier (Yan *et al.* 2007), stimulation of mucus secretion by goblet cells (Dogi and Perdigón 2006; Ohland and MacNaughton 2010), competition for nutrients, secretion of antimicrobial substances (bacteriocins) and the modulation of mucosal and systemic immune responses (Lebeer *et al.* 2008; Castillo *et al.* 2012).

Lactic acid bacteria (LAB) have acquired 'generally recognized as safe' status after a long history of use as food supplements, as agents for fermenting natural products, as starter cultures and as probiotics (Franz *et al.* 2010). In most animals, these beneficial gut bacteria interact actively with other micro-organisms belonging to the indigenous microbiota and with transient pathogens, inhibiting the installation of exogenous or uncontrolled multiplication of commensals (Fagundes *et al.* 2011). It has been demonstrated that probiotics can replace conventional growth promoters, such as antibiotics, especially in newborn animals (Jouany and Morgavi 2007; Castillo *et al.* 2012).

New commensal bacteria consisting of defined strains from food, human or animal sources, with adequate evidence of safety and efficacy to the host's health, are so-called probiotics (Hill *et al.* 2014). The selection criteria of new strains for application as probiotic involves *in vitro* testing for adequate gastrointestinal transit, antimicrobial activity against microbial pathogens, susceptibility to most antimicrobial agents, and survival during processing and storage (Silva *et al.* 2013). Subsequently, the promising strains tested *in vitro* undergo subsequent *in vivo* challenge. However, some doubts persist regarding the real value of some of the *in vitro* selection criteria for micro-organisms to be used as health-promoting agents. A critical rethinking of these selection criteria seems necessary to improve the process of development of better probiotics (Morelli 2000).

These criteria include functional characteristics, such as the ability to resist environmental conditions found in the digestive tract (low gastric pH and bile salts) and the ability to antagonize or competitively exclude pathogens via the secretion of antimicrobial substances or competition for nutrients and epithelial adhesion sites. Lactic acid bacteria produce different antimicrobial components, such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins and adhesion inhibitors (Servin 2004; Ripamonti *et al.* 2011). The adhesiveness of LAB can involve passive forces, electrostatic interactions, hydrophobic steric forces, lipoteicoic acids and lectins (Servin 2004). The hydrophobic nature of the outermost surfaces of micro-organisms facilitates the adhesion of bacteria to the host epithelium, thereby conferring competitive advantages during colonization of the gastrointestinal tract (Vinderola and Reinheimer 2003). The antimicrobial susceptibility of intestinal micro-organisms is an important criterion for the selection of probiotic strains, likely because of the possibility of lateral gene transfer to the potentially pathogenic or commensal bacteria that inhabit the gastrointestinal tract (Mathur and Singh 2005; Ripamonti *et al.* 2011).

There are several scientific evidence-based indications for probiotics to prevent or treat gastroenterological disorders, but these indications rely on specific strains and optimal dosages and on their mechanisms of action (Song *et al.* 2011; Tsai *et al.* 2011; Ringel *et al.* 2012; Alvim *et al.* 2016). Unfortunately, many so-called probiotics have not been properly proved in veterinary and medical practice, yet various producers make generic health claims that lead consumers to believe that they are using safe, reliable and efficacious products. However, the generalized prophylactic use of these 'probiotics' by consuming 'functional' foods or dietary supplements could establish a scenario in the host that is favourable to initial infection by and spread of some pathogens. It could occur not only as a result of changes in the 'healthy' microbiota leading to or associated with a disease but also by altering locally and systemically the immunological status of the host (Reid *et al.* 2003).

Probiotic products applied for horses generally comprise live bacterial cultures and they are intended to be used to prevent the undesirable consequences of stress due to failure to ingest the colostrum, weaning, switching of feeds, transportation, adverse weather, disease recurrence, nutritional debilitation and prolonged antibiotic therapy (Montes and Pugh 1993). In this work, LAB strains were isolated from the stool samples of young horses to select new probiotics, and *Pediococcus pentosaceus* strain 40, the most promising *in vitro*-tested strain, was chosen for subsequent *in vivo* challenge in a mouse model of *Salmonella* infection.

Materials and methods

Isolation of LAB and molecular identification

The faeces of two suckling foals and two 1-year-old foals (hay feeding) of the Mangalarga Machador breed were individually processed and used in this study. These animals were not treated with any type of medication. To isolate LAB and to assess their functional properties, the faecal specimens were homogenized using a glass rod in sterile tubes containing 0·9% saline. Serial saline dilutions were plated onto de Man, Rogosa and Sharpe (MRS) agar (Difco, BD Biosciences, Franklin Lakes, NJ) and were incubated for 48 h at 37°C in an anaerobic chamber (Forma Scientific Inc., Marietta, OH) containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. Distinct colonies were selected and processed by Gram staining and bacterial morphology examination. Gram-positive bacteria with a bacillus or coccobacillus morphology were assayed for catalase activity, and those that were negative were considered to be probable LAB. The isolates were regularly cultured by inoculation in MRS broth with 1% v/v of a fresh stationary culture and incubation in anaerobic conditions at 37°C for 18 h. *Lactobacillus* isolates were identified at the species level by 16S-23S rRNA ARDRA (amplified ribosomal DNA restriction analysis), or when necessary, the LAB isolates were identified by 16S rRNA gene sequencing as reported by Sandes *et al.* (2014). Bacterial lineages were clustered by rep-PCR (repetitive extragenic polymorphic-based polymerase chain reaction) fingerprinting, using the $(GTG)_5$ primer (5′-GTG GTG GTG GTG GTG-3′) according to Versalovic *et al.* (1994).

In vitro criteria for the selection of probiotic candidates

Potential probiotic micro-organisms should fulfil several selective criteria, which include an autochthonous origin, gastric acid and intestinal bile tolerance, and the ability to adhere to epithelial tissue, the production of substances to antagonize pathogens and to avoid infection, as well as being non-pathogenic, non-toxigenic and non-invasive, devoid of transmissible antibiotic resistance genes, resistant to technological processes, transiently persistent in the gastrointestinal tract, and able to modulate host immune responses and to influence the microbiota balance positively (Lahteenmaki and Ledeboer 2006). Selective criteria comprising cell surface hydrophobicity, antagonistic activity against bacterial pathogens, microbial susceptibility to antibiotics and artificial gastric juice and bile salt susceptibility were applied according to the methodologies described by Silva *et al.* (2013).

Cell surface hydrophobicity

Cell surface hydrophobicity was assessed by the microbial adhesion to solvents (MATS) approach. The LAB cultures in stationary phase were centrifuged, washed twice in PBS and adjusted to an $OD_{600\ nm}$ of 0·6 with 0·1 mol l^{-1} of KNO_3 pH 6·2 (A_0). Next, xylene was added to the bacterial suspensions, forming a two-phase system. The aqueous phase was removed and the $OD_{600\ nm}$ was measured (A1). The MATS was calculated by the percentage of LAB associated with xylene according to the formula: $[(A_0-A_1/A_0)] \times 100$. The adhesiveness capability of LAB strains indirectly assessed by MATS, instead of using intestinal epithelial cells obtained from animal small intestines, was preferred in this prospective study with many bacterial isolates because it is less time-consuming and less expensive.

Antagonistic activity against bacterial pathogens

Five microlitres of LAB cultures in the stationary phase were spotted onto MRS agar and incubated in anaerobic conditions for 18 h at 37°C. Next, the cells were killed by exposure to chloroform vapour for 20 min. In brain heart infusion (BHI; Acumedia Neogen Corp., Lansing, MI), six pathogenic bacteria (*Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Salmonella enterica* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 27853) were cultured to the stationary phase. These micro-organisms were inoculated in BHI soft agar which was used to overlay the surface of MRS plates containing dead LAB. After incubating at 37°C for 24 h, the LAB antagonistic activity was determined by measuring the growth inhibition zone using a digital pachymeter (Mitutoyo Sul Americana Ltd, São Paulo, Brazil).

Antibiotic microbial susceptibility

An antibiogram was created for each LAB strain by the disc diffusion method. Bacterial suspensions were adjusted to 10^8 viable cells according to the McFarland nephelometric scale. Next, the strains were spread onto MRS agar and antibiotic discs (amikacin 30 μg, ampicillin 45 μg, ceftriaxone 30 μg, chloramphenicol 30 μg, erythromycin 15 μg, oxacillin 1 μg, penicillin G 10 U and vancomycin 30 μg; Oxoid Ltd, Basingstoke, UK) were dispensed onto the surface using an antibiotic disc dispenser. After incubation at 37°C for 24 h, inhibition zones around the discs were measured using a digital pachymeter (Mitutoyo Sul Americana Ltd). The LAB strains were qualitatively classified as resistant, sensitive

or moderately sensitive strains according to the cut-off levels proposed by Charteris *et al.* (1998).

Gastric juice and bile salts susceptibility

Gastric juice susceptibility (GJS) and bile salts susceptibility (BSS) were assessed by taking into account their interference in the growth rate, being performed only for faecal strains. For GJS, LAB strains in the stationary phase were suspended in either PBS or artificial gastric juice (2 g l^{-1} NaCl, pepsin 3·2 g l^{-1}, pH 2·5) and incubated at 37°C for 3 h. The samples were centrifuged and the pellets were suspended in MRS broth. For BSS, LAB strains in the stationary phase were centrifuged and suspended in either MRS broth or MRS broth supplemented with 0·3% ox gall (Oxoid Ltd). The bacterial suspensions were aliquoted into sterile microwell plates and incubated for 12 h in a thermoregulated spectrophotometer at 37°C (Microplate Spectrophotometer System SpectraMax 340; Molecular Devices, Sunnyvale, CA). The $OD_{600\ nm}$ was measured at 30 min intervals. The GJS and BSS were calculated according to the formula: $(1-area_S/area_{CT}) \times 100$, where $area_S$ and $area_{CT}$ are those under growth curve for the stressed strain (artificial gastric juice or bile salts) and the control cells (PBS), respectively.

In vivo trials

Bacterial strains and culture conditions

The *Ped. pentosaceus* isolate 40 (henceforth referred to as Pp40) was chosen after *in vitro* screening for desirable physiological properties. The *Salm. enterica* serovar Typhimurium strain was kindly provided by Fundação Ezequiel Dias (FUNED, Belo Horizonte, MG, Brazil). Due to the dissimilar levels of virulence and/or invasiveness in mice of different strains of *Salm.* Typhimurium, a well-characterized strain was chosen in our laboratory that has already been used in several works published over the years and that infects and kills mice at low doses (Silva *et al.* 2004; Martins *et al.* 2010). The bacteria were stored at −80°C in MRS or BHI broth (Accumedia, Neogen Corp., Lansing, MI) with 15% glycerol, and for reactivation, the cultures were grown at 37°C for 24 h under aerobic conditions.

Mice

This work used 140 (60 for survival evaluation, 80 for relative cytokine-encoding genes mRNA expression) 3- to 5-week-old BALB/c mice of both sexes (Taconic, Germantown, NY). Water and a commercial autoclavable diet (Nuvital, Curitiba, PR, Brazil) were sterilized by steam and were administered *ad libitum.* The animals were housed in plastic mini-isolators in ventilated racks (Alesco, São Paulo, Brazil) and were maintained in animal housing under controlled lighting (12/12 h light/dark cycles) and temperatures (22–24°C), and they were handled according to the standards outlined in the 'Guide for the Care and Use of Laboratory Animals' (National Research Council 2011). The Institutional Ethics Committee on Animal Experimentation (CETEA/UFMG) approved all the experiments under agreement numbers 203/09 and 96/11.

Infection with Salmonella *Typhimurium and survival evaluation*

The assays to evaluate the effects of treatment with *Ped. pentosaceus* Pp40 on morbidity and mortality during an experimental bacterial challenge were performed using two groups of 10 mice each: mice pretreated with the Pp40 strain for 10 days (10^8 CFU daily, 0·1 ml by intragastric gavage) or not (sterile saline) and challenged with *Salm.* Typhimurium (10^6 CFU single dose, 0·1 ml by intragastric gavage) were weighed, and death events were recorded until 28 days after infection.

Liver histopathological analysis

The assay to evaluate the effects of treatment with *Ped. pentosaceus* Pp40 on liver histological analysis during experimental bacterial challenge were performed using four groups: mice pretreated with the Pp40 strain for 10 days (10^8 CFU daily, 0·1 ml by intragastric gavage) or not (sterile saline) and challenged with *Salm.* Typhimurium (10^6 CFU single dose, 0·1 ml by intragastric gavage) or not. All of these mice were euthanized 10 days after *Salmonella* challenge or not (day 21). Tissue samples were excised from the highest lobe of the liver. The samples were fixed, embedded in paraffin wax and 4 μm thick tissue sections were cut and stained with haematoxylin and eosin. A single pathologist in blinded experimental conditions evaluated the overall architecture of the liver. A numerical value was attributed to the changes observed in the liver of each animal that received a score value as described by Mathur *et al.* (2012). The liver tissues were evaluated by the following parameters: 0 (no changes in hepatocytes or tissue architecture, normal cellularity), 1 (presence of discrete inflammatory foci, maximum 1 for 10× field, associated with the lamina propria vessels congestion), 2 (1–2 inflammatory foci per field 10×, associated with discrete hepatocyte changes), 3 (inflammatory foci in number of 3 or more per field 10× with moderate degenerative changes, such as hepatocyte vacuolation), 4 (foci inflammatory infiltrate coalesced associated with severe degenerative changes of hepatocytes; intense intracellular vacuolization). The results were expressed as the median and standard deviation of the scores.

Relative expression of the mRNA of cytokine-encoding genes

At 1, 4, 7 and 10 days after ingestion of *Ped. pentosaceus* Pp40 ($n = 10$) and the untreated controls ($n = 10$), mice

were anaesthetized and euthanized by cervical dislocation. Fragments measuring 1–2 cm in length were obtained from sections of the small intestines (proximal, middle and distal) of these animals. The intestinal content was removed and the fragments were stored in RNAlater (Ambion, Austin, TX) and frozen at −20°C for subsequent extraction of total RNA. The total RNA was isolated using Trizol (Life Technologies Corp., Grand Island, NY) according to the manufacturer's instructions. Genomic DNA was removed using Turbo DNase I, and reverse transcription was performed using high capacity cDNA Reverse Transcription, both according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Relative quantification of the mRNA levels of genes encoding the cytokines IFN-γ (forward primer: 5′-TCAAGTGGCATA GATGTGGAAGAA-3′ and reverse primer: 5′-TGGCT CTGCAGGATTTTCATG-3′), TNF-α (forward primer: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ and reverse primer: 5′-TGGGAGTAGACAAGGTACAACCC-3′), IL-6 (forward primer: 5′-GAGGATACCACTCCCAACAGAC C-3′ and reverse primer: 5′-AAGTGCATCATCGTTGTT-CATACA-3′), IL-10 (forward primer: 5′-GGTTGCCAA GCCTTATCGGA-3′ and reverse primer: 5′-ACCTGCTCC ACTGCCTTGCT-3′) and TGF-β (forward primer: 5′-TG ACGTCACTGGAGTTGTACGG-3′ and reverse primer: 5′-G GTTCATGTCATGGATGGTGC-3′) were performed, accord ing to Steinberg *et al.* (2014). Gene-specific primers for references genes *Actb* (forward primer: 5′-AGAGGGAAAT CGTGCGTGAC-3′ and reverse primer: 5′-CAATAGTGATG ACCTGGCCGT-3′) and *Gapdh* (forward primer: 5′-TCAC CACCATGGAGAAGGC-3′ and reverse primer: 5′-GCTAAG CAGTTGGTGGTGCA-3′) (Sigma-Aldrich, St Louis, MO) were used to normalize the expression data according to the method of Giulietti *et al.* (2001). The expression levels in the noninfected control group were used as the calibration data, and the relative level of mRNA (RLmRNA) for each cytokine was obtained by the method of relative derived quantification, described by Hellemans *et al.* (2007). The results are expressed graphically using the means and standard deviations of the RLmRNA for each cytokine, normalized against the reference gene expression level.

Statistical analysis

The Shapiro-Wilk test was the normality statistic used to determine the data distribution. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer test or two-way ANOVA followed by Bonferroni's post-test was used for the parametric samples, and the Kruskal-Wallis test followed by Dunn's test was used to compare the nonparametric samples. The long-rank test was performed for the survival evaluation test. Data were considered significantly different at $P < 0{\cdot}05$.

Results

Thirty-three LAB isolates were obtained from the foal faecal samples, and they were confirmed as Gram-positive, non-sporulating, catalase-negative with a diverse range of sizes. Molecular characterization of these isolates at the species level conducted by PCR-ARDRA could identify two *Lactobacillus* species (*Lactobacillus reuteri* and *Lactobacillus crispatus*) and one *Weissella* species (*Weissella confusa*). The other species (*Ped. pentosaceus*, *Lactobacillus equi*, *Enterococcus casseliflavus*) were identified by 16S rRNA sequencing analysis. The LAB species were identified, in the following descending order of abundance: *W. confusa* (64%), *Ped. pentosaceus* (9%), *E. casselifavus* (9%), *Lact. reuteri* (6%), *Lact. crispatus* (6%) and *Lact. equi* (6%) (Table 1, Fig. 1). *Weissella confusa* was the most frequently isolated species in the equine faeces. Eighteen strains were found by GTG_5 PCR profiling (Fig. 2).

In vitro assessment of the functional properties of the isolates obtained in this work was performed based on the following criteria: artificial gastric juice and bile salt tolerance, cell surface hydrophobicity, antagonistic activity against pathogens and antibiotic resistance patterns. Table 1 summarizes the characteristics of these isolates. Almost all of the isolates (95%) were highly resistant to an acidic pH, with little (less than 10%) or no decrease in the viable cell numbers after 3 h of incubation at a pH of 2·5, and the remaining isolate was found to be tolerant to an acidic pH (Table 1). In contrast, the sensitivity to bile salts varied markedly between strains of the different species (Table 1). Five of the 18 strains showed little growth inhibition in MRS broth supplemented with 0·3% ox gall (<40% inhibition), and they were considered resistant. Another five strains were moderately tolerant to bile salts (40% >inhibition ≤60%), whereas the remaining nine strains were very susceptible (>60% inhibition).

The MATS method was used to evaluate the hydrophobic/hydrophilic cell surface properties of the LAB isolates and for comparison with other probiotic bacteria. Bacterial adhesion to xylene at a high ionic strength of 0·1 mol l^{-1} (pH 6·2) reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent. Our results indicated that only three LAB isolates had highly hydrophobic surfaces (≥70% adhesion), 13 strains were considered moderately hydrophobic (40% >adhesion<60%), and the remaining three strains had hydrophilic surfaces (<35% adhesion) (Table 1).

The inhibitory capacity against pathogens was evaluated using *in vitro* antagonism tests. The results showed diverse antagonistic activity against the six bacterial pathogens tested (*Salm. enterica* serovar Typhimurium, *E. coli*, *Ps. aeruginosa*, *Staph. aureus*, *Ent. faecalis* and *L.*

Table 1 Physiological properties of lactic acid bacterial strains isolated from foals

	Physiological features†			Antagonism‡						Antibiogram§							
LAB strain*	AGJ (%)	BS (%)	MATS (%)	Se	Ec	Pa	Sa	Ef	Lm	Ac	Ap	Er	Ct	Ch	Ox	Pe	Va
Pediococcus pentosaceus 40	0	39·6	56·5	+	+	+	+	+	+	R	S	S	S	S	R	S	R
Ped. pentosaceus 26	4·7	35	13·4	+	+	+	+	+	+	R	S	S	S	S	R	S	R
Ped. pentosaceus 27	0	25	35·1	+	+	+	+	+	+	R	S	S	M	S	R	S	R
Weissella confusa 1	2·1	56·6	52·3	+	+	+	+	+	+	R	S	S	S	S	R	S	R
W. confusa 2	5·7	60·5	49·4	–	–	–	–	–	–	R	S	S	S	S	R	S	R
W. confusa 3	3·5	70·6	53·5	+	+	+	+	–	+	R	S	S	S	S	R	S	R
W. confusa 4	6·7	74·4	17·1	–	–	–	–	–	+	M	S	S	S	S	R	S	R
W. confusa 9	0	68·7	51·2	–	–	–	–	–	–	M	S	S	S	S	R	S	R
W. confusa 17	11·1	72·6	55·2	+	+	+	+	–	+	R	S	S	S	S	R	S	R
W. confusa 19	4·2	59	53·7	+	+	+	+	–	+	R	S	S	S	S	R	S	R
Enterococcus casseliflavus 10	5	57	40·6	–	–	–	–	–	+	R	S	S	S	S	R	S	R
Ent. casseliflavus 14	1·2	54·9	54·8	–	–	–	–	–	+	R	S	S	S	S	R	S	R
Lactobacillus equi 28	3·2	93·9	43·4	+	+	+	+	+	+	R	M	S	S	S	R	S	R
Lact. equi 33	4·4	99·8	71·4	–	–	–	–	–	–	R	S	S	S	S	R	S	R
Lactobacillus crispatus 31	0	93·6	93·3	–	–	–	–	–	–	R	S	S	S	S	R	M	R
Lact. crispatus 44	0	97·5	93·6	–	–	–	–	–	–	R	S	S	S	S	R	S	R
Lactobacillus reuteri 45	2·5	22·4	59·9	–	–	–	–	–	–	R	S	S	S	S	R	S	R
Lact. reuteri 46	2·5	16·3	56·2	–	–	–	–	–	–	R	S	S	S	S	R	S	R

*Lactobacilli typed by 16S-23S rRNA ARDRA; *L. plantarum/L. paraplantarum* differentiated by multiplex PCR of the recA gene; *Weissella* isolates identified by DNA sequencing of the 16S rRNA gene.

†Physiological features: AGJ, artificial gastric juice inhibition; BS, bile salt inhibition; MATS, surface hydrophobicity (adhesion to xylene); +, presence of substance; –, absence of substance.

‡Microbial antagonisms: Ef, *Enterococcus faecalis* ATCC 19433; Lm, *Listeria monocytogenes* ATCC 15313; Sa, *Staphylococcus aureus* ATCC 29213; Pa, *Pseudomonas aeruginosa* ATCC 25853; Ec, *Escherichia coli* ATCC 25922; Se, *Salmonella enterica* serovar Typhimurium ATCC 14028; +, inhibition haloes ≥15 mm; –, inhibition haloes <15 mm.

§Microbial susceptibilities to antibiotics: Ac, amikacin (30 μg); Ap, ampicillin (45 μg); Er, erythromycin (15 μg); Ct, ceftriaxone (30 μg); Ch, chloramphenicol (30 μl); Ox, oxacillin (1 μg); Pe, penicillin G (10 U); Va, vancomycin (30 μg); R, resistant; M, moderately sensitive; S, sensitive; cut-off levels proposed by Charteris *et al.* (1998).

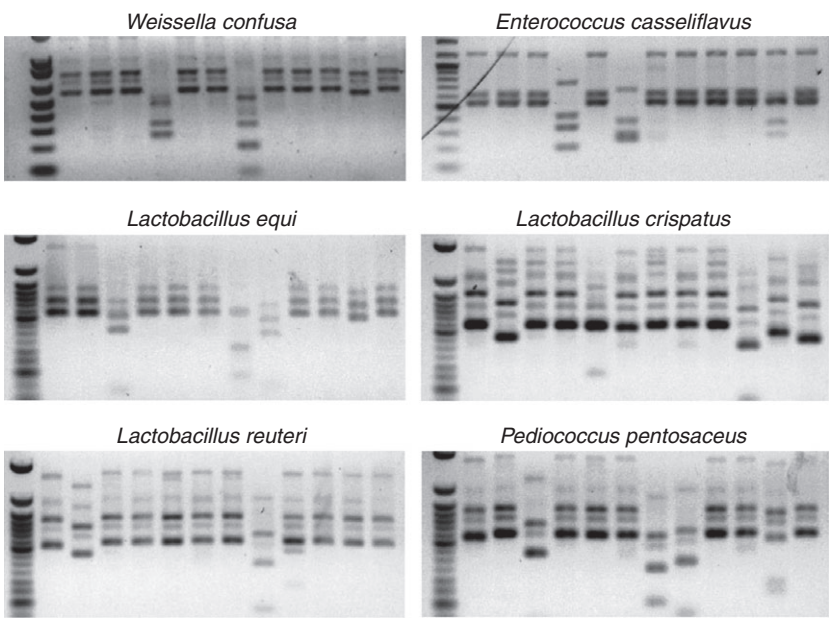

Figure 1 Restriction digestion profile of 16-23S rRNA intergenic transcribed sequences ARDRA to species identification. *Weissella confusa*, *Enterococcus casseliflavus*, *Pediococcus pentosaceus* and *Lactobacillus equi* typing was validated by DNA sequencing of the 16S rRNA gene. Lane 1, 100 bp molecular weight ladder (Invitrogen, Carlsbad, CA, USA), lanes 2–13 *Sph*I, *Nco*I, *Nhe*I, *Ssp*I, *Sfu*I, *Eco*RV, *Dra*I, *Vsp*I, *Hinc*II, *Eco*RI, *Avr*II and *Hind*III, respectively.

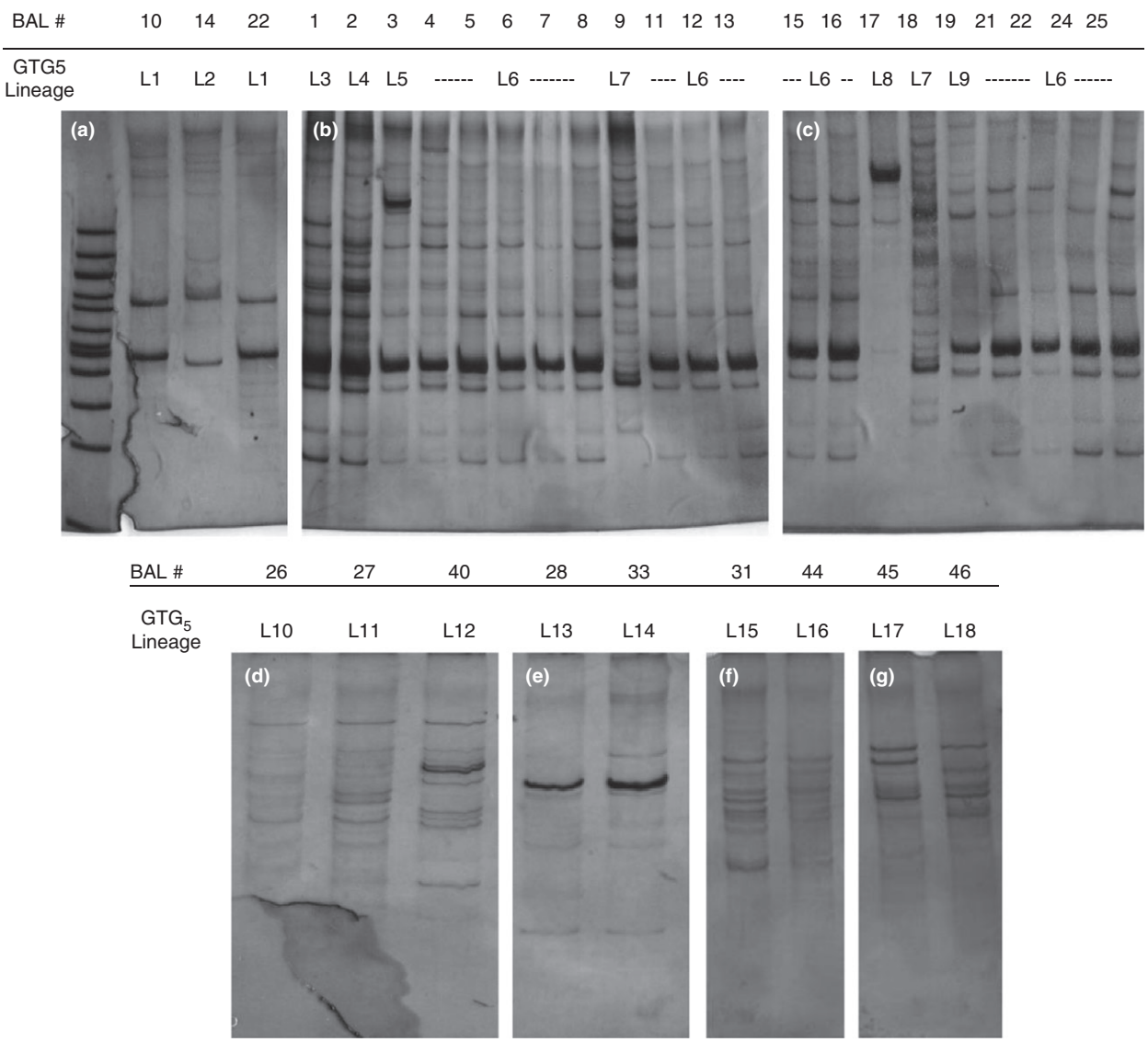

Figure 2 PCR GTG_5 fingerprinting of lactic acid bacteria isolated from young foals in 8% polyacrylamide gel silver stained. (a) *Enterococcus casseliflavus* isolates identified by their numbers and GTG_5 patterns. (b, c) *Weissella confusa* isolates. (d) *Pediococcus pentosaceus* isolates. (e) *Lactobacillus equi* isolates. (f) *Lactobacillus crispatus* isolates. (g) *Lactobacillus reuteri* isolates. First lane, panel (a) corresponds to the 100 bp molecular weight ladder (Invitrogen). Each isolated micro-organism (BAL #) was grouped in lineages (L-1 to L-18).

monocytogenes). The isolates from horse faeces that showed antagonism to all of the pathogens were *W. confusa* strain 1, *Ped. pentosaceus* strains 26, 27 and 40, and *Lact. equi* strain 28 (Table 1). In this study 45% of the LAB isolates showed antagonistic activity towards the *Salm. enterica* serovar Typhimurium.

The LAB isolates were evaluated for their resistance to some antibiotics according to the cut-off values proposed by Charteris *et al.* (1998), with the strains considered resistant if the inhibition zone diameters were equal to or smaller than 19 mm for oxacillin, ampicillin and penicillin G, 14 mm for amikacin, vancomycin and tetracycline, and 13 mm for ceftriaxone, chloramphenicol and erythromycin. All the isolates exhibited the same antimicrobial susceptibility pattern of resistance to vancomycin, oxacillin and amikacin but sensitive to erythromycin, penicillin, ceftriaxone, ampicillin, tetracycline and chloramphenicol (Table 1).

By analysing their functional characteristics, only *Ped. pentosaceus* strain 40 showed no inhibition by an acidic pH, low inhibition by bile salts, a hydrophobic cell surface, strong antagonism against bacterial Gram-negative and Gram-positive pathogens and antimicrobial resistance that was intrinsically encoded only (vancomycin, oxacillin

and amikacin) not associated with mobile genetic elements (Table 1). Thus, *Ped. pentosaceus* strain 40 was selected to be tested *in vivo* in a mouse model of *Salmonella* infection. A higher survival rate of *Salm.* Typhimurium-infected mice was observed in control animals compared with the Pp40-pretreated animals on postchallenge day 12 (50% and 10% survival, respectively, $n = 10$ mice per group), as well as a delay of 1 day to the beginning of animal deaths (Fig. 3). This finding was replicated in three independent experiments. We also tested two other putative strains, *W. confusa* 1 and *Lact. equi* 33, and the same survival rates were observed (data not shown).

Concerning the safety of *Ped. pentosaceus* strain 40, we performed a liver histopathological score analysis and it was observed that the groups of mice that just received these bacteria by gavage did not show a significant difference to the control group of mice, that just received saline (0·22 ± 0·44 *Ped. pentosaceus* 40 group; 0 ± 0, untreated group). Otherwise, challenged mice groups treated with *Ped. pentosaceus* 40 and not treated showed a significant increase in histopathological scores values when compared with the other non-challenged groups (3·77 ± 0·44, *Ped. pentosaceus* 40 group; 3·66 ± 0·5 untreated group).

To investigate further whether *Ped. pentosaceus* 40 could induce cytokine expression *in vivo*, the BALB/c mice were administered bacteria intragastrically for 1, 4, 7 and 10 days. The relative quantification of mRNA levels for TNF-α, IFN-γ, TGF-β, IL-10 and IL-6 in the intestinal tracts of mice treated or not with *Ped. pentosaceus* Pp40 are shown in Fig. 4. Treatment with *Ped. pentosaceus* revealed an inflammatory profile, inducing a significant increase in the production of IFN-γ throughout the experiment and of TNF-α at day 10 ($P < 0·05$). For the IL-6, IL-10 and TGF-β cytokine analyses, there were no significant differences between the experimental groups (Fig. 4).

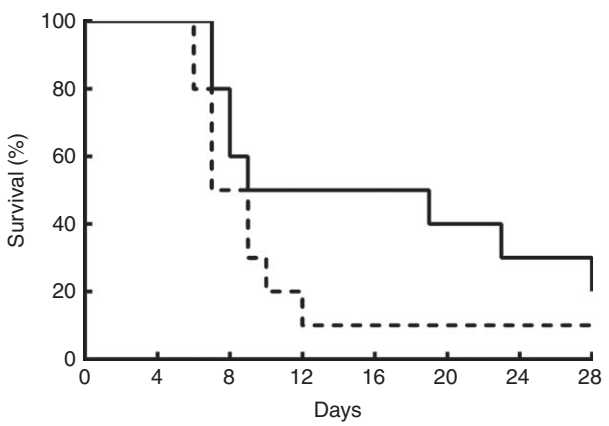

Figure 3 Survival curve of mice non-treated (—— control) or treated (- - - - Pp40 pretreated) with *Pediococcus pentosaceus* 40 by 10 consecutive days before *Salmonella*-challenging and during the course of infection.

Discussion

Amplified ribosomal DNA restriction analysis is an efficient method for species-level identification among *Lactobacillus* species (Stoyancheva *et al.* 2006). Sandes *et al.* (2014), based on the presence of polymorphisms in the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes, showed that 29 of 45 *Lactobacillus* species (64%) displayed unique restriction patterns by this method and therefore could be precisely identified. ITS amplification could establish three groups of LAB genera: *Streptococcus*/*Lactococcus* with one amplicon, *Enterococcus* with two amplicons of different sizes and *Lactobacillus*/*Weissella*/*Pediococcus* with three amplicons of different sizes. In this study, ARDRA was used to identify LAB isolated from the faeces of young foals. Of the 33 LAB isolates, 30 had three amplicons and belonged to the *Lactobacillus*/*Weissella*/*Pediococcus* group, whereas three had two amplicons and belonged to the *Enterococcus* group. *Weissella confusa* was the most prevalent species, accounting for more than 50% of LAB isolates. This result agreed with the findings of Endo *et al.* (2009), in which *W. confusa* was one of the most prevalent pathogens in horse faeces. Additionally, *Lact. equi*, *Lact. reuteri* and *Lact. crispatus* have previously been detected in the faeces of horses by other authors (Morotomi *et al.* 2002; Endo *et al.* 2007, 2009). The species *Lact. equi,* according to the literature to date, is considered exclusive to equine species (Morotomi *et al.* 2002). Among the genus *Pediococcus*, only *Pediococcus denticolens* was previously isolated from the faeces of horses (Endo *et al.* 2009). *Pediococcus pentosaceus* has not yet been isolated from horse faeces. Species of the genus *Enterococcus* from the faeces of horses were described by Niederhausern *et al.* (2007), including strains of *Ent. casseliflavus* like those found in this study.

The possibility that bacterial isolates from the same animal were clonally related was verified by genomic fingerprinting using repetitive element sequence-based polymerase chain reaction (rep-PCR) with the GTG_5 primer. This analysis employs oligonucleotide primers that target repetitive DNA elements distributed throughout the bacterial genome (Versalovic *et al.* 1994). These elements contain conserved regions that allow the primers to hybridize to the target sequences under more stringent conditions (Stephenson *et al.* 2009; Markiewicz *et al.* 2010; Lee *et al.* 2012). The rep-PCR fingerprinting technique using $(GTG)_5$ PCR is a rapid, easy-to-perform and reproducible tool for the differentiation of a wide range of lactobacilli at the species, subspecies and potentially

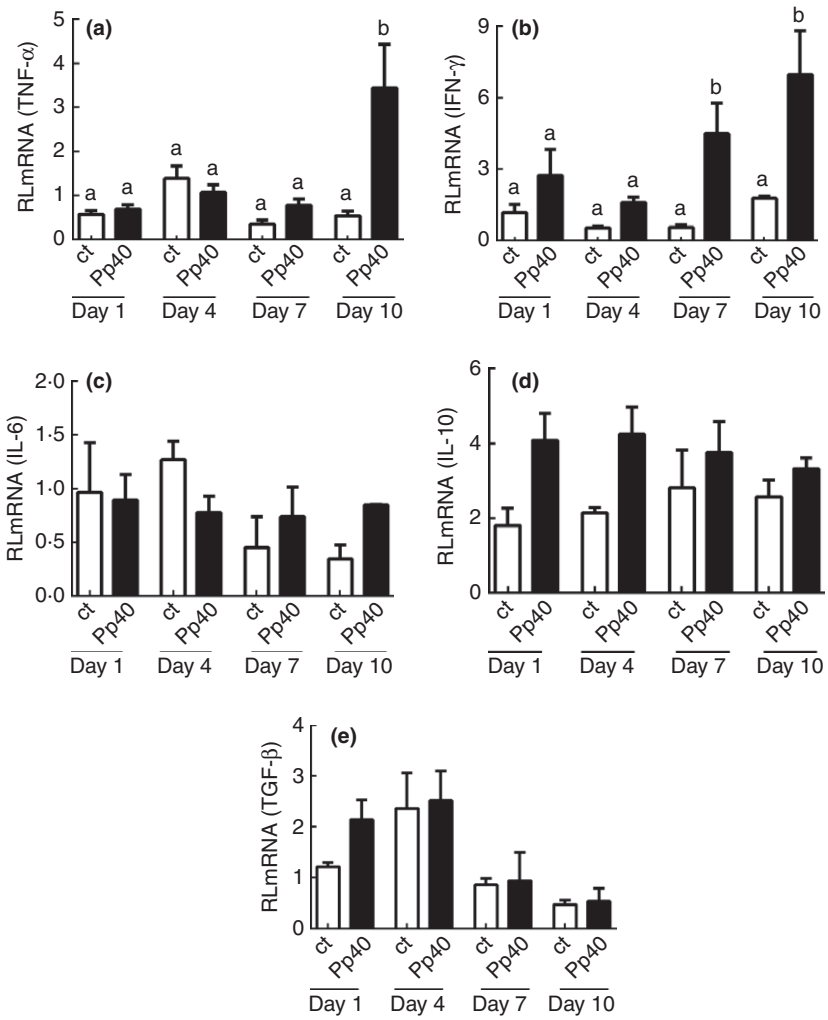

Figure 4 Relative gene expression levels (RLmRNA) of cytokines TNF-α (a), IFN-γ (b), IL-6 (c), IL-10 (d), and TGF-β1 (e) in the small intestine of mice treated for 1, 4, 7 and 10 days with *Pediococcus pentosaceus* 40; ct, saline control; Pp40, Pp40-treated mice. The data are expressed as mean mRNA amounts relative to the expression levels in Ct mice on day 1 (n = 10 per group). The vertical bars indicate the standard deviations of the means. Different letters above the bars indicate a statistically significant difference between the experimental groups (one-way ANOVA, Tukey-Kramer post test).

strain levels (Gevers *et al.* 2001; Lee *et al.* 2012). Bacteria isolates with identical GTG_5 patterns were considered to be the same strain, and 18 distinct profiles were observed in our screening.

Cell viability is probably a fundamental test of the efficacy of LAB as probiotics or vehicles for the oral delivery of beneficial proteins. Thus, an important characteristic of a new probiotic strain is its ability to resist acid and bile (Guo *et al.* 2010; Ripamonti *et al.* 2011). The pH of the stomach of horses ranges between 2·5 and 5·6, and ingested feed persists for approximately 2–6 h in the stomach of the animal (Voros 2008; Botha 2011). Weese *et al.* (2004) tested the feasibility of using LAB as probiotics for healthy horses, and they reported a survival rate of approximately 25% after bacterial incubation at a pH of 2·0 for 24 h at 37°C. This survival rate was considered adequate, and some isolates were considered acid-tolerant. Most of the isolates in the current study (97%) showed a percentage of inhibition at a pH of 2·5 for 3 h at 37°C of <10%. This rate of inhibition could also be considered adequate. This finding agreed with other authors who found that bacteria with an intestinal origin tended to be more resistant to stomach acids (Morelli 2000).

Resistance to bile salts is also an important parameter for selecting probiotic strains because bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes. The toxic effects of bile on bacterial cells are not well understood, but bile salts are surface-active, amphipathic molecules with potent antimicrobial activity and they act as detergents that disrupt biological membranes (Lebeer *et al.* 2008). The mean bile concentration in the small intestine is 0·3% w/v, and it was used to select new probiotic strains (Mainville *et al.* 2005). The percentage of resistance to bile salts has also tended to vary among LAB and even between strains of the same species (Mirlohi *et al.* 2009; Silva *et al.* 2013). In equines, bile is continuously released in the small intestine because horses do not have a biliary vesicle in the gastrointestinal tract (Botha 2011). We considered an isolate to be resistant

if it exhibited inhibition rates of <40%, tolerant if the inhibition rates ranged from 40% to 60%, and sensitive if the inhibition rates were >60%. Weese *et al.* (2004) challenged equine LAB with bile salts at 0·3% for 24 h and reported that the majority of the isolates (64%) had <25% inhibition, whereas the remaining isolates had higher inhibition between 25% and 50%, so they considered all their isolates to be tolerant to bile salts.

Bacterial cell hydrophobicity is directly related to the ability of strains to adhere to surfaces. This ability is determined by hydrophobic components present in the outer membrane of micro-organisms, and it is known that these interactions play an important role in the adhesion of bacteria to the epithelium (Guo *et al.* 2010). Testing the adhesion to solvents (MATS) facilitates a qualitative assessment of the polarity or nonpolarity of the bacterial surface, which is important because it indicates the potential for probiotic adhesion to apolar surfaces in the mucosal epithelia. However, this test is only a primary indicator of the adherence of micro-organisms (Guo *et al.* 2010). Our results of the MATS for the LAB strains showed that only 17% of the isolates exhibited high hydrophobicity (70–100%). Botha (2011) tested four strains of LAB isolated from horses, one of which showed hydrophobicity of 50% and was considered to have a medium hydrophobic surface, while the others had low hydrophobicity of 0–8%.

In the present study, the agar spot assay was used for the initial determination of antagonistic activity via diffusible compound(s) produced by LAB. This test indicates the activity against various Gram-positive and Gram-negative bacteria. This inhibitory effect might be due to H_2O_2, lactic acid, bacteriocins, antibiotic-like substances, or a combination of these compounds (Nardi *et al.* 2005). Five of the 18 isolates showed antagonistic activity against six bacterial pathogens. *Salmonella* infection is the most common cause of acute diarrhoea in foals, and it has zoonotic potential (Mastroeni and Grant 2011). It is therefore the most important pathogen to be antagonized by our candidates for probiotics. In this study, 45% of the isolates from equine faeces showed antagonistic activity towards the *Salm. enterica* serovar Typhimurium (ATCC 14028), consistent with the literature claiming that these bacteria produce lactic acid, which would be sufficient to inhibit the growth of this pathogen (Weese *et al.* 2004). Additionally, Weese *et al.* (2004) reported that lactic acid could increase the permeability of the outer membrane of Gram-negative micro-organisms, thus inhibiting the cellular functioning of these micro-organisms. However, the study did not test if the *in vitro* inhibitory activity of these isolates against *Salmonella* ATTC 14028, including *Ped. pentosaceus* 40, can be applicable to the strain used for *in vivo* assays. In the present study, it was also found that 28% of isolates exhibited antagonistic activity against *Ent. faecalis*, which is another LAB. This finding suggested that substances other than lactic acid were acting as antagonists.

Lactic acid bacteria that are widely used as probiotics or in starter cultures have the potential to host antibiotic resistance genes, thus presenting a risk of transferring such genes to many LAB and other pathogenic bacteria (Mathur and Singh 2005). Plasmid-linked antibiotic resistance is not very common among LAB, but it does occur, and safety implications should be considered. Strains harbouring resistance plasmids should not be used as human or animal probiotics. Assessing the capacity of a proposed probiotic strain to act as a donor for conjugative antibiotic resistance genes could be a sensible precaution in some instances (Ripamonti *et al.* 2011). Knowledge of the intrinsically coded resistance of LAB to common antibiotics is necessary for recognizing acquired resistance traits. All the isolates exhibited the same antimicrobial susceptibility pattern. All of them were resistant to vancomycin, oxacillin and amikacin but sensitive to erythromycin, penicillin, ceftriaxone and ampicillin. Micro-organisms belonging to other genera of LAB (*Enterococcus, Leuconostoc, Pediococcus* and *Weissella*) have been emphasized as having intrinsic resistance to vancomycin (Wright 2003; Mathur and Singh 2005). The results were consistent with numerous studies often reporting a high degree of resistance of LAB to the antibiotic classes of glycopeptides (vancomycin), aminoglycosides (amikacin) and oxacillin (Bywater 2005; Ammor *et al.* 2007). The strains analysed were susceptible to ampicillin, ceftriaxone, chloramphenicol and erythromycin. Lactic acid bacteria sensitive to these antibiotics have been commonly reported in scientific articles (Klare *et al.* 2007). Danielsen and Wind (2003) reported high sensitivity of LAB to the presence of inhibitors of cell wall synthesis, such as ampicillin and penicillin.

Consideration of the physiology of *in vitro*-specific strains to be used has been an efficient method for the preliminary selection of probiotic bacteria (Morelli 2000; Food and Agricultural Organization/World Health Organization 2002; Mirlohi *et al.* 2009). Thus, the criteria used in this work for the evaluation of probiotic bacteria were based on *in vitro* evaluation of functional properties, as well as the known behaviour of this strain under each of the evaluated conditions. By analysing their functional characteristics, *Ped. pentosaceus* strain 40 was chosen for an *in vivo* trial in a mouse model of *Salmonella* infection.

We believe that *Ped. pentosaceus* strain 40 did not colonize the gut, being a transient micro-organism. For this reason, we chose daily gavage of this strain for the entire experimental period. *Salmonella* Typhimurium have the ability to translocate to the visceral organs of mice

causing a systemic infection, a major determinant of this micro-organism's pathogenicity (Grassl *et al.* 2008). So an increase was observed in the mice challenge groups liver score, as expected. Hepatomegaly and splenomegaly are often reported as indicative of indirect bacterial infection. In this work mice treated with only *Ped. pentosaceus* 40 showed no macroscopic changes in the liver. Based on liver histological score analyses, we believe that *Ped. pentosaceus* 40 alone is safe, but has a synergistic effect on *Salmonella* pathogenesis, which could include increase gut permeability (discussed below).

Our study also showed a significant increase in the proinflammatory cytokine IFN-γ throughout the experiment and TNF-α after 10 days due to *Ped. pentosaceus* 40 treatment, indicating a proinflammatory profile of the treatment. TNF-α and INF-γ are key mediators of cell recruitment into the lesions, and they induce activation and recruitment of neutrophils involved in local inflammatory processes. Some authors have reported that regulatory cytokine expression profiles were found in probiotic bacteria, affording gastrointestinal disease protection to mice (Silva 2004; Alvim *et al.* 2016), while others have reported that the ideal was that the tested bacterial strain induced the expression of both regulatory cytokines (TGF-β and IL-10) as inflammatory cytokines (IFN-γ, IL-12, IL-17), contributing to an adequate balance between the two types of immune response (Galdeano and Perdigón 2004; Steinberg *et al.* 2014). There have been many studies showing probiotic strains that induce the expression of pro-inflammatory cytokines, such as IFN-γ and TNF-α, and that are capable of inhibiting viral replication and protecting animals against viral infections, such as the *Lactobacillus casei* Shirota strain, which protects against influenza viruses and is widely used in fermented milk for human consumption (Hori *et al.* 2001). Tanabe *et al.* (2014) showed that a mix of five horse probiotics, consisting of *Lactobacillus ruminis*, *Lact. equi*, *Lact. reuteri*, *Lactobacillus johnsonii* and *Bifidobacterium boum*, regulated intestinal function and contributed to diarrhoea prevention in neonatal Thoroughbreds. However, other studies have shown that the introduction of live, beneficial micro-organisms orally into the animal gut yielded variable results, with limited potential for therapeutic modification of foals' gastrointestinal microbiota and even adverse effects noted, with animals becoming more likely to develop diarrhoea requiring veterinary intervention (Schoster *et al.* 2015, 2016). Data in the literature have also reported the use of LAB that induced an inflammatory response to immune adjuvants in influenza, polio, rotavirus and cholera vaccines (Paineau *et al.* 2008; Zhang *et al.* 2008; Davidson *et al.* 2011).

The equine strain *Ped. pentosaceus* 40, as well as other four LAB strains, exhibited promising *in vitro* functional properties, including no inhibition by acids, moderate inhibition by bile salts, medium hydrophobicity of cellular surfaces and strong antagonism against bacterial pathogens. Nevertheless, it did not show resistance to antimicrobials that were not intrinsically encoded on LAB. Analysis of the cytokine mRNA expression induced by treatment with *Ped. pentosaceus* revealed an inflammatory cytokine pattern, with significant increases in IFN-γ and TNF-α gene expressions. On day 10 post-Pp40 inoculation, mRNA levels of *TNFa* and *IFNg* were higher in the treated group than in the control group. Probably, this inflammatory scenario in the intestine was responsible for increasing the gut permeability and in consequence, facilitating the *Salmonella* infection. However, based on data in the literature, it is possible that the *Ped. pentosaceus* strain could work as adjuvant immune or check front protection against important viral infections in horses, such as diarrhoea in foals caused by Rotavirus or Coronavirus, or respiratory or systemic infections, such as equine influenza, equine viral arthritis and equine viral encephalitis. However, in an experimental mouse infection with *Salm.* Typhimurium, previous treatment for 10 days with *Ped. pentosaceus* 40 was prejudicial to the animals, accelerating the infection rate and the number of deaths. Thus, the health claims for a probiotic strain should be firstly established in *in vivo* experimental models that provide evidence of the strain's efficacy and then precisely labelled in the final probiotic products.

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Conflict of Interest

The authors declare that they have no competing interests.

References

Alvim, L.B., Sandes, S.H.C., Silva, B.C., Steinberg, R.S., Campos, M.H.A., Acurcio, L.B., Arantes, R.M.E., Nicoli, J.R. *et al.* (2016) *Weissella paramesenteroides* WpK4 reduces gene expression of intestinal cytokines, and hepatic and splenic injuries in a murine model of typhoid fever. *Benef Microbes* **7**, 61–73.

Ammor, M.S., Florez, A.B. and Mayo, B. (2007) Antibiotic resistance in nonenterococcal lactic acid generate food grade vectors. *Food Microbiol* **24**, 559–570.

Botha, M. (2011) *Selection of probiotic lactic acid bacteria for horses based on in vitro and in vivo studies*. Master Thesis, University of Stellenbosch, Stellenbosch, South Africa.

Bywater, R.J. (2005) Identification and surveillance of antimicrobial resistance dissemination in animal production. *Poult Sci* **84**, 644–648.

Castillo, N.A., LeBlanc, A.M., Galdeano, C.M. and Perdigón, G. (2012) Probiotics: an alternative strategy for combating salmonellosis: immune mechanisms involved. *Food Res Int* **45**, 831–841.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1998) Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J Appl Microbiol* **84**, 759–768.

Chaves, S., Perdigón, G., Moreno, De. and de Leblanc, A. (2011) Yoghurt consumption regulates the immune cells implicated in acute intestinal inflammation and prevents the recurrence of the inflammatory process in a mouse model. *J Food Prot* **74**, 801–811.

Cremonini, F., Di Caro, S., Covino, M., Armuzzi, A., Gabrielli, M., Santarelli, L., Nista, E.C., Cammarota, G. *et al.* (2002) Effect of different probiotic preparations on antihelicobacter pylori therapy-related side effects: a parallel group, triple blind, placebo-controlled study. *Am J Gastroenterol* **97**, 2744–2749.

Danielsen, M. and Wind, A. (2003) Susceptibility of *Lactobacillus* ssp. to antimicrobial agents. *Int J Food Microbiol* **82**, 1–11.

Davidson, L.E., Fiorino, A.M., Snydman, D.R. and Hibberd, P.L. (2011) *Lactobacillus* GG as an immune adjuvant for live-attenuated influenza vaccine in healthy adults: a randomized double-blind placebo-controlled trial. *Eur J Clin Nutr* **65**, 501–507.

Dogi, C.A. and Perdigón, G. (2006) Importance of the host specificity in the selection of probiotic bacteria. *J Dairy Res* **73**, 357–366.

Endo, A., Okada, S. and Morita, H. (2007) Molecular profiling of *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species in feces of active racehorses. *J Gen Appl Microbiol* **53**, 191–200.

Endo, A., Futagawa-Endo, Y. and Dicks, L.M. (2009) *Lactobacillus* and *Bifidobacterium* diversity in horse feces, revealed by PCR-DGGE. *Curr Microbiol* **59**, 651–655.

European Union (2003) *Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition*. Available at: http://europa.eu/legislation_summaries/food_safety/animal_nutrition/l12037d_en.htm (accessed on 20 June 2016).

Fagundes, C.T., Souza, D.G., Nicoli, J.R. and Teixeira, M.M. (2011) Control of host inflammatory responsiveness by indigenous microbiota reveals an adaptive component of the innate immune system. *Microbes Infect* **13**, 1121–1132.

Food and Agricultural Organization/World Health Organization (2002) *Report of a Joint FAO/WHO. Expert Consultation on Guidelines for the Evaluation of Probiotics in Food*. London, ON, Canada: FAO/WHO of the United Nations.

Franz, C.M.A.P., Cho, G.-S., Holzapfel, W.H. and Galvez, A. (2010) Safety of lactic acid bacteria. In *Biotechnology of Lactic Acid Bacteria: Novel Applications* eds. Mozzi, F., Raya, R.R. and Vignolo, G.M. pp. 341–360. Ames, IA, USA: Wiley-Blackwell.

Galdeano, C.M. and Perdigón, G. (2004) Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J Appl Microbiol* **97**, 673–681.

Gevers, D., Huys, G. and Swings, J. (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31–36.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. and Mathieu, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**, 386–401.

Grassl, G.A., Valdez, Y., Bergstrom, K.S., Vallance, B.A. and Finlay, B.B. (2008) Chronic enteric *Salmonella* infection in mice leads to severe and persistent intestinal fibrosis. *Gastroenterology* **134**, 768–780.

Guo, X.H., Kim, J.M., Nam, H.M., Park, S.Y. and Kim, J.M. (2010) Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. *Anaerobe* **16**, 321–326.

Hellemans, J., Mortier, G., Paepe, A.D., Speleman, F. and Vandesompele, J. (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* **8**, R19.

Heselmans, M., Reid, G., Akkermans, L.M., Savelkoul, H., Timmerman, H. and Rombouts, F.M. (2005) Gut flora in health and disease: potential role of probiotics. *Curr Issues Intest Microbiol* **6**, 1–7.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B. *et al.* (2014) New commensals and consortia comprising defined strains from human samples, with adequate evidence of safety and efficacy, are 'probiotics' (Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic). *Nat Rev Gastroenterol Hepatol* **11**, 506–514.

Hori, T., Kiyoshima, J., Shida, K. and Yasui, H. (2001) Effect of intranasal administration of *Lactobacillus casei* Shirota on influenza virus infection of upper respiratory tract in mice. *Clin Diagn Lab Immunol* **8**, 593–597.

Jouany, J.P. and Morgavi, D.P. (2007) Use of natural products as alternatives to antibiotic feed additives in ruminant production. *Animal* **1**, 1443–1466.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S. *et al.* (2007) Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J Antimicrob Chemother* **59**, 900–912.

Lahteenmaki, L. and Ledeboer, A.M. (2006) Probiotics – the consumer perspective. *Food Sci Technol Bull: Functional Foods* **3**, 47–50.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C. (2008) Genes and molecules of lactobacilli supporting probiotic action. *Microbiol Mol Biol Rev* **72**, 728–764.

Lee, C.M., Sieo, C.C., Cheah, Y.K., Abdullah, N. and Ho, Y.W. (2012) Discrimination of probiotic *Lactobacillus* strains for poultry by repetitive sequenced-based PCR fingerprinting. *J Sci Food Agric* **92**, 660–666.

Mainville, I., Arcand, Y. and Farnworth, E.R. (2005) A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *Int J Food Microbiol* **99**, 287–296.

Maragkoudakis, P.A., Chingwaru, W., Gradisnik, L., Tsakalidou, E. and Cencic, A. (2010) Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection. *Int J Food Microbiol* **141**(Suppl. 1), S91–S97.

Markiewicz, L., Biedrzycka, E., Wasilewska, E. and Bielecka, M. (2010) Rapid molecular identification and characteristics of *Lactobacillus* strains. *Folia Microbiol* **55**, 481–488.

Martins, A.K.S., Martins, F.S., Gomes, D.A., Elian, S.D.A., Vieira, A.T., Teixeira, M.M., Cara, D.C., Nardi, R.M.D. *et al.* (2010) Evaluation of *in vitro* antagonism and of *in vivo* immune modulation and protection against pathogenic experimental challenge of two probiotic strains of *Bifidobacterium animalis* var. *lactis*. *Arch Microbiol* **192**, 995–1003.

Mastroeni, P. and Grant, A.J. (2011) Spread of *Salmonella enterica* in the body during systemic infection: unravelling host and pathogen determinants. *Expert Rev Mol Med* **13**, 1–15.

Mathur, S. and Singh, R. (2005) Antibiotic resistance in food lactic acid bacteria – a review. *Int J Food Microbiol* **105**, 281–295.

Mathur, R., Oh, H., Zhang, D., Park, S., Seo, J., Koblansky, A., Hayden, M.S. and Ghosh, S. (2012) A mouse model of *Salmonella* Typhi infection. *Cell* **151**, 590–602.

Mirlohi, M., Soleimanian-Zad, S., Dokhani, S., Sheikh-Zeinodin, M. and Abghary, A. (2009) Investigation of acid and bile tolerance of native lactobacilli isolated from fecal samples and commercial probiotics by growth and survival studies. *Iran J Biotechnol* **7**, 233–240.

Montes, A.J. and Pugh, D.G. (1993) The use of probiotics in food-animal practice. *Vet Med* **88**, 282–288.

Morelli, L. (2000) *In vitro* selection of probiotic lactobacilli: a critical appraisal. *Curr Issues Intest Microbiol* **1**, 59–67.

Morotomi, M., Yuki, N., Kado, Y., Kushiro, A., Shimazaki, T., Watanabe, K. and Yuyama, T. (2002) *Lactobacillus* equi sp. nov., a predominant intestinal *Lactobacillus* species of the horse isolated from faeces of healthy horses. *Int J Syst Evol Microbiol* **52**, 211–214.

Nardi, R.M., Santoro, M.M., Oliveira, J.S., Pimenta, A.M., Ferraz, V.P., Benchetrit, L.C. and Nicoli, J.R. (2005) Purification and molecular characterization of antibacterial compounds produced by *Lactobacillus murinus* strain L1. *J Appl Microbiol* **99**, 649–656.

National Research Council (2011) *Guide for the Care and Use of Laboratory Animals*. 8th Edn. Washington, DC: National Academies Press.

Niederhausern, S., Sabia, C., Messi, P., Guerrieri, E., Manicardi, G. and Bondi, M. (2007) Van A-type vancomycin resistant enterococi in equine and swine rectal swabs and in human clinical samples. *Curr Microbiol* **55**, 240–246.

Ohland, C.L. and MacNaughton, W.K. (2010) Probiotic bacteria and intestinal epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol* **298**, G807–G819.

Paineau, D., Carcano, D., Leyer, G., Darquy, S., Alyanakian, M.A., Simoneau, G., Bergmann, J.F., Brassart, D. *et al.* (2008) Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. *Fems Immunol Med Mic* **53**, 107–113.

Reid, G., Jass, J., Sebulsky, M.T. and McCormick, J.K. (2003) Uses of probiotics in clinical practice. *Clin Microbiol Rev* **16**, 658–672.

Ringel, Y., Quigley, E.M.M. and Lin, H.C. (2012) Using probiotics in gastrointestinal disorders. *Am J Gastroenterol Suppl* **1**, 34–40.

Ripamonti, B., Agazzi, A., Bersani, C., De Dea, P., Pecorini, C., Pirani, S., Rebucci, R., Savoini, G. *et al.* (2011) Screening of species-specific lactic acid bacteria for veal calves multi-strain probiotic adjuncts. *Anaerobe* **17**, 97–105.

Sandes, S.H.C., Alvim, L.B., Silva, B.C., Zanirati, D.F., Jung, L.R.C., Nicoli, J.R., Neumann, E. and Nunes, A.C. (2014) *Lactobacillus* species identification by amplified ribosomal 16S-23S rRNA restriction fragment length polymorphism analysis. *Benef Microbes* **5**, 471–481.

Schoster, A., Staempfli, H.R., Abrahams, M., Jalali, M., Weese, J.S. and Guardabassi, L. (2015) Effect of a probiotic on prevention of diarrhea and *Clostridium difficile* and *Clostridium perfringens* shedding in foals. *J Vet Intern Med* **29**, 925–931.

Schoster, A., Guardabassi, L., Staempfli, H.R., Abrahams, M., Jalali, M. and Weese, J.S. (2016) The longitudinal effect of a multi-strain probiotic on the intestinal bacterial microbiota of neonatal foals. *Equine Vet J* **48**, 689–696.

Servin, A.L. (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev* **28**, 405–440.

Silva, A.M., Barbosa, F.H., Duarte, R., Vieira, L.Q., Arantes, R.M. and Nicoli, J.R. (2004) Effect of *Bifidobacterium*

longum ingestion on experimental salmonellosis in mice. *J Appl Microbiol* **97**, 29–37.

Silva, B.C., Jung, L.R.C., Sandes, S.H.C., Alvim, L.B., Bomfim, M.R.Q., Nicoli, J.R., Neumann, E. and Nunes, A.C. (2013) *In vitro* assessment of functional properties of lactic acid bacteria isolated from faecal microbiota of healthy dogs for potential use as probiotics. *Benef Microbes* **4**, 267–275.

Song, H.J., Kim, J.Y., Jung, S.A., Kim, S.E., Park, H.S., Jeong, Y., Hong, S.P., Cheon, J.H. *et al.* (2011) Effect of probiotic prevention of antibiotic-associated diarrhea: a prospective, randomized, double-blind, multicenter study. *J Korean Med Sci* **25**, 1784–1791.

Steinberg, R.S., Silva, L., Souza, T.C., Lima, M.T., de Oliveira, N.L., Vieira, L.Q., Arantes, R.M., Miyoshi, A. *et al.* (2014) Safety and protective effectiveness of two strains of *Lactobacillus* with probiotic features in an experimental model of salmonellosis. *Int J Environ Res Public Health* **11**, 8755–8776.

Stephenson, D., Moore, R. and Allison, G. (2009) Comparison and utilization of repetitive-element PCR techniques for typing *Lactobacillus* isolates from the chicken gastrointestinal tract. *Appl Environ Microbiol* **75**, 6764–6776.

Stoyancheva, G.D., Danova, S.T. and Boudakov, I.Y. (2006) Molecular identification of vaginal lactobacilli isolated from Bulgarian women. *Antonie Van Leeuwenhoek* **90**, 201–210.

Tanabe, S., Suzuki, T., Wasano, Y., Nakajima, F., Kawasaki, H., Tsuda, T., Nagamine, N., Tsurumachi, T. *et al.* (2014) Anti-inflammatory and intestinal barrier-protective activities of commensal Lactobacilli and Bifidobacteria in Thoroughbreds: role of probiotics in diarrhea prevention in neonatal Thoroughbreds. *J Equine Sci* **25**, 37–43.

Tsai, C.C., Liang, H.W., Yu, B., Hsieh, C.C., Hwang, C.F., Chen, M.H. and Tsen, H.Y. (2011) The relative efficacy of different strain combinations of lactic acid bacteria in the reduction of populations of *Salmonella enterica* Typhimurium in the livers and spleens of mice. *FEMS Immunol Med Mic* **63**, 44–53.

U.S. Food and Drug Administration (2012) *The judicious use of medically important antimicrobial drugs in food-producing animals; New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209*. Rockville, MD, USA: U.S. Food and Drug Administration.

Versalovic, J., Schneider, M., de Bruijn, F.J. and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Method Mol Cell Biol* **5**, 25–40.

Vinderola, C.G. and Reinheimer, J.A. (2003) Lactic acid starter and probiotic bacteria: a comparative '*in vitro*' study of probiotic characteristics and biological barrier resistance. *Food Res Int* **36**, 895–904.

Voros, A. (2008) *Diet related changes in the gastrointestinal microbiota of horses*. Master thesis, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweeden.

Weese, J.S., Anderson, M.E., Lowe, A., Penno, R., da Costa, T.M., Button, L. and Goth, K.C. (2004) Screening of the equine intestinal microflora for potential probiotic organisms. *Equine Vet J* **36**, 351–355.

Wright, G.D. (2003) Mechanism of resistance to antibiotics. *Curr Opin Chem Biol* **7**, 563–569.

Yan, F., Cao, H., Cover, T.L., Whitehead, R., Washington, M.K. and Polk, D.B. (2007) Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* **132**, 562–575.

Zhang, W., Azevedo, M.S., Wen, K., Gonzalez, A., Saif, L.J., Li, G., Yousef, A.E. and Yuan, L. (2008) Probiotic *Lactobacillus acidophilus* enhances the immunogenicity of an oral rotavirus vaccine in gnotobiotic pigs. *Vaccine* **26**, 29–30.