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Compatibility and safety of five lectin-binding putative probiotic strains for the development of a multi-strain protective culture for poultry

J.D. Babot[1*], E. Argañaraz-Martínez[1,2], L. Saavedra[1], M.C. Apella[1,2] and A. Perez Chaia[1,2*]

[1]Centro de Referencia para Lactobacilos (CERELA-CCT Tucumán-CONICET), Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina; [2]Universidad Nacional de Tucumán, Ayacucho 471, T4000ILC San Miguel de Tucumán, Argentina; jbabot@cerela.org.ar; apchaia@cerela.org.ar

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RESEARCH ARTICLE

Abstract

The ban on the use of antibiotics as feed additives for animal growth promotion in the European Union and United States and the expectation of this trend to further expand to other countries in the short term have prompted a surge in probiotic research. Multi-species probiotics including safe and compatible strains with the ability to bind different nutritional lectins with detrimental effects on poultry nutrition could replace antibiotics as feed additives. *Lactobacillus salivarius* LET201, *Lactobacillus reuteri* LET210, *Enterococcus faecium* LET301, *Propionibacterium acidipropionici* LET103 and *Bifidobacterium infantis* CRL1395 have proved to be compatible as evaluated through three different approaches: the production and excretion of antimicrobial compounds, growth inhibition by competition for essential nutrients and physical contact, and a combination of both. The safety of *P. acidipropionici* LET103 was confirmed, since no expression of virulence factors or antibiotic resistance was detected. The innocuity of *E. faecium* LET301 should be further evaluated, since the presence of genes coding for certain virulence factors (*gelE*, *efaAfm* and *efaAfs*) was observed, albeit no expression of *gelE* was previously detected for this strain and there are no reports of involvement of *efaAfm* in animal pathogenicity. Finally, a combination of the five strains effectively protected intestinal epithelial cells of broilers from the cytotoxicity of mixtures of soybean agglutinin, wheat germ agglutinin and concanavalin A. To our knowledge, this is the first time that a combination of strains is evaluated for their protection against lectins that might be simultaneously present in poultry feeds.

Keywords: feed additives, antibiotic ban, poultry, agglutinins

1. Introduction

During the last decades, the increase in world population and income has led to a higher demand of animal protein. In order to fulfil this increasing demand, the use of antibiotics in animal production as feed additives for growth promotion has boomed (Gonzalez Ronquillo and Angeles Hernandez, 2017). Nevertheless, concerns over the antibiotic feed additives remaining in the food supply and the increase in the number of multi-resistant strains led to their ban as animal growth promoters by the European Parliament and the Council of the European Union on Additives for use in Animal Nutrition in 2006 (EC, 2003). Furthermore, the practice of routinely feeding antibiotics to livestock has been sharply reduced in the United States due to new rules issued by the Food and Drug Administration that went into effect at the beginning of 2017 (FDA, 2013). This trend is expected to further expand to other countries in the short term, which prompted a surge in probiotic research in anticipation to the impending loss of these important drugs (Vuong *et al.*, 2016).

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host in a safe and efficacious manner (Hill *et al.*, 2014). *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Propionibacterium* and *Bifidobacterium* are among the main probiotic genera included in feed additives (Chaucheyras-Durand and Durand, 2009). On the other hand, multi-species probiotic products can confer an extra benefit on

the host due to synergism among the beneficial effects of the different strains included in the formulation. Furthermore, bacterial activity can also be stimulated by symbiosis among the strains. Therefore, development of multi-species formulations where strain-specific properties are carefully selected to obtain synergism is encouraged (Timmerman *et al.*, 2004). Besides, the safety of strains is a critical selection criterion when screening for novel probiotic microorganisms (Sanz *et al.*, 2016). Currently available probiotic products for broilers have been mainly developed to inhibit infections by pathogenic bacteria (e.g. *Salmonella* Enteritidis and *Campylobacter jejuni*). However, probiotics can promote growth of broilers through other mechanisms, such as elimination of antinutritional factors included in the diets. These are biological compounds present in food that reduce nutrient utilisation or food intake, thereby inducing impaired gastrointestinal and metabolic performance (Dunlop, 2004), and include protease inhibitors, tannins, phytates and lectins, among others. Lectins are proteins/glycoproteins of non-immune origin that have at least one non-catalytic domain that binds reversibly to specific monosaccharides or oligosaccharides (Sarup Singh *et al.*, 2016). Most plant-based feeds include appreciable amounts of lectins because they are widely distributed among plants (Vojdani, 2015). Worldwide, soybean is the main source of protein of vegetable origin incorporated in poultry feeds (Valentine *et al.*, 2017) while wheat is commonly used as an energy source for poultry (Adebiyi and Olukosi, 2015), mainly in western Canada and parts of Europe, but also throughout the world when maize is scarce. Therefore, poultry can be routinely exposed to two lectins with proven detrimental effects on their nutrition: soybean agglutinin (SBA) and wheat germ agglutinin (WGA). Besides, incorporation of alternative seeds in diets for broilers has been evaluated during the last few years (Bingol *et al.*, 2016; Ditta and King, 2017; Leiber *et al.*, 2017), so poultry could be exposed to additional lectins.

Thus, the objective of this work was to assess the safety of *Enterococcus faecium* LET301 and *Propionibacterium acidipropionici* LET103, as well as the compatibility of a mixture of five putative probiotic strains including *Lactobacillus salivarius* LET201, *Lactobacillus reuteri* LET210 and *Bifidobacterium infantis* CRL1395 – strains previously selected for their ability to bind SBA, WGA or concanavalin A (ConA) (Babot *et al.*, 2014, 2016, 2017). We analysed the compatibility of growth as well as the compatibility of the functional property (i.e. the binding of lectins) based on which the above strains were selected. To this end, the protection of a mixed culture containing the five strains against the deleterious effects of a mixture of SBA, WGA and ConA on intestinal epithelial cells (IEC) of broilers was investigated. Inclusion of ConA in the lectin mixture aims to evaluate the protection of the five strains against agglutinins from alternative seeds with the same affinity for carbohydrates as this lectin.

2. Materials and methods

Bacterial strains and growth conditions

L. salivarius LET201, *L. reuteri* LET210, *E. faecium* LET301 (Babot *et al.*, 2014) and *P. acidipropionici* LET103 (Arganaraz-Martinez *et al.*, 2013), previously isolated from the intestines of healthy broilers and hens, were obtained from the LET Culture Collection (Laboratorio de Ecofisiología Tecnológica, Centro de Referencia para Lactobacilos, CERELA, Tucumán, Argentina), while *B. infantis* CRL1395, previously isolated from the intestine of a healthy infant (Reuter, 1971), was obtained from CRL Culture Collection (CERELA). The strains were stored at -70 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 0.5% yeast extract and 15% glycerol. Before use, they were activated by three successive transfers for 24 h at 37 °C in the following culture media and incubation conditions: lactobacilli in MRS broth (Rogosa *et al.*, 1951) in a chamber gassed with 10% CO_2 (Nuaire Co., MN, USA), *E. faecium* LET301 in LAPTg broth (Raibaud *et al.*, 1961) in aerobiosis, *B. infantis* CRL1395 in MRS broth supplemented with 0.04% cysteine (Guo-wei *et al.*, 2012) in an anaerobic atmosphere provided by Anaerocult A (Merk KGaA, Darmstadt, Germany) in an anaerobic jar (AnaeroGen System, Oxoid, UK), and *P. acidipropionici* LET103 in LAPTg broth in an anaerobic atmosphere. Cysteine was included in media for *B. infantis CRL1385* only when it was cultured in stressing conditions different from the oxygen presence as seeded media were incubated in anaerobic conditions.

Compatibility of strains

The compatibility among the five strains was studied through three different approaches: the production and excretion of antimicrobial compounds (agar diffusion method), growth inhibition by competition for essential nutrients and physical contact (joint growth in solid media), and a combination of both approaches (co-culture in liquid medium).

Agar diffusion method

The protocol described by Rahimifard and Naseri (2016) was followed, with some modifications. Each strain was grown in two different culture media to test if their composition had an influence on the compatibility between bacteria. Briefly, active cultures of the five strains were centrifuged twice (10,000×*g*, 10 min, 4 °C) and the supernatants were neutralised with 1 N NaOH and stored at 4 °C until use. Simultaneously, soft (7.0 g/l agar) LAPTg agar or soft MRS agar were melted, inoculated with each strain (0.1%) and poured onto petri dishes containing a solidified hard agar (15.0 g/l) base. Unlike the other strains, *B. infantis* CRL1395 was tested in soft MRS agar and in

soft LAPTg agar supplemented with 10 g/l lactose. 5 µl of each cell-free supernatant was spotted on the plates. Plates were incubated at 37 °C in aerobiosis (*E. faecium* LET301), in a chamber gassed with 10% CO_2 (lactobacilli) or in an anaerobic atmosphere (*B. infantis* CRL1395 and *P. acidipropionici* LET103) for 1, 2 or 5 days, respectively. After incubation, the presence or absence of inhibition zones around the spotted areas was determined.

Joint growth in solid media

This test was performed according to the technique described by Chapman *et al.* (2012). Briefly, active cultures of the five strains were washed twice (10,000×*g*, 10 min, 4 °C) with sterile phosphate buffered saline (PBS), pH 7.20, and seeded with a sterile swab in a straight line in petri dishes containing the same media as the previous assay. The remaining four strains were seeded with a sterile swab in the same plates in a straight perpendicular line. After incubation of the plates at 37 °C for 5 days in an anaerobic atmosphere, growth stimulation, growth inhibition or absence of interaction between the colonies was determined.

Co-culture in liquid medium

Active cultures of the five strains were washed twice (10,000×*g*, 10 min, 4 °C) with sterile PBS, pH 7.20. MRS broth was inoculated with approximately 10^6 cfu/ml of each of the five strains and incubated at 37 °C for 36 h with pH fixed at 6.30. At regular time lapses, culture aliquots were taken, properly diluted with sterile PBS pH 7.20 and plated onto selective media for each strain: KF *Streptococcus* agar (Merck) supplemented with 0.1 g/l 2,3,5-triphenyltetrazolium chloride (Sigma) for the enumeration of *E. faecium* LET301; RCA agar supplemented with LiCl and propionic acid (meat extract 10 g, peptone 10 g, NaCl 5 g, glucose 5 g, yeast extract 3 g, sodium acetate 3 g, soluble starch 1 g, cysteine-HCl 0,5 g, agar 15 g, distilled water 1000 ml, 12.5 mol/l LiCl 5 ml, propionic acid 5 ml, pH 6.60-7.00) for the enumeration of *B. infantis* CRL1395; modified LBS agar [tryptone 10 g, yeast extract 5 g, fructose 2 g, KH_2PO_4 6 g; ammonium citrate 2 g; sodium acetate 17 g; $MgSO_4$ 0,575 g; $MnSO_4$ 0,12 g; $FeSO_4$ 0,034 g; bromocresol green 0.33% (p/v) 20 ml; agar 20 g; Tween 80 1 ml; distilled water 1000 ml] for the differential enumeration of homofermentative *L. salivarius* LET201 and heterofermentative *L. reuteri* LET210 (green and blue colonies, respectively). Colonies were counted after the incubation of plates under the above conditions. The fluorescent *in situ* hybridisation protocol described by Babot *et al.* (2011) was used for the enumeration of *P. acidipropionici* LET103, using the FITC-labelled probe Pap446 (5´-ACACCCCAAAACGATGCCTTCGCC-3´) (Lorenzo-Pisarello *et al.*, 2010).

Safety of strains

Hemagglutination ability, production of gelatinase and cytolysin (haemolysin) and antibiotic resistance of *P. acidipropionici* LET103 were tested. A hemagglutination assay was performed according to Babot *et al.* (2014), and production of gelatinase and cytolysin (haemolysin) were assessed according to Eaton and Gasson (2001) and Elsner *et al.* (2000), respectively. Antibiotic resistance was evaluated by the disc diffusion method according to the specifications of the Clinical and Laboratory Standards Institute (CLSI, 2006). Bacterial suspensions (3×10^8 cfu/ml) were inoculated in LAPTg and LSM agar plates (Klare *et al.*, 2005) using sterile cotton swabs. Antibiotic discs containing ampicillin (10 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), streptomycin (300 µg), tetracyclin (30 µg) and vancomycin (30 µg) were placed on the surface of the plates, which where incubated for 7 days at 37±0.5 °C in an anaerobic atmosphere, and the diameters of the inhibition zones were measured. *P. acidipropionici* LET103 was classified as sensitive (S) or resistant (R) according to CASFM (2010), Abdel Fattah and Darwish (2013), and Zandi *et al.* (2011).

The presence of genes related to vancomycin resistance and virulence factors in *E. faecium* LET301 was assessed by PCR, using the primers indicated in Table 1. DNA extraction was carried out according to Pospiech and Neumann (1995) with some modifications. PCR reactions for genes *vanA*, *vanB* and *vanC1*, *cylA* and *hyl*, and *agg*, *ccf*, *cob*, *cpd*, *efaAfm*, *efaAfs*, *esp* and *gelE* were performed according to Kariyama *et al.* (2000), Vankerckhoven *et al.* (2004) and Eaton and Gasson (2001), respectively. Strains *E. faecium* CRL1492 and *E. casseliflavus* CRL1488 were included as positive controls. Amplification products were separated by electrophoresis at 90 V on 2% (w/v) agarose stained with SYBR® Safe DNA Gel Stain (Invitrogen, San Diego, CA, USA) in 1×TAE buffer (40 mM Tris acetate, 1 mM EDTA). PCR products were purified using the AccuPrep Gel Purification Kit (Bioneer, Alameda, CA, USA) according to the manufacturers instructions. DNA sequencing of amplified fragments was carried out by Sequencing Service of CCT-CONICET-Tucumán (Tucumán, Argentina). The fragments of sequences were assembled and edited with DNAMAN software (Version 4.03, Lynnon Biosoft, Vaudreuil, Quebec, Canada) and consensus sequences were compared with other gene sequences in the EMBL/GenBank/DDBJ database using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their identity.

Lectin toxicity protection by a mixed culture

Protection exerted by a five-strain mixed culture against cytotoxicity of a mixture of three dietary lectins (ConA, SBA and WGA) on IEC of broiler chicks was assessed according to Babot *et al.* (2016). Briefly, each active culture of the

Table 1. Primers used for safety assessment of *Enterococcus faecium* LET301.

Gene		Primer sequence	Size of amplification product (bp)
agg	aggF	AAGAAAAAGAAGTAGACCAAC	1,553
	aggR	AAACGGCAAGACAAGTAAATA	
ccf	ccfF	GGGAATTGAGTAGTGAAGAAG	543
	ccfR	AGCCGCTAAAATCGGTAAAAT	
cob	cobF	AACATTCAGCAAACAAAGC	1,405
	cobR	TTGTCATAAAGAGTGGTCAT	
cpd	cpdF	TGGTGGGTTATTTTTCAATTC	782
	cpdR	TACGGCTCTGGCTTACTA	
cylA	cylAF	TGGATGATAGTGATAGGAAGT	688
	cylAR	TCTACAGTAAATCTTTCGTCA	
hyl	hylF	ACAGAAGAGCTGCAGGAAATG	276
	hylR	GACTGACGTCCAAGTTTCCAA	
efaAfm	efaAfmF	AACAGATCCGCATGAATA	735
	efaAfmR	CATTTCATCATCTGATAGTA	
efaAfs	efaAfsF	GACAGACCCTCACGAATA	705
	efaAfsR	AGTTCATCATGCTGTAGTA	
Esp	espF	TTGCTAATGCTAGTCCACGACC	933
	espR	GCGTCAACACTTGCATTGCCGAA	
gelE	gelEF	ACCCCGTATCATTGGTTT	419
	gelER	ACGCATTGCTTTTCCATC	
vanA	vanAF	CATGAATAGAATAAAAGTTGCAATA	1,030
	vanAR	CCCCTTTAACGCTAATACGATCAA	
vanB	vanBF	GTGACAAACCGGAGGCGAGGA	433
	vanBR	CCGCCATCCTCCTGCAAAAAA	
vanC1	vanCF	GGTATCAAGGAAACCTC	822
	vanCR	CTTCCGCCATCATAGCT	

five strains, containing 1×10^8 cfu/ml, was washed three times with sterile PBS pH 7.40 (10,000×*g*, 10 min, 4 °C) and resuspended in one fifth the original volume in RPMI 1640 medium supplemented with 1% foetal bovine serum (RPMI/FBS). The suspensions were mixed and incubated with a mixture of ConA, SBA and WGA (final concentration of each lectin: 16.7 or 50 µg/ml) for 1 h at 41.5±0.5 °C to allow the attachment of the lectins to the bacterial surface. Then, the suspension was centrifuged (10,000×*g*, 10 min, 4 °C) and the supernatants containing the remaining lectin were collected and stored at 4 °C until use. Simultaneously, IEC from the distal portion of the jejunum of 14-day-old broiler chicks were obtained exactly as described by Babot *et al.* (2016) and adjusted to 5×10^5 cells/ml in cold RPMI/FBS. Then, cells were incubated with the solution containing the remaining lectins (1:1) for 2 h at 41.5±0.5 °C under a 5% CO_2 atmosphere. Finally, suspensions were stained as described by Babot *et al.* (2016), and the viable (green) and non-viable (red) IEC were counted with a conventional fluorescence microscope (Axio Scope A1; Carl Zeiss, Oberkochen, Germany) to determine the percentage of dead cells.

Statistical analysis

Three independent assays were performed for each experiment and the mean values ± standard deviations (SD) were obtained for each sample. Significant differences were determined by Tukey´s test after analysis of variance (ANOVA) with OriginPro 8 SR0 v8.0724 (OriginLab Corporation, Northampton, MA, USA). A value of $P<0.05$ was considered statistically significant.

3. Results

Compatibility of strains

All combinations of cell-free neutralised supernatants and strains assayed using the agar diffusion method evidenced lack of growth inhibition zones in both culture media. On the other hand, interaction between lactic acid bacteria (LAB) in LAPTg or MRS solid media had no inhibitory or stimulating effects on their growth. Regarding joint growth of LAB and *P. acidipropionici* LET103 or *B. infantis* CRL1395, inhibition of the latter was observed in MRS solid medium. In contrast, no inhibition of these strains by LAB was evidenced in LAPTg (*P. acidipropionici* LET103) or LAPTg supplemented with 10 g/l lactose (*B. infantis* CRL1395) solid media. Moreover, joint growth of *P. acidipropionici* LET103 and *B. infantis* CRL1395 had no effect on their growth in either solid media. With respect co-culture in MRS broth, LAB strains and *B. infantis* CRL1395 showed rapid growth, reaching approximately 10^8 cfu/ml at 6 h of incubation (Figure 1). In contrast, the growth of *P. acidipropionici* LET103 was slow, the log phase being reached after 12 h of incubation, while counts of *E. faecium* LET301 markedly diminished after 10 h of assay.

Safety of *Propionibacterium acidipropionici* LET103 and *Enterococcus faecium* LET301

Hemagglutination activity and production of haemolysin and gelatinase were absent in *P. acidipropionici* LET103. Moreover, this strain was sensitive to the seven antibiotics tested both in LAPTg and LSM agar. On the other hand, amplicons were obtained for primers targeted to *efaAfm*, *efaAfs* and *gelE* in *E. faecium* LET301. Sequencing of these PCR products confirmed the presence of such genes. Nevertheless, no amplification products were obtained for the remaining primer sets.

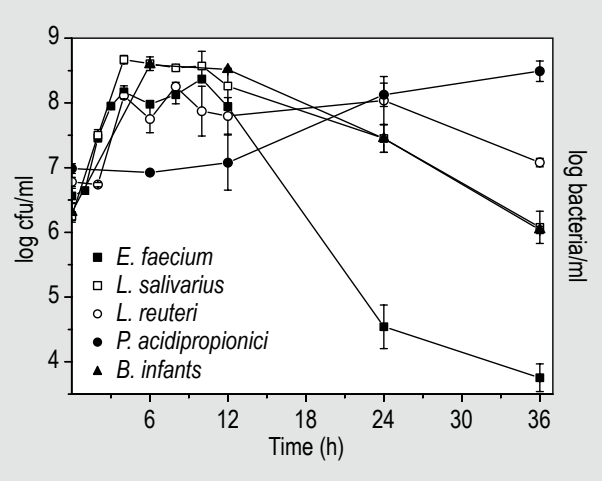

Figure 1. Growth kinetics of *Enterococcus faecium* LET301, *Lactobacillus salivarius* LET201, *Lactobacillus reuteri* LET210, *Propionibacterium acidipropionici* LET103 and *Bifidobacterium infantis* CRL1395 in co-culture. Counts of *P. acidipropionici* LET103 are represented as log bacteria/ml, while counts of the remaining strains are represented as log cfu/ml.

Protection of intestinal epithelial cells against lectins cytotoxicity by a mixed culture

Protection exerted by a suspension containing the five strains on the cytotoxicity of two mixtures with different concentrations of ConA, SBA and WGA on IEC of broilers is shown in Figure 2. Regarding the lectin mixture with a final concentration of each agglutinin of 16.7 µg/ml, the multi-strain suspension led to a marked reduction (56%) in its cytotoxicity on IEC of broilers. Likewise, a lower but still remarkable reduction of 41% in IEC death due to the lectin mixture with a final concentration of each agglutinin of 50 µg/ml was achieved after incubating it with the suspension containing the five strains.

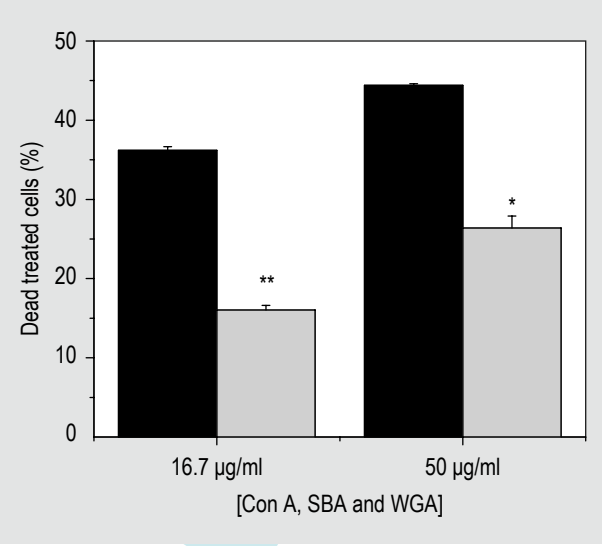

Figure 2. Cytotoxicity of a mixture of Concanavalin A (ConA), soybean agglutinin (SBA) and wheat germ agglutinin (WGA) on intestinal epithelial cells (IEC) before (black bars) and after (grey bars) incubation with the five-strain mixed culture. Significant differences are indicated with one (P<0.05) or two (P<0.01) asterisks.

4. Discussion

Multi-strain probiotic products could be more effective than single-strain ones (Fredua-Agyeman *et al.*, 2017). In fact, the latter preparations have fewer probabilities of colonising the gastrointestinal tract (Famularo *et al.*, 1999). Furthermore, each probiotic strain in a multi-strain formulation exerts strain-specific effects on the host (Sanders and Huis, 1999). Therefore, Dunne *et al.* (1999) and Rolfe (2000) suggested that probiotic products should consist of a combination of strains. In this case, compatibility between strains should be carefully assessed to avoid inhibition of one of the strains included in the formulation (Fredua-Agyeman *et al.*, 2017). Recently, Fredua-Agyeman *et al.* (2016) reported that none of the multi-strain probiotic products available in the United Kingdom contained all the labelled strains, probably due to inhibition amongst the strains. In our study, we detected absence of inhibition by the neutralised supernatants of the five strains on the growth of the remaining cultures in both solid media, which suggests that these strains do not secrete inhibitory compounds. On the other hand, other authors evaluated the growth in co-culture of bifidobacteria and lactobacilli (Fooks and Gibson, 2002), bifidobacteria and propionibacteria (Wu *et al.*, 2012), and enterococci and lactobacilli (Kos *et al.*, 2011) in MRS broth. They reported that this medium does not negatively affect the growth of strains of those genera. Therefore, we assayed the growth of the five strains in a co-culture in MRS broth. In agreement with the result of the previous assay, all strains were able to grow normally in a co-culture in MRS broth. All strains, with the exception of *P. acidipropionici* LET103, showed a short lag phase followed by the exponential and stationary phases. The long lag phase of *P. acidipropionici* LET103 is due to the typical slow growth of propionibacteria (Hettinga and Reinbold, 1972). pH was fixed at 6.30 in this study to avoid inhibition of this strain by acidification of the culture media as result of the fast growth of the other strains, since optimal pH for growth of *P. acidipropionici* ranges between 6.00 and 7.00 (Hsu and Yang, 1991). In the distal portion of small intestine of broilers, pH is relatively constant with small variations between 6.30 and 7.20 (Herpol and Van Grembergen, 1967; Sturkie, 2012). Therefore, growth of *P. acidipropionici* LET103 *in situ* should not be hindered by acidity. The colonies interaction assay showed inhibition of *P. acidipropionici* LET 103 and *B. infantis* CRL1395 by all LAB in MRS but not in LAPTg or LAPTg supplemented with 10 g/l lactose. These different effects may be due to the fact that MRS is a rich culture medium, supplemented with salts and mineral ions which favour the fast growth

of nutritionally exigent LAB (De Man *et al.*, 1960) over propionibacteria and bifidobacteria. Similarly, Chapman *et al.* (2012) reported inhibition of bifidobacteria strains by several lactobacilli in MRS. Altogether, these results suggest that *P. acidipropionici* LET103, *L. salivarius* LET201, *L. reuteri* LET210, *E. faecium* LET301 and *B. infantis* CRL1395 are compatible and can be included in a multi-strain formulation. This agrees with Timmerman *et al.* (2004), who analysed several published articles about multi-strain probiotics and concluded that strains of *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bifidobacterium* and *Propionibacterium* usually show symbiotic relations among them, which improves their growth and metabolic activity. Therefore, they stated that research on multi-strain probiotics including the above genera should be encouraged.

Lactobacilli, bifidobacteria and dairy propionibacteria are generally recognised as safe (GRAS) microorganisms (FAO/WHO, 2002; Meile *et al.*, 2008) and have received the Qualified Presumption of Safety (QPS) qualification by the European Food Safety Agency (EFSA, 2011). Although they are not GRAS, other microorganisms, such as enterococci, streptococci and *Bacillus* have been used as probiotics and are considered safe if they have a long history of innocuity (EFSA, 2011; Mogensen *et al.*, 2002). However, given the importance of this property in probiotic products, the safety of new putative probiotic strains requires a careful study, mainly for species lacking a long history of safe use or that are not GRAS. Safety of *L. salivarius* LET201, *L. reuteri* LET210 (Babot *et al.*, 2014) and *B. infantis* CRL1395 (Babot *et al.*, 2016) has already been demonstrated, as well as lack of hemagglutination activity and production of cytolysin (haemolysin) and gelatinase by *E. faecium* LET301 (Babot *et al.*, 2014). Yet, the safety of *P. acidipropionici* LET103 remained to be elucidated. In the present study, *P. acidipropionici* LET103 evidenced no hemagglutination ability, nor production of gelatinase or cytolysin (haemolysin), and was sensitive to the seven antibiotics tested on both culture media although natural resistance to oxacillin, aminoglycosides, first and second generation quinolones, colistin, metronidazole and fosfomycin on dairy propionibacteria has been reported (Chamba *et al.*, 2004; Madec *et al.*, 1994). On the other hand, in spite of being part of the normal intestinal microbiota of humans and animals, pathogenic or potentially pathogenic strains with haemolytic activity or antibiotic resistance exist among enterococci (Eaton and Gasson, 2001; Mannu *et al.*, 2003). Vancomycin resistance, which can be transferred by conjugation to other species of the same genus and even to *Listeria*, *Staphylococcus* or *Lactobacillus* (Conwell *et al.*, 2017; Jahan and Holley, 2016; Mater *et al.*, 2008; Weigel *et al.*, 2003), has been considered a critical factor in the selection of putative probiotic enterococci. Nevertheless, we detected absence of *vanA*, *vanB* and *vanC1* genes in *E. faecium* LET301. However, we observed the presence of the *gelE* gene in this strain. Gelatinase activity had not been detected in this strain in previous assays (Babot *et al.*, 2014), therefore expression of this gen could be silenced or it could codify for an inactive product (Eaton and Gasson, 2001). *gelE* is widely distributed among avian *Enterococcus*. Doğru *et al.* (2010) detected it in 30.2% of *E. faecium* and *E. faecalis* strains isolated from chicken skin and faeces. Besides, *efaAfm* and *efaAfs* genes were detected in *E. faecium* LET301. Up to now, only involvement of *efaAfs* in pathogenicity has been shown in animal trials (Singh *et al.*, 1998), while the role of *efaAfm* has not been demonstrated yet. On the other hand, *agg*, *ccf*, *cob*, *cpd* and *esp* genes were absent in *E. faecium* LET301.

Cytotoxicity of SBA and WGA on IEC of broilers and its protection by *B. infantis* CRL1395 and *L. salivarius* LET201, *L. reuteri* LET210 or *E. faecium* LET301 has been previously reported by Babot *et al.* (2016, 2017), respectively. Likewise, we observed cytotoxicity of ConA on IEC of broilers and protection by *P. acidipropionici* LET103 in previous assays (unpublished data). Nevertheless, the protection exerted by each strain could be enhanced or diminished in the presence of other strains and lectins. Therefore, we evaluated the protection exerted by the five-strain formulation against the cytotoxicity of a mixture of SBA, WGA and ConA. The assay here performed simulates the events occurring in the gut after ingestion of both lectins and lectin-binding strains: first, interaction of lectins and bacteria in the lumen of the gut; then, elimination of bacteria and bacteria-bound lectins along with the normal transit of digesta; and finally, interaction of the remaining lectin molecules with IEC. We observed reductions of 56% and 41% in IEC death due to the mixture of lectins (each lectin 16.7 or 50 µg/ml, respectively) when these glycoproteins were previously incubated with suspensions of the five strains. These results suggest that binding of lectins by the five strains is not negatively affected by the presence of the other strains and lectins, since the cytotoxicity protection observed agrees with values achieved for each strain individually: 49% reduction in cell death due to ConA by *P. acidipropionici* LET103 (unpublished data), 33% due to SBA by *B. infantis* CRL1395 (Babot *et al.*, 2016), and 54, 45 and 39% due to WGA by *L. salivarius* LET 201, *L. reuteri* LET 210 and *E. faecium* LET 301, respectively (Babot *et al.*, 2017). Carbohydrates on the surface of these bacteria (GlcNAc-β-1,4-GlcNAc or NeuNAc in *L. salivarius* LET201, *L. reuteri* LET210 and *E. faecium* LET301, GalNAc in *B. infantis* CRL1395, and α-D-mannose or α-D-glucose in *P. acidipropionici* LET103) would bind cognate lectins and prevent their interaction with IEC surface receptors (Babot *et al.*, 2016, 2017; Zárate *et al.*, 2017). Accordingly, protection against the deleterious effects of dietary lectins by bifidobacteria and propionibacteria was previously reported by Zárate *et al.* (2017). The effectiveness of a feed additive containing the five strains studied in this work would depend on their regular administration to broilers. Considering that these

strains are able to resist the harmful conditions in the gastrointestinal tract (Arganaraz-Martinez *et al.*, 2013; Babot *et al.*, 2014, 2016), a steady population of them would develop in the gut of the birds and their interaction with lectins included in the diet would be possible. On the other hand, the interaction between IEC of broilers and each of the lectins evaluated in the present study was previously analysed individually (Babot *et al.*, 2016, 2017). In these studies we observed that adherent and non-adherent strains could prevent eukaryotic cells-lectin interactions by different mechanisms depending on the location of these bacteria in the gut. The protection mediated by adherent bacteria was assayed by allowing their adhesion to IEC prior to the addition of agglutinin to interfere in the interaction between the lectin and eukaryotic cells. Despite bacterial binding to IEC surfaces, in some cases these surfaces remained vulnerable to damage probably due to the small size of bacteria compared to cells, or because bacteria bind to the cells by surface molecules other than agglutinin receptors. Conversely, many adherent and non-adherent strains protected the cells against lectin cytotoxicity under conditions that simulate the capture and removal of agglutinin in the intestinal lumen. Furthermore, the percentage of enterocytes with at least one bound bacterium decreased significantly after incubation with lectin in almost all the adherent strains assayed. These detached bacteria would still bound agglutinin in the intestinal lumen and be eliminated along with the lectin adsorbed to their surface during the normal transit of the intestinal content.

In conclusion, our study demonstrated that five putative probiotic strains, each with the ability to bind dietary lectins, are compatible and could be administered to broilers with no safety concerns. Furthermore, suspensions of the five strains successfully protected IEC of broilers against cytotoxicity of mixtures of ConA, SBA and WGA *ex vivo*. These results pave the way for the design of a feed additive for broilers with protective effect against dietary lectins. Nevertheless, additional assays are needed to unravel the mechanism of protection and to confirm the *in vivo* effectiveness of the five-strain mixture.

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