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antagonistic effects against swine-associated
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Isolation of lactic acid bacteria from swine milk and characterization of potential probiotic strains with antagonistic effects against swine-associated gastrointestinal pathogens

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Abstract

Probiotics are usually isolated from the gastrointestinal tract of humans and animals. The search of probiotics in human milk is a recent field of research, as the existence of the human milk microbiome was discovered only about a decade ago. To our knowledge, no reports regarding the potential probiotic effect of bacteria from swine milk were published. In this work, we isolated several lactic acid bacteria from swine milk and evaluated them for their potential as probiotics. Among the isolated strains, *Lactobacillus curvatus* TUCO-5E showed antagonistic effects against swine-associated gastrointestinal pathogens. TUCO-5E was able to reduce the growth of enterotoxigenic and enterohemorrhagic *E. coli* strains as well as pathogenic *Salmonella*. *In vitro* exclusion and displacement assays in intestinal epithelial cells showed a remarkable antagonistic effect for *L. curvatus* TUCO-5E against *Salmonella* TUCO-I7 and *S. enterica* ATCC 13096. Moreover, by using a mice model of *Salmonella* infection we were able to demonstrate that *L. curvatus* TUCO-5E preventive administration during 5 consecutive days was capable of decreasing the number of *S. typhimurium* in the liver and spleen of treated mice when compared to controls, and avoided dissemination of the pathogen to the blood stream. Then, we demonstrated here that swine milk is an interesting source for finding beneficial bacteria. In addition, the results of this work suggest that *L. curvatus* TUCO-5E is a good candidate for *in vivo* studying the protective effect of probiotics against intestinal infection and damage induced by *Salmonella* infection in the porcine host.

Running title: Isolation of probiotic bacteria from swine milk

Keywords: Probiotics, pigs, swine milk, intestinal infection, *Salmonella*, *Lactobacillus curvatus* TUCO-5E

Introduction

Antibiotics have played a major role in the growth and development of the swine industry for more than 50 years. Their efficiency in increasing growth rate, improving feed utilization and reducing mortality from clinical disease is well documented (Cromwell 2002, Emborg *et al.* 2004). However, the excessive use of antibiotics as growth promoters in the pig industry results in the development of antibiotic resistant strains of human and animal pathogens (Emborg *et al.* 2004). Moreover, such antibiotics can be lethal to beneficial microorganisms in the gastrointestinal tract of human and animals and, they may also enter the food chain and accumulate in human body as undesirable chemical residues. Therefore, there is a need for development of alternative control methods to the antibiotics used in feed to protect the animals from diseases such as infectious diarrhea (Thacker 2013). One of the alternative methods is biological control using probiotics (Villena and Kitazawa 2014), that are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO).

Several studies have shown that probiotics can beneficially improve health status of pigs and reduce susceptibility to infections (Villena and Kitazawa 2014; Suda *et al.*, 2014). In this regard, Qiao *et al.* (2004) conducted experiments to evaluate the effects of a complex *Lactobacilli* preparation on performance, resistance to *E. coli* infection and gut microbial flora of weaning pigs. The mix of four lactobacilli (*L. gasseri*, *L. reuteri*, *L. acidophilus* and *L. fermentum*) isolated from weaning pigs was able to reduce *E. coli* and anaerobe counts in the gut, and decrease diarrhoea. Herfel *et al.* (2013) examined the impact of a novel probiotic strain of *Bifidobacterium longum* AH1206 on the health, growth and development of neonatal pigs. Authors found that AH1206 supplementation modulated the inflammatory tone of the intestinal mucosa of suckling piglets. However, no differences were found between AH1206-treated and control piglets when comparing body weight gain, feed efficiency, and histological and physiological modifications in intestines. In addition, some studies have specifically evaluated the capacity of probiotics to improve the

resistance of piglets against enterotoxigenic *Escherichia coli* (ETEC). It was shown that the probiotic strain *L. plantarum* CJLP243 may serve as a potential alternative to antibiotic supplementation to improve the growth and health performance of weaning pigs because of its capacity to reduce the severity of ETEC-induced diarrhea (2012). Li *et al.* (2012) also showed that pretreatment of piglets with *L. rhamnosus* ATCC7469 ameliorates F4⁺ETEC-induced diarrhoea. More recently, it was proposed that *Enterococcus faecium* NCIMB 10415 exerts an immune modulatory effect by increasing the numbers of intraepithelial lymphocytes in weaned pigs and improves protection against *Salmonella enterica* serovar Typhimurium (Rieger *et al.* 2015). These results demonstrate that is possible to modulate piglets' gut microbiota and immunity and improve growth performance by using appropriate probiotics strains.

Probiotic strains have been usually isolated from the gastrointestinal tract of humans and animals. On the other hand, probiotics in human milk are a very recent field of research, as the existence of the human milk microbiome was discovered only about a decade ago (Martin *et al.*, 2003; Latuga *et al.* 2014; Jost *et al.* 2015). Current research is focusing on bacterial diversity and the influence of the maternal environment as well as the mode of delivery on human milk microbiota, the pathways of bacterial transfer to milk ducts, possible benefits of specific bacterial strains for the treatment of mastitis in mothers, and disease prevention in children (for a review see Bergmann *et al.* 2014). Moreover, recent advances in the assessment of early host-microbe interactions suggest that early colonization of infant gut by milk bacteria may have an impact on later health (Bergmann *et al.* 2014). To our knowledge, no reports regarding the potential probiotic effect of swine milk bacteria were published. Then, the objective this work was the isolation of beneficial lactic acid bacteria (LAB) inhabiting swine milk, which can be used as potential probiotics against swine gastrointestinal bacterial pathogens.

Materials and Methods

Isolation of LAB from swine milk

Ten swine milk samples were collected aseptically by trained personnel in the rotahouse from Sucesión Salvador Yanine Abadi, Bulnes, Chile. The samples were transported to the Laboratory of Food Microbiology, Faculty of Veterinary Sciences, University of Concepcion (Chillan, Chile), in cold containers. For the isolation of LAB from swine milk, resistance to pH 3 was used as a selection criterion. For this purpose, 100 µl of each milk sample were placed in MRS broth and, incubated at 37 °C, 5% CO₂ for 12 hours. Then, 100 µl of each tube they were transferred to MRS broth pH 3 (HCl, 5N) and incubated for 3 hours under the same conditions. After that period, the cultures were transferred to MRS agar to verify their viability. The grown colonies were subjected to Gram staining and catalase test. Colonies of Gram positive and catalase negative microorganisms resistant to pH 3 were selected for subsequent studies.

Identification of isolated strains

The isolates confirmed as Gram-positive and catalase-negative bacilli were biochemically identified using the API test as described elsewhere. Bacteria were further identified using species-specific primers (Table 1). Genomic DNA extraction was performed using the ZR Fungal/Bacterial DNA Miniprep Kit commercial kit (Catalog D6005). In a first step, the samples were amplified using the primers Lac (forward: 5' ACA GAC TGA AAG TCT GAC GG and reverse: 5' GCG ATG CGA ATT TCT ATT ATT) and LbG (forward: 5' AGA AGA GGA CAG TGG AAC and reverse: 5' TTA CAA ACT CTC ATG GTG TG). A total volume of 25 µl was used for amplifications (12.5 µl of Master Mix, 0.2 µl of each primer and 5 ng of total DNA from each strain). As a positive and negative controls genomic DNA from *Lactobacillus* GG and PCR mix without DNA were used respectively. Amplification consisted of an initial step of 94 °C for 60 s, followed by 30 cycles of 98 °C for 60 s, 55 for 40 s and, 72 °C for 40 s. Positive strains for the

genus *Lactobacillus* were subjected to PCR analysis using the species-specific primers as follow: 10 µl of DNA, 2.5 µl of buffer (10X), 1.25 µl of MgCl₂ (50 mM), 0.5 µl dNTPs (10 µM), 1.25 µl of each primer (0.5 µM) and, 0.125 µl Taq (500U), in a final volume of 25 µl. *Salmonella* Typhimurium DNA and *Lactobacillus fermentum* UCO-979C DNA were used as negative and positive controls respectively. Two bacterial strains TUCO-7A and TUCO-5E, showing potential probiotic properties, were further identified by 16s RNA sequencing.

Gastrointestinal bacterial pathogens

Several bacterial pathogens were used to evaluate the antagonistic effect of isolated LAB. *Escherichia coli* ATCC 25922 and *Salmonella enterica* ATCC 13076 were obtained from Oxoid. *Salmonella* sp. TUCO-I7 (isolated from intestines of infected pigs), enterotoxigenic *E. coli* TUCO-I5 and enterohemorrhagic *E. coli* TUCO-I6 provided by the Faculty of Veterinary Sciences, University of Concepcion (Concepcion, Chile). *Escherichia coli* strains and *Salmonella* strains were grown in BHI broth.

Antibiotic resistance and hemolysins and gelatinases detection

To assess resistance to antibiotics, the selected strains were cultured in Mueller Hinton agar from a suspension of 0.5 Mc Farland. The sensidiscs of the following antibiotics were used: ciprofloxacin (5 µg), vancomycin (30 µg), amoxicillin (25 µg), ampicillin (10 µg), gentamicin (10 µg), erythromycin (15 µg) and doxycycline (30 µg). Plates were cultured at 37 °C during 48 hour. The inhibition halo was measured in cm and the criterium of Vlkova' *et al.* (2006) and CLSI (2008) were used to determine resistance or susceptibility to antibiotics.

For hemolysins and gelatinases detection strains were cultured in Columbia blood agar or in gelatin at 35°C, 5% CO₂ during 48 hours, respectively.

Resistance to NaCl and Oxgall

The selected strains were cultivated in MRS broth containing 3%, 6.5% or 9% w/v NaCl or in MRS broth containing 0.3, 0.6, 2, 3 or 5% w/v Oxgall, as described by Cai *et al.* (1999). The tubes were incubated for 5 days at 37 °C, 5% CO₂ and the growth of the strains was observed. Then, 100 µl of each tube were streaked on MRS agar to test viability.

Hydrophobicity evaluation

As described by Gusils *et al.* (2002) strains were grown in MRS broth and centrifuged at 1500 rpm for 10 min. The pellets were washed twice with buffer Butterfield (pH 7.2) and resuspended in 1.2 ml of the same buffer. The absorbance was measured at 600 nm (0.4 – 0.6). A volume of 0.3 ml of xilol was mixed with 1.2 ml of the bacterial suspension in a vortex during 1.5 min. After the separation of the phases (20 min) the aqueous phase was removed and the absorbance was measured again. The hydrophobicity percentage was calculated by the formula $(A_i - A_f) / A_i \times 100$, being A_i and A_f the initial and final absorbances respectively.

Inhibition of bacterial pathogens

Study of bacterial pathogens inhibition was performed according to Moore *et al.* (2013). A 10 µl loop of a two day culture on MRS agar of LAB strains were streaked on a line on MRS agar and incubated at 37 °C, 5% CO₂ for 24 hours. After this period, a 10 µl loop of overnight cultures from the pathogens strains were streaked perpendicular to the LAB strains lines. The plates were incubated for 37 °C during 48 hours in aerobic conditions. The inhibition zones were measured in centimeters.

Exclusion and displacement assays

The Caco-2 cell line were cultivated in complete Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with heat-inactivated fetal bovine serum (10% v/v), 2,5 µg/ml streptomycin/amphotericin, 2 mM L-glutamine, 1% non-essential amino acids. The medium was replaced every two days. Cells were grown at 37°C, 5% CO₂, 95% humidity in T25 flasks. After 6 days, cells were washed with PBS buffer and transferred into the culture medium without antibiotic solution. Cell were grown for another two days until 80% of confluence to be used for adhesion experiments.

Bacterial assays on Caco-2 cells were evaluated using 24-well plates. Suspensions of LAB and pathogens strains were prepared with DMEM medium.

For the exclusion assays, bacterial cells were washed twice with PBS. A volume of 200 µl of LAB suspension (1×10^8 ufc/ml) was added to each well and the plates were incubated at 35 °C for 45 min. After that period, a volume of 200 µl of pathogen bacterial suspension (1×10^8 ufc/ml) was added to each well. After 45 min of incubation, the monolayers were washed two times with PBS to eliminate unbound bacteria. Following the last wash, Caco-2 cell monolayers were covered with 200 µl distilled water and mechanically agitated by vigorous pipetting.

For displacement assays, a volume of 200 µl of pathogen bacterial suspension (1×10^4 ufc/ml) was added to each well. The plates were incubated at the same conditions described above. Then, 200 µl (1×10^8 ufc/ml) of LAB suspension was added to each well and incubated for 45 min. The monolayers were washed two times and after the last wash, they were mechanically agitated by vigorous pipetting to detach bacteria and cells.

For both assays, the adherent bacteria were counted on Chromocult agar (Merck) (previously serial dilution) and incubated at 35 °C for 24 hours.

Animals, feeding procedures and infection

Male 6-week-old Balb/c mice were obtained from CERELA and animal studies and protocols were approved by the CERELA Ethical Committee of Animal Care. Mice were housed in plastic cages at room temperature. Animals were housed individually during the experiments and the assays for each parameter studied were performed in 5–6 mice per group. *L. curvatus* TUCO-5E were administered to different groups of mice for 5 consecutive days at dose of 10^7 , 10^8 or 10^9 cells/mouse/day in the drinking water. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*. Treated and control mice were challenged with 50 μ l of 10^7 cells/ mouse of *S. typhimurium* (20LD50) by oral administration. Mice were sacrificed on days 2 post-infection. The liver and spleen of mice in the different experimental groups were removed. Organs were homogenized in 0.1% peptone water, diluted appropriately and plated in MacConkey agar. Plates were incubated at 37°C for 48 h. Results were expressed as the number (log) of CFU/g of organ. Bacteremia was monitored by blood samples obtained by cardiac puncture which were plated on MacConkey agar. Results were expressed as the number (log) of CFU/ml of blood.

Statistical analysis

Experiments were performed in triplicate and results were expressed as mean $\pm$ standard deviation (SD). After verification of the normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $p < 0.05$.

Results

Isolation and identification of bacterial strains

The aim of the present study was to isolate and identify potential probiotic LAB from swine milk. A total of 47 different bacterial colonies were isolated from 10 samples of swine milk. Among these, 10 strains showed the capacity to grow in MRS broth at pH 3. Preliminary studies evaluating the capacity of LAB strain to inhibit *E. coli* ATCC23922 allowed us to select two strains for further research: TUCO-7A and TUCO-5E. The two bacterial strains were Gram+ bacilli (Figure 1). API analysis and molecular biology studies allow us to determine that both strains belong to genus *Lactobacillus* (data not shown). Moreover, 16s RNA sequencing indicated that the strain TUCO-7A showed a 99.4% of identity with *Lactobacillus brevis* ATCC14869 (GeneBank: M58810) while strain TUCO-5E showed a 99.7% of identity with *Lactobacillus curvatus* ATCC25601 (GeneBank: M113777). Therefore, the strains were designated as *Lactobacillus brevis* TUCO-7A and *Lactobacillus curvatus* TUCO-5E.

Antibiotic resistance and safety evaluation of selected strains

An important requirement of probiotic strains is that they should not take genes that confer antibiotic resistance. Then, the antibiotic susceptibility of the two selected strains was determined using various antibiotic agents. The results showed that the strains were susceptible to amoxicillin, ampicillin, gentamicin, and erythromycin while both lactobacilli were resistant to vancomycin (Table 2). *L. curvatus* TUCO-5E was susceptible to ciprofloxacin while *L. brevis* TUCO-7A was resistant to that antibiotic. In addition, results showed that TUCO-7A strain was moderately susceptible to doxycycline (Table 2).

Absence of haemolytic activity is also considered as a safety prerequisite for the selection of a probiotic strain. None of the examined strains exhibited α or β -haemolytic activity when grown in Columbia human blood agar (Table 3). Additionally, the two lactobacilli were negative for the detection of gelatinase activity (Table 3).

Studies on resistance to gastrointestinal conditions and hydrophobicity

L. curvatus TUCO-5E and *L. brevis* TUCO-7A were tested for their ability to grow in presence of bile salts (0.3, 0.6, 2, 3 and 5 % w/v) and NaCl (3, 6.5 and 9 % w/v). Both strains were found to be resistant to bile salts even after several hours of exposure. Lactobacilli retained their viability as they were recovered on MRS agar after the exposure of the conditions described above (Table 4). In addition, we observed that TUCO-5E and TUCO-7A strains only resisted a 3 % of NaCl (Table 4). The study of hydrophobicity showed that and *L. brevis* TUCO-7A presented a medium cell surface hydrophobicity while *L. curvatus* TUCO-5E strain showed a high value in this parameter (Table 4).

Inhibition of bacterial pathogens

As we mentioned previously, the two strains were able to inhibit the growth of *E. coli* ATCC23922 in a similar trend (Table 5). The capacity of these strains to inhibit the growth of pathogenic *E. coli* and *Salmonella* was also evaluated. As shown in Table 5, the two strains effectively reduced the growth of enterotoxigenic *E. coli* UCO-I5 (ETEC) and enterohemorrhagic *E. coli* TUCO-I6 (EHEC). *L. curvatus* TUCO-5E showed the most remarkable effect against EHEC. The two strains were also capable of reducing the growth of *Salmonella* TUCO-I7 and *Salmonella enterica* ATCC 13096 (Table 5), being the TUCO-5E strain the one with the highest activity. Neutralization of acid did not reduce the capacity of lactobacilli to inhibit pathogens growth indicating that other factors rather than lactic acid were the responsible for the beneficial affect (data not shown).

Exclusion and displacement assays

We further analyzed whether *L. brevis* TUCO-7A or *L. curvatus* TUCO-5E were able to protect intestinal epithelial cells against bacterial pathogens. The exclusion assay showed that *L. brevis* TUCO-7A was able to reduce bacterial cell counts of *S. enterica* ATCC 13096. Interestingly, *L. curvatus* TUCO-5E significantly reduced counts of pathogenic *E. coli* and *Salmonella* (Figure 2). In addition, displacement assays showed no capacity for *L. brevis* TUCO-7A to reduce intestinal pathogens counts, on the contrary, *Salmonella* TUCO-I7 and *S. enterica* ATCC 13096 cells counts were significantly reduced in cells treated with *L. curvatus* TUCO-5E (Figure 2). Then, considering these results, *L. curvatus* TUCO-5E was selected for further studies on the protection against *Salmonella* infection.

In vitro and in vivo anti-Salmonella effect of L. curvatus TUCO-5E

The capacity of *L. curvatus* TUCO-5E to reduce *Salmonella* infection was studied *in vitro* by using electron microscopy (Figure 3). Photographs show the integrity of cell monolayers before pathogen challenge and a significant alteration of monolayer integrity after 45 minutes of *Salmonella* infection. The epithelium is disrupted and some cell lost the attachment to the wells and developed a round shape. In addition, *Salmonella* infection induced ruffles and microvilli disruption. On the contrary, in *L. curvatus* TUCO-5E-treated cells, most monolayer conserved the structure and only few round shape cells were observed (Figure 3). Moreover, TUCO-5E-treated cells showed minor surface alterations.

Finally we evaluated *in vivo* the effect of TUCO-5E strain by using a mice model. *L. curvatus* TUCO-5E was administered to different groups of mice for 5 consecutive days at doses of 10^7 , 10^8 or 10^9 cells/mouse/day in the drinking water, and orally challenged with pathogenic *Salmonella typhimurium* as described previously (Salva *et al.* 2010). The administration of *L. curvatus* TUCO-5E in a dose of 10^7 did not induce significant modifications in the response of mice to *Salmonella* infection. On the contrary, TUCO-5E administered in doses of 10^8 or 10^9 for 5 days induced a

significant decrease in the number of *S. typhimurium* in the liver and spleen of treated mice when compared to controls (Figure 4). Besides, in the animals that received 10⁹ cells of *L. curvatus* TUCO-5E, the pathogen was not detected in the blood (Figure 4).

Discussion

Prerequisites for the healthy and efficient growth of young pigs are the rapid maturation of the gut mucosa and the mucosa-associated lymphoid tissue, and the formation of a local stable and complex bacterial community (Bosi and Trevisi, 2010). Therefore, in the past twenty years, an intensive amount of research has been focused on the development of alternatives to antibiotics to maintain swine health and performance and the most widely researched alternatives include probiotics and prebiotics (Thacker 2013, Villena and Kitazawa 2014, Suda 2015). On the other hand, it was reported that human neonatal gut microbiota establishment represents a crucial stage for gut maturation, metabolic and immunologic programming, and consequently short- and long-term health status. Human milk beneficially influences this process due to its dynamic profile of age-adapted nutrients and bioactive components and by providing commensal maternal bacteria to the neonatal gut. These include *Lactobacillus* spp., as well as obligate anaerobes such as *Bifidobacterium* spp., which may originate from the maternal gut via an entero-mammary pathway as a novel form of mother-neonate communication (Bergmann *et al.* 2014; Jost *et al.* 2015). Therefore, current research is focusing on human milk bacteria as a source of potential probiotics strains for disease prevention in children. In neonatal pigs, suckling and the maternal environment shape the gut microbiota. Later, when weaning stress causes a transient drop in favorable bacteria, the oral supply of microbes could contribute to re-establish the microbiota balance (Bosi and Trevisi, 2010). Then, similar to human milk, we hypothesized that swine milk could be and excellent source for potential probiotic strains with the capacity to improve protection against bacterial gastrointestinal pathogens in pigs.

Here, we isolated several LAB strains from swine milk and evaluated their potential as probiotics. Among the isolated strains, *L. curvatus* TUCO-5E showed several characteristics that would allow it to be used as a swine probiotic. To remain and exert probiotic potential within their host, several factors are usually considered. Probiotic strains must possess the ability to overcome the extremely low pH of gastric juice and the detergent effect of bile salts and arrive at the site of action in a viable physiological state (Kim *et al.* 2007). *In vitro* experiments demonstrated that *L. curvatus* TUCO-5E posse those characteristics.

In addition, an important requirement of probiotic strains is their safety, as they should not take genes encoding detrimental factors such as haemolysins or genes that confer antibiotic resistance (Kim *et al.* 2007). We demonstrated that *L. curvatus* TUCO-5E is a safe strain since no α or β -haemolytic or gelatinase activities were shown for this strain, and no side effects were observed after its administration to adult mice (data not shown). Notably, the TUCO-5E strain is resistant to vancomycin, supporting the native resistance of several lactobacilli species to vancomycin which is consistent with previous reports (Liu *et al.* 2009; Temmerman *et al.* 2002; Danielsen and Wind, 2003, Zhou *et al.* 2005).

Weaning-associated intestinal inflammation occurs in various animal species including the pig. Intensification of the pig industry has brought increased risks of both clinical and sub-clinical enteric disease. Piglets are vulnerable to potentially harmful microorganisms such as pathogenic *E. coli*, and *Salmonella* spp. (Williams *et al.* 2001).

Several studies reported the ability of probiotics to improve resistance against pathogenic *E. coli*. Probiotic LAB are able to reduce ETEC counts in the gut, decrease diarrhea (Qiao *et al.* 2004; Herfel *et al.* 2013) and beneficially modulate the inflammatory response against this intestinal pathogen (Li *et al.* 2012; Shimazu *et al.* 2012; Villena *et al.* 2012; Tomosada *et al.* 2013). Those beneficial effects of probiotics allow an improvement in the growth and health performance of weaning pigs (Li *et al.* 2012; Suda *et al.* 2014). We showed here that *L. curvatus* TUCO-5E significantly inhibit the growth of two pathogenic *E. coli* strains: TUCO-I5 and TUCO-I6 that are

EHEC and ETEC respectively. Moreover, *L. curvatus* TUCO-5E was able to significantly reduce EHEC and ETEC bacterial cell counts when evaluated in exclusion assay using an intestinal epithelial cell line. Then, similarly to other probiotic lactobacilli, TUCO-5E would have the potential to induce protection against ETEC and EHEC. In this work, we were not able to develop a mice model to test *in vivo* the protective effect of *L. curvatus* UCO-5E against pathogenic *E. coli*. Those studies are an important point for future near research.

In addition ETEC and EHEC, *Salmonella* infection is common intestinal pathogen in pigs, especially in the weaning stage (De Busser *et al.* 2013). *Salmonella* outbreaks and subclinical infections are often not only the cause of economic and animal welfare costs, but also a source of contamination of pork products entering the food chain since it has long been recognized that swine may be asymptomatic carriers of *Salmonella* (Funk *et al.* 2004). Therefore, several studies also focused in the use of probiotic microorganisms to control *Salmonella* infection in pig and some interesting results have been reported. It was reported that *L. zeae*- or *L. casei*-fermented feeds are able to reduce severity of diarrhea in pigs after the challenge with *Salmonella* and, that those effects were related to improvements in the inflammatory response (Yin *et al.* 2014). Further, Rieger *et al.* (2015) showed that *E. faecium* NCIMB is able to increase intraepithelial lymphocytes numbers in weaned pigs and enhance protection against *Salmonella enterica* serovar Typhimurium. These studies demonstrate that is possible to modulate pigs' resistance against *Salmonella*. We showed here that *L. curvatus* TUCO-5E was capable of reducing the growth of *Salmonella* and protect intestinal epithelial cells against this bacterial pathogen. Moreover, by using an infection mice model (Salva *et al.* 2010), we demonstrated that the administration of *L. curvatus* TUCO-5E induced a significant decrease in *S. typhimurium* counts in infected organs and blood when compared to controls, demonstrating *in vivo* the protective effect of TUCO-5E strain. Although it should be tested in the porcine host, these results suggest that *L. curvatus* TUCO-5E feed supplementation could be used for the prevention of intestinal infections in the pig. In addition, the precise mechanism of action of TUCO-5E strain has to be clarified.

389 Reports highlighting the positive effects of probiotics on the performance of pigs or on *in*
390 *vitro* traits are now quite frequent, but the use of probiotics in feed compounds has not been
391 widespread (Bosi and Trevisi, 2010). Therefore, the search of probiotic strains and the development
392 of functional feeds for the porcine host is still a challenge. In this regard, there is a need for
393 development of alternative control methods to the antibiotics used in feed to protect the animals
394 from gastrointestinal infectious diseases. One of the alternative methods is biological control by
395 probiotics. We demonstrated here that swine milk is an interesting source for finding beneficial
396 bacteria. In addition, the results of this work suggest that *L. curvatus* UCO-5E is a good candidate
397 for *in vivo* studying the protective effect of probiotics against intestinal infection and damage
398 induced by *Salmonella* infection in the porcine host.

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Figure legends

Figure 1. Characterization of *Lactobacillus* strain. A) *Lactobacillus brevis* TUCO-7A strain is a Gram positive bacterium with short rods shape and white and star shaped colony in MRS agar. B) *Lactobacillus curvatus* TUCO-5E strain is a Gram positive bacterium with rods shape and white pearl like colony in MRS agar. Photos represent data from three independent experiments.

Figure 2. Exclusion and displacement assays. Effect of *Lactobacillus brevis* TUCO-7A and *Lactobacillus curvatus* TUCO-5E on bacterial cell counts of intestinal pathogens: *E. coli* TUCO-I5 (EHEC), *E. coli* TUCO-I6 (ETEC), *Salmonella* TUCO-I7 and, *Salmonella enterica* ATCC 13096. The results represent data from three independent experiments. Asterisks indicate significant differences * ($P < 0.05$).

Figure 3. Electron microscopy analysis. Effect of on Caco-2 epithelial cells protection against *Salmonella enterica* ATCC 13096. A) uninfected monolayer, B-C) epithelial cell monolayer after 45 minutes of *Salmonella* infection, D) epithelial cell monolayer treated with *L. curvatus* TUCO-5E and then infected with *Salmonella*. Photos represent data from three independent experiments.

Figure 4. *In vivo* protective effect of *Lactobacillus curvatus* TUCO-5E. *L. curvatus* TUCO-5E was administered to different groups of mice for 5 consecutive days at dose of 10^7 , 10^8 or 10^9 cells/mouse/day in the drinking water. Treated and control mice were challenged with 10^7 cells/mouse of *S. typhimurium* (20LD50) by oral administration. Mice were sacrificed on days 2 post-infection. Liver, spleen and blood samples were taken for bacterial cell counts. The results represent data from three independent experiments. Asterisks indicate significant differences * ($P < 0.05$), ** ($P < 0.01$).

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Lactobacillus brevis UCO-7A

Lactobacillus curvatus UCO-5E

213x177mm (150 x 150 DPI)

Displacement assay

Uninfected control cells

Salmonella infected cells

Salmonella infected cells

L. curvatus TUCO-5E + *Salmonella*

10^7 cells

10^8 cells

10^9 cells

Control *L. curvatus* TUCO-5E

Table 1. Primers used for the identification of lactobacillus species.

<i>Lactobacillus</i> spp.	Primers	Sequences
<i>L. acidophilus</i>	Aci 16SI	5' AGC TGA ACC AAC AGA TTC AC
	16SII	5' ACT ACC AGG GTA TCT AAT CC
<i>L. crispatus</i>	Cri 16SI	5'GTA ATG ACG TTA GGA AAG CG
	Cri16SII	5'ACT ACC AGG GTA TCT AAT CC
<i>L. gasseri</i>	Gas I	5'GAG TGC GAG AGC ACT AAA G
	Gas II	5'CTA TTT CAA GTT GAG TTT CTC T
<i>L. johnsonii</i>	Joh 16SI	5'GAG CTT GCC TAG ATG ATT TTA
	Joh 16SII	5'ACT ACC AGG GTA TCT AAT CC
<i>L. plantarum</i>	Lfpr	5'GCC GCC TAA GGT GGG ACA GAT
	PlanII	5'TTA CCT AAC GGT AAA TGC GA
<i>L. casei</i>	PrI	5'CAG ACT GAA AGT CTG ACG G
	CasII	5'GCG ATG CGA ATT TCT TTT TC
<i>L. zeae</i>	ZeaI	5'TGT TTA GTT TTG AGG GGA CG
	ZeaII	ATG CGA TGC GAA TTT CTA AAT T
<i>L. rhamnosus</i>	PrI	5'CAG ACT GAA AGT CTG ACG G
	RhaII	5'GCG ATG CGA ATT TCT ATT ATT
<i>L. reuteri</i>	Lfpr	5'GCC GCC TAA GGT GGG ACA GAT
	Reu	5'AAC ACT CAA GGA TTG TCT GA
<i>L. fermentum</i>	Lfpr	5'GCC GCC TAA GGT GGG ACA GAT
	Fermi	5'CTG ATC GTA GAT CAG TCA AG
<i>L. sharpeae</i>	ShaI	5'GAT AAT CAT GTA AGA AAC CGC
	ShaII	5'ATA TTG TTG GTC GCG ATT CG
<i>L. salivarius</i>	Lsal I	5'ATT CGC TAA ACT CAT AAC CT
	Lsal II	5'CAC TCT CTT TGG CTA ATC TT

Table 2. Antibiotic resistance of lactic acid bacteria from swine milk

Antibiotic	Concentration	<i>Lactobacillus</i>	<i>Lactobacillus</i>
		<i>brevis</i> TUCO-7A	<i>curvatus</i> TUCO-5E
Ciprofloxacin	5 µg	Resistant	Susceptible
Vancomycin	30 µg	Resistant	Resistant
Amoxicillin	25 µg	Susceptible	Susceptible
Ampicillin	10 µg	Susceptible	Susceptible
Gentamicin	10 µg	Susceptible	Susceptible
Erythromycin	15 µg	Susceptible	Susceptible
Doxycycline	30 µg	Intermediate	Resistant

Table 3. Hemolysins and gelatinases detection in lactic acid bacteria from swine milk

	<i>Lactobacillus</i> <i>brevis</i> TUCO-7A	<i>Lactobacillus</i> <i>curvatus</i> TUCO-5E
α -haemolysis	Negative	Negative
β -haemolysis	Negative	Negative
gelatinase activity	Negative	Negative

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Table 4. Cell surface hydrophobicity and resistance to gastrointestinal conditions of lactic acid bacteria from swine milk

	<i>Lactobacillus brevis</i>	<i>Lactobacillus curvatus</i>
	TUCO-7A	TUCO-5E
NaCl (%)		
3.0	+++	+++
6.5	++	+
9.0	+	+
Oxgall (%)		
0.3	+++	+++
0.6	+++	+++
2.0	+++	+++
3.0	+++	+++
5.0	+++	+++
Hydrophobicity (%)	47.4 (medium)	87.2 (high)

Low growth: +, Medium growth: ++, High growth: +++

Low: 0 – 35%, Medium: 36 – 70%, High: 71 – 100.

Table 5. Inhibition of bacterial pathogens by lactic acid bacteria from swine milk

Strain	<i>E. coli</i> ATCC 23922	<i>E. coli</i> TUCO-I5 (EHEC)	<i>E. coli</i> TUCO-I6 (ETEC)	<i>Salmonella</i> TUCO-I7	<i>S. enterica</i> ATCC 13096
<i>Lactobacillus</i>					
<i>brevis</i> TUCO-7A	2.00 ± 0.23	1.53 ± 0.36	1.98 ± 0.19	2.23 ± 0.39	2.38 ± 0.21
<i>Lactobacillus</i>					
<i>curvatus</i> TUCO-5E	1.98 ± 0.33	1.78 ± 0.28	1.93 ± 0.30	2.88 ± 0.10	3.03 ± 0.22