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Sequencing and evaluation of the antimicrobial effect of lactic acid bacteria against strains of *Salmonella Tiphymurium* isolated from intensive and extensive pig production

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SUMMARY

The intensive and extensive production of pigs in Argentina and Spain, respectively, present different production factors. However, when dealing with pathogen threats, specifically *Salmonella*, both are affected by them. *Salmonella* is a common pathogen in food related to food outbreaks that cause serious problems in public health. Lactic acid bacteria, specifically the genera *Lactobacillus* and *Pediococcus*, have been characterized in the past few years for being of great importance as antagonists of different pathogenic bacteria. One control alternative is the application of *Lactobacillus* throughout the pig production chain, thus preventing the arrival of this pathogen to consumers. A preliminary assessment was made of the antagonistic capacity of lactic acid bacteria identified after sequencing as *Lactobacillus plantarum* and *Pediococcus acidilactici* against *Salmonella* Tiphymurium isolated from intensive pig production in Argentina, and *Salmonella* Tiphymurium isolated from extensive pig production in Spain. The results obtained showed the high antimicrobial capacity of the LAB selected against both pathogenic strains (93.75%). Thus, they can be proposed as potential biopreservative bacteria in the production of pig meat and its subproducts.

Palabras clave adicionales

Pediococcus.
Lactobacillus.
Salmonella Typhimurium.
Control biológico.

Secuenciación y evaluación del efecto antimicrobiano de bacterias acidolácticas frente a cepas de *Salmonella* Tiphymurium

RESUMEN

La producción intensiva y extensiva de cerdo en Argentina y España respectivamente presenta factores productivos diferentes. Sin embargo, cuando se trata de amenazas ante patógenos, específicamente *Salmonella*, ambos se ven afectados. *Salmonella* es un patógeno común en los alimentos relacionado con las toxiinfecciones alimentarias que provoca graves problemas en la salud pública. Las bacterias acidolácticas, específicamente los géneros *Lactobacillus* y *Pediococcus* se han caracterizado en los últimos años por tener gran importancia como antagonistas de diferentes bacterias patógenas. Una alternativa de control es la aplicación de *Lactobacillus* a lo largo de la cadena productiva de cerdo, evitando así la llegada de este patógeno a los consumidores. Se realizó una evaluación preliminar de la capacidad antagónica de bacterias acidolácticas identificadas tras secuenciación como *Lactobacillus plantarum* y *Pediococcus acidilactici* ante *Salmonella* Tiphymurium aislada de producción intensiva de cerdo de Argentina y *Salmonella* Tiphymurium aislada de producción extensiva de cerdo en España. Los resultados obtenidos reflejaron una alta capacidad antimicrobiana de las BAL seleccionadas ante ambas cepas patógenas (93.75%). De esta manera, se proponen como potenciales bacterias biopreservadoras en la producción de carne de cerdo y subproductos.

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INTRODUCTION

Pig production systems vary according to the region, pig raising method, climate, breeds, economic possibilities, production regulations and, above all, the culture and preferences of consumers. All these factors guide producers towards doing their work in a certain way.

Thus, in Spain, extensive Iberian pig production is based on natural means and without being governed by production programs, important infrastructures or external components (treatments, supplements) that

change or compromise the pigs' quality of life (Bolancé García, 2012). Their food is based on taking advantage of nuts, especially acorns obtained from the Holm, cork and gall oak trees, wild fruit, roots and grass. The older the animal, the greater the quality that is transferred to the final product. The pig is sacrificed at from 18 months to 2 years of age with a live weight of 160 to 180 kg.

In the other hand, in Argentina, the production system is intensive and the principal breed is the Landrace, that proliferates with a good carcass yield and low fat values. Over there, production has considerably varied during the past few years. The arrival of technified intensive raising systems has obliged producers to apply biosafety and disease prevention plans for their production to be profitable and sustainable. Any effective internal biosafety program should contain the cleaning, disinfection and complete dry-out of facilities for groups, with a depopulation process of at least four days. In this way, the probability of the pathogenic agents surviving in the environment is reduced (SENASA, 2018). Nutrition in this system is based on balanced food. Conversion into optimal food happens at around 6 to 7 months old and the animal is sacrificed with 100 to 120 kg live weight, lower values than those of the Iberian pig.

In both production systems pathogenic bacteria signify a hazard for the sector. Of the greatest concern is *Salmonella*, this being the potential causal agent of food-borne diseases. The genus Salmonella is constituted by two species: *S. enterica* and *S. bongori*, within which over 2,400 serotypes (Echeita, 2005) have been described. Zoonotic serovarieties have been defined as *Salmonella* (non-Typhi), and they are widely spread over the animal kingdom, with birds, pigs and their derivatives being the most common sources of infection and the cause of bouts of food-borne diseases (FBD). In most cases they may present themselves as self-limited gastroenteritis but, in some cases, the invasion of microorganisms may cause septicemia (Uribe, 2013).

In the two production systems mentioned above, serotypes virulent for humans have been isolated. For that reason, the need arises to contemplate other mechanisms to control these bacteria without employing chemical hygienization products. Lactic acid bacteria (LAB) represent a group of bacteria with different food uses, *Lactobacillus* and *Pediococcus* being some of its representative genera. The ability to metabolize sugars in lactic acid triggers an acidic environment, which inhibits the growth of pathogenic bacteria, as well as also the production of antimicrobial metabolites like hydrogen peroxide, diacetyl and diverse bacteriocins, among others. For that reason, they are being implemented as a biopreservation alternative in multiple food production systems. Pig farm production, whether it be intensive or extensive, is not exempt from this situation. In this study, it is proposed to analyze, after sequencing them to confirm their identification and as a first approach to pathogen control, the inhibitory effect of strains of lactic acid bacteria from pigs against *Salmonella* Tiphymurium isolated from intensive pig production in Argentina (*S*. Tiphymurium AR), and *Salmonella* Tiphymurium isolated from extensive pig production in Spain (*S*. Tiphymurium ES).

MATERIAL AND METHODS

Bacterial strains and culture conditions

Eight strains of LAB isolated from the pig chain production were selected for this study. Isolations were obtained from the farm (gestation, farrowing, weaning and growing/finishing), carcasses reception room, slaughterhouses, sausage preparation room and boning room. The origins of the strains were rectal swab, sausages raw material, and final product. LAB strains were supplied by the Laboratory of Immunochemistry and Biotechnology of the Faculty of Veterinary Sciences of Tandil, Bs. As., Argentina (Ruiz et al., 2017). The LAB strains were cultured in MRS broth (Man, Ragosa and Sharp, Oxoid) at 37°C for 24 h and kept as glycerol stocks until later use in the Laboratory of Food Microbiology sited at Department of Food Sciecne Technology of the University of Córdoba, Spain.

The bacterial pathogens used in this study were two strains of *S*. Tiphymurium. *S*. Tiphymurium AR was isolated from an intensive pig production system (80 pigs) by the Immunochemistry and Biotechnology Laboratory of the Faculty of Veterinary Medicine of at the University Center of the Province of Buenos Aires, Argentina. And *S*. Tiphymurium ES was supplied by the Department of Animal Health of the Faculty of Veterinary Medicine of at the University of Córdoba, Spain. This strain was isolated from an Iberian pig in a system of extensive production. *S*. Tiphymurium AR and *S*. Tiphymurium ES were grown in XLD broth (Xylose, Lysine, Deoxycholate) at 37°C for 24 h.

Identification of LAB by Sanger Sequencing

LAB identification by Sequencing was done in the Central Research Support Service (SCAI) of the University of Córdoba, Spain. DNA was extracted from 1.5 ml culture using the Higher Purity Bacterial Genomic DNA Isolation Kit (Canvax Ref. AN0067). For PCR analysis, 25 ng of DNA are taken to amplify by PCR the 16S ribosomal DNA with the universal primers: 16SF 5'-AGAGTTTGATCCTGGCTCAG-3' and 16SRv: 5'-GAAAGGAGGTGATCCAGCCG-3' (Sigma).

The reaction was performed in 20 µl total volume with 1x reaction buffer, 2.5 mM MgCl 2, 100 µM of each dNTP, 0.25 µM of each primer, 1% DMSO and 1 U of Taq Polymerase BioTaq (BioTools).

The amplification program consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, and a final extension of 7 min at 72°C.

The PCR reactions were purified by precipitation with AcNa/EtOH and resuspended in 15 µl of sterile mQ water. 6 µl of the purified DNA was taken for each sequencing reaction (16SFw and 16SRv).

The sequences obtained from each isolate were aligned to obtain the complete 16S ribosomal sequence (Megalign program of DNAstar) and analyzed by

BLAST in the GeneBank database (https://blast.ncbi.nlm.nih.gov).

Inhibition assay

The assay was performed following the stabbing inoculation procedure proposed by Gagliano and Hinsdill (1970), with modifications based on the study of Schillinger and Lücke (1987). The strains *P. acidilacitici* and *L. plantarum* were sown by stabbing on plates of MRS agar plates, and then incubated at 37ºC during 24 h under anaerobiosis conditions. The plates were exposed to fumes of chloroform for 1 hour to inactivate the bacteria that might have grown. Later, they were covered with a weak layer of xylose-lysine-deoxycholate agar (XLD) (0.75% agar) containing the pathogenic microorganisms (*S*. Tiphymurium AR and *S*. Tiphymurium ES) on which their inhibitory activity was assessed. The reading was performed by the observation of translucid areas indicating the inhibition of the pathogens' growth due to the LAB action. The presence of inhibition halos of over 1 mm around the stab cultures was a positive test (Daeschel and Klaenhammer, 1985).

RESULTS

Seven of the eight LAB strains sequenced were identified as *Lactobacillus plantarum* (strains 2, 3, 4, 5, 6, 7 and 8). One strain was identified as *Pediococcus acidilactici* (strain 1). All the identifications presented a homology of 99 to 100%.

The analysis of the inhibitory effect against *S*. Tiphymurium AR was positive in 7 of the 8 LAB, which represents 87.5%. Regarding the inhibitory effect against *S*. Tiphymurium ES, 8 positive inhibition halos were obtained, i.e. 100% of the strains studied (**Tables I** and **II**). **Table II** includes percentages of strains in terms of their inhibition potential (low, medium or high), taking as a reference the largest halo obtained.

DISCUSSION

Many studies have isolated LAB from pork like present study. Rodríguez-Membibre (1995) used *L. acidophilus* isolated from pig colon mucosa to determine its effect on animal production. Gámez et al. (2009) characterized the antibacterial activity of *L. plantarum* of the same origin and Verso et al. (2017) showed that intestinal pig LAB can produce antibacterial substances capable of inhibiting the growth of opportunistic enteric pathogens and could have potential as probiotic additives to prevent gastrointestinal diseases as an alternative or complement to the use of antibiotics. Jurado et al. (2009) and Zea et al. (2015) have isolated and characterized strains of the large intestine and colostrum of sows respectively to determine their bactericidal activity against *S.* Typhimurium. Dowarah et al. (2018) determined the probiotic properties of LAB isolated from piglet feces and concluded that *P. acidilactici* has potential in vitro probiotic characteristics.

Table I. Inhibitory effect of LAB strains studied against *S*. Tiphymurium AR and *S*. Tiphymurium ES (Production costs composition of the ostrich farm, in %, for condition incubation of eggs on their own or outsourced hatchery).

LAB isolates		ZDI (mm) for *S.* Tiphymurium	
		S. Tiphymurium AR	*S.* Tiphymurium ES
1	*P. acidilactici*	9 (40.91)*	15 (68.18)*
2	*L. plantarum*	7 (31.82)*	22 (100)*
3	*L. plantarum*	9 (40.91)*	18 (81.82)*
4	*L. plantarum*	8 (36.36)*	17 (77.27)*
5	*L. plantarum*	0 (0.00)*	14 (63.64)*
6	*L. plantarum*	8 (36.36)*	12 (54.55)*
7	*L. plantarum*	13 (59.09)*	13 (59.09)*
8	*L. plantarum*	12 (54.55)*	7 (31.82)*

ZDI: zone diameter of inhibition.
* Percentage of inhibition potential.

Identification in the genus and species of LAB has an increasing application in the food industry. It is one of the first steps for its characterization as probiotic bacteria to elaborate functional foods in great demand due to its beneficial properties in animals and humans. The basic biochemical tests allow a first approximation to the taxonomic group. The phenotypic characterization could be more specific if biochemical criteria based on macro and micro morphological, physiological and complete biochemical characteristics were used. But the possibility of using molecular techniques allows LAB to be identified at the species level reliably, accurately and in less time.

Numerous studies have shown the antagonistic activity of LAB against various pathogens. The LAB possessing the capacity to alienate bacterial pathogens through the production of some antimicrobials, such as H_2O_2, organic acids (mainly, lactic acid), bacteriocin, acquire the enviable property for probiotic potentiality and are a maintainable substitute for synthetic antibiotics. Ji Yeu Kim et al. (2015) demonstrated the antimicrobial activity of species of *Lactobacillus* and *Leuconostoc* against *Salmonella*. In another work, Murry et al. (2004) studied the inhibition of pathogens, among them *Salmonella*, due to the potential lactic and acetic acids produced by *L. plantarum* and *L. salivarius*. The lactobacilli strains, including *L. rhamnosus* and *L. plantarum* (procured from commercially available yoghurt and cheese) and rumen contents of cow, did not show growth inhibitory activity against *E. coli*; while *Salmonella menston* was found to be sensitive to all the test lactobacilli (Jose et al., 2015). The probiotic Lactobacillus strains: *L. fermentum*, *L. casei* and *L. acidophilus*, isolated from buffalo milk showed growth inhibitory activity against *Vibrio cholerae*, *S. typhi*, *E. coli*, and *Shigella* species having ZDIs 10–22 mm (Rahman et al., 2015). Many lactobacilli isolates, including *L. plantarum* obtained from traditional fermented foods prepared with the combination of cereal and dairy materials, had an excellent antibacterial activity against *E. coli* with ZDIs 20 mm (Mashak et al., 2016).

In this study, the antibacterial action of the LAB was confirmed, specifically with the genera *Pediococcus* and *Lactobacillus*. The ZDIs against *S*. Tiphymurium AR was 8.25 mm on average, whereas the ZDIs against *S*. Tiphymurium ES were, on average, 14.75 mm. A difference was observed between the ZDIs against the two strains of *S*. Tiphymurium, with the strain isolated from extensive production (*S*. Tiphymurium ES) being the most sensitive one. The answer may lie in the resistance acquired by the pathogens throughout the time

Table II. Efficacy (%) of LAB strains studied against *S*. Tiphymurium AR and *S*. Tiphymurium ES (Production costs composition of the ostrich farm, in %, for condition incubation of eggs on their own or outsourced hatchery).

	S. Tiphymurium AR	*S*. Tiphymurium ES
Positive	87.50%	100%
Low potential[a]	25.00%	12.50%
Medium potential[b]	75.00%	37.50%
High Potential [c]	0.00%	50.00%

[a] Percentage of between 0 and 7.33

since *S*. Tiphymurium AR and the LAB were isolated from the same production system.

There are several ways to perform inhibition tests. Cadirci et al. (2005) demonstrated in their study that the most effective method in the evaluation of inhibitory activity was the superposition of agar. On the other hand, Rahimifard and Naseri (2016) reported that well diffusion is better for determining antagonism than the other two methods (disc diffusion and spot on the turf), using the probiotic strain *Bifidobacterium bifidum* against *Salmonella enterica* serovar Enteritidis. The procedure used in our study gave us optimal results, presenting high antagonistic levels.

The in vitro assays presented here permit one to make a first approach to an alternative control of these pathogenic bacteria. Although Spain is a leader in the production and exportation of pigs, both countries are experiencing a rise in production, which adds to the importance of the high consumption per inhabitant of pig meat products, and demands a good organoleptic quality and, especially, microbiological safety. The great antagonistic capacity of these LAB against *S*. Tiphymurium is reflected in the results (93.75%). Therefore, the future application of these beneficial bacteria throughout the production chain of pig meat and its subproducts for the biological control of pathogenic bacteria, will allow us to advance towards obtaining safe foods. The inhibition tests carried out indicate that these bacteria can be widely used in the food industry as biopreservatives due to their broad spectrum of inhibition. In this way, the meat products commercialized would be safe, and the probability of contracting food-borne diseases would be reduced, hence favoring Public Health.

CONCLUSIONS

The results obtained reflected the high antimicrobial capacity of the LAB selected against both pathogenic strains (93.75%). Thus, they can be proposed as potential biopreservative bacteria in the production of pig meat and its subproducts.

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