Revista Latinoamericana de Microbiología

Volumen *Volume* **46** **Número** *Number* **1-2** **Enero-Junio** *January-June* **2004**

Artículo:

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Revista Latinoamericana de MICROBIOLOGÍA
ALAM

Vol. 46, Nums. 1-2
January - March. 2004
April - June. 2004
pp. 5 - 11

ORIGINAL ARTICLE

Resistance of *Enterococcus* strains isolated from pigs to gastrointestinal tract and antagonistic effect against *Escherichia coli* K88

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ABSTRACT. The intestinal flora plays an important role in health and wellbeing of different organisms. Indigenous microflora can be innocuous or pathogenic. Consumption of food supplemented with beneficial microorganisms as probiotics provides a good health state and this can be maintained and recovered. Currently, probiotic strains of *Bifidobacterium* and *Lactobacillus* are widely used in humans as well as animals. Swine industry would benefit with the application of probiotics, mainly to overcome diarrheal diseases produced by different causes, as a pathogenic *E. coli* K88. The aim of this work was to isolate strains of *Enterococcus* from gastrointestinal tract of pigs to use them as probiotic. Two strains of *E. faecalis*, 2 of *E. mundii* and 7 of *E. faecium* were isolated with characteristics of resistance to acid pH, tolerance to biliary salts and a high antagonistic activity (>80%) against *E. coli* K88. Based on their characteristics and species affinity, we believe that these strains could be administered to pigs as a probiotic.

Key words: *Enterococcus*, probiotic, acid, biliary salts, antagonism.

RESUMEN. La flora intestinal de los diferentes organismos juega un papel importante en el estado de salud de los individuos. Los microorganismos que forman parte de la flora intestinal pueden ser tanto inocuos como patógenos. Con la ingesta de alimentos adicionados con microorganismos benéficos para el organismo (probióticos), se puede mantener y restaurar un estado favorable de salud. En la actualidad se utilizan cepas de *Bifidobacterium* y *Lactobacillus* como probióticos tanto en humanos como en animales. Una de las áreas con mayor potencial de beneficiarse con la aplicación de probióticos es la industria porcícola, principalmente, para evitar el problema de las enfermedades diarreicas ocasionadas por el patógeno *E. coli* K88. Con base en lo anterior, el objetivo del presente trabajo fue aislar cepas de *Enterococcus* del contenido gastrointestinal de cerdos sanos para utilizarlas a futuro como probióticos en ellos mismos. Se aislaron 2 cepas de *E. faecalis*, 2 de *E. mundii* y 7 de *E. faecium* con características de resistencia a ácido, tolerancia a sales biliares y una fuerte actividad antagónica superior al 80% contra *E. coli* K88. Considerando el principio de afinidad de especie, se encontraron cepas con posible valor probiótico para aplicarse en cerdos.

Palabras clave: *Enterococcus*, probiótico, ácido, sales biliares, antagonismo.

INTRODUCTION

The intestinal flora plays an important role in maintaining health and wellbeing of organisms. Several bacterial species have been identified from vertebrate gastrointestinal tract.[11] From large intestine, 400 to 500 different bacterial species have been isolated.[30] Microorganisms found in gastrointestinal tract could be innocuous or pathogenic. Pathogens do not produce adverse effects in organism where there is an adequate balance between them.[12] Consumption of supplemented food with certain ingredients could benefit the suitable intestinal microbial balance, which will be reflected in a state of favorable health for the organism.[14]

Consumption of fermented foods is being conducted since ancestral times ignoring their benefits.[39] At the beginning of the XIX century, Metchnikoff proposed the existence of a strong association between health and consumption of fermented foods.[17] According these observations, the concept of functional food was developed.[11]

Functional food is defined as those foods products which objective is to contribute with physiological benefits, as well as improving nutritional and microbial balance.[14,28,34] This kind of nutraceutics, have two basic aims: a) to improve some physiological function and b) to diminish risk of diseases.[14]

Functional foods are a prophylactic alternative for obtaining and maintaining a favorable health state in humans and animals.[6] In the pig industry, this is of great importance since one of its main problems is gastrointestinal infection originated by bacteria. *Escherichia coli* K88 is one of the most common causes of infections in pigs.[21] The use of antibiotics is very common in order to treat these infections. One of the problems with this procedure is the development of bacterial resistance. For example, in 1979 less of 1% of *Salmonella* strains isolated from pigs were resistant to penicillin. In 1996, *Salmonella* strains resistant to

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penicillin increased to 34%.[8] Development of resistance to drugs induced by inadequate use implies a constant search of new antibiotics; and this search increases costs of production.[27,41] It is very important to search for an alternative to the use of antibiotics. A kind of functional food is one which is supplemented with probiotics.

Probiotics are live microorganisms established in the gastrointestinal tract and that are able to compete against pathogenic bacteria.[5,14,34] If an overgrowth of pathogens occurs, an intestinal infection appears. There are three proposed mechanisms by which probiotics improve intestinal balance: a) adhesion to intestinal mucus, b) production of inhibitory substances and c) stimulation of immune system.[12,35,36] Several species of probiotic bacteria belong to the *Lactobacillus* and *Bifidobacterium* genera. Currently just one species of the genus *Enterococcus* is used as probiotic.[1-3,6,13,15,25,29]

Probiotics are a feasible solution against gastrointestinal infections. An important number of bacterial strains are used for this purpose.[14] The genus *Enterococcus* could be an alternative in the search for probiotics. In some studies *E. faecium* showed antagonistic effect against some bacteria, and it is of great relevance to look for other species, from the same genus, with probiotic characteristics.[1,2,25]

For a bacterial strain to be considered as probiotic, needs to fulfill several requirements.[5,37] Two important criteria must be considered: to survive the gastrointestinal tract and to have an antagonic effect against pathogenic bacteria.[37] Lab tests that resemble gastrointestinal tract consist in survival of the bacterium in acidic pH and survival in biliary salts.[33] These determinations serve to simulate adverse conditions that the probiotic has to endure, when ingested by oral route until reaching the colon.[16,23] Bacterial antagonism provides information about inhibitory properties against some pathogens.[22] Probiotic bacteria produce inhibitory substances as organic acids, peroxide and bacteriocins.[17,22,42]

The main goal of this work was to characterize the diverse phenotypes of *Enterococcus* strains, isolated from healthy pigs, to determine their survival in acidic conditions and resistance to biliary salts and to evaluate antagonistic effect against *Escherichia coli* K88.

MATERIAL AND METHODS

Sampling. 37 samples from stomach, 66 samples of small intestine and 85 samples of large intestine were collected. Samples were collected from intestine and stomach of pigs taken from a Federal Inspection Type plant of pig meat, in Hermosillo, Mexico. Whole gastrointestinal tract of slaughtered pigs were removed. Selected portions were cut with sterile scissors and placed in bags containing Stuarts media (Difco, México) and transported on ice for their posterior analysis. The laboratory samples were placed in stomacher (Stomacher, IUL) during 2 minutes to release bacterial cells adhered to tissues and were streaked on M17 agar (Difco, México), and incubated at 37° C in atmosphere containing 5% CO_2, for 24 hours. Colonies with typical morphology of *Enterococcus* were reinoculated in the same media, to obtain pure cultures, gram stain and catalase tests among others were done from isolated colonies. Other 5 strains from *Enterococcus* sp with high antagonistic activity against several pathogens isolated from healthy rabbits were donated by Laboratorio de Bacterias Lácticas del Instituto de Agroquímica y Tecnología de los Alimentos, del CSIC, Spain (Perez-Martinez, personal communication).

Bile esculin hydrolysis test. Strains were inoculated on bile esculin agar (Difco, México). Plates were incubated as previously described.[26]

Growth in 6.5% NaCl. Brain Heart Infusion (BHI) media (Difco, México) was supplemented with 6.5% NaCl. Strains were inoculated in BHI and incubated in previously described conditions during 24 hours.[26]

PYR test. L-Pyrrolidonyl-β-naphthylamide was added to a final concentration of 0.01% into Todd Hewitt broth (Sigma, Co.) and dispensed into 96 well microplates. 3 morphologically similar colonies were emulsified into PYR broth and incubated for 2 hours. After this time, 1 drop 0.01% *p*-dimethylaminocinnamaldehyde was added.[26]

Carbohydrate fermentation tests. Carbohydrates analyzed were: arabinose, glucose, inositol, inulin, mannitol, melibiose, melezitose, lactose, raffinose, ribose, sorbitol, sucrose and trehalose. Fermentation tests were made in tryptone, peptone and yeast broth (Difco, México) supplemented with 1% carbohydrate and bromocresol purple as indicator.[20]

Qualitative test of survival to gastrointestinal tract. Previous probiotic characterization studies reported the gastrointestinal tract test only in a quantitative way.[16,33] In the present study, with the purpose of optimization of resources, a qualitative test was made as it will be described:

Strains were inoculated in M17 broth and incubated at 37° C in an atmosphere containing 5% CO_2 for 24 hours. M17 agar was inoculated from this culture (Plate A). The rest of the culture in suspension was centrifuged at 2600 x g (Beckman GS 6R), during 30 minutes, 4° C, supernatant discarded and pellet resuspended in acid M17 broth (M17 broth and 4N HCl until pH 3.0). Acid culture was incubated for 1 hour (Maximum time of meals in pig stomach).[43] From this culture a M17 agar plate was inoculated (Plate B). Both plates (A and B) were incubated as previously described. Strains with growth only in plate A were discarded.

Strains with growth in plate B were inoculated in new M17 broth and incubated for 24 hours. This culture was inoculated into two plates of M17 agar. One of them containing original media formula (plate C), whereas the other contained M17 agar, with 0.5% pig biliary salts (Sigma, Co.) (plate D). Both plates (C and D) were incubated as previously described. Strains with growth only in plate C were discarded.

Resistance to acidic pH. An overnight culture of M17 broth was used to make serial dilutions, using 0.85% NaCl (Difco, México), and plate count on M17 agar was performed (NOM-092-SSA1-1994).[31] Plates were incubated for 48 hours as described above. An aliquot of culture was centrifuged at 2600 x g, 4° C, for 30 minutes. Supernatant was discarded and bacterial pellet resuspended in acidic M17 broth (pH 3.0) Acidic culture was incubated for 1 hour. Serial dilutions were made and viable count on M17 agar was performed. All plates were duplicated. Percentage of resistance to acidic pH was determinated according to the equation designed by Kociubinsky.[23] %Resistance = 100 ($CFU_{sample}/CFU_{control}$).

Tolerance to biliary salts. An overnight culture of M17 broth was used to make serial dilutions, using 0.85% NaCl (Difco, México), and plate count on M17 agar was performed.[31] An additional count was performed, but the agar was added with 0.5% pig biliary salts. All plates were duplicated. Percentage of tolerance to biliary salts was determinated according to the equation designed.[23] %Tolerance = 100 ($CFU_{sample}/CFU_{control}$).

Bacterial Antagonism. Strains of *Enterococcus* as well as *E. coli* K88 were inoculated in separated BHI broth supplemented with 1% lactose. Cultures were incubated for 24 hours. All cultures were adjusted with tube number 3 of a MacFarland Nephelometer. *E. coli* K88 was massively inoculated over MRS agar (Difco, México) as recommended.[18] Five holes of 6 mm diameter were made at equal distances and filled with supernatant of *Enterococcus* strains cultures. Plates were incubated for 24 hours as previously described.[9,17]

Quantification of Bacterial Antagonism. Mixed cultures were prepared.[17] Cultures of 24 hours of *Enterococcus* strains and *E. coli* K88 in BHI broth supplemented with 1% lactose were adjusted with tube number 3 of McFarland nephelometer, and serial dilutions were made from *E. coli* K88 culture. Equal volumes of both cultures in a 1000:1 ratio (*Enterococcus: E. coli* K88) were mixed an incubated for 6 hours as previously described. A control tube was made containing just *E. coli* K88. Viable count was determined in Endo agar (Difco, México) at time of inoculation for each strain as well as mixed cultures after 6 hours of incubation. Percentage of antagonism was quantified based on the following equation: %AB = 100 – 100(Time $6_{mixed\ culture}$ – Time $6_{control}$). Where %AB is the percentage of bacterial antagonism for each strain, Time $6_{mixed\ culture}$ is the viable count obtained in mixed culture and Time $6_{control}$ is the viable count obtained in control. Times 0 were used as inoculation control.

Statistical Analysis. Mean comparison of percentage of resistance to gastrointestinal tract and percentage of antagonism in identified species in pig was made, as well as a comparison between same parameters in pig and rabbit species. Tukey-Kramer mean comparisons were performed using the statistical package NCSS 6.0.[19]

RESULTS

Strains and Biochemical Identification. 195 strains from gastrointestinal tract of pig were isolated. These strains had typical *Enterococcus* sp characteristics: gram-positive cocci, grouped in chains, negative to catalase, positive to hydrolysis of esculin, growth in salty BHI and positive PYR reaction. Finally, from the carbohydrate fermentation tests we identified 3 different species: *E. faecium, E. faecalis* and *E. mundii* from pig and rabbit.[20]

Gastrointestinal tract test. From 195 strains assayed just 11 fulfilled the criteria established for this study to consider a strain as possible probiotic bacteria. Although the study started with 195 isolated strains from pig, qualitative survival in acid pH discarded 118 strains, test for tolerance to biliary salts discarded another 19 strains. At the end of the qualitative test, only 58 pig strains were left. For a strain to be considered as probiotic resistance to acid pH is required at least in 20%, In the same way tolerance to biliary salts must be superior to 20%.[16,23,33] Resistance to acid pH test was done and 32 strains survived in a percentage superior to 20%. From these strains just 11 had tolerance to biliary salts superior to 20%. Isolated strains were grouped in 3 species: *E. faecium* 7, *E. faecalis* 2 and *E. mundii* 2. Fig. 1 shows the species identified from pig and percentages of resistance and tolerance to gastrointestinal tract. Fig. 2 shows species identified from rabbit and percentages of resistance and tolerance to gastrointestinal tract.

Bacterial antagonism. All the strains isolated from pigs and rabbits showed good antagonistic effect against *E. coli* K88. Figs. 1 and 2, illustrate the percentage of antagonism of pig and rabbit strains respectively. Fig. 1 shows that most of the strains, but one species of *E. faecium*, have tolerance to biliary salts above 20%. This single *E. faecium* strain presents a percentage of tolerance to the biliary salts of 12%, however its antagonistic effect was higher than 90%, thus the decision to include it in the study. Fig. 2 depicts some strains that do not offer resistance to acidic conditions and another one with no toler-

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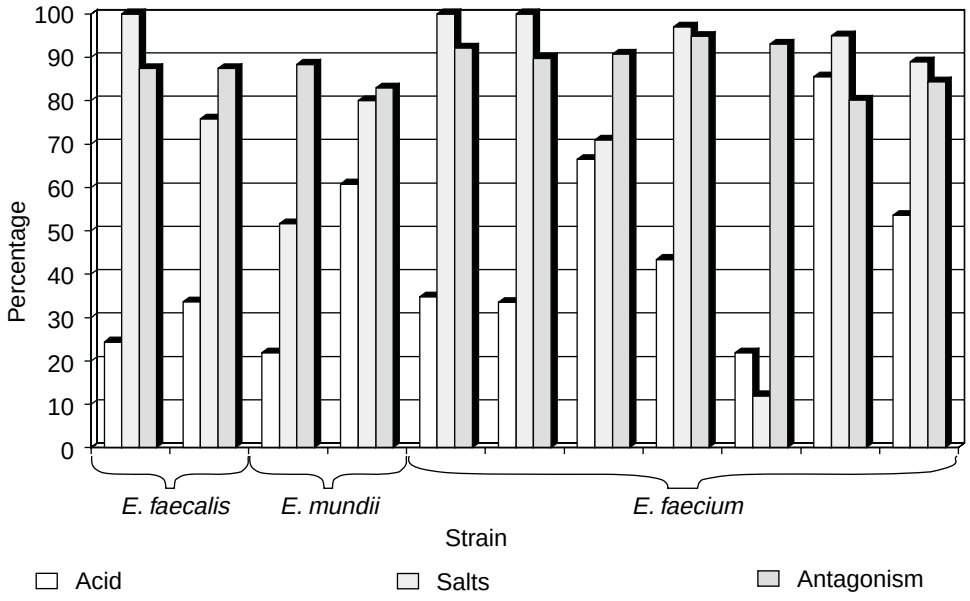

Figure 1. *Percentage of acid resistance, tolerance to biliary salts and bacterial antagonism against* E. coli *K88 in species isolated from pigs.*

ance to biliary salts, nevertheless, they were maintained in the study for comparison reason, considering that they were isolated from rabbits.

Statistical Analysis. Tukey-Kramer test ($p<0.05$) was applied to percentage of resistance to acids and tolerance to biliary salts, as well as the percentage of bacterial antagonism among isolated species of *Enterococcus* in pig. The same test was done to compare the same parameters between hosts (pig and rabbit). Tables 1 and 2, show the arithmetic means, as well as standard errors of comparisons. It can be observed in table 1 that *E. faecium* is the species that offers the greatest resistance to acid pH, as well as greater bacterial antagonism than the others. Nevertheless, statistical analysis did not reveal a significant difference in these parameters in comparison with other species also isolated from pigs. When comparisons of isolated strains from different hosts were made (Table 2). It was not observed a significant difference among the isolated species from both mammals.

It is important to indicate that for the accomplishment of the comparisons with rabbit strains, just 2 strains were considered whose fulfilled the three criteria used in the study to consider a strain like possible probiotic.

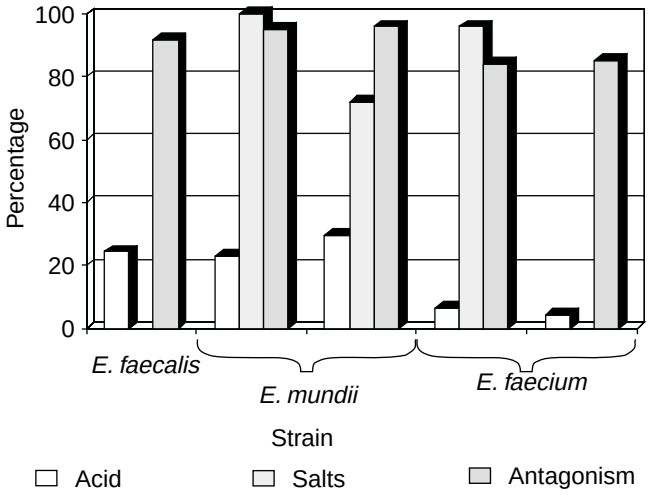

Figure 2. *Percentage of acid resistance, tolerance to biliary salts and bacterial antagonism against* E. coli *K88 in species isolated from rabbits.*

DISCUSSION

Recovery. The percentage of strains obtained as candidate probiotic from pig in the present study was 6% (11 out of 195 strains). It is a low percentage, if compared with other studies also made in pigs. In a previous study some strains of *Bifidobacterium* (16 of 123 strains) were characterized and it was obtained a percentage of recovery of 13%.[7] Whereas, another study elaborated by Pacheco,[32] using gastrointestinal tract of pigs, the percentage of recovery in *Lactobacillus* strains was 20% (28 out of 140 strains). Species of *Bifidobacterium* have special nutritional requirements for their growth and are more fastidious than those of *Enterococcus* strains.[4,12] A possible explanation for the low percentage of recovery could be that *Enterococcus* strains were not isolated in specially designed media. Current media used in this study are designed for iso-

Table 1. Percentages of acid resistance, biliary salts tolerance and bacterial antagonism against *E. coli* K88 (mean ± standard error) in species isolated from pigs.

	Acid resistance	Biliary salts tolerance	Bacterial antagonism
E. faecalis	29.05 ± 15.13	87.89 ± 20.60	87.4 ± 3.31
E. mundii	41.33 ± 15.13	65.85 ± 20.60	85.7 ± 3.31
E. faecium	48.47 ± 8.08	80.57 ± 11.01	89.31 ± 1.77

Table 2. Percentages of acid resistance, tolerance to biliary salts and bacterial antagonism against *E. coli* K88 (mean ± standard error) in strains isolated from pigs and rabbits.

	Acid resistance	Biliary salts tolerance	Bacterial antagonism
Pigs	43.64 ± 5.57	79.22 ± 10.61	88.30 ± 1.44
Rabbits	17.45 ± 8.26	53.60 ± 15.74	90.49 ± 2.13

lation of *Enterococcus* and *Streptococcus* sp. from feces. Whereas, there are culture media specially designed for isolation of *Bifidobacterium*, *Lactobacillus* and other lactic acid bacteria.[4,7]

Resistance to acid pH. In order to exert their beneficial functions in host, probiotic bacteria need to survive against natural defenses of the organism. Acid pH from stomach is the first one of these. Optimal pH for *Enterococcus* growth is near neutrality. However, *Enterococcus* has fermentative characteristics of acid production without gas; and these microorganisms can remain viable up to pH 4,0.[24] In the present study a simulation of the acidic condition of pig stomach was done, which pH appears to be near 3.0,[16,43] this pH value is below of the reported for this genus survival. Thirty-two strains were able to survive in this condition, with a resistance superior to 20%. Some authors had recommended a concentration of viable probiotic bacterial cells between 10^6 to 10^8 CFU/g per consumed food, This seems to be safe and it is required for an effective probiotic product.[10] This concentration has been suggested to compensate the reduction suffered during its passage through the stomach and small intestine.[16,38,39] Percentage of resistance to acid superior to 20% is useful to ensure that enough live cells will be able to survive the biliary salts in small intestine.[39]

Tolerance to biliary salts. The next natural barrier which bacteria has to support when entering gastrointestinal tract are biliary salts. In order to evaluate the potential role of bacteria as probiotics, it is necessary to test their ability to resist biliar salts. Biliary acids are synthesized in the liver from cholesterol and they are secreted by the gallbladder to small intestine in conjugated form. Biliary acids undergo several chemical modifications in large intestine due to activity of indigenous bacteria. Biliary acids in conjugated, or deconjugated form, present antimicrobial activity.[10] Pig bile is more inhibiting against bacteria than human or bovine bile.[40] It was expected that strains isolated in the present study would be used in near future as probiotics in pigs, thus it was decided to use biliary salts from pig origin, to show a situation closer to the real environment.

It has been observed that resistance to bile is related to bacterial species,[23] however, in the present work, of the 3 different species isolated from gastrointestinal tract from pigs, there was not significant difference in tolerance to biliary salts, as it is shown in Table 1. Even when comparing isolated species from pigs, with those isolated from rabbits, it could be observed that did not exist any significant difference among them. These observations suggest that the capacity to survive to biliary salts is independent of the origin strain. They have similar capacities to survive its passage by gastrointestinal tract.

Bacterial antagonism. There were no significant differences in percentages of bacterial antagonism against *E. coli* K88 among isolated species in pig as shown in Table 1 neither between different hosts, as shown illustrated in Table 2. This could constitute an alternative for treatment of diarrhea in pigs. *E. coli* K88 is a pathogen that can cause severe diarrheas.[21] Newborn pigs ingest food up to 7 times/day[43] and would be easy to feed *Enterococcus* strains to them in food (as fermented or supplemented forms) or water, to exert a protective effect. Although, some strains have low resistance to gastrointestinal tract, it can be easily assumed that probiotic effect expected did not appear, because strains are not reaching the intestine of pig in a viable way. On the other hand, it has been postulated that in-

testinal infections arise by food contaminated with pathogens ingestion.[12] If food is previously fermented with antagonistic strains, it can be assumed that a pathogens-free food was obtained.

This study shows that genus *Enterococcus* is a source of strains that can survive to gastrointestinal tract and present antagonistic activity, fundamental characteristics of bacterial strains used as probiotics.

It is necessary to continue with probiotic characterization of the *Enterococcus* strains isolated, such as stimulation of immune system, detoxificant effect, determination of pathogenicity factors, as well as molecular identification of strains. The molecular approach will allow us to track strains for *in vivo* studies and to verify their implantation in the gastrointestinal tract.

REFERENCES

1. Audisio, M.C., G. Oliver, and M.C. Apella. 1999. Antagonistic effect of *Enterococcus faecium* J96 against human and poultry pathogenic *Salmonella* spp. J Food Prot. 62(7):751-755.
2. Audisio, M.C., G. Oliver, and M.C. Apella. 2000. Protective effect of Enterococcus faecium J96, a potential probiotic strain, on chicks infected with *Salmonella* Pullorum. J Food Prot. 63(10):1333-1337.
3. Audisio, M.C., G. Oliver, and M.C. Apella. 2001. Effect of different complex carbon sources on growth and bacteriocin synthesis of *Enterococcus faecium*. Int J Food Microbiol. 63:235-241.
4. Beerens, H. 1991. Detection of Bifidobacteria by using propionic acid as a selective agent. Appl Environ Microbiol 57(8):2418-2419.
5. Collins, J.K., G. Thornton, and G.O. Sullivan. 1998. Selection of probiotic strains for human applications. Int Dairy J. 8:487-490.
6. Collins, M.D., and G.R. Gibson. 1999. Probiotics, prebiotics, and symbiotics: approaches for modulating the microbial ecology of the gut. Am J Clin Nutr. 69(suppl):1052-1057.
7. Corona, V. 2001. Aislamiento y caracterización de *Bifidobacterium* sp a partir del contenido gástrico del cerdo. Tesis. Universidad de Sonora.
8. CSPI news. 1999 FDA should ban the use of certain antibiotics to fatten farm animals.http://www.cspinet.org/new/antibiotics.htm
9. De Martinis, E., V. Alves, and B. Franco. 2002. Fundamentals and perspectives for the use of bacteriocins produced by lactic acid bacteria in meat products. Food Reviews Int. 18(2):191-208.
10. Dunne, C., L. Murphy, S. Flinn, L. O´Mahony, S. O´Halloran, M. Feeney, D. Morrisey. 1999. Probiotic: from myth to reality. Demonstration of functionally in animal models of disease and in human clinical trials. Anton Van Leeuwenhoek. 76:279-292.
11. Falk, P.G., L.V. Hooper, T. Midtvedt, and J.I. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. Microbiology and Molecular Biology Reviews. 62(4):1157-1170.
12. Forbes, B.A., D.F. Sahm, and A.S. Weissfeld. 1998. Bailey & Scott´s diagnostic microbiology. 10th ed. Mosby. USA.
13. Gardiner, G., C. Stanton, P.B. Lynch, J.K. Collins, G. Fitzgerald, and R.P. Ross. 1999. Evaluation of cheddar cheese as a food carrier for delivery of a probiotic strain to the gastrointestinal tract. Journal of Dairy Science. 82:1379-1387.
14. Gibson, G., D. Heber, S. Meydani, E. Postaire, and M.E. Sanders. 2000. Functional Dairy Products. John Libbey Eurotext. France.
15. Gomes, A. and F. Malcata. 1999. *Bifidobacterium* spp and *Lactobacillus acidophilus:* biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends in Food Science and Technology. 10:139-157.
16. Gómez. A., G. Kociubinski, P. Pérez, and G. De Antoni. 1998. Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J Food Prot. 61(7): 865-873.
17. González, S.N., M.C. Apella, N.C. Romero, M.E. Nader de Macias, and G. Oliver. 1993. Inhibition of enteropathogens by lactobacilli strains used in fermented milk. J Food Prot. 56(9):773-776.
18. Hernández, F. 2003. Algunos aspectos de investigación básica y aplicada sobre probióticos. Memoria Electrónica del 3er Simposio Mexicano de Probióticos. México, D.F.
19. Hintze, J.L. 1997. NCSS 97 u*ser's guide—II. [Computer software* manual.] Kaysville, UT: Number Cruncher Statistical Systems.
20. Holt, J., N.R. Krieg, P.H. Sneath, J.T. Staley, and S.T. Williams, 1994. Bergey´s manual of determinative bacteriology. 9th ed. William & Wilkins. USA.
21. Izeta M., J. 1992. Agentes etiológicos más comunes en el síndrome diarréico de los lechones. Síntesis Porcina. 11(2):36-40.
22. Kociubinski, G., P.F. Pérez, M.C. Añon, and G.L. De Antoni. 1996. A method of screening for highly inhibitory lactic acid bacteria. J Food Prot. 59(7):739-745.
23. Kociubinski, G., P. Pérez, and G. De Antoni. 1999. Screening of bile resistance and bile precipitation in lactic acid bacteria and bifidobacteria. J Food Prot. 62:905-912.
24. Koneman, E.W., S.D. Allen, W.M. Janda, P.C. Schreckenberger, and W.C. Winn. 1994. Introduction to diagnostic microbiology. Lippincott Company. USA
25. Laukova, A., S. Czikkova, Z. Vaslikova, P. Juris, and M. Marekova. 1998. Ocurrence of bacteriocin production among environmental enterococci. Letters in Applied Microbiology, 27:178-182.
26. MacFaddin, J. F. 2000. Biochemical Tests for Identification of Bacteria of Medical Importance. Lippincott Williams & Wilkins. 3rd. edition. USA.
27. McKellar, Q.A. 1998. Antimicrobial resistance: a veterinary perspective. British Medical Journal. 317:610-611.
28. Naidu, A.S., W.R. Bidlack and R.A. Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). Critical Rev. in Food Sci. and Nutrition. 38:13-126.
29. Netherwood, T., H.J. Gilbert, D.S. Parker, and A.G. O´donnell. 1999. Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. Appl Environ Microbiol. 65(11): 5134-5138.
30. Nielsen, O.H., S. Jorgensen, K. Pedersen, and T. Justesen. 1994. Microbiological evaluation of jejunal aspirates and faecal samples after oral administration of bifidobacteria and lactic acid bacteria. Journal of Applied Bacteriology. 76:469-474.
31. NOM-092-SSA1-1994. Norma Oficial Mexicana. Bienes y Servicios. Método para la cuenta de bacterias aerobias en placa.
32. Pacheco, M. 2002. Aislamiento e identificación de *Lactobacillus* sp del intestino y contenido intestinal del cerdo, por medio de técnicas bacteriológicas clásicas y caracterización parcial de sus propiedades probióticas. Tesis. Universidad de Sonora.
33. Prasad, J., H. Gill, J. Smart, and P.K. Gopal. 1998. Selection and characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. Int Dairy J. 8:993-1002.
34. Roberfroid, M.B. 2000. Prebiotics and probiotics: are they functional foods?. Am J Clin Nutr. 71(suppl):1682-1687.
35. Rolfe, R.D. 2000. The role of probiotic cultures in the control of gastrointestinal health. Journal of Nutrition. 130:396-402.
36. Salminen, S., A.C. Ouwehand, and E. Isolauri. 1998. Clinical applications of probiotic bacteria. Int Dairy J. 8:563-572.
37. Salminen, S., A. von Wright, L. Morelli, P. Marteau, D. Brassart, W.M. de Vos, R. Fondén, M. Saxelin, K. Collins, G. Mogensen, S. Birkeland, T. Mattila. 1998b. Demonstration of safety of probiotics – a review. Int J Food Microbiol. 44:93-106.

38. Shah, H., J. Lee, J. Pestka, and Z. Unstund. 1999. Viability of bifidobacteria in commercial dairy products during refrigerated storage. J Food Prot. 63(3):327-331.
39. Shortt, C. 1999. The probiotic century: historical and current perspectives. Trends in Food Science & Technology. 10:411-417.
40. Thornton, G., M. O´Sullivan , G. O´Sullivan, and J. Collins. 1993. Probiotic properties of Lactobacilli and Bifidobacteria. In Report of the Second Annual Meeting of Contractans. National Dairy Products Research Center.
41. Trieu-Cuot, P. y C. Poyart. 1999. Visita con guía al centro del arsenal bacteriano. Mundo Científico. 197:46-50.
42. Vandenbergh, P.A. 1993. Lactic acid bacteria, their metabolic product and interference with microbial growth. FEMS Microbiology Reviews. 12:221-238.
43. Yen, J. 2001. Digestive System. In Biology of the Domestic Pig. Edited by W. Pond and H. Mersmann. Cornell University. Press USA.

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