BIOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF PROBIOTIC *Lactobacillus* FOR SWINE

ELIZETE DE FÁTIMA REQUE PANCHENIAK*
CARLOS RICARDO SOCCOL**

The aim of the present work was to select lactic acid bacteria from the gastrointestinal tract of pigs and establish their biochemical characterization. Strains P01-001 and P08-002 were selected out of a total of 33 bile resistant strains (5% of bile) because they presented the best survival rate at room temperature and after freeze-drying. Complementary studies on aspects like the ability to grow at different temperatures and resistance to inhibitory substances such as bile 10%, phenol 0.4%, NaCl 4% and 8% were performed. The selected strains P01-001 and P08-002 were identified biochemically through the API 50 test as *Lactobacillus* and classified as heterofermentative bacteria by gas production from glucose and by the presence of lactic and acetic acids in Man, Rogosa and Sharpe (MRS) supernatants detected by HPLC. The strains P01-001 and P08-002 growing at 45°C and tolerant to 10% bile, 0.4% phenol and 4% NaCl will be studied in animal trials as potential probiotic agents.

KEY-WORDS: Lactobacillus; PROBIOTIC; SWINE.

1 INTRODUCTION

In pork production, one of the biggest problems is the high mortality of pigs. Particularly after weaning, about 41% of deaths are due to diarrhea. One of the most important causes of this is the enterotoxigenic *Escherichia coli*. Antibiotics, such as neomycin (PFIZER, 2005), have been used in the treatment of colibacillosis with rather variable results. This happens because of the *E. coli* 's ability to develop resistance to antibiotics.

* Doutoranda em Tecnologia de Alimentos, Setor de Tecnologia, Divisão de Engenharia de Processos & Biotecnologia, Universidade Federal do Paraná (UFPR), Curitiba-PR, Brazil (e-mail: efreque@terra.com.br).
** Professor titular, UFPR, Curitiba (e-mail: soccol@ufpr.br).

Strains with antibiotic resistance represent a serious health problem. The ratio of enteric bacteria that carry plasmid for resistance to multiple drugs has been gradually increasing during the last twenty-five years (SALDARRIAGA; CALLE; CAMACHO, 2000).

Probiotics are live microbial food supplements (FULLER, 1989) which, when administered in adequate amounts (FAO/WHO, 2005) beneficially affect the host by improving its microbial balance. They have undergone a rapid increase in importance as functional foods recently.

Many efforts have been made to find out potential probiotic *Lactobacillus* strains isolated from humans and animals. For this purpose, simple and reliable identification methods are required. Conventional biochemical and physiological tests (DAVIS, 1955) clearly have some limitations in discriminating large numbers of isolates that present similar physiological characteristics (SONG et al., 2000; KWON et al., 2004). FAO/WHO (2005) recommended that this genotypic technique should be combined with phenotypic tests for confirmation.

The aim of the present work was to select lactic acid bacteria from the gastrointestinal tract of pigs and establish their biochemical characterization.

2 MATERIAL AND METHODS

2.1 BACTERIA

The 33 strains employed in this work belong to the Bioprocesses Engineering & Biotechnology Division of Federal University of Paraná (UFPR).

The selection of probiotic strains for swine was carried out in accordance with bio-safety aspects, bacterioscopy (morphology), Gram staining, viability of strains kept in Man, Rogosa and Sharpe (MRS) agar while stored at 4-7°C (REQUE et al., 2000), bile tolerance (5%), and best survival rate at room temperature and after freeze-drying.

2.1.1 Strains Survival Subjected to Freeze-Drying and Storage

The strains were grown in MRS broth (Merck) for 24 h at 37 °C. The cells in culture were harvested by centrifugation at 6 000 rpm for 30 min. The

cell pellets were washed once with a sterile saline solution and then suspended in a small amount of sterile 10% (w/v) skim milk solution. The cell suspensions were frozen and freeze-dried. The number of viable cells before and after freeze-drying was determined in colony forming units per mL (CFU/mL). Decimal serial dilutions were prepared out of the skim milk suspension before freezing, and plated on MRS agar plates. The freeze-drying samples were ressuspended in peptone water 0.1%, diluted and subsequently plated. The agar plates were incubated at 37 °C for 48 h. The survival rate was calculated as CFU/mL one month after freeze-drying and storing at room temperature divided by CFU/mL before freezing (PALMFELDT; HAHN-HÄGERDAL, 2000).

2.2 BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE STRAINS

2.2.1 Organic Acid Identification and Quantification

Overnight isolated cultures were inoculated at 10% (v/v) in MRS broth and incubated at 37ºC for 12 h. To obtain the test materials, fermented MRS broth was centrifuged (6 000 rpm for 30 min) to remove the microbial cells. The resulting liquid was filtered through sterile 0.22 µm membrane filters (Millipore). Organic acid identification and quantification was performed through high performance liquid chromatography (REQUE, 1999), operated under the conditions presented in Table 1. Under those conditions the retention time of each component can be expressed as shown in Table 2.

TABLE 1 - OPERATION CONDITIONS IN THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FOR THE QUANTIFICATION OF ORGANIC ACIDS

Chromatograph	*Operation Conditions*
Column Temperature	60 °C
Mobile-eluant Phase	H_2SO_4 5 mM
Outflow of the mobile phase	0.6 mL/min
Pump pressure	48 kg/cm^2
Injected volume	50 µL
Dilution	1:5

TABLE 2 - RETENTION TIME OF SOME STANDARDS, MONITORED IN CHROMATOGRAPHIC COLUMN, UNDER OPERATIONAL CONDITIONS

Component	*Retention time (min)*
Glucose	9.38
Lactic acid	12.74
Acetic acid	14.92
Ethanol	21.76

2.2.2 Gas Production from Glucose

Overnight isolated cultures were inoculated at 10% (v/v) in MRS broth containing inverted Durham tubes and incubated at 37ºC. The gas production from glucose was observed after 24 h (DAVIS, 1955).

2.2.3 Growth at Different Temperatures

Overnight isolated cultures were inoculated at 10% (v/v) in MRS broth and incubated at 15°C, 37°C and 45ºC for 24 h. Total populations were determined by the pour plate method, incubating the plates at 37ºC for 48 h (BUCHANAN & GIBBONS, 1974).

2.2.4 Tolerance to Inhibitory Substances

Inhibitory substances such as bile (Sigma), phenol (Merck), and sodium chloride (Biotec) were tested. MRS broth containing 10% bile, 0.4% phenol, and 4% and 8% sodium chloride (DAVIS, 1955) were determined by inoculation at 10% of the overnight isolated cultures and incubated at 37ºC for 24 h. Total populations were determined by the pour plate method, incubating the plates at 37ºC for 48 h.

2.2.5 Fermentation of Different Carbon Sources

A suspension was made in the medium with the microorganism to be tested and each tube of the strain was inoculated. During incubation, carbohydrates are fermented to acids, which cause a decrease in the pH, detected by the color change of the indicator (BIOMËRIEUX, 2005).

3 RESULTS AND DISCUSSION

3.1 ELECTION OF STRAINS WITH PROBIOTIC POTENTIAL FOR SWINE

Out of a total of 33 bile resistant strains (5% of bile) obtained from the Division of Biotechnology, 29 presented bacillary morphology and four presented a coccus form. All strains reacted positively to Gram staining.

A superior concentration of bile recommended for the election of strains for human beings was used. The concentration of bile to be used in the election of probiotic species for human beings must be 0.3% (w/v) (GILLILAND; STAEY; BUSH, 1984; PENNACCHIA et al., 2004). This is so because the isolated microorganism may present tolerance to high concentrations of bile and may grow in the intestinal tract of swine. The daily average of biliary flow is very high in swine, around two liters for each 40 kg of swine (GILLILAND; WALKER, 1990; FULLER, 1992) as compared to that of an adult human (70 kg) that produces 400 to 800 mL of bile daily (SECRETION, 2005).

PATEL et al. (2004) have isolated species of *Lactobacillus reuteri* from human intestines that were highly resistant to 2% of bile.

GILLILAND (1979) proved that lactobacilli isolated from animal intestines, such as *L. fermentum*, were more tolerant to biliary salts than species isolated from milky products.

Based on the bacillary morphology, Gram positive staining (KANDLER & WEISS, 1989), and viability of strains kept in MRS agar during refrigerated storage (4-7°C), nine strains were selected. These strains were lyophilized and had their survival verified after one month of storage at room temperature. Out of those nine, only two were chosen, because of their best survival rates. Strains P01-001 and P08-002 presented survival rates respectively of 62.21% and 71.80%. These strains that were lyophilized and kept at refrigeration temperature (4-7°C) during a month period presented higher survival rates, between 93 and 100%.

The storage temperature after lyophilization was the one recommended by KANDLER and WEISS (1989). In order to obtain high viability of the bacterial cells at room temperature, it was necessary to investigate other methods of cellular conservation (DZIEZAK,1988; FULLER, 1992).

3.2 STUDIES ON THE PHYSIOLOGICAL AND BIOCHEMICAL BEHAVIOR OF THE SELECTED STRAINS

3.2.1 Organic Acid Identification and Quantification

Strains P01-001 and P08-002 are lactic acid bacteria as they produce lactic acid (as detected in HPLC, 12 g/L and 10 g/L, respectively). They are heterofermentative due to the production of other products beside lactic acid, including acetic acid (9.5 g/L the P01-001 and 6.6 g/L the P08-002) and ethanol from glucose (KANDLER & WEISS, 1989).

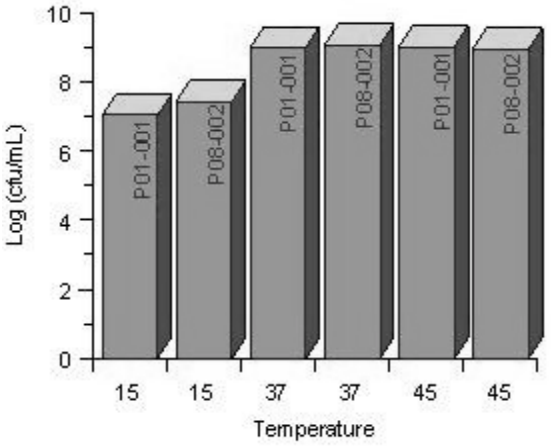
3.2.2 Presence of Gas by Glucose Fermentation

Strains P01-001 and P08-002 produce CO_2 from hexose (KANDLER; WEISS, 1989; TARANTO; VALDEZ; PEREZ-MARTINEZ, 1999).

3.2.3 Tolerance to Extreme Temperatures

Strains P01-001 and P08-002 presented better growth at 37-45 °C (FIGURE 1) and small growth at 15 ºC. Lactobacilli grow at temperatures between 2-53°C, where the optimal temperature generally is between 30-40°C (KANDLER & WEISS, 1989; CHERUBIN, 2003). According to *Bergey's Manual* (BUCHANAN & GIBBONS, 1974), the heterofermentative species that grows at 45°C and does not present any growth at 15 °C are *Lactobacillus fermentum*.

FIGURE 1 – INFLUENCE OF TEMPERATURE ON THE GROWTH OF STRAINS P01-001 AND P08-002 IN MRS BROTH INCUBATED AT 15°C, 37°C AND 45°C FOR 24 h



3.2.4 Tolerance to Inhibitory Substances

FIGURE 2 presents Log (UFC/mL) values of strains P1-001 (a) and P8-002 (b) in relation to the inhibitory substances: 10% of bile, 0.4% of phenol, 4 and 8% of NaCl. It can be observed that both strains P1-001 (a) and P8-002 (b) showed better tolerance to 10% of bile, 0.4% of phenol, 4% of NaCL, but not to 8% of NaCl when compared to the control (MRS), with characteristics of *Lactobacillus fermentum*, according to DAVIS (1955).

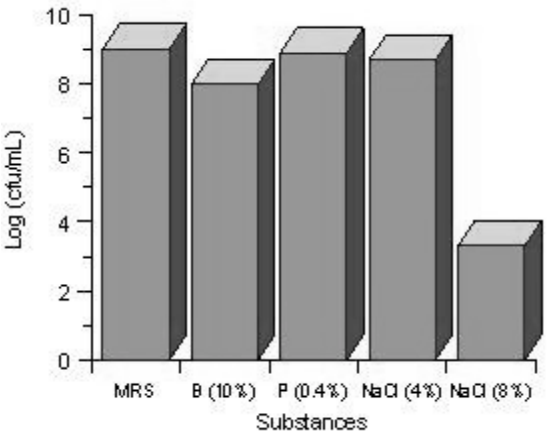
Tolerance to bile and phenol (phenols can be formed in the intestines by bacteria that desaminate some aromatic amino acids delivered by the diet or produced by endogenous proteins) is an important characteristic for a better survival ability, not necessarily of multiplication, in the intestines.

Some authors suggest that a 0.4% concentration of phenol causes a bacteriostatic action in some microorganisms (XANTHOPOULOS; LITOPOULOU-TZANETAKI; TZANETAKIS, 2000).

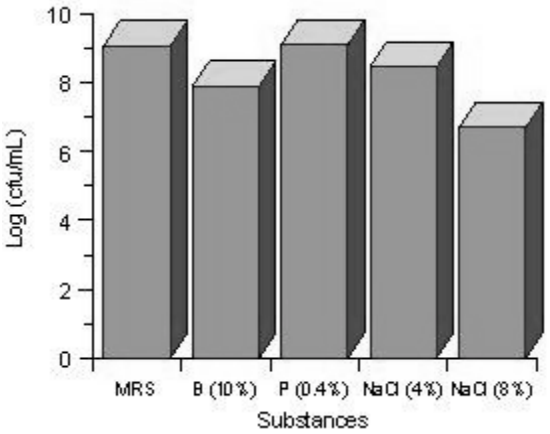
XANTHOPOULOS, LITOPOULOU-TZANETAKI and TZANETAKIS (2000) have isolated *Lactobacillus reuteri* DC423 with a high resistance (0.4%) to phenol from children's faeces. The counting of viable cells was not affected at all by phenol.

FIGURE 2 – INFLUENCE OF INHIBITORY SUBSTANCES ADD: MRS (CONTROL), BILE (B), PHENOL (P) AND NaCl ON THE GROWTH OF STRAINS P01-001 (a) AND P08-002 (b) IN MRS BROTH INCUBATED AT 37ºC FOR 48 h

(a)



(b)



3.2.5 Fermentation of Different Carbon Sources

The fermentation of selected carbohydrates of the API 50 CH gallery (BIOMÉRIEUX, 2005) was carried out, that is, the catabolism of these sugars that results in the production of organic acid and are detected by the pH turning point.

The results obtained constitute the biochemical profile of the strain and are also of some use for its identification or for determining its type.

Strains P01-001 and P08-002 were identified as *Lactobacillus fermentum*, although not conclusively. According to *Bergey's Manual* , the *Lactobacillus fermentum* species cannot be distinguished from the *Lactobacillus reuteri* species by means of simple physiological tests. Other tests are necessary in order to distinguish these two species, such as: determination of % mol G+C (guanine+cytosine), diamino acid of peptidoglycan or electrophoretic mobility of lactic acid dehydrogenase (LDH) (KANDLER & WEISS, 1989).

4 CONCLUSION

Strains P01-001 and P08-002 belong to the *Lactobacillus* genus. The *Lactobacillus sp* P01-001 and P08-002 are strains that ferment several

types of sugars and produce lactic and acetic acids, and thus are substances with possible antimicrobial activity.

The *Lactobacillus sp* P01-001 and P08-002 strains are heterofermentative, presenting better growth at 37 °C and 45 ºC and small growth at 15ºC.

The *Lactobacillus sp* P01-001 and P08-002 are strains with probiotic potential as they are resistant to high concentrations of bile and phenol. Tolerance to bile and phenol are important characteristics for a better survival ability in the intestines.

Resumo

CARACTERIZAÇÃO BIOQUÍMICA E IDENTIFICAÇÃO DE Lactobacillus PROBIÓTICOS PARA SUÍNOS

O objetivo deste trabalho foi selecionar bactérias ácido lácticas provenientes do trato gastrintestinal de suínos e caracterizá-las bioquimicamente. Do total de 33 cepas resistentes à bile (5% de bile), as cepas P01-001 e P08-002 foram selecionadas por apresentarem a melhor taxa de sobrevivência em temperatura ambiente após liofilização. Avaliaram-se em estudos complementares o comportamento fermentativo em diferentes temperaturas e tolerância a substâncias inibitórias (10% de bile, 0,4% de fenol, 4 e 8% de NaCl). As cepas selecionadas P01-001 e P08-002 foram identificadas bioquimicamente mediante galeria API 50 CHL como *Lactobacillus* e classificadas como bactérias heterofermentativas por produzirem gás proveniente da glicose e pela presença de ácidos lácticos e acéticos detectados por HPLC. As cepas P01-001 e P08-002 crescem em temperatura de 45º C, são tolerantes a 10% de bile, 0,4% de fenol e 4% de NaCl, devendo ser estudadas em experimentos com animais como potencial agente probiótico.

PALAVRAS-CHAVES: Lactobacillus; PROBIÓTICO; SUÍNO.

REFERENCES

1 BIOMÉRIEUX. **Api 50 CHL médium.** France. Disponível em: < http://courses.ag.uidaho.edu/fst/fstmmbb417/api50_CHL_medium.pdf> Acesso em: 20 set. 2005.

2 BUCHANAN; R. E.; GIBBONS, N. E. **Bergey's manual of determinative bacteriology.** 8th. ed. Baltimore: Williams & Wilkins,1974. 1268 p.

3 CHERUBIN, R. A. **Efeitos da viabilidade da levedura e da contaminação bacteriana na fermentação alcoólica.** Piracicaba, 2003. 137 p. Tese (Doutorado em Agronomia), Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo.

4 DAVIS, G. H. G. The classification of *Lactobacilli* from the human mouth. **Journal of General Microbiology**, v. 13, n. 3, p. 481-493, 1955.

5 DZIEZAK, J. D. Microencapsulation and encapsulated ingredients. **Food Technology**, Chicago, v. 42, n. 4, p.136-151,1988.

6 FAO/WHO. Food and Agriculture Organization of the United Nations/World Health Organization. **Guidelines for the evaluation of probiotics in food.** Ontario, Canada, 30 abr. – 1 maio, 2002. Disponível em: <http:www.fao.org/es/esn/food/foodandfood_probio_en.stm>. Acesso em: 17 jan. 2005.

7 FULLER, R. Probiotics in man and animals. **Journal of Applied Bacteriology,** Oxford, v.66, p. 365-378, 1989.

8 FULLER, R. **Probiotics the scientific basis**. London: Chapman & Hall, 1992. 398 p.

9 GILLILAND, S. E. Beneficial interrelationships between certain microorganisms and human: candidate microorganisms for use as dietary adjuncts. **Journal of Food Protection,** Des Moines, v. 42, p. 164-167, 1979.

10 GILLILAND, S. E.; STAEY, T. E.; BUSH, L. J. Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. **Journal of Dairy Science**, Champaign, v. 67, p.30-45, 1984.

11 GILLILAND, S. E.; WALKER, D. K. Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. **Journal of Dairy Science**, Champaign, v. 73, n. 4, p. 905-911, 1990.

12 KANDLER, O.; WEISS, N. Genus Lactobacillus Beijerinck 1901. In: BERGEY'S manual of systematic bacteriology. Baltimore: Williams & Wilkins, 1989. v. 2, p. 1209-1234.

13 KWON, H-S; YANG, E-H; YEON, S-W; KANG, B-H; KIM, T-Y. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. **FEMS Microbiology Letters,** Amsterdam, v. 239, p. 267-275, 2004.

14 PALMFELDT, J.; HAHN-HÄGERDAL, B. Influence of culture pH on survival of *Lactobacillu reuteri* subjected to freeze-drying. **International Journal of Food Microbiology**, Amsterdam, v. 55, n. 1–3, p. 235-238, Apr. 2000.

15 PATEL, H. M.; PANDIELLA, S. S.; WANG, R.H.; WEBB, C. Influence of malt, wheat, and barley extracts on the bile tolerance of selected strains of lactobacilli. **Food Microbiology,** London, v. 21, p. 83-89, 2004.

16 PENNACCHIA, C.; ERCOLINI, D.; BLAIOTTA, G.; PEPE, O.; MAURIELLO, G.; VILLANI, F. Selection of Lactobacillus strains from fermented sausages for their potential use as probiotics. **Meat Science**, Barking, v. 67, p. 309-317, 2004.

17 PFIZER. **Sulfato de neomicina**. Disponível em: <http://www.pfizer-ahg.com.br/pharmacia_produtosSui_neobiotic.asp#2>Acesso em: 11 fev. 2005.

18 REQUE, E. F. **Isolamento, identificação e estudos fisiológicos da bactéria de ação probiótica (*Lactobacillus fermentum* LPB) para uso em frangos de corte.** Curitiba, 1999. 94 p. Dissertação (Mestrado em Tecnologia Química), Universidade Federal do Paraná.

19 REQUE, E. F.; PANDEY, A.; FRANCO, S. G.; SOCCOL, C. R. Isolation, identification and physiological study of *Lactobacillus fermentum* LPB for use as probiotic in chickens. **Brazilian Journal of Microbiology**, São Paulo, v. 31, p. 303-307, 2000.

20 SALDARRIAGA, F. T.; CALLE, S. E.; CAMACHO, C. S. Resistencia antimicrobiana de cepas de *Escherichia coli* provenientes de lechones lactantes criados en una granja tecnificada de Lima. **Revista de Investigaciones Veterinárias del Perú**, Lima, v. 11, n. 2, p.195-200, 2000.

21 SECRETION of bile and the role of bile acids in digestion. Disponível em:< http://arbl.cvmbs.colostate.edu/hbooks/pathphys/digestion/liver/bile.html> Acesso em: 7 jun. 2005.

22 SONG, Y-L; KATO, N.; LIU, C-X; MATSUMIYA, Y.; KATO H., WATANABE, K. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group-and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA**. FEMS Microbiology Letters,** Amsterdam, v. 187, p.167-173, 2000.

23 TARANTO, M. P.; VALDEZ, G. F.; PEREZ-MARTINEZ, G. Evidence of a glucose proton motive force-dependent permease and a fructose phosphoenolpyruvate: phosphotransferase transport system in *Lactobacillus reuteri* CRL 1098. **FEMS Microbiology Letters,** Amsterdam, v. 181, p. 109-112, 1999.

24 XANTHOPOULOS, V.; LITOPOULOU-TZANETAKI, E.; TZANETAKIS, N. Characterization of *Lactobacillus* isolates from infant faeces as dietary adjuncts. **Food Microbiology**, London, v. 17, p.205-215, 2000.