

## BIOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF PROBIOTIC *Lactobacillus* FOR SWINE

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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.

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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 resuspended 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 37°C 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**

<i>Chromatograph</i>	<i>Operation Conditions</i>
<b>Column Temperature</b>	60 °C
<b>Mobile-eluant Phase</b>	H <sub>2</sub> SO <sub>4</sub> 5 mM
<b>Outflow of the mobile phase</b>	0.6 mL/min
<b>Pump pressure</b>	48 kg/cm <sup>2</sup>
<b>Injected volume</b>	50 µL
<b>Dilution</b>	1:5

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

<i>Component</i>	<i>Retention time (min)</i>
<b>Glucose</b>	9.38
<b>Lactic acid</b>	12.74
<b>Acetic acid</b>	14.92
<b>Ethanol</b>	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 37°C. 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 45°C for 24 h. Total populations were determined by the pour plate method, incubating the plates at 37°C 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 37°C for 24 h. Total populations were determined by the pour plate method, incubating the plates at 37°C 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 (BIOMÈRIEUX, 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).

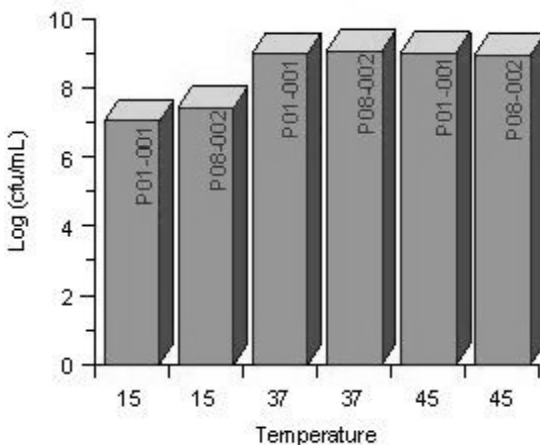
### 3.2.2 Presence of Gas by Glucose Fermentation

Strains P01-001 and P08-002 produce CO<sub>2</sub> 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 °C. 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).

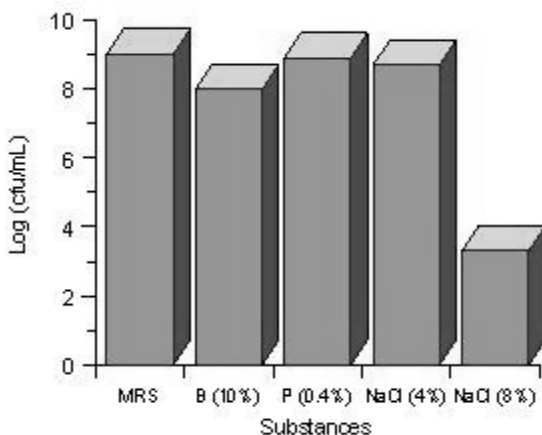
Tolerance to bile and phenol (phenols can be formed in the intestines by bacteria that desaminate some aromatic amino acids delivered by the diet or produced by endogenous proteins) is an important characteristic for a better survival ability, not necessarily of multiplication, in the intestines.

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

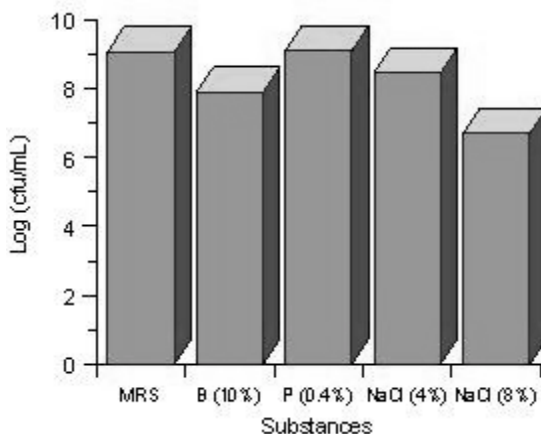
XANTHOPOULOS, LITOPOULOU-TZANETAKI and TZANETAKIS (2000) have isolated *Lactobacillus reuteri* DC423 with a high resistance (0.4%) to phenol from children's faeces. The counting of viable cells was not affected at all by phenol.

**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 37°C 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 °C and small growth at 15°C.

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 gastrointestinal 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 45° 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.

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