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BIODEV MASTER COURSE

Development of bioprocess to produce a new natural biopromoter for animal feeding

MASTER

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1. INTRODUCTION

The demand for animal protein for human consumption is currently on the rise and is largely supplied by livestock farms. This activity requires high-quality feeds for the animals with high protein content, which should contain not only necessary nutrients but also complementary additives to keep animals healthy and favor growth. Some of the most utilized growth-promoting additives include hormones, antibiotics, ionophores and some salts.

Although these additives promote increasing in growth, on the other hand their improper use can result in adverse effects on the animal and final consumer. In the case of antibiotics it can lead for example to resistance on pathogenic bacteria.

Diet, stress, antibiotics and modern husbandry practices have been identified as factors capable of affecting animal health and animal growth performance. However, it is known that antibiotics have indirect adverse side effects with implications for human health.

In this contest, probiotics, organics acids and food supplements have deserved considerable attention of researchers as a possible alternative to the substitution of the traditional promoters of growth.

1.1 SWINE

Pork meat is the most consumed animal protein in the world. In 1999, pork producers throughout the world produced 88.4 million tones of meat, in the following order, according to volume of production: Asia, EU, Americas and Africa + Australasia, with 53.2%, 28.9%, 16.3% and 1%, respectively. The worldwide consumption of pork, based on a population of 6.0 billion people, can be estimated at 14.7 kg/person, which makes it the most consumed meat in the world. The consumption of pork in Brazil can be estimated at 11 kg/person/year. The pig production industry in the south of Brazil can be considered the most technical pig production industry in South America, with good productivity rates, placing our country among the world's seven largest producers, with 1.75 tones of pork per year (Silveira, 2005).

One of the biggest problem of swine production is the mortality that is around 20%, especially after weaning. Precociously weaned pigs precociously experience

nutritional and environment stress with limited digestion capacity (due to insufficient production of chloride acid and digestive enzymes as amylase, lipase and tripsin pancreatic and also to the sudden alteration in the composition of the diet). Immunological problems can also affect the performance after weaning, since its immunity is not completely effective yet (APCS, 2005).

Diarrhea is the main cause in 41% of the deaths and it contributes significantly for the decrease in performance and health of swines (Larpent *et al*., 1994). According to Jonsson (1992), the main etiological agent of diarrhea in swines is the enterotoxigenic bacteria *Escherichia coli*. Infection rates are reported to vary between 31 in U.S.A. and 82 cases in Australia.

During the first three weeks of age (wean period included), swines are highly susceptible to the diarrhea. *Escherichia coli* bacteria adhere to the corrugated mucosal folds of the host intestine and produce enterotoxins that stimulate enterocit cells to pump liquid into the lumen, increasing intestinal contractions and resulting diarrhea.

Neomycin antibiotic has largely been used for colibacilose treatment (OURO FINO ANIMAL HEALTH, 2005), in which it acts inhibiting protein synthesis of the microorganism. This antibiotic binds to bacterial ribosome provoking the incorrect reading of the genetic code and consequently, defective amino acids are incorporated into the polypeptide chain. The defective protein formed is then used by the metabolism of the bacterium, resulting in its death.

This aforementioned antibiotic suffers little adsorption although it presents intense activity in the host intestine lumen. This antibiotic is specific for intestinal infections and afterwards it is eliminated with feces, in an active form.

E. coli has high ability to develop resistance to drugs due to its genetic exchanges from generation to generation. The mutational exchange that confers resistance can simultaneously modify the virulence factors and affect the pathogenicity of the microorganism. *E. coli* carries R-plasmids which regulates the resistance to drugs. Although this resistance happens, in the last 25 years the ratio of enteric bacteria that load plasmids for resistance to a multitude of drugs has increased quite slowly (Saldarriaga *et al*., 2000).

Swine is monogastric and harbors a complex bacterial flora in its intestine tract that basically suppresses undesirable bacteria. The predominant bacteria of this microflora are mainly those lactic acid types such as *Lactobacillus* sp. and *Streptococcus* sp. (Rojas *et al*., 2002).

Administration of antimicrobial agents may disturb the microbial flora. This and other factors that may lead to the microflora imbalance may directly reflect in the host heath. The use of an improper antibiotic for example can cause a stress of any nature that will allow pathogenic microorganisms to install and multiply.

1.2 PROMOTERS OF GROWTH IN THE SWINECULTURE

The gastrointestinal tract has the function to convert ingested food in necessary nutrients to the growth, maintenance, production and reproduction. The gastrointestinal tract is extremely exposed to action of exogenous agents that may be ingested with foods. To assure that the food will be properly ingested and converted into nutrients, some chemical additives are added to the diet. Some of them such as antimicrobials, acid substances (formic acid, ascetic, citric, lactic), probiotics and enzymes are used to promote the animal growth (Roth, 2005).

Modern growth-promoters can improve the animal performance by using low dosages without increasing resistance and crossovers with other promoters. They are non-toxic, non-mutagenic and consequently keep the normal gastrointestinal flora on balance. In addition, these promoters are biodegradable and then they prevent environmental pollution (Lancini, 1994).

1.3 PROBIOTIC

The term probiotic is a relatively new word that means “for life” and it is currently used to nominate those bacteria associated with benefit effects for humans and animals. The original observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, a Russian born scientist who won the Nobel Prize in the beginning of the last century when he worked at the Pasteur Institute. He suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes"(FAO/WHO, 2001).

At this time Henry Tissier, a French pediatrician, observed that children with diarrhea had in their stools a low number of bacteria characterized by a peculiar Y shaped. In contrast, these “bifid” bacteria were abundant in healthy children. Afterwards he suggested that these bacteria could be administered to patients with diarrhea to help to restore a healthy intestine flora.

In order to redefine the microbial nature of probiotics, Fuller (1989) point out the word as "A live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance". More recently, but probably not the last, a new definition for probiotic is given by Guarner and Schaafsma (1998) as being "live microorganisms that, when consumed in adequate amounts, confer a health effect on the host".

Lactobacilli are the most common bacteria used as probiotics in animal feeds and human foods. The use in animal feed is under regulations especially concerning to the additives.

Members of *Lactobacillus* and *Bifidobacterium* genera are also largely employed, but not exclusively as probiotic microorganisms. The number of probiotic foods available to the consumer is highly increasing in number. However, some ecological considerations for the intestine flora has to be taken into account. It is necessary to understand more about the relevance of probiotic food concept for humans.

When incorporated to the food as part of the elaboration or as additive, probiotics generate a functional food, i.e., it presents particular characteristics, nutritional or not, that promote a physiological effect on the organism in a positive way improving the traditional nutrition (Gonzaález-Martínez *et al*., 2001).

Health benefits by probiotics use include: the relief of symptoms of lactose intolerance, enhancement of the immune system, reduction in duration of diarrhea caused by rotavirus, decrease of faecal bacterial enzyme activity and mutagenicity, prevention of recurrence of supercial bladder cancer, and prevention of atopic diseases (Naidu, Bidlack, & Clemens, 1999; Kalliomaki et al., 2001).

Probiotic lactic acid bacteria are commercialized mainly as food supplements for daily use (Heller, 2001). Currently, probiotic milk drinks and yogurts are manufactured in different ways. Bacteria can be added to fresh milk without any fermentation (for instance, the sweet AB Milk®), or the milk can be fermented with the probiotic bacteria (for instance Yakult®). A third type of product is the mild yogurt added *Streptococcus thermophilus* and a probiotic *Lactobacillus* used as strain starters. The probiotic *Lactobacillus*, more often *L. acidophilus*, replaces *L. delbrueckii* subsp**.** *bulgaricus* that is the normal starter (together with *St. thermophilus*) for the manufacture of yoghurt (Heller, 2001). The advantage of using those two combined bacteria is that the probiotic bacterium may produce functional metabolites such as bacteriocins during fermentation.

The volume of fermented broth represents more than 95% of the biomass production. This fermented broth contains functional metabolites, not fermented sugar and mineral salts. In the conventional process this broth is considered a residue and generally is discarded. Due to the high concentration of the organic material, the broth naturally presents Biochemical Oxygen Demand/Chemical Oxygen Demand (BOD/COD) in 20.000 mg O_2/L. It may cause an overload in the station of treatment of the industry. The international literature is scarce in references about valuation and/or the use of this bioresidue. The development of a simple and low cost technology process to use this bioresidue is necessary to facilitate the transformation of it in new products.

1.4 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are Gram-positive, catalyze-negative, oxidase negative and non-sporulating microaerophilic bacteria whose lactate is the main fermentation product from carbohydrates. The lactic acid bacteria comprise both types cocci (e.g. *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Stretococcus*, *Enterococcus*) and rods (*Lactobacillus*, *Carnobacterium*, *Bifidobacterium*). Many of these lactic acid bacteria are generally recognized for their contribution to the flavor and aroma developed and to spoilage retardation (De Vuyst *et al.*, 1994). Therefore, the traditional use of these microrganisms in the fermentation of foods and beverages has resulted in their application in many starter cultures currently involved in the fermentation of a wide variety of agricultural raw materials such as milk, meat, fruit, vegetables and cereals (Buckenhüsk, 1993). The lactic acid bacterial strains present in these starter cultures contribute to the organoleptic properties and the preservation of the fermented products by *in situ* production of antimicrobial substances such as lactic acid and acetic acid, hydrogen peroxide and bacteriocins (Vandenbergh, 1993). Because of the general tendency to decrease the use of chemical additives, such natural inhibitors could replace the use of chemical preservatives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate and nitrite. For this reason, bacteriocins produced by lactic acid bacteria may be very promising as biological food preservatives in future food preservation (Lewus *et al.*, 1991). Some bacteriocins produced by LAB, such as nisin, inhibit not only closely related species but are also effective against food-borne pathogens such as *Listeria monocytogenes* and many other gram-positive spoilage microrganisms (Tagg *et al.*, 1976). For this reason, bacteriocins have attracted

considerable interest for use as natural food preservatives in recent years, which has led to the discovery of an ever increasing potential 'arsenal' of these protein inhibitors.

Undoubtedly, the most extensive studies are concentrated in the bacteriocin nisin, which has gained widespread application in the food industry. Bacteriocin is FDA (Food and Drug Administration) and GRAS (Generally Recognized as Safe) approved. For instance, *Lactococcus lactis* is largely used as a food additive in many countries, particularly for cheese process, dairy products and canned foods (Delves-Broughton, 1990). An alternative approach to introduce bacteriocins to food is the use of live cell cultures that produce bacteriocins *in situ* in the food.

Furthermore, certain lactic acid bacteria, especially some lactobacilli and bifidobacteria, are believed to play a beneficial role in the gastro-intestinal tract (Marteau *et al*., 1993). It is worth noticing that Lactobacilli are also potentially useful as carriers for oral immunization. Once orally administered lactobacilli trigger both a mucosal and systemic immune reaction against epitopes associated with these organisms (Norton *et al*., 1997).

The lactic acid bacteria are able to grow in a range of acid band of pH and in presence of organic acids. The mechanism of tolerance to the acidity is not completely understood, but one believes that passive diffusion occurs and the intracellular accumulation reduces pH affecting the membrane permeability (Mcdonald *et al*., 1990). The production of facts acids in the plasmatic membrane of the bacteria determines its resistance to stress caused for the acids. Therefore, organic acid contributes in a way for the inhibition of the microrganism growth by increasing energy consumption that keeps the homeostasis of pH (Gonzalo *et al*., 1998).

1.5 ORGANIC ACIDS

The way that organic acid works is attributed by the direct reduction of the substrate pH. The explanation is related to the reduction of the internal cell pH for ionization of acids that do not dissociate or for the transport interruption of the substrate due to the permeability alteration of the cell membrane. This transport inhibition of substrate occurs since organic acid can inhibit NADH oxidation eliminating supplements of reduction agents in the electron transport system (Beuchat *et al*., 1989). Organic acids are related to physiological effects with implications to the immune system, empting gastric content, intestinal motilities, and water and mineral absorption, especially calcium. Formiate is important for metabolism on the transport of substances

that contain carbon generated mainly during the amino acid metabolism. Formic acid is an efficient acidificant compound that presents independent antimicrobial action of pH and inhibits descarboxilases and porfirinics enzymes such as catalase. The antimicrobial action of the lactic acid is relatively small, acting mainly against anaerobic bacteria. Its antimicrobial action is more related to the reduction of pH (Lück, 1981).

1.6 BACTERIOCINS

Bacteriocins are proteinaceous compounds produced by bacteria, both Gram positive and Gram-negative, and they are active chiefly against closely related bacteria (Tagg *et al.*, 1976). The discovery of bacteriocins dates back to 1925, when *E. coli* V was shown to produce an antimicrobial compound active against *E. coli F*. These antimicrobial substances produced by *E. coli* were named colicins and 17 different types, based on their adsorption, were later reported.

Like the colicins (25–90 kDa, produced by *E. coli* and active against other *Enterobacteriaceae*) and microcins (<10 kDa, produced by *Enterobacteriaceae* and active against other Gram-negative bacteria), the bacteriocins produced by Gram-positive bacteria were defined as proteinaceous compounds that kill only closely related species (Jack *et al.*, 1995). Although it is true for the majority of compounds, it is now evident that bacteriocins produced by lactic acid bacteria display bactericidal activity beyond species that are closely related (Klaenhemmer, 1993). The first report of production of a bacteriocin produced by lactic acid bacteria was in 1928 by Rogers. The substance was determined as a polypeptide and subsequently named nisin (Mattick *et al.*, 1947). Since that time, bacteriocin field has expanded exponentially and now bacteriocins produced by all genera of the lactic acid bacteria have been reported. Bacteriocin production has been widely found among strains of *L. acidophilus* complex isolated from the intestinal tract (Itoh *et al.*, 1995). In contrast, information about bacteriocin production by strains of *L. casei* complex remains scarce (Cuozzo et al., 2000).

The majority of bacteriocins from lactic acid bacteria have been characterized according to the early definition of a proteinaceous inhibitor, estimation of their molecular mass, and determination of their inhibition spectrum. Recent developments in biochemical and molecular biology have improved the characterization of many compounds working to elucidate their genetic organization, structures and mode of action. Despite their heterogeneity, bacteriocins produced by lactic acid bacteria were

subdivided into three distinct classes based on these genetic and biochemical resemblances: Lantibiotics (class I); Small, Heat-Stable, Non-Lanthionine containing, Membrane-Active Peptides (class II) and Large Heat-Labile Proteins (class III) (Nes *et al.*, 1996).

The class I bacteriocin, nisin, and some of the class II bacteriocins have been shown to be membrane-active peptides that destroy the integrity of the cytoplasmic membrane via the formation of membrane channels (Figure 1). In doing so, they alter the membrane permeability and therefore cause leakage of low molecular mass metabolites or dissipate the proton motive force, thereby inhibiting energy production and biosynthesis of proteins or nucleic acids. Most bacteriocins produced by lactic acid bacteria display a bactericidal effect on the sensitive cells, all or not resulting in cell lysis (Bhunia *et al.*, 1991; Sahl, 1991). On the other hand, other bacteriocins, such as lactocin 27, leucocin A and leuconocin S have been reported to act bacteriostatically. However, the designation of lethal versus static effect can be dependent upon aspects of the assay system, including the number of arbitrary units, the buffer or broth, the purity of the inhibitor, and the indicator species and cell density used (De Vuyst *et al.*, 1994). The mode of action of numerous bacteriocins has been reported and, therefore, only a few of them, representing the different classes are described in this section.

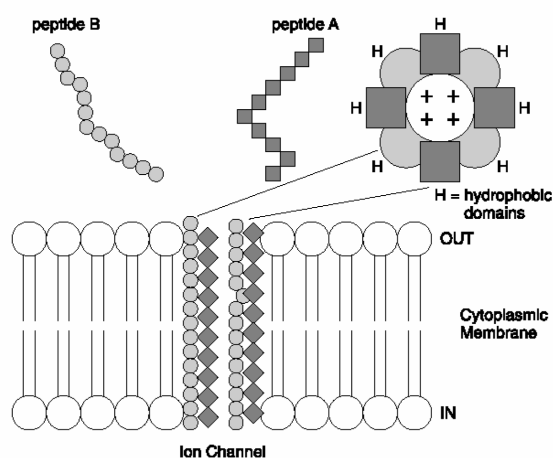

FIGURE 1 - Barrel-stave poration complexes proposed for class II bacteriocins. (Klaenhammer, 1993)

The class IA_I lantibiotic nisin was shown to form ion-permeable channels in the cytoplasmic membrane of susceptible cells, resulting in an increase in the membrane permeability, disturbing the membrane potential and causing an efflux of ATP, amino acids, and essential ions such as potassium and magnesium. Ultimately, the biosynthesis of macromolecules and energy production are inhibited resulting in cell death. Nisin

does not require a membrane receptor but requires an energized membrane for its activity, which appeared to be dependent on the phospholipid composition of the membrane (Sahl, 1991).

The class IIA pediocins PA-1/AcH and JD were reported to exhibit their bactericidal action at the cytoplasmic membrane and to cause a collapse of the pH gradient and proton motive force (Christensen *et al.*, 1992). As for the mechanism of action of the class III bacteriocins, it still remains to be elucidated.

Many of the bacterial metabolic pathways are induced by various extracellular stimuli. Those environmental conditions are sensed and signaled through, by means of signal transduction systems. Many of these systems consist of two components, a sensor, often located in the cytoplasmic membrane and a cytoplasmic response regulator (Parkinson *et al.*, 1992). They are, therefore, generally called two-component systems. The environmental sensor acts as a histidine protein kinase (HPK) and modifies the response regulator (RR) protein, which in turn triggers an adapting response, in most cases by gene regulation. Most histidine protein kinases consist of an *N*-terminal sensory domain and a cytoplasmic *C*-terminal transmitter. These findings could then result in the following hypothetical model for bacteriocin biosynthesis (Figure 2).

Firstly an inducing signal activates, via the two-component signaling pathway, the promoters responsible for the expression of the operons involved in bacteriocin production. In the case of nisin, production and immunity have shown to be auto regulated. Transcription results in the concerted production of the proteins constituting the modification and secretion machinery, together with the inactive bacteriocin precursor molecule.

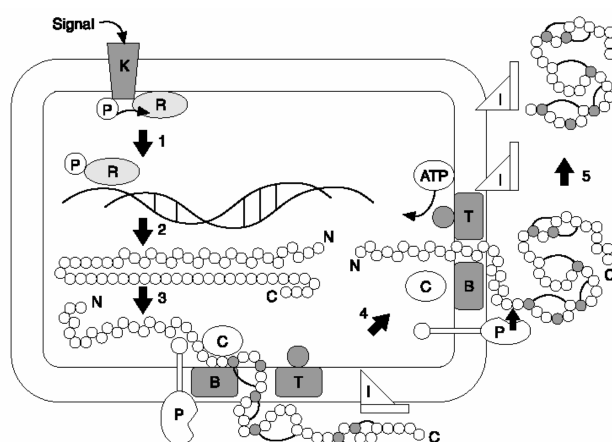

FIGURE 2 - A conceptual maturation pathway for nisin is given as a 5-step process. A two component signal transduction system induces transcription (*step 1*). Translation

results in an inactive unmodified precursor peptide (*step 2*). The leader peptide is proposed to play a role in targeting of the precursor to a membrane-located modification complex (*step 3*). Dehydration and lanthionine and dehydro-lanthionine formation (*step 4*) is followed by extracellular processing and secretion (*step 5*). (De Vos *et al.*, 1995)

1.7 ANTIBIOTIC

The antibiotics are chemical composites used to control infectious organisms. Initially they were employed on therapeutic usages and later they became widely used to prevent and promote animal growth. It can guarantee high rates of productivity with reduction in mortality and morbidity and maintain the animal welfare. Thereby, antibiotics use is essential for the current systems of production.

Although the relation that associates the antibiotic use in the units of animal production and the development of resistance and its transference to humans is not clear, some epidemiological studies suggest that the consumption of animal derivatives is a possible way of pathogenic bacteria acquisition. These evidences have led international organizations on human and animal health to recommend prudence for antibiotics use.

According to Padilha (2003), the bacterial resistance has been controlled due to great demand in the development of new composite families or simply by modifying already existing composites.

European countries were the first to establish programs for controlling antibiotics use in animal production (Wegener *et al.*, 1997). In the United States, the "National Program of Monitoring of the Antimicrobial Resistance" has started in 1996 as a resulting collaboration among institutes such as the Center for Disease Control (CDC), Food and Drug Administration (FDA) and the Department of Agriculture. This program was responsible for modifications on the antimicrobial susceptibilities status of zoonotic enteric-pathogens in humans and animals (illness and healthful) and in carcasses of animals. In January 1999, the FDA approved a regulation to vanish the use of the majority of antibiotics that promotes animal growth.

In Brazil, the use of cloranfenicol and nitrofuran in animals whose meat is destined to human consumption is prohibited by the Ministry of Agriculture. The antibiotics, which use is still allowed, will be forbidden from January of 2006. Consequently, an enormous demand for natural promoters of animal growth has been developed with scope to minimize or eliminate the formation of residues in foods.

2 OBJECTIVE

The purposes of the present work were to isolate and to characterize probiotic bacteria and to check the potential use of fermented broth produced by probiotic bacteria during the biomass formation in order to develop a new biopromoter for the animals (pigs) and:

- Determine the gain of suckling pig weight;
- Check the feed consumption;
- Estimate the feed conversion;
- Compare the action of the natural biopromoter with the probiotic in the fist weeks of pigs life;

It is important to notice that this biopromoter was adsorbed in natural support sugarcane bagasse, the residue of milled cane.

3 MATERIAL AND METHODS

3.1. MICRORGANISMS ISOLATION

The microorganisms were isolated from healthy swine never treated by antibiotics. Immediately after the pigs were slaughtered, microbial flora was collected from stomach, and from small and large intestine, places where a great number of bacteria from *Lactobacillus* genus are found. (Pancheniak, 2005). Samples were diluted and distributed, according to the *pour plate* technique, into plates with MRS agar (Difco Laboratories, Richmond, CA, USA). The plates were incubated for 48 hours at 37 °C (Reque *et al.*, 2000).

3.2. MICRORGANISM CHARACTERIZATION

3.2.1. Biochemistry and Physiology Characterizations

Biochemistry and Physiology characterizations followed Pancheniak (2005) where the method is based on the following exam types:

- Morfology and optical microscope ;
- Gram stain ;
- Fermentation in different carbon sources (API 50 CHL, BIOMËRIEUX) ;

3.2.2. Molecular Identification

The genotype profile for the isolated lactic bacteria were analyzed and compared to standard strains. The microbial DNA was amplified using RAPD-PCR technique (Random Amplification of Polymorphic DNA-Polymerase Chain Reaction).

3.2.2.1. DNA Extraction from Lactic Bacteria

Genomic DNA was extract from overnight cultures. The method of DNA extraction was performed in four steps: cellular lysis, deproteinization, precipitation and quantification of the extracted DNA.

Cellular lysis – lysis incubation was performed for 30 min at 55 °C using 500 µL of lysis buffer (Tris-HCl 10mM pH 8,0; EDTA 1mM; SDS - Sodium Dodecyl Sulfate 5%). Three cycles of frost (-196 °C/5min) and defrost (37 °C/5min) were made. Afterwards, samples were digested by 20 mg/mL of Proteinase K for 30 min at 55 °C and RNAase (20mg/mL) for 1hour at 37 °C.

Deproteinization – proteins were eliminated by 03 phenol extractions followed by 01 extraction adding phenol/chloroform/isoamyl alcohol (25:24:1) and 01 addidional step in chloroform (removes trace phenol). Each extraction was followed by homogenization and centrifugation at 14,000g for 5 min at 20 °C. DNA was always kept in the aqueous phase.

Precipitation – 10% of final volume of sodium acetate (3M) and two volumes of cold ethanol were added to the last upper aqueous phase placed in fresh tube. This mixture was kept for 1 hour at -70 °C. After centrifugation at 14,000g at 4 °C, the precipitated DNA was washed twice with 70% ethanol. The DNA was dried for 10 min in 37°C oven to allow ethanol evaporation, and then, the pellet was rehydrated in 100 µL of ultra pure water.

DNA dosage – the last step of DNA purification was to verify the purity and determine DNA concentration. DNA samples were quantified by agarose gel electrophoresis (0.8% agarose) and by UV spectrometer.

3.2.2.2. RAPD Technique

Amplification was carried out in 25µL reaction volume by thermocycler with a hot bonnet lid. Seven different primers (M13, M14, COC, A2, A3, A9 e A10) were singly employed in seven series of amplifications (Annex 1). To prevent evaporation 25µL of mineral oil was added to each reaction tube.

Amplification products were resolved by electrophoresis in 1.6% (w/v) agarose TAE (40mM tris/acetate, 10mM EDTA pH8.0) gels stained with ethidium bromide and photographed.

Numerical Analysis of the patters were performed by Vilber Lourmat® software. Comparisons of RAPD-PCR patters were made using Jaccard Coefficient and unweight pair group method using arithmetic averages (UPGMA).

3.3. MICROORGANISM SELECTION

The probiotic bacterium used in the biopromoter study was from strain *P01-001*. This bacterium was isolated from swine intestine. The strain was subcultured anaerobically in customized brown sugarcane (sugarcane brown sugar medium) for 24 hours at 36 °C. For the stock culture it was centrifuged (7.000 g at 4°C for 20 min), rehydrated in 25% (v/v) of skimmed milk and kept frozen at -4°C, and finally, it was lyophilized.

3.4. MEDIUM

The biomass production was carried out in customized 3% brown sugarcane (sugarcane brown sugar medium) and 1% yeast extract. This medium was sterilized at 121^0C for 20 min. To obtain a fresh culture, strains were grown in 50 ml of the medium that was used during the fermentation. The transfer volume was 10% (v/v), and the incubation was at 37^0C for 24 hours.

3.5. FERMENTOR-SCALE STUDIES

Fermentation was carried out in a 5 L bioreactor with a working volume of 4 L. The fermentation media was sugarcane brown sugar 3% and yeast extract 1% medium at initial pH 6.5. The pH was not kept constant and the fermentation medium was sterilized *in situ*. The fermentation temperature was kept constant at 37°C without agitation. The kinetic of the bacteria growth was carried out in a 5 L bioreactor with agitation of 12 rpm (Figure 3). To determine biomass dry weight (g/L), pH and bacteriocin activity, samples were taken out before inoculation, and after inoculation following 24 hours of incubation. Bacteriocin activity of the cell-free culture supernatant was measured by bioassay.

The supernatant of the fermentation was adsorbed in the sugarcane bagasse support, and the biomass was used as probiotic. This support impregnated with fermented broth was dried at 50°C in a ventilated oven.

FIGURE 3 – Bioreactor BioTecnal –TE 2005, five-liter capacity.

3.5.1. SUGAR CONTENT IN FERMENTATION PROCESS

In the present work, Somogyi-Nelson method (Nelson, 1944; Somogyi, 1945) was employed to determine reducing sugars and, for the total sugars, the method employed was Phenol-Sulfuric acid (Dubois *et al*., 1956).

3.6. ORGANIC ACIDS QUANTIFICATION IN FERMENTATION PROCESS

To quantify organic acids present in the lactic fermentation and in natural biopromoter the High Performance Liquid Chromatography (HPLC) method was used and its operating conditions are shown in table I.

TABLE I – Operating conditions on HPLC to quantify organic acids in fermented products.

Chromatography	Operating conditions
Column	Amine x HPX 87H
Column temperatura	60 °C
Mobile phase – eluent	H_2SO_4
Mobile phase concentration	5 mM
Mobile phase flow rate	0.6 mL/min
Pressure pump	48 kg/cm^2
Injection volume	20 µL

According to these conditions table II shows the retention times of each component.

TABLE II – Retention times of some organic acids on the HPLC column.

Standard organic acid	Retention time (min)
Glucose	8.96
Formic acid	13.33
Lactic acid	12.65
Acetic acid	14.78
Ethanol	21.50

3.7. ANTIMICROBIAL TEST - BIOASSAY

3.7.1. Antimicrobian activity of the biopromotor

Plates with Agar Muller Hinton Broth were used for growing bacteria culture. Bacteria *Escherichia coli* ATCC 25922 were activated in BHI (Brain Heart Infusion) broth, and incubated at 37 ºC for 24 hours. After activation, they were diluted to 10^{-3} with 0.1% peptone water. This 10^{-3} dilution was spread out onto the surface of each agar plate using a sterile spatula and adding 50 mg of biopromotor /well. In another plate an antibiogram test using Neomycin stander antibiotic (NEO 30 MCG) was added onto the agar surface with biopromotor (50mg/well) followed by incubation at 37 °C for 24 hours. HPLC was used to analysis of biopromotor concentration. After incubation, the diameter of the inhibition halo formed around the disk was measured.

3.8. INCOME COEFICIENT AND KINETIC PARAMETERS IN THE BIOMASS OF *Lactobacillus P01-001*

The income coefficient of the biomass production of *Lactobacillus P01-001* was obtained through of the equation (1); the biomass productivity for the equation (2) and the specific growth speed for the equation (3).

$$Y_{X/S} = \frac{X_o - X_i}{S_i - S_o} \quad (1)$$

Where:

X_o = final biomass
X_i = initial biomass
S_i = initial sugar
S_o = final sugar

The biomass productivity was obtained by the following relation:

$$P_X\,(g/L.h) = \mu.X \quad (2)$$

Where:

μ = specific growth factor (h^{-1})
X = final biomass (g/L)

3.9. DEVELOPMENT OF THE NATURAL BIOPROMOTOR

Several steps are necessary for developing the natural biopromoter, such as microorganism selection and fermentation. In the end of the lactic fermentation centrifugation (7.000 g at 4°C for 10 min) done in Falcon tubes, it was carried out to separate broth from the cells.

The broth was separated to be adsorbed in the support, while the cells were rehydrated in 25% (v/v) of skimmed milk and kept frozen at -4°C, and afterwards these cells were lyophilized to obtain the Probiotic PP-LPB. The process of biopromoter and probiotic production are summarized in figure 6.

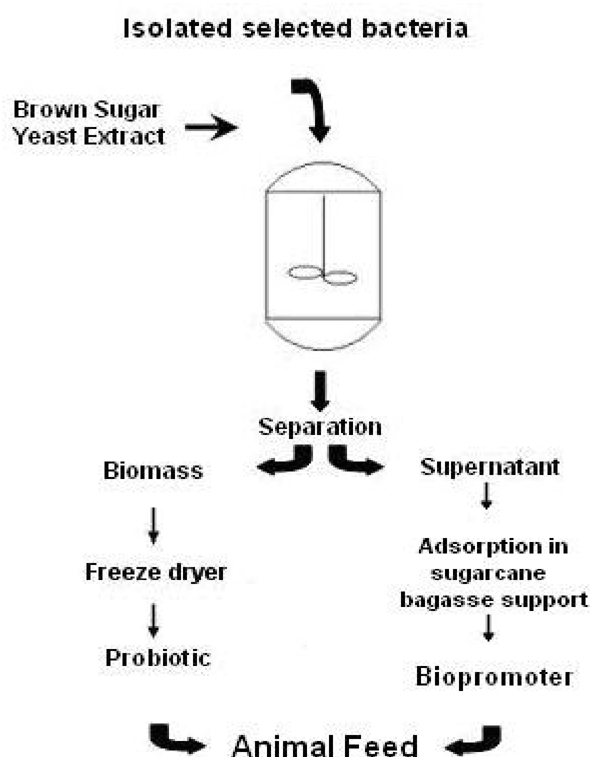

Figure 6 – Bioprocess production of the probiotic and biopromoter.

3.9.1. THE SUPPORT PREPARATION

The less fibrous internal part of the sugarcane was used because the adsorption is better in this portion. The bagasse was washed three times and after dried at 70 °C it

was ground on knife mill and the product was passed through sieves with mesh from 6 to 14.

3.9.2. FERMENTED BROTH APPLICATION ON SUPPORT

The fermented broth was mixtured to the support by an air compressor connected to an injector pistol until it became saturated but without droplet formation, i.e., when it achieved around seven times of its initial weight. Additionally, it was observed that a support weighting 143g could adsorb approximately 1000mL of fermented broth. Therefore, each gram of biopromotor retained 7 mL in fermented broth that means 7 times more concentrate. This way, for the amount of support available in the present work, the fermented broth volume used was 88 L (figure 4).

FIGURE 4 – Trays containing the biopromotor and sugarcane bagasse support.

3.9.3. BIOPROMOTOR ON DRYING TRAYS

Biopromotor was placed on trays and kept to dry at 50°C in a ventilated oven TE 394/3 TECNAL for approximately five hours by each dry cycle. The trays were made of wood with dimensions 0.75m x 0.55m x 0.05m. A *mesh* 50-screen was attached to the bottom of each tray to allow the air circulation in between of the support more fermented broth.

3.9.4. ADSORPTION EVOLUTION OF SUGARCANE BAGASSE/BROTH FERMENTED

An experiment was realized to determine the saturation region and the adsorptive capacity of the support. Three grams of the support were adsorbed several

times with fermented broth and dried. After each cycle of adsorption and drying the support was weighed to determine the weight gain.

3.9.5. BIOPROMOTOR PREPARATION

The biopromotor was grounded several times in microniser (pin mill) until *mesh* 30 granulometry powder was obtained. After micronisation the biopromotor was homogenized in shaped Y mixer.

3.9.6. BIOPROMOTOR ORGANIC ACID ANALYSED BY HPLC

From the final biopromotor produced it was taken 1g and added to 9 mL of ultra pure water. After shaking in vortex, the sample was kept for 3 hours in the fridge for dissolution of the soluble composts present in the sample. The sample was filtered in sieve and diluted again to 1:10 proportion. Then, the diluted sample was filtered in 0.22μm filter and injected on HPLC for organic acids analysis.

3.10. BIOPROMOTER ANIMALS TEST

The effect of the probiotic and of the natural biopromoter was evaluated based on the animal performance of 25 days suckling pigs diet.

3.10.1. Animals and Facilities

Forty eight (48) suckling pigs with 25 days old were used, accommodates in a nursery pig with 12 suspended compartments (1,50 x 2.00 m each one). All suckling pig received enough feed and water and the same conditions of handle, as well as Ouro Fino Ivermectin 1%, in the beginning of the experiment.

3.10.2. Studied Variables

The animal performance was studied based on the daily weight gain, daily feed consumption and feed conversions. The present work was divided into phases according to the age of the animals: phase 1 (25 to 35 days), phase 2 (35 to 54 days).

3.10.3. Treatments

The different treatments for the suckling pigs can be seen in the table III. The basal feed composition for the suckling pigs can be seen in annex 2.

TABLE III – Series of treatments given to suckling pigs.

Treatments	Description
T1	Basal feed (Negative control)
T2	Basal feed + Growth promoter[1] (Positive control)
T3	Basal feed + Probiotic PP-LPB 1,0%
T4	Basal feed + Natural Biopromoter 0,50%

[1] Neomicin sulphate Ouro Fino® (56 ppm)

3.10.4. Statistics and Experimental Analysis

The experimental analysis was in casual blocks (DBC) with 3 repetitions of 4 animals each (two male and two female). The results were analysed by of the procedure "General Linear Model" (GLM) of the software "Statistical Analysis Sistem" (SAS®-1998), and the averages compared with the Tukey test with 5% probability.

3.11. ECONOMIC ANALYSIS OF THE BIOMASS PRODUCTION OF *Lactobacillus P01-001* IN ALTERNATIVE MEDIUM

The comparison between medium for biomass production was realized using a commercial medium culture MRS (DifcoTM Lactobacilli) and an alternative medium in brown sugar 3% and yeast extract 1%. The economic analysis was performed to produce 1 g/L of biomass:

- To prepare 1 liter of MRS broth it costs $ 19.80
- To prepare 1 liter of alternative medium (brown sugar 3% and yeast extract 1%) it costs $ 0.28

4. RESULTS

4.1. MICROORGANISM ISOLATION

Thirty three strains were isolated after tolerance tests to extreme temperatures, different pH concentrations, and bile, an inhibitory substance. Three strains were selected (*P01-001*, *P08-002* and *LPB-05*) since they presented the following characteristics: bacillary bacteria, Gram positive and strong viability during the process. The isolates *P01-001* showed the best viability compared to the others.

4.2. MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS

4.2.1. Morphology and Physiology in Optical Microscope

The isolates are slightly irregular in shape, similar to bacillus form separated or linked in chain, generally present dimensions with width 0.7-1.0µm and length 2.0-5.0µm (Figure 5). These bacteria are heterofermentatives by producing other metabolites such as lactic acid, acetic acid, propionic acid, formic acid and ethanol. Therefore, according to these aforementioned characteristics, they can be considered as lactic bacteria.

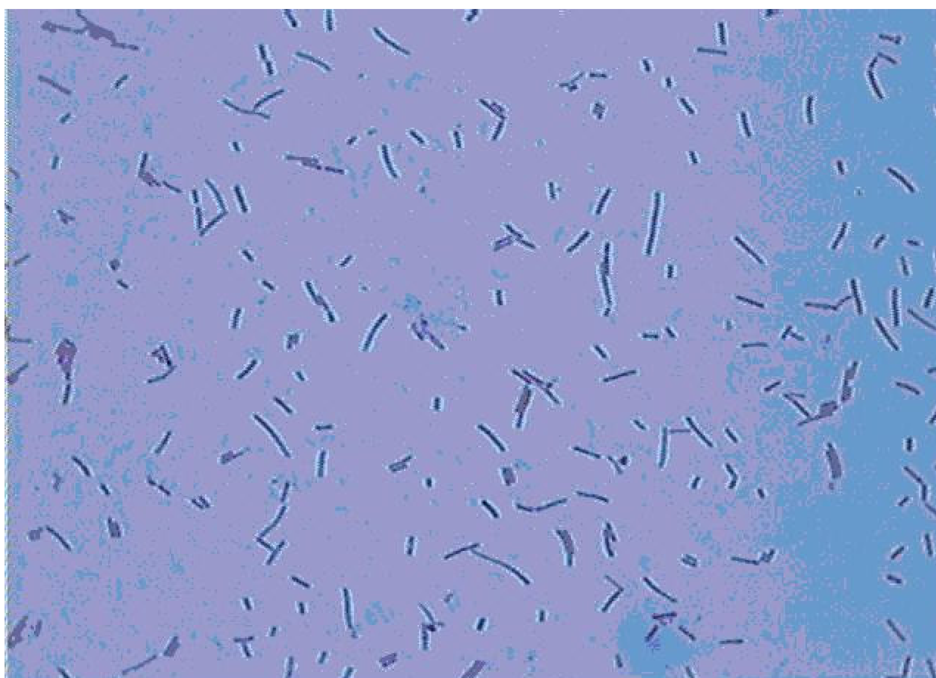

FIGURE 5 – Isolate *P01-001* morphology viewed by optical microscope (400x).

4.2.2. Sugar Fermentation

According to the gallery API 50 CHL (Biomérieux) of sugar fermentation, the isolates *P01-001* and *P08-002* were identified as *Lactobacillus fermentum* with probability 95%. As for the isolate *LPB-05*, it was identified as *L. acidophilus*.

4.3. MOLECULAR CHARACTERIZATION

In the present work, the obtained isolates (*P01-001*, *P08-002* e *LPB-05*) were genetically compared to reference standard strains (Table IV) by analyzing the electrophoretic profile of amplified DNA fragments using RAPD-PCR technique. The

outcome of screening test showed that informative patterns were obtained with M14 and COC primers, already applied for typing in several microorganisms (Paffeti et al., 1995, Zaparolli et al., 2000, Torriani et al., 2001). However, in this study the M14 primer did not show difference between Leuconostoc and L. sakei (figure 6). In addition, by using M13 primer no difference was seen between *P01-001* and *P08-002*, but difference was detected when A3 primer was used (annex 1).

TABLE IV – List of standard strains and isolated strains including the individual enter codes of the stock banks.

SPECIES	CODE
Lactobacillus alimentarius	ATCC 29643
Lactobacillus curvatus	ATCC 514369
Lactobacillus casei shirota	YIT – 9018
Leuconostoc mesenteroides	INRA 23
Lactobacillus lactis	INRA 18
Lactobacillus sakei	ATCC 15521
Lactobacillus fermentum ?	P01-001
Lactobacillus fermentum ?	P08-002
Lactobacillus fermentum ?	P01-001 05
Lactobacillus fermentum	ATCC 9338
Lactobacillus acidophilus	ATCC 43121

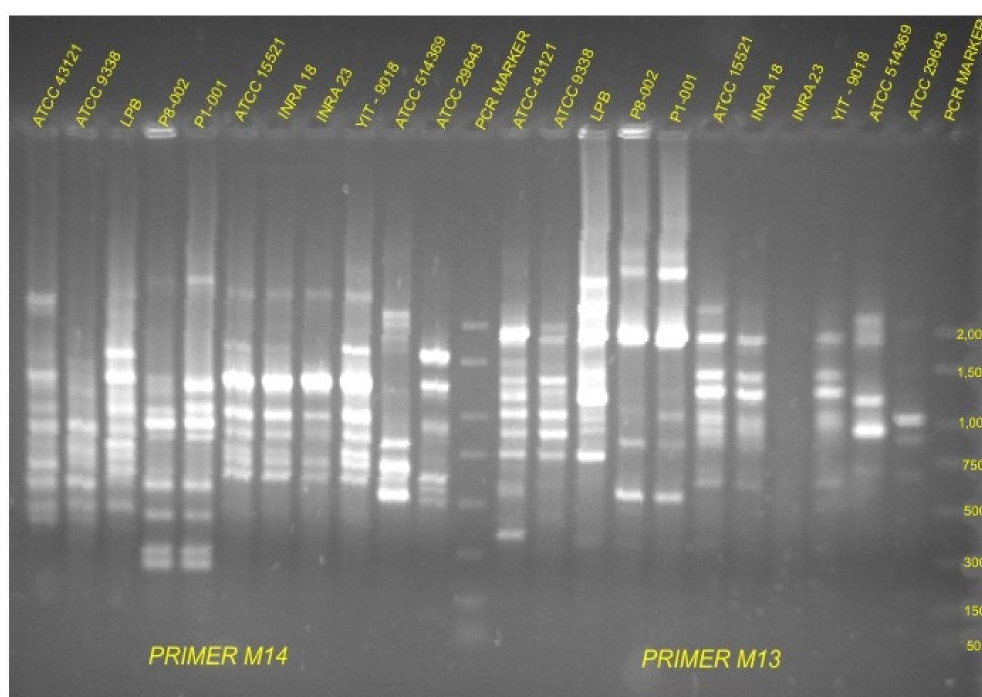

FIGURE 6 – Agarose gel electrophoresis showing amplified DNA using M13 and M14 primers in RAPD-PCR technique.

The philogenetic relation among the isolates and the reference strains can be observed by the built dendogram in figure 7.

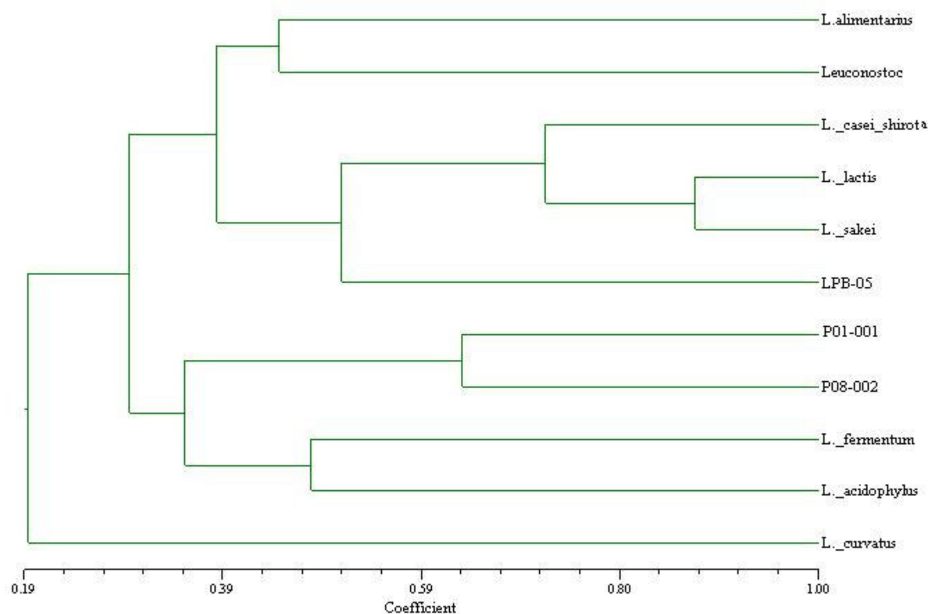

FIGURE 7 – UPGMA dendogram derived from the combined patterns using seven primers (A2, A3, A9, A10, COC, M13 and M14. Five groups were indicated: I, *Leuconostoc mesenteroides* group, II, *L. casei* group, III, strains *P01-001, P08-002,* IV, *L. acidophilus* and *L. fermentum,* and V, *L. curvatus.*

The isolated strains (*P01-001, P08-002 and LPB-05*) showed according to their profiles a clear separation from the reference strains. Although RAPD-PCR technique has not shown homology to ATCC 9338 strain, the *L. P01-001* strain was identified as being *L. fermentum* by biochemical characterization. By RAPD-PCR technique this strain forms a separated cluster. Both isolated *L. P01-001* and *P08-002* strains presented a coefficient of similarity equal to 0.62. It was observed 46% of genetic similarity between *LPB-05* and the strains YIT-9018, ATCC 15521 and INRA 18. Five distinct clusters where obtained in the present work. Cluster I include *Leuconostoc mesenteroides* group, cluster II, *L. casei shirota* group, cluster III, *P01-001* and *P08-002* strains, cluster IV, *L. acidophilus* and *L. fermentum,* and cluster V includes *L. curvatus.*

The importance of the molecular approaches to identify bacterial strains goes beyond of the classical morphology, biochemistry or physiology, so that, this approach might play a pivotal role in the understanding about genetic relations among bacterial strains, principally those of industrial interest. Several authors have used RAPD-PCR or

others molecular methodologies to study genetic diversity of *Lactobacillus* spp. Using specific amplification of 16S Ribosomal DNA, Heilig *et al.* (2001) studied the diversity of *Lactobacillus* group from fecal samples and found that molecular approaches were a strong tool to show genetic diversity of lactobacilli in the human GI tract. Torriani et al (2001) had used RAPD-PCR method in their work and concluded that this technique allowed distinguishing genetic variability in intra-specific level.

In our study, molecular methods have established a great genetic diversity among isolated strains, which was not possible to evidence through morphological and biochemical studies.

4.4. ADSORPTION OF THE FERMENTED BROTH IN THE SUGARCANE BAGASSE SUPPORT

The adsorption of sugarcane bagasse support by fermented broth and its maximum saturation capacity were evaluated in the present work. It was observed that initially the support adsorbs around 7 times of its weight.

The relation between adsorbed volume and the weight gained by the support after each adsorption step is shown in figure 10. It was observed that after 33 cycles of adsorption, 3g of support could achieve 13.41g. The total volume adsorbed was 438ml of fermented broth. After 12 adsorption cycles, the support looses adsorptive capacity and the increasing in volume are lesser in each new cycle. Thus, the saturation starts with 12 cycles of adsorption. Moreover, it was possible to observe that after six cycles of adsorption the support weight duplicates and after 12 cycles it triplicates. It is important to notice that for this experiment the condition given to support was the same given to biomioprotor, i.e., either on 50 ºC.

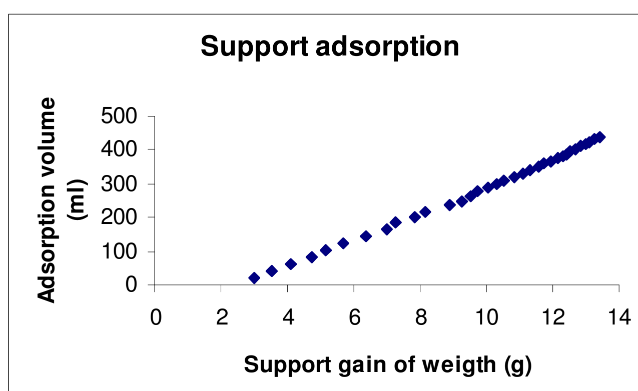

FIGURE 9 – Linear relation between volume and weight gained by support.

4.5. ORGANIC ACID AND BIOPROMOTER QUANTIFICATION IN THE FERMENTATION PROCESS

The strain *Lactobacillus P01-001* produces several organic acids such as lactic acid, formic acid acetic acid, propionic acid and ethanol. The concentrations of glucose and organic acids are presented in table V.

Table V – Glucose and organic acids concentrations in the fermentation broth after 24h and in the biopromoter product.

HPLC sample	Fermentation broth 24h Concentration (% w/w)	Biopromoter Feed (% w/1g of biopromoter)
Glucose	8.51	59.02
Lactic acid	33.84	13.33
Formic acid	-	7.06
Acetic acid	19.93	10.87
Propionic acid	9.93	9.70
Ethanol	15.45	-

In this research, the chromatographic profile of the organic acids presents in the fermented broth was similar to that obtained by Pancheniak (2005). One gram of biopromoter adsorbed 6.99 ml of fermented broth. However, since 50°C is the temperature kept during the dry cycles, there is a possibility that some of these organic acids may evaporate or degrade.

4.6. ANTIMICROBIAL ACTIVITY OF THE BIOPROMOTER

The zone of biopromoter inhibition can be observed in figure 10a. Table VI shows the diameter of each clear halo that represents the zone of inhibition. It was observed that increase in biopromoter concentration (adsorption) leads to increase in the inhibition zone reflected by the enhancement in diameter. Comparison between the clear halo formed by biopromoter and Neomicin (30MCG) treatment can be seen on figure 10b. The clear zone of inhibition formed to the biopromoter was 13.0 mm while the zone of inhibition formed to the antibiotic was 18.0 mm. It was verified that inhibition capacity is higher in the biopromoter however similar to that found for the commercial promoter Neomycin. It is clear from this result that the biopromoter is a bacteriostatic and not bactericidal. Then, it may be concluded that the biopromoter is highly effective antimicrobial agent against *Escherichia coli* ATCC 25922.

TABLE VI – Antimicrobial activity of the biopromoter against the bacteria *Escherichia coli* ATCC 25922.

Adsorption number	Clear zone of inhibition diameter (mm)
Sugarcane bagasse	-
Biopromoter	13.03

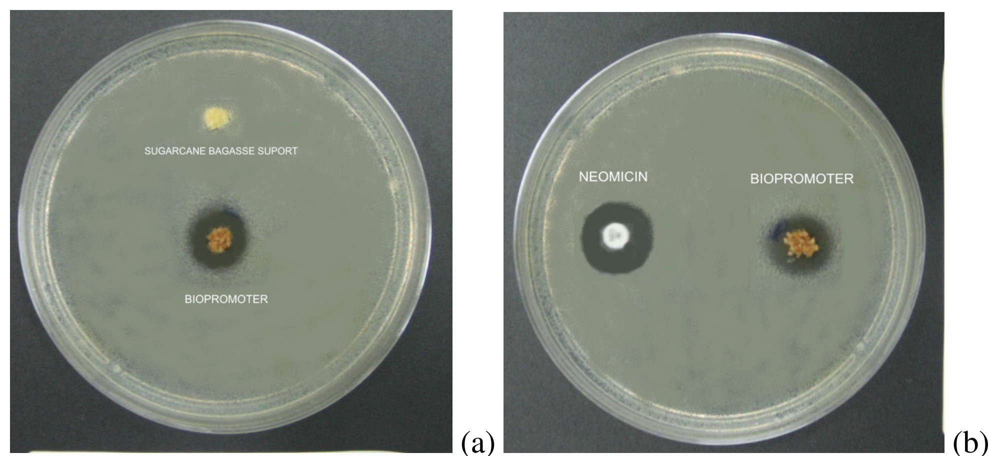

FIGURE 10 – (a) The clear zone of inhibition against the bacteria *Escherichia coli hemolítica.* (b) Comparison of the inhibitory effect of the biopromoter with Neomicin.

4.1. MODELING OF THE BACTERIAL GROWTH

The model for bacteria growing developed in the present work was based on the growth kinetic and the substrate consumption analysis. The growth kinetic results were given in g/l (figure 11). The values obtained to μ (h^{-1}), P_X (g/L.h) and $Y_{X/S}$ are shown on table VII.

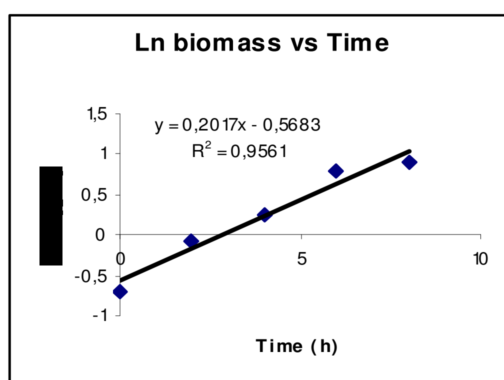

FIGURE 11 – Growth kinetic of *Lactobacillus P01-001* in bioreactor. The medium composition was 3% of brown sugar and 1% of yeast extract.

The growth kinetic and substrate consumption data are plotted on figure 12.

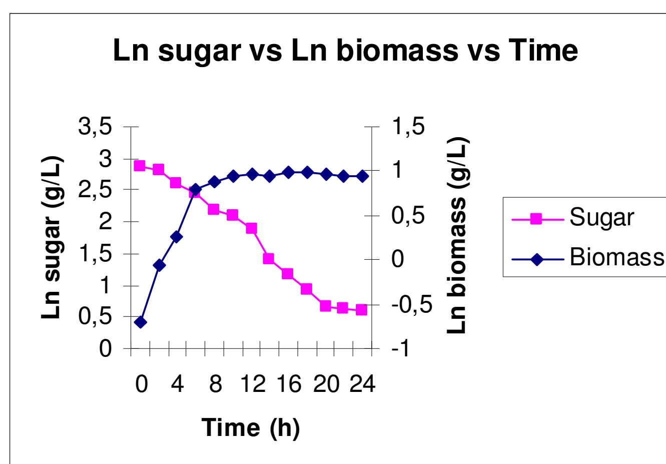

FIGURE 12 – Growth kinetic of *Lactobacillus P01-001* and the substrate consumption. The medium composition was 3% of brown sugar and 1% of yeast extract.

TABLE VII – Specific growth speed (μ), biomass production (P_X), and yield ($Y_{X/S}$), during eight hours of growth.

Coeficients	Values
μ (h^{-1})	0.2017
P_X (g/L.h)	0.348
$Y_{X/S}$	0.198

This kinetic economic model shows that the fermentation can be stopped after 10 hours of culture. It is observed in figure 12 an excess of sugar. The explanation is that the analysis takes into account the total sugars and it is worth noticing that the microorganism is able to consume only reducer sugars such as glucose and fructose, present in the brown sugar in the proportion of 1.5%. The larger percentage available is sucrose, however, it is not immediately ready for microorganisms consumption.

4.2. BIOPROMOTER ANIMALS TEST

The first week after weaning is considered the most risky phase for piglets. The change from a high nutritive liquid meal (milk) to a solid feed with different digestible proteins that can cause to piglets frequent digestive disturbs.

In the present work, results obtained in this phase did not show significant difference among the piglets, however it was noticed for the daily weight gain (DWG) a difference in 50g more for the positive control related to the negative control (Figure 13). In addition, a reduction in feed conversion in 9.5% was also observed, which indicates the effect of the promoter in the animals growth (Figure14). When daily

weight gain (DWG) was compared between animals treated with the Probiotic PP-LPB and with the natural Biopromotor related to the negative control. It was noticed that half of the effects was attributed to the growth Promoter, although the feed conversions were small showing 11.17 and 16.76% respectively.

In the second part of this assay, the feed conversion of the piglets that received the natural Biopromoter was significantly smaller than the negative and positive controls. Probiotic was in an intermediate level. In this phase, the growth Promoter did not result in improvement of the studied rates.

In a general analysis of both phases it was verified an increase in feed consumption on animals treated with Biopromotor (Figure 15). However, concerning the weight gain and food conversion for animals treated with Probiotic, the results were better compared to negative control and were similar to positive control.

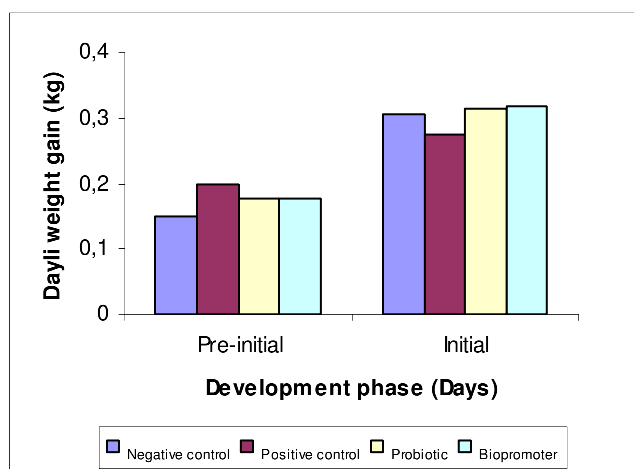

FIGURE 13 – Weight gain of piglets by treatment.

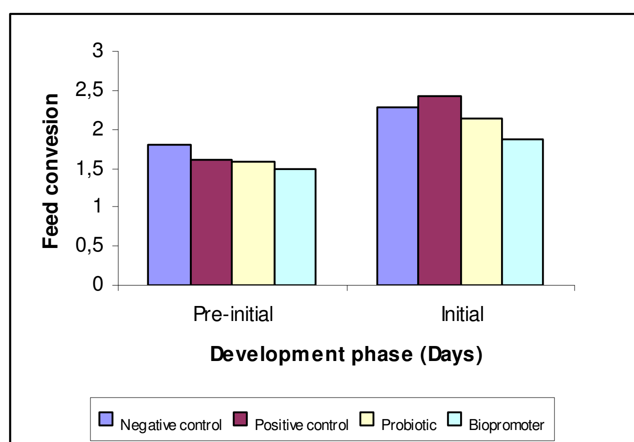

FIGURE 14 – Feed conversion from piglets by treatment

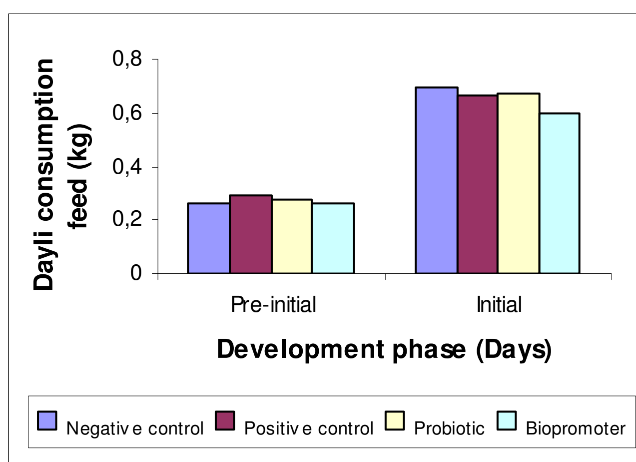

FIGURE 15 – Feed consumption from piglets by treatment.

4.3. ECONOMIC ANALYSIS OF BIOMASS PRODUCTION OF *Lactobacillus P01-001* IN ALTERNATIVE MEDIUM

The cost of biomass production comparisons were carried out using a commercial medium culture MRS (DifcoTM Lactobacilli) and a alternative medium based on brown sugar 3% and yeast extract 1% (TABLE VIII). The economic analysis was performed for 1 g/l of biomass production taking into account the following values:

- To prepare 1 litter of MRS broth it costs $ 19.80
- To prepare 1 litter of alternative medium (brown sugar 3% and yeast extract 1%) it costs $ 0.28

TABLE VIII – Economical comparison of biomass production in alternative medium.

Composition	Dry weight (g/L)	Production cost 1 g/L (R$)
MRS broth	1.348	36.72
Alternative broth	1.73	0.40

The formulation in alternative medium is much more efficient than the commercial MRS broth. The alternative medium can become incredibly cheaper when used in industrial scale since production costs can be reduced in 92%.

5. CONCLUSION

Five distinct clusters were obtained by RAPD-PCR technique and the isolated strain bacteria from swine used in this study was included into *L. fermetum* cluster. However, the similarity coefficient in this cluster is only 35%.

This strain is a potential probiotic bacterium because it can resist under high bile concentrations and produce organic acids and bacteriocins. Therefore, it is effective in the animal improvement, as it was observed in the present study concerning the given concentration. *L. P01-001* is a heterofermentative bacterium, with microaerophilic characteristic.

The fermented broth used in the natural biopromoter showed similar effect against *Escherichia coli* ATCC 25922 compared to the growth promoter Neomycin (30 MCG) concerning the formation of the inhibition halo.

The biopromoter tested in animals showed improvement in the zootecnic rates, daily weight gain, daily feed consumption and feed conversion, for the used dosage.

In the diet of the suckling pigs, the biopromoter can be used as growth promoter in substitution of the antibiotic Neomycin. Since the biopromoter is a natural composite, it follows the rules for antibiotic controls from Department of Agriculture making available a better quality of product for consumers.

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ANNEX 1

List of primers used in the RAPD reaction for identification of *Lactobacillus* isolates.

Primers	Sequence 5'-3'	No. Bases	%C+G
M13	GAGGGTGGCGGTTCT	15	66.7
M14	GAGGGTGGGGCCGTT	15	73,3
COC	AGCAGCGTGG	10	70
A2	TGCCGAGCTG	10	70
A3	AGTCAGCCAC	10	60
A9	GGGTAACGCC	10	70
A10	GTGATCGCAG	10	60

Components of the reaction DNA amplification of *Lactobacillus* and concentration used in the RAPD reaction for primers A2, A3, A9 and A10.

Components	Concentration	Reaction volume (µL)
Milli-Q water	---	18.25
Buffer	10X	2.5
DNTP MIX	5 mM	1.0
Primer	10 µM	1.0
$MgCl_2$	50 mM	0.75
Taq polimerase	5 U/µL	0.5
DNA	---	1.0
Total	---	25.0

Components of the reaction DNA amplification of *Lactobacillus* and concentration used in the RAPD reaction for primers M13 and M14.

Components	Concentration	Reaction volume (µL)
Milli-Q water	---	18.1
Buffer	10X	3.0
DNTP MIX	5 mM	1.2
Primer	10 µM	1.2
$MgCl_2$	50 mM	4.0
Taq polimerase	5 U/µL	0.5
DNA	---	2.0
Total	---	30.0

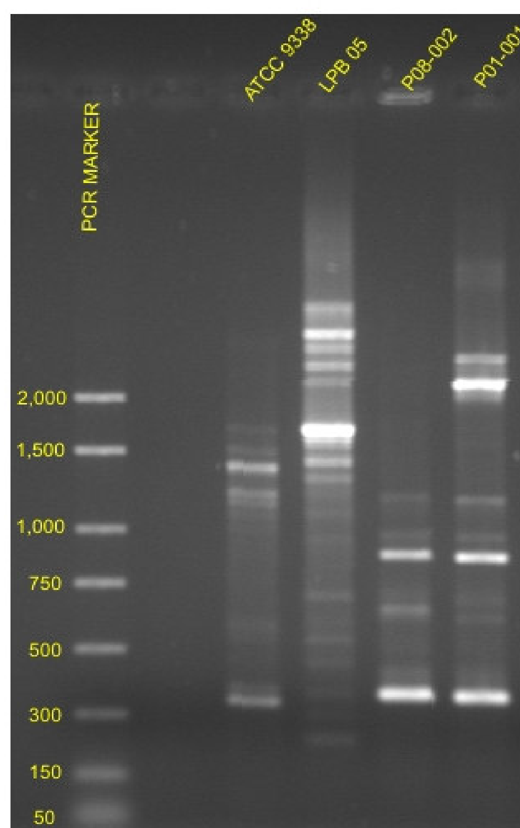
Components of the reaction DNA amplification of *Lactobacillus* and concentration used in the RAPD reaction for the primer COC.

Components	**Concentration**	**Reaction volume (µL)**
Milli-Q water	---	40.1
Buffer	10X	6.0
DNTP MIX	5 mM	1.2
Primer	10 µM	1.2
$MgCl_2$	50 mM	7.0
Taq polimerase	5 U/µL	0.5
DNA	---	4.0
Total	---	60.0

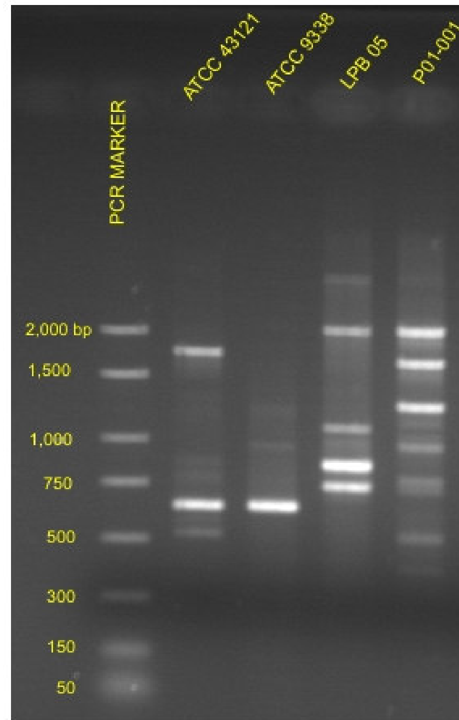
Conditions of amplification for the different primers.

Cycle number	Primer M13 e M14 Temperature./Time	Primer A2/A3/A9 e A10 Temperature/Time	Primer COC Temperature/Time
1 cycle	94º.C / 2min.	94º.C / 5min.	94º.C / 2min.
M13/M14 45 cycle	94º.C / 1min.	94º.C / 1min.	94º.C / 1min.
COC,	29º.C / 1min.	36º.C / 1min.	45º.C / 20seg..
A2/A3/A9/A10 40 cycle	72º.C / 2 min.	72º.C / 2 min.	72º.C / 2 min.
1 cycle	72º.C/ 5min.	72º.C/ 7min.	72º.C/ 10min.

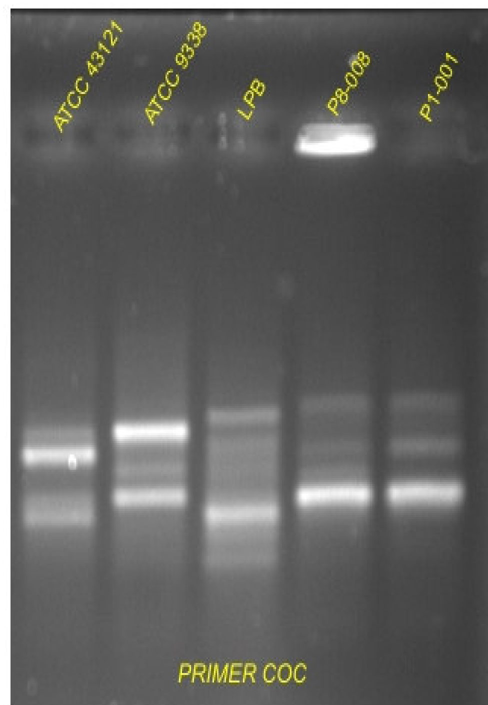
RAPD profile obtained for amplification with primer A3.



RAPD profile obtained for amplification with primer A9.



RAPD profile obtained for amplification with primer COC.



ANNEX 2

List of feed composition for the suckling pigs.

Feed composition		
Ingredients	**Pre-start**	**Start**
Corn	48.336	57.49
Defatted soybean cake	26.105	30.61
Lactic product	20.00	3.00
Sugar	-	2.00
Dicalcium fosphate	2.178	0.267
Calcarium	0.468	0.781
Inert	2.00	1.50
Soy oil	-	1.00
Antioxidant	0.010	-
Metionin	0.312	0.100
Lisin	0.236	0.077
Treonin	0.068	0.045
Sodium chloride	0.156	-
Supplement Min/Vit[1]	0.100	-
Supplement Min/Vit[2]	-	3.13
Total	**100.00**	
Composition		
Brute Protein (%)	21.00	19.00
Digestive Energy (kcal/kg)	3411.0	3336.0
Lactose (%)	8.00	1.200
Calcium (%)	0.900	0.840
Available Phosphorus (%)	0.500	0.440
Sodium (%)	0.220	0.180
Metionin (%)	0.629	0.396
Metionin + Cistin (%)	0.810	0.650
Lisin (%)	1.360	1.200
Treonin (%)	0.910	0.790
Triptophan (%)	0.279	0.250

[1] Mineral and vitaminic supplement without growth promoter. To guarantee levels for Kg of feed: Vit. A – 4.000 U.I.; Vit. D3 – 220 UI.; Vit. E – 22 mg; Vit. K – 0,5 mg; Vit. B2 – 3,75 mg; Vit. B12 – 20 mcg; Calcium Pantotenate – 12 mg; Niacin – 20 mg; Colin – 60 mg; Iodine – 140 µg; Selenium – 300 µg; Manganese – 10 mg; Zinc – 100 mg; Cupper – 10 mg; Iron – 99 mg.

[2] Given for pigs in initial phase.