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OBTAINING A FEED ADDITIVE BASED OF *Lactobacillus plantarum* STRAIN

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Abstract

The aim of the study was to isolate, identify and preliminary characterize strains of Lactobacillus spp. from the gastrointestinal tract of piglets. Three isolated strains were assessed morphologically, culturally, biochemically and enzymatically (amylase, celullase, protease). Successive replications were performed on MRS + $CaCO_3$ medium. The identification and analysis of the biochemical characteristics was performed by oxidase, catalase and API 50 CHL tests. From the all isolates, the Lactobacillus plantarum strain presents some potentially probiotic characteristics. The ability to synthesize organic acids (lactic, phenyl-lactic, hydroxyphenyl-lactic, acetic and propionic) was tested as well. The development of Lactobacillus plantarum was assessed on 4 variants of culture media containing different concentrations of ingredients (corn, soybean meal, substitute of milk). It was selected the first variant when was obtained 1.05 x10^{10} CFU/ml of L. plantarum. The feed additive was based on LABs, by following the biotechnological process steps. The procedure was performed in the biotechnological installation (IL 00). Subsequently, the optimum level of feed additive will be established in weaned piglet's diet.

Key words*: Lactobacillus plantarum, feed additive, weaning piglets, strain characteristics.*

INTRODUCTION

Currently, animal husbandry is focused towards to a more natural strategy. Utilization of feed additives in their livestock diets may have an impact on the environment and animal health (Kiarie et al., 2016).

The administration of microbial preparations has started to be used in animal nutrition in, as early as, 1960 (Ahasan et al., 2015). Due to these concerns, from 1969, the use of antibiotics as „growth promoters" in animal nutrition was banned, thereby inducing resistance to infection and diseases (Balasingham et al., 2017).

The administration of live microbial preparations as „dietary supplements" provides a strategy for animal's breeders to raise up to the level of market requirements (high level of meat production, increasingly number of animals, early weaning etc.).

In the early 1990's, a drastic reduction of microbial preparations as living microorganisms in animal feed was noticed, which can be explained by misunderstanding the mechanism of action and the scarce scientific data (Simon et al., 2001).

In 2004, Caramia relaunched the use of living microorganisms as feed additives, process stimulated by the European Union, which decreed in 2006 the administration of antibiotics as growth promoters. The microorganisms used, belong to bacterial genera such as: *Bacillus* (Gram-positive spore forming bacteria), *Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Streptococcus* and yeasts such *Saccharomyces*. The microorganisms mainly used in animal nutrition are: *Lactobacillus*, *Bacillus, Enterococcus* and *Saccharomyces* (Roselli et al., 2005; Stein and Kil, 2006; Musikasang et al., 2009, NRC, 2012).

The lactic acid bacteria (LAB) strains form a large group, characterized by a great diversity of species (*L. plantarum, L. acidophilus, L. rhamnosus, L. reuteri, L. casei, L. fermentum, L. johnsonii, L. salivarius* etc.). Around 71% of the LABs are used as feed additives in poultry

and swine diets, while only about 29% of the species from *Bacillus* and *Enterococcus* groups are used (Khunajakr et al., 2008). For good results, a number of factors should be observed, such as: tolerant to acid in the stomach and bile salt in the intestine (Idoui, 2014); capacity to adhere to the host intestinal epithelium, to present antagonistic activity against pathogenic bacteria, to keep their viability during processing and storage (Lin et al., 2007); general health, feed conversion ratio, growth rates, resistance to diseases, promoting body weight, high quality of animal products etc. (Ahmad, 2006).

Microbial bioproducts based on: *Lactobacillus farciminis* and *L. rhamnosus* inhibit *in vitro* the viability and adhesion of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* (pathogens in pigs and poultry) (Bernardeau et al., 2009); *L. johnsonii* and *L. pentosus* decreases the colonization of *Salmonella* spp. from pigs (Casey et al., 2004); *L. salivarius* and *L. pentosus* reducing the number of *Enterobacteriaceae* from pigs faeces (Gardiner et al., 2004); *Enterococcus faecium* increases the weight gain, feed conversion ratio and the dimensions of ileal villi in poultry (e.g. chicken) (Samli et al., 2007); *Bacillus subtilis* and *B. licheniformis* do not have a particular impact on the growth performance, weight, length, robustness and percentage of tibia calcium in broiler chickens, but improve the thickness of the medial and lateral wall of the tibia and the percentage of dry ash (Mutus et al., 2006); *L. johnsonii* controls the endemic necrotic enteritis caused by *Clostridium perfringens* in broiler chickens, reducing the economic losses and the use of antibiotics (La Ragione et al., 2004); the LABs inhibit *in vitro*, *Eimeria tenella* which is responsible for coccidiosis (Tierney et al., 2004); some *Lactobacillus* spp. increase the production of eggs, decrease the mortality, improve feed conversion ratio in laying hens (Yoruk et al., 2004); *Bacillus* strains, more stable due to spore forming, presents resistance to the processes of feed incorporation and granulation parameters, ensure long-term storage of forages (Simon, 2001).

The microbial candidates used as feed additives with probiotics role was defined, in 1992, by Fuller as „a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Idoui, in 2014, completed Fuller's probiotic definition: „microorganisms administered in adequate amounts, confer health benefits to the host and, as living microorganisms, induces no drug resistance or drug residues". Callaway et al. (2012), includes the term of probiotic as a „natural" strategy, to use microbial native population for diminished the pathogens bacteria.

Probiotic microorganisms can interfere by maintaining animal health, reducing the concentration of some toxic substances such mycotoxins (Trufanov et al., 2008; Niderkorn et al., 2009).

This study was conducted to obtain LABs with high probiotic properties and to use them as feed additive in weaned piglet's diet. This organism is a significant bacterium of the normal gut microbiota of piglet's and can induce a decrease in the number of pathogenic bacteria from animal gastro intestinal tract.

MATERIALS AND METHODS

Materials

Segments of gastrointestinal tract of healthy piglets were collected immediately after slaughter. The isolate was inoculated and grown in deMan, Rogosa and Sharpe (MRS, Oxoid), specific medium for *Lactobacillus* spp. Chemical reagents were used to assess the enzymatic activity of the isolates, which were selected and identified. The biotechnological product support was produced from raw forages (malt, soybean meal, milk substitute).

The research was carried out at the Laboratory of Biotechnology of the National Research and Development Institute for Animal Biology and Nutrition Balotești (INCDBNA), Romania.

Methods

Sample collection

LABs were obtained from intestine segments which were washedbefore with sterile physiological saline (NaCl 0.85%), until the all intestinal content was removed. The intestinal samples were cut into pieces of approximately 1 cm^2 and were inoculated in MRS broth medium (Oxoid), at 37°C, for 24 h, in anaerobic conditions.

Decimal dilutions (10^{-8}) were produced. From 10^{-4}-10^{-8} it was cultivated in Petri dishes with MRS agar medium, at 37°C, for 24 h. A pure culture was obtained by successive replications.

Identification of strain isolates

Isolation and selection of lactobacilli strains was done on MRS agar (Oxoid) supplemented with 0.5% $CaCO_3$ according to Jimenez et al. (2008).After incubation at 37°C, in anaerobic atmosphere (Jar with Anaerogen 2.5L from Oxoid), the medium, by nutrient fermentations, produce organic acids which determine an area of clearance around the bacterial colonies.

The isolated strains were stored at -80°C, in MRS medium with 20% glycerol.

The taxonomic identification was performed by culturally, morphologically and biochemically tests (Raducanescu and Bica Popii, 1973). Isolated in pure culture, the bacterial strains were analyzed macroscopically, followed by morphology, consistency, type and contour of colonies, the presence or absence of pigments (Garrity et al., 2002).

Gram staining was used to distinguish Gram - positive from Gram-negative bacteria (Garrity et al., 2002).

Biochemical tests

The isolates were assayed by oxidase test which consist in the presence of cytochrome C-oxidase by used impregnated strips in N,N,N,N-tetra Methyl-PhenylenediamineDihydrochloride(TMPD)(Sigma)(www.tgw1916.net).

According to Sagar (2015), the catalase test was determined, as well. It showed the presence of catalase, an enzyme that catalysis the release of oxygen from hydrogen peroxide (H_2O_2). A concentration of 3% H_2O_2 was used.

The isolate was identified using the API 50 CHL strips according to manufacturer's protocol (BioMerieux, Marcy l'Etoile, France). The strips are read after 48-72h incubation at 37°C. The results performed the biochemical profile of LABs, which were identified with API 50 CHL V5.1 and ABIS online software (Stoica and Sorescu, 2017).

The capacity of synthesizing organic acids (lactic acid, phenyl-lactic acid, hydroxylphenyl lactic and acetic acid) was determined by High-Performance Liquid Chromatography HPLC (Surveyor Plus – Thermo - Electron Corporation, Waltham, MA). 2.0 ml of monoculture was dispersed on MRS broth with addition of $CaCO_3$ and harvesting the cells by centrifugation at 5000 x g for 5 min., to pellet precipitate. The supernatant was removed and debris suspension was re-suspended in 2.0 ml distilled water (DW) and from this volume, 50 µl was transferred to be used in column separation of the acids.

Colony forming unit count (CFU)

To assess growth rate, the *L. plantarum* was cultivated on MRS medium (broth and agar) and Malt extract medium (broth and agar), at 37°C, for 48 h. Successive replications were done to determinethe bacterial density at 48 h.

Assessing the enzymatic activity

It was used for development and production of enzymes biosynthesis by our isolated strains, a culture medium consisting of malt, soybean meal, milk substitute, molasses and water (Table 5, variant 1).

The enzymatic activity of strain's isolates was determined by the following methods: Hostettler for amylase activity, Petterson and Porath for celullase activity and Anson modified on casein substrate for protease activity (Dumitru et al., 2016).

Hostettler's method is based on the action of α-amylase on starch and determination of maltose released after the enzymatic hydrolysis process, with 3.5-dinitrosalicylic acid (DNS). The optical density (OD) of the sample was read at 546 nm, compared to the control. An amylase unit corresponds to a quantity of maltose (µmol) released in one minute under the action of 1 ml of enzyme preparation, at 30°C.

Petterson and Porath's method consists in the enzymatic hydrolysis of carboxymethyl-cellulose (CMC) and dosing of reductant groups released with 3.5-DNS. A cellulolytic activity unit represents the amount of enzyme which releases from a CMC solution, an amount of reducing carbohydrates, which form with the DNS reagent, the same OD, similar to a milligram of glucose (Dumitru and Jurcoane, 2017). OD of the sample was read at 640 nm.

Protease activity was determined by Anson method, modified on casein substrate. Method is based on the determination of tyrosine,

resulting from the action of proteases on the casein substrate. In order to highlight the reaction product, was used Folin-Ciocalteu (FC) reagent, which forms a colored compound with tyrosine. OD was determined at 660 nm (Jurcoane et al., 2006).

Inoculum preparation of L. plantarum

Inoculum preparation at laboratory level

Tubes with MRS and malt extract medium (agar) were inoculated with lactic acid bacteria at 37°C, for 48 h. Successive passages were performed to determine the density of bacteria, until a yield of grows was reached.

In order to obtain the inoculum, several variants, based on natural medium (corn flour, malt natural medium, soybean meal, molasses and water) were prepared, which differed by the quantity of ingredients used (Table 1).

The amount of each version was calculated for 250 ml of water, dispensed in Erlenmeyer flasks of 500 ml, homogenized and sterilized 15 min., at 121°C. For each variant was added molasse at source of carbon.

Table 1. Variants of medium for obtaining the inoculum

Ingredients % (w/v)	Variant 1	Variant 2	Variant 3	Variant 4
Malt	20	15	15	10
Soybean meal	2	2.5	2	2.5
Milk substitute	1	1	1	2
Molasses	2	2	2	2
Water	100	100	100	100

The natural medium was autoclaved 15 min., at 121°C. After cooling to 45-50°C, the medium was inoculated with the pure culture of 25 ml *L. plantarum* and incubated at 37°C, for 48-72 h.

Four successive passages were done to determine the concentration of lactic bacteria.

Inoculum preparation in Biotechnological Installation

The procedure development in the lab was performed in the Biotechnological Installation (IL 00) from INCDBNA Balotesti, following the biotechnological process steps.

For the all variants of medium, it was selected variant 1 (Table 1).

Corn flour was added, to increase the lactic bacteria numbers. The used culture medium consisted of 1000 g corn flour, 400 g soybean meal, 200 g milk substitute, 4000 g malt flour and 400 g molasses, at 20 L of water. All ingredients were homogenized, sterilized by autoclaving, at 110°C, 1 h. After cooling to 40-45°C, the mixture was transfused in the fermenter, over which was added 2 L of inoculum. The inoculated medium was stored in the fermenter at 35°C, for 72h. To reduce the moisture content, the product was mixed with 6 kg of wheat bran.

The optimum level of feed additive obtained will be established in weaned piglet's diet, as well.

RESULTS AND DISCUSSIONS

Three LAB strains were isolated from gastrointestinal tract of healthy piglets and analyzed. The samples were purified by passing on MRS agar supplemented with 0.5% $CaCO_3$. After purification, the strains were stored at -80°C in MRS broth with 20% of glycerol.

Cultural characteristics

The isolated strains were presumptively identified as lactobacilli. It presents the ability to grow on MRS medium + 0.5% $CaCO_3$. The colonies appear white, cream, translucent, with regularly shaped and smooth surface and produce a clear zone around, as a result of the release of organic acids, that degrade $CaCO_3$ (Figure 1).

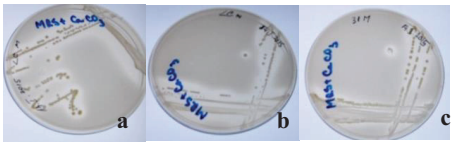

Figure 1. The *Lactobacillus* colonies on MRS agar + $CaCO_3$ (a. *L. plantarum*; b. *L. acidophilus;* c. *L. paracasei*)

The isolates are facultative anaerobic. In the MRS broth medium was noticed an intense turbidity, with abundant deposits, without surface formations.

Morphological identification

Gram-positive rods with rounded ends (0.7-0.9 x 1.2-1.5 μm), was measured by Zeiss Microscope which presents gradation on the

eyepiece. Mostly of the colonies have convex form, appear structures of coccobacilli and bacilli in short and long chains, non-spore forming, non-motile (Figure 2).

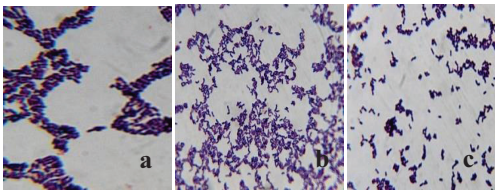

Figure 2. The morphological identification of *Lactobacillus* spp.
(a. *L. plantarum;* b. *L. acidophilus;* c. *L. paracasei*)

Biochemical characters

The isolated strains produced negative results in the catalase and oxidase tests.

The strains were identified and characterized using the bioMerieux API 50 CHL test kit. This kit consists of 49 tests and is based on the capacity to metabolize 49 different carbohydrates (Figure 3).

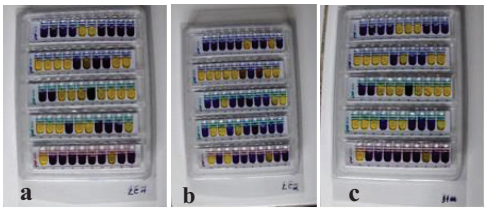

Figure 3. API 50 CHL strips inoculated with *Lactobacillus* spp. (a. *L. plantarum;* b. *L. acidophilus;* c. *L. paracasei*)

According to the fermentation profile, the isolates were identified as *L. plantarum*, *L. paracasei* and *L. acidophilus* with a percentage of 93%-95% (Table 2).

Table 3 presents the results from the preliminary conventional taxonomic analysis of LABs strains.

Table 2. The results obtained with API 50 CHL test for *Lactobacillus* spp. based by biochemical fermentation

Item	*L. plantarum*	*L. paracasei*	*L. acidophilus*
Glycerol	–	–	–
Erythritol	–	–	–
D-arabinose	–	–	–
L-arabinose	–	–	–
D-ribose	+	–	+
D-xylose	+	+	+
L-xylose	–		+
D-adonitol	–	–	–
Methyl-βD-xylopyranoside	–	–	–
D-galactose	–	+	–
D-glucose	+	–	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbose	-	+	+
L-rhamnose	±	+	–
Dulcitol	–	+	+
Inositol	–	±	–
D-mannitol	+	–	–
D-sorbitol	+	±	+
Methyl-αD-mannopyranoside	–	+	+
Methyl-αD-glucopyrano side	–	+	–
N-acetylglucosamine	+	–	–
Amygdalin	+	–	+
Arbutin	+	+	+
Esculin	+	–	+
Salicin	+	–	+
D-cellobiose	+	+	+
D-maltose	+	–	+
D-lactose	+	–	+
D-melibiose	+	+	+
D-saccharose	+	+	+
D-trehalose	+	–	+
Inulin	–	+	+
D-melezitose	+	+	–
D-raffinose	+	–	+
Starch	–	+	+
Glycogen	–	–	–
Xylitol	–	–	–
Gentibiose	+	–	–
D-turanose	+	–	+
D-lyxose	–	+	+
D-tagatose	–	–	–
D-fucose	–	+	–
L-fucose	–	–	–
D-arabitol	–	–	–
L-arabitol	–	–	–
Potassium gluconate	+	±	±
Potassium2-ketogluconate	–	–	–
Potassium5-ketogluconate	–	–	–

Table 3. Results of preliminary taxonomic analysis

Item	Strain 1	Strain 2	Strain 3
Gram staining	+	+	+
Catalase test	-	-	-
Peroxidase test	-	-	-
API 50 CHL	*L.plantarum*	*L.paracasei*	*L. acidophilus*

The ability to synthesize organic acids (lactic phenyl-lactic, hydroxyphenyl-lactic and acetic) was tested, as well (Table 4).

Table 4. Evaluation of the capacity of the synthesis organic acids

The strains	Lactic acid (g/l)	Phenyl lactic acid (g/l)	Hydroxi phenyl lactic acid (g/l)	Acetic acid (g/l)
L. plantarum	5.83	0.01	0.00	0.25
L. paracasei	3.50	0.00	0.00	0.20
L. acidophilus	6.40	0.02	0.01	0.45

The ability to synthesize organic acids differs in the various strains (Figure 4).

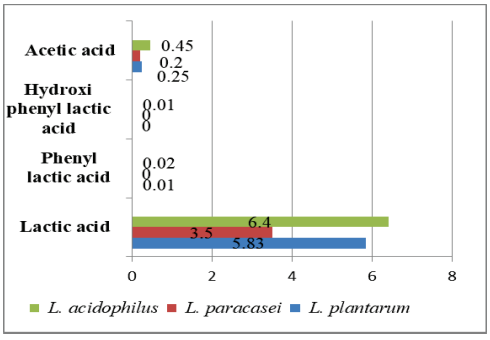

Figure 4. Optimal quantity of organic acids produces by LABs strains on Oxoid MRS medium supplemented with 0.5% $CaCO_3$

L. acidophilus presents the highest level to produce lactic acid (6.40 g/l) compared to *L. plantarum* (5.83 g/l). The minimal quantity of lactic acid is produced by *L. paracasei* (3.50 g/l). Also, the *L. acidophilus* produces acetic acid (0.45 g/l) compared to *L. paracasei* and *L. plantarum.* None of the isolated strains had the capacity to produce hydroxyphenyl and phenyl lactic acid.

According to the specialty data (Okorhi, 2014), the results obtained were classified based on quantities produced by the majority of LABs which was assessed for each type of acid (lactic acid 2.9-7.8 g/l, phenyl-lactic acid 0-0.05 g/l; hydroxyphenyl-lactic acid 0-0.22 g/l; acetic acid 0-0.65 g/l).

The isolated strains didn't have the capacity to produce exogenous enzymes (amylases and proteases) (Table 5).

Table 5. The enzymatic activity of isolate strains

The strains	Amilolytic activity	Celulolytic activity	Proteolytic activity
L. plantarum	0	1.5	0
L. paracasei	0	0.34-0.35	0
L. acidophilus	0	0	0

Regarding to the production of cellulase enzyme, *L. plantarum* shows a cellulolytic activity of 1.5 (U DNS/ml), compared with *L. paracasei* which had a low cellulolytic activity of 0.34-0.35 (U DNS/ml).

L. plantarum possesses some probiotic property and was selected for the next steps. The isolate registred the capacity to synthesis organic acids (5.83 g/l lactic acid and 0.25 g/l acetic acid) and a cellulolytic activity of 1.5 (U DNS/ml).

The development of *Lactobacillus plantarum* was assessed on 4 variants of culture media containing different concentrations of ingredients (corn, soybean meal, substitute of milk) (Table 1). Variant 1 presents the highest density (1.05 x 10^{10} CFU/ml), compared to variant 2 (6.5 x 10^9 CFU/ml), variant 3 (8.5 x 10^9 CFU/ml), respectively the last variant of medium (5.5 x 10^9 CFU/ml).

It was selected the first variant to manufacture the product at biorector level in the Biotechnological Installation (IL 00).

The obtained product was packaged in polyethylene bags and stored at 18-20°C (Figure 5). The finished product has a characteristic smell of lactic acid, sour taste and a concentration of 1.5 x 10^{11}CFU/g.

The production of lactic acid after fermentation process was determined and a similar value, compared with the quantity of organic acids produces by *L. plantarum* strain was detected.

The final product has been stored for a period of 3 weeks. The concentration of LABs from product storage was determined in this time.

Figure 5. Biotechnological product based on *L. plantarum* strain

In the first week, we noticed a slight increase of lactic bacteria numbers (1.75 x 10^{11}CFU/g), compared with the second week, when was registered a decrease of this (1.35 x 10^{11}CFU/g). In the last week, the lactic bacteria were around 9.5 x 10^{10}CFU/g. This decrease is due to the loss of bacterial viability which defined the ability of bacterial cells to form colonies on selective medium agar in suitable conditions (Figure 6).

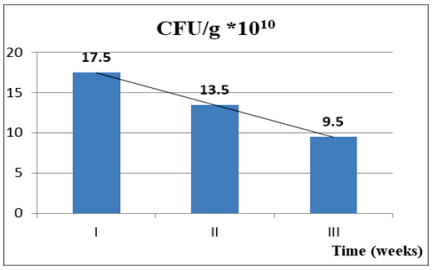

Figure 6. The concentration of bacteria time of 3 weeks in product storage

Lactobacillus spp. has been reported as one of the major bacterial groups from gastrointestinal tract of pigs (Dibner and Richards, 2005).

CONCLUSIONS

The carbohydrate fermentation tests using API 50 CHL kit was used to identify some species of lactic acid bacteria isolated from gastrointestinal tract of piglets. The isolated strains were identified as *L. plantarum, L. paracasei* and *L. acidophilus*.

From the all strains, *L. plantarum* was selected to be further assessed as feed additive in swine nutrition.

The ability of lactic acid bacteria to produce organic acids was tested, *L. plantarum* registered 5.83 g/l. The enzymatic activity level of *L. plantarum* compared with *L. paracasei* and *L. acidophilus* shows a cellulolytic activity of 1.5 (U DNS/ml).

The results suggest that *L. plantarum* strain had some probiotic traits and can be further assessed for other characteristics (resistance to pH 2.0, resistance to bile acids and salts, antibacterial activity, induction of local immune response etc.), in order to evaluate its probiotic utility in pigs nutrition.

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