Article

Evaluation of Lactic Acid Bacteria Isolated from Piglets Tract and Encapsulation of Selected Probiotic Cells

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Abstract: Fourteen lactic acid bacteria (LAB) strains were isolated from the intestinal tract and feces of piglets. Among these isolates, only twelve were biochemically (API 50 CHL and ABIS online for bacterial identification) and genetically (16S rRNA sequencing) confirmed as *Limosilactobacillus fermentum*, *Lactobacillus acidophilus*, and *Lactiplantibacillus plantarum*. Experiments to evaluate the probiotic potential of the isolates including pH tolerance (pH 2.0 and 3.0), bile salts (0.3% ox gall) resistance, hemolysis activity, antibiotic susceptibility, and high-temperature resistance were tested. Only two isolates from identified strains exhibited high survival rates when exposed to low pH and bile salts, these were *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84. The antibiotic test presented 100% resistance of both strains to gentamicin, kanamycin, lincomycin, colistin sulfate, erythromycin, amikacin, oxytetracycline, enrofloxacin, streptomycin, and tilmicosin, lower than the 0.6 mm inhibition zone diameter. Promising isolates (*L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84) were exposed to the spray-drying technique based on visible probiotic potential and survival rates. Carrier matrix material was used as a maltodextrin-glucose solution. The encapsulation probiotic isolated survived both over 67% and 77%, corresponding to a decrease in strain viability from 10^9 to 10^7 CFU/g. After further in vitro evaluations, the findings of this study showed that, from all LAB strains, *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84 may be considered probiotic candidates for animal nutrition and may have promising performance in piglet feed due to their origin of isolation.

Keywords: lactic acid bacteria; probiotics; spray-drying; viability; piglets

Citation: Dumitru, M.; Lefter, N.A.; Habeanu, M.; Ciurescu, G.; Vodnar, D.C.; Elemer, S.; Sorescu, I.; Georgescu, S.E.; Dudu, A. Evaluation of Lactic Acid Bacteria Isolated from Piglets Tract and Encapsulation of Selected Probiotic Cells. *Agriculture* **2023**, *13*, 1098. https://doi.org/10.3390/agriculture13051098

Academic Editor: Zhigang Song

Received: 23 March 2023
Revised: 8 May 2023
Accepted: 19 May 2023
Published: 21 May 2023

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1. Introduction

Lactobacillus spp. belong to the lactic acid bacteria (LAB) group with a long history of utilization [1,2]. Among other benefits, these bacteria are one of the most significant groups due to their capability to transform lactose into a lactic acid compound, stimulate the immune system, and prevent and treat diarrhea incidences [3]. Due to their administration as technological products, LAB species can attribute health benefits to the host if they are used in adequate quantities [4,5]. Described as a heterogeneous group, LAB bacteria are not sporulating [6] and can be found in different habitats, ranging from soil, plants products, waste and municipal (treated) water, the gastrointestinal tract (GIT) of animals and humans, as well in food and dairy products [7,8].

Since 2006, the European Union (EU) has banned the use of antibiotics as antimicrobial growth promoters (AGP) in animal nutrition, due to the increase of antibiotic-resistant

pathogens and the potential of transmission to humans after animal product consumption [9,10]. Based on recent global research, the current trend is to find a natural alternative to the diminishing effectiveness of antibiotics [9,10]. Promising interest was taken in probiotics. Based on FAO/WHO [11] affirmation, probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host". Most LAB strains, such as *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, *L. fermentum*, *L. brevis*, *L. casei*, *L. salivarius*, and *L. bulgaricus*, are generally used as probiotic sources [1,12] in the form of pure or combined bacteria [13].

Recently, data have demonstrated that some LAB have attracted increasing attention due to their beneficial effect on the host's gut microbiota [13]. Instead, until they can be defined as probiotics, each candidate strain should be isolated, identified, and evaluated for its probiotic traits [14]. However, the probiotic potential of LAB differs from one candidate to another, depending on the isolation sources and their capacity to stay alive [15]. According to the literature data, a good probiotic must be viable at a rate in the intestine level from 10^7 to 10^9 CFU/g [8,12,16]. Survivability through the host GIT must be determined through strain production, storage, and resistance [15]. Harsh environmental conditions such as pH, bile salts, hydrogen peroxide production, oxygen content, higher temperatures, buffering capacity, and citric acid concentration can involve the loss of strain viability and bioactivity [15,17].

Microencapsulation is a good technique by which to protect the viability of the probiotics, especially LAB strains, which present higher sensibility during processing and within host GIT [15,17]. Moreover, microencapsulation is known to be an excellent substitute for transforming the liquid form into a stabilized powder (solid compound) for easier handling, allowing for a longer period of preservation [18]. Several dry processes have been used for the microencapsulation of probiotics (vacuum drying, freeze drying, and spray drying methods). The drying technique is known as the most widely used procedure, and it is highly efficient due to its low operational costs and effectiveness, the time to obtain the product of interest, and easily available suitable equipment [19,20]. Furthermore, as a preferred process technique for producing dried microorganisms' cells [21], spray drying is used for converting a bacterial culture suspension into powder form, starting from a laboratory scale characterized by higher productivity [22]. However, the high temperature used in the spray drying process can spoil the probiotic bacteria, determining cell modifications due to thermal, osmotic, oxidative, and dehydration stresses, resulting in a decrease in cell survival [21]. In addition, during spray drying, LAB biomass and suitable thermoprotectants are atomized into micron-sized fine particles and combined with warm air flow to implement fast dehydration [23].

For beneficial effects to take place, the survivability of LAB is often considered a key criterion [24] due to their stability in the carrier matrix after spray drying [25]. Though it is not always possible to choose a process with a constant strain, the reason for which is the carrier media used as encapsulation material to help to progress a stable matrix structure [26]. The carrier medium or various thermoprotectant materials, such as sugars (lactose, glucose, trehalose, saccharose, dextrose, maltodextrin, etc.), are used as agents and protective substances [12] in the dehydration of bacteria providing optimal cell survival after spray drying (22,25]. Therefore, the present study was directed to isolate, identify, and characterize some LAB from piglet GITs. Based on their higher viability and in vitro probiotic properties, the selected strains were subjected to spray drying to use them as possible candidates in animal diets.

2. Materials and Methods

2.1. Ethical Statement

Animal experimental procedures were in agreement with Romanian Law no. 199/2018, respecting the Council Directive 2010/63/EU legislation for scientific purposes. In this study, piglets were kept in the Experimental Biobase of the National Research-Development Institute for Biology and Animal Nutrition (IBNA), Balotesti, and were randomly selected at

different times. Efforts were made to diminish animal stress and the farm's hygiene status following the protocol authorized by the IBNA Balotesti Ethical Committee (no. 5183/2018). The in vitro tests were conducted in the Biotechnology Laboratory of IBNA Balotesti in partnership with the University of Agricultural Sciences and Veterinary Medicine (US-AMV), Cluj, specifically the Fermentative Biotechnologies Department, and the University of Bucharest, particularly the Faculty of Biology, Biochemistry, and Molecular Biology Department.

2.2. Reagents and Materials

Brain Heart Infusion broth (BHI Basingstoke, Hampshire, UK), Man, Rogosa, Sharpe agar (MRS, CM0361), a jar with Anaerogen (2.5 L for anaerobic growth), phosphate buffer saline (PBS, Dulbecco A, Livingstone Ltd., London, UK), and bile salts (ox gall powder) were purchased from Oxoid. API 50 CHL kits were kindly provided by BioMérieux (Marcy l'Etoile, France) and API 50 CHL V 5.1 and ABIS online software were used according to the manufacturer's instructions [27].

2.3. LAB Isolation, Culture Conditions, and Phenotypic Identification

A total of 14 LAB isolates were selected from feces and piglet GITs (ileum and cecum content). The method by Sorescu et al. [8] was applied. An amount of 1 g of intestinal content from 14 healthy Topigs hybrid piglets [female Large White × Hybrid (Large White × Pietrain) × male Talent (mainly Duroc)] was well homogenized with 7.0 mL of BHI broth and 2.0 mL of sterile glycerol and was then rapidly frozen at −20 °C. After unfreezing, the ileum and cecum content was serially diluted in PBS (pH 7.0) and grown on MRS agar (three Petri plates) followed by anaerobic incubation at 37 °C for 24–48 h. After incubation, the purity of each culture strain was checked on MRS agar plates. Moreover, before experiments, the strains were activated, passaged 2–3 times, and then cultivated (1:10, *v/v*) in MRS broth for 24 h at 37 °C. The physiological traits of isolates were assayed (i.e., morphological, Gram-positive, rod-shaped, catalase test).

2.4. Biochemical Identification

API 50 CHL (BioMérieux, Marcy l 'Etoile, France), API 50 CHL V 5.1, and ABIS online software were used for *Lactobacillus* isolates [12].

2.5. Molecular Strains Identification

The isolation of genomic DNA from 14 isolates was carried out using the Wizard SV Gel and PCR Clean-Up System DNA Purification Kit (Promega, Corporation, WI, USA). The quality and quantity of DNA samples were evaluated using a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). Molecular identification of the strains was carried out based on 16S rRNA gene sequence analysis. Two pairs of primers were selected to amplify and sequence the gene from different regions. The forward and reverse primers Lac1 F (5′-AGCAGTAGGGAATCTTCCA-3′) and Lac1R (5′-ATTYCACCGCTACACATG-3′) were used to amplify a fragment of 380 bp of the V3-to-V4 region of the 16S rRNA gene [28]. The forward and reverse primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify the entire 16S rRNA gene when the amplification and sequencing of the 380 bp fragment were ambiguous. The amplification by PCR was carried out using an AmpliTaq polymerase assay (Applied Biosystems, Wilmington, DE, USA). Each PCR mixture (25 μL) presented 2.5 μL PCR buffer solution 10×, 1.5 μL 25 mM $MgCl_2$, 2 μL dNTPs mix (each dNTP contained a concentration of 2 mM), 0.5 μL of individual primer (with 20 pmols/μL), 0.1 μL of AmpliTaq polymerase (5 U/μL), and the corresponding volumes of sterile Milli-Q water and DNA diluted solution. Amplification was carried out in a Veriti™ 96-Well Thermal Cycler (ThermoFisher Scientific) under the following conditions: one cycle of 10 min at 95 °C (initial denaturation), followed by 30 cycles of 30 s at 95 °C (denaturation), 30 s at 60 °C (annealing), 1 min at 72 °C (extension), and a

final extension of 10 min at 72 °C. PCR products were checked using the electrophoresis technique in 2% (*w/v*) agarose gels, imagined by ethidium bromide staining, and purified with the Wizard SV Gel and PCR Clean-Up system (Promega, Corporation, WI, USA). The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Wilmington, DE, USA) following the standard procedure. The automated sequencing of both strands of the PCR products was carried out on an ABI Prism 3130 system (Applied Biosystems). The Basic Alignment Search Tool (BLAST) tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 16 December 2022) was used to evaluate the sequence similarity degree with 16S rRNA sequences from the GenBank database. BLAST is an online program belonging to the NCBI (National Center for Biotechnology Information) that finds regions of similarity between biological sequences by comparing the subject nucleotide database sequences and calculating the statistical significance [29].

2.6. Conservation and Bacterial Strains Viability

Lactobacillus strains were frozen and stored at −80 °C in MRS broth medium with 20% (*v/v*) sterile glycerol reagent. Bacteria cells were subcultured to the stage of top log-phase in MRS broth. MRS agar was used for further colony growth. The data were presented as Log_{10} CFU per gram.

2.7. Low pH Resistance

The tolerance of *Lactobacillus* isolates to a low pH was assessed in vitro according to Dumitru et al. [12] with minor modifications. Overnight active cultures (10 mL, 20 ± 2 h) were used for this purpose, with a growth of 8–9 Log_{10} CFU/mL. Each isolate was grown in MRS medium until optical density (OD 600 nm) attained 1.2 and contained around 10^{10} CFU/mL. The centrifugation of fresh culture was carried out at $8000\times g$ for 10 min at 4 °C. Biomass cells were harvested from the suspensions and resuspended in 9 mL of MRS broth; the pH was adjusted with 1 N HCl to 3.0 and 2.0 values. At different incubation times (0, 1:30, and 3 h), the viability was evaluated and decimal dilutions, up to 10^{-10}, were carried out on MRS agar, followed by anaerobic incubation at 37 °C for 24–48 h. Cell viability was counted and evaluated in triplicate.

The total plate count (TPC, CFU/mL) was determined for cell enumeration and transformed into the logarithmic value. Furthermore, the survival rate was expressed according to the formula:

$$\text{Survival } (\%) = \frac{\text{Log } number\ of\ cells\ survived \left(\frac{\text{CFU}}{\text{mL}}\right) \times 100}{\text{Log } number\ of\ initial\ cells\ inoculated \left(\frac{\text{CFU}}{\text{mL}}\right)}$$

2.8. Bile Salts Resistance

According to Prasad et al. [30], gastrointestinal bile absorption is around 0.3% and the feed layout is predicted to be 3–4 h. To reproduce these conditions, ox gall powder was used to perform the bile salts test at 0.3% (*w/v*). The overnight culture (7–8 Log_{10} CFU/mL) of LAB isolates was centrifuged at $8000\times g$ for 10 min at 4 °C. The cells from fresh culture were harvested and washed in PBS three times and resuspended in fresh MRS broth with 0.3% bile salt inclusion. After incubation at 37 °C for 24–48 h (0, 1:30, and 3 h), the viable counts were anaerobically estimated by decimal serial dilution in sterile PBS (up to 10^{-10}) on MRS agar discs. The blank was signified by MRS culture (pH = 6.5 ± 0.2) without bile salts addition [12].

2.9. Safety Evaluation

Trypticase soy agar (TSA, Sanimed) with 5% (*w/v*) sheep blood was used to determine the hemolytic activity of LAB isolates. The existence of hemolysis was evaluated as follows: green zones around colonies (α-hemolysis), clear zones around colonies (β-hemolysis), and

no zones around colonies (γ-hemolysis—i.e., no hemolysis). The interpretation was visually observed after incubation at 37 °C for 24 h. Only γ-hemolysis is considered safe [31].

2.10. Susceptibility Antibiotic Test

The susceptibility of LAB to diverse antibiotics (Oxoid, Basingstoke, Hampshire, UK), including amoxicillin (AMX, 25 µg), gentamicin (GN, 10 µg), kanamycin (K, 30 µg), lincomycin (MY, 10 µg), tetracycline (TE, 30 µg), penicillin (P, 10 µg), vancomycin (VA, 5 µg), colistin sulfate (CT, 10 µg), clindamycin (DA, 2 µg), erythromycin (E, 15 µg), amikacin (AK, 30 µg), chloramphenicol (C, 30 µg), oxytetracycline (OT, 30 µg), enrofloxacin (ENR, 5 µg), streptomycin (S, 10 µg), and tilmicosin (TIL, 15 µg), was determined using the agar disc diffusion method [32]. Our LABs were grown in MRS broth at 37 °C for 24 h. One hundred microliters of each bacterial suspension corresponding to McFarland Standard 1 turbidity and an optical density (OD 600 nm) of 0.8 (more than 1×10^8 CFU/mL) was placed on an agar media Petri dish with a sterile cotton swab. After incubation (37 °C for 24 to 48 h) in anaerobic conditions, the diameter of the transparent area was measured with a ruler in mm. Strains with an area diameter less than or equal to 6.0 mm are resistant (R).

2.11. Bioreactor Batch and Fermentation Process

Each selected strain was fermented in a 5 L bioreactor (Eppendorf, type BioFlo 320, one unit, Hamburg, Germany) with an operating volume of 2 L, 150 rpm, at 37 °C for 22 ± 2 h. The inoculum (200 mL with a concentration of 10^{10} CFU/mL) from each selected strain was used as a starter. The process of fermentation was fitted with a temperature sensor, rotation speed control, and a pH sensor, which maintained the medium constant at 6.5 ± 0.2. Automatically, a peristaltic pump adjusted the pH value by adding 20% NaOH (*w*/*v*) and 1 N 37% HCl (*v*/*v*). From time to time, an antifoaming Antifoam 204 agent (Sigma-Aldrich, St. Louis, MO, USA) was included as required (0.01%, *v*/*v*).

2.12. Spray Drying Process and Cell Viability

The microencapsulation of LAB strains was effectuated with a BÜCHI Mini Spray Dryer B-290 Swiss-made (Labortechnik AG, Flawil, Switzerland). Maltodextrin-glucose (24% maltodextrin and 4% glucose, *w*/*v*) was prepared in sterile distilled water (autoclave, 121 °C for 15 min). Overnight LABs cultures incubated at 37 °C, 150 rpm in the bioreactor, were harvested by centrifugation at 8000 rpm at 4 °C for 10 min. After removing the supernatant, the cell suspension (biomass) was cleaned three times by vertexing with PBS buffer to remove the nonadherent cell. Aseptically, the adherent cells were resuspended in the sterile maltodextrin-glucose solution. Through the spray drying process, the culture solution was well homogenized with magnetic stirring, under continuous agitation, at room temperature. The working conditions were applied in accordance with the study by Dumitru et al. [12]. The survival of microencapsulated LAB (*L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84) was carried out in PBS (pH = 7.0 ± 0.2) by serial dilutions. Briefly, one gram of powder strain was added in 9 mL of MRS broth (pH = 6.5 ± 0.2) and vortexed. Serial dilutions in 9 mL of PBS from powder were carried out and plated out on MRS agar (37 °C, 48 h, anaerobically). The counts were stated as CFU per gram of powder (Log CFU/g).

The encapsulation efficiency was calculated according to the equation by Bhagwat et al. [33] (EE):

$$EE = 100 \times Nr/Nf$$

where, Nr = Log CFU/mL before spray drying; Nf = Log CFU/mL after spray drying.

2.13. Powder Stability During Storage

2.13.1. Storage of Powders

In a Ziplock pouch, the powder was deposited at room temperature (25 ± 2.0 °C). The encapsulated LAB viability was periodically evaluated (0, 5, 10, 15, 20, 25, and 30 days).

2.13.2. Lab Powder Enumeration

LAB isolates were immediately enumerated after the spray drying process. One gram of dried powder was rehydrated with 9 mL of MRS broth followed by 1 min of homogenization at 200 rpm by magnetic stirring. The suspension was carried out in PBS buffer and spread onto MRS agar follow by incubation at 37 °C for 48 h. The viability of LAB were calculated and stated as CFU/gram powder.

2.13.3. Total Coliform Count

Lauryl sulfate-tryptose broth (LS, BK010HA) was used as a selective enrichment medium for the presumptive determination of Coliform numbers. Serial dilutions were ended in PBS (10^{-1}–10^{-5}) [34]. Each LS tube contained a Durham tube. A quantity of one mL from each dilution was pipetted into 5 LS tubes, followed by incubation at 37 °C for 48 h. Gas production in the Durham tubes was examined. The positive test (gassing) was well homogenized and 1 mL from the suspension was transported to another tube with brilliant green bile broth at 37 °C, 24 h. The Durham tubes with the gas formation and slight turbidity were noted as positive results. A McCrady table was used for interpretation (MPN—the most probable number of bacteria).

2.14. Statistical Analysis

Variance analysis (one-way ANOVA) was used for statistical analysis of the data. All experiments were conducted in triplicate, with three independent measurements. Results are stated as mean values and standard deviation of the mean (SD). The significant differences between treatments were considered statistically significant at $p < 0.05$. Multiple comparisons were evaluated by the Tukey LSD test for the untitled compact variable. The graphics were generated using GraphPad Prism software V. 9.1.2 (Inc., La Jolla, CA, USA).

3. Results

3.1. Lactic Acid Bacteria Isolation, Culture Conditions, and Phenotypic Identification

A total of 14 pure bacterial colonies were obtained from the feces and various GI compartments of piglets at different stages of age (Table 1). These colonies were isolated from the cecum (three isolates), ileum (six isolates), and feces (five isolates). The isolates were examined for morphological properties.

Table 1. Origin of LABs existence in piglets GIT.

Strains/IBNA Code	Source/Area of Isolation and Piglet Age
L. fermentum biotype 1, IBNA	
71	Cecum, 81 days old
75	Ileum, 81 days old
78	Cecum, 107 days old
85	Ileum, 107 days old
90	Feces, 30 days old
95	Feces, 30 days old
L. acidophilus, IBNA	
76	Ileum, 81 days old
81	Ileum, 81 days old
94	Feces, 30 days old
64	Ileum, 45 days old
70	Cecum, 81 days old
L. delbrueckii, IBNA 77	Cecum, 81 days old
L. plantarum group, IBNA 84	Ileum, 107 days old
L. salivarius, IBNA 87	Feces, 30 days old

The results of the phenotypic identification presented Gram-positive and negative catalase tests for all 14 isolates (without effervescent on the glass slide). Furthermore, the strains were coco-bacilli and non-motile with the capacity for growing in anaerobic conditions.

Based on cultural traits, the isolates occurred either isolated or in clusters of three to six cells with creamy, white, and opalescent colonies on MRS agar. Some LAB rods were noted with thin and long features; in other cases, the rods were stout, which confirmed that all of them belonged to the LAB group.

3.2. Biochemical Identification

The isolates were subjected to carbohydrate fermentation pattern analysis using API 50 CHL Biomerieux test strips; their capacity to utilize sugars (50 substrates) is summarized in Table S1. According to the above findings, following API 50 CHL and ABIS online soft confirmation, the isolates were identified as LABs. From fourteen isolates, six were identified as *L. fermentum* (IBNA 71, IBNA 75, IBNA 78, IBNA 85, IBNA 90, and IBNA 95), five were confirmed as *L. acidophilus* (IBNA 76, IBNA 81, IBNA 94, IBNA 64, and IBNA 70), and the last three showed characteristics of *L. delbrueckii* (IBNA 77), *L. plantarum* (IBNA 84), and *L. salivarius* (IBNA 87).

3.3. Molecular Strains Identification

A preliminary description of isolates was made based on the phenotype characteristics. For a more reliable identification, we added a molecular approach to the biochemical characterization. For the selected isolates, an analysis of 16S rRNA gene sequences was performed for molecular identification purposes using BLAST. Compared with the biochemical results, from a total of 14 isolates that were evaluated based on API 50 CHL, only 12 of them were confirmed by 16S rRNA sequencing, as follows: *L. fermentum* (IBNA 71, IBNA 75, IBNA 78, IBNA 85, IBNA 90, IBNA 95, 6/14 isolates; 42.85%), *L. acidophilus* (IBNA 76, IBNA 81, IBNA 94, IBNA 64, IBNA 70, 5/14 isolates; 42.85%), *L. plantarum* (IBNA 84, 1/14 isolates; 7.14%), and species belonging to the LAB genus (IBNA 77, IBNA 87; 2/14 isolates; 14.28%) (Table 2).

Table 2. Molecular identification of LAB from piglets.

Total Isolates	Technique Identification		
	API 50 CHL Biomerieux	**16S rRNA Gene Sequencing (Primers Lac1F/Lac1R)**	**16S rRNA Gene Sequencing (Primer 27 F/1492R)**
14	*L. fermentum* biotype 1 IBNA	identified as:	
	71	*L. fermentum*	*L. fermentum*
	75	*L. fermentum*	*L. fermentum*
	78	*L. fermentum*	*L. fermentum*
	85	*L. fermentum*	*L. fermentum*
	90	*L. fermentum*	*L. fermentum*
	95	*L. fermentum*	*L. fermentum*
	L. acidophilus IBNA	identified as:	
	76	*L. acidophilus*	*L. acidophilus*
	81	*L. acidophilus*	*L. acidophilus*
	94	*L. acidophilus*	*L. acidophilus*
	64	*L. acidophilus*	*L. acidophilus*
	70	*L. acidophilus*	*L. acidophilus*
	L. delbrueckii IBNA 77	identified as:	
		Lactobacillus spp.	*Lactobacillus* spp.
	L. plantarum IBNA 84	identified as:	
		L. plantarum	*L. plantarum*
	L. salivarius IBNA 87	identified as:	
		Lactobacillus spp.	*Lactobacillus* spp.

Two strains were only confirmed as *Lactobacillus* spp. (IBNA 77 and IBNA 87) since our sequences showed high similarity with sequences from the GenBank database from more than one species of LAB.

For the rest of the isolates (12 strains), the BLAST technique, which includes a complete 16S rRNA sequence, confirmed identification according to the API 50 CHL (Table 2). Regarding the strains, all 14 isolates were grouped into the regular non-spore-forming rods *Lactobacillus* spp. The next order of the frequency of our isolate identification was noted: *L. fermentum*, *L. acidophilus*, and *L. plantarum*. After molecular and biochemical identification, 12 LABs (IBNA 71, IBNA 75, IBNA 78, IBNA 85, IBNA 90, IBNA 95, IBNA 64, IBNA 70, IBNA 76, IBNA 81, IBNA 94, and IBNA 84) were subjected to their capacity for possessing maximum probiotic properties and their viability during batch fermentation and the spray drying process.

3.4. Conservation and Bacterial Strains Viability

LABs strains were preserved in MRS broth at −80 °C with a 20% addition of glycerol for long-time conservation. Under the representative identification code, all isolates can be found in the IBNA Balestri Intern Collection.

3.5. Low pH Resistance

Based on phenotypic and molecular identification, all strains were assessed in vitro for selecting prominent strains based on probiotic properties. LABs were exposed to pH 3.0 and pH 2.0, during 3 h of growth at 37 °C. The results are reported in Figure 1.

After exposure to pH 3.0 (0 h, 37 °C), IBNA 77, IBNA 81, and IBNA 94 lost total viability. The viability rate for some of the 14 isolates was higher at pH 3.0 compared to pH 2.0. Furthermore, after 3 h of incubation, the isolates' exposure to pH 3.0 and 2.0 involved a decrease in survivability, ranging from 9% to 100%. At pH 3.0, compared with an environment without acidic conditions (pH 6.5 $\pm$ 0.2), a decline in IBNA 71 (11.43%), IBNA 75 (26.47%), IBNA 76 (9.07%), IBNA 84 (14.44%), IBNA 87 (8.48%), IBNA 90 (33.41%), IBNA 95 (7.47%), IBNA 64 (21.79%), IBNA 70 (112.56%), IBNA 78 (11.53%), and IBNA 85 (100%) was found. Moreover, the strains' survivability at pH 2.0 was strongly modified, followed by a decrease in growth rate for IBNA 71 (100%), IBNA 75 (45.90%), IBNA 76 (35.08%), IBNA 81 (100%), IBNA 84 (33%), IBNA 87 (100), IBNA 90 (54.56%), IBNA 95 (100%), IBNA 64 (100%), IBNA 70 (29.11%), IBNA 78 (100%), and IBNA 85 (100%). Importantly, after exposure at pH 2.0 (3 h), there was a sharp decline in the survival rates of eight isolates where no significant difference in viable cells was registered ($p > 0.05$).

As shown in Figure 2, IBNA 77 and IBNA 81 did not tolerate the growth medium with bile salts addition in comparison with all the other isolates, where the survival rate was 45% and over. The results obtained after 3 h found insignificant differences between IBNA 87 and IBNA 95, respectively, IBNA 84, IBNA 90, and IBNA 78. Based on the results presented in Figure 2, 3 h of incubation at low pH and with bile salts involves a decrease in the viability of most of the isolates. Furthermore, the 0.3% bile salt concentration involved an acceptable resistance for IBNA 71, IBNA 75, IBNA 76, IBNA 84, IBNA 87, IBNA 90, IBNA 94, IBNA 95, IBNA 64, IBNA 78, and IBNA 85 at 3 h, which showed a decrease in survival rate between 1–3 Log_{10}. Instead, IBNA 70 and IBNA 78 were drastically affected by bile salts, losing total viability, which corresponds to a reduction of more than nine cycles in viable cell counts. Overall, we can infer that isolates IBNA 76 and IBNA 84 showed the most promising results among the pH and bile salt tests, involving higher probiotic resistance (Figure 3).

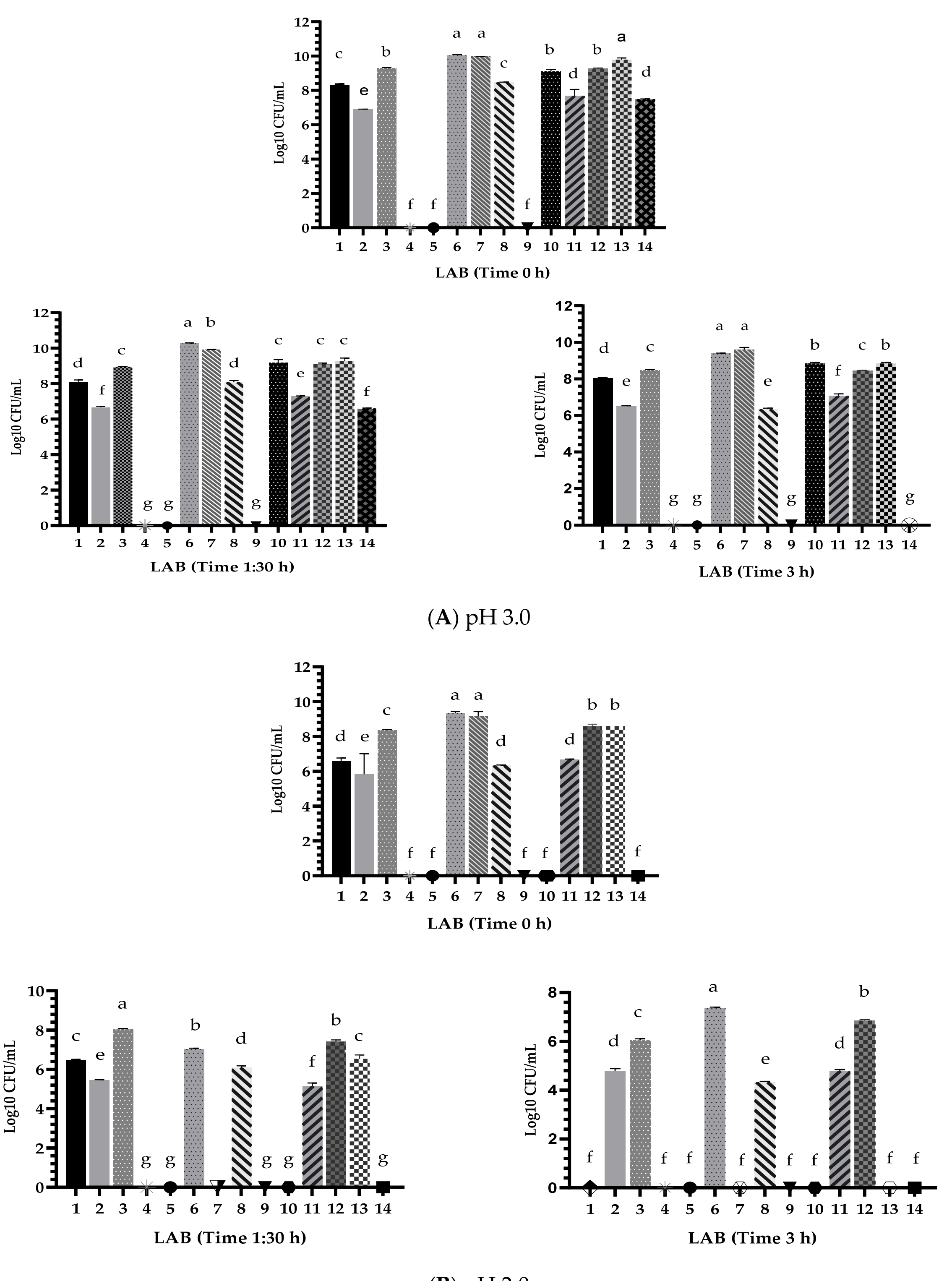

Figure 1. LAB viability after 3 h of exposure to low pH (3.0 and 2.0). Isolates code: 1: *L. fermentum* 1, IBNA 71; 2. *L. fermentum* 1, IBNA 75; 3. *L. acidophilus* IBNA 76; 4. *L. delbrueckii*, IBNA 77; 5. *L. acidophilus* 3, IBNA 81; 6. *L. plantarum* 1, IBNA 84; 7. *L. salivarius*, IBNA 87; 8. *L. fermentum* 1, IBNA 90; 9. *L. acidophilus* 3, IBNA 94; 10. *L. fermentum* 1, IBNA 95; 11. *L. acidophilus* 1, IBNA 64; 12. *L. acidophilus* 1, IBNA 70; 13. *L. fermentum* 1, IBNA 78; 14. *L. fermentum* 1, IBNA 85. Results are expressed as a mean ± SD of three independent experiments (n = 3). Means represent viable count (Log_{10} CFU mL^{-1}). $^{a–g}$ Means with different superscripts within each figure bar represent significant differences between treatments at $p < 0.05$.

3.6. Bile Salts Resistance

Figure 2 presents the resistance of isolate strains to bile salts concentration. After 3 h, only 12 isolates were able to grow in the presence of 0.3% Oxgall.

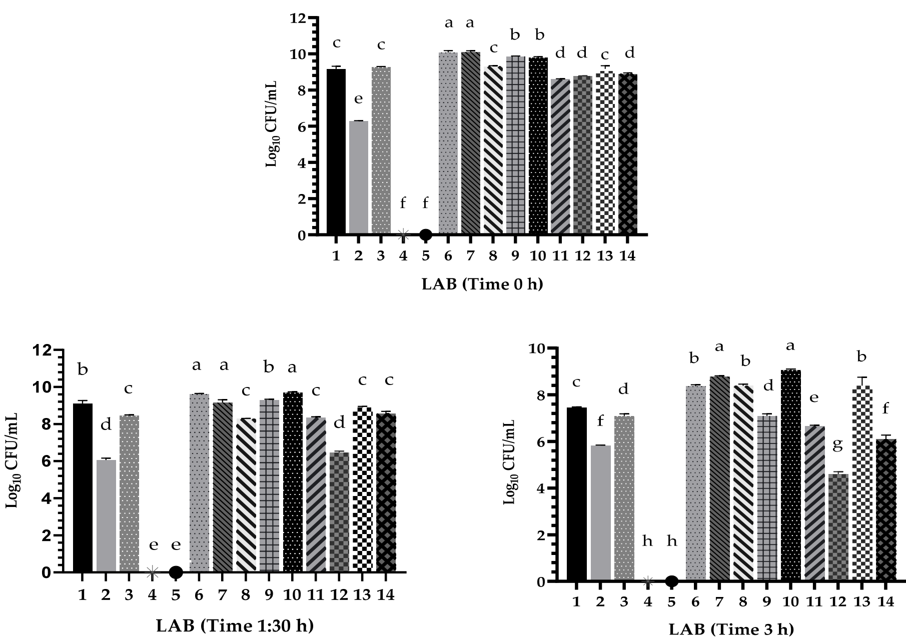

Figure 2. Strain viability after 0, 1:30, and 3 h of incubation in 0.3% bile salts. Isolates code: 1: *L. fermentum* 1, IBNA 71; 2. *L. fermentum* 1, IBNA 75; 3. *L. acidophilus* IBNA 76; 4. *L. delbrueckii*, IBNA 77; 5. *L. acidophilus* 3, IBNA 81; 6. *L. plantarum* 1, IBNA 84; 7. *L. salivarius*, IBNA 87; 8. *L. fermentum* 1, IBNA 90; 9. *L. acidophilus* 3, IBNA 94; 10. *L. fermentum* 1, IBNA 95; 11. *L. acidophilus* 1, IBNA 64; 12. *L. acidophilus* 1, IBNA 70; 13. *L. fermentum* 1, IBNA 78; 14. *L. fermentum* 1, IBNA 85. Results are expressed as a mean ± SD of three independent experiments (n = 3). Means represent viable count (Log_{10} CFU mL^{-1}). $^{a\text{–}h}$ Means with different superscripts within each figure bar represent significant differences between groups at $p < 0.05$.

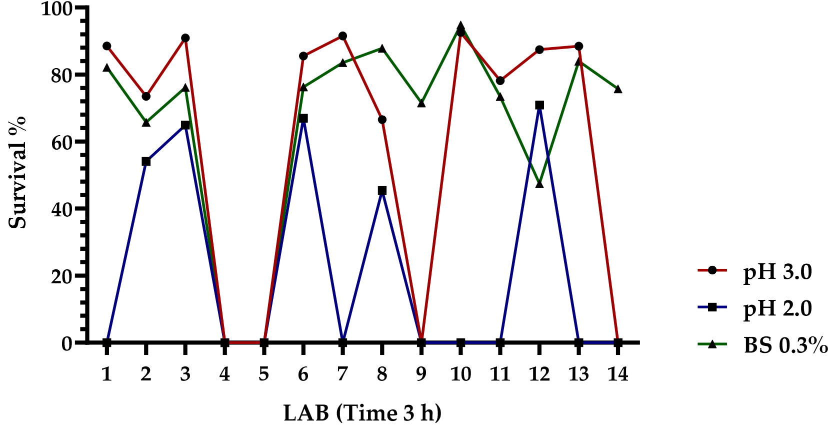

Figure 3. Strain survival after 3 h of incubation at low pH and with 0.3% bile salts. Isolates code: 1: *L. fermentum* 1, IBNA 71; 2. *L. fermentum* 1, IBNA 75; 3. *L. acidophilus* IBNA 76; 4. *L. delbrueckii*, IBNA 77; 5. *L. acidophilus* 3, IBNA 81; 6. *L. plantarum* 1, IBNA 84; 7. *L. salivarius*, IBNA 87; 8. *L. fermentum* 1, IBNA 90; 9. *L. acidophilus* 3, IBNA 94; 10. *L. fermentum* 1, IBNA 95; 11. *L. acidophilus* 1, IBNA 64; 12. *L. acidophilus* 1, IBNA 70; 13. *L. fermentum* 1, IBNA 78; 14. *L. fermentum* 1, IBNA 85. Results are expressed as percentages (%).

In addition, both strains were confirmed by phenotypical and molecular identification as *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84. Our findings showed that the survival of selected strains during 3 h of incubation after exposure to low pH and bile salts confirmed their resistance. Moreover, due to their ability to tolerate harsh conditions, IBNA 76 and IBNA 84 were selected for subsequent screenings and were subjected to the next step of examination.

3.7. Safety Evaluation

To be safe for use as a possible candidate, a probiotic strain must be nonhemolytic. To ensure this, all 14 *lactobacilli* isolates were tested on TSA agar medium and were found to be nonhemolytic and safe for administration as probiotic candidates.

3.8. Susceptibility Antibiotic Test

Table 3 presents the results regarding the susceptibility profile of all the LAB isolates compared to frequently used antibiotics (n = 16). Using disk diffusion methods to determine the antibiotic resistance of LAB, the strains were noted as resistant (R) for an inhibition diameter of ≤6.0 mm.

Table 3. Antibiotic susceptibility profile of *Lactobacillus* isolates.

	Inhibition Diameter (mm)															
Strains	**AMX**	**GN**	**K**	**MY**	**TE**	**P**	**VA**	**CT**	**DA**	**E**	**AK**	**C**	**OT**	**ENR**	**S**	**TIL**
1	≤6.0	≤6.0	≤6.0	18.0	14.0	24.3	13.3	≤6.0	17.3	12.3	10.0	8.3	15.0	≤6.0	≤6.0	≤6.0
2	30.0	≤6.0	≤6.0	≤6.0	26.0	40.0	10.0	≤6.0	9.0	14.6	10.0	23.6	21.6	7.0	≤6.0	≤6.0
3	≤6.0	≤6.0	≤6.0	≤6.0	6.5	11.6	22.0	7.0	16.0	≤6.0	20.0	20.0	≤6.0	≤6.0	10.0	11.0
4	30.0	≤6.0	≤6.0	20.0	22.3	31.3	16.0	12.3	15.0	14.0	10.0	24.6	≤6.0	≤6.0	7.1	9.0
5	14.0	≤6.0	≤6.0	≤6.0	≤6.0	14.0	19.0	≤6.0	7.0	16.3	≤6.0	23.6	11.3	≤6.0	7.0	10.0
6	31.0	≤6.0	≤6.0	≤6.0	16.0	29.0	14.0	≤6.0	9.0	14.6	≤6.0	24.0	20.0	≤6.0	≤6.0	≤6.0
7	≤6.0	≤6.0	30.0	≤6.0	14.0	33.6	15.0	≤6.0	13.0	19.0	24.0	17.0	≤6.0	≤6.0	≤6.0	≤6.0
8	32.0	≤6.0	≤6.0	30.0	≤6.0	29.0	12.3	≤6.0	24.0	17.3	≤6.0	25.0	21.0	≤6.0	≤6.0	≤6.0
9	24.3	≤6.0	≤6.0	7.3	≤6.0	31.0	15.0	≤6.0	15.3	20.0	≤6.0	30.0	16.0	≤6.0	10.0	7.0
10	26.6	≤6.0	≤6.0	21.3	7.0	9.6	10.0	≤6.0	22.0	15.0	≤6.0	21.0	13.0	≤6.0	≤6.0	≤6.0
11	15.6	≤6.0	≤6.0	9.0	≤6.0	29.3	15.0	≤6.0	10.0	19.0	≤6.0	17.3	8.3	≤6.0	≤6.0	8.0
12	25.6	≤6.0	≤6.0	8.6	≤6.0	31.3	17.0	≤6.0	14.3	21.3	≤6.0	34.6	8.3	≤6.0	6.6	11.6
13	31.0	≤6.0	≤6.0	30.0	16.6	35.3	11.3	≤6.0	24.0	17.6	≤6.0	23.0	20.0	≤6.0	≤6.0	≤6.0
14	20.0	≤6.0	≤6.0	11.6	19.6	25.0	10.6	≤6.0	10.0	12.0	≤6.0	≤6.0	17.3	13.6	≤6.0	≤6.0

Where: amoxicillin (AMX) 25 µg; gentamicin (GN) 10 µg; kanamycin (K) 30 µg; lincomycin (MY) 10 µg; tetracycline (TE) 30 µg; penicillin (P) 10 µg; vancomycin (VA) 5 µg; colistin sulfate (CT) 10 µg; clindamycin (DA) 2 µg; erythromycin (E) 15 µg; amikacin (AK) 30 µg; chloramphenicol (C) 30 µg; oxytetracycline (OT) 30 µg; enrofloxacin (ENR) 5 µg; streptomycin (S) 10 µg; tilmicosin (TIL) 15 µg. Resistant (R): ≤6.0 mm. Isolates code: 1: *L. fermentum* 1, IBNA 71; 2: *L. fermentum* 1, IBNA 75; 3: *L. acidophilus* IBNA 76; 4: *L. delbrueckii*, IBNA 77; 5: *L. acidophilus* 3, IBNA 81; 6: *L. plantarum* 1, IBNA 84; 7: *L. salivarius*, IBNA 87; 8: *L. fermentum* 1, IBNA 90; 9: *L. acidophilus* 3, IBNA 94; 10: *L. fermentum* 1, IBNA 95; 11: *L. acidophilus* 1, IBNA 64; 12: *L. acidophilus* 1, IBNA 70; 13: *L. fermentum* 1, IBNA 78; 14: *L. fermentum* 1, IBNA 85.

We found that 100% of isolates showed resistance to gentamicin and kanamycin, 21.42% to amoxicillin, 35.71% to lincomycin and tetracycline, 85.71% to colistin sulfate and enrofloxacin, 7.14% to erythromycin and chloramphenicol, 64.28% to amikacin and streptomycin, 28.57% to oxytetracycline, and 57.14% to tilmicosin.

3.9. Bioreactor Batch and Fermentation Process

After biochemical and molecular confirmation by API 50 CHL, respectively 16S rRNA, the strains were evaluated for a selection of probiotic properties. Viable isolates with beneficial probiotic potential were nominated for further investigation. Between all fourteen strains, only two isolates (*L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84) had the ability to present high resistance at low pH and bile salts concentration. Furthermore, the strains, after exposure to the fermentation process in the bioreactor at a final volume of 2 L, involved bacterial growth ranging from 9.39 ± 0.088 for *L. acidophilus* IBNA 76 and 10.97 ± 0.019 for *L. plantarum* IBNA 84, respectively.

3.10. Spray Drying Technique and Cell Viability

The application of spray drying was only applied for strains that involved significant tolerance at low pH and bile salts, respectively *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84. After spray drying, the strain powder obtained at the lab-scale was evaluated for cell viability. Encapsulation in dextrin and maltodextrin as a cryoprotectant material provided cell protection during the process. The survival of the encapsulated cells is presented in Figure 4.

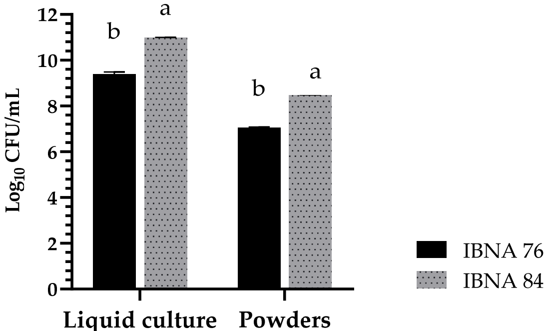

Figure 4. The viability of LAB strains before and after the spray drying process. Results are expressed as a mean ± SD of three independent experiments (n = 3). [a,b] Means with different superscripts within each figure bar represent significant differences between the groups at $p < 0.05$. **black bar**: *L. acidophilus* IBNA 76; **grey bar**: *L. plantarum* IBNA 84.

More specifically, viability reduction after spray drying exposure was 7.07 ± 0.040 for *L. acidophilus* IBNA 76 and 8.47 ± 0.017 for *L. plantarum* IBNA 84. Instead, the encapsulation process generated around 2 Log ten-fold lowest viability, as follows: IBNA 76 presented survivability around 67% (initial viability 2.5×10^9 CFU/mL) and 71% for IBNA 84 (initial viability 9.4×10^{10} CFU/mL).

3.11. Powders Stability during Storage

A representative selection based on in vitro assays was performed to choose the probiotics bacteria for the process of encapsulation. After spray drying, powder viability was evaluated at 0, 5, 10, 15, 20, 25, and 30 days. Figure 5 shows the growth rate of strains exposed to the spray drying method.

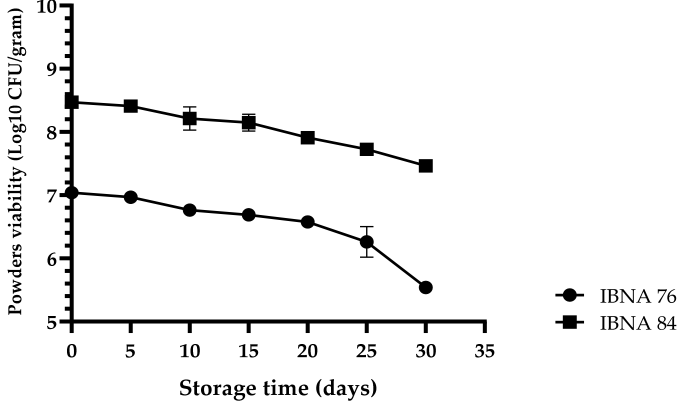

Figure 5. *Lactobacillus* (*L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84) powder stability for 30 days at room temperature (25 °C). Data are represented as means ± SD of three independent experiments (n = 3).

During 30 days of storage, the viability of *Lactobacillus* powders implied a daily decrease.

3.12. Total Coliform Count

As contamination indicators, coliforms were evaluated in the powder strains. The results confirmed that *Lactobacillus* in microencapsulated form did not register living or the presence of coliform bacteria.

4. Discussion

4.1. Lactic Acid Bacteria Isolation, Culture Conditions, and Phenotypic Identification

Recently, increasingly more probiotics have been used in the context of improving growth performance and the health status of animals. It is extremely important that the probiotic bacteria remain alive to obtain the benefits to the health status of the host. Therefore, the present study focused on the evaluation of certain properties of some LAB strains isolated from piglets. Based on the probiotic potential and the survival rate, the selected strains could be included in the design of a high-quality probiotic in the pig feed industry. It is known that intestinal mucosa, tract content, and livestock feces are alternative and strong sources of LAB with promising functional probiotic properties [35]. Additionally, a probiotic strain will act much better in a similar environment to that from where was originally collected and isolated [36]. Moreover, along with other probiotic traits, host-origin bacteria are preferred because they are well familiar with the GIT environment conditions and can proliferate and express their biological activity much more efficiently compared to bacteria from any other source [37].

A total of 14 Gram-positive, catalase-negative, and non-sporulated isolates were revealed from the intestinal content and feces collected from healthy piglets. To obtain a wide diversity of LAB, the samples were collected from different piglets. Therefore, from a security perspective, the original source of the selected lactobacilli is safe. Grown colonies appeared as individuals or in a cluster of three or more cells with white or cream colonies on MRS agar plates, all of which were culturally confirmed to be members of the *Lactobacillus* group [37]. The LAB isolates were evaluated for potential probiotic properties. According to the literature data [38,39], it is noteworthy that the assessment of the function and safety of *Lactobacillus* strains based on in vitro properties, which were evaluated in our study (pH and bile salts tolerance, hemolytic activity, antibiotics susceptibility, strains growth rate within batch fermentation, strains ability during the spray-drying process, and powder stability), are considered relevant aspects for selection of the best probiotic candidate with applicability in animal nutrition.

4.2. Biochemical Identification

The biochemical traits and distributions of the LAB isolated from piglets (intestinal content and feces) based on carbohydrate fermentation are profiled in Table S1. Based on phenotypic traits (Gram staining and morphology), catalase tests, API 50 CHL, and ABIS online software, the isolates belonged to the *Lactobacillus* genus. Based on physiological and biochemical characteristics, from the 14 isolates, most of them were classified as *L. fermentum* (6/14 isolates), *L. acidophilus* (5/14 isolates), *L. delbrueckii* (1/14 isolates), *L. plantarum* (1/14 isolates), and *L. salivarius* (1/14 isolates). One of the relevant characteristics that make LAB an ideal source for fermentation is due to their ability to ferment different ranges of carbohydrates [40]. Although all 14 LAB isolates could ferment sugars as maltose, only 13/14 isolates ferment glucose as the basic carbon source. It is known that glucose is the most utilized monosaccharide included in most commercial selective media [41]. Regarding the sucrose substrate, after 24 h of incubation, only 12/14 isolates were capable of growth. Previously, this disaccharide has been studied as a carbon source for bacteria proliferation and the synthesis of *Lactobacillus* spp. [41,42].

The literature data report similar observations of *Lactobacillus* spp. based on sugar fermentation [8,12], which was isolated from animal origin. Metabolically, LABs are known to produce high quantities of lactic acid, classified as heterofermentative bacteria [43]. In addition, species from the LAB group can synthesize secondary metabolites, including enzymes, bacteriocins, and exopolysaccharides, which are used to improve the host's

digestive process [43]. Therefore, based on their sugar fermentation ability using the API 50 CH kit, the isolates were subjected to molecular identification and evaluated for various contemporary in vitro probiotic properties.

4.3. Molecular Strains Identification

Lactobacillus are the largest and best-considered probiotics group with positive effects that are associated with the capacity for pathogen inhibition, influencing the GIT microflora and immune system of the host [44]. Based on phenotypical results, the bacteria isolated from piglets were LAB members. However, the strains were systematically identified to ensure the efficiency of the isolates. After phenotypical characterization using API 50 CH, isolates were identified by a molecular approach using 16S rRNA sequencing, which has become an established molecular identification technique since its introduction in the 1980s [45]. The results confirmed our isolates as *L. fermentum* (six out of fourteen isolates), *L. acidophilus* (five out of fourteen isolates), *L. plantarum* (one out of fourteen isolates), and species belonging to the LAB genus (two out of fourteen isolates). To identify bacteria including LAB, the 16S rRNA gene is the most commonly used marker. Over time, the analysis of this molecular marker replaced identification based on phenotypic features. The method is rapid and has good accuracy since the great number of reference sequences in databases (e.g., GenBank) simplifies the molecular diagnostics of LAB. However, 16S rRNA gene sequencing has some limitations. Our study showed that two isolates were only identified as LAB genus. Furthermore, *L. plantarum* and other closely related species of the *L. plantarum* group seem to be indistinguishable using 16S rRNA analysis and additional tests are required to accurately identify them [46]. Thus, we considered both of the results of biochemical molecular identification for a reliable assignment of LAB isolates.

Hence, the aim of our research was to evaluate the potential probiotic characteristics and screen the safety of the LAB in order to explore their viability during in vitro gastric conditions for the development of new probiotic sources for animal nutrition.

4.4. Conservation and Bacterial Strains Viability

As live microorganisms, probiotics are very difficult to incorporate and, most importantly, keep alive at the time of consumption. As a cryoprotectant for longer viability, the LAB strains were preserved with glycerol and keeping them at a low temperature (−80 °C).

4.5. Low pH Resistance

The continuous growth of uncharacterized bacterial probiotic products and their subsequent administration to improve zootechnical performance and resistance in animals [47] led us to study the profile of the LAB isolates presented in Table 1 that could be included in the design of a high-quality probiotic for piglets in weaning crisis and whose enzyme system is very poorly developed. Moreover, probiotics inclusion plays a vital role with a beneficial effect on intestinal immunity [38] by equilibrating the composition and activity of intestinal microflora, improving the digestion and absorption of nutrients. Scientists, based on in vitro assay, select potential probiotic strains according to their capacity to tolerate harsh GIT conditions, safety aspects, and technological properties [48,49]. In order to include beneficial effects, probiotic survivability during GIT is relevant to colonize the host's GIT [12,50]. According to the report of the FAO and WHO [51], a probiotic product must be consumed at a minimum concentration of 10^7 CFU to be effective in terms of the host's health status. This statement was also supported by Arepally et al. [52] and Soltani Lak et al. [53], who affirmed that the inclusion of the probiotic must be achieved at a value of at least 10^6 CFU/g. Probiotics are defined as "live microorganisms" that should reach their suitable action site in the GIT to efficiently apply their positive effects [54–56]. Generally, probiotic bacteria survivability is highly strain-dependent [57]. Their resistance in the GIT is low (especially *Lactobacillus* strains), and enhancing their viability in an adequate way during process conditions until they reach the host body represents a real challenge [53]. One of the important factors pertaining to probiotic viability is GI pH. High acidity in

the stomach is the first barrier that influences the selection of a probiotic strain [12,58]. In this research, the viability of unencapsulated LAB isolates was investigated under several conditions, pH 2.0, 3.0 and 7.0, which mimic the animal stomach [59]; the obtained results are shown in Figure 1. Tolerance to low pH values is recognized as a premise for probiotics to employ their beneficial effects on the host gut, since they must maintain viability after ingestion [60]. Our results suggested that some of the isolated strains could not survive during their journey along the host's digestive tract due to decreased viability following in vitro exposure to low pH; therefore, the maximum count was observed at pH 7.0 for all 14 isolates, which was 9.35 $\pm$ 1.04. The minimum strain growth was observed at pH 2.0 after 3 h of incubation (4.80 Log_{10} CFU/mL for IBNA 75, 6.05 Log_{10} CFU/mL for IBNA 76, 4.34 Log_{10} CFU/mL for IBNA 90, 6.86 Log_{10} CFU/mL for IBNA 70, and zero percentage viability for the rest of the strains). It was also revealed that the strains' cell viability at acidic pH values was significantly ($p \leq 0.05$) lower than that at 7.0 pH in MRS broth, which shows an extra reduction in viable cells to unfavorable conditions for all the strains. Sanhueza et al. [61] reported that the acid stress not only induces changes in the bacteria components, but also disturbs the proliferation of Gram-positive bacteria, causing them to be unable to survive at low pH after 3 h of exposition. In this study, the idea to select the best strains after exposure to extreme conditions, including an acidic environment, could be a good option to improve GI probiotic performance [62]. Strains such as *L. fermentum* and *L. acidophilus* isolated from piglets involved resistance after exposure to gradual pH reduction. This is very important if a strain is to be used as a possible probiotic candidate for animal nutrition, the reason for which the respective bacterial source needs to resist GI conditions where 1.5 to 2.0 pH can be found [12,63]. Moreover, the origin of bacteria is preferred due to their being quite familiar with the GIT environment. In the present study, the *Lactobacillus* strains were isolated from intestinal content (ileum and cecum) and piglet feces, and these candidates could proliferate and show their biological activity at a comparatively high level to bacteria from different niche sources [4,8]. Therefore, during their journey through the stomach, probiotic bacteria must resist low pH levels before passing into the lower tract and stay viable for 3 h or more [64,65]. It is known that the number of CFU is in more of a continuous decline at pH 2.0 than at 3.0; our results were similar to those of Hacin et al. [66], where lactobacilli isolate from the mucosa of weaned piglets recorded a low growth rate. Furthermore, a strain should retain more than a 60% viable cell count after 3 h of incubation at low pH, which corresponds to a reasonable percentage when encountering the harsh conditions of the host's stomach [67]. In this regard, the most acid-resistant isolate was 3/14, since the survival efficiency was noted for *L. acidophilus* IBNA 76 (65%), *L. plantarum* IBNA 84 (68%), and *L. acidophilus* IBNA 70 (71%).

4.6. Bile Salts Resistance

Ingested bacteria during transition through the GIT need to face the challenge of toxic compounds such as bile secretions [67,68]. According to Guzior and Quinn [69], bile acids are defined as metabolic end products produced from cholesterol in the liver that are coupled with glycine or taurine followed by secretion into the duodenum through the gallbladder. The presence of bile acids can break bacterial membrane structures, so in order to resist, they must allow high levels of bile acid in the GIT [69,70]. Along with other characteristics in vitro, resistance to bile salts represents an important criterion for bacterial strains before they can be termed probiotics [37]. Furthermore, as beneficial property, bile salt is one of the major factors responsible for the probability of survival of an exogenous culture along the host's GIT environment [36]. The ability of our isolates to survive stimulated intestinal content is in agreement with other previously reported probiotic bacteria [12,65,67]. When the isolates were subjected to a concentration of 0.3% bile salts, a large variation in survival was observed. Therefore, some of the isolates showed very poor tolerance. For two out of the fourteen total isolates, the growth rate was not present after 3 h of incubation at 37 °C, making them unable to tolerate the MRS medium with bile salts addition. Based on Figure 2, the isolates' exposure to bile salts involves a

decrease in proliferation of around 6.27 ± 2.93 for all *Lactobacillus* species. Furthermore, slow growth was observed in the presence of bile salts, with significant variation among the isolates (7.46 Log_{10} CFU/mL for IBNA 71, 5.85 Log_{10} CFU/mL for IBNA 75, 7.10 Log_{10} CFU/mL for IBNA76, 8.37 Log_{10} CFU/mL for IBNA 84, 8.78 Log_{10} CFU/mL for IBNA 87, 8.38 Log_{10} CFU/mL for IBNA 90, 7.10 Log_{10} CFU/mL for IBNA 94, 9.05 Log_{10} CFU/mL for IBNA 95, 6.65 Log_{10} CFU/mL for IBNA 64, 4.59 Log_{10} CFU/mL for IBNA 70, 8.39 Log_{10} CFU/mL for IBNA 78, and 6.10 Log_{10} CFU/mL for IBNA 85), which is indicative of their possible survival in the small intestine [71]. Most of the strains were found to be resistant to 0.3% bile salts; thus, we only selected strains with a growth of over 6 Log CFU/mL to obtain the strongest probiotics. In this study, differences in bile salt sensitivity between *Lactobacillus* isolates were consistent with the literature, which reported similar results [6,12,35,72,73]. The greater resistance was found for *L. fermentum* and *L. acidophilus* species, which confirms their capacity to tolerate a wide bile concentration (0.3%), indicating that organisms have good probiotic characteristics [74]. Moreover, the reason for the decrease in the bacteria growth rate with the increase in the level of bile salts could be due to the relationship between the probiotic strain and the bile salts [75]. In our research, most of the isolates registered a decrease in cell survival carried by 0.3% bile salts in the growth phase with increasing incubation time. Thus, considering the results obtained after evaluating the survival of the isolates, only two strains were identified as *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84, and showed better resistance to gastrointestinal stress during 3 h of exposure (>50%).

4.7. Safety Evaluation

The probiotic properties of 14 *Lactobacillus* strains isolated from the intestinal content and feces of piglets were evaluated. The resistant bacteria source could potentially be used as a probiotic for porcine. Regarding the *Lactobacillus* group, these species are recognized as probiotic candidates since they possess probiotic traits [76]. Certain probiotic strains, determined by the Generally Recognized as Safe (GRAS) status implemented by the U.S. Food and Drug Administration (FDA), are permitted to be administrated as feed additives [49], and which indicate no or fewer risks to the host's health upon consumption [76]. In addition, a strong probiotic can adhere to the intestinal epithelium, preventing the population from pathogenic microorganisms, a phenomenon well known as competitive exclusion [77]. Several types of *Lactobacilli* are marketed as probiotics in animal nutrition [50,54]. Typically, the administration of a new bacterial probiotic in animal feed is analyzed based on viability tests under in vitro standard GIT, processing, and storage [51]. Due to safety considerations, the isolates from our study were also evaluated for hemolytic activity. The absence of hemolysis is considered a safety prerequisite for the selection of probiotic bacteria [78]. Although most probiotics, including LAB, before their use must be evaluated for their functionality [70] and safety [79]. In the current study, none of the presented isolates showed β-hemolytic activity on TSA agar plates without any change in blood agar color, which would correspond to possible hemolytic susceptibility. The lack of recorded hemolysis of blood cells attests to the security of our LAB strains for application in the organisms of animals. Although the absence of hemolytic activity for bacterial strains is not considered harmful [80]. According to Rajab et al. [81], maximum caution and attention are necessary when consuming α-hemolytic strains. Thus, the LAB isolates exhibited no hemolytic activity, which is a real advantage [82,83]. Furthermore, de Melo Pereira et al. [43] stated that it is expected that the bacterial probiotic source does not have the ability to lyse red blood cells when ingested by an animal [84]. Instead, Jin et al. [82] reported that the presence of hemolysis in *Lactobacillus* species is rare. In our work, all 14 isolates have the potential for application as probiotic candidates in animal nutrition.

4.8. Susceptibility Antibiotic Test

Regarding the safety of probiotic products, one of the current concerns is the presence of antibiotic resistance [70]. Furthermore, probiotics are known to have genetic factors that enable the development of various antibiotic resistance [84]. Based on the results from Table 3, the isolated bacteria showed a fluctuating degree of resistance to most of the antibiotics that were verified. All strains showed complete resistance to gentamicin and kanamycin. Most of them exhibited resistance to colistin sulfate, enrofloxacin, amikacin, streptomycin, and tilmicosin (more than 50%). According to previous studies, *Lactobacillus* species are resistant to kanamycin [13,81]. It is recommended that, before using bacteria as a probiotic source for animal feed, they be thoroughly investigated for antibiotic resistance [85]. Furthermore, instances in the literature have affirmed that antibiotic resistance is an inherent property of most lactic acid bacteria [85–87] and strains that are intended to be used as probiotics should be carefully evaluated for their safety [88]. A major health concern worldwide is acquired antimicrobial resistance (AMR) among LAB strains [89]. Commensal bacteria, such as LAB strains, could act as a reservoir of transferable antibiotic resistance genes to pathogens bacteria [89], thus impairing the successful antibiotic treatment of public microbial infections [90]. Therefore, as a crucial safety issue, the evaluation and approval of LAB strains as feed additives should be an assessment of their AMR [91]. Thus, due to the high resistance of the strains, and also to clinically relevant antimicrobials, further research regarding the presence of developed resistance determinants is recommended in order to eliminate the incidence of potentially transferable AMR genes [90].

4.9. Bioreactor Batch and Fermentation Process

The selected isolates, based on probiotic properties, were exposed to fermentation in a 5 L bioreactor using an MRS broth medium. The growth observation of *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84 was examined after 22 ± 2 h. Instead, LAB strains could produce acid very quickly, which caused the pH to drop below 5.0 during the first 6 h of fermentation [41]. Moreover, in the stationary phase, the cells remained viable for more than 18 h, and after that, the viability dramatically decreased [41]. Since the inoculum ration was 1:10 (*v/v*) for a 2 L working volume, 200 mL of both strains was needed for batch fermentation. As an observation, the number of colonies obtained from the bioreactor was higher than that from the tubes. This aspect may be due to the consecutive transfer of the bacterial inoculum in the same culture medium, with respect to the growth parameters. In addition, successive passages were performed at least twice before the actual inoculation in the bioreactor. This action prepares the bacterial cells for new cell division on a laboratory scale.

4.10. Spray Drying Technique and Cell Viability

The inclusion of probiotic powder in animal feed, if orally taken, will have to deal with the heat stimulus caused by the host's internal body temperature (around 37.5 °C), forcing them to exhibit resistance to all antimicrobial factors [43] from the stomach (gastric acid, pepsin, low pH) and intestines (bile salts, trypsin, high pH). Furthermore, the International Dairy Federation (IDF) recommend a minimum level of 10^7 live bacterial cells per gram or milliliter at the time of ingestion of the probiotic product [25]. The microencapsulation of probiotic bacteria enables cell protection against unfavorable conditions, preserving the viability during processing, delivery, storage, and expressly along the host GIT [78,92].

Generally, after exposure to spray drying, the probiotic bacteria cannot withstand the harsh conditions that prevail during the spraying process. In addition, a strategy employed to enhance the cells' viability during drying is necessary. In our work, among the protective agents of carbohydrate type known for their protective traits, maltodextrin and dextrose were included in the culture medium as drying matrices [93]. According to the literature, due to the capacity of hydrolyzed starch, in the encapsulation process, the maltodextrins are the most used wall materials [93,94]. Selected LAB isolates, with probiotic potential, were cultivated with selective media enriched with carrying agents (dextrin and

maltodextrin) and subjected to the spray drying technique to extend their functionality in dried powders [25]. Moreover, a carrying agent can act as a natural delivery system [25], improving the cell viability during the host GIT and making probiotic powder able to resist once it is digested. Spray drying reduced *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84 around 2 Log ten-fold, but the final powder concentration met the minimum value of viable bacteria (10^7 CFU mL^{-1}), which are suggested to provide significant results as probiotic candidates [92,95]. For this reason, it can be concluded that both LAB strains successfully survived during encapsulation and the matrix was used to confer a protective role.

4.11. Powder Stability during Storage

The stability of formulation during storage and the viability of cells should constitute important criteria when selecting probiotic bacteria for obtaining probiotic products [25,92]. Moreover, in order to provide benefits to the host, a probiotic should maintain the minimum level recommended (10^6 CFU/mL or g^{-1} feed). Further, the FAO/WHO [96] published the "Guidelines for Evaluation of Probiotics in Food", where several criteria are suggested for selecting a probiotic bacterium for administration and, especially, to resist gastric and intestinal conditions (acidity, bile salts, and endogenous enzymes) in order to populate the gut epithelium host [12,97].

Based on our results, the selected strains exert desirable properties that can positively influence the status and enhance the host's resistance against enteric pathogens [8], including young animals such as piglets, where the enzymatic system is poorly developed [98]. Thus, *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84 in the microencapsulated powders were tested during storage at room temperature for up to 30 days to test the cell viability. As can be observed in Figure 4, during the first 10 days of storage, the powder showed good stability, followed by a slow decrease in viability with increasing storage time, especially after 15 days. After 30 days, the cell viability of *L. acidophilus* IBNA 76 registered a decrease of approximately 2 Log, while the strain of *L. plantarum* IBNA 84 showed a reduction of viability of 1 Log compared to the initial value. Puttarat et al. [98] reported that the microencapsulation technique is very relevant to increase the survivability and stability of probiotic products under various adverse conditions. Under storage at 25 °C after 30 days, free cells showed a trend in reduction of 27.05% from 7.04 to 5.54 Log_{10} CFU/g for IBNA 76, and 13.46% from 8.48 to 7.46 Log_{10} CFU/g for IBNA 84. These results demonstrated that after 30 days of storage, encapsulated cells under 25 °C involve the minimum requirement of probiotics, which require at least 10^5 CFU/mL of cells [99].

The current findings substantially add to our agreement of microencapsulation techniques can protect bacterial strains and increase cell survivability under storage. This validates the positive effect on the survivability rate when incorporating LAB strains as possible probiotic candidates into the carrier matrix during spray drying [12,99].

4.12. Total Coliforms Count

To target a probiotic strain, as an indicator of contamination, coliform counts were assessed in the powders after spray drying. The number of coliforms in the encapsulated strains (*L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84) was not detected. The reason was attributed to the competence of LAB to produce anti-compounds (bacteriocins, organic acids, and hydrogen peroxide), making them unsuitable for growth [97]. Moreover, according to the literature data, coliform bacteria is sensitive to spray drying stress [25]. In addition, the presence of coliforms implied common microbiological spoilage [100], constituting a major concern regarding food safety control in industrial-scale production [101]. Furthermore, the coliform numbers were undetected in the powders due to their sensitivity to spray drying heat treatment.

Our results are comparable to those obtained in the literature data [25,99,102,103], which reported that coliform counts less than 100 cells/mL are considered acceptable, but colonies of less than 10 are achievable and desirable [12].

5. Conclusions

As a result of this topic, 14 LAB strains were isolated from piglets (ileum, cecum, and feces). Among all the isolated LAB strains, three species of bacteria were identified (*L. fermentum*, *L. acidophilus*, and *L. plantarum*). The isolated strains were identified based on phenotypical and molecular traits. All LAB were evaluated for the potential to survive during GIT conditions and it was affirmed that *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84 possessed this ability and had notable probiotic properties. Microencapsulation could maintain high viability for the selected strains. In addition, the matrix used in the spray drying process had better survival than free cells, indicating the importance of encapsulating probiotics to avoid gastrointestinal environment conditions. Moreover, powder cell viability was greatly maintained during storage and satisfied all of the necessary criteria to be considered a useful probiotic source. Thus, it could be stated that *L. acidophilus* IBNA 76 and *L. plantarum* IBNA 84 in microencapsulated form could be used as sources of probiotics for animal nutrition and could have promising performances in the diet of weaned piglets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13051098/s1, Table S1: Morphological, biochemical and percentage of identification by API 50 CHL, and ABIS online for *Lactobacillus* strains isolated from piglets GIT.

Author Contributions: Conceptualization, M.D.; methodology, N.A.L., M.D. and G.C.; software, M.D., D.C.V. and S.E.; validation, M.D., I.S., S.E.G. and A.D.; formal analysis, M.D., I.S., S.E.G., D.C.V., S.E. and A.D.; investigation, M.D., I.S. and G.C.; resources, M.H.; data curation M.D.; writing—original draft preparation, M.D.; writing—review and editing, M.D. and N.A.L.; project administration, M.H.; funding acquisition, M.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Romanian Ministry of Research, Innovation, and Digitalization, through grant 8 PFE/2021 and Project PN 23-20.04.01.

Institutional Review Board Statement: This study was approved by the Ethics Commission of IBNA Balotesti, Romania (protocol No. 5183/2018) in compliance with European Directive (2010/63/EU) and Law 43/04.2014 regarding the protection of animals used for scientific purposes.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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