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Production of Lanhouin—A Fermented Catfish (*Clarias gariepinus*) Using the Selected *Lactiplantibacillus pentosus* Probiotic Strain

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Abstract

Lactic acid bacteria (LAB) preserve many foods and play a vital role in fermented food products. This study designed a controlled biotechnological process of catfish (*Clarias gariepinus*) fermentation with a LAB starter culture isolated from corn hydrolysate. The BY (Barbu-Yelouassi) LAB strain was characterized regarding fermentative and antimicrobial potential, and its adaptability in the simulated gastrointestinal system (SGIS). After 10–12 h of cultivation on MRS broth (De Man Rogosa and Sharpe), the strain achieved the maximum exponential growth, produced maximum lactic acid (33.04%), and decreased the acidity up to pH 4. Also, the isolated strain showed increased tolerance to an acidic pH (3.5–2.0), high concentrations of salt (2–10%), and high concentrations of bile salts (≤2%). The behavior in SGIS demonstrated good viability after 2 h in artificial gastric juice (AGJ) (1×10^7 CFU/mL) and up to 2×10^3 CFU/mL after another 6 h in artificial intestinal juice (AIJ). The characterized BY strain was identified with the API 50CHL microtest (BioMerieux) as *Lactiplantibacillus pentosus* (*Lbp. pentosus*) (90.9% probability), taxon confirmed by genomic DNA sequencing. It was also demonstrated that *Lbp. pentosus* BY inhibited the growth of pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and sporulated bacteria, such as *Bacillus cereus*. Additionally, it suppressed the sporulation of fungi like *Aspergillus niger*, *Fusarium* sp., and *Penicillium* sp. Furthermore, the *Lbp. pentosus* BY strain was used to ferment catfish, resulting in three variants of lanhouin (unsalted, with 10% salt, and with 15% salt), which exhibited good microbiological safety.

Keywords: catfish; *Lactoplantibacillus pentosus*; probiotic properties; functional food; lanhouin; sustainability

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1. Introduction

Lactic acid bacteria (LAB) are versatile microorganisms with many practical applications in food production, health, and biotechnology. These bacteria play a crucial role

in the production of fermented foods and beverages and contribute to their preservation, flavor development, but also to the consumer's well-being. Numerous studies have shown that the production of lactic acid and other metabolites can influence the taste of fermented foods, helping to inhibit the growth of spoilage organisms and pathogens [1]. Many LAB species are considered probiotics, with beneficial effects for gut microbiota health. By producing bacteriocins, they can fight harmful microorganisms, helping the digestive system's microbiota balance and improving immune system function.

Fermented fish foods are generally considered to belong only to the cultural areas of Southeast Asia, including the nuo-cman of Vietnam [2], or the plaa-som of Thailand [3]. In some West African countries, such as Benin or Senegal, but also in Central Africa, certain artisanal fish processing has gained an important place in the fish industry and thus in the country's economy. These include lanhouin (Benin and Togo), momoni (Ghana), guedj (Senegal), adjuevan (Côte d'Ivoire), metora, tambadiang, ketiakh, sali, and makayaku, which are fermented fish products [4–6]. Lanhouin was thus introduced, which is fermented, salted, and dried fish widely consumed as a condiment in the Gulf of Benin, particularly in Ghana, Togo, and Benin [4]. In Côte d'Ivoire, fermented fish is frequently used in dishes for its flavor, and studies have been carried out on the production system, its marketing, and on the identification of contamination and pathogenic flora. Similar research has focused on the guedj in Senegal [7] and the momoni in Ghana [8] and Benin [6].

Lactiplantibacillus pentosus (*Lbp. pentosus*), previously classified under the *Lactobacillus* genus, is a significant species of lactic acid bacteria recognized for its probiotic potential and diverse applications in food fermentation. The metabolic versatility of *Lbp. pentosus* allows it to thrive in various environmental niches, including fermented foods and the gut microbiota of mammals and insects [9,10]. *Lbp. pentosus* is commonly employed in the fermentation of various food products, including olives, sausages, and dairy products. Its role in the fermentation of table olives is particularly significant, as it helps lower the pH to safe levels, thus preventing the growth of spoilage organisms [11,12]. The species has also been identified in the fermentation processes of other traditional foods, indicating its broad applicability in food technology [13–16]. Furthermore, the strain's ability to produce biosurfactants has been linked to its antimicrobial activity, making it a candidate for use in food preservation [17].

Fermented fish products are a significant aspect of culinary traditions in various cultures, and the role of lactic acid bacteria, particularly *Lactiplantibacillus pentosus*, in these fermentation processes is crucial. *Lbp. pentosus* is known for its ability to ferment a wide range of carbohydrates, which is essential for the development of flavors and preservation of fermented fish products [18–21]. The fermentation process not only enhances the sensory qualities of the fish but also contributes to its safety by lowering the pH and inhibiting pathogenic microorganisms [22,23].

Different studies have shown that *Lbp. pentosus* is frequently isolated from traditional fermented fish products, such as pla-paeng-daeng in Thailand and suanzhayu in China. For instance, the use of *Lbp. pentosus* in fermented suanzhayu has been associated with increased umami flavors and improved overall sensory quality [24,25].

Moreover, the probiotic potential of *Lbp. pentosus* has garnered attention due to its health benefits, including immunomodulatory effects and cholesterol-lowering properties. These attributes make fermented fish products not only a source of unique flavors but also a functional food that can contributes positively to health [26,27]. The presence of bioactive compounds, such as gamma-aminobutyric acid (GABA), produced during fermentation further enhances the health benefits associated with these products [27].

The probiotic properties of *Lbp. pentosus* have been extensively studied, revealing its immunomodulatory effects and potential health benefits. For instance, strains of *Lbp.*

pentosus have demonstrated the ability to enhance salivary immunoglobulin A secretion, which is crucial for mucosal immunity [11,23]. Additionally, certain strains have been shown to produce gamma-aminobutyric acid (GABA), a compound associated with various health benefits, including antihypertensive effects [27–29].

In Scandinavian countries, the preparation of fermented raw salmon fillets and fermented raw fish canapés involves lactic acid fermentation, which is considered to extend the shelf life of seafood in the fresh food field. Indeed, the inoculation of tuna mince with strains of *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaeus* makes it possible to control spoilage and the growth of pathogenic bacteria by extending the shelf life to four weeks at 8 °C [30,31]. In Benin, to valorize maize by-products, and suspecting the presence of lactic acid bacteria in maize hydrolysate, it was used as a ferment to control the production of *lanhouin* (fermented fish consumed in Benin) [32]. Microbiological analysis of fermented maize hydrolysate produced in Benin showed the presence of *Lactiplantibacillus plantarum* in the range of 10^5 to 10^6 CFU/mL [33].

Indeed, it is known that especially marine fish (mackerel, sea bass, etc.) are usually used for fermentation in Africa, especially in Ghana, Togo, and Benin [4]. But the current trend is to ferment freshwater fish, such as *Clarias gariepinus* [34–37]. The preservation techniques of *Clarias gariepinus* depend on the culinary habits of each country. For example, in Asian countries (Vietnam, Thailand, Indonesia, Philippines, Malaysia, etc.), *Clarias* sp. is subjected to fermentation [38–42] more than smoking [43]. Instead, in Nigeria, it is often smoked and/or sun-dried [44–46]. Studies on the microbiological characterization of fermented catfish from Indonesia revealed the presence of lactic acid bacteria and the inhibition of pathogenic flora [39].

Often, microbiological analysis of traditional African products (lanhouin, guedj, and momoni) revealed the presence of a rich microbiota, including species such as *Bacillus*, *Pseudomonas*, *Pediococcus*, *Staphylococcus*, *Klebsiella*, *Debaryomyces*, *Hansenula*, *Aspergillus*, *Lactobacillus*, *Enterobacter*, and *Salmonella* [7,8]. The main analysis regarding the enumeration of total coliforms and fecal coliforms indicated that traditional African products were frequently contaminated with enteric bacteria [32].

In summary, *Lbp. pentosus* plays a vital role in the fermentation of fish products, contributing to flavor development, preservation, and health benefits. Its application as a starter culture in traditional fish fermentation processes highlights its significance in enhancing both the sensory and nutritional qualities of these foods. Its metabolic capabilities, probiotic properties, and role in enhancing food safety and quality underscore its importance in both traditional and modern food systems.

To the best of our knowledge, there is limited literature addressing both Benin catfish fermentation and its autochthonous heterofermentative bacteria. Thus, to isolate and characterize lactic acid bacteria from the native microbiota of catfish and using them as starter cultures is of technological interest for fermentation process control.

Food sustainability refers to practices that aim to minimize negative environmental impacts, preserve resources, and promote social and economic well-being throughout the food supply chain. Purchasing locally grown and seasonal foods helps reduce carbon footprints by cutting down on transportation and promoting regional economies.

This study aimed to isolate, characterize, and identify lactic acid bacteria from the microbiota of fish resources in Benin and to use them as a starter culture in controlled fermentation of freshwater catfish. Obtaining safe products from a microbiological perspective, while also ensuring they are functional and beneficial not only for special categories of consumers (with anemia, immunodeficiency, or digestive diseases) but also for the overall population, is crucial for consumer confidence. Functional foods have a potentially positive effect on health beyond basic nutrition. Following these general directions, in the present

study, functional foods were designed that use African catfish from the natural ecosystems of the country of Benin, but which, unlike traditional production technologies, possess rigorous control of the production process, to eliminate possible microbiological risks.

Our study makes a significant contribution to the sustainable exploitation of local natural resources in Benin, but in a controlled and safe way, to combine local traditional elements with those of modern technologies to obtain functional foods.

The ongoing need to ensure safe, sustainable foods with high nutritional and biological value is a major current concern in food biotechnology. Sustainability and functional foods are two key concepts that are increasingly relevant in today's food industry, as consumers become more aware of the environmental impact of food production and the potential health benefits of the foods they consume.

2. Materials and Methods

2.1. Plant Material

The grains of maize (corn) (*Zea mays* L.) variety BEMA14 B-09 used to produce the fermented maize hydrolysate were from the local market in Abomey-Calavi.

2.2. Animal Material

The African sharptooth catfish *Clarias gariepinus* (Burchell, 1822) was purchased from a fish farming center in Abomey-Calavi, Benin. It was frozen (−12 °C), transported, and then analyzed at the BioAliment-TehnIA Research Platform from Dunarea Jos University of Galati, Romania.

2.3. Media and Reagents

The culture media used for microbiological analyses were Plate Count Agar (PCA) (Biokar, Allonne, France), Malt Extract Agar (MEA) (Biolife, Monza, MI, Italy), Bacteriological Agar (Scharlau, Barcelona, Spain), De Man Rogosa and Sharpe (MRS) broth, Lactose Broth (LB), Brilliant Green Bile Lactose Broth (BGBL), and $CaCO_3$, which were purchased from Sigma Aldrich (Taufkirchen, Germany). Columbia agar supplemented with sheep blood (5%) (Oxoid, Hampshire, UK) was used for hemolytic activity determination. Brain Heart Infusion (Oxoid, Hampshire, UK) was used for *Listeria monocytogenes* cultivation and Muller–Hinton (Scharlau, Barcelona, Spain) for *Escherichia coli* and *Staphylococcus aureus*. Ox Bile was provided by Merck (Darmstadt, Germany). Pepsin, pancreatin, glycerol, HCl 0.1 N, NaOH 0.1 N, a 0.22 µm filter membrane, and phosphate-buffered saline (PBS) were purchased from Sigma Aldrich (Taufkirchen, Germany) and used for the preparation of artificial gastric and intestinal juices (AGJ and AIJ, respectively). The standard antibiotic discs from Sigma Aldrich (Taufkirchen, Germany) were used for antimicrobial susceptibility analysis. API 50CHL Kit (BioMérieux, Craponne, France) was used to identify the LAB strain. The Maxwell®16 Cell DNA purification kit (Promega, Madison, WI, USA) was used for DNA extraction, and all reagents for the PCR of 16S rDNA were purchased from Thermo Scientific, Waltham, MA, USA.

2.4. The Isolation of Lactic Acid Bacteria (LAB)

First, an amount of 3 kg of corn was soaked for two hours in 9 L of boiled water (100 °C). Secondly, the corn flour obtained after grinding the grains was mixed with tap water in a ratio of 1:3 (w/v). Then, the mix was filtered and the supernatant, which contained a high concentration of heterofermentative LAB, was maintained at 25 °C for 48 h to favorize the fermentation process (Figure 1).

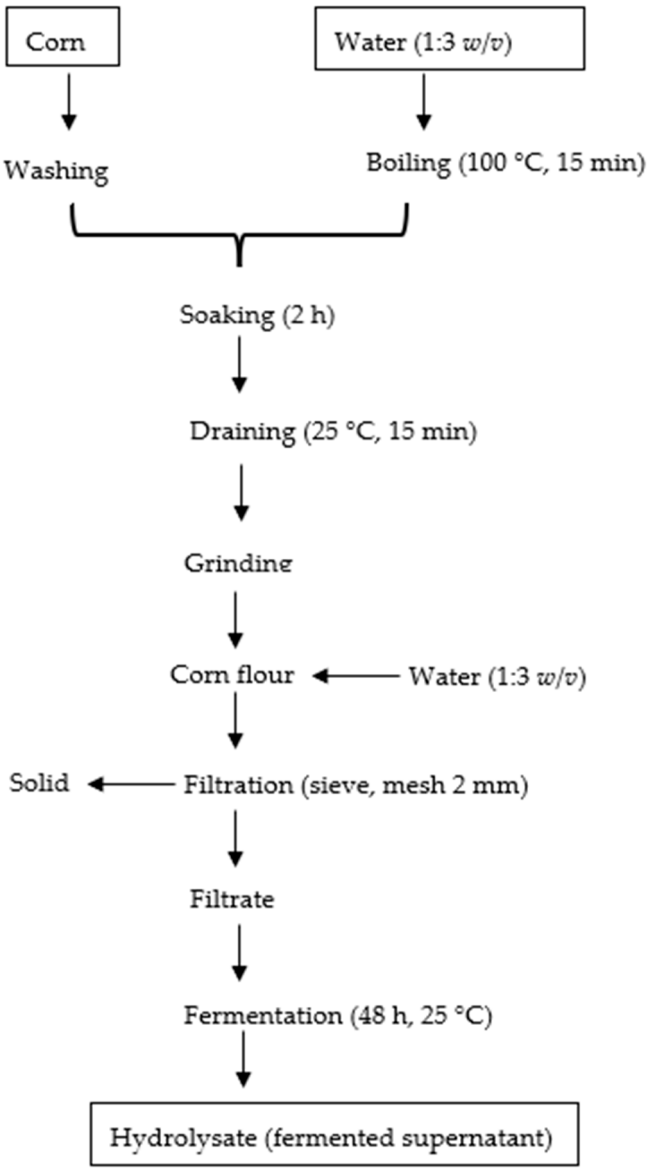


Figure 1. Diagram for obtaining corn hydrolysate.

From the corn hydrolysate, decimal dilutions were made, and 1 mL of each dilution was inoculated in MRS medium with 1.2% agar and 1.5% $CaCO_3$, using the double-layer method. Lactobacilli are microaerophilic and generally require layer plates for aerobic cultivation on solid media. When the medium was set, another layer of uninoculated MRS agar was poured over the surface to produce a layered plate. After 48 h of incubation at 37 °C (binder incubator BD 400, Tuttlingen, Germany), the typical LAB colonies were identified (white–cream, with a clear halo around them). The isolated colonies were recultivated on the same selective media (MRS 1.2% agar and 1.5% $CaCO_3$). From colonies with slightly different morphologies, Gram-stained microscopic smears were made to confirm culture purity. The samples were observed with a Carl Zeiss Axio Observer Z1 inverted microscope (Walpole, MA, SUA) (×100), and the images were analyzed by ZEN 2012 SP1 Blue edition software (Zeiss, Oberkochen, Germany). The biomass of each colony microscopically analyzed was resuspended in MRS broth media to obtain pure cultures. Then, the stock culture was preserved in 40% (*w*/*w*) glycerol at −80 °C (Angelantoni Platinum 500+, ALS, Massa Martana, Italy). Furthermore, the pure culture was freeze-dried at 10 mBar and −42 °C, for 48 h, using CHRIST ALPHA 1–4 LD plus

equipment (Martin Christ, GmbH, Osterode am Harz, Germany) on medium with 8% inulin in skim milk to create a cryoprotective environment and a source of prebiotics for the strain during preservation.

2.5. LAB Growth Kinetics and Lactic Acid Production

From the pure stock culture, 1% inoculum was obtained, and after 24 h of incubation at 37 °C, the optical density (OD_{600}) of the inoculum was spectrophotometrically adjusted (Libra S22 spectrophotometer, Biochrom Ltd., Cambridge, UK) using MRS broth, to a range of 0.8–1, a value equivalent to 10^9 CFU/mL [47]. A total of 1 mL of this inoculum was added to 100 mL of MRS broth and incubated at 37 °C for 48 h. Samples were taken every 2 hours during the 48 h of incubation, and the main analyses regarding the OD_{600}, pH (Mettler Toledo pH meter, Shanghai, China), and the acidity were performed. The total acidity was measured by the titrimetric method with a Tritroline Esay Schott instrument, using a 0.1N NaOH solution and phenolphthalein as an indicator. The total acidity was expressed as a percentage (%) of lactic acid ($CH_2CH(OH)$-COOH, MW = 90) with a reproducibility error limit of ±0.01% acid, and was determined with the formula from method 947.05, the Association of Official Analytical Chemists (AOAC, 1990) [48].

$$\% \text{ total acidity} = (V_{NaOH} \times N \times 90 \times 100)/(V \times 1000) \times DC \quad (1)$$

V_{NaOH} = volume of NaOH solution added in mL.
N = normality of NaOH solution.
V = volume of LAB suspension to be analyzed.
DC = dilution coefficient.
N = normality of NaOH solution.

The dilution coefficient was 20 (5 mL of bacterial suspension in 100 mL distilled water). Note that 1 mL of 0.1N NaOH is equivalent to 0.009 g of lactic acid.

2.6. LAB Stress Tolerance

The acid pH tolerance was evaluated according to the method described by Tian et al. [49] with some modifications. LAB isolates were incubated in MRS at 37 °C for 24 h to promote active growth. An acidic environment was created by adjusting the pH of the MRS broth to 1.5; 2.0; 2.5; 3.0; and 3.5, with 0.1N HCl. Also, a control tube with a pH of 6.2 was maintained in the same conditions. All tubes were inoculated with 1 mL of LAB suspension (10^9 CFU/mL) and incubated for 3 h at 37 °C. After incubation, decimal dilutions were immediately made from each sample, and the number of viable LAB cells was indirectly evaluated by the cultivating method (spreading in Petri dishes on double-layer MRS with 1.2% agar and 1.5% $CaCO_3$). The plates were incubated at 37 °C for 48 h.

The bile salt tolerance of the strains was assessed following the procedure described by Tian et al. [49], with some modifications. Bile salts were added to MRS broth to obtain concentrations of 0.1%, 0.5%, 1%, 1.5%, and 2%. A standardized 1% inoculum (v/v) (approximately 10^9 CFU) was added to each tube, and all samples were incubated at 37 °C for 3 h. Then, the number of viable LAB cells from each sample was evaluated by the method described above.

Sodium chloride tolerance of the LAB strain was performed following an adapted method from the one proposed by Khushboo et al. [50]. To the MRS media, different NaCl concentrations were added as follows: 2%; 4%; 6%; 6%; 8%; and 10%. Then, each variant was inoculated with 10^9 CFU/mL of pure LAB culture and the tubes were incubated under the same conditions. Furthermore, the viable LAB strains were evaluated using the indirect counting method described above.

All the experiments were conducted in triplicate. Colonies were counted on plates corresponding to the dilutions in which the number of colonies was in the range of 30–300 CFU. The average of the triplicates was then calculated. The results are expressed as the mean number of CFU per mL (CFU mL^{-1}). The plate counting technique was used to determine cell viability, and the results are represented as $\log_{10}$ CFU/mL.

$$\text{Survival rate } (\%) = \frac{\log \text{CFU/mL(F)}}{\log \text{CFU/mL(I)}} \times 100 \quad (2)$$

where log CFU/mL (F) represents the final bacteria number after the acid tolerance test and log CFU/mL (I) represents the initial bacteria number (in the control tube).

This experimental technique determined the ability of LAB isolates to tolerate and survive in acidic or high-salinity environments, revealing valuable biotechnological information about their probiotic character.

2.7. Viability in the Simulated Gastrointestinal System (SGIS)

LAB strain tolerance in the SGIS was determined according to the method reported by Sağlam et al. [51]. A volume of 100 mL of simulated gastric juice (SGJ) or artificial gastric juice (AGJ) was prepared using sterile phosphate-buffered saline (PBS: 8.0 g/L NaCl, 0.2 g/L KH_2PO_4, and 1.15 g/L Na_2HPO_4, pH 7.2) and 0.3% pepsin and was adjusted to 2.5 pH with 0.1 mol/L HCl (0.1N). The resulting artificial gastric fluid was filtered through a 0.22 µm filter membrane, and a volume of 10 mL was distributed in sterile test tubes.

For both the simulated intestinal juice and artificial intestinal juice (SIJ/AIJ), a volume of 100 mL of sterile PBS with 2% pancreatin was prepared, and the pH was adjusted to 8 with 0.1 mol/L NaOH [47]. The resulting artificial intestinal fluid was then filtered through a 0.22 µm filter membrane, and a volume of 10 mL was distributed in sterile test tubes.

Then, 1% standardized inoculum of the LAB strain (*v*/*v*) (approximately 10^9 CFU/mL) was added to the SGJ and static-incubated at 37 °C. After 2 h of incubation, decimal dilutions were made and spread on Petri dishes on a double layer of MRS media with 1.2% agar and 1.5% $CaCO_3$ to evaluate the cells' viability in AGJ [49]. Three plates were used for each dilution of each sample.

The rest of the AGJ left after the 2-hour incubation was mixed in a 1:1 ratio with AIJ and incubated further at 37 °C for 2 h, 4 h, and 6 h. Decimal dilutions were made each time and seeded on a double layer of MRS with 1.2% agar and 1.5% $CaCO_3$. The LAB cells' viability in AIJ was determined with the method described above by Tian et al. [49], and their survival rate in the SGIS was calculated according to Formula (2).

2.8. Antimicrobial Susceptibility Test (AST)

The in vitro antimicrobial activity was evaluated by two methods: the agar well diffusion methods proposed by Ye, Li, and Gu, in 2021 [52] and Kumar, Ruhal, and Kataria, in 2023 [53], and the adapted assay described by El Hammoudi et al. [54], Pereira et al. [55], and Ryu et al. [56].

Briefly, 1 mL of the selected LAB inoculum (BY strain) was added to 100 mL of MRS broth and incubated at 37 °C for 24 h (binder incubator BD 400, Tuttlingen, Germany). After 24 h, centrifugation (Universal Centrifuge 320R Hettich GmbH, Tuttlingen, Germany), for 10 min at 500 rpm, was performed to separate the biomass from the cell-free supernatant (CFS). The supernatant was stored in a −20 °C freezer (Angelantoni Platinum 500+, ALS, Massa Martana, Italy) for subsequent use in assessing antimicrobial activity.

The CFS with its antimicrobial molecules (organic acids, peptides, and/or bacteriocin) could inhibit the growth of test microorganisms, such as molds: *Aspergillus niger*,

Penicillium sp., and *Fusarium* sp.; yeasts: *Candida* sp.; Gram-positive bacteria: *Bacillus cereus*, *Listeria monocytogenes* Scott A, and *Staphylococcus aureus* ATCC 25923; and Gram-negative bacterium: *Escherichia coli* ATCC 25922. The molds, yeasts, and *Bacillus subtilis* strains belong to the MIUG microorganisms' collection of the BioAliment-TehnIA Research Platform, Faculty of Food Science and Engineering from Galati. Yeasts and molds were grown on MEA medium and the PCA medium was used for *Bacillus cereus* growth. The non-selective media and Brain Heart Infusion (Oxoid, Hampshire, UK) were used for *Listeria monocytogenes* cultivation, and Muller–Hinton (Scharlau, Barcelona, Spain) for *E. coli* and *S. aureus*. According to the CLSI standards [57], inoculum dosing was performed using the 0.5 McFarland standard corresponding to (1–2) $\times$ 10^8 CFU/mL for bacteria and (1–5) $\times$ 10^6 CFU/mL for yeasts and molds [58,59].

Furthermore, the antibacterial activity was tested against one Gram-negative bacterium (*Escherichia coli* ATCC 25922) and three Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus* ATCC 25923, and *Listeria monocytogenes* Scott A), based on the agar well diffusion test. One colony from each bacterium was inoculated on the broth formula of the specified medium and grown at 37 °C for 18 h, to reach the stationary phase. A layer of a specific culture medium with 1.5% agar was poured into sterile Petri dishes. Then, 10 µL from each of the overnight cultures were inoculated in 7 mL of the melted agar medium (0.75% agar) and poured onto the first layer medium, to reach a final concentration of 10^7 CFU/plate. Moreover, after media solidification, wells with 9 mm diameters were aseptically created into which 100 µL of the CFS was added. After incubation conditions at 37 °C for 24 h, the diameter of the inhibition zones was measured (DIZ, mm) [60].

A volume of 500 µL CFS was incorporated into each Petri dish with 10 mL MEA medium at 45 °C to reach a final concentration of 5% (*v*/*v*) [54]. Using the Thoma cytometer, a suspension of 10^4 CFU/mL was made from each test strain: *Aspergillus niger*, *Penicillium* sp., *Fusarium* sp., and *Candida* sp. [55,56]. In the center of each Petri dish with MEA medium, a micro-drop with a volume of 10 µL of the test strain was placed. The plates were incubated for 3 days at 25 °C. All analyses were performed in triplicate. The diameter of the central colony of yeast or fungus developed on MEA with CFS was measured compared to control plates in which the medium did not contain CFS. The percentage of growth inhibition (I) % was calculated according to the following formula:

$$I = 100 \times (AC - AT)/AC \quad (3)$$

where AC and AT are the diameters of colony growth in treated and control plates, respectively [54].

2.9. Antibiogram Analysis

The Clinical and Laboratory Standards Institute (CLSI) has published a series of guidelines to assist in preparing annual cumulative antimicrobial susceptibility test data, known as antibiograms. CLSI's 2022 M39 5th edition consensus document entitled "Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data" guides clinical laboratories and antimicrobial stewards. (CLSI. Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data. 5th ed. CLSI guideline M39. Clinical and Laboratory Standards Institute; 2022) [61].

The Kirby–Bauer diffusimetric method first obtained the inoculum of LAB by reactivating the stock cultures (preserved in 40% (*w*/*w*) glycerol solution, at a temperature of −80 °C) on the MRS broth medium, for 48 h, at a temperature of 37 °C. Later, the inoculum was spectrophotometrically dimensioned at a 0.5 OD_{600} value (that corresponding to 1–2 $\times$ 10^8 CFU/mL for bacteria). Then, 1 mL of the LAB inoculum was transferred to sterile Petri dishes over which 15 mL of 1.5% agar MRS was added. After homogenization

and solidification of the medium, the 14 antibiotic discs, Amikacin (AK 30 µg), Ampicillin (AM 10 µg), Ceftazidime (CAZ 30 µg), Cefoxitin (FOX 30 µg), Chloramphenicol (C 30 µg), Cifloxacin (OFX 5 µg), Ciprofloxacin (CIP 5 µg), Cotrimoxazole (SXT 25 µg), Fosfomycin (FF 200 µg), Gentamicin (GM 1 µg), Levofloxacin (LEV 5 µg), Penicillin (P 10 µg), Vancomycin (Va 5 µg), and Tetracycline (TE 30 µg), were aseptically placed. The plates were incubated for 48 h at 37 °C, and the results were interpreted by measuring the diameter of the inhibition zone (DIZ, mm) [61]. The antibiogram was created in duplicate. The correlation between the diameters of the inhibition zones and the sensitivity of the bacteria was made by consulting the corresponding tables from the CLSI standards [61]. The results are expressed as sensitive, resistant, and moderately sensitive.

2.10. Hemolytic Activity

The LAB strain was streaked on Columbia agar culture medium supplemented with sheep blood (5%) (Oxoid, Wesel, Germany). The plates were incubated at 37 °C for 72 h, and the plates were examined for a hemolytic reaction. The presence of hemolysis was indicated by the formation of greenish or clear areas in the inoculated plates [62]. The plates were observed for the formation of any clear zones (β-hemolysis), greenish hemolytic zones (α-hemolysis), or no such zones (γ-hemolysis) around the LAB colonies.

2.11. Identification of the Isolated Strain

The Biomérieux API 50CHL microassay was used to identify LAB isolates. The LAB strain was phenotypically characterized by this standard commercial identification system that analyzes carbohydrate fermentation properties. A positive result means a color change in the bromocresol violet indicator from violet to yellow, except for probe no. 25 (for the esculin hydrolysis test, which changes color from violet to black). No color change indicated a negative result. The first well on the strip was used as a control. The result was analyzed using the api-web identification software database version 4.0 (BMX-424275 BioMerieux, Craponne, France) to identify the species.

2.12. Molecular Identification

Genomic DNA of the LAB isolate strain was extracted using automated ADN Maxwell®16 equipment (Promega, USA) with a Maxwell®16 Cell DNA purification kit (Promega, USA), with the procedure performed according to the manufacturer's instructions. The PCR of the 16S rDNA was performed using the following two primers: 27F (5′- AGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [49,63]. The PCR Master Mix (25 µL) included 0.5 µL of DNA template (20–50 ng/µL), 2.5 µL of 10x PCR buffer (with 50 mol/L Mg^{2+}), 1.0 µL of dNTP (2.5 mmol/L each), 0.2 µL of DreamTaqTM DNA Polymerase (5U/µL) (Thermo Scientific, MA, USA), 0.5 µL of primers (10 µmol/L each), and ddH_2O, added to achieve a final volume. PCR was performed on a T100 thermal cycler (BIORAD, Hercules, CA, USA) based on the following procedure: initialization step of 5 min of denaturation at 95 °C; 30 amplification cycles (denaturation at 94 °C for 30 s; annealing primers at 57 °C for 30 s; extension at 72 °C for 90 s); and final extension at 72 °C for 10 min.

The amplified fragments were sequenced using the Sanger method in the Spectrum Compact CE System (Promega, Madison, WI, USA). The nucleotide sequences were aligned with the available sequences in the BLASTn (Basic Local Alignment Search Tool nucleotide) database through NCBI blasting (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 4 December 2024) and using MEGA software (version 11.0; Biodesign Institute, Tempe, AZ, USA, Acession no: OK325938.1; RID: N3VUW1MC016) to determine the species of the bacterial strain [63].

2.13. Biotechnological Design for Obtaining Lanhouin Using *Lactiplantibacillus pentosus*

Based on the methods described by Yelouassi et al. in 2018 [64] and Dossou-Yovo et al. in 2010 [6], an improved lanhouin production technology was designed. To control the process from a microbiological point of view, a standardized inoculation of the previously sterilized corn hydrolysate with a biotechnologically characterized starter culture, *Lactiplantibacillus pentosus* BY, was performed (Figure 2).

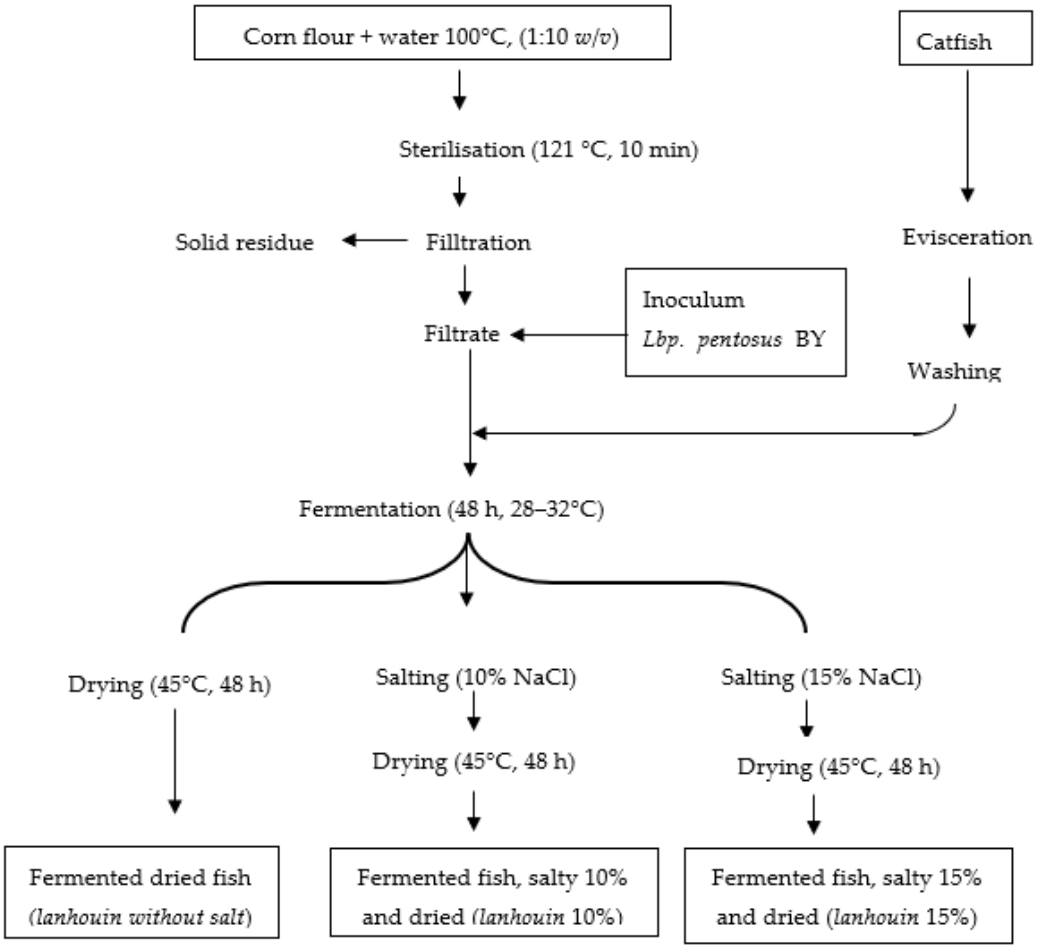


Figure 2. Biotechnological design of lanhouin obtained with starter *Lactiplantibacillus pentosus* BY strain.

Unlike the traditional product, in which the catfish was fermented with a complex, unknown microbiota, which involves microbiological risks, the new technological scheme designed to obtain lanhouin was based on directed lactic fermentation, with a probiotic LAB starter culture, for 48 h at 28–32 °C (binder incubator BD 400, Tuttlingen, Germany). The drying of lanhouin was carried out for two days at temperatures of 45 °C using a Sanyo drying oven MOV112 (Sanyo, Osaka, Japan).

2.14. Microbiological Analysis of the Products

The microbiota of all the products (corn hydrolysate, fresh catfish, and traditional and functional lanhouin variants) were quantified by the indirect counting method during the biotechnological process. The samples were homogenized with Pulsifier equipment (Microgen Bioproduct, London, UK) for 5 min at medium speed. Ten-fold serial dilutions in 0.85% sterile saline solution were plated out following the standard methodology [65]. The determination of the total number of aerobic mesophilic bacteria was performed on PCA after incubation for 48 h at 37 °C. The evaluation of total number of yeasts and molds

was performed on MEA after incubation at 25 °C for 3–5 days. The conventional method was used to determine the probable number of coliforms. The Most Probable Number (MPN) method is a statistical assay consisting of presumptive (on Lactose Broth) and confirmed (on Brilliant Green Lactose Bile broth) stages for 48 h each, at 35 °C (3 tube MPN analysis). The McCrady statistical table was used to interpret the results with de Man's confidence intervals.

The number of LAB was determined by inoculation on MRS media with 1.2% agar and 1.5% $CaCO_3$ using the double-layer method. After 48 h of incubation at 37 °C, the LAB colonies that appeared surrounded by a clear halo were counted.

All the experiments were conducted in triplicate considering each dilution. Colonies were counted on plates that had 30–300 CFU. The results are expressed as the mean number of the colony forming units per mL (CFU mL^{-1}).

2.15. Statistical Analysis

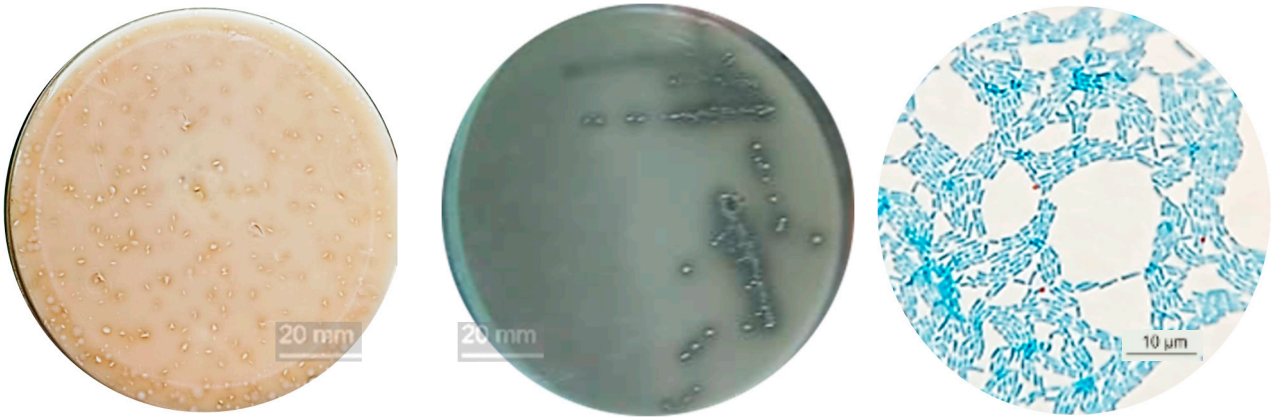
All the data reported in this study represent the averages of triplicate analysis and are reported as mean ± standard deviation. The results are compared statistically with one-way analysis of variance (ANOVA) using the software SAS version 8.2 ($p < 0.05$), which was carried out to assess the significant differences between values. The linear and quadratic effects of the predictor variable levels were determined by using a contrast of orthogonal polynomials. For highlighting the differences between lanhouin samples, ANOVA was employed after checking the normality and equality of variances, by using the Minitab statistical software package (Minitab 19, Minitab LLC., State College, PA, USA). As the test for equality of variances was not passed, the post hoc test employed to identify the differences among samples at $p < 0.05$ in ANOVA was the Games–Howell method.

3. Results

3.1. LAB Strain Isolation and Identification

The corn hydrolysate, obtained according to the diagram presented in Figure 1, was assessed for its LAB content. The results show a high LAB count of 4×10^5 CFU/mL. After repeated scarification on MRS in a double layer, the BY strain of LAB was obtained in pure culture. From a cultural point of view, it appears as convex, smooth, creamy colonies, buttery white, with a straight outline and 1–2 mm in diameter, surrounded by a clear halo on the $CaCO_3$ medium (Figure 3a,b). By performing Gram-stained smears and their microscopic analysis, the BY strain was determined to be Gram-positive bacteria, with short rod-shaped cells without spores, and tending to a palisade arrangement (Figure 3c).

Following the analysis of the biochemical profile of the strain using the API 50CHL kit from BioMerieux, it emerged that the BY strain was *Lbp. pentosus* with 90.9% probability, a taxon also confirmed by genetic analysis. The BLAST function on the NCBI database showed that the analyzed strain was part of the *Lactobacillaceae* family having a high degree of similarity (>99%). Based on the LAB 16S rDNA sequence, the BY LAB strain isolated from maize hydrolysate was *L. pentosus* with a 99.72 percent identity (accession no: OK325938.1; RID: N3VUW1MC016).



(**a**) (**b**) (**c**)

Figure 3. Colony and morphological appearance of isolated BY strain: (**a**,**b**) colony morphology; (**c**) cell morphology.

3.2. Characterization of the Biotechnological and Probiotic Potential of Lbp. pentosus by Strain

3.2.1. Lactic Acid Production and Evolution of pH and Optical Density

The graphs in Figure 4 show the curves of lactic acid production, pH evolution, and optical density during 48 h of cultivating the BY strain of *Lbp. pentosus*. The isolated BY strain quickly reaches the maximum exponential growth after 10–12 h of cultivation (OD_{600} = 2.256 ± 0.08) correlated with the maximum lactic acid production (33.06 ± 0.02%) and with the significant reduction in the pH of the medium (4.25) compared to the initial moment. After 12 h of cultivation at 37 °C, the bacterial population enters the stationary phase of growth (the plateau stage), which is maintained up to 48 h of cultivation. Throughout this interval, the pH values, the multiplication rate, as well as the production of lactic acid through the fermentation process fluctuate insignificantly. The correlation of the optical density values OD_{600} with the number of CFUs resulting from the colony counting method on MRS agarized medium showed that a unit value of OD_{600} means 1.3×10^3 CFU/mL.

3.2.2. Tolerance of *Lbp. pentosus* by Strain in Acidic Environments

The analysis of the survival rate of the *Lbp. pentosus* BY strain in acidic media demonstrated a good tolerance to this biochemical stress (Figure 5). The pH value of the medium exhibited both linear and quadratic effects on the colony count and survival rate of *Lbp. pentosus* BY ($p < 0.001$). The best viability of the BY strain was recorded at pH values between 3.5 and 3.0, but even at pH 2.5 and 2.0, the survival rate was over 50%. This ability is reduced as the pH decreases, and at pH 1.5, it is even lost (no microbial growth is observed at this pH value) (Table 1).

Table 1. Tolerance to acidic pH of the *Lbp. pentosus* BY strain.

	pH of MRS					
	6.2	**3.5**	**3.0**	**2.5**	**2.0**	**1.5**
Colony count (log CFU/mL)	9.00 ± 0.01 ^{a}	8.00 ± 0.03 ^{b}	8.15 ± 0.01 ^{c}	5.00 ± 0.08 ^{d}	5.60 ± 0.05 ^{e}	-

Note: Data are expressed as mean ± standard deviation (SD). In the same row, values with no letter or the same-letter superscripts represent no significant difference ($p > 0.05$), while those with different small-letter superscripts indicate a significant difference ($p < 0.05$).

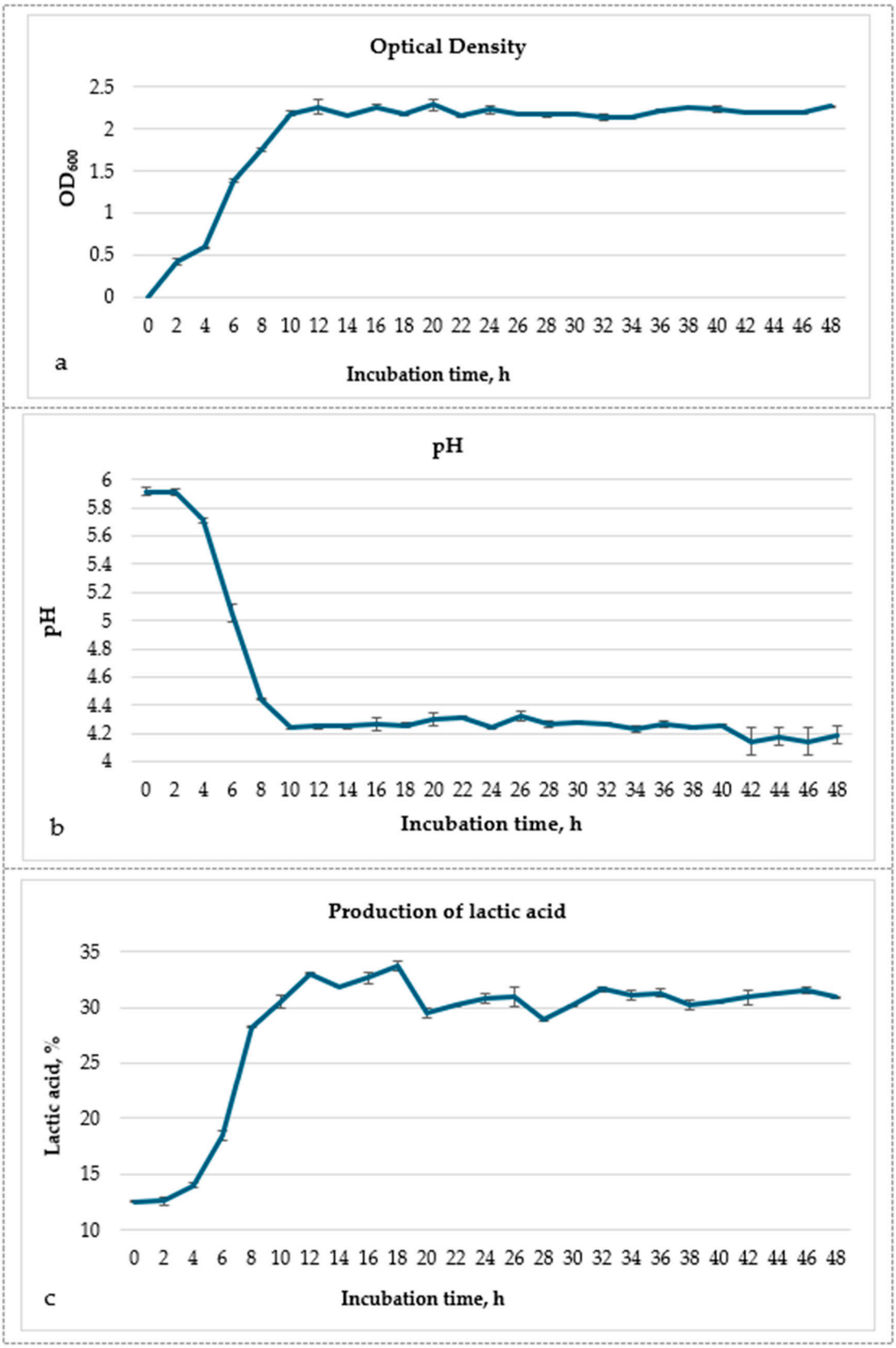


Figure 4. Growth curve, pH, and lactic acid concentration changes in *Lbp. pentosus* BY strain at 48 h of cultivation in a stationary batch system: (**a**) growth curve (1 OD_{600} = 1.3 × 10^3 CFU/mL); (**b**) pH of the medium; (**c**) lactic acid concentration produced by lactic fermentation.

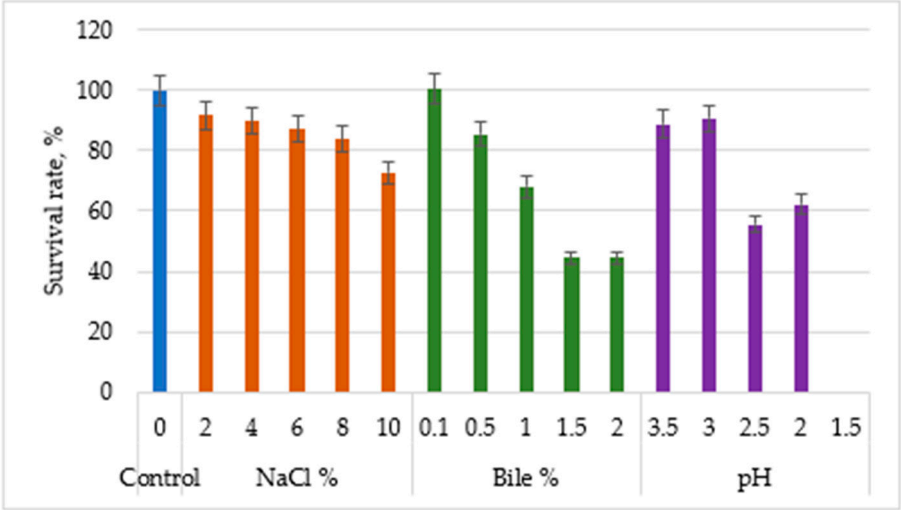


Figure 5. Biochemical stress tolerance of *Lactiplantibacillus pentosus* BY strain.

3.2.3. Salt Stress Tolerance of *Lbp. pentosus* by Strain

The BY strain analyzed exhibits excellent tolerance to salt stress at sodium chloride concentrations between 2% and 10% (Table 2). A slight negative correlation is evident, but even at the maximum salt concentration analyzed (10%), the survival rate is over 70% (Figure 5). When the NaCl concentration increased to 10%, the colony count decreased to 7.54 log CFU/mL and 72.68% ($p < 0.05$). Salt exhibited both linear and quadratic effects on the colony count and survival rate of the BY strain ($p < 0.001$).

Table 2. Salt tolerance of *Lactiplantibacillus pentosus* BY strain.

	Concentrations of NaCl, %					
	0	**2**	**4**	**6**	**8**	**10**
Colony count (log CFU/mL)	9.00 ± 0.01^{a}	8.25 ± 0.01^{b}	8.08 ± 0.01^{c}	7.83 ± 0.03^{d}	7.54 ± 0.02^{e}	6.54 ± 0.02^{f}

Note: Data are expressed as mean ± standard deviation (SD). In the same row, values with no letter or the same-letter superscripts represent no significant difference ($p > 0.05$), while those with different small-letter superscripts indicate a significant difference ($p < 0.05$).

3.2.4. Bile Salt Tolerance of *Lbp. pentosus* by Strain

The presence of low concentrations of bile salts in the medium, up to 1%, allows the maintenance of good growth of the *Lbp. pentosus* BY strain ranging between 100% and 67.98% ± 0.30. At 1.5% and 2.0% bile salts in the culture medium, ~10^4 CFU/mL (44.44 ± 0.5% of the cells) remains viable after 3 h of incubation (Table 3, Figure 5). Bile salts exhibited both linear and quadratic effects on the colony count and survival rate of the *L. pentosus* BY strain ($p < 0.001$). This indicates that *Lbp. pentosus* BY exhibits favorable tolerance to bile salt concentrations similar to those in the digestive tract.

3.2.5. *Lbp. pentosus* by Strain Behavior in a Simulated Gastrointestinal System (SGIS)

Figure 6 shows the survival rate of the *Lbp. pentosus* BY strain in a complex system that simulates the gastric and intestinal environment, in order to analyze its stability in the digestive tracts of consumers. After 2 h in AGJ (the average period of food retention in the stomach), the number of surviving bacteria is 3.7×10^7 CFU/mL (84.05 ± 0.20%). If simulated digestion is continued for another 2 h in AIJ, the bacterial population reaches 1×10^7 CFU/mL (77.78 ± 0.02%). The number of live bacteria after 6 h in the intestinal juice

remains 2 × 10^3 CFU/mL (after 8 h of total simulated digestion). The isolated BY strain has good adaptability in the gastrointestinal tract ecosystem and survives at a percentage of 36.68 ± 0.03% during 8 h of digestion (Figure 6, Table 4).

Table 3. Bile salts tolerance of *Lactiplantibacillus pentosus* BY strain.

	Concentrations of Bile Salt (%)					
	0	**0.1**	**0.5**	**1.0**	**1.5**	**2.0**
Colony count (log CFU/mL)	9.00 ± 0.01 ^{a}	9.04 ± 0.02 ^{a}	7.70 ± 0.01 ^{b}	6.11 ± 0.05 ^{c}	4.00 ± 0.03 ^{d}	4.00 ± 0.01 ^{d}

Note: Data are expressed as mean ± standard deviation (SD). In the same row, values with no letter or the same-letter superscripts represent no significant difference ($p > 0.05$), while those with different small-letter superscripts indicate a significant difference ($p < 0.05$).

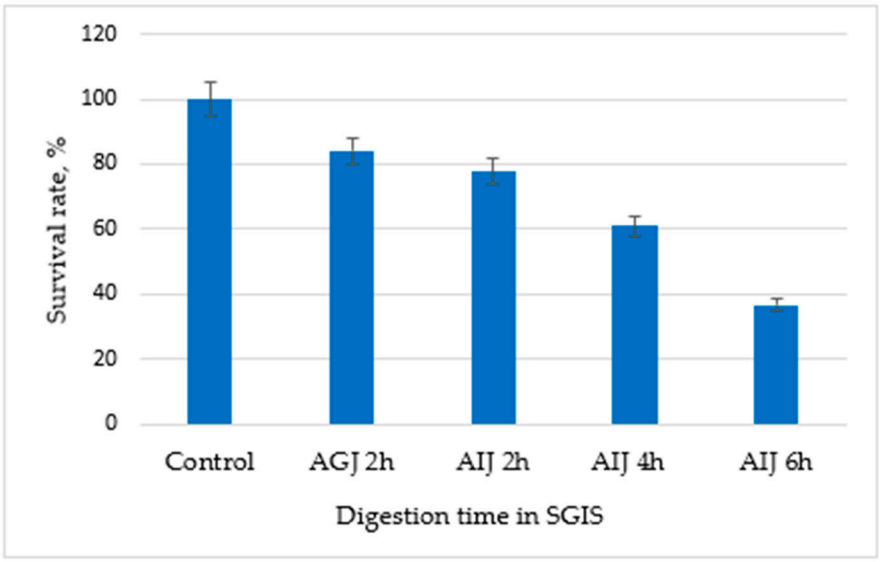


Figure 6. Viability of *Lactiplantibacillus pentosus* BY strain in the simulated gastrointestinal system.

Table 4. The evolution of the *Lbp. pentosus* BY population during eight hours of digestion in SGIS.

	Simulated Digestion Time, h				
	AGJ		**AIJ**		
	0	**2**	**2**	**4**	**6**
Colony count (log CFU/mL)	9.00 ± 0.01 ^{a}	7.56 ± 0.05 ^{b}	7.00 ± 0.01 ^{b}	5.48 ± 0.04 ^{c}	3.30 ± 0.06 ^{d}

Note: Data are expressed as mean ± standard deviation (SD). In the same row, values with no letter or the same-letter superscripts represent no significant difference ($p > 0.05$), while those with different small-letter superscripts indicate a significant difference ($p < 0.05$).

3.2.6. Antimicrobial Properties of *Lbp. pentosus* by Strain

The antimicrobial activity evaluation of CFS (obtained from the cultivation of *Lbp. pentosus* BY strain) against pathogenic or potentially pathogenic bacteria demonstrated a phenotype with inhibitory potential against all the tested strains. The CFS showed good activity against *L. monocytogenes*, followed by *E. coli*, *S. aureus*, and *B. cereus* (Figure 7 and Table 5).

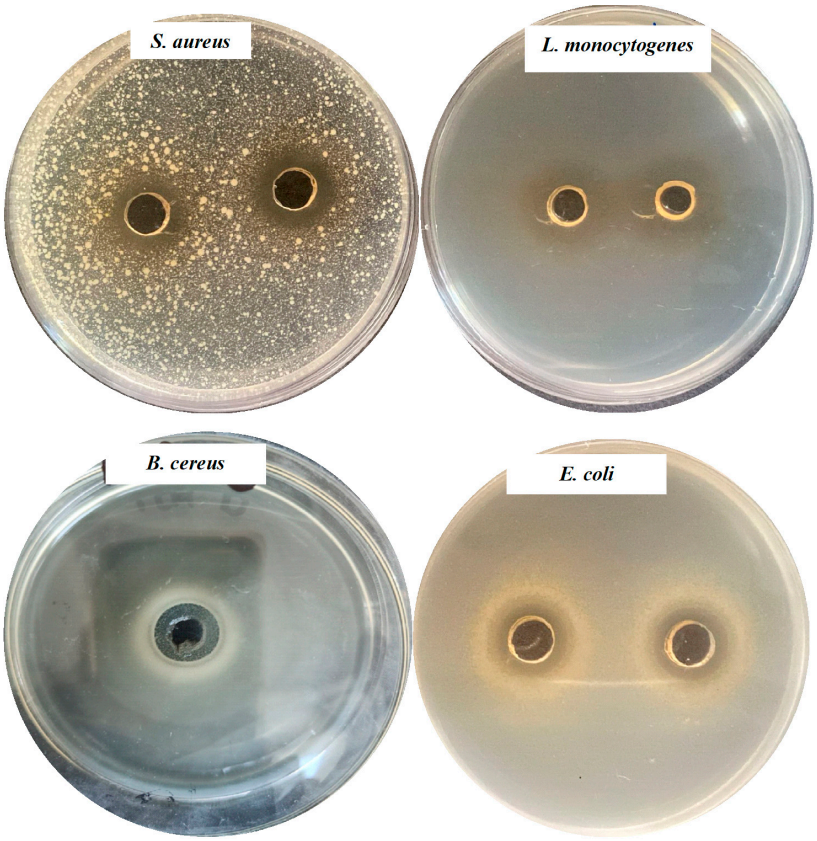


Figure 7. Antibacterial activity against *S. aureus*, *L. monocytogenes*, *B. cereus*, and *E. coli*.

Table 5. The diameter of the inhibition zone against bacteria species with a potential risk.

Bacteria	*B. cereus*	*E. coli*	*L. monocytogenes*	*S. aureus*
DIZ (mm)	17.05 ± 0.05	18.05 ± 0.07	22.9 ± 0.14	17 ± 0.00

The values include the well diameter.

Slight antifungal activity was also observed against *Fusarium* sp. and *Penicillium* sp. The BY strain inhibited the sporulation of *Aspergillus niger*. Furthermore, antimicrobial property against *Candida* sp. was not evident (Figure 8, Table 6).

Table 6. The diameters of the yeast and mold colonies grown on MRSA medium with 5% CFS.

Yeasts and Molds	*A. niger*		*Candida* sp.		*Fusarium* sp.		*Penicillium* sp.	
	C	S	C	S	C	S	C	S
Diameter (mm)	62.08 ± 0.05 ^{a}	67.12 ± 0.15 ^{a}	11.22 ± 0.02 ^{b}	12.18 ± 0.07 ^{b}	60.17 ± 0.02 ^{c}	48.15 ± 0.05 ^{d}	15 ± 0.08 ^{e}	13.15 ± 0.05 ^{e}

Note: C = Control and S = Sample with 5% CFS in MRS. Data are expressed as mean ± standard deviation (SD). In the same row, values with no letter or the same-letter superscripts represent no significant difference ($p > 0.05$), while those with different small-letter superscripts indicate a significant difference ($p < 0.05$).

3.2.7. Antibiotic Susceptibility of the Strain

The antibiotype of the BY strain was evaluated due to its ability to limit the horizontal transmission of antibiotic resistance genes to pathogens or opportunistic bacteria. Based on the zones of inhibition, the BY strain was designated as resistant (R), intermediate (I), and susceptible (S) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [61].

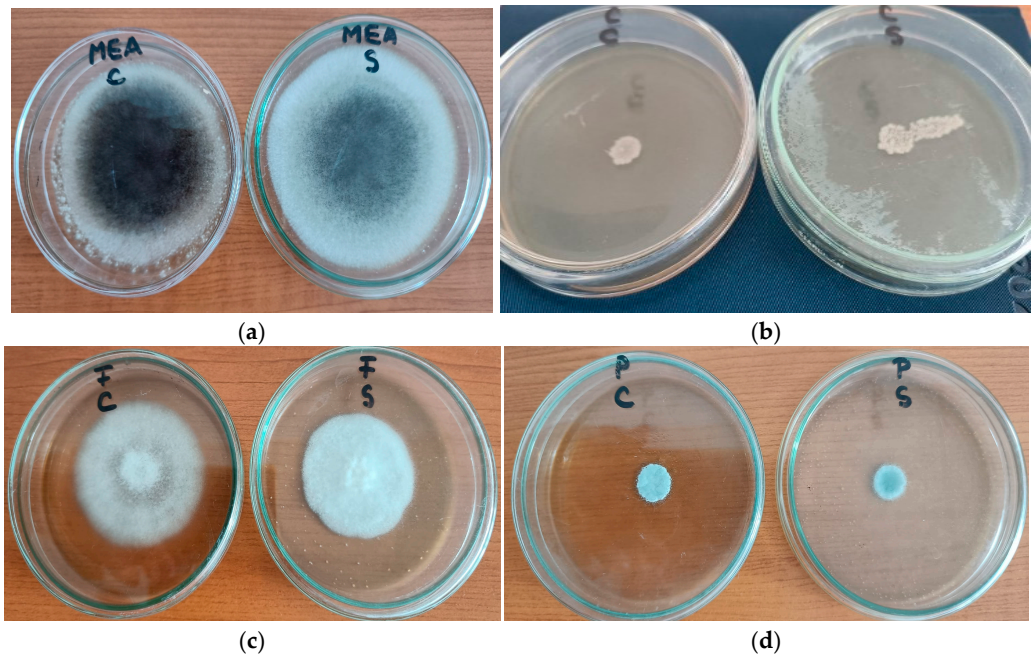


Figure 8. Antifungal activity of CFS of *Lactiplantibacillus pentosus* BY against *Aspergillus niger* (**a**), *Candida* sp. (**b**), *Fusarium* sp. (**c**), and *Penicillium* sp. (**d**). C—Control and S—Sample with 5% CFS in MRS.

The results of the antibiogram illustrated in Figures 9 and 10 demonstrate that the *Lbp. pentosus* BY strain is sensitive to 8 of the 14 standardized antibiotics tested, namely Levofloxacin (LEV 5 µg–25 mm), Ciprofloxacin (CIP 5 µg–24 mm), Fosfomycin (FF 200 µg–20 mm), Cifloxacin (OFX 5 µg–18 mm), Ceftazidin (CAZ 30 µg–18 mm), Vancomycin (VA 5 µg–17 mm), Cefoxitin (FOX 30 µg–14 mm), and Cotrimoxazol (SXT 25 µg–12 mm). The greatest sensitivity is observed with Levofloxacin, which comes after that of Ciprofloxacin and finally that of Fosfomycin. Vancomycin sensitivity should be noted in the current global context of the alarming increase in multidrug-resistant (MDR) and vancomycin-resistant strains.

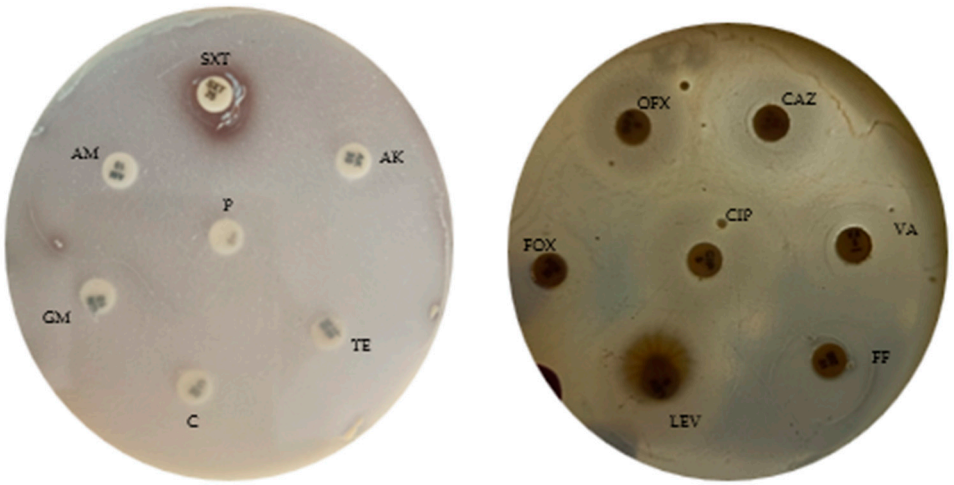


Figure 9. Susceptibility of the BY strain to different antibiotics.

The BY strain is resistant to six antibiotics: Amikacin (AK), Tetracycline (TE), Ampicillin (AM), Gentamicin (GM), Penicillin (P), and Chloramphenicol (C) (Figure 9).

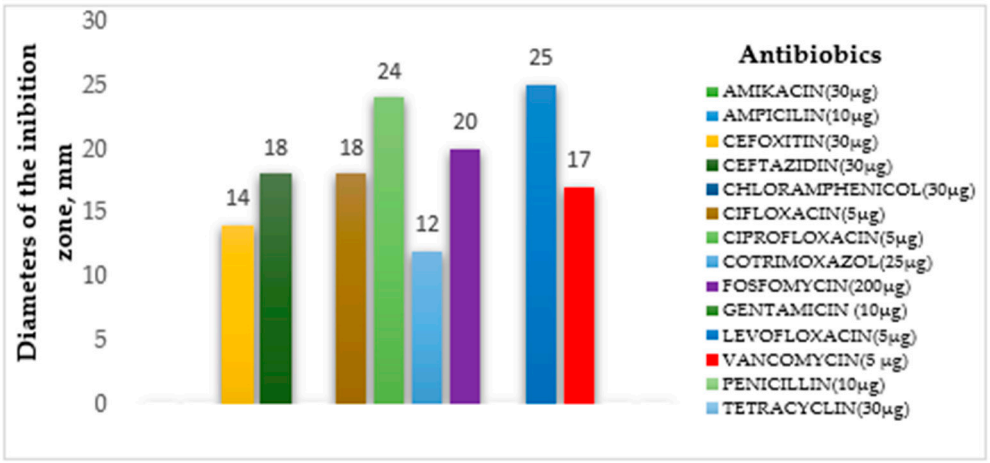


Figure 10. The degree of sensitivity/resistance of the *Lactiplantibacillus pentosus* BY strain to antibiotics (diameters of the inhibition zones (mm)).

3.2.8. Hemolysis Test

Concerning the production of hemolysis, the BY strain presented a partial degradation of the blood, emphasized through the greenish area around the colonies. The BY strain possesses α-hemolytic activity (Figure 11).

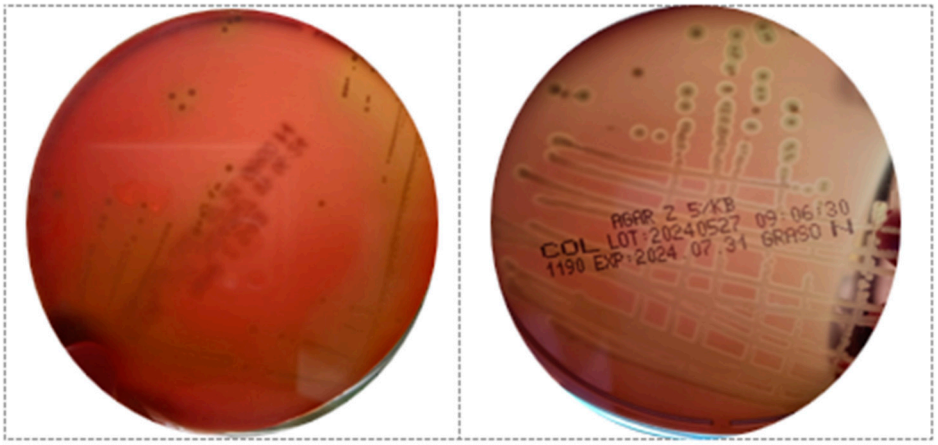


Figure 11. The BY strain colonies exhibiting areas of α-hemolysis on Columbia agar culture medium supplemented with sheep blood (5%).

3.3. Microbiological Analysis of Lanhouin Functional Product

Following the quantitative microbiological analysis, it was observed that the corn hydrolysate has very rich and diverse microbiota. Aerobic mesophilic bacteria (9.9×10^8 CFU/mL = 8.99 log CFU/mL) predominate (Figure 12), among which, by analyzing the colony characters and the microscopic appearance, the presence of the genera *Bacillus*, *Pseudomonas*, and *Actinomyces* was predominantly reported. Among the more common molds and yeasts are the genera *Aspergillus*, *Penicilium*, *Alternaria*, *Fusarium*, *Candida*, *Rhodotorula*, and *Saccharomyces*. We should emphasize the absence of coliform bacteria in the maize hydrolysate and the presence in high numbers of LAB (4×10^5 CFU/mL–5.6 log CFU/mL), from which the BY strain used in the present study and in obtaining the functional variants of lanhouin was isolated.

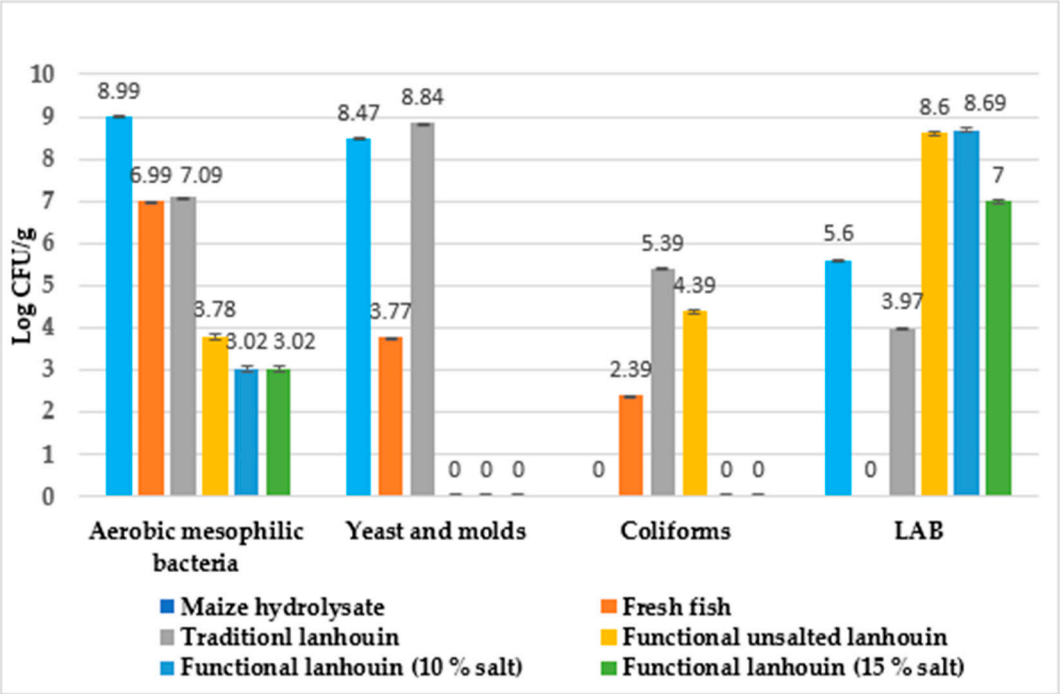


Figure 12. Quantitative evaluation of the microbiota of lanhouin variants and raw materials.

Compared to fresh catfish, traditionally obtained lanhouin has a significantly enriched microbiota in which the number of aerobic mesophilic bacteria rises to 8×10^7 CFU/g = 7.09 log CFU/g and is accompanied by many yeasts and molds (7×10^8 CFU/g = 8.84 log CFU/g). If the most probable number (MPN) of coliforms (2.5×10^2 CFU/g (2.39 log CFU/g)) were reported in fresh catfish, in traditional lanhouin, their number increased 10^3 times (5.39 log CFU/g). It should be emphasized that the traditional technological process for obtaining lanhouin does not use an analyzed, controlled starter culture, and drying is carried out outdoors, in the sun, at approximately 32 °C for 21 days [64]. However, compared to fresh fish that does not contain LAB, the presence of LAB in the traditional product was reported at an amount of 9.5×10^3 CFU/g (Figure 12).

Unlike the traditional version of lanhouin, the innovative functional products designed by controlling the biotechnological process parameters (according to the technological diagram in Figure 2) have significantly improved microbiological innocuity. The statistical comparisons (post hoc tests by the Games–Howell method) between traditional and functional lanhouin products for all microbiological parameters demonstrates the number of aerobic mesophilic bacteria is reduced by 10^4 times, yeasts and molds are no longer found, and coliforms are present only in the salt-free version (Table 7). We should note the presence in large numbers (10^7–10^8 CFU/g) of LAB from the *Lactiplantibacillus pentosus* BY strain, which was inoculated as a starter culture to trigger the fermentation process.

Table 7. Statistical comparison (post hoc tests, Games–Howell method) between traditional and functional lanhouin products.

Parameters	Aerobic Mesophilic Bacteria (10^5 CFU/g)	Yeasts and Molds (10^5 CFU/g)	Coliforms (10^2 CFU/g)	LAB (10^5 CFU/g)	Dry Matter (%)
Traditional lanhouin	803 ± 10^{a}	7016 ± 175^{a}	2500 ± 0^{a}	0.095 ± 0.02^{d}	82.75 ± 0.01
Functional unsalted lanhouin	0.06 ± 0.001^{b}	0^{b}	250 ± 0^{b}	4030 ± 190^{b}	66.44 ± 0.02

Table 7. *Cont.*

Parameters	Aerobic Mesophilic Bacteria (10^5 CFU/g)	Yeasts and Molds (10^5 CFU/g)	Coliforms (10^2 CFU/g)	LAB (10^5 CFU/g)	Dry Matter (%)
Functional lanhouin (10% salt)	0.01 ± 0.001^{c}	0^{b}	0^{c}	5003 ± 175^{a}	79.02 ± 0.01
Functional lanhouin (15% salt)	0.01 ± 0.001^{c}	0^{b}	0^{c}	102 ± 6^{c}	80.20 ± 0.1

Note: Data are expressed as mean ± standard deviation (SD). Means for the same parameter that do not share the same letter (a, b, c, d) are statistically significant at $p < 0.01$ based on the Games–Howell method and 95% confidence.

Regarding the dry matter (see Table 7), functional lanhouin with 15% salt (obtained by drying at 45 °C for two days) reaches a similar value (80.20%) to that of the version obtained by drying for 21 days in the open air (82.75%), calculated by Yelouassi et al. [64].

4. Discussion

The permanent need to ensure safe, sustainable food with high nutritional and biological value is a major current concern in food biotechnologies. Sustainability and functional foods are two key concepts that are increasingly relevant in today's food industry, as consumers become more aware of the environmental impact of food production and the potential health benefits of the foods they consume.

Sustainability in food refers to practices that aim to minimize negative environmental impacts, preserve resources, and promote social and economic well-being throughout the food supply chain. Purchasing locally grown and seasonal foods helps reduce carbon footprints by cutting down on transportation and promoting regional economies. Functional foods have a potentially positive effect on health beyond basic nutrition. These foods contain bioactive compounds that offer benefits like improved immune function, digestive health, heart health, and more. Following these general directions, in the present study, functional foods were designed that use African catfish from the natural ecosystems of the country of Benin, but which, unlike traditional production technologies, possess rigorous control of the production process, to eliminate possible microbiological risks.

Microbiological standards for fish products are set to ensure that fish and fish-based products are safe for consumption, minimizing the risk of foodborne illness caused by harmful microorganisms. These standards typically focus on both pathogenic microorganisms (such as bacteria, viruses, and parasites) and indicator organisms (which suggest potential contamination or poor hygiene practices during processing). These standards are established by the Codex Alimentarius Commission, the Food and Drug Administration (FDA) and the EU Food Safety Authority (EFSA). The Regulation (EC) No 2073/2005 provides microbiological criteria for fishery products [66]. These include limits for *Salmonella*, *Listeria*, and other pathogens (Table 8).

Table 8. Summary of key pathogens and limits.

Pathogen/Indicator	Fish Products Standard	Notes
Salmonella spp.	Absence in 25 g	For all types of fish products
L. monocytogenes	Absence in 25 g (for RTE products)	Especially for ready-to-eat fish
E. coli	<10 CFU/g	Indicator of fecal contamination
Vibrio spp.	Absence in 25 g	Critical for raw fish products
Total Plate Count	$<10^5$ CFU/g	Fresh fish products
Staphylococcus aureus	$<10^4$ CFU/g	Indicator of poor handling

Adhering to these microbiological standards and following proper handling and storage practices is essential to minimize the risk of foodborne illness and ensure the safety of fish products for consumers [67].

From the quantitative microbiological analysis carried out on fresh catfish and traditionally obtained lanhouin brought from Benin, it can be observed that the microbiota far exceed the food safety limits for these products. The total number of aerobic mesophilic bacteria (Aerobic Plate Count—APC) is more than 10^3-times higher. The Third Amendment Regulation, from 2017, related to microbiological standards for fish and fish products (Final Gazette Notification of Food Safety and Standards—Food Product Standards and Food Additives) [68] allows 20–40 MPN/g (most probable number) coliform bacteria and up to 5×10^2 CFU/g for yeasts and molds in fermented or dried fish. Traditional fermented and dried lanhouin records 10^6-times more in the yeasts and molds category. The most worrying is the presence of coliforms well above the allowed microbiological limits in fresh fish but especially in fish obtained through the traditional process, where 2.5×10^5 MPN/g (5.39 log CFU/g) is recorded (Table 7). As per the many national standards, fresh fish should ideally have <100 CFU/g of coliforms and <10 CFU/g of *E. coli*. These results confirm that good hygiene practices were not followed, which allowed fecal contamination of the fish during processing. Indeed, these data are consistent with the results of Dossou-Yovo et al. [6,32,33] and Yelouassi [69] who analyzed artisanal lanhouin-based fishmeal and samples of artisanal, fermented, salted, and dried fish from Benin. Similar results were obtained through a microbiological analysis of other traditional African fish-based products, such as adjeuvan from the Ivory Coast [4], guedj from Senegal [7], and momoni from Ghana [8].

To ensure and maintain fermented and dried catfish products within the limits of the microbiological standards, controlled lactic fermentation using a LAB starter culture isolated from corn hydrolysate, characterized and identified *Lactiplantibacillus pentosus* BY, was conceived as a biotechnological solution. The process of the lactic fermentation of fish can vary depending on the culture, method, and region. It is common in some traditional cuisines, particularly in East and Southeast Asia, as well as in parts of Africa and Europe [18]. Surströmming is a well-known fermented fish dish from Sweden, where herring is fermented for several months [70]. Pla-Ra is a traditional fermented fish product in Thai cuisine. Fish, usually small fish such as anchovies or tilapia, are fermented with rice or rice bran and salt [71]. Ancient Roman garum involved fermenting fish similarly, using salt and sometimes herbs. It was a common condiment and was made from fish intestines and other parts that would ferment in the sun [72]. In addition to extending the shelf life, some of the key characteristics of lactic fermented fish are an improved flavor profile, smoother texture, and more pronounced taste from spicy, salty, or slightly sour to intensely pungent (depending on the duration of fermentation). But, the live probiotics in this type of product provide the main benefits for the consumer's gut health, as it has recently been demonstrated to modulate the gut microbiota of healthy individuals. Studies have also shown its potential probiotic characteristics, proving to be an anti-inflammatory agent, lowering cholesterol levels, inhibiting foodborne pathogens, and adhering to Caco-2 cells [11,73].

According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), the minimum recommended number of probiotic products is 10^6 CFU/mL or CFU/g. Therefore, the viability of probiotics and the dose level of probiotic products are the most important factors when considering their function in the colon [74]. We can emphasize that in all variants of functional lanhouin obtained as a result of this study, the lactic microbiota fall within this range at values in the range of 10^7–10^8 CFU/g (Table 7).

As we demonstrated in the previous chapter of results, the BY strain of *Lactiplantibacillus pentosus* has a rapid fermentative capacity, reaching after 10–12 h the maximum number of CFU/mL, inversely correlated with pH and lactic acid production (Figure 4). The same observations were made by Tian et al. [49] with the lactic acid bacterium *L. pentosus* R26. Lactic acid is a primary metabolite that is produced in the exponential growth phase of the LAB population and is one of the important factors that ensure the inhibition of potentially risky microbiota in fermented foods. After 12 h, the microbial culture already enters the stationary phase of growth in which it is known that secondary metabolites are synthesized, such as bacteriocins that complete the antimicrobial potential of lactic acid bacteria. It is known that fish have been identified as reservoirs of bacterial pathogens linked to human diseases, including *Mycobacterium* sp., *Vibrio* sp., *Escherichia coli*, *Aeromonas* sp., *Salmonella* sp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium botulinum*, *Clostridium perfringens*, *Campylobacter jejuni*, and *Legionella pneumophila* [67]. Lashani et al. [75] mentioned that LAB can secrete compounds, including bacteriocins, biosurfactants, H_2O_2, and organic acids, contributing to their antimicrobial activity. The main mechanisms of action against pathogens involve the disruption of cell membranes, interference with septum formation, blocking of replication and transcription processes, suppression of protein synthesis, and inhibition of the synthesis of cell wall components (peptidoglycan units) [49]. The production of organic acids (such as lactic acids) and/or bacteriocins could limit or inhibit the growth of certain highly pathogenic bacteria.

These biotechnological characteristics of the BY *Lbp. pentosus* strain correlate very well with the proven antimicrobial capacity of CFS against pathogenic microorganisms such as *Lysteria moncytogenes*, *Escherichia coli*, and *Staphylococcus aureus*, and against sporulated bacteria *Bacillus cereus* (Figure 7, and Table 5), and our results are consistent with other studies [49,63,76–80]. This strain has several genes that are involved in adhesion, biofilm formation, bacteriocin production, carbohydrate degradation, and the metabolism of phenolic compounds, among these important technological characteristics [81]. As shown by the post hoc test (Games–Howell method) used to identify the statistical differences between functional lanhouin variants compared to the traditional variant, the antimicrobial properties of the *Lbp. pentosus* BY strain explain the significant reduction in the microbiota of functional lanhouin obtained by controlled lactic fermentation (Figure 2). Thus, the decrease in the number of APC ~ 10^3 CFU/g, the absence of yeasts and molds, as well as coliform bacteria (except for the unsalted product, where it still exceeds the permitted limits) are noted (Table 7). In this way, the salted, fermented, lanhouin variants are safe for consumption and can have a prolonged shelf life, if the storage conditions are respected. While lactic fermentation can extend the shelf life of fish and make it easier to store, it is important to ensure a proper environment and handling to avoid contamination by harmful bacteria. Careful attention to salt concentration, temperature, and hygiene is necessary to prevent spoilage and ensure the safety of the final product.

Although *Lactiplantibacillus pentosus* BY did not demonstrate in vitro a special antifungal capacity against the tested yeast and mold species, in the controlled biotechnological process of catfish fermentation, it managed to eliminate eukaryotic microbiota (yeasts and molds are absent from the functional variants of lanhouin) (Table 7). The literature often cites that the antifungal activity of LAB depends on the composition of the culture medium, on the microbial consortia in the fermentation medium, and on the species. Liu et al., in their study from 2023, showed that *Lactiplantibacillus pentosus* 86 excellently inhibits *Alternaria* species [82]. Lipińska et al., in 2017, show that the supplementation of the bacterial culture medium with polyols (erythritol, lactitol, maltitol, mannitol, sorbitol, and xylitol) or their galactosyl derivatives (gal-erythritol, gal-sorbitol, and gal-xylitol) enhanced the antifungal properties of *Lactoplantibacillus pentosus* ŁOCK 0979 [83]. So, although the

results of our study show weak antifungal activity, we probably did not identify the most suitable evaluation method, and it opens avenues for a more detailed analysis.

In demonstrating the probiotic character of a LAB strain, an extremely important role is played by the analysis of tolerance to various environmental factors that define the gastrointestinal ecosystem (acid pH, bile salts, digestive enzymes, etc.) or different fermentative media used in food processing (high salinity).

The *Lactiplantibacillus pentosus* BY strain has a survival rate of over 70% at all tested sodium chloride concentrations up to 10%, at pH levels of 3.5 and 3.0, but also at bile salt concentrations of 0.1 and 0.5%. The good viability (67.8%) of the BY strain was also recorded at a 1% bile salt concentration in the culture medium. Tian et al. (2024) observed a survival rate of 86.66% after 3 h at a bile salt concentration of 0.5% (Figure 5) [49]. Typically, *Lactiplantibacillus pentosus* can tolerate low-to-moderate concentrations of bile salts (usually up to a range of 0.3–0.5% *w*/*v*) but may show reduced growth or viability at higher concentrations. Higher concentrations of bile salts (1.5% and 2.0%) are more difficult to tolerate by the BY strain (remaining viable at a rate of 44.5%). Under physiological conditions, bile acids (which represent 67% of bile components) usually conjugate with glycine or taurine, giving rise to the bile salts cholylglycine or cholyltaurine. The normal human ratio of glycine and taurine conjugates of bile acids is 3:1 but can be modified under the influence of dietary and hormonal factors, in some liver diseases [84]. Different parts of the animal gastrointestinal tract contain certain amounts of bile salts (0.3–3.0 g/L) [85]. Bile salts are substances with detergent-like activity and can disrupt the structure of microbial cell membranes and cause DNA damage, indicating strong antimicrobial activity [86]. So, to exert their effects, the probiotics must exhibit tolerance to bile salts. *Lactobacillus* genus genome harbor genes for high bile salt tolerance, and their expression controls the synthesis of bile salt hydrolases and bile salt transporters, which block the negative effects of bile salts [87,88]. We assume that the *Lactiplantibacillus pentosus* BY strain also has mechanisms to maintain intracellular homeostasis in the presence of bile salts. As the pH of the culture medium decreased, the viable cell counts and survival rates of *Lbp. pentosus* BY fell significantly ($p < 0.001$). If in an environment with pH 3.0 or 3.5, the BY strain can adapt its metabolism; in a highly acidic environment (pH 1.5) the BY strain cannot adapt and does not survive. At pH 2.0 and 2.5, over 50% of the initial number of cells remained viable after 3 h (Figure 5). The *Lactiplantibacillus pentosus* R26 strain analyzed by Tian et al. demonstrated an excellent survival rate of 86.29%, even at pH 1.5 [49]. Das also characterized strains of *Lbp. pentosus* tolerant to pH 1.5 [88]. The genus *Lactobacillus* possesses a dynamic array of mechanisms to withstand an acidic pH. These include the generation of alkali via the arginine dihydrolase system to neutralize acidic compounds, the creation of biofilms to protect cells from adverse environments, and the efficient regulation of intracellular and extracellular H^+ concentrations via proton pumps [81,89].

In addition, the isolated BY strain of *Lbp. pentosus* showed remarkable salt tolerance, even when exposed to salinity ranging from 2% NaCl (8.25 log CFU/mL) to 10% NaCl (6.54 log CFU/mL) (Figure 5). This result is in agreement with that of Kushuboo et al. [50]. Das et al. also characterized strains of *L. pentosus* tolerant even to 14% salt [88]. For example, *Leuconostoc*, *Pediococcus*, and *Lactiplantibacillus* are three genera that can grow and thrive when fermented at high salinity [88]. This adaptive capacity of LAB strains is of interest for the promotion of "green technologies" of preservation in the food industry as well as in various other biotechnologies (cosmetics, pharmaceuticals, etc.). Previous studies have found similar results [76–78].

The behavior of the BY strain in SGIS (Figure 6) proved its very good adaptation capacity both to pH conditions and to the presence of specific enzymes. The electrolyte system of PBS in AGJ exerted a protective role on the bacterial plasma membrane, so

that, even at pH 2 and in the presence of gastric pepsin, after two hours (how long the gastric stage of digestion lasts on average), 77.77% of the LAB cells remain viable (more compared to the result of tolerance to the pH 2 environment). Continuing digestion in AIJ, in the presence of 2% pancreatin and at pH 8, presented very good adaptive mechanisms in the bacterial cell that determined survival rates of 73% and 60.88% after 2 and 4 h, respectively, after simulated gastrointestinal digestion of 8 h 2×10^3 CFU/mL (survival rate of 36.6%). Probably, the microencapsulation of the BY strain in biopolymer matrices would result in improved viability in the small intestine, where probiotics create biofilms by adhering to the enterocyte mucosa and exert their protective role. Furthermore, Ye et al. [52] reported that *Lbp. pentosus* isolated from the feces of healthy infants after exposure to simulated gastrointestinal juices under the same conditions yielded the same results. Raethong et al. [27] analyzed *L. pentosus* strain 9D3, which also showed good survival in the hostile stomach condition, pH 2.0 in the presence of the digestive enzyme pepsin for 1 h, with a reduction of approximately 1 log. In the intestinal phase, where acid-stressed cells encountered more digestive enzymes and bile salts, as well as a slightly acidic condition at pH 6.0, the *Lbp. pentosus* 9D3 strain showed a moderate survival capacity, with a reduction of approximately 2.4 log.

Antibiotic susceptibility is considered the most important safety criterion in the selection of strains with probiotic potential. It has been noted that the BY strain has a sensitivity to eight antibiotics with high sensitivity to Levofloxacin, Ciprofloxacin, and Fosfomycin. The highest sensitivity was recorded for Levofloxacin (Figures 9 and 10), an antibiotic from the third generation of the fluoroquinolone group, FDA-approved for the treatment of nosocomial diseases. The proven sensitivity of the *Lactiplantibacillus pentosus* BY strain to a wide spectrum of antibiotics is a characteristic that excludes its belonging to the MDR strain group. The phenomenon of multiple antibiotic resistance is of great relevance due to the risk of horizontal transmission (by conjugation, transduction) of the responsible genes in the microbial populations that make up the intestinal microbiota. From this point of view, it is useful that probiotics do not possess such genes that could transmit this property to enterobacteria involved in the dissemination of nosocomial infections. Numerous studies have shown that lactobacilli have a high natural resistance to Vancomycin due to the presence of highly conserved non-transferable genes in the chromosome [88]. The sensitivity of the BY strain to Vancomycin should be particularly noted, as the current concern in the medical world regarding vancomycin-resistant enterobacteria (VRE) strains is well known. These results are different from those obtained by Guo et al. who found 19 Vancomycin-resistant strains out of 33 LAB isolated from fermented milk [90]. These results are contrary to those of Das et al. [88], Bazireh et al. [89] and Guo et al. [90]. So, it seems that the sensitivity of the BY strain is due to the lack in the genome of the resistance genes *van*X and *gyr*A responsible for resistance to Vancomycin and Ciprofloxacin, respectively. To our knowledge, this is the first time that a LAB strain with simultaneous high susceptibility to both levofloxacin and vancomycin has been reported, although strains with separate susceptibility to one of these two antibiotics are often encountered.

At the same time, probiotics resistant to certain antibiotics can be administered concomitantly with those antibiotics in various infections with germs sensitive to them, because the LAB will remain alive and will exercise their protective role in the gastrointestinal tract. The BY strain is resistant to Amikacin, Tetracycline, Ampicillin, Gentamicin, Penicillin, and Chloramphenicol, so it can be administered even during treatments with these antibiotics (partially similar results to those obtained by Raethong et al. [27], on the *Lbp. pentosus* 9D3 strain). Antibiotic resistance is due to the presence in the genome of resistance markers, usually located on plasmids. These genes are responsible for the absence or biochemical/conformational modification of membrane target sites for antibiotics, or

which control mechanisms that reduce the cell membrane permeability to antibiotics or even their inactivation.

To ensure this safety, it is recommended to select strains that do not have hemolytic activity, indicating their non-virulent nature. The results in Figure 11 show a partial degradation of the blood, manifested by greenish areas around the colonies—α hemolysis and the absence of ß hemolysis. This result reveals that our selected strain does not present a major risk to the host during its application. This makes the strains suitable candidates for use as probiotics. Many researchers have reported similar results, indicating that probiotics do not possess hemolytic activity, which reduces their safety of use as probiotics [91–94]. This result aligns with that of Xue et al. [95], who mentioned that *Lbp. pentosus* is α hemolytic.

In summary, the results of this study highlight the clear advantages of using the BY *Lbp. pentosus* starter culture in the production of lanhouin, including microbiological safety and the functional benefits provided by the probiotic properties of the strain—such as proven antimicrobial activity, tolerance to gastrointestinal tract conditions, sustained viability in finished products, and sensitivity to Vancomycin and other antibiotics. However, potential drawbacks may include the higher cost of storing and maintaining the starter culture, as well as the need for additional training for personnel in standard microbiological techniques to prevent contamination during the production process (food safety management).

5. Conclusions

The *Lactiplantibacillus pentosus* BY strain exhibits unique probiotic properties, making it valuable across various industries, including agriculture, pharmaceuticals, cosmetics, and particularly the food sector. Its use as a starter culture for fermented catfish (Lanhouin) has demonstrated excellent microbiological safety results, with no coliforms, molds or yeasts, and a low level of APC. Additionally, it ensures the superior viability of *Lbp. pentosus* in the final products. Thus, the isolated *Lbp. pentosus* BY strain positively impacts the processing and production technologies of salted and dried fermented fish.

The results obtained by our team are important both among the scientific community in the field of food biotechnology, but also among the local economic community in Benin.

The convergence of sustainability and functional foods is set to define the future of the food industry. As consumers place greater importance on both personal health and environmental impact, the demand for foods that benefit both personal well-being and the planet is expected to grow. Sustainable production practices coupled with the increasing interest in foods offering health benefits will likely fuel innovations in functional and sustainable food systems.

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