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RESEARCH PAPER

Selection of *Lactobacillus plantarum* TN627 as a New Probiotic Candidate Based on *in vitro* Functional Properties

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Abstract Nine lactobacilli previously selected for high antagonism against food borne bacterial pathogens were identified *via* 16S rRNA gene sequencing and screened for probiotic potential for use in poultry production. The lactobacilli were subjected to a subtractive *in vitro* analysis system using a certified probiotic as reference. This allowed for selection of a milk-derived *Lactobacillus plantarum* strain, termed TN627. This organic acid-producing bacterium was free of harmful enzymatic activity and sensitive to several antibiotics. It also showed good growth at pH 4 and in the presence of bile. *L. plantarum* TN627 also exhibited high efficacy of adhesion to chicken enterocytes, which correlated with detecting genes encoding the mucus-binding, adhesion-promoting proteins (Mub and MapA) and the adhesion-like factor EF-Tu, commonly involved in adherence of lactobacilli to mucosal surfaces. Taken together, our findings suggest that TN627 is a promising probiotic candidate with high potential for application as a supplement in the animal feed industry.

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1. Introduction

The steady resistance to "antibiotics of last resort" has become one of the major human health and safety concerns throughout the world and constitutes one of the underlying reasons behind the current attempts to restrict the use of antibiotics as growth promoters in animal feed [1]. With this concern in mind, researchers in the animal feed industry have realized that the key lies in looking for alternative non-therapeutic products that can help maintain animal gut health and prevent or reduce the prevalence of pathogens in the food chain. Currently available reports indicate that probiotics, prebiotics, synbiotics, feed enzymes, organic acids, essential oils, and immunostimulants represent some of the key substitutes worthy of consideration [2].

Probiotics constitute an exceptional class of microorganisms with infinite and promising potential. The Food and Agriculture Organization and the World Health Organization define probiotics as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host [3]. Most probiotics include lactic acid bacteria (LAB) predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, which are health-promoting bacteria that form a major part of balanced intestinal microbiota [4]. The use of probiotics in farm animal feed helps improve gut microbial balance and, hence, the natural defense of the animals against pathogenic bacteria [5-8]. Feeding beneficial bacteria is also a natural solution for improving feed conversion performance

(productive weight gain) and increasing economic profitability [9].

Moreover, the literature often highlights a number of criteria that a probiotic microorganism must meet to be considered beneficial for health. The list of criteria includes the following: (i) recognition as safe; (ii) inhibition of potential intestinal pathogenic bacteria, a mechanism involved in the restoration of gut microbiota balance; (iii) survival under gastrointestinal conditions (acid pH and bile salt), particularly when administered orally; (iv) ability to adhere to host mucosal surfaces; and (v) stability during processing and storage [10]. Probiotic microorganisms should also be free of undesirable traits, such as transmissible antibiotic resistance (to avoid spreading resistance determinants in intestinal pathogenic or opportunistic bacteria), virulence factors, toxin production, and harmful biochemical activities such as α-chymotrypsin, β-glucosidase, β-glucuronidase, and N-acetyl-β-glucosaminidase activities, which are often associated with intestinal diseases and involved in generating carcinogens and tumor promoters [11,12].

Probiotics also have good adhesion properties. In fact, adhesion to host mucosal surfaces contributes to the efficacy of a probiotic, as it promotes gut residence time, which allows temporal colonization of mucosal surfaces, competitive exclusion of pathogenic bacteria, and close contact with host cells to protect epithelial cells or for immunomodulation [10,13,14]. The mucus-binding protein (Mub) and mucus adhesion-promoting protein (MapA) are among the most documented factors involved in the adhesion of lactobacilli to the gastrointestinal tract [13]. Surface layer proteins, such as CbsA, Slp, and SlpA, as well as peculiar cytoplasmic proteins, such as the elongation factor Tu (EF-Tu) and the heat shock protein GroEL, have also been reported to play a role in adhesion [13].

We previously screened 54 lactobacilli, newly isolated from various niches, for antagonistic activity against several indicator strains, including intestinal bacterial pathogens. This allowed for the selection of nine isolates, designated TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN644, and TN653, which exhibit large spectra of inhibitory effects. Among them, TN635 producing a bactericidal bacteriocin called "BacTN635" has been identified as a *Lactobacillus plantarum* strain [15].

The present study was undertaken to taxonomically identify these nine lactobacilii *via* 16S rRNA gene sequencing and to screen their probiotic potential for application in poultry production. Hence, the nine isolates were subjected to an *in vitro* analysis subtractive system to assess their probiotic properties using *Pediococcus acidilactici* MA 18/5M as a reference, a probiotic that is certified by the scientific committee for animal nutrition within the EU as a safe feed additive for broilers [16].The TN627 isolate exhibited the best *in vitro* functional properties and was subjected to further investigations including precise taxonomic identification, determination of the biological nature of the antibacterial compounds it produces, and assessment of the efficacy of adhesion to chicken enterocytes.

2. Materials and Methods

2.1. Bacterial strains

The nine lactobacilli TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN644, and TN653 used in the probiotic property screening were previously selected by Smaoui *et al.* [15] for their strong inhibiting effect against several bacterial pathogens. The commercial probiotic *P. acidilactici* MA 18/5M was purchased from Lallemand (Barenbrug, France) by the Nutrisud/Medimix company. MRS broth (Difco, Detroit, MI, USA) was used for LAB propagation.

2.2. DNA Isolation and manipulation

Chromosomal DNA was prepared from lactobacilli, as described previously by Serror *et al*. [17]. The general molecular biology techniques were performed as described by Sambrook *et al*. [18]. Polymerase chain reaction (PCR) amplifications were conducted using *Pfu* DNA polymerase from BIOTOOLS (Madrid, Spain). PCR products were purified from agarose gels using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced. The nucleotide sequences were determined with an automated 3100 Genetic Analyzer (Applied Biosystems, Courtaboeuf, France). A homology search was performed using the BlastN basic local alignment search tool.

2.3. PCR amplification and sequencing of LAB 16S rRNA genes

The 16S rRNA genes of the LAB isolates were amplified by PCR using the forward primer 27F and the reverse primer 1542R, as previously reported by Shin *et al*. [19]. The cycling conditions were 30 cycles of 30 sec at 94°C, 45 sec at 55°C, and 95 sec at 72°C. The primers used for sequencing of the PCR-amplified products were 27F, 1542R, 515R, 536F, 926F, and P4 (A) [19,20] in addition to P4, which was complementary to the latter one.

2.4. Antagonistic activity of the commercial probiotic

Antibacterial activity assays for the commercial probiotic *P. acidilactici* MA 18/5M were performed as described by Smaoui *et al*. [15]. The potential intestinal pathogens, namely *Staphylococcus aureus* ATCC 6538, *Listeria ivanovii*

BUG 496, *Escherichia coli* ATCC 8739, and *Salmonella enterica* ATCC 43972 were used as bacterial indicator strains.

2.5. Enzyme activities

The enzymatic activities of the TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN644, and TN653 isolates, as well as those of *P. acidilactici* MA 18/5M, were assayed using the API-ZYM System (bioMérieux, Montalieu-Vercieu, France), as recommended by the manufacturer. Inocula (65 μL of McFarland standard 1 suspension) were deposited in each well. Enzyme activity readings were taken after 4 h of anaerobic incubation at 37°C and expressed as approximate nmoles of substrate hydrolyzed during 4 h of incubation (from 0 to ≥ 40 nmol), according to the manufacturer's directions.

2.6. Growth at different pHs and bile concentrations

Lactobacilli with no harmful enzymatic activities were tested for tolerance to acidic pH and bovine bile and compared to the certified probiotic, as described by Todorov *et al*. [21]. The strains were grown in MRS broth, adjusted to pH 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, and 13.0, respectively, before autoclaving. Resistance to bile was assayed by growing the cells in MRS broth adjusted to 0.3, 0.6, 0.8, 1.0, 2.0, or 5.0% (w/v) bovine bile (Oxygall; Sigma-Aldrich, Mannerheim, Germany). All tests were conducted without shaking in 10 mL of MRS broth (with different bovine bile concentrations or pH values) that had been inoculated at a final concentration of 0.2 OD_{600} with 16 h-old cultures grown on MRS broth. The OD readings (at 600 nm) were recorded every hour throughout a 10 h cultivation period. Cultures grown in MRS broth without bile served as the control.

2.7. Detection of the Mub, MapA, and EF-Tu genes by PCR

The presence of genes corresponding to the lactobacilli adhesion proteins Mub, MapA, and EF-Tu was checked by PCR in the strains retained after the undesirable enzyme investigation. The oligonucleotides MapA D (5' GAGCGACAGGTTGACGATCG 3'), MapA R (5' CCGTCTTGACGGAGTTCTTG 3'), Mub D (5' GGTAGTTACTCAGTGACGATC 3'), Mub R (5' CGACGTTCGTGTCAGTCGTC 3'), EF-Tu D (5' CCGGCTTCACCTAAGTCAAG 3'), and EF-Tu R (5' CTTTGCTTCTATCGATGCTGC 3') used for PCR amplification of the genes encoding MapA, Mub, and EF-Tu, respectively, and were designed from the putative *MapA* (locus lp_3214; GenBank accession no. AL935261), *Mub* (locus lp_1643; GenBank accession no. AL935256), and *EF-Tu* (locus lp_2119; GenBank accession no. AL935258) genes of the *L. plantarum* WCFS1 strain whose genome sequence (GenBank accession no. AL935262) has been published [22]. The amplification conditions were 30 cycles of 30 sec at 94°C, 45 sec at 45°C, and 45 sec at 72°C. The PCR products of the expected sizes were purified from agarose gels and sequenced to confirm that they corresponded to the genes being amplified.

2.8. Sensitivity to antibiotics

An overnight culture of the isolates, free of undesirable enzymatic activities, was embedded in MRS soft agar (1%, m/v) to a final concentration of 10^6 CFU/mL. Antibiotic disks were placed on the agar surface and incubated for 48 h at 30°C. Growth inhibition was recorded by measuring zone diameters. The antibiotics analyzed included inhibitors of cell wall synthesis (β-lactams: penicillin G and oxacillin; cephalosporins: cefoxitin; and glycopeptides: vancomycin), protein synthesis (chloramphenicol, tetracycline, erythromycin, pristinamycin, and lincomycin), aminosides, such as streptomycin, gentamicin, kanamycin, and tobramycin, and the quinolone family, such as oflaxacin, as well as other antibiotics, such as nitrofuranes, cotrimoxazole, and rifampicin.

2.9. Taxonomic identification of the selected strain

To definitively identify the selected strain at the species level, additional PCR assays were performed wherein the *recA* gene-based primers planF/pREV and pentF/pREV, previously reported by Torriani *et al*. [23], were used to allow for differentiation of *L. plantarum* and *L. pentosus,* respectively. PCR products were visualized on a 1.4% agarose gel. The purified amplicons were sequenced to confirm their identities.

2.10. Effect of different treatments on the antibacterial activity of the selected strain

To determine the biological nature of the antibacterial compounds produced by the selected strain, the cell-free supernatant from culture of this isolate grown on MRS at 30°C for 18 h were subjected to different treatments, including a pH adjustment to 6.5 and an investigation of heat sensitivity (100°C for 60 min), catalase (3 h at 37°C at 1 mg/mL) and proteolytic enzymes (2 h at 37°C in the presence of 1 mg/mL of trypsin or proteinase K). Immediately after each treatment, residual antibacterial activity was determined against the same indicator strains used for the certified probiotic. The untreated cell-free supernatant served as a control.

2.11. Adhesion properties of the selected strain

An *in vitro* test for adhesion to epithelial cells from different parts of the chicken intestine was performed as described by Jakava-Viljanen and Palva [24], with minor modifications. Segments of duodenum, jejunum, and ileum

were opened, maintained in phosphate-buffered saline (PBS) at 4°C for 30 min to loosen the surface mucus, and washed three times with PBS. Enterocytes were scraped off gently with a spoon, and the scrapings were suspended in PBS. All cell suspensions were pre-examined microscopically to ensure that adhered commensal bacteria had been removed. Cells were diluted to approximately 5×10^6 cells/mL. The enterocyte suspensions were used for adhesion studies within 1 h. The overnight broth culture of the selected strain was centrifuged at 3,000 × *g*, resuspended in PBS, and diluted to 1×10^9 cells/mL in PBS. The bacterial suspensions (0.5 mL) and enterocytes (0.5 mL) were mixed and incubated with slow shaking (20 rpm) at 37°C for 60 min. After incubation, the mixtures were centrifuged and washed three times with sterile PBS to remove unattached bacteria. The pellets were resuspended in half the initial volume, and bacterial binding to the cells was examined by light microscopy (Giemsa staining) using an Olympus BX50 phase contrast microscope equipped with an Olympus DP 70 digital camera (Olympus, Tokyo, Japan). Bacterial adhesion was evaluated in 10 random microscopic fields of each intestinal segment, and each segment contained about 100 epithelial cells. The percentage (%) of adhesion was defined as the number of cells with adherent bacteria divided by the total number of cells observed × 100.

3. Results and Discussion

3.1. Genotypic identification of the LAB isolates to be screened for probiotic potential and the antagonistic effect of the certified probiotic

The lactobacilli designated TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN644, and TN653 exhibit high antagonistic effects against several bacterial pathogens. Additionally, the TN635 strain was identified as *Lactobacillus plantarum* and produced a bactericidal bacteriocin called "BacTN635 [15]. Prior to the screening for probiotic properties, the TN600, TN606, TN615, TN618, TN623, TN627, TN644, and TN653 strains were subjected to genotypic identification using a 16S rRNA gene nucleotide sequence analysis (EMBL accession nos. FN667920, FN667915, FN667921, FN667918, FN667914, FN667917, FN667916, and FN667919, respectively). As shown in Table 1, while the TN615, TN627, and TN644 isolates belonged to the genus *Lactobacillus*, TN600, TN606, TN618, TN623, and TN653 were most closely related to species in the *Weissella* genus, which was separated from the genus *Lactobacillus* due to developments in DNA technology [25].

P. acidilactici MA 18/5M was chosen as the reference during the subtractive screening of the nine lactobacilli for probiotic potential. Accordingly, this probiotic was subjected to antibacterial activity assays against a representative number of potential intestinal pathogens. As expected, *P. acidilactici* MA 18/5M exhibited strong inhibitory activity against all undesirable bacteria tested. The antagonistic effect was comparable to that of the nine lactobacilli (Table 2).

Table 1. Identification of the selected lactic acid bacteria (LAB) isolates by 16S rRNA gene sequencing

Strain	Origin	Identification
TN600	Fermented vegetable	*Weissella* sp. TN600
TN606	Fermented vegetable	*Weissella* sp. TN606
TN615	Cow milk	*Lactobacillus* sp. TN615
TN618	Bovine meat	*Weissella* sp. TN618
TN623	Goat milk	*Weissella* sp. TN623
TN627	Cow milk	*Lactobacillus* sp. TN627
TN644	Fermented vegetable	*Lactobacillus* sp. TN644
TN653	Fermented vegetable	*Weissella* sp. TN653

Table 2. Inhibition of probiotics against pathogenic bacteria by the agar spot test

Strain name	Inhibition zone (mm) of indicator strains			
	Gram-positive bacteria		Gram-negative bacteria	
	S. aureus ATCC 6538	*L. ivanovii* BUG 496	*E. coli* ATCC 8739	*S. enterica* ATCC 43972
P. acidilactici MA 18/5M	16	17	17	22
TN600	12	12	5	22
TN606	15	18	22	16
TN615	16	6	13	18
TN618	14	16	15	16
TN623	9	20	15	18
TN627	16	9	23	12
TN635	22	22	22	21
TN644	21	16	13	6
TN653	8	16	22	17

3.2. Enzymatic activities

Table 3 summarizes the enzymatic activities of the TN600, TN606, TN615, TN618, TN623, TN627, TN635, TN644, and TN653 isolates in comparison to *P. acidilactici* MA 18/5M as determined by the API-ZYM system, which is widely used to examine probiotic candidates for harmful enzymatic activity [26,27]. The strains displayed diverse enzymatic profiles; moderate-to-high Leu-arylamidase peptidase and β-galactosidase activities and no α-mannosidase and α-fucosidase activities. In addition to the commercial probiotic, only the TN600, TN627, and TN635 strains exhibited no harmful activities such as α-chymotrypsin, β-

Table 3. Enzymatic activities of the selected lactic acid bacteria (LAB) isolates assayed using the API-ZYM system (bioMérieux)

Enzyme strain	Al. phos	Est. (C 4)	Est (C 8)	Lip (C 14)	Leu	Val	Cys	Try	α-chy	A. phos	Naph	α-gal	β-gal	β-glucu	α-glu	β-glu	N-a-β-glu
P. acidilactici MA 18/5M	0	0	0	5	40	40	5	0	0	5	30	0	0	0	0	0	0
TN600	0	0	0	0	2.5	0	0	0	0	40	20	0	0	0	0	0	0
TN606	5	20	10	5	40	2.5	2.5	2.5	0	20	5	0	40	10	5	0	0
TN615	5	10	5	10	40	20	5	0	2.5	5	2.5	5	40	0	10	40	40
TN618	2.5	5	20	5	30	5	2.5	0	0	10	10	0	10	0	30	5	20
TN623	5	2.5	10	5	40	40	10	0	0	20	20	2.5	40	0	30	40	0
TN627	0	0	0	0	30	20	2.5	0	0	5	20	0	10	0	10	0	0
TN635	5	0	2.5	0	5	0	0	0	0	40	10	5	40	0	5	0	0
TN644	10	20	5	5	40	20	5	0	0	10	10	0	40	0	20	20	40
TN653	0	0	2.5	2.5	30	30	5	0	5	20	30	0	10	0	10	5	0

Enzymatic activity measured as the approximate nmol of substrate hydrolyzed during a 4 h incubation.
α-mannosidase and α-fucosidase activities were never recorded.
Al. phos, alkaline phosphatase; Est (C4), esterase (C4); Est (C8), esterase-lipase (C8); Lip (C14), lipase (C14); Leu, leucine arylamidase; Val, valine arylamidase; Cys, cystine arylamidase; Try, trypsin; α-chy, α-chymotrypsin; A. phos, acid phosphatase; Naph, naphtol-AS-BI-phosphohydrolase; α-gal, α-galactosidase; β-gal, β-galactosidase; β-glucu, β-glucuronidase; α-glu, α-glucosidase; β-glu, β-glucosidase; N-a-β-glu, N-acetyl-β-glucosaminidase.

glucuronidase, β-glucosidase, and N-acetyl-β-glucosaminidase activities (Table 3). Accordingly, the remaining isolates, *i.e.*, TN606, TN615, TN618, TN623, TN644, and TN653 were no longer considered in subsequent screening assays.

3.3. Growth at different pH values and bile concentrations

The TN600, TN627, and TN635 strains, which were free of harmful biochemical activities, and *P. acidilactici* MA 18/5M, were screened for their ability to grow in MRS broth media at different initial pH values and in the presence of bile. Except for the TN635 isolate, generally good growth was recorded in the MRS control media and the MRS media with initial pH values ranging from 5 to 10 over the 10 h incubation time (Fig. 1A). Moreover, none of the isolates (including the reference probiotic) initiated growth at pH 3.0, which agreed with results previously reported that focused on screening LAB isolates with probiotic potential [21,26]. TN635 was the most susceptible to low pH, as it was the only strain that showed an inability to grow at pH 4 and a limited tendency to growth at pH 5 (Fig. 1A).

Fig. 1B shows that although the TN600, TN627, TN635, and *P. acidilactici* MA 18/5M strains exhibited growth rates in the presence of bovine bile that were generally slower than those of the MRS control, they were able to grow at the highest bovine bile concentration of 5%, which is actually much higher than what is normally observed in animal intestines. In fact, resistance to bile varies largely not only among lactobacilli strains [26] but also with bile sources, and porcine bile is much more inhibitory than bovine or human bile [10].

3.4. Screening of genes involved in adherence

The TN600, TN627, and TN635 isolates were evaluated for adherence ability using a PCR approach that amplified internal fragments specific for the genes encoding the Mub, MapA, and EF-Tu proteins, which are among the principle lactobacilli adhesion factors [13]. The findings revealed that only the TN627 and TN635 isolates exhibited bands of the expected size corresponding to *EF-Tu* (663 bp), *Mub* (651 bp), and *MapA* (609 bp) (data not shown). Sequencing of these PCR products confirmed that they were specific to the indicated genes. In fact, the DNA sequences of the *EF-Tu*, *Mub*, and *MapA* amplified products exhibited 98 ~ 100% identity with their putative counterparts from the *L. plantarum* WCFS1 strain and also from the *L. plantarum* JDM1 strain (GenBank accession no. CP001617), a probiotic whose genome sequence has been determined [28]. These results clearly indicate the high conservation of the three *L. plantarum* adhesion genes in TN627 and TN635.

3.5. Growth inhibition by different antibiotic classes

Further studies were conducted to assess the inhibitory spectrum of these isolates to representative antibiotics. The results indicated that TN635 was resistant to five among the 17 antibiotics analyzed (penicillin G, oxacillin, cefoxitin, vancomycin, and oflaxacin), whereas TN600 was resistant to all tested antibiotics (Table 4). Interestingly, the TN627 strain was sensitive to the majority of the antibiotics (16 of 17), as only resistance to cefoxitin was observed. In fact, cell wall impermeability seems to be the main mechanism

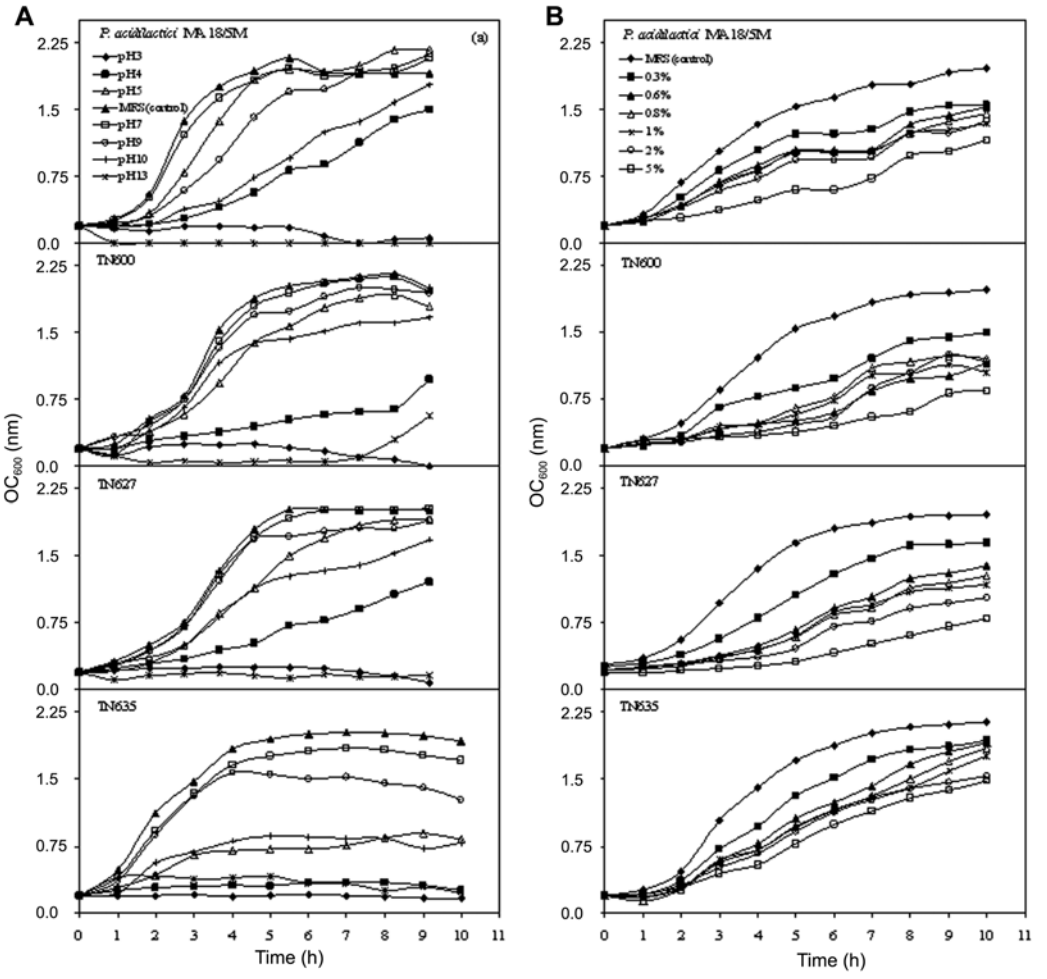

Fig. 1. Effect of MRS medium pH (A) and bovine bile concentrations (B) on growth of *Pediococcus acidilactici* MA 18/5M, TN600, TN627, and TN635. Cultures were incubated at 30°C for 10 h without shaking.

of intrinsic resistance to the cephalosporin cefoxitin because lactobacilli, similar to many other LAB, lack a cytochrome-mediated electron transport system [29].

Taken together, our findings revealed that only TN627 satisfied the majority of the *in vitro* potential probiotic candidate selection criteria among the nine lactobacilli screened [3,10]. Thus, this strain was subjected to further investigations to substantiate its probiotic potential for use as a supplement in the animal feed industry.

3.6. Taxonomic identification of the TN627 strain

Determining a microorganism's identity is the first cornerstone in the safety assessment process [30] and helps to avoid introducing potentially harmful microorganisms into the food chain. Table 1 shows that the TN627 strain was most closely related to *Lactobacillus* species. The DNA sequence of the TN627 16S rRNA gene (EMBL accession no. FN667917) showed 98% identity with that of *L. plantarum* JDM1 (GenBank accession no. CR001617), *L. pentosus* YM2-2 (dbj accession no. AB488810), and *L. plantarum* LpT1 (GenBank accession no. GQ166662). In agreement with previous reports, our results showed that 16S rDNA sequencing has limited resolution potential [31], and is not a sufficiently definite criterion for discriminating closely related species (The TN627 species could be *plantarum* or *pentosus*). Therefore, complementary information from other ancillary molecular methods may be required. Accordingly, additional PCR assays were performed with the *recA* gene-based primers, which discriminate between *L. plantarum* and *L. pentosus* [23]. This permitted designating TN627 as a *L. plantarum* strain, as an amplicon with a size (318 bp) corresponding to that of the expected *L. plantarum* product was obtained (data not shown). The fragment was sequenced (EMBL accession no. FN667925) and revealed a 99% identity with the DNA sequences of the *recA* genes in several *L. plantarum* strains.

3.7. Nature of *L. plantarum* TN627 antibacterial activity

The production of inhibitory substances, such as organic acids, bacteriocins, or hydrogen peroxide (H_2O_2), which

Table 4. Sensitivity of the TN600, TN627, and TN635 isolates to several antibiotics

Antibiotic name	Dose	Diameter of inhibition zone (mm)		
		TN600	TN627	TN635
Penicillin G	6 UI	0	30	0
Oxacillin	5 μg	0	20	0
Cefoxitin	30 μg	0	0	0
Streptomycin	10 UI	0	11	15
Gentamicin	10 UI	0	28	46
Kanamycin	30 UI	0	10	15
Tobramycin	10 μg	0	15	22
Chloramphenicol	30 μg	0	36	32
Tetracyclin	30 UI	0	35	16
Erythromycin	15 UI	0	45	44
Pristinamycin	15 μg	0	34	44
Lincomycin	15 μg	0	44	48
Oflaxacin	5 μg	0	19	0
Vancomycin	30 μg	0	7	0
Nitrofuranes	300 μg	0	30	28
Cotrimoxazole (Trimethropim-Sulfamethoxazole)	1.25 μg / 23.75 μg	0	30	28
Rifampicin	30 μg	0	44	28

inhibit undesirable and pathogenic bacteria, is a desirable property for probiotics [10]. Accordingly, the culture supernatant was subjected to different treatments, and residual antibacterial activity was determined to elucidate the type of antibacterial metabolites produced by *L. plantarum* TN627. The antagonistic activity of TN627 against *Listeria ivanovii* BUG 496 (and the other bacterial indicator strains tested; data not shown) was produced primarily by organic acid release, because the inhibitory effect was entirely destroyed by adjusting the supernatant pH to 6.5 (Fig. 2).

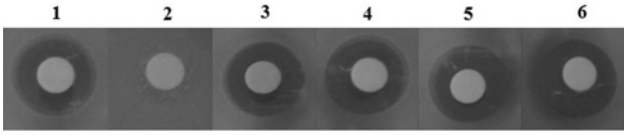

Fig. 2. Effect of different treatments on the antibacterial activity of *Lactobacillus plantarum* TN627 against *Listeria ivanovii* BUG 496. 1: untreated cell-free supernatant; 2 ~ 6: different supernatant treatments including a pH adjustment to 6.5 (2), heating for 1 h at 100°C (3), and the actions of catalase (4), proteinase K (5), and trypsin (6).

The antibacterial activity was insensitive to heat, catalase, trypsin, and proteinase K. In many studies, the production of organic acids was the major factor responsible for the antagonistic activity of several probiotic lactobacilli [32, 33]. Bacterial growth inhibition by lactic acid can be explained by efficient leakage of hydrogen ions across the cell membrane of indicator strains, causing acidification of cytoplasm and dissipation of the pH gradient, as suggested by Blom and Mørtvedt [34].

3.8. Adhesion of *L. plantarum* TN627 to enterocytes

Adhesion is one of the desired properties to select adequate probiotic microorganisms. In agreement with the detection of genes encoding the Mub, MapA, and EF-Tu proteins in *L. plantarum* TN627, we also observed that this milk-derived strain, although not isolated from the target animal

Table 6. Adhesion of *Lactobacillus plantarum* TN627 to chicken enterocytes collected from the duodenum, jejunum, and ileum

Intestine segment	% of adhesion
Duodenum	95
Jejunum	91
Ileum	83

Table 5. Summary of the *in vitro* tests performed during the screening procedure to select the TN627 probiotic candidate

Strain	Antagonistic effect	Harmful enzymatic activities	Growth in the presence of 5% bovine bile	Growth at pH 4	Detection of adhesion genes	Sensitivity to antibiotics
TN600	+	0/4	+	+	0/3	0/17
TN627	+	0/4	+	+	3/3	16/17
TN635	+	0/4	+	–	3/3	12/17
TN606	+	1/4				
TN615	+	3/4				
TN618	+	2/4				
TN623	+	1/4				
TN644	+	2/4				
TN653	+	2/4				

The harmful Enzymatic activities tested were α-chymotrypsin, β-glucuronidase, β-glucosidase, and N-acetyl-β-glucosaminidase.
The adhesion genes screened were: *MapA*, *Mub* and *EF-Tu*.
The antibiotics analyzed were penicillin G, oxacillin, cefoxitin, vancomycin, chloramphenicol, tetracycline, erythromycin, pristinamycin, lincomycin, streptomycin, gentamicin, kanamycin, tobramycin, oflaxacin, nitrofuranes, cotrimoxazole, and rifampicin.

species (ecological niche where the probiotic product will be applied), exhibited good adhesion efficacy to chicken enterocytes collected from the duodenum, jejunum, and ileum (Table 6). Nevertheless, the TN627 strain seemed to adhere slightly better to the epithelial cells from the duodenum and jejunum (adhesion of 95 and 91%, respectively) compared to that of the ileum (adhesion of 83%), and the bacterial distribution on the surface of all epithelial cells was generally uniform (data not shown). This property could avoid expulsion, and constitutes an important step for the subsequent colonization and formation of a barrier on the intestinal surface to prevent incoming pathogenic microorganisms [10,13,14].

4. Conclusion

Following the subtractive screening strategy, the *L. plantarum* TN627 strain was finally selected based on its desirable *in vitro* probiotic characteristics, including inhibition of intestinal pathogens, lack of harmful enzymatic activity, growth at low pH and in the presence of bile, sensitivity to several antibiotics, and good adhesion to chicken enterocytes. Considering the promising properties and attributes of this strain, further *in vivo* poultry production tests are necessary to support its potential as a feed supplement.

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