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Assessment of *Enterococcus durans* F21 isolated from *Lben*, a Moroccan fermented milk as a biopreservative agent

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Abstract

Background and Objective: Lactic acid bacteria used in food processing for a long time are known for their benefits to consumers and their ability to produce natural antimicrobial compounds used as bio-preservatives in foods. The aim of the present study was the characterization and assessment of F21 strain, isolated from *Lben* (a traditional Moroccan fermented milk), as food biopreservative.

Material and Methods: Isolate F21 was isolated from *Lben*, subjected to screening of inhibitory activity production, and identified based on morphological, biochemical and molecular identification. Then, the production and physicochemical characterization of the antagonistic substance were determined. Also, the safety profiling and biotechnological properties of isolate were evaluated. Finally, a biopreservative powder with antimicrobial activity was produced and assessed in various food systems (milk, ground beef and fresh cheese).

Results and Conclusion: Of the isolated lactic acid bacteria, *Enterococcus durans* F21, isolated from *Lben* (a traditional Moroccan fermented milk), was remarkably endowed with interesting enterocin-like substance (heat stable and pH resistant) active against potentially pathogens and food spoilages (*Listeria monocytogenes*, *Listeria innocua*, *Enterococcus faecalis*, *Brochothrix thermosphacta*, and *Mycobacterium smegmatis*). Concerning the safety properties, *Enterococcus durans* F21 was not hemolytic, sensible to antibiotics tested, unable to produce biogenic amines and other virulence enzymes (gelatinase, DNase and urease). In addition, *Enterococcus durans* F21 showed satisfactory biotechnological characteristics such as acidification power, exopolysaccharides production and antioxidant activity. The biopreservative powder containing enterocin-like substance F21 that was achieved via freeze-drying showed a minimum inhibition concentration of 60 AU ml^{-1} against *Listeria monocytogenes* in culture media. In addition, this biopreservative powder (at 665 AU ml^{-1}) was able to improve safety and shelf-life of numerous foods (milk, *Jben* and ground beef). Thus, these results provided foundations for further uses of *Enterococcus durans* F21 as producer of potential food biopreservative agent.

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1. Introduction

Despite modern techniques used in food preservation, number of foodborne outbreaks is still increasing. As reported by the World Health Organization, 600 million foodborne illnesses were reported in 2010; from which, 420000 deaths were reported [1]. In Morocco, 1371 cases of foodborne diseases were reported in 2019. In fact, foodborne diseases are the second leading cause of intoxication with 17.9% within all the reported cases [2]. Use of chemical preservatives and physical treatments becomes essential to prevent the growth of spoilers and pathogens improving general safety and to extend shelf-life of various foods. In addition to consumers who are scared by the potential toxicity of the chemical preservatives, these methods can negatively affect organoleptic characteristics of the foods. Therefore, challenge includes use of natural and promising alternatives by the food suppliers for biopreservation such as bacteriocins or their producers of lactic acid bacteria (LAB) [3]. Throughout the history, LAB were closely associated

with human culture and well-being. In addition, use of LAB as starter, protective and probiotic culture have greatly been interested within the last decades [4].

Nowadays, use of *Enterococcus* spp. in foods is controversial unlike other LAB [5]. In fact, several researchers agree enterococcal roles in dairy and meat products and vegetables [6-8]. Furthermore, *Enterococcus* spp. may be found in foods at high levels, leading to the formation of flavors [9]. They can be used as starters, bioprotectives against spoilage bacteria and pathogens [10], silage inoculants [11] and probiotics [12]. Moreover, *Enterococcus* spp. Include extracellular antagonistic substances (bacteriocins) such as enterocins, exhibiting effectiveness, specificity and low toxicity [13]. These characteristics make these bacteria essential in fermented foods. In addition, this has been extended to diverse therapeutic purposes [14-16]. In contrast, few researchers argue enterococci due to reported human infections and present antibiotic resistance in some cases (especially vancomycin resistance) [17].

In Morocco, traditional fermented dairy products are highly consumed. However, a few studies have addressed microbiota characterization of the most popular Moroccan fermented products. Examples include *Lben* as a fermented milk, *Rayeb* as a coagulated fermented milk, *Smen* as a fermented butter and *Jben* as a fresh cheese. In fact, these products are appropriate for the selection of starters, protective strains and probiotics [18]. These strains can include several enzymatic activities leading to the formation of flavors with antagonistic activity that make them essential in fermentation and preservation processes [3,18,19]. Discovery of natural antimicrobial substances is potentially interested by the food industries, which is appropriate to improve safety and shelf-life of various foods and consumer's health. Therefore, the aim of the present study included characterization and assessment of F21 strain, isolated from *Lben* (a traditional Moroccan fermented milk), as food biopreservative. Furthermore, bioactive powder was produced and assessed in various food systems (milk, ground beef and fresh cheese).

2. Materials and Methods

2.1. Bacterial strains

Bacterial strains used as indicator strains in this study included *Enterococcus* (*E.*) *faecalis* S5, *Pseudomonas fragi* S23, *Mycobacterium smegmatis* MC_2-155, *M. aurum* A+ and *Escherichia coli* DH5α from BM2B laboratory collection (Faculty of Science and Technology of Fez, University of Sidi Mohamed Ben Abdellah, Morocco); *Salmonella enterica* B801 as well as *Staphylococcus aureus* B804, *Bacillus cereus* B1167 and *Listeria* (*L.*) *monocytogenes* B806 from CNRST (Rabat, Morocco). Moreover, *L. innocua* DSM 20649, *E. coli* DSM 613, *Brochothrix thermosphacta* DSM 20171 and *S. enterica* DSM 11320 were provided by DSMZ (German Collection of Microorganisms and Cell Cultures, Germany).

2.2. Isolation of lactic acid bacteria and screening for anti-listerial activity

The LAB were isolated from samples of the Moroccan traditional products of *Lben* (fermented milk), *Smen* (fermented butter), *Rayeb* (coagulated milk), *Zebda beldiya* (raw fresh butter) and *Jben* (fresh cheese). All samples were purchased from artisanal producers of various urban local shops in Fez City, Morocco, and produced following traditional procedures without addition of commercial starter cultures. Briefly, 1 g of each sample was aseptically mixed with sterile physiological saline solution (0.85%) at 1:10. The homogenate was ten-fold serially diluted, plated on De Man, Rogosa and Sharpe (MRS) agar (Biokar Diagnostics, France) and incubated at 37 °C for 48 h. Typical LAB-colonies on each plate were purified on similar media and then subcultured in MRS broth for further screening of inhibitory activity production. Screening of anti-listerial activity in solid media was carried out via agar diffusion assay and in liquid media using agar well diffusion method as previously described by Ananou et al. [19,20]. For agar diffusion assay, petri dishes containing LAB colonies were covered with pre-inoculated molten soft buffered BHI (0.8% agar, pH 7.2). For agar well diffusion, stainless steel cylinders with 8 mm of outer diameter and 10 mm of height (Scharlau, Spain) that previously were deposited on buffered Mueller-Hinton agar (Biokar Diagnostics) were used for the creation of wells. The pre-inoculated molten soft buffered BHI was added and cylinders were removed after solidification. Then, 100 µl of the LAB supernatants (or fractions), sterilized by filtration through 0.45-µm cellulose filters (Millipore, USA), were transferred into the wells. Petri dishes from the two techniques were incubated at 37 ºC for 16–24 h and the inhibition zones were measured and expressed in mm. In the two assays, *L. monocytogenes* B806 ($\approx 10^6$ CFU ml^{-1}) was used as standard indicator strain.

2.3. Identification of the bacteriocinogenic isolates

Identification of the selected bacteriocinogenic isolate was carried out based on phenotypic characteristics described by Carr et al. [21], including Gram reaction, colonial morphology in MRS agar and physiological/ biochemical characterization. Physiological/biochemical identification was carried out based on catalase reaction (Solvapur, France), oxidase reaction (Sigma-Aldrich, Germany), gas production from glucose, ability to grow at various temperatures in MRS broth (10, 15, 37 and 45 °C) in presence of 6.5% of NaCl (Riedel-de Haen, Germany) at pH 9.6, ability to degrade esculin in presence of 4% bile (Fluka Biochimika, India), sodium azide (0.04%) resistance (Polysciences, USA), thermo-resistance (60 °C for 30 min)

and carbohydrate fermentation (glucose, lactose, mannitol, trehalose, raffinose, sorbitol and arabinose). In addition, species identification of the selected isolate (F21) was carried out using API 20 Strep Gallery (Biomerieux, France) for streptococci and other germs.

2.4. Molecular identification

In general, DNA from the selected strain was extracted using bacterial genomic DNA extraction kit using manufacturer's instructions (GenElute Kit; Sigma-Aldrich, Germany). Briefly, the selected strain was lysed using chaotropic salt-containing solution supplemented with lysozyme (at 10 mg ml^{-1}). Then, DNA was precipitated with ethanol, washed and dissolved in 200 µl of Tris-EDTA solution. The fragment of 16S rDNA gene was then amplified using polymerase chain reaction (PCR), Mastercycler ProThermal Cycler (Eppendorf, Germany) and bacterial universal primers of WO1 (5'-AGAGTTTGATC [A/C]TGGCTC-3') and WO12 (5'-TACGCATTTCACC [G/T]CTACA-3'). Amplification was carried out with an initial denaturing step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s as well as a final extension step of 72 °C for 2 min. Amplicons were used in purification step using ExoProStart kit (GE Healthcare, UK). Then, DNA sequencing was carried out by an external service (Sistemas Genomicos, Spain). The resulting sequence was analyzed using local alignment, NCBI BLASTN online software (https://blast.ncbi.nlm.nih-gov/).

2.5. Characterization of the antagonistic substance produced by the selected strain

2.5.1. Antagonistic substance production and growth kinetics of the selected strain

Growth kinetics of F21 strain and antimicrobial activity production in MRS broth (Biokar Diagnostics, France), inoculated at 5% (v v^{-1}), was followed at 37 °C for 24 h. Cell growth was assessed at 620 nm using BK-UV1000 spectrophotometer (Biobase, China). Production of the antagonistic substance by F21 strain was assessed against *L. monocytogenes* using agar well diffusion technique [wells filled with 100 µl of SN sterilized by filtration through 0.45-µm cellulose filters (Millipore)].

2.5.2. Sensibility to proteolytic enzymes

To assess protein nature of the inhibitory compounds, proteolytic enzyme preparations [proteinase K (Invitrogen, USA), pepsin (Polysciences, USA), papain, chymotrypsin and protease of *Streptomyces griseus* (Sigma-Aldrich, Germany)] at a final concentration of 50 mg l^{-1} were added to the supernatant (sterilized by filtration) of an overnight culture of the selected strain and incubated at 37 °C for 2 h. After incubation, remaining SN activity was assessed against *L. monocytogenes*.

2.5.3. Thermostability

To assess thermoresistance, SN was heated at various temperatures (60, 80 and 100 °C) for various times (30, 5 and 1 min) or at 120 °C for 20 min. The SNs were immediately cooled and assessed for anti-listerial activity using agar well diffusion technique [22].

2.5.4. Stability under various pH values

The pH-stability was assessed at various pH values (4,7,8) as previously described by Ananou et al. [19]. Adjusted preparations were assessed for anti-listerial activity using agar well diffusion technique after 2 h of incubation at room temperature (RT).

2.6. Antimicrobial spectrum

Inhibitory activity of BLIS (bacteriocin-like inhibitory substance) produced by F21 strain against several indicator strains of *L. innocua* DSM 20640, *L. monocytogenes* B806, *B. cereus* B1167, *S. aureus* B804, *P. fragi* S23, *E. faecalis* S5, *M. smegmatis* MC_2 155, *M. aurum* A^+, *E. coli* DH5α, *E. coli* DSM 613, *S. enterica* B801, *S. enterica* DSM 11320 and *B. thermosphacta* DSM 20171 was assessed using agar well-diffusion technique.

2.7. Production and recovery of enterocin-like substance produced by F21 strain

2.7.1. Enterocin production and recovery

Production of enterocin-like substance was carried out using bioreactor (Bioflo 2000, USA) and 1 L of MRS broth that was inoculated with F21 strain at 5 % (v v^{-1}) and incubated at 37 °C for 16 h. Semi-purified fractions of the enterocin like substance were recovered using cation exchange chromatography on carboxymethyl Sephadex CM-25 (Sigma-Aldrich, Germany) added to the bacterial culture at 20% (v v^{-1}). After agitation at RT for 30 min, mixture (bacterial culture and CM-25 resin) was set for 15 min. Then, resin was recovered and washed with distilled water (DW). Elution (at a flow rate of 10 ml min^{-1}) was carried out using three volumes of NaCl solution (2 M). Then, eluted fractions of enterocin-like were dialyzed against DW under refrigeration for 24 h using 2000-Da cut-off membranes (Sigma-Aldrich, Germany) and sterilized by filtration through 0.45-µm filters (Millipore, USA). Yield of the recovered enterocin was calculated using the following formula [19]:

Enterocin yield (in %) = 100 × activity recovered (in AU ml^{-1}) / total activity (in AU ml^{-1})

2.7.2. Bacteriocin quantification

Bacteriocin quantification was estimated from the titration curve achieved from the diameter of the inhibition zone of semi-purified fractions (expressed in mm) and their inhibitory activities (expressed in arbitrary units per ml) as previously described by Ananou et al. [19]. The inhibitory activity (AU ml^{-1}) assessed via well diffusion assay is

defined as the reciprocal of the highest dilution presenting anti-listerial activity [23].

2.7.3. Bacteriocin molecular weight estimation

For molecular weight estimation, the dialyzed free-cells fraction was filtered using 10000 and 5000-Da cut-off tubes (Millipore, USA). The filtrates were submitted to agar well diffusion technique for antimicrobial activity quantification as described previously.

2.8. Production of biopreservative powder

Sterilized fractions of the semi-purified enterocin (with activity of 689 AU ml^{-1}) were frozen at -20 °C for 24 h and then freeze-dried using Lyovapor L-200 equipment (Buchi, France) operating at vacuum pressure of 0.1 mbar and condensing temperature of -54 °C for 48 h. Dried preparation was weighed and stored at 4 °C for future assessment. Absence of viable cells of F21 strain in powder was verified by reconstitution of the dried preparation in MRS broth. Reconstituted preparation was incubated at 37 °C for 48 h and then plated in MRS agar in triplicate. Enterocin activity quantification was carried out after the reconstitution of powder in DW as explained previously.

2.9. Assessment of F21 strain safety

2.9.1. DNase, urease, gelatinase and hemolytic activities

Assessment of the safety of F21 strain was carried out based on various assays previously described by Varada et al. [24]. Ability of the isolate to hydrolyze DNA was assessed on DNase agar plate (Thermo-Fisher Scientific, USA). Urease and gelatinase activities were respectively assessed on urea tryptophan media (Merck, Germany) and Luria-Bertani agar plates supplemented with 3% of gelatin (Thermo-Fisher Scientific, USA). Hemolytic activity was assessed on blood agar plates (Thermo-Fisher Scientific, USA).

2.9.2. Production of biogenic amines and antibiotic susceptibility

Production of biogenic amines (histamine and tyramine) by F21 strain was assessed as previously described by Ananou et al. [25] and antibiotic susceptibility was assessed using disc diffusion method as described by The European Committee on Antimicrobial Susceptibility Testing [26]. Antibiotic discs included ampicillin (Am, 10 µg/disc), vancomycin (Vm, 30 µg/disc), gentamicin (GM, 10 µg/disc) and tobramycin (TOB, 10 µg/disc) (Bioanalyse, Turkey). According to EUCAST [26], F21 strain was classified as sensitive, intermediate or resistant to the antibiotics.

2.10. Biotechnological profiling of F21 strain

2.10.1. Acidification activity

Acidifying power of F21 strain was assessed by measuring pH of the culture in MRS media after 24 and 48 h using pH-meter (Eutech Instruments, USA).

2.10.2. Antioxidant activity

Antioxidant activity of the strain was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (Sigma Aldrich, France) assay as described by Zhang et al. [27]. Briefly, 1 ml of DPPH solution (0.2 mmol l^{-1} in ethanol) was added to 1 ml of the cell suspension with approximately 10^6 CFU ml^{-1}, previously prepared from an overnight culture of F21 strain, washed twice with PBS and resuspended in similar buffer) and set to react at ambient temperature within 30 min in dark. In addition, control included 1 ml of DPPH solution and 1 ml of PBS. After centrifugation, DPPH was monitored by measuring optical density (OD) at 517 nm and antioxidant activity (expressed in %) was calculated using following formula:

Antioxidant activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

2.10.3. Production of exopolysaccharides

Exopolysaccharide (EPS) production was assessed using colorimetric assay as described by Taj et al. [28]. Cell free supernatant (200 µl) of the overnight culture of F21 strain in Luria-Bertani broth (Biokar Diagnostics, France) was mixed with 200 µl of phenol solution (5%) (Thermo-Fisher Scientific, USA) and 1 ml of sulfuric acid (Sigma-Aldrich, Germany). The OD was measured at 490 nm after setting for 30 min in dark. In addition, negative control with DW was prepared. A standard graph was plotted with absorbance at 490 nm against concentrations of glucose as standard.

2.11. Antimicrobial activity assessment of enterocin-like powder

2.11.1. Assessment in vitro

Assessment of the antimicrobial activity of F21-biopreservative powder was carried out *in vitro* using minimum inhibitory concentration (MIC). Various concentrations of the biopreservative powder (10, 25, 33, 60, 83 and 167 AU ml^{-1}) were assessed against the indicator strain in tryptic soy broth (Biokar Diagnostics, France). Microplate was incubated at 37 °C for 24 h and OD measurements were carried out at 600 nm in MultiScan Sky spectrophotometer (Thermo-Fisher Scientific, USA).

2.11.2. Assessment in food matrices

Antimicrobial activity of the entrocin-like powder was assessed against pathogen and spoilage bacteria using milk (whole and skimmed milks), fresh cheese and ground beef.

2.11.2.1. Milk

The UHT-milk samples (whole or skimmed milk), inoculated with *L. monocytogenes* B806 at approximately 10^5 CFU ml^{-1}, were treated with F21-powder (at final concentrations of 167 and 665 AU ml^{-1}) and incubated at 30 °C for 24 h.

Viable cell counts (CFU ml^{-1}) were carried out on PALCAM agar (Biokar diagnostics, France) at selected times (0, 3, 6 and 24 h) by counting colonies after 48 h of incubation at 37 °C. Furthermore, negative control included no F21-powder.

2.11.2.2. Jben

Jben (a Moroccan fresh cheese) was prepared with commercial pasteurized whole milk using calf rennet for the coagulation, as previously described by Achemchem et al. [29]. Curd was recovered by filtration and drained overnight under normal storage conditions. After draining, curd was weighed, added with sodium chloride (at 1.5 %, w w^{-1}) and divided into two batches of control batch (inoculated with *L. monocytogenes* B806 at 10^3 CFU g^{-1}) and treated batch (inoculated with *L. monocytogenes* B806 at 10^3 CFU g^{-1} and added with F21-biopreservative powder at a final concentration of 665 AU g^{-1}). Fresh cheese was stored at 8°C for 15 d. Viable cell counts (CFU g^{-1}) were carried out at selected times (0, 2, 5, 10 and 15 d) on plate count agar incubated for 2 d at 30°C for mesophilic microorganisms, on MRS agar incubated for 3 d at 30 °C for LAB and on PALCAM agar incubated for 3 d at 37 °C for *L. monocytogenes*.

2.11.2.3. Ground beef

Ground beef (as a meat model) was produced using meat ground through a 6-mm plate, tempered at -1/0 °C, mixed for 1 min and divided into two batches of control batch (without powder addition); and treated batch (added with biopreservative powder at a final concentration of 665 AU g^{-1}). Ground beef was stored at 8 °C for 10 d using sterile plastic petri dishes. At selected times (0, 2, 4, 7 and 10 d), viable cell counts (in CFU g^{-1}) were carried out on plate count agar, MRS and violet red bile lactose media (Biokar Diagnostics, France) for mesophilic microorganisms, LAB and coliform bacteria, respectively.

2.12. Statistical analysis

All assessments were carried out in duplicate and results were average of the values. Standard deviations were calculated using Excel (Microsoft, USA). Statistical analysis, especially ANOVA, was carried out using trial version of SPSS software v.17.0 (IBM, USA) and differences were considered statistically significant when $p \leq 0.05$.

3. Results and Discussion

3.1. Screening for the lactic acid bacteria producer of antimicrobial compounds and identification of the isolate with anti-listerial activity

From 674 LAB isolates isolated from various Moroccan traditional products of *Lben, Smen, Rayeb, Zebda beldiya* and *Jben*, 21 isolates (3.11%) showed inhibitory activities against *L. monocytogenes* used as indicator strain in solid media. This especially included F21 strain isolated from *Lben*, which included the highest anti-listerial activity in liquid media (15-mm of inhibition diameter) and was selected for further characterization (Table 1). Furthermore, antagonistic activity of the supernatant was damaged by the proteolytic enzymes (Table 1), demonstrating that the antimicrobial activity was due to a protein/peptide substance. Moreover, identification of F21 isolate was carried out using API 20 Strep system and completed using 16S rRNA gene sequencing. Thus, F21 isolate was identified as *Enteroccus (E.) durans* (GenBank accession no. OQ572352). Previous studies reported Moroccan traditional fermented foods as important naturally sources of bacteriocin producing LAB strains [18].

3.2. Characterization of antagonistic substance produced by *E. durans* F21

E. durans F21 was able to multiply from 7.7 log units CFU ml^{-1} at 0 h to 8.97, 8.95 and 8.92 log CFU ml^{-1} after 20, 22 and 24 h, respectively, possibly due to the lack of nutrients (Figure 1A). Beginning of the antagonistic peptide production was detected after 2 h and reached its maximum (15 mm) after 16 h of incubation and decreased to 12 and 11 mm after 22 and 24 h, respectively ($p \leq 0.05$) (Figure 1A). Decreases in antimicrobial activity during the stationary phase could be attributed to the adsorption of molecules to producing-cells, endogenous proteases released after producing cells destruction and/or aggregation [19]. Thus, these results suggest that the antagonistic peptide, produced as primary metabolite pattern by *E. durans* F21 with molecular weight of 2000-5000 Da, was a bacteriocin-like inhibitory substance (BLIS) that belonged to the broad family of enterocins [30]. In addition, antibacterial peptide produced by *E. durans* F21 was thermostable and preserved 83, 82 and 54% of its activity after 30 min of heat treatments at 60, 80 and 100 °C, respectively (Figure 1B). However, antibacterial activity could not be detected after treatment at 120 °C. This natural peptide was relatively stable at pH 4, 7 and 8 (Figure 1C). These characteristics (thermal and pH stabilities) have frequently been referred for enterocins [30]. The enterocin-like substance produced by *E. durans* F21 was active against *L. monocytogenes, L. innocua, E. faecalis, B. thermosphacta* and *M. smegmatis* (Table 2). However, *B. cereus, S. aureus, M. aurum, P.fragi, E. coli* and *S. enterica* were resistant. Previous studies reported the primary target of LAB-bacteriocins (the cytoplasmic memb-rane) and protective effects of the outer-membrane of Gram-negative bacteria [3]. Thus, *E. durans*, naturally present in several foods [31,32] can produce protein substance with antagonistic effects against Gram-positive bacteria (*L. monocytogenes* and *L. innocua*) and acid-fast bacteria (*M. smegmatis*). To use enterococci in foods, it is necessary to assess their safety and technological characteristics. Hence, it was essential to submit *E. durans* F21 for safety and biotechnological assays.

3.3. Enterocin-like substance recovery

After cation exchange chromatography on carboxymethyl Sephadex CM-25, recovered yield of F21-enterocin-like

activity was reported as 62% with 640000 total AU in culture SN and 398 560 total AU recovered after chromatography (data not shown).

Table 1. Morphological, physiological and biochemical identification of the isolate F21.

Parameter	Result
Origin	Lben (a fermented milk)
Gram stain test	+
Shape	Cocci
Catalase	-
Oxidase	-
Gas production	-
10°C	-
15°C	-
45°C	+
Ability to hydrolyze esculin in presence of bile	+
Ability to grow in presence of 6.5 % NaCl	+
Resistance to heat at 60°C for 30 min	+
Glucose	+
Lactose	+
Mannitol	-
Trehalose	+
Raffinose	-
Sorbitol	-
Arabinose	-
Sensibility of supernatant to proteolytic enzymes	+
Identification of isolate F21	*Enterococcus durans*

: No growth/ No activity; +: growth/activity.

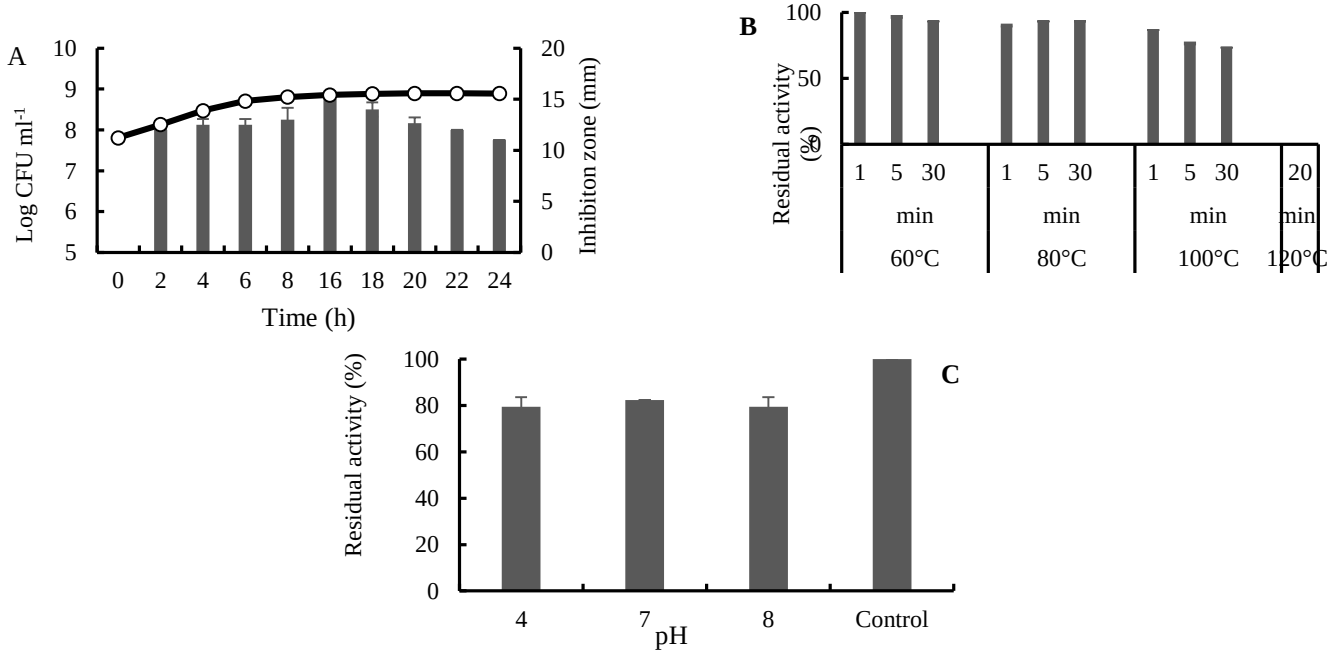

Figure 1. Characterization of the antagonistic substance produced by *Enterococcus durans* F21. Growth kinetics and production **(A)**; thermo-stability **(B)**; and pH-stability (**C**)

As a generally cationic nature, various cation exchange resins (e.g. Sepharose, carboxymethyl Sephadex CM-25 and Amberlite IRC-50) have been used to achieve semi-purified enterocins (e.g. enterocins A, B, P, AS-48, F-58 and OS1) at variable yields, ranging 15-75% [20,22,33,34]. Nevertheless, the recovered enterocin could be affected by nature, type and quantity of cation exchange resins as well as elution solution.

Table 2. Antimicrobial spectrum of antagonistic substance produced by *E. durans* F21.

	Inhibition diameter (mm)
Enterococus faecalis S5	Indicator strain
Listeria monocytogenes B806	15
Listeria innocua DSM 20649	14
Brochothrix thermosphacta DSM 20171	15
Mycobacterium smegmatis MC_2 155	13
Bacillus cereus B1167	0
Staphylococcus aureus B804	0
Mycobacterium aurum A+	0
Pseudomonas fragi S23	0
Escherichia coli DH5α	0
Eschirichia. coli DSM 613	0
Salmonella enterica B801	0
Salmonella enterica DSM 11320	0

- : Without activity +: With activity

3.4. Assessment of E. durans F21 safety

Safety profiling of *E. durans* F21 was assessed using DNase, urease, gelatinase, hemolytic and decarboxylases activities (Table 3) as well as its antibiotic resistance (Table 4).

Table 3. Safety profiling of *E. durans* F21.

Safety profiling	Results
Urease activity	-
DNase activity	-
Hemolytic activity	-
Production of histamine	-
Production of tyramine	-

- : Without activity +: With activity

Neither DNase nor gelatinase, urease and hemolysin were produced by this strain. No histidine and tyrosine decarboxylases were produced, meaning that this isolate could be used without risks of producing associated biogenic amines (histamine and tyramine) (Table 3). Moreover, the present results indicated that *E. durans* F21 was sensitive to ampicillin and vancomycin. This strain included a naturally low-level resistance against the tested aminoglycosides (gentamicin and tobramycin), (Table 4) and hence did not included problems of high-level resistance. Indeed, assessment of antibacterial susceptibility in entericocci is essential as they can be host of antibacterial resistance genes and allow horizontal gene transfer to patho-genic bacteria. In fact, ampicillin and vancomycin resistance in enterococcal species was emerged and frequently associated with antibacterial use [35]. For aminoglycosides, all enterococci included intrinsic low-level resistance. However, *Enterococcus* strains with high-level aminoglycoside resistance have been reported. For example, Li et al. [36] reported that *E. durans* isolated from a fermented cream in China was resistant to gentamicin. Yangzom et al. [37] reported that 3.6% of *E. durans* isolated from human samples were resistant to gentamicin.

3.5. Biotechnological profiling of E. durans F21

3.5.1. Acidification activity

In this study, *E. durans* F21 was able to decrease pH of the media (MRS) from 6.80 to 4.64 and 4.42 after 24 and 48 h of incubation, respectively (Figure 2A). In fact, acidification ability is important for food fermentation and industries. Fast decreases in pH, resulting from excessive accumulation of lactic acid and other organic acids, enhances safety (providing an unfavorable environment for the multiplication of pathogenic and/or spoilage microorganisms) of foods. Moreover, these organic acids lead to the formation of specific flavors and textures (e.g. sourness and acidic characteristics of cheeses, sausages and yogurts), positively affecting organoleptic characteristics of various fermented foods [38,39] that make them essential in fermentation and preservation processes. Therefore, *E. durans* F21 can be used as protective and starter culture in fermented products.

3.5.2. Antioxidant activity

The DPPH scavenging assay of *E. durans* F21 showed important antioxidant activity of 58%. In fact, LAB with antioxidant activity can be considered as one of the most important tools that significantly improve functional and organoleptic characteristics of several fermented foods [27]. Previous studies demonstrated antioxidant ability of *E. durans*, *E. faecium*, *E. faecalis* [40-42], *Lactobacillus* and *Leuconostoc* strains [27,43].

3.5.3. Production of exopolysaccharides

The amount of EPS produced by *E. durans* F21 was about 12.7 g l^{-1}. This value is high (increase of seven folds) with respect to 1.6 g l^{-1} referred for an *E. durans* strain isolated from Algerian fermented wheat [44]. However, this value represents nearly half of 23 g l^{-1} reported by Mostafa et al. [45]. In fact, these EPSs are widely used in food industries for their textural characteristics and as functional additives as well as packaging materials). Moreover, EPSs are used in various pharmaceutical and biomedical fields. Technically, EPSs need to be extracted because they are involved in structural components of the extracellular matrices, where they can play protective roles from the hard environmental conditions. In addition, EPSs affect physicochemical characters of the bacterial cell surface [46].

3.6. Production and inhibitory assessment of enterocin-containing powder

3.6.1. Assessment in vitro

Biopreservative powder of F21 that was recovered after lyophilization preserved 96.5% of its activity and was active against *L. monocytogenes* with a MIC of 60 AU ml^{-1}.

Therefore, this enterocin-containing powder was still active after the freeze-drying step and could successfully be used to produce bio preservative powder with stable activity. Indeed, the freeze-drying use can achieve high quantities of stable bio preservative powder, compared to that the liquid preparation can. These preparations are easily handled with doses well established and easily stored that are further appropriate for use in foods. In fact, this preparation can be used as additional hurdles in various minimally processed, non-thermal and thermal foods. Previous studies have shown that bacteriocin-powders (containing nisin A, bavaricin MN, lactocin GI3, pediocin A, curvaticin A, curvaticin L442 and curvacin A), achieved by freeze-drying, could remain active and preserve their anti-listerial activities after lyophilization [47,48].

3.6.2. Assessment in food matrices

3.6.2.1. Effects of biopreservative powder on milk

The F21-powder (at final concentrations of 167 and 665 AU ml^{-1}) was assessed against *L. monocytogenes* in milk (whole and skimmed milks) incubated at 30 °C for 24 h (Figure 3 A,B).

Table 4. Antibiotic susceptibility test results for *E. durans* F21

Strain	Antibiotics			
Enteroccus durans F21	Ampicillin	Vancomycin	Gentamicin	Tobramycin
	S ZOI=22±1 mm	S ZOI=14.33 ±0.57 mm	S ZOI=9.33 ±1.52 mm	S ZOI=8.33 ±0.57 mm

ZOI: Zone of inhibition; S: sensitive
, I: intermediate, and R: resistant to an antibiotic.
The breakpoints for the antibiotic susceptibility in mm zone of inhibition: Ampicillin (≥10/<8) and Vancomycin (≥12/<12); the breakpoints for the Gentamicin and Tobramycin natural resistance at low levels/high levels in mm zone of inhibition: Gentamicin (≥8/<8) and Tobramycin (≥8/<8).
Data were expressed as mean ± standard error of three independent experiments

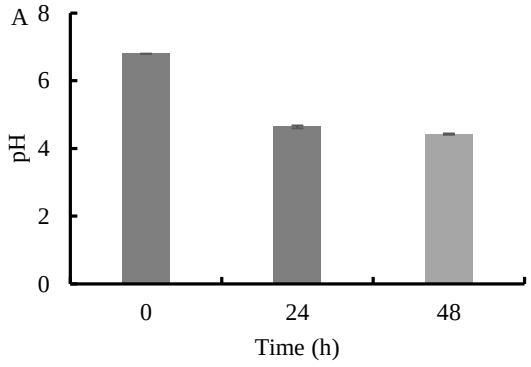

Figure 2. Acidification ability of *E. durans* F21.

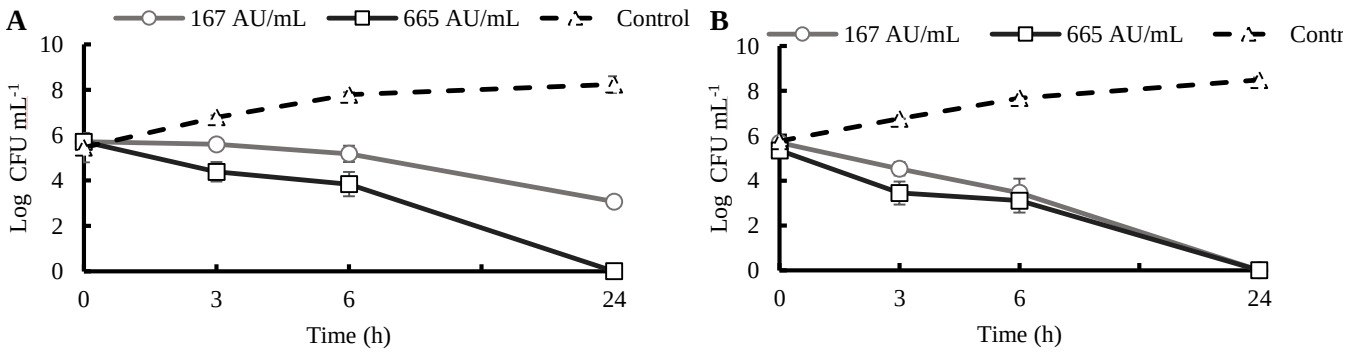

Figure 3. Effect of F21-bio-preservativepowder (at 167 and 665 AU ml^{-1}) against *Listeria monocytogenes* in whole milk **(A)** and skimmed milk **(B)** incubated at 30°C.

In whole milk, *Listeria* propagated from 5.45 to 8.23 log CFU ml^{-1} after 24 h of incubation. However, addition of F21-powder at 167 AU ml^{-1} decreased *L. monocytogenes* counts with 1.21, 2.61 and 5.16 log units ($p \leq 0.05$) after 3, 6 and 24 h respectively. Especially, addition of F21-powder at 665 AU ml^{-1} increased inhibitory effects and decreased viable counts to undetectable values after 24 h (Figure 3A). In skimmed milk, *L. monocytogenes* was able to achieve 8.48 log CFU ml^{-1} after 24 h. However, addition of the biopreservative powder at a final concentration of 167 AU ml^{-1} decreased *Listeria* counts by 2.23, 4.22 and 8.48 log units ($p \leq 0.05$) after 3, 6 and 24 h, respectively (*Listeria* was undetectable at 24 h). In addition, the value of 665 AU ml^{-1} was most effective and increased inhibitory effects of the whole milk (differences of 0.94 and 0.73 log units at 3 and 6 h of incubation respectively) and decreased the viable counts to undetectable value after 24 h (Figure 3B). Thus, the enterocin powder was still active in milk and preserved its anti-listerial activity. Concentrations that decreased *Listeria* counts to undetectable levels included 665 and 167 AU ml^{-1} for whole and skimmed milks, respectively. Therefore, inhibitory effects were negatively affected by the fat levels. In fact, concentrations in milks (167 and 665 AU ml^{-1}) were much higher than the MIC achieved in culture media (60 AU ml^{-1}). Indeed, verified inhibitory increases in powdered enterocin could be attributed to the adsorption to fat globules of milk, which made them unavailable to destroy pathogen cells [49]. However, the fat content did not affect *L. monocytogenes* propagation ($p > 0.05$).

3.6.2.2. Effects of biopreservative powder on Jben

Mesophilic bacteria and LAB counts in the control sample increased from 3.76 and undetectable level to reach 7.15 and 4.80 log CFU g^{-1} within 15 d of storage at 8 °C, respectively. However, addition of F21-powder at a final concentration of 665 AU g^{-1} decreased the mesophilic bacteria counts with 1.21, 2.55 and 3.50 log units ($p \leq 0.05$) and LAB counts with 1.10, 1.29 and 2.17 log units ($p \leq 0.05$) with respect to control after 5, 10 and 15 d, respectively (Figure 4A, B). Concerning *L. monocytogenes* (Figure 4C), viable counts increased in control sample from 3.21 to 7.48 log CFU g^{-1} within 15 d of storage. However, *Listeria* counts were significantly lower ($p \leq 0.05$) in treated sample than the control sample.

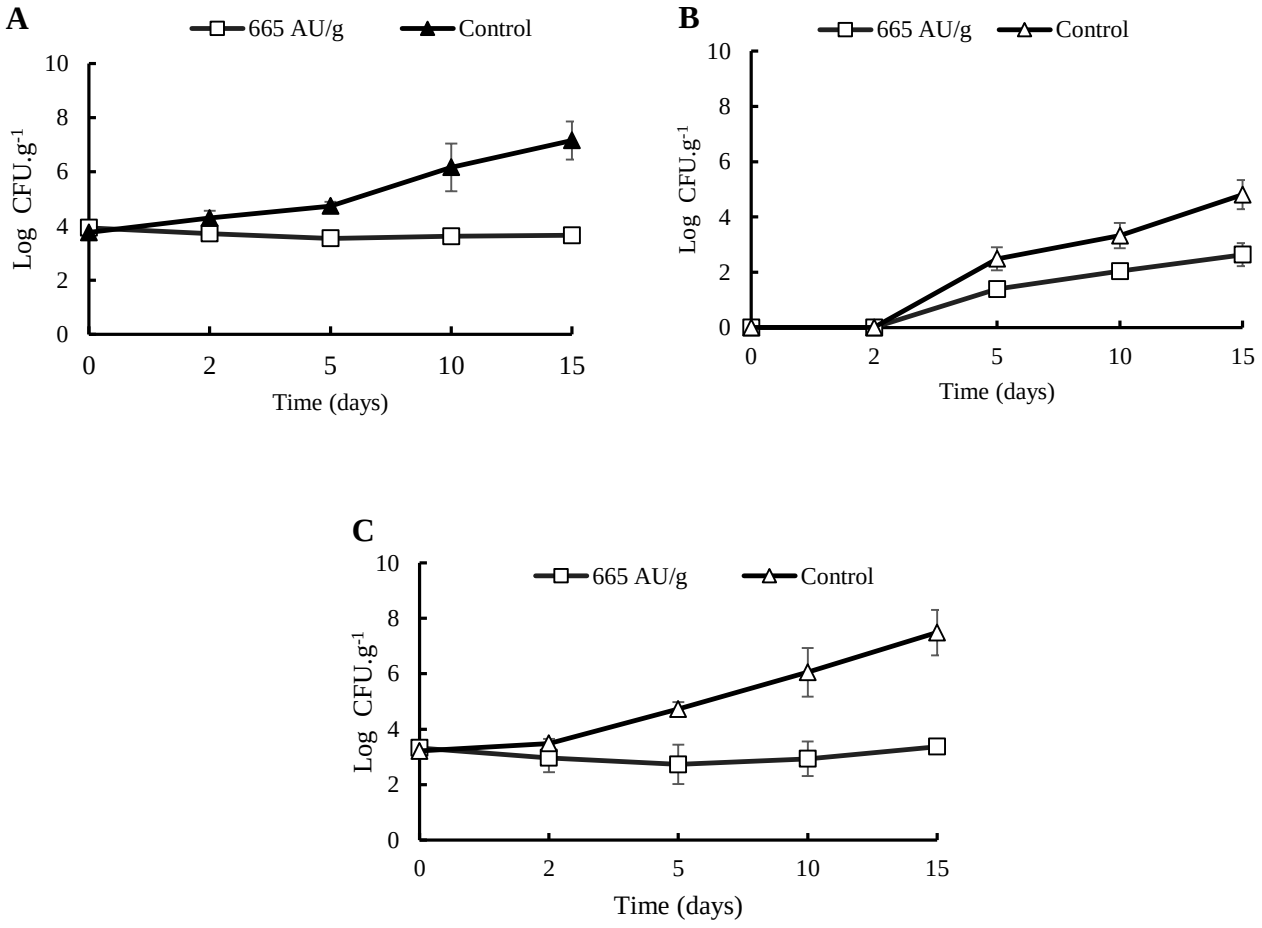

Figure 4. Effect of F21-bio-preservative powder (at 665 AU g^{-1}) in fresh cheese stored at 8 °C during 15 days. Effect on the viabiliy of mesophilic **(A)**; LAB **(B)**; and *Listeria monocytogenes* **(C).**

In fact, inhibitory effects of biopreservative powder was especially observed during the first 5 d. Moreover, *Listeria* slowly resumed its propagation after 10 d of storage. In fact, addition of enterocin-like powder significantly decreased the viable counts with 0.52, 1.99 and 3.12 log units ($p \leq 0.05$) after 2, 5 and 10 d, respectively. Use of biopreservative powder (665 AU g^{-1}) was unable to completely remove *L. monocytogenes* from the cheese samples due to the greater complexity of this matrix with respect to milk, leading to use of higher concentrations of F21-powder. Moreover, a few authors reported use of bacteriocin powder from the lyophilization process as biopreservative agents in cheeses. Indeed, use of freeze-dried bacteriocin at 500 AU g^{-1} was effective to decrease listerial propagation with approximately 3 log units in cheeses stored at 4 °C, compared to untreated samples [47,48]. Effectiveness of bacteriocins in foods has been reported previously. Indeed, it can be affected by the adsorption of bacteriocins to food constituents (e.g. lipids), slower dispersion/diffusion and solubility, irregular distribution in solid matrices, environmental conditions (not appropriate for biological activities), inactivation by other additives and development of resistance [3].

3.6.2.3. Use of biopreservative powder for ground beef preservation

Mesophilic bacteria counts in the control sample increased from 4.07 to 10.38 log CFU g^{-1} within 10 d of storage under refrigeration with an increase of 6.31 log units within 10 d, compared to 0 d. However, addition of enterocin powder (665 AU g^{-1}) significantly decreased mesophilic bacteria multiplication during the storage ($p \leq 0.05$). In fact, addition of biopreservative powder decreased 0.93, 0.75, 2.48 and 2.65 log units after 2, 4, 7 and 10 d respectively, compared to the control sample (Figure 5A). For LAB population, viable counts increased from 3.38 to 5.68 log CFU g^{-1} in the control sample after 10 d of refrigeration at 8 °C with an enhance of 2.30 log units within 10 d, compared to 0 d. However, LAB propagation was lower, compared to the control sample ($p \leq 0.05$) with F21-powder addition. Powder added at 665 AU g^{-1} decreased 0.32, 0.67, 0.76 and 1.14 log units $p > 0.05$), compared to the control after 2, 4, 7 and 10 d, respectively (Figure 5B).

For coliform bacteria, the viable counts increased from 1.58 to 7.89 log CFU g^{-1} in control sample after 10 d of storage at 8 °C with an increase of 6.31 log units within 10 d, compared to 0 d. However, addition of biopreservative powder (665 AU g^{-1}) completely decreased the coliform counts to undetectable levels through the storage time ($p \leq 0.01$) (Figure 5C).

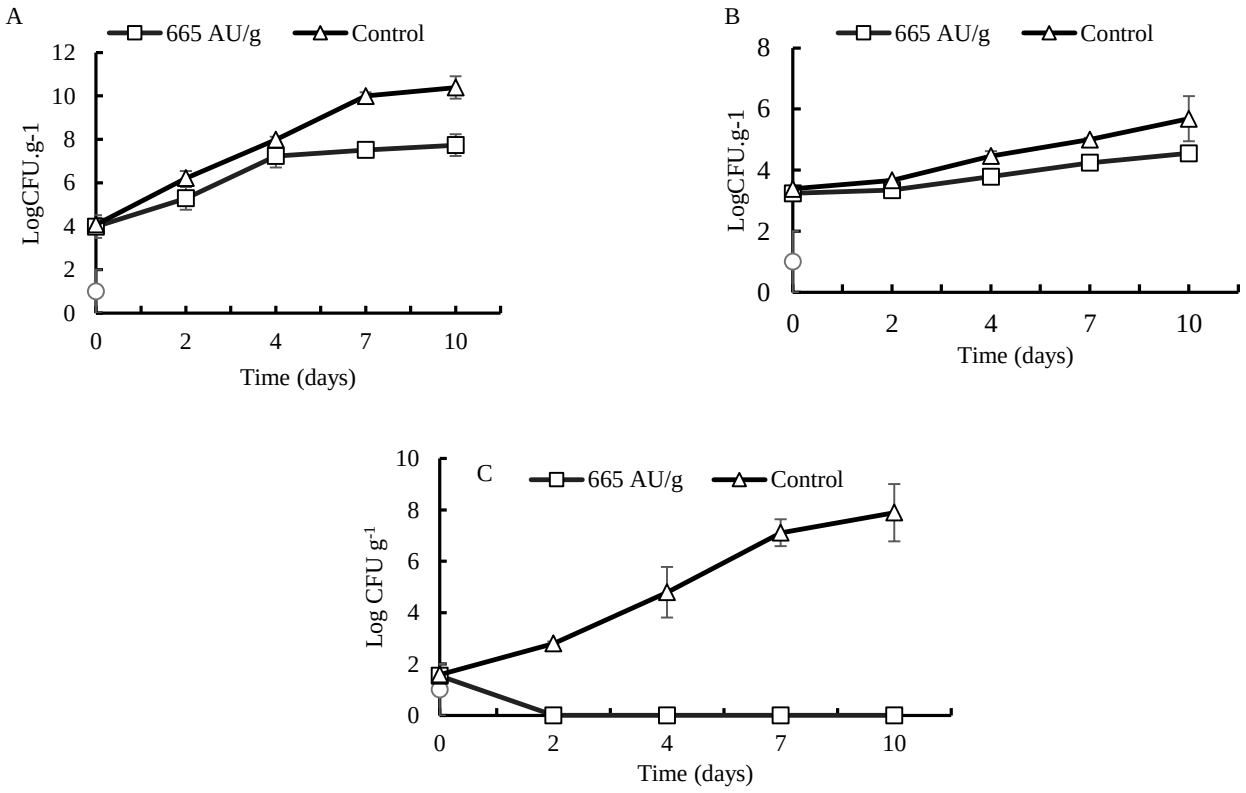

Figure 5. Effect of F21-bio-preservative powder (at 665 AU/g) in ground beef stored at 8°C during 10 days. Effect on the viabiliy of mesophilic (A) ; LAB (B) ; and coliform bacteria (C).

This result is interesting because F21-biopreservative powder was active against coliforms of Gram-negative rod-shaped bacteria, which are generally considered as resistant microorganisms to LAB-bacteriocins. In addition, these decreases in mesophilic bacteria and LAB counts of the ground beef were lower than those of the fresh cheese. Indeed, LAB bacteriocin efficacy could negatively be affected by the complexity of ground beef matrices, slower diffusion and solubility, irregular distribution in solid matrices, retention in food components and/or unfavorable environmental conditions [3].

Moreover, 6.69 log CFU g^{-1} of the total microorganisms has been considered the upper microbiological limit established by the Moroccan legislation; greater than which, the ground beef seems unacceptable for human consumption (*Bulletin Officiel* no. 5214, 20/05/2004). The mesophilic bacteria counts in control ground beef increased during the storage from 4.07 to 6.21, 7.98, 9.99 and 10.38 log CFU g^{-1} after 2, 4, 7 and 10 d, respectively. Therefore, the shelf-life was suggested as 2 d for the control ground beef. However, these counts increased to 5.28, 7.23, 7.51 and 7.73 log CFU g^{-1} after 2, 4, 7 and 10 d respectively in treated ground beef with 665 AU g^{-1} of enterocin F21 powder. Therefore, shelf life of the treated ground beef was estimated at least as 3 d (F21-treated ground beef was acceptable for human consumption at Day 3) with an increase of more than 1 d in shelf-life, compared to the control sample.

4. Conclusion

Due to the controversial use of enterococci as starter, protective culture and probiotic strains, it is necessary to assess all the characteristics (technological, probiotic and safety characteristics) for every selected strain. The present study assessed *E. durans* F21 isolated from *Lben* (a Moroccan fermented milk) as a biopreservative agent and starter culture. This strain was active against various pathogens and food spoilages and lacked undesirable characteristics, including virulence factors and the major antibacterial resistance. Indeed, *E. durans* F21 was not hemolytic, susceptible to antibacterials commonly used for the treatment of enteroccocal infections and did not produce biogenic amines and other virulence enzymes. In addition, this strain showed satisfactory biotechnological characteristics (high acidification power and antioxidant activity). These characteristics facilitate its potential use as a biopreservative agent and starter culture. Furthermore, production of anti-listerial powder (cells free) as a biopreservative agent via freeze-drying can greatly be interested to improve the food safety and extend the shelf-life of food products. This use guarantees high quantities of biopreservative powders appropriate for future uses. In fact,

this biopreservative powder was satisfactory assessed in various food systems (milk, fresh cheese and ground beef) and its addition enhanced safety and shelf life by decreasing microbiological levels of these food products to a limit less than the allowable limit established by the Moroccan legislation. Moreover, efficacy of the bacteriocin powder still needs assessments in several food systems because these powdered preparations could be affected by the physicochemical characteristics each food. However, further studies are necessary to purify and identify this enterocin and study its potential toxicity.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. Authors contributions

G.B. designed, carried out the experiments and wrote the manuscript; L.M. and E.D. contributed to sample preparation; N.C. contributed to the interpretation of the results; S.A. and A.G. conceived the original idea, planned the experiments, verified the analytical methods and supervised the project. All authors discussed the results and contributed to the final manuscript.

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ارزیابی *انتروکوکوس دورانز* F21 جدا شده از لبن، یک شیر تخمیر شده مراکشی به عنوان یک عامل نگهدارنده زیستی

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- خشک‌کردن انجمادی
- گوشت چرخکرده گاو
- لبن
- باکتری‌های لاکتیک اسید
- *لیستریا مونوسایتوژنز*

***نویسنده مسئول**
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چکیده

سابقه و هدف: باکتری‌های لاکتیک اسید که مدت طولانی در فرآوری مواد غذایی مورد استفاده قرار می‌گیرند، به‌دلیل فوایدشان برای مصرف‌کنندگان و توانایی آنها در تولید ترکیبات ضدمیکروبی طبیعی، به‌عنوان نگهدارنده‌های زیستی در غذاها استفاده می‌شوند. هدف از مطالعه حاضر شناسایی و ارزیابی سویه *F21* جدا شده از لبن (یک شیر تخمیر شده سنتی مراکشی) به عنوان نگهدارنده زیستی غذایی بود.

مواد و روش ها: جدایه *F21* از لبن جدا شد، تحت غربالگری به لحاظ فعالیت مهارکنندگی قرار گرفت و بر اساس شناسایی مورفولوژیکی، بیوشیمیایی و مولکولی شناسایی شد. سپس، تولید و ویژگی‌های فیزیکوشیمیایی ماده آنتاگونیست تعیین شد. همچنین پروفایل ایمنی و خواص بیوتکنولوژیکی جدایه مورد ارزیابی قرار گرفت. در نهایت، یک پودر نگهدارنده زیستی با فعالیت ضد میکروبی تولید و در سامانه‌های غذایی مختلف (شیر، گوشت چرخ کرده و پنیر تازه) مورد ارزیابی قرار گرفت.

یافته‌ها و نتیجه‌گیری: از باکتری‌های لاکتیک اسید جدا شده، *انتروکوکوس دورانز* F21، جدا شده از لبن (شیر تخمیر شده سنتی مراکشی)، به میزان قابل‌توجهی ترکیبات جالب و مشابه انتروسین (پایدار در برابر حرارت و مقاوم در برابر pH) فعال در برابر عوامل بیماری‌زای بالقوه و فساد مواد غذایی (*لیستریا مونوسایتوژنز*، *لیستریا اینوکوآ*، *انتروکوکوس فکالیس*، *بروکوتریکس ترموسفاکتا*، و *مایکوباکتریوم اسمگماتیس*) داشت. با توجه به ویژگی‌های ایمنی، *انتروکوکوس دورانز* F21 همولیتیک نبود، نسبت به آنتی بیوتیک‌های آزمایش شده حساس بود، قادر به تولید آمین‌های بیوژن و سایر آنزیم‌های فعال (ژلاتیناز، DNase و اوره‌آز) نبود. علاوه بر این، *انتروکوکوس دورانز* F21 ویژگی‌های زیستی رضایت‌بخشی مانند قدرت تولید اسید و اگزوپلی‌ساکاریدها و فعالیت ضداکسایشی را نشان داد. پودر نگهدارنده زیستی حاوی ماده مشابه انتروسین F21 که با خشک‌کردن انجمادی به‌دست آمد، حداقل غلظت بازدارندگی ۶۰ $AU\ ml^{-1}$ را در برابر *لیستریا مونوسیتوژنز* در محیط کشت نشان داد. علاوه بر این، این پودر نگهدارنده زیستی (با ۶۶۵ $AU\ ml^{-1}$) ایمنی و ماندگاری بسیاری از مواد غذایی (شیر، لبن و گوشت چرخ کرده گاو) را بهبود بخشد. بنابراین، این نتایج پایه‌هایی را برای استفاده بیشتر از، *انتروکوکوس دورانز* F21 به‌عنوان نگهدارنده بالقوه مواد غذایی فراهم کرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.