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Identification of lactobacilli with inhibitory effect on biofilm formation by pathogenic bacteria on stainless steel surfaces

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ABSTRACT

Two hundred and thirty individual clones of microorganisms were recovered from milk tanks and milking machine surfaces at two distinct farms (Bejaja City, Algeria). Of these clones, 130 were identified as lactic acid bacteria (LAB). In addition *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* species were identified in the remaining 100 isolates—spoilage isolate. These isolates were assayed for ability to form biofilms. *S. aureus*, *Lactobacillus brevis* strains LB1F2, LB14F1 and LB15F1, and *Lactobacillus pentosus* strains LB2F2 and LB3F2 were identified as the best biofilm formers. Besides, these LAB isolates were able to produce proteinaceous substances with antagonism against the aforementioned spoilage isolates, when grown in MRS or TSB-YE media. During the screening, *L. pentosus* LB3F2 exhibited the highest antibacterial activity when grown in TSB-YE medium at 30 °C. Additionally, *L. pentosus* LB3F2 was able to strongly hamper the adhesion of *S. aureus* SA3 on abiotic surfaces as polystyrene and stainless steel slides. LAB isolates did not show any hemolytic activity and all of them were sensitive to different families of antibiotic tested. It should be pointed out that LB3F2 isolate was not cytotoxic on the intestinal cells but could stimulate their metabolic activity. This report unveiled the potential of LB1F2, LB14F1, LB15F1, LB2F2, and LB3F2 isolates to be used as natural barrier or competitive exclusion organism in the food processing sector as well as a positive biofilm forming bacteria.

1. Introduction

Biofilms are sustainable sources of contaminations that are responsible of food-related illness and important economic losses. This lifestyle confers protection to bacterial cells and decreases the efficiency of cleaning and disinfection procedures. Consequently, the need for the development of new strategies and anti-biofilms agents allowing the prevention and control of biofilm-formation by various pathogens is of major importance in order to reduce the risk of contamination in food sector.

The process of bacterial biofilm formation is occurring in four dependent stages (Donlan, 2002). The bacterial adhesion stage is associated with the production of exopolyscharides, DNA and proteins. The initial stage of bacterial adhesion was reported to be reversible because of the weakness of the interactions between bacteria and surfaces, however this stage becomes irreversible as a result of anchoring by appendages and/or production of extracellular polymers mainly exopolysaccharides. Industrial formulations acting on the bacterial adhesion to food contact surfaces such as stainless steel are needed to reduce cross-contamination, food spoilage and transmission of diseases due to biofilms. Stainless steel is largely used in the food industries sector because of its mechanical strength, resistance to corrosion, and longevity (Marques et al., 2007).

Staphylococcus aureus is among the most common pathogenic bacteria isolated from different surfaces such as stainless steel in food processing plants (Pastoriza et al., 2002), where it can adhere and forms biofilms (Kunigk and Almeida, 2001; Archer et al., 2011; Brooks and Jefferson, 2012; Abdallah et al., 2014). Foodborne disease caused by *S. aureus* is typically intoxication due to the ingestion of enterotoxins preformed in food by enterotoxigenic strains (Normanno et al., 2007). The development of concepts and the discovery of novel anti-staphylococcal biofilm agents are expected to be promising issues in food safety. Related to this topic, different metabolites such as oregano essential oil, bacteriophage-derived peptidase, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic staphopains, β-2,2-amino acids derivatives, phenolic compounds and flavonoids have recently been described as potent anti-staphylococcal biofilm agents (Schillaci et al., 2013; Fenton et al., 2013; Luis et al., 2013; Manner et al., 2013). Strategies using biofilms produced by the competitive exclusion microorganisms to

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inactivate foodborne pathogens in the food processing environments are of major importance. Kim et al. (2013) showed the inactivation of *Escherichia coli* O157:H7 on stainless steel upon exposure to *Paenibacillus polymyxa* biofilms. Zhao et al. (2013) reported the reduction of *Listeria monocytogenes* in a ready-to-eat poultry processing plant by LAB. Pérez-Ibarreche et al. (2014) reported that lactobacilli with biofilm forming aptitudes were able to control *L. monocytogenes* biofilms.

Lactobacilli is an important part of the natural LAB consortium. They are considered as potentially antagonistic to spoilage and pathogenic bacteria because of their capabilities to produce an array of inhibitory substances such as organic acids, H_2O_2 and bacteriocins. Lactobacilli have been reported to compete with pathogens in urogenital and intestinal tracts (Reid et al., 1988), and interfere with their adhesion on catheters device (Hawthorn and Reid, 1990; Rodrigues et al., 2004). The use of lactobacilli as anti-biofilm strategies is a promising hope in food safety. Here, two groups of microorganisms were isolated from the industrial samples: LAB and non-LAB. Considering LAB natural and friendly environmental microorganisms, we term the remaining non-LAB as "spoilage," bearing in mind their possible unfriendly nature and recognizing that some of them may in fact be foodborne pathogens, such as *Salmonella*.

The objectives of this study were to explore novel and atypical ecological niches, isolate LAB with potential applications as hurdles against biofilm forming pathogens and spoilage strains.

2. Materials and methods

2.1. Isolation of lactic acid bacteria and spoilage bacteria

One hundred and twenty of samples (materials) were collected from the inner surfaces of milk tanks and milking machines in two distinct farms located at Bejaia city (Algeria). The samples were serially diluted (0.85% m/v NaCl) and plated onto the surface of MRS medium (de Man et al., 1960) (Merck, Germany), and M17 agar (Merck, Germany) plates, respectively. Plates were then aerobically and anaerobically incubated at 30 °C for 24–48 h. Gram-positive and catalase negative isolates were assumed as LAB. Basic microbiological methods were employed for characterization of non-LAB isolates. The presence of *E. coli* was tested on Eosin Ethylene Bleu agar plates (Merck), *Salmonella* clones were identified using Hektoen agar plates (Merck), and *S. aureus* isolates were selected on Chapman and Baird Parker media (Merck), followed by the assay for coagulase and DNase activities. *Pseudomonas* isolates were identified on nutritive agar (Fluka, Spain) and characterized for their pigmentation.

To keep the viability of the newly isolated strains, all of them were periodically streaked in MRS or Nutrient agar (Fluka). All isolates were kept at −80 °C in the presence of 20% glycerol (Sigma, France).

2.2. Assessment of LAB antagonism and preliminary of characterization of the inhibitory substances

The antagonism of 130 LAB isolates were tested against the aforementioned "spoilage" isolates using the spot method (Jacobsen et al., 1999) with some modifications. Briefly, 5 μl of overnight MRS cultures of LAB isolates grown in anaerobic jars (GasPak, BBL) to avoid the effect of H_2O_2, were spotted onto 1.5% MRS agar. Plates were dried for 30 min at room temperature, incubated under anaerobic conditions at 37 °C for 18 h; and overlaid with 10 ml of Brain Heart Infusion (BHI) (Merck) medium containing 0.75% agar seeded with 1% (v/v) of overnight culture of the indicator strain, leading to a final concentration of about 10^6 cells/ml. The incubation was carried out aerobically at 37 °C for 18 h. The sterile MRS broth was tested as a negative control.

To identify the inhibitory substances secreted into the growth medium, LAB isolates presenting antagonism were grown overnight at 30 °C in 20 ml MRS broth. The cell-free supernatant (CFS) obtained by centrifugation (8000 ×*g*, 20 min, 4 °C), filtered with 0.22 μm-pore-size Acrodisc® syringe filters (Pall Gelman Laboratory, USA) was separated into three samples named 1, 2 and 3. Sample 1 was directly tested; sample 2 was adjusted to pH 6.5 with 1 N NaOH (Merck-eurolab, Briare Le Canal, France) to rule out the hypothesis of acid inhibition. Sample 3 was treated with catalase (300 U/ml) (C-3515, Sigma-Aldrich Chemie, Steinheim, Germany) to rule out the hypothesis of inhibition by the H_2O_2. The antagonistic activities of these three samples were determined at least in triplicate for each LAB isolate using the well diffusion assay (Ennahar et al., 1998). The absence or presence of any inhibitory zone was recorded after 18 h of incubation at 37 °C. The CFS obtained from the antagonistic LAB isolates were treated with proteases such as α-chymotrypsin, proteinase K and papain (Sigma-Aldrich Chemie, Steinheim, Germany). Each CFS was adjusted to pH 6.5 with 1.0 mol/l NaOH (Sigma), filter sterilized (0.22 μm), treated with proteases at 1.0 mg/ml and left for 1 h at 30 °C. The treated and untreated CSF (control samples) were heated at 100 °C for 5 min and then immediately cooled in ice to inactivate the proteases. The residual activity of treated and untreated samples was determined by measuring the diameter of inhibition zones according to the above cited methods.

2.3. Biomolecular typing of the antagonistic LAB isolates

The identification of the antagonistic LAB isolates was carried out by the API 50 CHL system (Biomérieux, France), and the 16S rDNA sequence analysis. For the last method, total DNA was extracted from LAB isolates with the Wizard® Genomic DNA purification Kit (Promega Corp., France). The amplification of 16S rDNA was done with primers S1 and S2 (Table 1), and the following PCR program: denaturation at 95 °C/3 min, 29 cycles at 94 °C/40 s, annealing at 55 °C/50 s and extension at 72 °C/1 min, followed up by a final extension cycle at 72 °C/10 min. The PCR amplicons were separated on 0.6% (w/v) agarose gel upon electrophoresis carried out at 100 V for 1 h. The amplicons were purified, cloned into the pGEM®-T Easy Vector System (Promega Corp., France), and transferred to *E. coli* JM109 strain. The recombinant plasmids containing the 16S rDNA were extracted by GeneJET plasmid Miniprep (Fermentas), and sequenced at Eurofins MWG Operon (Germany). The resulting sequences were assembled into a unique contig with BioEdit sequence alignment software and analyzed using the NCBI-Standard Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov). Alignment with known 16S rDNA sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BL ASTU) was done with the basic local alignment search tool, an online software. The sequences have been deposited in gene banks and were assigned the following accession numbers: KF923749, KF923750, KF923751, KF923752 and KF923753.

LAB isolates with the highest antibacterial activity were characterized at the molecular level using a specific Lactobacilli PCR previously described by Dubernet et al. (2002), and a REP-PCR fingerprinting technique (Al Kassaa et al., 2014). The specific Lactobacilli PCR required the use of primers LbLMA1 and R16-1 (Table 1) and the following PCR program: (i) denaturation at 95 °C/5 min, (ii) 34 cycles of denaturation at 94 °C/1 min, annealing at 55 °C/1 min and extension at 72 °C/1 min, followed by (iii) a final extension at 72 °C/5 min. The 50 μl reaction mixture contained 25 μl of DreamTaq™ Green PCR Master Mix 2X (dNTPs, $MgCl_2$ 1.5 mM), reaction buffer DreamTaq™ polymerase (Fermentas), 2 μl of each primer (20 mM) and 5 μl of DNA sample of

Table 1
Primers used in this work.

Primers	Sequences 5′-3′	References
Primer S1	5′-AGAGTTTGATC(A,C)TGGCTCAG-3′)	Dubernet et al., 2002 /
Primer S2	5′-GG(A,C)TACCTTGTTACGA(T,C)TTC-3′	Messaoudi et al., 2011
LbLMA1	5′-CTCAAAACTAAACAAAGTTTC-3′	Dubernet et al., 2002 /
R16-1	5′-CTTGTACACACCGCCCGTCA-3′)	Messaoudi et al., 2011
$(GTG)_5$	5′-GTGGTGGTGGTGGTG-3′	Al kassaa et al., 2014