Exploratory Tests of Crude Bacteriocins from Autochthonous Lactic Acid Bacteria against Food-Borne Pathogens and Spoilage Bacteria

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Abstract—The aim of the present work was to test in vitro inhibition of food pathogens and spoilage bacteria by crude bacteriocins from autochthonous lactic acid bacteria. Thirty autochthonous lactic acid bacteria isolated previously, belonging to the genera: Lactobacillus, Carnobacterium, Lactococcus, Vagococcus, Streptococcus, and Pediococcus, have been screened by an agar spot test and a well diffusion assay against Gram-positive and Gram-negative harmful bacteria: Bacillus cereus, Bacillus subtilis ATCC 6633, Escherichia coli ATCC 8739, Salmonellatyphimurium ATCC 14028, Staphylococcus aureus ATCC 6538, and Pseudomonas aeruginosaa under conditions means to reduce lactic acid and hydrogen peroxide effect to selected bacteria with high bacteriocinogenic potential. Furthermore, crude bacteriocinssemiquantification and heat sensitivity to different temperatures (80, 95, 110°C, and 121°C) were performed. Another exploratory test concerning the response of St. aureus ATCC 6538 to the presence of crude bacteriocins was realized. It has been observed by the agar spot test that fifteen candidates were active toward Gram-positive targets strains. The secondary screening demonstrated an antagonistic activity oriented only against St. aureus ATCC 6538, leading to the selection of five isolates: Lm14, Lm21, Lm23, Lm24, and Lm25 with a larger inhibition zone compared to the others. The ANOVA statistical analysis reveals a small variation of repeatability: Lm14: 0.56%, Lm23: 0%, Lm25: 1.67%, Lm14: 1.88%, Lm24: 2.14%. Conversely, slight variation was reported in terms of inhibition diameters: 9.58± 0.40, 9.83± 0.46 and 10.16± 0.40 mm. Anova analysis in the second trials, for Lm23, Lm25, Lm14 and Lm24 indicating that the observed potential showed a heterogeneous distribution (BMS = 0.383, WMS = 0.117). The repeatability coefficient calculated displayed 7.35%.

As for the bacteriocinssemiquantification, the five samples exhibited production amounts about 4.16 for Lm21, Lm23, Lm25 and 2.08 AU/ml Lm14. The repeatability coeffici ent calculated displayed 7.35%. The ANOVA statistical analysis reveals a small variation of repeatability: Lm14: 0.56%, Lm23: 0%, Lm25: 1.67%, Lm14: 1.88%, Lm24: 2.14%. Conversely, slight variation was reported in terms of inhibition diameters: 9.58± 0.40, 9.83± 0.46 and 10.16± 0.40 mm for, Lm23, Lm25, Lm14 and Lm24 indicating that the observed potential showed a heterogeneous distribution (BMS = 0.383, WMS = 0.117). The repeatability coefficient calculated displayed 7.35%.

I. INTRODUCTION

Lactic acid bacteria constitute a group of genus that have in common a number of characteristics: cocci, rods, with a basic composition of DNA below 50 mol % G+C. Gram-positive, typically mesophilic which can grow at temperatures ranging from 5 to 45°C, under aerobic, anaerobic or microaerobic terms and are asporogenous. Furthermore, they are oxidase and catalase negative, do not reduce nitrate to nitrite, and do not produce indole or hydrogen sulfide. This group consists of a number of genus: Lactobacillus, Leuconostoc, Pediococcus, and Lactococcus, among others, inhabit a wide range of natural environment such as various food products as well as members of the normal flora of mouth, intestinal and vaginal flora of mammals [1]-[3]. Lactic acid bacteria are harmless for human which has led to the recognition of their G.R.A.S status "Generally Recognized as Safe" [4]-[6]. Otherwise, some usually associated with food have been implicated in food diseases [1]. However, it should be noticed that this is probably opportunist infections [7]. Since they have beneficial effects, medical and food industries are best known for their use: as probiotic cultures, suggesting the use of them to provide a transient intestinal flora, to compete with potentially harmful bacteria, and to prevent from some diseases [1]. So exert health benefits beyond inherent basic nutrition [8]; as protective cultures that have gained increasing attention as means of naturally enhancement of food safety and stability. Their application is a promising tool [5], [8]-[10]. These abilities are often due to, or enhanced by, the production of potent antimicrobial agents. The most commonly known are bacteriocins [11]; as starter cultures when are used to change the sensory properties and to provide food diversity [8], [10]. Bacteriocins have often been mootted as potentially food-grade to improve food safety and reduce the prevalence of foodborne diseases [11] also could help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties [10]. These bio tools can be used at least by three ways among theme as a crude bacteriocin-preparation concentrated, though not purified [11], [12]. Many lactic acid bacteria genera excrete bacteriocins, described as "Proteins or protein complexes with bactericidal activity against species usually closely related to the producer bacterium" [13], or "confined within the same ecological niche" [14], a definition which is the most widely accepted [6]. Through biochemical and genetic characterizations, four major classes of...
bacteriocins have been listed [14]: (I) lantibiotics, (II) Small heat stable peptides (III) Large heat-labile proteins (IV) complex/bacteriocins. The majority fall into classes I and II. [11] These ribosomally synthesised proteinaceous compounds are bactericidal only toward Gram-positive bacteria, which can be explained by the protective barrier of the additional layer of Gram-negative composed of phospholipids, proteins and mainly by lipopolysaccharides “L.P.S” [6], [15], [16]. It is generally accepted that bacteriocins exert their inhibitory action by formation of pores in the cytoplasmic membrane of Gram-positive bacteria. These cells differ in their sensitivity mainly because of difference in membrane composition and fluidity [8], [11]. Self-evidently, bacteriocin producers exhibit specific immunity against their bacteriocin. This is accomplished by the production of dedicated immunity proteins. [17], [11]. Moreover, several methods to determine their activity have been described. However, growth inhibition techniques are still the most commonly used in usual trials relying on tests performed either in solid medium, as the Spot on the lawn and well diffusion assay in which a microorganism is challenged for an arbitrary period; or in broth by measure turbidity increases over time[18]-[22]. Both techniques help to determine the approximate effective concentration and evaluate the effect of the antimicrobial compound on microorganism growth. These provide preliminary information about the potential antimicrobial activity and the usefulness of the test compound in a food. [22]. This study gather exploratory tests for activity assays of crude bacteriocins from autochthonous lactic acid bacteria, by screening with endpoint tests to provide primary qualitative information on a possible antimicrobial activity followed by a descriptive test leading to quantitative information on the food pathogen and spoilage growths.

II. MATERIAL AND METHODS

A. Target Strains and Growth Media

Active or test strains used were isolated in a previous study, these are lactic acid bacteria belonging to genera: Lactobacillus, Carnobacterium, Lactococcus, Vagococcus, Streptococcus, and Pediococcus. The stock culture collection was maintained frozen at -18°C in 20% of glycerol [23]. Through these cultures, the working ones were prepared as slants agar on MRS (De Man., Rogosa& Sharpe, Fluka®). Then, maintained at 4°C for short-term use and transferred monthly for a maximum of six transfers before a new working culture was performed. Passive or targets strains were six indicator pathogens mentioned I1, I2, I3, I4, I5, I6, which are considered as pure cultures from the west unit of National Laboratory of Pharmaceutical Products “L.N.C.P.P” (ORAN) including Gram-positive and Gram-negative strains: B. cereus (I1), B. subtilis ATCC 6633(I2)and St. aureus ATCC 6538 (I5). The well diffusion assay has been used [30]. Test strains were subject to culture at 37°C for 24h on MRS broth (MRS containing only 0.2% of glucose) to minimizing the production of lactic acid and select a bacteriocin producer. The cultures were centrifuged 6000 rpm i.e. 3461×g for 20min [31], crude bacteriocin solutions obtained were sterilized by heated at 80°C for 10min followed by a rapid cooling at 4°C to eliminate vegetative forms [32]. 10ml of TSAYE base agar (1.5% agar) were poured into petri dishes. Each plate was seeded by six candidates spaced approximately 3cm apart. The incubation was performed at 30°C for 24h, under microaerophilic conditions to minimize the formation of H2O2 and organic acids such as lactic acid. The lecture of results was performed by placing the petri plates with the lid down on the colony counter equipped with a lighting system. The presence of a clear zone of 0.5mm or larger around the spots reflected antagonist activity [20]. Diameters were measured with a millimetre ruler, the experiment was repeated twice.

D. Well Diffusion Assay

Test strains, showing activities in the previous screening, were investigated on their antibacterial properties towards B. cereus (I1), B. subtilis ATCC 6633(I2) and St. aureus ATCC 6538 (I5). The well diffusion assay has been used [30]. Test strains were subject to culture at 37°C for 24h on MRS broth (MRS containing only 0.2% of glucose) to minimizing the production of lactic acid and select a bacteriocin producer. The cultures were centrifuged 6000 rpm i.e. 3461×g for 20min [31], crude bacteriocin solutions obtained were sterilized by heated at 80°C for 10min followed by a rapid cooling at 4°C to eliminate vegetative forms [32]. 10ml of TSAYE base agar (1.5% agar) were poured into petri dishes. After solidification of the agar at room temperature, 10ml of TSAYE top agar (0.75% agar) was seeded with 0.3ml of indicator culture with a bacterial load comparable to 0.5McFarland standard and added to the agar surface. A short incubation at room temperature at about 20°C served to
harden the gel, which facilitates the subsequent cutting of wells [33]. Seven wells of 5mm diameter per plate spaced approximately 3cm apart were realized. Two lactic acid bacteria cultures were tested per plate, three wells each, filled with a volume of 30µl of culture supernatant containing bacteriocin. The seventh well in the centre as a control was filled with 30µl MRSⅠ₂. The plates were then put at +4°C for a period of pre-diffusion in room temperature at 20°C for 2h [21]. The plates were incubated at 30°C for 24h under microaerophilic conditions. Lecture of results was made on the colony counter equipped with a lighting system. Finding of an inhibition zone at the periphery of the wells of 0.5mm or larger suggests antibacterial activity due to the production of antimicrobial compounds of a protein nature. The experiment was repeated twice.

E. Bacteriocin Semi quantification

Test strains showing proteinaceous antibacterial activity have been subject to determine their bacteriocin's concentration, expressed in Arbitrary Units per milliliter (AU/ml), against St. aureus ATCC 6538. Crude bacteriocin solutions have been prepared as indicated before. The well diffusion assay as described above was used. Nine wells of 5mm in diameter per plate spaced approximately by 3cm were made. Two-fold dilutions of crude bacteriocin solutions were successively released using MRSⅠ₂ broth as diluent (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64). Each well was filled with 30 µl of supernatant containing bacteriocin diluted. The ninth well in the centre as control was filled with same volume of MRSⅠ₂ Dilution that resulted in the disappearance of the inhibition zone corresponds to the minimum inhibitory dose of pathogen growth indicator [34], [35].

F. Temperature Sensitivity

Crude bacteriocin solutions were tested for their sensitivity to different temperatures. The well diffusion assay as described above was used. Nine wells with a diameter of 5mm spaced approximately by 3cm were realized, then filled with 30µl of Crude bacteriocin solutions treated at different temperatures: 80, 95, 110°C in a water bath and at 121°C using an autoclave for 10min then followed by a flash-cooling at +4°C. The ninth well located at the centre served as a control filled with the same amount of MRSⅠ₂ broth.

G. Growth Kinetics Measurements

To prepare inocula, test strains were grown overnight at 37°C in MRS broth and crude bacteriocins solutions were done as mentioned above. St. aureus ATCC6538 used as a challenge strain has been regenerated in TSB at 37°C for 24h to obtain a young culture used to inoculate two batches of 30 ml of TSB broth so as 0.5 McFarland standard adjusted at A 450nm. Each batch was added with crude bacteriocin solutions at 6.24 AU/ml and 4.2 AU/ml for Le21, Le23, Le25 and Le14, Le24 in the first and at 12.48 AU/ml and 8.4 AU/ml for Le21, Le23, Le25 and Le14, Le24 in the second. These batches and a control batch were carried out in 100-ml Erlenmeyer flasks, clogs with cotton and incubated at 30°C with agitation (Reciprocating shakers GFL® 3006) at 100 rpm in order to give homogeneity and to reduce formation of PO₂ gradients. [36]. Periodic sampling of 1ml during each hour were removed, to determine changes on growth over 10 hours with a spectrophotometer (Spectronic© 1201) at A 450nm. TSB broth was used for calibration.

H. Statistical Analyses

One-way ANOVA analysis of variance was used to evaluate differences between means square (WMS and BMS) of the antibacterial activity. Calculations and growth curves were carried out using OriginLab© Pro 8 SRO Version 8.0724 (B724) [37].

III. RESULTS AND DISCUSSION

At present, there is great concern that lactic acid bacteria could act as a reservoir for antibacterial bio agents as an alternative to conventional preserves to improve stability and safety of foods. For this purpose, 30 lactic acid bacteria obtained from food habitat were characterized of their potent antagonistic towards spoilage and pathogenic bacteria to select candidates most active. Moreover, Listeria monocytogenes and St. aureus are the two bacterial species commonly used as target strain for screening antimicrobial agent generally, and specifically the bacteriocins produced by lactic acid bacteria in question [7]. Furthermore, to the best of our knowledge, many antagonism studies toward pathogens remain sporadic in Algeria. Thus, the selection of the most promising candidates is based on screening in vitro providing qualitative information on primary possible antimicrobial activity [38]. The screening is carried out firstly on solid medium in order to detect inhibition of growth of an indicator strain caused by the test strain [28]. In this context, different techniques are available, which most of them share two variants of protocols originally described; spot on the lawn and well diffusion assay [19], [20], [21]. However it is important to test the inhibitory activity by both techniques [28]. When evaluating the antibacterial activity under conditions of palliative effect of lactic acid and hydrogen peroxide (TSAYE, MRSⅠ₂, and semi anaerobic), the agar spot test indicates a half of the isolates with antagonist activity appears bacteriocenogenic toward one or more indicator strains, mainly Gram-positive: B. cereus (11), B. subtilis ATCC 6633(12) and St. aureus ATCC 6538 (15). In fact, this result is not surprising, given the ineffectiveness of bacteriocinogenic cultures toward the Gram-negative which has been reported previously [39] and that this inefficiency is attributed to the disqualification of such molecules to disrupt the protective barrier provided by lipopolysaccharids "L.P.S" [15], [16]. In contrast, all bacteriocins produced by lactic acid bacteria described so far have an activity against Gram-positive [6]. In addition, the well diffusion assay indicates the ability of third isolates to show a sure potent bacteriocenogenic; Pediococcus spp: Le14, Le21, Le23, and Le25 and Lactobacillus: Le24, these five isolates thus limit the growth of pathogen St. aureus ATCC6538.
Fig. 1 Well Diffusion assay of (a) Lm14 and Lm25, (b) Lm23 and Lm21, (c) Lm24 and Lm30 against Staphylococcus aureus ATCC 6538. A clear zone of 0.5 mm or more radially extending at the periphery of the wells after incubation for 1 day at 30°C was measured (two perpendicular diameters).

Similarly, this result is not surprising due to the fact that, generally, the technique spot on the lawn always reveals antagonistic activity with a higher proportion compared to that observed by the well diffusion assay [19]. As reported by [16] that St. aureus CTC33 is the most sensitive indicator comparing to the others. It was cited that lactic acid bacteria have the potential antagonist activity against St. aureus through bacteriocins [7], [38].

The evaluation of the antimicrobial activity revealed a small variation of repeatability (giving lower limit of the variability of results) indicating a fairly inner dispersion until null among these results in homogeneous coefficients of repeatability: Lm21: 0.56%, Lm23: 0%, Lm25: 1.67%, Lm14: 1.88%, Lm24: 2.14%. Conversely, slight variation was reported in terms of inhibition diameters: 9.58 ± 0.40, 9.83 ± 0.46 and 10.16 ± 0.24 8.5 ± 0.40 10 mm for, Lm21, Lm23, Lm25, Lm14 and Lm24, indicating that the observed potential knows a heterogeneous distribution (BMS = 0.383, WMS = 0.117). The repeatability coefficient showed 7.35%. As for the bacteriocins semiquantification, the five candidates exhibited production amounts of 4.16 and 2.08 AU/ml for Lm21, Lm23, Lm25 and Lm14, Lm24 in the same order. According to a recent study; [16], 93.8% of their strains proved producing bacteriocins active against St. aureus CTC33, with 93% having a mean inhibition in the interval (3mm < area < 5mm) and only 0.8% represented by one strain shows a high inhibition (area > 5mm). In addition, [40] accomplish after antagonism test on an inhibition zones up to 4 and 8mm (diameter wells not included), which agrees with our results.

Quantification of bacteriocins of the five samples was 4.16 and 2.08 AU/ml for Lm21, Lm23, Lm25 and Lm14, Lm24 in the same order [34], [35]. These results are similar to those of strains having bacteriocin activity reaching from 2 to 8 AU/ml [16]. Concerning the temperature sensitivity, the crude bacteriocins were fully insensitive to heat inactivation, until 121°C, preserving the same inhibition diameter, suggesting that these bacteriocins belonging to the class II [5], [6], [14], [15], [41]-[42] which is among the most likely to be used in food applications due to their specificity [5].

Secondly a descriptive test was carried out in order to determine the effectiveness of cultures, most efficient with a potent bacteriocinogenic. Such determination seemed necessary for quantitative information of the impact of on the dynamics of pathogen growth in question. In addition, growth of bacterial populations is tri-phasic taking into account the positives phases: lag phase, acceleration phase and exponential phase. These phases can be described quantitatively by Lag time, the maximum specific growth rate "μmax" and the asymptote "ymax" defined as the maximum microbial load reached during bacterial growth [43]-[44].

<table>
<thead>
<tr>
<th>Lactic acid bacteria</th>
<th>Diameter ± DS (mm)</th>
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<tbody>
<tr>
<td>L14</td>
<td>8.5 ± 0.40 (1.88%)</td>
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<tr>
<td>L21</td>
<td>10.16 ± 0.24 (0.56%)</td>
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<tr>
<td>L23</td>
<td>10 ± 0 (0%)</td>
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<tr>
<td>L24</td>
<td>9.83 ± 0.46 (2.14%)</td>
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<tr>
<td>L25</td>
<td>9.58 ± 0.40 (1.67%)</td>
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*Coefficients of repeatability
The case of this study $\mu_{max}$ was selected, being sufficient to appreciate responses of the pathogen in the presence of crude bacteriocins. Further, different classes of predictive models for the growth of animal/microbial population exist, either for the study of population in exponential growth or microbial sigmoidal. As these models estimates this slightly different [45]. Thereby these growth parameters can be used to describe the inhibitory effect of some bioactive products [46]. Moreover, food extension shelf life and safety are dictated by the time required by spoilage or pathogenic microorganisms to reach a critical level, this is why the estimation of $\mu_{max}$ is both necessary and of great importance in predictive microbiology [36]. Our results confirmed the desired effect: first a dose-response relationship clearly detectable between the two trials of the crude bacteriocins, at 6.24 AU/ml and 4.2 AU/ml for Lm21, Lm23, Lm25 and Lm14, Lm24 in the first and at 12.48 AU/ml and 8.4 AU/ml for Lm21, Lm23, Lm25 and Lm14, Lm24 in the second. The second trials showeda high potential as inhibitors compared to the first. The $\mu_{max}$, estimated by the
Baranyimodel, for the control was 0.699, those for Lm21, Lm23, Lm25, Lm14, and Lm24 were of: 0.532, 0.460, 0.545, 0.714, 0.656 in the first trails, and of about 0.399, 0.111, 0.080, 0.377, and 0.489 in the second trails. This showed reductions in pathogens load for Lm21, Lm23, Lm25, Lm14, and Lm24 of a 23.89%, 34.19%, 22.03%, 0%, 29.97% in the first trails, and of 42.92%, 84.12%, 88.55%, 54.95%, 29.97% in the second trails, compared to the control. The growth rate of batches Lm23 and Lm25 seemed similar. The largest bacterial regression trails, compared to the control. The growth rate of batches Lm21, Lm23, Lm25, Lm14, and Lm24 were of: 0.532, 0.460, 0.545, 0.714, 0.656 in the first trails, and of about 0.399, 0.111, 0.080, 0.377, and 0.489 in the second trails. This showed reductions in pathogens load for Lm21, Lm23, Lm25, Lm14, and Lm24 of a 23.89%, 34.19%, 22.03%, 0%, 29.97% in the first trails, and of 42.92%, 84.12%, 88.55%, 54.95%, 29.97% in the second trails, compared to the control. Similar results were observed for batches Lm23 and Lm25. It is important to mention that adding those cured bacteriocins kill (destroy is better than kill) a great deal of the pathogenic St. aureus ATCC 6538, mainly in the two hours of growth. Certain antimicrobials may still cause reduced maximum specific growth rate or even initial lethality followed by growth[22].

IV. CONCLUSION

The main purpose of this study was to characterize the bacteriocinogenic potential of 30 autochthonous lactic acid bacteria, against food pathogens and spoilage bacteria, to use them or their metabolite namely bacteriocins as antibacterial bio agents in order to improve stability, extension shelf life, and safety of foods. Hence, this screening showed antagonistic activity with proteinaceous compounds only towards Gram- and safety of foods. Hence, this screening showed antagonistic activity with proteinaceous compounds only towards Gram-

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