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Exploratory Tests of Crude Bacteriocinsfrom 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 aeruginosa* under conditions means to reduce lactic acid and hydrogen peroxide effect to select 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: L_{m14}, L_{m21}, L_{m23}, L_{m24}, and L_{m25} with a larger inhibition zone compared to the others. The ANOVA statistical analysis reveals a small variation of repeatability: L_{m21}: 0.56%, L_{m23}: 0%, L_{m25}: 1.67%, L_{m14}: 1.88%, L_{m24}: 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, L_{m21}, L_{m23}, L_{m25}, $L_{m14}$and L_{m24}, 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 L_{m21}, L_{m23}, L_{m25} and 2.08 AU/ml for L_{m14}, L_{m24}. Concerning the sensitivity the crude bacteriocins were fully insensitive to heat inactivation, until 121°C, they preserved the same inhibition diameter. As to, kinetic of growth , the μmax showed reductions in pathogens load for L_{m21}, L_{m23}, L_{m25}, L_{m14}, L_{m24} of about 42.92%, 84.12%, 88.55%, 54.95%, 29.97% in the second trails. Inversely, this pathogen growth after five hours displayed differences of 79.45%, 12.64%, 11.82%, 87.88%, 85.66% in the second trails, compared to the control. This study showed potential inhibition to the growth of this food pathogen, suggesting the possibility to improve the hygienic food quality.

***Keywords*—**Exploratory test, lactic acid bacteria, crude bacteriocins, spoilage, pathogens.

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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 genera: *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 mooted 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

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bacteriocins have been listed [14]**:** (I) lantibiotics, (II) Small heat stable peptides (III) Large heat-labile proteins (IV) complexbacteriocins.The majority fall into classes I and II. [11] Theseribosomally 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 cytoblasmic 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 Control of Pharmaceutical Products "L.N.C.P.P" (ORAN) including Gram-positive and Gram-negative strains: *B. cereus* (I1), *B. subtilis*ATCC 6633 (I2), *E. coli* ATCC 8739 (I3), *S. typhimurium*ATCC 14028 (I4), *St. aureus*ATCC 6538(I5), *P. aeruginosa*(I6). These strains were regenerated in TSB (Trypticase Soy Broth, Difco®) at 37°C for 24h, subcultured on plates containing nutrient agar at the same temperature and maintained on nutrient agar slants at 4°C for short-term use, then transferred for every six months [24]. The strains were subjected to Gram staining and tested for catalase production[25], [26].

B. In vitro Screening

This step involved the selection of test strains able to inhibit the growth of the pathogenic and spoilage bacteria mentioned above. This screening was used for the selection of bacteriocinogenic candidates, initially using a direct test by the spot agar test followed by another no direct test as the well diffusion assay [27].

C. Agar Spot Test

This initial screening was achieved to confirm the presence of a putative antagonistic action among the test strains toward the six pathogens, as mentioned in [16], [20]. 12ml of TSA-YE (Tryptic Soy Agar: pancreatic digest of casein17g/l, NaCl 5g/l, papaic digest of soybean meal 3g / l, K_2HPO_4 2.5g/l, glucose 2.5g/l, agar 15g/l; supplemented with 0.6% Yeast Extract), was poured in petri dishes and 100ml aliquots of bacterial suspension of the passive culture was spread on surface, with a microbial load adjusted to 0.5 McFarland [28] at absorbance of 450nm corresponding to a cell concentration of 1 to 2×108 CFU [29]. Passive cultures were previously seeded in TSB at 37°C for 24h. Their standardization was done according to the principle of dilution using saline-peptone water (Na Cl 8.5g/l; bactopeptone 1g/l) as a diluent. Active cultures, previously inoculated in MRS broth at 30°C for 48h, were then inoculated by single spot on the surface. 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 H_2O_2 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 $MRS_{-0.2}$ broth (MRS containing only 0.2% of glucose) to minimizing the production of lactic acid and select a bacteriocin producer. The cultures were centrifugedat 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

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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 $MRS_{-0.2}$. The plates was 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 showingproteinaceous antibacterial activity have been subject to determinate theirs 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 $MRS_{-0.2}$ 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 $MRS_{-0.2}$. 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 $MRS_{-0.2}$ broth.

G. Growth Kinetics Measurements

To prepare inocula, test strains were grown overnight at 37°C in MRS broth and crude bacteriocins solutions was done as mentioned above.*St. aureus*ATCC6538used 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 L_{m21}, L_{m23}, L_{m25} and L_{m14}, L_{m24} in the first and at 12.48 AU/ml and 8.4 AU/ml for L_{m21}, L_{m23}, L_{m25} and L_{m14}, L_{m24} 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 PO_2 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, $MRS_{-0.2}$, 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* (I1), *B. subtilis*ATCC 6633(I2)*and St. aureus*ATCC 6538 (I5).In fact, this result is not surprising, given the ineffectiveness of bacteriocenogenic 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 bacteriocinogenic;*Pediococcus* spp: L_{m14}, L_{m21}, L_{m23}, and L_{m25} and *Lactobacillus*sp: L_{m24},these five isolates thus limit the growth of pathogenic *St. aureus*ATCC6538.

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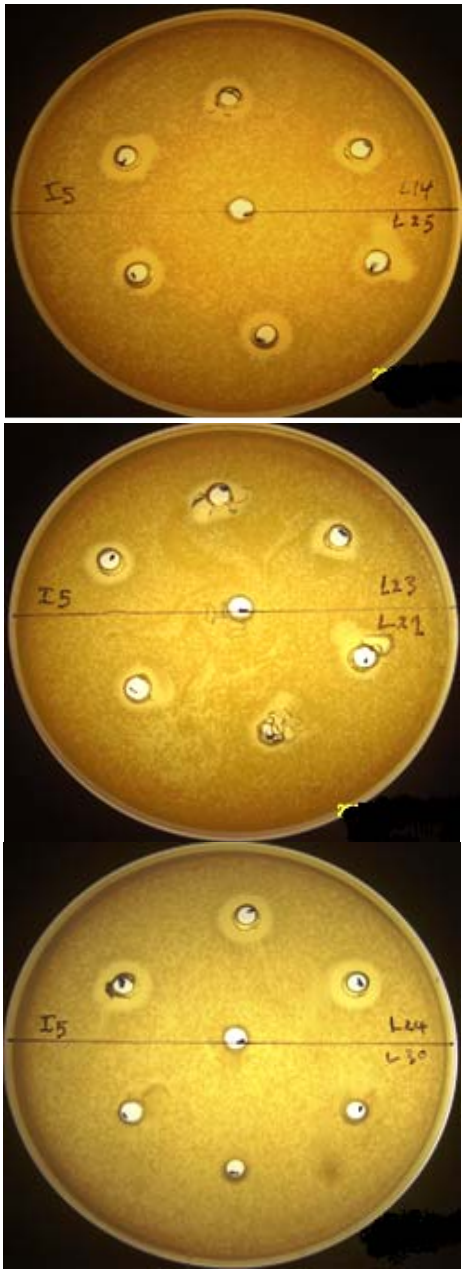


Fig. 1 Well Diffusion assay of (a) L_{m14} and L_{m25}, (b) L_{m23} and L_{m21}, (c) L_{m24} and L_{m30} against *Staphylococcus aureus*ATCC 6538. A clear zone of 0.5 mm or more radially extending at the periphery of the wells after incubation for 1 day at 30°C was measured (two perpendicular diameters)

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: L_{m21}: 0.56%, L_{m23}: 0%, L_{m25}: 1.67%, L_{m14}: 1.88%, L_{m24}: 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, L_{m21}, L_{m23}, L_{m25}, $L_{m14}$and L_{m24}, indicating that the observed potential knows a heterogeneous distribution (BMS = 0.383, WMS = 0.117). The repeatability coefficient showed 7.35%.As for the bacteriocinssemiquantification, the five candidates exhibited production amountsof 4.16 and 2.08 AU / ml for L_{m21}, L_{m23}, L_{m25} and L_{m14}, L_{m24} in the same order. According to a recent study; [16], 93.8% of their strains proved producing bacteriocins active against *St. aureus* CTC 33, 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.

Quantificationof bacteriocins of the five samples was 4.16 and 2.08 AU/ml for L_{m21}, L_{m23}, L_{m25} and L_{m14}, L_{m24} 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 those 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 I
DIAMETER OF THE INHIBITION ZONE, WELL DIFFUSION ASSAY AFTER 1 DAY AT 30 ° C

Lactic acid bacteria	Diameter ± DS ([a])
L14	8.5 ± 0.40 (1.88%)
L21	10.16 ± 0.24 (0.56%)
L23	10 ± 0 (0%)
L24	9.83 ± 0.46 (2.14%)
L25	9.58 ± 0.40 (1.67%)

[a]Coefficients of repeatability

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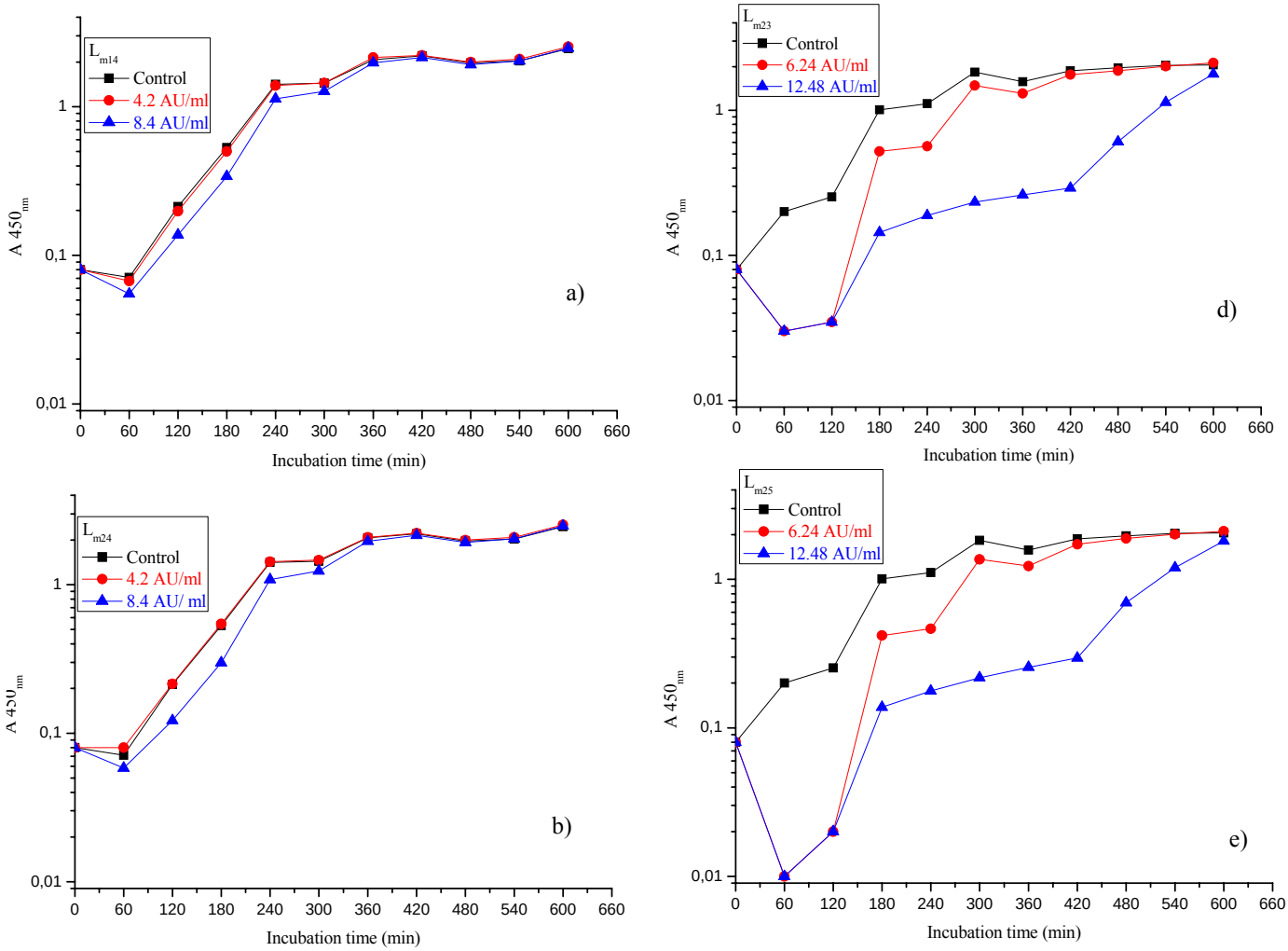


Fig. 2 Growth curves inhibition of *Staphylococcus aureus*ATCC 6538by crude extract of a) L_{m14}, b) L_{m24}, c) L_{m21}, d) L_{m23} and e) L_{m25}. Incubated at 30 °C with agitation at 100 rpm

The case of this study *μmax* 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 *μmax* 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 L_{m21}, L_{m23}, L_{m25} and L_{m14}, L_{m24} in the first and at 12.48 AU/ml and 8.4 AU/ml for L_{m21}, L_{m23}, L_{m25} andL_{m14}, L_{m24} in the second. The second trials showeda high potential as inhibitorscompared to the first. The*μmax*, estimated by the

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Baranyimodel, for the control wasof 0.699, those for L_{m21}, L_{m23}, L_{m25}, L_{m14}, and L_{m24} 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 L_{m21},L_{m23},L_{m25}, L_{m14}, L_{m24} 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 L_{m23} and L_{m25} seemed similar. The largest bacterial regression was attributed to the crude baceriocin of L_{m25} and the smaller to L_{m24}. Inversely, this pathogen growth after five hours displayed differences for L_{m21}, L_{m23}, L_{m25}, L_{m14}, L_{m24} of 91.22%, 80.76%, 74.28%, 0%, 0% in the first trails and of 79.45%, 12.64%, 11.82%, 87.88%, 85.66% in the second trails, compared to the control.Similar results were observed for batches L_{m23} and L_{m25}.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-positive undesirable bacteria, leading to the selection of five potent autochthonous lactic acid bacteria L_{m14}, L_{m21}, L_{m23}, L_{m24} and L_{m25} as anti- staph. These findings have opened interesting prospects for local food application asfood-grade. Applied tests shall be done in order to show the effectiveness of cultures or their bacteriocins in food system. Others studies are recommended to select others autochthones lactic acid bacteria more effective, while trying to improve the screening tests employed by the present investigation by using a wider range of undesirable micro-organisms, to obtain strains with broad spectrum action.

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