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Preservative potentials of crude bacteriocins produced by Lactobacillus tucceti CECT 5920 and Lactobacillus mindensis TMW on Escherichia coli 0157:H7 and Staphylococcus aureus NCTC 8325

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Abstract: *Preservation potentials of lactic acid bacteria (LAB) isolated from traditional fermented foods were evaluated by inoculating 0.1ml aliquots of appropriate dilution of the samples on De Man Rogosa Sharpe (MRS) agar fortified with 50mg of nystatin for the isolation of LAB. The LAB isolates recovered were screened for bacteriocin production by the Agar Well Diffusion assay and two best bacteriocin producers characterized by (GTG)5-PCR and 16S rDNA protocol as Lactobacillus tucceti CECT 5920 and Lactobacillus mindensis TMW. They were tested for their bio-preservative potentials against Staphylococcus aureus NCTC 8325 and Escherichia coli 0157:H7 in "Five Alive" and La casera fruit drinks. L. tucceti and L. mindensis had the same level of bacteriocin production and antimicrobial activity (P<0.05). Temperature had more effect on bacteriocin activity on L. tucceti CECT 5920 against S. aureus and E. coli while pH had same effects on both LAB isolates and pathogens. L. tucceti had highest reduction effect (90.03±0.04%) on S. aureus in "La casera" and 78.07±0.09% effect against E. coli in "Five Alive" while L mindensis had highest reduction effect (90.12±0.03%) in "La casera" against S. aureus and 77.80±0.01% E. coli in "Five Alive". L. tucceti bacteriocin synergy with onion had highest activity against S. aureus while with ginger gave highest activity against E. coli. L mindensis had highest activity against S. aureus with onion spice but with ginger against E. coli. Storage had decreasing effect on bacteriocin activity from both LAB isolates. L. tucceti gave overall better results, but the two LAB isolates possess bio-preservative potentials that could be exploited in bio-preservation against the use of chemical preservatives.*
Key words: *Bacteriocins, bio-preservation, fermented foods, LAB, pathogens.*

I. Introduction

Traditional fermented foods have been preserved by bioactive substances produced mainly by lactic acid fermentation in which lactic acid bacteria (LAB) predominantly produce lactic acid besides certain compounds such as bacteriocin, which has antimicrobial activity against other groups of microorganisms. Traditional food fermentation is a food preservation method intended to extend shelf-life, improve palatability, digestibility and the nutritive value of food by the activities of naturally occurring microorganisms especially Lactic acid bacteria (LAB). Fermentation of various foods by lactic acid bacteria (LAB) is one of the oldest forms of bio-preservation practiced by mankind.

Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria. The antimicrobial activity of bacteriocins produced by LAB has been detected in foods such as dairy products, meats, barley, sourdough, red wine, fermented vegetables (Yildirim and Johnson, 1998; Bromberg et al., 2004). Therefore, the strains of lactic acid bacteria have also potential to act as a bio-preservative or natural food preservative (Klaenhammer, 1998; Luchansky, 1999). The bacteriocins produced inhibited food spoilage and pathogenic bacteria such as Staphylococcus aureus, Escherichia coli, Bacillus cereus, B. subtilis, Listeria monocytogenes and Clostridium perfringens which are recalcitrant to traditional food preservation method (Bizani and Brandelli, 2002). The interest in the application of LAB and their metabolites in the prevention of the food spoilage and the extension of the shelf life of foods have increased during the last decade (Stiles, 1996).

Currently, different chemical food additives are used in many processed foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as bio-preservatives. The use of bacteriocins or the microorganisms that produce them is attractive to the food industry in the face of increasing consumer demand for natural products and the growing concern about foodborne diseases. It has also necessitated the need to exploit the biologically derived antimicrobial substances produced by LAB which are digested by the human body without any side effect.

Thus, in this research work, traditional fermented foods were used as good sources of bacteriocins and lactic acid that could be of bio-preservative effects and evaluating their bio-preservative potentials.

Rationale

The remedy to the problem presented by chemically based food additives is the use of antimicrobial metabolites of fermentative microorganisms. Many antimicrobial substances have been in use for some time now without any known adverse effect. Many of these organic compounds which have stirred interest are bacterial metabolites used to produce or associated with fermented foods. Hence, it is strongly believed that microbial metabolites will become the next generation of food additives and the current interest in LAB is a step in the right direction.

II. Materials And Methods

2.1 Sample Collection and analyses.

Twenty samples each of traditional fermented food: pap ("akamu"), "ugba", "kunu-zaki", fermenting cassava and "ogiri" were collected from the local producers into universal sterile bottles. The samples were packaged inside a cooler containing ice cubes and quickly transported to the laboratory for analyses. One gram each of the food samples was homogenized in 0.1% peptone water, serially diluted and 0.1ml aliquots of appropriate dilutions inoculated by streaking onto De Man Rogosa and Sharpe (MRS, Oxoid, England) agar medium fortified with 50mg of nystatin (Roissart and Luguet, 1994) for the isolation of lactic acid bacteria (LAB). The plates prepared in triplicates were incubated at 35°C for 48 hrs anaerobically (using anaerobic gas packs) for isolation of mesophilic LAB. The mixed isolates were sub-cultured on MRS agar plates and the pure cultures were stored on MRS agar slants at 4°C. All the isolates recovered were maintained by by-weekly sub-culturing on MRS agar for 48hrs (Cheesbrough, 2004).

2.2 Culture Identification.

The cultures were identified by observing the colonial morphologies, microscopy, biochemical, sugar fermentation tests (Cheesbrough, 2004) and by (GTG)5-PCR and 16S rDNA protocol.

2.3 Growth at different temperatures.

Ten ml of MRS broth containing bromocresol purple indicator was prepared in 10 test tubes and 0.1ml of overnight culture broth was inoculated into the tubes and incubated for 7 days at 10, 15 and 45 °C. Growth at any temperature was observed by the change of the cultures from purple to yellow (Cheesbrough, 2004).

2.4 Identification of test bacterial pathogens.

Cultures of Staphylococcus aureus NCTC 8325 and Escherichia coli 0157:H7 were used as test isolates in this work. They were sub-cultured onto appropriate media, gram stained and subjected to the necessary biochemical and sugar fermentation test to confirm their identity.

2.5 Preparation of Mcfarland Standard for inoculum preparation.

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard was used. It was prepared as follows:

1. A 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
2. The Barium Sulfate suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. These tubes were tightly sealed and stored in the dark at room temperature.
3. The correct density of the turbidity standard was verified using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm was 0.008 to 0.10 for the 0.5 McFarland Standard.

2.6 Selection of bacteriocin-producing LAB.

Two hundred LAB isolates recovered from the fermented food samples collected were screened and narrowed down to 10 LAB after gram staining, biochemical and sugar fermentation tests. They were screened for bacteriocin production by the Agar Well Diffusion (AWD) assay (Lasta et al., 2008) and two LAB isolates were picked as the best bacteriocin producers. These two isolates were identified as Lactobacillus tucceti (strain CECT 5920) and Lactobacillus mindensis (Strain TMW) through (GTG)5-PCR and 16S rDNA protocol and were used for further studies in the work.

2.6.1 Production and purification of bacteriocin sample.

The LAB isolates were each propagated in 1000ml MRS broth (pH 7.0; Oxoid, England). A cell-free solution was obtained by centrifuging the culture (10,000 rpm for 20 min, at 4 ^oC) and was adjusted to pH 7.0 by means of 1M

NaOH to exclude antimicrobial effect of organic acids. The cell-free solution obtained was precipitated with ammonium sulphate (40% saturation). The mixture was stirred for 2 hr at 4 ^{o}C and later centrifuged at 20,000 rpm for 1 hr at 4 ^{o}C. The precipitates were re-suspended in 25ml of 0.05M Potassium phosphate buffer (pH 7.0). The new precipitates were collected and used for further analyses (Jimenez-Diaz et al., 1993).

2.6.2 Determination of bacteriocin antimicrobial activity.

The test pathogens (S. aureus and E. coli) were grown in 100ml of peptone water for 18 hr and the concentration was matched against 0.5 Mcfarland Standard to obtain a concentration of $1.0x10^6$ CFU/ml. 18 hr old culture broths of LAB isolates grown in MRS broth were centrifuged at 5000 rpm for 15 min and the pH of the cell free supernatant was adjusted to pH 6.5-7.0 with 1N NaOH to neutralize the effects of the organic acid. The LAB isolate was seeded on the surface of Mueller-Hinton Agar (Oxoid, England) using sterile swab sticks. 3mm deep wells were made on the Mueller-Hinton agar using sterile cork borer and the diluted test bacteria broths were placed into each agar well using sterile pipette. The plates were kept at room temperature for 2 hr and then incubated at 37 °C for 24 hr. The antagonistic activity of bacteriocins was determined by measuring the diameter of the inhibition zone around the wells (Vinod et al., 2006).

2.6.3 Determination of Titratable Acidity (T.A) of LAB isolates.

MRS broths of the LAB isolates were incubated for 24 hrs at 35^{o}C. The T.A of the broths was determined according to A.O.A.C, (1990) by titrating 25ml of the MRS broth against 0.1M Sodium hydroxide (NaOH) using phenolphthalein as indicator until a pink colour appeared. Each ml of 0.1M NaOH is equivalent to 90.08mg of lactic acid. Total titratable acidity of lactic acid (mg/ml):

$$= \frac{\text{ml NaOH x N NaOH x M.E}}{\text{Volume of sample used}}$$

Where, ml NaOH = Volume of NaOH used,
N NaOH = Molarity of NaOH used,
M.E = Equivalent factor = 90.08mg

2.6.4 Effect of temperature on bacteriocin activity.

The pH-adjusted culture supernatants of the LAB isolates were heated for 10 mins at 30, 40, 60, 80, 100 ^{o}C and for 15 mins at 121 ^{o}C respectively and the bacteriocin activity was tested against the pathogens as described earlier (Vinod et al., 2006).

2.6.5 Effect of pH on bacteriocin activity.

Culture supernatants of LAB isolates were adjusted to pH range of 2-12 using diluted 1M HCL and 1M NaOH solutions and were allowed to stand at room temperature for 2 hr. The residual bacteriocin activity was then determined against the indicator organisms as described earlier (Vinod et al., 2006).

2.6.6 Preservative effect of bacteriocin.

Commercially sold fruit drinks (La casera and "Five Alive") were sterilized and inoculated with cultures of S. aureus and E. coli at 10^6 CFU/ml (determined with 0.5McFarland standard after 18 hr incubation). Initial counts of inoculated samples were recorded and bacteriocin supernatant at a concentration of 0.05 to 0.5 % was added and incubated at 37 °C. The plate counts were determined after 72 hr and the values were compared with the control (without bacteriocin) (Vinod et al., 2006).

$$\text{*Reduction of population (\%)} = \frac{\text{Reduction in microbial count}}{\text{Total count in control}} \times 100$$

2.6.7 Effect of spices used as Food Additives on bacteriocin activity (Synergism).

The possible effects of spices (ginger, onion and garlic) used as food preservatives were tested according to the method of Verluyten et al., (2004) with slight modification. 1g of each of the spices was weighed and dissolved in 50 ml of sterile warm distilled water and mixed overnight by stirring. Spice solutions were centrifuged and filter-sterilized. The cell free supernatants (CFS's) were filter sterilized, to which different volumes of the spice extracts were added independently to give a final concentration of 0.1, 0.5 and 1% (v/v) then inoculated with the indicator pathogens. Controls were prepared by growing the indicator strains in the CFSs excluding the spices. The bacteriocin activity was determined by determining the Optical density of the test pathogens at 600nm.

2.6.8 Effect of Storage on bacteriocin activity.

Purified bacteriocin samples (0.1%, w/v) dissolved in peptone water were tested every 24 hr for 14 days to determine their antimicrobial activities against the test isolates as described earlier (Vinod et al., 2006).

2.7 Statistical Analyses

Data collected were subjected to Analysis of Variance (ANOVA). Mean separation was done using Duncan Multiple range test using Statistical Package for Social Sciences (SPSS) version 20. Differences in statistical significance were considered at $P \leq 0.05$ and n=3.

III. Results And Discussion

The present investigation was aimed at determining the preservative potentials of lactic acid bacteria isolated from traditional fermented food samples. Results showed that 120 isolates were recovered from the 100 samples of traditional fermented food collected and these were screened down to 10 isolates after morphological, biochemical and sugar fermentation tests. They were then evaluated for bacteriocin production and the two isolates that showed best bacteriocin production result were then identified by (GTG)5-PCR and 16S rDNA sequencing (data not shown) as Lactobacillus tucceti CECT 5920 and Lactobacillus mindensis TMW. These two isolates were used for further studies in the work while Staphylococcus aureus NCTC 8325 and Escherichia coli 0157:H7 were used as test pathogens (Table 1).

Assessment of bacteriocin production by the two isolates showed that they have statistically similar results (0.06^{a}±0.02 and 0.08 ^{a}±0.02mg/ml; Fig.1). Bacteriocin production is considered a very biotechnologically important attribute of lactic acid bacteria in the present search for an alternative to chemical food preservatives.

When the crude bacteriocin samples produced by the two isolates were tested for antimicrobial activities, L. tucceti CECT 5920 had statistically same inhibition on S. aureus and E. coli (19^{a}±0.08 and 18^{a}±0.08mm respectively, P<0.05) while L. mindensis TMW also had statistically same inhibitory effects on the two test pathogens (18^{a}±0.08 and 17^{a}±0.07mm, P<0.05) respectively (Table 2). This also revealed that the bacteriocins produced by these isolates have broad spectrum activity against gram positive and gram negative bacteria (S. aureus and E. coli respectively). Similar results were recorded by Adesokan, et al., (2009) against S. aureus, E. coli and Pseudomonas aeruginosa. The result is a welcome development in modern food industry as the high level of susceptibility showed by the two test pathogens to the bacteriocins produced by the two LAB isolates is an advantage in food preservation and a possible good replacement for chemical preservatives. According to Ogunbanwo et al., (2004), Lactic acid bacteria have potentials to inhibit the growth of pathogenic and spoilage bacteria and the possibilities exist for using them to improve the shelf life of different foods. Their antagonistic property is attributed to the low pH, the un-dissociated acid and production of other primary and secondary antimicrobial metabolites (Ten Brink et al., 1994).

Screening of the two isolates for acidification potential after 72 hrs of growth showed that Titratable Acidity (T. A) was maximum (2.94±0.01%) for L. tucceti CECT 5920 after 48hrs of growth and was also maximum (1.48±0.02%) for L. mindensis TMW after 48 hrs of growth. This result is in agreement with Adesokan, et al., (2009) who recorded that as the incubation period increased, the quantity of lactic acid produced by LAB isolates also increased until there was a gradual decline after 48 hr of incubation. This result showed that both isolates will be good in food preservation where the rapid lowering of the medium's pH by the production of organic acid is necessary for the elimination of food borne pathogens and spoilers as well as in prolonging the shelf life of the food material. In the above result however, L. tucceti CECT 5920 had higher T. A. than L. mindensis TMW (Table 3).

The result of effect of temperature on bacteriocin activity showed bacteriocin activity up to the temperature of 30^{o}C. L. tucceti CECT 5920 gave 18mm and 17mm zones of inhibition against S. aureus NCTC 8325 and E. coli 0157:H7 respectively while L. mindensis TMW had 17mm inhibition for the two test pathogens respectively. The LAB isolates exhibited mesophilic inhibitory activities (≤30^{o}C, Table 4) and the mesophilic temperature of activity of the isolates showed that they could only be useful in food preservation in traditional food fermentation where the temperature is within that which the isolates can tolerate. However, this narrow range of temperature tolerance will be a disadvantage in modern food industries where higher temperatures are encountered. The increase in temperature beyond 30^{o}C could have possibly destabilized the 3-Dimensional structure of the bacteriocins (which are proteins) resulting in their denaturation. Djadouni and Kihal (2013) reported two bacteriocins that were thermally stable over a wide temperature range up to 100°C for 15 mins. This could be due to the ecological conditions prevalent where the lactic acid bacteria were isolated. Significant reduction in the bacteriocin production as the temperature increased was reported by Meera and Devi (2012) and this is in agreement with the findings in this work. Growth temperature seems to play an important role in bacteriocin activity.

Result from Fig. 2 showed that when incubated at a range of pH, L. tucceti CECT 5920 had a maximum inhibitory activity of 12.06±0.08mm and 11.81±0.01mm against S. aureus NCTC 8325 and E. coli respectively at pH of 7, while L. mindensis TMW had maximum inhibitory activity (12.81±0.01mm) against S. aureus NCTC

8325 at pH of 8 and 11.02±0.01mm against E. coli 0157:H7 at pH of 7. However, both isolates showed wide range of pH tolerance and activity as they inhibited the test pathogens between the pH range of 2-10: an indication of acidophilic and slightly alkalinophilic activity. Both isolates showed optimum inhibitions at or near neutral pH. It has been reported that some bacteriocins retained their activity at pH 2.0 to 6.0 (Djadouni and Kihal, 2013) and this report is in agreement with the findings here. Djadouni and Kihal (2013) stated that microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH.

When the bacteriocin was tested for its preservative activity using two fruit drinks: "La casera" and "Five Alive", result showed that the removal of the test pathogens from the fruit drinks was directly proportional to the concentration of the bacteriocin tested (0.05-0.5%). At 0.5% concentration, L. tucceti CECT 5920 against S. aureus NCTC 8325 had a preservative effect of 77.82^{a}±0.03 on "La casera" and 78.07^{a}±0.09 on "Five Alive" while against E. coli 0157:H7, it had a preservative effect of 90.03^{a}±0.04 and 61.05^{a}±0.06 on "La casera" and "Five Alive" respectively. L. mindensis TMW when tested against S. aureus NCTC 8325 had a preservative effect of 69.12^{b}±0.02 and 77.80^{a}±0.01 on "La casera" and "Five Alive" and against E. coli 0157:H7it had a preservative effect of 90.12^{a}±0.03 and 61.22^{a}±0.02 on "La casera" and "Five Alive" respectively. The two fruit drinks tested (control) did not show any inhibitory activity against the test isolates (Fig. 3). Vinod et al., (2006) in their work reported that partially purified bacteriocin was found to be preservative against B. cereus and clearly the preservative effect in juice, wine and pulp increased with the increase in the concentration of bacteriocin. They also reported that maximum reduction of Bacillus cereus population of 92% was observed in wine followed by juice (87%) and pulp (63%) at a concentration of 0.5%. The findings here indicate that bacteriocin possessed several desirable characteristics of a bio-preservative.

Determination of the possible synergism in activity between the bacteriocin and local food spices was evaluated using ginger, onion and garlic. There was a corresponding increase in antimicrobial activity with increase in the concentration of the spices (0.1, 0.5 and 1.0%). Result from Table 5 showed that L. tucceti CECT 5920 at 0.1% had highest synergistic activity against S. aureus NCTC 8325 from combination of ginger and onion (2000.00^{a}±200.00) while it was from ginger (2533^{a}.33±251.66) against E. coli 0157:H7. With L. mindensis TMW at 0.1% concentration, the highest activity (2000.00^{a}±300.00) was with garlic against S. aureus NCTC 8325 and with ginger (1533.33^{a}±450.92) against E. coli 0157:H7. Results have earlier shown that the spices tested in this work had various degrees of antimicrobial activities. So, the activities recorded from them in combination with the bacteriocins will be an added advantage in food preservation especially in hurdle technology where food borne pathogens and spoilers face double challenge of overcoming the combination of bacteriocin and the food spices. Djadouni and Kihal (2013) reported that all the spices they tested in their study in concentrations of 5% did not affect bacteriocins activity differently. But that the addition of less than 5% solution of spices to bacteriocins resulted in a significant activity against the indicator strain. This indicates that small amounts of additives should be used in some foods to promote the activity of the bacteriocins applied, especially when more than one spice is used. Ettayebi et al., (2000) and Singh et al., (2001) described that the combination of spices together with bacteriocins, however in a lower amount may lead to a synergistic effect, rendering pathogens susceptible to bacteriocin-spice combination. Such a synergistic inhibition has been shown between nisin on the one hand and garlic extract or thymol on the other. The spices used (red pepper, black pepper, and garlic) ranging from 0.5% affected the bacteriocins activity differently. The addition of 0.5% spices solution to both bacteriocins resulted in a significant activity against the indicator strains (up to 94% reduction of growth).

Evaluation of the effect of storage over a period of 14 days on bacteriocin's activity showed that there was a gradual decrease in the activity of the bacteriocin as storage progressed for both LAB isolates. However, there was bacteriocin activity recorded over the 14days of storage against the two test isolates. For L. tucceti CECT 5920, the highest bacteriocin activity (19.01^{a}±0.01) was recorded within the first 1-3days against S. aureus NCTC 8325 and 18.05^{a}±0.07 against E. coli 0157:H7. The highest activity recorded from L. mindensis TMW against S. aureus NCTC 8325 was 18.02^{a}±0.02 while it was 17.05^{a}±0.07 against E. coli 0157:H7. There was about two-third loss in activity of bacteriocin of L. tucceti CECT 5920 on the 14^{th} day of storage compared with the first three days of storage. But for L. mindensis TMW, it was almost a four-fifth loss in activity (Table 6). Hamdi et al., (2015) reported that extracted bacteriocin from L. acidophilus at 4^{o}C when fresh, after 15 days and 30 days of storage with the diameter of inhibition zone of 14, 12 and 10 against Bacillus subtilis, S. aureus and E. coli, respectively. However, the diameter of inhibition zone decreased to 11, 9.5 and 6.5 mm after 60 days of storage and reached to 7.5, 6 and 4mm of diameter after 90 days of storage for Bacillus. subtilis, S. aureus and E. coli., respectively. These results and the findings from this work are in accordance with those reported by Malini and Savitha (2012) as they found that the bacteriocin activity produced by L. paracasei subsp. tolerans isolated from locally available cheese was more stable at 4^{o}C for 30 days. By this result, it is found that bacteriocin will retain its antimicrobial activity when used as a bio-preservative in foods for up to 90 days in storage.

IV. Conclusion and Recommendation

The studies so far on the bio-preservative potential of the two lactic acid bacteria: L. tucceti CECT 5920 and L. mindensis TMW revealed that both isolates had rapid acidification ability as well as production of bacteriocin with varied levels of antimicrobial activities against the two test pathogens: S. aureus NCTC 8325 and E. coli 0157:H7 used in this work. Much of the interest in the analysis of LAB produced bacteriocins is driven by their potential applications. The properties of the bacteriocins studied, like the inhibition of pathogenic strains, their stability over a wide pH range, heat resistance and high salt tolerance makes them promising agents in food preservation. In overall performance, both LAB isolates would make good research organisms of study in future works as regards the use of lactic acid bacterial metabolites as better substitutes in food preservation that the conventional chemical preservatives which have several health issues.

In a way of recommendation, the government and private bodies should invest more in researches involving the preservative potentials of lactic acid bacteria that abound in our traditional fermented foods so as to encourage the application of the bio-active substances of the organisms that emerged from this work in our local industries. Bacteriocins can be used to preserve our locally made fruit juices and possibly vegetable pastes thereby prolonging the availability of such foods round the year and also improving our health status as the use of chemical preservatives which have been associated with several health challenges will be by-passed since bacteriocins are metabolically digested by the human body without any side effect.

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Table 1: Morphological and biochemical identification of test Pathogens

S/N	Morphology	Gram reaction	Catalase	Coagulase	Glucose	Lactose	M-R	V-P	Indole	Citrate	Isolate
1	Yellow, discrete colonies on Mannitol Salt Agar	Gram + cocci in clusters	+	+	+/-	+/-	-	+	-	-	**S. aureus**
2	Pinkish raised colonies in singles on MacConkey Agar.	Gram + short rods in singles	-	-	+/+	+/+	+	-	+	-	**E. coli**

+/+ : Acid and gas production.

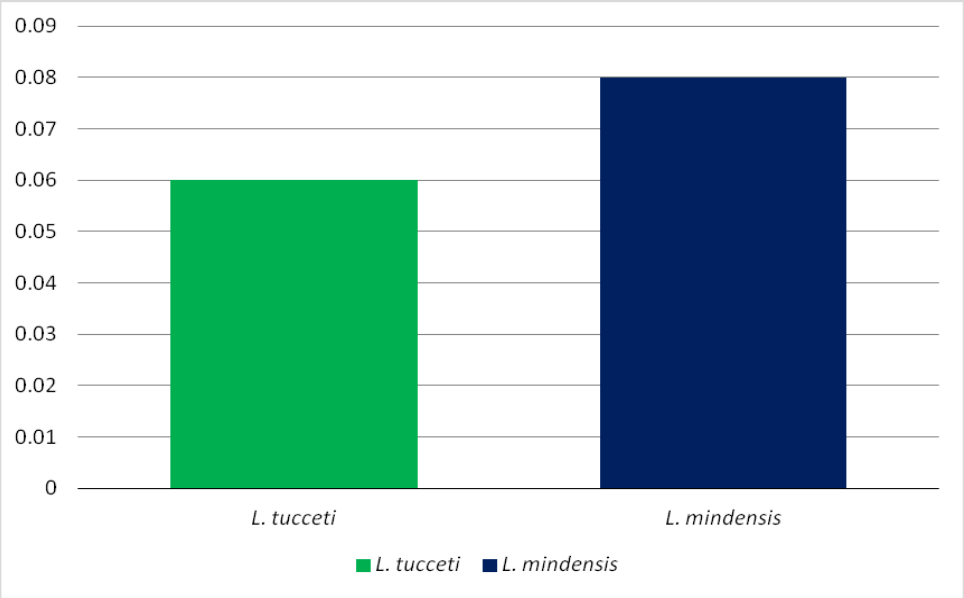


Fig. 1: Bacteriocin production by LAB isolates (mg/ml)

Table 2: Antimicrobial activity of crude bacteriocin (mm).

[a]Data values with the same letters are not significantly different ($p<0.05$; n=3).

*Control: MRS broths without LAB isolates

INTERPRETATIVE REFRENCE RANGE

SENSITIVE	INTERMEDIATE	RESISTANT
≥17	11 – 15	≤10

Table 3: Titratable Acidity of LAB isolates during 72hrs growth.

ISOLATE	T. A (%) 24hrs	48hrs	72hrs
Lactobacillus tucceti CECT 5920			
S. aureus	E. coli		
19[a]±0.08	18[a]±0.08		
Lactobacillus tucceti CECT 5920	0.85[c]±0.01	2.94[a]±0.01	2.54[b]±0.02
Lactobacillus mindensis TMW			
S. aureus	E. coli		
18[a]±0.08	17[a]±0.07		
Lactobacillus mindensis TMW	1.05[b]±0.08	1.48[a]±0.02	1.36[a]±0.01

*Control	0	0	0

*Control:MRS broth without LAB isolates.
[a,b,c]Data values with the same letters on the same columns are not significantly different ($p<0.05$), (n=3)

Table 4: Effect of temperature on bacteriocin activity (mm)

Temp	L. tucceti CECT 5920		L. mindensis TMW	
(for 10mins)	S. aureus	E. coli	S. aureus	E. coli
30	18	17	17	17
40	-	-	-	-
80	-	-	-	-
100	-	-	-	-
121^{o}C (for 15mins)	-	-	-	-
*Control	-	-	-	-

***Control: MRS broths without LAB isolates**
INTERPRETATIVE REFRENCE RANGE (mm)

SENSITIVE	**INTERMEDIATE**	**RESISTANT**
≥17	11 – 15	≤10

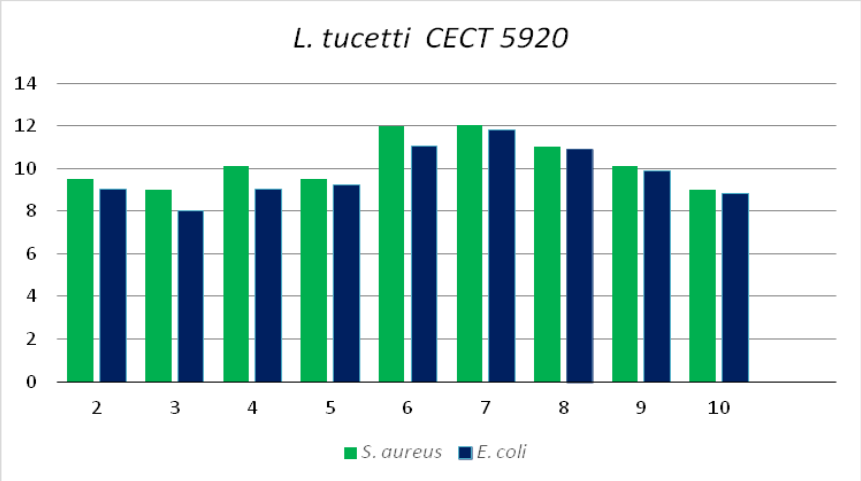
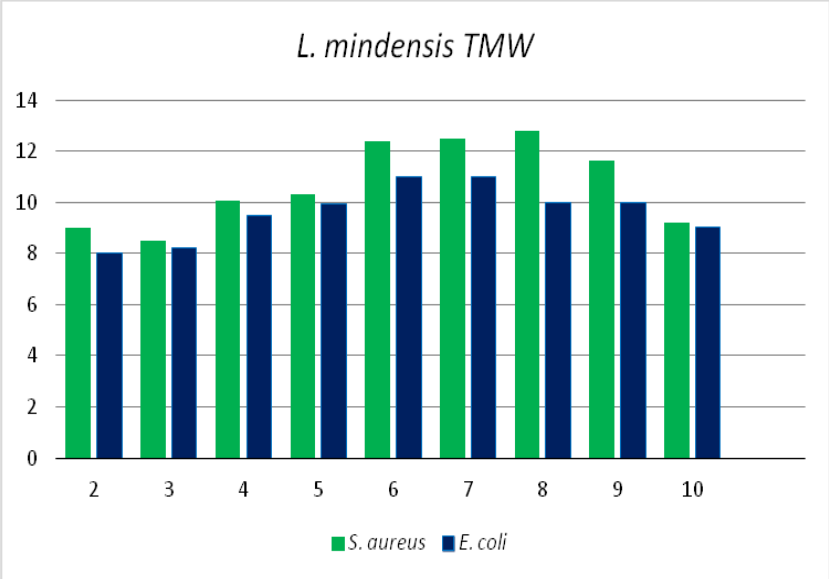
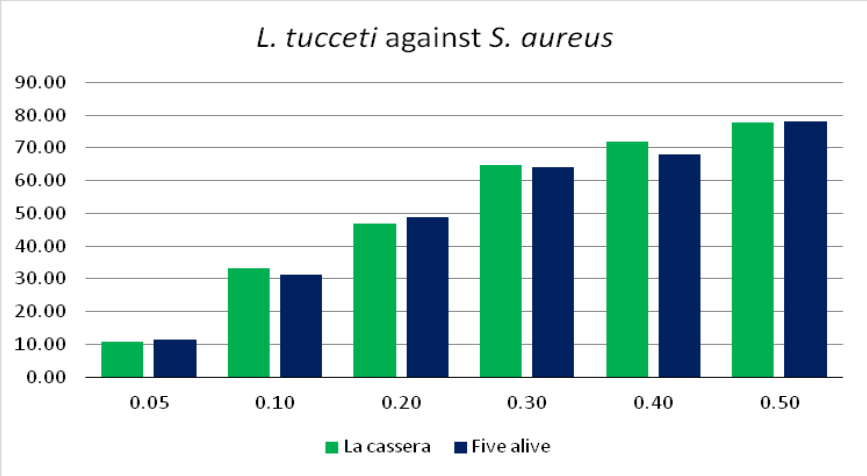


Fig. 2: Effects of pH on bacteriocin activity (mm)





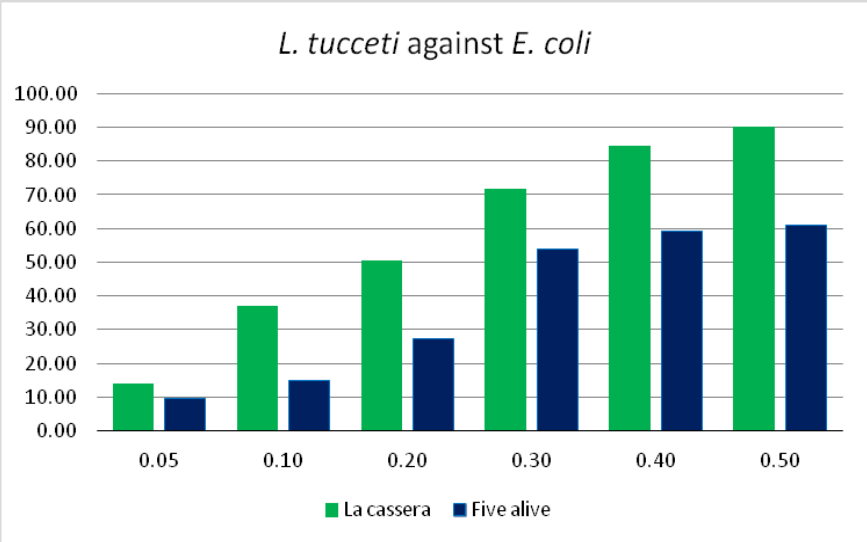
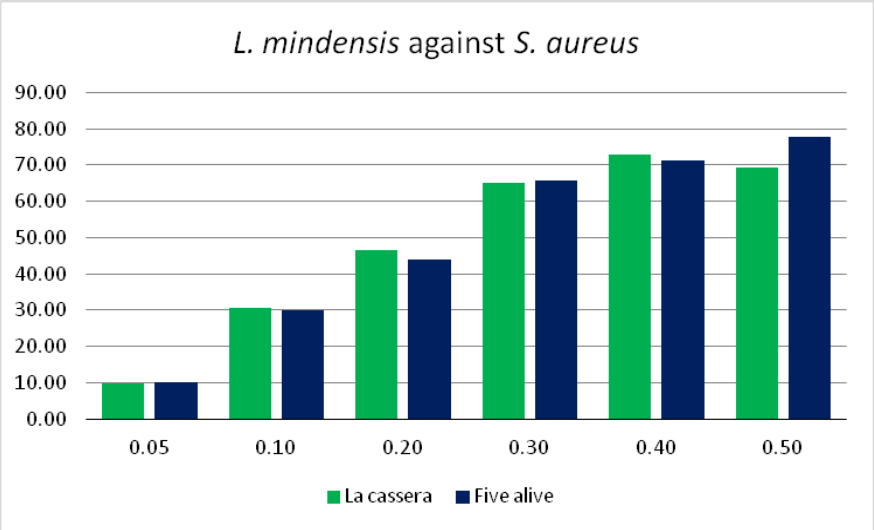


Fig. 3a: Preservative effect of *L. tuceti* CECT 5920



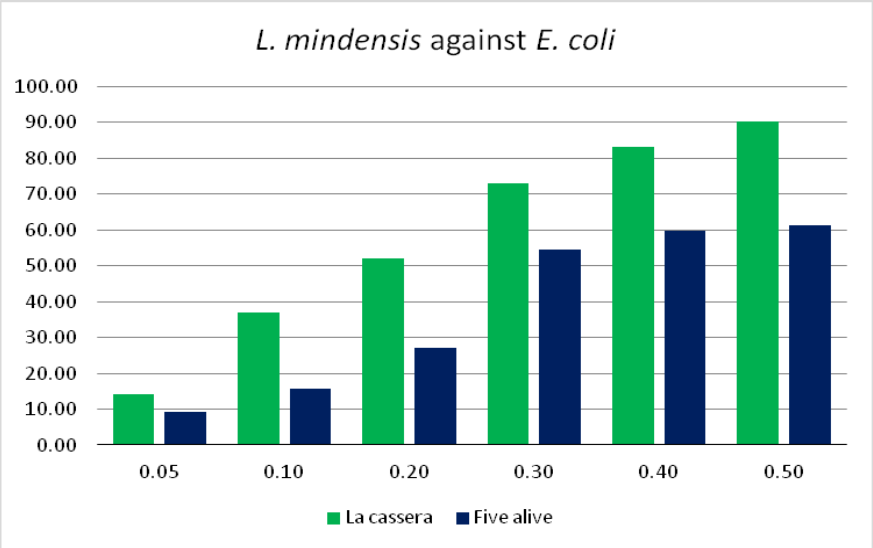


Fig. 3b: Preservative effect of *L. mindensis* TMW

Table 5: Effect of spices used as food additives on bacteriocin activity (AU/ml)

Spices (%)			L. tucceti CECT 5920		L. mindensis TMW	
			S. aureus	E. coli	S. aureus	E. coli
Bacteriocin + Ginger		0.1	2000.00^{a}±200.00	2533^{a}.33±251.66	2200.00^{a}±300.00	1533.33^{a}±450.92
		0.5	1500.00^{ab}±400.00	2000^{b}.00±100.00	1400.00^{b}±200.00	1300.00^{a}±400.00
		1.0	1000.00^{b}±100.00	1000^{c}.00±100.00	1000.00^{b}±200.00	1000.00^{a}±200.00
Bacteriocin + Onion		0.1	2000.00^{a}±200.00	3000.00^{a}±300.00	2500.00^{a}±300.00	2400.00^{a}±100.00
		0.5	1300.00^{b}±200.00	3000.00^{a}±100.00	2000.00^{ab}±400.00	2066.67^{a}±208.17
		1.0	1000.00^{b}±200.00	1600.00^{b}±300.00	1400.00^{b}±300.00	1000.00^{b}±200.00
Bacteriocin + Garlic		0.1	3000.00^{a}±100.00	3000.00^{a}±300.00	2000.00^{a}±300.00	2000.00^{a}±200.00
		0.5	2000.00^{b}±200.00	2000.00^{b}±100.00	2000.00^{a}±400.00	2000.00^{a}±500.00
		1.0	1033.33^{c}±152.75	1000.00^{c}±300.00	1000.00^{c}±100.00	1000.00^{c}±200.00

Table 6: Effect of storage time on bacteriocin activity (mm)

	L. tucceti (strain CECT 5920)		L. mindensis (strain TMW)	
Storage time (days)	S. aureus	E. coli	S. aureus	E. coli
1	19.01^{a}±0.01	18.05^{a}±0.07	18.02^{a}±0.02	17.05^{a}±0.07
3	19.01^{a}±0.01	18.02^{a}±0.02	18.05^{a}±0.07	17.01^{a}±0.01
7	10.05^{b}±0.07	11.01^{b}±0.01	10.01^{b}±0.01	10.05^{b}±0.07
10	7.01^{c}±0.01	8.05^{c}±0.07	4.05^{c}±0.07	5.01^{c}±0.01
12	6.05^{d}±0.07	7.06^{d}±0.08	4.06^{c}±0.08	5.06^{c}±0.08
14	6.02^{d}±0.02	6.01^{e}±0.01	3.01^{d}±0.01	3.07^{d}±0.09

^{a,b,c,d,e}Data values with the same letters on the same columns are not significantly different ($p<0.05$; $n=3$)

INTERPRETATIVE REFRENCE RANGE

SENSITIVE	**INTERMEDIATE**	**RESISTANT**
≥17	11 – 15	≤10