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PRODUCTION OF CRUDE BACTERIOCINS BY SELECTED LACTIC ACID BACTERIA ISOLATED FROM SOME FERMENTED FOODS

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ABSTRACT
Isolation of lactic acid bacteria (LAB) from cheese, pickles, palm wine and yoghurt (fermented foods) were carried out. The LAB were cultivated on De Man Rogosa Sharpe agar and were identified based on their colony and cell morphology, Gram's staining and biochemical tests. The LAB were screened for potential to produce crude bacteriocins in De Man Rogosa Sharpe (MRS) broth using standard techniques. The efficacy of the crude bacteriocins produced by the LAB were tested against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus pyogenes*. Cheese had the highest LAB counts (3.12×10^7CFU/g) than other fermented foods analysed. The lactic acid bacteria isolates were probably identified as *Lactococcus lactis, Lactobacillus bulgaricus*, *Leuconostoc mesenteroides and Pediococcus halophilus*. Out of the twelve (12) LAB screened for bacteriocin production in MRS broth (in test tubes), six (6) tubes revealed the produced bacteriocins. The result of the efficacy of the crude bacteriocins on the test bacteria showed that *Escherichia coli* and *Streptococcus pyogenes* had clear zones of inhibition ranging from 9 to 17mm for *Escherichia coli* and 9 to 16mm for *Streptococcus pyogenes* while *Bacillus cereus* and *Pseudomonas aeruginosa* were completely resistant to the test bacteriocins. The presence LAB in fermented food products and other food products will enhance preservation (shelf life extension) of the products and still maintain their keeping quality.

Keywords: fermented foods, LAB, MRS, CFU, bacteriocins.

INTRODUCTION

Fermentation is a metabolic process that converts sugar to acids, gases, or alcohol. It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation (Klein *et al.,* 2006). Or fermentation is a process that involve the utilization of substrate compounds by microorganisms to carry out their metabolism, generating final byproducts that can be exploited in various fields such as the pharmaceutical, chemical, and food industries (Yulma *et al.,* 2024).

Yoghurt is produced through the fermentation of milk by lactic acid bacteria, usually *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* other lactobacilli and bifidobacteria are also sometimes added during or after culturing yoghurt. The milk is firstly heat treated, homogenised and is then cooled to allow the addition of bacteria or starter culture. Given the right conditions, i.e. correct temperature and moisture, the bacteria are able to ferment the milk sugar (lactose), producing lactic acid. The milk proteins then coagulate and set, to form yoghurt (Miller *et al.,* 2000).The bacteria used to make yoghurt are known as "yoghurt cultures". Fermentation of lactose by these bacteria produces lactic acid, which acts on milk protein to give yoghurt its texture and characteristic tang (McGee, 2004). Cow's milk is commonly available worldwide, and, as such, is the milk most commonly used to make yoghurt. Milk from water buffalo, goats, ewes, mares, camels, and yaks is also used to produce yoghurt where available locally. Milk used may be homogenized or not (milk distributed in many parts of the world is homogenized); both types may be used, with substantially different results (McGee, 2004).

Pickling is the process of preserving or expanding the lifespan of food by either anaerobic fermentation in brine or immersion in vinegar (Chou, 2003). The resulting food is called a pickle, or, to prevent ambiguity, prefaced with the adjective pickled. The pickling procedure will typically affect the food's texture and flavour. Pickles are usually made from a mixture of vegetables and fruit (Chou, 2003). They are eaten as a savoury, spicy accompaniment to a meal. Pickles are preserved by a combination of increased acidity (reduced pH), added salt, reduced moisture and added spices. Pickles can be prepared using one of two main methods: lactic acid fermentation of vegetables, either with or without the addition of salt the preservation of vegetables in acetic acid (vinegar). The products made by these two methods are very different, each one has its own distinctive taste and texture (Chou, 2003). Vegetables such as cucumber, cabbage, olive and onion are fermented by lactic acid bacteria which can grow in low concentrations of salt. The bacteria ferment sugars in the food to form lactic acid, which then prevents the growth of food poisoning bacteria and moulds. The amount of salt added controls the type and rate of the fermentation. If 2-5% salt is used, the fermentation is carried out by a series of bacteria that produce lactic acid. The pickle is preserved by the high level of acidity. If higher levels of salt are used (up to 16%) the product is preserved by the high salt concentration rather than by fermentation and is known as a salt-stock pickle (Chou, 2003). Fruit and vegetables can be semi-processed and stored for many months by preserving in a high salt solution. They can be further processed into pickle later in the season. Sometimes sugar is added to increase the rate of fermentation or to make the product sweeter (Chou, 2003). Pickles prepared by fermentation are not heated. The concentration of salt, pH of the mixture and temperature of fermentation must all be controlled to ensure a good fermentation and to prevent the growth of undesirable bacteria. Vegetables pickled in acetic acid (vinegar) have salt and sugar added. They are not fermented and therefore have a different texture and flavour (Chou, 2003).

Cheese is a food derived from milk that is produced in a wide range of flavours, textures, and forms by coagulation of the milk protein

casein (Fankhauser, 2007).It comprises proteins and fat from milk, usually the milk of cows, buffalo, goats, or sheep. During production, the milk is usually acidified, and adding the enzyme rennet causes coagulation. More commonly starter bacteria are employed instead which convert milk sugars into lactic acid. The solids are separated and pressed into final form (Fankhauser, 2007). Cheese is said to be a concentrated dairy food made from milk, is defined as the fresh or mature product obtained by draining the whey (the moisture or serum of the original milk) after coagulation of casein, milk's major protein. Casein is coagulated by acid produced by select microorganisms and/or by coagulating enzymes resulting in curd formation (Fankhauser, 2007). Milk may also be acidified by adding food-grade acidulants in the manufacture of certain varieties of cheese, such as cottage cheese. Almost all cheese manufactured are made from cow's milk (whole, reduced fat, non-fat, buttermilk, cream, whey, non-fat dry milk solids, or a combination of some or all of these products). However, other sources of milk (sheep, goat) can be used in cheese-making. Cheese may be unripened (fresh) or ripened (matured) (Lenoir *et al.,* 2000).

Palm wine is an alcoholic beverage created from the sap of various species of palm tree such as the date palms, and coconut palms (Rundel, 2002).Palm wine is the fermented sap of various palm trees especially Palmyra, silver date palm and coconut palms Palm wine can be obtained from the young inflorescence either male (or) female one's palm wine is alcoholic beverage that are made by fermenting the sugary sap from various palm plants. It is collected by tapping the top of the trunk by felling the palm tree and boring a hole into the trunk it is a cloudy whitish beverage with a sweet alcoholic taste and very short shelf life of only one day, the wine is consumed in a variety of flavours varying foam sweet unfermented to sour, fermented and vinegary there are many various of the products and no individuals method or recipe palm wine is particularly common in parts of Africa, South India , Mayanmar and Mexico some of the local names for the product include emu and ogogoro in Nigeria and Nsafufuo in Ghana, kallu in south India and tuba in Mexico. Palm sap can be fermented (or) processed into an alcoholic beverage it just needs the correct the yeasts, temperature and processing conditions (Ogbulie *et al.,* 2007).

The Lactic Acid Bacteria (LAB) was gradually accepted in the beginning of the 20th century (Carol *et al.,* 2010). Lactic Acid Bacteria (LAB) are group of Gram positive, acid-loving, non-sporulating, either rod or cocci shaped bacteria (Axelsson, 2004). Lactic acid bacteria (LAB), can be found in the form of cocci or non-sporulating rods. They can produce lactic acid through carbohydrates fermentation, which serves as their sole or primary source of carbon . During this fermentation process, LAB induce a rapid acidification of the substrate by producing organic compounds such as lactic acid, acetic acid, ethanol, enzymes, and bacteriocins (Yulma *et al.,* 2024). Lactic acid bacteria (LAB) or Lactics constitute a diverse group of microorganisms associated with plants, meat, and dairy. They are used in the manufacture of dairy products such as acidophilus milk, yoghurt, buttermilk, and cheeses. The Lactics are also important commercially in the processing of meats (sausage, cured hams), alcoholic beverages (beer, fortified spirits), and vegetables (pickles, and sauerkraut) (Carr *et al.,* 2002). Classification of LAB genera was based on morphology, mode of glucose fermentation, growth at certain temperatures, and range of sugar utilization. Lactic acid bacteria constitute a group of bacteria that have morphological, metabolic and physiological similarities, and they are also relatively closely related phylogenetically (Jin *et al.,* 2009).The common agreement is that there is a core group consisting of five genera; *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* .Recent taxonomic revisions have proposed several new genera and the remaining group now comprises of the following: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus,* and *Weissella. Lactobacilli, Carnobacteria* and some *Weissella* are rods while the remaining genera are cocci (Jin *et al.,* 2009).

Bacteriocins, as a group of diverse antimicrobial peptides, are known as an inhibitory agent against closely related organisms such as Gram-positive and Gram-negative bacteria, they are produced by various bacterial species, specifically lactic acid bacteria (LAB) (Darbandi *et al.,* 2022). Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are similar to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse. Applications of bacteriocins are being tested to assess their application as narrow-spectrum antibiotics (Cotter *et al.,* 2012). They are gotten from Lactic acid bacteria. Bacteriocins are bacterial ribosomally synthesized peptides or proteins with antimicrobial activity. As ribosomally synthesized peptides, bacteriocins are encoded by a plasmid or chromosome-borne structural gene which is often clustered with genes coding for immunity protein(s) and dedicated transport. Bacteriocins are proteinaceous toxins produced as a tiny molecule by bacteria to inhibit the growth of similar or closely related bacterial strain(s) produced by a wide array of bacteria. They are typically considered to be narrow spectrum antibiotics, though this has been debated. They are phenomenologically (is the study of structures of consciousness as experienced from the first person point of view) analogous to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse.
Modern society is more conscious of the importance of food safety, as many of the chemical additives used in food may elicit toxic concern (Deegan *et al.,* 2006; Settanni and Corsetti, 2008).Long-term consumption of this products can affect human health as they reduce the counts of bacteria in the gut and are carcinogenic to human body. Therefore, it is beneficial to claim natural resources and health benefits of diets. The health benefits of natural foods without chemical additives have become more popular; however, most commercially available preservatives and antibiotics are produced by chemical synthesis. Moreover, the use of antibiotics or residues in food is illegal. Unlike chemical preservatives and antibiotics, "generally recognized as safe" (GRAS) (Deegan *et al.,* 2006; Settanni and Corsetti, 2008). This research therefore aimed at production of crude bacteriocins by selected lactic acid bacteria isolated from some fermented foods.

MATERIALS AND METHODS

Collection of Samples

A total of 3 samples each of cheese, pickles and yoghurt were purchased from Dalema super market, Kaduna State Nigeria while a sample of fermented palm wine was purchased from Narayi Market, Kaduna State. The samples were then transferred to the Microbiology laboratory in sterile sample bottles for isolation of lactic acid bacteria.

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Media preparation
All media were prepared according to manufacturer's instructions. The media include De Man Rogosa Sharpe (MRS) broth, De Man Rogosa Sharpe (MRS) agar, Nutrient broth Mueller Hinton agar (MHA) and Some biochemical test media.

Isolation of Lactic Acid Bacteria (LAB) from Fermented Foods
Ten (10) gram of cheese and pickles were weighed separately and made into different 90mL of sterile distilled water while ten (10) mL of palm wine and yoghurt also dispensed separately into 90mL of sterile distilled water. Ten folds serial dilutions were carried out for each food samples. Dilutions which include 10^{-5}, 10^{-6} and 10^{-7} in duplicate were pour plated on De Man Rogosa Sharpe (MRS) agar and incubated at 37°C for 24 hours. Colonies that appeared on the plates were counted using the colony counter and the result recorded as Colony Forming Unit per millilitre (CFU/mL). Pure culture were obtained by sub-culturing and maintaining the selected lactic acid bacteria on fresh media of De Man Rogosa Sharpe agar slant at 4°C further analysis (Cheesebrough, 2003; Mohammed and Ijah, 2013).

Identification of Lactic Acid Bacteria (LAB) from Fermented Foods
The LAB were identified based on cell morphology, Gram's staining and biochemical tests. The biochemical tests include motility, catalase test, Mannitol activity, Indole test and sugar utilization test (Cheesebrough, 2003; Oyeleke and Manga, 2008; Mohammed, 2012).

Production of Crude Bacteriocins
The lactic acid bacteria were inoculated into nutrient broth for 24 hours at 37°C separately. After 24 hours of incubation, the cultures were compared with McFarland turbidity standard and one (1) mL was inoculated into MRS broth separately in test tubes and were incubated at 37°C for 24 hours. The colourless appearance of the MRS broth is an indication of the production of bacteriocin (s) (Tserovska *et al.,* 2002).

Preparation of McFarland Turbidity Standard
One percent (1%) solution of sulphuric acid was prepared by adding 1ml of concentrated H_2SO_4 into 99ml of water. One percent (1%) solution of barium chloride ($BaCl_2$) was also prepared by dissolving 0.5g of dehydrated barium chloride in 50ml distilled water. Exactly 0.5mL of Barium chloride solution was added to 99.5ml of sulphuric acid solution to yield 1.0% barium sulphate suspension. The turbid solution formed was transferred into a test tube as the standard for comparison (Cheesebrough, 2006).

Efficacy of the Crude Bacteriocins on Selected Bacteria Isolates
The efficacy of the bacteriocins were assessed on Gram-positive and Gram-negative organisms : *Bacillus cereus, Streptococcus pyogenes, Escherichia coli and Psuedomonas aeroginosa*. The agar well diffusion technique as described by Biradar *et al.* (2008) was employed to test the efficacy of the bacteriocins against the test organisms. The Muller-Hinton agar was poured into sterilized Petri-dishes and were allowed to solidify for 30 minutes. One (1) mL broth of the test organisms were inoculated onto the sterile agar plates using a sterile bent glass rod aseptically. After 15 minutes of the inoculation, four wells of 8.0 mm in diameter each were aseptically bored using a sterile cock borer on each agar plate. On each agar plate, the crude bacteriocins were poured into the holes. The plates were then incubated at 37°C for 24 hours. The efficacy of the bacteriocins were assessed by measuring the diameters of zone of inhibition (mm) around the wells.

RESULTS
Count of Lactic Acid Bacteria in Fermented Foods
The range of count of lactic acid bacteria varies for all samples with cheese having the highest lactic acid bacteria count of 3.12×10^{7}CFU/g as presented in **table 1**.

Presence of Lactic Acid Bacteria in Fermented Foods
The LAB were present in varying amount in all the fermented food samples analysed .The cheese samples contain *Lactococcus lactis*, pickles *contain Leuconostoc mesenteriodes*, palm wine contain *Pediococcus halophilus* and yoghurt contain *Lactobacillus bulgaricus and Streptococcus thermophilus as* presented in the **table 2**.

Cultural, Morphological and Biochemical Identification of Lactic Acid Bacteria
The cultural, morphological and biochemical identification revealed the presence of the following LAB: *Lactococcus lactis, Lactobacillus bulgaricus, Leuconostoc mesenteroides* and *Pediococccus halophilus* (Table 3).

Production and Efficacy Studies of Bacteriocins of Lactic Acid Bacteria on *Escherichia coli*
The results of efficacy of the bacteriocins produced by *Lactococcus lactis, Lactobacillus bulgaricus, Leuconostoc mesenteroides* and *Pediococccus halophilus* on *Escherichia coli* are shown in Fig 1**.** The result showed that all the bacteriocins produced by lactic acid bacteria isolated from fermented foods (C1, C2, P2, PW1, Y1 and Y3) had zone of inhibition with 17mm as the highest while 9mm been the lowest zone of inhibition observed as shown in **fig 1.**

Efficacy of the Bacteriocins Produced by some Lactic Acid Bacteria on *Streptococcus pyogenes*
The efficacy of the bacteriocins produced by *Lactococcus lactis, Lactobacillus bulgaricus, Leuconostoc mesenteroides* and *Pediococccus halophilus* on *Streptococcus pyogenes* are shown in Fig 2. The result showed that all the bacteriocins produced by the lactic acid bacteria isolated from fermented foods with codes C1, C2, P2, PW1, Y1 and Y3 had zone of inhibition with 16mm as the highest while 9mm been the lowest zone of inhibition observed as shown in **fig 2.**

While the result showed that *Bacillus cereus* and *Pseudomonas aeroginosa* were resistant to all the bacteriocins produced by *Lactococcus lactis, Lactobacillus bulgaricus, Leuconostoc mesenteroides* and *Pediococccus halophilus*.

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Table 1: Count of Lactic Acid Bacteria in Fermented Foods

Fermented foods	No of samples analysed	No of LAB	No of test tubes with potential bacteriocin	Range of counts of LAB X10^7 CFU/g/mL
Cheese	1	1	2	2.68- 3.12
Pickle	1	1	1	1.50- 2.52
Palm wine	1	1	1	1.40-2.12
Yoghurt	1	1	2	2.0 -2.72
Total	4	4	6	

LAB: Lactic acid bacteria, CFU/mL: Colony Forming Unit per millimetre
CFU/g: Colony Forming Unit per gram

Table 2: Presence of Lactic Acid Bacteria in Fermented Foods

Lactic acid bacteria	Fermented foods			
	Cheese	Pickle	Palm wine	Yoghurt
Lactococcus lactis	+	-	-	-
Lactobacillus bulgaricus	-	-	-	+
Leuconostoc mesenteroides	-	+	-	-
Pediococcus halophilus	-	-	+	-

+: Present, - : Absent

Table 3: Cultural, Morphological and Biochemical Identification of Lactic Acid Bacteria with Potential to Produce Bacteriocin

Isolates codes	Colony Morphology	Cell Morphology	Gram Reaction	Indole Motility test		Mannitol activity	Catalase	Sugar utilization			Probable LAB
								Glucose	Sucrose	Lactose	
PW1	Grey, white convex	Cocci in two's	G+	-	-	-	-	A	AG	AG	*Pediococcus halophilus*
P2	Circular convex	Cocci in pairs	G+	-	-	-	-	AG	AG	AG	*Leuconostoc mesenteroides*
Y1	Circular slightly convex	Rods	G+	-	-	-	-	G	-	AG	*Lactobacillus bulgaricus*
Y3	Circular slightly convex	Rods	G+	-	-	-	-	G	-	AG	*Lactobacillus bulgaricus*
C1	Circular convex	Cocci in chains	G+	-	-	-	-	AG	A	AG	*Lactococcus lactis*
C2	Circular convex	Cocci in chains	G+	-	-	-	-	AG	A	AG	*Lactococcus lactis*

Key+= Positive, - =Negative, A=Acid production, G=Gas production, AG=Acid and Gas production, G+=Gram Positive, C, P, PW, and Y=Denote isolates from Cheese, Pickle, Palm wine, and yoghurt respectively

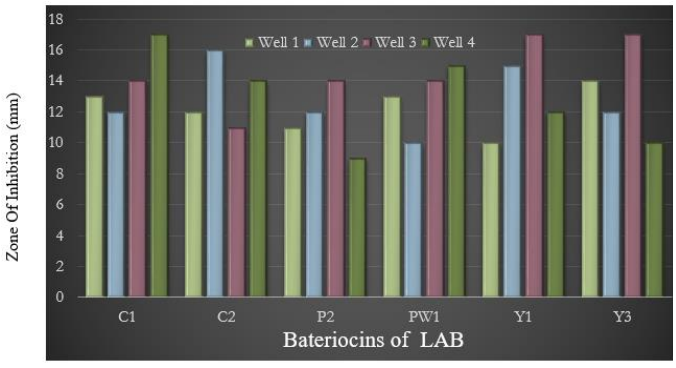

Fig 1: Efficacy of the Bacteriocins Produced by some LAB on *Escherichia coli*
Key: Bacteriocins from LAB isolated from Cheese = C1 and C2, Yoghurt= Y1 and Y3,
Pickles=P2 and Palm wine=PW1

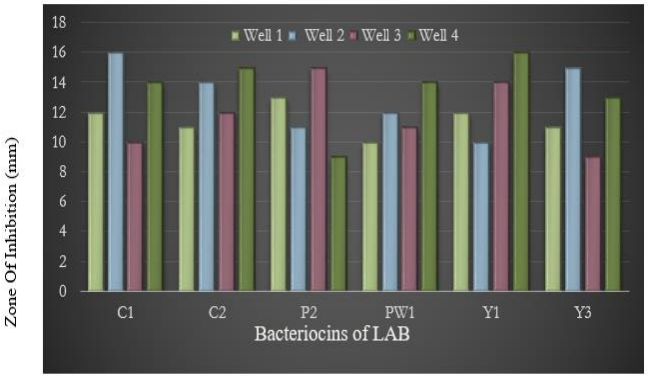

Fig 2: Efficacy of the Bacteriocins Produced by some LAB on *Streptococcus pyogenes*
Key: Bacteriocins from LAB isolated from Cheese = C1 and C2, Yoghurt= Y1 and Y3, Pickles=P2 and Palm wine=PW1

DISCUSSION

The fermented food products (yoghurt, cheese, pickle and palm wine analysed contained lactic acid bacteria (LAB) in different numbers with (cheese) having the highest lactic acid bacteria counts than others. This is in conformity with the report of Oyeleke *et al.* (2006) and Mohammed *et al.*(2012). The LAB isolated in the present studied include *Lactococcus lactis, Lactobacillus bulgaricus, Leuconostoc mesenteriodes* and *Pediococccus halophilus.* Mohammed *et al.* (2012) had earlier reported the isolation of these organisms from burkutu, fufu and palm wine. *Streptococcus thermophillus* and *Lactobacillus bulgaricus* are used as starter culture for commercial production of yoghurt. *Lactobacillus* are important in food fermentation as well as the production of bacteriocins as according to Adam and Moss (2008). The starter organisms used in most cheese-making and are described as mesophilic starters, strains of *Lactococcus lactis* and other subspecies as stated by Adam and Moss (2008). It was reported that *Leuconostoc* sp. and *Pediococcous* sp. are the most predominant lactic acid bacteria in pickles and palm wine which can produce bacteriocin according to Todorov *et al.* (2006) and *Lee et al.* (2011). The efficacy of the bacteriocins on *Escherichia coli* and *Streptococcus pyogenes* were observed with visible zone of inhibition while *Bacillus cereus* and *Pseudomonas aeruginosa* were completely resistant. This could be as a result of *Bacillus cereus* spore formation ability which has resistance to some bacteriocins while *Pseudomonas aeruginosa* could be due to low-level of iron which confer the resistance to some bacteriocins as reported by Cherif *et al.* (2008). Many strains of lactic acid bacteria related to food groups could produce bacteriocins or antibacterial proteins highly effective against foodborne pathogens such as *Staphylococcus aureus*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Clostridium botulinum* (Darbandi *et al.,* 2022). However it was generally observed that bacteriocins from the producer organisms help to inhibit the growth of other pathogenic organism. The implication is that both the bacteriocin and the bacteriocin producing LAB could be used for bio preservation of foods.

Conclusion

In conclusion, it was observed that not all species of lactic acid bacteria are good bacteriocin producers. Although samples of cheese, pickles, palm wine and yoghurt had potential bacteriocin producing LAB.

The efficacy results indicated that all the bacteriocins produced by the lactic acid bacteria isolated from the various samples possesses a wide spectrum of inhibitory activity against *Escherichia coli* which is an indicator organism, and *Streptococcus pyogenes* which is a pathogenic organism of the throat.

Recommendations

The following recommendations were made:

1. This study demonstrates that species of LAB identified were potential bacteriocin producers and are recommended for food processing industries to be employed in bio preservation of foods to enhance extension of shelf life for food products.

2. Extensive studies and in depth understanding of these antimicrobials mechanisms of action could enable scientists to determine their production in specific probiotic lactic acid bacteria, as they are potentially crucial for the final preservation of functional foods or for medicinal applications.

3. The use of bacteriocins and the bacteriocin producing LAB for food preservation's could reduce risks of the use of chemical preservatives and additives, as they could pose health risk generally

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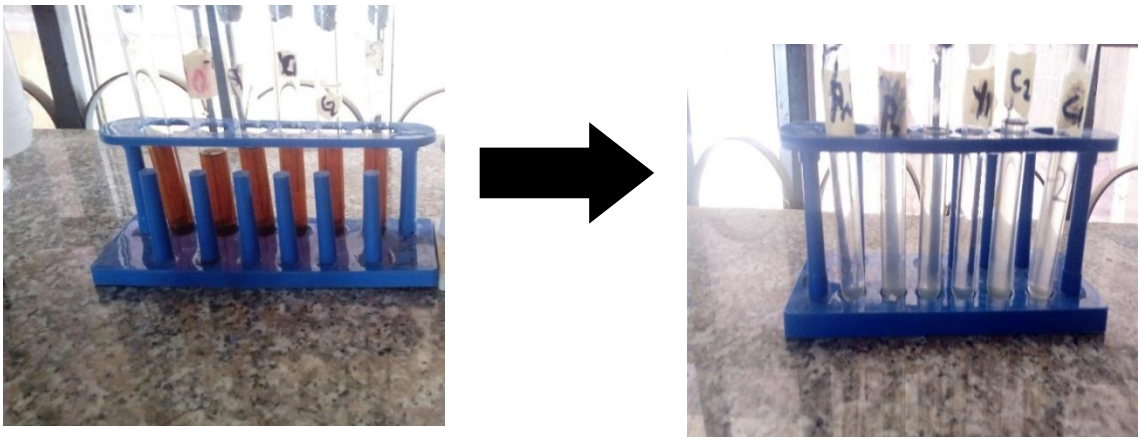

Appendix 1

Plate 1: Production of Bacteriocins