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Characterization and Antilisterial Potential of Lactic Acid Bacteria and Yeasts Isolated from Fermenting Cow Milk

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Abstract

Scientists and the food business are currently interested in a vast range of microbes called lactic acid bacteria (LAB) and yeasts. Despite playing a significant role in the taste improvement of fermented foods, their antimicrobial qualities have recently come to light. Standard techniques were used in this investigation to describe the LAB and yeasts derived from the fermented milk primarily on their morphological, microscopic, and biochemical characteristics. Furthermore, antilisterial activities of lactic acid bacteria and yeasts were performed. Also, the antilisterial activities of neutralized LAB and yeasts and the effect of treatment of pH, NaCl, and enzyme activities against *Listeria monocytogenes* were performed. Lastly, a co-culture of LAB and yeast with *Listeria monocytogenes* in skimmed milk was done. Twenty lactic acid bacteria isolates obtained from fermented cow milk were characterised as *Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus delbrueckii, Lactobacillus brevis, Lactobacillus casei, Lactobacillus fermentum,* and *Lactococcus lactis* while twenty- five yeast isolates obtained from fermented cow milk were identified as *Saccharomyces cerevisiae, Debaryomyces hansenii, Pichia anomala, Kluyveromyces lactis, Trichosporon mucoides, Saccharomyces barnettii* and *Saccharomyces bayanus* with different frequency. The secretion of metabolites like organic acids, hydrogen peroxide, diacetyl, and bacteriocin by lactic acid bacteria led to the inhibition of Listeria monocytogenes, whereas the synthesis of organic and volatile acids, substantial quantities of ethanol, hydrogen peroxide, while mycocins by yeasts led to their inhibition. When treated with -amylase and at pH 4–7, bacteriocins and mycocins remained constant, but part of their activity was reduced when treated with protease. As pH and NaCl concentration increased, the activity of bacteriocins and mycocins decreased. In the presence of the LAB and yeasts, *Listeria monocytogenes* showed a delay in growth and low microbial count for the control during the co-culturing experiment.

Keywords: *Listeria monocytogenes,* Lactic acid bacteria, Yeasts, cow milk, antilisterial activities.

Introduction

The fluid produced after fully milking a cow is known as cow milk. It is an excellent growth medium for microorganisms at suitable temperatures (Bahanullah et al., 2013). Cow milk is a highly nutritious food that has a nutritional composition of water 87.5%; sugar (majorly lactose) 5%; fat 3.6%; protein 3.2%; mineral 0.7%; pH 6.7-6.9. It also contains vitamins A, B complex, C, and D in varying amounts (Nudda et al., 2006).

Various microbes, including yeast, mold, and bacteria, may be present in raw milk.

LAB are well recognised for creating lactic acid by digesting milk sugar. According to Sezer and Güven (2009), lactic acid bacteria can synthesize antibacterial substances that can inhibit pathogenic microbes.

Listeria monocytogenes is a pathogenic microorganism that is naturally present throughout the ecosystem and is commonly found in food. Raw milk can be contaminated by L. monocytogenes, which can thrive at temperatures ranging from four to thirty-seven degrees Celsius. Listeria species are gram-positive and catalase positive rod. According to Campagnollo et al. (2018), listeriosis can cause significant sickness in females during pregnancy, babies, and old people, and could lead to a gastrointestinal disorder in immuno-compromised individuals.

Extreme temperatures or chemical substances may alter the essential nutrient content of food, but they are particularly effective in reducing Listeria monocytogenes in food preparation. In addition to these conventional techniques, a possibly novel strategy for limiting L. monocytogenes in food production can involve the use of LAB with antibacterial properties. Lactic acid bacteria can synthesize antimicrobial peptides (bacteriocin), diacetyl, reuterin, hydrogen peroxide, and lactic acids. The antibacterial components may be employed in the manufacturing of food as partly purified components or may be introduced to food as supplemental components that also produce antibacterials (Ivanovic et al., 2021).

As a result, Lactic acid bacteria are regarded as probiotics since they confer significant health benefits to individuals. Lactic acid bacteria are employed in a variety of food industries for fermentation processes and are widely used globally because they confer significant health benefits. As a result, some Lactobacillus and Bifidobacterium species, possess a long history of healthy usage, particularly in milk. (Moulay et al., 2013).

We have seen the usage of antagonistic yeast culture during the past few decades in a number of food-related industries. It is generally recognized that in sectors like sausage and winemaking, baking, and the "fermentation" of cacao and coffee beans, the growth of spoilage bacteria directly impacts the quality of the product. Antagonistic yeast starter culture improves the sensory quality and shelf life of the product while also suppressing spoilage microorganisms and limiting pathogen development throughout the fermentation process (Hatoum et al., 2012).

Mycocin-producing yeasts have been suggested for use as starter cultures in multiple research projects to stop the growth of pathogenic yeasts in beverages. However, it has been discovered that Saccharomyces cerevisiae strain(s) that generate mycocins may effectively stop the growth of malignant yeasts strain(s), other yeasts found on the outermost layer of grape(s) are resistant to the mycocins produced by yeast. The mycocins released by Kluyveromyces species are capable of inhibiting pathogenic microbes which may provide beneficial bioprotective properties for the beverage industries (Hatoum et al., 2012).

The quest for nutrient(s), the alteration in Hydrogen ion concentration of the substrates brought on by the synthesis of organic and volatile acids, hydrogen peroxide, and inhibitory compounds produced by microbes are the major causes of the inhibiting effect of fungi. The fungi have been utilised in food industries due to their antimicrobial effect towards harmful microbes, and a few yeasts were believed to confer significant health benefits. (Hatoum et al., 2012).

This study was done to characterise and determine the antilisterial properties of LAB and yeast derived from fermenting cow milk toward Listeria monocytogenes.

Materials and Methods

Materials

Cow milk was obtained from the Akinyele market and at the Cattle and Dairy unit at the University of Ibadan. The fermenting cow milk was taken to the laboratory in a clean and sterile container for microbiological analysis. Pure cultures of Listeria species were also cultured from Listeria monocytogenes given by the Department of Microbiology, University of Ibadan which were used for further microbiological analysis.

Methods

Isolation Procedure

Serial dilution was done for the Cow milk samples. Nine ml of water were dispensed into sterile test tubes. The cow milk was subjected to fermentation at various hours (0, 24, 48, and 72 hours). A volume of the sample was introduced into a sterile test tube with nine milliliters of distilled water and shaken vigorously to obtain dilution 10-1. This process was repeated until dilution 10-10 was obtained. Subsequently, 1ml of 10-2, 10-4, and 10-6 diluents were introduced into well-labeled sterile petri dishes respectively which were done twice since two media were involved De Mann Rogosa and Sharpe Agar (MRS) and Yeast Extract Agar (YEA). After that, sterilized and cooled MRS agar and YEA were poured into the plates aseptically. The plates were carefully swirled in clockwise and anti-clockwise directions to ensure uniformity and even distribution of inoculums throughout the growth medium. Control plates in which the inoculums were not introduced were also prepared to detect any form of contamination from the media used. The inoculated plates were then allowed to set and solidify on a flat surface before being inverted and placed in the incubator at 37°C for 24 to 48 hours anaerobically for MRS agar Petri dishes and at twenty-five degrees Celsius for 72 hours for YEA plates (Fossi et al., 2015; Olowonibi, 2017).

Identification of Lactic Acid Bacteria Isolates

The morphology, gram staining, and biochemical features of isolated microorganisms were used to describe them. Twenty-four hours old cultures were used in carrying out all procedures for the characterisation.

Endospore Test

This test was done by making a smear of pure cultures of the microorganisms on a clean grease-free slide. The smears were left to air dry, after which they were heat-fixed, flooded with malachite green, and heated for 5 minutes. After cooling, the slides were rinsed and stained with a secondary stain. This was cleaned and observed under the microscope. The spores showed green and the bacteria vegetative cells stained red (Olutiola et al., 2000).

Motility Test

Inoculation loops were utilised to inoculate day-old microorganisms into MRS agar in vials. The vials were later inserted in the incubator at thirty-seven degrees Celsius for twenty-four to forty-eight hours. After incubation, a restricted growth of microorganisms in the inoculated part denotes a negative outcome. However, a dispersed growth of microorganisms in the inoculated area denotes a positive outcome (Olutiola et al., 2000).

Catalase Test

A minute amount of three percentage of Hydrogen peroxide (H2O2) was added to clean grease-free slides. Then, inoculation loops were used to inoculate microorganisms into clean grease-free slides containing the H2O2. The presence of bubbles indicates catalase enzyme production and hence positive results. However, the absence of bubbles indicates catalase enzyme production that has negative results (Cheesbrough, 2006).

Citrate Utilization Test

The method used determines a microbe's capacity to use citrate as its only energy supply. Three grams of culture medium was put in one hundred milliliters of purified H2O. Every test tube containing 10 milliliters of the prepared culture media later undergoes sterilization and cooling process in a tilted balance. Then, the microbe was streaked into all test tubes on the outermost layer. Blue colouration indicates a positive result and hence, it implies that the citrate has been utilised. However, green coloration indicates a negative result and hence, it implies that the citrate has not been utilised (Abiola and Oyetayo, 2016).

Indole Test

Five milliliters of Tryptone broths were poured into various vials. Then, inoculation loops were used to transfer bacteria into the sterile Tryptone broths. No microorganism was transferred into one vial to serve as a negative indicator. All the vials were in the incubator for forty-eight hours at thirty-seven degrees Celsius. Then, half a milliliter of Kovacs' reagents were added, slowly mixed, and left for twenty minutes. A red colour at the uppermost layer of the vials implies that the microbes had converted tryptophan to indole, whereas, a yellow colour at the uppermost layer of the vials implies that the microbes hadn't converted tryptophan to indole (Cheesbrough, 2006).

Methyl Red Test

One gram of C6H12O6, 0.50% potassium dihydrogen phosphate, 0.50% peptone, and one hundred milliliters of purified H2O were mixed to make 5 milliliters of broth medium and later underwent a sterilisation process. Following the microbial inoculation, the vials underwent a forty-eight hours' incubation period at thirty-seven degrees Celsius. Minute amounts of methyl red mixture were added to all the vials and a color difference was noticed. A reddish colour denotes a positive result, while a yellowish colour denotes a negative result (Olutiola et al., 2000).

Voges-Proskauer test

One gram of C6H12O6, 0.50% potassium dihydrogen phosphate, 0.50% peptone, and one hundred milliliters of purified H2O were mixed to make 5 milliliters of broth medium and later underwent sterilisation process. Following the microbial inoculation, the vials underwent a forty-eight hours' incubation period at thirty-seven degrees Celsius. After the vials had been incubated, six percentages of 1-naphthol and NaOH were dispensed in one milliliter of the glucose phosphate medium. A reddish colour denotes a positive outcome, while a yellowish-brown colour denotes a negative result after some time (Olutiola et al., 2000).

Sugar Fermentation Test

The potential of microbes to break down carbohydrates was tested using this biochemical method. In this biochemical method, the components utilized were peptone water, phenol red, and sugars. All the tubes received around 10 mL of prepared liquid medium each and the Durham tube(s) were gently reversed to capture any Oxygen liberation. Each of the tubes was sterilised, filled with the microbes, and cultured for 120 hours at thirty-seven degrees Celsius and was monitored every day. According to Fawole and Oso (2004), yellowish coloration denotes acid generation.

Identification of Yeasts Isolates

Each microorganism was examined visually for its colony's elevation, color, and distinctive, and special traits. All microbes were also examined under a microscope using microbial smear(s) on grease-free slides. The smear(s) containing the microorganisms were allowed to dry, and methylene blue dye was added, followed by microscopic observation (Olowonibi, 2017). Then, the sugar

fermentation test was done as described above.

Starch Hydrolysis

Starch hydrolysis assesses a microbe's capacity to engage in enzymatic action that breaks down carbohydrates. The carbon compounds were mixed with de Mann Rogosa de Sharpe medium and autoclaved. The sterilised liquid media were poured into Petri dishes and the microbes were streaked following the solidification of the media. The petri dishes received a twenty-four-hour incubation period at thirty-seven degrees Celsius before the addition of iodine. The blue colour indicates a carbohydrate that hasn't been hydrolysed in the presence of iodine. A clear zone indicates hydrolysed starch caused by the action of α-amylase whereas partial hydrolysis of starch is indicated by reddish-brown zones (Abiola and Oyetayo, 2016).

Urea Hydrolysis

This method helps determine microorganisms' ability to produce urease. The liquid medium was prepared with urea and five milliliters were dispensed into each vial and sterilised. Microorganisms were streaked into the medium and then placed into the incubator at thirty-seven degrees Celsius. A reddish coloration indicates a positive result, while a yellowish coloration indicates a negative result (Sulieman et al., 2015).

Antilisterial Activity of Lab and Yeast Against Listeria

Species

The antilisterial activities of the LAB and Yeast isolates against Listeria monocytogenes were done utilising the agar diffusion technique. The microorganisms were cultured in 10mls of MRS broth and YEA broth and incubated anaerobically at thirty-seven degrees Celsius for one day and at twenty-eight degrees Celsius for three days respectively. Each of the incubated broth media later received a centrifugation process at four thousand revolutions per minute to obtain cell-free supernatants (CFS). Twenty-four hours old cell suspension of the broth culture of the pathogens was adjusted to 0.5 McFarland turbidity standards and used to seed 20mls Muller-Hinton agar using a sterile swab stick on Petri dishes. The Muller-Hinton agar plates were allowed to dry, and four wells, each with a diameter of 7mm were bored on each plate using a sterile cork borer, and 0.1ml of CFS from each isolate was pipetted into the bored wells and labeled accordingly. Each petri dish was placed in an incubator at thirty-seven degrees Celsius for one day and at twenty-eight degrees Celsius for three days for LAB and yeasts respectively and the inhibition zone was monitored. The width of the inhibition zone around all wells was assessed in millimetres and recorded (Balqis et al., 2015).

Determination of bacteriocin and mycocin production (Neutralization)

The supernatants of each isolate were prepared via a centrifugation process. The LAB and yeasts were cultured in de Mann Rogosa de Sharpe broth (Oxoid) at thirty-seven degrees Celsius for twenty-four hours and in Yeast extract broth at twenty-five degrees Celsius for seventy-two hours respectively. Cultures received centrifugation process at four thousand revolutions per minute to obtain cell-free supernatant (CFS) for fifteen mins. The supernatant was later treated with Sodium Hydroxide to maintain a neutral pH and was subjected to a filtration process using filter paper for its collection. The antilisterial activity of the isolate utilising the supernatants were done through the agar diffusion technique as described above (Cocolin et al., 2007).

Effect of enzymes on bacteriocin and mycocin activity

The stability of the bacteriocin and mycocin was done after treatment with

protease obtained from Streptococcus griseus and α-amylase from Aspergillus oryzae. Each enzyme was put in one milliliter of supernatants with neutral pH and one hundred microliters were dispensed into the agar well of the petri dishes (Cocolin et al. 2007). The Petri dishes were inserted in the incubator at thirty-seven degrees Celsius for twenty-four hours and twenty-five degrees Celsius for 72 hours for LAB and yeast respectively, and the antilisterial potential was done by assessing the inhibitory zone compared to that of Listeria monocytogenes (Banwo et al., 2012).

Effect of pH on bacteriocin and mycocin activity

The Hydrogen ion concentration of the isolates' supernatant was adjusted between four and ten with one mole per liter of Sodium Hydroxide and Hydrochloric acid to study the impact of pH on bacteriocin and mycocin effectiveness (Cocolin et al., 2007). The Petri dishes were inserted in the incubator at thirty-seven degrees Celsius for twenty- four hours and twenty-five degrees Celsius for 72 hours for LAB and yeast respectively, and the antilisterial potential was done by assessing the inhibitory zone compared to that of Listeria monocytogenes (Banwo et al., 2012).

Effect of NaCl on bacteriocin and mycocin activity

To detect the effect of NaCl on the bacteriocin and mycocin activity, the isolate supernatants of LAB and yeasts were prepared and supplemented once with different NaCl concentrations (2 and 4%). The Petri dishes inserted in the incubator at thirty- seven degrees Celsius for twenty-four hours and twenty-five degrees Celsius for 72 hours for LAB and yeast respectively, and the antilisterial potential was done by assessing the inhibitory zone compared to that of Listeria monocytogenes (Al-Qaysi et al., 2017).

Co-culture Experiments

Co-culturing of Listeria monocytogenes with yeasts and lactic acid bacteria was done independently in milk (two hundred milliliters) for around twenty-four hours at thirty- seven degrees Celsius. Another test-tube exclusively received Listeria monocytogenes. Lactic acid bacteria and yeast growth were later checked after co-culturing using de Mann Rogosa de Sharpe and Yeast Extract Agar. The Petri dishes were inserted in the incubator at thirty-seven degrees Celsius for twenty-four hours and twenty-five degrees Celsius for 72 hours for LAB and yeast respectively. Additionally, Listeria monocytogenes growth was checked using nutrient agar, and the petri dishes were placed in the incubator at thirty-seven degrees Celsius for two days. The Hydrogen ion concentrations were checked for all samples and recorded (Cocolin et al., 2007).

Results and Discussion

Lactic acid bacteria Count

The total LAB count on MRS medium plates was documented. A high LAB count of

3.21 × 108 cfu/ml was recorded in the fermented cow milk from the Akinyele market compared with the University of Ibadan dairy unit with a LAB count of 2.88 × 108 cfu/ml.

Morphological Characteristics of Lactic Acid Bacteria

All the isolates were Gram-positive rods and a coccus shape, non-motile and non-spore formers. The morphological features of the LAB pure cultures were circular colonies that were opaque with colors varying from white to cream on the MRS agar plate. Their surfaces were smooth while some were rough with small, medium, and big sizes.

Biochemical Characteristics of Lactic Acid Bacteria

The biochemical test revealed that all lactic acid bacteria isolated were catalase-negative, positive to methyl red, negative to indole test, Voges-Proskauer, and citrate with only one citrate positive as represented in Table 1. The twenty LAB isolates obtained from fermented cow milk were characterised as Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus delbrueckii, Lactobacillus brevis, Lactobacillus casei, Lactobacillus fermentum and Lactococcus lactis with different frequency.

In this study, twenty LAB isolates from fermented milk were identified using the standard biochemical test result of the LAB utilising Bergey's Manual of Systematic Bacteriology (Second Edition) as a guide. The adaptability of LAB is a crucial quality, according to some scientists, who also noted that despite requiring selective nutrition, LAB can inhabit a variety of ecosystems (Moulay et al., 2013).

Table 1: Microscopy and biochemical characterization of lactic acid bacteria obtained from cow milk

Isolatecodes	Gram' sStain	Shape	Catalase	SporeStain	Motility	Citrate	Indole	Methyl Red	Voges Proskauer	Sucrose	Galactose	Lactose	Glucose	Fructose	Mannitol	Maltose	Sorbitol	Inositol	Probable Organisms
L3U11	+	R	-	-	-	-	-	+	-	+	+	-	+	+	+	+	-	+	*Lactobacillus brevis*
L2U31	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
L1U33	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*
L1U22	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
L3U31	+	R	-	-	-	-	-	+	-	+	+	-	+	+	+	+	-	+	*Lactobacillus brevis*
L0U11	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
L1U32	+	R	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	*Lactobacillus delbrueckii*
L1U14	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*
L1U12	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
LIU31	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
L2A31	+	R	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	*Lactobacillus delbrueckii*
L1A21	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
L2A13	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus rhamnosus*
L1A11	+	R	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	*Lactobacillus delbrueckii*
L1A34	+	R	-	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	*Lactobacillus fermentum*
L1A13	+	C	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	*Lactococcus lactis*
L2A34	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	-	+	-	*Lactobacillus casei*

L1A12	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*
L1U21	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*
L2U32	+	R	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	*Lactobacillus plantarum*

Table 2: Antilisterial potential of lactic acid bacteria obtained from fermenting cow milk

Isolate codes	Antilisterial activity	ZI$_{Lm}$
Lactobacillus brevis L3U11	-	NZI
Lactobacillus rhamnosus L2U31	-	NZI
Lactobacillus plantarum L1U33	+	9.0 mm
Lactobacillus rhamnosus L1U22	-	NZI
Lactobacillus brevis L3U31	-	NZI
Lactobacillus rhamnosus L0U11	-	NZI
Lactobacillus delbrueckii L1U32	+++	17.0 mm
Lactobacillus plantarum L1U14	-	NZI
Lactobacillus rhamnosus L1U12	+	4.0 mm
Lactobacillus rhamnosus L1U31	++	12.0 mm
Lactobacillus delbrueckii L2A31	-	NZI
Lactobacillus rhamnosus L1A21	+	4.0 mm
Lactobacillus rhamnosus L2A13	++	10.0 mm
Lactobacillus delbrueckii L1A11	+	8.0 mm
Lactobacillus fermentum L1A34	-	NZI
Lactobacillus lactis L1A13	+	6.0 mm
Lactobacillus casei L2A34	-	NZI
Lactobacillus plantarum L1A12	-	NZI
Lactobacillus plantarum L1U21	-	NZI
Lactobacillus plantarum L2U32	-	NZI

Key: - = no activity; + (4.0 - 9.0 mm) = low activity; ++ (10.0 - 14.0 mm) = moderate activity; +++ (15.0 - 20.0 mm) = high activity; mm = millimetres; NZI = No zone of inhibition; ZI$_{Lm}$ = Zone of inhibition for *Listeria monocytogenes.*

Antilisterial Potential of Lactic acid bacteria

The eight LAB pure cultures that inhibited *Listeria monocytogenes* were *Lactobacillus rhamnosus* L1U12, L1U31, L1A21 and L2A13, *Lactobacillus plantarum* L1U33, *Lactococcus lactis* L1A13 and *Lactobacillus delbrueckii* L1U32 and L1A11 as illustrated in Table 2

Some of the LAB isolates were shown to have antilisterial potential against *Listeria monocytogenes* regarding this research. This is consistent with research by Bellil *et al*. (2014) who found that *Listeria monocytogenes* cannot thrive in fermented milk due to antilisterial compounds generated by LAB. This inhibitory property is a result of antilisterial compounds such as lactic acid, hydrogen peroxide, diacetyl, and bacteriocin. Also, the LAB isolates were observed to have inhibited *Listeria monocytogenes* at varying degrees. The varying degrees of inhibition are dependent on the concentration or quantity of the antimicrobial compounds produced (Afolabi *et al.*,2008).

Yeasts Count

The total yeast count on yeast extract agar (YEA) plates was recorded. A high yeast

count of 2.78 × 10^8 cfu/ml was observed in the fermented cow milk from the Akinyele market compared with the University of Ibadan dairy unit with a yeast count of 1.51 ×10^8 cfu/ml.

Morphological Characteristics of Yeasts

The physical features of the yeast isolates were spherical and circular colonies which were opaque with colors varying from white to cream on YEA plates. All their surfaces were smooth and had entire margins with small, medium, and big sizes.

Biochemical Characteristics of Yeasts

The biochemical tests revealed that all the yeast isolated were either positive or negative for urea hydrolysis, starch hydrolysis, and sugar fermentation as represented in Table

3. The twenty-five yeast isolates obtained from fermented cow milk were identified as *Saccharomyces cerevisiae, Debaryomyces hansenii, Pichia anomala, Kluyveromyces lactis, Trichosporon mucoides, Saccharomyces barnettii* and *Saccharomyces bayanus* with different frequency.

In this study, twenty-five yeast isolates from fermented cow milk were identified using standard biochemical test results of yeasts regarding the Taxonomic Study of Yeasts (Fourth Edition). Qvirist (2016) reported that there were various yeasts involved in thefermentation of milk which conferred significant health benefits.

Table 3: Microscopy and biochemical characterization of yeasts obtained from cowmilk

Isolatecodes	Shape	Urea hydrolysis	Starch hydrolysis	Sucrose	Galactose	Lactose	Glucose	Fructose	Mannitol	Maltose	Sorbitol	Inositol	Probable organisms
Y2A14	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y2A24	C	+	+	-	+	+	+	+	+	-	+	+	*Trichosporon mucoides*
Y2A22	C	-	+	+	+	+	+	+	+	+	+	-	*Kluyveromyces lactis*
Y2A13	C	+	+	-	+	+	+	+	+	+	+	+	*Trichosporon mucoides*
Y0A11	S	+	+	+	+	-	+	+	-	-	-	-	*Saccharomyces barnettii*
Y3A32	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y0A21	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y2A33	S	-	-	+	+	+	+	+	+	+	+	-	*Debaryomyces hansenii*
Y0A14	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y2A21	S	-	-	+	+	+	+	+	+	+	+	-	*Debaryomyces hansenii*
Y0A32	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y3A34	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y0U11	C	-	+	+	+	+	+	+	+	+	+	-	*Kluyveromyces lactis*
Y1U22	S	-	-	-	-	-	+	+	-	+	-	-	*Saccharomyces cerevisiae*
Y0U33	C	-	+	+	+	+	+	+	+	+	+	-	*Kluyveromyces lactis*
Y0U34	S	+	-	+	-	-	+	+	+	+	-	-	*Saccharomyces bayanus*
Y2U34	S	-	-	+	+	+	+	+	+	+	+	-	*Debaryomyces hansenii*
Y2U22	S	-	-	+	+	+	+	+	+	+	+	-	*Debaryomyces hansenii*
Y2U32	C	-	-	+	+	+	+	+	+	+	-	-	*Pichia anomala*
Y0U14	C	-	+	+	+	+	+	+	+	+	+	-	*Kluyveromyces lactis*
Y3U11	C	-	-	+	+	+	+	+	+	+	-	-	*Pichia anomala*

Y3U21	C	-	-	+	+	+	+	+	+	+	-	-	*Pichia anomala*
Y3U12	C	-	-	+	+	+	+	+	+	+	-	-	*Pichia anomala*
Y0U12	C	-	-	+	+	+	+	+	+	+	-	-	*Pichia anomala*
Y0U31	S	-	-	+	+	+	+	+	+	+	+	-	*Debaryomyces hansenii*

Table 4: Antilisterial potential of yeasts obtained from fermenting cow milk

Isolate codes	Antilisterial activity	ZILm
Saccharomyces cerevisiae Y2A14	-	NZI
Trichosporon mucoides Y2A24	-	NZI
Kluyveromyces lactis Y2A22	-	NZI
Trichosporon mucoides Y2A13	-	NZI
Saccharomyces barnettii Y0A11	-	NZI
Saccharomyces cerevisiae Y3A32	-	NZI
Saccharomyces cerevisiae Y0A21	-	NZI
Debaryomyces hansenii Y2A33	-	NZI
Saccharomyces cerevisiae Y0A14	+	8.0 mm
Saccharomyces cerevisiae Y2A21	-	NZI
Kluyveromyces lactis Y0A32	++	14.0 mm
Saccharomyces cerevisiae Y3A34	+	8.0 mm
Kluyveromyces lactis Y0U11	+++	17.0 mm
Saccharomyces cerevisiae Y1U22	+	12.0 mm
Kluyveromyces lactis Y0U33	+	8.0 mm
Saccharomyces bayanus Y0U34	-	NZI
Debaryomyces hansenii Y2U34	++	12.0 mm
Debaryomyces hansenii Y2U22	++	14.0 mm
Pichia anomala Y2U32	-	NZI
Kluyveromyces lactis Y0U14	-	NZI
Pichia anomala Y3U11	-	NZI
Pichia anomala Y3U21	-	NZI
Pichia anomala Y3U12	-	NZI
Pichia anomala Y0U12	-	NZI
Debaryomyces hansenii Y0U31	-	NZI

Key: - = no activity; + (4.0 - 9.0 mm) = low activity; ++ (10.0 - 14.0 mm) = moderate activity; +++ (15.0 - 20.0 mm) = high activity; mm = millimetres; NZI = No zone of inhibition; ZILm = Zone of inhibition for *Listeria monocytogenes.*

Antilisterial Potential of Yeasts

The eight yeast isolates that inhibited *Listeria monocytogenes* were *Saccharomyces cerevisiae* Y0A14, Y0A32, Y3A34 and Y1U22, *Debaryomyces hansenii* Y2U34 and Y2U22 and *Kluyveromyces lactis* Y0U11 and Y1U22 as illustrated in Table 4.

It was observed that the yeast isolates from the cow milk samples also possessed antilisterial potential. This inhibitory potential was observed by Mohamudha and Ayesha (2010) and is due to changes in pH and the secretion of biological substances such as vitamins, enzymes, amino acids and killer toxins (mycocins) (Lopitz-Otzoa *et al*., 2006; Mohamudha and Ayesha, 2010).

Effect of Neutralization, pH, NaCl, and Enzyme on Bacteriocin and Mycocin

The antimicrobial activities of neutralized LAB and yeasts and effects of treatment of pH, NaCl, and enzyme activities against *Listeria monocytogenes* were illustrated in

Table 5 in which *Lactobacillus rhamnosus* L2A13 and *Saccharomyces cerevisiae* Y1U22 were the most stable to have inhibited *Listeria monocytogenes* after their cell- free supernatant were treated with different NaCl concentration, pH and enzyme activities and were used for co-culturing.

In this work, the LAB and yeasts showed decreased action at pH eight and ten but continued to function well at pH four to seven. Similarly, the findings of Parente and Ricciardi (1999) and Al-Quaysi *et al*. (2017) proved that from pH four and above, bacteriocin and mycocin activity prolong the log phase(s). According to Du Toit *et al*. (2000), the reduction in action was attributed to the adhesion of bacteriocins and mycocin substances on the cell's membrane, which relies on the Hydrogen ion concentration of the cellular component and is very prominent at increased pH level.

This project examined the impact of enzymatic activity on the production of bacteriocins and mycocin. Following the protease activity on the LAB and yeasts, the action of the bacteriocin and mycocin were eliminated. This suggests that the mycocin and bacteriocin generated proteins. Moreover, after being treated with alpha-amylase, the bacteriocin and mycocin generated in this research maintained their complete action. As reported by Du Toit *et al*. (2000), the neutralized culture supernatant that was not inactivated by a-amylase implies that carbon compounds weren't a requirement forthe LAB and yeast action.

The effects of different concentrations of NaCl on bacteriocin and mycocin activity against *Listeria monocytogenes* were studied in this study. It was observed that the inhibition by lactic acid bacteria and yeasts reduced with increasing NaCl concentration which is consistent with the study of Karaoglu *et al*. (2003) whereby *Lactobacillus gasseri* showed great action in the presence of 3% NaCl concentration but the action was stopped at four percentage of Sodium Chloride concentration. This suggests that the bacteriocin and mycocin produced by LAB and yeasts respectively in this study arenot effective at higher NaCl concentration.

Table 5: Effect of Neutralization, pH, NaCl, and enzyme on bacteriocins and mycocins

Isolate codes	Neutralization	Protease	Amylase	2% NaCl	4% NaCl	pH 4	pH 8	pH 10
Lactobacillus rhamnosus L2A13	++ 12.0	+++15.0	+++10.0	+++20.0	+++16.0	+++17.0	++ 11.0	++ 10.0
Lactobacillus delbrueckii L1U32	- NZI	+ 6.0	- NZI	- NZI	- NZI	++ 14.0	++ 12.0	+ 9.0
Lactobacillus rhamnosus L1U31	+ 8.0	- NZI	- NZI	- NZI	- NZI	+++15.0	++ 12.0	+ 9.0
Kluyveromyces lactis Y0U11	- NZI	- NZI	- NZI	+ 6.0	- NZI	- NZI	- NZI	- NZI
Saccharomyces cerevisiae Y1U22	+ 6.0	++ 10.0	+++15.0	+ 4.0	- NZ	+++20.0	+++17.0	+ 6.0
Debaryomyces hansenii Y2U34	+ 6.0	- NZI	- NZI	- NZI	- NZI	+++20.0	+++17.0	+ 6.0
Debaryomyces hansenii Y2U22	+ 6.0	++ 10.0	+ 8.0	+ 6.0	+ 4.0	+ 8.0	+ 6.0	+ 5.0
Saccharomyces cerevisiae Y0A32	+ 5.0	+ 7.0	++ 12.0	+ 5.0	++ 10.0	++ 12.0	++ 12.0	++ 10.0

Key: - = no activity; + (4.0 - 9.0 mm) = low activity; ++ (10.0 - 14.0 mm) = moderate activity; +++ (15.0 - 20.0 mm) = high activity; mm =millimetres; NZI = No zone of inhibition

Co-culturing of Yeasts and Lactic acid bacteria

The colonies were counted after co-culturing LAB and yeast separately in a flask with *Listeria monocytogenes* in skimmed milk. The initial count of *Listeria monocytogenes* which was 4.06×10^5 cfu/ml reduced to 1.02×10^5 cfu/ml in co-culture with *Lactobacillus rhamnosus* L2A13 and also to 2.03×10^5 cfu/ml in co-culture with *Saccharomyces cerevisiae* Y1U22 after 24 and 72 hours respectively.

The antilisterial effect of LAB and yeasts as co-cultures was also examined in this study. The co-culture demonstrated a remarkable inhibitory action against *Listeria monocytogenes*. When *L. monocytogenes* were grown independently their development wasn't exclusively slowed, their final colonies were lower than their starting colonies. This concurs with the research of Cocolin *et al*. (2007) who reported similar observations.

The initial pH of the skimmed milk was 6.48 (slightly acidic) which reduced to 4.20 (acidic) and 4.60 (acidic) after co-culturing *Lactobacillus rhamnosus* L2A13 and *Saccharomyces cerevisiae* Y1U22 separately with *Listeria monocytogenes* in skimmed milk respectively but no significant decrease in pH of 6.39 (slightly acidic) when only *Listeria monocytogenes* was inoculated into the skimmed milk.

Conclusion

Listeria monocytogenes is a pathogen that is resistant to various food preservation methods which pose a risk to human wellbeing. Lactic acid bacteria and yeasts in this research have great antilisterial potential, and it suggests that they are suitable microorganisms in fermentation processes without causing any harm.

References

1. Abiola, C. and Oyetayo, V.O., 2016. Isolation and biochemical characterization of microorganisms associated with the fermentation of Kersting's groundnut (*Macrotyloma geocarpum*). Research Journal of Microbiology 11(2-3): 47-55.
2. Afolabi, O. R., Bankole, O. M. and Olaitan, O. J., 2008. Production and characterization of antimicrobial agents by lactic acid bacteria isolated from fermented foods. The International Journal of Microbiology 4: 2.
3. Al-Qaysi, S.A., Al-Haideri, H., Thabit, Z.A., Al-Kubaisy, W.H., and Ibrahim, J.A., 2017. Production, characterization, and antimicrobial activity of mycocin produced by *Debaryomyces hansenii* DSMZ70238. International Journal of Microbiology pp 1-9.
4. Bahanullah, K., Hamid, I., Sikaudar, K., Shah, M., Khan, A., Saifullah, Arras, M., Qaiser, J. and Shzad, M., 2013. Microbial analysis and quality control of milk collected from various districts of Khyber Pakhtunkwa and Shahzad Munir. International Journal of Pharmaceutical Research and Biosciences 4:243-252.
5. Balqis, P., Noriham, A., Khalilah, A.K. and Lilis, N., 2015. Antimicrobial activity of lactic acid bacteria isolated from different stages of soybean Tempe production. Australian Journal of Basic and Applied Sciences 9(28): 230-234.
6. Banwo, K., Sanni, A. and Tan, H., 2012. Technological properties and probiotic potential of *Enterococcus faecium* strains isolated from cow milk. Journal of Applied Microbiology 114: 229--241
7. Bellil, Y., Benmechernene, Z., Chahrour, W., Naoui, N. and Kihal, M. 2014. Selection and evaluation of antilisterial activity of *Leuconostoc mesenteroides* wild strains isolated from Algerian raw dromedary milk. Journal of Food Processing and Technology 5: 398.
8. Campagnollo, F.B., Margalho, L.P., Kamimura, B.A, Feliciano, M.D., Freire, L., Lopes, L.S., Alvarenga, V.O, Cadavez, V.A., Gonzales-Barron, U., Schaffner, D.W. and Sant'Ana, A.S., 2018. Selection of indigenous lactic acid bacteria presenting anti- listerial activity,

and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. Food Microbiology 73: 288-297.
9. Cheesbrough, M., 2006. District laboratory practice in tropical countries. Cambridge University Press.
10. Cocolin, L., Foschino, R., Comic, G. and Fortina, M.G., 2007. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goatmilk. Food Microbiology 24: 752–758.
11. Du Toit, M., Franz, C.M.A.P., Dicks, L.M.T. and Holzapfel, W.H. (2000). Preliminary characterization of bacteriocins produced by Enterococcus faecium and *Enterococcus faecalis* isolated from pig feces. Journal of Applied Microbiology, 88: 482–494.
12. Fawole, M.O. and Oso B.A., 2004. Characterization of bacteria. Laboratory manual of Microbiology 4th Edition Ibadan, Nigeria. Spectrum book Limited pp. 24-33.
13. Fossi, B.T., Ekue, N.B., Nchanji, G.T., Ngah, B.G., Anyangwe, I.A. and Wanji, S., 2015. Probiotic properties of lactic acid bacteria isolated from fermented sap of palm tree (*Elaeis guineensis*). Journal of Microbiology and Antimicrobials 7(5): 42-52.
14. Hatoum, R., Labrie, S. and Fliss, I., 2012. Antimicrobial and Probiotic Properties of Yeasts: from Fundamental to Novel Applications. Frontiers in Microbiology 3:421.
15. Ivanovic, M., Mirkovic, N., Mirkovic, M., Miocinovic, J., Radulovic, A., Knudsen, T.S. and Radulovic, Z., 2021. Autochthonous *Enterococcus durans* PFMI565 and *Lactococcus lactis* subsp. *lactis* BGBU1–4 in Bio-Control *of Listeria monocytogenes* in Ultrafiltered Cheese. Foods 10: 1448.
16. Karaoglu, S.A., Aydin, F., Kilic, S.S. and Kilic, A.O., 2003. Antimicrobial activity and characteristics of bacteriocins produced by vaginal lactobacilli. Turkish Journal of Medical Sciences 33: 7-13.
17. Lopitz-Otzoa, F., Rementeria, A., Elguezabal, N. and Garaizar, J., 2006. Kefir: a symbiotic yeasts-bacteria community with alleged health capabilities. Revista Iberoamericana de Micologia 23: 67-74.
18. Mohamudha, P.R. and Ayesha, B.J., 2010. Production and effects of killer toxin by *Saccharomyces cerevisiae* on sensitive yeast and fungal pathogens. International Journal of Pharmaceutical Sciences Review and Research 3(1): 127-129.
19. Moulay, M., Benlancen, H., Aggad and Kihal, M., 2013. Diversity and technological properties of predominant lactic acid bacteria isolated from Algerian raw goat milk. Advances in Environmental Biology 7: 999-1007.
20. Nudda, A., Bencini, R., Mijatovic, S. and Pulina, G., 2006. The yield and compositionof milk in Sarda, Awassi and Merino sheep milked unilaterally at different frequencies. Journal of Dairy Science 85: 2879-2884.
21. Olowonibi, O.O., 2017. Isolation and characterization of palm wine strains of *Saccharomyces cerevisiae* potentially useful as bakery yeasts. European Journal of Experimental Biology 7(2): 11
22. Olutiola, P.O., Famurewa O. and Sonntag, H.G., 2000. Introduction to general Microbiology: A Practical Approach. 2nd Edn., Bolabay Publications, Ikeja, Nigeria.
23. Parente, E. and Ricciardi, A., 1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. Applied Microbiology and Biotechnology 52, 628–638.
24. Qvirist, L.A., De Filippo, C., Strati, F., Stefanini, I., Sordo, M., Andlid, T., Felis, G.E.,Mattarelli, P. and Cavalieri , D., 2016. Isolation, identification and characterization of yeasts from fermented goat milk of the Yaghnob

valley in Tajikistan. Frontiers in Microbiology 7: 1690.
25. Sezer, C. and Guven, A., 2009. Investigation of bacteriocin production capability of lactic acid bacteria isolated from foods. Journal of the Faculty of Veterinary Medicine15:45-50.
26. Sulieman, A.E., Esra, A.M. and Abdelgadir, W.S., 2015. Isolation and identification ofyeasts from the different stages of Hulu-mur fermentation. Journal of Microbiology Research 5(2): 71-76.