Cameroon Journal of Biological and Biochemical Sciences, 2025, 33, 1-13.
DOI: https://doi.org/10.63342/cjbbs2025.543

CAMEROON JOURNAL OF BIOLOGICAL AND BIOCHEMICAL SCIENCES
Published by the Cameroon Biosciences Society (CBS)

Research Article

Production of acidified cow- and soy-milk with antimicrobial properties using lactic acid bacteria from indigenous fermented foods

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Received: 07 Nov 2024, Reviewed: 04 Jan 2025, Revised: 11 Jan 2025, Accepted: 15 Jan 2025, Published: 11 Mar 2025

ABSTRACT

Indigenous fermented milk in most West African countries including Nigeria is produced by spontaneous fermentation which results in products lacking uniformity, predictability, and safety. In this study, lactic acid bacteria (LAB) with antimicrobial properties from indigenous fermented foods were investigated for use as fermenting organisms for the acidification of cow and soy milk products. Twenty-two LAB isolates inhibited test organisms with diameter clear zones ranging from 3.67 to 28.00 mm. *Lactobacillus fermentum* (pH 4.46, 27.00 mm), *L. acidophilus* (pH 3.38, 28.00 mm), *L. plantarum* (pH 4.24, 25.67 mm), and *L. coryniformis* (pH 4.70, 23.33) with highest acidifying properties and inhibitory zones respectively, significantly impacted the sensory and proximate quality of the milk samples after fermentation. The texture of all the milk samples was altered through the appearance of curds and the beany aroma of soy milk was significantly suppressed. However, there was no significant change in the colour of the fermented milk compared to the original milk samples. Respectively for the cow and soy milk samples, the crude fibre (14%, 21%) and ash contents (27.36%, 56.04%) were depleted due to fermentation, while lipids (11.03%, 7.79%) and proteins (18.28%, 25.48%) were significantly improved. Optima acidification was obtained at 30 °C and 40 °C. The study concluded that the LAB isolated from Nigeria's indigenous foods has antimicrobial and acidifying properties and can be used to produce quality fermented milk. The study recommends a detailed optimization of the fermentation parameters and a comprehensive analysis of the antimicrobial substances and other metabolites of value that may be produced by the isolated LAB.

Keywords: milk, acidification, antimicrobials, sensory, proximate analysis.

RÉSUMÉ

Le lait fermenté indigène dans la plupart des pays d'Afrique de l'Ouest, y compris le Nigeria, est produit par fermentation spontanée, ce qui donne des produits manquant d'uniformité, de prévisibilité et de sécurité. Dans cette étude, des bactéries lactiques (BL) possédant des propriétés antimicrobiennes provenant d'aliments fermentés indigènes ont été étudiées pour être utilisées comme organismes fermentants pour l'acidification des produits laitiers de vache et de soja. Vingt-deux isolats BL ont inhibé les organismes testés avec des zones claires de diamètre allant de 3,67 à 28,00 mm. *Lactobacillus fermentum* (pH 4,46, 27,00 mm), *L. acidophilus* (pH 3,38, 28,00 mm), *L. plantarum* (pH 4,24, 25,67 mm) et *L. coryniformis* (pH 4,70, 23,33) avec respectivement les propriétés acidifiantes et les zones inhibitrices les plus élevées, et ont eu un impact significatif sur la qualité sensorielle et immédiate des échantillons de lait après fermentation. La texture de tous les échantillons de lait a été modifiée par l'apparition de caillé et l'arôme de haricots du lait de soja a été considérablement supprimé. Cependant, il n'y a eu aucun changement significatif dans la couleur du lait fermenté par rapport aux échantillons de lait d'origine. Respectivement pour les échantillons de lait de vache et de soja, les teneurs en fibres brutes (14%, 21%) et en cendres (27,36%, 56,04%) ont été réduites suite à la fermentation, tandis que les lipides (11,03%, 7,79%) et les protéines (18,28%, 25,48%) ont été significativement améliorés. Une acidification optimale a été obtenue à 30 °C et 40 °C. L'étude a conclu que les BL isolées des aliments indigènes du Nigéria possèdent des propriétés antimicrobiennes et acidifiantes et peuvent être utilisés pour produire du lait fermenté de qualité. L'étude recommande une optimisation détaillée des paramètres de fermentation et une étude approfondie des substances antimicrobiennes et autres métabolites de valeur qui peuvent être produits par les BL isolées.

Mots clés : lait, acidification, antimicrobiens, sensoriel, analyse nutritionnelle.

1. INTRODUCTION

Milk primarily refers to secretions of the mammary glands of mammals that serve as a source of nutrients for their new-borns until they can digest solid foods (Van Winckel et al., 2011) Several plant materials are processed

into milk-like juice and are referred to as non-dairy milk. The rich nutritional composition of milk makes it an easy target for microbial contamination resulting in spoilage and serving as a medium for transmission of diseases (Quigley et al., 2013). Milk is therefore unstable and requires a high level of preservation if not used immediately. Fermentation is one of the means by which milk preservation is achieved. Fermentation of milk involves acidification by lactic acid bacteria resulting in products with improved taste and aroma such as Yoghurt, Kefir, Koumiss, buttermilk, and a variety of others named by the region where the milk is produced (Akabanda et al., 2013). The most common sour milk product in Nigeria and neighbouring West African countries is called "nunu". Nunu is a yoghurt-like traditional milk product that is produced through spontaneous fermentation of cow milk (Owusu-Kwarteng et al., 2017). Lactic acid bacteria have been attributed to the acidity and other properties that improve the taste and shelf quality of nunu (Akabanda et al., 2013). Soymilk fermentation has also resulted in products of improved physicochemical and sensory properties, and other beneficial effects such as cholesterol-lowering; prevention of hepatic lipid and visceral fat accumulation; and prevention of cardiovascular diseases, obesity, cancer, and inflammation (Fukuda et al., 2017). Soymilk is used in making imitation dairy products such as soy yoghurt, soy cream, soy kefir, and soy cheeses. It is also used as an ingredient for making milkshakes, pancakes, smoothies, and mayonnaise (Bharti, 2019). Soy cheese known as "beske" which is synonymous with tofu is made in Nigeria (Raji et al., 2023).

Many lactic acid bacteria that are involved in the fermentation of milk are known to possess antimicrobial activities which help to prevent spoilage of the milk and ensure its safety (Goa et al., 2022). However, the spontaneous nature of fermentation in most developing countries affects the standardization of the products since different kinds of organisms with varying metabolic activities are often involved (Owusu-Kwarteng et al., 2017). Additionally, the safety of the product of such fermentation cannot be ascertained as diseases such as mastitis caused by many pathogens including *Staphylococcus aureus*; and tuberculosis, a lung disease caused by *Mycobacterium tuberculosis* and *M. bovis* are transmitted through milk consumption (Owusu-Kwarteng et al., 2020). This study therefore aims to investigate lactic acid bacteria with high acidifying potentials and antimicrobial properties in indigenous fermented foods, with the hope of developing a high throughput culture for the production of acidified cow and soy milk.

2. MATERIALS AND METHODS

2.1. Milk samples

Cow milk drawn from a healthy animal in Ilorin, Kwara State, Nigeria was collected into a sterile container. The sample was kept under a cold chain (0 ºC – 5 ºC) and transported to the laboratory for analysis. Soy milk was prepared following a previously described procedure by Deepika et al., (2017). Briefly, soybean was soaked in water for 18 hours. The seed coat was removed manually and the seed was ground to smoothness using a mechanical milling machine; the milled product was sieved using muslin cloth (approximately 100 µm); and boiled on low heat for 20 minutes while stirring continuously. The milk was left to cool, filled into sterile containers, and refrigerated at temperatures below 10 ºC until required.

2.2. Microorganisms

Lactic Acid Bacteria (LAB) were isolated from Indigenous fermented foods including Ogi, Fura, Maishanu (milk fat), Nunu, Palm-wine, and Kunu-Zaki. Serially diluted samples of the foods were inoculated on de Mann Rogosa and Sharpe (MRS) Agar by spread plate method. Distinct bacterial colonies were transferred to fresh agar plates by streaking until pure cultures were obtained. The pure isolates were transferred to agar slants and stored at 4 ºC until required. They were characterized by their morphology and biochemical reactions. Organisms that were used for antimicrobial sensitivity test were known foodborne pathogens (clinical isolates from the Department of Microbiology and Parasitology, University of Ilorin Teaching Hospital, Ilorin) and food spoilage organisms isolated in previous studies and maintained in the culture collection unit of the Department of Microbiology, University of Ilorin.

2.3. Assay for antimicrobial activity of the isolates

Cell-free supernatants of the isolated organisms were prepared from a 24-hour MRS broth culture by centrifuging at 10,000xg, 4 ºC for 20 minutes and were used to challenge test organisms using the agar well diffusion method. Briefly, test organisms of 0.5 McFarland were inoculated on Mueller Hinton agar plates by spreading; wells measuring 5 mm were bored in the seeded agar; and 50 µL of the CFS of isolated organisms was filled into the wells. Two drops of molten agar were added to seal the wells before incubation at 37 ºC (Balouri et al., 2016). Plates were examined for the growth of test organisms and inhibition zones around the loaded wells after 24 hours of incubation. The diameter of inhibition zones around wells was measured in mm using the meter rule.

2.3.1. Determination of rate of acidification by isolates

Isolates were grown in MRS broth for 24 hours and centrifuged at 10,000xg, 4 ºC for 20 minutes. The pH of the supernatant was measured using a hand-held pH meter calibrated to pH 4.0, 7.0, and 9.0. The supernatants were titrated against 0.1 N sodium hydroxide using a phenolphthalein indicator to obtain the titratable acidity of the isolates following the American Dairy Products Institute's analytical method ADPI (2023). The lactic acid concentration was calculated with a note that 1.0 ml of 0.1 N NaOH corresponds to 9.008 mg of lactic acid:

$$\% \text{ Lactic acid} = \frac{\text{Volume of 0.1N NaOH used X Normality of NaOH X 9.008}}{\text{Volume of culture supernatant used}} \text{ X } 100$$

2.3.2. Fermentation of cow and soy milk using the LAB isolates

Lactic Acid Bacteria isolates that demonstrated antimicrobial activities and a high rate of acidification in MRS broth was used to ferment fresh cow and soy milk. The milk samples, 100 ml in 250 ml Erlenmeyer flasks were pasteurized by heating to 65 ºC and holding for 30 minutes in a water bath before cooling rapidly in an ice bucket (Chi et al., 2024). Inoculum was prepared from 24 hours' broth culture of the organism by centrifugation at 10,000xg, 4 ºC for 20 minutes. The pellet was harvested and re-suspended in normal saline to obtain 0.5 McFarland which was used to inoculate the milk sample at 5% v/v. Flasks were incubated at 37 ºC without shaking. Samples were withdrawn at intervals to measure the growth of the inoculum, rate of acidification, and sensory evaluation of the milk. Fermentation was terminated after 24 hours and the samples were analysed for crude fibre, lipid, moisture, ash, protein, and carbohydrate composition following standard procedures of AOAC as described by Ajayi (2015). Inoculated flasks were also incubated at 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, and 50 ºC for 16 hours and pH was measured using a hand-held pH meter to determine the optimum temperature for the acidification of the milk samples.

2.3.3. Determination of the growth of LAB isolates and rate of acidification of cow and soy milk samples during fermentation

Samples of the fermenting cow and soy milk were withdrawn at 4-hour intervals to measure the rate of acidity and growth of the inoculum. A hand-held pH meter was used to measure the acidity by dipping the electrode directly into the milk samples. The growth rate of the fermenting organisms was measured by inoculating an appropriately diluted sample of the fermenting milk on MRS agar using the pour plate method. Colonies were counted on plates after 48 hours of incubation at 37 ºC.

2.4. Sensory evaluation of cow and soy milk samples during fermentation:

Samples were evaluated on the nine-point hedonic scale for colour, aroma, and texture by a panel comprising five persons who are very familiar with the sensory properties of yoghurt. Milk samples that were not inoculated with any organism served as the control. Each member of the panel was mandated to assess the samples and record their observation in a questionnaire developed for the evaluation.

2.5. Statistical analyses

Values were measured in triplicate and means ± standard deviation, ANOVA test for significance ($p < 0.05$) with post hoc (Duncan), and paired sample t-test were obtained using the IBM SPSS statistics version 27

3. RESULTS

3.1. Antimicrobial characteristics of isolated organisms

Among 72 bacteria that were isolated on MRS agar, 33 that were Gram-positive, catalase, and oxidase-negative, non-spore-forming, non-motile, facultatively anaerobic, and able to produce acid from lactose, glucose, sucrose, fructose, and maltose (Table 1) were selected as potential lactic acid bacteria. They were screened for antimicrobial activities and 22 inhibited test organisms with diameter inhibition zones ranging from 4.67 to 28.00 (Table 2). To compare, test organisms were challenged with common antibiotics using the disc diffusion method and the result is also included in Table 2. Isolates that produced inhibition zones greater than 15 mm in the agar well diffusion antimicrobial assays were selected for further studies.

Table 1: Biochemical Characteristics of the Lactic Acid Bacterial Isolates

Isolates	Gram reaction	Catalase	Oxidase	Citrate	Urease	Indole	Methyl Red	Voges Proskauer	Starch	TSI-H_2S	TSI-Gas	TSI-Reaction	Lactose	Sucrose	Fructose	Mannitol	Growth at 45°C	Growth in 6.5% NaCl	O_2 relationsh	Probable Organism
L1	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus cremoris*
L5	+	+	-	-	+	-	-	+	-	+	+	KA	-	-	A	-	+	+	Fa	*Lactobacillus brevis*
L6	+	-	-	+	-	-	-	-	-	-	-	KA	A	A	A	A	+	+	Fa	*Lactobacillus fermentum*
L7	+	-	-	+	-	-	-	-	-	-	-	KK	A	-	-	A	+	+	Fa	*Lactobacillus delbrueckii*
L8	+	-	-	+	-	-	-	-	+	+	-	AK	-	A	A	A	+	+	Fa	*Enterococcus faecalis*
L10	+	-	-	-	-	-	-	-	-	-	-	AA	A	A	A	A	-	-	Fa	*Lactobacillus acidophilus*
L14	+	-	-	-	-	-	-	-	-	-	-	KA	-	AG	A	-	+	+	Fa	*Streptococcus salivarius*
L15	+	-	-	-	-	-	-	-	+	-	-	KA	AG	AG	AG	A	+	-	Fa	*Streptococcus uberis*
L16	+	-	-	-	-	-	-	-	-	-	-	KK	A	AG	A	A	+	+	Fa	*Lactobacillus plantarum*
L17	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus fermentum*
L20	+	-	-	-	-	+	+	-	-	+	-	AK	AG	-	A	AG	+	-	Fa	*Enterococcus faecalis*
L24	+	-	-	-	-	-	-	-	+	-	-	AK	A	A	A	A	+	+	Fa	*Lactobacillus fermentum*
L25	+	-	-	-	-	-	-	-	+	-	-	AK	A	A	A	A	+	+	Fa	*Lactobacillus rhamnosus*
L27	+	-	-	-	-	-	-	-	-	-	-	KA	-	AG	A	-	+	+	Fa	*Lactobacillus leichmannii*
L34	+	-	-	-	-	-	-	-	-	-	--	KA	A	A	AG	-	+	+	Fa	*Lactobacillus plantarum*
L28	+	-	-	-	-	-	-	-	+	-	-	KA	AG	AG	AG	A	+	-	Fa	*Lactobacillus cellobiosus*
L38	+	-	-	-	-	-	-	-	-	-	-	KA	-	AG	A	-	+	+	Fa	*Enterococcus faecalis*
L39	+	-	-	-	-	-	-	-	+	-	-	KA	AG	AG	AG	A	+	-	Fa	*Lactobacillus rhamnosus*
L40	+	-	-	-	-	-	-	-	+	-	-	AK	A	A	A	A	+	+	Fa	*Streptococcus thermophilus*
L41	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus coryniformis*
L45	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus hilgardii*
L49	+	-	-	-	-	-	-	-	+	-	-	AK	A	A	A	A	+	+	Fa	*Streptococcus salivarius*
L50	+	-	-	-	-	-	-	-	-	-	-	AA	A	A	A	A	-	-	Fa	*Lactobacillus plantarum*
L52	+	-	-	-	-	-	-	-	-	-	-	AA	A	A	A	A	-	-	Fa	*Enterococcus faecium*
L56	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Streptococcus thermophilus*
L57	+	-	-	-	-	+	+	-	-	+	-	AK	AG	-	A	AG	+	-	Fa	*Lactobacillus casei*
L58	+	+	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus paracasei*
L59	+	+	-	-	-	+	+	-	-	+	-	AK	AG	-	A	AG	+	-	Fa	*Lactobacillus fermentum*
L61	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus plantarum*
L68	+	+	-	-	-	-	-	-	-	-	-	KK	A	AG	A	A	+	+	Fa	*Lactobacillus acidophilus*
L69	+	-	-	-	-	-	-	-	+	-	-	AK	A	AG	AG	AG	+	-	Fa	*Lactobacillus plantarum*
L70	+	-	-	-	-	+	+	-	-	+	-	AK	AG	-	A	AG	+	-	Fa	*Lactobacillus pentosus*
L72	+	-	-	-	-	+	+	-	-	+	-	AK	AG	-	A	AG	+	-	Fa	*Enterococcus faecium*

"+": Positive, "-": Negative, AK: Acid/Alkaline, KK: Alkaline/Alkaline, KA: Alkaline/Acid, AA: Acid/Acid, A: Acid, AG: Acid and gas, Fa: Facultative anaerobe, TSI: Triple sugar iron test

Table 2: Diameter zone of inhibition of test organisms by LAB Isolates

LAB Isolates	Diameter of inhibition zone (mm)								
	YE	SL	ECL	EC	BS	SE	SA	KO	CF
L. cremoris	+	-	-	-	-	+	-	-	-
*L. fermentum*1	-	++++	-	-	-	+++	-	++++	-
*E. faecalis*1	++	-	-	-	-	-	-	-	+++
L. acidophilus	++++	-	-	-	-	+++	+++	-	-
S. salivarus	+++	++	-	+++	-	-	-	-	+++
*L. plantarum*1	-	++	-	-	-	-	-	-	-
*L. fermentum*2	-	-	-	-	++	+	++	-	+
*E. faecalis*2	-	++++	+	++	+	-	-	-	+
*L. fermentum*3	-	++	-	-	-	+++	-	-	-
*L. rhamnosus*1	-	-	-	-	-	-	-	-	+
*L. plantarum*2	-	++++	-	++++	-	+++	-	-	++++
L. cellobiosus	+	+	-	-	-	-	++	-	-
*E. faecalis*3	-	+	-	++	-	-	-	-	-
*L. rhamnosus*2	-	++	-	-	-	-	-	-	-
S. thermophilus	+++	-	+++	-	+	++	-	++	+
L. coryniformis	++++	-	-	+++	+++	+++	-	-	+++
L. hilgardii	-	-	-	-	-	-	-	-	++
*L. plantarum*3	-	+++	-	-	-	++	+++	-	++++
S. thermophilus	++	-	-	-	-	-	-	+	+
L. casei	-	++	-	+++	-	++	+++	-	++++
*L. plantarum*4	-	-	-	-	-	++	-	-	-
L. pentosus	++++	-	-	-	-	++	-	-	-
Pefloxacin	++++	-	-	++++	-	++++	++++	-	-
Gentamycin	-	-	-	-	-	-	++++	-	-
Ciprofloxacin	++++	++++	-	++++	-	-	-	-	++
Streptomycin	-	-	-	-	-	-	-	++++	-
Septrin	-	-	-	++	-	-	++++	-	-
Ofloxacin	+++	-	-	-	-	-	-	-	-
Chloramphenicol	-	++	+++	++	-	++++	-	-	-
Sparfloxacin	++++	++++	-	++++	-	-	-	-	-

Diameter inhibition zone values are: "+" ≤ 10 mm; "++" 10 < 15 mm; "+++" 15 < 20 mm; "++++" > 20 mm; "-" no inhibition. YE: *Yersinia enterocolitis;* SL: *Serratia liquefaciens;* ECL: *Enterococcus cloacae;* EC: *Escherichia coli;* BS: *Bacillus subtilis;* SE: *Salmonella enteritidis;* SA: *Staphylococcus aureus;* KO: *Klebsiella oxytoca;* CF: *Citrobacter freundii*

3.2. Acidification properties of isolates

A measure of the pH and titratable acidity was used to determine the acidification properties of the isolates as presented in Table 3. Four isolates identified *as Lactobacillus fermentum, L. acidophilus, L. plantarum, and L. coryniformis* with highest titratable acidity and corresponding lowest pH were selected for use for the fermentation of cow and soy milk.

Table 3: pH and titratable acidity of cell-free supernatant of the broth culture of selected LAB isolates

S/N	Organisms	pH	% Lactic Acid
1.	*L. fermentum*1	4.46	0.90 ± 0.00
2.	*E. faecalis*1	6.10	0.42 ± 0.02l
3.	*L. acidophilus*	3.38	0.89 ± 0.00
4.	*S. salivarus*	5.28	0.66 ±0.03
5.	*E. faecalis*2	4.77	0.84 ±0.01
6.	*L. fermentum*3	5.04	0.76 ±0.03
7.	*L. plantarum*2	4.70	0.89 ± 0.00
8.	*S. thermophilus*	5.79	0.49 ±0.01
9.	*L. coryniformis*	4.89	0.87 ±0.00
10.	*L. plantarum*3	4.24	0.80 ±0.00

For % lactic acid, values are means ± SD; n=3

3.3. Growth and acidification rates of the LAB isolates in cow- and soy-milk during fermentation:

The rate of acidification and growth of bacteria during fermentation of the cow and soy milk samples were measured and presented in Figure 1a and b respectively. Bacterial growth in the two milk samples was initially slow during fermentation in line with the growth curve, an indication that the bacteria were adapting to the new medium. An exponential growth occurred thereafter. Acid production in the samples also followed the growth pattern as the change in the pH of the samples was negligible during the first 8 hours of incubation but more pronounced from 12 hours. Fermentation was terminated after 24 hours to avoid conversion of the lactic acid to undesirable secondary metabolites by hetero-fermentative LAB or other organisms that may be present in the sample.

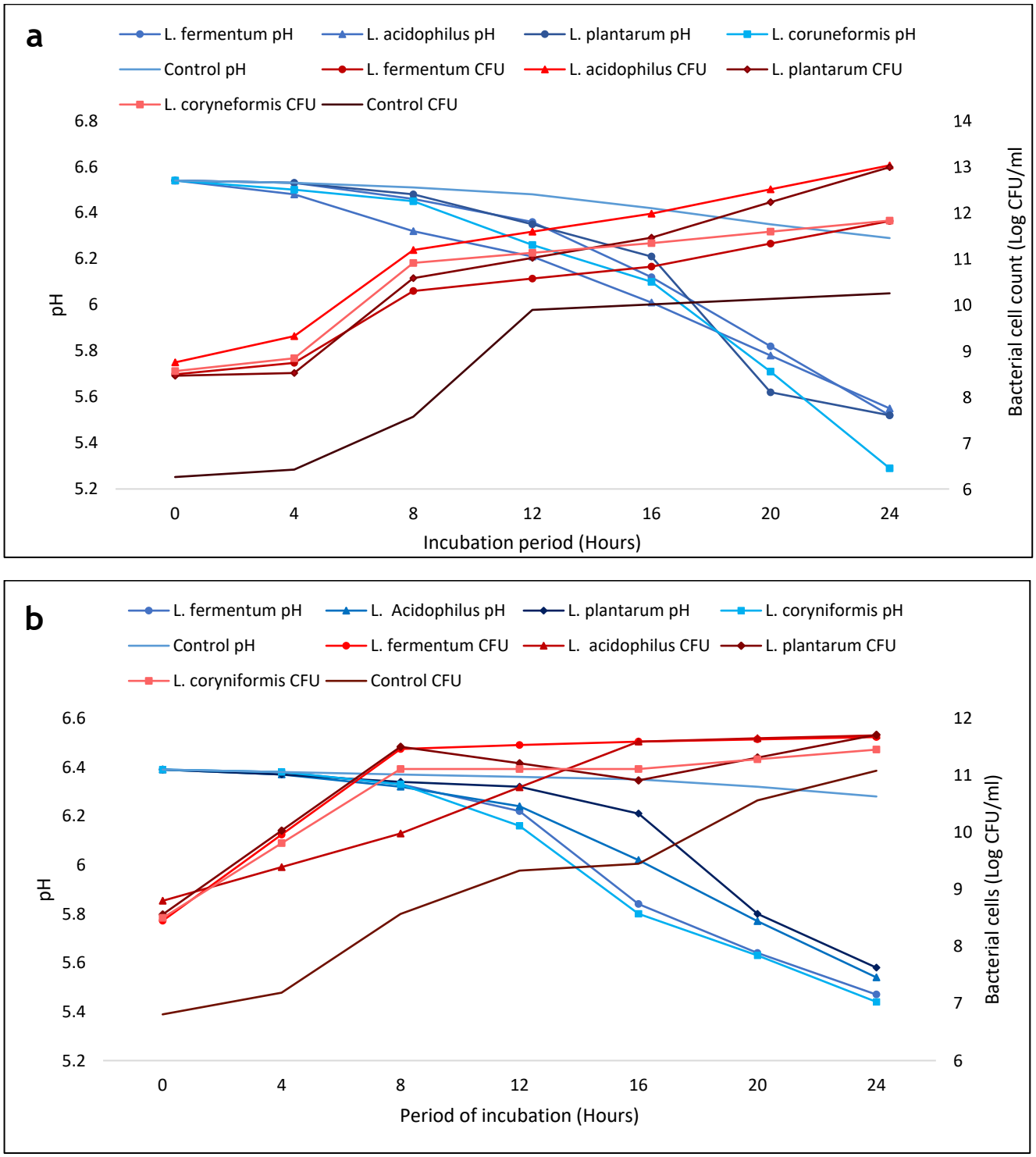


Fig. 1: Bacterial growth and rate of acidification of (a) cow and (b) soy milk during fermentation with the four LAB isolates

3.4. Sensory quality of cow and soy milk fermented with LAB isolates

As shown in Table 4, the colour of the cow and soy milk samples did not differ significantly due to fermentation. The difference in the aroma was also insignificant for the cow milk samples. The beany odour of soy milk was however suppressed by a significantly pleasant acidic aroma in the 16-hour fermented samples. The texture of the milk changed significantly due to fermentation with the appearance of tiny curds within 8 hours of fermentation. The curd's thickness increased, and an unpleasant appearance occurred in the 24-hour fermented samples.

Table 4: Mean ± standard deviation of nine-point hedonic sensory evaluation of Cow and soy milk during fermentation with LAB isolates

Organisms	Time (Hour)	Cow Milk			Soy Milk		
		Colour	Aroma	Texture	Colour	Aroma	Texture
Lactobacillus fermentum	0	8.0±0.45^{a}	6.8±0.45^{a}	8.4±0.55^{a}	8.6±0.55^{a}	5.4±0.55^{a}	7.6±0.55^{a}
	8	7.4±0.55^{a}	7.6±0.55^{a}	7.4±0.55^{ab}	8.6±0.55^{a}	5.6±0.55^{a}	7.8±1.09^{a}
	16	7.2±0.55^{a}	7.4±0.55^{a}	6.4±0.45^{b}	7.8±0.45^{a}	4.8±0.84^{b}	4.0±0.71^{b}
	24	7.0±0.84^{a}	7.8±0.84^{a}	7.6±0.45^{ab}	7.4±0.89^{a}	4.2±0.45^{b}	3.8±0.45^{b}
Lactobacillus acidophilus	0	8.0±0.45^{a}	6.8±0.45^{a}	8.4±0.55^{a}	8.6±0.55^{a}	5.4±0.55^{a}	7.6±0.55^{a}
	8	7.4±0.55^{a}	7.4±0.55^{a}	6.6±0.45^{b}	7.8±0.89^{a}	5.4±0.71^{a}	7.5±0.84^{a}
	16	7.4±0.84^{a}	7.2±0.84^{a}	6.2±0.84^{b}	7.6±0.45^{a}	4.6±0.55^{b}	4.6±0.55^{b}
	24	7.4±1.00^{a}	7.0±1.00^{a}	5.2±0.45^{c}	7.8±0.55^{a}	4.2±0.84^{b}	4.2±0.45^{b}
Lactobacillus plantarum	0	8.0±0.45^{a}	6.8±0.45^{a}	8.4±0.55^{a}	8.6±0.55^{a}	5.4±0.55^{a}	7.6±0.55^{a}
	8	7.6±0.89^{a}	7.4±0.89^{a}	6.6±0.45^{b}	7.8±0.84^{a}	5.8±0.45^{a}	7.0±1.00^{a}
	16	7.8±0.84^{a}	7.2±0.84^{a}	4.8±0.71^{c}	6.6±0.55^{b}	4.6±0.55^{b}	4.8±0.45^{b}
	24	7.6±0.71^{a}	7.0±0.71^{a}	4.6±0.84^{c}	7.2±0.45^{b}	4.0±0.71^{b}	4.4±0.55^{b}
Lactobacillus coryniformis	0	8.0±0.45^{a}	6.8±0.45^{a}	8.4±0.55^{a}	8.6±0.55^{a}	5.4±0.55^{a}	7.6±0.55^{a}
	8	7.6±0.71^{a}	7.0±0.71^{a}	7.0±0.55^{a}	7.8±0.84^{b}	5.6±0.89^{a}	7.4±0.89^{a}
	16	7.4±0.55^{a}	7.2±0.84^{a}	4.4±0.45^{b}	6.8±0.84^{b}	4.4±0.55^{b}	4.4±0.89^{b}
	24	7.6±0.84^{a}	7.2±0.84^{a}	4.2±0.45^{b}	6.8±0.45^{b}	4.0±0.71^{b}	4.6±0.55^{b}
Un-inoculated milk as Control	0	8.0±0.45^{a}	6.8±0.45^{a}	8.4±0.55^{a}	8.6±0.55^{a}	5.4±0.55^{a}	7.6±0.55^{a}
	8	7.8±0.55^{a}	6.8±0.55^{a}	7.8±0.45^{a}	8.2±0.45^{a}	5.8±1.30^{a}	8.0±0.71^{a}
	16	7.6±0.45^{a}	6.6±0.45^{a}	4.8±0.55^{b}	8.6±0.55^{a}	5.6±0.55^{a}	7.4±0.55^{a}
	24	7.6±0.55^{a}	6.4±0.55^{a}	4.6±0.45^{b}	6.2±0.84^{b}	4.0±0.71^{b}	4.8±0.45^{b}

Means in homogeneous subsets with different superscripts are significant ($p < 0.05$)

3.5. Proximate composition of cow and soy milk before and after fermentation:

The result of the proximate analysis of the milk samples is presented in Table 5. Data were analysed using a paired sample t-test and the effects of fermenting the cow and soy milk samples with the isolated LAB, on the proximate composition of the milk was shown. Values obtained from the individual raw milk samples were compared with those of the fermented products at a 95% confidence interval with p-value set at 0.05. For cow milk, the crude fibre of the raw milk was higher than all the fermented products with statistical significance in the *L. fermentum* and *L. plantarum* ferments (Table 5). The lipid content was significantly improved by the fermentation except for the *L. coryniformis* ferment. The moisture content of the milk fermented with *L. fermentum* was insignificantly reduced while the increase observed in the others was significant. Ash content was significantly reduced in all the fermented samples except the *L. fermentum* product which gave an insignificant p-value. The protein content was improved significantly in all the fermented samples. Carbohydrate was used up in the fermentation and the reduction in its value was significant in all the fermented samples.

The crude fibre was significantly improved in all the fermented soy milk while the lipid content was reduced significantly in samples fermented with *L. fermentum* and *L. acidophilus* and higher for the other two ferments although with insignificant difference in the *L. coryniformis* fermented sample. The moisture content of all fermented samples was significantly higher compared to the raw milk except the *L. coryniformis* ferment which gave an insignificant p-value. The ash content was significantly reduced in all the fermented samples. Similar to the cow milk samples, the protein content of the soy milk samples was improved significantly by fermentation, and the carbohydrate content was also reduced significantly in all the fermented samples.

Table 5: Proximate composition of cow and soy milk fermented using *Lactobacillus fermentum* (LFFM), *L. acidophilus* (LAFM), *L. plantarum*; LPFM and *L. coryniformis* LCFM

Milk Samples	Crude-fibre (%)		Lipid (%)		Moisture content (%)		Ash content (%)		Protein (%)		Carbohydrate (%)	
	Cow	Soy	Cow	Soy	Cow	Soy	Cow	Soy	Cow	Soy	Cow	Soy
Raw Milk	1.00±.15^{d}	0.79±.01^{a}	3.47±.02^{b}	2.13±.02^{c}	85.82±.09^{a}	90.57±.01^{ab}	1.06±.05^{d}	0.91±.01^{d}	3.13±.03^{a}	2.59±.15^{a}	5.52±.05^{e}	3.01±.04^{d}
LFFM	0.89±.10^{b} (0.016)	0.99±.06^{b} (0.002)	3.80±.02^{c} (0.003)	1.29±.02^{a} (<0.001)	86.38±.02^{a} (0.009)	92.11±.01^{b} (<0.001)	0.94±.04^{c} (0.014)	0.50±03^{c} (0.003)	3.75±.04^{d} (0.004)	3.17±.02^{c} (0.015)	4.24±.07^{b} (<0.001)	1.94±.06^{b} (<0.001)
LAFM	0.98±.10^{c} (0.250)	1.00±.01^{c} (<0.001)	3.77±.01^{c} (<0.001)	1.89±.01^{b} (<0.001)	87.40±.15^{b} (0.006)	91.27±.17^{ab} (0.017)	0.77±.01^{a} (0.010)	0.40±.01^{a} (<0.001)	3.83±.03^{e} (<0.001)	3.01±.03^{b} (0.027)	3.26±.01^{a} (<0.001)	2.43±.01^{c} (0.002)
LPFM	0.86±.10^{a} (<0.001)	0.99±.06^{b} (0.001)	3.90±.01^{d} (<0.001)	2.31±.04^{e} (0.002)	86.33±.74^{a} (0.902)	91.87±.03^{b} (<0.001)	0.83±.01^{b} (0.010)	0.45±.01^{b} (<0.001)	3.49±.01^{c} (0.002)	3.25±.04^{c} (0.024)	4.59±.01^{c} (<0.001)	1.13±.02^{a} (0.002)
LCFM	1.00±.10^{d} (0.840)	0.99±.06^{b} (0.001)	3.20±.02^{a} (0.002)	2.19±.02^{d} (0.057)	86.73±.46^{a} (0.106)	92.04±1.15^{b} (0.142)	0.78±.02^{a} (0.017)	0.52±.02^{c} (<0.001)	3.32±.01^{b} (0.008)	3.17±.05^{c} (0.010)	4.97±.05^{d} (0.002)	1.10±.04^{a} (<0.001)

Values are means % ± SD, and p-values in brackets. Means in homogeneous subsets with different superscripts are significant (ANOVA, $p < 0.05$), p-values in brackets show the significance of the paired sample t-test between the means of the raw milk and each of the fermented milk samples. LFFM – milk fermented using *L. fermentum*, LAFM – milk fermented using *L. acidophilus*, LPFM – milk fermented using *L. plantarum*, and LCFM – milk fermented using *L. coryniformis*.

3.6. Temperature optima for the acidification of cow and soy milk by the LAB isolates

The optimal temperature for the acidification of the cow and soy milk samples was investigated. *Lactobacillus acidophilus* fermentation was optimum at 40 ºC while the other LAB produced highest acidity at 30 ºC in the two milk samples. Acidification was very low at 20 ºC and 50 ºC (Fig. 2).

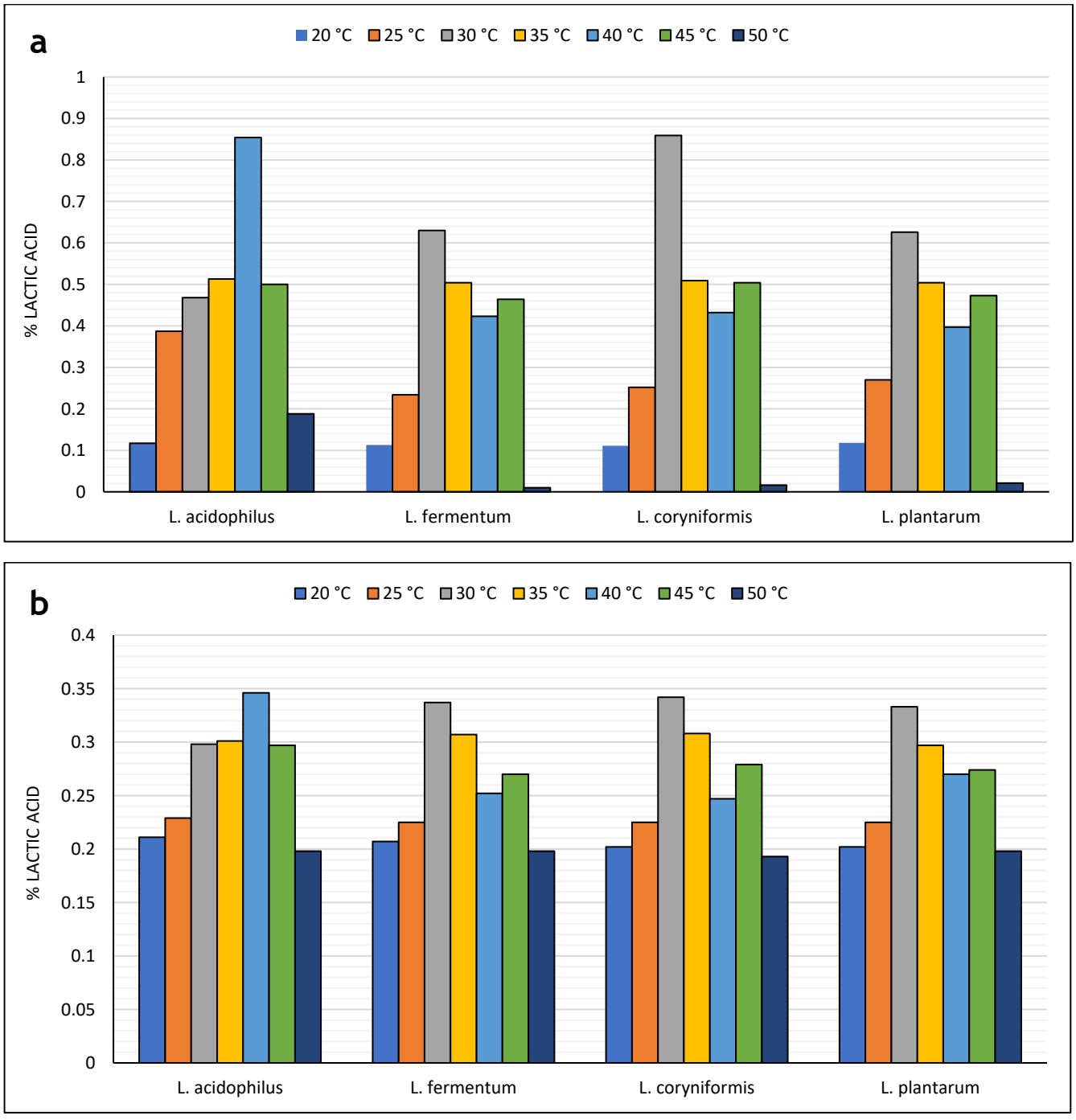


Fig. 2: Effect of temperature on the acidification of (a) cow milk and (b) soy milk by LAB isolates

4. DISCUSSION

In this study, most of the Gram-negative bacteria were found to be susceptible to the cell-free supernatant of the isolated LAB (Table 2) e.g., *Yersinia enterolitica, Serratia liquefaciens, Salmonella enteritidis, and Escherichia coli* were inhibited by 8, 11, 12 and 6 CFS respectively. In contrast, Enterococcus faecium, Bacillus subtilis, and Staphylococcus aureus which are all Gram-positive were susceptible to just 1, 3, and 5 of the CFS respectively. Similar studies of lactic acid bacterial inhibition of *Yersinia enterolitica* (Angino et al., 2015), *Serratia liquefaciens* (Rao et al., 2023), *Salmonella enteritidis* (Lando et al., 2023), *Escherichia coli* (Ren et al., 2019), *Bacillus subtilis* (Digaitiene et al., 2012) and *Staphylococcus aureus* (Ayeni et al., 2009) have been

reported. The CFS of 12 of the isolates inhibited at least 3 test organisms albeit some with diameter inhibition zone less than 10 mm. Inhibitory activities of many of the LAB CFS compared favourably with some of the known antibiotics used regarding the diameter zone of inhibition and activity spectrum. Previous studies have reported diverse inhibitory spectra. While some highlighted stronger inhibitory effects against Gram-positive bacteria (Cui et al., 2021), for others Gram-negative bacteria were more susceptible (Alakomi et al., 2000).

The inhibition of Gram-negative bacteria in this study is an indication that metabolites such as lactic acid produced by the isolates permeate the cell wall of the affected test organisms making their cell membrane vulnerable to the antimicrobial substances. This mechanism was reported by Wang et al. (2022) in a synergistic study combining plantaricin with lactic acid against *Aeromonas hydrophila*, a Gram-negative plantaricin-resistant bacterium, whereby, lactic acid caused a release of lipopolysaccharides from the organism paving the way for plantaricin to contact the cell membrane and cause damage to the cell. In addition, lactic acid produced by the LAB also functions as an antimicrobial agent due to its ability to penetrate the cytoplasmic membrane of the affected bacteria, lower the pH of the cytoplasm, and cause a disruption in the transmembrane proton motive force (Alakomi et al., 2000).

The pH of a food determines the type of microorganisms that can grow in it. Most bacteria have their optimum growth at pH between 6.5 to 7.5 i.e., neutral, while yeasts and molds grow at lower pH values. Food spoilage by bacteria and foodborne pathogens can be controlled by acidifying the foods. High-acid foods have a pH of less than 4.6 and include most fruits, acidified milk, some vegetables, and vinegar while most vegetables and meat are low-acid foods. Fresh cow and soy milk have a pH between 6.5 and 7.0 which is the optimum for most bacteria. This explains their vulnerability to spoilage bacteria and food-borne pathogens. Lowering the pH of milk through fermentation has been used to preserve the milk against the above-mentioned groups of bacteria. Acidification of milk by LAB therefore serves a dual purpose of improving the sensory quality and extending the shelf life of the milk. The four bacteria used for the fermentation of cow and soy milk in this study were *Lactobacillus fermentum, L. acidophilus, L. plantarum,* and *L. coryniformis*. They were selected based on their rate of acidification as exemplified by the comparative low pH and high acid titer (Table 3). The bacteria were able to use the sugar in milk and produce lactic acid as one of the metabolites. Cow milk contains mainly lactose, a disaccharide that is broken down to glucose and galactose by the enzyme lactase (Gambelli, 2017). The ability to use lactose in milk is therefore an indication that the organisms secretes lactase. The sugars in soymilk are starchyose, raffinose, and sucrose. Fermentation of soymilk by the LAB isolates in this study therefore shows that the organisms were able to metabolize the oligosaccharides, starchyose, and raffinose due to galactosidase activity (Surmarna, 2008).

Proximate composition is a reflection of the nutritional quality of foods. Among the very crucial goals of food processing, including fermentation, is the improvement of the nutritional value of the foods. In the current study, the proximate analysis was based on crude fibre, lipid, moisture, ash, protein, and carbohydrate composition. The improved protein content of the fermented milk samples in this study indicates low utilization by the organisms during fermentation. Also, enzymes secreted by the LAB during fermentation along with the organisms may have contributed to the total protein in the fermented milk. In similar studies, significant protein content and indeed other nutritional components were reported for the products of fermentation of milk from various sources (Ladokun and Oni, 2014; Abdulrahman and Sanmi (2021); Saliu et al., 2021; De et al., 2022; Obi et al., 2022).

The decline in the colour of fermented milk has been attributed to oxidation, dehydration, and enzymatic browning (Micheni et al., 2024). The colour of the two milk samples used in this study did not change significantly during fermentation probably due to the short fermentation time whereby the amount of enzyme and acid produced was not sufficient to cause the biochemical changes or the acid had not been oxidized. Also attributable to the short fermentation period, is the response of panelists to the aroma of the cow milk samples in the current study. The slight differences in the aroma may be attributed to the ability of the organism to produce substances such as acetaldehyde, diacetyl, and butanediol (Wang et al., 2021) within the short fermentation time. Suppression of the beany smell by a pleasant acidic aroma after fermentation in soy milk samples also attests to the aroma-producing capability of the organisms, some of which have been reported in previous studies to degrade beany flavour and produce volatile compounds such as acetic acid, diacetyl, and acetoin (Du et al., 2022) Another study reported a pleasantly sour taste of 24-hour fermented milk which deteriorated to off-taste and off-flavour by 48 hours (Chi et al., 2024). Significant changes occurred in the texture of the milk samples in the current study by the 16th hour and deteriorated further up to the 24th hour, another reason that led to the termination of fermentation since the desire was to produce yoghurt-like acidified milk. The tiny lumps that were noticed in the 16th-hour ferment which resulted in syneresis by 24 hours were

attributable to increased acidification which may lead to the breakdown of polysaccharides similar to an earlier reported study (Micheni et al., 2024).

Lactobacillus acidophilus is a thermophilic LAB that has its optimum growth at temperatures between 35 and 45 ºC and since lactic acid is produced as primary metabolites, the optimum acidification at 40 ºC obtained in the current study is justified. The other LAB isolates acidified the milk optimally at 30 ºC. There is a paucity of information relating to temperature optimization for the acidification of milk by the LAB used in this study. However, in the production of fermented milk beverages that used the same LAB, the milk was incubated at 37 ºC after inoculation [Abdel-Rahman et al., 2013; Hati et al., 2019; Fonseca et al., 2020; Meng et al., 2023). The low percentage acidity at 20 and 50 ºC can be attributed to inhibition of growth and consequently, low metabolic activities since these temperatures fall outside the optimal growth temperatures of most lactic acid bacteria.

5. CONCLUSION

In this study, lactic acid bacteria (LAB) with antimicrobial properties were isolated from Nigeria's indigenous fermented foods. Four of the isolated LABs with the highest antimicrobial and acidity potentials were used to ferment cow and soy milk to a product with significant proximate and sensory quality. This study recommends a detailed optimization study of the fermentation parameters such as incubation period and temperature, the substrate-to-inoculum ratio, and a comprehensive study of the antimicrobial substances and other metabolites of value including bacteriocins, organic acids, hydrogen peroxide, exopolysaccharides, amino acids and vitamins that may be produced by the isolated LAB.

ACKNOWLEDGEMENT. The authors acknowledge the technologists of the Department of Microbiology, University of Ilorin for graciously supplying the test organisms used in this study and for the opportunity to use the departmental laboratory and the equipment for the bench work.

AUTHORS CONTRIBUTION. Bolanle Kudirat Saliu: Conceptualisation, Supervision, Resources, Data analysis, Writing and compilation, review and editing of the manuscript. Rukayat Ibiyemi Salami: Data collection, Formal analysis, Resources, Writing the original draft of the manuscript. Abdulwaheed Ayodeji Alabi: Data collection, Formal analysis, Resources. Vanessa Omotola Dadzie: Data collection, Formal analysis, Resources. Adebisi Elijah Ajewole: Data collection, Formal analysis, Resources. Bashirat Olamide Yusuf-Salihu: Resources, Writing of parts of the manuscript.

CONFLICT OF INTERESTS. The authors of the manuscript declare no conflict of interest.

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