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Development of Starter Culture for Soymilk Fermentation using Lactic Acid Bacteria Isolated from Raw Milk

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Abstract

The use of unique microbial culture with desirable qualities as starter is very essential for the processing of fermented products with exceptional and uniform organoleptic properties. Fermentation enhances the formation of organic acid in food and thus promotes shelf life. The development of starter culture from Lactic acid bacteria (LAB) recovered from raw milk for soymilk fermentation was highlighted. The LABs were isolated from raw milk using pour plate techniques on MRS agar (MRSA). Characterization and identification of isolates were performed through morphological and biochemical tests. The LABs obtained were randomly mixed and used as starter culture to initiate fermentation in five sets of freshly prepared soymilk from soya beans for four hours at laboratory conditions, and the products were subjected to proximate and organoleptic assessments following standard methods. A total of four Lactobacillus species and one Streptococcus species were obtained, all isolates were acid formers and positive to phenol tolerance. The proximate content of the fermented soymilk showed that the moisture content ranged from 93.78±0.18 to 92.38±0.04 %, the protein content ranged from 3.52±0.04 to 2.30±0.01 %, the nitrogen-free extract ranged from 0.27±0.14 to 0.99±0.00. The titratable acid was between 25.53 and 15.04, while the pH values ranged from 4.20 to 3.19, the organoleptic scores were between 53 to 84 %. The study underscores the potential of the isolated lactic acid bacteria to produce fermented soymilk with good nutritional quality that could be harnessed for product development in the local food processing industries.

Keywords: Soy milk, Fermentation, Proximate, Lactic acid bacteria, Starter culture

INTRODUCTION

The food and beverage industry usually develops new products with desirable nutritional and organoleptic qualities and enhanced shelf life through microbial fermentation. However, fermentation in the local cottage food processing settings is mostly at a preliminary or small-scale level and is often brought about using primitive fermentation techniques such as spontaneous fermentation, back-slopping, or loop fermentation (Adedayo and Abdulkarreem, 2024). Products of such fermentation often lack uniformity in essential properties and might be difficult to reproduce. To mitigate these challenges and produce local food with acceptable qualities that are

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stable over a long period, it is essential to induce fermentation with microbial cultures of known noble characteristics that could also impact food safety. Microbial preparations of known desirable characteristics, able to produce advantageous compounds or develop useful secondary metabolites through their enzymatic processes, added to food to initiate fermentation are known as starter cultures (Fox *et al*., 2017; Adedayo and Abdulkarreem, 2024).

Potential starter culture organisms must meet the status of Generally Regarded as Safe (GRAS) before they can be considered, such organisms, such as bacteria, mold, or yeast, and used singly or in mixed culture formulations. The credentials of such organisms usually considered before choosing include the ability to produce and tolerate acid which will increase food storage ability and stability over a long period (Taskila *et al*., 2019). Furthermore, they must be non-toxigenic, non-pathogenic, and not easily mutated, they must grow and develop rapidly and not be fastidious, and possess the ability to develop the desired specific property in food, enhance food safety and fortify the nutritional and health-promoting status of the fermented food (Hammami *et al*., 2017). Starter culture is widely applicable in food processing industries globally and the involvement in food preparation is globally accepted (Ashaolu *et al*., 2020). The search for starter organisms is continuous to meet the demands in the food industries, and it is usually from the naturally occurring microbial communities of fermented foods or the gut of animals (Pereira *et al*., 2020). The ability to produce and tolerate organic acid resulting in the low pH in fermented foods, and the production of other biological preservatives such as bacteriocins, diacetyl, peroxides, and antioxidants in food help to enhance bio protection against common food-borne pathogens and hence, protect the food consumer from cases of food poisoning (Laranjo *et al*., 2017; Adedayo and Abdulkareem 2024).

Lactic acid bacteria (LAB) are among the prominent microbiota with dynamic and important metabolic activities essential in food processing, safety, and preservation. Their ability to produce lactic acid among other organic acids, bacteriocins, and diacetyl in food, stands them out in the dairy and other food processing industries, they are also known to enhance food nutrients and bioavailability, and could also serve as probiotics (Bintsis, 2018). Due to the relative importance of this group of bacteria in unique product development, uniformity, and stability of fermented food, it is a worthy venture to search for new strains with good potential for food fermentation. Soymilk made from soya beans is an inexpensive plant protein that has gained popularity as a suitable alternative to the more expensive cow milk. It is usually recommended for people with lactose intolerance, milk allergy, and vegetarians (Nabulsi *et al.,* 2014).

Soymilk is very nutritious and rich in essential vitamins, minerals, amino acids, and antioxidants. However, despite the nutritional and health benefits of this drink, poor consumer acceptability is a major setback because of the characteristic odour and unusually short shelf life (Wang *et al*., 2004). The use of fermentation to improve the sensory properties of soymilk has been reported (Akabanda *et al*., 2010; Nabulsi *et al*., 2014).

The process is thought to be able to improve the taste and colour of fermented soymilk. This study aims to develop a starter culture for soymilk fermentation using lactic acid bacteria (LAB) isolated from raw milk.

MATERIALS AND METHODS

Sample Collection

Raw cow milk samples were collected from the Federal College of Animal Health and Production Technology, Ibadan, Nigeria. The udder of the cow were sterilized using 70% alcohol before the milk sample was collected aseptically into a sterilized sample bottle and brought to the laboratory in an ice box to preserve its integrity.

Isolation of Lactic Acid Bacteria from Raw Cow Milk

The isolation of lactic acid bacteria was done with 10 ml of milk sample homogenized with 90 ml of sterile peptone physiological saline solution (1% peptone and 0.9% NaCl) for about 1-3 minutes. A ten-fold serial dilution was prepared from the homogenate. A volume of 0.1 ml of appropriate dilutions was spread plated on MRS agar medium. The plates were incubated for 48 hours at 37°C. Distinct colonies were subcultured using streaking method repeatedly until pure cultures were obtained.

Morphological Characterization of Isolates

The colonial morphology was described based on colony size, colour, shape, elevation, margin, texture, and consistency (Sulmiyati *et al*., 2019). Gram's reaction and motility test were also performed on the isolates (Fawole and Oso 2004).

Biochemical Characterization of the Isolates

Biochemical tests performed on the bacterial colonies for proper characterization and identification were Catalase test, indole, oxidase, citrate Utilization test, sugar fermentation and Voges Proskaeur Test. The tests were done according to the methods of (Fawole and Oso, 2004; Lundov and Zachariae, 2008).

Sugar Fermentation Test

The ability of the LAB isolates to ferment sugars such as glucose, lactose, sucrose, and maltose was determined. Ten (10) % solution of each of the sugar was added to nutrient broth using phenol red as an indicator. About 10 ml of the solutions were dispersed into test tubes containing inverted Durham tubes. It was ensured that the inverted Durham tube contained no air bubbles to avoid getting a false result concerning gas production. The sugar-indicator broth was sterilized in the autoclave at 121 °C for 15 minutes. Each of the test tubes was inoculated with a loopful of the LAB isolates. However, a test tube was left un-inoculated to serve as the control. The tubes were incubated at 37 °C for 48 hours. Gas production was shown by the presence of air space in the Durham tube which was due to displacement of some liquid in the tube. The utilization of sugar was also indicated by a change in the colour of the broth (Egbere *et al*., 2008).

Diacetyl Production Test

About 5 ml of peptone water, 1 ml alpha naphthol, and 1 ml KOH was dispensed into clean sterile test tubes. The tubes were inoculated with the test organisms and incubated at 37 ^{o}C for 48 hours; a test tube was left un-inoculated to serve as the control. A strong red ring formed indicated a positive reaction while no formation of a red ring indicates a negative reaction.

Phenol Tolerance Test

Fresh and sterile MRS broth containing 0.1, 0.2, 0.3, and 0.4 % of phenol concentration in test tubes were prepared for the determination of phenol tolerance. Fresh cultures of the isolated LAB were

inoculated into the tubes and incubated at 37°C for 48 hours; a test tube was left un-inoculated to serve as the control. Positive results were determined by observing turbidity after 24 and 48 hours and lack of turbidity was taken as no growth.

Preparation of Soymilk

Freshly harvested soybean (200g) was obtained, sorted, and cleaned before soaking in water for 12 hours. A small amount of sodium bicarbonate (0.3 %) was added to the water to remove the bitterness and anti-nutritional factors (trypsin inhibitor) inherent in the soya beans. Soaked soybean husks were removed using the pressure of two hands and cleaned with a continuous flow of fresh water. The clean dehulled soybeans were ground with 100 ml of water in a blender for 10-15 minutes at low speed. The homogenized mass was strained through a fine sterile cloth to separate the milk from the residue. The obtained soymilk was boiled at 100 °C for 15 minutes with constant stirring (Afroz *et al.,* 2016).

Inoculum Preparation

The inocula was prepared by subculturing the LAB isolates into freshly prepared peptone water for 18 hours before use to obtain actively growing cells. The isolates were centrifuged at 4000 rpm for 10 minutes. The supernatant were decanted and the residue cells were washed twice using sterile distilled water to remove the media from the cells. Cells were suspended in sterile MRS broth before use.

Inoculation of LABs Isolates into the Soymilk

The inoculum was standardized by adjusting to 0.5 % McFarland turbidity which provides an optical density comparable to the density of a bacterial suspension of approximately 1.5x 10^8 colony-forming units (CFU/ml). The isolates were randomly combined in a two-culture fermentation treatment for three sets of soymilk in a fermenter. Ten milliliters of each of the inoculum were used at a ratio of 1:1 v/v under sterile conditions to inoculate 100 ml of soymilk. There was no inoculum added to the negative control and all five isolates were added to the positive control. The fermenters were sealed to avoid contamination. The mixture was left to ferment for 4 hours (Adedayo and Abdulkareem, 2024). After fermentation, the soymilk produced was analyzed.

The inoculums were prepared randomly by combining the isolates as described for each treatment:

SOY1= Isolated LAB1+ LAB3, SOY2= Isolate LAB4+LAB2, SOY3= Isolate LAB5+LAB1 SOY4=Positive Control, SOY5=Negative Control

Physicochemical Tests of Fermented Soymilk

pH Test

The pH of the soymilk was determined using a pH meter (Denver model 20). The pH meter was turned on and the electrode was cleaned and calibrated. The electrode was placed in 25 ml of the soymilk samples and the reading was recorded. The same procedure was repeated for all the samples (Cartwright *et al*., 2006).

Titratable Acidity

The amount of acidity in the soymilk samples was determined by titrimetric method as described by (Egbere *et al*., 2008). Ten millimeters each of the soymilk produced were added to 90 ml of

distilled water, 25 ml of this was measured into a conical flask. Two drops of phenolphthalein were added as an indicator, titration was done against 0.1 M NaOH solution. The total titratable acidity was determined by the amount of 0.1 M sodium hydroxide titrated against 25ml of the sample. The titratable acidity was calculated by:
Titratable Acidity=Titre value x 0.09 (Adesokan *et al*., 2013).

Proximate Analysis of Fermented Soymilk
Samples of fermented and unfermented soymilk were analyzed for the following parameters; crude fat, crude protein, moisture content, crude fiber, total ash, and nitrogen-free extract using the methods of the Association of Official Analytical Chemists (1995). The nitrogen-free extract (carbohydrate content) was determined by the difference between 100 and the total sum of the percentages of other parameters examined (AOAC, 1995).

Organoleptic Assessment of fermented soymilk
The organoleptic assessment was done according to the method described by Cartwright *et al*. (2006). This assessment was done using a seven-member panel. The characteristics considered were taste, appearance, smell, consistency, and general acceptability on a hedonic scale of 1-5, where 1 was the least and 5 was the highest.

RESULTS

Total Viable Bacterial Count of Isolates from Cow Milk Sample
A total of five isolates were obtained from the samples, and the result is presented in Table 1.

Table 1: Bacterial Isolates from Raw Milk Sample

Sample	Total LAB Count ($x10^6$ CFU / ml)
Cow Milk	5.0

Morphological Characteristics and Gram's Reaction of Lactic Acid Bacterial Isolates
All the isolates were Gram positive, four were rod shaped and one was a coccus in chains. All the isolates were creamy with punctiform size (Table 2).

Table 2: Colonial Morphology, Biochemical Characteristic and Gram's Reaction of the LAB Isolates

Isolates	Size(mm)	Shape	Pigmentation	GM	Cat	Oxi	Ind	Mot	DA	VP	PT	Isolated LAB
L1	0.9	Rod	Creamy	+	_	_	_	_	_	_	+	***Lactobacillus* sp**
L2	1.2	Rod	Creamy	+	_	_	_	_	_	+	+	***Lactobacillus* sp**
L3	0.5	Cocci	Creamy	+	_	_	_	_	_	_	+	*Streptococcus* sp
L4	0.9	Rod	Creamy	+	_	_	_	_	_	_	+	***Lactobacillus* sp**
L5	1.5	Rod	Creamy	+	_	_	_	_	_	_	+	***Lactobacillus* sp**

KEYS: + = Positive, - = Negative
VP = Voges Proskaeur, PT = Phenol Tolerance, Mot = Motility, DA = Diacetyl production, Cat = Catalase, Oxi = Oxidase, Ind = Indole GM = Gram's reaction

Biochemical Characteristics of Lactic Acid Bacterial Isolates

All the lactic acid bacteria isolated were non-motile and negative to catalase, oxidase, indole, and diacetyl production tests. However, they were positive for sugar fermentation with the production of acid and/or gas, and phenol tolerance test. Four isolates could not utilize citrate but one was positive (Table 3).

Table 3: Sugar Fermentation Characteristic of the Lactic Acid Bacterial Isolates

	Sugar Fermentation				
Isolates	**Glucose**	**Sucrose**	**Fructose**	**Galactose**	**Isolated LAB**
L1	A	A+G	A	A	*Lactobacillus* sp.
L2	A	A	A+G	A	*Lactobacillus* sp.
L3	A+G	A	A	A+G	*Streptococcus* sp.
L4	A+G	A	A	A	*Lactobacillus* sp.
L5	A+G	A+G	A	A+G	*Lactobacillus* sp.

Key
A - Acid Production, A+G- Acid and Gas Production

Proximate Analysis of Fermented Soymilk

The proximate analysis of fermented soymilk showed that the moisture content ranged from 93.78±0.18 to 92.38 ±0.04 %. SOY3 recorded the highest value of 93.78±0.18 while the least was in SOY5 (92.38 ±0.04 %). The crude protein content ranged from 3.52± 0.04 to 2.30±0.01 %, SOY4 had the highest protein content and SOY3 had the lowest. The crude fat in the products ranged from 2.09±0.03 to 1.23±0.00 % with SOY3 having the highest value while SOY1 had the lowest value. There was no detection of crude fibre in the fermented soymilk. The ash content ranged from 1.02±0.03 to 0.70±0.02 %, SOY5 had the highest value and SOY2 had the least value. The NFE (carbohydrate) ranged from 1.84±0.09 to 0.27±0.14 (%) with SOY1 having the highest and SOY4 having the least (Table 4a).

Titratable acidity ranges of the fermented soymilk samples ranged from 25.53 ±0.08 to 15.04±0.01, the fermented soymilk SOY5 has the highest value while the lowest was recorded SOY4. The pH values ranged from 3.64±0.01 to 2.0±0.00, highest value was recorded from SOY4 while the lowest value was recorded from sample SOY5 (Table 4b).

Table 4a: Proximate Composition of Fermented Soymilk

FS	Moisture	Protein	Fat	Fiber	Ash	NFE (%)
SOY1	92.98±0.11	3.01±0.06	1.23±0.00	0.00±0.00	0.94±0.04	1.84±0.09
SOY2	93.36±0.16	3.45±0.04	1.72±0.03	0.00±0.00	0.70±0.02	0.77±0.13
SOY3	93.78±0.18	2.30±0.01	2.09±0.03	0.00±0.00	0.86±0.01	0.53±0.14
SOY4	93.38±0.21	3.52±0.04	1.99±0.05	0.00±0.00	0.91±0.05	0.27±0.14
SOY5	92.38±0.04	3.42±0.06	2.06±0.02	0.00±0.00	1.02±0.03	0.99±0.00

Note: Data are means of replicates ±SD

KEYS

FS = Fermented samples, NFE = Carbohydrate, SOY1= Isolate L1+ L3, SOY2= Isolate L4+L2
SOY3= Isolate L5+L1, SOY4=Positive Control, SOY5=Negative Control

Table 4b: pH and Titratable Acid of Fermented Soymilk

Fermented Samples	Titratable Acid	pH
SOY1	16.71±0.24	3.64±0.011
SOY2	17.91±0.14	3.58±0.001
SOY3	15.04±0.01	3.58±0.001
SOY4	16.67±0.08	3.19±0.00
SOY5	25.53±0.084	2.0±0.00

Note: Data are means of replicates plus or minus standard deviation
Key:
FS = Fermented samples SOY1= Isolate L1+ L3 SOY2= Isolate L4+L2 SOY3= Isolate L5+L1
SOY4=Positive Control SOY5=Negative Control

Sensory Score of the Fermented Soymilk

The sensory property showed that the fermented soymilk scored between 53 to 84 %. The highest score was in soymilk SOY4 and the least percentage score were recorded in SOY1 (Table 5)

Table 5: Sensory Evaluation of fermented soymilk

Isolates	Sensory Evaluation (%)
SOY1	53
SOY2	79
SOY3	56
SOY4	84
SOY5	70

Key:
FS = Fermented samples SOY1= Isolate L1+ L3 SOY2= Isolate L4+L2 SOY3= Isolate L5+L1
SOY4=Positive Control SOY5=Negative Control

DISCUSSION

The increase in protein demand especially in developing countries has led to increased efforts in searching for alternative sources of protein especially proteins of plant origin which are usually less expensive than animal sources of protein and provide almost the same amount of protein as animal sources. The use of the lactic acid bacteria species in food processing is important, the use in food is due mainly to their safe metabolic activity while growing in foods, utilizing available sugar for the production of lactic acid and other secondary metabolites that are of benefit to the food and hence, the ultimate consumers (Khalid, 2011). The detection of this group of bacteria in the raw milk sample corroborates their association with milk and other dairy products where they catabolise the conversion of lactose; the milk sugar into lactic acid. The production of lactic acid and other organic acids during fermentation results in lowering the inherent pH of food, the low pH favours the growth and activities of the lactic acid bacteria but is a deterrent to food spoilage and pathogenic bacteria which are mostly neutrophilic. In this study, all isolates were members of the homolactics producing acid and gas from sugar fermentation (Table 3b). The ability to produce organic acids during fermentation of soymilk was a major activity required for the change in consistency and taste (Tables 4a and b). Fermentation of milk always resulted in products with thicker consistency with the development of a characteristic astringent/sour acidic taste desirable in fermented milk, the taste is however, directly dependent on the period of fermentation (Hayek and Ibrahim, 2013). The isolated lactic acid bacteria species were able to influence the proximate content of the soymilk samples after fermentation. There was no loss in nutritional content after fermentation. The non-detection of crude fiber could be a result of the processing method used, which involves removing the crude fiber during sieving. Moreover, the relatively high moisture content of the fermented soymilk is traceable to the naturally high

moisture content in soymilk and it could also be a result of the partial coagulation of protein due to low pH, thus leading to water expulsion (Udeozor, 2012). The decrease observed in the carbohydrate content of the fermented soymilk during fermentation when compared to the control was due to the individual ability of the isolated lactic acid bacteria to utilize the metabolizable sugar in the soymilk converting or transforming them into energy for growth and other cellular activities (Osundahunsi *et al*., 2007).

The variation recorded in the proximate content of the fermented soymilk was due to the use of the nutrients available in the soymilk for growth and metabolic activities by the different species of the lactic acid combination used for the fermentation, and the difference in the requirements for growth and metabolism by each of the isolates (Bintsis, 2018). The use of more than one species of bacteria as a starter culture in food processing was well documented. Different species of lactic acid bacteria could work synergistically when grown together and cooperate to achieve a peculiar task. Such synergism is of advantage and is usually harnessed in the food industry for creating products with unique organoleptic properties. During such association, the metabolic product of one could serve as the raw material for the other organism, or one organism might produce what the other requires for growth. The overall effect of synergism is rapid growth and product formation. On the other hand, two or more co-cultures of lactic acid bacteria might result in antagonism, where the metabolic product of one is detrimental to the growth and activity of the other organism. Antagonism usually retards the growth of one population at the expense of the other, or one bacterial population might eliminate the other. Product development is usually not rapid when there is antagonism. In this research, using the five species for fermentation of the soymilk yielded the best scores in terms of proximate and organoleptic properties (Tables 4a and b, 5). The five species of lactic acid bacteria differed in their ability to produce acid during fermentation of the soymilk. This observation is similar to the finding of Michon et al. (2016). The characteristic odour of soymilk was reduced after fermentation as the metabolism of the fermenting bacteria led to their breakdown into less pungent substances. Thus, the fermented soymilk was more acceptable.

CONCLUSION

The isolated lactic acid bacteria have the potential of producing lactic acid which is a major requirement in milk fermentation. The research proved that there was relative stability of nutrients in the fermented soymilk, the soymilk fermented with all the isolated lactic acid bacteria had the highest protein content and also exhibits more desirable sensory properties than others. The increase in acidity during fermentation could inhibit the growth of pathogenic and spoilage bacteria which could lead to short shelf life of soymilk. More research should be carried out on culture conditions for best-fermented soymilk products.

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