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Production of fermented soymilk and its preservation using essential oils from the leaves of *Hoslundia opposita*

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Abstract

Fermented Soymilk (FSM), a protein rich beverage is highly prone to microbial contamination. Its preservation is therefore, key to its availability and safety. Soymilk was prepared by aqueous extraction of soybeans and allowed to ferment spontaneously. Samples of FSM were treated with essential oils from the leaves of *Hoslundia opposita* (LEOHO) at 5% and 10% (v/v) and stored at ambient temperature. The organoleptic, pH, microbiological and biochemical qualities of the samples were evaluated periodically during storage. The protein content was significantly ($p<0.05$) improved from 8.18±0.06% to 12.62±0.12% by fermentation. Compared to the untreated, samples treated with LEOHO had higher sensory ratings. The pH of the samples decreased from 6.9 to 5.7 during fermentation; and further to 4.9 during storage. Overall, the bacterial load was significantly ($p<0.05$) reduced (by up to 80.04%) during storage; while the fungal load was reduced to zero immediately after fermentation. The bacterial isolates were *Bacillus wiedmannii* FSL W8-016, *Micrococcus luteus* NCTC 2665, *Lactobacillus algidus* M6A, *Lactobacillus sakei* NBRC 15893, *Lactobacillus apodemi* ASB1, *Shigella* sp., *Staphylococcus aureus* and *Escherichia coli*, while the fungi were *Candida parapsilosis* IQMustafa31, *Penicillium citrinum*, *Aspergillus flavus* and *Fusarium verticilloides*. All the bacterial isolates were sensitive to LEOHO with the zone of inhibition ranging from 10.00±1.00mm to 46.33±1.53mm. This study shows that LEOHO was effective in preserving the sensory and nutritional value as well as reducing the microbial population of FSM. LEOHO is therefore recommended as a preservative to increase the shelf life of FSM.

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1. Introduction

Soybean is the seed of the leguminous plant, *Glycine max*. It has been identified over the years as a cheap and readily available source of protein. It is particularly rich in lysine, arginine, cysteine, leucine, and methionine amino acids (Rastogi and Singh, 1989; Riaz, 1999), minerals and vitamins such as calcium, iron, vitamin A, riboflavin and other trace elements (Adegoke *et al*., 2012). Its lipid content is rich in polyunsaturated fatty acids such as linoleic acid and linolenic acid (Agboke *et al*., 2011). Further, it contains a low amount of fat with no cholesterol and therefore, is known to reduce the risk of heart disease.

Soybean is processed to soymilk by aqueous extraction of whole beans. Soymilk can serve as a substitute for milk from cow, sheep, goat etc. It is safe for people who are allergic to milk protein, lactose intolerance and for children with galactosemia (Obadina *et al*., 2013; Subrota *et al*., 2013). Its consumption is however limited due to the presence of undesirable beany flavour attributed to several factors including the presence of aldehydes and alcohols such as n-hexanal, flatulence caused by indigestible galacto-oligosaccharides, and digestive problems associated with the presence of raffinose and stachyose (Subrota *et al.*, 2013; Horáčková *et al.*, 2015). Fermentation has been used to overcome the problems associated with soymilk by removing the beany flavour (Wang *et al*., 2006), making the protein contents more digestible (Ishibashi and Shimamura, 1993), and reducing oligosaccharides, raffinose and stachyose thereby improving its nutritional characteristics and acceptance.

Soybeans and soymilk have been fermented traditionally in many parts of the world, to products such as Buckwheat sokseongjang, Cheonggukjang, Miso, Douchi, Natto, Kinema, Tempeh, (Mora-Escobedo *et al*., 2018), soy cheese (Schnurer and Magnusson, 2005), soymilk-kefir (Silva *et al*., 2018), soy yoghurt (Osundahunsi *et al*.,2007) etc. Fermented soymilk (FSM) may contain probiotic lactic acid bacteria which can confer health benefits such as high antioxidant capacity, production of antimicrobial metabolites, improved digestibility and reduced metabolic disorders on the consumers (Zielinska and Kolozyn-Krajewska, 2018). Soy-yoghurt has a yoghurt-like flavour and can serve as a refreshing and nutritious beverage. It is however prone to microbial contamination. Its preservation is therefore key to its availability and safety.

Preservation can be achieved using physical methods such as exposing to extreme temperature, radiation etc. Chemical preservation is very common with beverage foods such as the FSM. However, only chemicals that have the GRAS status are permitted in foods. Such chemicals could be synthetic or natural. The use of most synthetic chemicals is being discouraged due to the associated health risks such as carcinogenic properties etc (Gutlekin *et al*., 2015). This highlights the importance of using natural chemicals such as essential oils.

Essential oils comprise a complex combination of bioactive chemicals mostly terpenes, terpenoids, other aromatic and aliphatic compounds. Many essential oils exhibit antibacterial, antifungal, antiviral and insecticidal activities and are being used both in medicine and as food additives (Djenane *et al.*, 2012; Stefanakis *et al*., 2013). Usually, two or three of the numerous chemical compounds which are present in a relatively large quantity of about 20-70% account for the antimicrobial activities of the oils (Pandey *et al*., 2015). *Hoslundia opposita* which is commonly known as orange-bird berry is a perennial herbaceous plant with tasty fruits that attracts birds but has an unpleasant scent that repels bees (Sadri, 2017).The essential oils from the leaves of *Hoslundia opposita* LEOHO) contains 1, 8-cineole, α- terpineol, sabinene, thymol and car-3-ene (Usman *et al.*, 2010; Akolade *et al*., 2014; Babarinde *et al*., 2017) which have been shown to exhibit antimicrobial activities against spoilage and pathogenic bacteria and fungi (Gundidza *et al*., 1992). There is however a paucity of information on its use as food preservatives.

Production of dairy milk in Nigeria is rudimentary and the populace depends largely on foreign supplies. The health benefits derivable from soymilk in addition to its nutritional composition makes it a suitable substitute for dairy. The soybean plant thrives well in Nigeria with a yield as high as 12,951 hectograms per hectare and a further capacity to increase production (www.factfish.com). Processing of soybeans to soymilk requires simple and cheap technology and fermentation can be used to overcome the problem of acceptability due to the beany flavour, flatulence and digestive issues. In addition, preservation with the use of natural products such as essential oils can ameliorate the risk associated with chemical preservatives. This work therefore focused on the production of fermented soymilk, and its preservation using the essential oils from the leaves of *Hoslunda opposita.*

2. Materials and Methods

2.1 Soymilk production

Soybeans were purchased from the market, cleaned, dehulled, wet milled into a slurry, sieved through a muslin cloth and boiled at 100 ºC for 15 minutes. The resultant soymilk was allowed to cool after which samples were withdrawn for pre-fermentation analysis which included sensory evaluation, proximate analysis and pH measurement.

2.2 Fermentation

The soymilk was distributed into four 250 ml Erlenmeyer's flasks in 100 ml portions. Sucrose, 8% w/v was added into two of the flask while the other two were not supplemented. One flask from each of the supplemented and non-supplemented was incubated on a rotatory shaker at 150 rpm while the others were left on work bench. The soymilk samples were allowed to ferment for 24 hours during which samples were withdrawn every 3 hours for the analysis.

2.3 Preservation

The essential oils from the leaves of *Hoslundia opposita* (LEOHO) was prepared by hydro distillation using a Clevenger-type apparatus. The oil was added to fermented soymilk (FSM) at 5% and 10% (v/v) in sterile sample bottles. All samples (treated and untreated) were stored at 28 ±2ºC (room temperature) and aliquots were withdrawn periodically for analysis

2.4 Sensory evaluation

A panel of five volunteers evaluated the sensory quality using five-point Hedonic scale. Attributes evaluated for the soymilk were colour, odour, taste and consistency.

2.5 Proximate analysis

The moisture, ash, crude fat, crude fibre, protein and carbohydrate contents were evaluated using standard procedures (AOAC, 2003).

2.6 Titratable acidity

Samples were titrated against NaOH using standard procedures (AOAC, 2003).

2.7 pH

The pH of the samples was measured periodically during fermentation using a digital hand held pH meter. The pH meter was standardized to pH 4.0, 7.0 and 9.0 using appropriate buffers.

2.8 Microbiological analysis

Bacteria were isolated on Nutrient Agar, de Man Rogosa Sharpe (MRS) medium and McConkey Agar; and fungi on Potato Dextrose Agar using pour plate method. Briefly, 1 mL of sample was added to 9 mL sterile distilled water in a test tube and serially diluted up to 10^{-3}. Aliquots, 100 µL from the last diluents was placed in the sterile plate, 15 mL of molten agar was poured on it and the plate was covered immediately. The sample was mixed with agar by rocking the plate gently on the work bench. Plates were incubated at 37 ºC for bacterial isolation and 28 ºC for fungi. After growth, a colony counter was used to enumerate total aerobic bacteria on Nutrient Agar, lactic acid bacteria on de Man Rogosa Sharpe (MRS) medium and enteric bacteria on McConkey Agar. Fungal colonies were also enumerated on PDA. Bacterial identification was based on morphological, biochemical and molecular characterization using standard procedures. Molds were identified by morphology while for the yeasts, physiological and molecular characterization was used in addition.

2.9 Antimicrobial sensitivity test

The sensitivity test was performed using the agar well diffusion method. Aliquots, 100 µL of isolate at 0.5 McFarland standard was inoculated on Mueller Hinton agar plates using the spread plate method. Wells measuring 6 mm with a space not less than 20 mm around it, were bored in the seeded agar. The LEOHO was diluted using Tween 80 and loaded into wells at 25%, 50% and 100%. Tween 80 was loaded as the control. Plates were incubated at 37ºC and observed after 24 hours for bacterial growth and clearance around wells. Diameter zones of clearance were measured at three planes around wells.

2.10 Statistical Analysis

Data were recorded as means with standard deviations of triplicate measurements. ANOVA was conducted using SPSS 20 software.

3. Results

3.1 Proximate composition of soybeans

The nutritional composition of the soybean samples is given in Table 1.

3.2 Sensory quality of soymilk

The taste rating of the sugar supplemented soymilk samples (SSM) was significantly ($p < 0.05$) higher than those of the non-supplemented samples (PSM). Apart from this, there was no significant difference ($p < 0.05$) in the consumer ratings of the other sensory attributes between the SSM and PSM (Table 2) by a five member panel.

3.3 Changes in the pH of soymilk during fermentation

The pH of soymilk decreased during fermentation from a value near neutral to as low as 5.7 within 24 hours (Figure 1).

3.4 Bacterial growth in soymilk samples during fermentation

The growth of lactic acid bacteria, enteric bacteria and total bacteria represented as the number of colonies on de Man Rogosa Sharpe (MRS) agar, McConkey agar and Nutrient agar are presented in Figures 2, 3 and 4 respectively.

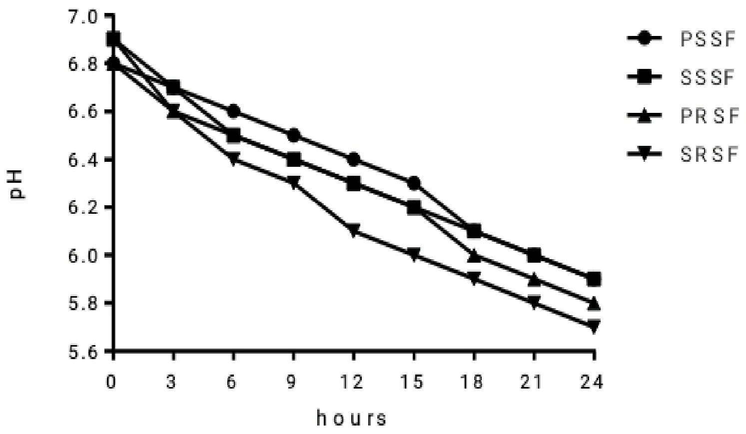
3.5 Growth of fungi in soymilk samples during fermentation

Fungal growth, measured as the number of colonies on Potato Dextrose agar is presented in figure 5.

Table 1: Proximate composition of soybeans samples

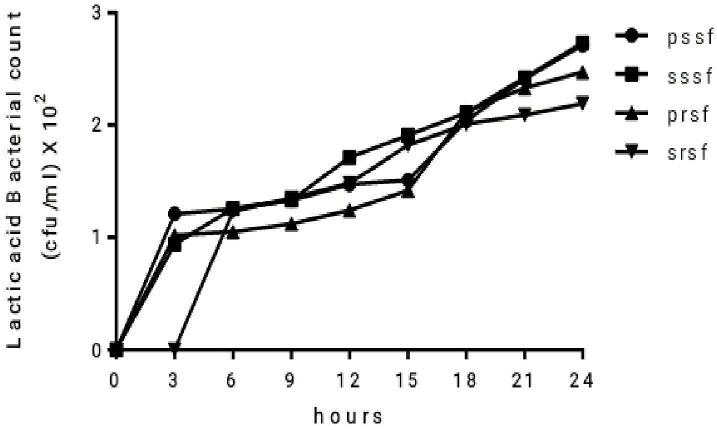
Parameters	Soybeans
Moisture (%)	7.93±0.09
Ash (%)	3.83±0.01
Carbohydrate (%)	28.09±0.5
Total Protein (%)	41.48±0.08
Crude Lipids (%)	11.83±0.69
Crude fibre (%)	6.83 ± 0.02
Calorific Value KJ/100g	1607.21±15.68
Metabolizable Energy Kcal/kg	384.37 ± 3.68
Metabolizable Energy Protein ratio	9.26±0.11
Titrable acidity (%)	0.067±0.02

Values are means ± standard deviations of three independent analysis.



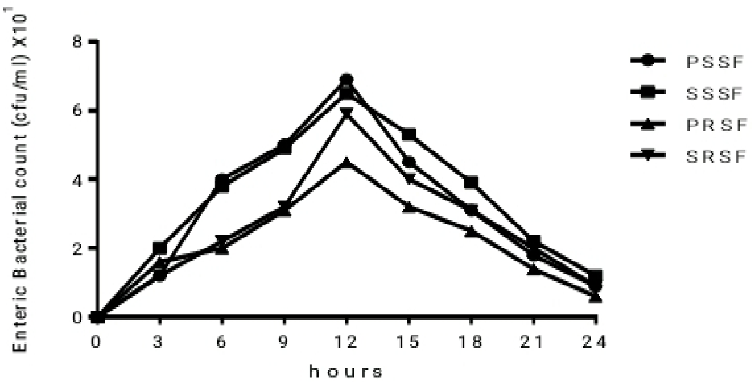
PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Figure 1: Changes in the pH of soymilk samples during fermentation



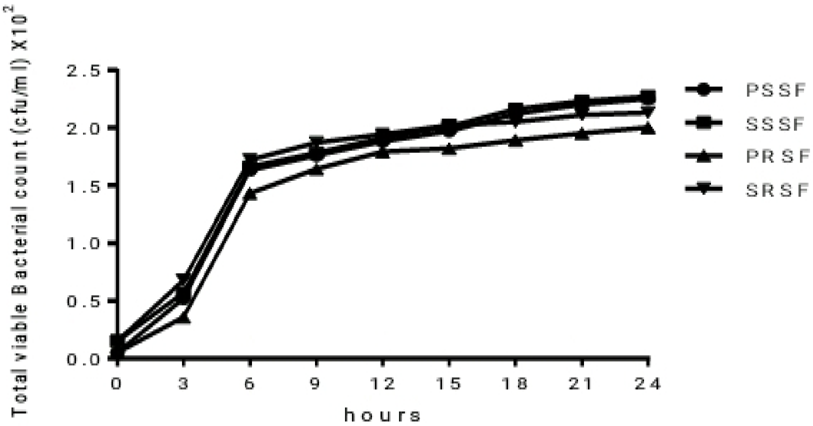
PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Figure 2: Growth of Lactic acid bacteria in soymilk samples during fermentation



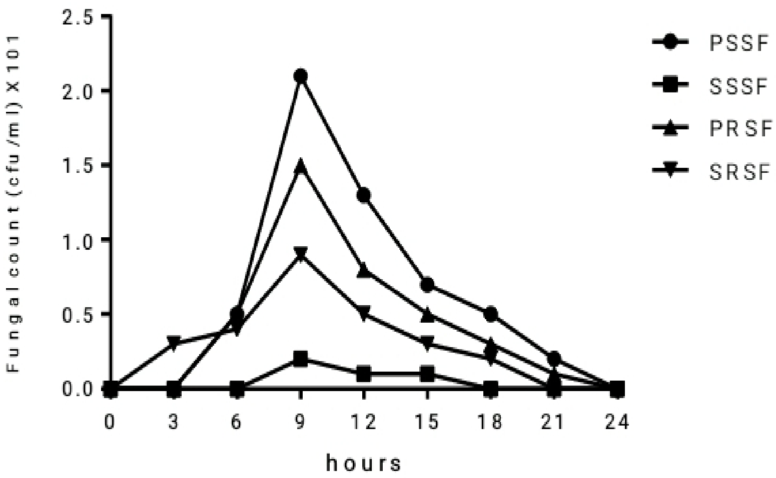
PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Figure 3: Growth of Enteric bacteria in soymilk samples during fermentation



PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Figure 4: Total bacteria growth in soymilk samples during fermentation



PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Figure 5: Total fungal growth in soymilk samples during Fermentation

Table 2: Sensory evaluation of soymilk samples

Samples	Taste	Odor	Texture	Color	Overall Acceptance
SSM	$4.3{\pm}0.3^a$	$4.6{\pm}0.2^a$	$4.6{\pm}0.2^a$	$4.8{\pm}0.1^a$	$4.73{\pm}0.1^a$
PSM	$3.4{\pm}0.2^b$	$4.4{\pm}0.2^a$	$4.4{\pm}\ 0.2^a$	$4.8{\pm}0.1^a$	$4.2{\pm}0.2^b$

Values represent mean of ratings by five member panel ± standard deviations. Values for overall acceptance are mean ± standard deviations of all sensory evaluations taken. Values in the same column with different superscripts are significantly ($P< 0.05$) different. SSM=Soymilk containing 8g of sucrose, PSM=Soymilk without sucrose.

3.6 Microbial isolates

A total of 12 microorganisms comprising 8 bacteria and 4 fungi were isolated and characterized. These are described below.

3.6.1 Bacteria

The morphological and physiological characteristics of the isolates are presented in Table 3. The bacteria were identified after analysis of their sequences using the BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/) (Table 4)

3.6.2: Fungi

The morphological characters of the fungal isolates on which their identification was based is presented in table 5. The fungi were identified as *Candida parapsilosis, Aspergillus flavus, Penicillium critinum* and *Fusarium verticilloides*. The yeast, *Candida parapsilosis* was identified to the strain level along with some of the bacterial isolates (Table 4)

3.7: Effect of fermentation on the proximate Composition of Soymilk Samples

The changes that occurred with the soymilk samples after fermentation is presented in table 6

3.8: Effect of the leaf essential oil of *Hoslundia opposita (LEOHO) on the sensory quality of fermented soymilk during storage*

The acceptance rating of the fermented soymilk remained high up to the 8th day of storage for all the samples that were treated with LEOHO while the control sample was rated low from the 2nd day onwards (Table 7).

3.9: Effect of the leaf essential oil of Hoslundia opposita on the bacterial load of fermented soy milk samples during storage

There was significant ($p < 0.05$) reduction in the bacterial load of fermented soymilk samples in the first 24 hours after treatment with LEOHO. The reduction in bacterial load was significantly ($p < 0.05$) more pronounced in the samples that were treated with 10% oil compared to that treated with 5%. Counts were kept low throughout the storage although there were significant ($p < 0.05$) increases after the initial reduction. In comparison, counts from the control sample increased significantly ($p < 0.05$) with the storage period and the sample deteriorated between the 4th and 5th day to an extent that they had to be discarded and hence bacterial loads were not determined (Table 8).

3.10: Effect of the leaf essential oil of Hoslundia opposita on the fungal load of fermented soy milk samples during storage

No fungi were recovered from most of the soymilk samples after fermentation. Sparse growth however occurred during storage. Fungal growth inhibition by the 10% LEOHO was significantly ($p < 0.05$) higher than that in the 5% oiland more significant compared to the untreated samples (Table 9).

3.11: Sensitivity of bacterial isolates to the leaf essential oil of Hoslundia opposita

All the isolated bacteria were sensitive to the LEOHO at the lowest concentration used (25%). The oil had cidal effects on all the isolates at 100% concentration; on four and three of the isolates at 50% and 25%, respectively, while a static effect was exerted on the others (Table 10).

4. Discussion

The total protein content of soybeans was recorded as 41.48% in the present study which falls within earlier reported values (Rathi *et al*., 2015; Etiosa *et al*., 2017; Useh *et al*., 2017), corroborating the claims that soybeans are rich sources of protein and further justifies its candidature in the production of milk.

The changes that occurred in the pH of soymilk samples during the fermentation process are indicative of the presence of microorganisms such as the lactic acid bacteria (LAB) which ferment sugar in the milk samples to produce organic acids mostly lactic acid. A similar pH decrease was reported by Obadina *et al.* (2013) during the fermentation of soy nono. The highest reduction occurred in sample D which was supplemented with sugar and fermented on rotary shaker. This shows that sugar supplementation may be necessary not only for taste enhancement but also to aid fermentation since the fermenting organisms require sugar for metabolism.

The growth of lactic acid bacteria during fermentation was exponential within the first 3 hours. This leaves out the lag phase of growth and thus can be attributed to the high presence of the LAB on the soybean used to produce the soymilk. Since the soybeans serve as the source of the LAB, the organisms may have adjusted to the nutrient accounting for the lack of the lag phase of growth. This concept can also lead to the efficient production of lactic acid which is a primary metabolite of the LAB (Figure 2). In addition, the growth of LAB in the soymilk sample indicates its rich nutrient composition since the organisms are fastidious.

The enteric bacteria grew steadily for the first 12 hours of incubation and dropped to almost zero within the next 12 hours, this being attributed to the production of antibacterial compounds such as lactic acid, hydrogen peroxide and bacteriocins in the fermentation medium which causes inhibition of this category of organisms and thereby facilitating the proliferation of the LAB.

The total bacterial growth as obtained on nutrient agar may have left out the fastidious LAB, and hence follows the growth curve pattern in a closed system (Willey *et al*., 2008). The organisms grew slowly during the first 3 hours of fermentation but exponentially thereafter for the next 3 hours. There was however no significant increase in growth thereafter until fermentation was terminated.

Table 3: Morphological and biochemical characterization of Isolates from Soymilk and Fermented Soymilk samples

Characters	**Isolates**							
	BA	BB	BC	BD	BE	BF	BG	BH
<u>Colony Characteristics</u>								
Shape	Circle	Circle	Swarm	Irregular	Circle	Circle	Circle	Circle
Margin	Entire	Entire	Undula	Undula	Entire	Entire	Entire	Entire
Elevat	Raise	Convex	Flat	Flat	Convex	Conve	Convex	Convex
Size	Small	Small	Spread	Large	Small	Large	Small	Small
Texture	Smooth	Smooth	Smoot	Rough	Smooth	Rough	Smooth	Smooth
Appear	Dull	Shiny	Shiny	Dull	Shiny	Shiny	Shiny	Shiny
Pigment	Cream	Cream	Cream	Cream	Yellow	Golden	Greyish	Cream
Optical	Transluc	Transluc	Opaqu	Transluc	Opaqu	Opaqu	Transluc	Transluc
<u>Cellular Morphology</u>								
Gram	+	+	+	+	+	+	-	-
Shape	Rod	Rod	Rod	Rod	Coccus	Coccus	Rod	Rod
Arrang	Single	Single	Single	Single	Tetrad	Cluster	Single	Single
Motility	-	-	-	+	-	-	-	+
Spore	-	-	-	+	-	-	-	-
<u>Biochemical Characteristics</u>								
Urease	+	+	+	+	+	+	-	-
Catalase	-	-	-	+	+	+	+	+
Starch	-	-	-	+	-	-	-	-
Oxidase	-	-	-	-	+	-	-	-
Citrate	-	-	-	+	+	+		-
Gelatin	-	+	+	+	+	+	-	-
Indole	-	-	-	-	-	-	-	+
MR	-	-	-	+	+	+	+	+
VP	-	-	-	+	-	-	-	-
Nitrate	-	-	-	+	+	+	+	+
Mannito	+	-	+	+	-	+	+	+
TSI	K/A	K/A	K/A	K/A	K/A	A/A	K/A	A/A, G
H_2S	-	-	-	-	-	-	-	-
NaCl	+	-	+	+	-	+		+
Gr10	+	+	+	+	+	+	+	+
Gr45	+	+	-	-	+	-	+	-
Casein	+	+	+	+	+	+	-	-
Probable identity	*Lactobacillus sakei*	*Lactobacillus algidus*	*Lactobacillus apodemi*	*Bacillus wiedmannii*	*Micrococcus luteus* strain	*Staphylococcus aureus*	*Shigella sp.*	*Escherichia coli*

+ denotes Positive, - denotes Negative, Elevat – Elevation, Arrang – Arrangement, MR - Methyl Red, VP - VogesProskauer, Mannito – Mannitol, TSI - Triple Sugar Iron, H_2S - Hydrogen Sulphite, NaCl – Growth in 6.5% Sodium chloride solution, Gr10 – Growth at 10^oC, Gr45 – Growth at 45^oC, K - Alkaline, A - Acid, G - Gas,

Table 4: Molecular characterization of Isolates from Soymilk and Fermented Soymilk samples

Isolate	**Organism**	**Number of Bases**	**Identity (%)**	**Accession Number**
BA	*Lactobacillus sakei* strain NBRC 15893	1012	91	NR 113821.1
BB	*Lactobacillus algidus* strain M 6 A9	853	92	NR 028617.1
BC	*Lactobacillus apodemi* strain ASB1	1012	89	NR 042367.1
BD	*Bacillus wiedmannii* strain FSL W8-0169	984	99	NR152692.1
BE	*Micrococcus luteus* strain NCTC 2665	887	77	NR 075062.2
FB	*Candida parapsilosis* strain IQMustafa31	558	95	LT 577616.1

Table 5: Colonial and Microscopic description of Fungi isolates

Characters	Isolates FA	FB	FC	FD
Colony Characteristics				
Shape	Round	Flat	Flat	Flat
Color	Creamy white	Yellowish green	Bluish green	White
Color change	None	Greenish brown	Pale green	Brown
Reverse color	No color	White	Blue brown	Colorless
Texture	Smooth	Granulated	Velvety	Cottony
Growth rate	Fast	Fast	Fast	Moderate
Microscopic features				
Hyphae	None	Septate and hyaline	Septate and branch	Septate hyaline and branched
Spores	Budding cells	Conidia	Conidia	Conidia: micro- and macro-conidia
Shape of spores	Oval	globose to subglobose	Spherical	Spherical
Phiallide	None	Biseriate,directly to vesicle	Branched, biverticillate	verticillate
Probable organism	*Candida Parapsilosis*	*Aspergillus flavus*	*Pencillium citrinum*	*Fusarium verticilloides*

Table 6: Proximate Composition of Soymilk and Fermented Soymilk Samples

Parameters	Soymilk	Fermented soymilk			
		PSSF	PRSF	SSSF	SRSF
Moisture %	88.54 ± 0.08^{b}	70.65 ± 8.48^{a}	71.47 ± 6.80^{a}	64.14 ± 4.40^{a}	63.26 ± 3.99^{a}
Ash %	9.92 ± 0.04^{b}	5.97 ± 0.02^{a}	5.98 ± 0.046^{a}	6.00 ± 0.06^{a}	$6.020.04^{a}$
CHO %	17.9 ± 2.4^{b}	12.20 ± 6.30^{a}	10.57 ± 6.80^{a}	17.49 ± 4.49^{a}	17.95 ± 4.10^{a}
Total Protein %	8.178 ± 0.06^{a}	12.53 ± 0.71^{c}	11.90 ± 0.07^{b}	12.18 ± 0.16^{b}	12.62 ± 0.12^{c}
Crude Lipids %	0.20 ± 0.03^{b}	0.25 ± 0.10^{b}	0.17 ± 0.01^{a}	0.19 ± 0.08^{ab}	0.16 ± 0.02^{a}
Crude fibre %	1.921 ± 0.02^{b}	1.24 ± 0.02^{a}	1.26 ± 0.01^{a}	1.24 ± 0.03^{a}	1.25 ± 0.03^{a}
Calorific Value KJ/100g	1478.59 ± 7.2^{a}	4196 ± 1145^{b}	3779 ± 1130^{b}	5025 ± 717^{b}	5164 ± 666^{b}
Metabolizable Energy Kcal/kg	353.97 ± 1.68^{b}	108.7 ± 24.0^{a}	92.5 ± 27.1^{a}	125.67 ± 15.6^{a}	128.21 ± 15.8^{a}
Metabolizable Energy Protein ratio	4.32 ± 0.01^{a}	8.10 ± 2.19^{b}	7.78 ± 2.31^{b}	10.32 ± 1.32^{b}	10.17 ± 1.34^{b}
Titrable acidity (%)	0.050 ± 0.01^{a}	1.63 ± 0.03^{b}	1.80 ± 0.01^{b}	1.76 ± 0.02^{b}	2.37 ± 0.02^{b}

Values are means of triplicate readings ± standard deviations. Values in the same row with different superscripts are significantly ($P< 0.05$) different, CHO=Carbohydrate, PSSF=Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Table 7: Sensory evaluation of LEOHO treated Fermented Soymilk samples during storage

Samples	Conc	Storage period (days)					
		0	2	5	8	11	14
PSSF	10%	5.00^{a}	4.00^{b}	3.33^{b}	3.33^{b}	3.33^{b}	2.33^{a}
	5%	5.00^{a}	4.33^{a}	3.67^{a}	3.67^{a}	3.67^{a}	2.33^{a}
	C	4.67^{b}	2.67^{c}	1.33^{c}	1.33^{c}	1.00^{c}	1.00^{b}
PRSF	10%	5.00^{a}	4.00^{b}	3.33^{b}	3.33^{b}	3.33^{b}	2.33^{a}
	5%	5.00^{a}	4.33^{a}	3.67^{a}	3.67^{a}	3.67^{a}	2.33^{a}
	C	4.67^{b}	2.67^{c}	1.33^{c}	1.33^{c}	1.00^{c}	1.00^{b}
SSSF	10%	5.00^{a}	4.00^{b}	3.33^{b}	3.33^{b}	3.33^{b}	2.33^{a}
	5%	5.00^{a}	4.33^{a}	3.67^{a}	3.67^{a}	3.67^{a}	2.33^{a}
	C	4.67^{b}	3.67^{c}	1.33^{c}	1.33^{c}	1.00^{c}	1.00^{b}
SRSF	10%	5.00^{a}	4.00^{b}	3.33^{b}	3.33^{b}	3.33^{b}	2.33^{a}
	5%	5.00^{a}	4.33^{a}	3.67^{a}	3.67^{a}	3.67^{a}	2.33^{a}
	C	4.67^{b}	3.67^{c}	1.33^{c}	1.33^{c}	1.00^{c}	1.00^{b}

Each value represents mean of three sensory parameter (odor, color and texture) evaluated by five individuals on five point Hedonic scale. Values in the same column for a particular sample with different superscripts are significantly ($P< 0.05$) different. C = Control, Conc = Concentration.
PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker

Table 8: Effect of leaf essential oil of *Hoslundia opposita* on the bacterial count of fermented soymilk samples during storage

P	**Bacterial count (CFU mL^{-1})**											
	PSSF			PRSF			SSSF			SRSF		
	5%	10%	Ctrl	5%	10%	Ctrl	5%	10%	Ctrl	5%	10%	Ctrl
0	5.06±0.02^{h,a}	5.06±0.02^{g,a}	5.06±0.02^{a,a}	4.52±0.02^{g,a}	4.53±0.02^{i,a}	4.52±0.02^{a,a}	5.12±0.01^{I,a}	5.12±0.01^{h,a}	5.12±0.01^{a,a}	4.86±0.01^{I,a}	4.86±0.01^{I,a}	4.86±0.01^{b,a}
1	2.20±0.10^{f,b}	0.97±0.01^{a,a}	7.41±0.04^{d,c}	2.17±0.01^{f,b}	0.92±0.01^{a,a}	7.76±0.01^{d,c}	2.17±0.01^{f,b}	0.71±0.06^{a,a}	9.25±0.02^{e,c}	2.12±0.02^{f,b}	0.62±0.04^{a,a}	8.89±0.02^{e,c}
2	2.33±0.01^{g,b}	1.28±0.02^{b,a}	6.25±0.02^{c,c}	2.25±0.05^{f,b}	1.20±0.01^{b,a}	6.77±0.02^{c,c}	2.21±0.01^{g,b}	1.16±0.02^{b,a}	8.37±0.45^{c,c}	2.17±0.02^{g,b}	1.11±0.01^{b,a}	7.57±0.02^{d,c}
3	2.38±0.02^{g,b}	1.85±0.02^{c,a}	5.73±0.04^{b,c}	2.29±0.10^{f,b}	1.62±0.02^{f,a}	5.92±0.02^{b,c}	2.27±0.02^{h,b}	1.93±0.03^{f,a}	7.12±0.02^{b,c}	2.24±0.01^{h,b}	1.87±0.02^{g,a}	6.47±0.02^{c,c}
4	1.98±0.01^{e,b}	1.82±0.06^{d,a}	ND	1.93±0.03^{e,a}	2.12±0.02^{h,b}	ND	1.88±0.01^{e,a}	2.05±0.01^{g,b}	4.93±0.12^{a,c}	1.78±0.01^{e,a}	2.03±0.02^{h,b}	4.23±0.01^{a,c}
5	1.81±0.04^{d,a}	1.77±0.02^{e,a}	ND	1.75±0.02^{d,b}	1.69±0.01^{g,a}	ND	1.72±0.02^{d,a}	1.88±0.01^{e,b}	ND	1.55±0.01^{d,a}	1.75±0.01^{f,b}	ND
8	1.65±0.01^{c,b}	1.42±0.02^{f,a}	ND	1.52±0.03^{c,b}	1.36±0.03^{e,a}	ND	1.54±0.01^{c,a}	1.62±0.03^{d,b}	ND	1.35±0.02^{ca,}	1.44±0.01^{e,b}	ND
11	1.49±0.03^{b,b}	1.35±0.01^{f,a}	ND	1.38±0.05^{b,b}	1.31±0.01^{d,a}	ND	1.34±0.02^{b,a}	1.42±0.01^{c,b}	ND	1.21±0.01^{b,a}	1.23±0.01^{d,a}	ND
14	1.20±0.02^{a,a}	1.26±0.03^{b,b}	ND	1.17±0.02^{a,a}	1.24±0.04^{c,b}	ND	1.26±0.02^{a,b}	1.18±0.01^{b,a}	ND	1.01±0.01^{a,a}	1.16±0.05^{c,b}	ND

Values are means ± standard deviation of three independent experiments. Values were compared based on period of incubation and volume of essential oil applied for each category of fermented soymilk. Values with different superscripts along the same column, and on the same row for each category of sample are significantly different ($p < 0.05$).

PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker
P – Period of storage after addition of essential oil (days);
Ctrl – Control (Untreated samples);
ND – Not determined (bacterial counts were not determined because the samples had deteriorated)

Table 9: Effect of leaf essential oil of *Hoslundia opposita* on the fungal count of fermented soymilk samples during storage

P	Fungal count (CFU mL^{-1})											
	PSSF			PRSF			SSSF			SRSF		
	5%	10%	Ctrl	5%	10%	Ctrl	5%	10%	Ctrl	5%	10%	Ctrl
0	NG	NG	NG	NG	NG	NG	NG	NG	0.20±0.00^{a}	NG	NG	NG
1	NG	NG	0.30±0.00^{a,b}	0.10±0.00^{a,d}	0.13±0.06^{b,b}	0.20±0.10^{a,c}	0.07±0.06^{a,a}	0.20±0.00^{c}	0.60±0.00^{b,e}	0.13±0.06^{a,b}	0.30±0.10^{a,d}	NG
2	0.07±0.06^{a,a}	0.13±0.06^{a,b}	0.67±0.12^{b,f}	0.17±0.06^{b,b}	0.23±0.06^{b,c}	0.50±0.10^{b,e}	0.20±0.00^{b,c}	NG	1.00±0.00^{c,g}	0.27±0.12^{b,d}	0.50±0.10^{b,e}	NG
3	0.17±0.06^{b,b}	0.23±0.06^{b,c}	1.30±0.10^{c,g}	0.23±0.06^{c,c}	0.07±0.06^{a,a}	0.80±0.10^{c,e}	0.23±0.06^{b,c}	NG	1.30±0.10^{d,g}	0.33±0.06^{b,d}	0.80±0.10^{c,d}	NG
4	0.33±0.06^{c,b}	NG	ND	0.30±0.00^{d,b}	0.20±0.00^{a,a}	ND	0.40±0.00^{c,c}	NG	1.50±0.10^{e,e}	0.33±0.02^{b,b}	1.00±0.00^{d,d}	NG
5	0.20±0.00^{b,b}	NG	ND	0.23±0.06^{c,c}	0.13±0.06^{a,a}	ND	0.20±0.00^{b,b}	NG	ND	0.17±0.06^{a,a}	NG	ND
8	0.07±0.06^{a,a}	NG	ND	NG	NG	ND	NG	NG	ND	0.10±0.00^{a,b}	NG	ND
11	NG	NG	ND	NG	NG	ND	NG	NG	ND	NG	NG	ND
14	NG	NG	ND	NG	NG	ND	NG	NG	ND	NG	NG	ND

Values are means ± standard deviation of three independent experiments. Values were compared based on period of incubation and volume of essential oil applied for each category of fermented soymilk. Values with different superscripts along the same column, and on the same row are significantly different ($p < 0.05$).
PSSF: Non supplemented soymilk fermented on stationary bench
PRSF: Non supplemented soymilk fermented on rotary shaker
SSSF: Soymilk supplemented with sucrose and fermented on stationary bench
SRSF: Soymilk supplemented with sucrose and fermented on rotary shaker
P – Period of storage after addition of essential oil (days);
Ctrl – Control (Untreated samples);
ND – Not determined (bacterial counts were not determined because the samples had deteriorated);
NG – No growth (no fungal growth occurred on agar medium after incubation for seven days)

Table 10: Sensitivity of bacterial Isolates to the leaf Essential oil of *Hoslundia opposita*

Isolates	Diameter zone of inhibition (mm)		
	100%	50%	25%
Bacillus wiedmannii strain FSL W8-0169	29.00±1.00[bc]	21.00±1.00[de]	15.67±2.08[bcd]
Micrococcus luteus strain NCTC 2665	32.00±2.00[b]	26.00±1.00[b]	21.33±1.53[a]
Lactobacillus algidus strain M 6 A9	43.33±3.06[a]	31.33±1.53[a]	17.00±1.00[bc*]
Lactobacillus sakei strain NBRC 15893	46.33±1.53[a]	31.67±1.53[a]	19.00±1.00[b]
Lactobacillus apodemi strain ASB1	30.33±2.00[bc]	23.67±1.53[ac]	13.00±1.00[e*]
Escherichia coli	30.67±2.52[bc]	23.00±2.00[cd*]	17.00±1.00[bc*]
Shigella sp.	28.00±2.00[c]	19.33±0.58[e*]	14.33±1.53[de*]
Staphylococcus aureus	29.00±1.00[bc]	16.00±1.00[f*]	10.00±1.00[f*]

Values are means ± standard deviations of readings taken in three planes. Values in the same column with different superscripts are significantly ($P< 0.05$) different. *bacteriostatic.

Fungal growth in all the soymilk samples was highest at 9 hours of fermentation and dropped sharply to zero by the 24th hour. This shows strong inhibitory activities of the metabolites such as the pH reducing lactic, acetic, propionic and formic acids as well as hydrogen peroxide produced in the samples during fermentation (Schnurer and Magnusson, 2005; Siedler *et al*., 2019).

The majority of the bacterial isolates belonged to the genus Lactobacillus. Other species recorded were *Bacillus, Staphylococcus, Micrococcus, Escherichia and Shigella*. *Lactobacillus sakei*, which was one of the isolates is a facultative heterofermentative LAB comprising strains used as a starter culture (Armour *et al*., 2005); and is used for the production of bacteriocins such as saucisson for meat preservation (Bredholt *et al*., 2001), sakacin P which inhibits Listeria monocytogenes (Carvalho *et al.*, 2009) and lactocin S (Mortvedt *et al*., 1991). Lactobacillus algidus is a group B lactobacilli that was first reported by Kato *et al*. (2000) as a fastidious, meat-derived, psychrophilic lactic acid bacterium implicated in spoilage of meat. L. apodemi was also reported as a novel species in 2006 (Osawa *et al*., 2006).

Bacillus weidmannii was first isolated from dairy milk and dairy environments. It is a member of the *Bacillus cereus* group which includes pathogenic and non-pathogenic strains as well as food spoilage organisms (Miller *et al.*, 2016). This makes its isolation from the fermented soymilk samples significant as it may be responsible for spoilage and may constitute a potential threat to the health of consumers. *Micrococcus luteus* may have contaminated the soymilk during processing or may be present on the soybean since it occurs in the soil, dust, water, air and as part of the normal flora of human skin. It was first isolated by Alexander Flemming in 1928. It has also been isolated from foods such as milk, cheese, meat and cassava (Laurie, 2015) that were contaminated through handling. *Staphylococcus aureus*, Escherichia coli and *Shigella* sp are contaminants that can constitute a potential health risks to consumers of the product. *Staphylococcus aureus,* is a normal flora of human and animal skin and can contaminate foods during processing. Its presence in foods is very important because it causes food borne diseases due to the production of toxins (Kadariya, *et al.*, 2014). Escherichia coli is a member of the gastrointestinal flora. Its detection in food signifies fecal contamination. Most strains are harmless, but some including shiga-toxin producing E. coli O157:H7 cause serious food poisoning and it's frequently associated with milk and dairy products (WHO, 2018). Shigella cause an acute disease involving the large and distal small intestine that is characterized by diarrhoea, vomiting, and abdominal pain (Yousefi *et al*., 2018).

All the fungal isolates are important spoilage organisms with implications in food-borne diseases. The yeast Candida parapsilosis is among other fungi that commonly cause spoilage of yoghurt and other dairy product (Akabanda *et al*., 2013). It is a commensal on human skin and has been isolated from human hands, soil, insects, domestic animals and marine environments. It was considered non-pathogenic but later found to cause blood sepsis as well as wound and tissue infections (Trofa *et al*., 2008). It is the second most common pathogen in superficial candidiasis after C. albicans (Feng *et al*., 2012).

The molds *Aspergillus, Penicillium and Fusarium* secrete mycotoxins in addition to changing the aesthetic nature of foods. *Aspergillus flavus* is a common saprophytic fungus that is also pathogenic to many crops such as cereals, legumes and tree nuts. They produce aflatoxin, a carcinogenic mycotoxin and also cause aspergilloses in humans (Saori and Keller, 2011). *Penicillium critinum* is also a contaminant of agricultural products and have been isolated from cereals and spices. They secrete a number of secondary metabolites including citrinum, a nephrotoxic mycotoxin (Houbrraken *et al*., 2010). *Fusarium verticilloides* also produces the mycotoxin fumonisin in addition to fusarin C which is a mutagenic chemical (Ortiz *et al.*, 2015)

After fermentation, there was significant increase in the total proteins (54%), calorific value (71%) and titratable acidity (98%) of the milk while the moisture (29%), ash (40%), carbohydrate (41%, crude lipids (20%), crude fibre (35%) and metabolizable energy (74%) were significantly reduced. This result which is similar to that reported by Obadina *et al*. (2013) shows an improvement in the nutritional composition due to fermentation particularly with regard to the protein content which is a major nutrient, and the main reason for consumption of fermented soymilk.

The acceptance rating of the LEOHO treated fermented soymilk was considerably significantly higher than the untreated samples. This improvement in acceptance further attests to the preservative properties of the LEOHO.

The preservative effect of the LEOHO was demonstrated with a general reduction in the bacterial load of the fermented soymilk samples after treatment. In addition, a residual effect occurred as there was no significant increase in the population of bacteria recovered during storage. This preservative effect is a result of the antimicrobial activities of the oil as reported earlier (Gundidza *et al*., 1992; Ojo and Anibijuwon, 2010).

The fungal growth was generally low in the fermented soymilk samples. No growth was obtained from the samples treated with LEOHO until the 2nd and 3rd day of storage while only one of the control samples had fungal growth immediately after fermentation. The fungal load was also low during fermentation and this was carried over to the storage period accounting for the initial zero counts. In addition, the LEOHO may have inhibited fungal growth as its antifungal activities has been demonstrated earlier (Gundidza *et al*., 1992; Zolo *et al.*, 1998). The growth that occurred shortly during storage also shows that the oil exhibited fungi-static activity at the concentration used.

The LEOHO significantly inhibited the bacterial isolates at a concentration as low as 25% and with cidal effects at higher concentrations. There is a dearth of information on the antimicrobial activity of the LEOHO in literature. However activities against some bacteria including many Bacillus species such as *B. cereus, B. pumilus, B. subtilis*, and others such as *Staphylococcus aureus*, *Enterobacter feacalis* (Kapoor *et al*., 2015), and *Lactobacillus acidophilus* (Ocheng *et al*., 2015) had been reported.

5. Conclusions

Fermentation of soymilk yielded a product with improved nutritional composition and consumer acceptability. The leaf essential oil of *Hoslundia opposita* significantly inhibited growth of microorganisms and all the microbial isolates were found to be sensitive to the oil. This work therefore recommends the production of yoghurt-like fermented soymilk as a nutritious and refreshing beverage and its preservation using LEOHO after ascertaining its safety.

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