

Chemical characterisation and microbiological quality of naturally fermenting soy milk

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Received: 6 May 2013 / Accepted: 31 October 2013

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RESEARCH ARTICLE

Abstract

Soymilk is the rich creamy liquid extract of soybean and is the most available soy products. Changes that occur during natural fermentation of soymilk to soy-nono were evaluated. Clean soybean seeds were soaked, blanched and blended in order to obtain soymilk. The soymilk was filtered and allowed to undergo natural fermentation in order to obtain soy-nono. Soy-nono samples were taken at 6 h intervals from 0-72 h and analysed for their pH, titrable acidity and proximate composition. Also, the microbiological quality of the fermenting soymilk was carried out by enumerating the microbial population (total aerobic count, *Lactobacillus* spp., yeast and mould) and the isolated microorganism were characterised and identified. The pH of the fermenting soymilk decreased from 6.90 to 4.09 while the total titrable acidity increased from 0.42 to 1.82% (lactic acid equivalent) during the fermentation period. The crude fat and carbohydrate content decreased from 2.18 to 0.87% and 1.52 to 0.60%, respectively while total ash and crude protein content increased from 0.23 to 0.74% and 2.62 to 5.09%, respectively. Changes in the magnesium content, zinc content, total solid, moisture content, protein content, energy value of the soy-nono throughout the fermentation period were not significantly different at 5% confidence level. The increase observed in the calcium content of the soy-nono increased significantly after 36 h fermentation period. Based on the microbiological quality of soy-nono, the total aerobic, lactic acid bacteria and yeast count increased throughout the fermentation period from 5.60 to 7.30, 3.48 to 5.90 and 1.30 to 3.63 log cfu/ml, respectively. Mould counts increased from 1.00 to 3.07 log cfu/ml between 0 and 42 h and then decreased till the end of the fermentation period. The microorganisms present in the soy-nono were found to be *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus farciminis*, *Lactobacillus alimentarius*, *Saccharomyces cerevisiae* and *Candida pseudotropicalis*. This study shows that several microorganisms are involved in the natural fermentation of soymilk to soy-nono and lactic acid bacteria were the dominant microbial group in the soy-nono. Also, the protein and calcium content of the soy milk increased as a result of its conversion to soy-nono by natural fermentation.

Keywords: fermentation, microorganisms, soybeans, soy-nono

1. Introduction

Soybean (*Glycine max*) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses. Examples of soybean products are soymilk, soy vegetable oil, tofu, tempeh, natto, soy cheese and soy-nono. Soymilk is the rich creamy liquid extract of soybean (Tunde-Akintunde and Souley, 2009) and is the most available soy products. Soymilk is a popular nutritive alternative to cow milk and also cheaper than cow milk (Soya-Agrodok, 2005). It has become an important food product due to its extraordinary nutritive and medicinal. It

is a very rich source of highly valuable proteins, unsaturated fatty acids, soluble and insoluble dietary fibres, and isoflavones (Božanić, 2006). In some countries, soymilk are consumed by lactose intolerant individuals, vegetarians and those allergic to milk proteins, it also has limited consumer acceptability due to its undesirable beany flavour. However, its acceptability can be enhanced by modifying its processing methods such as cold-water extraction of soymilk, application of heat, soaking of soybean in ethanol or alkali and acid grinding. Several researchers have tried to ferment soy milk using either pure or mixed culture of the some microorganisms to improve its acceptability due

to reduction in objectionable flavour and oligosaccharides such as starchose and raffinose that cause flatulence (Wang *et al.*, 2002). Hui *et al.* (1996) reported that fermentation of soymilk can serve as a means of modifying or improving its flavour and texture for it to become more acceptable and also new types of soy products that resemble cultured dairy products.

Fermented milk products are sour tasting milk which has been made by either fermenting the milk naturally or by the use of starter culture to produce the desirable milk products example of such products are found in Africa, Syria, India, America, Nepal, etc., and this includes cheese, nono, buttermilk, yoghurt, etc. Two popular products produced by fermenting milk in Nigeria are wara, which is prepared by coagulating fresh soy milk using the leaf extract of the sodom apple plant (*Calotropis procerca*), and nono. Nono is the indigenous name for naturally fermented thick milk product sold in the Northern part of Nigeria. It is usually prepared by naturally fermenting cow milk or goat's milk in a covered calabash at room temperature (Atanda and Ikenebomeh, 1991). To prepare nono, fresh milk is left to ferment in a covered calabash at room temperature.

Recent research has further emphasised the importance of a fermented soymilk diet (Omojasola, 2000). Ohta *et al.* (2000) reported that high consumption of a high soy meal diet and soybean related products provide protection against breast cancer. Fermented soymilk has been shown to contain high levels of the anti-cancer chemo-preventive agent's genistein and isoflavone (Fututake *et al.*, 1996). Fermented soymilk products like soy-nono helps to build inner ecosystem, providing a wealth of friendly intestinal microflora which aids digestion, leads to nutrient assimilation and boost immunity. Soy-nono has also been reported to have higher ash, protein, sugar and amino acids than soy milk and have been rated to be superior to cow milk nono (Omojasola, 2000). Being of plant origin, they are cheaper, have lower cholesterol, richer in unsaturated fatty acid than cow milk nono.

Eka and Ohaba, (1977) stated that different bacterial species from various sources that naturally contaminate the milk might be responsible for its fermentation into soy-nono. Microorganisms that have been reported to be implicated in the fermentation of nono include *Lactobacillus acidophilus*, *Streptococcus cremoris*, *Micrococcus aureus* and *Streptococcus lactic* (Akinyanju, 1989) but literature is scarce of information on the traditional fermentative microflora that is involved in the production of soy-nono. Also, information on the changes (chemical, nutritional, microbiological) that occur during the process of fermenting soymilk to soy-nono is limited. This study was therefore aimed at evaluating the changes that occur during natural fermentation of soymilk to soy-nono and determining

the predominant microbial groups responsible for the fermentation of soymilk to soy-nono.

2. Materials and methods

Sample collection and preparation of soymilk

Soybean (*G. max*) was purchased from Lanfenwa market, Abeokuta, Ogun State, Nigeria. The seeds were sorted manually to remove stones, damaged and immature seeds. The clean soybean seeds were soaked in water at room temperature in a 2.0 l beaker for 18 h, drained, subjected to blanching at 98 °C for 2 minutes, dehulled manually to remove their testa and blended for 3 minutes. The slurry was filtered through two layers of 50 mesh cheese cloth and the resulting soymilk was boiled at 100 °C for 5 minutes and thereafter held at 5 °C.

Fermentation of soymilk

Freshly prepared soymilk was dispensed into clean, dry conical flasks. The samples were loosely covered with aluminium foil to keep out dust and insects, while allowing air circulation. The samples were then left on the bench to ferment at room temperature at 27±2 °C for 72 h.

Sample preparation

Fermented samples were collected at 6 h interval until the end of fermentation period of 72 h for analyses.

Chemical analysis

The pH value of the resulting soy-nono was determined immediately after production. 10g of the fermenting soy-nono was mixed with 100 ml of distilled water, the mixture was allowed to stand at room temperature for 3 minutes and the pH of the supernatant was then measured with a pH meter. Titratable acidity was determined by dispersing 10 g of the sample in 100 ml of distilled water and titrating 10 ml aliquots with 0.1 M NaOH.

Proximate composition

Samples of fermented and unfermented soy-nono (0 h) were analysed for the following parameters, total solid, fat, carbohydrate, protein, moisture and ash content according to AOAC (2000). The mineral content of the soy-nono was determined by the procedure of AOAC (2000). Magnesium, calcium, iron and zinc were determined using an atomic absorption spectrometer (Thermo scientific S Series Model GE 712354; Thermo Fisher Scientific Inc., Waltham, MA, USA) after digestion with a perchloric-nitric acid mixture (AOAC, 2000).

Isolation and identification of microorganisms

Ten ml of each fermenting Soy-nono milk samples were aseptically added into 90 ml of sterile 0.10% peptone water and mixed thoroughly. Serial dilutions (10^{-4} to 10^{-5}) was performed and 1 ml aliquots of appropriate dilution were directly inoculated on the following media using pour plate method:

- MRS agar (CM0361; Oxoid, Basingstoke, UK) incubated aerobically at 35 °C for 48 h for enumeration of lactobacilli (Owusu-Kwarteng *et al.*, 2010);
- plate count agar (CM0325; Oxoid) incubated at 32 °C for 48 h for enumeration of total aerobic bacteria (Owusu-Kwarteng *et al.*, 2010);
- Sabouraud dextrose agar (CM0041; Oxoid) supplemented with chloramphenicol incubated aerobically at 25 °C for 96 h for enumeration of yeast and moulds.

Statistical analysis

All analyses were carried out in triplicates and the data collected were subjected to analysis of variance (ANOVA) according to Steel and Torrie, (1980) using the SPSS statistical package version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used to separate the significantly different means of the parameters at 5% confidence level.

3. Results and discussion

The pH of soymilk produced at the initial stage of fermentation (0 h) in this study was 6.90 and decreased to 4.09 at the end of the fermentation period (72 h) while the total titratable acidity of the fermenting soymilk increased from 0.42 to 1.82% with the highest value recorded (1.82%) at 72 h of fermentation as shown in Figure 1 and this might be attributed to the increase in the number of acid producing bacteria which was favoured by the reduction in pH. Generally, decrease in pH and increase in total titratable acid in the soy-nono could be attributed to accumulation of organic acid resulting from fermentative organisms during natural fermentation. Similar sequence of decrease in pH and increase in acidity during fermentation was reported by Akabanda *et al.* (2010) in naturally fermented cow milk.

The effect of fermentation on the proximate composition of soy-nono is presented in Table 1. There was insignificant decrease in the moisture content of the soy-nono, the initial moisture content of soymilk (93.45%) reported in this study falls within the range and was similar to reports of Namrata and Gurmukh (2007), Tunde-Akintunde and Souley (2009), and Orhevba (2011). The moisture content decreased to 92.50% at 54 h of fermentation and then later peaked to 92.70% at the end of fermentation period (72 h). The

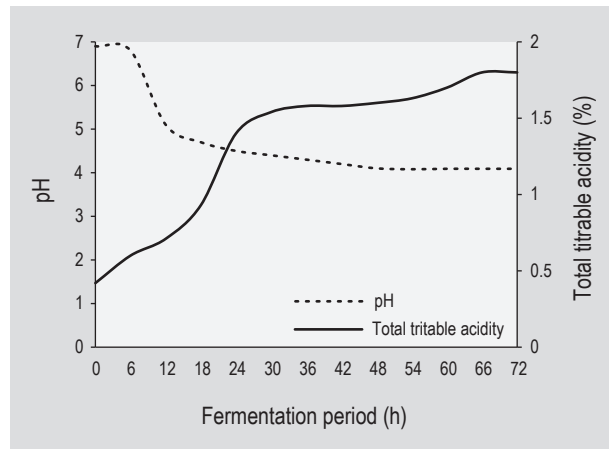


Figure 1. Changes in the pH and total titratable acidity of fermenting soy-nono during natural fermentation.

decrease observed in moisture content as fermentation time progressed could be due to increased dry matter content as a result of microbial cell proliferation. Decrease in moisture content was also reported by Omojola (2000) in soymilk fermented for 30 h.

The carbohydrate content recorded in this study decreased from 1.52 to 0.60% as fermentation period increased. The decrease observed could be as a result of the activities of the fermenting microorganisms which utilised and transformed the carbohydrate into energy for growth and other cellular activities. Osundahunsi *et al.* (2007) also reported a decrease in carbohydrate content of soy-yoghurt fermented with starter cultures.

Fat content as reported in this study varied from 2.18-0.87% (Table 1). Fat content of soymilk was found to decrease significantly as fermentation time increased and this might be attributed to the increased activities of the lipolytic enzymes during fermentation which hydrolyses fat components (triacylglycerol) into fatty acid and glycerol. The fatty acids were reported by Astuti *et al.* (2000) to be used as sources of energy by some microorganisms such as moulds resulting in lower fat content in fermenting soymilk at the end of fermentation.

This result shows an increase in protein content of soymilk (2.62-5.09%) as it was being fermented for the time under consideration. The improvement reported in protein content of the soy-nono as compared to soymilk can be attributed to microbial synthesis of proteins from metabolic intermediates during their growth cycles and the activities of the fermentative organisms to synthesis amino acids (Zamora and Fields, 1979).

Ash content of fermenting soymilk ranged from 0.23-0.74%. The ash content increased significantly as the fermentation period increased. The higher ash content of fermenting

Table 1. Changes in proximate composition of soymilk nono during natural fermentation.

Time (h)	Energy (Kcal/100 g)	Moisture content (%)	Ash content (%)	Fat content (%)	Protein content (%)	Carbohydrates (%)
0	36.18±1.41 ^a	93.45±0.07 ^a	0.23±0.14 ^a	2.18±0.01 ^f	2.62±0.14 ^a	1.52±0.14 ^d
6	38.85±2.82 ^a	93.45±0.35 ^a	0.25±0.02 ^{ab}	2.13±0.01 ^f	2.75±0.07 ^a	1.37±0.01 ^d
12	36.21±1.41 ^a	93.30±0.28 ^a	0.26±0.01 ^{abc}	2.08±0.00 ^f	3.12±0.02 ^a	1.23±0.14 ^d
18	31.91±1.41 ^a	93.25±0.14 ^a	0.56±0.14 ^{abcd}	1.43±0.14 ^{de}	3.75±1.41 ^a	1.01±0.00 ^{bc}
24	32.47±2.82 ^a	93.05±0.07 ^a	0.57±0.28 ^{abcd}	1.43±0.28 ^{de}	4.00±0.14 ^a	0.90±0.14 ^{abc}
30	33.48±1.42 ^a	92.90±1.41 ^a	0.58±0.28 ^{abcd}	1.48±0.00 ^{ce}	4.15±1.41 ^a	0.89±0.00 ^{ab}
36	32.90±1.41 ^a	92.80±1.41 ^a	0.60±0.14 ^{abcd}	1.25±0.70 ^{bcdde}	4.43±1.42 ^a	0.87±0.14 ^{ab}
42	32.40±1.41 ^a	92.75±1.41 ^a	0.65±0.07 ^{bcd}	1.20±0.14 ^{bcd}	4.53±0.00 ^b	0.87±0.00 ^{ab}
48	31.85±1.41 ^a	92.73±1.41 ^a	0.67±0.14 ^d	1.09±0.00 ^{abc}	4.72±0.70 ^b	0.79±0.14 ^{ab}
54	32.75±1.41 ^a	92.50±0.07 ^a	0.70±0.14 ^d	1.11±0.00 ^{ab}	4.96±1.41 ^b	0.73±0.28 ^{ab}
60	32.16±1.41 ^a	92.60±0.00 ^a	0.71±0.14 ^d	1.08±0.00 ^{ab}	4.96±1.41 ^b	0.65±0.14 ^a
66	31.61±1.41 ^a	92.65±1.41 ^a	0.71±0.28 ^d	1.01±0.00 ^{ab}	5.00±1.41 ^b	0.63±0.14 ^a
72	30.59±1.41 ^a	92.70±1.41 ^a	0.74±0.14 ^d	0.87±0.14 ^a	5.09±2.82 ^b	0.60±0.14 ^a
± SEM	0.75	0.16	0.04	0.09	0.24	0.06

Values followed by different superscript letters within a column differ significantly ($P<0.05$).

SEM = standard error of the mean.

soymilk compared with the unfermented soymilk could be due to reduction of certain chemical components. Similar and greater increase in ash content during fermentation have been reported by several researchers (Adebowale and Malik, 2011; Irtwange and Achimba, 2009).

Soymilk is rich in minerals such as calcium, iron, magnesium and zinc. The value of calcium, magnesium and zinc contents in the fermenting soymilk ranged from

52.86 to 65.00 mg/l, 7.66 to 7.83 mg/l and 4.42 to 6.75 mg/l, respectively as shown in Table 2. The calcium, iron and magnesium contents in soy-nono increased with increase in natural fermentation period up to 54 h and then decreased as the fermentation period increased to 72 h (Table 2). Increase in the zinc content of the fermenting soy milk was insignificant. Gabriel *et al.* (2011) reported increase in some mineral contents of jack beans as affected by the use of mould starter cultures for fermentation. The increase in

Table 2. Changes in the mineral content of Soymilk nono during natural fermentation.

Time (h)	Calcium (mg/l)	Iron (mg/l)	Magnesium (mg/l)	Zinc (mg/l)
0	52.86 ± 1.41 ^a	28.00±1.41 ^a	7.66±1.41 ^a	4.42±1.41 ^a
6	52.86±2.82 ^a	32.00±1.41 ^{ab}	7.91±2.82 ^a	4.65±1.41 ^a
12	58.57 ± 1.41 ^{ab}	32.00±1.41 ^{bc}	8.00±1.41 ^a	5.35±1.41 ^a
18	58.57±4.24 ^{ab}	32.00±2.82 ^{bcd}	8.13±1.41 ^a	5.58±0.00 ^a
24	60.00±2.82 ^{ab}	34.00±2.82 ^{ab}	8.16±2.82 ^a	5.58±0.00 ^a
30	60.14±0.00 ^a	36.00±1.41 ^{ab}	8.24±1.41 ^a	6.05±0.00 ^a
36	67.14±1.41 ^{bc}	36.00±1.41 ^{cd}	8.25±0.00 ^a	6.12±1.41 ^a
42	67.77±1.41 ^{bc}	37.00±1.41 ^{cd}	8.36±1.41 ^a	6.28±1.41 ^a
48	68.43±1.41 ^{bc}	37.00±1.41 ^{cd}	8.47±1.41 ^a	6.28±2.82 ^a
54	71.43±1.41 ^c	40.00±1.41 ^d	8.87±2.82 ^a	6.28±0.00 ^a
60	65.71±1.41 ^{bc}	38.00±0.00 ^{bcd}	8.00±1.41 ^a	6.74±1.41 ^a
66	65.00±1.41 ^{bc}	32.00±1.41 ^{ab}	7.87±1.42 ^a	6.75±0.00 ^a
72	65.00±0.00 ^{bc}	28.00±1.41 ^a	7.83±0.00 ^a	6.75±1.41 ^a
± SEM	1.45	0.77	0.26	0.24

Values followed by different superscript letters within a column differ significantly ($P<0.05$).

SEM = standard error of the mean.

some of the minerals of the fermenting soymilk compared to unfermented soymilk was an indication that these minerals were released from chelated complex compound through the activities of microorganisms responsible for the fermentation. The decrease in the magnesium, iron and calcium contents of soy-nono towards the end of the fermentation period could be an indication that certain organisms utilise them for their growth and metabolism (Hassan *et al.*, 2005).

The variations in the microbial counts of soy-nono are shown in Figure 2. The counts of aerobic bacteria, lactic acid bacteria, yeasts and moulds ranged from 5.60-7.30, 3.48-5.90, 1.30-3.63 and 1.00-2.04 log cfu/ml, respectively. The number of lactic acid bacteria increased throughout

the fermentation period. The yeast count did not increase until after 54 h and then increased marginally till the end of fermentation. The counts of moulds increased in a steady rate up to 42 h and then decreased till the end of fermentation period as shown in Figure 2. The dominance of lactic acid bacteria over other organisms can be explained by their tolerance to high acidity and their ability to utilise the substrate better, Table 3 shows the morphological and biochemical characterisation of the lactic acid bacteria that were found in the fermenting soy milk while Table 4 and 5 show the morphological and biochemical characterisation of the yeast and moulds isolated from the soy-nono. Lactic acid bacteria possess α -galactosidase and can therefore utilise galacto-oligosaccharides like raffinose and starchyose in soymilk for growth (Garro *et al.*, 2005).

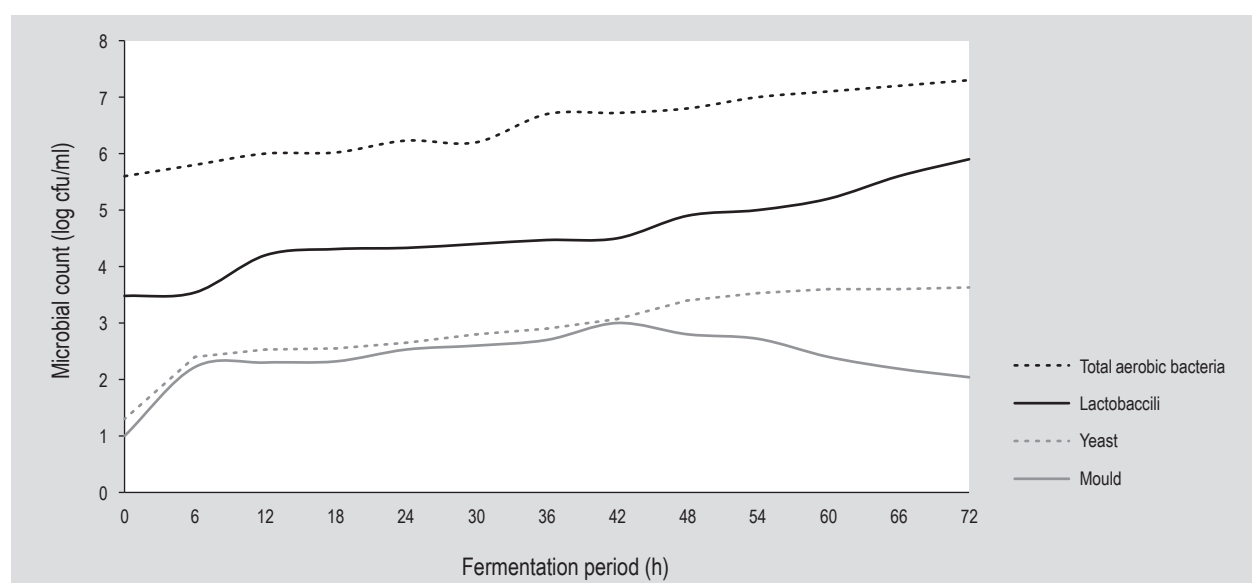


Figure 2. Microbiological changes in naturally fermenting soymilk

Table 3. Morphological and biochemical characterisation of lactic acid bacteria (LAB) isolates in naturally fermenting soymilk.

Isolate	Colony morphology	Cell	Biochemical test				Fermentation test								Growth		Probable organism		
			G	Cat	Oxi	Ho/He	Gl	L	Ar	Ma	Su	St	Tr	Sal	M	15 °C		45 °C	
LAB 1	small, round and shiny creamy colony with entire edge	SR	+	-	-	He	+	+	+	-	+	-	+	-	+	-	+		<i>Lactobacillus fermentum</i>
LAB 2	small, round and creamy colony with smooth edge	LR	+	-	-	Ho	+	+	-	-	+	-	+	+	+	-	+		<i>Lactobacillus acidophilus</i>
LAB 3	slightly large, round, concave, creamy colony with smooth edge	SR	+	-	-	Ho	+	+	-	-	+	-	+	+	+	+	+		<i>Lactobacillus farciminis</i>
LAB 4	Small, round, creamy with smooth edges	SR	+	-	-	He	+	-	+	-	+	+	+	+	+	-	+		<i>Lactobacillus alimentarius</i>

SR = short rod; LR = long rod; G = gram; Cat = catalase test; Gl = glucose; L = lactose; Oxi = oxidase test; Ar = arabinose; Ma = mannitol; Su = sucrose; St = starch; Tr = trehalose; Sal = salicin; Ho = homofermentative; He = heterofermentative; M = maltose.

Table 4. Cultural and morphological characteristics of yeast isolates in naturally fermenting soymilk.

Isolate	Colony	Cell	Fermentation/acid production							Growth at				Probable organism				
			Glu	Mal	Suc	Lac	Gal	St	Ra	Xy	25 °C	30 °C	37 °C		40 °C	N	C	U
Y1	white shiny smooth edge	small oval budding	+	+	+	-	+	-	-	-	+	+	+	-	-	-	-	<i>Saccharomyces cerevisiae</i>
Y2	smooth edge	small oval budding	+	+	-	-	+	-	-	-	+	+	+	+	-	+	-	<i>Candida pseudotropicalis</i>

Y = yeast; Glu = glucose; Mal = maltose; Suc = sucrose; Lac = lactose; Gal = galactose; St = starch; Ra = raffinose; Xy = xylose; N = nitrate test; U = urease test; C = citrate test.

Table 5. Cultural and morphological characteristics of mould isolates in naturally fermenting soymilk.

Isolate code	Cultural characteristics	Morphological characteristics	Probable identity
M1	white and later turn green to black with black reverse	conidiophores erect, non-septate, single and terminated with globose vesicle	<i>Aspergillus niger</i>
M2	yellow-green fluffy showing yellow reverse	conidiophores are thick-wall and roughened globose conidia bearing two series of series of sterigmata covering the entire vesicle	<i>Aspergillus flavus</i>
M3	white filamentous showing dark grey reverse	smooth wall conidiophore with sterigmata parallel rows of conidia	<i>Aspergillus fumigatus</i>
sM4	dry powdery surface bluish-green interior with a whitish surroundings, reverse is red	conidiophore upright simple not swollen at apex, arising from the mycelium singly spores in unbranched chains borne from bottle-shaped phialides	<i>Penicillium</i> spp.
M5	white fluffy filament showing brownish yellow reverse	branched aseptate mycelium with spherical Sporangiochore	<i>Mucor</i> spp.
M6	whitish, long-hyphae, growing rapidly	sporogium breaking apart releasing spores of the fungus	<i>Rhizopus</i> spp.
M7	white yeast-like and later turn cream dry powdery	blastoconidia arrangement along the hypha	<i>Geotrichum</i> spp.

M = mould.

Atanda and Ikenebomeh (1991) also reported large numbers of lactic acid bacteria to yeast and mould during natural fermentation of goats' milk (nono).

4. Conclusions

Several microorganisms including lactic acid bacteria, yeasts and moulds are involved in the fermentation of soymilk to soy-nono and the observed increase in the protein and calcium contents of the soy-nono during natural fermentation conferred nutritional advantages on the product and this could be used to improve the nutritional value of other food products.

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