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Isolation and characterization of microorganisms associated with soft cheese produced from sheep milk using different coagulants

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Abstract
Milk is an ideal medium for the growth of microorganisms because it is highly nutritious. Cow milk is the major milk used for the production of cheese although milk can also be gotten from other animals such as sheep, goat, camels and buffalo. Soft cheese was produced from sheep milk and the microorganisms associated with the soft cheese were isolated and identified using colonial, morphological and biochemical characterization. Different coagulants such as Calotropis procera, Carica papaya, lemon juice and steep water from maize, millet and sorghum were used for the production. The result revealed that six different bacteria were isolated and identified and they include Bacillus cereus, Bacillus subtilis, Lactococcus lactis, Lactobacillus casei, Pseudomonas aeruginosa, Staphylococcus aureus and 8 different fungi species isolated and identified include Aspergillus niger, Aspergillus fumigatus, Rhizopus stolonifer, Penicillium chrysogenium. Mucor hiemalis, Candida albicans, Fusarium verticillioides and Saccharomyces sp. The presence of these microorganisms indicated that the soft cheese produced was not sterile and highly contaminated and it can pose serious health risk to consumers. It is therefore recommended that soft cheese should be produced under hygienic condition since it's a ready to eat food.

Keywords: bacteria, fungi, soft cheese, sheep milk

Introduction
Milk is secreted by the mammary glands of mammals. It provides not only the nutritional requirements but added protection to new born. Milk is got from a healthy udder and it is sterile but may be contaminated by bacteria present in the tubules, from where milk flows and the storage space called "Cistern"(Otoikhan, 2012). Cow"s milk is the major milk used for the production of cheese although milk can also be gotten from other animals such as bufallo, goat, sheep, camel e.t.c. Sheep milk is an excellent raw material for the milk processing industry especially in cheese production (Park *et al*., 2007) [23]. Sheep milk has higher specific gravity, viscosity, refractive index, titratable acidity, and lower freezing point than average cow milk (Haenlein and Wendorff, 2006) [12]. Milk is usually coagulated with *Calotropis procera*, other coagulants such as *Carica papaya*, lemon juice and steep water from cereals (maize, millet and sorghum) can also be used (Ogunlade *et al*., 2020) [16]. Soft cheese is fresh cheese, that is, soft, moist curd that has been cut and drained of the whey but never ripened. The soft cheese produced in Nigerian farms especially in the northern part makes use of local ingredients. The vegetable rennet used for the production is made from a native plant *Calotropis procera* (Sodom apple), which can be cultivated, all year round (FAO, 1994). The presence of microorganisms in soft cheese can be from the rudimentary equipment used for the production of the cheese and also from environmental factors. Raw milk easily becomes sour when it is stored for a long period at high ambient temperatures prevalent in tropical and subtropical countries (Orhevba and Taiwo, 2016) [20]. This is because the inherent lactic acid bacteria and contaminating microorganisms from storage vessels or the environment break down the lactose in milk into lactic acid. Fresh milk drawn from a healthy cow normally contains a low microbial activity particularly with bacterial load of less than 10^3 cfu/mL (Chatterjee *et al*., 2006 [7], Lingathurai *et al*., 2009) [14], but the load may increase up to 100 fold or more once it is stored for sometime at ambient temperature (Lingathurai *et al*., 2009) [14]. Some of the factors that increase the bacterial activity in raw milk and its products include health of the animal, cleanliness of the housing area, the nature of feed, the water used at farm, the milk vessels / utensils for storage and essentially the hygiene of the milker / handler (Chatterjee *et al*., 2006, Ali and Abdelgadir, 2011 and Salman *et al*., 2011) [7, 26]. The presence of this pathogenic bacteria in raw milk and its products have been reported to be a major threat to human health especially those who still drink raw milk (Mubarack *et al*., 2010 [15], Lingathurai and Vellathurai, 2010) [13]. and also reduces the keeping quality of milk (Salman *et al*., 2011) [26]. Consumption of raw milk and its by-products is considered potentially hazardous and has been associated with several types of infections including *brucellosis, tuberculosis, salmonellosis, yersiniosis, Escherichia coli* O157 and *Staphylococcal* entero toxin poisoning (Baylis, 2009) [6].

Materials and Methods

Sample collection

The raw milk sample was collected from sheep from Fulani pastoralists at a local farm settlement, Ado Ekiti. It was collected aseptically and transported to the laboratory for analysis.

Plant collection

The leaves (*Carica papaya and Calotropis procera*) were collected from The Federal Polytechnic, Ado-Ekiti premises and the Authentications of the Plants were done at the Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti, Nigeria. The voucher specimens of UHAE 2018/022 for *Carica papaya* and UHAE 2018/023 for *Calotropis procera* have been deposited at the University herbarium.

Collection of coagulants:

Lemon fruits used were purchased from the market and steep water (effluent from pap produced from maize, sorghum, millet) were produced by steeping the grains in water for 3days after which it was milled and later steeped again for 2days. The steep water was then collected for use as coagulants.

Production of soft cheese

The milk was stirred gently during the heating process with a wooden spoon. About 4ml of the leaf extract of *Calotropis procera, Carica papaya*, Lemon juice and steep water were added to the warm milk and the mixture was heated the second time with intermittent stirring to about 45-50°C and was kept at this temperature until coagulation was achieved and the heating was stopped after the separation of curd and whey. The sign of coagulation was observed within the range of 10-15 min. It was transferred into a small raffia basket to facilitate whey drainage and characteristic shape, when the cheese was firm enough it was removed from the raffia basket and placed inside a covered plastic container for analysis.

Culture Media and Reagents

The following media were used for the isolation and characterization of the microorganisms from the soft cheese, Nutrient Agar (NA) was used for the isolation and enumeration of total viable mesophilic bacteria count; Plate Count Agar (PCA) for the enumeration of total viable mesophilic count; Potato Dexrose Agar (PDA) for isolation and enumeration of Yeast and Molds. Nutient agar was used for subculturing the bacteria isolates and PDA for Yeast and Molds. Each of the media used was prepared and sterilized according to manufacturer's specifications. The reagent used for chemical analysis and biochemical characterization was prepared according to the requirements of each analysis. The media were sterilized at 121^{o}C for 15 minutes in an autoclave at 1.0Nm^{-2}.

Isolation of microorganisms

This was done using the modified pour plate method (Olutiola *et al*., 2000) [19]. One gram (1 gram) of the cheese sample was weighed and introduced into 10mL of sterile distilled water to carry out a ten-fold serial dilution. Molten nutrient agar (NA), plate count agar (PCA) and Potato dextrose agar (PDA) which have been prepared and autoclaved, were allowed to cool to about 45^{o}C before they were dispensed aseptically into sterile Petri dishes containing 1mL of introduced inoculums from the dilutions. The plates were gently swirled and allowed to set. The inoculated plates were incubated in an incubator (Gallenkomp 9052A, England) at 37^{o}C for 18-24hours for bacteria while PDA plates were incubated at 28±2^{o}C for 72-120hrs for fungi. All the plates were observed for growth and counted after incubation.

Subculturing method

Pure colony was obtained from streak plate. The microbial mixture was transferred to the edge of an agar plate with an inoculating loop and then streaked out over the surface of fresh agar plate. Pure colonies obtained were maintained on agar slants at 4^{o}C and subsequently sub cultured before use. Colonies of fungal growth observed were sub cultured onto fresh potato dextrose agar (PDA) until pure cultures of the fungal isolates were obtained.

Cultural and Biochemical tests

The test carried out on the isolates include Gram's staining reaction, motility, spore staining, catalase, indole production, methyl red, Voges- Proskauer test, citrate utilization, urease test, oxidase and sugar fermentation tests according to the methods of Olutiola *et al*., 2000 [19].

Identification of Bacteria

Appearance of the colony of each isolate on the agar media was studied. Characteristics observed include shape, edge, colour, elevation and texture after 24 h of incubation. Staining reaction and other results of biochemical tests were interpreted for the tentative identification of bacterial isolates to specie level according to Cowan and Steel (1993) [8].

Morphological and Microscopic Identification of Fungi Isolates

Fungal mycelium (48 h) old was aseptically taken with needle and placed gently on a clean slide containing a drop of lacto-phenol blue, covered with cover slip and examined under the microscope. Microscopic identification was done according to (Barnett *et al*., 2000 [5]. and Samson *et al*., 2010) [27]. The identified fungi were maintained on PDA slants at 4^{o}C in refrigerator for subsequent use.

Results

Tables 1, 2, 3, and 4 shows the Cultural, Morphological and Biochemical characteristics of bacteria and fungi isolated from the soft cheese. Six bacterial species and eight different fungi species were isolated from the samples. The Bacteria includes *Bacillus cereus, Bacillus subtilis, Lactococcus lactis, Lactobacillus casei, Pseudomonas aeruginosa, Staphylococcus aureus* and the fungi species includes *Aspergillus niger, Aspergillus fumigatus, Rhizopus stolonifer, Penicillium chrysogenium. Mucor hiemalis, Candida albicans, Fusarium verticillioides* and *Saccharomyces* sp.

Table 1: Cultural and Morphological Characteristics of Microbial Isolates

Isolates	Colony colour/Shape	Cell Shape	Gram's reaction	Spore	Motility
1	White to Yellow	Long Rod	+	+	+
2	White Spreading	Long rod	+	+	+
3	Amber	Rod in chains	+	-	-
4	Grey beaded	Cocci in chains	+	-	-
5	Greenish blue-brown	Short rod	-	-	+
6	Golden Yellow	Cocci in clusters	+	-	-

Table 2: Morphological Characteristics of the Isolates

Isolates	Shape	Elevation	Opacity	Emulsification	colour
1	Rod	Flat	Translucent	E	White to yellow
2	Irregular Rod	Flat	Translucent	E	Cream
3	Rod	riased	Opaque	E	Amber
4	Cocci short chains	Flat	Translucent	E	Grey
5	Rod	Flat	Transparent	E	Greenish Blue-brown
6	Cocci in clusters	Flat	Translucent	E	Golden yellow

Table 3: Biochemical Characteristics of soft cheese Isolates

Iso	Ind	Cit	Nit	M/R	Vp	Oxid	Catal	H_2S	Glu	Malt	Suc	Lact	Man	Suspected Orgs
1	-	+	+	+	+	-	+	-	+	+	-	-	-	*Bacillus cereus*
2	-	+	+	-	+	-	+	-	+	+	+	-	-	*Bacillus subtilis*
3	-	-	+	-	-	-	-	-	+	+	+	+	+	*Lactobacillus casei*
4	-	-	-	-	-	-	-	-	+	+	+	+	+	*Lactococcus lactis*
5	+	+	-	-	-	+	+	-	-	-	-	-	-	*Pseudomonas aeruginosa*
6	-	+	-	-	-	-	+	-	+	+	+	-	+	*Staph. aureus*

Table 4: Cultural and Morphological characteristics of Fungi isolates

Cultural Characteristics	Morphological Characteristics	Tentative Identification
Brownish to black powdery	Conidiophores arising from long broad thick – walled end of the phialide	*Aspergillus niger*
Bluish green	Branched out from ends of phialide	*Aspergillus fumigatus*
White to brownish	Sporangiophores which are short ellipsoidal arising from the pointed	*Rhizopus stolonifer*
Grey –green reverse intensely yellow	Penicillate conidiophres branched out as verticillate phialide	*Penicillium chrysogenium*
White to yellow tater dark green black sporangia	Branched out from the phialides	*Mucor hiemalis*
Creamy	Ovoid shape produced laterally mycelium	*Candida albicans*
Salmon red smooth glossy mucous	Conidiophores richly branched but not in spirodochia usually one celled macro-conidia	*Fusarium verticillioides,*
Creamy	Ovoid shape produced laterally mycelium	*Saccharomyces sp*

Table 5 shows the occurrence of bacteria isolated from the soft cheese sample. *Lactobacillus casei* was present in cheese coagulated with steep water from sorghum (SSO), maize (SMA) and Lemon juice (SLJ), *Lactococcus lactis* was isolated from all the cheese samples except from sample coagulated with *Calotropis procera* (SCPR). *Staphylococcus aureus* was present in cheese coagulated with steep water from sorghum (SSO), maize (SMA), millet (SMI) and *Calotropis procera* (SCPR), *Bacillus cereus* was present only in sample coagulated with *Carica papaya.* (SCP). *Bacillus subtilis* was present in cheese coagulated with steep water from millet (SMI), *Calotropis procera* (SCPR) and *Carica papaya* (SCP) while *Pseudomonas aeruginosa* was present in cheese sample coagulated with *Calotropis procera* (SCPR) and *Carica papaya* (SCP).

Table 5: Occurrence of Bacterial Isolates in soft cheese sample

Sample Code	*Lactobacillus casei*	*Lactococcus lactis*	*Staphy aureus*	*Bacillus cereus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*
SSO	+	+	+	-	-	-
SMA	+	+	+	-	-	-
SMI	-	+	+	-	+	-
SLJ	+	+	-	-	-	-
SCPR	-	-	+	-	+	+
SCP	-	+	-	+	+	+

Key: **SSO –** sheep milk coagulated with steep water from sorghum**, SMA -** sheep milk coagulated with steep water from maize, **SMI -** sheep milk coagulated with steep water from millet, **SLJ -** sheep milk coagulated with steep water from lemon juice, **SCPR -** sheep milk coagulated with *Calotropis procera*,**SCP -** sheep milk coagulated with *Carica papaya*, +: present, -: absent

Table 6 shows the occurrence of fungal Isolates in soft cheese sample. *Aspergillus niger* was found in cheese coagulated with steep water from millet (SMI) and *Calotropis procera* (SCPR). *Aspergillus fumigatus* was isolated from all the cheese samples except in cheese sample coagulated with steep water from millet (SMI) and *Carica papaya* (SCP). *Rhizopus stolonifer* was also found in cheese coagulated with steep water from millet (SMI) only. *Penicillium chrysogenium* was isolated from cheese coagulated with *Carica papaya* (SCPR) and steep water from sorghum (SSO) and maize (SMA). *Mucor hiemalis* was isolated from cheese coagulated with steep water from sorghum (SSO), millet (SMI) and maize (SMA). *Candida albicans* was isolated from cheese coagulated with *Carica papaya* (SCP) and steep water from maize (SMA). *Fusarium verticillioides* was isolated from all the cheese samples except the ones coagulated with lemon juice (SLJ) and *Calotropis procera* (SCPR) while *Saccharomyces* sp was found only in two samples which are cheese samples coagulated with lemon juice (CLJ) and *Carica papaya*. (SCP)

Table 6: Occurrence of Fungi in soft cheese sample

Sample Code	*A. niger*	*A. fumigatus*	*R. stolonifer*	*P. chrysogenium*	*M. hiemalis*	*C. albicans*	*F. verticill*	*Sacch.*sp
SSO	-	+	-	+	+	-	+	-
SMA	-	+	-	+	+	+	+	-
SMI	+	-	+	-	+	-	+	-
SLJ	-	+	-	-	-	-	-	+
SCPR	+	+	-	-	-	-	-	-
SCP	-	-	-	+	-	+	+	+

KEY: S- sheep, SO- sorghum, MA- maize, MI- millet, LJ- lemon juice, CPR- *Calotropis procera*, CP- *Carica papaya*, +: present, -: absent

Discussion

Microbial analysis of soft cheese produced from sheep milk revealed the presence of the following bacteria; *Bacillus cereus, Bacillus subtilis, Lactococcus lactis, Lactobacillus casei, Pseudomonas aeruginosa, Staphylococcus aureus* and eight fungi species which includes *Aspergillus niger, Aspergillus fumigatus, Rhizopus stolonifer, Penicillium chrysogenium. Mucor hiemalis, Candida albicans, Fusarium verticillioides, Saccharomyces* sp.

Bacillus cereus, Staphylococcus aureus, Aspergillus niger, Aspergillus fumigatus, penicillium chrysogenium, Rhizopus spp isolated from this study are similar to those reported by Uzeh *et al*. (2006) [29]. in their work on nono and wara. Olatunji *et al*. (2006) [18]. had earlier reported that cheese harbors bacteria including LAB such as *Lactobacillus*, *Streptococcus* as well as yeast and moulds. *Bacillus cereus* which is known to be highly resistant to environmental stress due to its ability to produce spores was isolated and it is known to be of public health importance since it is pathogenic. The detection of *Staphylococcus aureus* is also of public health importance because of its ability to cause a wide range of infections especially food-borne intoxication. This organism was equally isolated by Olasupo *et al.* (2002) [17]. from cheese and kunun-zaki, a cereal based, non-alcoholic beverage.

The high bacterial load found in the cheese samples investigated during the present study is supported by findings of Elkhider *et al.* (2011) [9]. who reported that cheese samples collected from different producers in rural areas of Eastern Sudan indicate that the level of hygiene and production methods, source of raw milk and its handling could be the main factors responsible for the high microbial loads which might affect the quality of cheese. The decrease in the microbial load of cheese coagulated with lemon juice may be as a result of the acidic nature of the juice. Adetunji *et al*. (2007) [7]. had earlier reported a decrease in the microbial load of cheese produced with lemon juice. The increase observed in the microbial load of the cheese were higher when compared with those isolated from the raw milk and the coagulants used might serve as source of the contaminants (Adegoke *et al*., 1992) [1]. A similar increase in microbial counts after addition of coagulants was reported by Adetunji *et al*. (2006) [3]. Therefore some preventive measures should be taken at this stage such as washing the leaves thoroughly before extraction of the juice, covering the steep water so as to reduce contamination, monitoring the temperature of the milk and controlling the development of acidity. The result is different from the results obtained by Adegoke *et al*. (1992) [1]. who observed reduction in population of total aerobes at the curdling point during the manufacture of the cheese.

The presence of Lactic acid bacteria (LAB) and yeast indicates that the milk was not sterile. LAB are the predominant microorganisms in milk (Sawsan and Maymouna, 2010) [28]. *Lactobacillus casei* had the highest percentage occurrence (28.3%) followed by *Lactococcus lactis* (20%), *Staphylococcus aureus* (18.3%), *Bacillus cereus* (13.3%), *Bacillus subtilis* (11.7%) and *Pseudomonas aeruginosa* (8.4%). This is in line with the work of Guessas and Kihal (2004) [11]. who isolated *Lactobacillus* and *Leuconostoc* from raw goat milk. LAB improves food quality and also plays an important role in preventing the growth of undesirable bacteria. LAB creates an acidic environment conducive for the proliferation of yeasts, while yeasts provide growth factors such as vitamins and amino acids for LAB. Axelsson (2004) [2]. reported that LAB is generally associated with habitats rich in nutrients. It is well known that LAB produces a variety of antimicrobial substances with potential importance for food preservation.

The existence of mold and yeast in cheese observed in this study was not unusual. Other researchers have isolated *Penicillium* and *Aspergillus* spp. from West African cheese (Adegoke *et al.*, 1992) [1]. Oyeleke *et al*. (2006) [22]. in their study on 'nono', 'wara' 'mai-shanu', 'fufu' and 'kamu' isolated *Lactobacillus bulgaricus, L.*

lactis, L. acidophilus, Streptococcus thermophillus and *S. cremoris*. *Aspergillus* species which was isolated is a known spore-former. This means that it can easily contaminate the dairy products which are usually exposed during processing, storage and hawking. They are the major spoilage organisms of carbohydrate food. However, their growth can result in the production and accumulation of mycotoxins which are of public health and economic importance (Rhodes and Fletcher, 1966). The yeast and moulds counts in this study were lower than those reported by Roostita and Fleet (1998) [23]. who stated that yeast population greater than 6 log cfu/mL were found in 54 % out of 85 cheese samples examined in New South Wales, Australia.

Conclusion

In conclusion, result from this work showed that the presence of both bacteria and fungi isolated from the soft cheese produced indicated that the soft cheese was not sterile. Therefore regular sterilization of dairy equipments, washing of utensils and proper hygiene during production are some of the preventive measures that could be applied to avoid contamination of the soft cheese.

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