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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF MICROORGANISMS IN KAWAL (*CASSIA OBTUSIFOLIA*) FERMENTED LEAVES

[1,*]Nosyba A Ibrahim, [2]Elgasim A Elgasim and [3]Salah AMustafa

[1]Assistant professor; Alneelain University, Faculty of Agricultural Technology and Fish Sciences, Department of Food Technology
[2]Professor, National centre of Rehearses, General Director
[3]Associated Professor, Sinnar University, Faculty of Agriculture

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ABSTRACT

Kawal is an African and Sudanese meat substitute produced traditionally through solid state fermentation of sicklepod (*Cassia obtusifolia*) fermented leaves. The present study aimed to isolate and identify microorganism involved in kawal fermentation through conventional microbiological analysis and further molecular confirmation. Two samples of kawal were processed in Algeniana and Sinnar cities, Sudan (AK and SK respectively). Isolated bacterial groups were biochemically identified as *Bacillus subtilis*, *Staphylococcus scuiri* and *Lactobacillus plantarum* in AK, while only *Bacillus subtilis*, *Staphylococcus scuiri* appeared in SK. Yeasts, molds, *Coliform*, *E.coli* and Beta lactamase producing bacteria were not detected. Results were further confirmed using molecular techniques where DNA of isolate bacteria was amplified by PCR, electrophoresed on agarose gel with reference DNA of proposed bacteria. Results were in agreement with the biochemical identification of both AK and SK. Identified bacterial species can be used for further product development.

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INTRODUCTION

The word fermentation is derived from the Latin verb fevere which means "to boil" and fermentation was defined by Louis Pasteur as "La vie sans l'air" (life without air) (Bourdichon *et al.*, 2012). Food fermentation has a long history since ancient times which involves the chemical transformation of complex organic compounds into simpler compounds by the action of enzymes, organic catalysts produced by microorganisms including yeast, moulds and bacteria (Corma *et al.,* 2007). During fermentation, it is the unique property of the bacteria and fungi present that increase the level of proteins, vitamins, essential amino acids and fatty acids in the food. Some microorganisms produce flavor compounds, complex polysaccharides or organic acids (Khalid, 2003). Furthermore, safety and some nutritional benefits (degradation of anti-nutritional factors, improvement of protein digestibility) have also been attributed to fermented foods (Svanberg and Lorri, 1997). Dietary proteins represent key issue for the future regarding worldwide food security. Besides animal sources, plant proteins represent an opportunity to mainly contribute to protein demand. According to FAO, 1/7 of the world population suffers from hunger and 1 billion people have inadequate protein intake (Jean an Stéphane, 2016).

***Corresponding author:* Nosyba A Ibrahim,**
Assistant professor; Alneelain University, Faculty of Agricultural Technology and Fish Sciences, Department of Food Technology.

On other hand, vegetarian foods occupy a larger than ever shelf space in today's market due to the consumers' increasing health concerns (Craig, 2010; Istudor *et al*. 2010). Each nation has its own types of fermented food, representing the staple diet and the raw ingredients available in that particular place (Egwim Evans *et al*., 2013). In Africa, dawadawa, iru, and ogiri are Traditional fermented condiments based on vegetable proteins, and consumed by different ethnic groups (Ashi, 2005). Dawadawa for example, is the fermented seeds meal of *Parkiabiglobosa*. It is used mainly as a flavouring agent but also improves the nutritional composition of poor-protein diets (Teye *et al.*, 2013). Indigenous fermented foods of Sudan are numerous and varied. The raw materials from which these foods were prepared include sorghum, pearl millet, dates, honey, milk, fish, meat, wild plants, marginal food crops and even skins, hooves, bones, caterpillars, locusts, frogs and cow urine. Most of the foods are preserved by the double method of fermentation and sun drying. The indigenous fermented food of Sudan should have their share of modernization in the future through studies and knowledge on the indigenous processes of food fermentation. Now some of 80 fermented foods of Sudan may even be ready to produce on large scale. These are mostly prepared from a mixture of cereals, oil seeds, honey and green leaves (Dirar, 1993). Kawal (*Cassia obtusifolia*) fermented leaves is a well-knownmeat substitute in Western and other parts of Sudan and Africa.

It prepared by a solid-state process of fermentation of kawal leaves and used as an ingredient of sauces destined for consumption with porridge. It imparts a savory and a meaty flavor on the sauce (Dirar, 1993). Kawal is used in relatively large quantities in the preparation of sauces as a meat substitute or meat extender by poor people and in small quantities as a spice by some urban rich people (Osman, 2009). It has medicinal uses in folk medicine as anti-jaundice anti-diabetic, anti-malarial and other uses. Dirar *et al*. (1985) stated that the most important microorganisms of kawal fermentation are *Bacillussubtilis* and *Propionibacteriaspp*, followed by *Lactobacillus plantarm* and *Staphylocoscussciuri* sub *lentis*. In addition to two yeasts, which are found in even much lower counts, have been identified as *Candida krusei* and *Saccharomyces spp*. Dry kawal samples collected from Darfur and Kordofan almost containing the pure culture of *Bacillus subtilis* (Dirar, 1984). Very rare ones would encounter, in addition, a few cells of *Propionibactirum* (Dirar, 1993).The exact role played by each microorganism in kawal fermentation is unknown, but the result of concerted action of all organisms together is a foul-smelling, macerated (but not liquefied), coherent paste. The development of strong odors seems to be the rule in food fermentation in which B. *subtilis* dominates (Dirar, 1993). It is known to have powerful proteolytic enzymes (Mackie *et al.,* 1971).

Besides the beneficial group of microflora responsible for successful fermentation, certain microorganisms have been observed to cause spoilage of the fermenting kawal. The most commonly noticed kind of spoilage is complete liquefaction in the green leaf paste. It was found to be associated with the bacterium *Pseudomonas putida.* Sometimes dried kawal samples collected from Sudan were found to harbor this organism together with *B. subtilis*. Such product samples usually gave the lowest crude protein content and were shown to have concentrations of acetic acid and butyric acid that diverged markedly from the mean. Another organism sometimes found in liquefied pastes that is very difficult to isolate is most likely to be *Clostridium* (Dirar, 1993). As no starter is used in kawal fermentation, the microbes involved must have originated in the leaf, the utensils, the air and/or the human hands (Dirar, 1993).

MATERIALS AND METHODS

Kawal samples: Fresh green leaves of *Cassia obtusifolia* plant was collected from Algenina, Dar Fur State and Sinnar, Sinnar State, Sudan. The two samples were processed following the traditional method of processing described by Dirrar(1993). Completely dried kawal samples were designated as Algeninakawal (AK) and Sinnarkawal (SK). 300g of each sample were placed separately in a sterile plastic sampling bag and transported immediately to the laboratory, stored under refrigeration until analysis.

Microbiological analysis

Total viable count: Ten (10) g of representative *kawal* sample were dissolved in 90 mL of sterile Ringer's solution and homogenized. Appropriate dilutions of the sample homogenates were prepared and inoculated in duplicate in growth specific media to estimate microbial counts. The Total viable were counted on Plate Count Agar (PCA), after 48 hours of incubation at 37°C under aerobic conditions. Colony counter (Labtech) and hand-tally were used for the determination of the total bacterial counts in terms of colony-forming units per mL (CFU/mL).

Yeast and moulds count: Yeast and moulds were enumerated on Sabouraud Dextrose Agar (Biokar, France) after 3-5 days of incubation at 30°C under aerobic conditions (Lawane *et al.,* 2016).

Purification of colonies: Colonies were purified by twice sub-culturing using the streaking plate method. Young cultures were used for Gram staining and then subjected to primary and secondary biochemical identification tests.

Isolation by membrane filtration: For the two kawal samples, three volumes of 100 mL were filtered through 0.45 µm pore size filter (cellulose nitrate membranes, Millipore corporation) using a water pump (model Rocker4000). These membranes were aseptically placed up on plates with appropriate selective media as follows: mFC agar for fecal coliform(Britton and Greeson, 1987) and mEndo agar for total coliforms (McCarthy *et al*,1961). Fecal coliforms should appear as dark blue colonies on mFc agar while total coliforms should appear as metallic-sheen colonies on mEndo agar.

Detection of Extended Spectrum B-Lactamase (ESBL) production: Detection of ESBL phenotype was carried out using BD phonix ESBL automated system (Becton, Dickinson, Md., USA) A total of 20 bacterial isolates from kawal samples cultured on nutrient agar were subjected for study. The isolates were sub-cultured on Mac-Conkey agar to obtain pure culture for which a 0.5 McFarland suspension was obtained and tested according to the manufacturer provided protocol.

Biochemical and physiological identification: All the pure cultures isolates were observed for cell morphology, motility, and Gram staining, using microscope. Then the isolates were subjected to biochemical tests such as Oxidase, Catalase, gelatin hydrolyses, nitrate reduction, methyl red, urease and sugar fermentation ability following the criteria described in the Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994), for identification.

Molecular confirmation: Biochemical identification results obtained for the isolated bacteria was confirmed by using molecular methods for AlGenina and Sinnarkawal. Reference samples of genomic DNA of pure strains were run as same time as of proposed bacteria. The genomic DNA was amplified by PCR using 16srRNA universal primers and then electrophoresed on the agarose gel after checking its purity.

DNA isolation and purification: The Invitrogen PureLink™ Microbiome DNA Kits were used to enable fast and high-quality microbial DNA according to the manufacturer's recommendation to isolate and purify genomic DNA from three bacterial cultures which previously identified using biochemical methods. The purified genomic DNA was qualified by electrophoresis through an agarose gel (1 % (w/v)).

PCR: Thermo Scientific PCR Master Mix is a 2X concentrated solution of Taq DNA Polymerase, dNTPs, and all the components required for PCR, except DNA template and primers. The mix is optimized for efficient and reproducible PCR. Universal 16S rRNA bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-

CGGTTACCTTGTTACGACTT-3’) for isolate 1(Kumbar *et al.,* 2017) and isolate 3 (Lane, 1991).while BS-F (59-GAAGGCGGNACNCAYGAAG-39) and BS-R (59-CTTCRTGNGTNCCGCCTTC-39) for isolate 2 were used.

PROTOCOL: As recommended in the protocol the PCR Master (2X) Mix was gently vortexed and briefly centrifuged after thawing. Then a thin-walled PCR tube was placed on ice and the following components were added for each 50 µL reaction: PCR Master Mix (2X) 25 µL Forward primer 0.1-1.0 µM Reverse primer 0.1-1.0 µM Template DNA 10 µg - 1 µg Water, nuclease-free to complete 50 µl Total volume and gently vortexed and spinned down. PCR was performed using the recommended thermal cycling conditions outlined below: Step: Temperature °C: Time: Number of cycles: Initial denaturation 95°C: 1-3 min: 1. Denaturation: 95°C: 30 min: 40. Annealing 53°C: 30 min: 25-40. Extension 72°C: 1 min/kb Final Extension 72°C: 5-15 min: 10. The PCR products were electrophoresis in 1% (w\v) agarose gel stained with ethidium bromide (0.5mg\mL) with 1000pb ladder and visualized with an Ultraviolet Illuminator. Pure strains of pre-identified bacteria were used as reference samples.

RESULTS AND DISCUSSION

Colony characteristics: Colony count, size and shape are shown in Table (1). AlGeninakawal had a $6.8*10^5$CFU\mL total viable count (TVC). Three types of colonies were distinguished, the dominant one ($5.6*10^5$CFU\mL large colonies) were gram-positive bacilli with irregular margin followed by ($1*10^5$CFU\mL small colonies) gram-positive cocci with smooth margins followed by ($0.2*10^5$CFU\mL small) gram-positive bacilli with smooth margins. While TVC in Sinnar sample was $4.1*10^4$CFU\mL with two types of colonies. Dominant ($4*10^4$CFU\mL large colonies) were gram-positive bacilli with irregular margins and the other ($0.1*10^4$CFU\mL small colony) were gram-positive cocci with smooth margin. Both counts were very low compared with findings of Lawane *et al.* (2016) who reported TVC ranging between $9.7*10^9$and $2.7*10^{11}$CFU\mL. This may be due to the time of analysis and storage period and conditions.

Yeast, moulds and pathogens: Yeast and mould count in both samples were negligible which agreed with Lawane *et al.* (2016) finding that yeast and moulds in dry kawal samples from Burkina Faso were less represented or absent. coliform and E-coli were not detected using membrane filtration method which is in agreement with Dirar´s (1985) findings.

Detection of extended-spectrum Beta-lactamase production: No organisms were identified in both samples since the system was settled to detect penicillin-resistant pathogens. From detected organisms in both samples only *staphylococcus sciuri* has few strains with beta-lactams resistance and they seem to be absent in both samples.

Biochemical characterization: Cultural characteristics (shown in Table 2) and morphological examination showed three different groups of bacteria in AlGenina sample and two groups in Sinnar sample. A total of 51 colonies of bacteria were isolated and grouped into three groups. Most of them (the largest group) were rod-shaped, gram and catalase-positive, motile and spore-forming, these characteristics with other examination results indicate that the isolates were belonging to *Bacillus subtilis* spp.

The second group of isolates was smooth cocci, gram, catalase and oxidase-positive, spore forming with nitrate reductase activity, which identifies it as *Staphylococcus scuiri* spp. while the last small group of isolates was identified as *Lactobacillus plantarum* spp. from criteria of being smooth rods, gram-positive, immotile and non-spore forming, with ability to ferment different tested starch and sugar. The three isolated groups appeared in kawal sample from AlGenina, while only *Bacillus subtilis* and *Staphylococcus scuiri* appeared in kawal from Sinnar. Results were in agreement with results of Dirar *et al.,*(1985), who detected *Bacillus subtilis* spp, on dry kawal Samples. He mentioned that this organism was also the dominant or may be the principal organism of kawal fermentation due to its high tolerance to pH alteration andIts protein and starch hydrolysis ability.

Torino (2013) also used *Bacillus subtilis* as starter culture in successive solid state fermentation of lentils which resulted in enhanced anti-oxidant and anti-hypertensive properties. This organism is widely used as starter in solid state fermentation of different legumes and grains which almost result in enhancing phenolic compounds and antioxidant potential which goes in line with findings in other part of the present study which revealed that fermented kawal has high anti-oxidant and anti-microbial potential. Lawane *et al.* (2016) detected *Bacillus subtilis* as dominant organism from kawal samples from Burkina Faso in addition to lactic acid bacteria and *Staphylococcus* spp. It is clear that *Bacillus subtilis* isolated from kawal samples from widely different origins is important organism in kawal fermentation which can be used as starter culture in controlled fermentation of *Cassia obtusifolia* leaves in the future for product development.

The second isolate in this study was *Staphylococcussciuri*. It is commonly detected in natural fermentation of foods from different origins. Stepanovic *et al.* (2003) characterized it as a strong organism that can grow in nutritionally limited medium and tolerate pH alteration. It may also play an important role in fermentation and compete strongly throughout the course of fermentation. As for *Lacobacillusplantaram* which appeared only in Algenina sample and in very low count, this may be due to the competition within the fermentation community as well as fermentation and post fermentation conditions. It can be added as a functional starter in controlled fermentation. Identification was further confirmed using molecular techniques.

Molecular confirmation: As shown inn Fig 1 (Algeninakawal) Lane 1, 2 and 3 represent the reference strains *Bacillus subtilis*, *Staphylococcus scuiri* and L. *plantarum*. These bacteria showed bands as 595, 450 and 200 basepairs. While in Fig 2 (Sinnarkawal) Lane 1, 2 and 3 represent the reference strains *Bacillus subtilis*, *L. plantarum* and *Staphylococcus scuiri* respectively, which showed bands as595, 200 and 450 basepairsas the same manner. L lane was DNA ladder. Comparison of the bands obtained by the investigated isolates (lane 4,5,and 6) in Fig 1 and (lane 4 and 5) in Fig 2 to the bands given by the reference bacteria showed that they are representing*Bacillus subtilis*, *Stapylococcusscuiri*. and L. *plantarum* respectively in Fig 1 and *Bacillus subtilis* and *Stapylococcusscuiri* respectively in Fig 2. The results gained showed that two species (*Bacillus subtilis* and *Staphylococcus scuiri*) isolated from both AlGenina and Sinnarkawalsamples,while L. *plantarum* appeared only in AlGeninakawal sample.

Table 1. Colony characteristics of bacteria isolated from AlGenina and Sinnarkawal samples

sample	CFU\mL	types	number	size	shape	margin	Gram staining
AlGenina	$6.8*10^5$	3	56	large	bacilli	irregular	+
			10	small	cocci	smooth	+
			2	small	bacilli	smooth	+
Sinnar	$4.1*10^4$	2	4	large	bacilli	irregular	+
			1	small	cocci	smooth	+

+: positive reaction

Table 2. Biochemical characterization of bacteria isolated from AlGenina and Sinnarkawal samples

parameter	*Bacillus subtilis*	*Lactobacillus plantarum*	*Staphylococcus scuiri*
Catalase	+	-	+
Gas	-	+	ND
Gelatin hydrolysis	+	+	-
Gram staining	+	+	+
Indole	-	-	ND
Motility	+	-	ND
Methyl red	-	+	ND
Nitrate reduction	+	-	+
Oxidase	variable	-	+
Pigment	-	+	+
Shape	rods	rods	Cocci
Spore	+	-	+
Urease	-	-	-
Fermentation			
Fructose	+	+	+
Galactose	variable	+	Variable
Glucose	+	+	+
Glycerol	+	+	Variable
Glycogen	+	+	+
starch	+	+	+
Sucrose	+	+	+

+:positive reaction, -:Negative reaction, ND: Not detected.

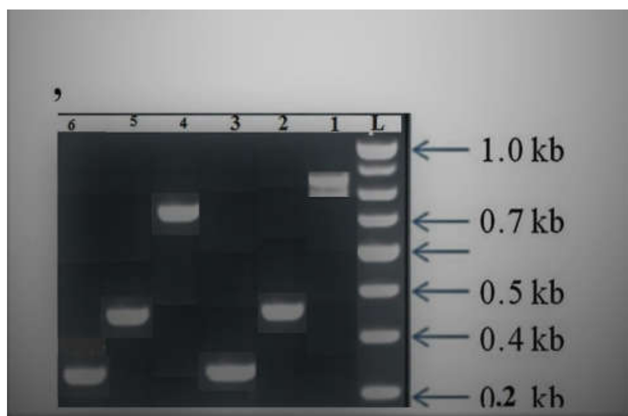

Figure 1. Gel electrophoresis of bacterial genome of isolates from Al Genina sample using 16srRNA primer

*lane L: Ladder (1000 bp)
*Lane 1: *Bacillussubtilis*(ref sample)
*Lane 2: *Staphylococcusscuiri*(ref sample)
*Lane 3: *Lactobacillusplantarum*(ref sample)
*Lane 4: Isolate 1
*Lane 5: Isolate 2
*Lane 6: isolate 3

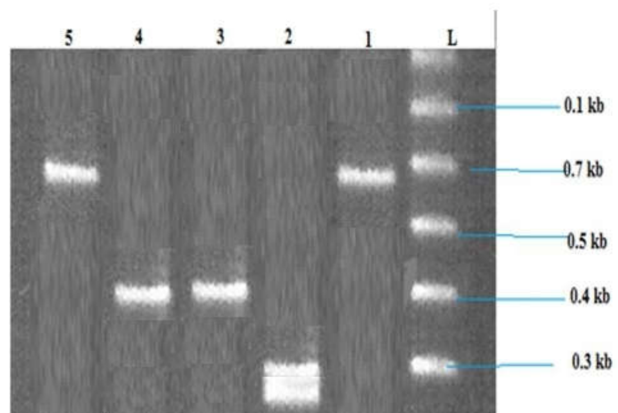

Figure 2. Gel electrophoresis of the bacterial genome of isolates from Sinnar sample using 16srRNA primer

*lane L: Ladder (1000 bp)
*Lane 1: *Bacillussubtilis*(ref sample)
*Lane 2: *Lactobacillusplantarum*(ref sample)
*Lane 3: *Staphylococcusscuiri*(ref sample)
*Lane 4: Isolate 1
*Lane 5: Isolate 2

These results were in agreement with results of Dirar*et al* (1985)who stated the mentioned species as the principal organisms in kawal fermentation. He also reported that dry kawal collected from Darfur and Kurdufan almost contain pure culture of *Bacillus subtilis* and very rare ones would encounter, in addition, a few cells of *propioni bacterium* which was not detected in the present study may be due to long time period between the two studies and to the competition between present species.

At the same time the presence of the same bacteria in different sample originated from different parts of Sudan band Africa confirmed that the fermentation of *kawal* is due to the activity of very specific bacterial strain whatever the location and condition is. No previous studies found in using molecular techniques in identifying microorganisms in kawal except that of Lawane*et al* (2016) who identified B.*subtilis* from kawal in Burkina Faso which also support the previous claim.

Conclusion

Isolated bacterial groups were identified as *Bacillus subtilis*, *Staphylococcus sciuri* and *Lactobacillus plantarum* in Algeninakawal and *Bacillus subtilis* and *Staphylococcus scuiri* in Sinnarkawal. No yeasts, moulds, *Coliform*, *E.coli* and Beta lactamase producing bacteria were detected. It is clear that *Bacillus subtilis* isolated from kawal samples from widely different origins is important organism in kawal fermentation which can be used as starter culture in controlled fermentation of *Cassia obtusifolia* leaves in the future for product development as meat substitute in large scale.

Conflicts of interest: The authors have no conflicts of interest to be declared.

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Key points

- No yeast, moulds or pathogens were detected.
- Fermentation of kawal is due to specific bacterial strain whatever the condition and the location is.
- *Bacillussubtilis*, *Staphylococcusscuiri* and *Lactobacillusplantarum* can be used as starter culture in controlled fermentation for product development.

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