

Original Research Article

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Identification and Biochemical Analysis of Microorganisms involved in the Fermentation of Kawal-A Traditional Fermented Sicklepod Leaves (*Senna obtusifolia*)

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ABSTRACT

Keywords

Kawal, chad, *Bacillus*, biochemical composition, characterization, PCR.

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This study was carried out to determine, using microbial and chemical standards methods, the chemical composition, the quality and the major microorganisms of *Kawal*, fermented *Senna obtusifolia* leaves consumed as a substitute of meat or an appetizing agent in Chad. The pH was ranged between 5.82 ± 0.19 and 7.22 ± 0.31 , the acidity between 0.31 ± 0.00 and 0.54 ± 0.00 %, the dry matter content, 90.00 ± 0.00 and 92.46 ± 0.43 %, the protein content, 15.52 ± 0.09 and 22.06 ± 0.07 %, the ash content, 17.12 ± 0.01 and 19.3 ± 0.00 %. The Total viable counts were between 9.7×10^9 and 2.7×10^{11} CFU / g; *Bacillus* sp., 3.7×10^8 and 9.1×10^9 CFU/g, lactic acid bacteria, 1.1×10^2 and 2.8×10^6 CFU/g; *Staphylococcus* sp., 1.5×10^3 and 3.7×10^7 CFU/g, *Micrococcus* sp. < 10 CFU/g and 7.4×10^4 CFU / g. Yeasts and molds as well as enterobacteria were less represented or absent in most samples analyzed. *Bacillus* strains, the dominant microflora were isolated and grouped into 15 groups. Their biochemical and molecular characterization showed a high diversity involved in the fermentation of *kawal* among them *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens* were identified.

Introduction

Fermented foods play an important role in providing food security, enhancing livelihoods and improving the nutrition and social well-being of millions of people around the world (Battcock, 1998). They also play a very important role in the socioeconomics of developing countries. Fermented foods are generally produced from plant or animal-based raw materials in combination with fungi or bacteria, which are either present in the natural

environment, or added intentionally by human to obtain the desirable end-products (Law *et al.*, 2011). Fermentation is a process that has been used by humans for thousands of years, as a method of food transformation and preservation. Fermentation improves nutritive value, safety, hygienic and organoleptic quality of the food and detoxification of anti-nutrient factors (AFNOR, 1986; Law *et al.*, 2011; Nnam and Obiakor, 2003; Nout *et al.*, 1987;

Sawadogo-Lingani *et al.*, 2008). 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, the art of fermentation is widespread including the processing of fermented cereals, legumes, and tubers used for the production of fermented foods, and other carbohydrate sources to yield alcoholic and non-alcoholic beverages. The fermented cereals are dominated by Lactic Acid Bacteria (LAB) associated with yeast. In other African indigenous alkaline-fermented foods, *Bacillus* sp is one of the microorganisms that dominates, with their major proteolytic activity to hydrolyze proteins into amino acids and ammonia.

Senna alata L., *Senna hirsuta* L. and *Senna obtusifolia* L. are the woody annual herbs or undershrub herbs which are native to Africa as medicinal species with active functions and therapeutic agents (Ayo 2010). *S. obtusifolia* leaves, seeds, roots and flower are used in folk medicine, primarily in Asia. It is believed to possess a laxative effect like *S. alata* compared to *S. hirsuta* as well as to be beneficial for the eyes (Oliver 2005, Sofowora 2008). *S. obtusifolia* is used as folk remedy, the seeds are often roasted, then boiled in water to produce a tea but in Java and in South-East Asia (Sofowora 2008). Within the Chad and the western Sudan, *Kawal* is fermented leaves of *Senna obtusifolia*, produced and consumed as a substitute of meat or an appetizing agent (Mbaiguinam, 2005). *Kawal* was found to be rich in proteins (30.20%), Carbohydrate (22.68%), Oil (4.12%) and minerals (18.74%) (Osman *et al.*, 2010). The production process is still naturally and traditionally with the rudimentary equipment. However, contamination of the products may take place at all stages of production by undesirable microorganisms

from the human and animal reservoir as well as other environmental. It is necessary to determine the biochemical and microbiological quality and to isolate and select *Bacillus* strains responsible for *kawal* fermentation which can be used as starter cultures, for their essential role in most fermented food, for their ability to produce various antimicrobial compounds promoting probiotic properties (Temmerman *et al.*, 2003).

This study was performed to investigate the microbiology and chemical content of fermented leaves of *S. obtusifolia* and characterize the dominant microflora of these products in order to improve safety and stability of the product.

Materials and Methods

Sampling

Twenty (20) *Kawal* samples were purchased from the markets of Abéché, Bokoro, Mandelia and N'Djamena in Chad. 200g of each sample were placed separately in a sterile plastic sampling bag and transported immediately to the laboratory, stored under refrigeration until analysis.

Physico-chemical analysis

The physico-chemical properties of the *kawal* samples were determined by using standard methods of analysis. The pH was measured using a digital pH-meter (Mettler-Toledo) calibrated with pH 4 and 7 buffers at 25°C according to AOAC (Feldsine *et al.*, 2002). Moisture content was determined by the difference between the known weight of *kawal* samples and the determined weight of the total solid after evaporating the liquid component of the *kawal* samples on a hot plate at 105°C for 3 hours (Helrich, 1990). The titratable acidity was determined by

titration with 0.1 N NaOH, using 1% phenolphthalein as the indicator (AFNOR, 1986). The result was expressed as g lactic acid/100g of sample. The ash content was determined by incineration of the sample placed in the muffle furnace at 550 °C for 8 hours (AOCS, 1990; Feldsine *et al.*, 2002; Helrich, 1990) and the crude protein content by kjedahl method (AOCS, 1990).

Microbiological analysis

Ten (10) g of representative *kawal* sample were placed in a sterile stomacher bag containing 90 mL of 0.1% peptone sterile physiological saline solution (0.9 % NaCl) and homogenized. Appropriate dilutions of the sample homogenates were prepared in sterile physiological saline solution (0.9 % NaCl) and inoculated in duplicate in growth specific media to estimate microbial counts.

The Total viable were counted on Plate Count Agar (PCA), after 24 hours of incubation at 30°C under aerobic conditions.

Lactic acid bacteria were enumerated and isolated using De Mann, Rogosa and Sharpe (MRS) agar(Sigma-Aldrich, USA) after 24–72 hours of incubation at 30°C anaerobically for 24-72 hours.

Yeasts and molds were enumerated on Sabouraud Dextrose Agar (Biokar, France) after 3-5 days of incubation at 30°C, under aerobic conditions. *Micrococcus* sp, and *Staphylococcus* sp, were enumerated and isolated on Mannitol Salt Agar (MSA) (Biokar, France)after 24 hours of incubation at 37°C, under aerobic conditions. Enterobacteriaceae were determined on violet red bile dextrose agar (VRBDA) (Biokar, France) after incubation at 37 °C for 24 hours.

Bacillus species were isolated over Plate Count Agar (PCA) (Biokar, France)

following heat treatment of the homogenate (80 °C for 10 min) and incubation at 37°C for 24 hours.

Isolation and purification of *Bacillus* strains

A total of 108 bacterial strains were randomly picked from petri dishes according to the colony characteristics, shape size, structure, elevation, edge and color. The isolated strains were sub-cultured onto tryptone Soy Agar (TSA) by the streaking method. Colonies with the same shape and appearance on agar were isolated for the morphological and biochemical characterization.

Biochemical and physiological identification

All the pure cultures isolates obtained by repeated streaking were observed for cell morphology, motility, and Gram staining, using microscope. Then the isolates were subjected to biochemical tests such as Oxidase, Catalase, oxidation/fermentation of glucose, and gelatin hydrolyses, nitrate reduction, amylase, lecithinase productions following the criteria described in the Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994), for identification to genus or species level. The presumptive *Bacillus* species were confirmed by endospore forming and catalase test. In parallel, the commercial API 20E (bio, France) and digitized probabilistic method for *Bacillus* identification were also used(Guech-Lamari and Kirane-Gacemi, 2012).

Molecular identification

DNA isolation and purification

The QIAamp DNA stool minikit (Qiagen, France S.A.) and protocol technics were

used according to manufacturer's recommendation to isolate and purify genomic DNA from bacterial cultures. The purified genomic DNA was qualified by electrophoresis through an agarose gel (1 % (w/v)).

Amplification and Electrophoresis

Two group-specific primer pairs were used for the identification of *Bacillus* strains. The first primers (B-K1/F {5'-TCACCAAGGC RACGATGCG-3'} and B-K1/R1 {5'-CGTATTCACCGCGGCATG-3'}) used to amplify a specific sequence of 16S rDNA from all *Bacillus* genus have been developed by Wu *et al.* (2006).

The second primers used for PCR were Bsub5F {5'- AAG TCG AGC GGA CAG ATG G-3'} and Bsub3R {5'- CCA GTT TCC AAT GAC CCT CCC C-3'} to amplify a specific sequence of 16S rDNA from *Bacillus Subtilis* group (Table 1).

For the amplification of the bacterial DNA, 25 μ L of reaction mixture containing 9.87 μ L of sterile deionised water, 5 μ L of PCR buffer (5X Green GoTaq Promega Corporation), 1.5 μ L of 25 mM $MgCl_2$ (Promega Corporation), 0.5 μ L of 10 mM dNTP (Promega), 1.5 μ L of forward and reverse primers, 0.13 μ L (5U) of Taq DNA polymerase (Promega,) and 5 μ L of DNA. The cycling program was started with an initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 58 °C for 45 s and elongation at 72 °C for 1 min. The PCR mixture and thermal cycling used for the amplification of *Bacillus Subtilis* group DNA was the same as for *Bacillus* genus except for the annealing temperature it was at 60 °C for 45 s. After complete reaction mixture were cooled to 4 °C. 7 μ L of the amplified products of PCR were analyzed by

electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide (0.5 mg/mL). The gels were visualized with an Ultraviolet Illuminator (TFX – 20.C).

Results and Discussion

The chemical parameters of collected *kawal* samples analysed were presented in table 2.

pH and Titratable acidity

The pH values were ranged between 5.87 ± 0.19 and 7.22 ± 0.31 and titratable acidity (as lactic acid) 0.31 ± 0.01 and 0.54 ± 0.01 % respectively (Table 2). These values are agreed with those reported (6.0 and 0.0 %) by Dirar *et al.* (1985). A remarkable characteristic of *Senna obtusifolia* fermented leaves is the little change in pH. These values were relatively stable, and varies between 5.87 and 7.22. This was due to the buffering effect of the calcium present in the leaves of *S. obtusifolia*.

The crude ash content of the *kawal* was 17.12 ± 0.01 and 19.3 ± 0.01 % (Table 2). These values agreed favourably with 18.0 and 18.74% value respectively reported in *kawal* by Mbaiguinam *et al.* (2005) and Osman *et al.* (2010). The values obtained for *S. obtusifolia* fermented leaves show a high level of ash in the fermented leaves than in the fresh leaves. This could be due to the migration of minerals from clay to the leaves during the fermentation.

The crude protein content was between 15.52 ± 0.09 and 22.06 ± 0.01 % (Table 2). The values obtained were however lower than those obtained by Nuha *et al.*, (2010) and Dirar *et al.*, (1985) they reported 26.2% and 30.20 % for crude protein respectively. The results obtained have been reported by Dirar *et al.*, (1985) and Osman *et al.*, (2010) indicated the crude protein content in

fermented leaves was higher than in the crude leaves. The difference observed may be explained by difference the crude protein with plant age, locations and environment (Mbaiguinam *et al.*, 2005). Several studies have reported increasing levels of free amino acids, and soluble nitrogen content during alkaline fermentation.

Our results show that the collected kawal samples contained 7.54 ± 0.43 to 10.00 ± 0.01 % moisture. However, water activity (aw) of the dried samples is enough to prevent the chemical changes, microorganisms growth and maintain the organoleptic quality during storage time. According to Mbaiguinam *et al.*, (2005) the soil type can affects chemical constituents of the plants.

Microorganisms isolates Numeration

The results of microbial flora analysis in the collected samples of *kawal* were presented in Table3. The number of Total Mesophilic Aerobic Flora (TMAF) was between 9.7×10^9 and 2.7×10^{11} CFU / g, with an average of $5,7 \times 10^{10}$ CFU /g. The Lactic acid bacteria (LAB) levels varied from $1,1 \times 10^2$ to 10^6 CFU/g with an average of 3.4×10^5 CFU/g. The presumptive *Micrococcus* sp (MI) counts varied from (<1CFU/g) to $7,4 \times 10^4$ CFU /g with an average of 4.1×10^3 CFU/g. The presumptive *Staphylococcus* sp. (Staph) counts varied from $1,5 \times 10^3$ to $3,7 \times 10^7$ CFU /g with an average of $2,5 \times 10^6$ CFU /g, The *Bacillus* sp between 3.7×10^8 and 9.1×10^9 CFU / g. Yeasts and molds (YM) counts varied from (<10 CFU/g) to $4,5 \times 10^2$ CFU /g. Enterobacteria (EB) ranged from (<10 CFU /g) to $1,6 \times 10^2$.

The microbial flora counted and isolated from the different samples of *kawal* includes different species of microorganisms among the TMAF. *Bacillus* species occurred most

consistently and dominated the fermentation of *kawal*. This could be due to the ability of the *Bacillus* species to grow at wide pH range (pH 5.5–8.5) and in addition, the production of several proteolytic enzymes and biologically active compounds enhance the growth of *Bacillus* species in fermented foods (Ayo, 2010; Mo *et al.*, 2010). Our results correspond to the study conducted by Dirar *et al.*, (1985) showing the *Bacillus* species as dominant bacteria in *kawal* samples. Their proteolytic activity with the production of the highest ammonia, characteristic of leguminous, contributes to the development of the texture, flour and organoleptic quality.

The lactic acid bacteria species were isolated and their levels depended on the samples. The variation in the proportion within species of lactic acid bacteria may be due to physicochemical changes during the fermentation on one hand and to the competition between these species on the other hand.

Yeasts and molds as well as enterobacteria are less represented or absent in most of the analyzed samples. In all samples tested Enterobacteria is only represented in two samples and absent in the others. Diar *et al.*, (1985) reported the isolation of *Staphylococcus* sp. throughout the fermentation of *kawal*. The *Staphylococcus* and *Micrococcus* species could have arisen from handling of the product.

The International Commission on Microbiological Specifications for Foods (ICMSF, 1998) does not recommend microbiological criteria for acceptance or rejection of fermented vegetables. However, the microbial quality for foods are rated as “good”, “average”, “poor” and “spoiled food”. In this study, the quality of the samples was regarded as “good”.

Isolation and Identification of the *Bacillus* strains

The morphological examination and cultural characteristics showed variable colony and cell morphology. A total of 108 colonies of bacteria were isolated on Plate Count Agar (PCA) and grouped into 15 groups. Most isolates were large flat, wide spreading, dull surfaced colonies. They are all Gram positive, catalase positive and endospore forming, most of them found to be motile, these indicates that the isolates were belonging to *Bacillus* genus. To confirm identification, the PCR amplification of the 16S rDNA gene have been used with specific primers to detect *Bacillus subtilis* group (Wattiau *et al.*, 2001). The amplification of the targeted 16S rDNA resulted in a single well defined bands of

about 595 bp when match with the band from the DNA Marker (Figure1).

These results confirm that the isolates are belonging to the *Bacillus subtilis* group. According to morphological and biochemical characterization (Table 4), the isolates showed a high diversity of species within the *Bacillus* genus, which could belong to the species: *B. subtilis*; *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens* with a percent similarity of 94.9%, 99.9%, 97.1% and 96.6% respectively obtained by API 20 E using a digitized probabilistic method for *Bacillus* identification and a search for additional test such as lecithinase, caseinase and amylase(Guech-Lamari and Kirane-Gacemi, 2012).

Table.1 Primers used in this study

Primers	Sequences	Size (bp)	Targettedspecies	References
B-K1/F B-K1/R1	TCA CCA AGG CRA CGA TGC G CGT ATT CAC CGC GGC ATG	1114	<i>Bacillus, Paenibacillus, Brevibacillus</i>	(Wattiau <i>et al.</i> , 2001)
Bsub5F Bsub3R	AAG TCG AGC GGA CAG ATG G CCA GTT TCC AAT GAC CCT CCC C	595	<i>Bacillus Subtilis</i> group (<i>Bacillus subtilis, licheniformis, amyloliquefaciens, pumilus, atropheus</i>)	(Wu <i>et al.</i> , 2006)

Table.2 Physico-chemical composition of dried collected kawal samples

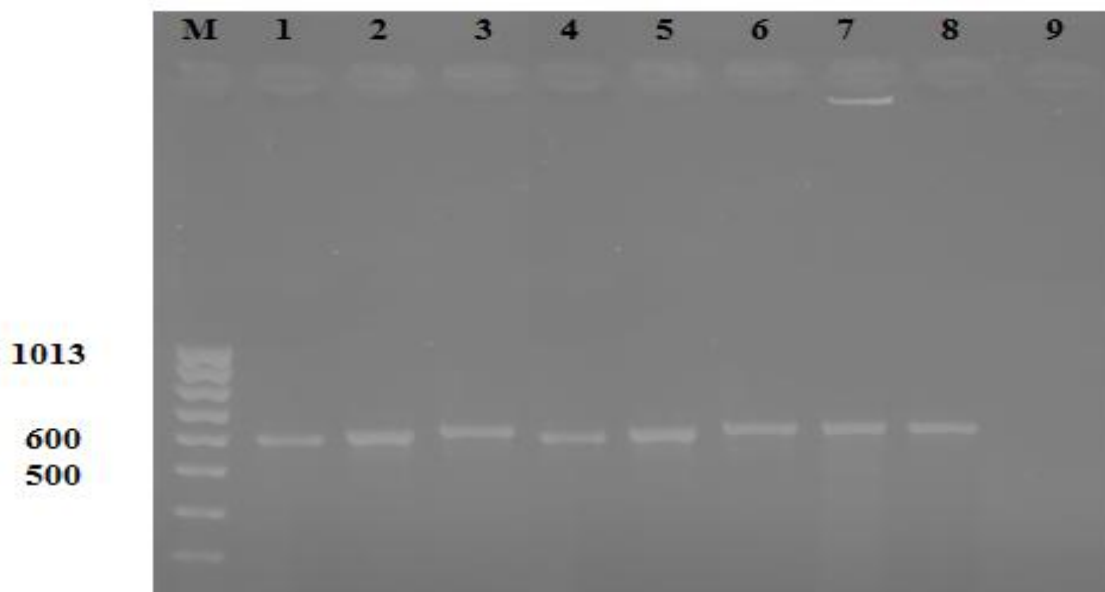
Parameters	Minimum±SD	Mean±SD	Maximum±SD
pH	5.87 ± 0.19	6.47±0.47	7.22± 0.31
Titratable acidity (%)	0.31 ± 0.01	0.40±0.07	0.54± 0.01
Crude Protein (%)	15.52 ± 0.09	19.23±2.16	22.06 ± 0.01
Total Ash (%)	17.12 ± 0.01	18.73±0.59	19.3 ± 0.01
Moisture (%)	7.54 ± 0.43	8.71±0.72	10.00 ± 0.01
Dry Matter (%)	90.00 ± 0.01	91.29±0.72	92.46 ± 0.43

Table.3 Microorganism count (CFU/g) in twenty collected samples

Samples	Microorganisms						
	TMAF	<i>Bacillus sp</i>	LAB	YM	Staph	MI	EB
E1	2,7×10 ¹¹	4,3×10 ⁹	2,8×10 ⁶	<10	1,1×10 ⁴	4,4×10 ²	<10
E2	3,5×10 ¹⁰	5,1×10 ⁹	8,8×10 ⁵	<10	1,3×10 ⁴	3,9×10 ²	<10
E3	1,1×10 ¹⁰	3,7×10 ⁷	2,4×10 ²	<10	2,1×10 ⁵	<10	<10
E4	2,3×10 ¹¹	6,7×10 ⁸	1,2×10 ⁶	<10	2,3 10 ⁴	9,2×10 ²	<10
E5	2,8×10 ¹⁰	6,1×10 ⁸	1,5×10 ²	<10	2,8×10 ⁴	8,4×10 ²	1,2 ×10 ²
E6	4,1×10 ¹⁰	2,8×10 ⁸	1,1×10 ²	<10	2,3×10 ⁴	1,8×10 ²	<10
E7	3,7×10 ¹⁰	2,0×10 ⁹	3,7×10 ³	4,5×10 ²	1,1×10 ⁷	<10	<10
E8	2,1×10 ¹⁰	1,2×10 ⁹	1,6×10 ²	<10	1,2×10 ⁵	4,5×10 ²	<10
E9	7,8×10 ¹⁰	9,4×10 ⁹	2,7×10 ⁴	<10	1,8×10 ⁴	5,4×10 ²	<10
E10	2,0×10 ¹⁰	6,2×10 ⁸	2,1×10 ²	<30	1,1×10 ⁴	2,2×10 ²	<10
E11	2,2×10 ¹⁰	1,1×10 ⁹	3,3×10 ⁴	<10	1,5×10 ³	<10	<10
E12	2,6×10 ¹⁰	1,7×10 ⁸	2,8×10 ²	<10	2,4×10 ⁴	<10	1,6 ×10 ²
E13	8,7×10 ¹⁰	6,2×10 ⁸	4,6×10 ²	<10	7,8×10 ³	3,1×10 ²	<10
E14	7,6×10 ¹⁰	2,1×10 ⁸	1,7×10 ³	<10	1,8 ×10 ⁶	<10	<10
E15	9,7×10 ⁹	5,3×10 ⁷	6,3×10 ²	2,2×10 ²	3,6×10 ⁴	<10	<10
E16	6,3×10 ¹⁰	1,2×10 ⁸	3,4×10 ⁵	<10	5,2×10 ⁴	4,1×10 ³	<10
E17	4,1×10 ¹⁰	4,3×10 ⁸	3,5×10 ⁴	<10	6,2×10 ³	<10	<10
E18	9,9×10 ⁹	1,7×10 ⁷	1,6×10 ⁶	<10	1,1×10 ⁵	1,1×10 ³	<10
E19	2,1×10 ¹⁰	2,2×10 ⁸	5,1×10 ³	<10	3,7×10 ⁷	7,4×10 ⁴	<10
E20	1,5×10 ¹⁰	4,3×10 ⁸	2,2×10 ²	<10	4,4×10 ³	1,7×10 ²	<10

TMAF: Total Mesophilic AerobicFlora;**LAB**: lactic acid bacteria; **Staph** :*Staphylococcus sp*; **MI** : *Micrococcus sp* ; **EB** : Enterobacteria

Fig.1 Electrophoresis gel analysis of PCR-amplified 16S rDNA fragments obtained with *Bacillus subtilis* Group-Specific Primer



Lanes 1 to 7: Isolates S₁, S₂, S₃, S₄, S₅, S₆ S₇, and S₈. Lanes 9: Negative control; M: ladder (595 pb)

Table.4 Biochemical characters of the isolates

Characteristics	Strains														
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₇	S ₈	S ₁₂	S ₁₃	S ₁₄	S ₁₅	S ₃₄	S ₄₀	S ₄₁
ONPG	-	+	±	+	+	-	+	+	+	+	+	+	+	-	-
ADH	-	+	-	-	+	+	+	-	-	+	+	+	-	-	-
LDC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	+	+	-	-	+	+	-	+	-	+	-	-	-
H ₂ S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
INDOLE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	-	+	+	±	+	+	+	±	±	+	±	±	+	±	+
Mannitol	-	+	+	-	+	+	+	±	±	+	±	+	+	+	+
Inositol	-	+	+	-	±	±	±	-	±	+	±	±	±	-	±
Sorbitol	-	+	+	-	+	±	±	-	±	+	±	±	±	±	±
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saccharose	-	-	+	±	-	+	+	+	+	+	+	±	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdaline	-	+	+	±	+	+	+	+	+	+	±	+	+	+	+
Arabinose	-	+	±	-	+	-	-	-	-	+	±	±	±	-	-
Nitrites	+	-	+	-	-	-	-	-	-	-	+	+	-	+	+
Oxydase	+	+	+	+	+	-	+	+	-	+	+	+	-	+	+
Motility	-	+	+	-	+	-	+	-	+	+	+	-	+	+	+
Lecithinase	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+
StarchHydrolysis	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Caseinase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gram Stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sporulation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: Positive reaction; -: Negative reaction; ±: variable.

The result shows that all the isolates of 595 pb were specific to *Bacillus subtilis* Group. Lanes 1, 2, 3, 4, 5, 6, 7 and 8 belongs to *Bacillus subtilis* group of isolates S₁, S₂, S₃, S₄, S₅, S₆, S₇, and S₈. Lanes 9 was the Negative control; M is the ladder (595 pb) (Figure 1). This group-specific primer pair (Bsub5F/Bsub3R) capable of amplifying a16S rDNA fragments from *Bacillus subtilis* group has been developed by Wattiau *et al.*, (2001), to identify *Bacillus subtilis*, *Bacillus*

licheniformis, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, and *Bacillus atrophaeus*. *Bacillus* species have been found to be associated with the fermentation of *Bikalga* and *Soumbala*, similar food condiments (Ouoba *et al.*, 2003; Savadogo *et al.*, 2011).

In conclusion, microbiological analyzes of different samples of *kawal* showed that the Total viable counts with the high number among them *Bacillus* sp., lactic acid

bacteria, *Staphylococcus* sp., *Micrococcus* sp., Yeasts and molds and enterobacteria. Biochemical and molecular characterization of the *Bacillus* strains isolated from our sample showed a high diversity of species within the genus *Bacillus*, which could belong to the species: *B. subtilis*; *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*. However, the present work reveals that *Bacillus subtilis* was dominant in *kawal*. The Lactic acid bacteria with ability to produce probiotics as well as *Bacillus* sp strains with their protease activity are important microorganisms which can be used as starter cultures in *Senna obtusifolia* leaves fermentations. Their addition to *kawal* may improve safety and stability of the product.

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