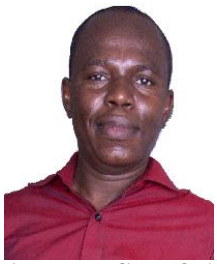
Journal of Food Technology Research
2019 Vol. 6, No. 2, pp. 57-71
ISSN(e): 2312-3796
ISSN(p): 2312-6426
DOI: 10.18488/journal.58.2019.62.57.71

TRADITIONAL TECHNOLOGIES AND PROBIOTIC PROPERTIES OF BACILLUS STRAINS ISOLATED FROM KAWAL -A CHAD TRADITIONAL FERMENTED FOOD CONDIMENT

Abakar Idriss L[1]
Abdelsalam Tidjani[2]
Essodolom Taale[3]
Abdoullahi Hissein O[4]
Abel Tankoano[5]
Savadogo Aly[6+]

[1,5,6]*LABIA, Laboratory of Biochemistry and Applied Immunology, Joseph KIZERBO University, Burkina Faso.*
[1]*Email: benlawane@gmail.com Tel: (+ 235) 66223171*
[5]*Email: tankoanoabel@gmail.com Tel: (+ 226) 70013879*
[6]*Email: alysavadogo@gmail.com Tel: (+ 226) 70 35 62 27*
[2]*Faculty of Human Health Sciences (FSSH), Laboratory of Research in Food Science and Nutrition (LaRSAN), University of N'Djamena, Chad.*
[2]*Email: abdelti@gmail.com Tel: (+ 235) 66352119*
[3]*Higher Institute of Agricultural Trades (ISMA) / University of Kara, Togo.*
[3]*Email: taaleernest12@hotmail.com Tel: (+ 228) 91182886*
[4]*LABIA, Laboratory of Applied Biochemistry and Immunology of Joseph KIZERBO University, Burkina Faso, Faculty of Human Health Sciences (FSSH), Laboratory of Research in Food Sciences and Nutrition (LaRSAN), University of N'Djamena, Chad, Faculty of Exact and Applied Sciences (FSEA), Laboratory for Research on Natural Substances (LaRSN), University of N'Djamena, Chad.*
[4]*Email: hisseinabdallah3@gmail.com Tel: (+ 235) 66231863*


(+ Corresponding author)

Article History
Received: 2 August 2019
Revised: 4 September 2019
Accepted: 10 October 2019
Published: 21 November 2019

Keywords
Kawal
Bacillus
Senna obtusifolia
Fermented food
Traditional
Probiotic.

ABSTRACT

Kawal is a condiment produced by the traditional fermentation of Sicklepod (*Senna obtusifolia*) leaves. Many people from the different part of Chad use it for flavoring as well as a substitute of meat. The fermentation techniques used in *kawal* production are often applied on a small scale and still mainly at the household level, characterized by the use of simple and rudiment equipment. This study was conducted firstly to investigate the *Kawal* traditional methods production. Secondly, to evaluate probiotic potential of *Bacillus* species isolated in order to develop starter cultures for *kawal* production. Protease, amylase and phytase activities of *Bacillus* strains were screened. The candidate were screened *in vitro* for antimicrobial activity against, *Micococcus luteus LMG3293, Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 8739 and Salmonella spp.* Biofilm synthesis, Antibiotics sensitivity, tolerance to simulated gastric and intestinal juice were also evaluated. The majority of selected strains formed biofilms, produced amylase and protease, some of the strain produced phytase by forming clearing zones around the growth of the colonies. For biochemical conditions for simulated gastric and intestinal juice, among the selected strains tested, some had the highest survival in simulated gastric juice and better viability in simulated intestinal juice. The selected strains showed sensitive to a panel of antibiotics and they presented resistance to penicillin G, oxacycline and bacitracin. Some strains showed an inhibitory effect on all of the indicator bacteria tested. Overall, some of these selected strains could be used as potential microbial starters for the controlled fermentation of *kawal.*

Contribution/Originality: This study is one of the very few studies, which have investigated the traditionnel technologies, and probiotic properties of *Bacillus* strains isolated from *kawal*, towards use as a starter culture for fermenting Sicklepod (*Senna obtusifolia*) leaves.

1. INTRODUCTION

Food quality is generally influenced by the processing techniques used. It was found to be largely influenced by a variety of factors and micro-organisms (Mohamadou *et al*., 2008). Since old time, various methods have been used to process and to preserve foods. Fermentation is a process that has been used by humans for thousands of years, with major roles in food preservation. This process is generally carried out to bring diversity into the kinds of foods and beverages available; make otherwise inedible foods edible; flavor dishes; enhance the nutritional value; decrease toxicity; preserve food; and decrease cooking times and energy requirements (Steinkraus, 2018). Many different products around the world are a result of fermentation, either occurring naturally or through addition of a starter culture (Marshall and Mejia, 2011). The African continent has a wide variety of traditional fermented foods ; especially those based on plant substrate materials. These usually foods have a large impact on the nutritional health and social economic status of Africa' people (Franz *et al*., 2014). Fermentation of African foods is usually performed using minimal technology often on a small scale and household basis, characterized by the use of simple, non-sterile equipment, chance or natural inoculas, unregulated conditions, sensory fluctuations and poor durability (Oguntoyinbo *et al*., 2016; Wafula *et al*., 2016). *Bacillus* spp. are commonly involved in African vegetable fermentation via alkaline hydrolysis of the proteins to amino acids and ammonia. *Bacillus* species are one of the most investigated bacteria for Africa vegetable alkaline fermented food and their utlisation as stater culture (Parkouda *et al*., 2010).

In Chad and other African countries, fermented fresh leaves of Sicklepod (*Senna obtusifolia*) name *kawal*, is used in relatively large quantities in the preparation of sauces as a meat substitute or as a spice by some people. *Kawal* is known to be rich in proteins and contains many nutrients such as essential amino acids, minerals and various organic acids (Mbaiguinam *et al*., 2005; Nuha *et al*., 2010). The women rural are often responsible for *kawal* processing. This production constitutes an importante economical source for the producers. The traditional methods used in the production varies amount producers and often still, at the household level under uncontrolled fermentation, and used rudiment equipment with poor hygenic conditions.

In many developed countries, traditional methods are now replaced with specific technologies for production of fermented foods, and an emerging industrial practice allows for higher quality standardization of food products in the market place (Borresen *et al*., 2012).

In order to enhance the fermentation process, the *kawal* production need to make better, like introducing starters culture of microorganisms with a potential technologies and probiotics proprieties. *Bacillus* species principally *B. subtilis* group are one of the most investigated bacteria for alkaline food fermentaton. This group, characterized by their ability to produce a high level of enzymes, antimicrobial compounds and others potential Probiotic (Savadogo *et al*., 2011; Olmos and Paniagua-Michel, 2014; Taalé *et al*., 2015).

In this sense, *B. subtilis* group isolated is a potential candidate to be used as starter cultures for production of *kawal*. This will guarantee the end- product safety and improve keeping the quality. Thus, the objective of this work was to study the technology of *kawal* in different places of Chad, and then to establish a general flow diagram of production as well as to screen probiotic properties of *Bacillus* strains, predominant microflora isolated from *Kawal* to be used as starter cultures.

2. MATERIALS AND METHODS

2.1. Investigation on Kawal Technologies

The survey was conducted to understand the *kawal* production technologies. It took place in the form of interview and observation during process of production. In total 186 interviewed person, among them 62 producers in plain activity, 54 who practiced technology in the past and 70 *kawal* producers and sellers were identified by the the surveys. Different productions sites in different places of Chad were identified. For that, a survey was conducted in the Abeché, East regions and Central region including Mongo, and Chari Baguirmi localities.The study

concerned the areas of high production and consumption of *kawal* in Chad. Others sites were divided by considering the large production of *kawal* in Chad involved by this survey included Sarh and Mayo-kebi area Figure 1. A flow diagram of each site was established, and then a general diagram for all production sites was proposed.

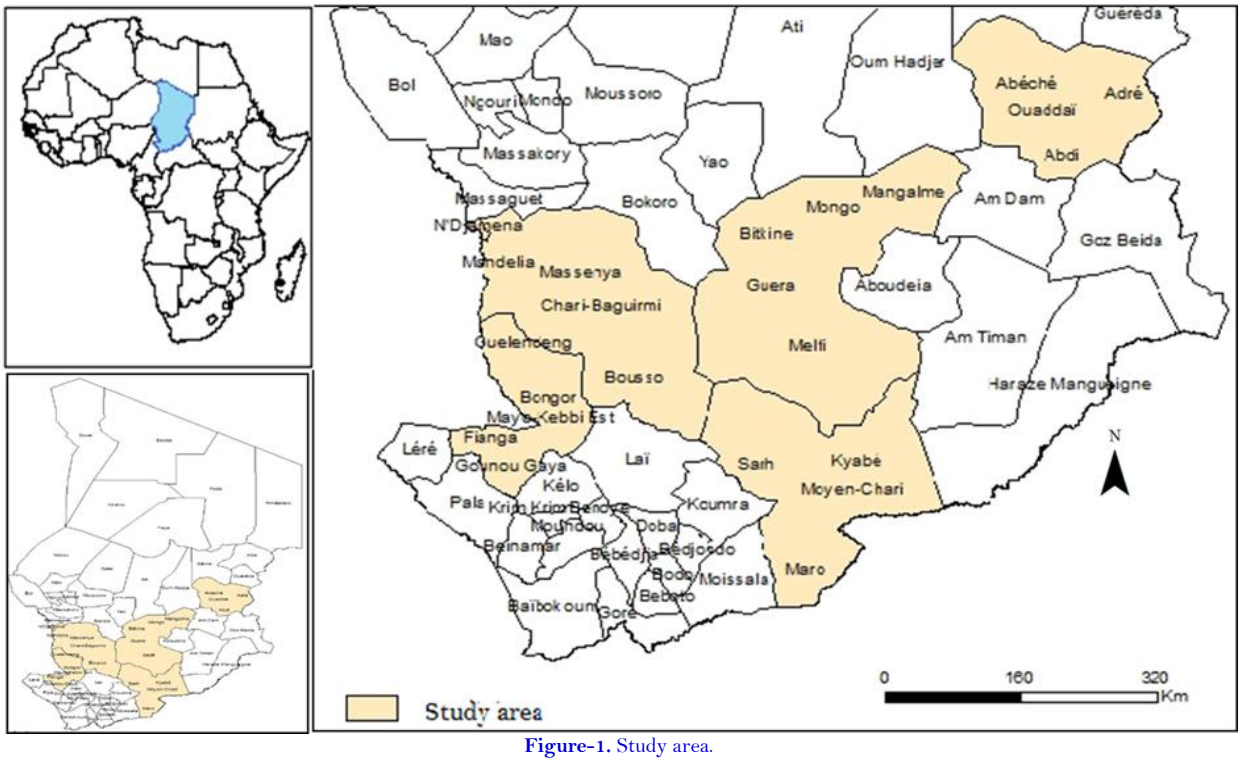


Figure-1. Study area.

2.2. Microbiological Analysis

2.2.1. Sampling and Bacillus sp. Characterization

Dry fermented *kawal* samples were collected from different *kawal* site productions and markets places at East, Central and South of Chad. A total 30 *kawal* samples (30 to 100 g) were collected in a sterilize plastic sampling bag and kept in coolers with ice and transported within 24 h to the laboratory for microbialogical analysis. *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 30 °C for 24 h. Then the isolated strains were sub-cultured onto tryptone Soy Agar (TSA) by the streaking method and incubated at 30°C. The strains were identified and selected according to their phenotypic characters : colony and cellular morphology, ability to grow under aerobic conditions, Gram and catalase tests.

2.2.2. Evaluation of Potential Probiotics of Bacillus Strains

In vitro, experiments were used to investigate the selected *Bacillus* strains for their probiotic properties Simulated gastric and intestinal juice, Antimicrobial activity, Resistance to antibiotics, Biofilm synthesis and Enzyme production : amylase, protease and phytase (Fakhry *et al.*, 2008; Merghni *et al.*, 2014; Prieto *et al.*, 2014; AlGburi *et al.*, 2016; Latorre *et al.*, 2016).

2.2.2.1. Antimicrobial Activity Assay

Antimicrobial activity of isolates was determined by the agar well diffusion assay (AWDA) in triplicate, as previously reported by Mishra and Prasad (2005) against indicator bacteria. These indicator strains included two Gram-positive and two Gram-negative pathogens (*Micococcus luteus LMG3293, Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 87 39 and Salmonella* spp. (isolated from local food)). The indicator bacteria were grown for 24 h in 10 mL of nutrient broth and then spread on nutrient agar plates and incubated at 30°C for 24 h. The colonies

from pure culture were suspended and diluted with 0.85 % sterilized saline water. 1 mL of broth indicator strains was spread over Muller Hinton Agar (MHA) plates. Then, four wells with a diameter of 6 mm were made in each MHA plates, and100 µL of antimicrobial filtrate containing the test compound from potential probiotic strains were introduced into the wells. For negative controls, well with DMSO was used as descriped by Nitiema *et al.* (2012). Plates were incubated at 37 °C for 24 h. After incubation, the plates were observed for growth inhibition zones formed around the well.

2.2.2.2. Antibiotic Susceptibility Assay

The antibiotic susceptibility test of studied *Bacillus* was carried out as described by Weber *et al.* (1988) using agar spread diffusion method. Overnight cultures of the each collected bacterial strains incubated at 37°C in TSB were diluted and adjusted to approximately 10^6 CFU/ mL and spread on Mueller-Hinton agar plates. Then serial antibiotics : ampicillin 10 µg, tetracycline 30 µg, erythromycin 15 µg, chloramphenicol 30 µg, kanamycin 30 µg, streptomycin 10 µg, oxacycline 30 µg, gentamycine 10 µg, amoxiciline 10 µg, imipenem 10 µg, Bacitracine10 µg and penicillin 10 IU, were dispensed onto the plates and incubated for 24 h at 37˚C as suggested by the National Committee for Clinical Laboratory Standards (NCCLS). The results were determined according to the inhibition zone diameter and expressed as susceptible, ≥ 16-mm diameter ; intermediate, 13- to 15-mm diameter ; resistant ≤ 12-mm that suggested by Weber *et al.* (1988) for *Bacillus* spp.

2.2.2.3. Determination of Biofilm Formation

The determination of biofilm synthesis was performed using a crystal violet staining method as described by Latorre *et al.* (2016) with slight modifications to observe biofilm formation and adherence to a surface. Indeed, 10 µL of overnight cultures of the *Bacillus* isolates growth in nutrient broth were inoculated into 0.5 mL Casein-Mannitol broth in 1.5 mL polypropylene tubes and incubated at 37°C for 12 h without shaking. After incubation the liquid was removed and the tubes were rinsed with distilled water. Then, 1 mL of crystal violet solution (1 % w/v) was added to the polypropylene tubes to fix the cells adhered to form a purple pellicule on the airsurface of the polypropylene tube. After 30 min, the tubes were washed with distilled water, after removing crystal violet. The measurement of biofilm synthesis was based on color intensity and size of the adherent crystal violet ring with a score ranging from negative (−) to strong (+++) biofilm formation. All the strains were tested in triplicate.

2.2.2.4. Tolerance to Simulated Gastric and Intestinal Juices

Resistance to artificial gastric juice of the *Bacillus* sp. was studied as previously reported by Charteris *et al.* (1998) the survival of *Bacillus* strains in simulated gastric juices was prepared by dissolving pepsin (0.3 %, w/v) in PBS buffer solution and adjusting the pH to 2.0 with hydrochloric acid and sterilization by filtration through a 0.22 µm pore filter. Briefly, each bacterial suspension (appropriate dilutions) was added to simulated gastric (pH 2.0) and to control (pH 7.0, without gastric juices). Viable count was done at 0.5, 1, 1.5, 2 and 3 h of incubation in the juice and spread onto nutrient agar plate and incubated at 30°C for 24 h.

Simulated small intestinal juice was prepared, by suspending pancreatin and bile salts in PB to final concentrations of 1 g /L and 4.5 g /L, respectively as previously reported by Huang and Adams (2004) and adding bile salts (4.5 % w v^{-1}). The pH was adjusted to 7.0 with 1 N sodium hydroxide (NaOH). The mixture was sterilized by filtering through 0.45 µm filter. The bacteria suspension was inoculated in this juice and incubated for 1 h, 2 h and 3 h. The survivability of of *Bacillus* species were determined by spread-plate technique after serial dilution.

2.2.2.5. Phytate Degrading Test

Phytase screening was carried out on agar plates, using phytate specific medium (Ehon *et al.*, 2015) containing [Phytase screening was carried out on agar plates, using phytate specipic medium [18] containing [glucose 15g, $(NH_4)_2SO_4$ 5, KCl 0.5 g, $MgSO_4$ 0.1 G .$7H_2O$, NaCl 0.1g, 0.01 % $CaCl_2$0.1g. $2H_2O$, $FeSO_4$ 0.01 g,

MnSO 4, 0.01 g, pH 6.5 with sodium phytate 5g and 20 g of noble agar] per 1000 mL of distilled water. The reaction mixture was autoclaved at 121°C for 15 min and poured into petri dishes. Then, 7 µL of bacterial suspension were spotted onto phytate agar medium. The Petri dishes were incubated at 37° C for 1- 3 days to observe the clear zones of hydrolysis around the colonies.

2.2.2.6. Determination of Amylase Activity

Amyliotic activity of the isolates was determined as described by Ibrahim *et al.* (2012) and Latorre *et al.* (2016) using basal medium with following composition: 10 g of tryptone, 5 g of KH_2PO_4 , 10 g of yeast extract, 15 g of noble agar, supplemented with 3 g of soluble starch and 1000 mL of distilled water. The pH of the reaction mixture was adjusted to pH 7 then the components of the starch agar medium were autoclaved at 121°C for 15 min and poured in Petri dishes. Each tested *Bacillus* strain was inoculated and incubated at 37°C for 24-48 h. The Petri dishes were flooded with Gram's iodine solution. The results were reported amylase positive, if the clear zone was appeared around inoculated colonies and amylase negative if the clear zone did not appear.

2.2.2.7. Determination of Protease Activity

Detection of the proteolytic activity of *Bacillus* strains, was performed as described by Latorre *et al.* (2016) with minor modifications. Protease production was screened on skim milk agar media. The skim milk agar media containing 25 g of skim milk, 25 g of noble agar and 1000 mL of distilled water was prepared. The mixture was autoclaved at 121°C for 15 min and poured into plates. Each selected *Bacillus* strain was inoculated on Petri dishes and incubated at 37°C for 24 h. Proteolysis activity was characterized by the formation of clear zone around the colonies.

2.3. Statistical Analysis

All data obtained from technologies questionnaire and resultats of microbiological analysis in laboratory were analysed using Excel (Microsoft office 2013) and SPSS for Windows, version 17.0. The results were expressed as mean ± standard deviation (SD) and significant difference was accepted at P <0.05 using analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1. Kawal Production Technology

The production of *kawal* is a traditional process involving different unit operations with the most important being fermentations and drying after harvesting of the leaves. The technologies used in *kawal* production are often applied on a small scale and still mainly at the household level, characterized by the use of simple and rudimentary equipments under poor hygenic conditions. These technologies remain traditional with the empirical knowledge and the know-how is transmitted from mother to daughter and from generation to generation. Different varieties of technologies processes in *kawal* production depending on the sites and producers Figure 3 was observed in this study and the essentiel operation are the fermentation and drying. The flow general chart of the processing line of *Senna obtusifolia* fresh leaves to *Kawal* food condiment is presented in Figure 4.

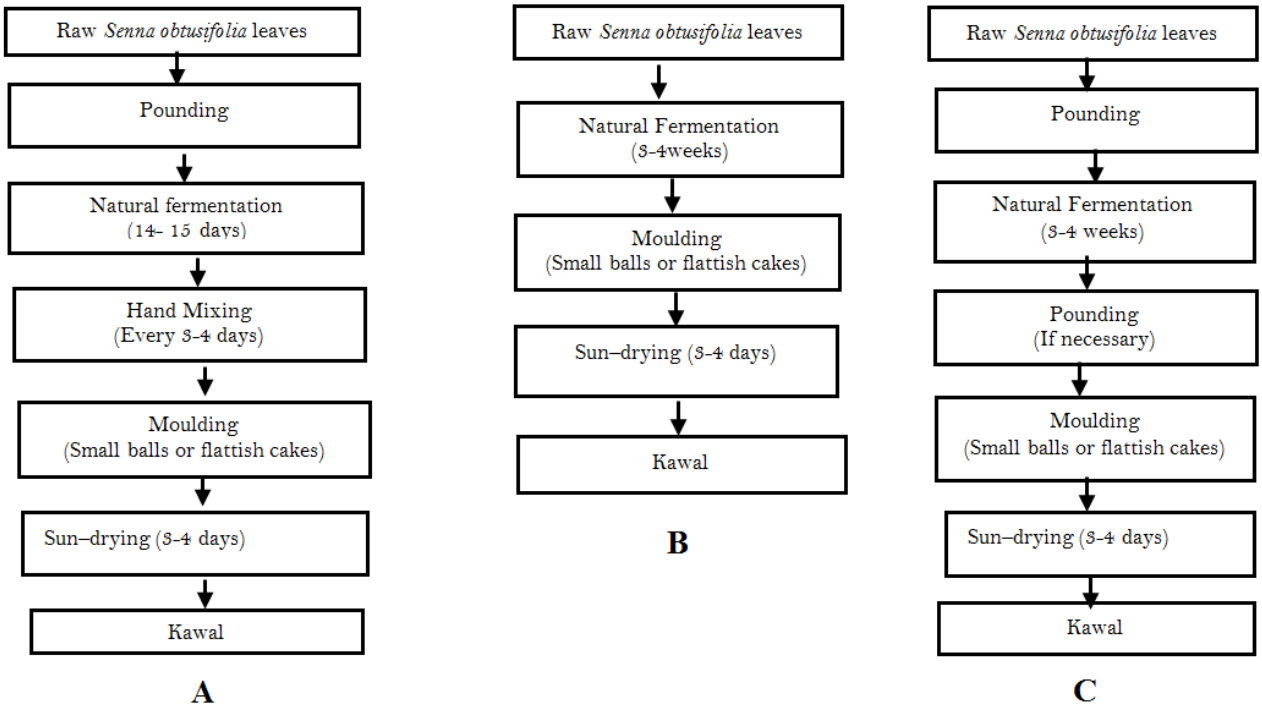


Figure-2. A ; B ; and C : Different varieties of technologies processes in *kawal* production from different sites.

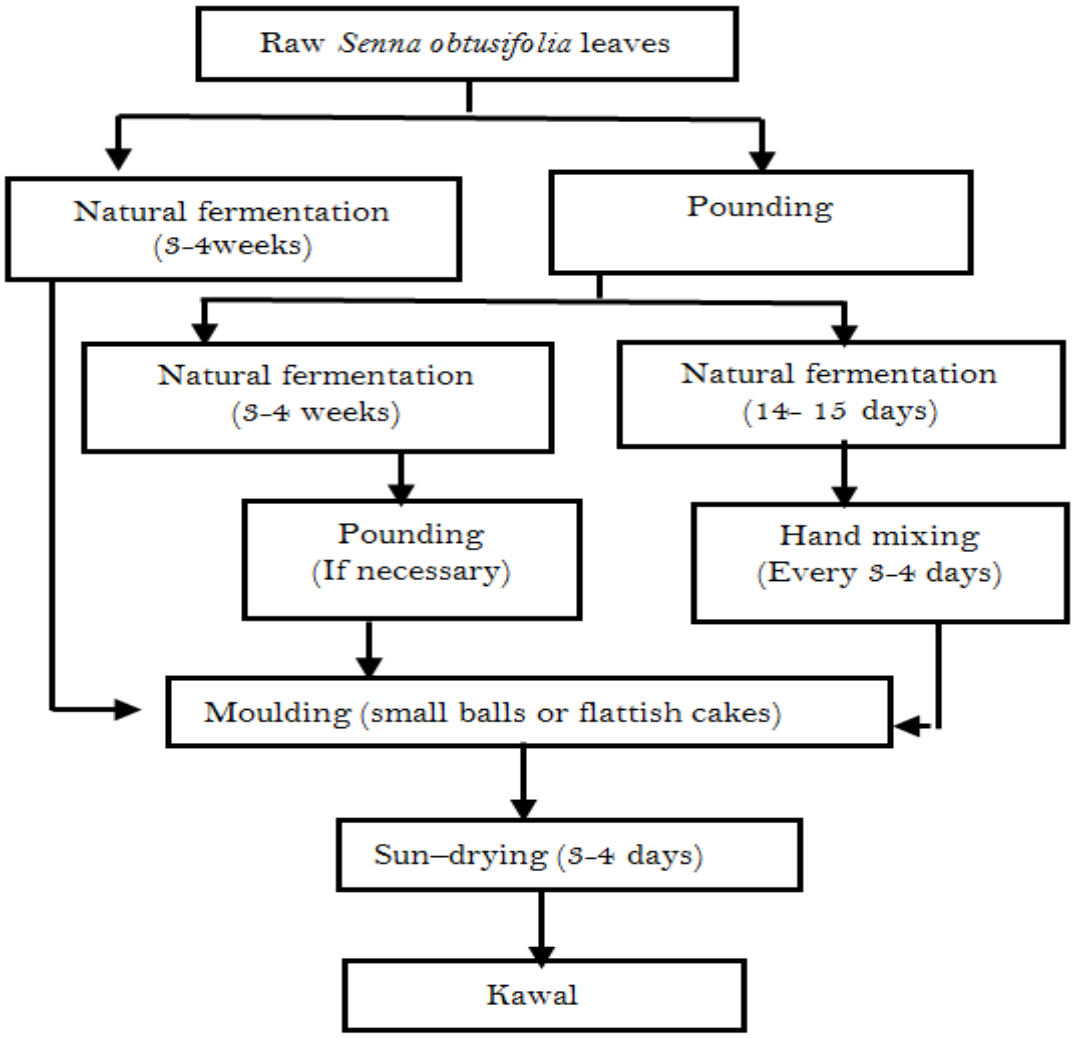


Figure-3. Flow general chart for *Kawal* production in Chad.

3.1.1. Preparation of Raw Material

The raw material (*Senna obtusifolia* leaves) was reaped in the rainy season when the plant is fully grown and pre-processed before the real production step. The pre-processing step consists of a selection by manually sorting. The green leaves are cleaned and the impurities, such as flower petals and leaves of other plant, worm, insects or leaves damaged by insects were remove.

3.1.2. Pounding

The unwashed, healthy green leaves are beaten manually in a wooden pestle and mortar to give a green paste. The leaves are usually pounded to destroy the filament, reducing and harmonizing until they formed a paste, without loss of juice. This hydration creates favorable conditions for the metabolic and microbial enzymes activities during the fermentation.

3.1.3. Fermentation

After a complete pounding, the paste is placed immediately in a plastic bag or earthenware. Meanwhile, a pit is dug in the ground in a shaded cool place, then the whole containers is fitted into the pit, leaving only the neck of the container above ground level. The containers is packed with cover to ferment at room temperature. Processing techniques used in *kawal* fermentation and procedures differ with localities, environmental factors and the types of processing equipment and technologies available. The duration of fermentation depends on the local conditions, the producer and the organoleptic quality of final product desired. However, for most producers, the fermentation was done at one step for 3 - 4 weeks according to the intensity of the desired ammonia-like flavour displayed by the fermented. This fermentation method is very popular in many parts of Chad. For others, the fermentation takes about 2 weeks or more and every 3-4 days the jar is opened and the contents are mixed by hand and repacked. Sometimes, after the first pounding of the process, the paste is not completly pounding, in this case at the end of the fermentation the pastes were removed and pounded again. At the end of the fermentation the product acquired a very pronounced and stronger ammonia-like odour.

3.1.4. Drying

At the end of the fermentation, the fermented paste is removed, moulded into small balls and sun-dried for 3 - 5 days according to the intensity of sunshine.

The process used in the production are often applied on a small scale by rural woman, characterized by the use of simple and rudimentary equipments, uncontrol fermentation with the empirical knowledge and the know-how is transmitted from mother to daughter and from generation to generation. The technology of *kawal* like most of the Africa fermented food remains traditional. In many developed countries, different traditional methods are now replaced with specific technologies for the production of fermented foods, and an emerging industrial practice allows for higher quality standardization of food products in the market place (Borresen *et al*., 2012). In recent times in Africa, a number of research works focused on developing starter cultures to upgrade the processing of traditionally fermented foods like dawadawa (Amoa-Awua *et al*., 2006). In the same line, this study aimed to the standardizing of the production of *kawal*, a traditional condiment produced by spontaneous fermentation of *Senna obbtusifolia* leaves. This study was to isolate and investigate *Bacillus* strains from *kawal* for potential starter culture selection.

3.2. Bacterial Isolation and Characterization

Ninety-seven (97) bacteria were isolated from the *kawal*. According the morphological and biochemical characters obtained, all isolates were belong to *Bacillus* spp. These strains were isolated by heat treatment of *kawal* samples. They were unicellular rod-shaped bacteria, rough, opaque colonies, Gram positive, catalase positive and

negative for others, and able to grow under aerobic conditions. They were endospore forming, and motile. According the phenotypic characters of the strains, we retained only 17 strains for probiotic properties evaluation.

3.3. Antimicrobial Activity

The selected *Bacillus* strains were screened for their antibiotic producing and inhibitory effect against Gram-negatives and Gram-positive bacterias. Majority of potential probiotic strains tested (58.82%) had activity against at minmum on one of the indicator bacteria : *Micococcus luteus LMG3293*, *Staphylococcus aureus ATCC 25923*, *Escherichia coli ATCC* 8739 and *Salmonella spp.* (isolated from local food). However, this selected strains exhibited antagonistic activity, but varied in their degree of inhibition and spectrum of activity. In particular, the strains SS6, SA9 and SG2 showed high inhibitory activity against the indicator bacteria, *Micococcus luteus LMG3293* and *Staphylococcus aureus ATCC 25923* respectively with inhibition zone more than ranged 3 mm. Some of the *Bacillus* strains showed moderate inhibition (2.1 to 3 mm) to the indicator bacteria. The others strains were least effective against the indicator bacteria. However, the weak inhibitions were observed with others against the indicators bacteria, as indicated in the Table 1.

Table-1. Antimicrobial activity of *Bacillus* Isolates using the agar-solid diffusion method.

Strain	Indicators bacteria			
	E. coli ATCC 87 39	**Salmonella sp.**	**S. aureus ATCC 25923**	**M. luteus LMG3293**
SM2	+	+	++	+
SM1	-	+	+	+
SG6	++	++	+	++
SL2	-	-	-	-
SS6	+	++	++	+++
SM6	-	+	-	-
SS2	-	-	-	+
SG2	+	+	+++	+
SG5	-	-	-	-
SG3	++	+	++	+
SN1	-	-	-	-
SL5	-	-	-	-
SK1	+	+	++	++
SK2	-	-	-	+
SA4	-	-	+	-
SA9	+	+	++	+++
SB12	-	-	-	-

Mean of inhibition zone : +,0.1 to 2 mm ; ++, 2.1 to 3 mm ; +++ >3 mm ; - no antimicrobial activity.

This research was also conducted to evaluate the antibacterial activity of the *Bacillus* strains isolated from *kawal*. The results of tests show good antibacterial activity of the tested strains against the indicator bacteria (*Micococcus luteus LMG3293*, *Staphylococcus aureus ATCC 25923*, *Salmonella* sp. and *Escherichia coli ATCC 87 39*) which resulted in inhibition zones Table 2. However, the strains SM2, SG6 and SK2 respectively showed high activity against the *M. luteus LMG3293* and *S. aureus ATCC 25923* with large inhibition zones more than 3 mm. The majority of the strains iolated were most effective against *M. luteus LMG3293* and *S. aureus ATCC 25923*. This inhibition may be due to the production of one or more antibacterial substances or active compounds. Several studies have reported about the genus *Bacillus* showing antagonism against Gram-negatives and Gram-positive bacterias (Korzybski *et al.*, 1978; Zweerink and Edison, 1987; Savadogo *et al.*, 2011; Taalé *et al.*, 2015). have reported isolation, characterization and pathways of antibacterial substances produce by *Bacillus* strains isolated from fermented food. Currently, the scientific works have been consecrated to the exploitation of antimicrobial metabolites of certain bacteria to improve the sanitary quality of foods (Desmazeaud and Cogan, 1996).

Bioprotection of food is based on the use of beneficial microorganisms that can control the presence of pathogenic bacteria by the production of specific metabolites.

3.4. Antibiotic Sensitivity of Bacillus Strains

The sensitivity of the *Bacillus* strains to a panel of antibiotics was determined using the agar disc-diffusion assay as recommended by the Use of the National Committee for Clinical Laboratory Standards guidelines for disk diffusion susceptibility testing in New York state laboratories. Ampicillin 10 µg, tetracycline 30 µg, erythromycin 15 µg, chloramphenicol 30 µg, kanamycin 30 µg, streptomycin 10 µg, oxacycline 30 µg, gentamycine 10 µg, amoxicilin 10 µg, imipenum 10 µg, Bacitracine10 µg and penicillin 10 IU were the antibiotics used in this study. The test results Table 2 revealed that the majority of the strains evaluated for the antibiotic susceptibility test had the same level of highest sensitivity to amoxicillin, kanamycin, chloramphenicol, streptomycin, and tetracyclin. All the *Bacillus* sp. tested were more susceptible to imipenum, erythromycin and gentamycin. Many isolates were resistant to penicillin G, oxacycline and bacitracin compared to their sensitivity profiles to other tested antibiotics.

Table-2. Susceptibility test of *Bacillus* sp. to antibiotics with diameter of inhibition (mm).

Antibiotic discs	Dose (µg)	Inhibition zone diameters by strains				
		BS1	SM1	SG1	SG2	SKB
Ampicillin	10	20 ± 0.4	2□□1 ± 0.4	20.4 ± 0.4	18 ± 1.2	21 ± 0.2
Chloramphenicol	30	29.5 ± 0.1	26 ± 0.3	24 ± 0.9	29 ± 2.1	25. ± 4.1
Tetracyclin	30	19 ± 1.4	25 ± 1.2	12.3 ± 0.3	30± 0.9	19.3 ± 06.
Erythromycin	15	34 ± 0.0	36 ± 0.5	33.5 ± 0.5	32 ± 0.6	30 ± 9.0
Kanamycin	30	25 ± 0.6	26 ± 1.1	22 ± 1.5	26.6 ± 0.4	24 ± 3.0
Penicillin	10	08 ± 1.3	06 ± 0.0	06 ± 0.0	08 ± 0.1	06 ± 0.0
Streptomycin	10	16 ± 0.7	15 ± 1.6	17 ± 1.7	16 ± 0.4	15 ± 0.5
Oxacycline	30	07 ± 0.2	06 ± 0.1	07 ± 0.2	06 ± 0.0	08± 0.9
Gentamycin	10	32 ± 0.4	35 ± 0.1	30 ± 0.4	35.2 ± 0.4	31 ± 0.8
Amoxicilin	30	22 ± 0.6	18± 2.4	15.6 ± 0.9	20 ± 1.3	24 ± 1.1
Imipenem	10	51 ± 2.1	53 ± 0.2	50.8 ± 0.1	51.6 ± 0.1	52 ± 3.2
Bacitracine	10	06 ± 0.2	06 ± 0.0	12 ± 0.4	06 ± 0.0	06 ± 0.0

Diameter of inhibition zone : susceptible, ≥ 16-mm diameter ; intermediate, 13- to 15-mm diameter ; resistant ≤ 12-mm.

Resistance profile of isolated, the present study showed that all the *Bacillus* isolates were highly susceptible to imipenum, erythromycin and gentamycin, and the results show that many of these *Bacillus* sp. tested were resistant to β-lactam antibiotics (penicillin G, oxacycline and bacitracin). The hight resistance of Bacillus strains to penicillin G, oxacycline and bacitracin probably in part by means of β-lactamase synthesis, on the other hand by biofilm production, that protects the bacteria (Philippon *et al.*, 1998; Chen *et al.*, 2004). The isolates strains were categorized as intermediately susceptible to amoxicillin, kanamycin, chloramphenicol, streptomycin, and tetracyclin. This observation is similar with that reported by Coonrod *et al.* (1971) to provide detailed antibiotic profiles of large number of well-characterized *Bacillus* spp. Coonrod et *al.* , reported that tetracycline, chloramphenicol, kanamycin, and gentamicin inhibited almost all *Bacillus* strains regardless of species.

3.5. Screening for Protease and Amylase Activities

The *Bacillus* strains were screened for protease and amylase respectively on skim milk agar media and on basal medium supplemented with soluble starch in order to detect their potential enzyme activity. Results showed that all selected strains are able to assimilate the protein and starch except two investigated becteria that were amylase negative. However The selected strains BL1 and BS23 showed the highest amylase and protease activities. These *Bacillus* strains tested gave different production levels of the enzymes studied, indicated by the presence of halos around the colonies Table 3.

3.6. Phytate Degrading Bacteria

All isolates were tested for their ability to phytase degradation, using sodium phytate media. 65.76 % of the strains produced zone of clearing on sodium phytate agar medium. Among them the strains BS23, SK23 and SB8 showed good phytase activities and could produce medium clear halo zone on phytase specific agar medium around colonies. The iolate BL1 was found to be the best strains and could produce large clear halo zone around the colony. Enzyme activities of *Bacillus* sp. selected as well as size of zone of clearance are shown in Table 3.

Table-3. Screening of different enzymes and biofilm formation of *Bacillus* sp. Isolates.

Enzymes and biofilm formation				
Strains	**Protease**	**amylase**	**Phytase**	**Biofilm**
BS23	+++	+++	++	+++
BS42	++	++	+	+
BS12	++	+	+	++
BS5	++	++	+	+
SM2	+	+	+	+
BL1	+++	+++	+++	++
SM3	+	-	-	+
SS7	+	+	+	+
BA21	++	++	-	++
AM9	+	+	-	+
SK11	+++	+	-	+
SK23	++	+++	++	+++
SB8	+	+++	++	+++
SL6	++	-	-	-
GS4	+	+	+	++
SL9	+	+	-	-
SA11	+	+	-	+

Highly (+++), medium (++), small (+) and (-) unable to synthese.

The production of enzymes, protease, amylase and phytase by *Bacillus* spp. showed different production levels of the studied enzymes indicated by the presence of halos diameters. The ability of *Bacillus* spp. to produce different hydrolytic enzymes is an interesting healthy and technological property. Phytic acid is an antinutritional factor that forms a complexe with proteins and also chelate metals making them unavailable to the organism (Quax, 1997; Nakamura *et al*., 2000). The presence of phytate in *Senna obtusifolia* leaves will be harmful for human health. Phytic acid is known as a food inhibitor which chelates micronutrient and prevents it to be bioavailabe for monogastric animals, including humans, because they lack enzyme phytase in their digestive tract (Gupta *et al*., 2015). Fermentation of *Senna obtusifolia* leaves to produce *kawal* was found to cause highly significant decrease in phytic acid content as reported by Algadi and Yousif (2015). The involvement of *Bacillus* genus with phytase activities in *kawal* fermentation could play a major role on phytate degradation. As concern amylase, this enzyme hydrolyzes the starch to produce sugar used by microorganisms involving in fermentation as energy source (Ketiku and Oyenuga, 1972) and improves digestibility of fermented food. In the alkaline or vegetable fermented food, the *Bacillus* genus hydrolyzes proteins to peptides and free amino acids and they also have a meat-like flavour, which contributes to food enjoyment (Sarkar *et al*., 2002; Ouoba *et al*., 2003).

3.7. Biofilm Synthesis

The ability of the tested strains to synthese biofilm was determined in liquid medium. All of *Bacillus* spp. strains tested were able to adhere to the polypropylene tubes giving a purple pellicule on the airsurface of the polypropylene tube, except to strains. However, the color intensity and size of the adherent crystal violet differ from one to another strain. In fact, BS23, BL1, BA21 and GS4 strains were identified as highly adherent, giving a large purple (+++). BS12, SK23, and BS5 strains were identified as faintly adherent showing a medium purple pellicule

(++). Others strains were identified with a very small purple pellicule (+). Whereas the strains SL6 and SL9 were unable to synthese biofilms in liquid Table 3.

The biofilm production ability of the *Bacillus* strains isolates was further assessed by generation of an adherent crystal violet-stained ring in polypropylene tubes. Majority of the screened *Bacillus* spp. strains produced biofilms. We found in this study a difference in the adherent crystal violet-stained ring in polypropylene tubes between the colonies. However among the isolates formed biofilm, some of the strains were identified as highly, medium or small adherent, others were unable to synthese biofilms. Biofilm synthesis, facilites *Bacillus* spp. adherence to intestinal epithelial cells and served as a mechanism of survival for some *Bacillus* to the harsh environmental conditions of the gastrointestinal tract (Barbosa *et al.*, 2005). These properties could facilite intestinal colonisation and also prevent enteropathogen (Barbosa *et al.*, 2005; Fakhry *et al.*, 2008; Latorre *et al.*, 2016).

3.8. Simulated Gastric and Intestinal Juices

One of the required criteria of probiotics microorganisms is their ability to survive in harsh environment of the gastrointestinal juice. Therefore, the survival ability of *Bacillus* strains was researched under simulated gastric juice at pH 2.0 and simulated intestinal juice (pH 7.0) conditions. The survival rate was compared with bacterial suspension (concentration between 1.0 x 10^8 and 1.0 x 10^9 CFU) before treatment and the viable counts of bacteria after incubation in gastric fluid and intestinal fluid for 3 h. Seventeen *Bacillus* strains were examined for gastrointestinal juice in this research. The results obtained after growing the strains in the harsh condition medium are shown in Figures 4 and 5. The viable counts of these 17 strains tested decreased at pH 2.0 of simulated gastric juice after 3 h compared with the control (S8). The strain S7 was the most acid tolerant of simulated gastric juice at 3h of incubation, with a survival rate of 7.05 log (CFU mL-1juice) among the seven strains (S1, S2, S3, S4, S5, S6 and S7) showed in Figure 4. The viabilities of the certains strains after incubation in intestinal juice at pH 7.0 for 3 h showed survival rates of 7.21 ± 0.11, 6.9 ± 0.13, 6.70 ± 0.1, 6.60 ± 0.1, 6.54 ± 0.1, 6.13 ± 0.1, and 5.80 ± 0.13 log (CFU mL-1juice), respectively for the strains SM3, SA1, LK3, KA, AKL, KBK, and KGA and considered intrinsically tolerant to gastrointestinal transit. All the strain tested showed decreasing numbers, except the control strain LK6 and S8 showed very good survival at 3 h of incubation in simple medium without simulated gastrointestinal juice Figures 4 and 5.

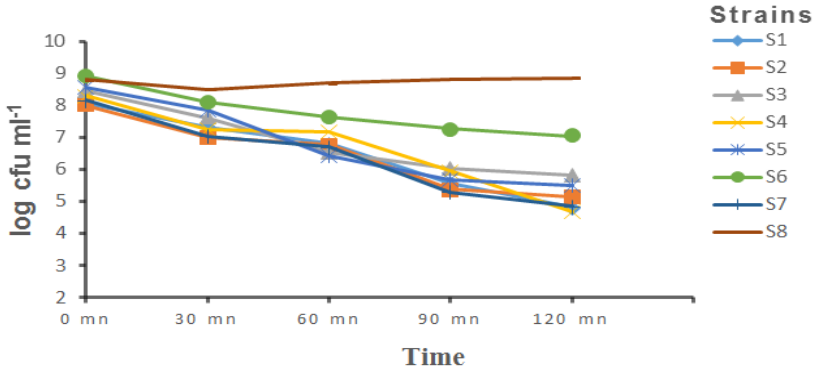


Figure-4. Tolerance of *Bacillus* strains to simulated gastric juice.

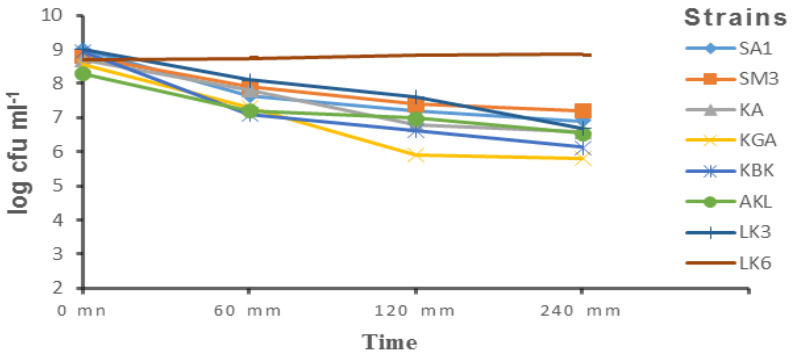


Figure-5. Tolerance of *Bacillus* strains to simulated intestinal juice.

The isolates of *Bacillus* sp. were examined under harsh environment of gastrointestinal conditions. Hence, evaluation of the survivability of the strains was carried out with regard to the cell counts in suspension obtained after the dilution as initial cells before the gastrointestinal treatment. The results obtained for simulated gastric and simulated intestinal conditions, indicates that all the cell densities of the strains tested reduced after treatment during the first 180 min. In that case the selected strains showed their capability to survive in simulated gastrointestinal conditions and showed differentes variations in the surviviabilities throught the treatment period. Among the strains tested, most of them gave the highest surival rate more than 7.0 log (CFU mL^{-1}) in gastric juice after 120 min Figure 4. At the same time the strains selected had better viability in simulated intestinal juice for 3 hours with survival rate 7,21 log (CFU mL^{-1} juice) Figure 5. The excellent resistance to stimulated gastric juice and stimulated intestinal juice of the *Bacillus* strains selected are similar with previous results obtained by Wang *et al.* (2010) and Hong *et al.* (2005). This finding suggests that these isolates have the potential to survive in the human gastrointestinal tract and can likely survive the passage through the stomach and small and large intestine (Jena *et al.*, 2013).

4. CONCLUSION

Different varieties of technologies processes in *kawal* production were identified in this study. The kawal production is still traditional with rudimentary equipment, natural fermentation, no standards, conducing to the variability of the nutritional and hygienic qualities as well as the stability of the final product. This alkaline fermentation of *Senna obtusifolia* leaves is a proteolotic enzyme process, and most important metabolism throughout the fermentation. The *Bacillus* spp. Screened showed high production levels of the studied enzymes. These selected *Bacillus* strains were very good proteolytic, phytasic and amylolytic properties as well as important antimicrobial activities against most common food spoilage and foodborne pathogens bacteria. The isolates susceptible to antibiotic tested, among them the isolates were susceptible to all types of antibiotic tested, biofilm synthesis and survive in harsh conditions. These isolates constitute then suitable candidates for the selection of starter cultures for controlled production of *kawal.*

Funding: This study received financial support from our Laboratory.
Competing Interests: The authors declare that they have no competing interests.
Acknowledgement: All authors contributed equally to the conception and design of the study.

REFERENCES

Algadi, M. and N. Yousif, 2015. Anti-nutritional factors of green leaves of cassia obtusifolia and kawal. Journal of Food Processing & Technology, 6(9): 1-3.Available at: https://doi.org/10.4172/2157-7110.1000483.

AlGburi, A., A. Volski, C. Cugini, E.M. Walsh, V.A. Chistyakov, M.S. Mazanko, A.B. Bren, L.M. Dicks and M.L. Chikindas, 2016. Safety properties and probiotic potential of Bacillus subtilis KATMIRA1933 and Bacillus amyloliquefaciens B-1895. Advances in Microbiology, 6(6): 432-352.Available at: https://doi.org/10.4236/aim.2016.66043.

Amoa-Awua, W.K., N.N. Terlabie and E. Sakyi-Dawson, 2006. Screening of 42 bacillus isolates for ability to ferment soybeans into dawadawa. International Journal of Food Microbiology, 106(3): 343-347.Available at: https://doi.org/10.1016/j.ijfoodmicro.2005.08.016.

Barbosa, T.M., C.R. Serra, R.M. La Ragione, M.J. Woodward and A.O. Henriques, 2005. Screening for bacillus isolates in the broiler gastrointestinal tract. Applied and Environmental Microbiology, 71(2): 968-978.Available at: https://doi.org/10.1128/aem.71.2.968-978.2005.

Borresen, E.C., A.J. Henderson, A. Kumar, T.L. Weir and E.P. Ryan, 2012. Fermented foods: Patented approaches and formulations for nutritional supplementation and health promotion. Recent Patents on Food, Nutrition & Agriculture, 4(2): 134-140.Available at: https://doi.org/10.2174/2212798411204020134.

Charteris, W., P. Kelly, L. Morelli and J. Collins, 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic lactobacillus and bifidobacterium species in the upper human gastrointestinal tract. Journal of Applied Microbiology, 84(5): 759-768.Available at: https://doi.org/10.1046/j.1365-2672.1998.00407.x.

Chen, Y., F.C. Tenover and T.M. Koehler, 2004. B-lactamase gene expression in a penicillin-resistant bacillus anthracis strain. Antimicrobial Agents and Chemotherapy, 48(12): 4873-4877.Available at: https://doi.org/10.1128/aac.48.12.4873-4877.2004.

Coonrod, J.D., P.J. Leadley and T.C. Eickhoff, 1971. Antibiotic susceptibility of bacillus species. The Journal of Infectious Diseases: 102-105.Available at: https://doi.org/10.1093/infdis/123.1.102.

Desmazeaud, M. and T.M. Cogan, 1996. Role of cultures in cheese ripening. Dairy starter cultures. New York: VCH Publishers. Inc. pp: 207-231.

Ehon, A.F., R.E. Krabi, A.A. Assamoi and L. Niamke, 2015. Preliminary technological properties assessment of bacillus spp. Isolated from traditional cassava starters used for attieke production. European Scientific Journal, 11(9): 1857- 7431.

Fakhry, S., I. Sorrentini, E. Ricca, M. De Felice and L. Baccigalupi, 2008. Characterization of spore forming bacilli isolated from the human gastrointestinal tract. Journal of Applied Microbiology, 105(6): 2178-2186.Available at: https://doi.org/10.1111/j.1365-2672.2008.03934.x.

Franz, C.M., M. Huch, J.M. Mathara, H. Abriouel, N. Benomar, G. Reid, A. Galvez and W.H. Holzapfel, 2014. African fermented foods and probiotics. International Journal of Food Microbiology, 190(2014): 84-96.Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.033.

Gupta, R.K., S.S. Gangoliya and N.K. Singh, 2015. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. Journal of Food Science and Technology, 52(2): 676-684.Available at: https://doi.org/10.1007/s13197-013-0978-y.

Hong, H.A., L.H. Duc and S.M. Cutting, 2005. The use of bacterial spore formers as probiotics. FEMS Microbiology Reviews, 29(4): 813-835.Available at: https://doi.org/10.1016/j.femsre.2004.12.001.

Huang, Y. and M.C. Adams, 2004. In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. International Journal of Food Microbiology, 91(3): 253-260.Available at: https://doi.org/10.1016/j.ijfoodmicro.2003.07.001.

Ibrahim, S.E., H.B. El Amin, H.E. N. and A.M.E. Sulieman, 2012. Amylase production on solid state fermentation by bacillus spp. Food Public Health, 2(1): 30-35.Available at: https://doi.org/10.5923/j.fph.20120201.06.

Jena, P.K., D. Trivedi, K. Thakore, H. Chaudhary, S.S. Giri and S. Seshadri, 2013. Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. Microbiology and Immunology, 57(6): 407-416.Available at: https://doi.org/10.1111/1348-0421.12054.

Ketiku, A. and V. Oyenuga, 1972. Changes in the carbohydrate constituents of cassava root-tuber (manihot utilissima pohl) during growth. Journal of the Science of Food and Agriculture, 23(12): 1451-1456.Available at: https://doi.org/10.1002/jsfa.2740231209.

Korzybski, T., Z. Kowszyk-Gindifer and W. Kurylowicz, 1978. Antibiotics isolated from the genus bacillus (bacillaceae). Antibiotics-Origin, Nature and Properties, 3(1978): 1529-1661.

Latorre, J.D., X. Hernandez-Velasco, R.E. Wolfenden, J.L. Vicente, A.D. Wolfenden, A. Menconi, L.R. Bielke, B.M. Hargis and G. Tellez, 2016. Evaluation and selection of bacillus species based on enzyme production, antimicrobial activity, and biofilm synthesis as direct-fed microbial candidates for poultry. Frontiers in Veterinary Science, 3: 95.Available at: https://doi.org/10.3389/fvets.2016.00095.

Marshall, E. and D. Mejia, 2011. Traditional fermented food and beverages for improved livelihoods. FAO Diversification Booklet, 21(2011): 1810-0775.

Mbaiguinam, M., Y. Mahmout, M. Tarkodjiel, B. Delobel and J.-M. Bessiere, 2005. Constituents of Kawal, fermented Cassia obtusifolia leaves, a traditional food from Chad. African Journal of Biotechnology, 4(10): 1080-1083.

Merghni, A., N. Leban, A. Behi and A. Bakhrouf, 2014. Evaluation of the probiotic properties of Bacillus spp. strains isolated from Tunisian hypersaline environments. African Journal of Microbiology Research, 8(4): 398-405.Available at: https://doi.org/10.5897/ajmr2013.5457.

Mishra, V. and D. Prasad, 2005. Application of in vitro methods for selection of lactobacillus casei strains as potential probiotics. International Journal of Food Microbiology, 103(1): 109-115.Available at: https://doi.org/10.1016/j.ijfoodmicro.2004.10.047.

Mohamadou, B.-A., C.M. Mbofung and D. Thouvenot, 2008. Bacterial fermentation induced mineral dynamics during the production of mbuja fromhibiscus sabdariffa seeds in earthen-ware pots. Annals of Microbiology, 58(3): 447-451.Available at: https://doi.org/10.1007/bf03175542.

Nakamura, T., T. Suzuki, J. Tokuda, N. Kato, Y. Sakai, D. Mochizuki and H. Takahashi, 2000. Patent and trademark office. Washington, DC: U.S. Patent No. 6,140,077.

Nitiema, L.W., A. Savadogo, J. Simpore, D. Dianou and A.S. Traore, 2012. In vitro antimicrobial activity of some phenolic compounds (coumarin and quercetin) against gastroenteritis bacterial strains. Int J Microbiol Res, 3(3): 183-187.Available at: 10.5829/idosi.ijmr.2012.3.3.6414.

Nuha, M., A. Isam and E. Elfadil, 2010. Chemical composition, antinutrients and extractable minerals of sicklepod (cassia obtusifolia) leaves as influenced by fermentation and cooking. International Food Research Journal, 17(3): 775-785.

Oguntoyinbo, F.A., G.-S. Cho, B. Trierweiler, J. Kabisch, N. Rösch, H. Neve, W. Bockelmann, L. Frommherz, D.S. Nielsen and L. Krych, 2016. Fermentation of African kale (Brassica carinata) using L. Plantarum BFE 5092 and L. fermentum BFE 6620 starter strains. International Journal of Food Microbiology, 238: 103-112.Available at: https://doi.org/10.1016/j.ijfoodmicro.2016.08.030.

Olmos, J. and J. Paniagua-Michel, 2014. Bacillus subtilis a potential probiotic bacterium to formulate functional feeds for aquaculture. J Microb Biochem Technol, 6(7): 361-365.Available at: https://doi.org/10.4172/1948-5948.1000169.

Ouoba, L., K. Rechinger, V. Barkholt, B. Diawara, A. Traore and M. Jakobsen, 2003. Degradation of proteins during the fermentation of african locust bean (parkia biglobosa) by strains of bacillus subtilis and bacillus pumilus for production of soumbala. Journal of Applied Microbiology, 94(3): 396-402.Available at: https://doi.org/10.1046/j.1365-2672.2003.01845.x.

Parkouda, C., L. Thorsen, C.S. Compaoré, D.S. Nielsen, K. Tano-Debrah, J.S. Jensen, B. Diawara and M. Jakobsen, 2010. Microorganisms associated with maari, a baobab seed fermented product. International Journal of Food Microbiology, 142(3): 292-301.Available at: https://doi.org/10.1016/j.ijfoodmicro.2010.07.004.

Philippon, A., J. Dusart, B. Joris and J.M. Frère, 1998. The diversity, structure and regulation of β-lactamases. Cellular and Molecular Life Sciences, 54(4): 341-346.Available at: https://doi.org/10.1007/s000180050161.

Prieto, M., L. O'Sullivan, S. Tan, P. McLoughlin, H. Hughes, M. Gutierrez, J. Lane, R. Hickey, P. Lawlor and G. Gardiner, 2014. In vitro assessment of marine bacillus for use as livestock probiotics. Marine Drugs, 12(5): 2422-2445.Available at: https://doi.org/10.3390/md12052422.

Quax, W., 1997. Merits of secretion of heterologous proteins from industrial microorganisms. Folia Microbiologica, 42(2): 99-103.Available at: https://doi.org/10.1007/bf02898715.

Sarkar, P., B. Hasenack and M. Nout, 2002. Diversity and functionality of Bacillus and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). International Journal of Food Microbiology, 77(3): 175-186.Available at: https://doi.org/10.1016/s0168-1605(02)00124-1.

Savadogo, A., A. Tapi, M. Chollet, B. Wathelet, A. Traoré and P. Jacques, 2011. Identification of surfactin producing strains in Soumbala and Bikalga fermented condiments using polymerase chain reaction and matrix assisted laser desorption/ionization-mass spectrometry methods. International Journal of Food Microbiology, 151(3): 299-306.Available at: https://doi.org/10.1016/j.ijfoodmicro.2011.09.022.

Steinkraus, K., 2018. Handbook of indigenous fermented foods revised and expanded. Food science and technology. 2nd Edn.: CRC Press.

Taalé, E., A. Savadogo, C. Zongo, M. Somda, S. Sereme, S. Karou, I. Soulama and A. Traore, 2015. Characterization of Bacillus species producing Bacteriocin-like inhibitory substances (BLIS) isolated from fermented food in Burkina Faso. International Journal of Advanced Research in Biological Science, 2(4): 279-290.

Wafula, E.N., C.M. Franz, S. Rohn, M. Huch, J.M. Mathara and B. Trierweiler, 2016. Fermentation of african indigenous leafy vegetables to lower post-harvest losses, maintain quality and increase product safety. African Journal of Horticultural Science, 9.

Wang, C.-Y., P.-R. Lin, C.-C. Ng and Y.-T. Shyu, 2010. Probiotic properties of Lactobacillus strains isolated from the feces of breast-fed infants and Taiwanese pickled cabbage. Anaerobe, 16(6): 578-585.Available at: https://doi.org/10.1016/j.anaerobe.2010.10.003.

Weber, D.J., S.M. Saviteer, W.A. Rutala and C.A. Thomann, 1988. In vitro susceptibility of bacillus spp. To selected antimicrobial agents. Antimicrobial Agents and Chemotherapy, 32(5): 642-645.Available at: https://doi.org/10.1128/aac.32.5.642.

Zweerink, M.M. and A. Edison, 1987. Difficidin and oxydifficidin: Novel broad spectrum antibacterial antibiotics produced by bacillus subtilis. The Journal of Antibiotics, 40(12): 1692-1697.