EVALUATION OF THE MICROBIOLOGICAL, CHEMICAL AND SENSORY ACCEPTABILITY OF FERMENTED *Parkia biglobosa* SEEDS (*Daddawa*) USING DIFFERENT DENSITIES OF *B. subtilis*

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Abstract
Indigenous fermentation of *Parkia biglobosa* seeds rely on back slopping, so there is no control of organisms that ferment the seeds or a definite standardization of innocula during the fermentation. This study is aimed at evaluating the physiochemical, microbiological, nutritional and sensory acceptability of fermented *Parkia biglobosa* seeds *(daddawa*) using different densities of *Bacillus subtilis***.** Isolation of *B. subtilis* was carried out using MYP agar and the identification was done using biochemical and molecular techniques. During the process of fermentation, the *Parkia biglobosa* seeds (*daddawa)* were assessed for pH, titrable acidity, temperature, aerobic mesophilic bacterial count, proximate composition and sensory evaluation using standard procedures. Results show that highest increase was observed in pH (6.3±0.01 to 7.41±0.01) and a corresponding highest decrease in titrable acidity was observed (0.05±0.00 to 0.01±0.00) in *Parkia* seeds innoculated with 6.0×10^8 CFU/ml during the fermentation. An increase in temperature (24 to 30ºC), aerobic mesophilic bacterial count (6.01±0.01 to 6.78±0.51) in uninnoculated *Parkia* seeds was also observed at 72h of fermentation. There was highest increase in protein content from 39.79±0.01 at 0hr to 47.73±0.00 in *Parkia* seeds innoculated with 3.0×10^8 CFU/ml at 72h of fermentation, but the content was not statistically different when compared with the other treatments. Highest decrease was observed in carbohydrate from 23.73±0.00 at 0hr to 17.70±0.00 in *Parkia* seeds innoculated with 6.0×10^8 CFU/ml at 72h of fermentation. Sensory evaluation showed that the judges preferred fermented *Parkia biglobosa* seeds treated with 1.5×10^8 CFU/ml of innoculum and control with a percentage likeness of 100% compared to the other treatments (3.0×10^8 CFU/ml, and 6.0×10^8 CFU/ml) with a percentage likeness of 40% and 0% respectively after 72h of fermentation. Statistically, there was no significant difference ($p>0.05$) between the different treatments in terms of protein contents. However, there is a general lower nutritional content observed in the uninnoculated *Parkia* seeds. Better texture, color and odor were also observed in the *Parkia seeds* innoculated with starter cultures as compared to the uninnoculated *Parkia* seeds. Conclusively, it was observed that the use of starter culture in higher density does not necessarily increase its nutritional value but rather, speed up the fermentation process.

Key words: condiments, fermentation, innoculum, Nigeria, Seeds

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INTRODUCTION

Traditional fermented condiments (*dadawa, iru, ogiri*) based on vegetable proteins and consumed by different ethnic groups in Nigeria have been the many pride of culinary traditions for centuries (Omodara, 2014). It is evident that these products have played a major role in shaping the food habits of communities in the rural regions not only by serving as nutritious non-meat proteins substitute but by also serving as flavoring and condiment agents in soups. These products are being increasingly marketed throughout the country and beyond in informal ways. Differences in the chemical compositions of fermented condiments are evident mainly because different ingredients have been used in their preparations. Emphasis is placed on the relevance of the role of starter cultures the traditional methods of manufacture to ascertain appropriate nutritional properties of the final product.

Parkia biglobosa is a multipurpose fodder tree that belongs to the family Mimosaceae (Sabiti and Cobbina, 1992) popularly called the "African locust bean tree". *P. biglobosa* is found in many countries of the world especially along the West African coast. The plant is locally used in northern Nigeria to treat diarrhoeal cases in both adults and infants (Agunu *et al.*, 2005). Several researches have

been conducted on the medicinal potentials of the plant (Udobi and Onaolapo, 2009). Recent interest on the preservative activity of the plant on foods has necessitated conduct of research on the plant. Bukar and Magashi (2008) have reported on the ability of *Parkia* pod aqueous extract to sanitize surfaces of fruits and vegetables. The phytochemical constituents and antimicrobial activity of the extract against some food-borne pathogens and spoilage microorganisms have also been reported (Bukar*et al.*, 2010).

For the use of starter cultures, naturally, the amount to be used is unique to each fermentation process but needs standardization. For the fermentation of *Parkia biglobosa* seeds (*daddawa)*, different densities have been applied in the fermenting process. There has been little works that are mainly concerned with perfecting the optimum densities. Yabaya (2006) used 10.05 $\log_{10}$CFU/g of *Bacillus subtilis* while producing *daddawa* seasoning with *Acacia nilotica* (Linn) seeds. Omodara (2014) used 1.0×10^7CFU/ml of *Bacillus subtilis* in her study of the effects of starter culture and different components of '*kuuru*' on the nutritional quality of fermented *Parkia biglobosa* and found it to be very effective. Furthermore, Kehinde *et al.* (2014) used 1.2×10^4 cell/ml of *Bacillus subtilis* in their study on the effects of fermentation on ground cooked lima bean seeds using *Bacillus subtilis* and *Bacillus primulus* and reported better yield of protein and ash contents of the product. Modupe (2016) carried out a study on the effect of *Bacillus subtilis* as starter culture and concluded its effectiveness during the fermentation of African locust (*Parkia biglobosa)* seeds. Bukar and Muhammad (2018) compared densities of *Bacillus subtilis* on the fermentation of *Parkia biglobosa* seeds and found out that there is no significant difference ($P > 0.05$) between the densities 1.5×10^8CFU/ml and 3.0×10^8CFU/ml in terms of the nutritional properties of the products after fermentation. The problem with indigenous fermentation has always been lack of standardization of starter culture that will produce a much desirable product of higher nutritional value and acceptability. Findings by Bukar and Muhammad (2018) have shown not significant difference in terms of nutritional content of the fermented product. It becomes imperative to conduct more research on the use of different densities of starter culture to optimize the nutritional content of the fermented product, which is the aim of this work.

MATERIALS AND METHODS

Source and identification of *Parkia biglobosa* Seeds

The *Parkia biglobosa* seeds were purchased from a local market in Kano state, Nigeria. The *Parkia* seeds were identified with a herbarium voucher number of BUKHAN 0262 at the Department of Plant Biology, Bayero University, Kano.

Starter Culture

Bacillus subtilis, which was isolated from previous fermented *Daddawa* was used as starter culture.

Isolation and Identification of *Bacillus* species

Bacillus species were isolated using Mannitol Egg Yolk Polymyxin (MYP) agar, incubated aerobically at 37°C for 24hours. At the end of the incubation period, the isolates that showed morphological appearance of *B. subtilis* were purified by repeated subculture and inoculated onto a slant and stored in the fridge for further analysis.

Identification of the *Bacillus subtilis* was carried out using Gram staining, catalase test, oxidase test, egg yolk emulsion test, citrate utilization test, spore staining, starch hydrolysis, gelatin hydrolysis, MRVP test, sporulation and motility tests. Further identification was done using molecular techniques that involve DNA extraction and purification, PCR, gel electrophoresis (DNA Labs, Kaduna State, Nigeria) and sequencing (at Inqaba, South Africa).

The Preparation of different densities of *B. subtilis* for the Fermentation

The innoculum was standardized using a 24hr culture of *Bacillus subtilis* at different densities, namely 1.5×10^8CFU/mL,

3×10^8CFU/mL and 6×10^8CFU/mL (Bukar and Muhammad, 2018).

The Preparation of *Daddawa*

The fermentation procedure was carried out according to the method of Kehinde *et al.* (2014) but with slight modification, which includes the addition of different starters and the autoclaving of the seeds before fermentation. Four kilograms (4Kg) of the *Parkia biglobosa* seeds were divided into four. Each portion, with the exception of the fourth (4^{th}) was cleaned with water and boiled in 1000ml of tap water for 30min. The bean was allowed to cool for one hour, pounded in a mortar and the seed coats were removed (dehulling). The beans was then sieved and boiled with another 1000ml of tap water for 12h and transferred into a clean cool water of 2000ml beaker. The dehulled seeds were sieved and autoclaved at 121°C. The seeds were then placed in a plastic container. The starter culture, *B. subtilis* was added to each of the three portions of *Parkia biglobosa* seeds using different densities (1.5×10^8CFU/mL, 3.0×10^8CFU/mL and 6.0×10^8CFU/mL, respectively. The fourth (4^{th}) has no *B. subtilis* added and, therefore, served as negative control. Aluminium foil was used to cover the surface of the plastic container firmly. The beans were incubated at 37°C for 72h. Temperature, pH, titrable acidity and aerobic mesophilic plate count were recorded at 24h, 48h and 72h periods during the fermentation.

Determination of Temperature of the fermenting *Daddawa* seeds

The temperature was monitored during the fermentation period by aseptically inserting the thermometer sterilized with 75% ethanol (Kehinde *et al.*, 2014) into the fermenting seeds at 0(at the beginning of fermentation), 24, 48 and 72 hours of fermentation. All measurements were conducted in triplicate.

Determination of pH of the fermenting *Daddawa* seeds

The pH was determined using a pH meter (model Denwer 20) calibrated with standard buffer (Kehinde *et al.*, 2014). The pH of the suspension was measured using pH meter at 0hr, 24hrhr, 48 and 72 hours. All measurements were conducted in triplicate.

Proximate Composition during Fermentation

Samples of *Parkia biglobosa* seeds fermented into *daddaw*a were taken periodically at 0hr, 24h, 48h and 72h. These were taken to the Department of Biochemistry, Faculty of Basic Medical Science for proximate composition. The parameters determined include; ash, moisture, protein, fat, crude fiber and carbohydrate contents (Association of Official Analytical Chemists, AOAC, 2002).

Statistical Analysis

Data generated from the viable cells count, physiochemical parameters, proximate composition and scores generated based on the assessment of judges from fermented *Parkia* seeds with different densities and control at 0hr, 24hr, 48hr and 72h were statistically analyzed using one way Analysis of Variance (ANOVA) at 5% probability level in accordance to the software package developed by Microsoft Corporation.

RESULTS AND DISCUSSION

Table 1 shows that the organism isolated is characterized by cream colored, central spore forming short rods and sparse colonies. The organism tested positive for Gram's reaction, endospore test, catalase test, motility test, citrate test, VogesProskeur test and starch hydrolysis. Methyl red, oxidase and indole tests showed negative results. The characteristics of the isolate agreed with those reported by many research works, which includes Kehinde *et al.* (2014) and Bukar and Mohammad (2018).

Plate 1 and Figure 1 both show the amplification product and DNA sequence of *B. subtilis*, which further confirms the isolate to be *B. subtilis.*

Table 2 showed that there was highest increase in pH from 6.36±0.01 to 7.41±0.01 ($P>0.05$) in *daddawa* innoculated with 6.0×10^8 density of *B. subtilis*. A number of research works corroborated with findings in this work, which include the work of Odunfa (1985), who reported that an increase in pH may be as a result of deaminase activity through proteolysis and the release of ammonia during the

fermentation. Bukar and Mohammad (2018) also agreed with this analysis in their research on the fermentation of *daddawa* using different densities (pH rose from 6.43 - 8.41). Temperature increased from 24 to 30^{o}C in all the treatments. Ibrahim and Antai (2005) did a similar report which reported that the increase in temperature during the fermentation might be due to the growth and development of microorganisms liberating heat in the process.

An increase in aerobic mesophilic bacterial counts was observed in all the treatments from 0-72h except in *daddawa* innoculated with 6.0 × 10^{8}CFU/mL, which recorded decrease from 6.49±0.69 to 6.43±0.65. Yabaya (2006) reported an increase in the microbial count from 2.8 × 10^{7} at 24h to 1.8 × 10^{10} at 72h of fermentation and attributed it to the breakdown of protein, lipid, starch and other nutrients to their simpler forms which the organisms use as their source of carbon and nitrogen. Bukar and Mohammad (2018) also observed an increase of microbial load from 4.49*log* to 4.70 *log* counts, but was slightly lower than the result that has been reported in this study which observed an increase from 6.01 - 6.78 during the course of the fermentation. This shows that even though precautions have been applied quite seriously, it is difficult to eradicate contamination completely under the traditional fermentation process.

There was general protein content increase in all the treatments with the highest observed in treatment with *daddawa* innoculated with 3.0 × 10^{8}CFU/mL from 39.79±0.01 to 47.03±0.00. Bukar and Mohammad (2018) corroborated this finding and reported that the increase may be due to the fact that the organisms breakdown the protein into amino acids.

Crude fibre content decreased from 0h to 72h in all the treatments. Ganiyu (2006) also reported a similar case when working on the nutrients and anti-nutrients of condiments produced from fermented legumes. However, this does not comply with the report of Bukar and Mohammad (2018) who observed neither an increase nor decrease during the production of locust and soy bean *daddawa* using different densities of *B. subtilis.*

Carbohydrate content decreased from 0h to 72h in all the treatments, which may be accredited to the utilization of sugar by fermenting organisms for growth and metabolic activities The result conforms with the report of Ibrahim and Antai (2005) during the fermentation of oil seeds and Bukar and Mohammad (2018) who observed a decrease in carbohydrate from 32.64% to 22.96%.

The research showed that 3.0 × 10^{8}CFU/mL is the best density which should be used during the fermentation of *Daddawa* as it had the highest overall nutritional boost which was revealed by the proximate composition. Omofuvbe *et al.* (2002) reported that *Bacillus subtilis* has been identified as being capable of producing organoleptically acceptable final products in alkaline fermented legumes. The use of *Bacillus* starters resulted in the highest degree of sliminess, softness and nutritional content of the substrates as reported by Kehinde *et al.* (2014). The sensory analysis has shown that the judges preferred the *daddawa* produced with 1.5×10^{8}CFU/mL density and control with a percentage likeness of 100. This may be attributed to the usage of reasonable amount of the organism in comparison with the amount of *Parkia* seeds used for the fermentation. The texture was light brown at the end of fermentation but gets darker with drying afterwards. The pungent smell accompanying fermentation is slightly less pronounced in the *daddawa* inoculated with starter culture than in control. The *daddawa* prepared with 3.0 and 6.0 x 10^{8}CFU/mL density of the *B. subtilis* were the least accepted by judges after fermentation. This may be attributed to the fact that it was observed that the fermentation was faster in these two treatments as compared to treatment with 1.5 x ^{8}CFU/mL and the control, which might have resulted in degradation of the end products of the fermentation by the high count of the *B. subtilis.*

Table 1: The Morphological and Biochemical Characteristics of the Isolate

Cell morphology	cream colored, gram positive, central spore forming rods
Cell colony	short rods, sparse colonies
Gram's reaction	+
Endospore test	+
Catalase test	+
Motility test	+
Citrate utilization test	+
Methyl red test	-
Vogesproskeur test	+
Starch hydrolysis	+
Oxidase test	-
Indole test	-
Egg yolk reaction test	+
Identity of organism	*Bacillus subtilis* suspected

Key: + = Positive
- = Negative

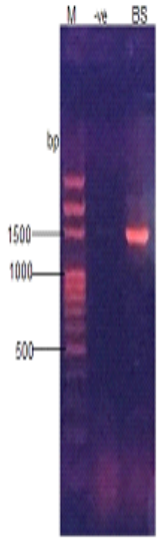

Plate 1: Primer amplification of *B. subtilis* gen sequencing result

Key: M = Molecular ladder, - Ve = negative control, BS = *Bacillus subtilis*

Bacillus subtilis strain 168 16S ribosomal RNA gene, complete sequence
Sequence ID: gi|507147976|NR_102783.1 Length: 1555 Number of Matches: 1

Range 1: 82 to 877 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1353 bits(1500)	0.0	778/797(98%)	1/797(0%)	Plus/Minus

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Query  17    CGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCAYTCATCGTTTACGGC  76
Sbjct  877   CGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGC  818
Query  77    GTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGT  136
Sbjct  817   GTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGT  758
Query  137   TACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCG  196
Sbjct  757   TACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCG  698
Query  197   CTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCT  256
Sbjct  697   CTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCT  638
Query  257   CCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACG  316
Sbjct  637   CCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACG  578
Query  317   CCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTT  376
Sbjct  577   CCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTT  518
Query  377   AGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTRCCGCCCTATTCGAACGGTACTTGTTC  436
Sbjct  517   AGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGCACTTGTTC  458
Query  437   TTCCCTAACAACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCG  496
Sbjct  457   TTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCG  398
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Sbjct  397   TCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCG  338
Query  557   TGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGT  616
Sbjct  337   TGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGT  278
Query  617   GAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCTRAA  676
Sbjct  277   GAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCG-A  219
Query  677   AGCCACCTTTTATGATTGAACCATGCGGTTCAATCAAGCATCCGGTATTAGCCCCGGTTT  736
Sbjct  218   AGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTT  159
Query  737   CCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCT  796
Sbjct  158   CCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCT  99
Query  797   GACCTAAGGGAGCAAGC  813
Sbjct  98    AACATCAGGGAGCAAGC  82
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Figure 1: ***Bacillus subtilis*** **strain 168 16S ribosomal RNA gene, complete sequence**

Table 2: pH and temperature recorded during the Fermentation of *Parkia biglobosa* Seeds Using Different Densities of *B. subtilis* at Different Periods

Physiochemical Parameters	Fermentation time (h)	Control	1.5×10^8 (CFU/ml)	3.0×10^8 (CFU/ml)	6.0×10^8 (CFU/ml)
pH	0	6.31±0.01	6.31±0.01	6.31±0.01	6.31±0.01
	24	6.91±0.01	6.07±0.07	6.83±0.02	6.98±0.01
	48	6.91±0.01	6.07±0.07	6.83±0.02	6.98±0.01
	72	7.10±0.06	7.30±0.06	7.33±0.06	7.41±0.01
Temperature (°C)	0	24±0.00	24±0.00	24±0.30	24±0.30
	24	28±0.33	27±0.88	28±0.33	29±0.58
	48	30±0.58	28±0.33	29±0.00	28±0.58
	72	30±0.58	30±0.33	30±0.58	30±1.00

Table 3: Aerobic Mesophilic Bacterial Count (log CFU/mL) during the Fermentation of *Parkia biglobosa* Seeds Using Different Densities of *B. subtilis* at Different Periods

Fermentation Time(h)	Control	1.5×10^8 (CFU/ml)	3.0×10^8(CFU/ml)	6.0×10^8 (CFU/ml)
0	6.01±0.00	6.01±0.00	6.01±0.00	6.01±0.00
24	6.45±0.57	6.65±0.58	6.36±0.40	6.35±0.61
48	6.61±0.71	6.72±0.59	6.47±0.48	6.49±0.69
72	6.78±0.51	6.63±0.57	6.75±0.65	6.43±0.65

Table 4: Proximate composition during Fermentation of *Parkia biglobosa* Seeds (*Daddawa*)

	Time (h)	Control	1.5×10^8 (CFU/ml)	3.0×10^8 (CFU/ml)	6.0×10^8 (CFU/ml)	Statistics
Moisture	0	14.70±0.00				
	24	14.30±0.00	12.74±0.00	12.01±0.00	12.70±0.00	
	48	11.30±0.00	9.97±0.00	9.01±0.00	9.70±0.00	
	72	4.89±0.00	4.20±1.00	8.00±0.00	4.40±0.00	NS
Protein	0	39.79±0.01				
	24	41.14±0.00	40.99±0.00	38.20±0.00	40.30±0.00	
	48	44.14±0.02	43.73±0.00	39.25±0.01	42.30±0.02	
	72	44.14±0.00	44.32±0.00	47.03±0.00	45.30±0.00	NS
Ash	0	2.81±0.00				
	24	8.85±0.00	6.49±0.00	8.33±0.00	8.88±0.00	
	48	6.85±0.00	8.48±0.01	7.30±0.00	7.88±0.00	
	72	2.60±0.00	1.61±0.00	7.09±0.00	4.24±0.00	NS
Crude fat	0	6.43±0.00				
	24	8.16±0.01	6.62±0.00	8.34±0.00	8.20±0.00	
	48	12.17±0.00	8.62±0.00	9.33±0.00	7.20±0.01	
	72	18.57±0.00	18.23±0.00	8.39±0.03	19.98±0.00	NS
Crude fibre	0	12.54±0.00				
	24	6.23±0.00	9.95±0.00	8.84±0.00	6.32±0.00	
	48	8.23±0.00	5.96±0.00	7.81±0.00	5.32±0.00	
	72	5.37±0.01	11.87±0.00	6.77±0.00	8.38±0.00	NS
Carbohydrate	0	23.73±0.00				
	24	21.32±0.00	23.21±0.00	24.28±0.00	23.60±0.00	
	48	17.31±0.00	23.24±0.01	27.30±0.00	27.60±0.00	
	72	14.71±0.00	19.77±0.00	22.72±0.00	17.70±0.00	NS

Key: NS = Not statistically significant, S = Statistically significant

Table 5: Sensory evaluation of *Parkia biglobosa* seeds (*Daddawa*) using different densities of *B. subtilis* after Fermentation for 72h

Densities	Sensory scores (% like) out of 9.00 scale
Control	8.6±0.2 (100)
1.5×10^8 (CFU/mL)	6.1±0.3 (100)
3.0×10^8 (CFU/mL)	4.3±0.2 (40)
6.0×10^8 (CFU/mL)	2.9±0.4 (0)

CONCLUSION

From the present findings, it can be concluded that the isolation of *B. subtilis* from previously prepared *daddawa* and its use as starter culture, is achieved.

The parameters checked at intervals during the fermentation showed an average pH and temperature of 7 and 30°C respectively at 72h of fermentation. Proximate composition showed an increase in protein from 39.79±0.01 at 0hr to 47.03±0.00 in 3.0×10^8CFU/ml density at the 72h of fermentation. A decrease was observed in carbohydrate from 23.73±0.00 at 0hr to 14.71±0.00 at 72h of fermentation. There was no significant difference between the densities in all the parameters which were observed during the fermentation ($p>0.05$). The organoleptic assessment showed the highest preference for 1.5×10^8CFU/ml density and control in terms of texture and aroma with a 100% like.

RECOMMENDATIONS

i. Further studies (mineral, vitamin, amino acid content of different densities) should be carried out to determine the optimum density of the starter culture.
ii. For the commercial production of *Daddawa*, it is advisable to use starter cultures with specific densities to speed up the fermentation process and to maintain consistency in quality.

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