Characterization of potential probiotics isolated from fermented under-utilized *Chrysophyllum albidum* Linn kernels using microbiological, biochemical and molecular techniques

O E Odutayo 1,*, B E Adegboye 1,2, E A Omonigbehin 2, O O Ogunlana 1 and I S Afolabi 1

1Biochemistry Department, College of Science and Technology, Covenant University, 100122 Ota, Nigeria; 2Molecular Biology Laboratory, College of Science and Technology, Covenant University, 100122 Ota, Nigeria;

*Correspondence: oluwatofummi.obaseki@gmail.com, Tel: +234-803-942-2498

Abstract. *Chrysophyllum albidum* is a crop of commercial value in Nigeria, however, the seeds are either used for local games or thrown away. This study aimed at exploring this under-utilized kernel as a novel source for obtaining health beneficial bacteria with desired probiotic characteristics. Isolation of potential probiotic bacteria from naturally fermented *C. albidum* seeds was carried out and followed by their safety evaluation, Gram staining, catalase test, acid tolerance, bile tolerance, cellular hydrophobicity and auto-aggregation assays. 16S rRNA sequencing and the detection of bile salt hydrolase (*bsh*) gene were the molecular methods applied for the bacteria characterization. Three potential probiotic bacteria were isolated from the fermented seeds. All isolates were non-haemolytic, Gram positive cocci, and catalase negative, grew in 1% bile, acidic pH of 3.5, and showed good auto-aggregation property. 16Sr RNA sequencing revealed isolates to be strains of *Enterococcus durans*, and the *bsh* gene was detected in all the strains. In conclusion, novel naturally fermented foods as seen in the fermented *C. albidum* kernels can serve as sources for the isolation of probiotic bacteria with great interest, and thus serve as starter culture to improve the organoleptic property of dairy and non-dairy foods.

1. Introduction

*Chrysophyllum albidum* Linn, commonly referred to as African star apple is a sweet palatable fruit tree belonging to the family Sapotaceae and habitually found throughout the Central, East, and West African regions. It is a crop of commercial value in Nigeria. The tree grows to about 8-36m in height, while the fruit being periodic, is annually available around December to April [1]. Usually, the fruits are not harvested from the trees, they are left to naturally drop to the forest floors and picked. The fleshy edible pulp is useful in the beverage industry for the formation of soft drink, it is used in table jelly manufacture [2], it is consumed as desired by people, and also taken to stop salivation, irritation, and loss of appetite [3]. *C. albidum* fruit is a berry with orange to yellow colour. When ripe, it is ovoid to subglobose, with a pointed apex. On the average, it is about 6 cm by 5 cm in length and diameter respectively. The succulent pulp within may be pink, or light yellow, or orange colour. Inside the pulp are about four to six seeds, and their coats are dark brown in colour, shiny, and hard. Breaking these coats reveal the white kernels present in the seeds are. *C. albidum* seed is under-explored such as being used for local games or thrown away. On another
hand, Probiotics are defined as living organisms which on administration in adequate amounts to a host confers a beneficial health effect [4]. To meet the demand of getting probiotic strains of better interest, there are continuous efforts on the characterization of probiotics. Since the probiotic characteristic of a strain cannot be extrapolated to another strain, it is of necessity that any promising probiotic strain is properly identified and characterized to get the best strains [5]. Naturally fermented foods are rich sources of microorganisms, some of which may exert probiotic qualities [4,6]. Due to the specific characteristics of strains, exploring the wealth of microorganisms from novel naturally fermented sources can bring the discovery of unique strains of probiotic bacteria with characteristics of great interest.

2. Methodology
2.1 Seeds collection and identification
Seeds collection and identification were carried out as earlier reported by Odutayo et al. [7]. The *C. albidum* fruits from which the seeds were collected were procured from the Afoaje market; one of the local markets in Ota, Nigeria. It is commonly called “Oja Ota” and geographically located on 6.6841° N, 3.2153° E. Identification and authentication was done at the Forest Research Institute of Nigeria (FRIN) with the given voucher number, FHI: 112774.

2.2 Flour preparation
Flour preparation from *C. albidium* kernels were done as reported earlier by Odutayo et al. [7]. *C. albidium* seeds were manually de-hulled to remove the kernels which were thereafter dried in the oven at 40°C till it was sufficiently crusty to be pulverized into smooth flour. This took place within 24 hours. kernels were ground into a smooth powder with the use of an electric blender.

2.3 Isolation of probiotic bacteria
Isolation of probiotic bacteria from the fermented seeds was carried out by applying the techniques reported by Odutayo et al. [7] and Devi et al. [8]. Ground kernels of *C. albidium* were steeped in distilled water (1:10w/v) and made to anaerobically ferment for 72 hours under normal room temperature. An aliquot of 20 µl of the fermentation mesh was conveyed by using a sterile nichome inoculating loop into sterile de Man Rogosa Sharpe (MRS) agar plates which had been earlier prepared following the manufacturer’s instruction. This was afterwards streaked to detect the growth of distinctly defined bacteria colonies. The plates were microaerophilically incubated at 37°C for 48 hours. At the end of incubation, single isolated colonies were picked, using a sterile inoculating loop and cultured by streaking repeatedly on sterile MRS agar plates, till pure cultures of the bacteria isolates were derived.

2.4 Maintenance of bacteria cultures
For immediate analyses and short term storage, the pure single isolated colonies were transferred into 3mls MRS soft agar stabs and preserved at 4°C, while for the long term storage of the cultures, the single isolated bacterial colonies were transferred into 3 ml MRS broth and glycerol (4:1v/v) and preserved at -80°C.

2.5 Safety evaluation
Safety evaluation of isolates was carried out to exclude haemolytical isolates from the study. This was done as described by Tallapragada et al. [9]. Blood agar was prepared by aseptically collecting and adding 5mls of healthy human blood into 100 ml of sterile Mueller-Hinton agar. The blood and agar mix was aseptically and gently shaken to obtain a homogenous blood agar. Sterile blood agar plates were thereafter prepared by aseptically pouring the blood agar into sterile petri dishes and left to gel. Spot inoculation of the freshly sub-cultured 24 hr old isolates was done on the sterile blood agar plates. The inoculated plates were microaerophilically incubated at 37°C for 48 hr and observed thereafter for zones of haemolysis.

2.6 Gram staining
Gram staining of isolates has been earlier reported by Odutayo et al. [7]. Isolates were freshly sub-cultured on pre-prepared sterile MRS agar plates, and thereafter incubated under microaerophilic conditions at 37°C
for 24 hr. The fresh cultures were smeared on microscopic slides and Gram stained with the use of a Gram staining kit to detect shape and Gram reaction.

2.7 Catalase test
Isolates were subjected to catalase test as earlier reported by Odutayo et al. [7]. Isolates were treated with hydrogen peroxide (H$_2$O$_2$); each of the isolates was picked with a sterile inoculating loop, and transferred to a microscopic slide. A drop of H$_2$O$_2$ (20 µl) was placed on each of the isolates to detect the formation of bubbles. Isolates that had bubbles formed in reaction with the hydrogen peroxide was an indication of a positive catalase reaction, while isolates that had no bubble formed in reaction with hydrogen peroxide was an indication of a negative catalase reaction.

2.8 Acid tolerance assay
Acid tolerance assay was carried out according to the method described by Tallapragada et al. [9]. MRS broth was adjusted to the following pH; 1.5, 3.5, 5.5, 7.5, and 9.5. The unadjusted broth (pH 6.2) served as the control. The broths were sterilized by autoclaving, and after cooling, 3mls each of the different broths were aseptically inoculated with 20 µl of each of the freshly sub-cultured 24 hr isolates adjusted to turbidity level equivalent to 2 McFarland standard. Isolates were allowed to grow microaerophilically in the incubator at 37°C for 48 hr. After incubation, the growth rate of the isolates was measured using the spectrophotometer at an absorbance of 600 nm.

2.9 Bile tolerance assay
Bile tolerance assay was carried out according to the method described by Tallapragada et al. [9]. MRS broth of the following cow bile salt concentrations; 0.1%, 0.3%, 0.5%, 0.7% and 1% was prepared. MRS broth without bile (0% bile) served as control. 3mls each of the different broths were aseptically inoculated with 20 µl of each of the freshly sub-cultured 24 hr isolates adjusted to turbidity level equivalent to 2 McFarland standard. Isolates were allowed to grow microaerophilically in the incubator at 37°C for 48 hr. After incubation, the growth rate of the isolates was measured using the UV-visible spectrophotometer by Thermo scientific at an absorbance of 600 nm.

2.10 Cellular hydrophobicity assay
Cellular hydrophobicity assay was done using the technique reported by Tallapragada et al. [9] with slight modification. The freshly sub-cultured 24hrs isolates were centrifuged at 6000 rpm for 15 minutes and the pellets were washed with 2 ml of sterile saline solution (pH 6). The washed pellets were afterwards resuspended into 2 ml of the sterile saline solution, and absorbance was measured at 580 nm. 1ml of each suspension was mixed with 1 ml of xylene, vortexed for 2 minutes, and incubated for 30 minutes. After 30 minutes of incubation, two phases separated, and the absorbance of the lower aqueous phase was measured at 580 nm.

The percentage of hydrophobicity was measured as:

$$\% \text{ Hydrophobicity} = \frac{(Abs_0 - Abs_{30})}{Abs_0} \times 100$$

Where Abs$_0$ and Abs$_{30}$ refer to initial absorbance and absorbance measured after 30 minutes respectively.

2.11 Cellular auto-aggregation assay
The cellular auto-aggregation assay was carried out as described by Tallapragada et al. [9]. The freshly sub-cultured 24 hr isolates were centrifuged at 6000 rpm for 10 minutes and the pellets were washed with 2 ml of sterile saline solution (pH 6). The washed pellets were diluted to an absorbance of 0.3 (using McFarland standard) with the sterile saline solution, and the absorbance was measured at 600 nm. An aliquot 1 ml of each suspension was taken and allowed to stand for 60 minutes under normal room temperature. After 60 minutes, the suspensions were centrifuged at 300 g for 2 minutes and absorbance of the supernatant was measured at 600 nm.
The percentage of auto-aggregation was calculated as follows:

\[
\% \text{ Auto-aggregation} = \left(\frac{\text{Abs}_0 - \text{Abs}_{60}}{\text{Abs}_0}\right) \times 100
\]

Where \(\text{Abs}_0\) and \(\text{Abs}_{60}\) refer to initial absorbance and absorbance measured after 30 minutes respectively.

2.12 Genomic characterization and detection of bile salt hydrolase (bsh) gene in isolates

Genomic characterization and detection of bsh gene was done according to the methods described by Tallapragada et al., Devi et al. and Turpin et al. [8–10] with slight modifications. DNA was isolated from the freshly sub-cultured 24 hour old isolates using Quick-DNA™ Miniprep Plus Kit by Zymo Research (Inqaba biotechnology South Africa). Procedure for isolation was carried out according to the manufacturer’s instructions. 16s forward Primer: 5’-AGGTTGTCTGCTCA-3’ and 16s reverse Primer: 5’-TCGGTCCTTTCTCAGCT-3’ (Inqaba biotechnology, South Africa) was used in the bidirectional sequencing. Also, bsh forward(a): 5’-TGGTTGTCCGGTCA-3’, bsh reverse(a) : 5’-TTAGGGCGGAACGTTA-3’ (Inqaba biotechnology, South Africa) were used for the detection of bile salt hydrolase gene in the isolates. A 100 bp DNA ladder was used as the molecular marker. Polymerase chain reaction (PCR) was carried out in total volumes of 25 µl containing 50 ng genomic DNA, 20 mM primers, 1X Taq buffer, 0.2 mM deoxyribonucleotide phosphate (dntp), 1.25 units Taq polymerase, and DNA and RNAase free water. PCR products were separated on an agarose gel prestained with ethidium bromide. Amplicons of 1500 bp obtained were sent to Inqaba biotechnology South Africa sequencing service lab to obtain the sequences of the isolates.

2.12.1 Sequencing of the 16S rRNA amplicons. PCR products were cleaned using ExoSAP-IT™ PCR product clean up reagent. Exo/SAP master mix was prepared by adding 50 µl of exonuclease I (20 U/ul) and 200 µl of shrimp alkaline phosphatase (1 U/ul) into a 0.6 ml microcentrifuge tube. 2.5 ul of the Exo/SAP mix was added to 10 ul of each amplified PCR product, mixed properly and incubated at 37°C for 30 min. The reaction was stopped by heating the mixture at 95°C for 5 min. Sequencing was thereafter carried out using the Applied Biosystems™ BigDye™ Terminator v3.1 cycle sequencing kit according to manufacturer’s instructions. The labelled products were cleansed using the ZR-96 DNA sequencing clean-up kit according to manufacturer’s instruction. The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7.

2.12.2 Computational analysis of the genomic sequences. Sequences were assembled using geneious prime (version 2021.0.1). Blasting was done for the assembled sequences using the Basic Local Allignment Search Tool for nucleotide sequences from the National Centre for Biotechnology Information (NCBI). The sequences have been submitted to the NCBI database and given their respective accession numbers.

2.13 Statistical analysis

One way analysis of variance (ANOVA) followed by Turkey’s test for the comparison of multiple means (P<0.05) was used to statistically analyse the results from the acid tolerance, bile tolerance, cellular hydrophobicity, and cellular auto-aggregation assays. Results are expressed as mean ± standard error of the mean (SEM) of triplicate readings. The statistical tool used was GraphPad prism 8.0.2 by Graphpad Software Inc. San Diego California USA.

3. Results

3.1 Safety evaluation, Gram staining and catalase test

Three potential probiotic bacteria coded A1, A2a and A2b were isolated from the naturally fermented C. albidum seeds. The three isolates were non-haemolytic, Gram-positive cocci [7] and catalase negative [7].
3.2 Acid tolerance
The acid tolerance values of isolates A1, A2a and A2b for the pH ranges 1.5 to 9.5 are shown in Figure 1. From the results none of the isolates grew under the acid condition of pH 1.5, but an increased growth rate with increase in pH values was observed from pH 3.5 to 9.5.

3.3 Bile tolerance
The bile tolerance values of isolates A1, A2a and A2b for the bile percentage (0 to 1) % are shown in Figure 2. From the results, all the isolates grew in all the bile concentrations, with an increased growth rate observed as bile concentration increased.

3.4 Cellular hydrophobicity and auto-aggregation
The results of the cellular hydrophobicity and auto-aggregation of isolates A1, A2a and A2b are shown in Figure 3. From results obtained, none of the isolates showed cellular hydrophobicity property, while they all exhibited cellular auto-aggregation property.
3.5 Genomic characterization and detection of bile salt hydrolase (bsh) gene in isolates

The gel images for the 16S rRNA characterization and detection of the bsh gene are presented in figures 4 and 5 respectively. Isolates A1, A2a and A2b have been previously identified as *Lactococcus raffinolactis*, *Lactococcus lactis*, and *Pediococcus pentosaceus* respectively, using biochemical characterization with API 50 CHL and reported by Odutayo et al. [7]. Table 1 thus presents the results from the genomic identification of the isolates and their accession numbers.

![Figure 4: 16S rRNA characterization of isolates](image1)

![Figure 5: Detection of bsh gene in isolates](image2)

*Note: Unlabeled bands are for another study, and are therefore not discussed in this report.*

Table 1: Genomic identification of probiotic isolates

| Probiotic Isolates | Genomic identification -accession numbers |
|--------------------|------------------------------------------|
| A1                 | Not identified                            |
| A2a                | *Enterococcus durans*-MW481700            |
| A2b                | *Enterococcus durans*-MW481702            |

4. Discussion

To the best of our knowledge, this is the first report on the isolation and characterization of probiotics from naturally fermented *C. albidum* seeds. The most commonly used probiotics in foods have originated from the lactic acid bacteria family. The Gram positive cocci and catalase negative results thus obtained are also notable to either the Lactococcus or Enterococcus genera of lactic acid bacteria. In choosing probiotics to be used in foods, safety is a first criterion to be considered, and this was shown in the non-haemolysis expressed by isolates A1, A2a and A2b in this study. For bacteria to be considered a prospective probiotic, they should have the ability to conquer the low pH surrounding in the gastrointestinal tract, and get to the site of action in a viable physiological state [8,9]. According to Beasly et al. [11], the more acidity a probiotic can tolerate, the better it is to perform its role, and this is because overcoming the highly acidic region of the stomach is imminent to it arriving at the site of action in a workable state. In this study, all the isolates grew in the pH 3.5 to 9.5. Also, the ability of the isolates to multiply and still remain viable after an incubation period of 48 hours in high acidity of pH 3.5 shows their high level of acid tolerance. Bile tolerance is another factor to consider when selecting probiotics for use. In the upper intestinal tract, the bile concentration might possibly be unpredictable, and could vary any time, therefore, tolerance to bile is a ‘must’ need by probiotics [11]. From our results, all the isolates grew in the different bile concentrations and expressed the highest growth rate in 1% bile during the 48 hour incubation time, aside these organisms being able to survive these conditions, the tolerance is also essential for easy colonisation of the GIT. Toleration of GIT stress conditions is also necessary for the retainment of cell integrity, and maintenance of
adhesiveness and metabolic activity of the probiotic strain [11]. The results of the in vitro acid and bile tolerance tests in this study are thus good prediction tools for the organisms to cope in the host’s body environment. Cellular hydrophobicity and auto-aggregation are essential probiotic characteristics, since these properties enable them to adhere strongly to the mucosal cells of the host intestine and exert physiological functions [8,9]. Furthermore, auto-aggregation enables cells to coexist with one another and aid the formation of biofilms [9]. From our study, Isolates A1, A2a and A2b expressed good auto-aggregation property, and on the contrary showed no hydrophobicity property. The no hydrophobicity property shown by the isolates could have been surprising, however according to the reports of Nakao et al. [12] a direct relationship between the auto-aggregative and hydrophobicity properties of cells as expressed in their study was suggested. On a contrary note, the same study also observed an inverse relationship between these two properties in some cells. Therefore, while it is expected that the strong hydrophobicity property of bacteria cells is essential for their attachment to mucosal surfaces, some other mechanism(s) that affect auto-aggregation still need(s) to be understood. Nakao et al. [12], also suggested that some changes in the outer membrane component of cells might affect their auto-aggregation property. The hydrocarbon used in determining the hydrophobicity property of cells have also reported varying values even for the same bacteria strain, and this might suggest the need for more accurate and definite method(s) to determine the hydrophobicity property of cells in vitro. For example, Zhao et al. [13] reported that the percentage aggregation of four lactic acid bacteria isolates differed as a result of the solvents used, and hydrophobicity of the same strain for different solvents were not similar. However, they concluded that combined strains with low adherence ability or high and low together could reliably enhance the adherence ability. Bile acids are synthesised in the liver from cholesterol and are conjoined with either glycine or taurine. They are stored and concerted in the gall bladder and excreted through the bile duct into the small intestine to aid the assimilation of dietary fat [5,14,15]. Conjugated bile salts have been known to inhibit the growth of some strains of bacteria [5]. The bile salt hydrolase gene detected in all the isolates is an indication that they all produced bile salt hydrolase and have the ability to hydrolyse bile salt. This is in agreement with reports that some probiotic bacterial strains can produce bile salt hydrolase and hydrolyse bile salts [5]. This ability could be a result of the tolerance of all the isolates to all the bile concentrations. This is also in agreement with the report that the ability of some probiotic bacterial strains to hydrolyse bile salt is associated with the resistance of the strains to the presence of bile salt, which seems to be a protective mechanism for the bacterial strains against conjugated bile salts [5]. The 16S rRNA sequencing identified isolates A2a and A2b as Enterococcus durans -MW481700 and Enterococcus durans-MW481702 respectively. This is an improvement over their earlier biochemical characterization as Lactococcus lactis, and Pediococcus pentosaceus [7]. Inconsistences with the phenotypic identification of bacteria has been reported [16], hence the the 16S rRNA gene which is the central structural component of the bacterial and archaeal 30S ribosomal subunit [17,18] has been a useful molecular tool to identify bacteria further from the specie to strain level. 16S rRNA gene sequence analysis is also a better tool for the identification of poorly described, rarely isolated, or phenotypically aberrant strains. It can be used routinely for the identification bacteria and can also, lead to the recognition of novel and noncultured ones [19]. Enterococcus durans -MW481700 and Enterococcus durans-MW481702 are of the Enterococcus genus belonging to the lactic acid bacteria group; it represents the third-largest lactic acid bacteria genus after Lactobacillus and Streptococcus [20]. Enterococci are widespread microorganisms that can be found in places such as gastrointestinal tract of humans and animals, water, soil, foods and plant [21]. They have been applied as probiotics and starter culture in fermentation of food because of their biotechnological traits like proteolytic activities or protective cultures in food biopreservation, which has risen from their ability to produce antimicrobial bacteriocins called enterocins [21]. Notwithstanding, in the past years, the use of enterococci in foods or as probiotics had caused an important debate, which resulted from their opportunistic pathogenicity [21]. Hence, developing new enterococcal probiotics must be strictly assessed with regards to safety aspects for the selection of truly harmless enterococcal strains for safe applications. As discussed
earlier, the non-haemolytic reaction expressed by the isolated enterococcal strains in this study was thus an indication of their safety.

5. Conclusion
Novel naturally fermented foods as seen in the fermented C. albidum kernels can serve as sources for the isolation of probiotic bacteria with great interest, and thus serve as starter culture to improve the organoleptic property of dairy and non-dairy foods. Furthermore, novel sources for the isolation of probiotics could also lead to obtaining novel probiotic strains with desirable attributes. The isolates from this study showed good probiotic characteristics and can be recommended for use as probiotics in foods and pharmaceuticals. However, further in vitro, in vivo and molecular studies are recommended to further ascertain their credibility as probiotics.

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