Heliyon 9 (2023) e14959

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Screening, isolation and biotechnological potentials of foodborne *Lactobacillus fermentum* strains MT903311 and MT903312

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ARTICLE INFO

Keywords:
Probiotic
Vitamins
Antimicrobial
Biotechnology

ABSTRACT

Vitamins are an essential food source with excellent roles in the cellular metabolism and other essential nutrients required in food intake but cannot be synthesized by humans. There have been reports of some lactic acid bacteria (LAB) abilities with probiotic activities to produce food-grade vitamins. Our study aimed to investigate lactic acid bacteria (LAB) possessing antimicrobial activities and extracellular production of folate from different Nigerian fermented foods. LAB was assayed for their antimicrobial activities against clinical isolates of *Escherichia coli* and *Salmonella typhimurium* and their extracellular production of essential vitamins. Among the 43 isolates of LAB, two strains of *Lactobacillus fermentum* showed the highest inhibitions against the test bacteria and demonstrated the highest concentrations of extracellular vitamins production. The range of vitamins produced at 24 h was between 12.23 and 801.79 μg/ml, while the highest vitamin production of 801.79 and 310.55 μg/ml was observed for folate and vitamin B12 respectively, the lowest production was for B1+B2. Consistent vitamin production was typical with only *L. fermentum* MT903311 and *L. fermentum* MT903312, so were their antimicrobial activities. The L. *fermentum* strains isolated in this study could be exploited and applied in food products to substitute synthetic vitamin enrichment and fortification.

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative, non-motile, and non-spore forming bacteria. They are known for their probiotic activities in improving intestinal microflora, food preservation, and other health benefits [1]. These bacteria are efficient in various capacities such as antioxidant effects, chelation of ferrous ions, degradation of nitrite and cholesterol, production of hydrogen peroxide and bacteriocin [2–5], their metabolites are generally regarded as safe [6]. With the current escalated problem of antimicrobial resistance due to the non-judicious use of antibiotics worryingly going out of control, alternative strategies for control of resistant bacteria have been requested, of which LAB are among the front liners [7].

The recent emphasis on food security as part of the United Nations goals has made it expedient to exploit various ways of food safety including nutrient improvement and promotion of sustainable agricultural products. Concerns have been raised over several food additives and supplements regarding their safety and health risk due to the addition of chemicals for preservation and formula. The

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https://doi.org/10.1016/j.heliyon.2023.e14959
Received 6 July 2022; Received in revised form 27 January 2023; Accepted 22 March 2023
Available online 28 March 2023

LAB fermentation roles in promoting healthy foods by inhibiting pathogenic and spoilage bacteria through acidifications have been previously documented [8]. More interests are now geared towards functional ingredients in food, i.e., nutraceuticals that provide health or medical benefits and are also capable of antimicrobial properties to prevent diseases. Such ingredients can either be macro or micronutrients naturally present in food or are added during processing. Of particular interest among these food supplements in the present study are natural vitamins as sources of food enrichment.

Vitamins are micronutrients essential for all living organisms' daily metabolism and are usually obtained through diets [9]. Humans cannot synthesize most vitamins required for growth; consequently, they have to be obtained exogenously. The use of vitamin-producing microorganisms might represent a more natural and consumer-friendly alternative to fortification using chemically synthesized pseudo-vitamins. The use of LAB to produce vitamins could suppress the expensive chemical production of these ingredients to enrich food or improve local food production. Studies worldwide have reported abilities of specific LAB isolated from fermented foods producing natural vitamins and other food supplements [10]. The projection of such studies is the biotechnological prospect of these industrial-friendly microbes for various purposes, including food enrichment and security.

Despite the awareness of this new strategy in and around the globe, there is a paucity of such data from Nigerian fermented foods despite assortments of fermented food production in Nigeria. Therefore, this study was designed to isolate LAB from locally fermented *Parkia biglobosa* (locust bean) and *Manihot esculenta* (cassava) and assess their *in vitro* antimicrobial and vitamins producing potentials.

2. Methods

2.1. Isolation of lactic acid bacteria

Forty (40) samples of fermented foods comprising of *P. bigblosa* (fermented locust beans) ($n = 10$), cheese ($n = 10$), uncooked fermented cassava (*M. esculenta*) ($n = 10$), and cooked cassava (fufu) ($n = 10$) were collected consecutively for a period of 3 months (June to August 2018) in various locations in Lagos and Ogun states. Samples were collected at these periods in sterile containers and immediately transferred to the Biotechnology Laboratory of the Nigerian Institute of Medical Research, Lagos, for immediate analysis. Ten (10) fold serial dilutions were made from the initial sample suspension (1 g) in ten (10) ml of sterile distilled water followed by inoculation by pour plate into sterile de Man, Rogosa and Sharpe (MRS) (Oxoid, UK) agar plates, and incubated micro-aerophilically for 48 h at 37 °C. Preliminary identification was carried out on single colonies using colonial morphologies such as Gram staining, catalase reaction and oxidase test. All Gram positive, catalase and oxidase negative, non-spore forming, large white/milky, creamy colonies were sub cultured for purity. Pure cultures of the isolates were obtained by streaking on MRS agar and isolates stored in 20% MRS-glycerol broth at −20 °C until further use.

2.2. Cell-free supernatant (CFS) preparation

The cell-free supernatant (CFS) was obtained by subculturing the LAB into MRS broth, incubated as stated above (at 37 °C for 24 h, microaerophilically). The cultures were then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were further sterilized by filtration through a sterile 0.22 μm membrane filter (Merck, Germany).

2.3. Evaluation of the antimicrobial effect of suspected LAB

Antimicrobial activities of the preliminarily identified LAB isolates fermented broths were demonstrated on agar well diffusion as previously described with modifications [11]). The test bacteria, which included the clinical strains of *S. typhimurium* NIM 2020 and *E. coli* NIM 3030 previously isolated, identified and stored for this purpose in the culture bank of the Molecular Biology & Biotechnology Department of the Nigerian Institute of Medical Research, were sub-cultured in *Salmonella-Shigella* and MacConkey agar respectively. After 24 h of incubation, single black and pink colonies were suspended in sterile normal saline to make 0.5 Mac Farland standard (approx. 10^8 CFU/ml) and swabbed on Mueller Hinton agar (Oxoid). With the use of a standard sterile cork borer, 6 mm diameter wells were made on the inoculated solidified agar plates, and a 100 μl of each CFS from isolated LAB were applied to the wells and allowed to stand for at least an hour before incubation in air atmospheres at 37 °C for 24 h. *Lactobacillus rhamnosus*GG was used as the positive control. Finally, inhibition zone diameter was measured after 24 h incubation. The LAB strains showing clear zones of inhibitions were further identified by sequencing the 16S rRNA genes.

2.4. Vitamins assays using high-performance liquid chromatography (HPLC)

Standard preparation: The reference standard for each vitamin was weighed (10 mg) and transferred into a 10 ml volumetric flask, and 6 ml of diluent (10% acetonitrile in water) was added. The contents were shaken to dissolve and made up to obtain a stock concentration of 1 mg/ml. This was used to prepare graded concentrations of mixed and separate standards to determine retention time and calibration curve.

Vitamins production and quantification of the isolated strains in this study were carried out according to the method of LeBlanc et al. [9] with modifications. Briefly, the CFS obtained from an overnight broth culture of LAB was reacted with chilled acetonitrile in the ratio of 1:2 to precipitate protein. After vortex mixing and sonication in an ultrasonic bath, the mixture was centrifuged at 10 min at 4 °C and membrane filter (0.22 μm pore size) sterilized before HPLC. The resulting supernatant aliquots were syringe filtered (0.45 μm) and injected at a 0.5 ml/min flow rate. The analysis, performed on the Agilent HPLC machine was driven by chemo station software

under mobile phase (6 mM of tetraoxosulphate (VI) acid solution at a flow rate of 0.6 mL/min with the column operating at 55 °C, pH 3.6, UV) at 280 nm wavelength, ambient temperature with Zorbax eclipse XDB T8 150/4.6 mm 5 μm column. The sterile MRS broths treated the same way as the test, but without LAB inoculation, served as controls.

2.5. Amplification and sequencing of the 16S rRNA genes of the selected isolates

Isolates with a consistent and appreciable production of vitamins and antimicrobial properties were selected for species identification which was carried out by sequencing the amplified 16S rRNA region. Briefly, LAB isolates were grown in MRS broth for 18 h at 37 °C, after which the DNA was extracted using the DNA extraction kit (Qiagen USA) following the manufacturer's instructions. The set of primers (0.2 μl each); BSF- 8 (51-AGAGTTTGATCCTGGCTCAG-31) and BSR-534 (5^1- ATTACCGCGGCTGCTGGC- 3^1) were employed [12] and 4 μl of 5× HOT Firepol master mix (Solis biodyne, Estonia) added to each PCR reaction tube in which 2 μl of template DNA has been added. The reaction mixture was made up to 20 μl with DNAse-free distilled water.

The PCR was carried out in a vapo. protect (Eppendoff, Nexus series, Germany) Mastercycler, at the following cycling parameters; initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30s, and elongation at 72 °C for 30s.The PCR product (5 μl) was loaded alongside a 100bp marker (Thermo Scientific, USA) on a 1% agarose stained with ethidium bromide (10 μl of 1 mg/ml) and electrophoresis run for 1hr at 100V (Bio-Rad, USA). The gel was viewed under ultraviolet (UV) ray in a gel photo documentation system (Clinix, China).

The amplicons showing bands corresponding to 526 bp were sent to inqaba Biotech, South Africa for commercial 16S rRNA sequencing. The sequence results were edited using the SeqManTM II program for the contig and peak verification. The nucleotide sequences generated were aligned with the ones in GenBank of the National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) algorithm. Multiple alignments and phylogenetic relationships with other species of *Lactobacillus* were performed using the ClustalX 2.1 program and MEGA version X program with the Neighbor-joining hood method [13].

2.6. Statistical analysis

The production of vitamins among the LAB at 24 and 48 h was compared at a significant level ($p < 0.05$) using the two-way analysis of variance (ANOVA).

3. Results

Different samples of fermented foods (cassava, locust beans, and cheese) assayed for the presence of LAB gave different numbers of isolates. The mean viable counts of LAB present in the food samples was 2.5×10^5, with microbial values ranging from 1.8×10^5 to $3.0 \times 10^{6.}$ The total viable count for uncooked cassava has 3.0×10^6, while fermented locust beans have 2.8×10^6. The least count was obtained from cheese 1.8×10^5 (Table 1). The isolates were initially identified based on cell morphology, gas production, and Gram reactions. There were mixtures of Gram-positive rods and cocci amongst the isolated LAB. Forty-three identified LAB isolates were recovered from the various samples and were assigned laboratory codes pending species identification. Uncooked fermented cassava has the highest number of isolates of 19, while the cooked cassava flour (fufu) yielded no growth. Fifteen isolates (15) were recovered from locust beans, while 9 isolates were recovered from cheese (Table 1).

The antimicrobial activities of the LAB CFS against the two gastrointestinal bacterial pathogens are shown in Table 2. Out of the total LAB investigated, 5 (12%) LAB isolated from fermented locust beans were active against *S. typhimurium* NIM 2020 and *E. coli* NIM 3030 while the remaining isolates were either not active or showed narrower zones of inhibition for any one of the tested pathogens. In this study, higher susceptibility to the LAB CFS was observed for *E. coli* NIM 3030 than *S. typhimurium* NIM 2020. All other LAB with no activities or appreciable zones of inhibitions were excluded from further studies.

3.1. Vitamins production

The vitamins contents produced after 24 h at 37 °C ranged from 12.23 to 801.79 μg/ml (Table 3) and 7.21–241.05 μg/ml at 48 h (Table 4). At 24 h, folic acid (vitamin B9) and vitamin B12 were produced at the highest concentration of 310.55 and 801.79 μg/L, respectively. The concentrations of B1+B2 produced at 24hr were very low compared to folic acid and vitamin B12 at 24 h. The standard controls were 275.18 and 88.41 μg/ml for folic acid and vitamin B12, respectively, at the same hours of observation. After 48 h, the concentration of folic acid was reduced to 241.05 μg/ml while B12 was reduced to 105.19 μg/ml. The concentration range after

Table 1
Population distribution of lactic acid bacteria from various fermented foods.

Sample	LAB Isolate	CFU/g	No. Isolates in total
Locust beans	*L. fermentum*	2.8×10^6	15
Cassava flour (cooked)	No growth	–	–
Cheese	*Lactobacillus* spp	1.8×10^5	9
Uncooked cassava	*Lactobacillus* spp	3.0×10^6	19

Table 2
Antimicrobial activities of cell-free-supernatant of the samples against test bacteria.

[a]Zone of Inhibition (mm)		
Strain	*E. coli* NIM 2020	*S. tyhimurium* NIM 3030
L. fermentum MT903311	**14**	**13**
Lactobacillus spp BOT 18	**13**	**11**
Lactobacillus spp BOT22	**14**	**10**
Lactobacillus spp BOT 08	**13**	**10**
L. fermentum MT903312	**14**	**11**

[a] Cork borer = 6 mm diameter.

Table 3
Concentration of vitamins present in each sample after 24 h.

LAB STRAINS *Concentration (ug/ml)	**B1+B2[a]**	**SD[b]**	**B3[c]**	**SD**	**Folic acid**	**SD**	**B12[d]**	**SD**
Lactobacillus BOT 18	15.13	+0.03	24.45	0.26	120.37	0.57	12.23	0.34
Lactobacillus BOT 22	89.49	+0.03	37.91	0.59	253.08	0.26	53.72	0.53
L. fermentum MT 903311	98.86	+0.02	39.93	0.53	310.55	0.67	801.79	0.65
Lactobacillus BOT 08	29.18	+0.02	39.93	0.74	287.11	0.74	320.80	0.38
L. fermentum MT 903312	87.82	+0.02	61.74	0.87	384.26	0.95	503.06	0.39
Control[e]	16.43	+0.02	0.87	0.03	275.18	0.36	88.41	0.31

Table 4
Concentration of vitamins present in each sample after 48 h.

LAB strains	Concentration (μg/ml)							
	B1+B2[a]	SD[b]	B3[c]	SD	Folic acid	SD	B12[d]	SD
L. fermentum MT 903311	29.95	0.04	42.76	0.02	241.05	0.05	67.03	0.04
Lactobacillus BOT 18	15.17	0.02	16.21	0.02	123.33	0.78	18.67	0.14
Lactobacillus BOT 22	13.14	0.07	20.15	0.02	135.73	0.40	17.99	0.54
L. fermentum MT 903312	22.05	0.30	24.62	0.06	142.85	0.59	105.19	0.03
Lactobacillus BOT 08	18.81	0.06	20.99	0.16	119.95	0.93	7.21	0.05
[e]Control	10.59	0.01	0.87	0.01	−1.27	0.01	133.86	0.01

[a] Vitamins B1 and B2 (Thiamin and Riboflavin).
[b] Standard Deviation.
[c] Vitamin B3 (Niacin).
[d] Vitamin B12 (Cyanocobalamin).
[e] Sterile MRS broth incubated as test cultures.
[a] Vitamins B1 and B2 (Thiamin and Riboflavin).
[b] Standard Deviation.
[c] Vitamin B3 (Niacin).
[d] Vitamin B12 (Cyanocobalamin).
[e] Sterile MRS broth incubated as test cultures.

48 h was between 7.21 and 241.05 μg/ml. Consistent high vitamins production was common with only two strains of *L. fermentum* isolated from locust beans compared with other investigated strains. This trend of increase in vitamins production was more pronounced at 24 h compared to 48 h of incubation (Test statistics, 0.665, CI 95%). Overall vitamin production by these two *L. fermentum* strains were seen to be higher than the control at 24 and 48 h, respectively.

3.2. 16S rRNA sequencing and phylogenetic analysis

After the sequence characterization and identification, the two strains were identified by sequencing of their 16S rRNA and showed 100% and 99.4% identities as *L. fermentum* and were assigned with ascension numbers MT903311 and MT903312 and have been deposited in GenBank Database maintained by the NCBI. These two are clades belonging to common ancestry (cluster) with *L. fermentum* strain NBRC 15885 and *L. fermentum* strain CIP 102980 (Fig. 1).

3.3. Statistical analysis

Statistical analysis showed the test statistic FA equals 0.665, at 95% confidence interval region of acceptance: [-∞: 1.9233]. Hence there was a significant difference in the production of the vitamin between 24 and 48 h of investigation.

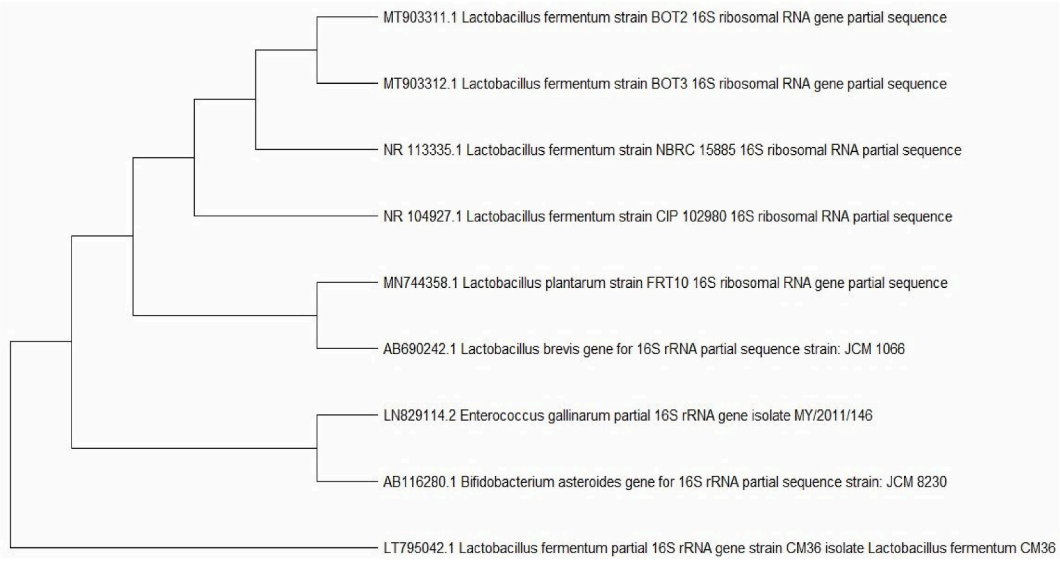

Fig. 1. Phylogenetic affiliation of *L. fermentum* MT903311 and MT903312 against other related species of Lactobacillus.

4. Discussion

We isolated LAB from various fermented foods to identify strains capable of inhibiting foodborne pathogens and endowed with extracellular production of vitamins as a form of food supplements. The total LAB detected among the investigated fermented foods in this study was in the range of 1.8×10^5 to 3.0×10^6 CFU/g. It was observed that uncooked cassava (*M. esculenta*) had the highest load of LAB followed by locust beans (*P. bigblosa*) while the cooked cassava flour popularly known as fufu yielded no growth. These observations reiterated the fact that cooking reduces the population of microorganisms, of which beneficial LAB are not exempted in such foods. Tamang et al. [14] previously reported LAB in a range of 10^7–10^9 from traditionally fermented vegetable products of the Eastern Himalayas, while Masuda et al. [15] reported a range of 10^7–10^8 from Japanese pickles. The low range of LAB from these studies may be due to differences in fermented foods studied and the processes involved. *Lactobacillus fermentum* was prevalent among the phenotypically identified isolates and based on the 16S RNA sequencing carried out on the most active strains. The dominance of *L. fermentum* in Nigerian fermented foods has been previously reported [16,17]. The result of the present study agrees with previous studies indicating higher prevalence of *L. fermentum* isolation from Nigerian fermented foods.

According to the agar well diffusion method, the CFS of *L. fermentum* strains showed antagonistic activities against the tested *E. coli* and *S. typhimurium* used in this study. Some previous studies on LAB isolated from fermented foods have also shown the antagonistic activities of CFS of LAB against *Samonella* sp. and outbreak strains of *Vibrio cholerae* O1 [4,18],. Interestingly, only *L. fermentum* MT 903311 and L. *fermentum* MT 903312 isolated from fermented locust beans in the present study showed the highest antagonistic activities against the tested pathogens, especially on *E. coli* (Table 2). The test pathogen selected for this study was based on their history of foodborne-related outbreaks and reports implicating them in various human infections [19]. Therefore, the result of this study suggests the LAB species from fermented food produces antimicrobial metabolites and may prevent the growth of these pathogens if present in the food and may consequently reduce foodborne infections. Food preservatives effect of LAB strains has been previously established and reported to be due to the presence of active metabolites such as bacteriocins, fatty acids, and other inhibitory substances that act by reducing the pH of the environment, inhibiting a variety of metabolic functions, and interfering with cell membrane potentials [20,21].

Strains with high extracellular production of vitamins and antimicrobial properties were identified in this study. Reports on LAB species and *Bifidobacterium* found in human guts producing vitamins in large quantities have been previously documented [22]. Additionally, the consumption of probiotics has also been shown to be effective in the treatment of various medical conditions such as colorectal cancer, lactose intolerance, gastroenteritis, genitourinary tract infections and for the prevention of colon cancer tumor [23, 24].

To the best of our knowledge, this is the first LAB report with antimicrobial properties against foodborne bacteria producing folates from fermented locust beans in Nigeria. It should be mentioned that all the vitamins assayed for in this study are of B types which are essential for the maintenance of body's homeostasis by playing major roles in metabolic processes such as energy production and red blood cell formation. The concentration of extracellular folate (310.79 μg/ml) and vitamin B12 (801.79 μg/ml) produced by *L. fermentum* strains isolated from locust beans in 24, and 48 h (Tables 3 and 4) were higher than the control standards in the present study. In a similar study carried out by Masuda et al. [15], the folate concentration produced (100 μg/L) was lower than the concentration observed in this study. However, the method and samples used in both studies were different, while the folate production in this study as compared with the standard, is lower than a previous study from Iran, which describes the production of vitamins B3, B6,

and B9 from fermented yogurt as high as 1566 μg/L [25]. It was observed in the present study that *L. fermentum* strains isolated from the fermented locus beans produced the highest concentration of the vitamins at 24 h fermentation which may be the optimal period for the production, although the fermentation was terminated at 48 h. This highest production may be LAB and substrate specific, for instance, Ngene et al. [26] reported the highest production of similar vitamins from traditional fermented Nigerian foods (Yoghurt, ogi, ogiri and ugba) by *L. plantarum* to be between 5.04 and 5.88 μg/100 ml at 24 h fermentation. In the present study, production up to 801.79 μg/ml was seen from fermented locust beans. It should be noted that this study employed ultra-sensitive methods of quantification (HPLC) and the LAB was identified using molecular methods rather than the spectrophotometric and phenotypic methods used by Ngene et al. [26]. The study suggests that the LAB obtained from the locust beans (*P. biglobosa)* are efficient producers of vitamins and can serve as food supplements for human consumption. *Parkia biglobosa* seed is a rich food seasoning obtained by fermentation. It is popularly known as *Iru* amongst the Yoruba people in Southwest Nigeria and known as *dawadawa* among the Hausa in the Northern Nigeria and Ashanti tribes of Ghana. Although the whole plant has been reported to possess certain health benefits [27,28], however from the results of the study, it was observed that the fermented seed is a rich source of support for LAB colonization with antimicrobial properties and at the same time producing vitamins which suggest it as a rich source of food additives. As a limitation, this study was unable to investigate samples from different parts of the country to ascertain the resident LAB communities and their vitamins producing abilities in Nigerian fermented foods. Another limitation of the present study is our inability to assay for other vitamins aside vitamin B-complex and this is due mainly to the unavailable reference standards and funding.

The two identified LAB species in this study produced folates at varying concentrations within 24–48 h of investigations. As it was also previously observed by Masuda et al. [15], the vitamins were produced at different levels by different LAB strains in the present study, and the production appears to depend on the strains but not species. The analysis of variance in the vitamins production between the 24 and 48 h among the LAB strains showed a significant difference at 95% confidence interval. The Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences constructed also showed the two strains are clades belonging to common ancestry (cluster) with *L. fermentum* strain NBRC 15885 and *L. fermentum* strain CIP 102980 despite being isolated from the same source of fermented food. This further suggests *L fermentum* ancestry compared to other isolated LAB in this study may possess more biotechnological potentials and antimicrobial activities based on the study results.

5. Conclusion

This study has demonstrated interesting biotechnological potentials of *L. fermentum* MT903311, MT903312 isolated from fermented locust beans in Nigeria. It is well noted that there are mandatory efforts in certain countries to fortify certain food substances with synthetic vitamins, while efforts to replace these synthetic supplements with naturally occurring ones because of the possibility of unwanted side effects associated with synthetic supplements are ongoing. The present study provides additional evidence of naturally occurring food supplements generally regarded as safe obtainable with LAB, suggesting an economic alternative to food enrichments. The findings in this study would pave the way for the use of natural additives as food supplements with future biotechnological use of these strains.

Author contribution statement

Bamidele Tolulope Odumosu: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tajudeen Akanji Bamidele: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Daniella Williams Ofem, Friday Agbozo: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Grace I. Olasehinde: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data associated with this study has been deposited at NCBI under the accession number [MT903312 & MT903311].

Declaration of competing interest

None.

Acknowledgements

The authors wish to acknowledge Covenant University Centre for Research Innovation and Development for the payment of Article Processing Charges.

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