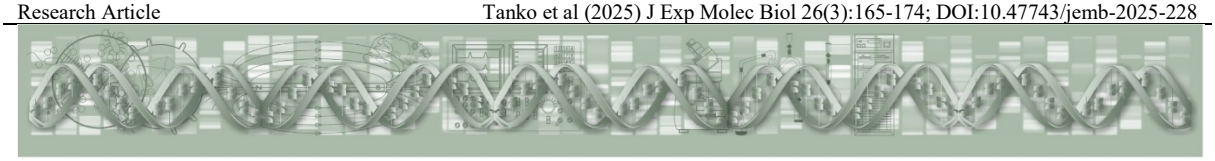

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF STARTER CULTURES IN THE CONTROLLED FERMENTATION OF CABBAGE AND SOYBEANS

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Abstract

The aim of this study was to characterize starter cultures of LAB (lactic acid bacterium) and *Bacillus* spp. for controlled fermentation of cabbage and soybeans to achieve desired products. Cultured isolates were studied morphologically and revalidated using biochemical and molecular methods. Extracted DNA was amplified using two specific primers. Banding pattern at 1500bp showed the presence of LAB and *Bacillus subtilis* DNA amplified. La57 primer amplified amino acid antiporter gene that identified LAB while ENIF primer amplified endoglucanase gene that identified *B. subtilis.* The16S ribosomal RNA was sequenced using the Sanger's method for strain specific identification. As a result, the test organisms were morphologically identified as *Pediococcus* spp and *B. subtilis.* They differed in the type enzyme production (4:7 respectively). Sequence alignment identified them as *Pediococcus pentosaceus* strain DSM20336 (LAB) and *B. subtilis* subsp. *subtilis* strain 168. This is the novelty in this work. Results showed that *P. pentosaceus* induced ferments contained the least number of isolates in cabbage (*Lactobacillus* spp., *Streptococcus* spp. and *Pediococcus* spp) and soybean (*Bacillus* spp., *Streptococcus* spp. and *Pediococcus* spp) unlike in spontaneous fermentation where 8-9 bacterial isolates were recorded. The study identified potential fermenting strains of bacteria that could be employed as potential starter culture in the industrial fermentation of vegetable and legume foods to boost food security in Nigeria. These findings have industrial and economic benefits.

Keywords: Characterization, Starter Culture, Fermentation, Vegetable, Legume, Food security

Introduction

Industrial fermentation of foods has played major roles in boosting global food security. This is achieved through preservation and conversion of plant materials into diverse edible products that are available for people to choose from. Also, controlled fermentation increases the nutritional values of products (Walters et al. 2016).

In addition to traditional methods, modern techniques such as molecular characterization are now trending in the analysis of fermenters. They are concerned with specific identification of microbial strains and the genes responsible for fermentation of a particular food (Vossen et al. 2019). Techniques in extraction of genomic and plasmid DNA, PCR (Polymerase Chain Reaction), gradient gel electrophoresis, and 16s rDNA sequencing are employed to characterize different type food fermenters (Walters et al. 2016, Hui et al. 2017, Vossen et al. 2019). Characterization of isolates by using DNA fingerprinting electrophoresis has revealed genetic variability within highly heterogeneous species (Swain et al. 2014). Genetic variability has been

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reported within a species of fermenting bacteria (Li et al. 2021). Knowledge of gene technology and genomics has played a major role in identification of new strains with potential fermenting capability of products and their use as probiotics (Li et al. 2021).

Identification of strains and variants of fermenting species of bacteria is very crucial in fermentation technology because of the need to ensure proper selection of starter culture needed to achieve desired results. Morphological identification of fermenters, that combines cultural and biochemical characterization, has been explored in this regard among authors (Emkani et al. 2022). This type of microbial systematic evidence provides baseline data of microbial identification that is limited to generic level and species taxa. However, starter culture needed in an elaborate fermentation of commercial product requires high level of microbial specificity and accuracy beyond species taxon. LAB and *Bacillus* spp. have been employed in the fermentation of vegetable and legume foods. However, lack of identity of strain specific variants make controlled fermentation experiments difficult to achieve. The aim of this study was to characterize starter cultures of a LAB and *Bacillus* spp. (in the controlled fermentation of cabbage and soybeans) using biochemical and molecular methods.

It was designed to explore the enzyme production differences and gene sequencing of 16s rDNA to identify the starter cultures to strain taxon.

Materials and Methods

Sample collection and preparation

Cabbage leaves and soybean seeds were purchased in sufficient quantities from major markets within the State Capital. All materials were packaged using sterile bags and transported to the laboratory for further processing and analysis. Cabbage leaves were cleaned by removing the damaged outer leaf cover. These were shredded after washing with sterile distilled water. Hydrated soybean seeds were dehulled manually by rubbing the seeds multiple times with the palms to get them ready for fermentation (Ahmad et al. 2015).

Source of organisms

LAB and *Bacillus* spp. were isolated from vegetable and legume sources. Stock cultures were also collected from the Microbiology Laboratory, Joseph Sarwuan Tarka University Makurdi, Nigeria. Sub-culturing was done on MRS agar using spread plate method followed by cultural characterization (Du et al. 2018).

Biochemical characterization of isolates

Isolates were revalidated using standard biochemical tests including Gram staining and enzyme production. The following tests were carried out: catalase, citrate utilization, urease, indole, amylase and gelatinase production tests using standard protocol as given by Florindo et al. (2018). Other enzyme production tests carried out were invertase, cellulase, lipase, phytase, protease and esterase tests. The bacteria isolates confirmed by biochemical tests were sub-cultured on Luria-Bertani (LB) media and incubated at 37℃ overnight from which broth cultures were prepared for further revalidation and characterization using molecular techniques (Pino et al. 2018).

Extraction of genomic DNA

Protocols described in Du et al. (2018) and Arteaga et al. (2021) were used. Bacterial DNA was extracted using boiling method. Exactly 1.5ml of the sample broth was centrifuged at 10,000rpm for 5 min. The supernatant was decanted while the pellets were washed twice with 200µL sterile deionized water. This was followed by homogenization step and boiling in a water bath at 100^{o}C for 10 min, while further centrifugation took place at 12,000 rpm for 5 min. The supernatant containing the DNA was transferred to another test tube and stored at -20℃. Purity of the extracted DNA was checked on a spectrophotometer at 280nm wavelength, using

quantitative approach that determines the quantity of DNA extracted. This served as the template for PCR (Du et al. 2018, Arteaga et al. 2021).

Polymerase chain reaction (PCR)

This step amplified the extracted DNA (genomic and plasmid) of test isolates for a more precise identification than biochemical tests. The protocols outlined in Du et al. (2018) were used. Amplification was carried out on a thermocycler loaded with 50 µl reaction volumes containing; 25 ul Dream Taq Master Mix (Thermo Fisher Scientific, USA), 15µl of nuclease free water, 2.5µl of each primer and 2.5 µl of extracted bacterial DNA. The primers used in the amplification and their sequences are given in Table 1. The Amplification was done in 35 cycles with an initial denaturation at 95 ℃ for 5 min. followed by a denaturation step of 95 ℃ for 2 min, primer annealing at 55ºC for 30s and primer extension at 72℃ for 1 min, with the final extension at 72 ℃ for 10 min. The amplified DNA products were loaded on electrophoresis machine (64 V for 2 h) in a 1.5% agarose gel medium stained with ethidium bromide. Lamda DNA Hind III Marker was used as DNA molecular weight marker. Separated DNA bands were visualized on a UV trans-illuminator.

Gene sequencing and strains identification

All PCR products were purified with Exo sap for Sanger Dideoxy sequencing for determination of nucleotide arrangements in the genes and specific identification of strains of fermenting species of *Bacillus* and LAB. Variable regions within the 16s rRNA (ribosomal RNA) regions were sequenced. This was compared with known 16s rRNA sequence at National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLASTn) algorithm using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Identification of the sequences at both genus, species and strain level were defined as a 16s rRNA sequence similarity at between 95- 100% with that of the phenotype strain sequence in GenBank. (Du et al., 2018).

Table 1. List of Specific Primers for PCR

Primer	Gene function	Sequence	Base pair	Target
La57	Amino acid antiporter gene	F- GGTCGGGGGATCTGAAAAGA R- GATTTGGGCAAGCACATTGG	274	LAB (lactic acid bacteria)
EN1F	Endoglucanase gene	F-CCAGTAGCCAATGGCCAGC R-GGAATAATCGCCGCTTTGTGC	124	*Bacillus subtilis*

(Du et al. 2018, Arteaga et al. 2021)

Identification of bacterial species in fermented products

Controlled fermentation of cabbage and soybean experiments were set up using the characterized isolates as starter cultures. Spontaneous fermentation and bacterial induced fermentation were allowed to progress simultaneously. Morphological observations were recorded on the agar culture media. These included the colour and outline of the colony of each bacterium. Motility test was done by adding a drop of peptone water on a glass slide containing bacterial colony covered with a slip and viewed under the microscope with high power objective lens (Cheesbrough 2006). Serial dilution, pour plates techniques and incubation (37ºC for 24 hours) methods employed (Cheesbrough 2006). Visible colonies on the plates were counted using Colony Counter. Biochemical methods were used in analysis of bacterial species present in fermented products at week 4 of the experiment (Li et al. 2019) as previously discussed.

Results and discussions

Table 2 gives the morphological identification of isolates based on cultural and microscopic characterizations as well as responses to Gram's staining. Grey, circular Gram-positive rods that possessed smooth entire edge were typical of *B. subtilis.* Opaque, and circular Gram-positive rods with smooth entire edges were characteristics of *Pediococcus* species. Cultural characteristics of the isolates were in tandem with previous findings on morphologies of the test and standard cultures (Li et al. 2019). However, systematic resolutions were delimited to the genus taxon in *Pediococcus* and species taxon in *B. subtilis*. The study aligns with the use of cultural characteristics as fundamental steps, not only in providing pure culture for further analysis, but also in the determination of identity of the collected bacterial samples (Kiczorowski et al. 2022). Cultural characterization provides baseline information in microbial analysis of samples. All the LAB and *B. subtilis* were first identified using their outline, colour, transparency and elevation.

Table 2. Cultural and Microscopic Morphology of Bacterial Sample

Organism	Morphology	Gram reaction	Outline	Status
B. subtilis	Grey, Circular, Smooth, entire edge	+	Rods	Test colony
Pediococcus species	White/opaque circular and entire, tops of colonies were raised, convex or umbonate.	+	Rods	Test colony

Further biochemical characteristics re-validated the colonies according to their reactions to catalase, urease, citrate and indole tests (Table 3). *B. subtilis* colonies were positive to catalase and indole tests. Out of ten (10) enzymes screened for the test isolates (Table 4), *Pediococcus* spp produced 4 enzymes (amylase, lipase, phytase and protease) while *B. subtilis* produced 7 enzymes (catalase, urease, amylase, cellulase, lipase, phytase and gelatinase). The validity of the bacterial cultures confirmed through the use of basic biochemical tests gave credence to the identity of the LAB and *B. subtilis* tested. This aligns with standard bacteriological practices because it takes advantage of the biochemical and physiological responses of the isolates to certain chemical compounds. Unlike the cultural method of identification, biochemical methods are more objective and reliable (Adedokun et al. 2016, Pino et al. 2018, Taddia et al. 2019). Among other biochemical tests, enzyme production is a good biochemical parameter that distinguishes the test isolates.

Enzymes are organic catalysts that speed the rate of metabolic reactions in all living cells including microbial cells. The presence of functional enzymes is crucial in the formation of fermented food products. Production of these enzymes is an indicator of fermentative ability of the test isolates. This is because fermenters require functional enzymes to carry out food fermentation processes through hydrolysis, liquefaction, saccharification, and modification of diverse food types (Taddia et al. 2019). The above explanation probably accounts for the fermentation of cabbage and soybeans by LAB and *B. subtilis* used as starter culture in this work. According to Akanni et al. (2018), LAB produce lactic acid, organic acids and carbon dioxide during enzyme-based fermentation while *B. subtilis* hydrolyze proteins to form constituent peptides, amino acids and ammonia in alkaline fermentation. Fermentation enhances food digestibility and the level of vitamin, amino acids and minerals (Taddia et al. 2019).

Table 3. Biochemical Characteristics of Test and Standard Isolates

S/N	Biochemical tests	*B. subtilis*	*Pediococcus* species
1.	Catalase	+	-
2.	Urease	-	-
3.	Citrate	-	-
4.	Indole	+	-

Key: + = Positive, - = Negative

Table 4. Screening for Enzyme Production in Test Isolates

	Enzyme	*Pediococcus* spp.	*B. subtilis*
1	Catalase	Negative	Positive
2	Urease	Negative	Positive
3	Invertase	Negative	Negative
4	Amylase	Positive	Positive
5	Cellulase	Negative	Positive
6	Lipase	Positive	Positive
7	Phytase	Positive	Positive
8	Protease	Positive	Negative
9	Esterase	Negative	Negative
10	Gelatinase	Negative	Positive
Total		4 enzymes	7 enzymes

The two test isolates were confirmed true as shown in the agarose gel image that revealed DNA bands of the two bacteria (Plate 1). Banding pattern at 1500bp showed the presence of LAB and *B. subtilis* DNA amplified using two different specific primers. La57 primer amplified amino acid antiporter gene that identified LAB while ENIF primer amplified endoglucanase gene that identified *B. subtilis.* The sequence alignment of 16S ribosomal RNA of the test strain identified the LAB as *Pediococcus pentosaceus* strain DSM20336. It was assigned sequence identification number of 343201332|NR_042058.1. It contained 910 bits (1008) with 94% identity and 0% gaps. Sequence alignment of 16S ribosomal RNA of the test strain identified the *B. subtilis* as *B. subtilis* subsp. *subtilis* strain 168. It was assigned sequence identification number of 1269801457|NR-102783.2. It contained 426 bits (472) with 99% identity and 0% gaps.

Molecular characterization provides a more reliable confirmation and re-validation of test isolates at the gene level. This also confirms the presence of two genes (La57 coding for amino acid antiporter gene and EN1F gene coding for endoglucanase enzyme) needed in lactic acid and alkaline fermentation carried out by LAB and *B. subtilis* respectively. Starter culture needed in an elaborate fermentation of commercial product requires high level of microbial specificity and accuracy beyond species taxon (Walters et al. 2016, Hui et al. 2017). Identification of strains and variants of fermenting species of bacteria is very crucial in fermentation technology because of the need to ensure proper selection of starter culture needed to achieve desired results. In this work, sequence alignment of 16S ribosomal RNA of the test strain confirmed the LAB as *Pediococcus pentosaceus* strain DSM20336 and *B. subtilis* subsp. *subtilis* strain 168. These strains may offer potential use as probiotics in line with the recommendations of Vossen et al. (2019).

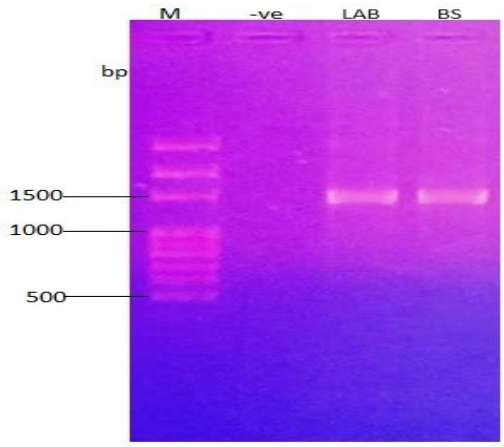

Plate 1. Agarose Gel Image of DNA Bands of LAB and *B. subtilis* Amplified by La57 and ENIF primers respectively
Legend: LAB (lactic acid bacteria); BS - *B. subtilis*

Figure 1 shows the number of bacterial isolates in fermented cabbage from week 1 to 4 of the experiments. Isolates consisted of 5-8 species in the first three weeks. In the final product (week 4), spontaneous fermented cabbage contained a total of 8 bacterial species of the genus *Bacillus, Escherichia*, *Staphylococcus*, *Pseudomonas*, *Micrococcus*, *Corynebacterium*, *Lactobacillus* and *Salmonella* (Table 5). Fermented cabbage with mixed culture of *Bacillus* + *Pediococcus* starter cultures contained 5 species: *Corynebacterium* spp., *Bacillus* spp., *Pediococcus* spp., *Leuconostoc* spp. and *Streptococcus* spp. Fermented cabbage with *B. subtilis* as starter culture contained 4 species of bacteria including *Bacillus* spp., *Streptococcus* spp., *Pediococcus* spp. and *Leuconostoc* spp. The 3 bacteria isolated from Pediococcus-ferment were *Lactobacillus* spp., *Streptococcus* spp. and *Pediococcus* spp.
There were evidences that unwanted bacteria were eliminated in cabbage and soybean ferments inoculated with single and mixed test strains especially when *P. pentosaceus* was applied as starter culture, thus confirming the antibacterial effects of the ferments. Results showed a clear elimination of *Escherichia*, *Staphylococcus*, *Pseudomonas*, *Micrococcus*, *Corynebacterium* and *Salmonella* found in products of spontaneous fermentation. These organisms were known to cause food spoilage and gastroenteric diseases. The present study conforms with the outcome of other studies since reports have shown that fermented vegetables and legumes may contain diverse micro-organisms strains of fermenting bacteria (Akanni et al. 2018, Kiczorowski et al. 2022).

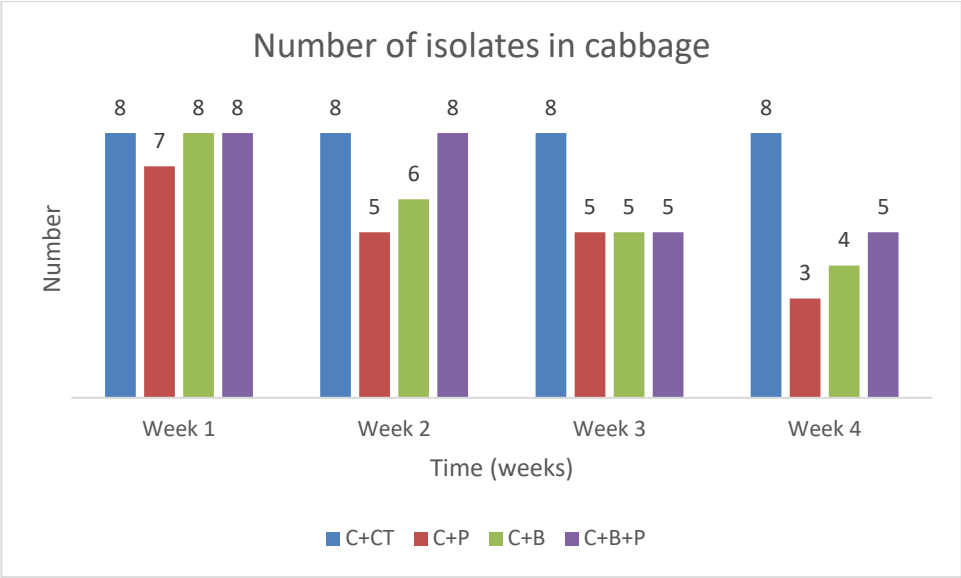

Figure 1. Number of Bacterial Isolates in Fermented Cabbage

Legend: C+CT= Control experiment (spontaneous fermentation); C+P = Cabbage + *Pediococcus*; C+B = Cabbage + *Bacillus*; C+B+P = Cabbage + *Bacillus* + *Pediococcus*

Table 5. Isolated Bacterial Species in Fermented Cabbage at Week 4

Treatments	C+CT	C+P	C+B	C+B+P
1	*Bacillus* spp.	*Lactobacillus* spp	*Bacillus* spp	*Corynebacterium* spp
2	*Escherichia coli*	*Streptococcus* spp	*Streptococcus* spp	*Bacillus* spp
3	*Staphylococcus* spp	*Pediococcus* spp	*Pediococcus* spp	*Pediococcus* spp
4	*Pseudomonas* spp		*Leuconostoc* spp	*Leuconostoc* spp
5	*Micrococcus* spp			*Streptococcus* spp
6	*Corynebacterium* spp			
7	*Lactobacillus* spp			
8	*Salmonella* spp			
Total isolates at week 4	8	3	4	5

Legend: C+CT= Control experiment (spontaneous fermentation); C+P = Cabbage + *Pediococcus*; C+B = Cabbage + *Bacillus* C+B+P = Cabbage + *Bacillus* + *Pediococcus*

Figure 2 shows the number of bacterial isolates in fermented soybeans from week 1 to 4 of the experiments. Isolates consisted of 4-9 species in the first three weeks. In the final product (week 4), spontaneously fermented soybean contained a total of 9 bacterial species of the genus *Bacillus, Escherichia*, *Staphylococcus*, *Pseudomonas*, *Micrococcus*, *Corynebacterium*, *Lactobacillus*, *Salmonella* and *Streptococcus* (Table 6). There were 5 species in two starter culture treated ferments: Bacillus+Pediococcus (S+B+P) ferment and Bacillus-ferment (S+B).

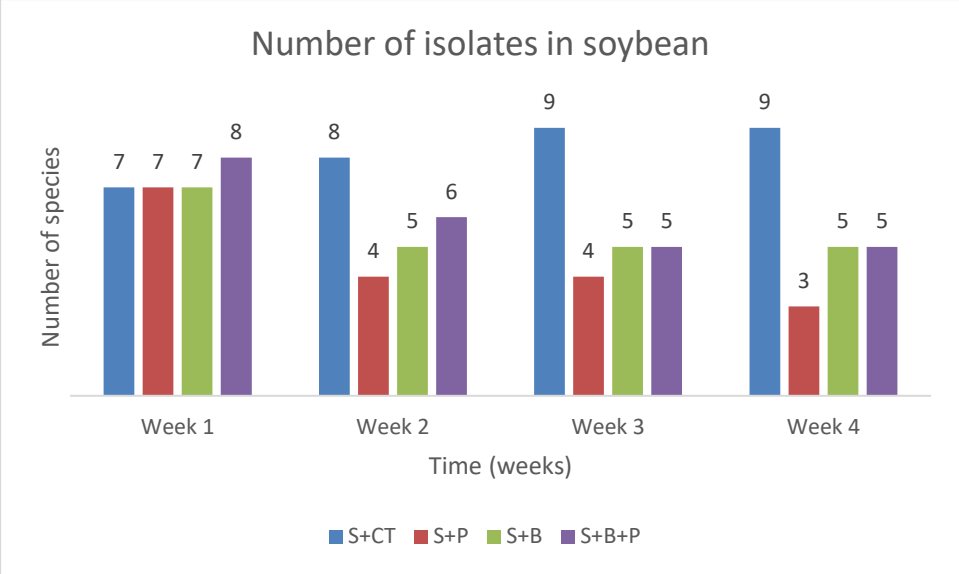

Figure 2. Number of Bacterial Isolates in Fermented Soybean

Legend: S+CT= Control experiment (spontaneous fermentation); S+P = Soybean + *Pediococcus*; S+B = Soybean + *Bacillus*; S+B+P = Soybean + *Bacillus* + *Pediococcus*

Table 6. Isolated Bacterial Species in Fermented Soybean at Week 4

Treatments	S+CT	S+P	S+B	S+B+P
1	*Bacillus* spp	*Bacillus* spp	*Bacillus* spp	*Lactobacillus* spp
2	*Escherichia coli*	*Streptococcus* spp	*Streptococcus* spp	*Bacillus* spp
3	*Staphylococcus* spp	*Pediococcus* spp	*Micrococcus* spp	*Pediococcus* spp
4	*Pseudomonas* spp		*Leuconostoc* spp	*Leuconostoc* spp
5	*Micrococcus* spp		*Lactobacillus* spp	*Streptococcus* spp
6	*Corynebacterium* spp			
7	*Lactobacillus* spp			
8	*Salmonella* spp			
9	*Streptococcus* spp			
Total at week 4	9	3	5	5

Legend: S+CT= Control experiment (spontaneous fermentation); S+P = Soybean + *Pediococcus*; S+B = Soybean + *Bacillus*; S+B+P = Soybean + *Bacillus* + *Pediococcus*

Although the aim of the study was achieved, some limitations were noted. These were lack of strain identity of the bacterial isolates as unwanted contaminants present as the experiment progressed, inability to introduce quality control points to eliminate undesired bacteria and lack of controlled optimized conditions for fermentation, as well as how these conditions affect bacterial population. The above should be included in future design in related work.

Conclusions

The two test strains are useful as starter cultures in the controlled fermentation of cabbage and soybeans to form useful products. Both *Pediococcus pentosaceus* and *B. subtilis* have capabilities to produce useful enzymes. Sequence alignment of 16S ribosomal RNA of the test strain confirmed the LAB as *Pediococcus pentosaceus* strain DSM20336 and *B. subtilis* subsp. *subtilis* strain 168. Results showed that *P. pentosaceus* induced ferments contained the lowest number of unwanted isolates in cabbage and soybean unlike in spontaneous fermentation where 8-9 unwanted isolates were found. The study identified potential fermenting strains of bacteria

that produced quality ferments, reduced spoilage and prevented unwanted bacteria. These strains could be employed as starter culture in the industrial fermentation of vegetable and legume foods to boost food security in Nigeria.

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