Screening and Identification of Predominant Lactobacillus spp. from Fermented Milled-Mixed Corn -Soybean Wastes

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Abstract

Most fermented edible plant products that contain *Lactobacillus* spp. have been considered as rich probiotic meal for human consumption. However, for the availability and cost-effectiveness of probiotic meal to the Nigerian populace, especially the low-income earners, the study was focused on screening for predominant *Lactobacillus* spp in fermented milled corn-soybean wastes. Washed yellow corn and white soybean (0.5Kg each) soaked in 1L of water (25^{o}C) for 12h were wet- milled and sieved to recover their wastes. The individual wastes were mixed (1:1) prior to their 72h- static fermentation at 30^{o}C. The fermented- milled wastes were first assessed for lactic acid bacteria (LAB) by culturing into De Man Rogosa and Sharpe (MRS) medium. Thereafter, the recovered LAB were subjected to phenotypic and molecular analyses. The isolated DNA molecules from the dominant LAB strains were amplified by polymerase chain reaction (PCR) and sequenced using 16SrRNA method. A significant count of ≥ 7.4x10^{8}±0.03cfu/ml of LAB at $p<0.05$ was recovered with *Lactobacillus* spp. being the most predominant genus. The 16SrRNA analysis assigned 83.3% of the Lactobacillus isolates to be *Lactobacillus plantarum*CS and 16.7% as *Lactobacillus fermentum* CS. The obtained result gave an insight that the developed fermented milled corn-soybean wastes are a rich source of L. *plantarum* CS strain.

Key words: Screening, Identification, Corn-soybean wastes, Fermentation, Lactic acid bacteria, *Lactobacillus plantarum.*

INTRODUCTION

Modern food biotechnology has moved a long way since ancient time from empirical food fermentations. Amongst all processed foods, fermented cereals have been very important in Nigerian diets. The most common organisms isolated during cereal fermentation are Lactic Acid Bacteria [1]. Among the Lactic Acid Bacteria (LAB), Lactobacilli present a diverse group of homo-fermentative and hetero-fermentative species which can produce a variety of substances such as lactic acid, ethanol, formic acid, acetone, hydrogen peroxide and diacetyl [2]. Among the Lactobacilli group that have been confirmed to be probiotic, *Lactobacillus plantarum* has gained populace as a probiotic strain [3]. *Lactobacillus plantarum* is Gram positive, catalase negative, oxidase negative, endospore forming, rod shaped bacterium [4], [5]. It has a relatively large genome compared to other *Lactobacillus spp*. Its genome consists of a 3.3 Mb circular chromosome, which is the largest sequenced genome of any lactic acid bacteria [6]. The genome of *L. plantarum* consists of 5 rRNA operons, which are evenly distributed around the chromosome [7]. A total of 62 tRNA encoding genes have been found and are related to some of the rRNA clusters. In addition, the genome encodes

two classes of transposase regions, which are thought to encode mobile genetic elements [8]. The genome consists of 3,052 protein-encoding genes and only 39 of these genes are pseudogenes [3]. *L. plantarum* proteins are very similar to other Gram-positive bacteria since they have low GC content, a peptidoglycan cell wall and are organized collinearly [9]. *L. plantarum* are commonly found in many fermented food products including sourdough, sauerkraut, pickles, brinded olives, Korean kimchi, Nigerian fermented corn ('akamu') and other fermented plant materials [10], [11] . The Nigerian fermented corn ('Akamu') is a cost effective meal consumed by many, including the adults. However, its high starch and low amino acid contents is a health concern to most aged populace, especially diabetic patients. Thus, there is need to consider the corn- waste which has low starch content and soybean-waste which contains some amino acids in the search for *Lactobacillus plantarum* strains that are considered probiotic. These wastes are commonly utilized as poultry feeds in Nigeria, confirming their safety for consumption. Hence, the aim of the present study was to screen the 72h-fermented corn-soybean wastes for *L. plantarum* since its presence in the fermented waste-meal will be beneficial to man. However, a full characterization of the *L. plantarum* isolates is of utmost importance so as not to confuse them with other isolates of the same genus.

MATERIALS AND METHODS

Samples and their Preparations

The dry white soybean (*Glycine max*) and yellow corn (*Zea mays*), were sorted to remove the unwanted debris and spoilt grains prior to use. Each type of the grain (0.5kg) was weighed, washed and soaked with cooled boiled water (25^{o}C) for 12h. Thereafter, they were respectively, wet-milled with sterile hand grinder (Corona Landersycia) and then sieved with sterile muslin cloth to recover their wastes. Each type of wastes was further wet-milled and the paste recovered. The milled wastes were subjected to proportionate mixing (1:1) to obtain a 10g mixed corn-soybean waste-paste. Similarly, the obtained 10g of unmixed waste-pastes of corn and that of soybean (10g) served as the reference samples. Summarily, 3 samples notably, soybean milled-waste, corn-milled waste and mixed corn-soybean milled wastes were prepared for fermentation.

Fermentation and Recovery of the Isolates

Each milled waste (0.2g.ml^{-1}) was homogenously mixed with water prior to static-fermentation at 30^{0}C under facultative anaerobic condition for 72h. Thereafter, the supernatant was decanted and the recovered fermented waste-pastes (1% w/v) were serially (10^{1}-10^{6}) diluted with sterile water and 0.2ml of the diluted samples were inoculated unto De Man Rogosa and Sharpe agar (Titan Biotech Ltd., India) and incubated at 37^{o}C/24h. The developed colonies were estimated using Quebec colony counter (Medifield Equipment and Scientific Ltd, England). Only the most dominant colonies were subjected to identification.

Phenotypic Characterization of the Isolates

Gram staining, oxidase, catalase, indole, coagulase, urease, gelatin liquefaction, methyl red and sugar fermentation tests were carried out on 24h culture of the isolates as described by Cheesbrough [12] and Collins *et al.*[13]. Peptone Water (LABM ™, U.K) and Nutrient agar/broth (Oxiod Ltd., U.K) were employed for some of the analyses.

Preparation of the Isolates for DNA Extraction

The glycerol-stock culture of the isolates were re-inoculated into freshly prepared sterile De Man Rogosa and Sharpe (MRS) broth and incubated at 37°C

for 24 h. Thereafter, 1ml of each active broth culture was re-inoculated into 5 ml sterile MRS broth and incubated at 37°C for 18 h. The broth cultures were then centrifuged at 1000 x g for 10min to recover the cells for DNA extraction.

DNA Extraction

The method of Pitcher *et al.* [14] was adopted with several modifications by using Zymo research reagents instead of guanidium thiocyanate and other reagents they used. Each re-suspended cell [50mg in 200µl sterile phosphate buffered saline (PBS)] was vortex and 1ml of it was added to a Zymo Reagent (ZR) Bashing bead™lysis tube (0.1mm and 0.5mm) containing 750µl Xperdition™ lysis solution. It was processed for 5min and centrifuged at 10000 x g for 1min. Then, the recovered supernatant (400µl) was further centrifuged at 7000 x g for 1min. This was followed by the addition of 1,200µl of the DNA lysis buffer to the filtrate. The obtained mixture (800µl) was centrifuged 2 times at 10000 x g/ 1min, discarding the flow that ran through the collection tube. Thereafter, washing was carried out with centrifugation at 10000 x g for 1min, using 200µl DNA pre-wash- buffer followed by 500µl DNA -wash- buffer. The DNA in the preparation was then eluted with DNA elution buffer (100µl) at 10000 x g for 30s and stored at -20°C for use.

DNA Sequencing Analysis

The 16SrRNA region sequencing analysis as described by Vesty *et al.* [15] was used with some modifications in reaction- time and reagents. The PCR reaction was performed with 20ng of genomic DNA as the template in a 30µl reaction mixture by using a EF – Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2min, 35 cycles of 95°C for 1min, 55°C and 72°C for 1 min and finishing with a 10 min-step at 72°C. The amplification products were purified with a multi-screen filter plate (Millipore corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator V3.1 cycle sequencing kit. The DNA samples containing the extension products were added to Hi – Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5min, followed by 5min on ice and then analyzed by ABI prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis:

The data on LAB yield were statistically analyzed using Statistical Packaged for Social Sciences (SPSS), version 20 software.

RESULTS AND DISCUSSION

LAB Yields from the Sampled Substrates

The result shown in table 1 indicated that mixed corn-soybean waste samples significantly registered higher LAB counts ($\geq$ 7.4 x10^8±0.03), than those of the control samples (≤2.4x10^7 ± 0.02) at P<0.05. Higher LAB recovered from the mixed samples could be explained from the fact that the mixture must have provided a good medium enriched with both carbon and nitrogen sources for the LAB proliferation. However, the recovery of some measurable number of LAB (6.8x10^4±0.03- 2.4x10^7±0.02) even from the controlled-sampled-wastes is interesting as it is comparable to LAB counts recovered from other sampled-fermented-corn products [5], [16]. The corn and soybean wastes have been used for a long time as part of poultry feeds in Nigeria. Thus, their transformation for possible human use through fermentation is very encouraging. This is because

most fermented meal rich in good LAB has been certified as functional food nutrients for man.

Table1: Lactic Acid Bacterial Yield from Fermented Mixed Corn-Soybean Wastes

No of Analysis	LAB Counts (Cfu/ml) +SD		
		Control Samples	
	CS-Mixed wastes	Corn wastes	Soybean wastes
1	$6.8 \times 10^9 \pm 0.01$	$2.4 \times 10^7 \pm 0.03$	$5.9 \times 10^5 \pm 0.01$
2	$7.0 \times 10^8 \pm 0.02$	$6.0 \times 10^6 \pm 0.01$	$6.2 \times 10^5 \pm 0.02$
3	$6.9 \times 10^9 \pm 0.01$	$1.4 \times 10^7 \pm 0.04$	$6.8 \times 10^4 \pm 0.03$
4	$7.4 \times 10^8 \pm 0.03$	$6.8 \times 10^6 \pm 0.02$	$7.0 \times 10^4 \pm 0.01$
5	$1.5 \times 10^9 \pm 0.04$	$9.6 \times 10^6 \pm 0.05$	$7.3 \times 10^5 \pm 0.03$

Legend: Individual corn and soybean wastes served as the control samples

LAB- Lactic acid bacteria, Cfu- Colony forming unit, SD-Standard deviation, CS- Corn-Soybean

Characterization of LAB Isolates

Identification through morphological and biochemical tests as stated in table 2 revealed that *Lactobacillus* isolates were catalase, oxidase, gelatin liquefaction, indole, methy- red, urease and coagulase negative. However, they were able to ferment glucose, sucrose, lactose, fructose, galactose and maltose with no gas formation. These findings are in line with those of Mohagnia *et al.*[3], Woo *et al.* [8], Lin *et al.* [17] that associated these characteristics with *Lactobacillus* spp.

Table 2: Phenotypic Characteristics of Bacterial Isolates

Colonial appearance	Gram reaction	Catalase	Oxidase	Gelatin	Liquefact	Indole	Methyl	Ureas	Coagulase	Sugar Fermentation						Suspected Organisms
										Glu	Sucr	Lact	Fruc	Gala	Malt	

NA	MRS														
Creamy colonies	Whitish colonies	+ Short rod in short chains	-	-	-	-	-	-	-	A	A	A	A	A	A	*Lactobacillus* sp1
Creamy colonies	Whitish colonies	+Short rod, Some in clusters and others in singles	-	-	-	-	-	-	-	A	A	A	A	A	A	*Lactobacillus* sp2
Creamy colonies	Whitish colonies	+very short rod in short chains	-	-	-	-	-	-	-	A	A	A	A	A	A	*Lactobacillus* sp3
Creamy colonies	Whitish colonies	+ Short rod in short chains	-	-	-	-	-	-	-	A	A	A	A	A	A	*Lactobacillus* sp4
Creamy colonies	Whitish colonies	+medium sized rod in chains	-	-	-	-	-	-	-	A	A	A	A	A	A	*Lactobacillus* sp5
Creamy colonies	Whitish colonies	+medium sized rod, not in chains	-	-	-	-	-	-	-	A	A	A	A	A	A	*Lactobacillus* sp6

Legend: ISTs- Isolates, NA-Nutrient Agar, MRS-De Man Rogosa and Sharpe, + positive, -Negative, A-Acid production, LS-*Lactobacillus* isolate, sp. – species

The genomic DNA extraction showed identical bands for 5 of the 6 isolates under identification with little variation only on one strain; LSP3 (fig.1). 1t is interesting to note that the identical genomic bands of the 5 isolates were confirmed to be *Lactobacillus plantarum* CS , with 1511 nucleotides, showing 99% similarity with *Lactobacillus plantarum* JCM1149 (table 3), determined from the constructed phylogenetic tree (Not shown). The obtained result of 83.3% *L. plantarum* from the fermented mixed waste-paste is comparable to 85% of *L. plantarum* recovery from fermented cereals after their genotypic characterization [18], [19]. The only isolate which had 99% identity of *L. fermentum* TW27-2 was observed to be *L. fermentum* CS (table 3).

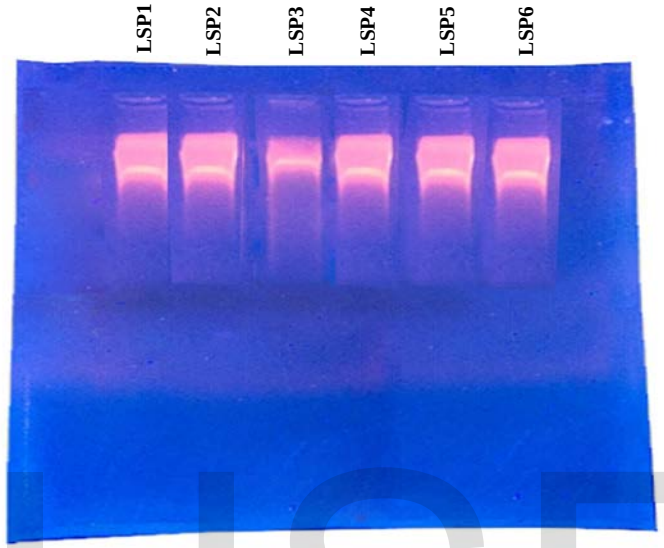

Fig. 1: Gel electrophoresis of the extracted DNA of *Lactobacillus* spp.

Legend: LSP- *Lactobacillus* spp.

Table3: Summary of Molecular Identification of Dominant Strains of *Lactobacillus* Isolates from Mixed Corn-Soybean Wastes

No of Examined Isolates	Length	Start	End	PCR Idenification	Nearest strain	% identity
5	1511	15	1511	*L. plantarumi* CS	*L. plantarum* JCM1149	99

1	1500	15	1500	L. *fermentum* CS	L. fermentum TW27-5	99

Legend: Sequencing Primer Name Primer Sequencinq

I -785F---------- 5^{I} (GGA TTA GAT ACC CTG GTA) 3^{I}

II - 27F---------- 5^{I} (AGA GTT TGA TCM TGG CTC AG) 3^{I}

III-907R---------- 5^{I} (CCG TCA ATT CMT TTR AGT TT) 3^{I}

IV- 1492R---------- 5^{I} (TAC GGY TAC CTT GTT ACG ACT T)3^{I}

% occurance of *Lactobacillus plantarum* among the recovered dominant species= 5/6 x100 = 83.3%

% occurance of *Lactobacillus fermentum* among the recovered dominant species =1/6 x 100= 16.7%

The low recovery of *L. fermentum* CS from corn-soybean wastes is contrary to many reports that had *L. fermentum* among the dominant strains in cereal products [20]. The low recovery could be due to the fact that the wastes could not support or stimulate the enzymatic activity to release enough energy for the growth of the organism. This, on the other hand, could have prolonged the lag growth phase of *L. fermentum* CS. The use of 16SrRNA genotypic characterization has enabled the authors to narrow down the dominant isolates from the waste-paste to species level and this particular genomic characterization has generally helped in the understanding of not only the microbial diversity within and across the group but also in identifying new strains with different potentials [21], [22], [23]. The method also solves the problem of misidentification as some LAB are closely related [24], [25]. The recovery of *L. plantarum* from various fermented foods of plant origin has been severally reported [26], [27], [28]. In Nigeria, these corn or soybean wastes recovered after fermentation have been used for the preparation of poultry feeds. Thus, the study revealing their importance when fermented as rich source of *L. plantarum* is a welcome development.

CONCLUSION

The results of this study indicated that *Lactobacillus plantarum* were the predominant Lactobacillus group in the fermented mixed corn-soybean wastes. However, since most strains of *Lactobacillus plantarum* have been associated with probiotics potentials, there is need for the recovered *L. plantarum* CS-strain from fermented corn-soybean wastes be further screened for their probiotic and related properties so that the corn-soybean wastes are exploited for their health and economic benefits.

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