Assessment of the Probiotic Potential of *Lactobacillus* species Isolated from Selected Brands of Yoghurt sold in Zaria, Kaduna State, Nigeria

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Abstract: Probiotics are non-pathogenic and non-toxigenic bacteria that serve as a natural barrier against pathogenic enteric bacteria. Yoghurt and other fermented dairy products are the most common source of probiotics.This study was carried out to assess the probiotic potential of *Lactobacillus* species isolated from different brands of yoghurt. Nine(9) yoghurt samples consisting of three (3) different brands were purchased from local vendors. The samples were serially diluted, inoculated onto De Man Rogosa and Sharpe(MRS) Agar and incubated anaerobically using a candle jar at 37^{o}C for 24 hrs. Colonies with characteristics colonial morphology of *Lactobacillus* species on MRS agar were sub-cultured fresh MRS agar. The isolates were further identified and characterized microscopically and biochemically. The isolates were screened for their ability to tolerate low pH, tolerate bile and exhibit antibacterial activity. The nine (9) isolates of *Lactobacillus* species obtained consist of 7(77.78%) *L. plantarum,* 1(11.11%) *L. bulgaricus* and 1(11.11%) *L. salivarius*. All the isolates tolerated low pH and bile salt at different concentrations. Two (2) of the isolates had antibacterial activity against *Escherichia coli*, however none of the isolates had antibacterial activity against *Salmonella* Typhi. Of the three *Lactobacillus* species isolated from yoghurt, *L. plantarum* had the highest occurrence with 77.78%.Two of the *Lactobacillus* species isolated from yoghurt tolerated low pH, bile salt at different concentrations and exhibited antibacterial activity hence possesses probiotic potential.
Keywords: *Lactobacillus* species, probiotic, yoghurt, antibacterial

INTRODUCTION

Recently, the importance of probiotics as part of healthy humans and animals diet have been reported by different researchers as they provide a natural, safe and effective barrier against microbial infections (Oh and Jung, 2015; Angmo *et al*., 2016). Lactic acid bacteria (LAB) found in yoghurt were first associated with health by Metchnikoff in 1907. This concept of probiotic microbes has led to the widespread consumption of food preparations containing LAB with the expectation that they will confer health benefits (Nagpal *et al.,* 2012).

World Health Organization (WHO) defined probiotics as ‘‘live microbial food supplements which, when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). The major group of probiotic bacteria usually used microorganisms are lactic acid bacteria (LAB) (Shehata *et al*., 2016). LAB are non-pathogenic, technologically suitable for industrial processes, acid tolerance, bile tolerance, produce antimicrobial substances (Mojgani *et al*., 2015) and they are classified as generally recognized as safe (GRAS) microorganisms because of their long and safe use as starter cultures in fermented products (Shehata *et al*., 2016). Probiotics bacteria must be non-pathogenic, non-toxigenic, withstand gastric acid and bile salts (Han *et al.,* 2017).

Most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium*. However, species belonging to the genera *Lactococcus*, *Enterococcus* and *Saccharomyces* are also considered as probiotic microorganisms (Shehata *et al*., 2016).

Lactobacillus species can be isolated from diverse sources such as the natural environment, intestinal tracts of mammals, and fermented foods. However, the main source of *Lactobacillus* species are fermented dairy products especially yoghurt (Ji *et al.,* 2015). *Lactobacillus* species and other LAB exert a positive role in human health following oral administration.

They improve the balance of the microbial community in the intestine, confer protection against potential pathogenic bacteria, and prevent and/or cure intestinal diseases by producing antimicrobial metabolites such as organic acids (e.g. lactate, acetate, and butyrate), hydrogen peroxide, and bacteriocins. They also compete with harmful bacteria for nutrients or adhesion receptors (Azat *et al.,* 2016).

According to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts (Vijaya *et al*., 2015; Shehata *et al*., 2016). In addition, functional properties such as production of antimicrobial compounds are also used to characterize probiotic bacteria (Xie *et al*., 2015). The mechanism through which probiotics may antagonize pathogens involves production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins (Shehata *et al*., 2016). Hence, this was study was carried out to assess the of probiotic potential of *lactobacillus* species isolated from different brands of yoghurt sold in Zaria, Kaduna state Nigeria.

MATERIALS AND METHODS

Collection of Samples and processing

A total of nine (9) yoghurt samples (three from each brand) were purchased randomly from local vendors. The manufacturing date and expiry date of all the samples were noted. The samples were transported in ice pack immediately after purchase to the laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for bacteriological analysis.

The yoghurt samples were first diluted by adding 10 mL of each sample into 90 mL of sterile physiological saline to give 10^{-1}dilution. After thorough mixing under aseptic condition, One (1) mL of the 10^{-1}dilution was serially diluted using nine (9) mL of physiological saline and 0.1mL of the 10^{-4}dilution was used as inoculum.

Isolation and Characterization of *Lactobacillus* species from Yoghurt

Exactly 0.1mL of the 10^{-4} dilution was inoculated onto already prepared sterile De Man Rogosa and Sharpe (MRS) agar using spread plate technique and incubated anaerobically using a candle jar for 24hrs at 37°C. The colonies with characteristic cultural morphology of *Lactobacillus* on MRS agar were sub-cultured on another MRS agar by streaking after which discrete colonies were stored on slants at 4°C and further characterized microscopically and biochemically (De *et al.,* 2016).

Pure cultures of suspected *Lactobacillus* species isolates were characterized on the basis of their microscopic and biochemical characteristics. These were Gram reaction and morphology, catalase, Methyl Red, Voges Proskauer and sugar fermentation.

Low pH tolerance test

The acidic pH tolerance test was performed by the viable plate count method as described by Maragkoudakis *et al.* (2006). Overnight bacterial cell culture of *Lactobacillus* species grown anaerobically in MRS broth at 37°C were harvested by centrifuging at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and cell suspension was washed twice with phosphate buffer saline(PBS) of pH7.22 and re-suspend in PBS of pH3.04 and pH6.5 for 3hrs each. Aliquot of the cell suspensions were plated on sterile MRS agar and incubated anaerobically at 37°C for 24hrs.

Bile tolerance test

The ability of the isolates to tolerate different concentrations of bile salt (0.3%, 0.5%, 1.0% and 1.5%) was determined by the viable plate count method. Overnight MRS broth culture of the bacterial cells were harvested at 10,000 rpm for 10min at 4°C, then washed twice with PBS of pH 7.22 before resuspension in PBS solution containing 0.3%, 0.5%, 1.0% and 1.5% bile salt.

Cell aliquotswere plated on sterile MRS agar and incubated anaerobically at 37^{o}C for 24hrs (Maragkoudakis *et al*., 2006).

Antibacterial activity of the *Lactobacillus* species against *Escherichia coli* and *Salmonella* Typhi

To determine the antibacterial activity of the *Lactobacillus* species, agar well diffusion method was used. Isolates of *Escherichia coli* and *Salmonella* Typhi were obtained from department of Microbiology and reconfirmed. *Lactobacillus* species isolates were inoculated in MRS broth and incubated overnight anaerobically at 37^{o}C. Intact bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min, the supernatant was used as cell-free supernatant.A 0.5 McFarland standardized inoculum of overnight cultures of *Escherichia coli* and *Salmonella* Typhi were spread onto Muller Hinton agar plates and wells were made on each of the plate. The wells were filled with 100µLof *Lactobacillus* cell-free supernatant. After diffusion, the plates were incubated for 24 hrs at 37^{o}C and observed for zones of growth inhibition (De *et al.,* 2016).

RESULTS

Table 1 shows the microscopic and biochemical characteristic of the isolates. Isolates of *Lactobacillus* species appeared as smooth, round, raised, moist, translucent and creamy/milky white in colour with varying sizes on MRS agar. The isolates were Gram positive rods, citrate negative, catalase negative, MR positive and VP negative. However, the isolates varied in their sugar fermentation ability based on their species; *Lactobacillus plantarum* isolates fermented all the sugars*, L. Salivarius* isolate fermented all the sugars except Arabinose while the *Lactobacillus bulgaricus* isolate was a non-fermenter of Arabinose, Maltose, Mannose and Sucrose.

Lactobacillus plantarum had the highest occurrence of 77.78%, while *Lactobacillus bulgaricus* and *L. salivarius* had the lowest occurrence of 11.11% each (Table 2).

Table 3 shows the tolerance of the *Lactobacillus* species isolates to pH 3.04, 6.5 and 7.22. All the 9 isolates of *Lactobacillus* species screened were able to tolerate pH 3.04, 6.5 and 7.22. Table 4 presents the tolerance of the isolates to different concentrations of bile salt. All the isolates tolerated bile salt at concentrations of 0.3%, 0.5%, 1.0% and 1.5%. Out of all the *Lactobacillus* species screened only two isolates (H1 and N3) showed antibacterial activity against *Escherichia coli* *w*ith isolate N3 showing a higher zone of inhibition (17mm) compared to isolate H1 (11mm). No zone of inhibition was observed against *Salmonella* Typhi as showed in Table 5 below.

Table 1:Cultural, microscopic and biochemical characteristics of bacterial isolates from different brands of yoghurt

Isolate codes	Cultural Characteristics	Gram Reaction	Cat	MR	VP	Cit	Glc	Lac	Suc	Mal	Dex	Man	Gal	Fru	Ara	Inference
H1	Milky white raised moist	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L.plantarum*
H2	Milky white raised moist	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L plantarum*
H3	Milky white raised moist	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L.plantarum*
N1	Milky white raised moist	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L.plantarum*
N2	Whitish, flat and moist	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	-	*L.salivarius*
N3	Milky white, raised moist	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L.plantarum*
S1	Milky white, smooth convex	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L.plantarum*
S2	Milky white, smooth convex	Gram positive rods	-	+	-	-	+	+	+	+	+	+	+	+	+	*L.plantarum*
S3	Creamy white moist	Gram positive rods	-	+	-	-	+	+	-	-	+	-	+	+	-	*L.bulgaricus*

Key: += Positive; - =Negative, Cat=catalase, Cit=citrate, Glc=Glucose, Lac=Lactose, Suc=Sucrose, Mal=Maltose, Dex=Dextose, Man=Mannitol, Gal=Galactose, Fru=Fructose, Ara=Arabinose

Table 2: Occurrence of *Lactobacillus* species Isolated from yoghurt sample

Isolates n = 9	**Number present**	**Occurrence (%)**
L. plantarum	7	77.78
L. salivarius	1	11.11
L. bulgaricus	1	11.11
Total	9	100

Table3: pH tolerance of *Lactobacillus* species isolated from different brands of yoghurt

Isolate codes	(pH 3.04)	(pH 6.5)	(pH 7.22)
H1	+	+	+
H2	+	+	+
H3	+	+	+
N1	+	+	+
N2	+	+	+
N3	+	+	+
S1	+	+	+
S2	+	+	+
S3	+	+	+

Key: +=Isolate tolerate the condition

Table 4: Bile tolerance of *Lactobacillus* species isolated from different brands of yoghurt.

Isolate codes	Bile tolerance (0.3%)	Bile tolerance (0.5%)	Bile tolerance (1.0%)	Bile tolerance (1.5%)
H1	+	+	+	+
H2	+	+	+	+
H3	+	+	+	+
N1	+	+	+	+
N2	+	+	+	+
N3	+	+	+	+
S1	+	+	+	+
S2	+	+	+	+
S3	+	+	+	+

Key: += Isolate tolerate the condition

Table 5: Antibacterial activity of Isolates against *Escherichia coli* and *Salmonella* Typhi

	Mean zone of Inhibition(mm)	
Isolate codes	***Escherichia coli***	***Salmonella* Typhi**
H1	11	0
H2	0	0
H3	0	0
N1	0	0
N2	0	0
N3	17	0
S1	0	0
S2	0	0
S3	0	0

Plate I: Colonial morphology of *Lactobacillus* species on MRS agar

Plate II: Antibacterial activity of *Lactobacillus* species against *Escherichia coli*

DISCUSSION

The three (3) different species of *Lactobacillus* isolated were *Lactobacillus Plantarum, L. salivarius and L. Bulgaricus* with *Lactobacillus plantarum* having the highest occurrence (77.78%). This result is in line with the research of Asmahan (2011) who also reported *Lactobacillus plantarum* with the highest occurrence in yoghurt samples analyzed.

All the *Lactobacillus* species were able to tolerate and survive low pH and the different concentrations of bile salt tested.This implies that the isolates can survive and grow under acidic environment as well as in the presence of bile salts which are the conditions of the small intestine.Similar results have been previously reported by other researchers working with strains of *Lactobacillus* from different environments (Zago *et al*., 2011; Ramos *et al*., 2013; Leite *et al.,* 2015).The major properties of bacterial strains with probiotic potentials are acidic pH tolerance, bile tolerance and antimicrobial activities. Since probiotics are usually administrated orally, they must have the ability to survive passage through the stomach and small intestine. A good probiotic bacteria must be able to overcome the primary defence mechanism of the stomach constituted by the secretion of gastric acid (Leite *et al.,* 2015). Bile salt tolerance is a prerequisite for colonization and metabolic activity of probiotic bacteria in small intestine of the host (Shehata *et al.,* 2016).

The acid tolerance ability of the *Lactobacillus* speciesis also an indication that they can be used as dietary adjuncts and can survive for longer period of time in high acid carrier food without a larger reduction in humans. So also, bile salt tolerance ability of the *Lactobacillus* species will help them to reach the small intestine and colon, thereby balancing the intestinal microflora.

Possession of antimicrobial activity is one of the most important selection criteria for probiotic. The two strains of *Lactobacillus plantarum* (H1 and N3) exhibited antibacterial activity against *Escherichia coli.*

The lack of antimicrobial activity observed in seven isolates could be attributed to the low concentration of the antimicrobial compounds produced which was not enough to inhibit the selected bacteria. This is in accordance with the study of Ashok *et al*. (2014) who reported that not all strains of *Lactobacillus* species have antibacterial activity.

The observed inhibitory activity is likely due to the production of antimicrobial compounds like organic acids, hydrogen peroxide, diacetyl and bacteriocins by LAB as well as their competition for nutrients (Shehata *et al*., 2016)

This property might provide the LAB isolates with an advantage in competing either in a food product or in the gut. So also, this protective property may be useful as a defense mechanism in the intestinal microbial ecosystem as reported by Leite *et al.* (2015).

The probiotic potential of the isolates observed is in line with reports that strains belonging to the *Lactobacillus* genus are commonly used as probiotics because they possess desirable probiotic-related properties (FAO/WHO, 2006; Zago *et al*., 2011).

In conclusion, *Lactobacillus plantarum* was found to be the dominant species of *Lactobacillus* in yoghurt. All the isolates were tolerant of low pH and bile salts, however only two of the isolates had antibacterial activity against *Escherichia coli* while none of the isolates inhibited *Salmonella* Typhi. These two isolates of *Lactobacillus plantarum* possess desirable probiotic related properties.

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