Screening, Antimicrobial Susceptibility and Gastrointestinal Tolerance of Phytase-Producing Bacteria Strain for Potential Use as Probiotic Feed Supplement

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Abstract: In order to utilize phosphorus and other nutrients efficiently, monogastric animals require an exogenous supply of phytase to hydrolyze phytate present in feed. However, the stability and efficacy of phytase may be grossly compromised in the event of non compliance by veterinary marts and farmers to storage directives from manufacturers. To overcome this challenge, it is envisaged that live phytase-producing microorganisms may be used as feed supplement. The aim of this study was to screen some phytase-producing bacteria strains for pathogenicity, and thereafter evaluate the antimicrobial sensitivity and gastrointestinal tract (GIT) tolerance of selected strain *in vitro*. Four phytase-producing bacteria strains previously isolated from top soils from Lagos dumpsites, and identified as *Enterobacter cloacae* ODS 29, *Bacillus amyloliquefaciens* ODS 33, *Bacillus amyloliquefaciens* FDS 10 and *Bacillus subtilis* FDS 16 (MH879829, MH879830, MH879831 and MH879832 respectively) were screened for pathogenic ability by microbiological methods. Pathogen-negative strain was evaluated for susceptibility against ten standard antimicrobials over 24 h and thereafter evaluated for tolerance to GIT conditions (pH 1-4 over 2 h, bile concentrations 0.1 to 2% (w/v) over 5 h, gastric juice over 6 h and simulated GIT condition over 4 h respectively). Results of pathogen test revealed only *Enterobacter cloacae* ODS 29 as non pathogenic strain of bacteria. Evaluation of its sensitivity to various antimicrobials revealed susceptibility to all ten antimicrobials. Result of GIT tolerance showed *E. cloacae* ODS 29 to survive pH < 2, bile concentration 2% (w/v), gastric juice and simulated GIT conditions. As such, *E. cloacae* ODS 29 is considered safe and having potential for probiotic use as feed supplement.

Key words: Antimicrobial sensitivity, *E. cloacae*, GIT condition, Hemolysin, Phytase.

INTRODUCTION

Feed for livestock are made from plant preparations and these contain phytate, the major storage form of phosphorus (70 to 80% of total phosphorus) and other minerals in many plant tissues (Blaabjerg *et al.,* 2011; Onawola *et al.* 2019). The phytate-bound nutrients are generally non bioavailable to monogastric animals due to inability to degrade phytate because they lack phytase-producing microorganisms in the digestive tract (Kumar *et al.,* 2015). Consequently, these nutrients remain unabsorbed and are excreted in the feces, leading to nutrient deficiencies. Phytase-producing microorganisms are a group of organisms with ability to produce phytases. They include yeasts such as *Pichia anomala* (Vohra et. al. 2011) and bacteria such as *B. subtilis* (Abd-Alhadi *et. al.,* 2015), *B. amyloliquefaciens* and *Enterobacter cloacae* (Onawola *et. al.,* 2019). Due to several biological characteristics which include substrate specificity and resistance to proteolysis, bacterial phytases are considered to be more appropriate in degrading phytates in nutritional applications than their fungal counterparts (Sreedevi and Reddy, 2013).

Phytases possess the ability to sequentially release phosphates from phytates, thereby functioning to improve feed nutritional value and decreasing phosphorus excretion (Sreedevi and Reddy, 2013; Onawola *et al.* 2019). However, its stability (percent of initial phytase activity) may be grossly compromised in the event of non compliance by veterinary marts and farmers to storage directives from manufacturers. As such, exogenous phytase may only be actively utilized at a reduced level of its optimal activity. To overcome a compromise in stability and efficacy of exogenous phytase, and reduce phosphorus excretion and its impact on the environment, it is envisaged that live enzyme sources such as bacteria may be used as feed supplement for the endogenous production of phytase.

The limitation may be getting an organism with potentials to survive the gastrointestinal tract (GIT) conditions without harming the host. The aim of this study therefore was to screen phytase-producing bacteria strains for pathogenicity, and evaluate the antimicrobial sensitivity and GIT tolerance of selected strain *in vitro*.

MATERIALS AND METHODS

Screening of Phytase-producing Bacteria Strains for Pathogenicity

Four phytase-producing bacteria strains previously isolated from top soils from Lagos dumpsites and identified as *Enterobacter cloacae* ODS 29, *Bacillus amyloliquefaciens* ODS 33, *Bacillus amyloliquefaciens* FDS 10 and *Bacillus subtilis* FDS 16 (Genbank Accession numbers: MH879829, MH879830, MH879831 and MH879832) (Onawola et al. 2019) were reconfirmed for phytase potentials on phytase specific medium.

The phytase-producing bacteria strains were screened for pathogenicity on Mannitol Egg Yolk Polymyxin (MYP) supplemented with polymyxin B and eggyolk, and Blood agar supplemented with sheep blood according to manufacturer's instructions respectively. Overnight cultures of the bacteria strains on plate count agar (PCA) were each sub cultured onto MYP and blood agars, and incubated at 37°C for 48 h respectively. The development of a halo or zone of precipitation on MYP agar and the presence of a halo or green colouration on blood agar were considered potentials to produce lecithinase and hemolysin respectively.

Evaluation of the Antimicrobial Sensitivity of Non Pathogenic Bacteria

Antimicrobial susceptibility was performed using the agar disc diffusion method as recommended by the National Committee for Clinical Laboratory Standards (2006). A culture of *E. cloacae* ODS 29 was prepared in 5 mL of Mueller-Hinton broth and incubated at 37°C for 24 h. A sterile cotton swab dipped into the inoculum (0.5 MacFarland Standard) was applied evenly onto the surface of Mueller-Hinton agar plates. Surfaces of plates were dried for 5 min under laminar air flow, after which antibiotic discs for Gram negative bacteria were placed on it and incubated at 37°C for 24 h. Ten antibiotic discs produced by Maxi care medical Laboratories Ltd., Nigeria were used for the test. These were Septrin (30 µg), Chloramphenicol (30 µg), Sparfloxacin (10 µg), Ciprofloxacin (10 µg), Amoxacillin (10 µg), Augmentin (30 µg), Gentamycin (10 µg), Pefloxacin (30 µg), Tarivid (10 µg) and Streptomycin (30 µg) respectively. The zones were measured in millimeters (mm) by placing the metric ruler across zones of inhibition at the widest diameter, and measuring from one edge of the zone to the other. The sensitivity pattern of the strain was interpreted as susceptible, resistant, or intermediate using the breakpoint tables for interpretation of minimum inhibitory concentrations and zone diameters of The European Committee on Antimicrobial Susceptibility Testing (2014 and 2018) respectively.

Evaluation of Tolerance of Non Pathogenic Bacteria to Acid Condition

Acid tolerance was carried out as described by Mathipa and Thantsha (2015) with modifications. A stock culture of the bacteria was prepared by cultivating a loopful in tryptic soy broth (TSB) at 30°C for 20 h, harvested by centrifugation, washed and suspended in sterile water. TSB were prepared and acidified to pH 1.0 to 4.0 using 5M HCl. To each 100 mL of acidified TSB was added 100 µL of the stock culture and incubated at 37°C for 2 h. One (1) mL samples were taken half-hourly, serially diluted and spread-plated on plate count agar (PCA). Plates were incubated at 37°C for 24 h for total viable counts. Results were expressed as colony forming units per milliliter (CFU)/mL).

Evaluation of Tolerance of Non Pathogenic Bacteria to Bile Salts

Bile tolerance was carried out as described by Buruleanu (2012). TSB was enriched with bile salts at concentrations of 0.1 to 0.3 % (w/v) and 1.0 to 2.0 % (w/v).

To each 100 mL of bile-enriched TSB was added 100 µL of the stock culture and incubated at 37°C for 3 and 5 h respectively. After incubation, 1 mL samples were taken from each flask and serially diluted for total viable counts on PCA after 24 h. Results were expressed as CFU/mL.

Evaluation of Tolerance of Non Pathogenic Bacteria to Gastric Juice

This was carried out as described by Buruleanu (2012). Gastric juice (0.3 g pepsin in 0.5 % (w/v) saline) was prepared and sterilized by membrane filtration using a 0.22 µm pore-sized membrane filter. Into 100 mL of the gastric juice was inoculated 100 µL of the stock culture and incubated at 37°C for 6 h. One (1) mL samples were taken from each flask hourly and serially diluted for total viable counts on PCA after 24 h. Results were expressed as CFU/mL.

Evaluation of Tolerance of Non Pathogenic Bacteria to Simulated GIT Conditions

This was carried out as described by Madureira et al. (2005) but with modifications. One hundred (100) mL of sterile gastric juice, pH 2.0 was inoculated with 100 µL of stock culture and thereafter incubated at 41 ± 1°C for 2 h (gastric condition). One (1) mL samples were taken from the flask at 0.5h intervals and serially diluted for total viable counts after 24 h. At the end of gastric condition, a 10 mL sterile solution of 0.3% (w/v) bile salts was added to the medium after which the resulting solution was incubated for 2 h (duodenal condition). One (1) mL samples were taken from the flask at 0.5 h intervals and serially diluted for total viable counts on PCA after 24 h. Results were expressed as CFU/mL.

Statistical Analysis of Data

Data were analyzed using SPSS package version 23.0. Comparative analyses were performed using the one-way analysis of variance (ANOVA). Results were expressed as Mean ± SEM and significant differences among the treatment means were evaluated at $p \leq 0.05$ using the Tukey HSD.

RESULTS

All four phytase-producing bacteria strains previously isolated from top soils from Lagos dumpsites were observed to retain their phytase-producing abilities when reconfirmed on phytase specific medium.

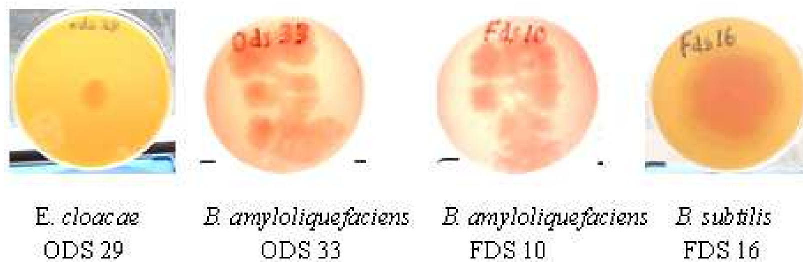

Plate 1. Growth of bacteria strains on MYP agar. *E. cloacae* ODS 29 shows no ability to produce lecithinase.

Plate 2. Growth of bacteria strains on blood agar. *E. cloacae* ODS 29 shows no ability to produce hemolysin.

Table 1. Antimicrobial sensitivity of *Enterobacter cloacae* ODS 29

Antibiotic	MIC (Disc content) (µg/mL)	Halo Diameter ± SEM (mm)	Zone Diameter Breakpoint (mm) S ≥	R ≤	S, I or R
Amoxicillin-Clavulanic acid (AU). (Augmentin)	20 – 10 (30)	20.3 ± 0.33	19.0	19.0	S
Gentamicin (CN)	10	26.0 ± 0.00	17.0	14.0	S
Pefloxacin (PEF)	10	28.0 ± 0.00	24.0	24.0	S
Ofloxacin (OFX). (Tarivid)	10	30.3 ± 0.33	24.0	22.0	S
Trimethoprim-sulfamethoxazole (SXT). (Septrin)	1.25-23.75 (30)	20.0 ± 0.00	14.0	11.0	S
Chloramphenicol (CH)	30	26.3 ± 0.33	17.0	17.0	S
Sparfloxacin (SP)	10	22.3 ± 0.33	19.0	15 .0	S
Ciprofloxacin(CPX)	10	32.0 ± 0.00	26.0	24.0	S
Amoxicillin (AM)	10	26.0 ± 0.58	14.0	14.0	S
Streptomycin (S)	30	22.3 ± 0.33	15.0	11.0	S

MIC = minimum inhibitory concentration, S = susceptible, R = resistant, I = intermediate.

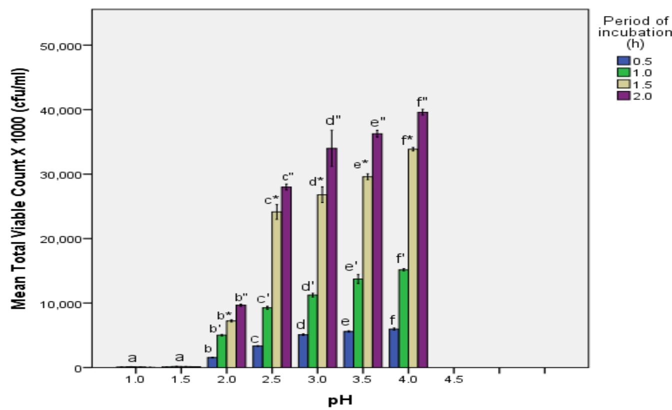

Fig. 1. Tolerance of *E. cloacae* ODS 29 to pH 1.00 to 4.00 over 2 h. *E. cloacae* ODS 29 survived pH < 2 with non significant increases in mean viable counts with increases in time. However, significant increases were observed in mean counts with increases in pH and time. Any two means not followed by the same letter are significantly different.

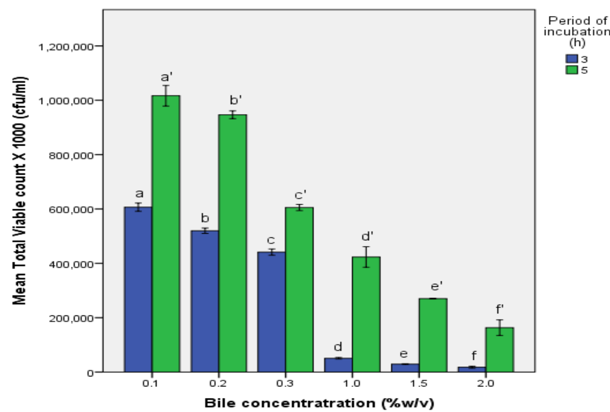

Fig. 2. Tolerance of *E. cloacae* ODS 29 to bile salt concentrations 0.10 to 2.00% (w/v) over 3 and 5 h. *E. cloacae* ODS 29 survived increases in bile salt concentrations up to 2% with significant decreases in viability for each period. Any two means not followed by the same letter are significantly different.

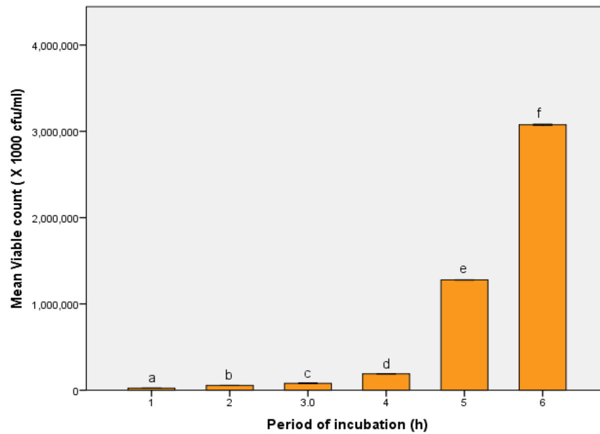

Fig. 3. Tolerance of *E. cloacae* ODS 29 to gastric juice over 6 h. *E. cloacae* ODS 29 survived in gastric juice with significant increases in mean viable counts with increases in incubation period. Any two means not followed by the same letter are significantly different.

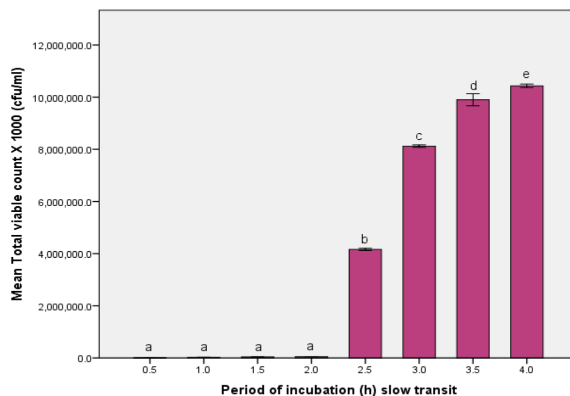

Fig. 4. Tolerance of *E. cloacae* ODS 29 to simulated GIT conditions over 4h. *E. cloacae* ODS 29 survived gastric conditions with non-significant increases in viability. However, mean viable counts increased significantly under the duodenal condition. Any two means not followed by the same letter are significantly different.

DISCUSSION

Lecithinases hydrolyze lecithin in cell membranes thereby damaging its integrity. The halos surrounding the colonies of *B. amyloliquefaciens* ODS 33 and *B. amyloliquefaciens* FDS 10 and a zone of precipitation around the colony of *B. subtilis* FDS 16 on MYP agar (Plate 1) indicate an ability to produce lecithinase. The observation of lecithinase production by these strains agree with Zhao et al. (2014) and Law et al. (2015) who reported that lecithinase production is typical of some pathogenic strains of *B. subtilis* and *B. amyloliqeufaciens.* The inability of *E. cloacae* ODS 29 to produce lecithinase suggests it to be non pathogenic.

Hemolysins cause lysis of erythrocytes by disrupting the cell membrane. The growth of *B. amyloliquefaciens* ODS 33, *B. amyloliquefaciens* FDS 10 and *B. subtilis* FDS 16 with α-hemolysis is indicative of the production of hemolysin, while the γ-hemolysis observed in *E. cloacae* ODS 29 is indicative of its inability to lyse erythrocytes (Plate 2). These findings agree with Pisano et al. (2014) who stated that hemolysin production is important for pathogenicity of bacteria, and Mezzatesta et al. (2012) who reported that the ability of *E. cloacae* to form biofilms and secrete hemolysin and various cytotoxins is important for its pathogenicity. Therefore, in consideration of its non lecithinase and non hemolytic activities, *E. cloacae* ODS 29 is considered non pathogenic.

The susceptibility of *E. cloacae* ODS 29 to amoxicillin and amoxicillin-clavulanic acid (Table 1) may be due to a lack or an inability to over-produce the β-lactamases required to degrade the drugs. This finding contradicts Mezzatesta et al. (2012) and Davin-Regli and Pagès (2015) who reported resistance of *E. cloacae* to ampicillin, amoxicillin and clavulanic acid due to an ability to over-produce the constitutive AmpC β-lactamases which confers an intrinsic resistance to these drugs. The susceptibility of *E. cloacae* ODS 29 to ciprofloxacin and the other fluoroquinolones is suggestive of a lack of fluoroquinolone-degrading enzyme or an inability to produce enough of the enzyme required for their degradation. This suggests that the strain may lack the fluoroquinolones-resistance gene. This finding contradicts Huang et al. (2012) who reported an enzymatic resistance to fluoroquinolones in *E. cloacae*.

E. cloacae strains are often isolated from human clinical specimens and numerous publications on the antibiotic resistance of these bacteria have been made (Salimiyan et al. 2019). However, information on the antimicrobial sensitivity of *E. cloacae* isolated from soil sources is scarce. As such, the susceptibilities to the β-lactam and fluoroquinolone drugs which contradict reported drug resistance may be attributed to the source of bacteria (soil) and possibly strain variation, as the strain under study may be different from those reported in literature. In addition, the use of broad spectrum antibiotics in treatment of infections might have led to drug resistance in reported strains. Salimiyan et al. (2019) reported that the unnecessary use of extended-spectrum cephalosporins and carbapenems in treatment of *Enterobacter* infections has led to emergence of resistant strains.

The susceptibility of *E. cloacae* ODS 29 to gentamicin and streptomycin may suggest a lack of aminoglycoside-modifying genes required to produce the enzyme needed to modify and inactivate the aminoglycosides. These findings agree with Kim et al. (2009) who reported susceptibility of some *E. cloacae* isolates to amikacin and gentamicin. The susceptibility of *E. cloacae* ODS 29 to all ten antimicrobials therefore suggests its safety in probiotic use.

Oral probiotic strains experience severe acidic conditions in the stomach, where the pH is close to 2 (Mathipa and Thantsha 2015). The viability at pH < 2 observed for *E. cloacae* ODS 29 (Fig. 1) may be suggestive of the possession of acid-inducible genes to overcome stress. Chung et al. (2006) reported that *E. coli* and other bacteria respond to stress conditions by activating small or large group of genes to allow cells cope with specific stress situations. Currently, information on the gastrointestinal tolerance of the *E. cloacae* species is scarce. As such, we compare our observations with Menconi et al. (2013) and Mathipa and Thantsha (2015) whose reports for probiotic *B. amyloliquefaciens* and some strains of *Lactobacilli* and *Bifidobacteria* strongly support our observations.

The reduction in viability of *E. cloacae* ODS 29 with increases in bile concentrations (Fig. 2) may be attributed to the toxic effects of bile salts. This finding is supported by Hassanzadazar et al. (2012) who reported that bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes. This finding also agree with Mathipa and Thantsha (2015), who reported similar decreases in viability of probiotic *Lactobacilli and Bifidobacteria* strains respectively. The ability of the strain to survive bile concentrations up to 2% may possibly be attributed to a gene expression mechanism. Goyal et al. (2020) reported that the methods by which bacteria cells counters bile stress is not well defined and may involve a complex gene expression. The survival of *E. cloacae* ODS 29 at 2% bile concentrations over 5 h is an advancement over the findings of Menconi et al. (2013) who reported bile tolerance at 0.3% for probiotic *B. amyloliquefaciens* over 2 h.

The significant increases in viability of *E. cloacae* ODS 29 in gastric juice (Fig 3) is

indicative of its non degradation by pepsin, implying that gastric juice has no debilitating effect on the vitality of the organism. The resistance to proteolysis may be attributed to a possible possession of a non pepsin-degradable capsule covering the outer layer of the cell wall. Mezzatesta et al. (2012) reported that the *Enterobacter* species do not form spores while some are encapsulated. The increases in viability of *E. cloacae* ODS 29 contradict Buruleanu (2012) and Nair and Dubhashi (2016) who reported decreases in viability of *Bifidobacterium animal is* and fluctuations in probiotic *Bacillus* species tested over same period.

Tolerance to simulated gastric and duodenal conditions is important for potential probiotic bacteria. The non significant increases in the viability of *E. cloacae* ODS 29 under simulated gastric condition (Fig. 4) may be attributed to its ability to survive acid stress and resist proteolysis. The significant increases in viability under the duodenal condition may be attributed to an increase in the pH of the medium at the introduction of bile salts. This might have neutralized the acid, reducing the stress on the bacteria. These findings agree with Ruiz et al. (2011) and Ashraf and Smith (2016) who reported similar increases in viability of commercial probiotics under similar conditions. Tolerance of *E. cloacae* ODS 29 to *in vitro* simulated GIT conditions therefore suggests its ability to survive the GIT condition *in vivo*.

CONCLUSION

Safety to health, survival at low pH and high concentrations of bile salts, as well as resistance to proteolysis is crucial for bacteria intended for probiotic use. To date, the antimicrobial sensitivity testing of *Enterobacter cloacae* has received much attention, but reports on its GIT tolerance has been scarce. As such, information on the probiotic potentials of a phytase-producing strain of *E. cloacae* (*E. cloacae* ODS 29) have been made available by the authors. The non pathogenic nature of *E. cloacae* ODS 29, its susceptibility to different classes of antimicrobials and tolerance to an *in vitro* assessment of gastrointestinal conditions makes it ideal for use as a probiotic feed supplement for the endogenous production of phytase.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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