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Bioinhbition of Diarrhogenic Gram-negative Bacteria using the Tukey-HSD Test

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Abstract: A variety of probiotics show promise as effective therapies in the control of diarrhoea, however, diets and geographical locations affect probiotic therapy. In this pilot study, four indigenous probiotic candidates, *Lactobacillus acidophilus* AAOOL4, *L. reuteri* AAOOCH1, *L. plantarum* AAOO25NN and *L. delbrueckii* AAOOT20 were investigated for their *in vitro* bactericidal effects on bacterial pathogens implicated in infantile diarrhoea using the Tukey test. The inoculum levels were between 5 $\log_{10}$ cfu g^{-1} at 1% (inoculum per *ogi* sample) for 96 h at 35°C. The bactericidal effect of the probiotic candidates on the microbial load of the inoculated samples was determined by plate counts 24 hourly. Significant differences ($p<0.05$) were observed between the control (4.79-5.28 $\log_{10}$ cfu ml^{-1}) and the inoculated samples AAOOL4 (<1.00-4.61 $\log_{10}$ cfu ml^{-1}), AAOON25 (<1.00-4.71 $\log_{10}$ cfu ml^{-1}), AAOOCH1 (<1.00-4.67 $\log_{10}$ cfu ml^{-1}) and AAOOT20 (<1.00-4.78 $\log_{10}$ cfu ml^{-1}), especially the mixed probiotic culture-inoculated samples, MLC (<1.00-4.56 $\log_{10}$ cfu ml^{-1}). The diarrhogenic candidates were not within detectable limits between 72 and 96 h in most of the samples, indicating their *in vitro* bactericidal effects. The indigenous probiotic candidates can therefore, serve in the control of infantile diarrhoea.

Key words: Antibiotics, bactericidal, biotherapy, diarrhoea, indigenous, infantile, probiotics, Tukey statistical test

INTRODUCTION

For more than a century, researchers have suggested that live bacterial cultures, such as those found in yogurt, might be useful in the prevention and treatment of gastrointestinal disorders[11]. The development of innovative biodrugs by using live microorganisms that are active in the human digestive environment has also been considered by various workers[2,3,5,8,9,12,18,20,22] and potential medical applications in few developed countries are numerous. However, studies on probiotics in Nigeria are still very scanty and yet mostly undocumented. This preliminary study is therefore, undertaken to investigate the *in vitro* bactericidal effects of locally developed probiotics on diarrhogenic bacteria implicated in infantile diarrhoea.

MATERIALS AND METHODS

Bacterial species: Diarrhogenic stool and vomitus specimens were obtained from pediatric patients at Oni Memorial Children Hospital, Ibadan, and from epidemic patients in Abia, Benue and Eboyin states of Nigeria. Final microbiological analyses were carried out at the Nigeria Institute of Medical Research (NIMR), Lagos and Department of Botany and Microbiology, University of Ibadan laboratories.

Lactobacillus strains were obtained from infantile faecal samples of healthy children, and indigenous fermented foods and beverages. Standard phenotypic taxonomic tools were employed for the identification of the bacterial species used in this study[1,7, 14].

Survival of diarrhogenic bacteria in *ogi*: The diarrhogenic bacteria and the *Lactobacillus* strains were separately grown using standard procedures and appropriate culture media. For each set up, the inoculum was prepared by separately washing the bacterial strains several times with sterile phosphate-buffer saline. The microbial load of the freshly prepared inoculum was adjusted to give about log 10^5 cfu ml^{-1} of the diarrhogenic bacteria and 10^7 cfu ml^{-1} of the *Lactobacillus* strains.

The method of Svanberg *et al*.[17] was adopted for the preparation of *ogi*. The appropriate volume of tap water was brought to a vigorous boil and the corresponding quantity of the *ogi* paste was added while stirring to avoid lumps. Cooking continued until

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the *ogi* paste became boiled. The probiotic candidates were then introduced at concentrations of 10^7cfu ml^{-1} into the cooled *ogi* (supplemented with 1% glucose) in the culture flasks (10% v/v) and left for about 6 h. Ten ml of the diarrhogenic cell suspensions (10^5 cfu ml^{-1}) was then added to culture flasks and the contents (1% v/v) were thoroughly homogenised to ensure even distribution of the inoculum. Incubation was at 32-35°C for 96 hours. Both the control samples (no single or mixed probiotic cultures) and the probiotic culture samples contained same inoculum levels at 0 h and growth of the diarrhogenic indicator bacteria was monitored throughout the experimental period. The quantitative determination of the surviving diarrhogenic bacteria was the plate count method.

Statistics: The bactericidal rates of the potential probiotic candidates were analysed with the chi-square test (2×3 Table) Tukey (Statview; SAS institute Inc., Cary, NC, USA). $p<0.05$ was considered significant. The results are presented as the median and range, based on the groups in homogenous subsets and on Type III sum of squares unless otherwise indicated.

RESULTS AND DISCUSSION

Table 1-8 show the growth kinetics (log cfu ml^{-1}) of the diarrhogenic bacteria inoculated into *ogi* already containing single and mixed probiotic candidates. Both the control samples (uninoculated cultures) and the probiotic culture samples contained same inoculum levels at 0 h and growth of the indicator bacteria was monitored throughout the experimental period. Although there were minor differences, a similar decline trend was observed among the growth kinetics of the diarrhogenic indicator isolates. Inoculation of the test diarrhogenic isolates, *Pseudomonas aeruginosa* 23S, *Klebsiella* sp. 24S, *Klebsiella pneumoniae* 33S, *Klebsiella pneumoniae* 411, *Citrobacter aerogenes* 55S, *Klebsiella pneumoniae* 57S and two reference strains, *Escherichia coli* NCTC11560 and V157 into laboratory prepared *ogi* previously inoculated with the probiotic candidates (*L. acidophilus* AAOOL4, *L. reuteri* AAOOCH1, *L. plantarum* AAOO25NN and *L. delbrueckii* AAOOT20) as single and mixed cultures showed significant inhibition of the various diarrhogenic bacterial isolates between 48 and 96 h, with the highest inhibition mostly produced by the mixed probiotic cultures. There was a sharp decrease in the number of the surviving diarrhogenic bacteria in the probiotic-inoculated *ogi*.

Within 72 h of incubation, all the diarrhogenic bacterial strains were within the limit of detection of less than 1.00 in the culture samples containing the mixed probiotic candidates (MLC) except for *Ps. aeruginosa* 23S, while at 96 h of incubation, in the single and mixed cultures, all the diarrhogenic bacterial isolates were within the limit of detection of less than

Table 1: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Pseudomonas aeruginosa* 23S inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.05±0.033^{a}	5.05±0.033^{a}	5.05±0.033^{a}	5.05±0.033^{a}	5.05±0.033^{a}	5.05±0.033^{a}
12	5.08±0.025	4.61± 0.031^{bc}	4.71±0.035^{cd}	4.67±0.047$^{c\ d}$	4.78±0.036^{d}	4.56±0.058^{b}
24	5.22±0.013	4.35±0.101^{ef}	4.61±0.026^{g}	4.53±0.053^{fg}	4.59±0.025^{g}	4.28±0.045^{e}
48	5.13±0.068	4.03±0.153^{hi}	4.28±0.040^{I}	4.23±0.136^{hi}	4.15±0.045^{hi}	3.93±0.167^{h}
72	5.17±0.129^{j}	3.49±0.200^{j}	4.07±0.115^{j}	3.88±0.191^{j}	3.69±0.210^{j}	1.00±1.732
96	4.79±0.067	<1.00^{k}	<1.00^{k}	<1.00^{k}	<1.00^{k}	<1.00^{k}

Values represent mean scores (n = 3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 = *Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection).

Table 2: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Klebsiella aerogenes* 24S inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.25±0.050^{a}	5.25±0.050^{a}	5.25±0.050^{a}	5.25±0.050^{a}	5.25±0.050^{a}	5.25±0.050^{a}
12	5.27±0.051	4.49±0.042^{c}	4.61±0.046^{d}	4.73±0.029^{cb}	4.77±0.050^{d}	4.51±0.045^{b}
24	5.32±0.047	4.41±0.035^{e}	4.43±0.035^{f}	4.59±0.070^{f}	4.63±0.050^{f}	4.26±0.095^{e}
48	5.27±0.090^{g}	4.14±0.231^{g}	4.25±0.058^{g}	4.22±0.135^{g}	4.60±0.061^{g}	3.91±1.905
72	5.19±0.060^{I}	3.75±1.732^{h}	3.65±0.075^{i}	3.90± 0.135^{g}	4.18±0.000^{i}	<1.00^{h}
96	4.92±0.038	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}

Values represent mean scores (n=3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection)

Table 3: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Klebsiella pneumoniae*.33S inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.15±0.050^{a}	5.15±0.050^{a}	5.15±0.050^{a}	5.15±0.050^{a}	5.15±0.050^{a}	5.15±0.050^{a}
12	5.28±0.051	4.41±0.042^{c}	4.59±0.046^{d}	4.38±0.029^{cb}	4.61±0.050^{d}	4.27±0.045^{b}
24	5.17±0.047	4.08±0.035^{e}	4.40±0.035^{f}	4.25±0.070^{f}	4.41±0.050^{f}	3.95±0.095^{e}
48	5.14±0.090^{g}	3.43±0.231^{g}	4.11±0.058^{g}	3.89±0.135^{g}	4.27±0.061^{g}	1.10±1.905
72	5.10±0.060^{i}	1.00±1.732^{h}	3.78±0.075^{i}	<1.00^{h}	3.85±0.000^{i}	<1.00^{h}
96	4.95±0.038	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}

Values represent mean scores (n = 3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection)

Table 4: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Klebsiella pneumoniae* 411 inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}
12	5.37±0.046	4.71±0.045^{b}	4.66±0.060^{b}	4.76±0.065^{b}	4.69±0.036^{b}	4.49±0.101
24	5.40±0.021	4.57±0.055	4.31±0.087^{a}	4.40±0.055^{a}	4.26±0.106^{a}	3.94±0.150
48	5.28±0.025	4.21±0.215^{d}	3.83±0.155^{c}	4.10±0.117^{d}	3.83±0.156^{c}	3.46±0.151
72	5.22±0.021	3.81±0.221^{b}	3.48±0.000^{ab}	3.46±0.151^{ab}	2.16±1.886^{a}	<1.00
96	5.07±0.055	<1.00^{a}	<1.00^{a}	<1.00^{a}	<1.00^{a}	<1.00^{a}

Values represent mean scores (n = 3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection).

Table 5: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Citrobacter aerogenes* 55S inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.15±0.015^{a}	5.15±0.015^{a}	5.15±0.015^{a}	5.15±0.015^{a}	5.15±0.015^{a}	5.15±0.015^{a}
12	5.17±0.025	4.45±0.130^{cd}	4.61±0.097^{d}	4.59±0.070^{d}	4.31±0.136^{bc}	4.03±0.114^{b}
24	5.07±0.050	4.10±0.156^{e}	4.26±0.146^{e}	4.23±0.185^{e}	4.14±0.131^{e}	3.54±0.280
48	5.09±0.042^{f}	3.46±0.151^{f}	3.79±0.200^{f}	3.58±0.173^{f}	3.46±0.151^{f}	1.00±0.173
72	5.26±0.026^{i}	2.16±1.886^{h}	3.48±0.00^{hi}	3.46±0.151^{hi}	<1.00^{g}	<1.00^{g}
96	4.88±0.026	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}

Values represent mean scores (n = 3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection)

1.00. It was noticeable that the diarrhogenic indicator isolates in the probiotic pre-inoculated *ogi* reached significantly lower levels faster than the uninoculated *ogi* samples (Table 1-8).

The anti-diarrhoeal properties of lactobacilli flora of the human intestine known as probiotics (health-promoting bacteria) have been investigated since the 1960s by various workers[2,8,13,19,21]. The beneficial properties of a probiotic however, should not only be based on theoretical aspects. Some scientific proof should be given that the probiotic improves the properties of the indigenous microflora and/or beneficially affects the host. The results of this present study therefore, showed a significant *in vitro* decline trend among the growth kinetics of the diarrhogenic bacterial indicator strains that were inoculated into boiled, supplemented *ogi* already containing single and mixed probiotics. The viability of the diarrhogenic bacteria, *Pseudomonas aeruginosa* 23S, *Klebsiella aerogenes* 24S, *Klebsiella pneumoniae* 33S, *Klebsiella pneumoniae* 411S, *Citrobacter* sp. 55S, *Klebsiella pneumoniae* 57S and two reference strains, *Escherichia coli* NCTC11560 and V157 inoculated into *ogi* already seeded with single and mixed selected probiotic candidates reduced significantly between 12 and 96 h of incubation, however, most of the indicator isolates were within the limit of detection of less than 1.00 by 96 h of incubation.

About 2.2 million children die from diarrhoeal diseases each year in developing countries, nearly all

Table 6: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Klebsiella pneumoniae* 57S inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}
12	5.37±0.046	4.71±0.045^{b}	4.66±0.060^{b}	4.76±0.065^{b}	4.69±0.036^{b}	4.49±0.101
24	5.40±0.021	4.57±0.055	4.31±0.087^{a}	4.40±0.055^{a}	4.26±0.106^{a}	3.94±0.150
48	5.28±0.025	4.21±0.215^{d}	3.83±0.155^{c}	4.10±0.117^{d}	3.83±0.156^{c}	3.46±0.151
72	5.22±0.021	3.81±0.221^{b}	3.48±0.000^{ab}	3.46±0.151^{ab}	2.16±1.886^{a}	<1.00
96	5.07±0.055	<1.00^{a}	<1.00^{a}	<1.00^{a}	<1.00^{a}	<1.00^{a}

Values represent mean scores (n = 3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection)

Table 7: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Escherichia coli* NCTC11560 inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Seeded *ogi* sample Incubation period (h)	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.08±0.015^{a}	5.08±0.015^{a}	5.08±0.015^{a}	5.08±0.015^{a}	5.08±0.015^{a}	5.08±0.015^{a}
12	5.17±0.025	4.45±0.130^{cd}	4.61±0.097^{d}	4.59±0.070^{d}	4.31±0.136^{bc}	4.03±0.114^{b}
24	5.07±0.050	4.10±0.156^{e}	4.26±0.146^{e}	4.23±0.185^{e}	4.14±0.131^{e}	3.54±0.280
48	5.09±0.042^{f}	3.46±0.151^{f}	3.79±0.200^{f}	3.58±0.173^{f}	3.46±0.151^{f}	1.00±0.173
72	5.26±0.026^{i}	2.16±1.886^{h}	3.48±0.00^{hi}	3.46±0.151^{hi}	<1.00^{g}	<1.00^{g}
96	4.88±0.026	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}	<1.00^{j}

Values represent mean scores (n = 3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection)

Table 8: Growth kinetics (log cfu mL^{-1}) with standard deviation of *Escherichia coli* V157 inoculated into laboratory prepared *ogi* pre-inoculated with single and mixed probiotic cultures

Incubation period (h)	Seeded *ogi* sample					
	UNS	AAOOL4	AAOON25	AAOOCH1	AAOOT20	MLC
0	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}	5.28±0.040^{a}
12	5.37±0.046	4.71±0.045^{b}	4.66±0.060^{b}	4.76±0.065^{b}	4.69±0.036^{b}	4.49±0.101
24	5.40±0.021	4.57±0.055	4.31±0.087^{a}	4.40±0.055^{a}	4.26±0.106^{a}	3.94±0.150
48	5.28±0.025	4.21±0.215^{d}	3.83±0.155^{c}	4.10±0.117^{d}	3.83±0.156^{c}	3.46±0.151
72	5.22±0.021	3.81±0.221^{b}	3.48±0.000^{ab}	3.46±0.151^{ab}	2.16±1.886^{a}	<1.00
96	5.07±0.055	<1.00^{a}	<1.00^{a}	<1.00^{a}	<1.00^{a}	<1.00^{a}

Values represent mean scores (n=3) with standard deviations. Sample means having the same letters for each incubation period are not significantly different. UNS = unseeded *ogi* sample; AAOOL4 =*Lactobacillus acidophilus*; AAOON25 = *Lactobacillus plantarum*; AAOOCH1 = *Lactobacillus reuteri*; AAOOT20 = *Lactobacillus delbruecki*; MLC = Mixed *Lactobacillus* cultures; <1.00 = less than 1.0 (limit of detection).

from dehydration as a result of persistent diarrhoea that is often aggravated by malnutrition[6]. The greatest mortality from diarrhoeal diseases and enteric infections occur in infants and small children, thus, over thirteen percent of the children born in certain parts of Latin America die before their fifth birthday with diarrhoea-associated diseases as the major cause of their death. In some cases, children of 36 months of age experience as many as 4 episodes of severe diarrhoea annually and, in this age group, gastroenteritis is reported to be a major cause of death[10]. Studies in Nigeria have also shown diarrhoea as the commonest cause of death among hospitalized children under-5 years of age[4], while the transmission of the pathogens can occur mostly through contaminated food or water or by person-to-person contact[10]. This study has demonstrated that *Lactobacillus* species with the ability to produce antimicrobial compounds against diarrhogenic bacteria are wide spread in Nigerian indigenous fermented foods and beverages as well as in infantile faecal samples of healthy children.

The development of rationally based applications of probiotic therapy could have very useful clinical contributions and their impact could be of very great importance in Nigeria, especially if they result in effective but low-cost ways of prevention and treatment of infantile diarrhoea. The basal food sample was studied through simulations of *ogi*, the most popular

non-industrial infant weaning food in Nigeria, and was shown to have good performances in the support of the probiotic bacterial candidates. In this study, none of the selected *Lactobacillus* strains, selected as potential probiotic candidates, *L. acidophilus* AAOOL4, *L. delbrueckii* AAOOT20, *L. plantarum* AAOON25 and *L. reuteri* AAOOCH1 gave any inhibitory activity towards other *Lactobacillus* strains, indicating possible lack of adverse effects in their selection as probiotic candidates against other closely related selected probiotic candidates. This research therefore, reports that some indigenous, non strain-specific probiotic candidates of Nigerian fermented foods and beverages as well as of infantile faecal specimen' origin were inhibitory against infantile diarrhogenic bacterial pathogens *in vitro*.

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