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Full Length Research Paper

Investigation on the safety and probiotic potentials of yoghurts sold in Owerri metropolis in Imo State Nigeria

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Investigation on the microbial safety and probiotic potentials of different yoghurt brands sold in Owerri, Imo State Nigeria, was carried out using standard microbiological procedures. Ten each, of five different brands of commercially available yoghurt packaged in plastic containers were purchased from the street vendors and shopping malls in Owerri metropolis. The mean total count of samples on Brain Heart Infusion (BHI) and De Mann Rogosa Sharpe (MRS) agar media ranged from 2.0×10^7 to 6.0×10^8 and 1.0×10^8 to 5.4×10^8 cfu/ml respectively. The yoghurt isolates were identified as *Streptococcus* and *Lactobacillus* species; these isolates were resistant to commonly used antibiotics and inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from clinical samples. No viable growth of isolates was observed in simulated gastric fluid of pH 1.5 to 2.5. Slight decrease in viable count of *Lactobacillus* spp. from 4.0×10^7 to 3.0×10^7 cfu/ml and *Streptococcus* spp. from 3.0×10^8 to 2.0×10^8 cfu/ml was observed in bile of pH 8.28 to 8.30. The isolates were recovered from faecal samples two weeks after ingestion with mean count ranging from no growth (zero) to 5.8×10^8 cfu/ml on MRS agar media. The isolates were found to exhibit some probiotic potentials and no pathogen was isolated from samples. It is recommended that strains of microorganisms that can deliver full probiotic potentials to consumers be used in commercial yoghurt production.

Key words: Yoghurt, microbial safety, probiotic potential, simulated gastric fluid, bile.

INTRODUCTION

Residing in the human gastrointestinal tract is a large and complex microbial ecosystem that develops through infancy and childhood to form a diverse, but relatively stable community in adults (Vaughan et al., 2002; Turnbaugh et al., 2009). These autochthonous bacteria interact with the diet and the host, contributing to protection against intestinal pathogens through colonization resistance and providing nutritional and colonic health benefits via their metabolic activities (Guarner and Malagelada, 2003; Sleator and Hill, 2008; Sleator, 2010). It has become clear that these bacteria also interact with the host's immune system and are essential for the maturation and homeostasis of a healthy immune system (Isolauri et al., 2001; Mishra et al., 2008; Ibrahim et al., 2010). Recognition of the importance of the intestinal microbiota to health has led to increasing interest in manipulating the composition and/or activity of the microbiota to improve both human and animal health (Corr et al., 2007; Raoult, 2009; Allen et al., 2010).

Probiotics are live microbial food supplements that are consumed with the aim of providing a health benefit to the host by contributing to an improved microbial balance within the intestinal microbiota (FAO/WHO, 2002; Sleator, 2010). Probiotic bacteria selected for commercial use in foods and in therapeutics must retain the characteristics for which they were originally selected (Sheehan et al., 2006, 2007). These include characteristics for growth and survival during manufacture and, after consumption, during transit through the stomach and small intestine. Importantly, probiotics must retain the characteristics that give rise to their health effects. Consequently, it is necessary to test the stability of these characteristics during manufacture (Lee and Salminen, 1995). Strains

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from the genera Lactobacillus and Bifidobacterium, both of which are indigenous to the human intestine, are the bacteria predominantly selected for use as probiotics (Reid et al., 2005; Ouoba et al., 2008; Hoveyda et al., 2009). Fermented dairy products (yoghurts and drinks) and capsules with freeze-dried bacteria are the most popular vehicles for delivering these organisms to the gastrointestinal tract.

Due to wide circulation of this drink in our market, it is necessary for a research to be carried out to determine the bacterial microflora of yoghurts in Owerri metropolis and to determine whether the isolates possess attributes of probiotics or not.

MATERIALS AND METHODS

Sample collection and storage

Sample selection was based on popularity (mostly demanded), and only samples within the expiry date as stipulated by manufacturer was analyzed. Ten each, of five different brands of commercial prepared yoghurt packaged in plastic containers were purchased from the street vendors and shopping malls in Owerri metropolis. The yoghurts were kept in refrigerator at 4°C before commencement of microbiological analysis. The information on their labels was recorded. These includes the manufacturers address, brand name, manufacture and expiry dates, batch number and the National Agency for food and Drug Administration control (NAFDAC) Number.

Culture media preparation

The culture media employed in this study was De Mann Rogosa Sharpe (MRS) agar for lactobacillus and Brain Heart Infusion (BHI) for fastidious and diverse microorganisms was prepared according to the recommendations of the manufacturer.

Sources of test pathogens

The pure isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* used as test pathogen were obtained from the Federal Medical Center (FMC) Owerri, Imo State. The purity and identification of the organisms was confirmed by using cultural, microscopic and biochemical methods as recommended by Cheesebrough (2004).

Microbiological analysis

The samples of yoghurt were brought out of the refrigerator and allowed to assume the laboratory room temperature of between 26 and 28°C, this is to avoid heat shock on the microbial content. They were then shaken vigorously to suspend the microbial content. Ten-fold serial dilution (10^{-1} to 10^{-5}) was carried out for all 5 products (A to E) using distilled water. The plates containing the media (MRS and BHI) were arranged in duplicate and labeled according to the samples and dilution factors to be used.

Determination of viable cell counts

Aliquot 0.1 ml of the sample (A to E) was dispensed into labeled Petri dishes. Spreader which was sterilized in 75% alcohol and flamed was allowed to cool and used to spread the inoculums on the plate. The BHI plates were incubated at 37°C for 24 h, while the MRS plates were incubated at 37°C for 48 h to five days in increased CO_2 atmosphere.

Morphological and cultural examination

After incubation, the macroscopic characteristics of the microbial growth on various media were observed and recorded. Colony forming units were counted and recorded as well. The isolates were purified using the repeated subculture streak plate technique after which the pure colonies were confirmed by Gram staining and subsequently stored in the refrigerator for further identification (Harrigan and Maccance, 2006).

Biochemical characterization of bacteria isolates

Biochemical tests such as catalase, citrate utilization, indole, oxidase, motility, temperature tolerance, growth on concentrations of NaCl, methylene blue, haemolysis on blood agar and sugar fermentation test described by Buchannan and Gibbbon (1974), and Cheesbrough (2004), were adopted to identify and differentiate the isolates.

Study on the antagonistic activity of yoghurt isolates

Culture BHI broths containing *Lactobacillus* and *Streptococcus* strains respectively isolated from yoghurt samples, and pure cultures of *S. aureus* and *P. aeruginosa* obtained from the Federal Medical Centre Owerri, Imo State, all inoculated in BHI broth were incubated at 37°C for 24 h. Nutrient agar media was used for the agar well diffusion assay and for spot inoculation. Aliquots of the target organisms was spread unto the surface of nutrient agar medium, four wells (5 mm diameter) were cut from the agar and aliquot of test culture broth was delivered into them, direct spot inoculation without wells was also carried out. After incubation for 24 h at 37°C, the plates were examined for any zone of inhibition/growth.

Susceptibility of yoghurt isolates to antibiotics using disc diffusion method

The method of Charteris et al. (1998) was adopted with modification. Cultures of the bacterial flora of yogurt and control virgin *S. aureus* were inoculated into BHI broth and were incubated at 37°C for 24 h, after which, 2 ml of the broth was inoculated on freshly prepared agar plate. It was rocked for two minutes, then the excess was decanted and multi anti bacterial disc were carefully and firmly placed on each inoculated agar for 24 h at 37°C, this was viewed for zone of inhibition/ growth around each anti bacterial disc, which was measured using transparent rule in millimetre (mm) (Forbes et al., 2007).

Test for tolerance to gastric acidity

Determination of the tolerance to simulated gastric transit was carried out by using artificially prepared simulated gastric fluid (SGF) peptone water plus concentrated hydrochloric acid (HCL) BDH laboratory grade of pH 1.5 to 2.5. Overnight, 1 ml culture of the isolate was subjected to 9 ml SGF. In controls, 1 ml of overnight cultures of 2.0×10^7 cfu/ml without SGF was used for determination of initial cell counts. Aliquot 0.1 ml culture broth treated with SGF

were removed after 30, and 40 min at 37°C and viable counts were determined by plating neat and serial 10 fold dilution on BHI agar media and the plates were incubated and observed after incubation of plates at 37°C for 48 h. Experiments were carried out in duplicate and were repeated. Results are expressed in mean value.

Test for tolerance to bile

The bile sac of ox was aseptically collected from the slaughter, dispensed into a beaker and 50 ml was measured and mixed with 50 ml of peptone water. The pH of bile was in the range of 8.29 to 8.30. Culture was made to test for sterility of the bile.

One millimeter culture of the isolate was subjected to 9 ml of the bile mixture and was dispensed in bijou bottle, 0.1 ml of the culture broth that was treated with bile mixture was removed, and viable counts were determined by plating neat and serial tenfold dilution on media and the plates were observed after incubation at 37°C for 48 h. This was done in duplicates and the mean value was recorded.

Test for colonization and attachment to intestinal mucosa

Yoghurts were administered to ten healthy volunteers (six males and four females) aged 16 to 30 years. Subject's faecal samples were screened for presence of test isolate prior to yoghurt administration and were to abstain from consumption of any other yoghurt/fermented products. After a period of two weeks, microbiological analysis of faecal samples was performed as previously described, by plating sample on MRS and BHI agar medium. Isolates were enumerated and recorded. In addition, further (biochemical) test were carried out to confirm the isolates as organisms of interest.

RESULTS

The total bacterial count ranges from 2.0×10^7 to 6.0×10^8 on BHI and from 1.0×10^8 to 5.4×10^8 on the MRS (Table 1), only sample E had no growth on the MRS agar medium after the period of incubation. The different characteristics of the colonies were observed and represented in the Table 1.

The susceptibility of the yoghurt isolates to commonly used antibiotics is shown in Table 2. *Streptococcus* species were resistant to amoxicillin and three other commonly used antimicrobials, while sensitive to gentamycin and ciproflaxacin. *Lactobacillus* species were resistant to gentmycin, streptomyc and amoxicillin.

The yoghurt isolates were found to posses varying inhibitory activity against *S. aureus* and *P. aeruginosa.* While the *Lactobacillus* spp. had inhibitory activity against *S. aureus* and *P. aeruginosa, Streptococcus* spp. had no effect against *S. aureus.*

The experiment shows that when yoghurt isolates was exposed to a simulated gastric fluid at the pH 1.5 to 2.5, all the yoghurt isolates (*Lactobacillus* spp. *and Streptococcus* spp.) after incubation period of 24 h at 37°C, were found to have no viable count.

Exposure of yoghurt isolates to bile at pH 8.29 to 8.30 shows that the isolates (*Lactobacillus* spp. *and Streptococcus* spp.) survived a period of 24 h incubation at 37°C. The viable counts of the *Lactobacillus* spp. isolates were, however, slightly decreased from 4.0×10^7 to 3.0×10^7 cfu/ml. While the *Streptococcus* specie decreased from 3.0×10^8 to 2.0×10^8 cfu/ml.

After a period of two weeks, viable growth of Lactobacillus on MRS was obtained from faecal samples of individuals that consumed yoghurt samples except for samples A and B (Super Sunnex and GT yoghurt) which had no growth. The mean total bacteria count ranges from zero to 5.8×10^8 cfu/ml (Table 3).

DISCUSSION

The study on safety and probiotic potentials of different yoghurt brands sold in Owerri has shown that the yoghurt producing companies provides information such as batch number, manufacturers address, NAFDAC number, but they do not give information on microbial composition/contents.

The study has also shown that some yoghurts circulating in Owerri south–east Nigeria contains viable micro organisms which are able to survive production process, preservation temperature and refrigeration temperature of 4°C. The gram reaction and microscopic examination of the yoghurt isolates reveals that all the isolates were gram positive and there was no trace of gram negative bacteria, which might indicate the presence of contamination from coliforms/ enterobacteriaceae. The microbiological and biochemical analysis reveals the identified organisms were *Lactobacillus* species and *Streptococcus* species.

Yoghurt isolates were resistant to some commonly prescribed antibiotics. Amoxicillin, streptomycin, ceftriazone, chloramphenicol, gentamicin and cotrimazole were not effective against all yoghurt microflora, while oflaxacin, pefloxacin, and erythromycin, were very active against the yoghurt microflora. Antibiotic resistant in bacteria may be intrinsic or acquired. Intrinsic resistance is a naturally occurring trait and may be considered as specie characteristics, whereas acquired resistance drives either from genetic mutation or acquisition of foreign DNA from other bacteria (Teuber et al., 1999; Belletti et al., 2009). The relationship between antibiotic use and resistance was reviewed by Singer et al. (2006).

In several fermented foods as in probiotic, Lactobacilli are often a relevant microbial component and can interact with gut microflora (Ammor, 2007).There is need for further investigation on the antibiotic resistance implication of LAB because in spite of the large consumption of live Lactobacilli, the presence of acquired antibiotic resistance is not crucial for their classification as generally recognized as safe by the U.S. Food and Drug Administration (Belletti et al., 2009). It has been reported that there is no barrier between pathogenic (for example, streptococci), potentially pathogenic (for example, enterococci), and commensal (for example, lactobacilli and lactococci) LAB regarding acquired

Table 1. Mean total count and colonial characteristics of isolates on BHI and MRS agar.

Sample code	Mean total count cfu/ml	Colony code	Size (mm)	Shape	Elevation	Margin	Colour	Surface appearance
				BHI medium				
YGA	3.0×10^{7}	A_1	5	Regular	Low convex	Entire	Cream	Moist, shiny and mucoid
		A_2	<1	Regular	Low convex	Entire	Yellow	Moist and shiny
YGB	5.0×10^{7}	B_1	1	Regular	Low convex	Entire	Cream	Moist and shiny
		B_2	<1	Regular	Low convex	Entire	Yellow	Moist and shiny
YGC	2.0×10^{7}	C_1	3	Regular	Low convex	Entire	Cream	Moist and shiny
		C_2	<1	Regular	Low convex	Entire	Yellow	Moist and shiny
YGD	6.0×10^{8}	D_1	8	Regular	Low convex	Entire	Cream	Moist, shiny and mucoid
		D_2	2	Regular	Low convex	Entire	Yellow	Moist and shiny
YGE	5.0×10^{8}	E_1	<1	Regular	Low convex	Entire	Cream	Moist and shiny
		E_2	6	Regular	Low convex	Entire	Yellow	Moist and shiny
				MRS medium				
YGA	2.0×10^{8}	A	4	Regular	Flat	Entire	Cream	Mucoid and dry
YGB	1.0×10^{8}	B	3	Regular	Low convex	Entire	Cream	Moist and shiny
YGC	5.0×10^{8}	C	5	Regular	Flat	Entire	Cream	Moist and shiny
YGD	5.4×10^{8}	D	3	Regular	Low convex	Serrated	Cream	Moist and shiny
YGE	NO GROWTH	-	-	-	-	-	-	-

Key: YGA = SUPER SUNNEX YG B = GARDEN CITY YGC = G T YG D = JOSSY YG E = KYLIN.

Table 2. Susceptibility of yoghurt isolates to commonly used antibiotics.

Organism		Streptococcus specie		Lactobacillus specie		Standard virgin
Antibiotics	**Conc. (µG)**	**Sensitivity**	**Inhibition zone (mm)**	**Sensitivity**	**Inhibition zone (mm)**	***S. aureus* zone (mm)**
Amoxycillin	25	r	8	r	8	10
Ofloxacin	5	s	20	s	20	15
Streptomycin	10	s	15	r	8	12
Chloramphenicol	30	s	12	r	9	14
Ceftriazone	30	r	6	s	16	9
Gentamycin	10	s	14	r	10	14
Pefloxacin	5	s	12	s	12	12
Cotrimaxozole	25	r	5	s	17	10
Ciproflaxacin	10	s	15	s	20	15
Erythromycin	5	s	14	s	12	11

Key: S = sensitive; R = resistant.

antibiotic resistance, and identical genes responsible for resistance are found among these organisms (Mathur and Singh, 2005; Belletti et al., 2009). Horizontal transfer of resistance factor from *Lactobacillus* to *Enterococcus* of a gene involved in the resistance towards erythromycin and tetracycline has been shown (Jacobsen et al., 2007; Ouoba et al., 2008).

The study has shown that the *Lactobacillus* species and *Streptococcus* species had inhibitory activity against *S. aureus* and *P. aeruginosa*; this means that the yoghurt isolates have the ability to inhibit the growth of some pathogenic organisms when found in the same habitat, such as the gastro intestinal tract. Several researches on effect of probiotics on pathogenic organisms reveals that regular intake of probiotics can reduce potentially pathogenic bacteria in upper respiratory tract and GIT (Ulrich and Jan-Olaf, 2003; Hoveyda et al., 2009; Allen et al., 2010). Beneficial effect of probiotics has been observed in several models of gastrointestinal infection, clinical trials in colonized human adults and children show that while probiotics do not completely eradicate pathogens such as *H. pylori*, they maintain significantly

Table 3. Mean total count and colonial characteristics of bacteria isolates from faeces on MRS agar.

Sample code	Yoghurt name	Mean total count cfu/ml	Colony code	Size (mm)	Shape	Elevation	Margin	Colour	Surface appearance
YGA	Super sunnex	No growth	-	-	-	-	-	-	-
YGB	GT	No growth	-	-	-	-	-	-	-
YGC	Kylin	5.0×10^8	C	5	Regular	Low convex	Entire	Cream	Moist and shiny
YGD	Garden City	1.0×10^8	D	7	Regular	Low convex	Entire	Cream	Moist and shiny
YGE	Jossy	4.8×10^7	E	3	Regular	Low convex	Entire	Cream	Moist and shiny

Key: YG = Yoghurt.

lower levels of the bacterium and thus, in combination with antibiotics, increase eradication rates and/or decrease antibiotics' adverse effects (Gotteland et al., 2006).

The application of probiotic cocktails (combinations of two or more strains with potentially different mechanisms of antimicrobial action), have been reported (Hickson et al., 2007; Sleator, 2010). Using a mixture of two strains of Lactobacillus *murinus* and one strain each of *Lactobacillus salivarius* subspecies *salivarius*, *Lactobacillus pentosus* and *Pediococcus pentosaceous* (collectively referred to as LIVE5) Casey et al. (2007) obtained significant reductions in both clinical and microbiological signs of *Salmonella typhimurium* infection in a porcine model animals treated with this cocktail. Hamilton-Miller (2003), Johnson-Henry et al. (2004) and Hoveyda et al. (2009) noted that a mixture of *Lactobacillus* strains reduces gastric inflammation and bacterial colonization in *Helicobacter pylori*-infection and in irritable bowel syndrome. Furthermore, probiotics have also been demonstrated to be effective against enteric viruses, particularly rotavirus (Heyman, 2000). Besides treating enteric infections, "designer probiotics" have been recruited to combat HIV (Rao et al., 2005; Lagenaur and Berger, 2005), and reduction in pregnancy related complications have also been reported (Braga et al., 2011; Myhre et al., 2011).

Exposure of the yoghurt isolates to simulated gastric fluid of pH 1.5 to 2.5 resulted in no growth. This indicates that the specific pH levels and incubation time condition affected the survival of the yoghurt isolates. In that case, strains with high acid tolerance should be used in the production of yoghurt, so as to enable them survive in the gastro intestinal tract and confer desired health benefits. Acid stability and intestinal mucosal adhesion properties are among the criteria used to select probiotic microbes (Salminens et al., 1998; Sheehan et al., 2006, 2007). Viability is an important factor, but not the only criteria for quality assurance (Elina et al., 2001). One of the limitations of probiotics in clinical application is that the most effective probiotic strains often prove to be fragile during industrial processing such as drying or heating (Sheehan et al., 2006). Improving the stress tolerance of probiotic strains is thus an important biological and clinical goal (Sheehan et al., 2007; Sleator and Hill, 2008).

The study showed the trend of tolerance of yoghurt isolates to bile at 8.29 to 8.30. Here, all isolates of *Lactobacillus* and *Streptococcus* species survived treatment with bile at the incubation period of 37°C for 24 h. The viable counts of the *Lactobacillus* specie and *Streptococcus* specie, however, decreased from 4.0×10^7 to 3.0×10^7 cfu /ml and from 3.0×10^8 to 2.0×10^8 cfu /ml respectively. Other studies on lactic acid bacteria corroborate with these findings (Haller et al., 2001). Bile production contributes to the antimicrobial arsenal developed by mammalian host to inhibit microbial colonization and potential infection; this could contribute to reduction in viable cell count as observed in this work. The bile resistant traits of intestinal yoghurt isolates are crucial for maintaining viability during GI transit and are desirable attributes of an orally administered probiotic (Charteris et al., 2000; Elina et al., 2001; Elkins and Lisa, 2004).

This study also showed that the yoghurt samples after being consumed by healthy volunteers, two weeks after the gastro intestinal tract sill had a good number of the yoghurt microflora. It could be explained that yoghurt microflora were able to adhere and colonize the intestinal mucosa, these characteristics are necessary for selecting probiotics for commercial use in foods and therapeutic, to ensure that they are retained inherently. The ability to adhere is also, to some extent likely to be connected to the ability to stimulate the immune defence (Ouwehand et al., 1999; Plant and Conway, 2002). However, permanent colonization by the probiotics is unwanted and remains unsolved whether colonization is critical for probiotics to have their effect at all (Ouwehand et al., 1999; Fedorak and Madsen, 2004). That the isolates were found/recovered from stool even though they did not survive the simulated gastric fluid after 24 h incubation, calls for further investigation.

It can be concluded that some yoghurts circulating Owerri in Imo State Nigeria, contain no contaminating gram negative microorganisms/coliforms and are thus acceptable. The starter culture used in the production possesses some desired attributes of a probiotic organism such as surviving production process, storage

temperature, resistance to bile and some commonly used antibiotics, ability to inhibit the growth of some pathogenic organisms, and colonization/adherence to intestinal mucosa (recovery from faecal samples). The use of strains of probiotic organisms better adapted to resist gastric acid and more of the commonly used antibiotic is advocated, further work in this regard is also necessary.

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