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Research Article

In vitro Inhibitory Effect of Probiotics on *Helicobacter pylori*

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ABSTRACT

The use of probiotics for the inhibition of *Helicobacter pylori*, could present a low-cost, large-scale alternative solution to prevent or decrease Helicobacter pylori colonization. This study is however designed to evaluate the antibacterial activity of probiotics (Lactobacillus) produced by Lactic acid bacteria against *Helicobacter pylori*. The study also focuses on the isolation and characterization of Helicobacter pylori and different lactic acid bacteria from stool sample of ulcer patients and local rice water respectively. The effect of probiotics on *Helicobacter pylori* growth and Helicobacter proliferation were examined in vitro of the isolates identified from this study, the probiotic *Lactobacillus acidophilus* strain NK-S10 had the highest zone of inhibition of 18mm against Helicobacter isolate Hp3 while *Lactobacillus spp* had the least inhibition zone of 2mm against *Helicobacter pylori* isolate Hp1. Therefore, this study demonstrates the lethal effect probiotics could have on *Helicobacter pylori* growth and proliferation. Thus, the administration of probiotics can decrease the frequency of diarrhea, a frequent side effect of traditional anti- *Helicobacter pylori* tri-therapy.

Keywords: *Probiotics; Helicobacter pylori; Antibacterial; Ulcer patients*

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INTRODUCTION

The genus *Helicobacter* is a Gram-negative bacterium possessing a characteristic helical shape. They were initially considered to be members of the genus *Campylobacter*, but in 1989, Goodwin et al., published sufficient reasons to justify the new genus name *Helicobacter* (Goodwin et al., 1989). Boyanova (2011) and Yamoaka (2008) reported that Helicobacter has about 35 species. Some species have been found living in the lining of the upper gastrointestinal tract as well as the liver of mammals and some birds (Ryan and Ray 2004). The most widely known species of the genus is *Helicobacter pylori*, which infects up to 50 percent of the human population (Yamoaka 2008). They are micro-aerophilic (optimal oxygen concentration between 5 and 14 percent) and are fast moving with their flagella (Rust et al., 2008). Helicobacter pylori is most likely transmitted from person to person (usually acquired in childhood).

Helicobacter pylori infection, a highly prevalent pathogen, is a major cause of chronic gastritis and peptic ulcer and a risk factor for gastric malignancies. Antibiotics-based Helicobacter pylori eradication treatment is 90% effective. However, it is expensive and causes side effects and antibiotic resistance. To quantify this interest, it is enough to look at a number of studies on the subject, keeping in mind that the organism was first named *Campylobacter pyloridis* and then *Campylobacter pylori* before taking its present moniker of Helicobacter pylori in 1998.

MATERIALS AND METHODS

Setting: The study was conducted at the University of Calabar, Calabar, Cross River State and all laboratory procedures were carried out in the Department of Microbiology of Same University.

Isolation of Helicobacter pylori from stool samples: A tenfold serial dilution for the two samples was carried out by dissolving 1g of each stool sample into a test tube containing 9ml of water to obtain a total of 10ml. the tubes were then used to run a 10- fold serial dilution after which 1ml from dilution 10^{-6} and 10^{-7} were plated using pour plate method.

Isolation of probiotics from rice water: The probiotics used were isolate from local rice water, using the method of David et al., (2013). Local rice was purchased from Goldie market, Calabar, washed with distilled water and the first two rinses of cloudy water was collected in a sterile glass jar to about 2/3 full. The jar was labeled and covered with breathable clothes and secured with rubber band to keep out insects and pests. The content of the jar was stored at room temperature away from sunlight for four days. After four days, there was a mat floating on top of the cloudy liquid. The cloudy liquid was collected into another sterile jar and a cup of milk was added to make 2/3 full. The jar was again covered with breathable cloth, secured with rubber bands to keep out insects and pests

and stored at room temperature away from direct sunlight without shaking or tilting for another four days. After this period, the content of the jar was separated into a thick mass of floating semi-solid material and a yellow liquid fraction. The yellow liquid which contains the LAB culture was poured off and 1.0ml pipetted into 99ml of distilled water for a 10-fold serial dilution. A 1.0ml of 10-8 diluent was pour plated in duplicated on De-Mann, Rogosa and Sharpe (MRS) agar, prepared according to the manufacturer's instructions and was sterilized by autoclaving at 1210C for 15 minutes then, incubated at 370Cfor 24- 48 hours.

Purification and maintenance of cultures: The plates were observed and inspected for visible growth and colonies. Morphologically, distinct colonies were picked from each plate and transferred to fresh sterile De-Mann Rogosa and sharpe (MRS) agar plates and Chocolate agar plates for Lactobacillus and Helicobacter pylori respectively, for further culture and analysis. The bacterial cultures were cultured using a sterile inoculation loop to pick morphologically distinct colonies and then a zig-zag streak was done on the surface of De-Mann Rogosa and sharpe (MRS) agar plates as described by De- Mann et al., (1979) and on the surface of Chocolate agar plate. The plates were labeled with identification marks and numbers for each isolate and were incubated anaerobically at 370C for 24 hours.
Pure cultures isolated after 24 hours incubation were maintained in De-Mann Rogosa and sharpe (MRS) and Chocolate agar stock bottles which were allowed to solidify in slant position. With a sterile wire loop, each isolate was streaked into approximately labeled De-Mann Rogosa and sharpe (MRS) and Chocolate agar slants from bottom to top. The slants were incubated for 24 hours at 370Cfor growth to take place before over-laying with sterile paraffin oil.

Biochemical characterization and identification of isolates: The following biochemical tests were carried out for further identification of bacterial isolates. They include; Gram reaction, catalase test, oxidase test, motility test, methyl red Voges Proskauer (MR-VP) test, carbohydrate fermentation tests which are; lactose, glucose, xylose, mannitol, sucrose, maltose, raffinose, fructose, and galactose.
The tests were carried out using specific methods by De-Mann et al., (1979. The tests were performed using 18-24 hours old culture of the isolates grown in MRS and chocolate agar broth to characterize and identify the pure isolates for easy identification to the lowest possible taxonomic group. Refer to appendix III for the principles of the specific biochemical test.

In vitro study protocol: The inhibition of Helicobacter pylori growth by probiotics and antibiotics was also investigated on culture plates. Well diffusion assay; Helicobacter pylori cultures were plated on fresh Chocolate agar without antibiotics and wells were drilled into the agar using a sterile cup borer. 5µl aliquots of fresh Lactobacillus strains re-suspended in fresh De- Mann Rogosa and Sharpe (MRS) broth was suspended in the agar wells. Plates were incubated for 48 hours under micro-aerophilic conditions at 370C and the diameters of inhibition zones around the wells were measured. Again, serial dilution for the two antibiotics; metronidazole and clarithromycin was carried out by dissolving 1 gram of each antibiotic in test tubes containing 9 ml of sterile distilled water for each antibiotic. Serial dilution was then performed by transferring 1 ml from the first tubes into other tubes to obtain 10-6 respectively. Wells were drilled into the agar using a sterile cup borer and then 5µl of the different concentrations diluted were suspended into the wells. Plates were incubated following the above procedure and observations showed no inhibition on the plates.

Genomic identification of selected isolates: Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) (Thomas Scientific, USA) was spun at 14000 rpm for 3 min. The cells were then re-suspended in 500 µL of normal saline and heated at 95 0C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5 mL micro-centrifuge tube and stored at -20 oC for other downstream reactions. The extracted genomic DNA was quantified using the NanoDrop 1000 spectrophotometer using the method Reyes-Escogido et al. (2010). The 16S rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR conditions were as follows: Initial denaturation, 95 ºC for 5 min; denaturation, 95 ºC for 30 s; annealing, 52 ºC for 30 s extension, 72 ºC for 30 s for 35 cycles and final extension, 72 ºC for 5 min. The product of the PCR amplification was resolved on a 1% agarose gel at 120 V for 15 min and visualized on a UV transilluminator as described by Reyes-Escogido et al. (2010). Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa (Reyes-Escogido et al., 2010)

RESULTS

Table 1 presents the macro-morphological characteristics of each distinct colony of respective isolates in terms of pigmentation, margin, elevation, surface and form. All the Helicobacter pylori isolates were found to be milky in color with an entire margin and raised elevation. The colonies were also observed to have a smooth surface and mucoid in texture. Table 2 shows the biochemical characterization and identification of Helicobacter pylori isolates. All the selected isolates were biochemically confirmed as *Helicobacter pylori* with the aid of Bergey's Manual of Determinative Bacteriology. The biochemical characterization and identification of the lactic acid bacteria isolates is presented in Table 4. The species isolated included Lactobacillus plantarum, Lactobacillus lactis, Lactobacillus fermentum and Lactobacillus acidophilus. The result of the antibacterial testing of the broth culture of Lactobacillus species against Helicobacter pylori is shown in Figure 1.

Table 1:
Macro-morphological characteristics of the bacterial isolates

Isolate code	Pigmentation	Margin	Elevation	Surface	Form	consistency
Hp1	Milky	Entire	Raised	Smooth	Irregular	Mucoid
Hp2	Milky	Entire	Raised	Smooth	Circular	Mucoid
Hp3	Milky	Entire	Flat	Smooth	Irregular	Dry
Hp4	Milky	Entire	Raised	Smooth	Irregular	Mucoid
Hp5	Milky	Entire	Flat	Smooth	Circular	Dry
Hp6	Milky	Entire	Flat	Smooth	Circular	Dry

Key:
Hp1-6 = *Helicobacter pylori* isolates

Table 2:
Biochemical characterization and identification of the isolates

Isolates code	Gram's reaction	Cell morphology and arrangement	Catalase	Motility	Oxidase	Coagulase	Indole	Citrate	H_2S	MR	VP	Nitrate	Urease	Glucose	Lactose	Sucrose	Growth at 0.5 and 0.75% NaCl	Growth at 1.00 and 1.25% NaCl	Growth at 1.5% NaCl	Probable organism
Hp1	-	Rods in pairs	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-	*Helicobacter pylori*
Hp2	-	Rods in pairs	+	+	-	-	-	+	+	-	+	+	+	+	-	+	+	+	-	*Helicobacter pylori*
Hp3	-	Rods in pairs	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	-	*Helicobacter pylori*
Hp4	-	Rods in pairs	+	+	-	-	±	-	+	+	-	+	+	±	-	-	+	+	-	*Helicobacter pylori*
Hp5	-	Rods in pairs	+	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	-	*Helicobacter pylori*
Hp6	-	Rods in pairs	+	+	-	-	+	-	+	+	-	+	+	+	+	±	+	+	-	*Helicobacter pylori*

Key:
+ = Positive ; - = Negative; ± = variable; Hp1-6 = *Helicobacter pylori* isolates

Table 3
Morphological and biochemical characteristics of *Lactobacillus* spp

Isolate	Gram reaction	Motility	Catalase test	Oxidase test	Indole test	Citrate test	Urease test	MR	VP	Glucose	Lactose	Sucrose	H_2S	Gas	Elevation	Shape	Consistency	Pigmentation	Texture	Probable organism
LAB_A	+	-	-	-	-	-	-	+	-	+	+	+	-	-	Raised	Circular	Dry	White	Rough	*Lactobacillus plantarum*
LAB_B	+	-	-	-	-	-	-	+	-	+	+	+	-	-	Raised	Circular	Moist	Creamy	Smooth	*Lactobacillus acidophil;us*
LAB_C	+	-	-	-	-	-	-	+	-	+	-	-	-	-	Raised	Irregular	Dry	Creamy	Rough	*Lactobacillus plantarum*
LAB_D	+	-	-	-	-	-	-	+	-	+	+	+	-	-	Flat	Circular	Moist	White	Smooth	*Lactobacillus* spp
LAB_E	+	-	-	-	-	-	-	+	-	+	+	+	-	-	Raised	Circular	Moist	Creamy	Smooth	*Lactobacillus fermentum*
LAB_F	+	-	-	-	-	+	-	+	-	+	-	+	-	-	Raised	Circular	Moist	White	Smooth	*Lactobacillus fermentum*

Key: - = Negative + = Positive

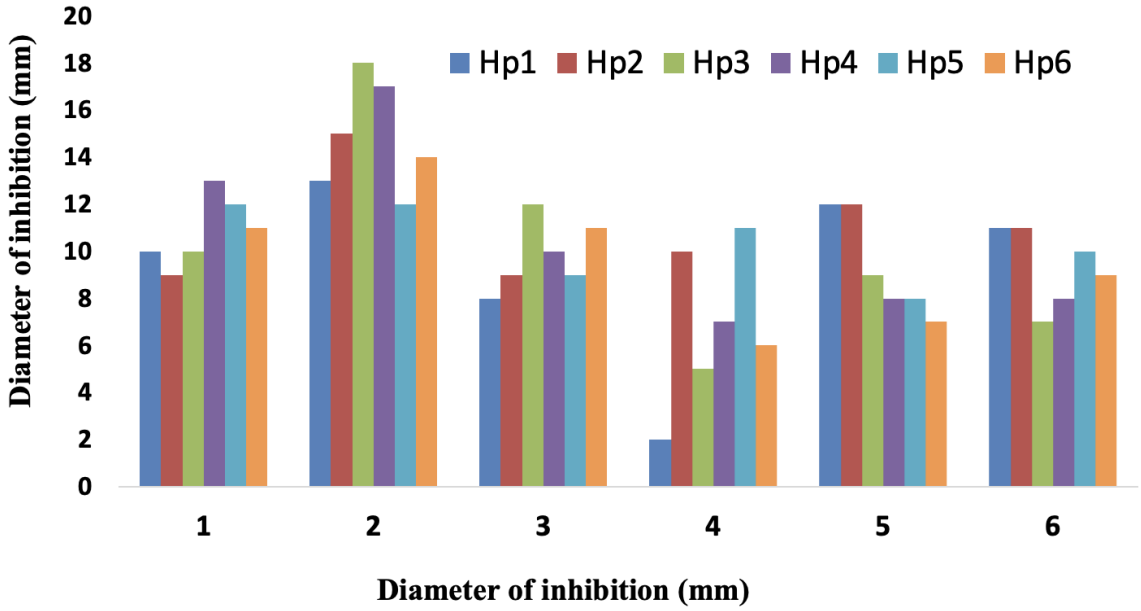

Figure 1:
Antibiogram of probiotic against *Helicobacter pylori*

Table 4:
Identification of the isolates by 16S rRNA genes amplification showing percentage similarities

Isolate code	Quantity (ng/µl) of bacterial DNA	Gene Bank isolates	Accession number	% Similarity
LAB_D	12.7	*Lactobacillus acidophilus* strain NK-S10	MH084833.1	96.8

The highest inhibition zone of 18 mm was recorded by LAB_B against Helicobacter pylori isolate Hp3 while the least inhibition zone of 2 mm was recorded by LABD against Helicobacter pylori isolate Hp1. LABB had the best inhibitory activity against all the Helicobacter pylori isolates used.

DISCUSSION

The inhibitory activity of *Helicobacter pylori* has been reported in several Lactobacillus spp., including L. acidophilus (Rolf, 2000), *Lactobacillus casei Lactobacillus johnsonii*, *Lactobacillus reuteri* and *Lactobacillus salivarius*. Lactic acid bacteria suppress the growth of human bacterial pathogens by secreting compounds such as antibiotic agents, organic acids, and bacteriocins and by decreasing environmental pH, thereby inhibiting gastrointestinal infections (Rolf, R. 2000). Other researchers reported also that probiotics alone cannot completely eliminate *Helicobacter pylori* but can reduce the amount of *Helicobacter pylori* growth (Salas-Jara et al., 2016); (Song et al., 2018).

The identification of five best bacteriocin positive LAB to determine the strain was done based on 16S rRNA gene. Table 4 below presents both the quantity and purity of genomic DNA that was extracted from the selected lactic acid bacterial isolate. The 16S rRNA of the isolate showed a percentage similarity of 96.8% to the gene bank isolate Lactobacillus acidophilus strain NK-S10. This also agrees with the work of Kawthar et al., (2018) and Negussie et al., (2016) where they obtained molecular result showing that the 16S rRNA gene sequencing produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. These results therefore, demonstrate that the intake of foods containing probiotics can inhibit *Helicobacter pylori* growth and is thus a promising treatment for patients with Helicobacter pylori infections. The use of antibiotics including metronidazole and clarithromycin against *Helicobacter pylori* for the in vitro study did not show any zone of inhibition. This might be as a result of loss of potency from the drugs used or due to the prevalence of the antibiotic resistance of Helicobacter pylori which vary within countries as has been observed by some researchers.

All the bacteriocins produced by the six isolates inhibited six tested organisms. The result equally shows that bacteriocins produced by all the *Lactobacillus species* inhibited the growth of the target organism with LABB having the highest zone of inhibition and LABD having the least zone of inhibition. The zone of inhibition exhibited by bacteriocin produced by six selected isolates extended to both Gram positive and Negative organisms. Klaenhammer (2000); Lash et al., (2005) reported that different Lactic acid bacteria synthesize bacteriocins that vary in their spectra of activity. A probable explanation of the small zone of inhibition obtained from the bacteriocin in this work is that the bacteriocins are

present in crude extract and not pure forms. If eventually, the bacteriocins are purified, larger zones of inhibition could be recorded.

In conclusion, this study has conclusively shown that *Helicobacter pylori* can be successfully isolated from stool samples obtained from peptic ulcer patients. It is important to note that this work demonstrates the inhibitory effect of probiotics on *Helicobacter pylori*. The administration of probiotics can decrease the frequency of diarrhea, a frequent side effect of traditional anti- *Helicobacter pylori* tri-therapy. Long-term intake of products containing probiotic strains namely; *Lactobacillus spp*, has a favorable effect on Helicobacter pylori infection in humans. Probiotics may provide a novel approach to the management of *Helicobacter pylori* infection. The risk of developing *Helicobacter pylori*-associated disease may increase with an increasing level of *Helicobacter pylori* density (Yamaoka et al., 1999). Numerous animal and human studies have demonstrated a decrease in *Helicobacter pylori* density and inflammation following the intake of probiotics (Felley et al., 2001).

It is recommended that further studies should be carried out to improve the production of foods containing probiotics as they will go a long way in reducing Helicobacter pylori colonization and also serve as satisfactory antibacterial drugs

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