IN VITRO EFFECT OF ANTIBIOTICS AND GUAVA LEAF EXTRACT ON *BACILLUS INFANTIS* AND *AEROMONAS JANDAEI* FROM NILE TILAPIA

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ABSTRACT

In this study, the impact of antibiotics and Guava leaf extract (GLE) on the growth of bacteria isolated from the gut of Nile tilapia was investigated. Specifically, the inhibitory potential of these additives was explored for both non-pathogenic and pathogenic organisms. The antibiotic susceptibility tests showed that *Aeromonas jandaei* GH-AS II was resistant to Amoxicillin-Clavulanic Acid (AMC 30). Both *Aeromonas jandaei* GH-AS II and *Bacillus infantis* GH-AS II were susceptible to Penicillin (P 10). However, *Bacillus infantis* GH-AS II showed resistance to Tetracycline (TE 30). Both organisms were susceptible to Erythromycin (E15), Chloramphenicol (C30), and Ampicillin (AMP 10). Additionally, GLE concentrations (ranging from 10mg/ml to 100mg/ml) were tested using the agar well diffusion method. GLE inhibited the growth of both bacteria, with the zone of inhibition increased with increasing concentration. *Bacillus infantis* GH-SA II, zones of inhibition ranged from 15.00mm to 23.33mm, while *Aeromonas jandaei* GH-SA II, from 11.67mm to 21.00mm. Zones of inhibition were higher for all GLE concentrations against *Bacillus infantis* GH-AS II. While in vitro assays suggest that antibiotics and GLE influence the gut microflora of Nile tilapia, in vivo investigations are essential to establish their true efficacy and risks in aquaculture systems.

Keywords: Tilapia, Guava, Antibiotics, Aeromonas, Bacillus

Introduction

Nile tilapia (*Oreochromis niloticus*) is the commonly cultured and consumed specie in Ghana (Abarike et al., 2022; Amenyogbe et al., 2018; Osei et al., 2019) and the third most-cultured fish in the world (Assane et al., 2021). Globally, Tilapia farming has grown tremendously over the years but continually plagued with the incidence of infectious diseases largely pathogenic bacteria culminating into massive economic losses. Paramount among them include *Streptococcus agalactiae*, *Aeromonas veronii*, *Aeromonas jandaei* , and *Aeromonas hydrophilia* (Abarike et al., 2018; Assane et al., 2022; Basri et al., 2020).

The fight against the proliferation of infectious pathogens in Tilapia culture is gravitating more to use of probiotic bacteria and medicinal plants (Hoseinifar et al., 2018). Some of the most studied probiotics with promise reported widely include Lactic acid-producing bacteria and *Bacillus spp* (El-Saadony et al., 2021), *Bacillus spp*. stand out when compared to others because they produce compounds that are non-toxic and non-pathogenic. Additionally, their ability to form spores provides them with a defense mechanism against heat and the environment of the gastrointestinal tract (Jlidi et al., 2022). Within the species of Bacillus, *Bacillus infantis* has been reported for its beneficial probiotics properties and significant

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antagonistic effect against fish pathogens that inhabit the gastrointestinal track (Dharmaraj & Rajendren, 2014)

The use of medicinal plants in aquaculture has been attributed to their inhibitory and bactericidal effects and also their ability to strengthen immunity and resistance to disease (Yue et al., 2023). Among the many discovered medicinal plants, the Guava (*Psidium guajava*) plant has been reported to be extensively used in folk medicine due to the presence of various active components that can be used to manage and treat various diseases (Omitoyin et al., 2018). Chemical analysis of Guava leaf extracts (GLE) shows the rich presence of phenolics, tannins and flavonoids, constituents which give GLE antimicrobial properties against bacteria (Ceballos-Francisco et al., 2020; Pereira et al., 2023). Although there are potential benefits of medicinal plant application in fish culture, there are also some potential health risks, thus the effects on non-targeted organisms (Zoral, 2023).

Antibiotics are substances with a direct action on bacteria that is used for the treatment or prevention of diseases. Antibiotics can inhibit bacterial growth as they are complex molecules of different functional groups. In aquaculture, they are used as prophylactics, therapeutic and metaphylactic against bacteria (Pepi & Focardi, 2021). There have been reported cases of abuse and misuse of a wide range of antibiotics conventionally in an attempt to control bacterial diseases in aquaculture with lots of mishaps (Chowdhury et al., 2022). That notwithstanding, there are reports on continuous use with no recourse to the effects on untargeted bacteria, particularly those which could be super beneficial (Özcan & Önalan, 2017).

The influence exerted by antibiotics and medicinal plants on both non-pathogenic/ beneficial and pathogenic organisms remains insufficiently investigated. This represents a significant gap in our current understanding and warrants further rigorous, systematic exploration. For sustainable fish production, it is essential to safeguard against diseases and foster growth, which can be facilitated through the use of medication. The inevitable use of antibiotics, probiotics and medicinal plants to promote sustainable production requires deeper a understanding of their effects on both targeted and non-targeted organisms and perhaps an interplay of their interactions. Given this context, this study aims to evaluate the inhibitory potential of commonly used antibiotics and GLE on the growth of a non-pathogenic bacterium *Bacillus infantis* and pathogenic bacterium *Aeromonas jandaei* in an in vitro controlled laboratory setting.

Experimental

Sampling

Intestinal samples from 50 healthy Tilapia ranging from 100 – 200 grams obtained from 10 randomly cage cultured farms on in Stratum II on Lake Volta were aseptically removed and streaked on nutrient agar plates (see details in section on media preparation) and stored in a sterile ice chest and transported to the Spanish Laboratory Complex of the University for Development Studies (UDS), Nyankpala campus for culture.

Bacteria identification

At the laboratory, a single, well-isolated strawberry-pink colony was selected based on its distinct morphology and dominance among the colonies, suggesting potential significance. This selection aligned with the study's objective, as the isolate was later confirmed to be pathogenic and relevant to aquaculture, whereas other colonies were non-pathogenic and of limited importance. The colony was picked, rinsed in 1 ml of Brain

Heart Infusion (BHI) broth, and incubated at 32 °C for 24 hours to obtain a pure culture for further analysis. The bacterium solution was streaked on BHI agar and cultured for another 24 h. From the culture plate a dominant pure colony was picked and mass cultured using BHI for use in morphometric and biochemical analysis.

Biochemical test

Citrate Test

The aim of this experiment was to determine the ability of the bacterium to use citrate as their sole source of carbon and energy. To do this, Simmons citrate agar (BIO MARK LAB. India) was used as the growth medium. The Simmons citrate agar was prepared by dissolving 24.28 g of the powder in 1 L of distilled water and boiled until completely dissolved. The solution was then sterilized by autoclaving at 121℃ for 15 minutes. After cooling, the agar was poured into sterile petri dishes and allowed to solidify. The experiment was conducted by inoculating each bacterial isolates onto a separate Simmons citrate agar plate using a sterile loop. The plates were then incubated at 36℃ for 24 hours. The results were recorded by observing the colour change of the medium. A blue colour indicated a positive citrate test, meaning that the bacteria could utilize citrate and produce alkaline by-products. A green colour indicated a negative citrate test, meaning that the bacteria could not utilize citrate and the pH remained unchanged. Protocol for citrate test was performed according to Cappuccino and Sherman (2014)

Triple sugar iron test

The experiment was to determine the ability of the bacterium isolate to ferment glucose, lactose, and sucrose and produce hydrogen sulfide (H_2S). To do this, triple sugar iron (TSI) agar was used as the growth medium. The TSI agar was prepared by following the manufacturer's instructions and sterilized by autoclaving. The agar was then poured into test tubes and allowed to solidify at an angle, forming a slant and a butt. The test tubes were capped with loose-fitting caps to allow gas exchange. The experiment was conducted by inoculating the bacterium isolate onto a separate TSI agar tube using a sterile loop. The loop was first stabbed into the butt and then streaked on the slant. The tubes were then incubated at 36℃ for 24 hours. The results were recorded by observing the colour change and gas production of the medium. A yellow slant and a yellow butt indicated glucose, lactose, and sucrose fermentation. A red slant and a yellow butt indicated glucose fermentation only. A red slant and a red butt indicated no carbohydrate fermentation. A black precipitate in the medium indicated H_2S production. A crack or a gap in the medium indicated gas production. Protocol for triple sugar iron test was performed according to Cappuccino and Sherman (2014)

Gram staining

Gram staining was done to significantly categorise the bacterium based on it cell wall composition. Thin smears of the isolate were prepared on a clean glass slide; heat-fixed slightly and allowed to air dry. The fixed bacterium was stained with crystal violet for three minutes and then washed with clean water. Iodine(mordant) treatment was added to the sample for two minutes and then washed with water. A decolorizing compound (acetone/alcohol) was added to the sample and was immediately washed away with water. A counterstain (safranin) was then added to the sample for thirty seconds and then washed away with water. The back of the slides was cleaned, and the smears were air-dried. A drop of oil was applied to the smear, and it was examined microscopically using the 100x

magnification to observe cell morphology, arrangement, and gram staining characteristics PCR Amplification, Gel Electrophoresis, and Sequence Analysis of Bacterium DNA

A colony PCR for the bacterium was performed using the following cycling conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The PCR reaction volume used was 10 µl, and it contained; 5 µl of premix, 3 µl of molecular biology water, 1 µl of universal primers (forward -27F-AGAGTTTGATCCTG GCTCAG and reverse -1492R- GGTTACCTTGTTACGACTT) and 1 µl of suspended bacterium as template. The PCR product (amplicons) was loaded on a 2% agarose gel. A ladder of 1kb was loaded alongside the amplified DNA and ran one hour at a voltage and current of 120. The bands were visualized using Gel Doc. DNA products were then sent to Inqaba Biotec West Africa Ltd in South Africa for sequencing. The sequencing analysis of the bacterium obtained was compared to the already existing results at the National Centre for Biotechnology Information (NCBI) using BLAST (Basic Local Alignment Search Tool).

Antibiotic sensitivity test

The Kirby- Bauer disc method was used for in-vitro antimicrobial sensitivity tests of the bacterium isolated alongside *Aeromonas jandaei* GH-AS II, a previous isolate from same fish sample (Abarike et al., 2022). Based on information on an earlier study (Abarike et al., 2023) regarding commonly used antibiotics in aquaculture operations in Ghana, the following antibiotics were tested: AMC 30 – Amoxicillin Clavulanic Acid (30mg), P 10 – Penicillin (10units), TE 30 – Tetracycline (30mg), E 15 – Erythromycin (15mg), C30 – Chloramphenicol (30mg), AMP 10 – Ampicillin (10mg). 10 ul each of the bacteria tested were spread on prepared BHI agar plates and impregnated various antibiotic discs (Himedia, India) and incubated at 37°C for 24 hs. Agar plates were then examined and the zones of inhibition measured in millimeters (mm) using a meter rule and interpreted according to the CLSI guidelines (CLSI, 2015).

Extraction of guava leaf extract (GLE)

Guava leaves of about 0.5 kg were obtained by plucking fresh leaves from a Guava tree from the UDS Nyankpala Campus. The leaves were blendered (Nasco Blinder Model BL9295A) in 1 litre of absolute ethanol (99%), the solution was stored overnight and sieved using 93-, 65-, and 23-µm meshes to separate liquid from the fibrous leaf materials. The guava leaf extract (GLE) filtrate was concentrated by heating in a water bath at 100 °C for 30 minutes to remove ethanol and water. Although rotary evaporation under reduced pressure at lower temperatures is the standard procedure for solvent removal, this modification was adopted due to equipment limitations in the laboratory setting. The approach provided a practical means of obtaining crude extract for preliminary antimicrobial assays, while acknowledging that prolonged heating may reduce the stability of certain heat labile phytochemicals. The extract was then put into a ziplock bag and stored at 4°C in the refrigerator until use.

Evaluation of inhibitory potential of GLE

Guava leaf extract (GLE) was used for bioassay against bacterium isolate and *Aeromonas jandaei* GH-AS II. Bacteria inocula were prepared from a 24-hour-old cultures in BHI broth. Nutrient agar plates were prepared, and the inocula were seeded by the spread plate method. Using the agar well diffusion method, wells of 6 mm diameter were punched into the agar plates and filled with solutions prepared by dissolving various amounts of the individual herbal leaf extracts

into sterile distilled water to make 10, 30, 50, 70, and 100 mg/ml concentrations of GLE alongside distilled water as a control. The plates were incubated at 37 ℃ for 18-24 hours. Antibacterial activity was evaluated by measuring the inhibition zone in millimeters in diameter. All the samples were done in triplicate.

Statistical analysis

Data on zones of inhibition for different concentrations of GLE were analyzed using one-way analysis of variance (ANOVA) in Minitab software (version 16). Differences among means were further evaluated using Tukey's Honestly Significant Difference (HSD) test, and results were expressed as mean ± standard error (SE). Data comparing the treatment groups for GLE for the bacterium isolate and *Aeromonas jandaei* GH-AS II were analyzed using an independent samples t-test.

Results

Identification of bacterium

Gram staining test showed the isolate was gram positive. Based on morphology, the results showed that bacterium isolate exhibited strawberry pink colour, rodlike shape, circular in form undulate in margin and flat in elevation. A blast analysis of the 16S rRNA gene sequences of the bacterium isolate showed a pairwise similarity of 99.72% to *Bacillus infantis* strains deposited at the Genbank. This suggested the isolate in this study belonged to the same genus but to different strains. Based on the test results for the bacterium isolated in this study, it was identified as *Bacillus infantis* GH-AS II under GenBank accession number PP494415. The bacterium with the greatest pairwise similarity to strain *Bacillus infantis* GH-AS II was MN629993.1. Molecular Evolutionary Genetics Analysis (MEGA) software version 6 was used to construct a phylogenetic tree (Figure 1) using the neighbour-joining method. The analysis indicated that *Bacillus infantis* GH-AS II belongs to the genus *Bacillus* but represents a distinct strain.

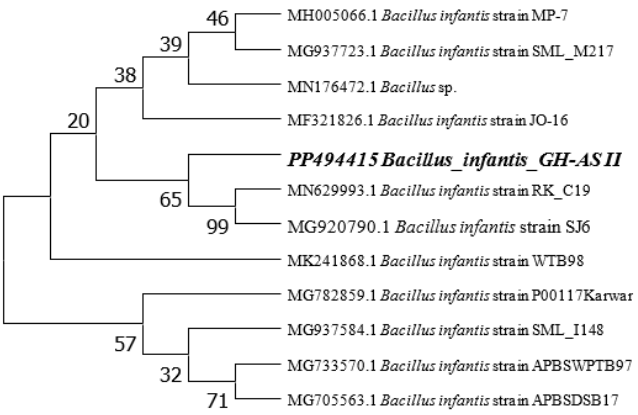


Fig.1: Phylogenetic tree based on 16S ribosomal DNA of *Bacillus infantis* GH-AS II inferred using the neighbour-joining method

Antibiotic Sensitivity Test

TABLE 1

Antibiotic sensitivity test for bacteria.

Antibiotic	Disc Content (*μg*)	CLSI Zone diameter interpretative criteria (mm)			Zone of inhibition		Interpretation	
		S	I	R	*Aeromonas jandaei* GH-AS II	*Bacillus infantis* GH-AS II	*Aeromonas jandaei* GH-AS II	*Bacillus infantis* GH-AS II
Amoxicillin Clavulanic Acid	30	≥ 7		≤ 6	20	6	S	R
Penicillin	10	≥ 12		≤ 12	18	34	S	S
Tetracycline	30	≥ 15	12-14	≤ 11	8	32	R	S
Erythromycin	15	≥ 16	13-15	≤ 12	24	24	S	S
Chloramphenicol	30	≥ 18	13-17	≤ 12	22	24	S	S
Ampicillin	10	≥ 32	14-16	≤ 8	14	32	I	S

Where: S – Susceptible, R – Resistant and I – Intermediate.

As shown in the table 1, *Aeromonas jandaei* GH-AS II was resistant to tetracycline and intermediate for Ampicillin and susceptible to Erythromycin and Chloramphenicol. Except Amoxicillin Clavulanic Acid, *Bacillus infantis* GH-AS II was susceptible to all antibiotics tested

Antibacterial activity of GLE on bacteria

TABLE 2

Zones of inhibited test on bacteria with different concentrations GLE

BACTERIA	CONTROL	10 mg/ml	30 mg/ml	50 mg/ml	70 mg/ml	100 mg/ml
Bacillus infantis GH-AS II	0.00l ± 0.00 a	15.00 ± 0.15 a	17.00 ± 0.11a	19.00 ± 0.24a	21.33 ± 0.03a	23.33 ± 0.34a
Aeromonas jandaei GH-AS II	0.00l ± 0.00a	11.67 ± 0.25b	14.33 ± 0.11b	15.67± 0.25b	17.67 ± 0.44b	21.00 ± 0.00b

ANOVA to test differences in concentrations within each bacterium in rows. T-test for zones of inhibition between bacteria in columns. Columns and rows with same letters are statistically similar

The result presented in table 2 showed that GLE under investigation exhibited antibacterial properties as evidenced by their zone of inhibitions. GLE extracts showed zone of inhibitions, 15.00mm – 23.33mm for *Bacillus infantis* GH-AS II, and 11.67mm – 21.00mm for Aromonas jandaei GH-AS II. Zone of inhibition increased as concentration levels also increased.

Discussion

In this study in an in vitro study, the antibacterial activities of antibiotics and Guava leaf extract (GLE) were tested against a *Bacillus infantis* GH-AS II, a non-pathogenic bacterium and *Aeromonas jandaei* GH-AS II, a pathogenic bacterium both of which originate from the gut of Nile tilapia reared in cages at Stratum II of the Volta Lake. Citrate and Triple Iron sugar tests confirmed that *Aeromonas jandaei* GH-AS II and *Bacillus infantis* GH-AS II are enterobacteria corroborating other earlier reports (Pakingking et al., 2015). The presence of both bacterial species demonstrates that the gut of cultured Nile tilapia harbours both non-pathogenic and pathogenic bacteria. An increase in pathogenic bacterial populations, coupled with a reduction in non-pathogenic bacteria, is detrimental to the host (Milutinović et al., 2021).

Globally, it has been proven that indiscriminate use of antibiotics in aquaculture to combat diseases is the result of a myriad developed antibiotic resistant bacteria (Okeke et al., 2022). In a previous report (Abarike et al., 2023), Tetracycline was purported to be the commonly used in the study area; this could explain the resistance exhibited by pathogenic bacterium, *Aeromonas jandaei* GH-AS II . It is important to note that antibiotics to which the pathogenic bacterium was susceptible to suggest these might not have been widely or indiscriminately used in the study area. Primarily, use of antibiotics in aquaculture is targeted at eliminating the proliferation of infectious bacteria, however their application does not discriminate against gut microflora which comprise both infectious and non-infection bacteria (Shi et al., 2022). This could explain the susceptibility of *Bacillus infantis* GH-AS II. More so, it has been reported that probiotic bacteria are generally more susceptible to antibiotics because they lack the defense mechanisms that many pathogenic bacteria have (Rabetafika et al., 2023). This suggest that the continues use of antibiotics in the culture of fish in might have serious deleterious effects on gut non-pathogenic bacterium such as *Bacillus infantis* GH-AS II which might be beneficial to fish.

GLE is a medicinal plant with a potential usage as a feed additive for increased disease resistance and improved growth performance (Omitoyin et al., 2018). As a medicinal plant, Guava is able to increase disease resistance by boosting the immune system, resulting in milder diseases with less mortality and economic loss (Hasani, 2022). The mechanism of action of combining medicinal plants and probiotic bacteria is not well understood. However, their synergistic effects have been widely advocated. Their use as feed additives in aquaculture shows strong potential. They can improve fish growth and enhance immune response. (Wei et al., 2022). The concentration of GLE used in this study inhibited the growth of both bacteria. This corroborates earlier reports (Chou et al., 2023) that GLE has an bactericidal properties. This could hold a promise in finding alternative treatment regimens to the conventional chemicals and antibiotics particularly against multidrug resistant strains in aquaculture (Jung et al., 2022). However, further research is needed to fully understand the potential benefits and drawbacks of using herbal extracts. For instance, the impact on the fish microbiome, which consists of both pathogenic and non-pathogenic bacteria, would need to be carefully studied.

In conclusion, antibiotic susceptibility testing revealed that *Aeromonas jandaei* GH-AS II was resistant to tetracycline, while *Bacillus infantis* GH-AS II exhibited resistance to amoxicillin-clavulanic acid. Both isolates were susceptible to the other antibiotics tested. GLE demonstrated dose-dependent inhibitory effects on both bacteria at concentrations

ranging from 10 mg/mL to 100 mg/mL. These findings highlight the potential of GLE as an alternative antimicrobial agent.

From a management perspective, the observed resistance to commonly used antibiotics such as tetracycline underscores the need for strengthened antibiotic stewardship in Ghanaian aquaculture systems, particularly within Lake Volta. The promotion of susceptibility-guided antibiotic use, alongside the integration of plant-based alternatives such as GLE, could reduce selective pressure for antimicrobial resistance and support sustainable fish health management. Nevertheless, further studies are necessary to comprehensively evaluate the combined use of antibiotics and plant extracts in Nile tilapia culture. While this in vitro study provides important preliminary insights, in vivo investigations are essential to better understand interactions within the whole organism and to assess the safety and efficacy of these interventions in aquaculture systems.

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