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Research Article

Ameliorative Activities by Ellagic acid on Gut dysbiosis during starvation induced gastric ulceration in Rats: The role of *Enterococcus feacium*

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Abstract

Starvations result to formation of gastric ulcer, and microbiome dysbiosis. During starvation, there is increased release of iron stores, which can either be beneficial for growth of some gut flora or impedance to activities of some polyphenols. Polyphenols and gut microflora homeostasis are pivotal during healing of gastric ulcers. Ellagic acid (EA); a plant-derived poly-phenol, has proven to be gastro-protective, but there is dearth of information on its activities on commensal *Enterococcus faecium* during starvation induced gastric ulcer and hence the aim of this study. Thirty rats were grouped into 6 as control, untreated or treated with cimetidine (50mg/kg), 75mg/kg, 50mg/kg or 25mg/kg EA for 4 weeks. Gastric ulcer was induced by depriving the animals of food for the last 6 days in groups 2 to 6. Ulcer index was determined and gastric biochemicals were estimated from stomach homogenate, while microorganisms were assessed in gastric sample as well as tissue histological studies. The data were analysed using ANOVA with Turkey's posthoc significance test at $p \leq 0.05$ significance.

Ellagic acid treatments significantly decreased mean ulcer index, lipid peroxidation, hydrogen peroxide levels and H+/K+-ATPase activity compared with starved-untreated. Gastric mucin, sulfhydryl, and catalase activities were significantly increased in low- and medium-EA treated groups compared with starved-untreated. Gastric carbonyl level significantly decreased only in low-EA group compared with starved-untreated. Nitric oxide levels significantly increased in medium- and high-EA treated groups. Gut derived commensal microflora *Enterococcus faecium*; and probiotic bacteria *Lactobacillus fermentum*, *Lactobacillus delbrueckii* and *Pedicoccus acidilactici* were up-regulated in all EA treatment groups.

In conclusion, EA attenuated starvation induced gastric ulceration via increased anti-oxidative mechanisms, nitric-oxide and reduced gastric H+/K+-ATPase activities, which enabled proliferation of gut-commensal microflora; *Enterococcus faecium*

Key Words: Ellagic acid, starvation induced gastric ulcer, *Enterococcus faecium*, gut microflora metabolite

INTRODUCTION

The stomach or gut is affected during certain adverse conditions, one of which is starvation (Mahadevan, 2014). Starvation occurs during environmental disaster or socio-economic imbalance and it leads to severe calorie restriction, which often progresses into decreased metabolic rate (Watts and Bohle, 2016; McCue, 2010). Anorexia nervosa is a pathological form of self-induced starvation involving changes within the gut and body systems (Love, 1986). Gut microbiome has been found to be altered during anorexia nervosa, a state of starvation or fast (David *et al*., 2014), which confers negatively on energy balance (Ley *et al*., 2005). Several gut microflora have been documented to also produce signalling hormones such as serotonin (Roshchina, 2010), GABA (Asano *et al*., 2012), insulin (Vrieze *et al*., 2012) as well as satiety hormone leptin (Queipo-Ortuño *et al*., 2013). Probiotics such as *lactobacillus* and *bifidobacterium* have been documented to increase leptin, but with reduced population of bacteroides however with *prevotella*, an inverse trend was observe in association with Ghrelin (Queipo-Ortuño *et al*., 2013) the hunger hormone. These micro-biome are important as they mediate alterations in nutritional regulation (Hildebrandt *et al*., 2009; Asolo *et al*., 2020), energy (Leulier *et al*., 2017), hormonal balance and communication between the brain and gut (Seitz *et al*., 2019), which are pivotal for growth, development and homeostasis (David *et al*., (2014); Storey and Storey 1990). Starvation has been documented to increase gut and or body stress (Mullane *et al*., 1974.) hence the stress hormone inevitably disrupting the biological rhythm. Starvation has also been researched to cause chronic gastric ulcers (Elegbe, 1978, Daneshi *et al*.,2017). Peptic ulcer disease (PUD) has greatly declined in prevalence (Salaric *et al*., 2022), however, management has become much more difficult to achieve, especially PUD due to *H.pylori* infection and non-compliance to medication (Kavitt *et al*., 2019) thus

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threatens the world's population (Malfertheiner *et al*., 2009; Lanas and Chan, 2017). The gastric mucosa integrity is maintained through a homeostatic balance between these aggressive and defensive factors (Hoogerwerf and Pasricha, 2001) as well as activities of the gut microflora recently (Khoder *et al*., 2016. Zhao *et al*., 2023). Starvation is known to have deleterious morphological and histological effects on stomach mucosa that often times clinically present as or show as ulcers (Al-Kawaz and Jawad, 2021).

However, the goals of treating gastric ulcers have not changed and it includes relief of pain, healing of the ulcer, and prevention of its recurrence (Sostres and Lanas, 2011). The types of drugs normally used include H2 receptor antagonists (e.g. cimetidine), proton pump inhibitors (e.g. omeprazole), and cytoprotective agents (e.g. sucralfate). However, most of these drugs show side effects like arrhythmias, gynecomastia, enterochromaffin-like cell hyperplasia, hematopoietic changes, and increased risk of virulent gut microbe *Clostridium difficile* infection (CDI) (Azab *et al*.,2017). These adverse effects poorly achieve the simple goal of total and complete gastric ulcer alleviation (Chan and Leung, 2002). There is, therefore, a need for safe therapies, which may not have a harmful side effect in patients. Polyphenols (Chiu *et al*., 2021) are abundant in mostly consumed fruits, vegetables and un-processed green tea consumed. They have been documented to confer gastroprotective activities mainly via antioxidative activities, examples of these polyphenols include protocatechuic acid, naringin, naringinin, ellagic acid (Santio *et al*., 2017; Luca *et al*., 2020). Ellagic acid is a naturally occurring polyphenol, or micronutrient, found in fruits vegetables and in a medicinal mushroom, *Phellinus linteus*. (Lee *et al*., 2008). Few foods contain a more complex version called ellagitannin which is an acid that is converted into ellagic acid in the body (Seigler and David, 1998). Ellagic acid has been shown to have a dose-dependent inhibition of hog gastric H+/K+-ATPase, (Murakami *et al*.,1991) and to potentially inhibit lipopolysaccharide-induced nitric oxide, prostaglandin E2, interleukin-6 production (BenSaad *et al*., 2017). It has been shown to significantly change nitric oxide, total sialic acid, malondialdehyde and ghrelin levels as well as stomach cyclooxygenase 2 activity of mice given indomethacin (Kaya *et al*.,2019). Ellagic acid has shown promising gastroprotective potentials in a wide range of gastric ulcer models (Lino *et al*.,2002; Angela *et al*.,2011; Selim, *et al*., 2016; Soheir *et al*.,2018). Ellagic acid has also been documented to be converted to urolithin A (Larrosa *et al*., 2010, Espin *et al*., 2013, Saha *et al*., 2016), which improves gut dysbiosis in various species (Qin *et al*., 2022,). However, urolithin performs optimally when it cannot bind with iron and its parent compound ellagic acid has also been documented to be inactive once it binds with iron (Saha *et al*., 2016). During starvation, there is increased release of iron stores (Lisiecki, 2010), indicating a barrier in conversion of ellagic acid to urolithin. However, iron is important for growth of some commensal probiotics (Lactobacillus sp and *Enterococcus faecium*), which are known to suppress aggressive microflora, which causes dysbiosis leading to ulceration during starvation.

However, there is dearth of information on activities of commensal gut microflora derived during ellagic acid treatment in starvation induced gastric ulcers which was evaluated in this study.

MATERIALS AND METHODS

Reagents and chemicals: Reagents used were all purchased from sole distributor for BDH Chemicals, England. All Agar (Nutrient Agar, Eosin Methylene Blue Agar, Macconkey agar, MRS agar) were from LAB M Limited, United Kingdom. Cimetidine was acquired from a commercial supplier, but manufactured by Maxheal Pharmaceutical (India) 301, Maxheal House, Bangur Nagar, Goregaon West, Mumbai whose sole agent in Nigeria is Nkoyo Chemicals Onitsha. The primary treatment used was ellagic acid and it was purchased from Rejuvenation® Therapeutics

Animal model, housing and feeding: Thirty 30 male Wistar rats (weighing 110-130g) were procured, acclimatised for two weeks and housed in animal house with 12-hour light/ dark cycles. The rats had free access to water (tap water) and commercial pelleted rodent feed (Ladokun feed, Nigeria Limited, Ibadan) throughout the experiment. The experiment was carried out in accordance with the National Institute of Health's guidelines and regulations for laboratory animal care and use (NIH, 1985) and the study was approved by University of Ibadan Animal Care Committee (ACUREC) with assigned number (UI-ACUREC/21/124).

Experimental design/ animal grouping:

A total of 30 rats were grouped into 5 Groups. The grouping is as follows:

Group 1 (control group) they were not starved or treated, they had unlimited access to food and water.

Group 2: (ulcerated untreated; SUTE) group, rats were food-starved for the last 6 days but had access to clean water for 4 weeks.

Group 3: (reference drug treated; SCIME) group; rats were treated with cimetidine (50mg/kg) daily for 4 weeks (28 days) but food-starved for the last 6 days (days 22-28).

Group 4: (high dose Ellagic acid treated; SEAH) group; rats were treated with 75mg/kg EA for 4 weeks (28days) but food-starved for the last 6 days (days 22-28)

Group 5: (medium dose Ellagic acid treated; SEAM) group; rats were treated with 50mg/kg EA for 4 weeks but food-starved for the last 6 days (days 22-28).

Group 6: (low dose Ellagic acid treated; SEAL) group; rats were treated with 25mg/kg EA for 4 weeks but food-starved for the last 6 days (days 22-28).

Starvation-induced ulceration: Gastric ulcer was induced by depriving the animals of food for the last 6 days of administration i.e. from the 22nd to 28th day, however, water was given ad-libitum all throughout the period of the fast as described by Onwuchekwa and Oluwole (2015).

Gastric ulcer index: The Ulcer was calculated using the following formula:

Ulcer index = Mean Ulcer Score x % of ulcerated animals (i.e. Mean Ulcer Score x Number of animals in a group/ 100)

Determination of preventive ulcer index (%): Preventive Ulcer Index (P.U.I %) was later calculated as follows:

P.U.I % = (Ulcer Index of Control – Ulcer Index of treated)/ Ulcer Index of Control x 100

Preparation of excised gastric tissue for biochemical assay: A part of the stomach tissue of each animal was excised, washed in normal saline, blotted on filter paper and weighed. Tissues were homogenised in phosphate buffer (pH adjusted to 7.4) using a teflon homogenizer. The homogenates were

cold centrifuged at 3600rpm for 15 minutes. The resulting supernatants were decanted, divided and stored at 4oC for biochemical estimation and gut microbial evaluations.

Biochemical assay: Gastric tissue protein concentration was by the biuret method as described by Gornal *et al*.,(1949). Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation and is expressed in terms of malondialdehyde (MDA) per mg protein following the method of Varshney and Kale (1990). Protein carbonyls in gastric tissue was determined by spectrometric DNPH assay according to Fagan *et al*.,(1999).Catalase activity was determined according to the method of Aebi (1974) while sulfhydryl content was also the method of Ellman (1959). The gastric nitrite concentration was measured as an indicator of nitric oxide production detected by the Griess reaction (Ignarro *et al*.,1987; Kuan *et al*.,2013) and gastric hydrogen peroxide level was determined by the method of Wolf *et al*. (1990). Gastric H+/K+ATPase pump assay was carried out by the method of Bewaji *et al*. (1985).

Evaluation of microorganisms in stomach samples: Deoxyribonucleic acid (DNA) extraction (isolation of prominent gastric microbiota): DNA was extracted using the protocol stated by Trindade *et al*., (2007). Briefly, single colonies grown on respective agar media were transferred to 1.5 mLs of their broth media and cultures were grown on a shaker for 48 hours at 28 ºC. Thereafter, grown cultures were centrifuged at 4600 rev for about 5 minutes. The resulting pellets were re-suspended in 520 µL of 10 mMTris-HCl, 1mM EDTA (TE), pH 8.0 buffer. Fifteen microliters of 20% SDS and 3 µL of proteinase K (20 mg/mLs) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µL of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 minutes at 65 ºC and kept on ice for 15 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifugation at 7200g for 20 minutes. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 hours. DNA was collected by centrifugation at 13000g for 10 minutes, washed with 500 µL of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µL of TE buffer.

Molecular identification polymerase chain reaction (PCR): PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mMs MgCl2, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water 8µL DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermocycler (Applied Biosystem Inc, USA) with a PCR profile consisting of an initial denaturation at 94°C for 5 minutes; followed by a 30 cycles consisting of 94°C for 30 seconds, 50°C for 60seconds and 72°C for 1 minute 30 seconds; and a final termination at 72°C for 10 minutes. And chill at 4oC.GEL (Warwick *et al*.,2005; Frank *et al*.,2008).

Integrity: The integrity of the amplified about 1.5Mb gene fragment was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (Warwick *et al*.,2005; Trindade *et al*.,2007) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well (Trindade *et al*.,2007). The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

Purification of amplified product: After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µL of Na acetate 3M and 240 µL of 95% ethanol were added to each about 40µL PCR amplified product in a new sterile 1.5 µL tube eppendorf, mix thoroughly by vortexing and keep at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µL of 70% ethanol and mix then centrifuge for 15 minutes at 7500 g and 4°C. Again, remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 minutes. then re-suspend with 20 µL of sterile distilled water and kept in -20oC prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nano-drop of model 2000 from thermo scientific.

Sequencing: The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

Preparations of sections for histology: Gastric tissue histological studies evaluation was processed for Hematoxylin and Eosin (H&E) stain were as documented by Avwioro (2002), while the mucous cells were stained by Period Acid Shiff Stain.

Statistical analysis: The mean and standard error of mean (SEM) of the variables were recorded. One way ANOVA with Turkey's post hoc significance test was used and significant at $p \leq 0.05$.

RESULTS

Effect of ellagic acid on starvation induced gastric ulcers: All rats treated with the different doses of ellagic oil had significantly reduced ulcer index relative to the ulcer untreated rats while there is no significant difference between the

starvation-induced ulcers treated EA and cimetidine treated groups (Table 1).

Table 1:
Effect of Ellagic Acid on starvation induced ulcers score, percentage protection and mean stomach weight in male Wistar rats

Groups	Mean ulcer index	Percentage Protection	Mean Stomach weight (g)
NOR	0	100	1.4
SUTE	9.3 ± 0.33	0^{acdef}	1.0
SCIME	0.8 ± 0.83^{b}	91.4	1.1
SEAH	0.3 ± 0.33^{b}	96.7	1.1
SEAM	0	100	1.1
SEAL	0.2 ± 0.17^{b}	97.8	1.2

Where **SUTE**- Ulcer Untreated Group, **SEAL**- Low dose ellagic acid group, **SEAM**- Medium dose ellagic acid group, **SEAH**- High dose ellagic acid group, **SCIME**- Cimetidine group.
Keys of significance; [a]- compared with the normal group, [b]- compared with low dose ellagic acid group, [c]- compared with medium-dose ellagic acid group, [d]- compared with high dose ellagic acid group

Effect of ellagic acid on gastric protein, MDA and carbonyl levels in starvation induced gastric ulcer in male Wistar rats: There was a significant increase in the gastric protein level in the low and medium doses of ellagic acid treated rats when compared with rats in the ulcer untreated group. A significant decrease in gastric MDA levels was observed in all groups, when compared with the ulcer untreated group except in the low dose ellagic acid and cimetidine treated groups, where there was no significant difference (Table 2). There was a significant increase in the gastric carbonyl level in rats treated with medium dose and high dose ellagic acid respectively compared with the ulcer untreated rats, but there was a significant decrease carbonyl level in the low dose ellagic acid treated rats relative to the ulcer untreated rats. There was no significant difference in the carbonyl levels between the low dose ellagic acid and cimetidine treated rats (Table 2).

Table 2:
Effect of Ellagic Acid on Gastric biochemical assay of Starvation induced ulcers in male Wistar rats

Groups	**Gastric Protein** (mg/mL)	**Gastric MDA X10^{-5}** (nmol/mg protein)	**Gastric Carbonyl** (nM/mg protein)	**Gatsric Catalase** (µmol H_2O_2 consumed/mim/mg protein)	**Gastric Sulfhydryl** (µ/ng Protein)	**Gastric Nitric oxide** (µM/g tissue)
Nor	0.34 ± 0.012^{bcdef}	8.01 -06 ± 4.46 -07 ^{bcdef}	2.3e-07 ± 1.47e-08	2137.88 ± 369.02^{de}	0.14 ± 0.015	53.78 ± 7.46^{bcdef}
Sute	0.15 ± 0.05^{adef}	5.18 ± 1.12-05	3.33E-07 ± 1.88E-08	24642.75 ± 909.37^{ade}	0.08 ± 0.002^{cdef}	88.73 ± 2.59^{ac}
Scime	0.22 ± 0.07^{b}	3.00 ± 5.00E-06^{b}	2.88E-07 ± 6.22E-09	20590.76 ± 32.19	0.18 ± 0.003	101.06 ± 1.97^{abc}
Seal	0.19 ± 0.06	3.00 ± 1.00E-06^{b}	2.32E-07 ± 8.34E-09^{bde}	21415.00 ± 287.09	0.18 ± 0.004	76.44 ± 1.85
Seam	0.26 ± 0.09^{b}	2.04 ± 6.59E-06^{b}	3.57E-07 ± 3.56E-08	17105.00 ± 193.66^{bf}	0.17 ± 0.007	98.28 ± 0.73^{abc}
Seah	0.20 ± 0.07^{b}	3.00 ± 2.00E-06^{b}	5.67E-07 ± 4.60E-08^{bcdf}	19601.00 ± 743.09^{bf}	0.495 ± 0.008 ^{abcdf}	115.20 ± 0.88^{abc}

Where **SUTE**- Ulcer Untreated Group, **SEAL**- Low dose ellagic acid group, **SEAM**- Medium dose ellagic acid group, **SEAH**- High dose ellagic acid group, **SCIME**- Cimetidine group. ***Keys of significance***; *[a]- compared with the normal group, [b]- compared with low dose ellagic acid group, [c]- compared with medium-dose ellagic acid group, [d]- compared with high dose ellagic acid group*

Effect of ellagic acid on gastric catalase, sulfhydryl levels in starvation induced gastric ulcer in male Wistar rats: There was a significant increase in the gastric catalase activity in all groups when compared with the ulcer untreated group (Table 2). Gastric tissue catalase activity was not significantly different in the low and high doses ellagic acid treated groups respectively compared with cimetidine treated group. A significant increase in sulfhydryl levels was observed in the medium and low doses ellagic acid treated groups compared with the ulcer untreated group but there was no significant different between the gastric sulfhydryl level of the low dose ellagic acid and cimetidine treated group (Table 2).

Effect of ellagic acid on gastric nitric oxide, mucin and hydrogen peroxide levels in starvation induced gastric ulcer in male Wistar rats: No significant differences were observed in the gastric nitric oxide levels in all the ellagic acid treated groups when compared with the ulcer untreated group (Table 2). However, gastric mucin significantly increases in all the ellagic acid treated groups, when compared with the ulcer untreated group (Figure 1). All ellagic acid treated groups had a significantly higher gastric mucin content compared to the cimetidine treated rats. On the other hand, there was a significant decrease in the gastric hydrogen peroxide levels in all ellagic acid treated groups compared with the ulcer untreated groups (Figure 2).

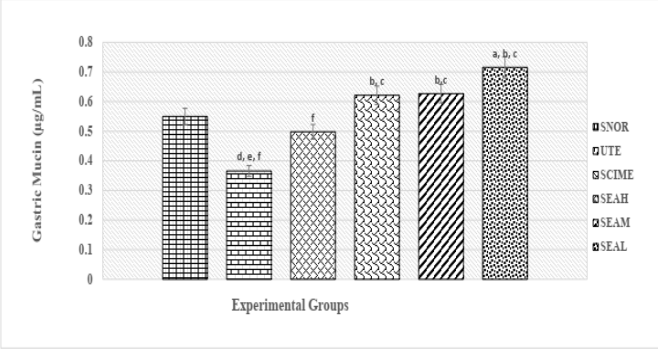

Figure 1:
Effect Of Ellagic Acid on Gastric Mucin in Starvation Induced Ulcer in Male Wistar Rats

Where **SNOR**- Normal Group, **UTE**- Starved Untreated Group, **SEAL**- Starved Low dose ellagic acid treated group, **SEAM**- Starved Medium dose ellagic acid treated group, **SEAH**- Starved High dose ellagic acid treated group, **SCIME**- Starved Cimetidine treated group.
Keys of significance; a- compared with the normal group. [b]- compared with Untreated group/non ulcer group, [c]- compared with the cimetidine group, [d]- compared with the high dose ellagic acid group, [e]- compared with the medium dose ellagic acid group, [f]-compared with the low dose ellagic acid group

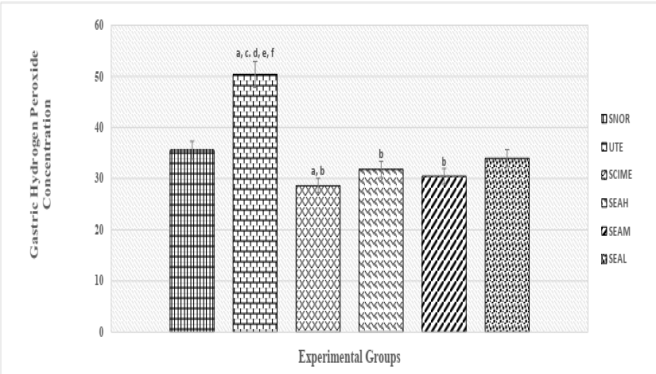

Figure 2:
Effect Of Ellagic Acid on Gastric Hydrogen Peroxide Levels in Starvation And Indomethacin Induced Ulcer In Male Wistar Rats

Where **SNOR**- Normal Group, **UTE**- Starved Untreated Group, **SEAL**- Starved Low dose Ellagic acid group, **SEAM**- Starved Medium dose Ellagic acid treated group, **SEAH**- Starved High dose Ellagic acid treated group, **SCIME**- Starved Cimetidine treated group.
Keys of significance; a- compared with the normal group. [b]*- compared with Untreated group/non ulcer group,* [c]*- compared with the cimetidine group,* [d]*- compared with the high dose ellagic acid group,* [e]*- compared with the medium dose ellagic acid group,* [f]*compared with the low dose ellagic acid group*

Effect of ellagic acid on gastric H+/K+ATPase activity in starvation induced ulcer in male Wistar rats: A significant decrease in the gastric H+/K+ ATPase activity was observed in all the ellagic acid treated groups, when compared with the ulcer untreated group (Figure 3). There was no significant difference between the gastric tissue H+/K+ ATPase activity of the medium dose ellagic acid treated group compared with the cimetidine group (Figure 3).

Effect of starvation induced gastric ulceration on microbial counts in Macconkey agar: Four pathogenic organisms were identified with distinct characteristics (Table 3) including: *Salmonella enterica*, *Escherichia coli*, and *Shigella dysenteriae*. There were decrease in microbial count of the Escherichia coli, and Salmonella enterica colony of the ellagic acid treated groups compared with the ulcer untreated group and cimetidine group. The *Escherichia coli* species was totally absent in the Ellagic (high and low doses) treated groups in the starvation induced ulcer model (Figure 4). However, commensal *Enterococcus faecium* was significantly increased in the ellagic acid treated groups especially the medium dose (Figure 4).

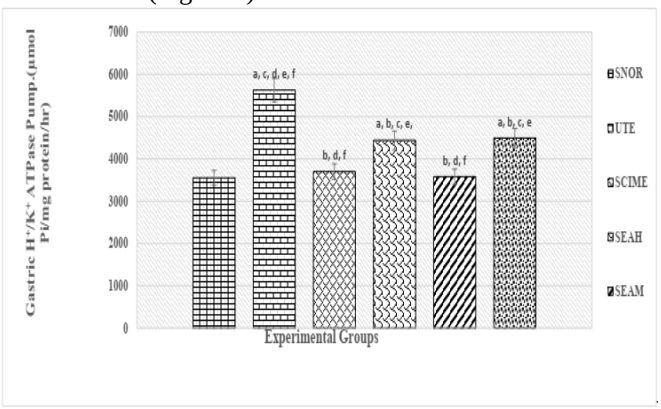

Figure 3:
Effect Of Ellagic Acid on Gastric H^+/K^+ Atpase Activity in Starvation And Indomethacin Induced Ulcer In Male Wistar Rats

Where **SNOR**- Normal Group, **UTE**- Starved Untreated Group, **SEAL**- Starved Low dose Ellagic acid treated group, **SEAM**- Starved Medium dose Ellagic acid treated group, **SEAH**- Starved High dose Ellagic acid treated group, **SCIME**- Starved Cimetidine treated group.
Keys of significance; a- compared with the normal group. [b]*- compared with Untreated group/non ulcer group,* [c]*- compared with the cimetidine group,* [d]*- compared with the high dose ellagic acid group,* [e]*- compared with the medium dose ellagic acid group,* [f]*compared with the low dose ellagic acid group.*

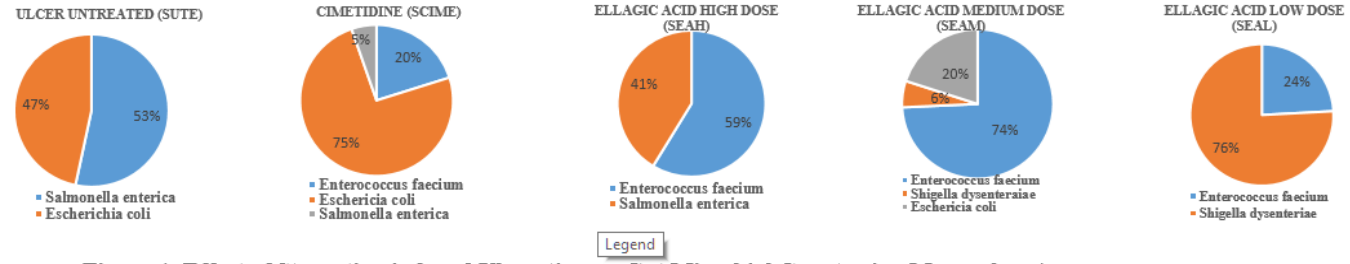

Figure 4: Effect of Starvation induced Ulceration on Gut Microbial Count using Macconkey Agar.

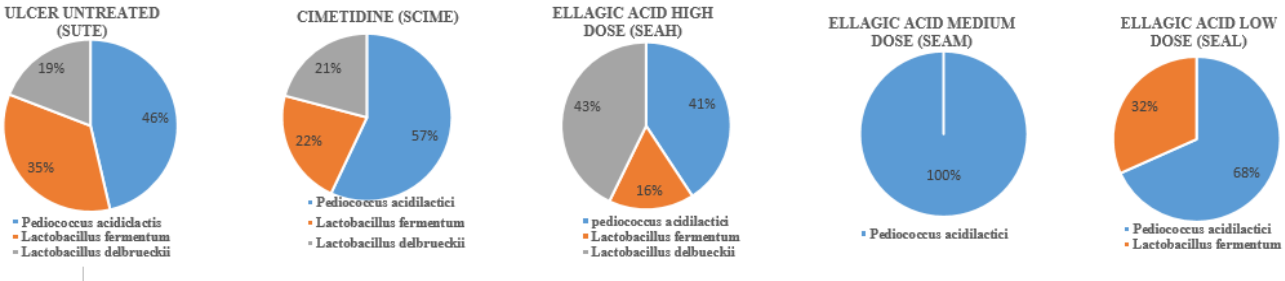

Figure 5: Effect Of Starvation Induced Ulceration On-Microbial Counts Of Lactic Acid Bacteria (Lab) On Mrs Agar

Effect of starvation induced gastric ulceration on microbial counts of Lactic Acid Bacteria (LAB), in MRS agar: In ulcer models three probiotic organisms were identified with distinct characteristics (Table 4) including: *Lactobacillus delbrueckii*, *Pedicoccus acidilactici*, and *Lactobacillus fermentum*. There was a significant increase in the microbial count (Table 4) of the *Pedicoccus acidilactici* species in all treatment group compared with the ulcer untreated group.

Table 4:
Effect of starvation induced ulceration on cultural and morphological characteristics of prebiotics microflora on MRS agar

Organisms	Gram staining	Colour	Shape	Motility	Lactose fermenter
Lactobacillus delbrueckii	Positive	Milky	Cocci	Non-motile	Positive
Pedicoccus acidilactici	Positive	Colourless	Cocci	Non-motile	Positive
Lactobacillus fermentum	Positive	Milky	Rod	Non-motile	Positive

Sequencing: The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using the manufacturers' manual. The sequencing kit used was the BigDye terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA 6 were used for all genetic analysis. The phylogenic relationship of gut microbial and Agarose gel electrophoresis shows the bands for varied bacteria amplifications (Table 5, Figures 6 and 7, respectively).

Effect of ellagic acid on gastric epithelium in starvation induced ulcers in male Wistar rats: Histology slide review showed that all ellagic acid doses and cimetidine treatment reduced erosion of surface mucous cells, distortion of pits, hyperplasia of neck mucous cells and multifocal necrosis of glandular (parietal) cells (Plates 1 and 2).

Table 5:
Blasted Result Showing Most Likely Organism and Percentage Similarity

Code	Description	Max Score	Total Score	Query Cover	E value	Percent Identity	Accession No.
MC	*Pediococcus acidilactici* gene for 16S ribosomal RNA, partial sequence, strain: JCM 2014	2115	2115	97%	0	97.88%	LC260021.1
MR	*Lactobacillus fermentum* strain 6998 16S ribosomal RNA gene, partial sequence	2656	2656	99%	0	99.52%	MT464045.1
CC	*Pediococcus acidilactici* 16S ribosomal RNA	2704	2704	99%	0	98.68%	M58833.1
CR	*Lactobacillus delbrueckii* subsp. indicus strain JCM 15610, complete genome	2726	24470	99%	0	99.67%	CP018614.1
CD	*Salmonella enterica* subsp. *enterica serovar* Heidelberg strain CVM N16S321 chromosome, complete genome	2665	18367	99%	0	99.73%	CP049313.1
MC	*Salmonella enterica* subsp. *arizonae* strain ATCC 13314 16S ribosomal RNA, partial sequence	2706	2706	99%	0	99.93%	NR_041696.1
MR	*Shigella dysenteriae* strain 6-CB-DE-D3-20 16S ribosomal RNA gene, partial sequence	2468	2468	99%	0	99.85%	MT903219.1
PR	*Escherichia coli* strain Gol11 16S ribosomal RNA gene, partial sequence	2577	2577	99%	0	99.44%	MT263026.1
MDC	*Salmonella enterica* subsp. *arizonae* strain ATCC 13314 16S ribosomal RNA, partial sequence	2702	2702	99%	0	99.73%	NR_041696.1
PC	*Enterococcus faecium* strain LMG 11423 16S ribosomal RNA, partial sequence	2830	2830	99%	0	99.93%	NR_042054.1
PDC	*Enterococcus faecium* strain A7 16S ribosomal RNA gene, partial sequence	2748	2748	99%	0	99.80%	KT924259.1

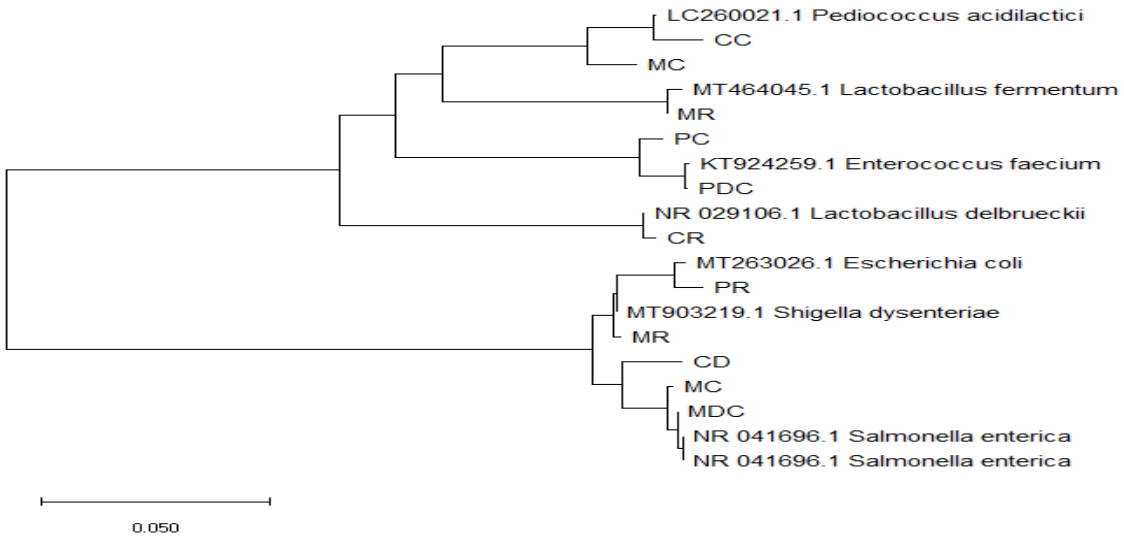

Figure 6:
Phylogenetic Relationship of Gut Microbiota in Starvation Induced Gastric Ulceration

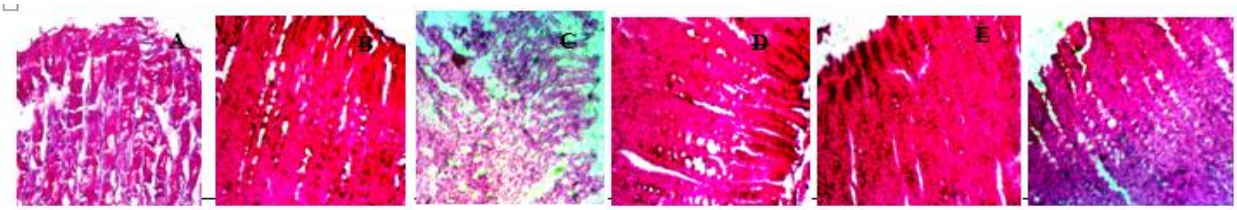

PLATE 1: PHOTOMICROGRAPH SECTION OF THE GASTRIC TISSUE STAINED WITH Periodic Acid Shicff (PAS (MAG X400) (AND MAGX100 in the box):SNOR (A): abundant surface epithelia mucin production (thin arrow), weakly stained foveolar and mucus neck cells. **SUTE (B)**: There is erosion of surface mucous, and distortion of gastric pit. **SCEM1 (C)**: There is hyperplasia of mucous neck cells. **SEAH (D)**: There is hyperplasia of mucous neck cells. **SEAM(E)**: There is no observable lesion. **SEAL (F)**: There is no observable lesion.

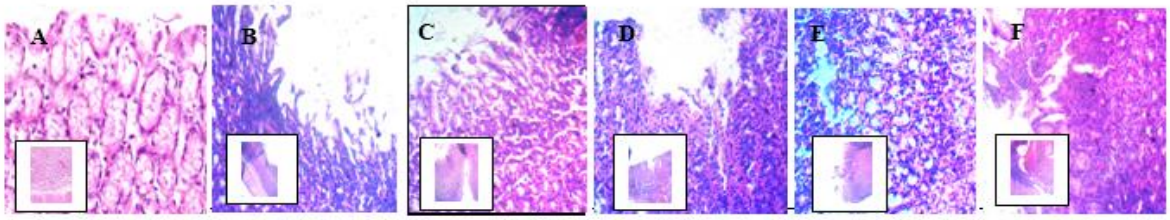

PLATE 2: PHOTOMICROGRAPH SECTION OF THE GASTRIC TISSUE STAINED WITH H&E (MAG X400) (AND MAGX100 in the box): SNOR (A): show normal mucosa, submucosa and muscularis. The surface epithelial is well preserved. No significant lesion seen **SUTE (B)**: There is distortion of gastric pit. **SCEM (C)**: There is severe mucosal defect (ulceration) characterized by loss of pit and glandular cells, and hyperplasia of mucous neck cells. **SEAH (D)**: There is focal mucosal defect (ulceration) characterized by loss of pit and glandular cells. **SEAM (E)**: There is no observable lesion. There is atrophy of gastric pit. **SEAL (F)**: There is no observable lesion. There is mucosal defect characterized by loss of pit and glandular cells.

DISCUSSION

Ellagic acid has been shown to exert gastro-protection in a few gastric ulcer models with varied or specific mechanisms of ulcer formation (Lino *et al*., 2002; Bessera *et al*., 2011; Selim *et al*., 2016; Soheir *et al*., 2018). Starvation (a form of malnutrition) being a risk factor in many developing countries has been well linked to peptic ulcer formations (Daneshi *et al*., 2017), with deleterious morphological, physiological, and histological effects on stomach mucosa (John*et al*.,1974; Al-Kawaz and Jawad, 2021). There were decreased gastric ulcer scored in the ellagic acid treated groups, suggesting gastro-protection against starvation induced gastric ulceration in this study and was as potent as cimetidine.

Proteins are important vital biomolecules of the cell (Tiwari *et al*., 2013, Asmat *et al*., 2016), they are involved in several physiological functions including cell signaling and transport across cells. Reactive Oxygen Species (ROS) target these proteins especially during uncontrolled disease conditions. Once ROS attacks the cell proteins, they become denatured, thus affecting the structure and functions of these cell proteins leading to adverse modifications (Auguste *et al*., 1988; Tiwari *et al*., 2013). Ellagic acid was shown to preserve the gastric epithelium protein content in the starvation induced ulcer models.

Malondialdehyde has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress (Shodehinde and Oboh, 2013). Malondialdehyde (MDA) produced ROS which damages lipids within the gastric cell membranes (Guo *et al*., 2012; Zhang *et al*., 2017). Normally fasting does not lead to a release of free fatty acid but during persistent fasts, the fatty acids become substrates for reactive oxygen species to give rise to peroxidation products including MDA and carbonyl (Asadi *et al*., 2015). When lipids are broken down or damaged especially by free radicals, they usually result in the production of malondialdehyde (MDA) molecules, thus explaining why MDA levels in the starvation models are higher. The ulcerated untreated group had elevated MDA level but were well attenuated in the ellagic acid treated groups. Carbonyls are the oxidation products of proteins (Kehn *et al*., 2021 Estevez *et al*., 2022) and are potent biomarkers of oxidative stress. Elevated protein carbonyl levels in the ulcer untreated group clearly depicts the denaturing of proteins ongoing at the cellular level. Increased gastric carbonyl levels were ameliorated by the ellagic acid doses, however, the low dose was more potent in reducing protein carbonyl levels and was at par with the cimetidine group in starvation induced ulceration. This reduction in gastric carbonyl level of ellagic acid treated groups further buttress the elevated gastric protein level obtained in this study. This is a pointer that ellagic acid has an anabolic effect on the gastric mucosa especially during mucosal injuries.

Catalase is an antioxidant enzyme which plays a role against oxidative stress generated in complications such as ulcers. Catalase acts as the main regulator of hydrogen peroxide metabolism (Takemoto *et al*., 2009) within the biological system. Catalase activity was reduced in the ulcerated untreated rats, but increased in the ellagic acid treated groups. The decreased catalase activity in the starved untreated groups could have resulted from decreased activity of functional enzymes (Geetha *et al*., 2003). Sulfhydryl is an important protective factor that contribute to the gastric mucosa integrity. It is a strong antioxidant that counteracts oxidative stress in gastric tissues by mopping up reactive oxygen species. An increase in sulfhydryl level is considered a strong protective mechanism (Batista *et al*., 2015) and this was observed in all the ellagic acid groups with the highest increase seen in the low ellagic acid dose. Higher lipid peroxidation has

also been linked to the impairment of the glutathione redox system (Virdju *et al*., 2018) and might have been responsible for the observed reduction of sulfhydryl observed in the untreated group.

Mucins are a family of high molecular weight, heavily glycosylated proteins (glycoconjugates) produced by epithelial tissues. Mucin can be both structural and secreted, within the gut. Mucin create the unstirred gastric mucus layer and maintain a stable pH above the gastric mucosa. This mucous layer prevents enzymatic attack by acid and pepsin. In this study ellagic acid was shown to improve gastric mucin content in the starvation induced ulcer models, which might be another mechanism by which ellagic acid exert some of its gastroprotective effect. However, it negates the submission by Beserra *et al*., (2011), whose study showed that ellagic acid had no effect on gastric mucin content, thus acting independent of prostaglandin E2 produced by cyclooxygenase-1 (COX-1). In this study, the increase in gastric mucin in the ellagic acid treated groups was further buttressed by the increase in mucous cells observed.

Gastric hydrogen potassium ATPase, (H+/K+ ATPase), is an enzyme which functions to acidify the stomach (Fujii *et al*., 2015, Han *et al*., 2021). A reduction in the expression of H+/K+ATPase would ultimately lead to a less acidic stomach environment which is pivotal in ameliorating the formation of ulcers. It was observed in this study that H+/K+ATPase levels were well reduced in the ellagic acid treated groups, thus posing it as a potent proton pump inhibitor. This result was similar to that obtained by Murakami *et al*., (1991) when ellagic acid aborted gastric lesions, which might have formed during stress ulcer. Starvation has been reported (Hung and Neu, 1997) to enhance gastric acidity which facilitates gastric ulcer formation, however, reduced gastric H+K+ATPase activity might be another mechanism by which ellagic acid reduced gastric acidity towards alkalinity, thus preventing gastric lesions.

Nitric oxide can posse to be ameliorative and detrimental vis-à-vis the level and oxidative/antioxidant status (Lundberg and Weitzberg, 2012). Nitric oxide has also been documented to be gastroprotective mainly due to vasodilatation of blood vessel, which aids blood flow to ulcerated site (Konturek *et al*., 1995). This increased blood flow enhances delivery of nutrient to ulcerated site, thus facilitating healing (Petersson *et al*., 2007, Liang *et al*., 2021), ridding the gastric mucosa of metabolic waste and enhancing secretion of mucus (Brown *et al*., 1993, El-Demerdash *et al*., 2010). It has also been documented that increased nitric oxide release facilitates alkaline reactions within the gastric lumen thus enhancing cell protection (Nobuhara *et al*., 1984, Takeuchi *et al*., 1986) and gastric mucosal angiogenesis (Tian *et al*., 2014). Nitric oxide has also been documented to prevent generation of oxidants, superoxides and hydrogen peroxide, which are known ulcerogens when increased (Vaananen *et al*., 1991; Liang *et al*., 2021). In this study, nitric oxide was increased dose dependently in the ellagic acid treated groups. It may probably be that it helps in vasodilatation and microcirculation within the gastric tissue thus maintaining normalcy and restoring the gastric epithelium during starvation. This is further buttressed by reduced oxidative stress markers (lipid and protein peroxidation), decreased gastric H+K+ATPase activity which accounts for gastric alkalinity, with enhanced antioxidative (catalase and sulfhydryl) status and gastric mucin levels.

During starvation, there is dysbiosis and increased oxidative stress (Knaus *et al*., 2017, Chen *et al*., 2022). Bacteria have been well established as a critical element in gastric pathology serving as the primary cause of chronic active gastritis, which can lead to serious consequences such as atrophic gastritis, peptic ulcer disease, and gastric cancer (Wang *et al*., 2014). Probiotics have been documented to help ameliorate dybiosis during gut inflammatory reactions (Salami *et al*., 2018) and starvation through certain signalling molecules they produce. Nitric oxide and hydrogen peroxide (are signalling gut molecules and) have been documented to be produced by certain gut microbes with beneficial activities towards maintain gut flora homeostasis (Rodríguez-Rojas *et al*., 2020). This study also shows that gram-negative pathogens such as *Escherichia coli*, *Salmonella enterica*, and *Shigella dysenteriae* were isolated and elevated in the ulcerated untreated groups which could be as a result of immune suppression. The roles of these bacteria might not be far-fetched from aggravated gastric ulcer (in the ulcerated untreated groups), however they outnumber the gram-positive bacteria 100 to 1 especially in the ulcer untreated groups. Two strains of commensal *Enterococcus faecium* a gram positive bacteria were isolated but elevated only in the ellagic treated groups, especially the medium group. Commensal *Enterococcus faecium* have been documented to thrive during increase iron availability thus mopping up free iron for their growth (Capcarova *et al*., 2010; Lisiecki 2010). However, *E. faecium* has also been documented to reduce aggressive gut microbiome (Szabo *et al*., 2009); and might have been responsible for the reduced *Escherichia coli*, *Salmonella enterica*, and *Shigella dysenteriae* observed in the ellagic treated groups of this study. *E. faecium* was completely absent in the ulcerated untreated group in this study; this further supports the efficacy of gut *E. faecium* in the groups treated with Ellagic acid.

Lactic acid bacteria have the ability to improve the gastrointestinal ecosystem (Tang *et al*., 2016). They function as a potent antioxidant agent for example the scavenging effects of lactic acid bacteria on hydrogen radicals is one of the important index antioxidation (Lin *et al*., 2018). A group of probiotics (lactic acid bacteria) with rod-like or coccoid shape were isolated and were prominent in the ellagic acid treated groups: Lactobacillus fermentum, *Lactobacillus delbrueckii* and *Pedicoccus acidilactici.* The *lactobacillus* species have been documented to be gastroprotective besides them secreting certain metabolites like hydrogen peroxide, ethanoic acid, organic acids, etc. which can inhibit or ward off harmful gut microorganism or microflora, (Zhang *et al*.,2013; De Paula *et al*.,2014, and Khoder *et al*., 2016). In this study, hydrogen peroxide level, was increased in the ulcerated untreated group which had higher percentages of gram negative pathogenic gut organisms (*Escherichia coli*, *Salmonella enterica*, and *Shigella dysenteriae*). These further collaborated the increased level of reactive oxygen species, dysbiosis and probably unhealed gastric ulcer. Lactic acid bacteria have also been reported to produce hydrogen peroxide and use them as bacteriocides against pathogenic gut microbes (Hertzberger *et al*., 2014; Pircalabioru *et al*., 2016). However, hydrogen peroxide level was reduced in the ellagic treated groups and probably might have been linked to these isolated lactic acid bacteria and commensal *E. faecium* thus preventing dysbiosis and attenuating starvation induced gastric ulcer.

This might be another mechanism by which ellagic acid ameliorates starvation induced gastric ulceration and facilitating the antioxidative activities observed. This means that ellagic acid showed prebiotic activities by promoting proliferation of gut lactic acid and *E. faecium* bacteria probably through the gastric alkaline medium, due to reduced

activities of the gastric H+K+ATPase besides increased gastric mucin of these groups, thereby preventing epithelial gut colonisation by gut gram-negative bacteria which causes dysbiosis. Dysbiosis has been documented to be due to increased reactive oxygen species as well as imbalance in two main signalling molecules such as nitric oxide and hydrogen peroxide (Knaus *et al*., 2017). Nitric oxide has also been documented to be produced by some lactic and commensal bacteria like *E. faecium* (Sobko *et al*., 2004; 2005; Maitreya *et al*., 2022) especially lactic acid bacteria (Li *et al*., 2016) which can either stimulate the gastric mucosa to or they themselves produce NO (Soboko *et al*., 2004; Lundberg and Weitzberg 2013). It may probably posse NO as a regulator of intestinal crosstalk in maintaining gut epithelial homeostasis during starvation as observed in this study.

However, though there was an increase in the nitric oxide, there were reduced hydrogen peroxide and higher percentages of isolated lactic acid bacteria in the ellagic acid treated groups, which enhanced gut microflora homeostasis. The reduced gastric ulcer observed might be linked to the cross talk between these microorganisms and gut epithelial through nitric oxide and hydrogen peroxide metabolites. The gap between this study and that of Bessera *et al*., (2011) could be these probiotics: *L. fermentum* which has particularly been shown to increase nitric oxide activity and decrease MDA activity (Suo *et al*., 2016). *E. faecium* has also been documented to enhance production of nitric oxide (Wang *et al*., 2013) and was observed in this study. In this study, the NO was increased dose dependently. This increase in NO might enhance vasodilatation of the blood vessels and oxygenation of the gut epithelial. The presence of *Lactobacillus delbrueckii* strain provides an anti-microbial, specifically anti-*H-pylori* gastric environment (Boyanova *et al*., 2009; Gou *et al*., 2019). Khoder *et al*., (2016) documented the roles of these lactic acid bacteria notably probiotics in restoring gastric mucosa barrier as observed in this study.

However, the gut derived *E. faecium* observed mostly in the fecal sample of the ellagic acid treated groups further buttresses the reports by Zhang *et al*., 2022. Despite the higher release of iron stores during starvation-induced gastric ulcer, the gut derived *E. faecium* might have transformed ellagic acid to urolithin A in this study as observed by Espin *et al*., (2013) and Saha *et al*., (2016), thus conferring gastroprotection. However, this iron would have been moped up by commensal *E. faecium*, and *Lactobacillus species* for their growth thus conferring antioxidative and ameliorative activities together with urolithin A as observed in this study.

CONCLUSION

This study revealed that Ellagic acid enhanced epithelia protective factors such as mucin and antioxidant enzymes while mitigating gastric aggressive factors such as lipid peroxidation and H+/K+pump activities as well as promoting gastric probiotic and commensal microflora. Ellagic acid promoted the growth of some probiotics such as Lactobacillus fermentum, *Lactobacillus delbrueckii*, *Pedicoccus acidilactici* and *Enterococcus faecium*, which are known to have antimicrobial potentials on the gastric microflora and inhibit the proliferation of pathogenic bacteria like Escherichia coli. Ellagic acid has gastroprotective potentials in starvation induced gastric ulceration basically from the gut derived *E. faecium* and *Lactobacillus* specie.

Conflict of interest:

Authors of this study have no conflicting interest.

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