RESEARCH ARTICLE

Protective potential of *Ganoderma lucidum* on human gut microbiota balance and rat intestinal morphology

Purna Nandi[1], Sudeep Mitra[2], Mousumi Mitra[1], Mousumi Chakraborty[1], Mrinal Kanti Paira[1], Dilip Kumar Nandi[2*]

ABSTRACT

Background: Ganoderma lucidum (GL) can help to control the gut microbiota, a dynamic microbial community that is crucial for human health. *Hypothesis:* This work investigates the dose-dependent effects of GL on gut microbiota composition using integrated *in-vitro* and *in-vivo* approaches, as well as its possible modulatory role. *Materials and Methods:* 150 female college students, aged between 19 to 21 years, participated in this study. They were given 500 and 1000 mg of GL capsules daily as supplements. Albino rats (n=12) were divided into control and GL-treated groups (n=6 each); the treated group received 112.12 mg/kg/day orally for 1 month. *In-vivo* human composition was examined by CFU enumeration, biochemical assays, Gram staining, and 16S rDNA PCR analysis of female-derived isolates. *In-vitro*, intestinal morphology was studied using SEM, and the lowest effective dose of GL was determined using MIC and antibiotic susceptibility testing. *Results:* Significant (p <0.05) increases in gram-positive lactic acid bacteria and *Bifidobacterium* sp. and decreases in gram-negative *Escherichia coli, Salmonella* sp., and Gram-positive total aerobes in the gut microbiota. GL was also shown to be sensitive to *Salmonella* sp., *E. coli*, and total aerobes and resistant to lactic acid and *Bifidobacterium* sp. Following supplementation, the SEM investigation also noted certain morphological changes. *Conclusion:* Our findings showed a novel role for GL in beneficially modifying the gut microbiota and maintaining small intestinal integrity through antimicrobial activity.

Keywords: Human gut microbiota, *Ganoderma lucidum*, Small intestinal morphology.

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INTRODUCTION

The gut microbiota is a highly diversified and metabolically active consortium of microorganisms that collectively encodes a gene pool far beyond that of the human genome and serves as an essential component of host physiology. Numerous physiological processes, such as the digestion of dietary components, host metabolism, immunological control, nutritional assimilation, energy conservation, the provision of (micro) nutrients, and the modification of xenobiotics, are aided by the metabolic activity of the gut microbiota.[1] Overall, a balanced composition of the gut microbiota helps the host, whereas microbial imbalances are linked to problems in the metabolism and immune system.[2] The protective, immunological, and metabolic processes that the gut microbiota produces significantly impact the host's nutritional and health state. Additionally, the gut microbiota influences the host's metabolism by providing additional enzymes and regulating the expression of genes involved in the metabolism of proteins, lipids, and carbohydrates.[3] Moreover, studies have shown the potential use of polysaccharides as a novel source of probiotics with protective benefits on intestinal barrier function, such as allowing the absorption of essential nutrients, and also maintaining gut homeostasis and preventing inflammation. Polysaccharides can reduce damage to the intestinal lining, contributing to a protective environment that favors probiotic colonization and downregulates inflammatory responses, thereby indirectly stabilizing the gut microbiota through the cytokine and SCFA (short-chain fatty acid) pathways.[4,5] Therefore, GL polysaccharides could be feasible for controlling the gut microbiota.

[1]Laboratory of Human Performance and Nutraceuticals, Department of Human Physiology (UG & PG) & BMLT, and Chemistry, Raja Narendralal Khan Women's College (Autonomous), Midnapore, India.

[2]Department of Paramedical & Allied Health Sciences and Biological Sciences, Midnapore City College, Kuturiya, Bhadutala, Salboni, Paschim Medinipur, 721129, West Bengal, India.

***Corresponding author:** Dilip Kumar Nandi, Department of Paramedical & Allied Health Sciences and Biological Sciences, Midnapore City College, Kuturiya, Bhadutala, Salboni, Paschim Medinipur, 721129, West Bengal, India, Email: dilipnandi2004@yahoo.co.in

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The composition of the host's gut microbiota can be influenced by polyphenols present in GL, which are known to regulate intestinal microbial fermentation and overall bacterial numbers. Notably, GL polysaccharides have been used for both therapeutic and nutritional perspectives, enriching some beneficial bacteria, increasing short-chain fatty acid synthesis, improving intestinal barrier function, and ameliorating dysbiosis.[6] GL polysaccharides undergo distal intestinal tract absorption facilitated by gut microbiota. A possible cancer chemoprevention strategy also involves altering the gut microbiota using polysaccharides, according

to some studies.[7] Human intestines have been shown to exhibit microbial dysbiosis or changes in the abundance of particular bacterial species. New research indicates that polysaccharides play a critical role in regulating the gut microbiota and preventing microbial dysbiosis.[7] According to prior research, GL extract altered the gut microbiota by increasing immunocompetent species (*Helicobacter*, *Rikenella*, and *Turicibacter*) and lowering immunosuppressive genera (*Bacteroides*). GL is most likely caused by suppressing co-inhibitory signaling (PD-1 (Programmed death-1) and CTLA-4 (Cytotoxic T-lymphocyte associated antigen-4)) pathways, restoring exhausted Tc cells, and significantly remodeling the gut microbiome.[8] Previous research shows that GL extract (GLE) treatment changed the makeup of the gut microbiota in a DSS (Dextran sulphate sodium)-induced colitis mouse model.[9] Despite emerging evidence that GL can impact gut microbial dynamics, studies are primarily descriptive. The molecular basis for dose-dependent effects is not fully known.

The growing demand for GL fruiting bodies in international markets has necessitated the artificial cultivation of this medicinal mushroom.[10] Active compounds account for its numerous pharmacological activities, including antitumor, immunomodulatory, cardiovascular, respiratory, antihepatotoxic, and central nervous system effects.[11;12] Although the fruiting body of GL, known as Ling-Zhi in Chinese and Reishi in Japanese, has been used for thousands of years in Asian traditional medicine to treat a range of illnesses, its precise most important role in modulating gut microbiota composition and function has received insufficient attention.[13]

Novelty of the work: The study systematically investigates the impact of GL on human gut microbiota and its broader implications for metabolic health. Additionally, it enhances beneficial probiotic groups, and it investigates a broader range of bacterial taxa, offering new insights into its modulatory effects, unlike prior work limited to animal models. Therefore, the study aids in determining if GL can be used as a nutraceutical for gut-microbiome health.

MATERIALS AND METHOD

Study Materials and Supplement Preparation

Supplements

GL capsules each contain 500 mg of GL extract with 9.5% of polysaccharide, coated with only gelatine. The capsules were provided by the Defense Institute of Physiology and Allied Sciences (DIPAS), Defense Research and Development Organization (DRDO), for the present study and were stored in dry, shaded locations. Commercially purchased placebo capsules consist only of empty gelatine-coated capsules. To reduce bias, account for physiological responses unrelated to the intervention, and ensure reliable evaluation of treatment-specific effects, one group received a placebo. It is an identical formulation devoid of the active ingredient. This group serves as a control, allowing us to observe the effects of *Ganoderma* extract.

Preparation of GL capsule

A wild reddish-brown form of GL was collected from Pithoragarh, Uttarakhand, India (North-West Himalayas, ~4000 m elevation). An ethnobotanist from the Defense Institute of Bio Energy Research (DIBER) in Haldwani, India, studied a voucher specimen (DIP-GL/2014) and dried it at 30 to 35°C to make dust. The GL aqueous extract was produced using accelerated solvent extraction (ASE). The samples were extracted at room temperature for 15 minutes before being cleaned. After extraction, the supernatant solution was lyophilized, and the dried extract was kept at 4°C for further investigation. The extract did not include steroids, but it did contain glycosides, flavonoids, phenols, polysaccharides, terpenoids, and tannins, which remained stable for up to 2 years at room temperature, 4°C, and -20°C. DIPAS, DRDO, provided GL capsules in airtight, high-density polyethylene (HDPE) bottles and stored them at or below 25°C, in a dry, cool place, and away from direct sunlight. A previous study describes the characterization, substance composition, processing, and storage.[14]

The GL capsules were developed and provided by Defense Institute of Physiology and Allied Sciences (DIPAS), Defense Research and Development Organization (DRDO), Ministry of Defense, India, with approval from the Ministry of Ayush, Government of Telangana, India (Letter No. 1052018/DA/2021) under license No. T-1632/Ayur. These groups had no involvement in the study's design, implementation, inquiry, or statistical analysis.

Human study

Ethical approval

According to the Indian Council of Medical Research's (ICMR) rules, the Institutional Review Board (IRB) and Institutional Ethical Committee (IEC) of Raja Narendra Lal Khan Women's College (Autonomous), Midnapore-721102, West Bengal, approved all of the research's activities (Ref. NO.-05/IEC/RNLKWC/20; dated 07/02/2024).

Study population

The study involved 150 volunteers, comprising female adults aged 19 to 21 years (BMI from 23.3–53 kg/m^2). Female subjects were carefully recruited, given known gender differences in gut microbiota composition, and the modulatory effects of female sex hormones on microbial diversity and metabolic function. Furthermore, a narrow age range was chosen to ensure reproductive-age hormonal stability while reducing variability associated with hormonal changes.[15] Furthermore, academic and career-related stress has been linked to changes in gut microbiota composition and increased susceptibility to dysbiosis.[16]

Inclusion criteria

include being healthy and disease-free, having a similar socioeconomic background (education, gender, housing, employment, amenities), and being female between the ages of 19 and 21 years.

Exclusion criteria

The study excluded participants with 1. A history of serious sickness, 2. An irregular menstrual cycle, 3. Students with known mushroom allergies and allergy status were determined based on responses to a pre-screening questionnaire (since all of the students live in the college residence hostel, their diets are the same), and 4. use of contraceptive pills or other supplements.

Sample size calculation

PS Power version 3.0.43 was used to determine the sample size, with a 95% confidence interval. 150 female college students were included in the study, accounting for the type I error probability, power, and the population means difference.

Human grouping according to dose

Female adults were divided into three groups:

- Placebo group: Normal control supplemented with placebo capsules.
- GL 500 mg group: Treated group, given 500 mg/day GL capsule for 30 days.
- GL 1000 mg group: Treated group, given 1000 mg/day GL capsules for 30 days.

These were chosen as the lowest effective dose to avoid any potential side effects from an overdose. The reported and recommended daily dosages of GL for humans were 500 and 1000 mg, respectively.[14] However, there is currently limited research on GL's efficacy in humans at these levels.

Fecal sample collection

Human fecal samples were collected from healthy volunteers with no recent history of gastrointestinal symptoms, no dietary restrictions, and no antibiotic use in the six months before the sampling date. The stool samples were collected in pre-weighted stool pots, prepared as a 20% fecal slurry in normal PBS (Phosphate Buffer Saline) (pH 7.4), and immediately stored on ice until processing. Appropriate dilutions were prepared from fecal samples.[17]

Microbiological Analysis

Microbial study

- Media for total lactic acid bacteria: Using HiMedia MRS (De Man, Rogosa, and Sharpe) agar, the following *lactobacilli* were examined. MRS agar was prepared as directed by the manufacturer. The identical samples and plating techniques were used for the total lactic acid bacteria. All plates were incubated at 37°C for 14 to 20 hours under anaerobic conditions (0% O_2) in an anaerobic chamber. Colonies were counted both overall and typically. The microscopic examination of all colonies with various morphologies. All straight rods that didn't develop spores were tentatively considered to be *lactobacilli*.[18]
- Media for *E. coli: E. coli* was determined by using HiMedia MacConkey agar, as per the manufacturer's instructions. All plates were incubated at 37°C for 14-20 hours for superior bacterial growth. Colonies were counted both overall and typically. All diluting and plating processes were carried out aerobically.[19] MacConkey agar contains bile salts and crystal violet to inhibit Gram-positive bacteria and *E. coli*; it mainly ferments lactose, producing pink/red colonies due to acid production (natural red indicator).
- Media for *Salmonella* sp.: *Salmonella* sp. was determined by using HiMedia Brilliant Green agar, as per the manufacturer's instructions. Plates were incubated at 37°C for 14-20 hours for superior bacterial growth. All dilution plates were counted following the CFU (Colony forming unit) method, and pinkish-white colonies were considered as *Salmonella* sp.[20]
- Media for total aerobes: Total aerobic bacteria's selective media is TSA (Trypticase soya agar). Using different dilution plates incubated at 37°C for 14-20 hours, white colonies were considered to be the bacteria of interest.[21]
- Media for *Bifidobacterium* sp. The selective medium used for *Bifidobacterium* sp. is HiMedia Bifidobacterium agar. After incubation for 16-20 hours at 37°C in an anaerobic chamber, black colonies were identified as Bifidobacterium sp. on different dilution plates.[22]

Colony-forming unit

In microbiology, dilution plating to quantify colony-forming units (CFUs) has long been the standard method for enumerating bacterial colonies in stool samples. An aliquot of culture was 10-fold serially diluted, and 100 µL was spread, and the CFU number calculated by colonies detected at 106-fold dilutions or lower is comparable to that obtained by the dilution plating method at 107-fold dilutions.[23]

Biochemical characterization

Gram's staining, the catalase test, and other secondary biochemical tests, such as indole, citrate utilization, triple sugar iron agar, and urease tests, were used to identify five distinct bacterial groups. Fifty isolates from each bacterial group were evaluated for their biochemical characteristics.[24;25]

Extraction and preparation of genomic DNA (Deoxyribonucleic acid) from fecal samples

Isolates were individually cultured for 12 hours in 1.5 mL of the corresponding selective broth at 37°C, and cells were individually collected by centrifugation at 5000 rpm for 3 minutes. The broth was removed, and genomic DNA was extracted from the cell pellets using InvitrogenTM Genomic DNA Extraction Kits. The extracted genomic DNA from each isolate was checked by horizontal gel electrophoresis at 60°C and 60 V for 16 hours, using 0.8% (w/v) agarose containing 0.5

mg/mL ethidium bromide in 0.5X TBE. Gels were stained with ethidium bromide in TAE buffer for 20 min, then destained with sterile deionized water for 10 min, and visualized using a GeNeiTM UV-transilluminator, college model TCM1.26. The DNA preparations were stored in 40 mL of nuclease-free water at -20°C. Nanodrops (BioSpectrometer, Eppendorf) (A260)[27] were used to assess DNA concentration before and after PCR, indicating that distinct bacterial groups were amplified by their respective primers.[28]

Amplification of 16s rDNA of isolates

The 16S rDNA fragment of each isolate was amplified by SEDI G Polymerase Chain Reaction-Wealtec PCR with the universal primer set of 27-f (59-AGT TTG ATC CTG GCT CAG-39) and 1492-r (59-GTT ACC TTG TTA CGA CTT C-39).[29] All reagents that were used in PCR amplification were purchased. Amplification was performed in 50 µL reaction volumes as described.[30] Each PCR reaction consisted of 200 mM of each dNTP, 1.0 mM of each primer, 2.5 mM $MgCl_2$, 1X PCR buffer, 2.5 U of Taq DNA polymerase, and 50 ng of DNA template. The thermal cycling included an initial denaturation step at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR product was checked using 0.8% (w/v) agarose gel electrophoresis TARSONS MC-01. The gel was visualised using a UV transilluminator.[31] The semi-quantitative measure of DNA PCR product refers to an approximate assessment of DNA concentration using the nanodrop (A260) method.[27]

In-vitro study

Antibiotic susceptibility test

The agar dilution and disk diffusion techniques outlined in the National Committee for Clinical Laboratory Standards (NCCLS) guidelines[32] were used concurrently to determine the antimicrobial susceptibilities of each isolate. The following 10 antimicrobial disks: Doxycycline (DO) 30 µg, Levofloxacin (LE) 5 µg, Amoxicillin (Amx) 30 µg, Amikacin (Ak) 30 µg, Ceftriaxone (CTR) 30 µg, Ciprofloxacin (CIP) 5 µg, Cefixime (CFM) 5 µg, Erythromycin (E) 15 µg, Meropenem (MRP) 10 µg, and Imipenem (IPM) 10 µg were included for susceptibility testing using the disk diffusion method, together with various agar. All of the disks were purchased, used as controls, and compared with the GL concentration (1000 mg/mL). Sterile paper disks containing 10 µL of each antibiotic were placed on a lawn culture plate inoculated with five groups of test bacterial isolates from a fecal sample. Twenty isolates were used for this test. Each isolate's susceptibility to each medication and GL (1000 mg/mL) was tested twice on the same day in identical settings. After being incubated at 37°C for 18 to 24 hours, plates were examined, and the diameter of the zone of inhibition was measured in mm using a digital caliper. Each test was performed in triplicate, and the mean value was recorded.[33] The results were interpreted according to the Clinical Laboratory Standards Institute.[34]

Minimum Inhibitory Concentration (MIC) Assay

For 16 to 18 hours, the inoculated plates containing five different bacterial types isolated from the fecal sample were incubated at 37°C. The lowest concentration of GL (three different concentrations) was used as the antimicrobial agent and prevented the organism's observable growth by the MIC assay. Ignored a solitary colony or a tiny, scarcely perceptible cloud. To assess the lowest effective dose of GL, the lowest concentration that visually inhibited ≥80% of bacterial growth was taken as the effective dose.[33] The dosages for MIC estimation were based on human supplement doses of 500 and 1000 mg, with a minimum dose of half the human dose, 250 mg, for detecting the lowest effective concentration of GL. First, the three measurements (2500, 5000, 10000 mg) were taken with a weighing machine and dissolved in 1mL of ddH2O. The 100 µL mixture was then plated against the five distinct gut microbiota groups previously isolated from the fecal sample.

In-vivo animal study

Animal ethical approval

Surgery and animal care for the experimental studies were performed as per the Animal Ethical Committee guidelines (Ref. No.: 03/IAEC (1)/S/RNLKWC/2023; dated 15.06.2023) and were maintained as per the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India (Registration no.:1905/PO/Re/S/2016/CPCSEA).

Animal selection, care, and supplementation for Scanning electron microscopy (SEM) study

About 12 adult male Wistar strain rats that were free of pathogens were supplied and approved for the study from Chakraborty Animal Suppliers in Kolkata (M/S Chakraborty Enterprise Registration no.:1443/PO/b/11/CPCSEA). The rats weighed 160 ± 10 g at 12 weeks of age. The rats were kept under typical laboratory conditions for the experiment, including 22 ± 4°C, 50 to 10% humidity, and appropriate food and water. The animals were kept in specially made cages, with six rats per cage. They were given the usual diet and unlimited water. For scanning electron microscopy, male albino rats were divided into two groups: 1. Control group, comprising 6 rats that served as a control group and received a normal diet, 2. Experimental group I- comprising 6 rats, each received 112.12 mg/kg/day once daily orally for thirty days, including a normal diet. In this study, different doses of GL were administered to human subjects and to rats, based on the specific objectives and physiological differences between the two models. The dose used in humans was selected based on tolerability, regulatory guidelines, and practical relevance for dietary supplementation. In contrast, the dose used in the rat model, particularly for morphological and histological analyses, was determined using FDA (Food and Drug Administration)-recommended body surface area (BSA) scaling based on K_m factors.[35] The K_m value is constant for each species. The equation is-

$$\text{Rat dose (mg/kg/day)} = \text{Human dose (mg/kg/day)} \times \frac{\text{Km human}}{\text{km rat}}$$

SEM study

Rats were anesthetized with chloroform, sacrificed after 30 days of supplementation, and a fresh small intestinal segment was taken and washed 3 times in regular PBS (pH 7.4). Specimens for electron microscopy were immersed in 2.5% buffered glutaraldehyde for 24 hours and then dehydrated using ascending grades of ethyl alcohol (50, 70, 80, 90, and 100%). The tissues were further dehydrated in a 1:1 mixture of absolute alcohol and acetone (100%) and then in 100% acetone twice (15 min each). The specimens were then dried by critical-point drying using liquid carbon dioxide. The specimens were fixed on aluminum stubs and then sputter-coated with gold. The intestine was examined using a scanning electron microscope, ZEISS, and GEMINI.2, MERLIN at the central research facilities (CRF, IIT Kharagpur).[36]

Statistical Analysis

The collected data were analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) and presented as the mean of three replicates. The Shapiro-Wilk test was used to check the normality of the data distribution before conducting parametric analyses. The parameters were examined using one ANOVA (Analysis of variance) and two-tailed t-tests. The change in bacterial quantity was tested using Bonferroni's post hoc test. Significant variation is accepted at the 5% level ($p < 0.05$).

RESULTS

The current study included 150 research participants, all female college students with an average age of 19 years and a maximum age of 21 years. The subjects were evenly distributed across the college hostel.

Human study

Figure 1 depicts the differences in gut microbial abundance across three human groups at three distinct GL dosage durations. Figures 1: a, b, c, d, and e demonstrate a significant difference in the GL 1000 mg/day group after thirty days of supplementation. *Bifidobacterium sp*. (1.a) and lactic acid bacteria (1.d) numbers have greatly increased, while *Salmonella sp*. (1.b), *E. coli* (1.c), and total aerobes (1.e) numbers have significantly decreased.

Table 1 shows that only total aerobes can produce urease. *E. coli* and total aerobes can hydrolyze tryptophan to indole. *E. coli*, *Salmonella* sp., and total aerobes exhibited immediate bubble formation during the catalase test, indicating catalase enzyme production. All gut microbiomes show a negative result in the triple sugar iron test. In the citrate utilization test, except for total aerobes, all gut microbial groups were negative. None of the bacterial groups from the intestines can ferment glucose.

Microscopic examination (100×; Oil immersion) of gram-stained isolated colonies from feces revealed a heterogeneous

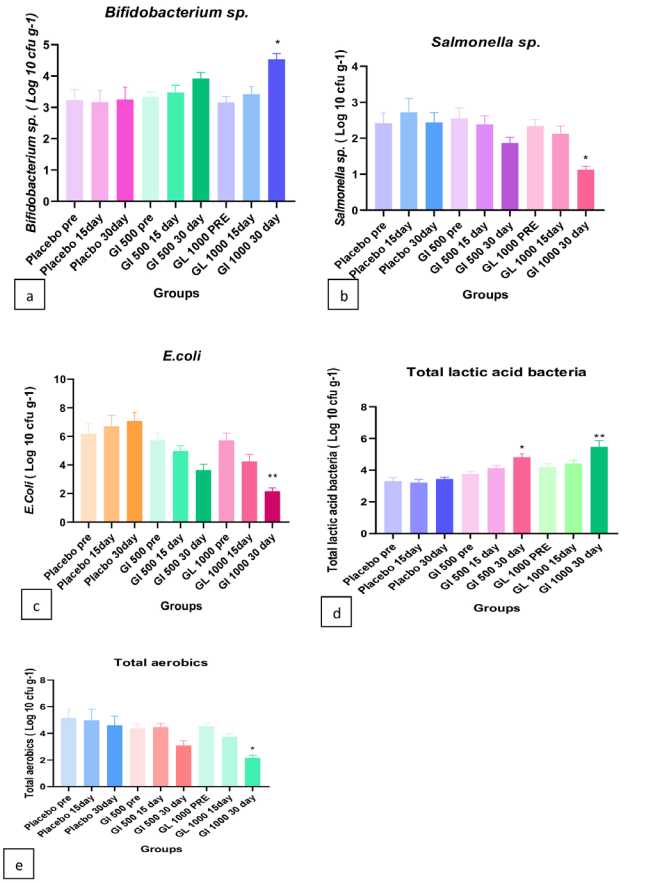

Figure 1: The graphical representation shows quantification of five different groups of gut microbiota: a. *Bifidobacterium* sp., b. *Salmonella* sp., and c. *E. coli,* d. Total lactic acid bacteria and E. total aerobes were isolated from a human fecal sample and cultured in different selective media for 24 hours to estimate colony-forming units. Number of bacteria represented as Log 10 cfu g^{-1}. Comparison of three groups: placebo, GL500, and GL1000 at 0 days, the 15th day of supplementation, and the 30th day of supplementation. The comparison was statistically analyzed using one-way ANOVA, followed by the Bonferroni test. *$p<0.05$ and * p <0.01 when compared between pre- and post-supplementation trials.

bacterial community. Colonies on MacConkey and brilliant green agar were gram-negative, whereas those on Bifidobacterium agar and MRS medium were gram-positive. The fraction of isolated colonies from TSA agar appeared, with some gram-positive and others gram-negative (Figure 2.a-e). The DNA concentration increases, as shown by gel electrophoresis (Figure 3. A, B, C, D). A significant ($p <$ 0.0001) increase in DNA copy number was observed using the Nanodrop (A260) before and after PCR processing, as determined by a two-tailed t-test (Table 2).

In-vitro study

Figure 4 represents the minimum effective dose of GL to act on five different microbiota groups *in-vitro.* Among three different concentrations of GL (250, 500, and 1000 mg), the largest zone of inhibition was observed only at the 1000 mg dose for *E. coli* (3.6 cm), *Salmonella* sp. (3.3 cm), and total

Table 1: Characterization of five different groups of bacteria isolated from different fecal samples by Biochemical characterization

Organism Name	*Urease test*	*Indole production*	*Catalase test*	*Triple sugar iron test*	*Citrate utilisation test*	*Glucose fermentation test*
E. coli	-	+	+	K/k -	-	+
Total aerobes	+	+	+	K/k -	+	+
Salmonella sp.	-	-	+	K/k -	-	+
Lactic acid bacteria	-	-	-	K/k -	-	+
Bifidobacterium sp.	-	-	-	K/k -	-	+

All results are from 50 isolates. '+' = Positive; '-'= Negative

aerobes (3.1 cm). However, total lactic acid and *Bifidobacterium* sp. did not show any zone of inhibition (Figure 4).

After determining GL's MIC value, Figure 5 explains how to assess the drug's effectiveness and compare it with that of other antibiotics. 1000 mg/mL GL exhibited marked antibacterial activity with a zone of inhibition (ZOI) of 3.6 cm against *E.coli*, 2.9 cm against *Salmonella* sp., and 2.5 cm against the total aerobes. In the case of the lactic acid bacteria and the *Bifidobacterium sp.* bacterial group, GL demonstrated complete resistance, as no inhibition zone was identified around the test sample. This implies that GL at 1000 mg/mL was as sensitive to *E. coli, Salmonella* sp., and total aerobes as other antibiotics at different dosages, while being resistant to lactic acid bacteria and *the Bifidobacterium* sp. bacterial group. Figure 6 demonstrates that among 20 isolates from five different bacterial gut microbial groups, *E. coli* (90%), *Salmonella* sp. (95%), and Total aerobes (95%) were susceptible to GL. Lactic acid bacteria and *Bifidobacterium sp.* were susceptible to GL (00%).

In-vivo animal study

Figure 7 shows that GL-supplemented small intestine villi were less damaged than the control group; the projection of villi of the small intestine showed a lesser number of parasite attachments and was also covered by regular enterocytes in the supplemented group's small intestine (A.1 and A.2). There was a significant difference in the shape of villi on the mucosal folds, and between the folds, gaps were irregular compared to the supplemented group. The average density significantly decreased in the control group compared to the

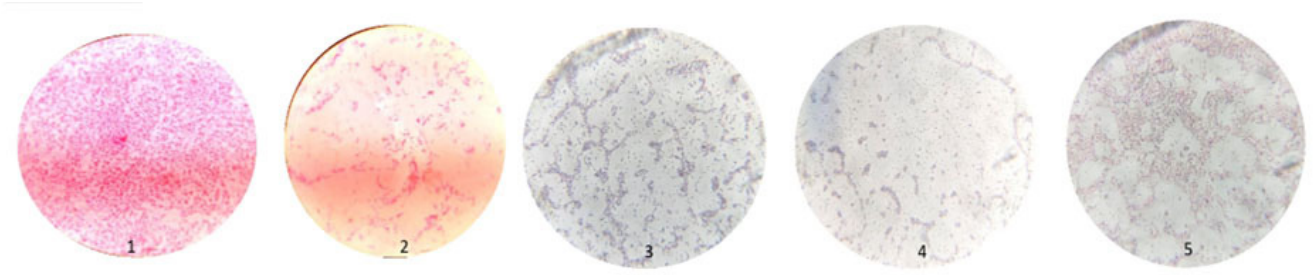

Figure 2: Gram staining of isolated fecal bacterial group, microscopic examination under 100× oil immersion- a. *E. coli*, b. *Salmonella* sp., c. *Bifidobacterium sp*., d. Total aerobes, and e. Total lactic acid bacteria.

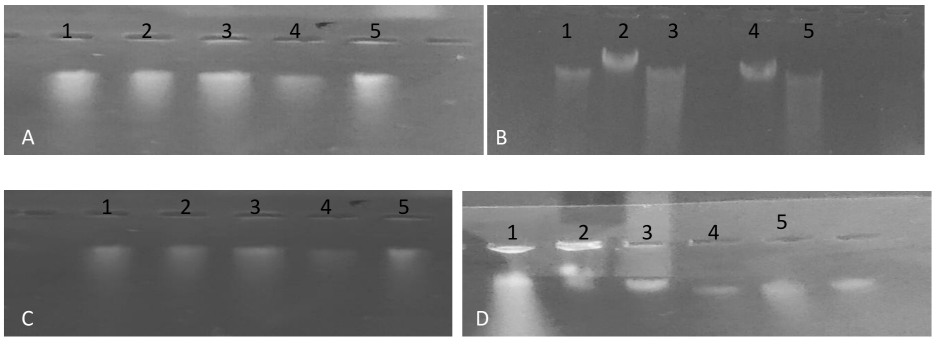

Figure 3: DNA isolated from fecal sample bacteria of the (A) 500 and (C) 1000 mg/day supplementary group as visualized by gel electrophoresis. Gel electrophoresis of the DNA PCR product isolated from bacteria of the fecal sample of the (B) 500 and (D) 1000 mg/day supplementary group. PCR was performed with a universal primer. (Lane: 1. E. coli, 2. Total aerobes, 3. Salmonella sp., 4. Lactic acid bacteria, and 5. Bifidobacterium sp.).

Table 2: The DNA concentrations of five different bacterial groups before and after PCR amplification

	DNA concentration (ng/µL)				
	E. coli	*Salmonella sp.*	*Bifidobacterium sp.*	*Total aerobes*	*Lactic acid bacteria*
Before PCR	84.19 ± 0.02	112.44 ± 0.03	218.11 ± 0.02	586.46 ± 0.01	216.32 ± 0.02
After PCR	124.97±0.01*	224.34±0.01*	370.95±0.02*	849.19±0.02*	333.47±0.01*

Data are mean ± SD; * indicates p <0.05

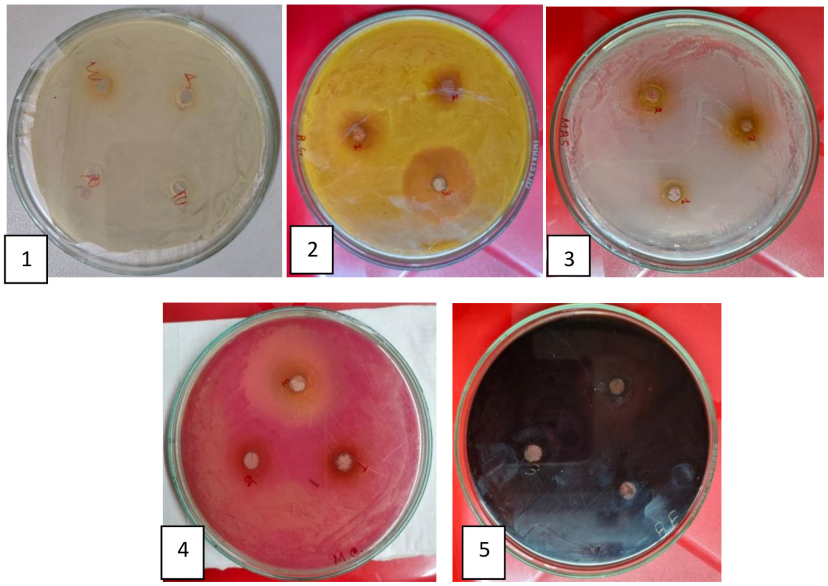

Figure 4: MIC of GL potentiality against five different gut microbiota (1: Lactic acid bacteria, 2: *Salmonella sp*., 3: Total aerobes, 4*: E. coli,* and 5: *Bifidobacterium sp*.) Gut microbiota. Three different concentrations of GL – 250, 500, and 1000 mg – were used.

supplemented group (B.1 and B.2). The cross-section shows no such significant difference, but villi were more numerous and in a regular position in the supplemented group, while the submucosa layer and muscular layer were the same in both groups (C.1 and C.2).

DISCUSSION

Current evidence indicates the critical role of the intestinal microbiota in supporting human health, suggesting a feasible strategy for developing new food products, such as functional foods, that can reshape the gut microbiota.[37] Bioactive compounds derived from natural sources for modulating the gut microbiota represent a promising, physiologically compatible approach. Consequently, the present study focused on the effect of GL on changes in the gut microbiota.

This study demonstrates that supplementing with GL can alter the composition of the human gut microbiota. GL 1000 mg/day supplementation results in a considerable rise in the number of *Bifidobacteria* sp. and lactic acid bacteria after 30 days. The 500 mg/day group also showed an increase in the overall lactic acid bacterial composition. In contrast to the previous study, which found only a few alterations following *Agaricus bisporus* consumption[38], the supplemented group in this study showed considerable modifications compared with the placebo and the pre-supplementary phase (0 days). The observed modulation of the gut microbiota may be attributed to triterpenoids from GL, biosynthesized via the mevalonate pathway, such as ganoderic acids. These compounds may support the growth of *Bifidobacterium* sp. *and* lactic acid bacteria by providing amino acids and vitamins. The nondigestible polysaccharide of GL functions as a probiotic, resisting upper gastrointestinal digestion and serving as a fermentable substrate to support beneficial gut flora. Previous discoveries imply that the gut microbiota can be modulated.[39] According to a previous study, *A. bisporus* polysaccharide has potent immune stimulatory and anti-tumor bioactivity both *in-vivo* and *in-vitro*.[40] Additionally, GL is abundant in polysaccharides that generate acetic acid and propionic acid, which lower colonic pH and create an environment that is unfavorable for *Salmonella* sp. and *E. coli* (Figure 8). *Salmonella*, total aerobes, and *E. coli* were not completely eradicated from the gut in the current investigation, despite a considerable drop in composition in the 1000 mg/day supplementation group after 30 days. Similarly, no trace of these harmful bacteria was observed

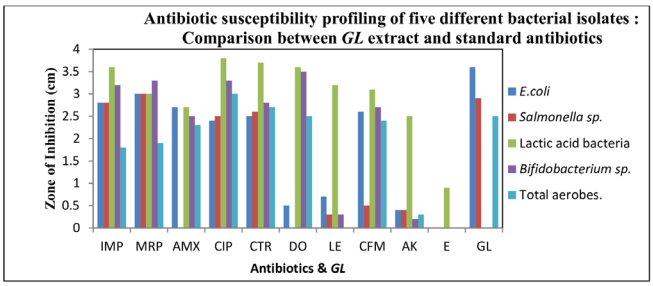

Figure 5: Doxycycline (DO) 30 µg is sensitive to Lactic acid bacteria and *Bifidobacterium* sp. and total aerobes. Levofloxacin (LE) 5 µg and Amikacin (AK) 30 µg are only sensitive to Lactic acid bacteria. Amoxicillin (Amx) 20 µg, Cefixime (CFM) 5 µg, and Ciprofloxacin (CIP) 5 µg are sensitive to Lactobacillus and Bifidobacterium sp., as well as total aerobes *and E. coli.* Ceftriaxone (CTR) at 30 µg is active against all five bacterial groups. Meropenem (MRP) 10 mcg and Imipenem (IPM) 10 mcg show sensitivity against *E. coli, Salmonella sp*., Lactic acid bacteria, and *Bifidobacterium* sp. Whereas Erythromycin (E) 15 µg did not show any sensitivity against the 5 different bacterial groups. GL 1000 mg/ml shows sensitivity against *E. coli,* total aerobes*, and Salmonella* sp.*,* and resistance to Lactic acid *bacteria* and *Bifidobacterium* sp. compared to antibiotics.

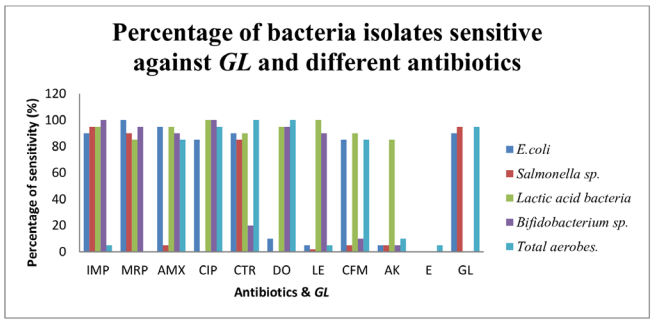

Figure 6: Percentage (%) of bacterial isolates sensitive to GL and various antibiotics.

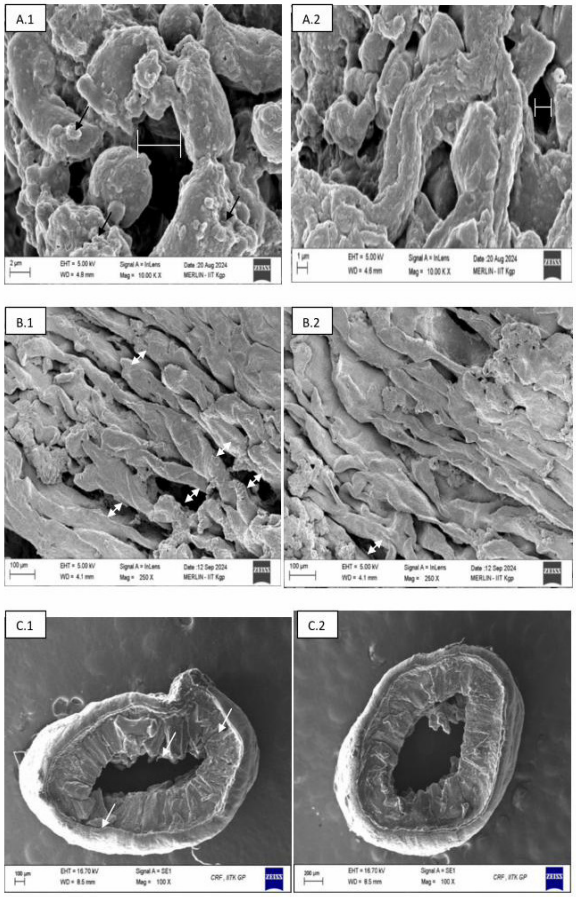

Figure 7: SEM study of the small intestine (rat) before and after GL supplementation. A.1, B.1, and C.1 figures represent the pre-supplementary phase, and A.2, B.2, and C.2 represent the post-supplementary phase. Cross-section, Villi morphology, Villi impactness, and regular enterocytes, as well as the presence of a mucus layer, were observed by SEM

in broiler chickens following supplementation with *Agaricus* sp. in a prior study.[41]

Gram-negative bacteria are categorized based on the color they take on following the application of a chemical technique known as Gram staining. Using this method causes Gram-negative bacteria to appear red. Only two of the five bacterial groups isolated from human fecal samples in this study, *E. coli* and *Salmonella* sp.*,* take on a red or pink hue when subjected to the gram-staining procedure; these were deemed to be gram-negative bacteria,[20;37,] which constitute a significant group of pathogens that impact a wide variety of hosts. Following gram staining, another three bacterial groups, *Bifidobacterium sp.* and total lactic acid bacteria, showed a violet color under a microscope. Gram stain indicated that some of the total aerobes were gram-negative, while others were gram-positive, consistent with a previous study.[42]

Previous studies have reported that *Lactobacillus* and *Bifidobacterium* sp. strains were short rods with regular ends that formed chains, whereas the total aerobes were spherical. Consistent with these findings, the present study also found similar morphological characteristics with positive gram reactions.[43] None of these five bacterial groups produced spores in the present study. Several biochemical assays further validated the identity of all isolated microorganisms. The synthesis of enzymes by all bacteria is significant. To test the presence of the tryptophanase enzyme, which produces indole from tryptophan, all the isolated bacteria indole production tests were performed, and *E. coli* [37] and total aerobes produced indole and appeared as a red-coloured ring at an upper layer, whereas in the case of *Salmonella* sp.[20], total lactic acid bacteria and *Bifidobacterium* sp.[44], a yellow-coloured ring appeared at the top and was referred to as an indole negative strain. To determine the presence of the urease enzyme, the isolated bacterial strains were tested, and only total aerobes produced ammonia from urea, thereby changing the phenol from red to pink color.[45] Whereas *E. coli, Salmonella* sp., *Bifidobacterium* sp.*, and* lactic acid bacteria appeared pale yellow in color due to the absence of the urease enzyme.[20,44,37] When five distinct

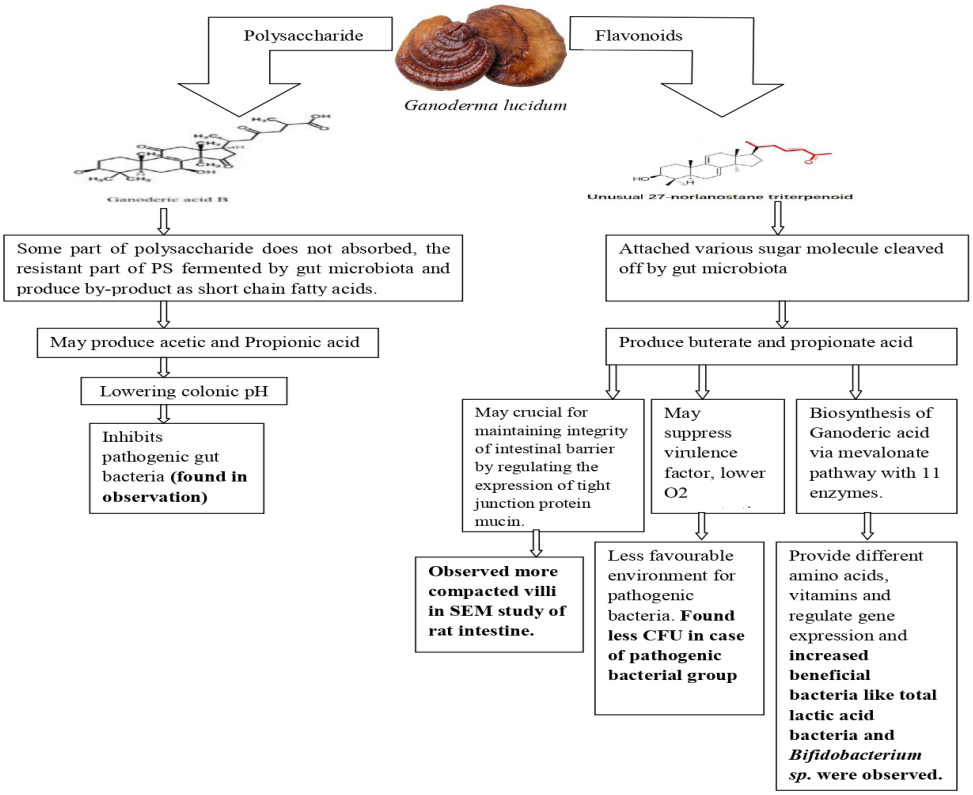

Figure 8: Probable Mode of action of polysaccharides and triterpenoids of GL on gut microbiota[58;59]

bacterial groups were treated using hydrogen peroxide, they generated the common antioxidant enzyme catalase. *E. coli, Salmonella* sp., and total aerobes all showed bubbling as soon as hydrogen peroxide was added[20,37,45], and these were regarded as catalase positive. Lactic acid bacteria and *Bifidobacterium* sp. are known as catalase-negative because they do not form bubbles.[44] It evaluates an organism's capacity to ferment glucose and break down pyruvic acid, the by-product of glycolysis, into gaseous by-products. Since all of the isolated fecal bacteria in this investigation were glucose-positive, similar to a previous study[20,37,44,45], it can be concluded that, because glucose fermentation is advantageous for both digestion and energy production, these bacteria are beneficial. This study found that only total aerobes can convert citrate to pyruvate.[45] After that, pyruvate can enter the organism's metabolic cycle and produce energy. The presence of a catalase-permease indicated that growth consumed citrate, an intermediate metabolite in the Krebs cycle; however, the other four isolated bacteria did not exhibit catalase activity.[20,37,44] Since all organisms in this study remained red in the media, none of the sugar was used by the microorganisms. [20,37,44,45]

Increased DNA yield indicates that PCR successfully amplified DNA from five separate groups of bacterial isolates from the fecal sample. Additionally, the UV illuminator observation showed that the DNA band in the gel was wider following the use of a 'universal' primer for the identification of certain bacteria than it was before PCR.[46;47;48] The DNA extraction and PCR product are critical for the ultimate characterization of bacteria isolated from human feces. As in this study, following PCR, the concentrations of the five bacterial groups significantly ($p < 0.0001$) increased, confirming bacterial identification.

GL's antimicrobial activity against both Gram-positive and Gram-negative bacteria was demonstrated in this study. At a minimum concentration of 1000 mg/mL, GL's antimicrobial activity against *E. coli*, *Salmonella* sp., and total aerobes was the most notable among the five bacterial groups.[49]

The antimicrobial assay demonstrated that the GL extract exhibited variable inhibitory activity against the tested bacterial groups. Among the tested strains, *E. coli, Salmonella* sp., and total aerobes showed a prominent zone of inhibition when treated with 1000 mg/mL, and the ZOI is closely related to the standards sensitive antibiotics ZOI, indicating strong antimicrobial activity and implying that mushroom terpenoids and polysaccharides interfere with bacterial cell wall or membrane integrity, which aligns with previous reports showing ethanol and methanol extracts of GL showing inhibition to pathogens such as *E. coli* and *Salmonella* sp.[50] In contrast, *Bifidobacterium* sp. and lactic acid bacteria had no susceptibility, indicating potential resistance. To date, the literature[51] lacks evidence indicating that GL exhibits resistance to lactic acid bacteria and *Bifidobacterium* sp. in antibiotic susceptibility tests. Instead, the literature

predominantly demonstrates that GL extracts either spare or promote these probiotic genera. Our observation, showing zero inhibition of probiotic isolates under standard antibiotic testing conditions, thus represents a novel observation. These findings emphasize the mushroom's potential as a natural antibacterial agent, but the dose is higher than that of commercial antibiotics. The antimicrobial assessment revealed that GL exhibited inhibitory effects of similar magnitude to those of standard antibiotics, suggesting it may serve as a natural alternative for controlling bacterial growth. In our study, several isolates exhibited multidrug resistance. The findings indicate that GL possesses selective antimicrobial properties, demonstrating sensitivity to 90 to 95% of pathogenic gut bacterial isolates, comparable to that observed with standard antibiotics. Notably, unlike most conventional antibiotics, which also affect beneficial gut microbiota, GL showed no sensitivity to 0% of isolates from beneficial bacterial groups. This suggests its potential for targeted modulation of gut microbiota with minimal disruption to commensal populations. As previously reported, GL may therefore be a restorative medication that allows for a large dosage reduction of harmful antimicrobial drugs without compromising antimicrobial action.[52]

The present investigation found that the concentration of the lactic acid bacterial group was higher in the GL-treated group than in the placebo group, and an earlier report has shown that Lactobacilli are the most advantageous strains. *Lactobacilli* may interact with enterocytes, influencing the immune system and strengthening the intestinal barrier, according to earlier research.[53] In this study, the GL-supplemented group outperformed the placebo group in terms of animal intestinal morphology. By regulating the expression of the tight-junction protein mucin, GL triterpenes, which also generate propionic and butyric acids, are essential for preserving intestinal barrier integrity (Figure 8). Nutrient absorption, hormone and enzyme release, and ongoing epithelium regeneration are all facilitated by the intestinal mucosa's vast surface area.[54] Compared to samples from the control group, the acellular matrix in the GL-treated group displayed a collagen fiber network that was more firmly compressed. As in previous studies, the intestinal villi in this investigation were reported to have finger-like protrusions.[55] The small intestine is the site of the majority of chemical digestion and a major site of nutrient absorption.[56] When compared to the control group, the GL group's small intestine tissue had more epithelial lining of villi throughout. Diet can change the small intestine's anatomy, according to several studies. There is evidence that a diet rich in fiber increases the number of villi. It was discovered that this included the growth of mucosal epithelial cells. It has also been demonstrated that nutrients impact gut structure.[57]

CONCLUSION

There is growing evidence that GL improves gut microbial complexity, suggesting that it may be more beneficial to focus on eating a varied, healthful diet. This study showed that GL can both reduce the proportion of harmful germs and increase the proportion of beneficial bacteria. These findings point to the potential application of GL as a nutritious nutraceutical. The present research also found that the antimicrobial components of the basidiomycetous fungus GL appeared to be a potential antibiotic compared with current commercial antibiotics. GL selectively resists pathogenic gut microbiota and is resistant to lactic acid bacteria and *the Bifidobacterium* sp. bacterial group; hence, it can shape the gut microbiota, adding beneficial activity as a natural antimicrobial agent with minimal impact on the probiotic gut microbiota. The outcomes of this study can be summarised as follows: GL is a multipurpose medicinal mushroom with a broad spectrum of beneficial activities. The study's main focus was GL's ability to alter gut microbial diversity. We have a long way to go before fully comprehending the intricacies of the microbiota and how microbes affect human health. To support the health claims of commercially marketed health supplements, far more thorough clinical testing will be needed. Some studies have shown the anticancer and anti-inflammatory properties of GL ethanol extract in animal models. This article discusses the unknown portion of GL, which has a regulatory impact on human gut microbiota. In today's world, characterized by widespread disease, humans depend on medication, although the overuse of drugs leads to resistance against numerous pharmaceuticals. A simple technique to get fit and healthy by ingesting natural products. This study demonstrates one of the most effective ways to stay healthy: simply modulating the gut microbiota with natural products that have no side effects. This study focuses on only five bacterial groups among the trillions of gut microbiota, including both beneficial and pathogenic groups that regulate several physiological processes. This study also discovered that GL can protect the intestinal lining from oxidative damage while maintaining intestinal structure, thereby increasing the number of beneficial bacteria in the gut microbiome and providing a healthy gut environment, both of which directly affect human physiological functions. In the future, GL can be used as a nutraceutical to improve health by modifying the gut microbiota. Further investigation at the molecular level is required to better understand the mode of action.

CONFLICT OF INTEREST

There are no disclosed conflicts of interest for the authors.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.