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Brewers' spent grain as fish feed ingredient: Evaluation of bio-safety and analysis of its impact on gut bacteria of *Cirrhinus reba* by 16S Metagenomic sequencing

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ABSTRACT

A comprehensive eight week feeding trial was conducted to investigate the potential of brewers' spent grain (BSG) as a sustainable fish feed ingredient. The study assessed both the biosafety of BSG and its impact on the gut microbiome of *Cirrhinus reba*, utilizing advanced 16S metagenomic sequencing techniques to analyze the composition and diversity of gut bacteria. A total of 90 healthy *C. reba* juveniles (average weight: 12 ± 1 g) were divided into two dietary groups [for control (C), for BSG meal (tB)] in triplicates. Feed prepared with conventional ingredients was used to feed the control group (C). The group tB was fed with BSG meal. After the feeding trial, the fish in tB group showed significantly higher ($p < 0.05$) growth parameters as compared to the control group. The results of bio-safety assessment indicated the absence of any pathological symptoms in the BSG meal fed carps. The fish in tB group didn't show any histopathological abnormality. Fish fed the Brewers' Spent Grain exhibited significantly elevated serum biochemical parameters, including alanine transaminase (ALT) and aspartate transaminase (AST), compared to the control group ($p < 0.05$). 16S Metagenomic sequencing of the fish gut microbiota provides insights into how BSG inclusion affects microbial diversity and composition within the digestive tract of *C. reba*. The analysis revealed the existence of 240 and 250 diverse bacterial genera in the gastrointestinal tract (GIT) of *C. reba* in dietary groups C and tB respectively. Importantly, the study found the gut of fish in tB group to be dominated by different beneficial genus including *Bacillus, Lactobacillus, Bifidobacterium, Paenibacillus,* and *Lysinibacillus*. Feeding *C. reba* with BSG meal significantly increased the alpha diversity of the gastrointestinal microbiota, as evidenced by elevated Chao 1 estimator and Shannon index values compared to the control diet ($p < 0.05$). This study provides comprehensive evidence for the bio-safety of BSG as a sustainable feed ingredient in aquaculture, demonstrating its potential to support healthy fish growth and development. Moreover, the prebiotic potential of BSG in fish has also been highlighted.

1. Introduction

In recent decades, the global demand for fish feed has surged drastically; leading to a sharp and sustained escalation in the prices of traditional fish feed products. Hossain et al. (2022) highlighted the essential role of fish feed in freshwater carp farming, indicating that it represents 57.3 % of the operating costs. Naz and Javed (2013) emphasized the crucial importance of protein in fish feed for optimal growth and health, highlighting its significance in aquaculture. However, the reliance on traditional protein sources such as fish meal and soybean meal in conventional fish feeds poses dual challenges. These ingredients not only contribute to higher production costs but also raise environmental concerns, highlighting the need for eco-friendly alternatives. Fish meal production heavily relies on pelagic fish stocks,

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raising sustainability issues (Luo et al., 2012). Similarly, utilizing soybean meal in aquatic feeds not only escalates production costs but also renders a valuable protein source away from human consumption (Jayant et al., 2018). Moreover, both fish meal and soybean meal have high carbon footprints, further complicating their environmental impact (Chang et al., 2017).

In response to these challenges, there is a growing global effort on finding sustainable cost-effective alternative protein sources for fish feed formulations. Brewer's spent grain (BSG), a proteinaceous by-product of the brewing industry, presents a promising candidate for sustainable use, with approximately 38.6 million tons generated annually worldwide, highlighting the significant concern for its disposal (Mussatto, 2014). The nutritional abundance of BSG has made it suitable for various animal feeds, including poultry, cows, and pigs (Eliopoulos et al., 2022).

In recent years, researchers have explored the potential of BSG as a novel protein source in aquaculture feeds. Studies have demonstrated that BSG can effectively replace traditional protein sources like soybean meal in fish diets, thereby reducing reliance on fish meal and mitigating the associated environmental footprint (Gokulakrishnan et al., 2022). Previous studies have documented the effective substitution of soybean meal with BSG in fish feeds, resulting in improved growth performance and feed digestibility in various fish species (Estévez et al., 2021; Chattaraj et al., 2024a). BSG typically contains around 47.26 % crude protein, comparable to soybean meal, along with other essential nutrients (Jayant et al., 2018). Hence, biotechnological implementation of this waste product as fish feed will be in line with the sustainable waste management (Janeeshma et al., 2023). Metagenomic analysis of fish intestines post-feeding with BSG is crucial for understanding the impact of this feed on gut microbiota. By exploring into the microbial community composition and functional potential, researchers can assess how this feed alters microbial diversity, enzymatic activity, and nutrient metabolism. This analysis helps in optimizing feed formulations for improved fish health and growth. It also provides insights into the sustainability and environmental benefits of using brewer's spent grains as a feed ingredient, making it an ultimate tool for both aquaculture innovation and ecological management.

The nutritional requirements and feeding habits of fish species like *Cirrhinus reba* (Hamilton, 1822) further highlight the need for sustainable and nutritious diets (Sarkar et al., 2022). *C. reba*, valued for its taste and nutritional content, is facing population declines due to diseases and overharvesting (Afroz and Begum, 2014; Gupta and Banerjee, 2016; Chattaraj et al., 2023a). Ensuring optimal through well-formulated feeds that meet specific protein and lipid requirements is crucial for conserving and sustainably managing these vulnerable fish populations. Ultimately, the success of alternative protein sources like BSG in fish feed formulations hinges on their ability to support growth, and improve overall fish health. Continued research into optimal inclusion levels, nutritional adequacy, and environmental impact will be pivotal in advancing sustainable aquaculture practices worldwide. The adoption of innovative protein sources like BSG represent a promising avenue for the future of aquaculture feed formulations by reducing reliance on costly, resource-intensive and environmentally taxing conventional protein sources, such as fish meal and soybean meal. Lao et al. (2020) identified brewers' spent grain (BSG) as a promising prebiotic, yet there is a notable absence of literature on the biosafety of BSG in fish. Additionally, the specific effects of BSG on gut microbiota modifications in fish have not been thoroughly investigated. Therefore, the present study seeks to address these scientific gaps by employing *C. reba* as the experimental model. This research aims to provide comprehensive insights into the biosafety aspects of BSG and its impact on the gut microbiota of *C. reba*, thereby contributing valuable knowledge to the field of aquatic nutrition and health management.

2. Materials and methods

2.1. Sample collection and processing

Brewers' spent grains (BSG) were procured from Yuksom Breweries Ltd. BSG were thoroughly rinsed with distilled water and subsequently grounded to fine powder to facilitate their incorporation into fish feed formulations. Fish meal and soybean were utilized as the conventional protein sources of the fish feed. Wheat meal, yellow corn flour and wheat bran were utilized as the basic carbohydrate source (Chattaraj et al., 2024a). The particular waypoint of fish sample collection has been recorded using handheld GPS device (Garmin e-Trex 10) (Suleiman et al., 2024). The GPS data is exported to Google Earth Pro for processing. For preparing the location map of the sample collection point and its surroundings, Google Earth Pro image of the area has been digitized in Arc GIS platform and layout is prepared. The individual categories of land use and land cover has been visually identified and digitized.

2.2. Experiment set up for biosafety assessment of BSG on C. reba

The *in vivo* biosafety assay of the BSG meal was executed by following the method of Abdel-Tawwab et al. (2020) with few modifications. One hundred healthy juvenile *C. reba* (average weight: 12 ± 1 g) were obtained from the sampling region (25°37′09.67″N latitude and 88°06′54.64″E longitude) and transported to the laboratory in oxygenated plastic bags filled with one-third water, ensuring minimal stress and optimal survival during transit. The fish underwent a 15-day acclimatization period in an aquarium setting, during which they were fed the control diet. After that, they were divided into two groups [for control (C), for BSG meal (tB)] having three replicates each and each set up had fifteen fish. All the fish were fed at 2 % body weight twice daily (9:00 and 16:00 hrs) for eight weeks. Feed prepared with conventional ingredients was used to feed the control group (C). The group tB was fed with BSG meal (Table 1). The feeding trials were conducted for 56 days (excluding acclimatization period) in glass aquariums (76 cm × 30 cm × 30 cm) provided with external aeration for 18 h and under 12 h of light and 12 h of darkness. The aquariums were supplied with 70 L of flowing water, with 15 L regularly replaced with fresh water. Routine cleaning and siphoning were conducted to remove uneaten debris and fecal waste matter. Water quality parameters were meticulously monitored and maintained within specified limits following standard protocol (Chattaraj et al. 2024a; Ganguly et al. 2018a). At the 57th day, fish in all the dietary groups were observed for any alteration in overall health parameters.

Table 1
Formulated feed with BSG meal as a substitute of soybean meal.

Ingredients (in gm)	Control meal (C)	BSG meal (tB)
Fish meal	5	5
Soybean meal	0	42.7
BSG meal	48.5	0
Wheat flour	16.11	18.14
Yellow corn	13.37	15.23
Wheat bran	12.02	13.93
Sunflower oil	3	3
Vitamin mineral mixture	2	2
	Nutrient analysis (%)[a]	
Crude protein	30.34	30.12
Fat	6.12	6.69
Crude Fiber	8.22	9.54
Ash	7.23	7.46
NFE[b]	48.09	46.19
Gross energy (MJ.Kg $^{-1}$)[c]	19.26	19.34

[a] Nutrient analysis has been performed on dry matter basis.
[b] NFE: dry matter (100) – (crude protein + fat + ash + crude fiber).
[c] Gross energy was evaluated according to gross caloric values of 23.6 kJ/g (for protein), 39.5 kJ/g (for fat), and 17.2 kJ/g (for carbohydrate).

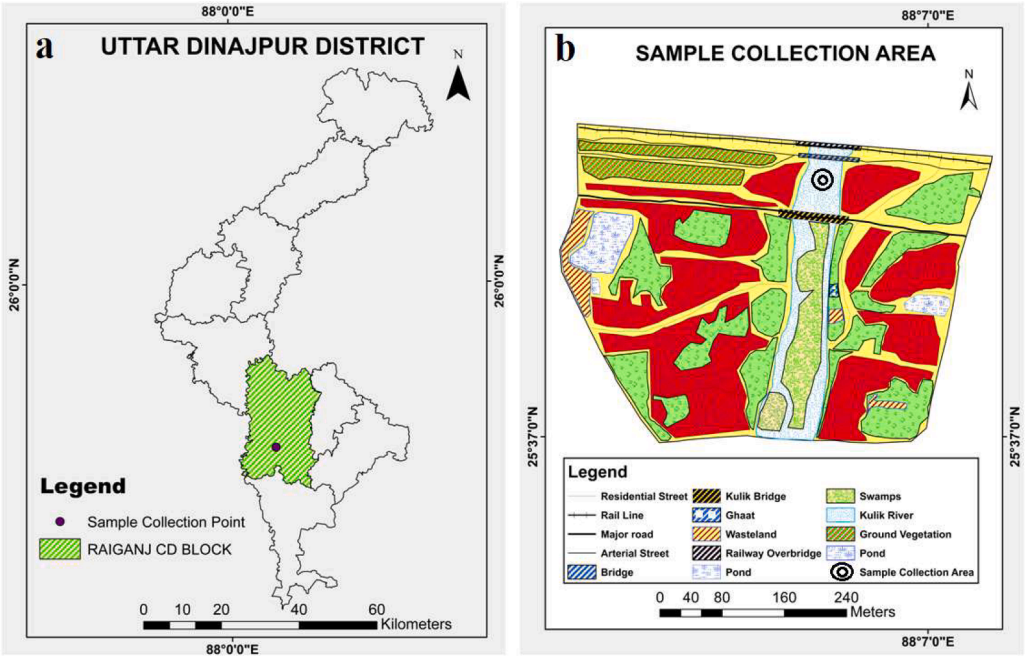

Fig. 1. Collection region of *C. reba*. a. stretch of Kulik River in point conjuncting at 25°37′09.67″N latitude and 88°06′54.64″E longitude, b. Land use land cover map of the sampling area.

2.3. Data analysis

Following parameters of fish growth are evaluated for assessing the dietary performances of the fishes (Ganguly et al., 2024).

$$Live\ weight\ gain\ (LWG):\ Final\ wt. - Initial\ wt.$$

$$Average\ Daily\ Growth\ (ADG):\ LWG/Duration\ of\ trial(days)$$

$$Feed\ Conversion\ Ratio\ (FCR):\ Feed\ intake\ /LWG$$

$$Protein\ Efficiency\ Ratio\ (PER):\ LWG\ /Protein\ intake$$

$$Specific\ Growth\ Rate\ (SGR):[\{ln(Final\ wt.) - ln(Initial\ wt.)\} / Duration\ of\ trial] \times 100$$

$$Survival\ Rate\ (SR):(Final\ number / Initial\ number) \times 100$$

$$Hepatosomatic\ Index\ (HSI\%) = [liver\ weight\ (g) / whole\ body\ weight\ (g)] \times 100$$

2.4. Serum biochemical parameters

Serum biochemical analyses were conducted on carp from both dietary groups. Nine fish from each group were randomly selected for blood collection *via* caudal vessel puncture without anticoagulants. Serum was obtained by centrifuging coagulated blood at 3000 g for 15 min. Total protein, albumin, globulin, nitric oxide, creatinine, and urea levels were determined using standard methods. Serum AST, alkaline phosphatase (ALP), and ALT activities were assessed using kinetic assays, following protocols outlined by Lavanya et al. (2011), Fadl et al. (2020), and Kathak et al. (2022).

Table 2
Growth indices and feed efficiency of *C. reba* in the dietary groups after trial.

Parameters	Control meal (C)	BSG meal (tB)
Initial wt	12.66 ± 1.06	12.27 ± 1.18
Final wt	17.68 ± 1.83	18.04 ± 1.42
LWG	5.02 ± 0.26^{b}	5.77 ± 0.48^{a}
ADG	0.9 ± 0.001^{b}	0.103 ± 0.001^{a}
FCR	1.92 ± 0.002^{a}	1.67 ± 0.003^{b}
PER	2.87 ± 0.01^{b}	2.92 ± 0.01^{a}
SGR	2.07 ± 0.004^{b}	2.14 ± 0.01^{a}
HSI	1.36 ± 0.02	1.33 ± 0.01
Survivability (%)	98.46 ± 1.2	98.07 ± 1.38

Data represented as Mean ± SD, $n = 3$; the mean values in the same row having different superscript vary significantly ($p < 0.05$).

2.5. Histopathology analysis

For the purpose of histopathological examination, internal tissues such as intestine, kidney and liver were collected from fish of both the groups and cut into 1–2 mm sections. These tissues were immersed in 10 % neutral buffered formalin (NBF) solution and fixed for 24 h. Subsequently, the tissues underwent dehydration using different concentrations of alcohol, followed by washing with xylene. They were then embedded in paraffin wax and left to solidify overnight. Once solidified, the tissues were sectioned to a thickness of 3 μm using a microtome and placed on clean glass slides coated with egg albumin. Haematoxylin and eosin stains were used for staining the sections. Pathological changes were examined under microscope (Magnus Opto Systems India Pvt. Ltd., MLXi-TR Plus 18E0608), and relevant photographs were taken (Behera et al., 2022).

2.6. 16S Metagenomic analysis of the gut microbiota after feeding trial

2.6.1. DNA extraction

Three fish from each group were selected for gut bacteria analysis. DNA was extracted using the gDNA Xploregen soil kit according to the manufacturer's guidelines. After extraction, the quality of the genomic DNA was assessed using Nano Drop and 0.8 % agarose gel electrophoresis (110 V for 35 min) prior to PCR amplification. The DNA samples were visualized using a GeNei™ transilluminator (Chattaraj et al., 2024b).

2.6.2. PCR based amplifying V3-V4 region of 16SGene

The hypervariable region (V3–V4) of the 16S rRNA encoding gene

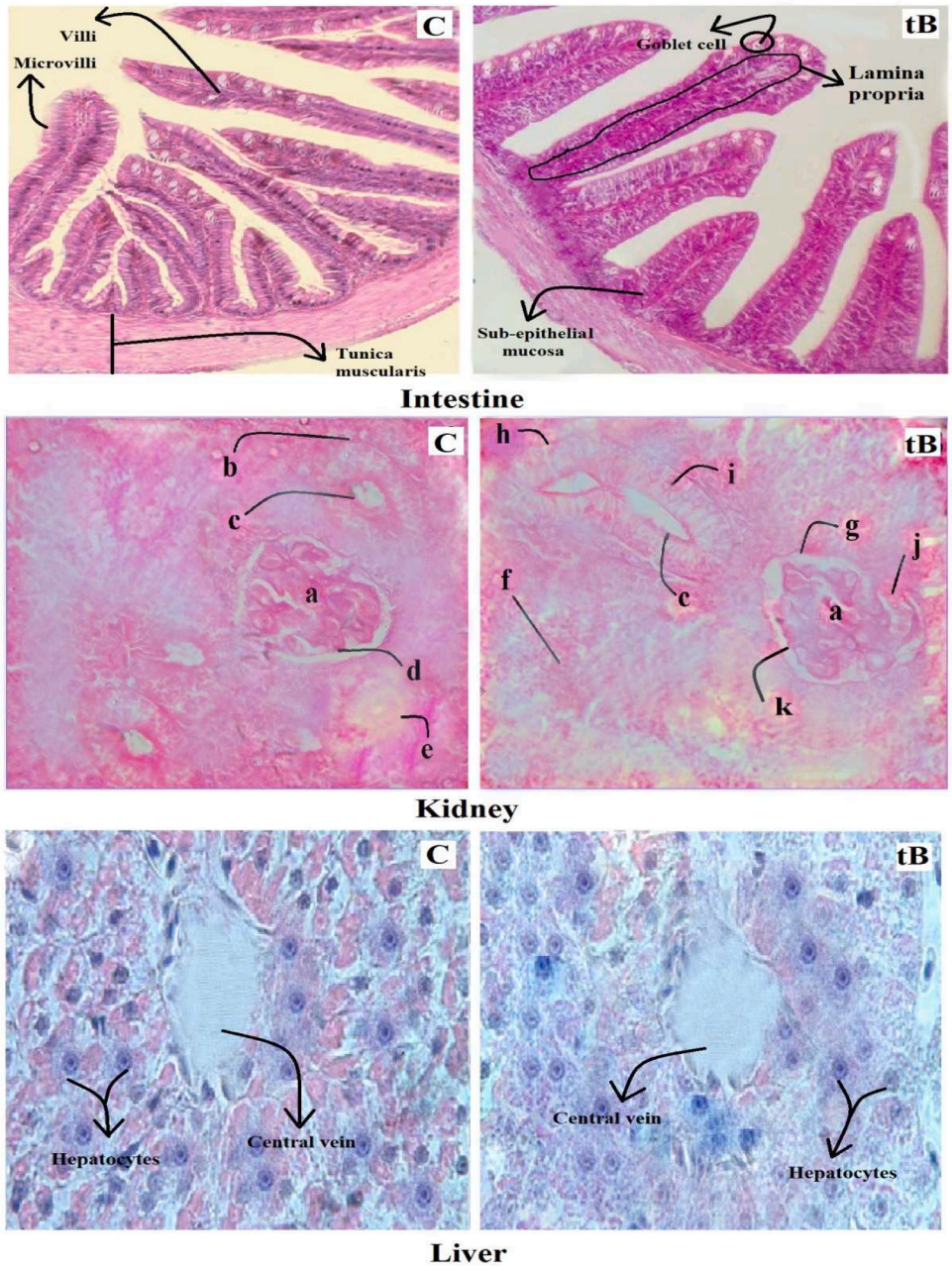

Fig. 2. Histopathology of the intestine, kidney and liver of *C. reba*. C (Control meal), tB (Treatment with BSG meal). a. well-vascularized glomeruli, b. TS of proximal convoluted tubule, c. microvilli that form the brush border extending into the lumen, d. prominent internal stratum of epithelial cells (podocytes), e. renal tubule, f. haemopoietic tissue, g. healthy parietal layer, h. proximal convoluted tubule (LS), i. epithelial cells, j. well network of capillaries, k. Bowman's space.

was amplified using V34F (5′-AGAGTTTGATGMTGGCTCAG-3′) and V34R (5′-TTACCGCGGCMGCSGGCAC-3′) primers. 40 ng of extracted DNA and 10pM of each primer were used for amplification. The amplification was carried out using TAQ Master MIX, which includes High-Fidelity DNA Polymerase, 0.5 mM dNTPs, 3.2 mM $MgCl_2$, and PCR Enzyme Buffer, in the AriaMx Real-Time PCR System (Agilent Technologies). The final reaction volume was 25 μL, and PCR was conducted over 26 cycles. The thermal cycling conditions included initial denaturation at 95 °C for 15 s, followed by annealing at 60 °C for 15 s, and elongation at 72 °C for 2 min. The integrity of the DNA throughout the process was assessed by agarose gel electrophoresis and quantification of double-stranded DNA concentration. After PCR, samples containing the PCR products were stored at -20 °C until further analysis. Prior to sequencing, the amplicon-containing samples were pooled together (Chattaraj et al., 2024b).

2.6.3. *Sequencing and data processing*

AMPure beads were used to eliminate unutilized primers to purify the amplicons from each sample. Eight more cycles of PCR were conducted using Illumina barcoded adapters to prepare sequencing

Table 3
Assessment of *in vivo* toxicity of BSG meal in *C. reba*.

Parameters	Control meal (C)	BSG meal (tB)
	Pathological symptoms	
Lesion/ abrasion	No	No
Hemorrhage	No	No
Edema	No	No
Loss of scale	No	No
Loss of mucus	No	No
Ataxia	No	No
Petechiae	No	No
	Tail and fin rot	
Dorsal	Normal	Normal
Caudal	Normal	Normal
Pectoral	Normal	Normal
Anal	Normal	Normal
Pelvic	Normal	Normal
	Swimming behavior	
Whirling	No	No
Side swimming	No	No
Erratic swimming	No	No
Lethargic swimming	No	No
	Abdomen, eyes and respiration rate	
Distended abdomen	No	No
Exophthalmos	No	No
Debris from anal orifice	No	No
Opercular respiration rate	62 ± 2.14	63 ± 2.05
	color	
Darkening body	No	No
Bluish body	No	No
Pale body	No	No
Grayish white spots	Absent	Absent
Skin erosion	Healthy skin	Healthy skin
Gill color	Red	Red
	Nutritional parameters	
Feeding activity	Normal	Normal
	(2 % of B/W)	(2 % of B/W)
Anorexia	NO	NO
	Post sacrifice analysis	
Intestine	Normal	Normal
Liver	Normal	Normal
Swim bladder	Normal	Normal
Gall bladder	Normal	Normal
Kidney	Normal	Normal
Gill	Normal	Normal
Ascites	NO	NO

NO- Not Observed; Normal- Healthy appearance and abnormalities not observed; B/W- Body Weight.

Table 4
Serum biochemical parameters of carp fed the control and experimental diets.

Serum biochemical parameters	Control meal (C)	BSG meal (tB)
TP (g/dl)	5.04 ± 0.69	5.23 ± 0.42
Albumin (g/dl)	4.51 ± 0.44	4.64 ± 0.71
Globulin (g/dl)	0.53 ± 0.26	0.59 ± 0.17
Creatinine (mg/dl)	1.89 ± 0.08	1.82 ± 0.04
Urea (mg/dl)	5.08 ± 0.13	5.19 ± 0.17
ALT (IU/L)	28.64 ± 0.37^{a}	27.15 ± 0.33^{b}
ALP (IU/L)	20.04 ± 0.16	19.97 ± 0.29
AST (IU/L)	77.14 ± 0.37^{a}	75.02 ± 0.18^{b}
Nitric oxide (μmol/L)	55.17 ± 0.4	55.52 ± 0.31

Data represented as Mean ± SD, $n = 3$; the mean values of the same row with different superscript vary significantly ($p < 0.05$).

libraries. Subsequently, the libraries were purified with AMPure beads and quantified using the Qubit dsDNA High Sensitivity assay kit (Chattaraj et al., 2024b). Sequencing was carried out on an Illumina Miseq platform using the 2 × 300PE V3-V4 sequencing kit. The raw bcl data obtained from the sequencer was de-multiplexed into .fastq format. TrimGalore (v0.6.0) (Kim et al., 2022) was employed for trimming adapters and filtering low-quality reads. FastQC (Version 0.11.9) (Andrews, 2010) and MultiQC (Version 1.10.1) (Ewels et al., 2016) were used to verify, visualize, and assess the quality of the raw fastq files. The trimmed reads were further processed, including merging of paired end reads, removal of chimeras, and calculation of Operational Taxonomic Unit (OTU) abundance and estimation correction using QIIME 2 (Bolyen et al., 2019) and KRAKEN (Lu et al., 2022) workflows. Amplicon sequence variants (ASVs) and their abundances were determined. Taxonomies were assigned to the generated ASVs using the weighted Naive Bayes classifier with the 16S rRNA silva 138 SILVA SSU gene database, implemented through the q2-feature-classifier plugin (Wilczynski et al., 2022), ensuring classification based on over 99 % coverage and identity. Alpha diversity analysis was conducted using Microbiomeanalyst (available online at https://www.microbiomeanalyst.ca/).

2.7. Statistical analysis

Statistical analyses utilized a significance level of $p < 0.05$, with data expressed as mean ± SD. Normality and homogeneity were assessed via Shapiro-Wilk and Levene's tests. ANOVA followed by Dunnett's test in GraphPad Prism 10.0.0.153 was employed. Mann–Whitney U Test compared values at $p < 0.05$. Confidence intervals for ASV mean differences used bootstrap resampling (1000 iterations), identifying significant effects if intervals excluded zero.

3. Result

3.1. Description of the fish collection area

The sample of fish has been collected from the stretch of Kulik River in point conjuncting at 25°37′09.67″N latitude and 88°06′54.64″E longitude (Fig. 1a). Kulik is a small stream draining through southern parts of Uttar Dinajpur district of West Bengal crossing the Raiganj Community Development Block, meets Nagar and finally drains into the Mahananda River. In the study area, the riparian fauna and swamps acted as natural absorbent of pollutants, and the algal blooms in the swamps provide essential food for fish growth (Fig. 1b). The construction of concrete revetment and riprap on either side of the bank of the river has prevented sedimentation, which is evident from the mud-free gills of the fish samples collected. The revetments also prevent the inflow of any runoff to the focused stretch of the river, providing a pollution free environment for healthy fish population.

3.2. Growth parameters of the carp

The BSG meal fed group (tB) exposed better growth performance as compared to the control group (Table 2). Fish in group tB revealed significant higher ($p < 0.05$) LWG, ADG, PER and SGR than the control group. BSG meal fed fish also showed significantly lower ($p < 0.05$) FCR. The hepatosomatic index was non-significant altered ($p > 0.05$) among the control and tB groups. A good percentage of survivability was observed in both the dietary groups.

3.3. Biosafety assessment of BSG meal on C. reba

The assessment of biosafety revealed that BSG meal didn't possess any toxicity in the experimental carp *C. reba*. Histopathological analysis of the intestine of the treatment group showed no significant alteration in the architecture of tunica muscularis, villi, microvilli, goblet cells, lamina propria etc. as compared to the control one (Fig. 2). The histopathological architecture of the kidney was also healthy in the treatment groups. Prominent bowman's space, proximal convoluted tubule, podocytes, renal tubule, haemopoietic tissue, parietal layer etc. were observed (Fig. 2). Again, the liver histopathology showed healthy hepatocytes and the central vein was prominent in all the groups (Fig. 2). The other parameters of bio-safety assessments are shown in Table 3.

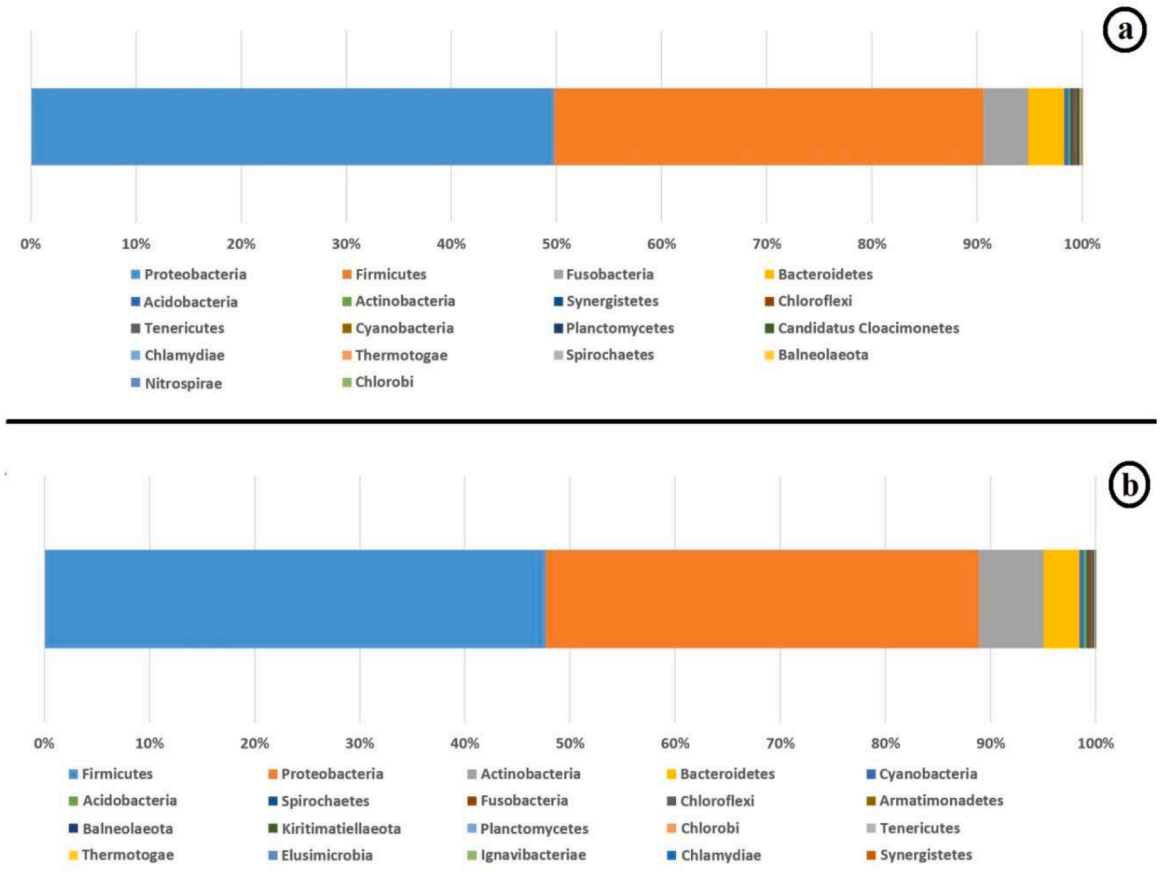

Fig. 3. Dominant bacterial phylum in the GIT of *C. reba* fed **a.** Control feed. **b.** BSG meal.

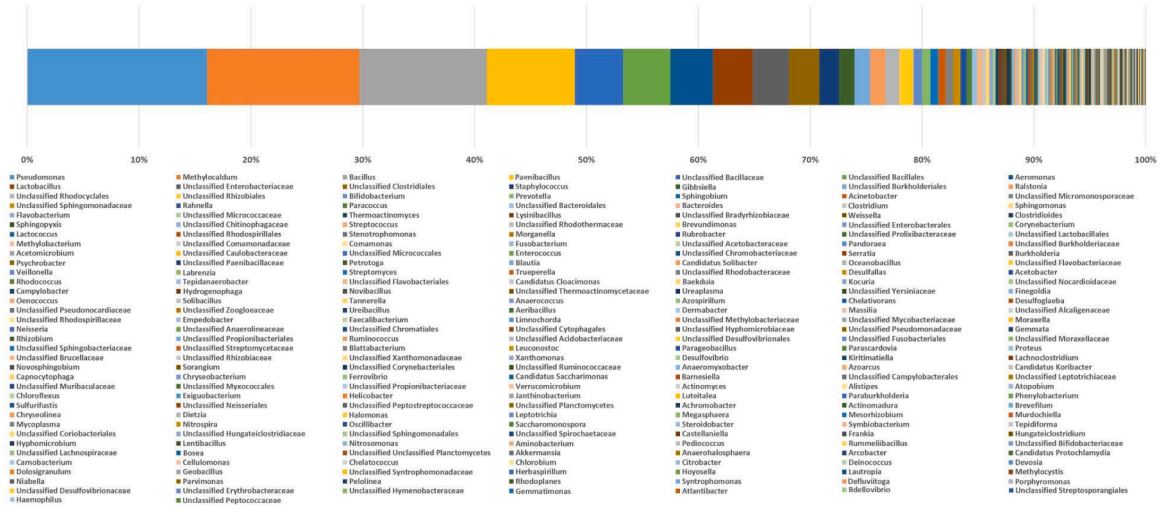

Fig. 4. Dominant bacterial genera in the GIT of *C. reba* fed control feed.

3.4. Serum biochemical parameters

The serum biochemical parameters got altered after dietary treatment of the fish with the BSG incorporated diet (tB) (Table 4). The tB group revealed non-significantly ($p > 0.05$) higher content of total protein, albumin, and globulin than the control group. Content of serum ALT and AST was significantly higher ($p < 0.05$) in the control group than the fish fed BSG diet. No significant ($p > 0.05$) alteration in the level of serum urea, creatinine, ALP and nitric oxide had been found in the groups.

3.5. 16S Metagenomic analysis of the carp intestine

At the phylum level, the GIT of the fish fed control meal showed dominance of *Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, Acidobacteria, Actinobacteria, Synergistetes, Chloroflexi, Tenericutes, Cyanobacteria* (Fig. 3a). However, *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Acidobacteria, Spirochaetes, Fusobacteria, Chloroflexi,* and *Armatimonadetes* are found to dominate the GIT of BSG fed fish (Fig. 3b).

At the genus level, the GIT of the fish fed control meal showed

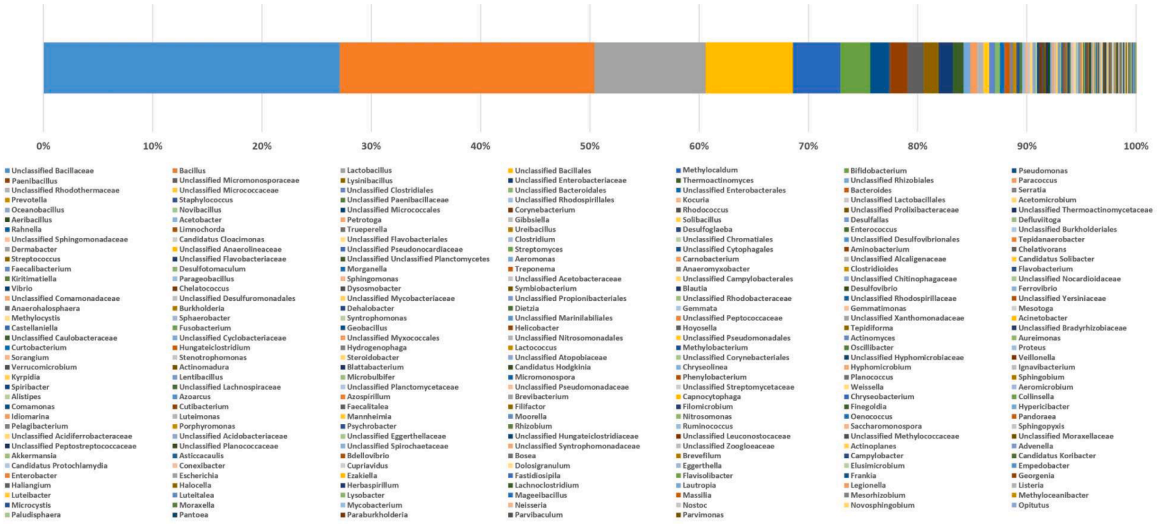

Fig. 5. Dominant bacterial genera in the GIT of *C. reba* fed BSG meal.

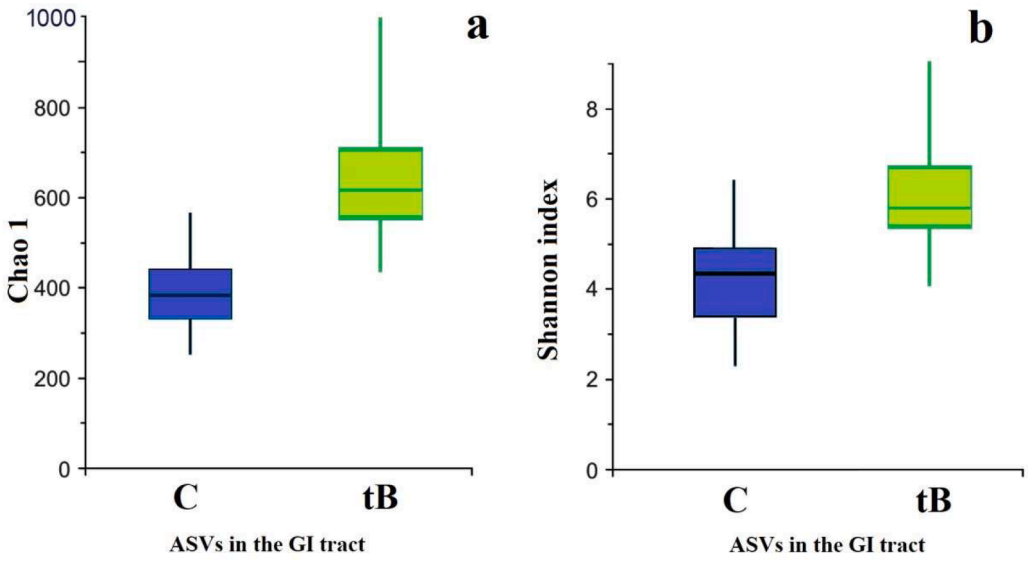

Fig. 6. Box-plot. a Chao 1 estimator. b Shannon index on the basis of the quantity and the proportional abundances of ASVs found in the gastrointestinal microbiota of *C. reba* in C and tB groups.

dominance of *Pseudomonas, Methylocaldum, Bacillus, Paenibacillus, Unclassified Bacillaceae, Unclassified Bacillales, Aeromonas, Lactobacillus, Unclassified Enterobacteriaceae,* and *Unclassified Clostridiales* (Fig. 4). However, *Unclassified Bacillaceae, Bacillus, Lactobacillus, Unclassified Bacillales, Methylocaldum, Bifidobacterium, Pseudomonas, Paenibacillus, Unclassified Micromonosporaceae,* and *Lysinibacillus* are found to dominate the GIT of BSG fed fish (Fig. 5).

3.5.1. α-diversity

The alpha diversity of the gastrointestinal microbiota in *C. reba* fed BSG meal was notably elevated in comparison to those fed the control diet. This was indicated by significant differences observed in both the Chao 1 estimator (Fig. 6a, U-12, Z-3.36, $p < 0.05$) and Shannon index (Fig. 6b, U-22, Z-2.14, $p < 0.05$).

4. Discussion

In this study, the biosafety of BSG was evaluated by replacing soybean meal with BSG meal in the diet of *C. reba*, demonstrating promising growth parameters with this substitution. Previous studies have also explored the use of BSG as a substitute for soybean meal (Hassan et al., 2016; Jayant et al., 2018; Estevez et al., 2021). Chattaraj et al. (2024a) found that complete substitution of soybean meal with BSG in the diet of experimental *C. reba* is viable. Jayant et al. (2018) reported a 2.66-fold enhancement in growth parameters of *Pangasianodon hypophthalmus* when soybean meal was replaced with BSG. BSG has been reported to contain adequate levels of limiting amino acids such as methionine (1.8 %), cystine (2.1 %), and lysine (5.5 %) (Hassan et al., 2016).

The *in vivo* bio-safety assay revealed the nontoxic nature of BSG meal and hence, can be applied as a constituent of fish feed in the cultivation of *C. reba*. In the histopathology study, no sign of necrosis in liver, kidney and intestine tissues of the BSG meal fed fish was observed. Renal tubules in the kidney tissue were intact, non-degenerated and interstitial haemorrhages were not observed. Dietary inclusion of BSG meal did not resulted in the formation of melanomacrophages center in the liver tissue of the carp. Hence, the histopathological observations pointed out that the test samples didn't distort the architecture of the intestine, kidney and liver of the carp. Estevão-Rodrigues et al. (2024) found an

improvement in intestinal histomorphology of European seabass juveniles fed with solid-state fermented BSG.

Serum biochemical analysis was utilized to assess overall health status and metabolic mechanisms in response to treatment conditions. Significant decreases in plasma protein levels typically indicate inhibition of liver protein synthesis in organisms (Pedrosa et al., 2019). The observed increase in total serum protein levels among fish in the tB groups suggests improved health status in carp fed experimental diets. Fish nutrition affects enzyme expression, especially in the liver, crucial for toxicity assessment. AST links gluconeogenesis, urea and citric acid cycles, and amino acid metabolism; ALP removes phosphates, and ALT aids in transamination, producing pyruvate and glutamate (Pedrosa et al., 2019). These enzymes' changes detect organ damage in treatments; serum biomarkers like AST, ALP, and ALT indicate liver damage and protein degradation (Lavanya et al., 2011). Importantly, in the current study, fish fed tB diet revealed slight decrease in serum ALT and AST than the control group. The observed values were within the range reported by Kavitha et al. (2012) for *Cyprinus carpio* and by Chattaraj and Das Mohapatra (2023) and Chattaraj et al. (2024a) for *C. reba*, indicating a positive effect of the test diet on liver health. Gyan et al. (2021) observed better serum biochemical indices in *Litopenaeus vannamei* fed with BSG meal. The non-significant alteration in the levels of creatinine and urea in the fish depicted the healthy conditions of the kidneys. Hence, the BSG meal didn't exhibit any toxicity and can be safely applied as feed ingredient for *C. reba*. The gut microbiome of a fish serves as a reliable indicator of its overall health status and can influence its response to environmental stresses and diseases (Li et al., 2023).

Bacteria play a very great role in different sectors of biotechnology like aquaculture, agriculture, biomedical science, pharmaceutical science (Thiagarajan et al., 2019; Banerjee et al., 2020; Khoshru et al., 2023; Sen et al., 2023; Samantaray et al., 2024). Recently, the scientific community has intensified its focus on gut microbial communities due to their essential roles in immunity, digestion, enzyme regulation, and responses to stress (Ganguly et al., 2018b; Ganguly et al., 2017; Chattaraj et al., 2023b). Factors such as nutrition, dietary supplements, environmental conditions, and trophic levels can lead to variations in gut microbial populations (Foysal et al., 2020). The study explored the capacity of BSG, a fiber-rich by-product from brewing, to influence the gut microbiome. The 16S rRNA metagenomic analysis identified bacterial phyla and genera present in the gastrointestinal (GI) microbiota of *C. reba* fed either a control meal or BSG meal. The major microbial groups identified in our research were in line with the usual microbiota observed in freshwater fish (Chattaraj et al., 2024b; Ganguly et al. 2018c). Substantial variations in taxonomic makeup were observed between the gastrointestinal microbiota of *C. reba* fed the control diet and those fed the BSG diet, confirming the initial hypothesis of distinct taxonomic structures between the two dietary groups. The prevalence of pathogenic genera in the GI tract of *C. reba* has been previously documented which is often stressful to the fish (Pramanik et al., 2023; Chattaraj et al., 2024b). Notably, the gastrointestinal tracts of *C. reba* fed the Brewers' Spent Grains (BSG) diet showed a higher abundance of beneficial probiotic bacteria and a lower presence of harmful pathogenic bacteria compared to those fed the control diet. Specifically, bacteria known for their probiotic properties, such as *Firmicutes, Actinobacteria*, and *Bacteroidetes*, were significantly enriched in the GI microbiota of fish fed the BSG meal. At the genus level, *Bacillus, Lactobacillus, Bifidobacterium, Paenibacillus,* and *Lysinibacillus*, were notably more abundant in the GI tract of BSG-fed fish and are recognized as probiotics (Chattaraj et al., 2022; Ganguly et al., 2019). The occurrence of lignocellulosic biomass in BSG likely modifies the gut physiology, potentially promoting increased growth of beneficial bacteria (Bonifácio-Lopes et al., 2023). Arabinoxylan from brewers spent grain is a potential prebiotic (Lynch et al., 2021). Additionally, BSG contains significant amounts of β-glucans, which can stimulate the host's gut microbiota (Chattaraj et al., 2024c). The second hypothesis, suggesting changes in taxonomic diversity within the GI microbiota of BSG-fed *C. reba* compared to controls, was substantiated. Measures of α diversity (Chao 1 estimator and Shannon index) were remarkably elevated in the GI microbiota of fish fed the BSG meal (tB) compared to those on the control diet (C), indicating a significant increase in microbial diversity in the GI tract of BSG-fed fish. Foysal et al. (2020) observed similar high alpha-diversity metrics in the gut of the mrigal carp. Chao1 estimator provides a reliable measure of species diversity, while the Shannon index calculates richness and evenness based on abundance levels (Wilczynski et al., 2022). The alterations in microbial diversity and taxonomic composition, characterized by an increase in probiotic taxa and a decrease in pathogenic taxa, suggest a more favorable gut environment, which is known to confer beneficial effects on host health. These beneficial changes in the GI microbiome of fish are correlated with enhanced health and performance metrics.

5. Conclusion

The feeding trial demonstrated that BSG is a viable and safe alternative ingredient for fish feed, leading to significant improvements in growth performance in *Cirrhinus reba* compared to traditional feeds. Biosafety assessments revealed no pathological symptoms or histopathological abnormalities in BSG-fed fish. Moreover, serum biochemical analysis showed improved profiles in BSG-fed fish, indicating enhanced physiological and metabolic function. 16S Metagenomic sequencing illustrated that BSG inclusion enriched the gut microbiota diversity of *C. reba*, with the dominance of Hamilton (1822) beneficial genera like *Bacillus, Lactobacillus, Bifidobacterium, Paenibacillus,* and *Lysinibacillus*. Increased alpha diversity, indicated by elevated Chao 1 estimator and Shannon index values, further supports BSG's potential as a sustainable feed ingredient with immense prebiotic benefits for aquaculture.

CRediT authorship contribution statement

Sourav Chattaraj: Conceptualization, Methodology, Data curation, Writing – original draft, Formal analysis. **Debasis Mitra:** Writing – review & editing. **Manasi Chattaraj:** Methodology, Formal analysis. **Arindam Ganguly:** Writing – review & editing. **Hrudayanath Thatoi:** Writing – review & editing. **Pradeep K. Das Mohapatra:** Supervision, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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