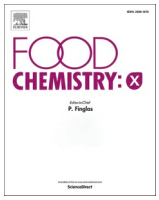
Food Chemistry: X 23 (2024) 101656

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Two-stage solid-state fermentation to increase the nutrient value of corn processing waste and explore its efficacy as a feed protein source

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ARTICLE INFO

Keywords:
Fermented by-products
Free amino acids
Simulated digestion
Gut microbiota
Gut development

ABSTRACT

Corn gluten meal-corn husk mixes (CCM) are an inexpensive and readily available agricultural by-product. This study explores a novel technique by converting CCM into high-value livestock feed protein sources through fermentation with *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS, aiming to sustainably meet future global protein needs. The process of fermentation significantly altered the structural composition of high molecular weight proteins, zein, and dietary fibers. This transformation resulted in a marked elevation in the concentrations of peptides, free amino acids, and polyphenols. The acidic environment produced during fermentation prevented lipid oxidation in CCM, thereby extending its storability. After fermentation, the content of anti-nutritional factors decreased, while its antioxidant capacity increased. *In vitro* simulated digestion suggested that fermentation improved the digestibility of CCM protein. *In vivo* animal experiments showed that fermented CCM (FCCM) promoted growth and gut health in chicks. This study provides new insights into the utilization of CCM.

1. Introduction

The increasing demand for food will inevitably lead to more waste in the processing process, resulting in environmental pollution and a waste of resources. Annually, China's corn processing industry generates over 80 million tons of Corn gluten meal (CGM), a by-product. CGM has a protein content as high as 60%, but its solubility in water is poor, and its amino acid profile is unbalanced, particularly lacking in lysine and tryptophan (Jiao et al., 2022). These limitations reduce its nutritional value and make it unsuitable for direct use as a food component. Rich in fiber and phenolic acids yet low in nutritional value, corn husk has limited use within the feed industry (Jiao et al., 2022). Combining these two by-products offers a potential source of nutrients for fermentation. Given the rising costs of feed and the shortage of protein resources, developing a high-quality feed protein source is of paramount importance (Sobhi et al., 2023).

Two-stage solid-state fermentation (SSF) is a unique technology that utilizes the physiological properties of bacteria being both aerobic and anaerobic. A cost-effective and straightforward approach has been shown to substantially enhance the nutritional profiles of agricultural by-products (Su, Jiang, Wang, Xu, et al., 2022). SSF has been reported to increase the concentrations of amino acids, peptides, organic acids, vitamins, and antioxidants in by-products (Wang, Pan, et al., 2023). *Aspergillus niger* fermentation generates numerous enzymes, promoting the breakdown of macromolecules, the conversion of bioactive ingredients, and the synthesis of novel molecules (Cui et al., 2021). This process increases the nutritional value of corn gluten meal-corn husk mixes (CCM). Extracellular enzymes from *Lactobacillus plantarum* can break down macromolecular proteins into amino acids and peptides (Abd Rashid et al., 2022). The lactic acids generated by *Lactobacillus*

Abbreviations: CCM, corn gluten meal-corn husk mixes; FCCM-1, CCM aerobic fermentation; FCCM-2, CCM anaerobic fermentation; CGM, corn gluten meal; SSF, solid-state fermentation; PDA, potato-dextrose agar plates; MRS, de Man Rogosa and Sharpe; DM, dry matter; CP, crude protein; EE, crude fat; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber; TCA-SP, trichloroacetic acid-soluble protein; FAA, free amino acids; SDS-PAGE, sulfate-polyacrylamide gel electrophoresis; SEM, scanning electron microscope; FITC, fluorescein isothiocyanate; ConA, concanavalin A; CW, calcofluor white; CLSM, confocal laser scanning microscope; FTIR, fourier transform infrared spectroscopy; FRAP, ferric reducing antioxidant power; DH, degree of hydrolysis; CON, untreated basal diet; FCCM, fermented CCM; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; HE, hematoxylin and eosin..

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https://doi.org/10.1016/j.fochx.2024.101656
Received 22 May 2024; Received in revised form 30 June 2024; Accepted 11 July 2024
Available online 14 July 2024

plantarum help reduce the antibacterial acidity of the two-stage SSF system and suppress the proliferation of certain pathogenic microbes (Su, Jiang, Wang, Zhang, et al., 2022). It was observed that *Lactobacillus plantarum*-inoculated soybean meal also increased the levels of amino acids, peptides, organic acids, and flavonoids post-SSF, significantly improving its nutritional value (Wang et al., 2022). Additionally, SSF of whole-oat with *Lactobacillus* and *Rhizopus oryzae* effectively increases total phenol and reducing sugar content (Wu, 2022). Furthermore, the mixed lactic acid bacteria can degrade cellulose effectively in wheat bran SSF, thus increasing the total phenol content, providing excellent antioxidant capacity to the substrates, and producing highly nutritive and palatable feed for animals (Li et al., 2024).

Enzyme preparations for use in fermented feeds are expensive to produce and utilize, limiting their application (Wang, Tang, et al., 2023). Given the costs associated with animal husbandry, a microbial two-stage SSF approach was chosen to maximize the benefits of strains and improve the utilization of agricultural by-products. In the gastrointestinal tract, nutrient absorption and energy regulation are significantly influenced by the gut microbiota, which also has strong links to various diseases, including mucosal immunity disorders and inflammatory bowel disease (Huang et al., 2022). However, the impact of fermented CCM (FCCM) as a feed protein resource on the growth performance and gut health of chicks has not been reported. Therefore, to better assess the feasibility of FCCM as a feed protein resource, it is necessary to conduct *in vivo* experiments on the biological utilization of FCCM in chicks.

This study evaluated the impact of two-stage SSF of CCM using *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS on protein quality and amino acid composition. The study also determined the contents of phenolics, flavonoids, carotenoids, and anti-nutritional factors. Using an *in vitro* simulated digestion method, the impact of SSF on the protein structure and digestibility of CCM was investigated. Additionally, the regulatory effect of FCCM as a substitute feed protein source on the gut microbiota, morphology, and barrier of chicks was examined. This research provides theoretical principles and experimental foundations for improving the nutrition and application of CCM.

2. Materials and methods

2.1. Microbial strains

Aspergillus niger AAX (CICC 41269) was cultivated on potato-dextrose agar plates (PDA, Solarbio) as the culture medium for 5 d at 30 °C. Spores were then carefully scraped from the plates using 3 mL of saline solution and transferred into 50 mL centrifuge tubes, where they were subjected to magnetic stirring for 30 min. A Neubauer counting chamber was used to quantify the spores, resulting in the preparation of *Aspergillus niger* AAX inoculum culture (10^7 spores/mL). *Lactobacillus fermentum* LLS (CICC 0813E3) was cultured in de Man, Rogosa, and Sharpe (MRS, Coolaber, Beijing, China) at 37 °C for 24 h at 150 rpm. The bacterial solution was then adjusted to 10^9 CFU/mL and stored at 4 °C for subsequent fermentation. All microorganisms mentioned above were screened and preserved in our laboratory (National Engineering Laboratory for Wheat and Corn Deep Processing, Jilin Agricultural University, Jilin, China).

2.2. Preparation of fermentation substrates

CGM and corn husk were purchased from Wellhope Foods Co., Ltd. (Changchun, China). Before fermentation, CCM (CGM: corn husk = 7:3, *w*/w) was placed in a fermentation tank and mixed with sterile water (CCM: sterile water = 5:3, w/w). *Aspergillus niger* AAX (10^7 spores/g) was then introduced to the mixture, which was covered with a sterile membrane allowing only air exchange, and subjected to the first-stage aerobic fermentation (FCCM-1) at 30 °C for 3 d. For the second stage (FCCM-2), *Lactobacillus plantarum* LLS (10^9 CFU/g) was added, the membrane was removed, and the flask was sealed with rubber stoppers for anaerobic fermentation at 37 °C for another 2 d. All samples were prepared in sextuplicate and stored at −80 °C for future use.

2.3. Microorganisms and chemical analysis

For sample preparation, 1 g was combined with 9 mL of sterile saline and agitated at 150 rpm for 15 min on a constant temperature air-bath shaker at 37 °C (RADOBIO Stab M1, Shanghai, China). The solution was rested for 10 min and the pH of the supernatant was measured using a pH meter (Thermo Fisher, LSTAR1118). The viable count of *Lactobacillus* was determined following a 10-fold gradient dilution in sterile saline and subsequent anaerobic incubation on MRS agar at 37 °C for 48 h. The content of ergosterol in CCM during the two-stage SSF was determined following the methodology outlined by Wu (2022).

Dry matter (DM), crude protein (CP), crude fat (EE), crude fiber (CF), ash content, neutral detergent fiber (NDF), acid detergent fiber (ADF), and trichloroacetic acid-soluble protein (TCA-SP) were quantified according to AOAC international guidelines. The concentration of reducing sugars was measured using the 3,5-dinitrosalicylic acid technique (Cui et al., 2021). Lactic acid content was evaluated using a commercial colorimetric kit based on the colorimetric method (Nanjing Jiancheng Bioengineering Institute, Nanjing). The samples were sent to the Jilin Academy of Agricultural Sciences (Changchun, China) for analysis of the composition of free amino acids (FAA) before and after fermentation using a fully automated amino acid analyzer (KNAUER Amino A200, Germany). Briefly, 1 g of samples were placed into a hydrolysis tube, to which 50 mL of HCl (6 mol/L) was added, followed by 15 min of ultrasound treatment. The tube was sealed under nitrogen and heated at 110 °C for 22 h for decomposition. After cooling to room temperature, the contents were transferred to a rotary evaporator and heated at 50 °C to remove HCl. The resulting residue was dissolved in 10 mL of sample buffer. After filtration through a 0.45 μm membrane, the samples were ready for analysis.

2.4. Electrophoresis and microanalysis

Proteins were analyzed using sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the protocol established by Gao et al. (2021). In parallel, the microscopic structural integrity of the samples was meticulously examined at a magnification level of 1000× through a scanning electron microscope (SEM, JSM-7610FPlus, JEOL, Japan), applying the method meticulously documented by Su, Jiang, Wang, Zhang, et al. (2022). Prior to observation under a confocal laser scanning microscope (CLSM, Stellaris5, Leica, Germany), the specimens were subjected to a pre-treatment regimen that included the application of fluorescein isothiocyanate (FITC), concanavalin A (ConA), and calcofluor white (CW), with their respective excitation wavelengths meticulously set at 518 nm, 555 nm, and 440 nm to facilitate an intricate visualization of the interaction among proteins, starches, and fibers. This comprehensive imaging technique was complemented by the analysis of infrared spectra, performed using a Fourier transform infrared spectrometer (FTIR, Bruker VERTEX 70, Germany), following the analytical procedures delineated by Zhao et al. (2022), which allowed for an in-depth assessment of the molecular composition and potential functional properties of the samples.

2.5. Digestive enzyme activity and anti-nutritional factors

An assessment of acid protease activity was conducted using a test kit provided by Boxbio, Beijing, China. This assay defines the enzymatic activity where the hydrolysis of one mole of tyrosine per minute per gram of tested material represents one unit of protease activity. Following the detailed methodology outlined by Wang et al. (2023), the activity of cellulase enzymes was detected. For the determination of malondialdehyde concentrations, a commercial kit from Solarbio,

Beijing, China, was utilized. Furthermore, the levels of phytic acid and trypsin inhibitors were quantified employing the analytical techniques described by Ranjan and Kumar (2019), ensuring precision in the measurement of phytic acid and trypsin inhibitors.

2.6. Antioxidation assay

2.6.1. Total phenol, flavonoid and carotenoid content

Quantification of total phenol and flavonoid content was carried out with a specialized assay kit procured from Sangon Biotech, located in Shanghai, China. A modified procedure described by Dulf et al. (2020) was employed to measure total carotenoid content. This modified protocol involved blending 1 g of the biological sample with 5 mL of extracting solvent. This mixture was then agitated at a speed of 150 r/min for 15 min using a temperature-controlled shaker (RADOBIO Stab M1, Shanghai, China). Following agitation, the sample was subjected to centrifugation at a speed of 7000 r/min at a temperature setting of 4 °C for 20 min. The brightly colored hexane layer that formed at the top was then carefully extracted and transferred into a 50 mL centrifuge tube, after which the volume was meticulously adjusted by adding additional hexane. The measurement was conducted at 450 nm, and carotenoid content was assayed in triplicate. The color changes of carotenoids in CCM before and after fermentation were measured using a colorimeter (MS3000, THREENH, China). Relevant information regarding color measurements is provided in Supplementary Materials S1.

2.6.2. Antioxidant capacity

Antioxidant capacities, including DPPH and ABTS radical scavenging capacities, ferric reducing antioxidant power (FRAP), and superoxide radical scavenging activity, were determined using commercial kits (BC4750, BC4770, BC1310, and BC1320, Solarbio, Beijing, China).

2.7. In vitro gastrointestinal digestion

Following the static *in vitro* digestion protocol established by Brodkorb et al. (2019), the experimental samples were processed. Initially, each sample was combined in a 1:1 ratio (*v*/v) with simulated gastric fluid, which contained 2000 U/mL pepsin, and the pH was stabilized at 3. This gastric phase proceeded with the samples being maintained at a constant temperature of 37 °C, allowing for a digestion period of 2 h. After completion of the gastric phase, the gastric digests were mixed with an equivalent volume of simulated intestinal fluid. This fluid was enriched with 100 U/mL pancreatin and 10 mM bile, and the pH was carefully adjusted to 7 to simulate intestinal conditions. The samples were then incubated for an additional 2 h at 37 °C to complete the intestinal digestion phase. Throughout the digestion process, samples of the digestive fluids were extracted at predetermined intervals: at 0, 10, 30, 60, and 120 min during the gastric digestion, and at 60 and 120 min during the intestinal digestion, in order to conduct subsequent analytical evaluations. Before subsequent analysis, all aliquoted samples were transferred into enzyme-free tubes and stored at −80 °C. All reagents for preparing digestive fluids were purchased from Sigma-Aldrich. SDS-PAGE was conducted as described in Section 2.4. Degree of hydrolysis (DH) determination was performed following the method outlined in previous studies (Zhao et al., 2022).

2.8. Chicks feeding experiment and sampling

Hy-line Brown chicks, provided by the Jilin Academy of Agricultural Sciences, were used in this study, which was conducted under the approval of the Animal Ethical Committee at Jilin Agricultural University (Authorization No. 201705001). The experiment involved a total of 180 chicks, which were methodically distributed into three distinct groups. The control group was fed an unmodified basal diet (CON), whereas the other two groups received diets enriched with 10% CCM (CCM) and 10% FCCM (FCCM), respectively. Each dietary group was further subdivided into three replicates, each consisting of 20 chicks. The formulation of these experimental diets was meticulously designed to be both isoenergetic and isonitrogenous, adhering to the standards set forth in the Nutrient Requirements of Poultry by the NRC (1994), as detailed in Supplementary Table S1. The chicks were housed in cages of uniform size (80 × 80 × 50 cm3) at the same altitude. The experiment lasted for 35 d, during which the chicks had unrestricted access to feed and water. Management practices followed the standards provided by Hy-line International Co., Ltd., USA (see Table S2). Temperature was gradually decreased during the rearing period (from 36 °C to 21 °C). Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated based on initial and final body weights, as well as feed intake records.

After the chick-feeding experiment, 5 chicks were randomly selected from each group for sampling post-euthanasia. Gut lengths were measured, and intestines were subsequently fixed in 4% paraformaldehyde to observe gut morphology. Jejunal samples were collected for 16S rRNA sequencing and western blot analysis.

2.9. Observations of gut morphology and western blot analysis

Hematoxylin and eosin (HE) staining was used on 5-μm thick paraffin-embedded and paraformaldehyde-fixed jejunum slices following conventional techniques. Villus length (measured from the base to the tip), villus width (measured perpendicular to the length), and crypt depth (measured from the base to the opening) were assessed using a microscope (Olympus, Japan) and ImageJ software (National Institutes of Health, DC, USA).

Proteins were extracted from jejunum tissue samples utilizing the RIPA buffer provided by Beyotime (Catalogue No. P0013B). Following extraction, the concentration of these proteins was accurately determined using Beyotime's BCA protein assay kit (Catalogue No. P0010). The proteins were then separated through SDS-PAGE and subsequently transferred onto PVDF membranes, which were prepared for analysis *via* western blotting. The primary antibodies employed included ZO-1 (ab307799), Occludin (ab216327), and β-actin (ab115777), each at a dilution of 1:1000, sourced from Abcam. Following the overnight incubation of these membranes with the primary antibodies at a temperature of 4 °C, they were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody at a dilution of 1:2000 (Abcam, ab6721). Subsequent to the antibody treatments, the membranes were rigorously washed with TBS-T buffer (MedChemExpress, HY-K1025) to remove any unbound antibodies. The visualization of protein blots was achieved using an advanced electrochemiluminescence detection system, and the intensities of the resultant bands were quantitatively analyzed with ImageJ software to assess protein expression levels.

2.10. Analysis of jejunum microbial diversity

Total microbial DNA was isolated from the contents of the jejunum using a dedicated DNA extraction kit (Model RM201–02, Vazyme, Nanjing, China), as per the specifications outlined by the manufacturer. This extracted DNA was subsequently stored at −20 °C in preparation for further detailed analyses. To evaluate the quality of the extracted DNA, assessments were conducted using both a Nanodrop NC2000 spectrophotometer for quantitative analysis and agarose gel electrophoresis for visual verification of integrity. The amplification process targeted the V3-V4 regions of the bacterial 16S rRNA, utilizing PCR with specific primers: forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3′) and reverse primer 806R (5'-GGACTACHVGGGTWTCTAA.

T-3′). The PCR amplifications were then purified using Agencourt AMPure Beads (Beckman Coulter, USA) and the concentration of the purified products was determined using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Subsequent sequencing of these amplicons was performed on the Illumina MiSeq platform,

employing the MiSeq Reagent Kit v3 supplied by Shanghai Personal Biotechnology Co., Ltd., which produced paired-end reads of 2 × 300 bp. The data from sequencing was analyzed utilizing the advanced capabilities of the QIIME2 platform to interpret and quantify microbial compositions.

2.11. Statistical analysis

Statistical analysis of all experimental data was conducted using SPSS software version 26.0 (SPSS Inc., Chicago, USA). The means of the data sets were statistically compared utilizing a one-way Analysis of Variance (ANOVA) followed by Duncan's multiple range test for post-hoc analysis. The results are displayed as Means ± SDs. Any differences between the means of the treatments were deemed statistically significant if the $p < 0.05$.

3. Results and discussion

3.1. Dynamic change of fermentation strains

Ergosterol is a marker used to assess fungal biomass (Zwinkels & Wolkers-Rooijackers, 2023). Ergosterol levels increased continuously during the two-stage SSF of CCM by *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS (Fig. 1A), with a more pronounced increase observed during the aerobic stage compared to the anaerobic stage, consistent with previous research findings (Wu et al., 2022). Changes in lactic acid and pH during the two-stage SSF of CCM are depicted in Fig. 1B and C. The lactic acid content gradually increased, leading to acidification of the fermentation environment. The acidic environment generated by fermentation inhibited mold growth and extended the storage period (Su et al., 2022). Additionally, lactic acid contributed a broader range of flavors to CCM products (Wu et al., 2022). The transformation of large molecules into smaller ones was promoted by the extracellular enzymes produced by the mold (Wu et al., 2022). Collectively, the substrate following the first fermentation stage is more favorable for the growth of *Lactobacillus fermentum* LLS and contributes to further improving the quality of CCM.

3.2. Chemical composition

The chemical compositions of CCM during the two-stage SSF by *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS are detailed in Table 1. FCCM exhibited higher levels of CP, TCA-SP, and EE, which increased by approximately 24.94%, 260.78%, and 62.50%, respectively. The increase in CP may be attributed to the concentration of single-cell protein generated by microbes and the reduction in DM (mostly carbohydrates) in the fermentation substrate (Wang et al., 2022). *Lactobacillus fermentum* LLS was predominantly used for fermentation (López-García et al., 2022), significantly contributing to the increase in TCA-*SP*. TCA-SP consisted of small peptides and FAA, most of which are readily absorbed by the gastrointestinal tract (Su et al., 2022). Small peptides were found to improve gut structure, promote gut growth and development, and interact with immune cells to strengthen immunity (Eugenio et al., 2023). In this study, the breakdown of CF, ADF, and NDF was primarily promoted by cellulase released by microorganisms during fermentation. After two-stage SSF, the reducing sugar content of CCM increased significantly. The conversion of cellulose to reducing sugars was accelerated by the cellulase enzyme produced by microorganisms, which is easily absorbed and utilized by the body (Cui et al., 2021). Overall, the nutritional quality of CCM is improved by the two-stage SSF process, resulting in FCCM having a higher application value in feed.

3.3. Electrophoresis and microscopic observation

The effect of *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS on the molecular weight of proteins during the two-stage SSF of CCM was analyzed using SDS-PAGE (Fig. 1D). Multiple bands in the 15–55 kDa range were observed in the protein profile of CCM. The protein bands in this range were noticeably degraded during the aerobic stage and nearly completely degraded during the anaerobic stage. The protein content of CGM predominantly consists of zein, which is characterized by poor water solubility, thus limiting its application (Jiao et al., 2022). Zein is classified into α- (19–24 kDa), β- (15 kDa), and γ- (43 kDa) fractions, with α-zein being the most abundant, constituting >80% of the total zein protein (Gao et al., 2021; Zhao et al., 2022). Our results indicated that the total FAA content and various FAA levels, except methionine, were consistently elevated throughout the fermentation process (Fig. 2A). The breakdown of high molecular weight proteins and zein correlated with an increase in TCA-SP and FAA levels during fermentation, suggesting that the quality of the proteins in CCM was improved, making them more digestible and absorbable by animals.

Fourier Transform Infrared Spectroscopy (FTIR) was utilized to examine the microstructural changes in samples, focusing on the alterations in the absorption spectra of CCM after engaging in a two-stage SSF, as depicted in Fig. 1E. The spectrum, covering the range from 4000 to 400 cm^{-1}, displayed significant transformations. Notably, the intense and broad absorption band ranging from 3300 to 3000 cm^{-1} is indicative of O—H stretching vibrations, primarily stemming from the degradation of polymers such as proteins and cellulose (Zhao et al., 2022; Mushtaq et al., 2023). The process of SSF induced a shift in these peaks from 3285 cm^{-1} to 3277 cm^{-1} and further to 3118 cm^{-1}, which suggests a disruption in the hydrogen bonding within the molecular structure of the protein and cellulose components of CCM. Further analysis within the spectral domain of 1125 to 1000 cm^{-1} revealed the stretching of C—O bonds and the bending of C-O-H bonds in molecules such as alcohols and phenols (Zhao et al., 2023), with a notable strong absorption peak appearing near 1027 cm^{-1}. This particular peak is associated with the deformation of aromatic ring C—H bonds, indicating substantial molecular rearrangements (Zhao et al., 2023). Post-SSF analysis also revealed that the peaks around 2900 cm^{-1}, which typically represent the stretching vibrations of C—H bonds in the carbon skeletons of proteins and cellulose, underwent shifts and decreased in intensity, highlighting the impact of SSF on these biomolecules (Dulf et al., 2020; Zhao et al., 2022). Additionally, the region reflecting amide I absorption (1600–1700 cm^{-1}) exhibited a minor yet significant shift from 1630 cm^{-1} to 1633 cm^{-1} and then to 1634 cm^{-1}, accompanied by an increase in intensity. This indicates enhanced structural rigidity or changes in the protein backbone. Similarly, the amide II absorption peaks (1500–1600 cm^{-1}) showed slight modifications, primarily due to alterations in the C—O stretching, N—H bending, and C—N stretching vibrations, suggesting nuanced changes in the secondary structures of proteins (Chen et al., 2019). This comprehensive analysis leads to the conclusion that the structural composition of CCM proteins and cellulose is notably influenced by the fermentation process, potentially altering their functional properties and interactions within cellular structures.

SEM was employed to examine the microstructures of CCM before and after two-stage SSF (Fig. 1F). At a magnification of 1000×, the surface structure of CCM appeared relatively compact and uniform. During the aerobic stage, the surface exhibited fragmented structures and increased porosity, forming a distinct contrast with the unfermented CCM. In the anaerobic stage, the surface of CCM showed even more fragmentation and porosity. This increased permeability of FCCM likely promotes greater penetration by digestive enzymes, thereby improving substrate breakdown efficiency. These surface changes are attributed to the action of extracellular enzymes and organic acids, which decompose the CGM and corn husk, making FCCM easier to digest than CCM (Su et al., 2022).

The microstructural changes of proteins, fibers, and starch were further investigated using CLSM (Fig. 1G). After staining with CW, FITC, and ConA, the fibers, proteins, and starch components emitted blue, green, and red fluorescence, respectively (Su et al., 2022). During the

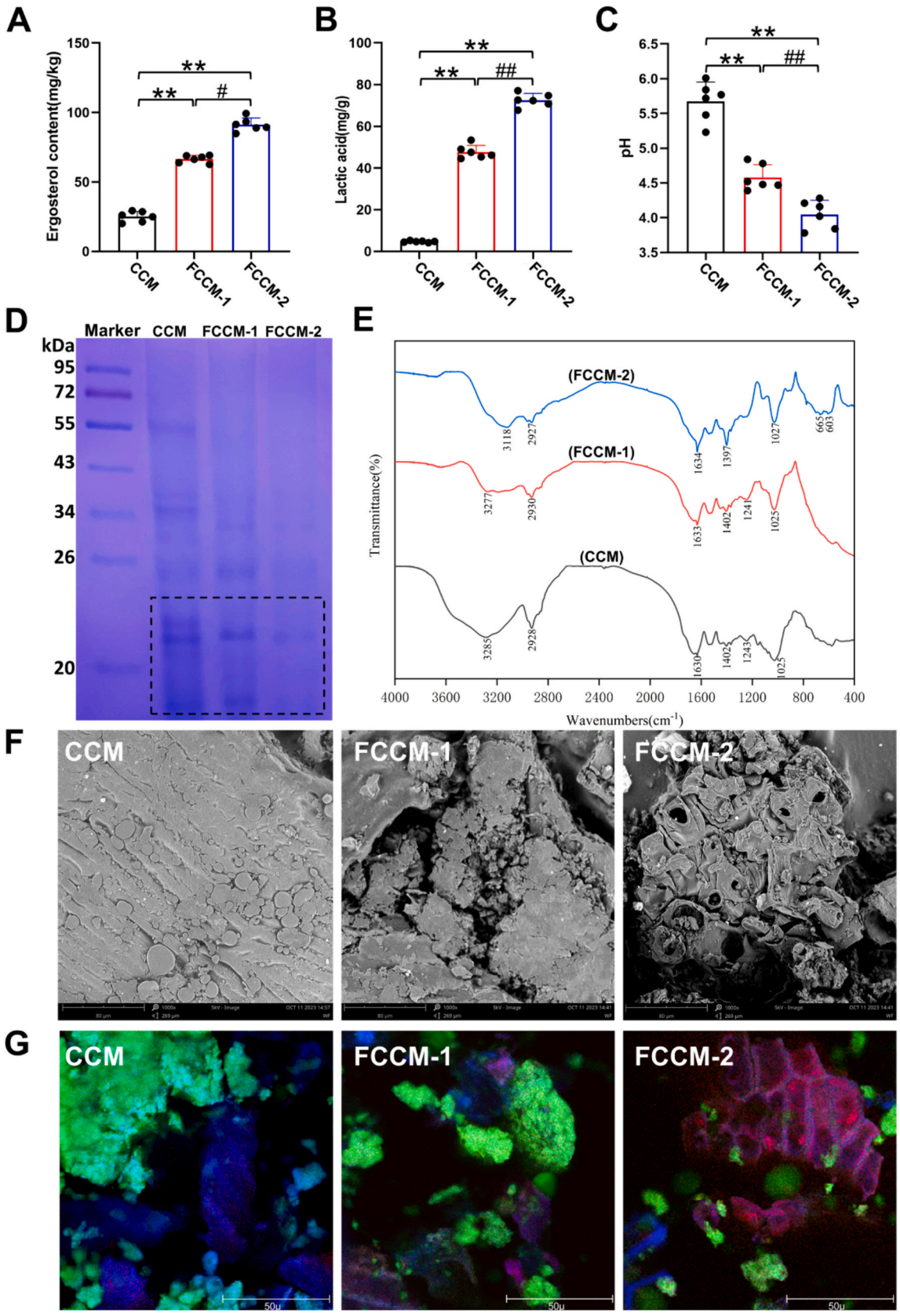

Fig. 1. Effect of fermentation on microorganisms and microstructure in corn gluten meal-corn husk mixes. Ergosterol content (A); Lactic acid content (B); pH value (C); SDS-PAGE protein profile (D); Fourier transform infrared spectra(E); SEM images (1000×) (F), CLSM images (G). Values are means ± SD, $n = 6$. CCM: corn gluten meal-corn husk mixes; FCCM-1: CCM aerobic fermentation; FCCM-2: CCM anaerobic fermentation. * $p < 0.05$, ** $p < 0.01$ compared with the CCM. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ for FCCM-1 *vs.* FCCM-2.

Table 1
Dynamic changes in the chemical compositions of corn gluten meal-corn husk mixes during fermentation.

Items	CCM	FCCM-1	FCCM-2
Dry matter, %	47.58 ± 0.48	41.47 ± 0.26	34.53 ± 0.37 **, ##
Crude protein, %	51.24 ± 0.24	59.85 ± 0.36**	64.02 ± 0.21**, #
TCA-SP, %	0.31 ± 0.03	1.27 ± 0.05**	1.84 ± 0.02**, #
Crude fat, %	1.52 ± 0.17	2.24 ± 0.13**	2.47 ± 0.12**
Crude fiber, %	7.97 ± 0.24	6.15 ± 0.17*	3.94 ± 0.36**, #
ADF, %	9.51 ± 0.67	7.78 ± 0.42*	5.53 ± 0.62**, ##
NDF, %	19.84 ± 0.81	17.43 ± 0.52*	14.74 ± 0.47**, ##
Ash, %	10.27 ± 0.19	5.52 ± 0.24**	4.37 ± 0.15**, ##
Reducing sugar, mg/g	0.07 ± 0.01	0.24 ± 0.03*	0.61 ± 0.05**, ##

Values are means ± SD, n = 6. CCM: corn gluten meal-corn husk mixes; FCCM-1: CCM aerobic fermentation; FCCM-2: CCM anaerobic fermentation. * $p < 0.05$, ** $p < 0.01$ compared with the CCM. # $p < 0.05$ and ## $p < 0.01$ for FCCM-1 *vs.* FCCM-2. Abbreviations: ADF, acid detergent fiber; NDF, neutral detergent fiber; TCA-SP, trichloroacetic acid soluble protein.

aerobic stage, the initially well-organized external structure of CGM and corn husk began to break down; macromolecular proteins on the surface of CGM were gradually decomposed, revealing the fibrous mesh of the corn husk. During the anaerobic stage, the breakdown continued with large proteins fragmenting into smaller peptides, fully exposing the fibrous mesh and the internal starch particles. These findings are consistent with increases in CP, TCA-SP, and FAA, and the reduction of CF, ADF, and NDF in the two-stage SSF-processed CCM.

3.4. Digestive enzyme activity and storability

During the two-stage SSF of CCM by *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS, the activity of digestive enzymes played a crucial role. Acid protease catalyzed the breakdown of peptide bonds, forming amino acids and peptides (Wang, 2023). In our study, the contents of TCA-SP (Table 1) and FAA (Fig. 2A) significantly increased. Compared to CCM, acid protease activity rose in both stages of fermentation, with the anaerobic stage reaching 11.33 U/g (Fig. 2B). The acidic protease promoted the degradation of macromolecular proteins in CCM, aligning with the observed microstructural changes. The high fiber content of corn husk hindered the full utilization of its nutritional components. Cellulase is an enzyme that catalyzes the breakdown of cellulose in fibers into monosaccharides (Cui et al., 2021). During fermentation, cellulase activity continued to increase, rising

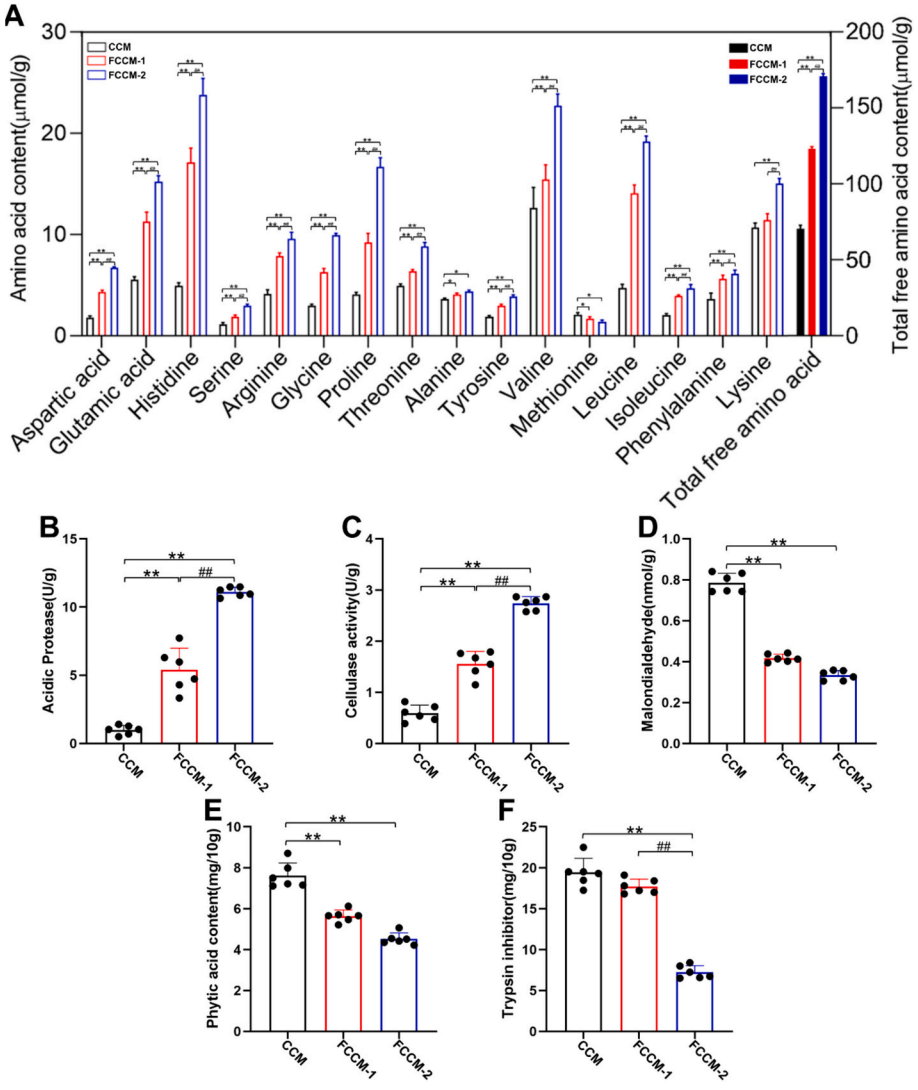

Fig. 2. Effect of fermentation on free amino acid, digestive enzyme activity, storability, and anti-nutritional factors content in corn gluten meal-corn husk mixes. Free amino acid content (A). Acidic protease (B); Cellulase activity (C); Malondialdehyde (D); Phytic acid content (E); Trypsin inhibitor content (F). Values are means ± SD, n = 6. CCM: corn gluten meal-corn husk mixes; FCCM-1: CCM aerobic fermentation; FCCM-2: CCM anaerobic fermentation. * $p < 0.05$, ** $p < 0.01$ compared with the CCM. # $p < 0.05$ and ## $p < 0.01$ for FCCM-1 *vs.* FCCM-2.

from 0.59 U/g to 2.74 U/g (Fig. 2C). The elevation in cellulase content was responsible for altering the surface microstructure of the corn husks, a finding that aligns with the observations reported by Su et al. (2022). Moreover, this escalation in enzymatic activity was linked to the diminished levels of CF, ADF, and NDF throughout the fermentation of CCM. In essence, the fermentation process facilitated the degradation of large molecular compounds within CCM, thereby enhancing its digestibility.

The high fiber content in corn husk made it susceptible to oxidative decay during storage (Wang et al., 2023). Malondialdehyde and other harmful intermediates were produced as a result of lipid oxidation in CCM during storage. The content of lipid oxidase was efficiently determined by detecting the level of malondialdehyde (Su et al., 2022). The two-stage SSF effectively decomposed >57.71% of malondialdehyde in CCM, with most decomposition occurring in the aerobic stage (Fig. 2D). Reports indicate that the acidic environment formed during fermentation inhibited the activity of lipid oxidase (Su et al., 2022). Therefore, the acidic environment produced during fermentation could prevent lipid oxidation of CCM during storage, which might be the primary reason for the decrease in malondialdehyde content. Additionally, the acidic environment inhibited the growth of spoilage bacteria, thus extending the storage stability of CCM (Wang et al., 2023).

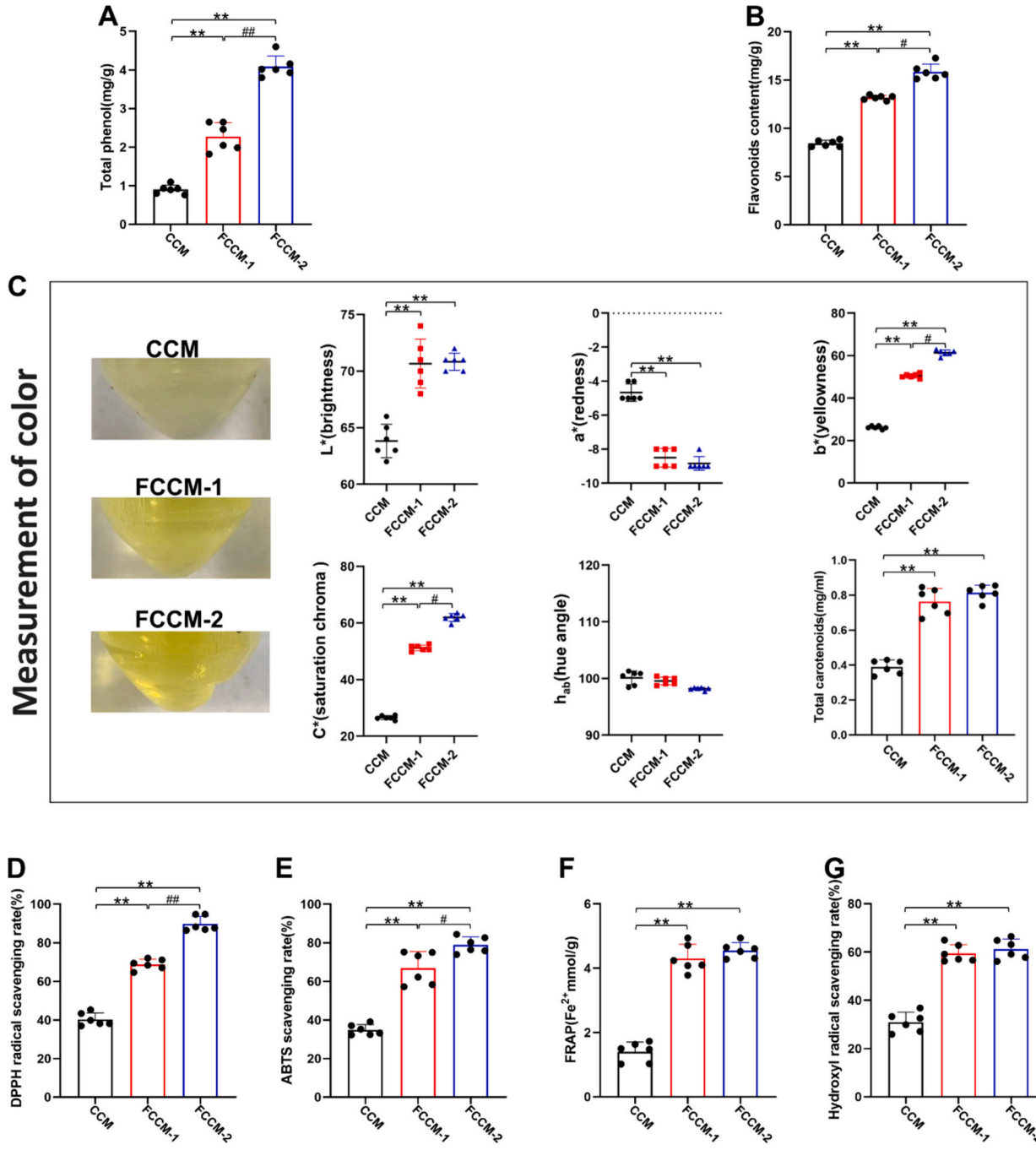

Fig. 3. Effect of fermentation on active ingredients and antioxidant capacity in corn gluten meal-corn husk mixes. Total phenol (A); Flavonoids content (B); Total carotenoids (C); DPPH radical scavenging rate (D); ABTS scavenging rate (E); Ferric reducing-antioxidant power (F); Hydroxyl radical scavenging rate (G). Values are means ± SD, n = 6. CCM: corn gluten meal-corn husk mixes; FCCM-1: CCM aerobic fermentation; FCCM-2: CCM anaerobic fermentation. * $p < 0.05$, ** $p < 0.01$ compared with the CCM. # $p < 0.05$ and ## $p < 0.01$ for FCCM-1 *vs.* FCCM-2.

3.5. Anti-nutritional factors

Anti-nutritional factors in corn husk hinder nutrient absorption and utilization (Su et al., 2022). Phytic acid and trypsin inhibitor (TI) are the most common anti-nutritional factors found in plant tissues. Phytate complexes chelate with metal-mineral ions, reducing digestive function by inhibiting the activity of related digestive enzymes (Ranjan et al., 2019). Following the two-stage SSF of CCM by *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS, the concentration of phytic acid in CCM decreased by 40.71%, with the most significant reduction observed in the aerobic stage (Fig. 2E). Previous research indicated a significant reduction in phytic acid content in millet bran during two-stage SSF, similar to our findings (Wang et al., 2023). TI inhibits digestive enzyme activity, reducing the organism's ability to digest, absorb, and utilize nutrients (Ranjan et al., 2019). After the two-stage SSF, the concentration of TI in CCM decreased by 62.72%, with the most significant reduction observed in the anaerobic stage (Fig. 2F). Wang et al. (2023) indicated that microorganisms producing cellulase effectively reduced TI content. In our study, cellulase activity increased from 0.59 U/g to 2.74 U/g, likely contributing to TI degradation. Overall, after two-stage SSF, the levels of anti-nutritional factors in CCM were significantly reduced, increasing the safety and nutritional availability of CCM.

3.6. Antioxidant ingredients and antioxidant capacity

SSF can increase the value of agricultural by-products. Su et al. (2022) found that SSF reduces fiber and anti-nutrient factors, thereby increasing the antioxidant capacity of fermented products. Phenolics and flavonoids are positively correlated with antioxidant capacity (Dulf et al., 2020). During the two-stage SSF of CCM by *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS, the total phenol and flavonoid content of FCCM significantly increased, with total phenols rising from 0.76 mg/g to 4.09 mg/g and flavonoids increasing from 8.42 mg/g to 15.85 mg/g (Fig. 3A-B). The polysaccharide chains in corn husk were cleaved by cellulase, resulting in increased total phenol and flavonoid content (Wang et al., 2023). In this study, cellulase activity increased continuously (from 0.59 U/g to 2.74 U/g), contributing to the increased total phenol and flavonoid content. Additionally, CCM is rich in lutein and zeaxanthin, which are carotenoids (Jiao et al., 2022). The color of carotenoids in CCM before and after fermentation was analyzed (Fig. 3C). The results indicated that after fermentation, redness decreased while yellowness and saturation chroma increased continuously, explaining the color difference compared to CCM. The content of carotenoids in CCM was assessed before and after fermentation, revealing an 88.24% increase in carotenoid content in FCCM. The active metabolisms during two-stage SSF, such as peptide and carotenoid production, were also correlated with increased antioxidant capacities (Jiao et al., 2022; Wang et al., 2023).

Due to the different mechanisms through which various antioxidant components act, we evaluated the overall antioxidant capacity of both CCM and FCCM. The results showed that the DPPH radical scavenging activity, ABTS radical scavenging activity, FRAP, and hydroxyl radical scavenging rate of FCCM were notably improved (Fig. 3D-G). In general, the increase in antioxidant metabolites improves the application value of CCM in animal feed.

3.7. In vitro simulated digestion

The profiles of digesta during the gastrointestinal digestion process are illustrated in Fig. 4. The differences in the CCM digestion process became more pronounced after two-stage SSF by *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS (Fig. 4A). Five bands ranging from 17 to 43 kDa were observed for digested CCM samples. The intensity of these bands significantly decreased during the aerobic stage and almost

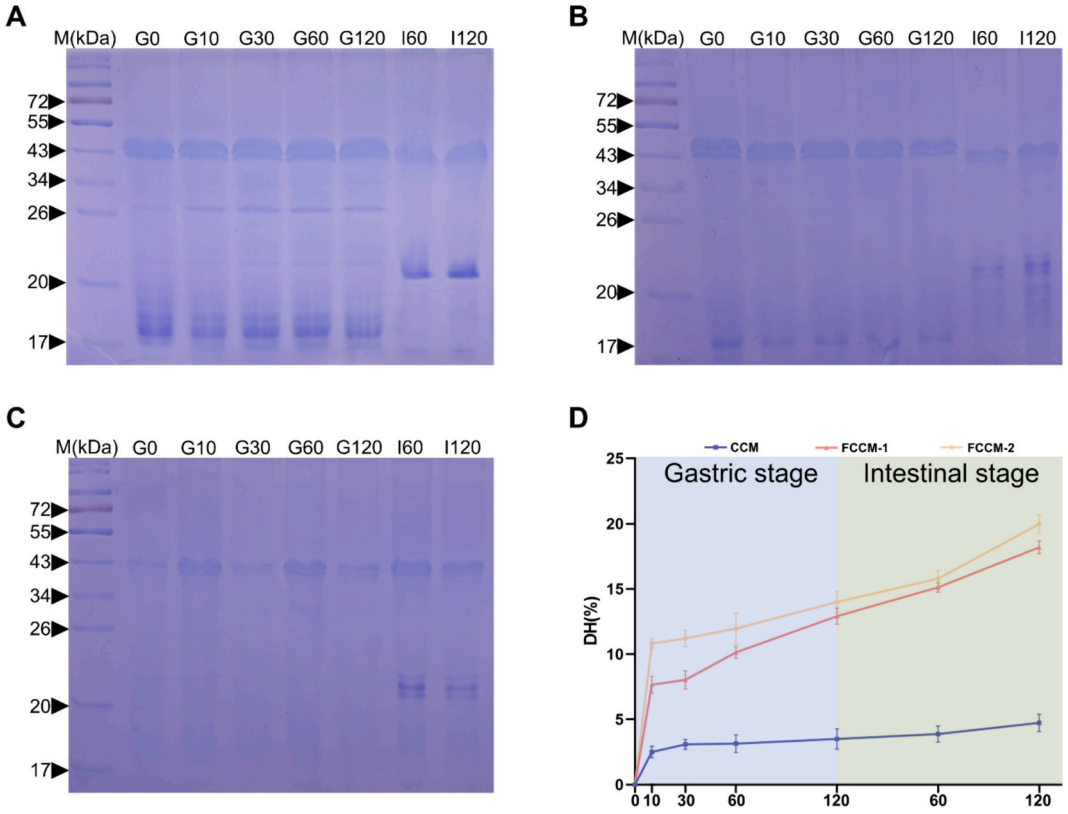

Fig. 4. Differences between *in vitro* simulated digestion of corn gluten meal-corn husk mixes before and after fermentation. SDS-PAGE profiles of proteins in the CCM (A), the FCCM-1 (B), and the FCCM-2 (C). Degree of hydrolysis after *in vitro* digestion of CCM, FCCM-1, and FCCM-2 (D). Values are means ± SD, n = 6. CCM: corn gluten meal-corn husk mixes; FCCM-1: CCM aerobic fermentation; FCCM-2: CCM anaerobic fermentation.

disappeared during the anaerobic stage compared to the CCM (Fig. 4B-C). α-zein (19–24 kDa, representing 80% of the zein content) and γ-zein (around 43 kDa, representing 10%–20% of the zein content) are known to exhibit poor water solubility but dissolve well in ethanol-water solutions and alkaline solutions, resulting in poor digestibility (Gao et al., 2021; Zhao et al., 2022). Previous studies have reported that proteins more readily hydrolyzed by enzymes possess higher nutritional value (Zhao et al., 2022). It was evident that CCM after two-stage SSF was more conducive to digestion, thereby increasing its nutritional value. Research has shown that the shift of zein towards lower molecular weights indicates a decrease in the molecular weight and aggregation degree of its subunits (Zhao et al., 2022), which is consistent with our findings.

The dynamic changes of CCM and FCCM in gastrointestinal digestion were simulated through *in vitro* digestion, and the digestibility and nutritional value of CCM and FCCM proteins were assessed using DH (Zhao et al., 2022). The DH of FCCM reached its peak after simulated gastrointestinal digestion during the anaerobic stage (19.97%), compared to 4.74% in the CCM (Fig. 4D). This suggests that the digestibility of CCM was increased after two-stage SSF. Additionally, the structural changes and lower molecular weight distribution of FCCM also promoted its digestibility, similar to the findings of previous studies (Sun et al., 2023).

3.8. Growth performance and gut microbiota

This investigation explored the potential to diminish the amount of soybean meal traditionally incorporated into chick diets by integrating CCM and FCCM, as depicted in Fig. 5A. Given the limited application of protein supplements in animal nutrition, soybean meal remains the predominant choice. This study assessed whether these alternatives could effectively reduce reliance on soybean meal in formulations for chicks. The final weight and ADG of chicks fed FCCM were significantly increased (Table 2). Several factors may contribute to this improvement.

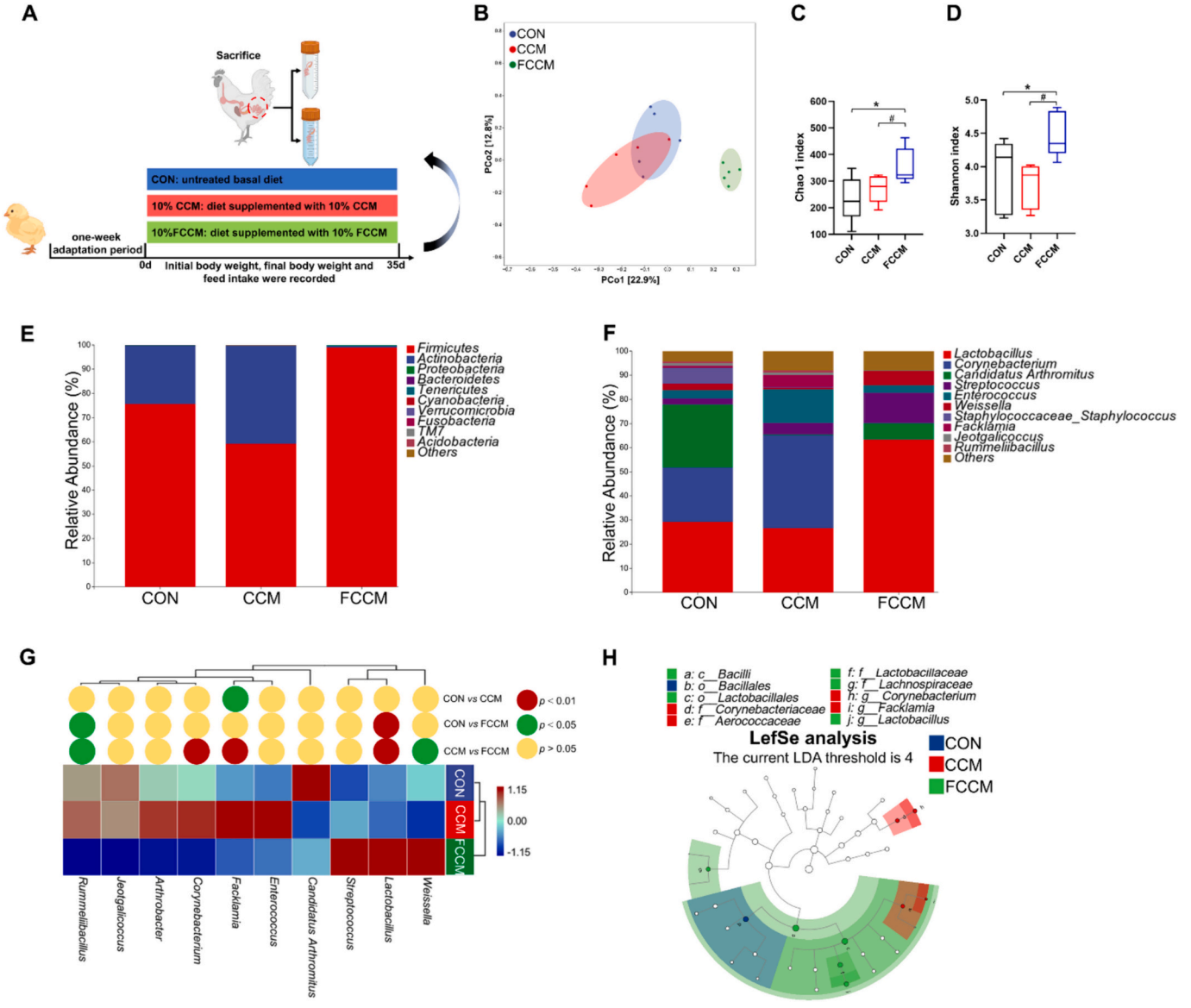

Fig. 5. Effect of corn gluten meal-corn husk mixes (CCM) on the gut microbiota of chicks before and after fermentation. Flow chart of the experimental groupings(A); PCoA based on the unweighted-UniFrac distance plot of OTUs (B); Alpha diversity analysis Chao1 (C) and Shannon (D); The relative abundance of the gut microbiota by phylum (E); Genus levels relative abundance (F); Genus levels heatmap (G); LEfSe taxonomic branching maps (LDA > 4.0) computed for OTU level characteristics (H). Values are means ± SD, $n = 5$. CON: untreated basal diet; CCM: diet supplemented with 10% CCM; FCCM: diet supplemented with 10% fermented CCM. * $p < 0.05$, ** $p < 0.01$ compared with the CON. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ for CCM *vs.* FCCM.

Firstly, the two-stage SSF process improved the palatability of CCM (Su et al., 2022), thereby increasing the starter intake of chicks. Secondly, FCCM exhibited higher contents of CP, FAA, small peptides, total phenols, flavonoids, carotenoids, and lactic acid compared to CCM. These components likely promoted the growth performance of chicks. Similar results were observed in previous studies where fermented wheat bran improved swine growth performance (Zhang et al., 2023).

The gut microbiome is crucial for digestion, regulating nutrient absorption and metabolism carried out by the host (Huang et al., 2022). To explore the overall differences in the gut microbiota of chicks fed with CCM and FCCM, principal coordinates analysis (PCoA) was conducted. The results revealed distinct microbial community compositions among the different treatment groups (Fig. 5B). Alpha diversity analysis indicated increased community richness and diversity in the FCCM group, evidenced by higher Chao1 and Shannon index values compared to the control group (Fig. 5C-D). As shown in Fig. 5E, analysis of the bacterial composition at the phylum level within the jejunal content of each experimental group revealed that the proportion of Firmicutes was substantially higher in chicks consuming FCCM (98.95%) compared to those in the control group (75.68%). Conversely, the presence of Firmicutes was notably lower in the chicks from the CCM group, registering at 59.17%, when contrasted with the control group. Chicks fed FCCM (0.66%) exhibited a lower abundance of *Actinobacteria* compared to CON (23.99%), whereas the CCM group (40.60%) had a notably higher abundance. Previous research has indicated that feeding fermented defatted rice bran to finishing pigs increases *Firmicutes* levels in their guts, correlating with improved growth performance (Su et al., 2022). Moreover, weight gain and increased energy harvesting capabilities have been associated with a *Firmicutes*-rich microbiome (Lu et al., 2019). In our study, the guts of chicks fed with FCCM exhibited an average relative abundance of *Firmicutes* as high as 98.95%. This finding may have contributed to the favorable growth performance observed in the FCCM group of chicks.

Differences in the abundance of the top 10 genera were observed among the three groups (Fig. 5F). Notably, the FCCM group showed a significant increase in *Lactobacillus* (63.39%) compared to the CON group (39.32%) and the CCM group (26.61%). Xiao et al. (2017) reported that *Firmicutes* is the dominant phylum, with *Lactobacillus*, commonly found in probiotics, being the predominant genus in the jejunal microbiota of broiler chickens. *Lactobacillus* can inhibit pathogen colonization, prevent intestinal diseases, improving intestinal barrier function, regulate the immune system, and show significant effects in cancer prevention (Huang et al., 2022). The heatmap at the genus level revealed distinct bacterial abundances in each group (Fig. 5G). The relative abundance of *Lactobacillus* ($p < 0.01$) and *Weissella* ($p < 0.05$) in the FCCM group showed a significant increase. *Weissella* offers antimicrobial and probiotic benefits in various areas including dental health, skin care, obesity, inflammatory conditions, and even cancer (Ahmed et al., 2022). Conversely, the CCM group displayed a notably higher relative abundance of *Facklamia* and *Corynebacterium* compared to the FCCM group ($p < 0.01$). A higher relative abundance of *Facklamia* has been associated with increased gut barrier permeability (Lee et al., 2023), while *Corynebacterium* is recognized as an animal pathogen with infective potential (Tresch et al., 2023). In our study, the relative abundance of *Facklamia* and *Corynebacterium* was low in the FCCM group. To further examine the impact of CCM and FCCM on chicks, Linear Discriminant Analysis Effect Size (LEfSe) analysis was employed to compare the characteristic flora of each group (Fig. 5H). *Lactobacillus* was significantly more abundant in the FCCM group ($p < 0.05$), whereas *Facklamia* and *Corynebacterium* were notably higher in the CCM group ($p = 0.01$). Our study found that fermentation increased the abundance of beneficial bacterial genera in CCM and decreased the abundance of harmful bacterial genera, thereby improving the utility of CCM.

Table 2
Effects of corn gluten meal-corn husk mixes (CCM) and fermented CCM (FCCM) on the chick's growth performance.

Items	CON	10%CCM	10%FCCM
Initial weight/g	51.36 ± 0.74	51.83 ± 0.52	51.45 ± 0.67
Final weight/g	500.22 ± 18.58	488.46 ± 13.68	598.65 ± 19.86*, #
ADG g/d	12.82 ± 0.21	12.48 ± 0.15	15.63 ± 0.27*, #
ADFI g/d	50.24 ± 1.37	47.75 ± 2.43	51.24 ± 1.52
FCR	3.92 ± 0.14	3.83 ± 0.15	3.27 ± 0.17*, #

CON: untreated basal diet; CCM: diet supplemented with 10% CCM; FCCM: diet supplemented with 10% FCCM. Values are means ± SD, n = 5. * $p < 0.05$, ** $p < 0.01$ compared with the CON. # $p < 0.05$ and ## $p < 0.01$ for CCM *vs.* FCCM.

3.9. Gut morphology and gut barrier

Building upon the favorable changes in chick gut flora induced by FCCM, we investigated the gut morphology in chicks (Fig. 6A). Parameters such as villus height, villus width, crypt depth, the ratio of villus height to crypt depth, and overall villus surface area are widely acknowledged as definitive indicators for evaluating gut health, as highlighted in the research by Ducatelle et al. (2018). The data from this study indicates that within the FCCM group, there was a statistically significant enhancement in key gut health markers. Specifically, the measurements for villus height and villus surface area showed remarkable improvements ($p < 0.01$). Additionally, the decrease in crypt depth ($p > 0.05$) and the ratio of villus height to crypt depth also registered significant growth ($p < 0.05$), pointing to superior structural integrity compared to the CON group. On the other hand, the CCM group displayed a notable increase in crypt depth ($p < 0.05$). However, this group's performance on other critical parameters fell below the levels observed in the FCCM and control groups (Fig. 6B-F). The increase in villus height and width indicated an expansion in the area of efficient nutrient absorption, thereby improving gut absorption function and ultimately benefiting chick growth (Chen & Yu, 2023).

The digestive absorption function of the gut relies on both normal gut morphology and an intact gut barrier. The intestinal barrier prevents pathogenic antigens from entering the intestine (Donaldson et al., 2021). The impact of CCM and FCCM on the intestinal barrier function of chicks was evaluated by measuring the expression levels of tight junction proteins (TJs) ZO-1 and Occludin (Fig. 6G). In our study, the levels of ZO-1 protein ($p < 0.05$) and Occludin protein ($p < 0.01$) in the FCCM group were significantly higher than those in the CON group, while in the CCM group, they were significantly lower (Fig. 6H-I). This indicates that TJs strengthen intestinal protective function by forming a physical barrier (Slifer & Blikslager, 2020).

In summary, our findings suggest that FCCM could increase villus surface area and improve gut barrier function. FCCM contains various bioactive compounds, such as polyphenols, flavonoids, peptides, and carotenoids, which may directly benefit gut health.

4. Conclusion

This study employed *Aspergillus niger* AAX and *Lactobacillus fermentum* LLS for two-stage SSF of CCM. The results demonstrated that two-stage SSF degraded macromolecular proteins, fibers, and anti-nutritional factors in CCM, while concurrently increasing CP, FAA, small peptides, reducing sugars, and lactic acid levels. Additionally, two-stage SSF increased antioxidant capability by elevating the levels of total phenols, flavonoids, and carotenoids, and inhibited lipid oxidation in CCM by reducing malondialdehyde levels. Furthermore, *in vitro* simulated digestion experiments showed a significant increase in the digestibility of FCCM. *In vivo* experiments revealed that FCCM improved the growth performance of chicks, increased the abundance of beneficial gut bacteria, decreased the abundance of harmful bacteria, and improved gut digestion, absorption, and barrier functions. This suggests that fermentation could increase the bioavailability of CCM *in vivo*. Overall, our findings contribute to the efficient utilization of agricultural by-products and the reduction of environmental pollution, offering a

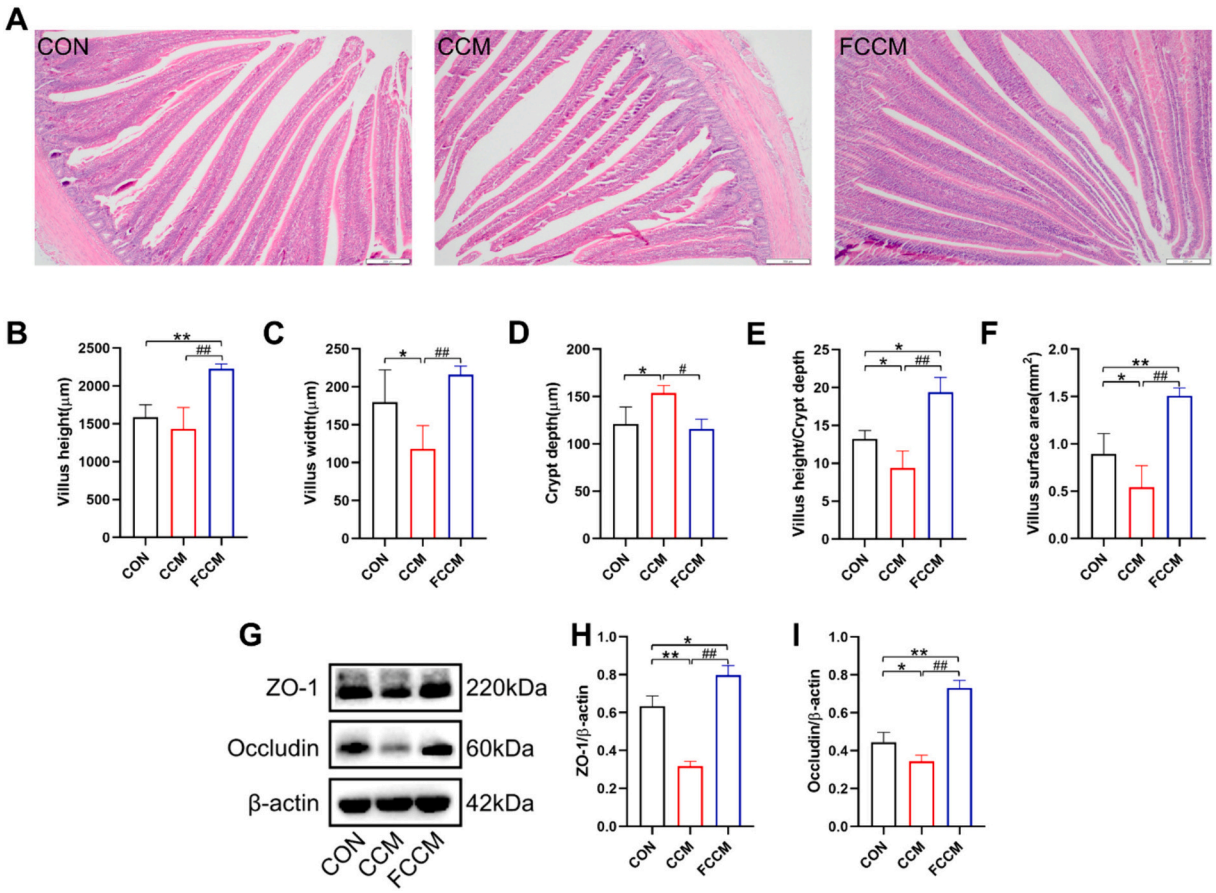

Fig. 6. Effect of corn gluten meal-corn husk mixes (CCM) on the gut morphology and barrier of chicks before and after fermentation. Representative H&*E*-stained jejunum sections, scale bar 200 μm (A); Villus height (B); Villus width (C); Crypt depth (D); Villus height/crypt depth (E); Villus surface area (F); Western blot analysis of tight junction proteins ZO-1 and Occludin (G-I). Values are means ± SD, n = 5. CON: untreated basal diet; CCM: diet supplemented with 10% CCM; FCCM: diet supplemented with 10% fermented CCM. * $p < 0.05$, ** $p < 0.01$ compared with the CON. # $p < 0.05$ and ## $p < 0.01$ for CCM *vs.* FCCM.

viable solution for the application of CCM in animal feed.

CRediT authorship contribution statement

Shuai Zhang: Writing – review & editing, Writing – original draft. **Zhaoxin Huang:** Investigation. **Qining Li:** Formal analysis. **Xin Zheng:** Methodology. **Jingsheng Liu:** Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by the National Key Research and Development Program of China (No. 2021YFD2101003-2), the supported by the earmarked fund for Agriculture Research System of China (CARS-02), the Science and Technology Development Plan Program of Jilin Province, China (No. 20220203096SF), and the scientific and technological innovation team project for outstanding young and middle-aged of Jilin Province (20230508014RC).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101656.

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