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Current Research in Food Science 12 (2026) 101357

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Characteristic of different thermal treatment-induced dough protein oxidation and its impact on human gut microbiota

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ARTICLE INFO

Handling Editor: Professor Alejandro G. Marangoni

Keywords:
Thermal processing
Dough
Protein oxidation
Amino acid oxidation
Gut microbiota

ABSTRACT

The thermal processing methods of flour products are diverse. This study aims to explore the effects of these methods (e.g., steaming, high-pressure, frying, baking, air frying, and microwave) on wheat dough protein oxidation and these oxidized proteins on human gut microbiota composition. We found that the different methods resulted in varying degrees of browning, decreased protein digestibility, and altered water distribution, protein noncovalent interactions, and surface hydrophobicity in the dough; the protein structure changed from α-helix and random coil to β-sheet and β-turn, and showed varying degrees of distinct aggregation and cross-linking. The protein oxidation products demonstrated elevated levels of carbonyl compounds, advanced glycation end products, and Schiff bases to varying extents, accompanied by a reduction in free amino and sulfhydryl group contents. Additionally, decreases in tryptophan and tyrosine concentrations were observed, while oxidation-derived amino acid products—namely dityrosine, kynurenine, and N'-formylkynurenine—displayed an increase in concentration. Under *in vitro* anaerobic fermentation conditions, thesev oxidized proteins further disrupted the human gut microbiota at different levels, notably raising the abundance of pro-inflammatory bacteria such as *Fusobacterium*, *Bilophila*, and *Sutterella*, and lowering the abundance of anti-inflammatory bacteria including *Bacteroides*, *Phascolarctobacterium*, and *Oscillibacter*. The findings indicate that different thermal processing methods induce protein oxidation to differing degrees, and these oxidative products consumption may lead to varying extents of gut microbiota dysbiosis, with baking and air frying exhibiting the most pronounced effects. Thus, the choice of processing methods for flour-based products should take into account the potential for protein oxidation.

1. Introduction

Wheat, one of the world's most extensively cultivated crops, is a staple food in many countries. Wheat flour typically contains 8–12% protein (Day et al., 2021), which, although not exceptionally high, constitutes a primary protein source for populations with wheat-based diets. Industrial advancements have introduced various thermal processing methods for flour products, such as steaming, high-pressure, frying, baking, air frying, and microwaving (Devi et al., 2020). These methods changed the flavor, texture, and structure of wheat-based products (Joycelyn et al., 2013; Kukurová et al., 2009; Öztürk Kerimoğlu et al., 2019; Zhou et al., 2021). Numerous studies have investigated the effects of thermal processing on the structure, functionality, and composition of animal proteins (Lu et al., 2025; Wen et al., 2023; Zhuang et al., 2023), particularly focusing on protein oxidation. This process involves protein cross-linking and modification, leading to the accumulation of oxidative products. Key representatives include carbonyl compounds (Tan et al., 2024), advanced glycation end

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https://doi.org/10.1016/j.crfs.2026.101357
Received 22 December 2025; Received in revised form 7 February 2026; Accepted 16 February 2026
Available online 17 February 2026

products (AGEs) (Liu et al., 2025), and dityrosine (Liu et al., 2015). Substantial evidence indicates that excessive dietary intake of these oxidized proteins can induce oxidative stress and chronic inflammation (Tovbin et al., 2002), which are associated with accelerated aging and the development of age-related diseases such as diabetes (Schröter and Höhn, 2018), Alzheimer's disease (Butterfield and Lauderback, 2002), Parkinson's disease (Butterfield and Kanski, 2001). In recent years, the effects of thermal processing methods on plant proteins have begun to attract the attention of researchers, especially given the importance of flour products as a staple food. For instance, heating wheat protein at 100 °C promotes aggregation, thereby impairing its digestibility (Rahaman et al., 2016). Elevated temperatures during hot-air drying enhance protein cross-linking and network formation (Wang et al., 2024), while frying induces covalent gluten polymerization, reducing protein extractability (Riley et al., 2024). Metabolomic analyses further reveal that prolonged baking drives the accumulation of metabolites derived from amino acids such as tyrosine and tryptophan, suggesting progressive thermal degradation (Cheng et al., 2022b). Collectively, these findings imply that thermal processing can markedly affect the structure, functionality, and oxidative state of dough proteins. However, previous research has typically examined only one or two processing methods in isolation. A systematic comparison and evaluation of the effects of various common thermal processing techniques on dough protein oxidation remain lacking, which is the focus of this study.

The gut microbiota is crucial for numerous physiological processes, including digestion, nutrient absorption, metabolism, and host health regulation (Ramakrishna, 2012). It is often referred to as the “second genome” of humans and plays a pivotal role in maintaining intestinal health and the host's metabolic homeostasis (Zhu et al., 2021). Furthermore, mounting evidence underscores the substantial impact of the gut microbiota on the prevention and management of a wide array of diseases, including chronic inflammation (Yan et al., 2021), aging (Lv et al., 2023), and age-related diseases (Cai et al., 2022). As a primary nitrogen source for intestinal microorganisms, dietary proteins are indispensable for the growth and multiplication of microorganisms (Dai et al., 2015). Throughout the digestive process in the small intestine, proteins are subject to hydrolysis by proteases and peptidases, which converts them into absorbable amino acids and oligopeptides (Yan et al., 2024). However, dietary proteins that have undergone oxidative aggregation are less readily absorbed, allowing them to transit to the colon where they can be metabolized into harmful compounds and alter the composition of the gut microbiota (Davila et al., 2013). This underscores that the composition and structural integrity of dietary proteins are key factors in regulating the microbial community and, consequently, host health (Li et al., 2023b). *In vitro* fermentation models have demonstrated that cooking-induced oxidation of chicken protein (e.g., baking, microwaving, frying) gives rise to a notable elevation in the abundance of *Fusobacterium*, which is associated with serious health risks (Lv et al., 2023). Similarly, Wu et al. (2025) found that refrigerated storage-induced oxidation in rice and steamed bread proteins increased the abundance of pro-inflammatory bacteria (e.g., *Sutterella* and *Tyzzerella*) while decreasing anti-inflammatory bacteria (e.g., *Bifidobacterium* and *Phascolarctobacterium*) during *in vitro* human colonic fermentation. Given the diversity of processing methods for flour products, each may induce varying degrees of change in protein structure. The subsequent intake of these modified proteins is likely to impact the composition of the gut microbiota. Nevertheless, the specific effects of different thermally processed dough proteins on the human intestinal microbiota remain to be systematically elucidated.

Therefore, we hypothesize that daily thermal treatments (steaming, high-pressure, frying, baking, air frying, and microwave) induce varying degrees of oxidation in dough proteins, and that these oxidized proteins differentially alter the gut microbiota of human. To test this hypothesis, we initially analyzed the color, water distribution and structure, non-covalent interaction force, surface hydrophobicity, protein digestibility, protein secondary structure, protein molecular weight distribution, and confocal laser scanning microscope (CLSM) images to evaluate the characteristic and structure of the dough proteins. Subsequently, key protein oxidation markers were determined, including representative protein oxidation products, amino acid composition, and specific amino acid oxidation products. Finally, we measured the gut microbiota composition to further analyze the effects of these oxidized dough proteins induced by different thermal treatments on the human gut microbiota using an *in vitro* anaerobic fermentation model. The outcomes of our research are capable of offering a theoretical foundation for the choosing of flour-product cooking methods that prioritize health. Moreover, they can facilitate a systematic comprehension of the correlation between the thermal processing of these products and human intestinal health.

2. Materials and methods

2.1. Materials

Wheat flour was obtained from a supermarket in Zhengzhou (Yihai Kerry Arawana Holdings Co., Ltd., Zhengzhou, China). Its proximate composition (per 100 g) was as follows: protein, 12.20 g; energy, 1496 kJ; fat, 1.60 g; moisture, 13.78 g; carbohydrate, 72.30 g; ash, 0.58 g. Soybean oil was sourced from the same supplier. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford kits were all purchased from Shanghai Biotechnology Bioengineering Co., Ltd (Shanghai, China). The HiPure Soil DNA Kit was supplied from Shanghai Maihe Biotechnology Co., Ltd (Shanghai, China). The QubitÂ® dsDNA HS Assay Kit was purchased from Thermo Fisher Scientific (Waltham, USA). All chemical reagents used were of analytical grade.

2.2. Sample preparation

The dough (15 g, water: flour = 1:2, v: m) was accurately weighed and pressed into pieces of uniform dimensions (same length, width, and height) using a mold. These dough pieces were randomly allocated to the subsequent treatment groups: control, steaming, high-pressure, frying, baking, air frying, and microwave. Thermal processing was applied based on common cooking methods for flour products: (a) control group, untreated dough; (b) steaming group, steamed at 100 °C for 20 min (Steam kettle, Midea Group Co., Ltd., Guangdong, China); (c) high-pressure group, treated at 121 °C for 20 min in an autoclave (LDZX-50KBS, Shanghai Shen'an Medical Instrument Co., Ltd., Shanghai, China); (d) frying group, fried at 180 °C for 10 min, after which surface oil was blotted (Magnetic stirrer heating pot ZNCL-DG, Bohua Instrument Equipment Co., Ltd., Zhengzhou, China); (e) baking group, baked at 200 °C for 20 min (Cabinet oven PT3540, Midea Group Co., Ltd., Guangdong, China); (f) air frying group, treated at 200 °C for 20 min (Air fryer AUX-3002; AUX Group Co., Ltd., Ningbo, China); (g) microwave group, treated in a microwave oven at 200 °C for 5 min (Microwave oven P20D20TJ-D3, Guangdong Galanz Group Co., Ltd., Guangdong, China).

All treatments were performed in triplicate. The processed samples were subsequently freeze-dried, ground into powder, and stored at −20 °C for further analysis. The crude protein content in the dough (calculated as total nitrogen × 6.25) was determined using the Kjeldahl method, while the protein content in solution was measured with a Bradford kit.

2.3. Dough characteristic index

2.3.1. Color

The dough color was detected using a chroma meter according to previously published literature (Liu et al., 2023a), with the lightness (L*), redness (a*), and yellowness (b*) values were recorded to represent the color changes.

2.3.2. Water distribution

The distribution of water in the dough was investigated using low-field nuclear magnetic resonance (LF-NMR) conforming to a documented experimental procedure (Hu et al., 2022). Briefly, a 0.7 g dough sample was introduced into an NMR tube, which was then situated within the NMR probe. The transverse relaxation time (T_2) was determined by employing the Carr-Purcell-Meiboom-Gill sequences with the following parameters: EchoCount = 13,000, EchoTime = 0.1 ms, SW = 200 kHz, TD = 259,996, TW = 2000 ms, NS = 64, and TE = 0.1 ms.

2.3.3. Non-covalent interaction force

Non-covalent interaction force in the dough was evaluated based on a previously described procedure (Yang et al., 2023). Four different extraction solutions (A: 0.05 M NaCl; B: 0.6 M NaCl; C: 0.6 M NaCl with 1.5 M urea; D: 0.6 M NaCl with 8 M urea) were prepared. Then, 5 mL of each solution was mixed with 250 mg of the sample. Subsequent to the incubation phase, a Bradford assay kit was utilized to quantify the soluble protein concentration within the mixtures. The contribution of hydrogen bonds was determined by subtracting the amount of soluble gluten protein extracted by solution B from that by solution C; for ionic bonds, it was calculated as the difference between the amount extracted by solution B and solution A; and for hydrophobic interactions, it was found by subtracting the amount extracted by solution C from that by solution D.

2.3.4. Protein surface hydrophobicity

The value was estimated according to the method put forward by Wang et al. (2023a). Specifically, 100 mg of the sample was dispersed in 2 mL of phosphate buffer solution (PBS). Subsequently, 20 μL of an 8 mmol/L 8-Anilino-1-naphthalenesulfonic acid solution was added into the dispersion. The mixture was gently stirred for 1 min and then incubated in the dark for 15 min. The fluorescence intensity then detected at an Ex (excitation) = 390 nm and Em(emission) = 470 nm.

2.3.5. In vitro protein digestibility

In vitro protein digestibility was evaluated using a two-step gastric-trypsin digestion protocol adapted from Sharma and Kapoor (1996).

2.4. Protein structure analysis

2.4.1. Secondary structure

Protein secondary structure was analyzed by Fourier transform infrared (FTIR) spectroscopy (ALPHA spectrometer, Bruker, Germany), in accordance with previously published procedures (Liu et al., 2017a).

2.4.2. SDS-PAGE

SDS-PAGE was performed according to a modified protocol reported by Yang et al. (2024). A discontinuous gel system was utilized, made up of a 5% stacking gel having a pH of 6.8 and a 12% separating gel having a pH of 8.8. An amount of 10 μL of the supernatant from dough protein extraction was pipetted into each well. Electrophoresis was performed at a constant electric potential of 150 V. Subsequently, the gels were stained with a 0.2% Coomassie Brilliant Blue R-250 preparation in 25% methanol/10% acetic acid and destained in a 5% methanol/10% acetic acid formulation.

2.4.3. CLSM

The microstructure changes of the samples were analyzed by CLSM (Li et al., 2023a). The freeze-dried dough was frozen at −80 °C for 12 h and cross-sectioned into slices with a thickness of 15 μm. The protein network was stained with a 0.0001% rhodamine B solution under dark conditions at room temperature for approximately 20 min. Excess dye was gently rinsed off with distilled water before observation. The gluten network structure was characterized using AngioTool software, which quantified the following parameters: total number of junctions, gluten area (10^4 μm^2), total gluten length (10^4 μm), and number of endpoints (10^2).

2.5. Analysis of protein oxidation products

2.5.1. Carbonyl content

Carbonyl content (nmol/mg) was quantified according to a published 2,4-dinitrophenylhydrazine (DNPH) method (Soglia et al., 2016). The absorbance of the obtained solution was measured at 367 nm using a spectrophotometer (UV-6100S, MAPADA, China).

2.5.2. Free SH and amino contents

The free SH content was measured in accordance with the 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) method detailed by Zhou et al. (2019). Refer to the above method to process the sample. SH content (nmol/g) = $73.53 \times A_{412}/C$; where C denotes the protein concentration (mg/mL). Free amino content was assayed using the o-phthalaldehyde (OPA) method adapted from Gujral and Rosell (Gujral and Rosell, 2004). A standard curve, which had been prepared with L-serine in the concentration range of 0–200 mg/L, was employed for the purpose of quantification.

2.5.3. AGEs content

AGEs content was evaluated by the fluorescence method of Wang et al. (2023b). Refer to the above method to process the sample. The supernatant was measured at Ex/Em wavelengths of 390/480 nm.

2.5.4. Schiff base content

The values was assessed according to the method described by Xia et al. (2021). 250 mg samples were suspended in 5 mL of PBS. The supernatant was analyzed by fluorescence spectroscopy with an Ex = 360 nm, and the Em spectrum from 380 to 600 nm.

2.6. Analysis of amino acid composition and amino acid oxidation products

2.6.1. Amino acid composition

Amino acid composition (except for tryptophan) was analyzed following a modified acid hydrolysis method (Zhou et al., 2015). Refer to the above method to process the sample. Amino acids were quantified using an automatic amino acid analyzer (Sykam S433D, Germany). Based on the method proposed by Mario et al. (2008), the tryptophan content was measured using fluorometry. Refer to the above method to process the sample. The fluorescence intensity of the supernatant was recorded with Ex = 283 nm and Em scanning from 250 to 400 nm. The intensity was used as an indicator of relative tryptophan content.

2.6.2. Dityrosine content

Dityrosine formation was assessed by acid hydrolysis coupled with fluorescence detection, adapted from Xiong et al. (2009). Refer to the above method to process the sample. The supernatant was analyzed for fluorescence at Ex/Em wavelengths of 292/410 nm.

2.6.3. Determination of kynurenine and N′-formylkynurenine

The oxidation products kynurenine and N′-formylkynurenine were measured according to Wang et al. (2023b). Refer to the above method to process the sample. Kynurenine was detected at Ex/Em wavelengths of 365/480 nm, and N′-formylkynurenine at 325/434 nm.

2.7. In vitro anaerobic fermentation assays

In vitro anaerobic fermentation was employed to evaluate the impacts of protein oxidation on human gut microbiota composition (Li et al., 2025; Tao et al., 2025). Proteins extracted from dough subjected to different thermal treatments served as the sole nitrogen sources in the fermentation medium.

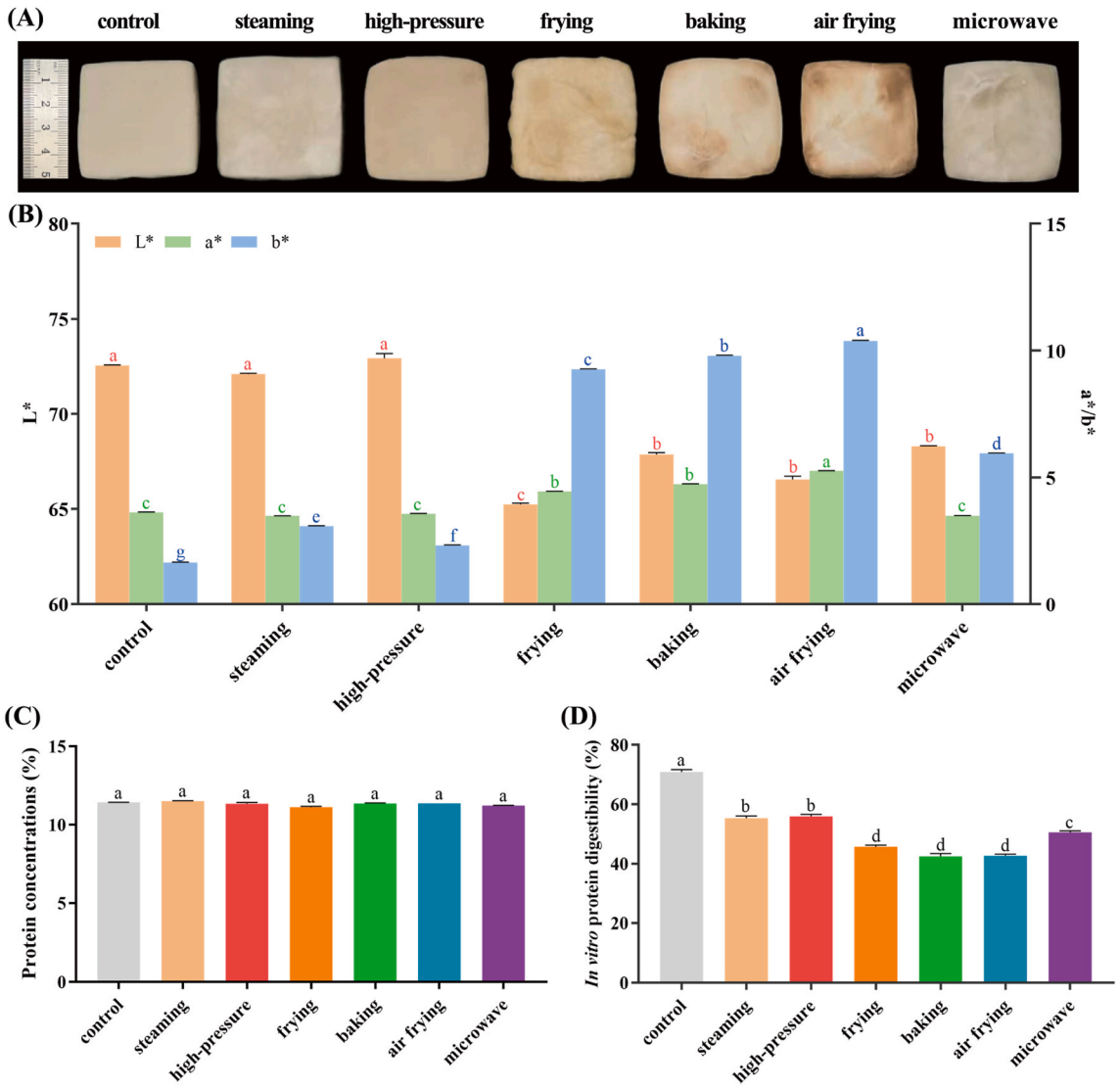

Fig. 1. Appearance (A), color (L*, a* and b*) value (B), protein content (C), *in vitro* protein digestibility (D) of dough under different thermal processing methods. Different letters indicate a significant difference ($p < 0.05$).

2.7.1. Preparation of dough protein

Dough proteins were extracted using the Osborne fractionation method (Osborne and Voorhees, 1894). The crude protein extract was further purified by ultrafiltration (Kumar et al., 2021), yielding a final product with a protein content above 95%. The resulting protein, after simulated gastric and intestinal digestion, was prepared as a 5 mg/mL solution for fermentation.

2.7.2. Preparation of basic medium

A modified basal medium was prepared according to Ji et al. (2024). Key components per 800 mL included: 2 g of yeast extract powder, 0.01 g of calcium chloride hexahydrate, 2 g of sodium bicarbonate, 0.1 g of sodium chloride, 0.04 g of potassium dihydrogen phosphate, 0.04 g of dipotassium hydrogen phosphate, 0.05 g of hemin, 0.5 g of L-cysteine, 0.01 g of magnesium sulfate heptahydrate, 2 mL of Tween 80, 0.5 g of bile salt, 4 mL of azure, and 10 μL of vitamin K1. The pH of the medium was regulated to 6.5. After sterilization treatment, 8 mL of the medium was allotted to each fermentation tube. Then, the medium was boiled, cooled, and purged with nitrogen.

2.7.3. Preparation of fecal inoculum

Fecal samples were gathered from six healthy volunteers aged 18-25 (3 males and 3 females) in strict accordance with ethical guidelines. Informed consent was meticulously obtained from each participant, ensuring their full understanding and voluntary participation. The study was thoroughly reviewed and approved by the Biomedical Ethics Committee of Henan University of Technology (Approval No. HAUTETHI-20230208). A 10% (w/v) fecal slurry was precisely prepared in PBS to generate the bacterial suspension, following the standardized protocols (Gong et al., 2025).

2.7.4. Intestinal microbial culture

Fermentation tubes containing basal medium were inoculated with bacterial suspension and the prepared protein solution at a ratio of 8:1:1. Each treatment was repeated three times. Fermentation was performed under anaerobic conditions at 37 °C for a period of 48 h. Once the fermentation process had ended, the samples were centrifuged and then preserved at −80 °C.

2.7.5. DNA extraction and sequencing

A commercial kit was utilized to extract the total microbial DNA from the fermentation pellets in a highly standardized manner. The DNA concentration was accurately measured using the Qubit® dsDNA HS Assay Kit. Only those samples that conformed to the pre-determined

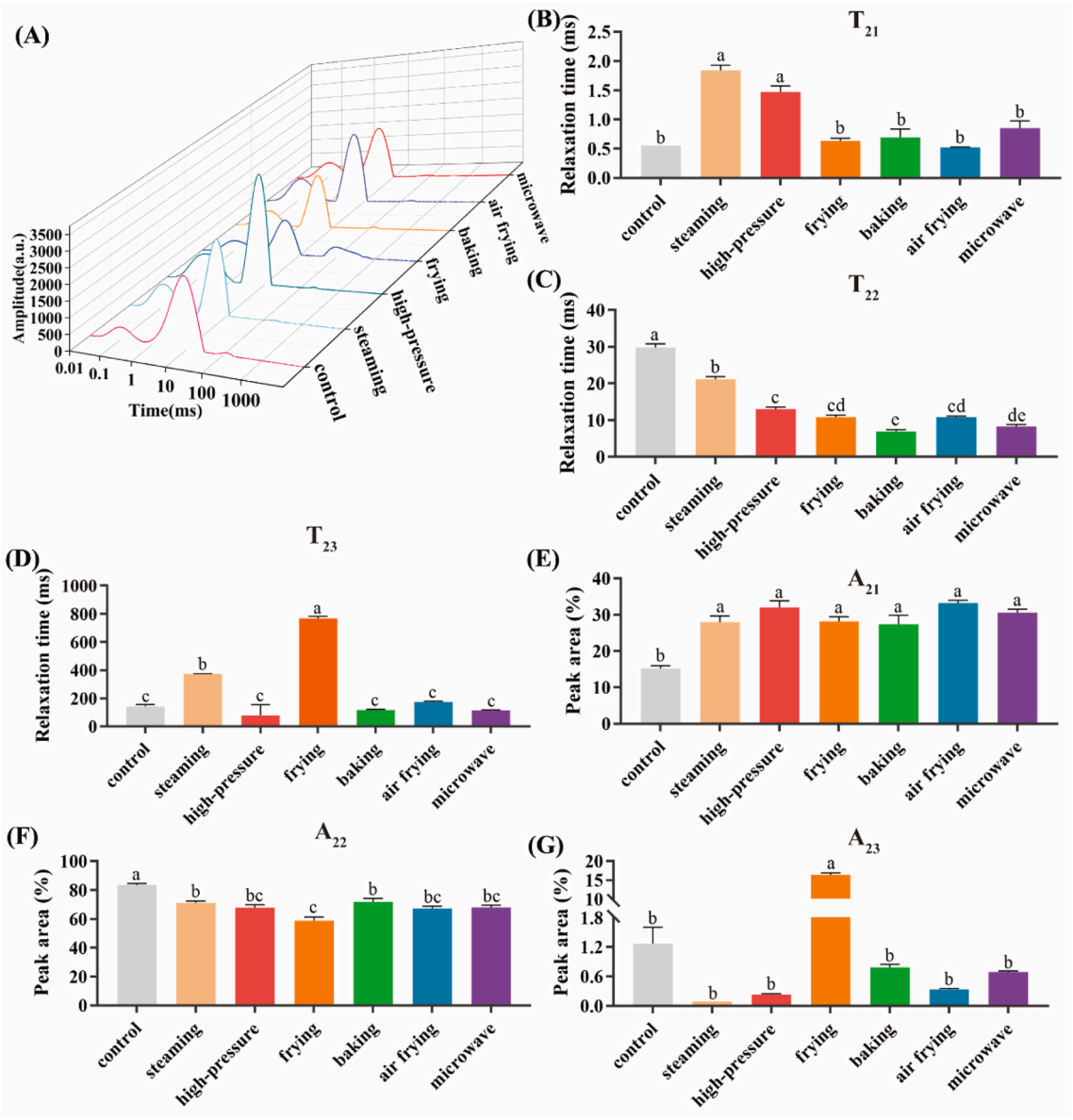

Fig. 2. Water distribution of dough under different thermal processing methods: relaxation time of dough distribution sequence (A); changes of the bond water (T_{21}), weakly bound water (T_{22}), and free water (T_{23}) proton transverse relaxation times (B-D); the peak area percentage of bond water (A_{21}), weakly bound water (A_{22}), and free water (A_{23}) proton (E-G). Different letters indicate a significant difference ($p < 0.05$).

quality parameters were advanced to the next steps, which consisted of PCR amplification, library construction, and sequencing on the Illumina MiSeq platform with the 16S rDNA gene as the specific target. Bioinformatics analysis, including the precise clustering of OTUs and in-depth alpha- and beta-diversity analysis, was performed with the application of QIIME (v1.9.1) and R (v3.1.1). The visualization of the results was carried out in a scientific and accurate way using HemI 1.0.3.3 and GraphPad Prism (v8.0) software.

2.8. Principal component analysis (PCA)

Differences among thermally treated groups were assessed using three-dimensional principal component analysis (3D-PCA) in SIMCA-P^+ software (v14.1, Umetries, Sweden). The analysis assimilated data on protein oxidation products, amino acid oxidation products, as well as the relative abundance of differential microorganisms from the fermentation broth. The integrity of the model was evaluated by means of the cumulative R^2X (which reflects the goodness-of-fit) and Q^2 (which reflects the predictive ability) parameters. The thresholds of $R^2X > 0.50$ and $Q^2 > 0.50$ were established, indicating robust model performance with satisfactory goodness-of-fit and predictive capability (Liu et al., 2023a).

2.9. Statistical analysis

All samples and tests were replicated three times. All experiment data were analyzed using SPSS Statistics 23, and the results were expressed as mean ± standard error mean (SEM). The one-way ANOVA and Tukey's HSD were used to analyze the differences between groups, and the results were expressed in different letters ($p < 0.05$).

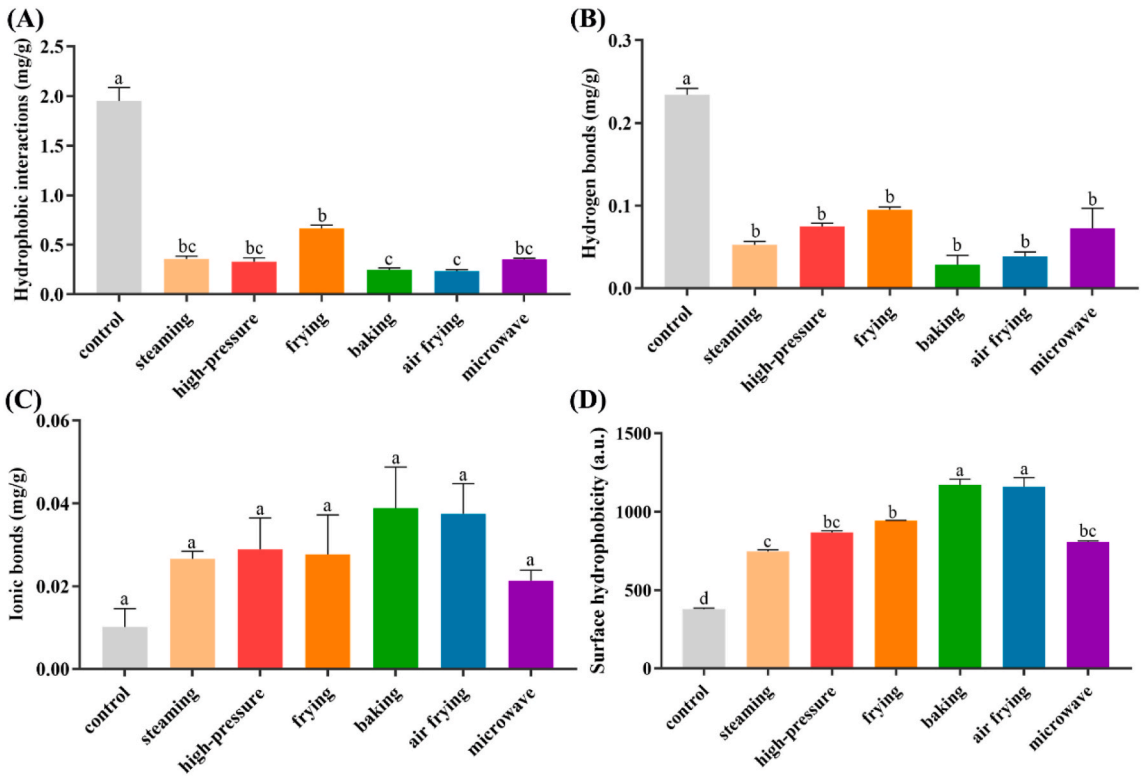

Fig. 3. Non-covalent interaction forces and surface hydrophobicity of dough protein under different thermal processing methods: Hydrophobic interactions (A), Hydrogen bonds (B), Ionic bonds (C); and surface hydrophobicity (D). Different letters indicate a significant difference ($p < 0.05$).

3. Results and discussion

3.1. Effects of different thermal processing methods on the color and protein content and digestibility of dough

Fig. 1A shows that the six different thermal processing methods have visibly altered the appearance of the dough, manifesting as different degrees of browning. As shown in Fig. 1B, compared with the control group, frying, baking, air frying, and microwave treatment generally reduced the L* value of the dough ($p < 0.05$). Meanwhile, the results showed that the a* values of frying, baking, and air frying groups increased significantly, while the b* values of dough increased generally ($p < 0.05$). The results supported that different thermal processing methods induced different degrees of browning of dough. This color development is attributed to pigment formation via the Maillard reaction between proteins and reducing sugars during heating, resulting in an attractive golden-yellow color (Liu et al., 2014). As shown in Fig. 1C, no significant difference in the crude protein content among the six thermal-treated samples and the control group, indicating that thermal processing did not significantly alter the protein content of the dough. However, the results found that all six thermal processing methods significantly reduced the digestibility of dough protein compared to the control group ($p < 0.05$, Fig. 1D). This phenomenon may occur because thermal-induced protein cross-linking promotes aggregation and denaturation, which masks the specific cleavage sites required for pepsin and trypsin activity (Guan et al., 2011). At the same time, structural modifications such as protein shrinkage, dehydration, and conformational changes have been shown to hinder the hydrolysis of gastrointestinal proteins (Bhat et al., 2021; Suleman et al., 2020). Semedo et al. (2018) explored the effects of boiling, baking, and frying on the *in vitro* digestibility of hairtail protein and found that all the three thermal processes reduced it, and this finding is similar to our results.

3.2. Effects of different thermal processing methods on the water distribution of dough

The effects of different thermal processing methods on water distribution in dough were analyzed using LF-NMR to measure water state and mobility. T_2 and corresponding peak area (A_2) were measured for doughs subjected to six thermal treatments (Fig. 2A). Based on the T_2 relaxation spectra (Fig. 2B–G), water in dough was categorized into three states: bound water (T_{21}, A_{21}), which is tightly associated with polar groups on protein surfaces; weakly bound water (T_{22}, A_{22}), which has restricted mobility within the dough matrix; and free water (T_{23}, A_{23}), which resides in interstitial spaces and flows freely (Jia et al., 2017; Lee et al., 2002; Marcone et al., 2013; Peters et al., 2023). Compared to the control group, all six thermal processing methods significantly decreased the T_{22} relaxation time ($p < 0.05$), while the effects on T_{21} and T_{23} varied. This decrease in T_{22} suggests that thermal processing restricts the mobility of weakly bound water, likely due to shrinkage of the dough's network structure. Analysis of the peak area ratios revealed that all thermal treatments significantly increased the proportion of bound water (A_{21}) and decreased the proportion of weakly bound water (A_{22}) ($p < 0.05$). The proportion of free water (A_{23}) remained largely unchanged, except for a significant increase in the frying group. These changes indicate that thermal processing generally promotes the conversion of weakly bound water to bound water. The unique increase in free water observed after frying may be attributed to the coating of proteins by oil, which enhances surface hydrophobicity and releases previously confined water. These findings are consistent with previous studies. Savadkoohi and Farahnaky (2012) confirmed that high temperatures induce the formation of protein polymers via covalent bonds, thereby strengthening water-binding capacity. Similarly, Yang et al. (2023) observed reduced water mobility and increased bound water content in wheat dough with increasing preheating temperature. Consequently, the six thermal processing methods tested here all enhance the protein-water binding strength to varying degrees,

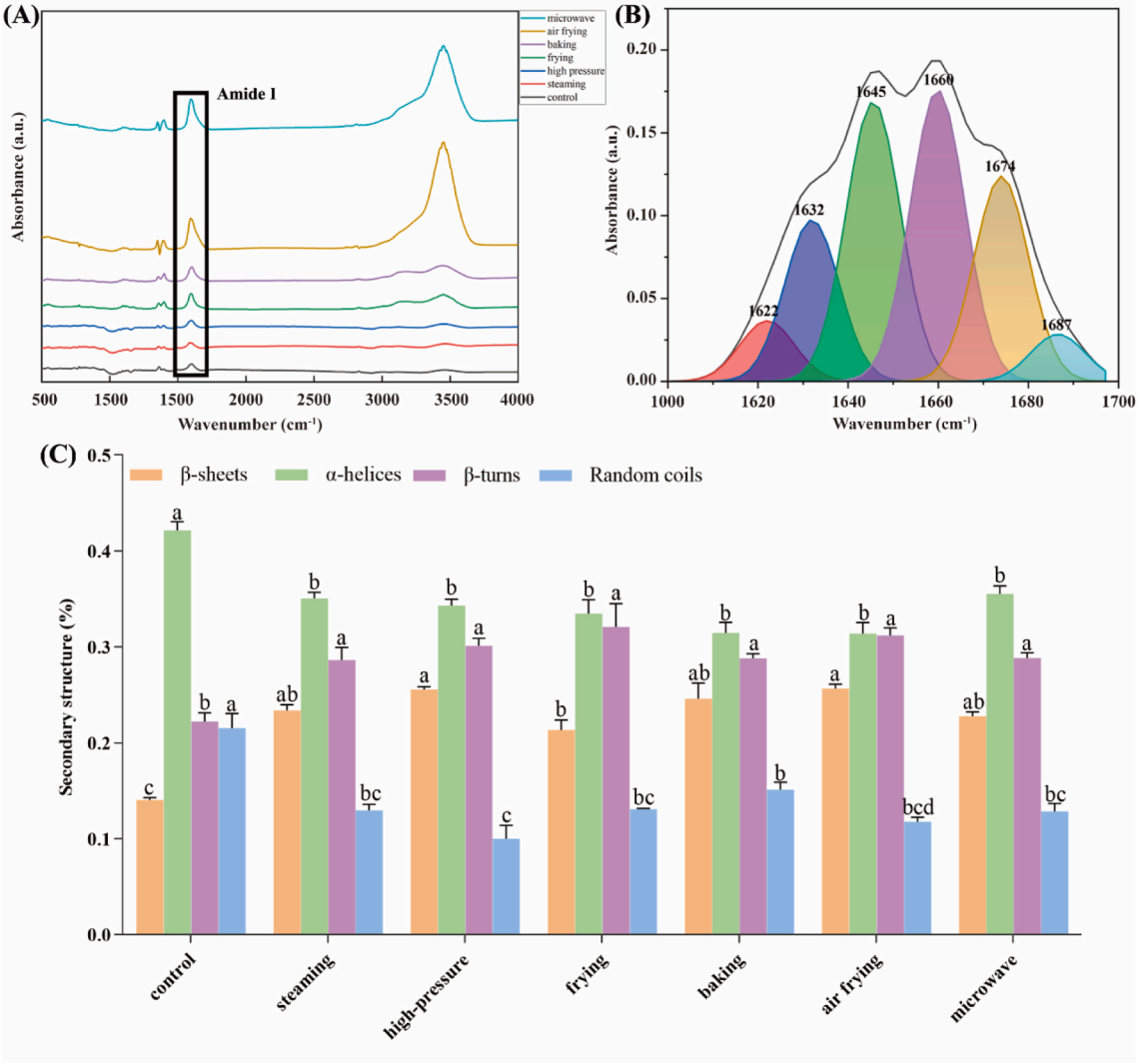

Fig. 4. Secondary structure of dough protein under different thermal processing methods: full-wavelength scanning of fluorescence spectra (A), secondary structure distribution simulation diagram (B), percentage of secondary structure (C). Different letters indicate a significant difference ($p < 0.05$).

indicating that protein aggregation and denaturation occurred.

3.3. Effects of different thermal processing methods on non-covalent interaction forces and surface hydrophobicity of dough protein

To quantify the relative strength of different non-covalent interactions that maintain protein structure in dough, ionic bonds, hydrogen bonds, and hydrophobic interactions were measured (Liang et al., 2024). Compared with the control group, the six thermal processing methods did not significantly alter ionic bond levels ($p > 0.05$, Fig. 3C) but significantly reduced both hydrogen bonds and hydrophobic interactions ($p < 0.05$, Fig. 3A and B). The contribution of ionic bonds to the overall protein stability was significantly lower than that of hydrophobic interactions and hydrogen bonds, indicating that the latter two play a dominant role in determining dough protein conformation (Kong et al., 2022). Hydrogen bonds are a primary force stabilizing protein secondary structure and are essential for maintaining the stability of carbonyl and amide groups in the protein backbone (Wang et al., 2007). Hydrophobic interactions, weak forces between non-polar amino acid residues, are crucial for the properties of most proteins and drive protein folding (Zhang et al., 2024b). The observed reduction in these forces suggests that thermal processing induces changes in the dough's protein structure. As expected, all six thermal processing methods significantly increased the surface hydrophobicity of the dough proteins compared to the control group ($p < 0.05$, Fig. 3D). An increase in surface hydrophobicity is directly related to protein unfolding. Common thermal processing methods have been reported to cause protein structure to unfold, thereby exposing internal hydrophobic groups and increasing surface hydrophobicity (Zhao et al., 2022). This phenomenon is further supported by studies demonstrating an inverse relationship between protein hydrophobic activity and the degree of polymerization, highlighting the connection between hydrophobicity and macromolecular structure (Liu et al., 2023b). During heating, the unfolding and exposure of hydrophobic domains can lead to protein aggregation, manifesting as increased hydrophobicity. Thus, the elevated surface hydrophobicity observed in this study supports the conclusion that different thermal processing methods induce varying degrees of structural change in dough proteins.

3.4. Effects of different thermal processing methods on the protein structure of dough

To further elucidate the effects of different thermal processing methods on dough protein structure, FTIR spectroscopy was employed to analyze changes in protein secondary structure. The amide I band (1600–1700 cm^{-1}), which reflects protein secondary structure, was

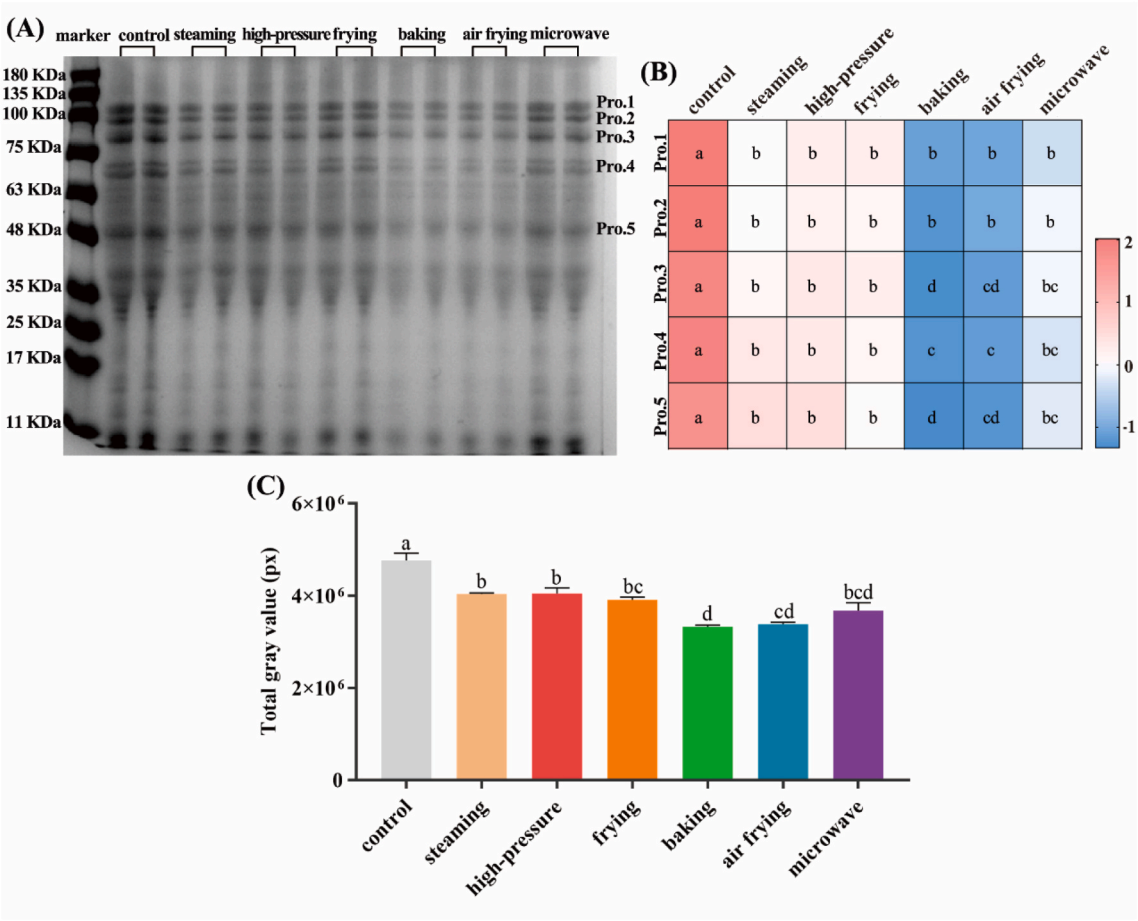

Fig. 5. Molecular weight distribution of dough protein under different thermal processing methods: SDS-PAGE protein profiles (A), band intensity heatmap of characteristic protein bands (statistical analysis of SDS-PAGE protein map using Image J software) (B), and the sum of the band intensity of the characteristic protein bands (C). Different letters indicate a significant difference ($p < 0.05$).

analyzed (Byler and Susi, 1986). Following baseline correction, Gaussian smoothing, and deconvolution of the amide I band (Fig. 4A and B), the relative content of each secondary structure component was calculated based on the ratio of its characteristic peak area to the total peak area (Choi and Ma, 2007). Compared with the control group, all thermal processing methods significantly altered the secondary structure composition ($p < 0.05$, Fig. 4C). A significant increase was observed in the proportions of β-sheet (by 7.3–11.63%) and β-turn (by 6.35–9.83%), accompanied by a significant decrease in the contents of α-helix (by 6.62–10.78%) and random coil (by 6.43–11.54%). This shift from α-helix/random coil to β-sheet/β-turn configurations indicates thermal-induced protein structural rearrangement, suggesting enhanced protein cross-linking (Liu et al., 2025). This structural transition likely occurs due to the unwinding of helices and subsequent exposure of previously buried amino acid residues, which facilitates cross-linking reactions.

To further assess the degree of protein cross-linking, SDS-PAGE was used to evaluate the protein molecular weight distribution (Li et al., 2022a). The number of electrophoretic bands was not significantly different between the control and treated samples ($p > 0.05$, Fig. 5A), indicating a consistent subunit composition. When the same amount of sample protein was loaded, the intensity of five specific protein bands was significantly reduced after all six thermal treatments ($p < 0.05$, Fig. 5B). These bands corresponded to various proteins, including non-storage proteins (10-30 kDa), α/β-glutenin and LMW-GS (30-50 kDa), γ-gluten (45-60 kDa), ω-gliadin (60-80 kDa), and HMW-GS (65-90 kDa). In addition, the total gray values of the characteristic protein bands in the samples were quantitatively analyzed. The results showed that the total band intensity of the electrophoresis bands was significantly reduced after the six thermal processing methods ($p < 0.05$, Fig. 5C). The attenuation of band intensity is likely attributable to thermal-induced protein cross-linking and aggregation, which forms large polymers too massive to enter the separation gel. This finding aligns with the study by Chu et al. (2025), who also observed a decrease in protein band intensity in cereals treated under high temperature and pressure.

3.5. *Effects of different thermal processing methods on the gluten protein network structure of dough*

To further clarify the effects of thermal processing on dough protein structure, the gluten network was analyzed using CLSM and quantitatively characterized with AngioTool software (Gao et al., 2022) (Fig. 6A). The key microstructural parameters evaluated were gluten area, total number of junctions, gluten length, and gluten end points (Fig. 6B–E). The control sample exhibited a uniformly dispersed and highly regular network structure with uniform pore size, indicating a well-connected gluten matrix. In contrast, all thermal processing methods altered the gluten network to varying degrees. Particularly under baking and air frying, significant protein aggregation was observed, accompanied by a notable increase in internal voids. Quantitative analysis further confirmed that thermal processing generally reduced the gluten area, total number of junctions, and gluten end points. By comparing these microstructural parameters, it was found that thermal processing had a significant effect on the gluten network, especially in the baking and air frying groups ($p < 0.05$), while

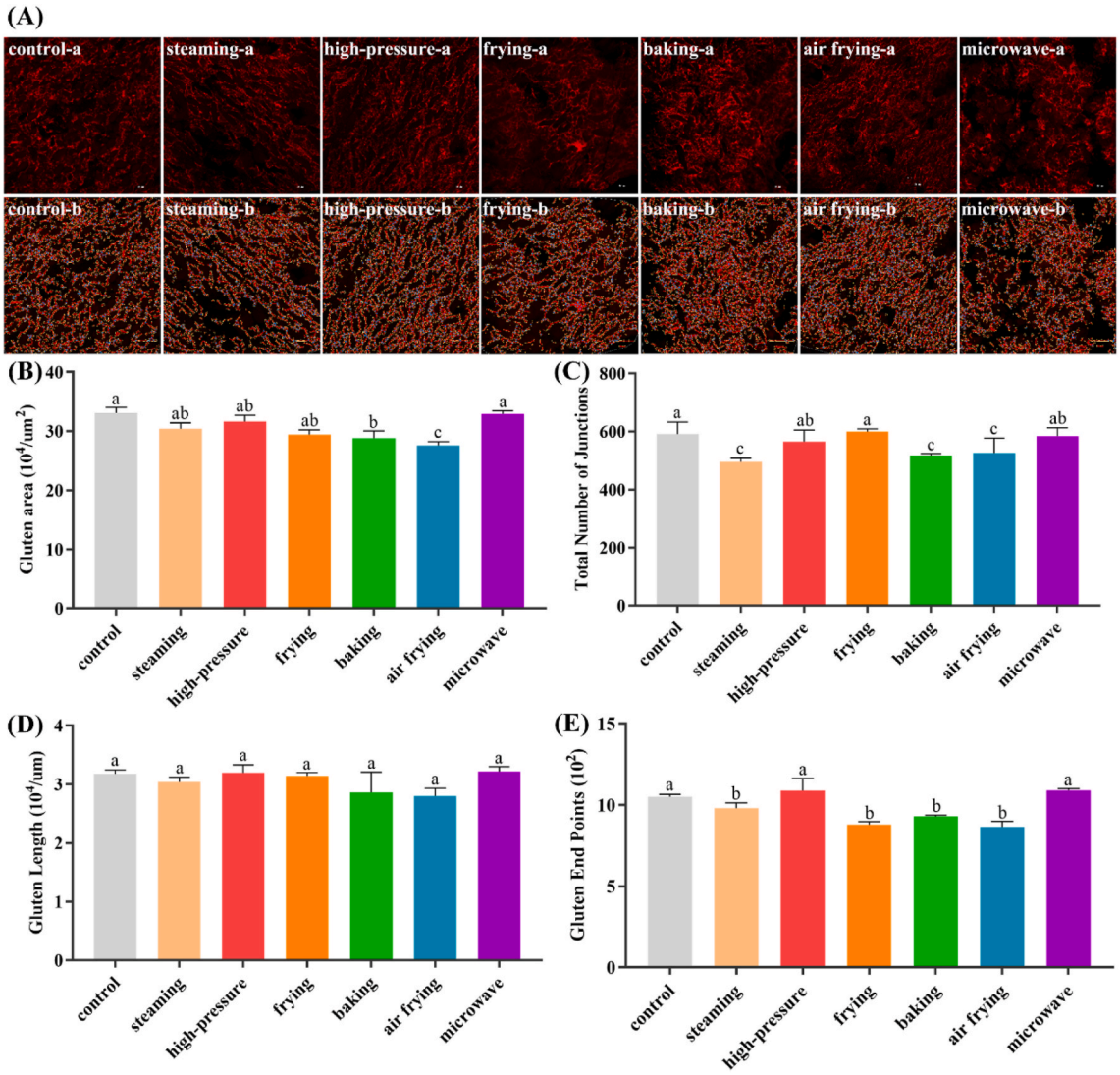

Fig. 6. Gluten protein network analysis of dough protein under different thermal processing methods: Gluten protein network analysis of doughs (a: the dough samples were stained with Rhodamin B to visualize proteins and analyzed by CLSM with the scale bar of 50 μm; b, images are the pictures processed with AngioTool, in which the junctions were shown in blue, protein skeleton in red, and protein outline/area in yellow) (A), gluten area (B), total number of junctions (C), gluten length (D), and gluten end points content (E). Different letters indicate a significant difference ($p < 0.05$).

microwave treatment had a relatively small effect on the gluten network ($p > 0.05$). These structural changes are consistent with the established principle that high-temperature treatment induces protein denaturation and aggregation (Yin et al., 2020). The findings align with those of Yang et al. (2023), who reported that increased temperature promoted a dendritic structure in the gluten network due to the aggregation of protein macromolecules. Given the reported relationship between macromolecular protein aggregation and protein oxidation (Guo et al., 2022), the results suggest that different thermal processing methods induce varying degrees of protein aggregation, implying the occurrence of protein oxidation.

3.6. Effects of different thermal processing methods on protein oxidation products of dough

To assess the impacts of different thermal processing methods on protein oxidation in dough, key oxidation markers were quantified, including carbonyl compounds, AGEs, Schiff bases, free SH groups, and free amino groups. Carbonyl compounds, which are formed by the oxidation of amino acids by reactive oxygen species or free radicals, serve as a primary indicator of protein oxidation (Li et al., 2022b). AGEs are stable end products generated by the non-enzymatic glycation of proteins with reducing sugars, while Schiff bases represent their unstable intermediate products that can further stabilize under thermal conditions (Chen et al., 2023). When proteins are attacked by free radicals, free SH groups can be oxidized to form disulfide bonds, and free amino groups may undergo oxidative deamination to yield carbonyl groups (Liu et al., 2023a). As shown in Fig. 7, all thermal processing methods significantly increased the levels of carbonyl compounds, AGEs, and Schiff bases compared to the control group ($p < 0.05$). The percentage increases relative to the control group were as follows: for carbonyls: air frying (123.33%) > baking (110.75%) > frying (98.45%) > high-pressure (85.75%) > microwave (78.54%) > steaming (70.43%); for AGEs: air frying (104.35%) > baking (73.62%) > frying (56.20%) > high-pressure (40.71%) > microwave (8.22%) > steaming (3.04%); for Schiff bases: baking (287.96%) > air frying (189.52%) > frying (90.40%) > microwave (80.80%) > steaming (63.36%) > high-pressure (54.53%). Conversely, the content of free SH groups and free amino groups decreased significantly ($p < 0.05$): for free SH groups: baking (39.92%) > air frying (36.71%) > frying (31.86%) > steaming (31.11%) > high-pressure (23.37%) > microwave (21.67%); for free amino groups: air frying (51.84%) > baking

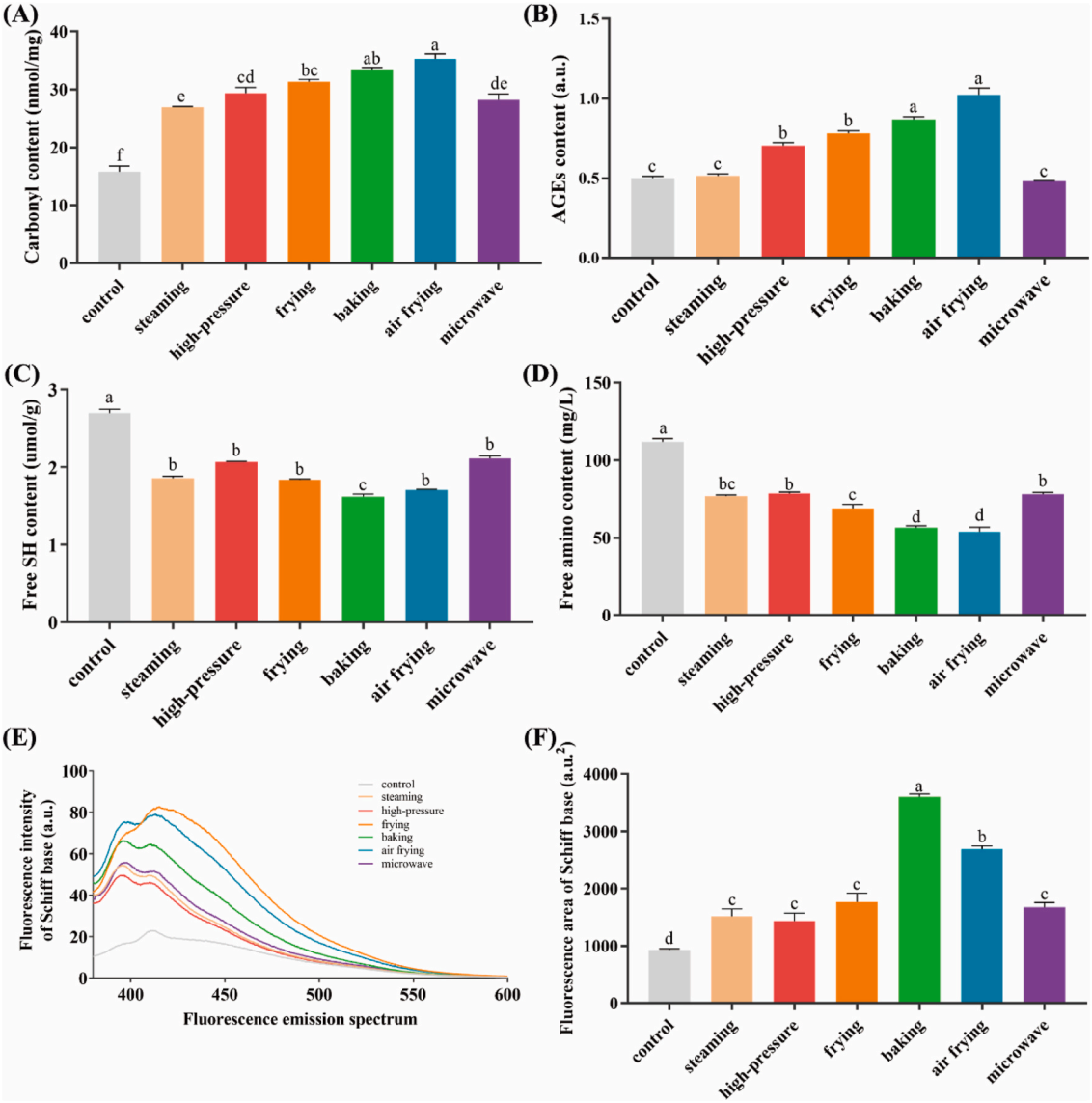

Fig. 7. Protein oxidation products analysis of dough under different thermal processing methods: Carbonyl content (A), AGEs content (B), free SH content (C), free amino content (D), fluorescence intensity of Schiff base (E), and Schiff base content (F). Different letters indicate a significant difference ($p < 0.05$).

(49.44%) > frying (38.37%) > steaming (31.26%) > microwave (30.15%) > high-pressure (29.71%). These results clearly demonstrate that different thermal processing methods induce varying degrees of protein oxidation, with baking and air frying having the most pronounced effects, while microwave treatment resulted in the least oxidation. These findings are consistent with those of Chu et al. (2025), who reported that high-temperature and high-pressure treatment of glutelin promoted protein oxidation, characterized by increased carbonyl, Schiff base, and AGEs content, along with decreased free SH and free amino groups.

3.7. Effects of different thermal processing methods on amino acid composition and amino acid oxidation products in dough

To evaluate the effects of different thermal processing methods on amino acids in dough, the amino acid composition and representative oxidation products were quantitatively analyzed (Hellwig, 2019). The results showed that the contents of asparagine, threonine, serine, glycine, alanine, cysteine, valine, isoleucine, tyrosine, histidine, and proline decreased significantly after thermal processing ($p < 0.05$, Fig. 8). Additionally, a concurrent analysis of tryptophan, based on its characteristic fluorescence intensity (which is positively correlated with concentration (Ouyang et al., 2023)), also revealed a significant reduction in its content ($p < 0.05$). These findings indicate that these amino acid residues serve as primary oxidation sites during thermal processing. Heat map analysis further demonstrated that baking and air frying had the most substantial impact on amino acid content, whereas microwave treatment resulted in relatively minor changes. Notably, tryptophan residues, which are predominantly located on the protein surface, are highly susceptible to oxidation (Estévez et al., 2008). Similarly, the phenolic hydroxyl structure of tyrosine makes it vulnerable to free radical attack (Davies, 2016).

To comprehensively assess the extent of oxidation, the levels of specific oxidation products derived from these amino acids were further quantified. The oxidation intermediates of tryptophan, N′-formylkynurenine and kynurenine, have been linked to gastrointestinal diseases and inflammation, serving as indicators of amino acid oxidation (Estévez and Luna, 2017). Dityrosine, a marker of tyrosine cross-linking, can induce oxidative stress, disrupt thyroid hormone function, and has potential carcinogenicity (Li et al., 2019). As shown in Fig. 8D–F, all thermal processing methods significantly increased the content of these oxidation products compared to the control group ($p < 0.05$). The

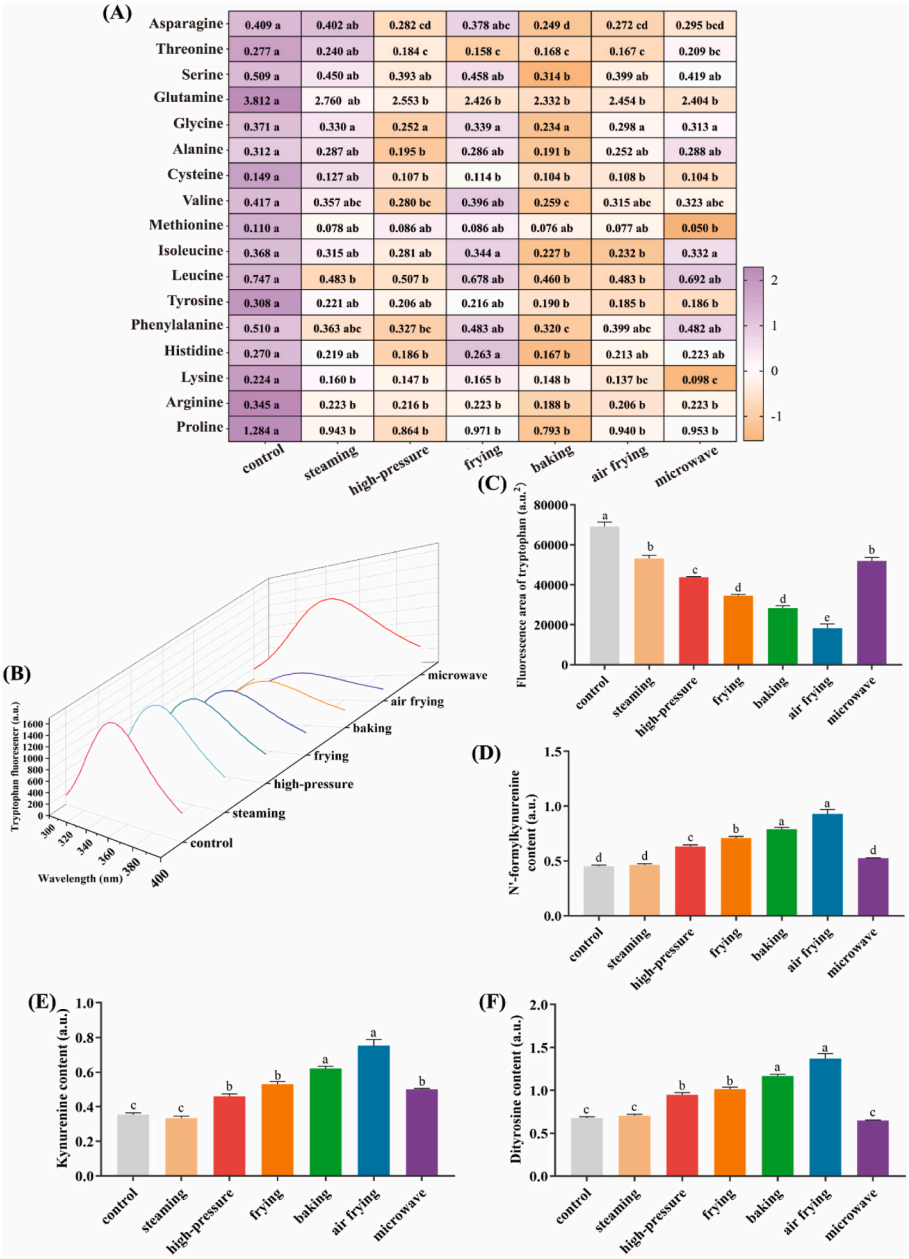

Fig. 8. Amino acid composition and amino acid oxidation products analysis of dough under different thermal processing methods: the amino acid content was shown on the heat map (g/100 g) (A), tryptophan fluorescence spectroscopy (B), tryptophan content (C), N′-formylkynurenine content (D), kynurenine content (E), and dityrosine content (F). Different letters indicate a significant difference ($p < 0.05$).

percentage increases were as follows: for N′-formylkynurenine: air frying (106.17%) > baking (75.39%) > frying (57.29%) > high-pressure (39.97%) > microwave (16.33%) > steaming (3.05%); for kynurenine: air frying (112.07%) > baking (75.17%) > frying (49.39%) > microwave (41.09%) > high-pressure (29.64%) > steaming (−5.87%); for dityrosine: air frying (102.30%) > baking (72.34%) > frying (50.00%) > high-pressure (40.07%) > steaming (4.11%) > microwave (−4.22%). Notably, air frying and baking treatments led to an accumulation of these products approximately 1.5 times that of the control group, whereas microwave and steaming had no significant effect ($p > 0.05$). These results indicate that thermal processing promotes the gradual oxidation of amino acids, leading to the accumulation of stable oxidation products in the dough matrix. In summary, based on the analysis of amino acid depletion and of oxidation product accumulation, air frying and baking were found to have the greatest impact on protein oxidation, while microwave processing had the least.

3.8. *Effects of oxidized dough proteins on human gut microbiota under in vitro anaerobic fermentation conditions*

3.8.1. *Effects of oxidized dough proteins on bacterial OTUs, alpha diversity, and beta diversity*

OTUs, defined by grouping 16S rDNA sequences at a 97% similarity threshold (Reddy and Armah, 2018), were used to analyze microbial diversity. A total of 564 OTUs were generated across all samples (Fig. 9A), with the following distribution: control (433 OTUs), steaming (431 OTUs), high-pressure (429 OTUs), frying (429 OTUs), baking (425 OTUs), air frying (425 OTUs), and microwave (416 OTUs). The rarefaction curve (Fig. 9B) indicated a linear increase in the number of OTUs as the sequence count grew to approximately 20,000. Beyond 60,000 sequences, the curve plateaued, suggesting that the sequencing depth

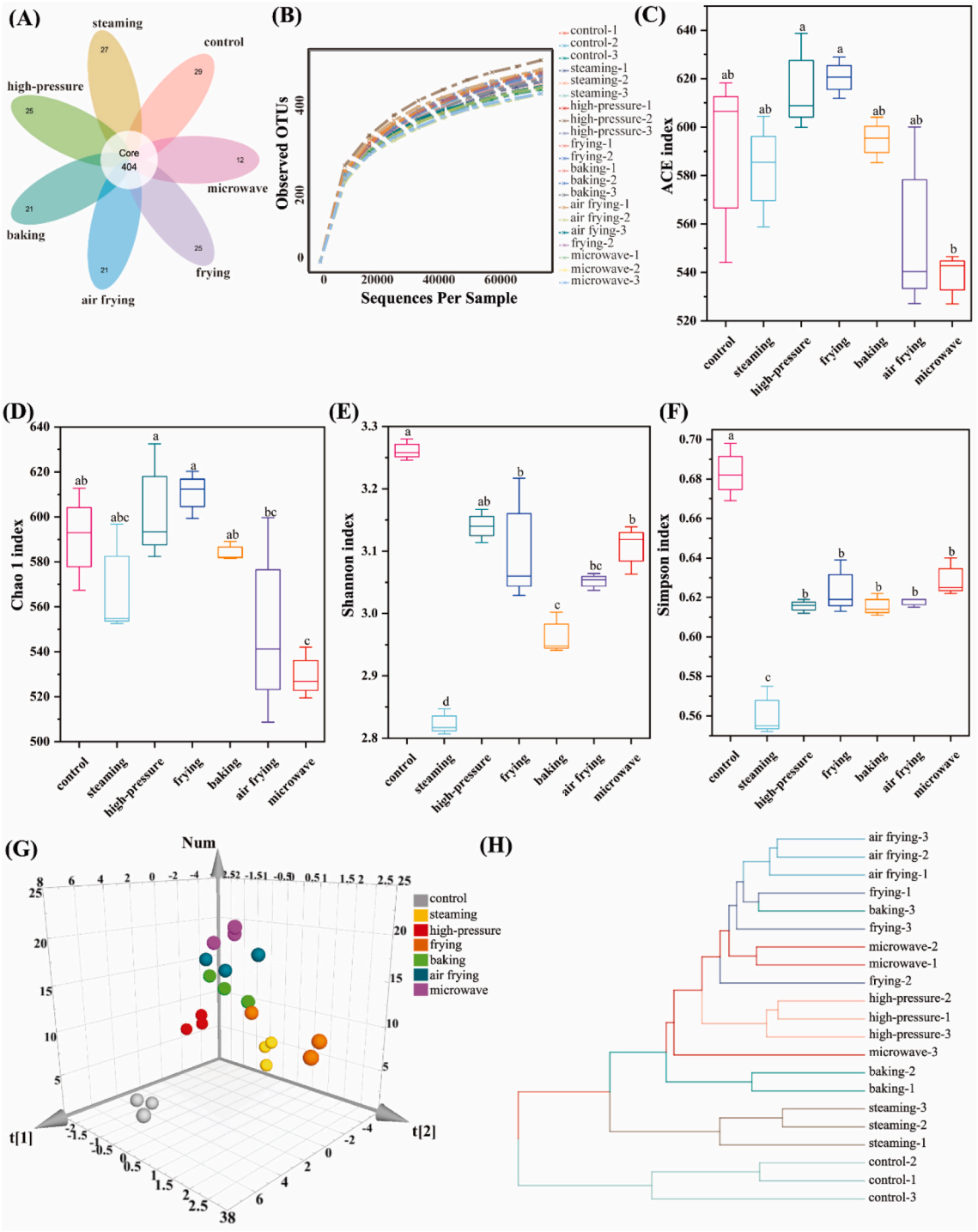

Fig. 9. OTUs, alpha-diversity and beta-diversity analysis: OTU Venn plots (A), dilution curve (B), ACE index (C), chao 1 index (D), Shannon index (E), Simpson index (F). 3D-PCA (G), sample aggregation class analysis (H). Different letters indicate a significant difference ($p < 0.05$).

was sufficient and the samples were representative of the microbial community (Zhang et al., 2024a). Alpha diversity, which reflects microbial diversity within samples, was assessed using indices for species richness (ACE and Chao1) and species diversity (Shannon and Simpson) (Liu et al., 2017b). Compared to the control group, the microwave treatment group showed a significant decrease in both the ACE and Chao1 indices ($p < 0.05$, Fig. 9C and D), while the other groups showed no significant change. In contrast, all six thermal processing methods resulted in a significant reduction in both the Shannon and Simpson indices ($p < 0.05$, Fig. 9E and F), with the steaming group exhibiting the lowest values. These results indicate that the oxidation of dough proteins induced by thermal processing reduces the α-diversity of human gut microbiota *in vitro*. Additionally, the beta diversity of the human gut microbiota was assessed using 3D principal component analysis (3D-PCA) and cluster analysis (Wei et al., 2021). The 3D-PCA model ($R^2X = 0.745$, $Q^2 = 0.558$) demonstrated a clear separation between the control group and the thermally processed groups. Furthermore, samples subjected to different thermal processing methods showed distinct clustering patterns. The R^2X and Q^2 values, both exceeding the threshold of 0.5, confirm the model's satisfactory robustness, goodness-of-fit, and

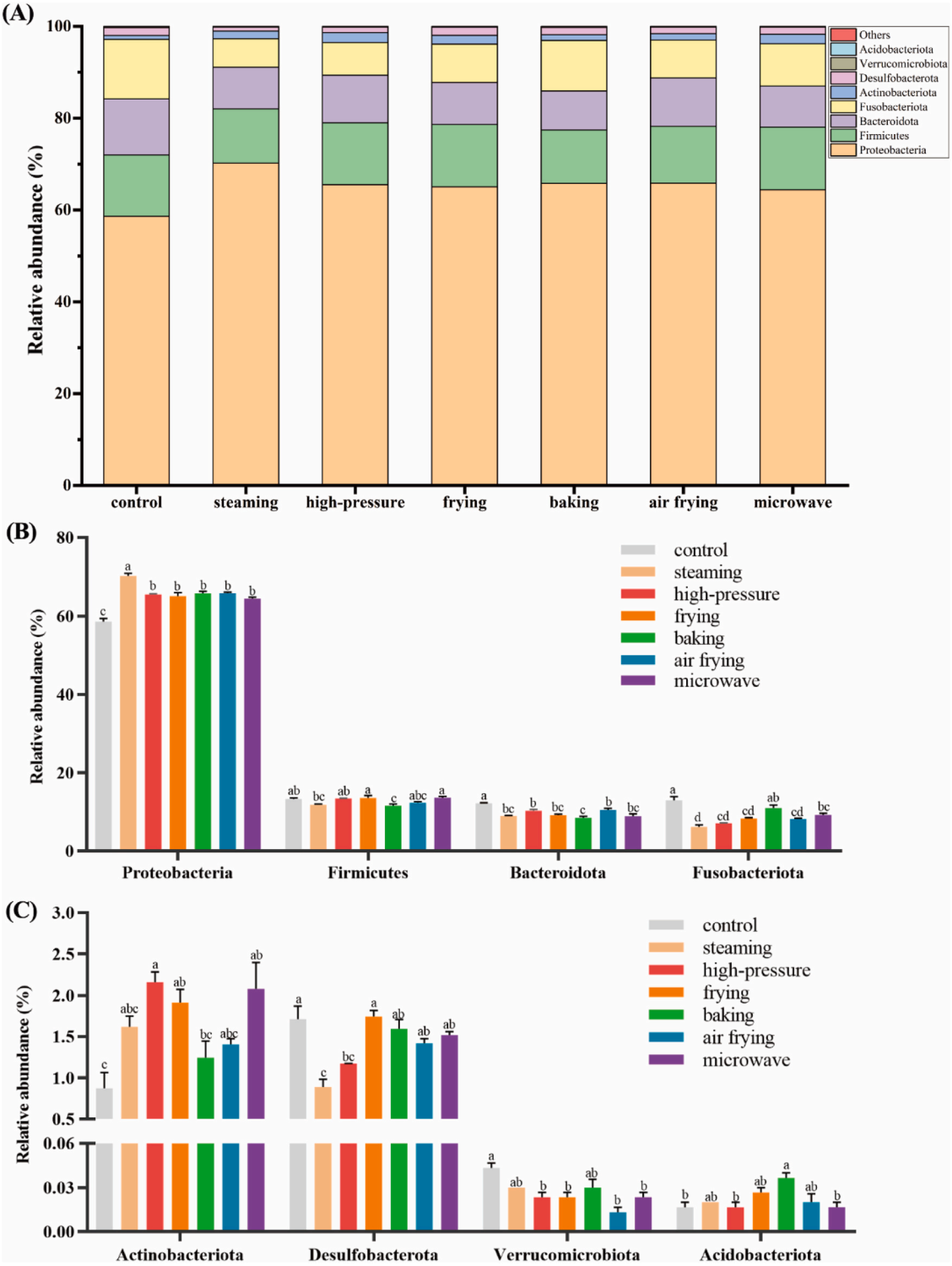

Fig. 10. Effects of oxidized dough protein on the relative abundance of human intestinal microbiota at the phylum level: relative abundance stacked bar chart (A), differential bacterial phyla (B, C). Different letters indicate a significant difference ($p < 0.05$).

predictive capability, thereby validating the rationality and reliability of the sample grouping.

3.8.2. *Effects of oxidized dough proteins on the relative abundance of major bacterial phylum*

To assess the impacts of thermally induced dough protein oxidation on the human gut microbiota, the bacterial composition at the phylum level was analyzed in fermentation broths. A total of 24 bacterial phyla were identified, and those with a relative abundance greater than 0.01% were selected for further analysis (Fig. 10). The predominant bacterial phyla included *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Fusobacteria*. Compared to the control group, steaming processing significantly increased the relative abundance of *Proteobacteria* and *Actinobacteriota*, while significantly reducing *Bacteroidota*, *Fusobacteriota*, and *Desulfobacterota* ($p < 0.05$). High-pressure processing led to a marked increase in *Proteobacteria* and *Actinobacteriota* but a significant decrease in

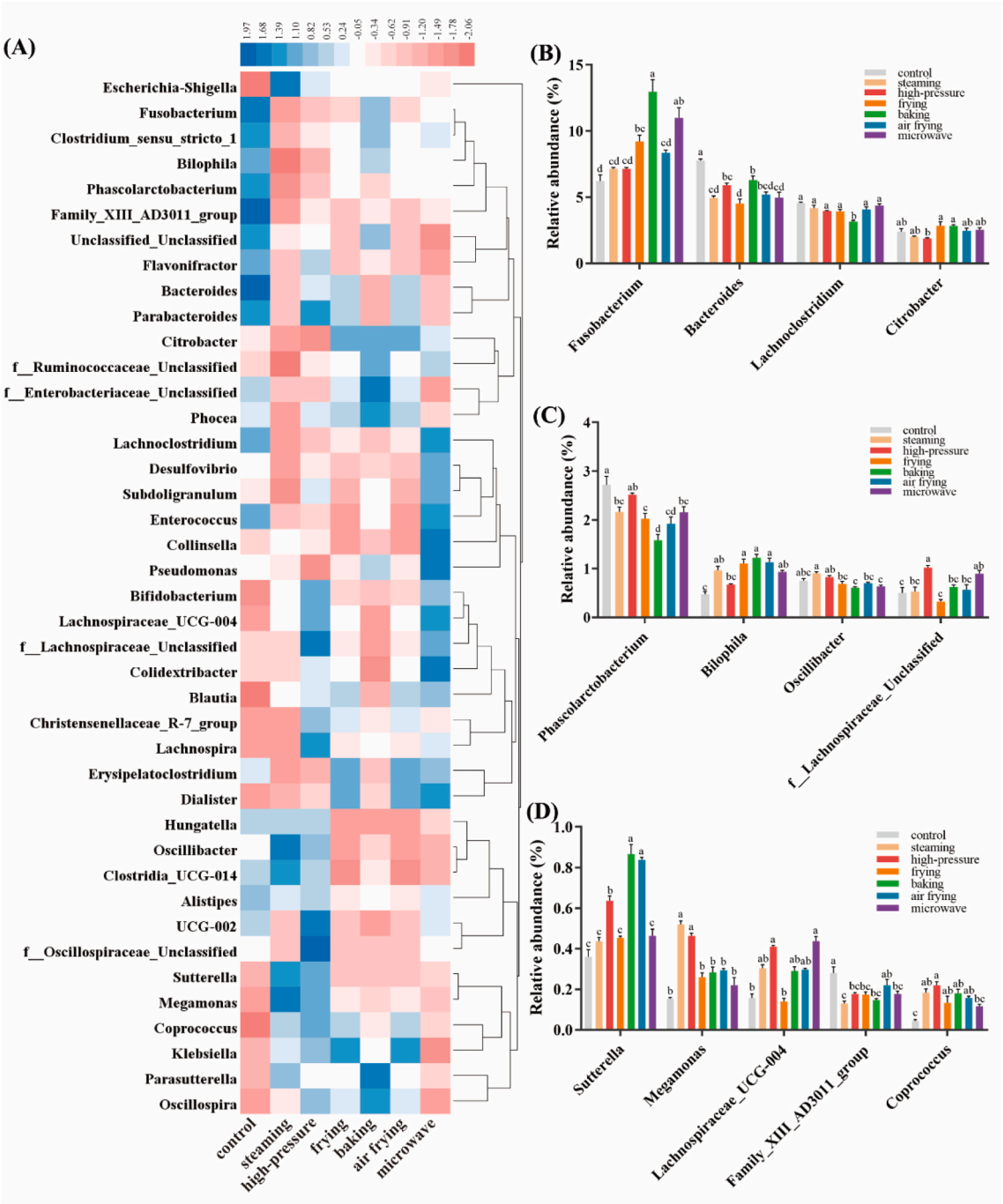

Fig. 11. Effects of oxidized dough protein on the relative abundance of human intestinal microbiota at the genus level: heat map with relative abundance greater than 0.1% (A), differential bacterial genera (B, C, and D). Different letters indicate a significant difference ($p < 0.05$).

Bacteroidota, *Fusobacteriota*, *Desulfobacterota*, and *Verrucomicrobiota* ($p < 0.05$). Frying processing notably elevated *Proteobacteria* and *Actinobacteriota* while reducing *Bacteroidota*, *Fusobacteriota*, and *Verrucomicrobiota* ($p < 0.05$). Baking processing significantly increased *Proteobacteria* and *Actinobacteriota* but decreased *Firmicutes* and *Bacteroidota* ($p < 0.05$). Air frying processing resulted in a significant rise in *Proteobacteria* but a decline in *Bacteroidota*, *Fusobacteriota*, and *Verrucomicrobiota* ($p < 0.05$). Similarly, microwave processing significantly enhanced *Proteobacteria* and *Actinobacteriota* while reducing *Bacteroidota*, *Fusobacteriota*, and *Verrucomicrobiota* ($p < 0.05$). Collectively, these results demonstrate that thermal processing-induced protein oxidation significantly alters the composition and relative abundance of the human gut microbiota under *in vitro* fermentation conditions.

3.8.3. *Effects of oxidized dough proteins on the relative abundance of major bacterial genus*

To illustrate changes in the human fecal microbiota at the genus level resulting from thermally induced protein oxidation in dough, a heatmap was constructed and differential analysis was performed on bacteria with a relative abundance exceeding 0.1% (41 genera, Fig. 11). The predominant bacterial genera included *Escherichia-Shigella*, *Fusobacterium*, *Bacteroides*, *Lachnoclostridium*, *Parabacteroides*, *Citrobacter*, and *Phascolarctobacterium*. Subsequent analysis focused on 13 genera showing significant differences. Compared to the control group, the abundances of *Bacteroides*, *Phascolarctobacterium*, and *Oscillibacter* were significantly reduced after all six thermal processing methods. *Bacteroides* have been reported to be significantly enriched in the gut

microbiota of centenarians (Luan et al., 2020; Wang et al., 2019). They engage in nutritionally mutualistic interactions with other bacteria, thereby enhancing host metabolic homeostasis and exerting synergistic benefits against obesity and dysregulation of glucose and lipid metabolism (Cheng et al., 2022a). *Phascolarctobacterium* contributes to the production of short-chain fatty acids (SCFAs) and modulates inflammatory cytokines, thereby reducing the risk of host metabolic disorders and inflammatory responses (Milton-Laskibar et al., 2022; Rondanelli et al., 2025). Furthermore, *Oscillibacter* utilizes specific enzymes, such as cholesterol dehydrogenase and isomerase to metabolize host cholesterol, thereby decreasing intestinal cholesterol absorption and the risk of cardiovascular diseases (Li et al., 2024). These findings indicate that dough proteins treated with different thermal processing methods, under *in vitro* fermentation conditions, reduce the abundance of anti-inflammatory bacteria in the fermentation broth to varying degrees. Consumption of such proteins may lead to a decrease in the abundance of these beneficial bacteria in the gut. Moreover, all six thermal processing methods significantly increased the abundance of *Fusobacterium*, *Bilophila*, and *Sutterella* compared to the control group. The proliferation of *Fusobacterium* and *Bilophila* is associated with intestinal inflammation, aggravating hepatic lipid accumulation and metabolic disorders (Wu et al., 2024; Zhong et al., 2022). Elevated abundances of *Sutterella* are positively correlated with inflammatory bowel disease, as their reduced SCFAs production compromises the host's anti-inflammatory capacity (Shimizu et al., 2019; Zhou et al., 2023). These results indicate that under *in vitro* fermentation conditions, dough proteins treated with different thermal processing methods increase the abundance of pro-inflammatory bacteria in the fermentation broth to varying degrees. Correspondingly, consuming such proteins may lead to an increase in the abundance of these harmful bacteria in the gut.

The observed changes in these genera following exposure to oxidized proteins may be linked to specific metabolic preferences. Protein oxidation can lead to the formation of cross-links and intermolecular aggregates at the protein level, as well as side-chain modifications and intermolecular bridges at the amino acid level. These adverse changes reduce the susceptibility of proteins to gastrointestinal digestive enzymes such as pepsin and trypsin, decrease the hydrolysis of proteins and the subsequent release of amino acids, thereby affecting the ability of intestinal microorganisms to utilize them for growth and reproduction (Li et al., 2022b). Based on a comprehensive review of extensive published literature on the relationship between protein oxidation and gut microbiota, it has been found that oxidized proteins may be less accessible to beneficial bacteria, while being more readily utilized by harmful bacteria (Wu et al., 2022). These chemically modified protein derivatives could potentially serve as preferred nitrogen or carbon sources for certain pro-inflammatory bacteria. Conversely, oxidation may degrade or mask protein structures that are normally metabolized by beneficial genera. The study has shown that dietary oxidized beef proteins can directly reshape gut microbiota, increasing pro-inflammatory bacteria and decreasing beneficial ones, leading to colonic inflammation in mice (Yin et al., 2022). Ge et al. (2020) demonstrated that feeding mice oxidatively damaged pork reduced the abundance of beneficial bacteria, increased pro-inflammatory bacteria, and caused intestinal barrier damage. Similarly, Shu et al. (2024) demonstrated that oxidized yak milk proteins significantly altered gut microbial diversity, modified community structure and function, and adversely affected metabolic status and health in mice. These independent findings corroborate the conclusion that protein oxidation disrupts gut microbiota homeostasis. In summary, thermal treatment induces oxidative modification of dough proteins, thereby disturbing the composition of the human intestinal microbiota under *in vitro* fermentation conditions. All six thermal processing methods increased the abundance of pro-inflammatory bacteria and decreased that of anti-inflammatory bacteria. Notably, baking and air frying exerted the most pronounced effects.

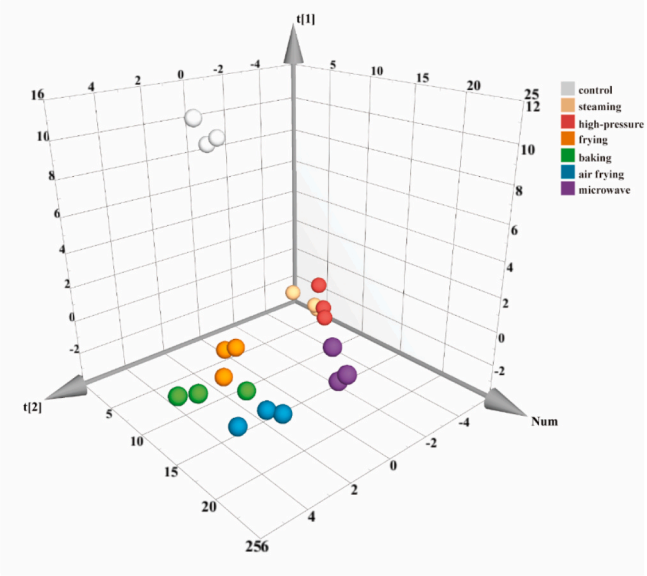

Fig. 12. 3D-PCA ($R^2X = 0.643$, $Q^2 = 0.545$) score plots. The data in the figure are based on the content of protein oxidation products and amino acid oxidation products in the dough, as well as the relative abundance of differential microorganisms in the dough protein fermentation broth.

3.9. Evaluation of protein oxidation degree

To further evaluate the differential effects of various thermal processing methods on the degree of protein oxidation in dough, a 3D-PCA model was constructed incorporating all measured variables. The model (Fig. 12) demonstrated satisfactory robustness, goodness-of-fit, and predictive capability, as indicated by R^2X and Q^2 values both exceeding 0.5. All data points were distributed within the confidence interval without outliers, confirming the statistical reliability of the data. Samples within each group showed a high degree of clustering, indicating good reproducibility. In contrast, clear separation was observed between different groups, with a particularly pronounced distinction between all thermally processed samples and the control group. This result strongly supports the significant effects of different thermal processing on dough protein oxidation. Furthermore, the three groups of frying, baking, and air frying were distinctly separated from the steaming, high-pressure, and microwave groups, suggesting differential effects of these processing methods on protein oxidation and gut microbiota. The 3D-PCA results are consistent with our experimental findings that the six thermal processing methods induced varying degrees of protein oxidation, with baking and air frying causing the most substantial oxidative modifications, while microwave treatment resulted in the least. Correspondingly, the oxidized proteins led to different degrees of gut microbiota dysbiosis, with baking and air frying showing the most pronounced effects. These findings provide novel mechanistic insights from the perspective of protein oxidation, substantiating global nutritional experts' concerns regarding the health implications of highly processed foods.

4. Conclusion

This study confirmed that six different thermal processing methods induce characteristic and structural changes in dough proteins, along with the accumulation of protein and amino acid oxidation products. When these oxidized proteins were subjected to *in vitro* anaerobic fermentation with human gut microbiota, they altered the microbial community composition by significantly promoting the proliferation of pro-inflammatory bacteria and suppressing beneficial anti-inflammatory bacteria. Moreover, these findings further indicate that

thermal processing promotes dough protein oxidation to varying degrees, with baking and air frying exerting the most substantial impact, while microwave treatment resulted in the least. Long-term intake of such oxidized proteins may induce gut microbiota dysbiosis and could potentially elevate the risk of chronic gut-related diseases. However, the speculative conclusions regarding the impacts of oxidized dough proteins on gut health are based on potential changes in microbial composition observed under *in vitro* fermentation conditions. This approach has inherent limitations, as it cannot fully replicate the complex human gut environment or directly observe associated inflammatory states. Therefore, from a health perspective, cooking methods that induce less protein oxidation, such as microwaving, may be preferable to high-impact methods like baking and air frying. Future research should focus on developing green, healthy, and sustainable processing strategies to mitigate protein oxidation in dough, which is crucial for producing flour-based products with lower oxidation levels and enhanced nutritional quality.

Declaration of competing interest

There are no conflicts of interest to declare.

Acknowledgments

This research was supported by Key R&D projects in Henan Province (No. 231111113300), Joint Research Fund for science and technology R&D Projects of Henan Province (No. 225200810066), National Natural Science Foundation of China (No. 32502230), Double First-Class Discipline Construction Program of Henan University of Technology (No. 0517-24410014), Cultivation Project of Tuoxin Team in Henan University of Technology (No. 2024TXTD08), and Cultivation Programme for Young Backbone Teachers in Henan University of Technology (No. 21421230).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2026.101357.

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