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Xanthan gum modified fish gelatin and binary culture modulates the metabolism of probiotics in fermented milk mainly via amino acid metabolism pathways

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ABSTRACT

^{1}H NMR combined with multivariate data analysis were applied to investigate the effects of fish gelatin (FG) addition and co-culture of *Lactobacillus acidophilus* LA-5 (La-5) and *Bifidobacterium lactis* BB-12 (Bb-12) on the growth and metabolic pathways of the probiotics themselves. The results showed that the addition of FG had no significant effects on the growth of probiotics, but co-culture did promote the growth of probiotics, especially for Bb-12 (up to 2 log CFU/mL). FG addition inhibited amino acids synthesis and TCA cycling in *Lacticaseibacillus paracasei* subsp. *paracasei* CASEI 431 (L431) to some extent. However, for the single La-5 strain, these pathways were promoted. As for mixed bacterial cultures, Bb-12 promoted amino acids metabolism, sugar transport and energy metabolism in La-5. These findings suggested that the metabolic profile of probiotic bacteria can be adequately explained by metabolic pathway analysis, which also provides theoretical guidance for the industrialization of functional fermented milk.

1. Introduction

Low-fat fermented milk has attracted the attention of consumers due to the global low-calorie diet trend. However, the taste of low-fat fermented milk is generally not as good as that of full-fat fermented milk. This may be because the fat content affects the flavor of the fermented milk itself, with the release time of flavor volatiles being significantly shorter in low-fat fermented milks compared to those with higher fat content (Brauss, Linforth, Cayeux, Harvey, & Taylor, 1999). In addition, low fat can affect the structure of the fermented milk, resulting in low viscosity and large particulate matter, which can affect the texture and taste of the fermented milk (Lin, Xu, Li, & Shao, 2022). Among all the methods to solve the poor taste of low-fat fermented, adding fat substitute gelatin is the most widely used method. Adding them to the milk base can maintain the texture and taste of yogurt and prevent the separation of whey. However, the commercial gelatin commonly used in fermented milk currently comes from pigs and cattle. These mammalian gelatins may have the potential to spread some diseases like bovine spongiform encephalopathy (Limpisophon et al., 2015). In addition, products made from mammalian sources are not accepted by religious consumers such as Mohammedanism, Muslims and Hinduism. Therefore, fish gelatin (FG) is considered to be a promising alternative to mammalian gelatin (You, Regenstein, & Liu, 2010).

FG is a kind of high molecular polypeptide polymer, which can be obtained by hydrolyzing fish collagen. And fish collagen is easily obtained from fish skin, fish scales, fish bones and other by-products discarded in the processing of fish (Yang, Chaieb, & Hemar, 2021). Therefore, the production of fish gelatin contributes to the consumption of fishing waste (Kittiphattanabawon, Sriket, Kishimura, & Benjakul, 2019). In previous studies, our team members found that FG modified with xanthan gum (XG) provides a more uniform structure to the yogurt and found that the modified FG yogurt has better water retention capacity than commercial gelatin yogurt. This study demonstrates that FG can replace the function and role of porcine gelatin in yogurt (Yin, Yang, Lai, & Yang, 2021). In addition, previous studies have shown that collagen and its hydrolysates and peptides can promote the growth of probiotics in fermented milk (Zhang, Zhang, Li, & Liu, 2020; Znamirowska, Szajnar, & Pawlos, 2020). Therefore, the addition of FG to fermented milk will not only improve the quality of the milk and make it more acceptable to consumers but will also make use of fish waste resources and may also have a catalytic effect on the growth of probiotics.

The quality of fermented milk is determined not only by its texture,

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but also by the status of the probiotics in it. Fermented products must have an adequate number of live probiotics to provide health benefits. The most used commercial probiotics for fermented milk are genera *Lactobacillus* and *Bifidobacterium* (Zúñiga, Monedero, & Yebra, 2018). However, *Bifidobacterium bifidum* lacks the protease enzyme to hydrolyze casein and, therefore, it tends to grow poorly in milk. In addition, the weak cell envelope protease (CEP) secretion capacity of *Lactobacillus acidophilus* makes it poorly utilized for macromolecular proteins, resulting in slower growth and longer fermentation time of single strain in milk (Li et al., 2020). It has been shown that peptide supplements like collagen and protein hydrolysates can be directly transferred to *L. acidophilus* cells and are hydrolyzed and utilized by the peptidases in the cells, thereby improving their growth and metabolic status (Zhang et al., 2020). Besides, the multi-strains probiotic fermentation can be better than single probiotic fermentation, it can increase the nutritional properties and texture of fermented milk, and that *Bifidobacterium* can also promote the growth of *L. acidophilus* (Soni et al., 2020). The target microorganisms in this study are*Lacticaseibacillus paracasei* subsp. *paracasei* CASEI 431 (L431), *Lactobacillus acidophilus* LA-5 (La-5) *and Bifidobacterium lactis* BB-12 (Bb-12). In practical studies, *L. paracasei* CASEI 431 is often used alone, whereas *B. lactis* BB-12 is always used in combination with *L. acidophilus* LA-5. Therefore, in this study, the effect of *L. acidophilus* LA-5 on its own metabolites and the regulation of related pathways after co-culture with *B. lactis* BB-12 was also investigated.

The growth of bacterial communities during the fermentation of yogurt is the result of the continuous interaction between the microorganisms in it and the metabolites in the milk (Bai et al., 2020). Additions to milk and environmental changes have the potential to affect the status and metabolism of probiotics (Baig, Turner, Liu, Al-Nabulsi, Shah, & Ayyash, 2021). Emerging metabolomics has been used to acquire “snapshots” of intracellular metabolites under different external stimuli (Chen et al., 2020). In metabolomics, nuclear magnetic resonance (NMR)-based metabolomics is a rapid analytical method that does not damage the sample and provides comprehensive information (Hatzakis, 2019). Many studies have been conducted to investigate the effect of gelatin addition on the quality of the yogurt itself (Shori, Baba, & Chuah, 2013) and on the extracellular metabolites of probiotics (Ayyash et al., 2021). However, there are no studies on the metabolic effects of added fish gelatin on probiotic bacteria in fermented milk. Moreover, systematic studies on the growth and metabolomics of mixed probiotic cultures during milk fermentation are still missing.

The objective of the present study was, therefore, to determine the effect of mixed bacterial fermentation and FG-XG addition on probiotics in fermented milk food matrices. Metabolites and metabolic pathways were identified to differentiate between mono- and multi-probiotic cultures through NMR-based metabolomics and compare the effects of different sorts of gelatin additions on the growth and metabolic profiles of probiotics. In addition, multivariate data analysis and pathway analysis were used to further establish the relationship between growth and metabolomic profiles to provide a theoretical basis and guidance for the industrialization of functional fermented milk.

2. Materials and methods

2.1. Materials

L. paracasei CASEI 431, *L. acidophilus* LA-5 and *B. lactis* BB-12 were obtained from Chr. Hansen Co. Ltd (Singapore). Skim milk powder (SMP; protein 33.4, moisture 3.9, fat 0.7, lactose 54.1, and ash 7.9 g/100 g) was purchased from Nature Ltd. (Singapore). FG (200 Bloom, 99 % purity, Mw ≈ 50 kDa) and bovine gelatin (BG) (200 Bloom, 99 % purity, Mw ≈ 50 kDa) were purchased from Chengdu Jingdian Co. Ltd. (Sichuan, China). Gun xanthan (XG) (Sigma®, G1253, from xanthomonas campestris, Mw ≈ 2000 kDa (Gagnon, Shen, & Arratia, 2013)) were purchased from Sigma-Aldrich Co. Ltd (Singapore).

2.2. XG modified fish gelatin

XG modified FG was prepared according to the method of Yin et al. (2021). FG and XG were hydrated in deionized (DI) water for 10 h, followed by magnetic stirring at a speed of 700 rpm for 10 min at 65 °C (water bath heating) until dissolution. The total concentration was brought to 2.5 g/100 g by heating at 60 °C (water bath heating) for 30 min, where the ratio of XG to FG (w/w) was 1:99. All prepared solutions had a pH about 6.3 and the solutions were all used on the same day.

2.3. Bacterial strains and culture conditions

The lyophilized probiotics *L. acidophilus* LA-5 (anaerobic), *B. lactis* BB-12 (anaerobic) and *L. paracasei* CASEI 431(facultative anaerobic) were inoculated in Deman-Rogasa (MRS) broth (Merck) and incubated at 37 °C for 24 h, individually. Subsequently, a second subculture was prepared in the same way and incubated at 37 °C for 48 h. Bacterial precipitates collected by centrifugation were washed twice with 0.1 mol/L phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 10,000 × *g* for 10 min at 4 °C and resuspended (Ozturkoglu-Budak, Akal, Buran, & Yetişemiyen, 2019).

2.4. Fermented milk

The solution prepared in section 2.2 was added to reconstituted skim milk to bring the concentration of XG-FG (abbreviated as FG) to 0.4 g/100 g (Yin et al., 2021). The same concentration was also applied to BG. The original fermented milk without any gelatin (OG) as control for BG and FG. Each strain (L431; La-5; La-5 & Bb-12) has three different gelatin treatments: OG, FG and BG. Then, the milk mixture was heated to 121 °C in an autoclave for 5 min and immediately cooled to 42 °C. At this point, the probiotics were inoculated into the skim milk mixture at a concentration of approximately 8 log CFU/mL and incubated at 42 °C for 24 h until the pH reached 4.5 to 4.6. The fermented milk was then taken out of the incubator and cooled rapidly and stored at a temperature of 4 °C for 48 h until the next step of analysis. A simplified scheme of the experimental approach is shown in Figure S1.

2.5. Enumeration of probiotic bacteria

The method used to enumerate probiotic bacteria with some modifications (Faraki, Noori, Gandomi, Banuree, & Rahmani, 2020). For each strain, 10 mL of milk samples were mixed with 90 mL DI water in stomacher bags, and then homogenized for 2 min. The mixed samples were used tenfold serial dilutions prepared in 0.1 % (w/v) buffered peptone water. Subsequently, L431 was cultured overnight under aerobic conditions in MRS agar. Single La-5 was cultured anaerobically in MRS agar. La-5 co-cultured with Bb-12 was selected by MRS-bile agar aerobically and Bb-12 co-cultured with La-5 was cultured anaerobically in MRS agar containing 0.3 % sodium propionate and 0.05 % L-cysteine. Probiotics were incubated at 37 °C for 72 h. The number of viable cells of probiotics was expressed in log CFU/g.

2.6. Extraction of probiotics metabolites

Extraction of the probiotic strains was performed separately and 30 mL of fermented milk containing probiotics was subjected to subsequent metabolic analysis. The samples were diluted 10-fold with 270 mL of 0.1 % peptone water. Subsequently, the probiotics in the fermented milk were immediately collected and centrifuged at 500 × *g* for 3 min at 4 °C (Zhao, Chen, & Wu, 2020). The cell suspensions obtained by the low-speed centrifugal separation method were centrifuged at 12,000 × *g* for 10 min at 4 °C, followed by washing twice with 5 mL of PBS and resuspending. The prepared probiotic pellets were mixed with about 1 mL of cold methanol-d_4 solution, immediately frozen in liquid nitrogen while maintaining low temperature, and then thawed on ice. The

freeze–thaw step was repeated three times to disrupt the cell membrane of the probiotic bacteria and allow the metabolites to flow out (Winder et al., 2008). The intracellular metabolites were subsequently extracted, and the metabolites were obtained by centrifugation at 12,000 × *g* for 20 min at 4 °C. Trimethylsilyl propionic acid (TSP) at a concentration of 1 mmol/L was added as an internal standard, which was used to determine the concentration of individual metabolites. Extraction of metabolites was performed three times (Wang et al., 2022).

2.7. NMR spectroscopic analysis

NMR tests were performed using a Bruker DRX-500 NMR spectrometer at 25 °C (Bruker, Rheinstetten, Germany) equipped with a triple inverse gradient probe with a ^{1}H resonance frequency of 500.23 MHz. The ^{1}H spectra of each sample were acquired using the first increment of a standard NOESY pulse sequence. The ^{1}H spectra have a width of 10 ppm and their spectra were recorded as 128 transients with a relaxation delay of 2 s. One-dimensional NMR spectra were FID multiplied by a broadening factor of 1 Hz exponential window function and corrected for phase and baseline. To further confirm the chemical signal of the metabolites, a two-dimensional NMR spectrum ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC) of the metabolite was acquired. Among them, the ^{1}H spectrum was collected in the F2 channel with a spectral width of 10 ppm and the ^{13}C spectrum was recorded in the F1 channel with a spectral width of 175 ppm. This procedure was performed according to Zhao, Zhao, and Wu (2019).

2.8. Data processing

The obtained NMR data were processed by the software Topspin 4.0.9 (Bruker). The phase and baseline of the spectrum were manually corrected, and the chemical shift of the TSP was referenced. The metabolites corresponding to the peaks were determined by referring to the 1D ^{1}H and 2D ^{1}H–^{13}C NMR database, Biological Magnetic Resonance Data Bank (https://www.bmrb.wisc.edu/), Madison Metabolomics Consortium Database (https://mmcd.nmrfam.wisc.edu/), and Human Metabolome Database (https://www.hmdb.ca/) and 2D ^{1}H–^{13}C NMR spectra analysis (https://www.bmrb.wisc.edu/metabolomics/) and some related reference materials (Chen et al., 2020; Li et al., 2020; Zhang et al., 2021a, 2021b). The region containing the methanol signal (3.29–3.35 ppm) was excised and the rest of the region was integrated by normalization to a width of 0.02 ppm.

Data were further analyzed using SIMCA software (version 13.0, Umetrics, Umeå, Sweden) for group separation by principal component analysis (PCA) and orthogonal projection to latent structure discriminant analysis (OPLS-DA) to determine the differences due to different treatments. R^2Y and Q^2 values were used to assess the reliability of the OPLS-DA model. In the coefficient loading plots, different colors were coded with different absolute correlation coefficient values. Warm colors contributed more to the differences between groups than cool colors. In our results, a correlation coefficient with an absolute value greater than 0.602 was significantly different, which corresponds to a significant difference level of $P < 0.05$. Furthermore, the importance of variables in the projection (VIP values) was also analyzed to determine the different metabolites. The increasing trend of the peaks in the spectrum in the correlation coefficient plot indicates that the amount of a metabolite in the former sample is lower than the amount of this metabolite in the latter sample; the decreasing trend of the peaks in the spectrum in the correlation coefficient plot indicates that the amount of a metabolite in the former sample is higher than the amount of this metabolite in the latter sample. The redder the color, the greater the apparent difference between the groups. The various metabolites contained in the samples were quantified by the ratio of the area of the proton signal to be detected in the ^{1}H NMR spectrum to the integrated area of the proton signal of the known concentration of the internal standard (TSP). Metabolic pathway analysis was performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/), respectively (He, Zhao, Chen, Zhao, & Yang, 2021).

2.9. Statistical analysis

All experiments were repeated independently in triplicate. The metabolite content obtained was statistically analyzed using the one-way ANOVA (Duncan's Multiple Range Test) model in SAS 8.0 with a significant difference level of $P < 0.05$.

3. Results and discussion

3.1. The growth of probiotics culture during fermentation

The effects of three different gelatin treatments (OG, FG and BG) on the growth of probiotic bacteria in fermented milk with L431 (Fig. 1A), La-5 (Fig. 1B), co-fermentation of La-5 (Fig. 1C1) and Bb-12 (Fig. 1C2) are shown in Fig. 1. Overall, each strain grew by more than 1.5 log CFU/mL during the fermentation process. Among these, La-5 grew by more than 3 log CFU/mL, probably due to the relatively low concentration in pre-fermented milk of La-5 at around 5 log CFU/mL (related to its bacterial viability). In contrast, the initial concentrations of both L431 and Bb-12 were above 6 log CFU/mL. It is also noteworthy that the growth of single *B. lactis* on milk was poor and almost non-existent, which is in line with previous studies (Gomes, Malcata, & Klaver, 1998). Therefore, to ensure consistency in our experiments, we did not study the effect of gelatin on the metabolism of probiotics in single Bb-12 fermented milk (in the absence of supplemental nutrient additions, Bb-12 was unable to grow).

During fermentation, the addition of FG did not have a significant effect on probiotics growth ($P > 0.05$), except for Bb-12 co-culture with La-5. Bb-12 co-cultured with La-5 grew significantly in FG group, reaching 2.04 log CFU/mL ($P < 0.05$), while it increased by 1.44 log CFU/mL in BG group. Although the growth of Bb-12 also reached 1.96 log CFU/mL in the OG group, which was not significantly different from the FG group. It may be because the CEP activity of *lactobacillus* was relatively low, thus, it did not have enough time to consume the gelatin nitrogen source in the fermented milk (Beganović et al., 2013). Bb-12, on the other hand, unlike *lactobacillus*, lacks CEP and therefore cannot directly utilize the proteins (Janer, Arigoni, Lee, Peláez, & Requena, 2005). The increase in Bb-12 following the addition of FG was because Bb-12 utilizes the nitrogen source obtained from the breakdown of La-5. Although it is not clear why Bb-12 is significantly increased in the presence of FG compared to BG, one possibility is due to the fact that the amino acid composition of FG may be better utilized by Bb-12 than that of BG. This result was also in line with previous studies where the addition of bovine gelatin had a more negative effect on the acidification and fermentation rate of fermented milk compared to fish gelatin (Ma, Zhao, & Zhao, 2019).

In mixed cultures fermentations, co-fermentation with La-5 promoted the growth of Bb-12 extremely significantly ($P < 0.05$) in three gelatin treatments. However, the growth of La-5 in co-culture did not appear to be affected by Bb-12 except the one in FG group ($P < 0.05$). It has been reported that the multi-strains probiotic fermentation can be better than single probiotic fermentation (Bujna, Farkas, Tran, Dam, & Nguyen, 2018) In addition, La-5 co-cultured with Bb-12 has been proved to maintain a high level of their activity during cold storage and had a synergistic effect on the growth of both of them (Ranadheera, Evans, Adams, & Baines, 2015).

3.2. Metabolic profiles of probiotics cultures in fermented milk

NMR-based metabolomics was used to explain the effects of treatment with different types of gelatins and fermentation with binary and single strains on the metabolic state of probiotic bacteria. As shown in

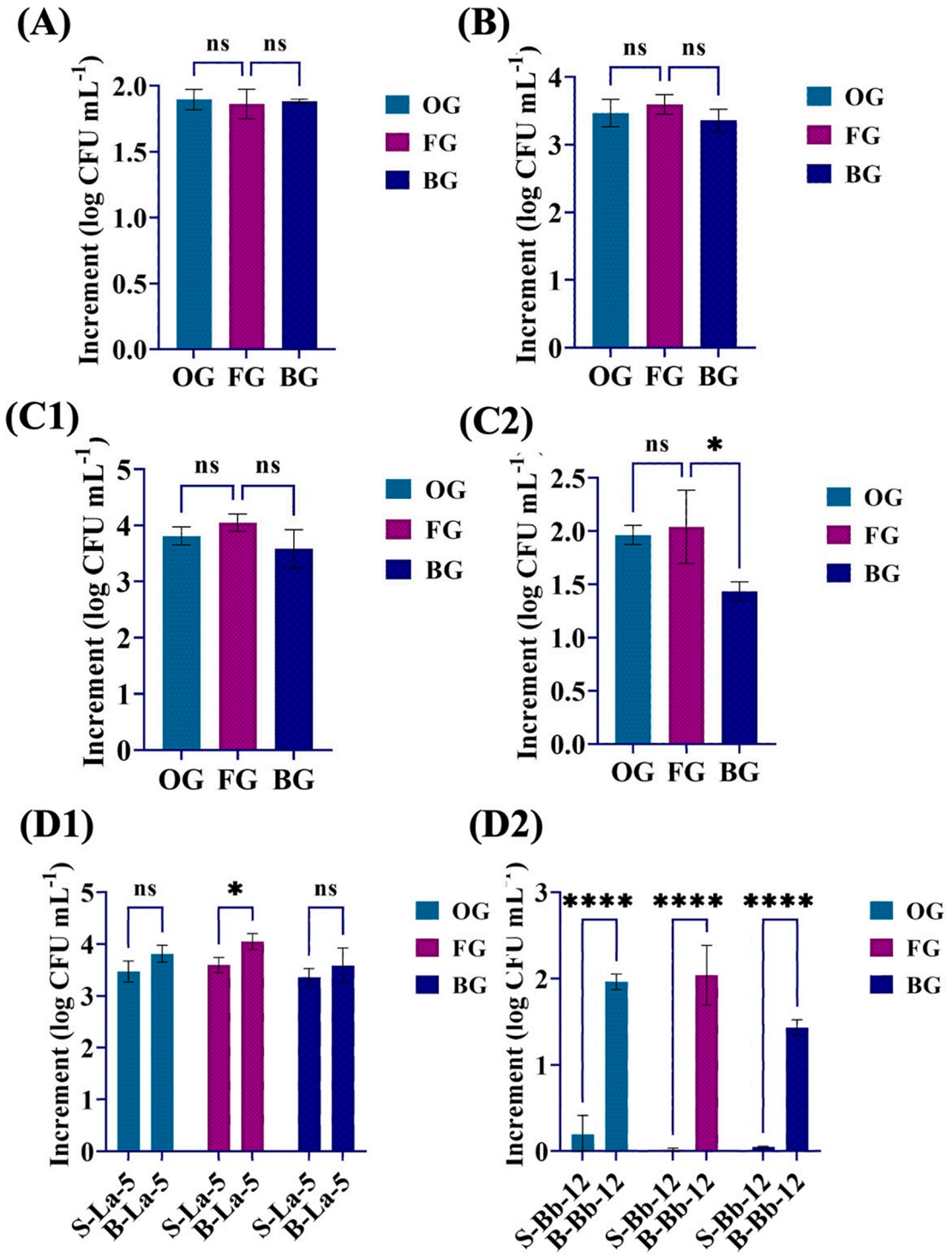

Fig. 1. Increment of probiotic cells (A: L431; B: La-5; C1: La-5 in binary culture; C2: Bb-12 in binary culture; D1: La-5 of single and binary culture; D2: Bb-12 of single and binary culture) under different gelatin treatments before and after fermentation. Data are expressed as mean ± standard deviation (n = 3). One-way ANOVA was used to assess statistical significance between conditions, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

Figure S2, similar patterns with varying peak intensities in these spectra were observed under same strains treatment. For instance, most of the peaks were clustered in the range 0.5–4 ppm, and these peaks were mainly classified as amino acids (Ile, Leu, Val, Thr, Ala, Glu, Pro, Met, Arg, Try, Gly, Asp, Phe), organic acids (lactic acid, acetic acid, pyroglutamic acid, succinic acid, oxoglutaric acid), and sugars (glucose and ribose 5-phosphate). Alcohols like ethanol also belonged in this region. Nucleotides (ADP, uridine, NADP, UMP, NAD, Cyclic AMP, adenosine and ATP) and others (betaine, choline) contributed the main signals from 4.0 to 10.0 ppm.

Within each strains group, the identified metabolites were different. In L431, a total 40 metabolites without overlapping chemical shifts were analyzed (Table S1), and a heatmap by using $\log_{10}$ transformation is shown in Fig. 2. The heatmap visualizes the relative abundance of metabolites in the strain on a red-blue scale, with redder color indicating higher concentrations and bluer color indicating lower concentrations.

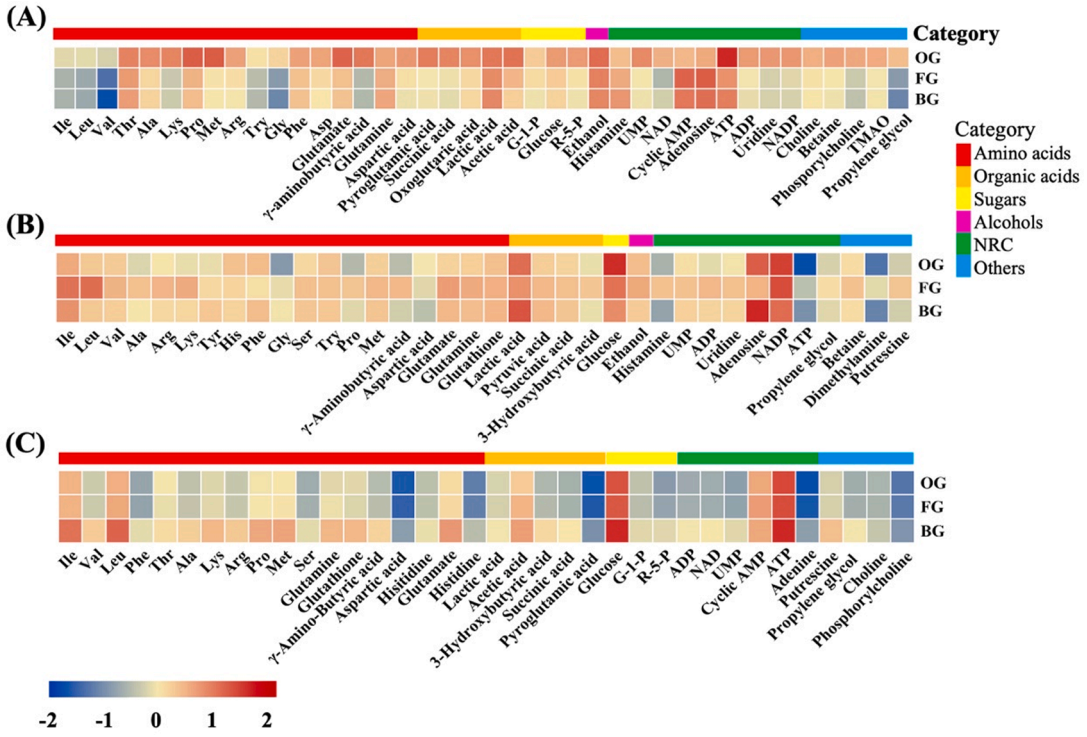

Fig. 2. Heatmap of metabolites of probiotic strains without gelatin, with physically modified fish gelatin and with bovine gelatin. Note: A: L431; B: La-5; C: Binary culture of La-5 and Bb-12.

Overall, the intracellular metabolites of L431 were better expressed in the fermented milk in OG than BG and FG groups (Fig. 2A). Besides, there was no significant difference in metabolite expression between the BG and FG groups. Almost all amino acids were increased in OG group, except for glutamine, which is probably due to the fact that glutamine is a substrate for pyruvate and ATP production, and its glutamine accumulation also helps probiotic bacteria to fight against acidic survival conditions (Aguilar-Toalá et al., 2019; Zhang et al., 2021a, 2021b). Therefore, it was expressed at a higher level among three gelatin groups. As with the expression of amino acids, almost all organic acids (except lactic acid) were expressed more in OG group. It may be because lactic acid is a product expressed by probiotics using carbohydrates from food to supply their own energy needs, independent of the nitrogen source provided by gelatin. For most of the identified nucleotides such as UMP, NAD, ATP, ADP, uridine and NADP, higher levels were observed in OG tan BG and FG groups. It may be due to the fact that the expression of extracellular enzymes and other components of the protein hydrolysis system of L431 is inhibited when there is a large and adequate source of proteins and amino acids in the growth environment (Alcántara et al., 2016).

As for La-5 (Fig. 2B), a total 36 metabolites were identified and the ^{1}H and ^{13}C peak assignments were displayed in Table S2. The graph shows that the FG metabolites were expressed more than both the OG and BG groups, with no significant difference in metabolite concentration between the latter two. Most of the amino acids (Ile, Leu, Val, Arg, Lys, Pro, Met, γ-aminobutyric acid, glutamate and glutamine) increased after the FG treatment, while Tyr, His and Phe decreased. Pyruvic acid, succinic acid and 3-Hydroxybutyric acid, which are organic acids, as well as ethanol also had higher concertation in FG than OG and BG groups. Besides, all NRC compounds and others (betaine, dimethylamine and putrescine) also showed an increase compared to OG and BG. The increase in intracellular metabolites may be due to the activation of key pathways and an increase in metabolic end products to meet the needs of active cell growth FG.

A total of 35 metabolites were identified in the fermented milk cultured with binary probiotics, and the assignment peak is shown in Table S3, the heatmap is shown in Fig. 2C. In contrast to the metabolic results of the first two strains, the treatment group with the addition of BG had the highest metabolite expression. All metabolites except Cyclic AMP were expressed at higher levels than in the OG and FG groups. This may be due to a key physiological role of cAMP, which ensures that proteomic resources are used in different metabolic sectors as needed in different nutritional environments (You et al., 2013). The results show that the variation in metabolites induced by different gelatins varied between different strains. It also further explains the reason that different gelatin types did not affect the growth numbers of probiotic.

3.3. *Differential analysis of probiotic strains under different gelatin treatments and between binary and monocultures*

Principal components analysis was performed with the first two principal components to show the differences in the overall metabolites of the different probiotics with different gelatin treatments. For multivariate analysis, the quality factors of PCA and OPLS-DA are R^2X and R^2Y, while Q^2 is the predictor. Usually, models with Q^2 greater than 0.4 and R^2 greater than 0.5 are considered reliable and robust (Lee & Lucey, 2010). For L431 (Fig. 3A1), the first two components explained 82.78 % of total variance (PC1: 72.01 %; PC2: 10.67 %). The R^2X and Q^2 of this model were 0.828 and 0.664, revealing that this model had good reproducibility and predictability. The score plot of PCA shows (Fig. 3A2) that there was a significant difference between the OG and other two groups. However, there was no significant difference between BG and FG. These two groups were both located in the negative sides of PC1, while the OG group were all gathered in the opposite sides of PC1. As for mono-culture LA-5, the first two components explained 75.9 % of variance (PC1: 58.5 %; PC2: 17.4 %) (Fig. 3B1-2). The model quality R^2X and Q^2 were 0.759 and 0.54. The result of the score plot shows that FG group is located on the negative side of PC1, while the BG and OG groups are both on the positive side of PC1. It shows that the metabolites of FG are significantly different from the other two groups. For binary cultures

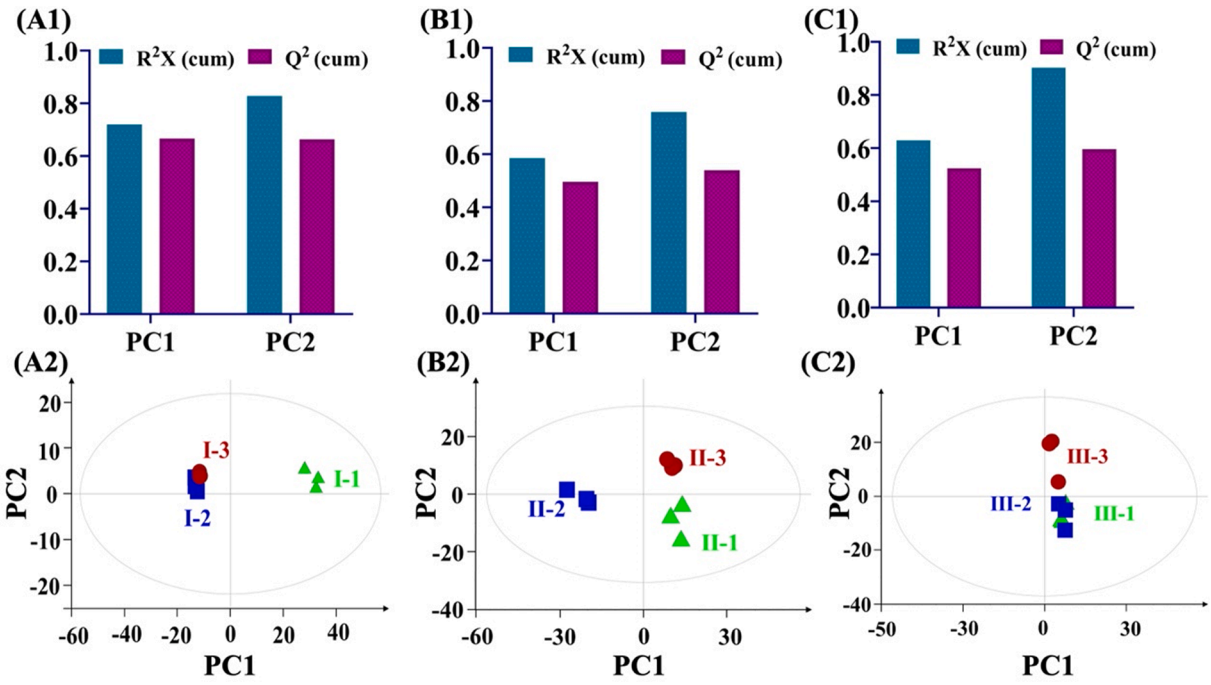

Fig. 3. Principal component analysis (PCA) of ^{1}H NMR spectra of probiotics under different treatments. variation explained by principal components in PCA (A1-C1); score plot for PCA (A2-C2). Note: I: L431; II: La-5, III: Binary culture (La-5 & Bb-12); 1: no gelatin treatment, 2: physically modified fish gelatin treatment, 3: bovine gelatin treatment.

(Fig. 3C1-2), the score plot shows that it explains more than 90 % of the variables with PC1 (62.9 %) and PC2 (27.1 %). In PC1, these three groups were located on the positive sides. As for PC2, the BG group was gathered on the opposite sides, while the FG and OG groups were both located on the negative sides. It shows that metabolites in BG have differences compared with the rest of the groups.

The PCA results above illustrated well the differences in the metabolic response of different probiotics to different gelatin treatments. Whereas OPLS-DA was able to illustrate well the differences between the two groups and to determine the biochemical alterations induced by different gelatin treatments. In general, all groups could be distinguished except for the OG and FG groups in the binary bacteria (L431 FG/OG: $R^2Y = 0.999$ and $Q^2 = 0.996$; L431 FG/BG: $R^2Y = 0.995$ and $Q^2 = 0.822$; La-5 FG/OG: $R^2Y = 0.992$ and $Q^2 = 0.952$; La-5 FG/ BG: $R^2Y = 0.999$ and $Q^2 = 0.995$; and binary probiotic FG/BG: $R^2Y = 0.836$ and $Q^2 = 0.468$). This OPLS-DA result shows that the effect of adding FG or not was detectable in the metabolome of probiotics (Fig. 4).

For L431, most of the amino acid metabolites were significantly higher in OG compared to that in FG (Fig. 4A1-b), such as Val, Ala, Lys, Glu and Pro, and organic acids such as lactic and acetic acid were also increased. In addition, there was a trend for elevated nucleotide-related compounds (e.g., UMP, ATP, ADP, uridine, NADP) and sugars (D-glucose and D-ribulose-5-phosphate). However, the S-lines of the BG group (Fig. 4A2-b) did not show any significant differences from the FG group. Only a few metabolites changed, with lower concentrations of valine, arginine, aspartate, acetate, NADP and propylene glycol and higher NAD concentrations in the BG group than FG group. As for the metabolic pattern of LA-5, the S-line of FG/OG (Fig. 4B1-b) showed that OG groups was associated with decreased levels of most amino acids, organic acids like lactic acid, NRC (histamine) and other compounds such as betaine and putrescine. In the FG/BG comparison group (Fig. 4B2-b), the BG treatment showed the same downward trend as the OG group, with a decrease in almost all the same metabolites, except for an increase in adenosine levels. To some extent, this result may indicate that the addition of FG had a favorable effect on LA-5 growth compared to the addition of BG and OG. As for binary cultures, R^2Y was 0.797 and Q^2 was −1.33. The negative value of Q^2 indicates a low level of variation between the data (Arredouani, A., Stocchero, M., Culeddu, N., Moustafa, J. E.-S., Group, D. S., Tichet, J., Balkau, B., Brousseau, T., Manca, M., Falchi, M. (2016), 2016), meaning that there was no significant difference between the FG and OG treatments. The S-line of FG/OG (Fig. 4C1-b) confirmed it because there were no correlation coefficients with values greater than 0.602. However, the situation of FG/BG was completely different from that of FG/OG. Most metabolites under the BG treatment showed an upward trend compared to the FG treatment (Fig. 4C2-b). Specifically, most amino acids such as isoleucine, leucine, alanine, lysine, arginine, proline, glutamic acid, glutathione, histidine, serine, and phenylalanine showed an upward trend, as did some nucleotide-related compounds (ATP and adenine). This phenomenon may be a survival strategy for Bb-12 and La-5 in the presence of BG and oxidative stress. Some amino acids (such as Val, Por and Gly) play an important role in protecting cell structure and maintaining normal physiological functions (Milner, McClellan, & Wood, 1987; Zhao et al., 2020). In addition, the increase in amino acids within the bacteria can be an alternative source of carbon to supplement cellular energy (Hai et al., 2022). Besides, levels of antioxidant compounds such as GSH are increased may allow them to survive in stressful environments (Sandoval, Arenas, & Vasquez, 2011).

In addition, to determine whether there would be metabolic differences between single and binary cultures, we also compared binary probiotics with single strain under different gelatin treatments, respectively. In OG group (Fig. 4Da-b), mono-bacterial probiotics could be clearly distinguished from multi-bacterial probiotics (R^2Y: 0.999, Q^2: 0.968). As compared to single culture group, a distinct increase in some amino acids (Ile, Val, Ala, Lys and Pro), lactic acid, succinic acid, ADP, Uridine, NADP and ATP and a slightly decrease in ethanol as well as 3-hydroxybutyric acid in binary group. In the fermented milk supplemented with FG (Fig. 4Ea-b), single strains could also be distinguished from co-cultured strains, where R^2Y was 0.943 and Q^2 was 0.705. Of these, Phe, Gly, Ser and uridine 5-monophosophate all showed a significant increase in multi-strains compared to mono-culture fermentations. Consistent with the results of the first two groups, there was also a

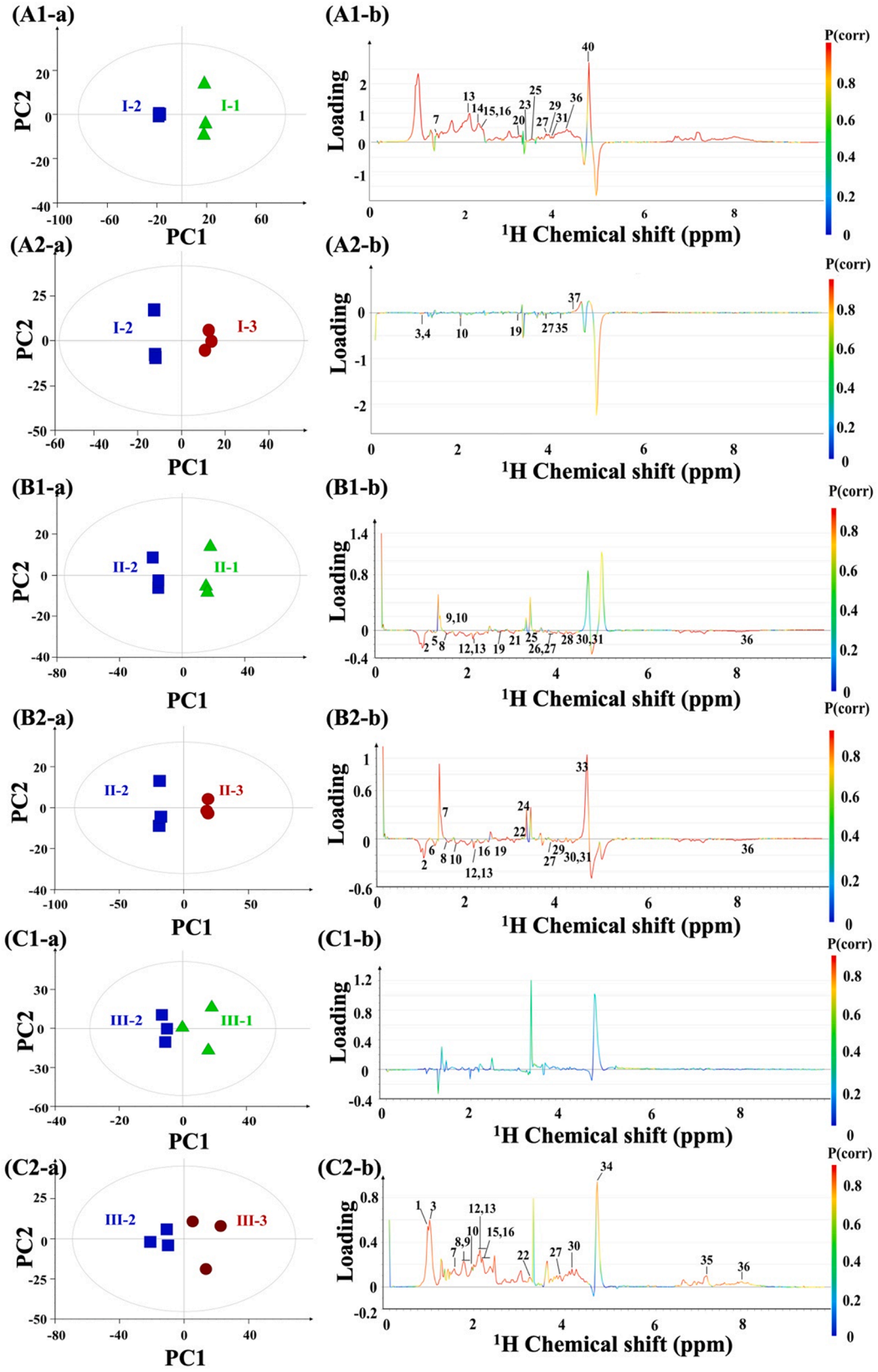

Fig. 4. Orthogonal projection to potential structure discrimination analysis (OPLS-DA) for comprising probiotics of modified fish gelatin and gelatin-free groups and modified fish gelatin and bovine gelatin groups as well as single and binary culture. OPLS-DA score plot (a); loading S-line (b); A, B, and C represents different probiotic strains; D, E and F represents La-5 under OG, FG and BG treatment; 1 is modified fish gelatin and gelatin-free groups; 2 is modified fish gelatin and bovine gelatin groups. Note: I: L431, II: La-5, III: La-5 & Bb-12; 1: gelatin-free treatment, 2: modified fish gelatin treatment, 3: bovine gelatin treatment.

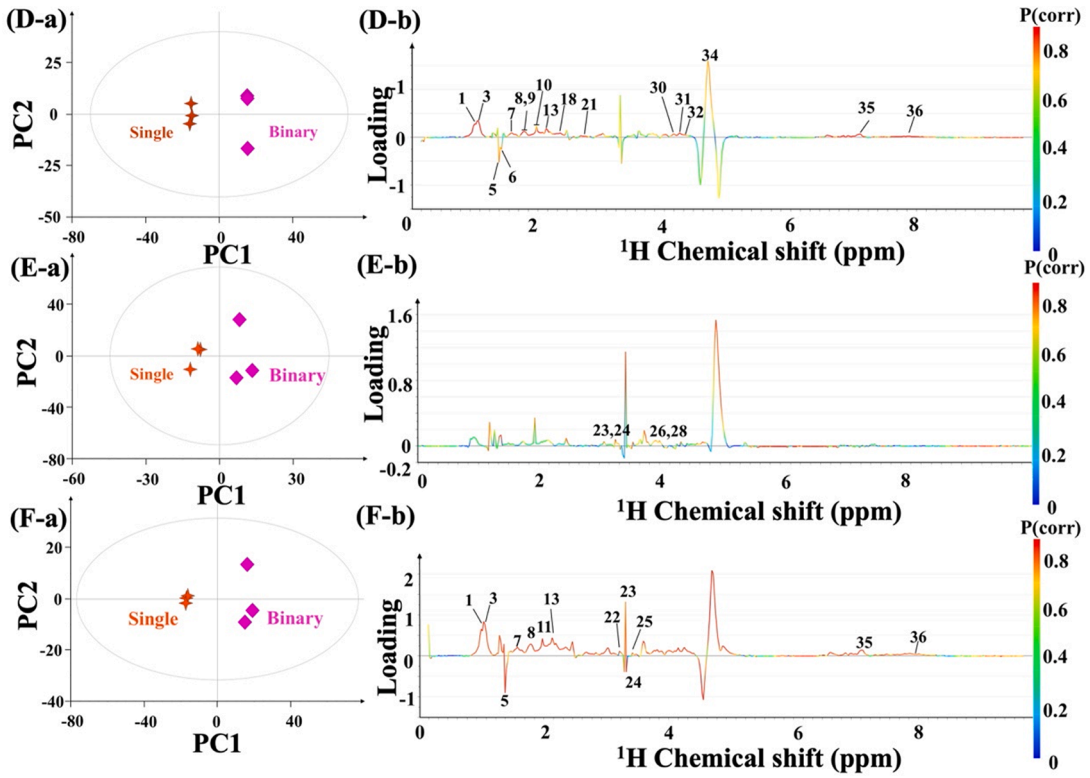

Fig. 4. (*continued*).

clear distinction between mono- and bi-colonial bacteria in the bovine gelatin fermented milk (Fig. 4F-a) (R^2Y was 0.994 and Q^2 was 0.966). The S-line of BG group (Fig. 4F-b) indicated that mixed cultures were associated with increased levels of glucose, ATP, putrescine, lactic acid some amino acids as well as depleted levels of phenylalanine and 3-hydroxybutyric acid. The higher concentrations of metabolites in the mixed strains may be because the number of bacteria was already higher than that of single strain; it was also possible that the production of additional metabolites further stimulates growth-related metabolic pathways.

3.4. Alternative metabolic pathway disturbed by different gelatin

Bacteria have a variety of sensors that enable them to monitor their environment, which also means that bacteria can regulate their physiology in ways that are necessary for survival changes (Camilli & Bassler, 2006). To identify metabolic pathways disturbed by gelatin, significantly different metabolites were screened. Within this discriminatory metabolite pool, the most critical metabolites with absolute correlation fold change greater than 1.20 or < 0.83, VIPs > 1 and P values < 0.05 were screened and summarized in the Table S4 and the P value of pathways were showed in Table S6-8. In addition, the metabolic pathways affected and the associated up- and down-regulated metabolites are shown in the Table S10 and marked in Fig. 5.

As for L431, the glycolysis, amino acid, TCA cycle, and cell membrane biosynthesis were the main pathways that were disturbed. Based on KEGG pathway databases, the disturbance in relevant metabolic pathways of L431 adding FG compared to OG and BG were summarized and presented in Fig. 6A. Initially, L431 converts lactose into glucose, which serves as a source of carbon and energy. Glycolysis, as a pathway of sugar catabolism, can provide such as G-6-P, R-5-P, and energy as precursors for cellular biosynthesis. In the present experiment, the decrease in ATP and R-5-P levels indicated that the addition of fish gelatin inhibited the energy replenishment of metabolic pathways. In addition to glycolysis, amino acid metabolism was also greatly affected by the fish gelatin treatment. Lower concentrations of amino acids (valine, proline, arginine, aspartic acid) were observed, which can be explained by the inhibition of CEP expression in *Lacticaseibacillus paracasei* when there is an abundant source of peptides and amino acids in the growth environment. (Alcántara et al., 2016).

The metabolism of amino acids provides the metabolic intermediates that enter the TCA pathway (Zhao et al., 2020). The precursors of valine, proline and arginine are α-ketoglutaric acid, which are important intermediates of the tricarboxylic acid cycle. Furthermore, in our study, there was a significant decrease in the synthesis of aspartic acid, a precursor of OAA, as well as Succinct acid and NAD, important intermediates of the TCA cycle. The above results indicate that the TCA cycle of L431 in FG group was indeed weakened and inhibited compared to the OG group. And some studies have confirmed that the TCA cycle of microorganisms can be affected by the stimuli generated by external environmental changes (Zhao et al., 2020). Simultaneously, it is also noted that Cyclic AMP and adenosine increased significantly after adding FG. The reason for the increased concentration of cAMP as we mentioned in 3.2, ensuring that proteomic resources are used in different metabolic sectors to meet their own growth metabolism in different nutritional environments (You et al., 2013). Betaine is thought to play an important role in the maintenance of structural integrity in cells as well as in the synthesis of cell membranes (Michel, Yuan, Ramsubir, & Bakovic, 2006). In addition, choline and betaine may also play a supporting role in the formation of microbial communities, as they enhance cell survival under stressful conditions (Wargo, 2013). In the present study, the decrease in phosphorylcholine acted as a precursor to synthetic choline and betaine content may indicate that the addition of FG does not stress the survival of cells. Whereas there was no significant difference between the FG-treated and BG-treated groups, the upregulation of acetic acid metabolites in BG fermented milk may have been an attempt to supplement the lack of TCA energy supply through the mixed acid fermentation pathway (Zhao et al., 2019). Specific analysis of metabolic pathways showed that FG, like BG, had adverse effect on the metabolism of probiotics to some extent. This may be due to

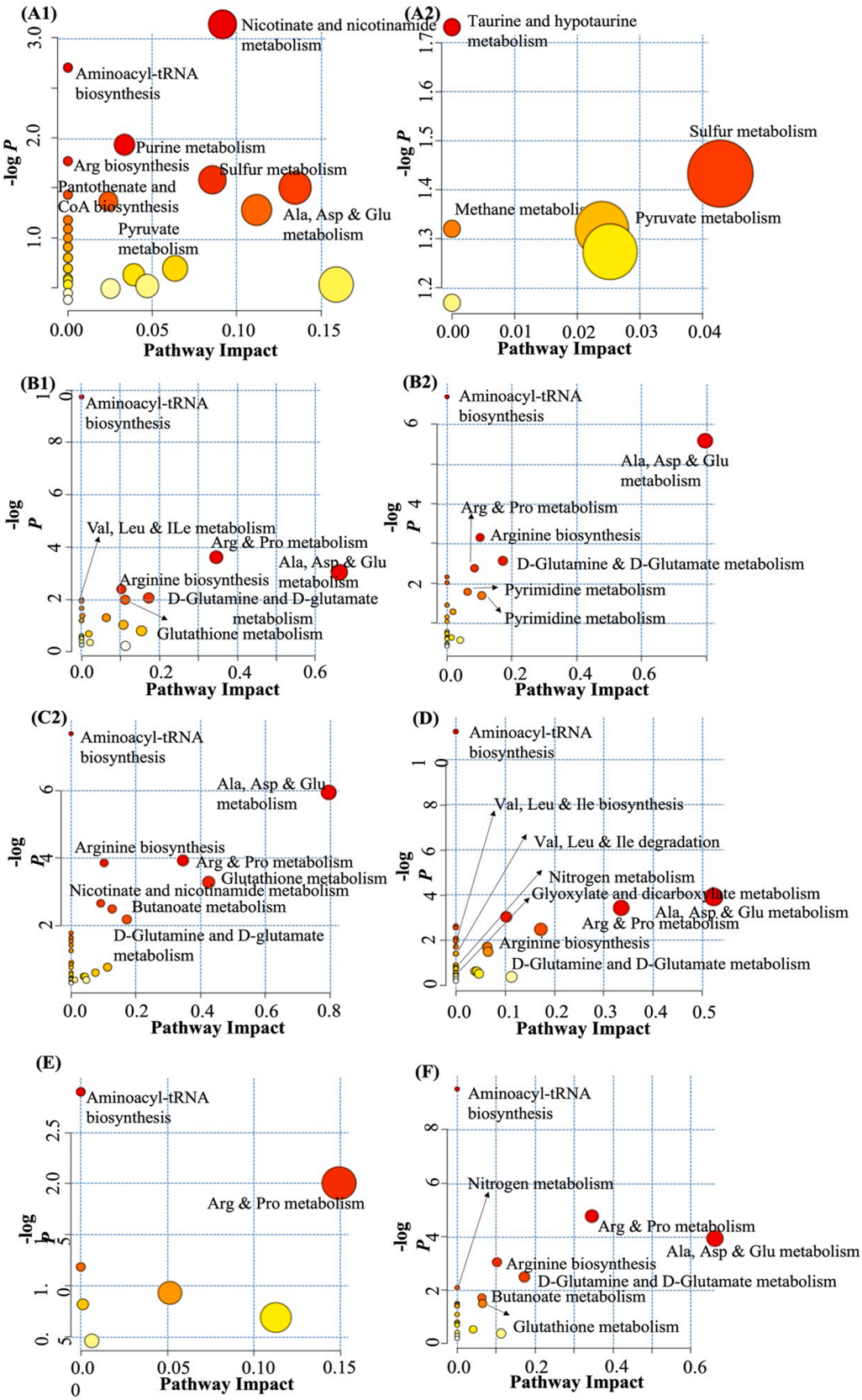

Fig. 5. Altered metabolic pathways between adding XG-FG and no adding gelatin (A1, B1); and between adding modified-fish gelatin and bovine gelatin (A2-C2); Altered metabolic pathways induced by monoculture versus binary cultures in different gelatin fermented milks (D: fermented milk without gelatin; E: fermented milk with modified fish gelatin; F: fermented milk with bovine gelatin).

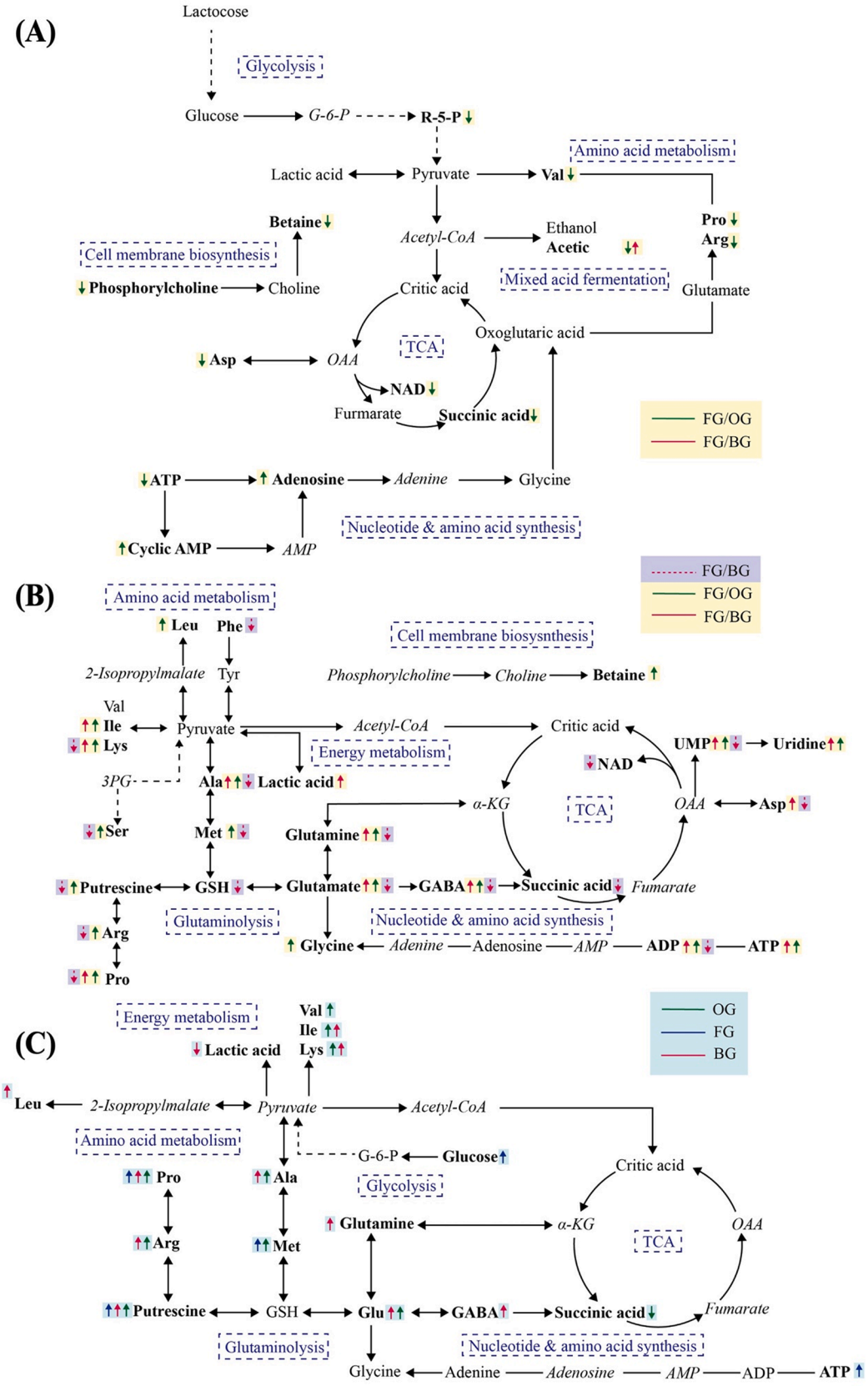

Fig. 6. Proposed schematic of metabolic alteration by different gelatin treatment and binary culture. Note: A: L431 in different gelatin treatment; B: La-5 in different gelatin treatment (light yellow arrow background indicates single La-5; light purple arrow background indicates La-5 co-cultured with Bb-12); C: light blue arriw background indicates binary culture (La-5 and Bb-12) compared with single culture (La-5); Arrows pointing upwards and towards represent increases and decreases in metabolite levels; metabolites in italics were not detected or no significant change in metabolites in bold ($P > 0.05$).

the presence of FG and BG, which allows L431 to survive with an adequate and complex source of nitrogen, and this may lead to the inhibition of its protein hydrolysis system and the expression of extracellular proteases (Marugg et al., 1995; Hebert, Raya, & De Giori, 2000). It is probably the inhibition of enzymes associated with protein digestion and absorption that allowed L431 to grow more rapidly in the OG group compared to the FG and BG groups. As for single La-5, the main pathways that are disturbed are amino acid metabolism, glutaminolysis, TCA cycle and energy metabolism. However, unlike L431, metabolites under all affected metabolic pathways were increased. The inferred metabolic pathway for La-5 is shown in the Fig. 6B. Analysis of the intracellular metabolite pool data showed increased production and accumulation of amino acids such as Ile, Lys, Ala, Pro, glutamine and glutamate in the FG group compared to the OG and BG groups, indicating an upregulation of amino acid metabolic pathways. The reason for the up regulation of these amino acids may be since proteins associated with the metabolism of these amino acids have been affected. Previous studies have shown that the expression of a glutamate-fructose-6-phosphate transaminase (GFAT), which plays a key role at the intersection of carbohydrate metabolism and protein metabolism, was increased in the treatment group supplemented with soy protein (Zhang et al., 2021a, 2021b). In addition, high levels of branched-chain amino acids, glutamate and phenylalanine can regulate metabolic pathways such as purine biosynthesis, amino acid synthesis, etc. (Kaiser & Heinrichs, 2018), and these pathways are mainly involved in the regulation of high growth rate of the probiotics. This also explains the higher accumulation of ADP and ATP in the FG group, as these metabolites contribute to the accumulation of basic microbial metabolites to increase the growth rate of microorganisms. In addition, upregulation of the glutaminolysis metabolic pathway was also associated with energy conversion and energy metabolism, as glutamate and glutamine can be phosphorylated at the substrate level to provide ATP (Drake, Sidorov, McGuinness, Wasserman, & Wikswo, 2012). An increase in ATP can reflect the transcriptional level of the organism and serve as an important marker reflecting external stimuli. There was a significant increase in UMP and uridine levels, which may be due to the enhanced nucleotide metabolism, thus promoting pyrimidine nucleotide synthesis (Lourenço, Kamnetz, Gadotti, & Diez-Gonzalez, 2017).

Unlike single La-5, La-5 co-cultured with BB-12 did not have significantly different metabolites in the comparison between FG and OG and therefore there is no different metabolic pathways. In contrast to the changes in the FG and BG metabolic pathways in single La-5, significantly lower levels of Phe, Arg, Lys, Met, Pro, Phe, glutamine, glutamate, gamma-Aminobutyric acid (GABA) and Asp were observed in binary La-5. Thus, the metabolism of amino acids was undoubtedly the most affected metabolic pathway. In addition to this, key intermediates such as NAD and succinate involved in the TCA cycle and nucleotide synthesis were observed to be significantly reduced, which also indicated a significant inhibition of the TCA cycle. It is also possible that the conversion of amino acids resulted in a weakening of the energy synthesis pathway. A greater accumulation of amino acids was observed in fermented milk supplemented with BG than OG and FG. In mono-La-5, FG fermented milk had a facilitative effect on probiotic metabolism, whereas in binary La-5, it was BG that had a facilitative effect on probiotics. This may be due to the richer nutritional source of proteins in FG and BG fermented milk. It is possible that the proteins provided by the gelatin were degraded to amino acids by the proteases of *Lactobacillus acidophilus* as a source of providing energy and carbon sources. The results are similar to previous studies that compared the similarities and differences in the metabolomics of *S. aureus* inoculated on chicken breast and broth (Dupre et al., 2019), and another study that compared the metabolic differences of *L. monocytogenes* between inoculation on shrimp and broth (Zhao et al., 2020). Bacteria on more complex substrates with nitrogen sources had better metabolic expression than those in broth only.

3.5. Alternative metabolic pathway disturbed by binary culture compare with single culture

Based on selected differential metabolites (Table S5), pathway analysis was carried out to investigate the reasons for the promotion of probiotic growth by binary cultures. A total of 28, 7, and 22 pathways were predicted, with 14, 2, and 12 pathways was significantly disturbed ($P < 0.05$) (Table S9) by binary culture in OG, FG and BG fermented milk (Fig. 5D-F) (Table S11), respectively.

The hypothetical metabolic pathway of La-5 affected by co-cultured with Bb-12 under different gelatin treatment is shown in Fig. 6C. Consistent with the results of previous studies, the mixed probiotic ferments had higher concentrations of probiotic metabolites, a result that suggests a coordinated promotion of probiotics by mixed fermentation (Peng et al., 2021). The most obvious changes in metabolic pathways in all three groups were mainly in amino acids metabolism pathways. Previous studies comparing the stimulatory effects of *S. thermophilus* on *L. bulgaricus* found that most of the differentially expressed genes were related to growth genes and the amino acid metabolism and nucleotide metabolism had the highest percentage of differentially expressed genes (Sieuwerts, Molenaar, van Hijum, Beerthuyzen, Stevens, Janssen, Ingham, de Bok, de Vos, & van Hylckama Vlieg, 2010). In this study, the higher concertation of Val, Ile, Lys, Leu, Pro, Arg, Ala, Met indicated that mixed culture indeed promoted amino acid biosynthesis.

In additions, glutamate, glutamine and GABA can be produced form putrescine, which was elevated in all three groups. And GABA can further supplement the TCA cycle as a source of carbon and nitrogen, providing energy for cell growth and vital activities. The decrease in succinate in the OG group could be that the cells are regulating carbon and nitrogen homeostasis through GABA shunting. In the BG group, a decrease in lactic acid was observed, accompanied by a rise in leucine, probably due to a decrease in lactic acid caused by the increased synthesis of leucine from their common precursor alanine. While in the FG group, a significant rise in glucose was observed, probably because the addition of protein resulted in an upregulation of β-phosphoglucomutase, which plays an important role in sugar transport and metabolism (Zhang et al., 2021a, 2021b). In addition, a higher ATP content was found in mixed strains of fish gelatin fermented milk than in single strains, the increased levels of ATP indicated that the binary culture enhanced replenishment of energy for metabolic pathways. In short, metabolic expression and growth of La-5 were promoted by co-culture mainly through three metabolic pathways: facilitation of amino acid metabolism, sugar transport and energy metabolism.

4. Conclusion

In this study, the effects of different types of gelatin addition and binary culture on the growth and metabolism of probiotic bacteria were investigated by NMR-based metabolomics. In general, FG addition did not significantly promote the growth of probiotics. From the metabolic point of view, the addition of FG affected the amino acid metabolism of L431 to some extent but did not negatively affect the survival status of L431. In contrast to the results for L431, La-5 was able to use the amino acids provided by FG to promote its own growth and metabolism. However, in La-5 cultured with Bb-12, it was BG that showed a facilitative effect on its amino acid metabolism. Furthermore, all metabolites of La-5 co-cultured with BB-12 containing different gelatins were highly expressed compared with single strain. Metabolic expression and growth of La-5 were promoted mainly through three metabolic pathways: facilitation of amino acid metabolism, sugar transport and energy metabolism. Overall, this study shows that NMR-based metabolomics can effectively elucidate the effects of different gelatin additions and binary cultures on the metabolic mechanisms of probiotics, providing theoretical guidance for the industrialization of fish gelatin in the fermented milk industry. Furthermore, this work provides a detailed

understanding of the different metabolic profiles and growth between single and binary probiotic cultures, which could aid the development of probiotic fermented milk with high probiotic viability and unique metabolomic profiles.

CRediT authorship contribution statement

Yi Le: Conceptualization, Methodology, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. **Hongshun Yang:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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