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Metabolic labeling of peptidoglycan enabled optical analysis of probiotic vitality†

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The growing awareness of the health benefits associated with probiotics has led to an increasing interest in probiotic products. To develop probiotic functional foods that deliver health benefits, it is essential to characterize both probiotic viability (the ability to survive) and vitality (the ability to remain active and effective). However, traditional probiotic assays only provide limited information about their survival state. To gain a comprehensive understanding of probiotic states, a D-amino-acid-based metabolic labeling strategy was applied to quantitatively depict probiotic vitality. In this approach, probiotics were first metabolically incorporated with azido-modified D-lysine and then labeled with dibenzocyclooctyne-sulfo-Cy5 through click chemistry. This two-step labeling process provides a visual representation of the metabolic levels of probiotics as well as the bacterial membrane integrity. Besides, this method is capable of characterizing the influence of various environmental conditions, from manufacturing to oral administration, on probiotic vitality. With its rapid detection process and general applicability, this strategy has the potential to be widely implemented in the food industry for probiotic vitality evaluation.

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1. Introduction

The term "probiotics" first came into public view in 1974 and was later officially defined by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".[1] Nowadays, probiotics are commonly used as supplements to promote overall wellness[2] and are found in various products such as drinks, yogurts, and cheese. The probiotic market is projected to increase to 91.9 billion USD by 2026 with a compound annual increase rate of 8.3% from 2021.[3]

To deliver health benefits, probiotics must be alive and metabolically active. An active metabolic state is essential for probiotics to adhere to and colonize the gut, avoid exclusion by resident microbes, and release beneficial metabolites.[4–6] However, the states of probiotics can change during the manufacturing, delivery, storage, and gastrointestinal transit processes,[7] before reaching their target in the large intestine. Therefore, it is crucial to assess both viability (the ability to survive) and vitality (the ability to remain active and effective) of the bacteria. Traditional probiotic assays, including the standard plate count of colony forming units (CFUs), nucleic acid staining, measurement of enzymatic activity, and quantification of the membrane potential, mostly focus on viability, providing limited information on the metabolic state of probiotics.[8–10] To evaluate vitality, the growth rate or production of target metabolites, such as organic acids, antimicrobial compounds, and enzymes, is usually quantified.[11] Nevertheless, these assays are time-consuming and a little complicated. Therefore, new methods are required to probe the vitality of probiotics more efficiently.

The biosynthesis of peptidoglycan (PG) plays a key role in regulating bacterial growth and involves the participation of various enzymes, amino acids, saccharides, and adenosine triphosphate (ATP) as an energy source. Hence, understanding this process can provide insights into both the dynamic growth and metabolic status of bacteria. It was reported that unnatural D-amino acids can be incorporated into newly synthesized PG,[12,13] leading to the use of fluorescent derivatives of D-amino acids as probes to visualize the growth pattern of various bacteria,[12] detect the existence of anaerobic commensal bacteria,[14] and assess the viability of transplanted bacteria.[15] As such, metabolic labeling that utilizes D-amino acids modified with functional molecules has exhibited great promise in the field of microbiology.[16–18]

Herein, we present a new method to quantitatively and comprehensively evaluate the vitality of probiotics in commercial products by exploiting the biological process of PG synthesis (Scheme 1). This approach involves the metabolic labeling of probiotics with azido-modified D-lysine, followed by

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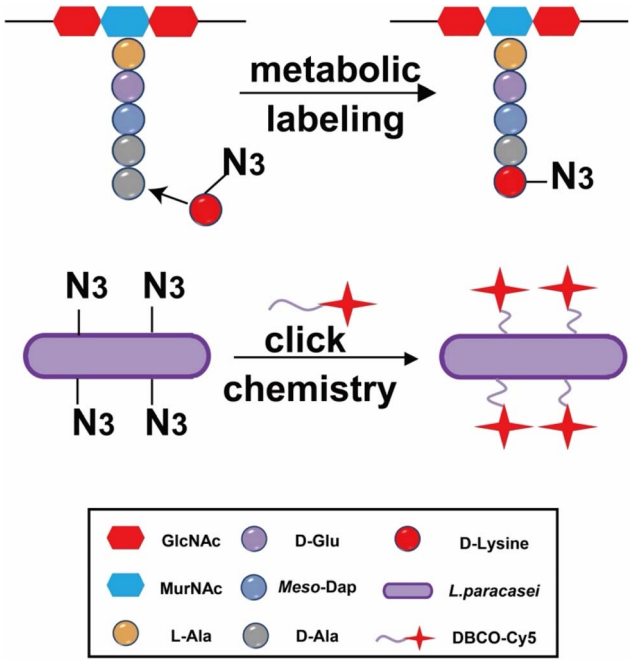

Scheme 1 Metabolic labeling of probiotics as a platform for assessment of probiotic vitality. The method involves the incorporation of azido-modified D-lysine into the peptidoglycan and subsequent labeling of the surface with DBCO–Cy5 through click chemistry, which introduces fluorescence to the probiotics, allowing for easy evaluation of the effects of temperature, lyophilization, prebiotics, gastrointestinal fluid, storage, and pH on the vitality of probiotics.

the conjugation of the fluorescent probe dibenzyl cyclooctyne (DBCO)-sulfo-Cy5 *via* click chemistry. The method was validated using the model bacterium *Lactobacillus paracasei* by comparing the degree of metabolic labeling with intracellular ATP levels, ensuring that the fluorescence intensity of Cy5 accurately reflected the metabolic activity of probiotics. It was then compared with traditional assays that quantify CFUs, membrane integrity, or enzymatic activity, demonstrating its ability to rapidly assess bacterial viability and metabolic activity at both the single cell and bulk levels. We also evaluated the effect of various conditions during probiotic manufacturing and administration, such as temperature, lyophilization, prebiotics, pH, and simulated gastrointestinal fluid. Our strategy is versatile and can be applied to other probiotic strains, with a commercial product containing *Lactobacillus* selected as a representative for assessing the vitality of probiotics.

2. Materials and methods

2.1. Reagents

6-Azido-D-lysine hydrochloride, 6-azido-L-lysine hydrochloride, D-lysine, sodium azide (NaN_3), MRS broth and agar (from De Man, Rogosa and Sharpe), LB broth and agar, Triton X-100, starch, inulin (M_w = 5000), pepsin, pancreatin from porcine pancreas and bile extract porcine were purchased from Sigma-Aldrich. An ATP determination kit (A33066), a LIVE/DEAD® BacLight™ bacterial viability kit, and 5-(and-6)-carboxyfluorescein diacetate [5(6)-CFDA] were obtained from Thermo Fisher. DBCO-sulfo-Cy5 was purchased from Jena Bioscience. Phosphate buffered saline (PBS, pH 7.4) was purchased from Gibco. Tris-acetate–EDTA (TAE buffer, pH 8.0, ultra-pure grade) was supplied by Vivantis.

2.2. Bacterial strains

L. paracasei (BNCC 192210) was obtained from the BeNa Culture Collection (BNCC). *L. rhamnosus* GG (ATCC 53103), *L. casei* (ATCC 393) and *B. subtilis* (ATCC 33677) were purchased from the American Type Culture Collection (ATCC). MRS broth and agar were used for the bacterial culture. The fourth generation of bacteria was used in all the involved experiments. *L. paracasei*, *L. rhamnosus* GG and *L. casei* were all purified from the isolated colony on the MRS agar and then cultured in the MRS broth in a CO_2 incubator at 37 °C. *B. subtilis* (ATCC 33677) was purified from the isolated colony on the LB agar and then cultured in the LB broth in the incubator at 37 °C.

2.3. Metabolic labeling of *L. paracasei*

L. paracasei was cultured overnight and diluted 100 times for further incubation for 6 h to reach the exponential growth phase. Next, 6-azido-D-lysine hydrochloride was added into 1 mL of culture medium with the final concentration being 1 mM and co-incubated with bacteria for 1 h. Afterwards, the labeled bacteria were harvested by centrifugation (3000 × *g*, 2 min) followed by washing with PBS three times to remove the excess 6-azido-D-lysine hydrochloride. Then the solution of DBCO-sulfo-Cy5 in PBS with a final concentration of 5 μM was added and incubated with azido decorated bacteria for another 1 h at 100 rpm at room temperature. Finally, the metabolically labeled bacteria were also acquired through centrifuging and washed with PBS three times followed by storage in PBS at 4 °C for further characterization.

2.4. The specificity of metabolic labeling

To investigate the specificity of metabolic labeling on probiotics, *L. paracasei* was treated with the different chemical structures of amino acids respectively for metabolic labeling. Then each group was treated with DBCO-sulfo-Cy5. Flow cytometry (Fortessa X20, BD) was performed to assess the fluorescence intensity of Cy5 at an excitation wavelength of 642 nm. And the metabolic labeling was visualized by means of a confocal microscope (LSM800, ZEISS).

2.5. The feasibility of metabolic labeling

Different metabolic states were characterized *via* metabolic labeling to verify the feasibility of the method for evaluating the probiotic physiological state. The bacteria in the lag phase, log phase and stationary phase with the same optical density (OD_{600} = 0.480) were selected for metabolic labeling. After that, flow cytometry and the confocal microscope were also used to evaluate different metabolic states. Meanwhile, the intracellular ATP levels of these three growth phases were determined to

reflect metabolic status directly. Briefly, *L. paracasei* in different growth phases were harvested *via* centrifuging and resuspended into the lysis solution (40 mM Tris-acetate, 1 mM EDTA, 1% Triton X-100) followed by boiling for 5 min to release the ATP. The supernatants were collected and added to the standard reaction solution prepared from the ATP determination kit with 1% of the final volume. The luminescence intensity was determined by using a microplate reader, and normalized luminescence intensity was calculated.

In addition, optical density (OD) at a wavelength of 600 nm was determined at predetermined time intervals after the overnight cultivation was diluted 100 times and at the same time the fluorescence intensity of metabolic labeling was also detected using a fluorospectrophotometer (excitation wavelength of Cy5: 642 nm; emission wavelength of Cy5: 665 nm).

4 mM of sodium azide (NaN_3), the metabolic inhibitor, was also co-incubated with 1 mM of 6-azido-D-lysine hydrochloride for metabolic labeling of *L. paracasei* in the log phase. Finally, the results were acquired from the microplate reader, flow cytometry and the confocal microscope. Similarly, the intracellular ATP level was also assessed as mentioned above with the group without NaN_3 as the control.

2.6. Comparison with traditional probiotic assays

2.6.1. Standard plate count. 100 μL of NaN_3 treated bacteria and untreated ones with appropriate concentrations were transferred to the MRS agar for spread plating separately after serial dilutions followed by incubation for 48 h at 37 °C. The information on CFU in original samples could be calculated by counting the number of colonies on the plates.

2.6.2. Bacterial live and dead assay. The membrane integrity of *L. paracasei* was assessed by applying the SYTO 9 and PI double staining method. Concisely, 1.5 μL of SYTO 9 solution (3.34 mM in dimethyl sulfoxide (DMSO)) and 1.5 μL of PI solution (20 mM in DMSO) were added together into 1 mL of NaN_3 treated bacterial or untreated one's PBS suspension and the solution was mixed thoroughly followed by incubation in a dark environment at room temperature for 15 min. Subsequently, the excess dye was removed by centrifugation and the stained bacteria were resuspended in PBS and detected both by using flow cytometry and the confocal microscope.

2.6.3. CFDA assay. The intracellular esterase activity was evaluated by utilizing 5(6)-CFDA as the indicator. In brief, 2 μL of CFDA solution (5 mM in DMSO) was added into 1 mL of NaN_3 treated or untreated bacterial culture medium, and the solution was stirred thoroughly. Then the labeled bacteria were acquired *via* centrifuging after being cultured for 30 min at 37 °C. Lastly, the organisms were washed with PBS three times and characterized by using flow cytometry and the confocal microscope.

2.7. Assessment of probiotic vitality under different environmental conditions

2.7.1. Effect of temperature. To verify the effectiveness of this method in the assessment of vitality, a series of temperatures was established to evaluate its effect on *L. paracasei*. In short, bacteria in the log phase were collected and resuspended in PBS buffer followed by treatment at different temperatures separately for 30 min.[19] After the treatment, the cells were harvested by centrifugation and redispersed in MRS broth for subsequent metabolic labeling. The effect of temperature was also assessed *via* flow cytometry, the confocal microscope, and the microplate reader. The SYTO 9 and PI double staining as well as the standard plate count were also applied for the 50, 70 and 90 °C treated groups.

2.7.2. Effect of lyophilization. Since lyophilization is commonly conducted in the probiotics industry,[20] its influence was also studied *via* metabolic labeling. Similarly, *L. paracasei* in the log phase was treated with lyophilization and the powders were re-dissolved in MRS broth followed by metabolic labeling. The vitality difference caused by lyophilization could be compared by conducting analysis of flow cytometry and using the confocal microscope and microplate reader.

2.7.3. Effect of prebiotics. To test the effect of prebiotics, two commonly added prebiotics-starch and inulin in the probiotic food were selected to evaluate their effects on the probiotics.[21] At this time, the starch and inulin were co-incubated with *L. paracasei* for metabolic labeling, respectively. The effect after adding the prebiotics was evaluated as stated above.

2.7.4. Effect of pH. To assess the effect of pH on the probiotic vitality, a wide range of pH was set to simulate possible situations in manufacturing. First, log phase bacterial broth cultures were centrifuged, and cells were redispersed in sterile, acidic, or basic PBS solution (adjusted to pH 1, 2, 3, 4, 5, 6, 8, and 9 with HCl or NaOH, PBS as the control), and allowed to incubate at 37 °C for 0.5 h.[22] Although the buffer capacity was limited when the pH was 1, 2, 3, 4, 5, and 9, there were nearly no factors causing the adjusted pH to change with a short treatment time since there were no nutrients in PBS for bacterial proliferation and metabolism. After that, the cultures were revived in MRS broth for metabolic labeling. The effect of pH was characterized by means of flow cytometry, the confocal microscope, and the microplate reader. In addition, the bacterial LIVE/DEAD staining and standard plate count were conducted for the pH 1 and 2 treated groups.

2.7.5. Effect of simulated gastrointestinal fluid. The simulated gastrointestinal fluid was used to mimic the gastrointestinal environment for the determination of its effect on probiotic vitality. And it should be noted that the following settings refer to the operations which researchers commonly adopted and cannot totally represent the real gastrointestinal conditions since it really depends on the administered food type and human state. But it could give us a preliminary judgement about the effect of harsh environments on the probiotic vitality within the GI tract. *L. paracasei* in the log phase was initially dispersed in the simulated gastric fluid (SGF, normal saline with 0.32 mg per mL pepsin and adjusting it to pH 2 with HCl) for 2 h or simulated intestinal fluid (SIF, normal saline with 0.1% of pancreatin and 3.6% w/v of bile acid) for 4 h at 37 °C, respectively.[23,24] Then, the bacteria were harvested for metabolic labeling and SYTO 9 and PI double staining was also conducted. The bacterial survival states were also checked with standard plate count. The evaluation of bacterial performance could be

achieved *via* flow cytometry, the confocal microscope, and the microplate reader.

2.8. General applicability in other probiotic strains

The general applicability in other probiotic strains was investigated by selecting *L. rhamnosus* GG, *L. casei* and *B. subtilis* as models.[25] These strains in the exponential phase were used for metabolic labeling followed by characterization *via* flow cytometry and the confocal microscope.

2.9. Assessment of probiotic products

The assessment was also conducted on probiotic products and commercial probiotic drinks containing *Lactobacillus* were selected as the model. The drinks were placed at 4 °C for conservation. In brief, an equivalent volume of MRS broth was mixed with drinks and the mixture was set aside for 1 h. Coagulation occurred *via* the electrostatic interaction between the negatively charged κ-casein in milk solids and positively charged metal ions such as Mg^{2+} and Mn^{2+} from MRS broth.[26] Subsequently, the sample was centrifuged (100 × *g*, 5 min) to remove the sediments and the supernatant was collected to harvest the probiotics. The acquired probiotics were then dispersed in fresh MRS broth and cultivated for 6 h followed by metabolic labeling. The drinks were further evaluated on day 1 and day 5 (the day of purchase was set as day 0) with the same treatment. Also, the change in probiotic vitality was characterized with the help of flow cytometry, the confocal microscope, and the microplate reader.

2.10. Statistical analysis

All the data were exhibited as mean ± SD, and an analysis was carried out with FlowJo and GraphPad Prism. The significant difference between two groups was considered only when the value of p was lower than 0.05 and presented as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

3. Results and discussion

3.1. Construction of a probiotic vitality assessment platform

The metabolic incorporation of azido-modified D-lysine into PG, followed by the bioorthogonal reaction with DBCO-sulfo-Cy5, is expected to serve as a probiotic vitality assessment platform, as the biosynthesis of PG reflects the vitality of bacteria. *L. paracasei*, one of the most commonly used probiotics in food production,[27] was selected as the study model. As presented in Fig. S1,† *L. paracasei* incubated with D-lysine–N_3 for 1 h showed intense fluorescence after reacting with DBCO-sulfo-Cy5, while negligible fluorescence was observed when D-lysine–N_3 was replaced with L-lysine–N_3 or D-lysine. This indicates that (1) D-amino acids rather than the L-type can be incorporated during the synthesis of PG, (2) the fluorescent dye cannot permeate an intact cell membrane for nonspecific staining, and (3) DBCO-sulfo-Cy5 specifically labels metabolically active bacteria *via* the copper-free click chemistry between azide and DBCO. Thanks to the introduction of fluorescent probes, the metabolic state of probiotics is revealed by the fluorescence intensity of the labeled bacteria, which can be measured at the single-cell level with flow cytometry and confocal microscopy and at the bulk level with microplate readers.

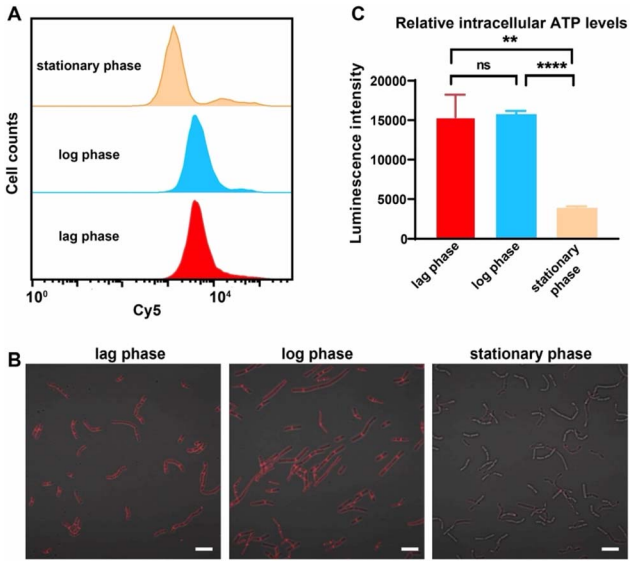

Fig. 1 (A) Representative flow cytometry results of metabolic labeling of *L. paracasei* in different growth phases. (B) Representative confocal images of *L. paracasei* through metabolic labeling in different growth phases. Red, DBCO-sulfo-Cy5. Scale bar, 5 μm. (C) Relative intracellular ATP levels in different growth phases ($n = 3$). $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

3.2. Feasibility of the probiotic vitality assessment platform

To determine whether metabolic labeling could quantitatively characterize probiotic vitality, we investigated metabolic labeling of *L. paracasei* in different growth phases, each characterized by a distinct metabolic profile. The results showed that the fluorescence intensity of bacteria in the log phase was similar to that of bacteria in the lag phase, but both were higher than that of bacteria in the stationary phase (Fig. 1A and B), indicating that the metabolic level of *L. paracasei* in the stationary phase was much lower than that of bacteria in the exponential and lag phases. This was further verified by the relative intracellular ATP levels in different growth periods. It is well established that ATP is the primary source of energy for biological processes in living organisms and its fluctuation could directly reflect the change in bacterial metabolism.[28] As shown in Fig. 1C, the lower ATP level in bacteria in the stationary phase (represented by the normalized luminescence intensity) was consistent with the results of metabolic labeling. The discrepancy among these three growth phases was due to different biological activities, such as necessary molecule synthesis for division in the lag phase, bacterial division in the log phase, and cessation of the biomass increase in the stationary phase.[29] This evidence demonstrates that metabolic labeling can accurately reflect the metabolic status in different physiological states.

Our results have shown that the change in fluorescence intensity was in line with the growth curve of *L. paracasei*

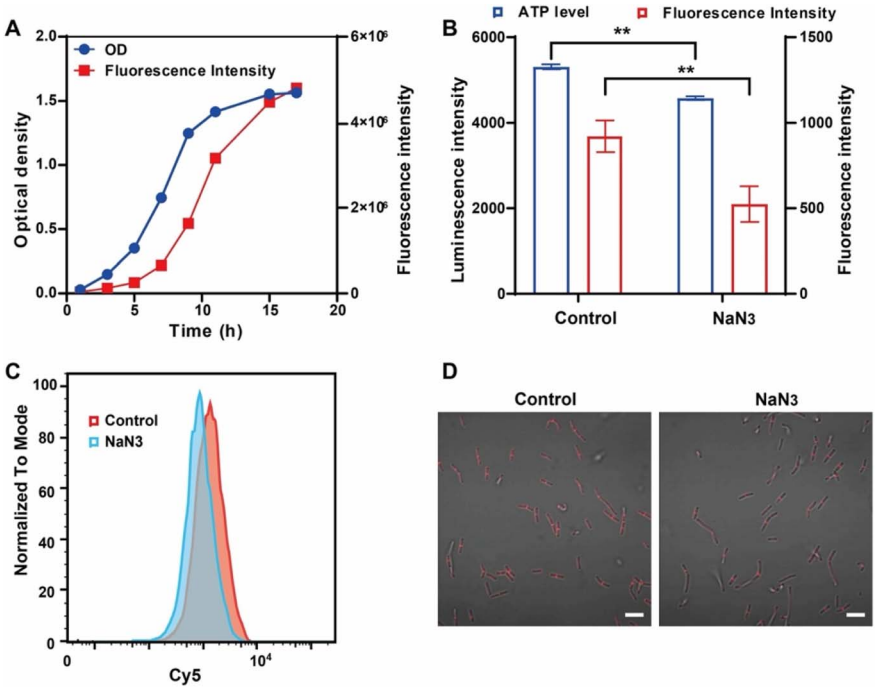

Fig. 2 (A) The growth curve of *L. paracasei* and change in fluorescence intensity at predetermined intervals ($n = 3$). (B) Relative intracellular ATP levels and fluorescence intensity of the bacteria after metabolic labeling in the absence and presence of NaN_3 ($n = 3$). $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. (C) Representative flow cytometry results of metabolic labeling of *L. paracasei* with and without the NaN_3 treatment. (D) Representative confocal images of *L. paracasei via* metabolic labeling with or without NaN_3 treatment. Red, DBCO-sulfo-Cy5. Scale bar, 5 μm.

(Fig. 2A), demonstrating that metabolic labeling can reflect the dynamic growth behaviors of probiotics. To further evaluate the feasibility of this probiotic vitality assessment platform, *L. paracasei* was treated with 4 mM NaN_3 to inhibit its metabolism. NaN_3 is known to interfere with the electrochemical proton gradients of bacteria, leading to a reduction in intracellular ATP levels.[30,31] The results clearly showed a reduction in ATP levels as well as a decrease in the overall fluorescence intensity of Cy5 (Fig. 2B). Flow cytometry showed the same trend at the single-cell level (Fig. 2C), which was further confirmed by confocal images (Fig. 2D).

3.3. Comparison with traditional methodologies

In comparison, the results of the standard plate count showed no significant difference in the number of CFUs between the NaN_3 treated group and the untreated group (Fig. S2A†). This suggests that NaN_3 at an appropriate concentration only affects bacterial vitality but not viability or the ability to replicate.[32] Although plate count can indicate the number of bacteria capable of reproduction, it does not reveal metabolic status and can be time-consuming to conduct with incubation time up to 48–72 h. Notably, our proposed method only took 8 h in total to get information about probiotic vitality, which is essential for the industry to develop probiotic foods. It can help the manufacturer quickly adjust the fermentation conditions in order to preserve the best probiotic vitality. In this case, the manufacturing process would be more efficient and hence a lot of manpower and resources would be saved to make the production economical and environment-friendly.

With regard to the live and dead staining, both the control and NaN_3 treated groups showed intact membranes, as indicated by SYTO-9 staining (Fig. S2B and D†). Therefore, this method provides limited information about bacterial vitality. As for CFDA staining, it showed that *L. paracasei* had similar esterase activity with similar fluorescence intensity in both groups (Fig. S2C and E†), indicating that intracellular esterase activity is not a reliable indicator of bacterial vitality. Overall, these methods only provide a partial understanding of the probiotic states and cannot provide a comprehensive overview.

3.4. Effect of temperature on *L. paracasei*

Temperature during manufacturing can significantly impact the vitality of probiotics. In general, temperatures higher than 45–50 °C are harmful to probiotic survival.[33] The results from metabolic labeling showed that treating *L. paracasei* at 4 °C and 25 °C for 0.5 h did not affect its metabolic state, as indicated by fluorescence intensity similar to that of the control group (37 °C) (Fig. 3A). In contrast, no Cy5 labeling was observed in the group treated at 50 °C; the fluorescence intensity was significantly lower than that of the other groups, suggesting a stagnant metabolic state after being cultured at 50 °C (Fig. 3B). And it was reasonable that the bacteria were metabolically inactive after the treatment at 50 °C, since the metabolic labeling was done quickly after the treatment and the duration for metabolic labeling was only 1 h, which is too short for the bacteria to

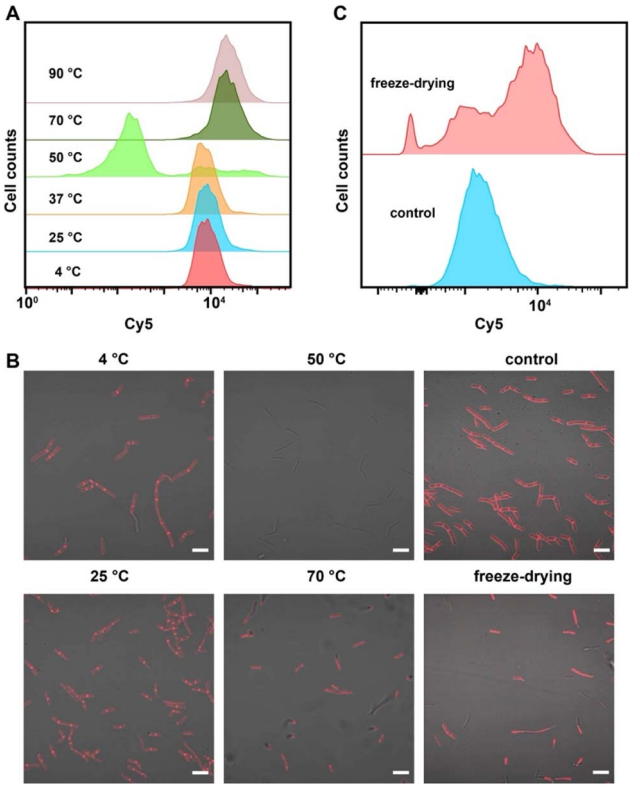

Fig. 3 (A) Representative flow cytometry results of metabolic labeling of *L. paracasei* after treatment at different temperatures. (B) Representative confocal images of *L. paracasei via* metabolic labeling after different temperature treatments and lyophilization. Red, DBCO-sulfo-Cy5. Scale bar, 5 μm. (C) Representative flow cytometry results of metabolic labeling of *L. paracasei* after treatment with lyophilization.

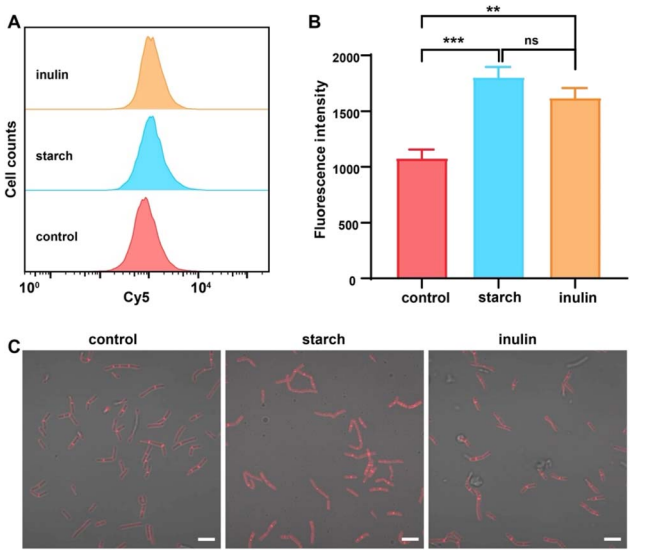

Fig. 4 (A) Representative flow cytometry results of metabolic labeling of *L. paracasei* under different prebiotic treatments, compared with the control. (B) Fluorescence intensity of the bacterial group through metabolic labeling under treatment with starch and inulin, respectively ($n = 3$). $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. (C) Representative confocal images of *L. paracasei* after metabolic labeling in the presence or absence of prebiotics. Red, DBCO-sulfo-Cy5. Scale bar, 5 μm.

restore the normal state, even though the bacteria were still alive to form the colony (Fig. S3†). On the other hand, treatment at 70 °C and 90 °C led to abnormally high fluorescence intensity, which was caused by non-specific dye adsorption in dead cells, as indicated by PI staining and plate count (Fig. S3†). Combined with the results of plate count (Fig. S3A†), close examination of the fluorescence images (Fig. 3B and S4B†) revealed that the bacteria treated at 70 °C or 90 °C were stained with Cy5 throughout the whole bacterial cell instead of being selectively stained in the cell wall, confirming that DBCO-sulfo-Cy5 could enter the dead bacterial cells for nonspecific staining. The design of such a probiotic vitality assessment platform not only reveals the metabolic level of viable cells but also the bacterial survival status.

3.5. Effect of lyophilization on *L. paracasei*

Lyophilization is commonly used in the probiotic industry, but it can cause damage to probiotics due to the formation of ice crystals during the freeze process.[20] To study its effect on the vitality of *L. paracasei*, metabolic labeling was utilized. The results showed that there were three bacterial cell subpopulations present compared with only one population in the absence of lyophilization treatment (Fig. 3C). Furthermore, it was not surprising that a remarkable higher overall

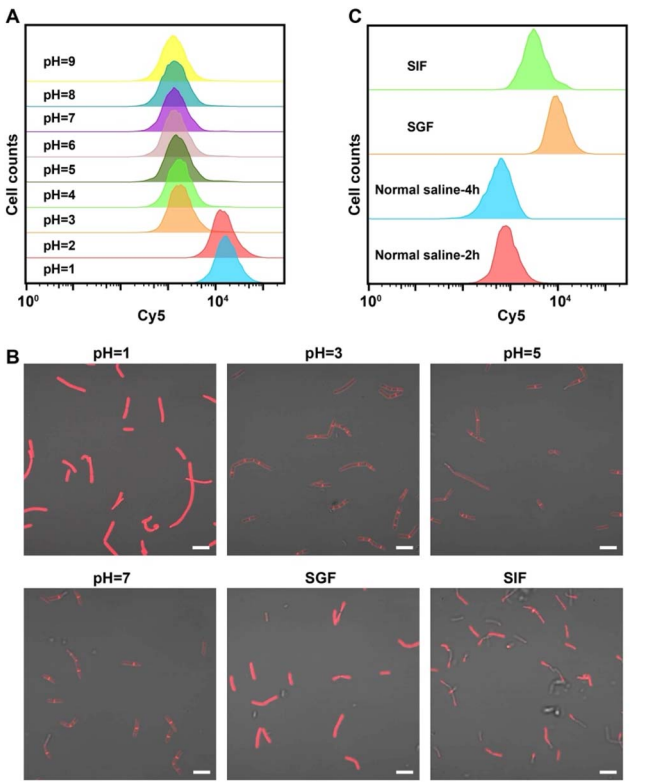

Fig. 5 (A) Representative flow cytometry results of metabolic labeling of *L. paracasei* after different pH treatments. (B) Representative confocal images of *L. paracasei* after metabolic labeling under different pH and simulated gastrointestinal fluid treatments. Red, DBCO-sulfo-Cy5. Scale bar, 5 μm. (C) Representative flow cytometry results of metabolic labeling of *L. paracasei* after the treatment with simulated gastric and intestinal fluids.

fluorescence intensity was observed at the bulk level after the lyophilization treatment (Fig. S4C†). As shown in Fig. 3B, both fully stained and normal metabolically labeled bacteria were present besides the non-labeled probiotics, consistent with the results of flow cytometry, which was attributed to the damage caused by lyophilization. After freeze drying, most probiotics were found to be dead by the penetration of ice crystals with few remaining metabolically active or dormant. Therefore, measures such as adding cryoprotectants are often implemented to increase the possibility of survival.[34]

3.6. Effect of prebiotics on *L. paracasei*

Prebiotics are defined as beneficial substances for human health and are frequently added to probiotic foods as supplements.[35] *L. paracasei* can utilize prebiotics such as starch and inulin by expressing enzymes such as amylopullulanase and β-fructosidase.[36] To examine the effect of prebiotics on probiotics, starch and inulin were selected for investigation. As presented in Fig. 4A and B, the fluorescence intensity increased after adding the prebiotics, with no significant difference when co-incubated with starch or inulin, suggesting that both prebiotics can equally enhance their metabolic activity. Direct observation of Cy5-labeled *L. paracasei* under the microscope (Fig. 4C) further confirmed this conclusion.

3.7. Effect of pH on *L. paracasei*

From manufacturing to processing, probiotics can be exposed to diverse environments with different pH values, such as sour fruit juice and alkaline fermentation environments.[33,37] Therefore, it is essential to study the influence of these pH treatments on the vitality of probiotics, which could provide some guidance for the production of probiotic foods. The flow cytometry results (Fig. 5A) showed that pH treatments from 3 to 9 had a minimal effect compared with the control group, exhibiting similar labeling intensity and characteristics. This demonstrates that *L. paracasei* had a high tolerance to both acidic and slightly alkaline environments.[38] In contrast, the fluorescence intensity of bacteria treated at pH 1 and pH 2 was significantly higher than that of bacteria in other groups according to flow cytometry (Fig. 5A), which was also reflected in the overall fluorescence measurement of the bulk bacterial solutions (Fig. S5A†). Combined with the results of live and dead assay and plate count (Fig. S6†), it was confirmed that the probiotics had died and were stained with Cy5 throughout the whole bacterial cells under extremely acidic conditions (Fig. 5B and S5B†).

3.8. Effect of simulated gastrointestinal fluids on *L. paracasei*

To confer health benefits on the human body, probiotics must survive the harsh conditions of the gastrointestinal tract. However, the normal state of the bacterial cells may be altered

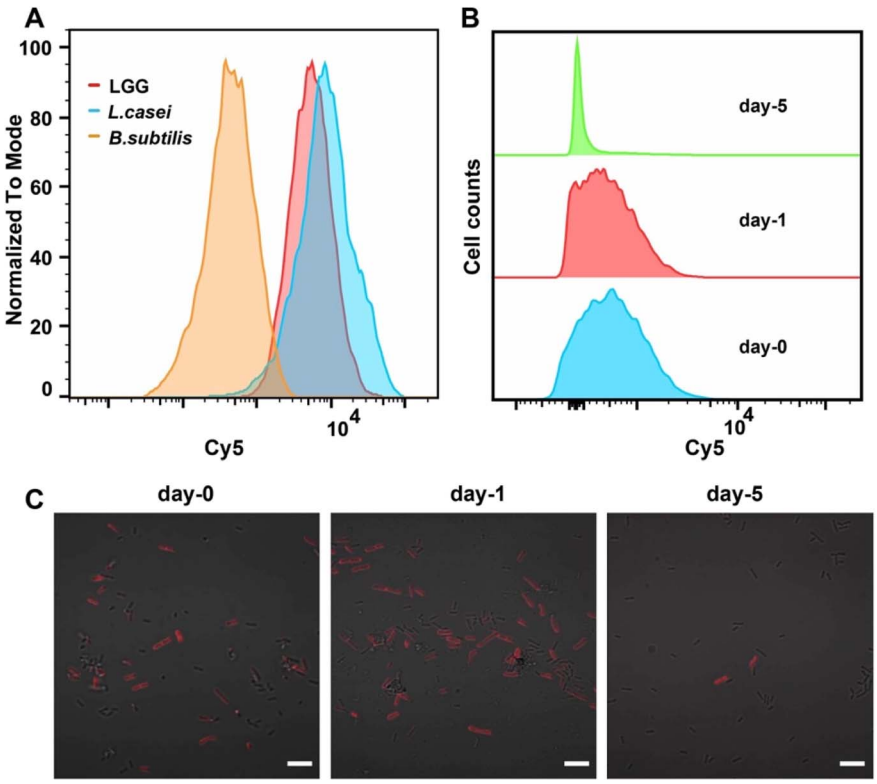

Fig. 6 (A) Representative flow cytometry results of metabolic labeling of LGG, *L. casei* and *B. subtilis*. (B) Representative flow cytometry results of metabolic labeling of the probiotics in commercial probiotic drinks after storage for 1 and 5 days. (C) Representative confocal images of probiotics in commercial probiotic drinks after metabolic labeling at different storage times. Red, DBCO-sulfo-Cy5. Scale bar, 5 μm.

before reaching their target location. To assess the influence of the complicated digestive system, incubation with simulated gastric fluid (SGF) for 2 h or simulated intestinal fluid (SIF) for 4 h was conducted to simulate the physiological environment.[39] As illustrated in Fig. 5C, there was a remarkable higher fluorescence intensity of Cy5 in both the SGF and SIF treated groups compared with the control. As clearly shown in Fig. 5B, the whole *L. paracasei* cell was stained with DBCO-sulfo-Cy5. Together with the results of live and dead assay and plate count (Fig. S7†), it can be inferred that it is difficult for *L. paracasei* to survive in the gastrointestinal tract for a certain period of time. In addition, it was notable that the fluorescence intensity of Cy5 after SIF treatment was lower than that after SGF treatment, suggesting that an environment with an extremely low pH value causes more damage to probiotics than bile acids.

3.9. General applicability assessment

To assess the general applicability of this vitality assessment platform, another three commonly used probiotics, *L. rhamnosus* GG (LGG), *L. casei* and *B. subtilis*, were studied under normal conditions. It was evident that metabolic labeling can be successfully performed on these three probiotics, as demonstrated by the flow cytometry and microscope results (Fig. 6A and S8A†). Therefore, this method has great potential for widespread use in various probiotic products.

3.10. Evaluation of commercial products

To evaluate the vitality of probiotics in drinks, a commercial product which represents the mainstream of probiotic drinks, was purchased from a local market in Singapore. The selected probiotic drink contains *Lactobacillus*, which is believed to help maintain a healthy gut environment using adequate amounts of probiotics. As shown in Fig. 6B, the distribution of bacterial population was quite broad on day 0, indicating the presence of different labeled states. This was further confirmed by the confocal images, which showed that some bacteria were metabolically labeled, similar to *L. paracasei* under normal status above, while others were unlabeled (Fig. 6C). This suggests that some probiotics were still metabolically active while others may have been forced into an inactive state by factors related to the manufacturing process. Moreover, with extended storage time, the fluorescence intensity of Cy5 decreased dramatically (Fig. S8B†), and even fewer bacteria were seen to be metabolically labeled. The increased percentage of inactive bacteria was probably due to the prolonged storage time at 4 °C, which causes the bacteria to enter a dormant state.[40]

4. Conclusion

In summary, to gain insight into the vitality of probiotics in commercial products during and after processing, we have developed an approach which can specifically label metabolically active probiotics *via* metabolic incorporation of functionalized D-amino acids during the biosynthesis of peptidoglycan and quantitatively displays the metabolic level of probiotics through fluorescence detection methods. Most importantly, this method is timesaving (around 8 h) and provides a more accurate reflection of probiotic vitality compared with traditional viability assays such as plate count, live/dead assay, and CFDA assessment. Although the design may not be state of the art, especially compared with that of microfluidic devices,[41] our method was much simpler to perform and interpret. And our method revealed that temperature, lyophilization, pH, prebiotics, the harsh gastrointestinal environment and the storage time of the products would have some effects on probiotic vitality. Admittedly, the advantage of this method is limited but it has the potential to provide a more comprehensive understanding of the states of probiotics in various products and offer guidance on probiotic preservation during the manufacturing process. Besides, metabolic labeling has provided a facile surface modification method for probiotics for future biomedical applications.

Data availability

The authors confirm that the data supporting the conclusions of this study are presented in the manuscript (and/or its ESI†). Requests to access additional data should be directed to the corresponding author.

Author contributions

Xinping Hu: conceptualization, methodology, investigation, data analysis, writing – original draft. Qirong Xiong: methodology, resources, software. Shuai Hou: conceptualization, software, investigation, writing – review & editing. Hongwei Duan: writing – review & editing, funding acquisition, supervision.

Conflicts of interest

The authors declare no competing financial interest.

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