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Article

A New Simple Passage Culture to Improve the Viability of Bifidobacteria

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Abstract: *Bifidobacterium* is the most beneficial flora of the human gastrointestinal tract, but there is a concentration threshold for *Bifidobacterium* to exert its beneficial properties. In this work, the effect of several simple culture methods on the number of viable bifidobacteria was discussed to improve its viable concentrations, and we unexpectedly discovered that bifidobacteria have inert growth. The concentration of viable bacteria of two isolated *Bifidobacterium animalis* subsp. *lactis*, BZ11 and BZ25 and *Lactiplantibacillus plantarum* SQ-4 were investigated using solid-liquid-liquid circulation subculture, solid-liquid-liquid subculture and solid-liquid-liquid circulation subculture. These 3 subculture methods increase the viable cell concentration, and the best is the solid-liquid-liquid subculture. The highest concentrations of third-generation BZ11 and BZ25 were 9.62×10^9 CFU/mL and 1.21×10^{10} CFU/mL in order by solid-liquid-liquid subculture method, which was 10.38 times and 42.31 times higher ($P<0.05$) than those of the first generation, respectively. However, none of these three subculture methods increased the concentration of SQ-4. These results suggested that *Bifidobacterium* has growth inertia and Solid-liquid-liquid passage culture inhibiting the growth inertia. This finding is valuable in promoting the industrial application of probiotic bifidobacteria to potential probiotic food production.

Keywords: *Bifidobacterium*; growth inertia; solid-liquid-liquid passage culture; viable bacterium concentrations; rapid growth

1. Introduction

Bifidobacterium is the most beneficial probiotic in the human intestine, and it has attracted the interest of researchers [1–4]. Although *Bifidobacteria* are strictly anaerobic bacteria, some can grow on agar plates in air, such as *Bifidobacterium animalis* subsp. *lactis* [5,6]. The oxygen tolerance ability of *Bifidobacterium* promotes its application in the food industry. *Bifidobacteria* are widely used in yogurt, probiotics, synbiotics, probiotics, fermented fruits, and vegetables [7]. These foods rich in *Bifidobacteria* help to enhance the value of products and people's health.

Concentration is an essential factor in determining how *Bifidobacteria* perform their probiotic functions, and probiotic *Bifidobacterium* products are typically required to contain more than 10^7 CFU/mL or 10^7 CFU/g product [8]. The concentration of probiotics is related to culture conditions. The traditional culture method is to increase the number of viable bacteria by optimizing the medium and culture environment [9–12]. Then, is there a more straightforward culture method to increase the viable concentration of *Bifidobacteria*?

In this work, the effect of several simple culture methods on the number of viable *Bifidobacteria* was discussed to improve its viable concentrations, and we unexpectedly discovered that *Bifidobacteria* have inert growth. Through a simple solid-liquid-liquid passage culture method, the growth inertia of *Bifidobacteria* was inhibited, and viable *Bifidobacterium* concentrations were significantly increased.

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2. Materials and Methods

2.1 Strains, Materials and Culture

The *Bifidobacterium animalis* subsp. *lactis* strains BZ11 and BZ25 were isolated by our lab. BZ11 and BZ25 are preserved in the China General Microbiological Culture Collection Centre (CGMCC) under CGMCC No. 10224 and CGMCC No. 10225, respectively. *Lactiplantibacillus plantarum* SQ-4 is another anaerobic bacterium isolated by our lab. SQ-4 is preserved in the China Center for Type Culture Collection (CCTCC) under CCTCC M 2016002. Modified PTYG [13] medium (mPTYG) was used, which contained tryptone, 5 g·L^{-1}; soy peptone, 5 g·L^{-1}; yeast extract, 10 g·L^{-1}; glucose, 10 g·L^{-1}; Tween 80, 1 mL; L-cysteine hydrochloride, 0.05 g·L^{-1}; fructooligosaccharides, 5 g·L^{-1}; and salt solution, 4 mL. The pH was adjusted to 6.5. The salt solution contained the following (in g·L^{-1}): K_2HPO_4, 1.0; KH_2PO_4, 1.0; Na_2CO_3, 10; NaCl, 2; $CaCl_2$, 0.2; and $MgSO_4 \bullet 7H_2O$, 0.48. Modified PTYG agar medium (PTYG-F) was prepared by adding 20 g agar per liter modified PTYG medium. MRS liquid medium was prepared as previously described [14]. The MRS agar medium was prepared by adding 20 g per liter of MRS medium. All chemicals used in this work were of analytical grade and commercially available.

All microbes were cultured at 37 °C in 20% (v/v) CO_2–80% (v/v) atmospheric air in an incubator. The seed liquid culture and fermentation liquid culture were carried out in a sealed erlenmeyer flask with medium added to 40% of the field capacity. *Bifidobacterium* was cultured in liquid mPTYG and on solid PTYG-F medium. *Lactiplantibacillus plantarum* SQ-4 was cultured in a liquid medium or on a solid MRS medium.

2.2. Passage Culture and Viable Count

2.2.1. Solid-Liquid-Liquid Circulation Subculture

Solid-liquid-liquid circulation subculture method (The first passage culture method, 1st) is shown in Figure 1(a). The bacterial strain BZ11, BZ25, or SQ-4 was streaked onto a plate medium from glycerine preservation at -20 °C and incubated for 48 hours. Then, a single colony was picked, streaked onto a plate, and incubated for 48 hours. The process was repeated three times for the purification and activation of *Bifidobacterium* or *Lactiplantibacillus plantarum*. A single active colony was selected and transferred into a sealed 50-mL Erlenmeyer flask containing 20 mL of medium and incubated for 48 hours as the seed culture. Subsequently, 2% of the seed broth was transferred into a sealed 250-mL Erlenmeyer flask containing 100 mL of liquid medium and incubated for 36 hours. The culture was used as the first generation of the fermentation culture and as the seed of the second generation of the streaked plates. Then, the streaking plates (here only once), seed cultivation, and fermentation incubation were repeated for 3 cycles. Thus, four generations of BZ11, BZ25, and SQ-4 cultures were obtained.

2.2.2. Solid-Liquid-Liquid Passage Culture

Solid-liquid-liquid passage culture (The second passage culture method, 2nd) is shown in Figure 1(b). The bacterial strain BZ11, BZ25, or SQ-4 was streaked from glycerine preservation at -20 °C onto a plate medium and incubated for 48 hours. Then, a single colony was picked and streaked onto a plate medium for a 48-hour incubation, and the process was repeated three times for the purification and activation of *Bifidobacterium* or *Lactiplantibacillus plantarum*. The active single-colony plate obtained after the third streak was recorded as plate 1.

A single colony from plate 1 was inoculated into a 50-mL triangular bottle with 20 mL medium for 48 hours as the seed culture (simultaneously, plate 1 was refrigerated at 4 °C). Subsequently, 2% of the seed culture was transferred into 250-mL sealed flasks containing 100 mL of medium and incubated for 36 hours. The culture was used as the first generation of the fermentation culture. Then, from plate 1, which was refrigerated for 3 days, a single colony was restreaked (here, only once), and the plate obtained after incubation for 48 hours was recorded as plate 2. A single colony from plate 2 was inoculated into 50-mL sealed flasks containing 20 mL medium for 48 hours as the second-generation seed culture (simultaneously, plate 2 was refrigerated at 4 °C), 2% of which was transferred into 250-mL sealed flasks containing 100 mL of medium and incubated for 36 hours. The culture was used as the second generation of the fermentation culture. In this way, 5 generations of fermentation cultures of BZ11, BZ25, and SQ-4 were obtained.

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2.2.3. Solid-Liquid-Liquid Continuous Passage Culture

Solid-liquid-liquid continuous passage culture (The third passage culture method, 3rd) is shown in Figure 1(c). The bacterial strains were activated using the same process described for the first subculture method. The activated single-colony plate obtained after the third streak was recorded as plate 1. A single colony was selected from plate 1 for transfer into a sealed 50-mL Erlenmeyer flask containing 20 mL of medium and incubated for 48 hours as the first-generation seed culture. At the same time, another colony was selected from plate 1 and streaked, and the plate obtained after incubation for 48 hours was recorded as plate 2. When the first-generation seed culture was prepared, 2% of the first seed broth was transferred into 50-mL sealed flasks containing 20 mL of medium and incubated for 36 hours as the first generation of fermentation culture. Similarly, after plate 2 was prepared, plate 3 and the second-generation seed culture were obtained from plate 2, and then the second generation of fermentation culture was obtained. This way, 4 generations of fermentation culture of BZ11, BZ25, and SQ-4 were obtained.

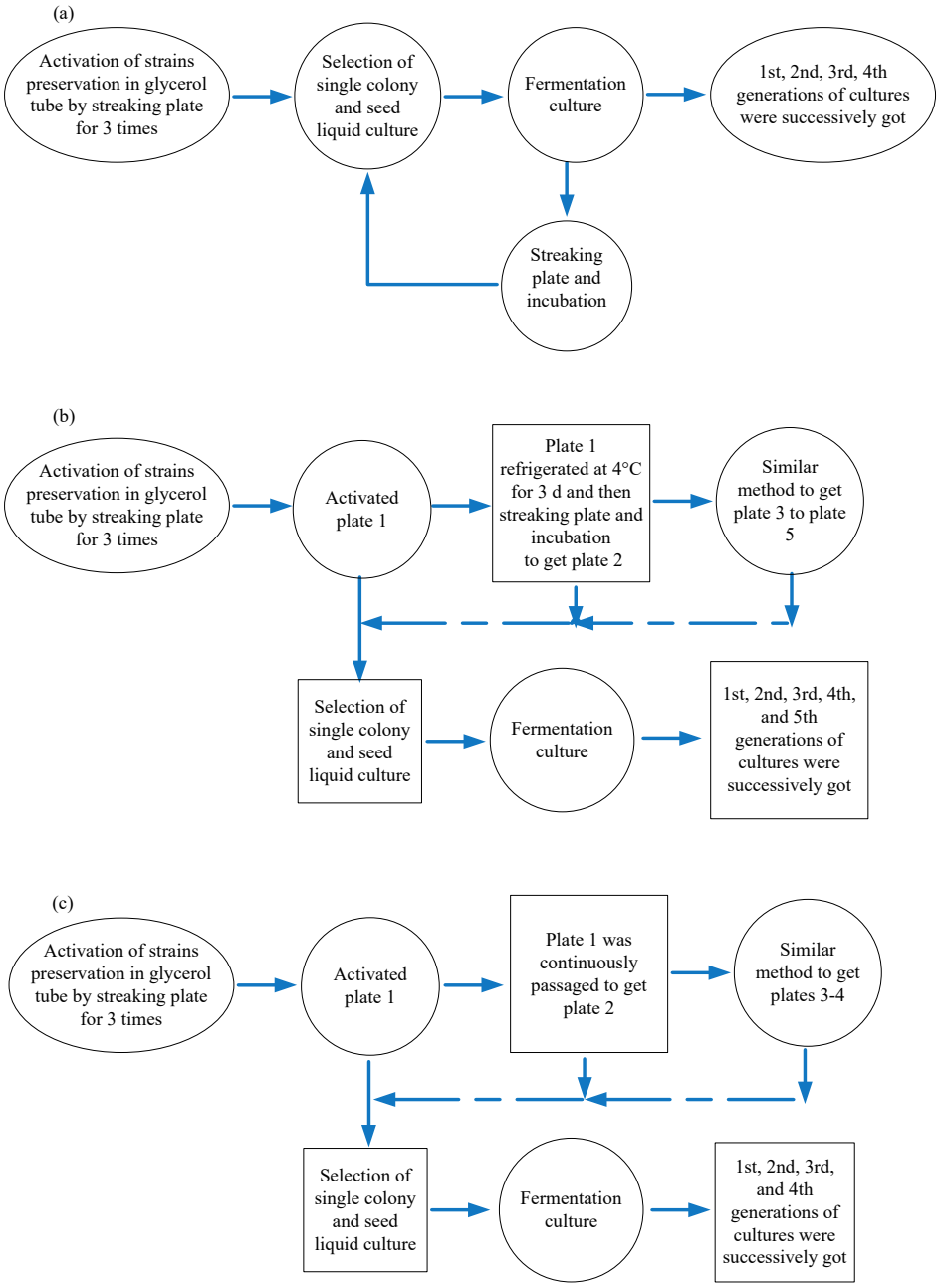

Figure 1. Different passage culture methods: (a) Solid-liquid-liquid circulation subculture, (b) Solid-liquid-liquid passage culture and (c) Solid-liquid-liquid continuous passage culture.

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2.2.4. Determination of Cell Concentration

During each subculture, samples were taken at different times to analyze the number of viable bacteria by spread plate and incubation for 48 hours.

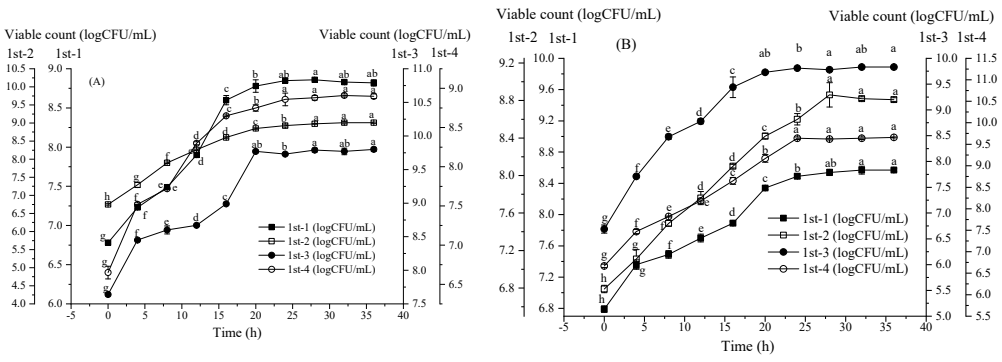
2.3. Data Analysis

All experiments were performed in 3 replicates, and the data are presented as the mean ± SD. One-way ANOVA was used for data analysis by IBM SPSS 20.0 software, and the differences between the mean values were significant at $P < 0.05$.

3. Results

3.1. Strain Growth Characteristics with the First Method of Passage Culture (Figure 2)

The common characteristics of the number of viable bacteria in the first passage culture (solid-liquid-liquid circulation subculture) were: the number of viable bacteria in different generations of BZ11, BZ25, and SQ-4 all increased first and then reached the stable stage ($P > 0.05$). With the increase of passage times, the time to reach the stable stage tended to shorten. However, *Bifidobacterium* BZ11 and BZ25's growth characteristics also showed apparent differences from those of *Lactiplantibacillus plantarum* SQ-4. Figure 2 shows that because the fermentation medium and seed medium is the same, the strains quickly enter the growth phase and reach the maximum and stable phase, but each strain also has its growth characteristics. Figure 2A shows that BZ11 reaches its maximum value (8.85 log CFU/mL) within 24 hours from 1ST-1 to 1ST-4 and then enters a stable period. At 1ST-2, the maximum value (8.98 log CFU/mL) was reached in 28 hours. At 1ST-3 hours, the maximum value was 9.77 log CFU/mL in 20 hours. At 1ST-4 hours, the maximum value (8.86 log CFU/mL) was reached in 24 hours. Figure 2B shows that BZ25 reaches its maximum value (8.54 log CFU/mL) within 24 hours at 1ST-1 and then enters a stable period. At 1ST-2, the maximum value (8.86 log CFU/mL) was reached in 28 hours; at 1ST-3, the maximum value was 9.73 log CFU/mL in 20 hours. At 1ST-4, the maximum value was 9.64 log CFU/mL in 24 hours. Figure 2C shows that SQ-4 reaches its maximum value (10.36 log CFU/mL) within 24 hours at 1ST-1, then enters a stable period. At 1ST-2, the maximum value (10.59 log CFU/mL) was reached in 24 hours. 1ST-3 hours, the maximum value was reached in 20 hours (9.03 log CFU/mL); At 1ST-4, the maximum value (8.94 log CFU/mL) was reached in 24 hours. Figure 2D indicates that the viable bacteria concentration of *Bifidobacterium* BZ11 and BZ25 increased ($P < 0.05$) with consecutive passages from 1 to 3. The highest concentrations of the first generations of BZ11 and BZ25 were 7.20×10^8 CFU/mL and 3.74×10^8 CFU/mL, respectively, while the concentrations of BZ11 and BZ25 in the third generations were 6.20×10^9 CFU/mL and 6.80×10^9 CFU/mL, respectively, showing significant ($P < 0.05$) increases of 8.6 times and 18.2 times compared with the first generation. However, in the fourth generation, the viable bacteria concentration of BZ11 and BZ25 decreased to 8.18×10^8 CFU/mL and 4.39×10^9 CFU/mL, respectively ($P < 0.05$). So, the number of passages was not as high as possible. *Lactiplantibacillus plantarum* SQ-4 exhibited another growth characteristic, which is the general microbial growth characteristic of not increasing with the increase of the number of passages but decreasing ($P < 0.05$) from 8.97×10^{10} CFU/mL to 9.06×10^8 CFU/mL (Figure 2).



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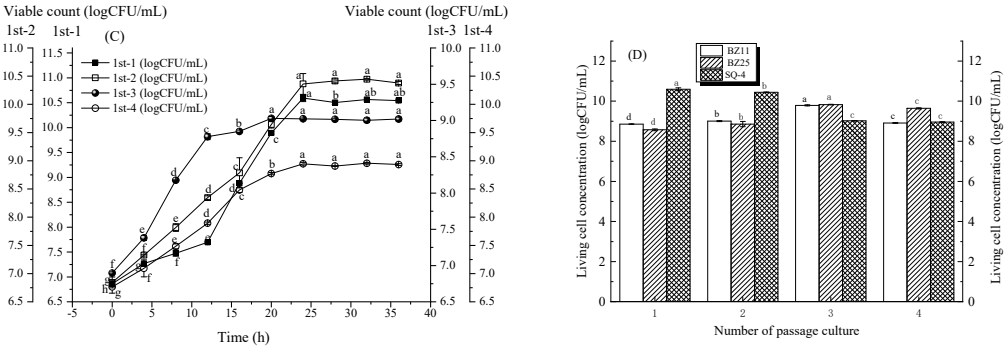

Figure 2. Changes in living cell concentrations of strains with the solid-liquid-liquid passage culture, (A) BZ11, (B) BZ25, (C) SQ-4. Means with dissimilar lowercase letters in the same sort of bar indicate significance ($P < 0.05$), the same as below.

3.2. Change in Strain Growth with the Second Method of Passage Culture

Figure 3 showed that similar to the first pass culture method, the second pass culture inoculated strains rapidly entered the growth phase and reached the maximum and stable phase. However, this pass culture method had its apparent characteristics. Figure 3A shows that at 2nd-1, BZ11 goes its maximum value (8.85 log CFU/mL) within 24 hours and then enters a stable period. The maximum value (9.01 log CFU/mL) is reached in 24 hours at 2nd-2. At 2nd-3, the maximum value (9.46 log CFU/mL) was reached in 24 hours. At 2nd-4 hours, the maximum value in 24 hours (9.79 log CFU/mL) was reached. At 2nd-5, the 24-hour maximum is reached (9.98 log CFU/mL). Figure 2B shows that at 2nd-1, BZ25 goes to its maximum value (8.42 log CFU/mL) within 28 hours and then enters a stable period. At 2nd-2, the maximum value (8.95 log CFU/mL) is reached in 24 hours. At 2nd-3 hours, the maximum value (8.97 log CFU/mL) was reached in 20 hours. At 2nd-4, the maximum value in 24 hours is 9.62 log CFU/mL. At 2nd-5, the maximum is reached in 32 hours (10.05 log CFU/mL). Figure 2C shows that at 2nd-1, SQ-4 reaches a maximum value (10.67 log CFU/mL) in 24 hours and then enters a stable period. At 2nd-2, the maximum value in 20 hours (10.56 log CFU/mL) is reached. At 2nd-3, it gets the maximum (10.71 log CFU/mL) in 20 hours and enters the decline period at 32 hours. At 2nd-4 hours, the maximum value (10.37log) was reached in 16 hours, and the decline period entered in 24 hours. At 2nd-5, it gets a maximum (10.00 log CFU/mL) in 20 hours and enters a recession in 36 hours. Therefore, with the increase of passage times, SQ-4 enters the stable period and decline period quickly, while BZ11 basically enters the stable period within 24 hours, while BZ25 enters the stable period between 24 and 32 hours, with some fluctuations. Figure 3D shows the common growth characteristics (Figure 3) of the three bacteria with the second passage culture (solid-liquid-liquid passage subculture) were similar to those with 1st passage culture. The difference is that the viable concentration of BZ11 and BZ25 gradually increased from 8.45×10^8 CFU/mL and 2.86×10^8 CFU/mL in the first generation, respectively, to 9.62×10^9 CFU/mL and 1.21×10^{10} CFU/mL in the fifth generation, respectively ($P < 0.05$) (Figure 3). The number of living bacteria of BZ11 and BZ25 increased by 10.38 times and 42.31 times, respectively. However, with the passage from the first generation to the third generation, the maximum viable count of SQ-4 remained unchanged ($P > 0.05$), but with the further increase in passage times, the maximum viable count of SQ-4 decreased significantly ($P < 0.05$), from 4.75×10^{10} CFU/mL in the third generation to 9.93×10^9 CFU/mL in the fifth generation (Figure 3).

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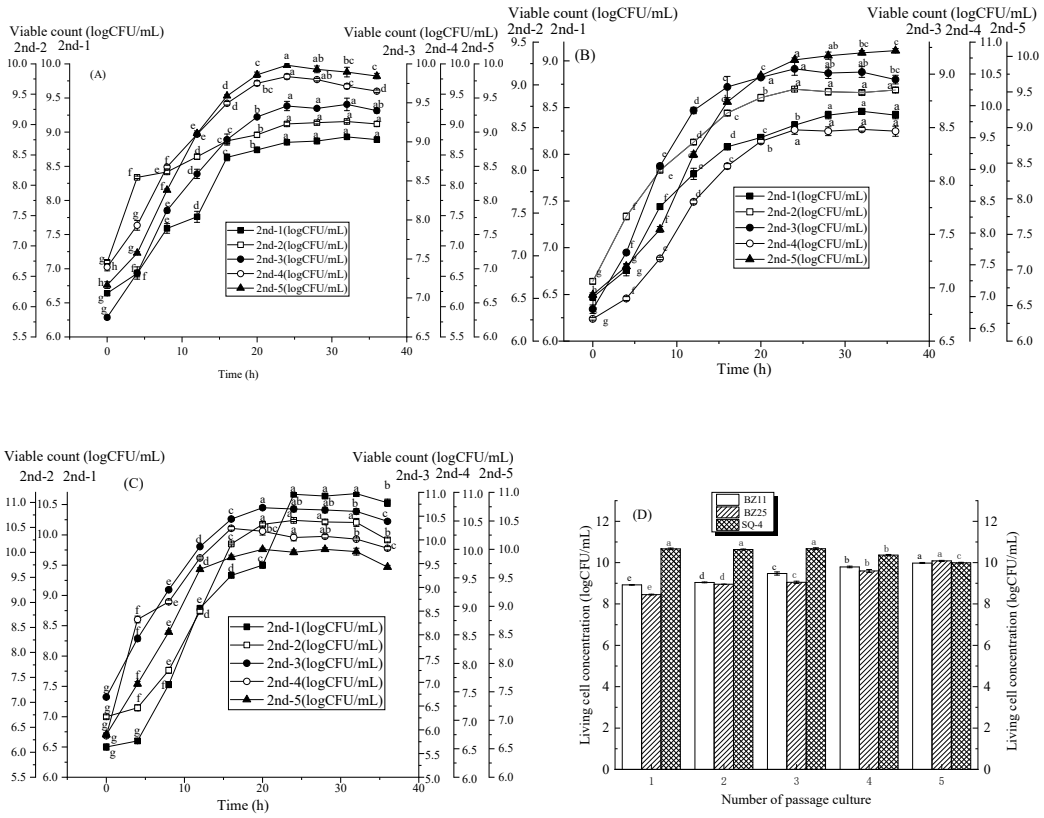

Figure 3. Changes in living cell concentrations of strains with the Solid-liquid-liquid passage culture, (A) BZ11, (B) BZ25, (C) SQ-4.

3.3. Change in Strain Growth with the Third Method of Passage Culture

Figure 4 shows that *Bifidobacterium* and *Lactiplantibacillus plantarum* with this passage culture method, also have their obvious characteristics. Figure 4A shows that at 3rd-1, BZ11 reaches its maximum value (8.73 log CFU/mL) within 28 hours and then enters a stable period. At 3rd-2, the maximum is reached in 28 hours (8.83 log CFU/mL); At 3rd-3, the maximum value is 9.06 log CFU/mL in 24 hours. At 3rd-4, the maximum is reached in 16 hours (8.69 log CFU/mL). Figure 4B shows that at 3rd-1, BZ25 reaches its maximum value (8.65 log CFU/mL) within 28 hours and then enters a stable period. At 3rd-2, it reaches its maximum value (8.71 log CFU/mL) in 24 hours; At 3rd-3, the maximum value is 9.12 log CFU/mL in 24 hours. At 3rd-4, the maximum value is 8.89 log CFU/mL in 24 hours. Figure 4C shows that at 3rd-1, SQ-4 reaches a maximum value (10.38 log CFU/mL) within 24 hours, then enters a stable period and a decline period after 32 hours. At 3rd-2, it reaches the maximum value (10.36 log CFU/mL) in 28 hours and enters the stable period. At 3rd-3, it reaches its maximum value (10.39 log CFU/mL) in 20 hours and enters a decline after 28 hours. At 3rd-4, it reaches a maximum (10.15 log CFU/mL) in 20 hours and enters a decline after 32 hours. Figure 4D shows these three methods of passage generally enter a stable period between 16 and 28 hours. The growth curves of BZ11 and BZ25 were similar. However, the earlier growth curves of SQ-4 were steeper with the third passage culture (solid-liquid-liquid continuous subculture). With the increase of passage times, the viable concentrations of BZ11 and BZ25 increased ($P < 0.05$) gradually from 8.783 log CFU/mL and 8.683 log CFU/mL at the first generation to 9.063 and 9.123 log CFU/mL at the third generation (Figure 4). The number of living bacteria of BZ11 and BZ25 increased by 0.9 times and 1.75 times, respectively. However, further increasing the passage times to the fourth generation, the concentrations of BZ11 and BZ25 decreased ($P < 0.05$) to 8.763 and 8.94 log CFU/mL in the fourth generation. SQ-4 showed another change in viable bacterial concentrations. The maximum viable count of SQ-4 remained unchanged ($P > 0.05$) with the passage from the first to the third generation. However, further increasing the passage times, the maximum viable count decreased significantly ($P < 0.05$), from 10.397 at the third generation to 10.163 at the fourth generation (Figure 4).

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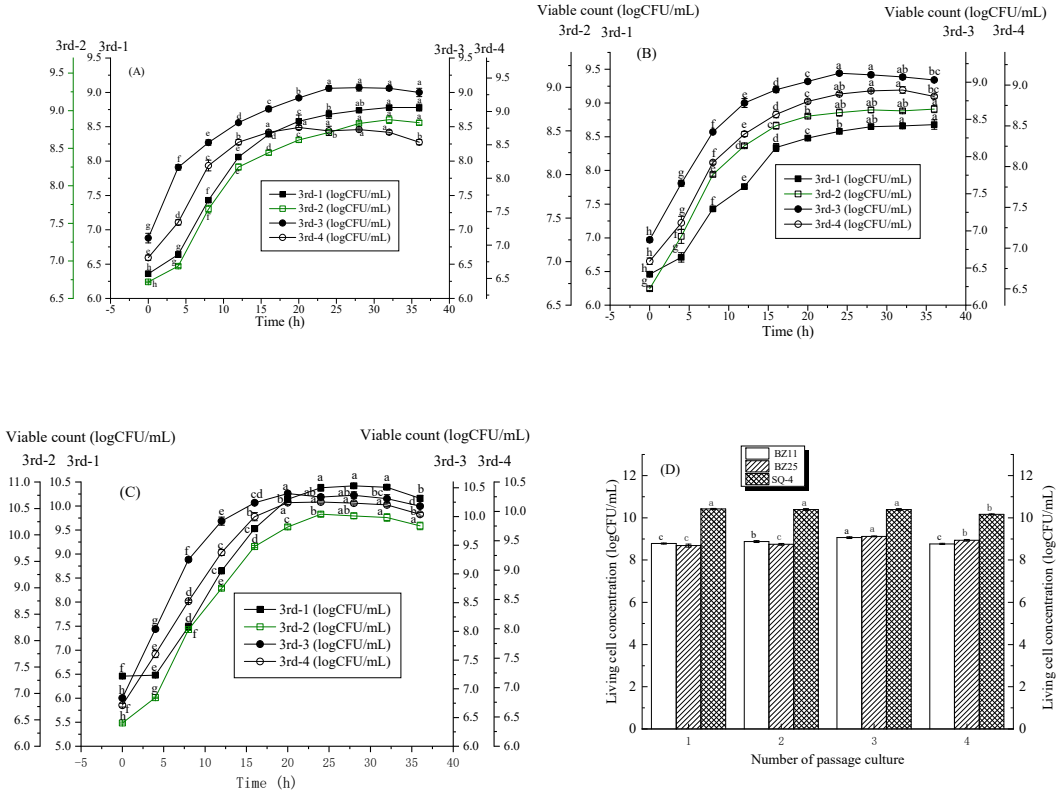

Figure 4. Changes in living cell concentrations of strains with the Solid-liquid-liquid continuous passage culture, (A) BZ11, (B) BZ25, (C) SQ-4.

4. Discussion

Bifidobacteria adjust the balance of the gastrointestinal tract and contribute to human health. Therefore, probiotic products are very popular among consumers. However, the probiotic concentration is a key that probiotic plays a role in the human body. Therefore, the increasing concentration of viable *Bifidobacteria* attracts the interest of scholars.

The first method and the third method were in the first three generations, the number of viable bacteria increased with the increase of passage times, but the number of viable bacteria decreased in the fourth generation. The first culture method had the highest number of viable bacteria in the third generation, and the second culture method had the highest number of viable bacteria in the fourth generation. The growth performance of *Bifidobacterium* will be degraded after three generations by the first and the third subculture methods because the number of viable bacteria is decreased. In the second culture, viable bacteria increased significantly in the fifth generation. However, these three methods did not increase the maximum viable count of SQ-4. The maximum viable count of SQ-4 was either stable or decreased with the increase in passage times, which was the same as that of the general microbial growth. Junyan Liu reported similar growing characteristics of a *Lactobacillus harbinensis* strain [15]. However, by comparing the growth characteristics of *Bifidobacterium* and *Lactiplantibacillus plantarum*, it can be found that *Bifidobacterium* has growth inertia, that is, the number of viable bacteria could have reached a high concentration. However, due to the growth inertia, the concentration of the first-generation culture is often low, but it can play its good inherent growth characteristics through proper passage culture. The improvement in growth performance is not due to the inactivation of the strain because the strain has been activated three times in succession. The results showed that the concentration of Bifidobacterium could be significantly increased by simple subculture. Despite all this, the number of passages needed to be controlled. The suitable number of passages was 3 for *Bifidobateria* with the first and third methods of passage culture. Further prolonging the number of passages, the growth performance of BZ25 and BZ11 would decrease.

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The comparison of the maximum viable concentration with the three passage methods for four generations of fermentation culture (Figure 5) was as follows: the third passage method had the lowest viable concentration, and there was no significant difference between the first and the second passage methods for BZ11. However, the maximum viable concentration with the first passage method was in the third-generation culture, while the maximum viable concentration with the second passage methods was still increasing in the fourth and fifth generations. In addition, the highest viable count of BZ25 was the best with the second method, while that of SQ-4 was the highest, and there was no significant difference between the first and second methods. Therefore, on the whole, the second culture method (Solid-liquid-liquid passage culture) is best for inhibiting the growth inertia of *Bifidobacerium* and increasing the number of viable bacteria.

The changes in the growth performance of *Bifidobacteria* are essentially adaptations to environmental changes, which are common in microorganisms(Figure 5). However, achieving a ten-fold increase in the living cell concentration of *Bifidobacteria* by simple subculturing without changing the medium and culture environmental conditions is a valuable finding. With only subculturing, the growth performance of microorganisms generally remains stable or declines with the increase of the passage number [15], but *Bifidobacteria* exhibited the opposite characteristic. Why did the previous subculture method not yield this phenomenon? The reason for this was maybe mainly because the traditional subculture method for *Bifidobacterium* generally first involves activating the strain, followed by selecting a suitably activated colony to inoculate the slant medium to obtain the slant seed, and then sequentially carrying out the liquid seed culture and liquid fermentation culture from the test tube slant. The culture of BZ25 that we previously reported was based on such a subculture method, and as a result, the concentration of the strain only reached 10^8 CFU/mL [13]. However, with the three subculture methods used in this work, the *Bifidobacteria* cell concentration came from 10^9 CFU/mL to 10^{10} CFU/mL, and the concentration of living cells increased by 0.9 to 42.31 times. Therefore, it was found that *Bifidobacteria* exhibited growth inertia with the traditional subculture method, and its growth characteristics could be activated by appropriate passage stimulation. The critical point of this appropriate continuous passaging fermentation culture method is that each batch of liquid fermentation began with activated plate seeds, and then liquid seed cultivation and fermentation culture were performed. Subsequently, the next batch of fermentation culture was carried out, and the fermentation broth was reactivated by plating and used for the seed culture and fermentation culture (the first method of passage culture), or plate seeds that had been refrigerated for three days were continuously subjected to solid-state plate activation, liquid seed culture, and fermentation culture (the second method of passage culture). Moreover, there should be a time interval between the solid plate seeds of different generations of fermentation culture; otherwise, the activation of *Bifidobacteria* will be weak (the third subculture method).

Bioinformatics technology based on multi-omics analysis plays a vital role in the study of the action mechanism of microorganisms [16–18]. The inhibitory mechanism of subculture methods on the growth inertia of *Bifidobacteria* is still unclear. The next step is to combine genomics and proteomics to analyze the inhibitory mechanism of subculture methods on the growth inertia of *Bifidobacteria*.

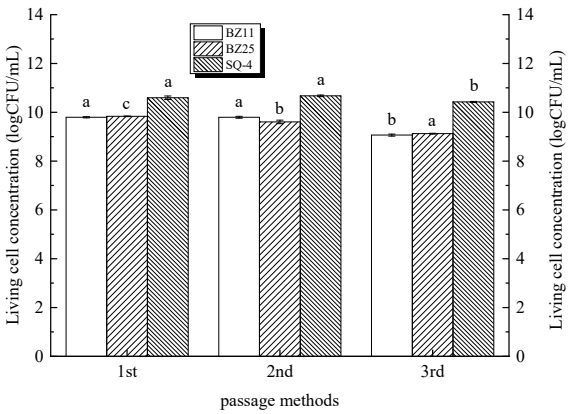

Figure 5. Comparison of several subculture methods.

5. Conclusion

The growth of *Bifidobacterium* is challenged by growth inertia, which could be overcome by proper passage culture to activate its potential. Thus, the ability to inhibit the growth inertia of *Bifidobacterium* effectively depended on the method of subculture. However, general microorganisms such as the lactic acid bacteria SQ-4 did not display such growth inertia. This finding is valuable in promoting the industrial application of probiotic *Bifidobacteria* in potential probiotic production. We will carry out a multi-omics analysis to explain the inhibitory mechanism of subculture methods on the growth inertia of *Bifidobacteria* in the future.

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