

# Study on Enhancement of Acid Production by Combination Fermentation of Caproic Acid-producing Bacteria and Lactic Acid-producing Bacteria

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**Abstract:** A new caproic acid bacteria 20-5 (*Caproicibacterium* sp.) was newly screened in the laboratory, and several high-quality lactic acid bacteria A2-3 (*Sporolactobacillus fermentans*), GCB-3 (*Pediococcus acidilactici*), KR-4 (*Lactiplantibacillus plantarum*), LY-5 (*Levilactobacillus brevis*), B2-1 (*Lactiplantibacillus plantarum*) were used for exploratory compound fermentation experiments. The compound fermentation was a caproic acid bacteria (20-5) alone plus four lactic acid bacteria (A2-3, GCB-3, KR-4, LY-5, B2-1), the experimental ratio was set up four groups of compound ratio of 1 : 2, 1 : 1, 5 : 1, 10 : 1. The optimum ratio of 20-5 to B2-1 was 10 : 1, and the total acid yield reached 26.75 g / L. The best caproic acid production capacity after compounding was the 10 : 1 group of 20-5 and B2-1, and the caproic acid yield was 7.22 g / L. The average lactic acid content in the fermentation broth was 4.52 g / L after compounding. After compounding, the acid production of the 1 : 1 and 5 : 1 groups of 20-5 : A2-3 and the 10 : 1 group of 20-5 : B2-1 increased compared with that of single bacteria. In the other compound groups, the acid production capacity decreased after the compound operation, and the lactic acid production generally decreased, but the caproic acid production increased, indicating that caproic acid bacteria can use lactic acid as a carbon source for fermentation and acid production activities, so that caproic acid bacteria can produce caproic acid.

**Keywords:** Compound Strains; Caproic Acid Bacteria; Lactic Acid Bacteria; Acid Production.

## 1. Introduction

Luzhou-flavor liquor, as one of the four basic flavor liquors in China, is widely welcomed by the public, and has the characteristics of strong cellar flavor, mellow sweetness, fullness and coordination [1,2]. The four major flavor substances in Luzhou-flavor liquor are ethyl caproate, ethyl acetate, ethyl lactate and ethyl butyrate, among which ethyl caproate is the highest content component except ethanol and water, and it is also the main flavor characteristic of Luzhou-flavor liquor [3,4]. Caproic acid bacteria, as the key functional bacteria of pit mud, play a decisive role in the production of caproic acid and ethyl caproate in traditional solid-state Luzhou-flavor liquor fermentation. Because some caproic acid bacteria such as *Ruminococcaceae* sp. CPB6 can use lactic acid to produce caproic acid, so it may play an important role in "increasing hexane and reducing milk" in the fermentation process; In addition, caproic acid bacteria can metabolize and produce other trace components such as butyric acid and caprylic acid, so the content and acid production of caproic acid bacteria in pit mud have an important influence on the formation of typical flavor substances and the improvement of liquor quality [5,6].

At present, the enhanced application of caproic acid bacteria mainly focuses on three aspects: aging of pit mud, maintenance of pit mud and enhanced fermentation [7,8,9]; At present, most wineries adopt caproic acid bacteria compound liquid prepared by single addition of caproic acid bacteria, combined fermentation of pure bacteria and mixed bacteria or enrichment culture of pit mud, which has no significant effect or unstable quality in actual production. How to effectively prepare multi-bacterial compound system

is a difficult point in the development of caproic acid bacteria. Studying the synergistic symbiotic relationship among microorganisms can improve the stability of caproic acid bacteria compound combination. Sean et al. Made the caproic acid-producing bacteria screened out from Yanghe old pit mud into pure bacteria enrichment solution and added it during the preparation of pit mud. After replacing the degraded old pit mud with the prepared new pit mud, the excellent wine production rate in the pit increased from the original 36.72% to 43.36% [8]; Wanlian compounded caproic acid bacteria isolated and screened from high-quality pit mud with yeast and butyric acid bacteria, and made pit culture solution to strengthen the pit culture of new pit mud, which significantly increased the content of ethyl caproate in wine produced in new pit [9]. Although caproic acid bacteria have achieved remarkable results in liquor fermentation production, it has been reported that it can produce caproic acid and can be used in actual brewing production. In this study, caproic acid-producing bacteria isolated from Luzhou-flavor white wine cellar mud were used as the research object, and they were mixed with lactic acid bacteria for fermentation, to construct a compound combination that can improve the yield of caproic acid and ethyl caproate. Related research can not only provide functional strains with high yield of caproic acid for the brewing production of Luzhou-flavor liquor enterprises, but also the compound bacterial liquid formula obtained from the experiment can be applied to pit mud maintenance, enhanced fermentation in traditional pits and automatic fermentation of Luzhou-flavor liquor.

## 2. Materials and Methods

### 2.1. Material

#### 2.1.1. Experimental Strains

The species information is shown in Table 1.

**Table 1.** Information of tested strains

Name of strain	species name
20-5D	<i>Caproicibacterium sp</i>
A2-3	<i>Sporolactobacillus fermentans</i>
GCB-3	<i>Pediococcus acidilactici</i>
KR-4	<i>Lactiplantibacillus plantarum</i>
LY-5	<i>Levilactobacillus brevis</i>
B2-1	<i>Lactiplantibacillus plantarum</i>

#### 2.1.2. Medium

RCM medium [10]: 10 g peptone, 1 g soluble starch, 3 g yeast powder, 5 g NaCl, 3 g NaAc, 5 g glucose, 0.5 g cysteamine, 10 g beef extract, 20 g agar powder.

RCM fermentation medium [10]: peptone 10 g, soluble starch 1 g, yeast powder 3 g, NaCl 5 g, NaAc 3 g, glucose 5 g, cysteamine 0.5 g, beef extract 10 g.

#### 2.1.3. Instruments and Equipmen

SW-CJ-2D ultra-clean worktable, Jiangsu Sujing Group Co., Ltd.; dI54DS high pressure steam sterilization pot, Chengdu Xiongyi Technology Co., Ltd.; cSYY-600, Shanghai Ruisai Scientific Instrument Co., Ltd.; 7890B gas chromatography, Agilent Technologies, USA; 2023C liquid chromatography, Shimadzu (Shanghai) Experimental Equipment Co., Ltd.; zWYR-D2403 thermostatic incubator, Shanghai Zhicheng Company.

## 2.2. Experimental Methods

### 2.2.1. Activation and Counting of Experimental Bacteria

In this experiment, a caproic acid bacteria 20-5D and five

lactic acid bacteria A2-3, GCB-3, KR-4, LY-5 and B2-1 were selected. The strains required for the experiment were activated and cultured using RCM solid medium. The colonies in the medium were inoculated into the RCM liquid fermentation medium at 37 ° C for 48 h in the anaerobic workstation. The seed solution was prepared at 37 ° C for 48 h in the anaerobic workstation. The seed solution was counted using the spread plate counting method. The number of colonies was counted at 37 ° C for 48 h in the anaerobic workstation to obtain the bacterial density of the seed solution.

### 2.2.2. Compound Fermentation Experimental Method

The seed liquid of the experimental strain was diluted to  $1 \times 10^8$  / ml under anaerobic conditions, and anaerobic fermentation was carried out using RCM liquid medium at 37 ° C in an anaerobic workstation, and the compound ratio was set as shown in the table. After the fermentation was completed, the fermentation broth was analyzed for fermentation products.

**Table 2.** Compound combination

compound proportion 20-5:X	compound strains				
	20-5+A2-3(A)	20-5+LY-5(B)	20-5+KR-4(C)	20-5+B2-1(D)	20-5+GCB-3(E)
1:2	A1	B1	C1	D1	E1
1:1	A2	B2	C2	D2	E2
5:1	A3	B3	C3	D3	E3
10:1	A4	B4	C4	D4	E4

Note: X is the mixed strains (A2-3, LY-5, KR-4, B2-1, GCB-3)

### 2.2.3. Determination of Organic Acid Production

#### 2.2.3.1 Detection of Volatile Fatty Acid Content

Under sterile and anaerobic conditions, 2 mL of the fermented sample was centrifuged at 13 000 r / min for 5 min in a high-speed centrifuge, and the supernatant was taken and filtered with 0.22 μm microporous membrane. The 1 mL filtered sample was taken into the injection bottle, and 10 μL of pre-configured internal standard n-pentyl acetate (21.9 mg / mL) was added and mixed well. The content of volatile substances was detected by GC-MS.

Gas chromatography conditions[11]: DB-WAX capillary column ( 60 m × 250 μm × 0.25 μm ), inlet temperature : 230 °C, flame ionization detector ( FID ) detector temperature : 220 °C, carrier gas : high purity helium ( He ) ( purity ≥ 99.999 5 % ), carrier gas flow rate : 30 mL / min, column flow rate : 1 mL / min, H2 flow rate : 40 mL / min, Air flow rate : 400 mL / min, tail flow rate : 30 mL / min. The splitless injection method was adopted. The initial temperature was maintained at 35 °C for 5 min, then increased

to 100 °C at a rate of 5 °C / min for 2 min, and then increased to 230 °C at a rate of 15 °C / min for 5 min.

Mass spectrometry conditions : electron ionization ( EI ), electron energy 70 eV, full scan acquisition mode, mass range 20 ~ 550 u, ion source temperature 230 °C, quadrupole temperature 150 °C, interface temperature 230 °C.

Qualitative and quantitative: The mass spectra were compared with the national institute of standards and technology (NIST) standard spectrum library 05a.L provided by Agilent, and semi-quantitative was performed by internal standard method.

#### 2.2.3.2 Detection of Non-volatile Fatty Acid Content

The liquid phase samples were prepared by the same method as the preparation of GC-MS samples. The non-volatile fatty acids in the samples were mainly used to detect the content of lactic acid, and the standard curve of lactic acid was drawn in advance. The content of lactic acid in the samples was detected by high performance liquid chromatography, and the external standard method was used

for quantification.

HPLC conditions: C18 chromatographic column (250 mm × 4.6 mm, 5 μm), column temperature 30 °C, mobile phase of 2 % methanol-0.12 % phosphoric acid aqueous solution, flow rate 0.8 mL / min, detection wavelength 214 nm, injection volume 10 μL.

### 3. Experimental Results and Analysis

#### 3.1. Seed Liquid Counting

The concentration of activated seed liquid is shown in Table 3.

**Table 3.** Seed concentration

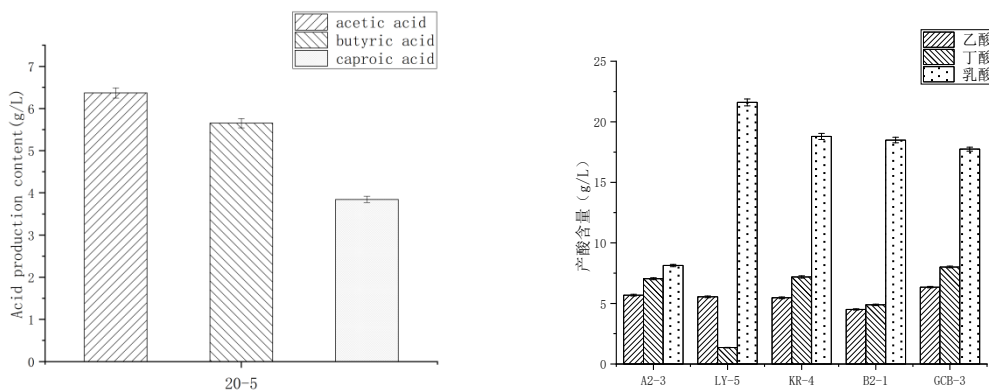
seed liquid	Concentration (cfu / ml)
20-5	$3.2 \times 10^8$
A2-3	$8.8 \times 10^8$
GCB-3	$4.0 \times 10^8$
KR-4	$5.6 \times 10^8$
LY-5	$4.0 \times 10^8$
B2-1	$6.2 \times 10^8$

From the above table, it can be seen that after the activation of the six strains used, the biomass of each bottle of activated seed liquid is  $10^8$  cfu / ml, and the dilution of the bacterial liquid concentration is diluted to  $1 \times 10^8$  cfu / ml, which is the

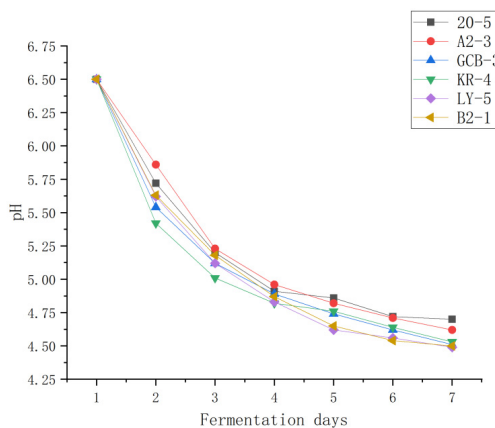
inoculation liquid.

#### 3.2. Analysis of the Results of Single Strain Fermentation Experiment

The pH of each acid-producing bacteria decreased rapidly in the first 4 days of fermentation, and the pH no longer decreased after the 6th day, showing a gentle trend, and the fermentation period was about 4-6 days. The results of single-strain fermentation of caproic acid bacteria showed that although caproic acid was produced, the yield of caproic acid was less. According to the results of single-strain fermentation of lactic acid bacteria, it can be seen that the lactic acid production ability of lactic acid bacteria GCB-3, KR-4, LY-5 and B2-1 selected in the experiment is relatively good, among which the lactic acid production ability of lactic acid bacteria LY-5 is outstanding, and the lactic acid production ability is 21.60 g / L. Although A2-3 produced lactic acid, its ability to produce acid was worse than that of other lactic acid bacteria, producing acid 8.13 g / L. The average lactic acid production of single lactic acid bacteria fermentation was 16.57 g / L. According to Cheng Fan 's research [8], caproic acid bacteria can use lactic acid to produce caproic acid in a higher concentration of lactic acid environment, in which the conversion rate of lactic acid-caproic acid can reach 44.4 %. According to the single-strain fermentation results of these lactic acid bacteria, their high-yield lactic acid characteristics provide a good lactic acid guarantee for the success of the compound experiment.



**Fig 1.** Fermentation acid production results



**Fig 2.** The change trend of pH value of single bacteria fermentation for 7 days

### 3.3. The Variation Characteristics of pH in Different Compound Combinations

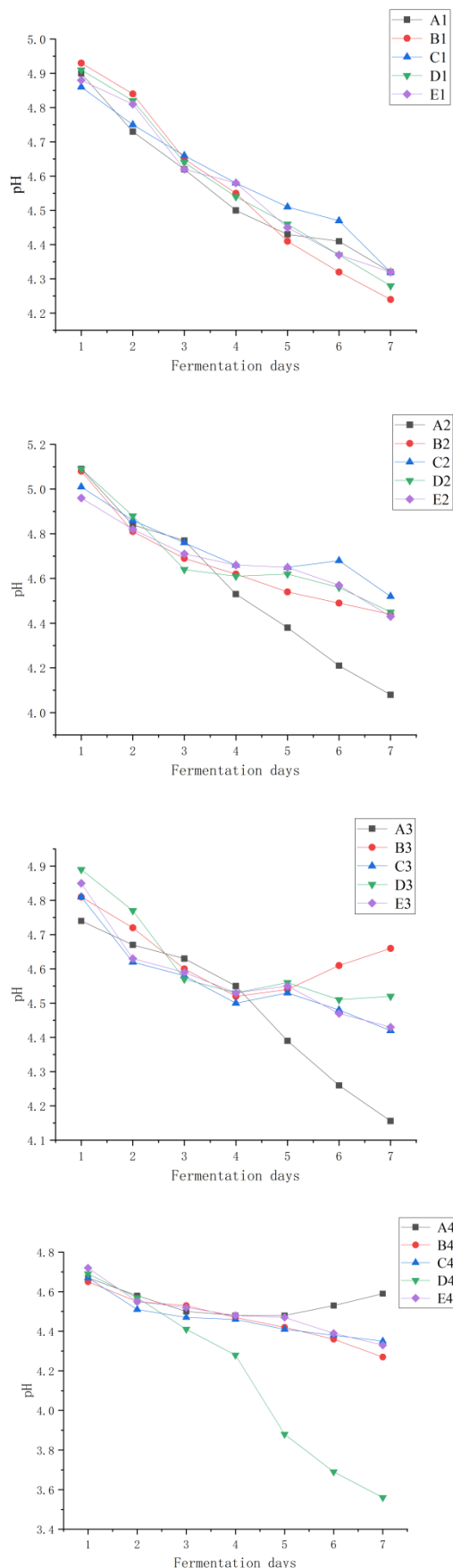


Fig 3. The pH change of different compound combination fermentation ratio 1 after 7 days of fermentation

The initial pH value of the medium was 6.5, and the pH value measured after 24 h fermentation was used as the first data to draw the line chart of the pH value of the compound fermentation and the number of fermentation days as shown in Figure 3.

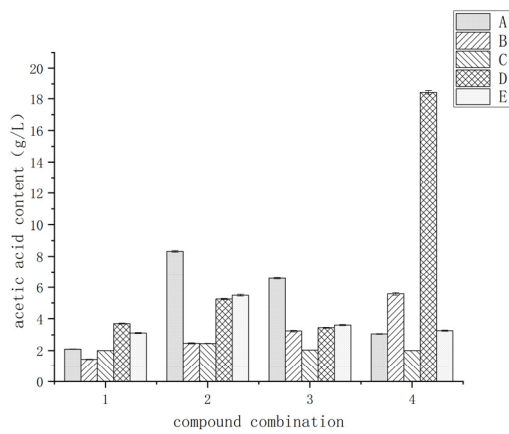
Through the analysis of the change of pH value, it can be known that according to the order of compound ratio 1 : 2, 1 : 1, 5 : 1, 10 : 1 ( compound ratio 1, 2, 3, 4 ), when the initial bacterial solution added increases, the final pH of fermentation will decrease, and the downward trend of pH in the fermentation process will accelerate. It shows that when the initial addition of bacterial solution increases, the added compound strain will adapt to the new growth environment more quickly, complete the growth and proliferation quickly, and start the acid production activity, and the amount of acid production increases from the final pH value. After the completion of fermentation, the pH value of most of the compound groups remained at about 4.3. It is speculated that when the amount of medium is constant, the ability of most of the compound groups to produce acid by using nutrition is not much different. A small number of groups such as A2-3 ( A2, A3 ) at 1 : 1 and 1 : 5 compounding ratios and B2-1 ( D4 ) at 1 : 10 compounding ratio The final pH of the fermentation broth can reach 4 and below. It is speculated that the ability of the compound strains to produce acid by using the same amount of nutrients in these compounding groups is improved. During the fermentation process, the pH of LY-5 ( B1 ) in the compound ratio 1 group showed the strongest downward trend, and it was speculated that the acid production capacity and acid production rate of LY-5 were the best under the compound ratio of 2 : 1. The pH decline trend of A2-3 ( A2, A3 ) in group 2 and group 3 was the strongest, and it was speculated that the acid production capacity and acid production rate of A2-3 were the best under the compound ratio of 1 : 1 and 1 : 5. The pH decline trend of B2-1 ( D4 ) in 4 groups was the strongest, and it was speculated that the acid production capacity and acid production rate of B2-1 were the best under the compound ratio of 1 : 10.

In the late stage of fermentation of group 3 and group 4, it can be observed that the pH value of the samples of several strains of compound bacteria solution has an upward trend. It is speculated that when these compound groups produce more acid in the later stage of fermentation, the acid produced in the later stage of fermentation reacts with other fermentation products to form compounds, which leads to the increase of pH value. Whether the chemical reaction occurs and which products are synthesized need to be further verified by experiments.

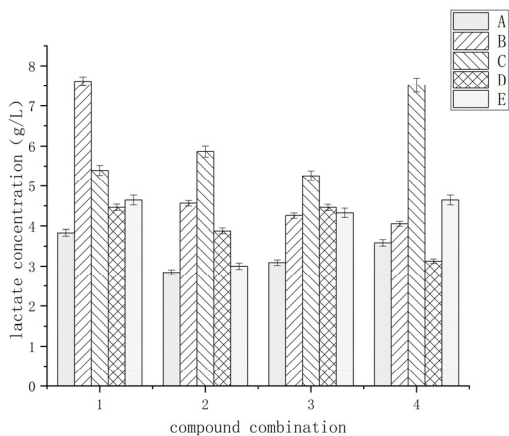
### 3.4. Comparison of Compound Fermentation Metabolites and Acid Production Capacity

After the compound fermentation, the fermentation metabolites were determined, and it was found that the main acid production included acetic acid, lactic acid and caproic acid. The results are shown in Figure 4 ~ 7.

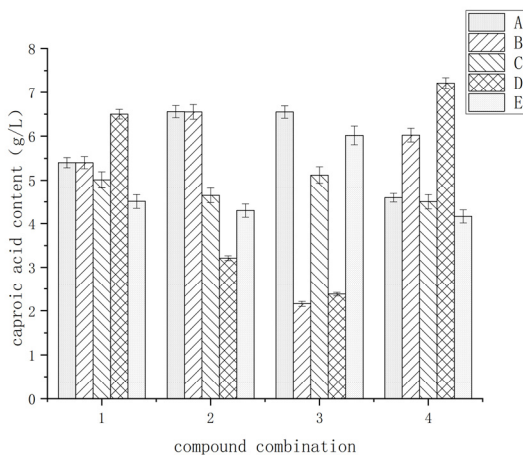
Through the above chart, it can be seen that through the compound experiment, the acid production capacity of some compound combinations has been significantly improved compared with the single strain fermentation, but the acid production capacity of most compound groups has decreased, with a maximum decrease of 39.14 %.



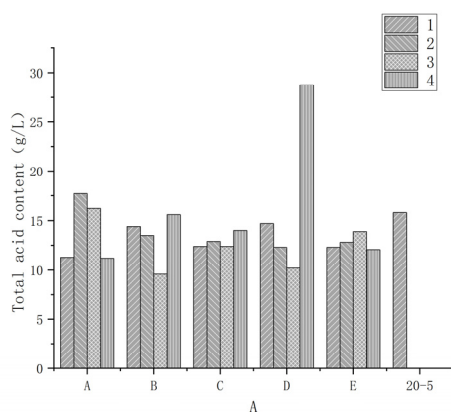
**Fig 4.** Compound ratio and acetic acid content



**Fig 5.** Compound ratio and lactic acid content



**Fig 6.** Hexanoic acid content



**Fig 7.** Total acid content

Among them, the 1:1 combination of 20-5 and A2-3 (A2), the 5:1 combination of 20-5 and A2-3 (A3), the 10:1 combination of 20-5 and LY-5 (B4), and the 10:1 combination of 20-5 and B2-1 (D4) have improved the acid production capacity compared with single bacteria, with a maximum increase of 81.21%. It shows that these compound groups have a mutual promotion effect in the fermentation broth system. Among them, the 10:1 (D4) combination of 20-5 and B2-1 has outstanding acid production ability and is the optimal compound group. In this group, the total acid reached 26.75 g/L, which was the highest acid production group in the experiment. The main acids produced were caproic acid and acetic acid. Combined with the comparison of the acid production ability of the two strains in the single strain fermentation experiment, the lactic acid produced by the lactic acid bacteria is utilized by the caproic acid bacteria and generates other acids, but the acetic acid production in the fermentation broth is very large, indicating that the caproic acid bacteria cannot use or difficult to use acetic acid, and the caproic acid bacteria and lactic acid bacteria used in the experiment can generate acetic acid, so acetic acid accumulates in large quantities. Compared with the single strain acid production, it is speculated that the two bacteria cooperate with each other under this compound ratio, which makes the fermentation acid production increase. However, why the specific acid production capacity is improved, and whether it is the compounding effect that increases the acid production capacity, further research is needed.

Except for several groups with increased acid production capacity after fermentation, the acid production capacity of the other compound groups decreased after fermentation, among which the 1:2 group of 20-5 and A2-3 (A1), the 5:1 group of 20-5 and LY-5 (B3), and the 5:1 group of 20-5 and B2-1 (D3) decreased the most. In the single-strain fermentation results, the acid production ability of LY-5 and B2-1 is very strong. It is speculated that the compound group of LY-5 has a faster reproduction speed and more production after the addition of the compound bacteria solution, which inhibits the growth of 20-5. The antagonistic effect between the bacteria in the fermentation broth leads to a decrease in the final acid production capacity. The compound group of A2-3 may be due to the weak acid production of A2-3 single bacteria, and the growth rate of the two bacteria is not much different when compounded, so that the two compete for nutrients, resulting in an antagonistic effect, resulting in a decrease in the acid production capacity of the final fermentation broth. Comprehensive analysis and speculation in these fermentation ratios of the strain occurred antagonism makes the strain acid production capacity decreased or may occur in the product of the chemical reaction to generate other compounds such as esters, the specific reasons need to be further studied.

The lactic acid yield of single bacteria fermentation and compound fermentation lactic acid were analyzed as shown in Fig 8, The yield of caproic acid by single strain fermentation and compound fermentation caproic acid were analyzed as shown in Fig 9.

Through the comparative analysis of the yield of lactic acid in single-strain fermentation and compound fermentation in Fig 9, it can be found that several strains of lactic acid bacteria produce more lactic acid in single-strain fermentation, and the average yield of lactic acid in single-strain fermentation is 16.57 g/L. However, after compounding with caproic acid bacteria 20-5, the yield of lactic acid in the final fermentation

broth decreased significantly, and the average yield of lactic acid after compounding was 4.52 g / L, which decreased by 73.02 %. It is speculated that the utilization of lactic acid in the fermentation broth by caproic acid bacteria leads to the decrease of lactic acid content in the fermentation broth. In addition, the reason for the decrease of lactic acid may be that lactic acid bacteria have antagonistic effect with caproic acid bacteria in the compound system, resulting in the decrease of lactic acid production by lactic acid bacteria. Through the comparative analysis of the yield of caproic acid in single-strain fermentation and compound fermentation in Fig 9, it can be obtained that the caproic acid production capacity of caproic acid bacteria 20-5 increased after compounding compared with single-strain fermentation. Among them, the

10 : 1 combination of 20-5 and B2-1 ( D4 ) produced the most caproic acid, which increased by 87.74 %. Therefore, it can be determined that caproic acid bacteria can indeed use higher concentrations of lactic acid as a carbon source for fermentation and acid production. However, there are also groups such as the 5 : 1 group of 20-5 and LY-5 ( B3 ) and the 1 : 1 group and 5 : 1 group of 20-5 and B2-1 ( D2, D3 ). After compounding, the ability to produce caproic acid decreased, up to 43.81 %, indicating that the two compounding strains had antagonistic effects during fermentation, and the acid production of the bacteria in the fermentation broth was inhibited, resulting in a decrease in caproic acid production

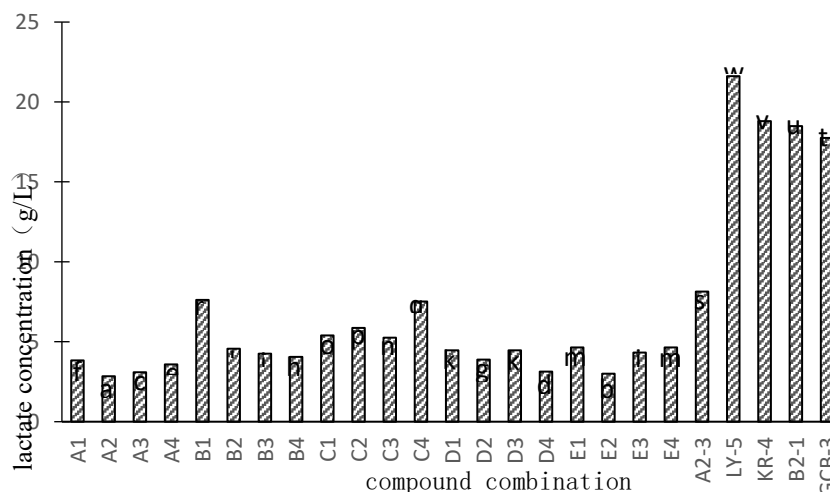


Fig 8. Comparison of lactic acid production by different compound combinations and single strain fermentation

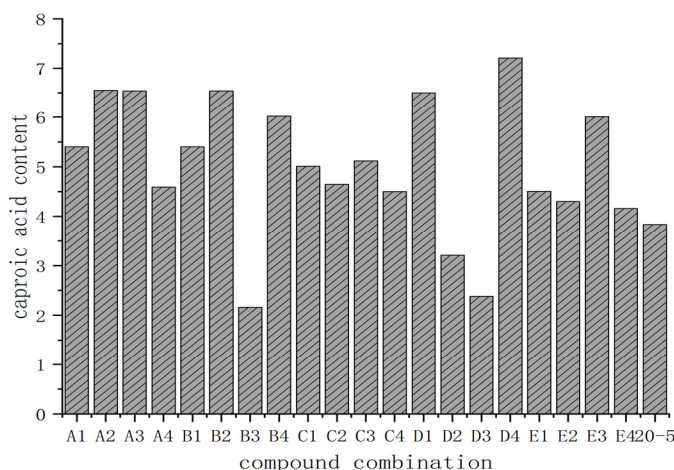


Fig 9. Comparison of caproic acid production by different compound combinations and single strain fermentation

#### 4. Conclusion

This experiment is an exploratory experiment to discuss the improvement of the acid production ability of the strain by the compound effect of the strain. Through a series of single bacteria and multi bacteria fermentation experiments, we can get some conclusions. After the combination of several high-yield lactic acid bacteria (A2-3, GCB-3, KR-4, LY-5, B2-1) and new caproic acid bacteria (20-5), the acid production ability of most combinations decreased, and only a few combinations such as 20-5 and B2-1 10: 1 combination (D4) increased the acid production ability, and the highest yield was 28.75 g/L, which was the optimal combination for this experiment. The total acid increased by 81.21%, and the

caproic acid increased by 87.74%. However, why the specific acid production capacity is improved, and whether it is the compounding effect that increases the acid production capacity, further research is needed. Through the comparison of the yield of two key acids-lactic acid and caproic acid before and after single-strain fermentation and compound fermentation, it can be determined that caproic acid bacteria can use lactic acid as a carbon source for fermentation and acid production, and its caproic acid production capacity has increased. The maximum increase was 3.37 g/L, an increase of 87.74%. However, there is also a compound group with a decrease in caproic acid production capacity, such as the 5: 1 combination of 20-5 and LY-5 (B3), with a maximum decrease of 1.68 g/L, a decrease of 43.81%. The experimental

results showed that the yield of organic acids increased in the process of compounding caproic acid bacteria with lactic acid bacteria, indicating the feasibility of compounding fermentation with different strains. This method is of great significance in pit mud maintenance, enhanced fermentation of traditional pits and automatic fermentation of Luzhou-flavor liquor.

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