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Original Research Article

Physicochemical Properties, Flavor and Microbial Community of Clean Low-Temperature Daqu Originated from Synthetic Autochthonous Microbiota

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Abstract: Based on the 23 strains of low-temperature Daqu, microbial inoculants were prepared and inoculated into crushed barley and pea to prepare clean low-temperature Daqu (XQ). The physicochemical properties, flavor profile, and microbial community of XQ were investigated, with traditional low-temperature Daqu (CQ) and production requirements as controls. The results indicated that there was no significant difference in moisture and acidity between the two types of Daqu: CQ exhibited a moisture of 10.6% and an acidity of 1.1 mmol/10 g, while XQ demonstrated a moisture of 10.9% and an acidity of 1.2 mmol/10 g. The fermenting activity (1.36 g/0.5 g·72 h) and liquefying activity (1.13 g/g·h) of XQ surpassed those of CQ; however, its saccharifying activity (780 mg/g·h) and esterifying activity (808 mg/50 g·7 d) were lower. In general, the physicochemical properties of XQ align with the production requirements. HS-SPME-GC-MS analysis indicated that the flavor profiles of the two types of Daqu were largely similar, with 84.85% of the flavor components of CQ being reproducible in XQ. Microbiota community analysis revealed there were some differences in relative abundance of microbes and COG (Clusters of Orthologous Groups) function between the two Daqu. The dominant microorganisms in XQ identified were *Bacillus*, *Pichia*, *Transversalis*, and *Monascus*, meanwhile the dominant microorganisms in CQ identified were *Pediococcus*, *Rhizomucor*, *Wickerhamomyces*. This study establishes a robust experimental basis for producing clean Daqu and provides valuable insights for the development of safer microbial fermented foods.

Keyword: Clean Daqu, Synthetic Microbiota, Physicochemical Properties, Flavor, Microbial Community, COG.

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INTRODUCTION

Daqu plays a crucial role in the Baijiu brewing process, as it directly influences its quality (Jiang Z Y, *et al.*, 2024)). Based on the fermentation temperature, Daqu can be mainly classified into high-temperature Daqu, medium-high-temperature Daqu, and low-temperature Daqu (Cui M J, *et al.*, 2024). Low-temperature Daqu, made from barley and pea, acts as a saccharifying and fermenting agent in the production of light-flavor Baijiu. The production of low-temperature Daqu involves five key stages: *Shangmei* (opening the windows of Daqu chamber to promote microbial proliferation), *Liangmei* (opening the windows of Daqu chamber for ventilation, allowing the Daqu to harden and take shape), *Chaohuo* (sealing the window of Daqu chamber to optimize the culturing temperature, which simultaneously raises the humidity within the chamber), *Dahuo* (alternating between opening and sealing chamber windows to regulate the temperature and ensure even heat distribution), and *Houhuo* (sealing the chamber windows to enhance thermal retention property, facilitate moisture evaporation, and generate aromatic components). This process provides a rich array of nutrients and flavor precursors for the subsequent fermentation of Baijiu, playing an essential role in determining its quality and flavor profile (Chen P, *et al.*, 2024)).

A diverse array of microorganisms is present in Daqu, including amylolytic molds, ethanol-producing yeasts, and aroma-generating bacteria. Among these,

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bacteria are considered the primary contributors to aroma production during Baijiu fermentation, while yeasts serve as the principal agents for fermentation, and molds function as the key catalysts for saccharification (Liu X G, *et al.*, 2022, Liang M H, *et al.*, 2023). Lei (2015) conducted an analysis of the microbial composition of low-temperature Daqu and fermented grains utilizing high-throughput sequencing technology, revealing *Bacillus* was the predominant strain in low-temperature fermented grains (Lei Z H, 2015). Wang *et al.* used high-throughput sequencing to analyze the microbial composition of Daqu prepared using different methods (traditional and new methods), and found that there were obvious differences in the microbial composition of the two types of Daqu. Notably, traditional Daqu exhibited a more balanced and diverse microbial community (Wang J Y, *et al.*, 2023). He *et al.*, (2024) discovered that the addition of *Bacillus velezensis* and *Bacillus amyloliquefaciens* derived from Daqu enhanced the quality of high-temperature Daqu in the region, and compared to the control group, the fortified Daqu exhibited superior performance in terms of physicochemical properties, microbial diversity, and flavor components, particularly pyrazines (He M C, *et al.*, 2024). Van-Diep L. (2011) identified 19 components that are closely associated with microbial changes during the mature stage of low-temperature Daqu. Furthermore, monitoring the physicochemical properties throughout the Daqu preparation process can effectively control its quality, ensure stability, and enhance production efficiency (Van-Diep L, *et al.*, 2011).

In this study, we investigated two types of Daqu produced through distinct processes: clean low-temperature Daqu (XQ) and traditional low-temperature Daqu (CQ) (Figure 1). By evaluating their physicochemical properties and flavor components, and employing high-throughput sequencing technology to analyze the microbial community, we preliminarily established the feasibility of XQ. This research provides an experimental foundation for the development of clean Daqu based on a more concise autochthonous microbiota. Additionally, it offers valuable insights into producing safer fermented foods.

1. MATERIALS AND METHODS

1.1. Strains

By reviewing relevant literature (Zhou S, *et al.*, 2019, Luo H B, *et al.*, 2014, Liu J, *et al.*, 2017, Du A M, *et al.*, 2021, Hu Y N, *et al.*, 2021, Wang Z M, *et al.*, 2016), and according to the principle of strains abundance ≥1% and its occurrence frequency in relevant the literature ≥60% (Wolfe B E, *et al.*, 2014), 23 functional strains were identified to constitute the synthetic autochthonous microbiota of XQ (Table 1). Among these, 20 strains were obtained from Redstarwine Co., Ltd, while *L. pseudomesenteroides* (Stn10.) and *P. kudriavzevii* (Stn16.) were isolated from Baijiu Daqu by Southwest Strain Station of the China Industrial Microbiological Culture Collection and Management Center (Wenjiang, Sichuan).

Table 1: The strains of autochthonous microbiota used in clean low-temperature Daqu

Genus	Species (No.)	Initial abundance	Potential function
Bacillus	*Bacillus licheniformis (Stn1.)*	3%	Amylase, protease, and flavor production, etc
	Bacillus subilis subspinaquosorum (Stn2.)	5%	
	Bacillus amyloliquefaciens (Stn3.)	5%	
	Bacillus megaterium (Stn4.)	3%	
Acetobacter	*Acetobacter aceti NBRC 14818(T) (Stn5.)*	5%	Acetic acid fermentation, flavor production, etc
	Acetobacter cerevisiae LMG 1625(T) (Stn6.)	3%	
Lactiplantibacillus	*Lactobacillus pentosus JCM 1558(T) (Stn7.)*	5%	Lactic acid fermentation, flavor production, lactic acid production, etc
	Lactobacillus Brevis ATCC 367(T) (Stn8.)	5%	
Weissella	*Weissella cibaria KACC 11862(T)(Stn9.)*	3%	Lactic acid fermentation, flavor production, etc
Leuconostoc	*Leuconostoc pseudomesenteroides (Stn10.)*	3%	Lactic acid fermentation, flavor production, etc
Candida	*Candida glabrata ATCC 90030 (T) (Stn11.)*	8%	Flavor production, etc
	Candida ethanolic ATCC 44956 (T) (Stn12.)	3%	
Saccharomyces	*Saccharomyces cerevisiae ATCC 9080 (T) (Stn13.)*	8%	Alcohol fermentation, alcohol production, flavor production, etc
Saccharomycopsis	*Saccharomycopsis fibuligera strain NRRL Y-2388 (Stn14.)*	3%	Flavor production, alcohol production, glysylenzymes, etc
Thermoascus	*Thermoascus aurantiacus (Stn15.)*	3%	Decompose sugar substances and, flavor production, etc

Pichia	*Pichia kudriavzevii (Stn16.)*	8%	Alcohol fermentation, flavor production, etc
	Pichia guilliermondii ATCC 20323(T) (Stn17.)	3%	
	Pichia pastoris (Stn18.)	3%	
Aspergillus	*Monascus anka Nakazawa et Sato (Stn19.)*	5%	Amylase, protease, and flavor production, etc
	Aspergillus chevalieri strain CBS 522.65 (Stn20.)	3%	
	Aspergillus oryzae (Stn21.)	5%	
Thermomyces	*Thermomyces lanuginosus (Stn22.)*	3%	Xylanase production, etc
Rhizopus	*Rhizopus oryzae (Stn23.)*	5%	Glycidase, amylase, flavor production, etc

1.2. Preparation of Microbial Inoculant

Twenty-three strains of bacteria, mold, or yeast were rejuvenated and expanded using NA (Nutrient Agar), YPD (Yeast Extract Peptone Dextrose), and PDA (Potato Dextrose Agar) media. Bacteria were cultured at 37 °C, whereas yeast and molds were cultured at 30 °C. Microbial suspensions were obtained by centrifuging the strains expanding mush, respectively. The suspensions for bacteria, mold, and yeast were then adsorbed onto a carrier based on the ratio for strains: carrier composed of 50% bran powder (50 mesh) and 50% corn straw powder (50 mesh) of 2:1 (volume to mass) and their respective initial abundances of Table 1, while a protective agent was added to the carrier. Subsequently, the protective agents were added according to the following mass fractions(Liu Q S, *et al*., 2023): mannitol (3.7% of the carrier), gelatin (4% of the carrier), trehalose (4% of the carrier), and glutamic acid (4.4% of the carrier) were mixed in a beaker. The aforementioned mixture was precooled in a refrigerator at -20 °C for 10 hours, and subsequently subjected to cryodesiccation in a vacuum freeze-dryer at -50 °C for 10 hours, preparing the initial microbial inoculant.

The three initial microbial inoculants were inoculated into crushed bran at a 1:100 mass ratio and incubated for 3 days at optimal temperatures (37 °C for bacteria, 28 °C for yeast and mold). During incubation, the bran was manually agitated twice daily to maintain a loose texture, ensuring favorable microbial growth subsequently, the secondary microbial inoculant was dried at 50 °C until its moisture < 10%, and then vacuum sealing of secondary microbial inoculant was performed on an ultra-clean bench.

1.3. Preparation of XQ in the Laboratory

The prepared process of clean low-temperature Daqu (XQ) was showed in Figure 1. Barley and pea were soaked and disinfected with food-grade chlorine dioxide (150 mg/L) in a ratio of 6:4 for 15 minutes. Subsequently, they were milled into particles with coarse exterior and fine interior. Then, 50% sterile water and 5% secondary microbial inoculant were incorporated into the mixture and thoroughly mixed. Subsequently, the resulting mixture was shaped into bricks, fermented for a duration of 26 days, and stored for a period of 3 months. The fermentation process consists of six stages. The process begins with the Shangmei Stage, during which the Daqu is incubated at 38℃ for 2 days. During this stage, a gradual increase in temperature fosters optimal mold growth until it is fully established. This is followed by the Liangmei Stage, lasting 3 days with the temperature maintained between 24℃ and 36℃. During this phase, the Daqu is turned daily, ensuring that the bottom edges of the lower layer are brought to the top and vice versa. The subsequent stage is the Chaohuo phase, during which the temperature gradually increases from 43℃ to 47℃ over a period of five days. During this period, microorganisms migrate inward from the surface of the Daqu, raising the core temperature significantly and releasing large amounts of moisture. To manage this, moisture is vented 2-3 times daily, and the Daqu is turned once a day. The Dahuo Stage comes next, with the temperature maintained at 43℃ for 7 days. The Daqu is turned daily for the first 3 days and every other day for the remaining 4 days. Subsequently, the Houhuo Stage requires controlling the temperature between 34℃ and 38℃ for 4 days. Finally, completion of fermentation occurs during the Developing Stage, where temperatures are sustained at 30℃ for an additional five-day period.

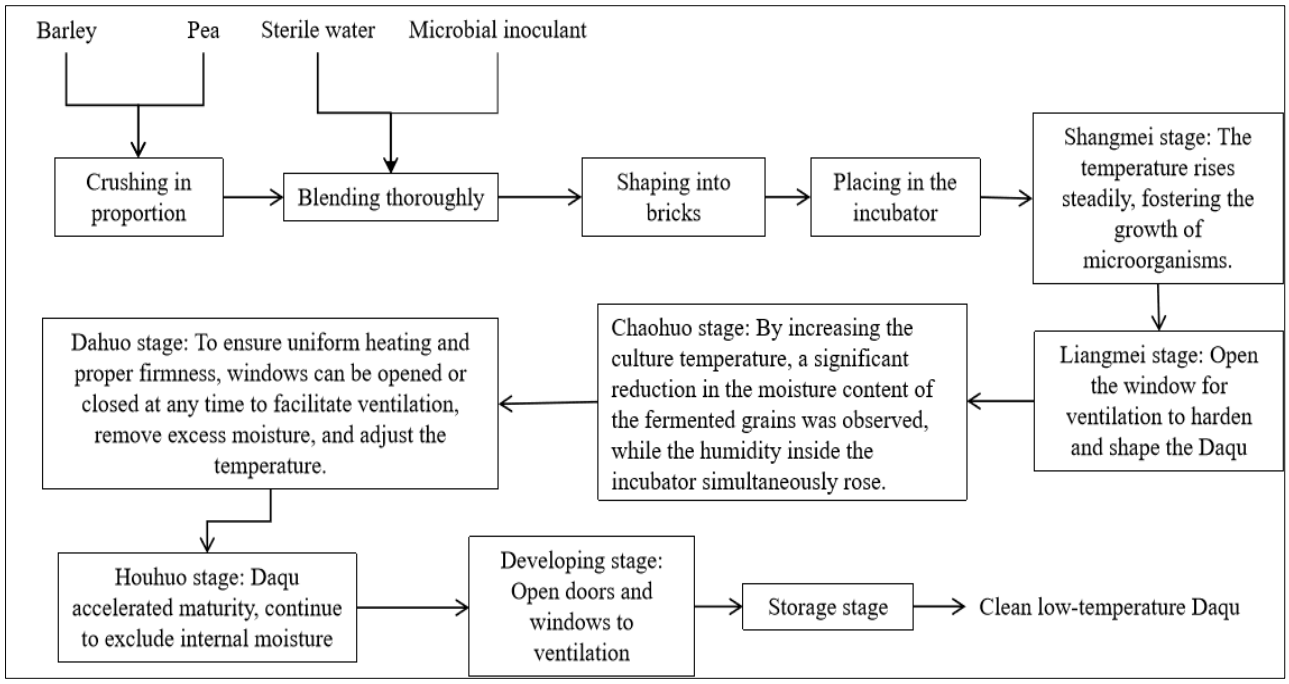

Figure 1: The preparation process for clean low-temperature Daqu

1.4. Sampling

XQ was prepared in the laboratory of the Baijiu Microbiology Research Team at Sichuan University of Science & Engineering, while CQ was gathered at Jinyuan Jinmei Qingxiang Baijiu Co., Ltd. in Shanxi Province, China. Each sample was collected using a five-point sampling method, ground into powder, and combined to obtain a total mass of 200 grams. These were then sealed in aseptic pouches and stored at -80 °C.

1.5. Determinations of Physicochemical Properties

Physicochemical properties, including moisture, acidity, starch, fermenting activity, liquefying activity, saccharifying activity, esterifying activity, and bulk density, were measured in accordance with the standards specified in *QB/T 4257-2011 General Methods of Analysis for Daqu* (China national light industry council, 2011).

1.6. Determination of Flavor Components in Daqu by GC-MS

Extraction method: 0.500 g of Daqu powder, 2 g of NaCl and 10 mL redistilled water were added into a 20 mL headspace bottle, then, 10 μL of the internal standards (2-octanol, 0.00274 g/100 mL) were added to the headspace bottles and sealed. The headspace bottle was then placed in a fully automatic solid-phase microextraction instrument at 60±1 °C. It was pre-balanced for 15 minutes, after which a solid-phase extraction fiber was inserted into the headspace bottle, positioned 2 cm above the liquid surface and absorbed flavor components for 45 minutes, and then discharged in the gas chromatography inlet at 230 °C for 5 min.

The GC-MS conducted as per the method of Fan *et al*., (2018) with some modifications. The chromatographic condition was as follows: a programmed temperature rise scheme was adopted: the initial temperature was 40 °C for 5 min, the temperature was increased to 100 °C at 4 °C/min and then increased to 230 °C at 6 °C /min for 10 min (Fan G S, *et al*., 2018). The inlet temperature was set at 250 °C, and the ion source temperature was maintained at 230 °C. The mass spectrometry scanning range was m/z 40–450. An HP-INNOWAX chromatographic column (0.25 μm × 60.0 mm × 0.25 mm) was employed. The detected mass spectra were compared against the NIST05 standard database. Flavor components with a matching degree of ≥80% were qualitatively and quantitatively analyzed using the internal standard method. The content of flavor components was calculated using the formula provided by Li *et al*., (2013), and the results were expressed in μg/kg.

$$\text{Concentration} = \frac{\text{peak area of flavor components}}{\text{peak area of internal standard}} \times \text{concentration of } 2-\text{octanol}$$

1.7. High-Throughput Sequencing of Daqu Microbiota

DNA extraction from Daqu: A specialized DNA extraction kit was used to purify the pre-treated samples. Each PCR sample was run in triplicates. After the product fragments were mixed, the quality of the extracted DNA was determined. The V3-V4 regions of the 16S rRNA of bacteria (341F 5' CCTAYGGGABGCASCAG3' and 806R 5' GGACTACNNGGGTATCTAAT3') and ITS1 and ITS4

regions of fungi (1737F 5' GGAGTAAAAGTCGTAACAGG-3' and 2043R 5' GCTGGTTCTTCATCGATGC-3') were amplified through PCR (Wang Y, *et al*., 2012). Finally, high-throughput sequencing analysis was conducted on the amplified fragments using an Illumina HiSeq sequencing platform (Beijing Qingke Biotechnology Co. Ltd.).

2. Statistical Analysis of Data

The data were processed using SPSS 26, and the results are presented as mean ± standard deviation. Heat maps were generated using TB tools. The processing and analysis of the amplified sequence data were conducted by Beijing Qingke Biotechnology Co. Ltd. The microbial composition of Daqu samples were determined using bioinformatic analysis of 16S rDNA and ITS high-throughput sequencing results. The UPARSE algorithm was used to cluster Operational Taxonomic Units (OTUs) with a 97% similarity level to identify representative OTU sequences and compare the database for species classification. The software QIIME2 2020.6 was utilized to assess the alpha diversity index of XQ and CQ samples, while Picrust 22.3.0 was employed to predict the functional profiles of Daqu samples.

3. RESULTS AND DISCUSSION

3.1. Analysis of Physicochemical Properties during the Fermentation of XQ

The physicochemical properties of XQ at each phase are shown in Table 2. Both moisture and starch consistently exhibited a decreasing trend from the initial fermentation stage to 90 days of storage. Over this period, moisture decreased by 36%, while approximately 19.8% of the starch was decomposed and converted into flavor components through microbial metabolism (Wan Z R, 2004). The acidity exhibited a continuous upward trend during the initial phases of fermentation, coinciding with temperature and increased moisture. During this period, mold hyphae had not yet developed, resulting in an anaerobic environment that was conducive to organic acid production by microorganisms (Zhang Q, *et al*., 2021). Acidity peaked on the fifth day, subsequently entering the Chaohuo stage, organic acids participated in protein degradation and ester synthesis. The temperature increase led to the inhibition of certain microbial activities, resulting in a decline in these microorganisms and a reduction in organic acid production. During storage, although the moisture of Daqu was lower, the ambient storage temperature was more favorable for microbial growth and metabolism compared to the Chaohuo stage, resulting in a slight increase in the acidity of Daqu. Before fermentation, the glucoamylase present in the barley and pea raw materials was activated by moisture, resulting in the newly formed Daqu exhibiting initial saccharifying activity. As fermentation progressed, the saccharifying activity initially increased, then decreased, and subsequently gradually increased again. During fermentation, the increasing incubator temperature accelerated the proliferation of saccharifying microorganisms, such as molds, leading to a rapid rise in saccharifying activity. However, once the temperature exceeded the optimal range and moisture decreased, microbial activity was inhibited or ceased. Additionally, the glucoamylase present in the raw materials is unstable at higher temperatures, resulting in a loss of enzymatic efficacy (Xing G, *et al*., 2014). After the Dahuo stage, as the temperature stabilized within a more suitable range, glucoamylase activity increased. During storage, despite the low activity of glucoamylase, saccharifying activity gradually increased to approximately 780 mg/g·h by the later stages. Fermenting activity exhibited a similar trend to saccharifying activity, initially increasing, then decreasing, and subsequently gradually increasing again, eventually stabilizing during late fermentation. During storage, the suitable temperature caused a slight upward trend in fermenting activity, consistent with the variations described by Xing *et al*., for low-temperature Daqu (Xing G, *et al*., 2014). Before fermentation, the Daqu exhibited minimal liquefying activity. As fermentation progressed, increased microbial metabolic activity enhanced liquefaction enzyme activity. By the tenth day, rising temperatures caused the death of certain microorganisms, leading to a reduction in liquefying activity. During storage, reduced acidity and temperature facilitated fungal growth (Shi S, *et al*., 2017), contributing to an increase in liquefying activity. However, a sustained decline in microbial counts following storage resulted in a slight variation in overall liquefying activity (Jiao M J, *et al*., 2015). Esterifying activity fluctuated during Daqu fermentation. In the initial stages of fermentation, increasing temperatures significantly enhanced esterifying activity, which can be attributed to the growth of molds and yeasts in the starter material (Wan Z R, 2004). As fermentation temperatures peaked, some microorganisms perished, and mold and yeast metabolism were inhibited (Shen C H, *et al*., 2005), leading to a decline in esterifying activity. In later fermentation and storage stages, as temperatures decreased, molds and yeasts resumed metabolic activity, resulting in an increase in esterifying activity, reaching 806 mg/50 g·7 d by the end of storage.

Table 2: Physicochemical properties of clean low-temperature Daqu during fermentation

Time (days)	Moisture (%)	Acidity (mmol/10g)	Starch content (%)	Saccharifying activity (mg/g·h)	Fermenting activity (g/0.5g·72h)	Liquefying activity (g/g·h)	Esterifying activity (mg/50g·7d)
0d	47.69±2.29	0.40±0.02	72.20±4.39	433.74±25.53	2.09±0.11	"0"	9.43±0.49
2d	42.02±1.99	0.79±0.04	68.70±4.42	514.24±33.86	3.23±0.19	0.59±0.03	68.84±4.09
5d	37.62±1.89	1.68±0.09	64.75±4.16	808.47±49.71	3.72±0.23	0.88±0.05	781.64±48.88
10d	28.48±1.41	0.99±0.06	62.21±4.18	684.67±40.99	3.12±0.20	0.87±0.06	308.71±18.96

17d	23.13±1.12	0.71±0.04	60.63±4.03	641.36±38.18	1.43±0.12	0.79±0.05	231.12±13.39
21d	17.30±0.82	0.98±0.05	58.44±3.88	622.76±39.96	1.23±0.08	0.84±0.06	318.66±16.83
26d	13.22±0.61	1.19±0.07	57.90±4.76	712.43±44.74	1.24±0.08	0.88±0.05	464.17±26.26
56d	11.01±0.53	1.30±0.08	56.07±4.61	765.16±39.19	1.28±0.07	1.02±0.06	760.32±39.61
116d	10.69±0.53	1.10±0.06	55.3±4.62	780.70±45.66	1.36±0.08	1.13±0.06	806.36±48.15

The physicochemical properties of XQ stored for 90 days and CQ are summarized in Table 3, facilitating a comparative assessment of the two types of Daqu. In the preparation of low-temperature Daqu, the following physicochemical properties are typically required: moisture ≤ 13%, acidity ranging from 0.90 to 1.30 mmol/10 g, starch ≤ 57.5 %, saccharifying activity ≥ 600 mg/g·h, fermenting activity ≥ 0.9 g/0.5 g·72 h, liquefying activity ≥ 1.0 g/g·h, esterifying activity within the range of approximately 800 to 1200 mg/50 g·7 d, and bulk density ≤ 0.72 g/cm³ (Shen Y F, 1998). From Table 3, it can be concluded that the XQ prepared in this study conforms to the stipulated production requirements. By comparison, the XQ (stored for 90 days) exhibited minimal differences from CQ in terms of moisture and acidity. However, the starch in XQ was detected to be lower than that in CQ, which may be attributed to variations in starch utilization rates resulting from differences in the Daqu preparation processes (Xing G, *et al.*, 2014). With respect to saccharifying activity and esterifying activity, XQ manifested conspicuously lower magnitudes than CQ. Saccharifying activity and esterifying activity respectively reflect the efficiency of saccharification and esterification of Daqu, which are influenced by enzymes present in barley and pea, as well as substantial enzymes produced by molds during fermentation (Chen P, *et al.*, 2024). Differences in the Daqu preparation environment may have contributed to a reduced quantity of molds in the XQ, thereby resulting in lower esterifying and saccharifying activities. Conversely, the fermenting and liquefying activities of XQ were higher than those of CQ. These activities indicated the capacity for Baijiu production and yield efficiency (Feng Y, 2022). Bulk density functions as an indicator of total material consumption throughout the Daqu preparation process and reflected the maturation state of Daqu fermentation. A lower bulk density indicated a lighter quality of Daqu per unit volume and suggested more thorough fermentation (Shen C H, *et al.*, 2005). In this respect, XQ is decidedly superior to CQ.

Table 3: Physicochemical properties of clean low-temperature Daqu and traditional low-temperature Daqu

	Moisture (%)	**Acidity (mmol/10g)**	**Starch content (%)**	**Saccharifying activity (mg/g·h)**	**Fermenting activity (g/0.5g·72h)**	**Liquefying activity (g/g·h)**	**Esterifying activity (mg/50g·7d)**	**Bulk density (g/cm3)**
XQ	10.6±0.98	1.1±0.06	55.3±4.28	780±55.04	1.36±0.08	1.13±0.07	808±66.26	0.676±0.04
CQ	10.9±0.63	1.2±0.09	53.2±3.65	856±66.27	1.28±0.08	1.02±0.06	873±56.17	0.811±0.05

Note: XQ: clean low-temperature Daqu; CQ: traditional low-temperature Daqu

3.2. Flavor Components of Two Types of Daqu Based on GC-MS

With the aim of exploring the disparities in flavor components among clean low-temperature Daqu that has undergone 26 days of fermentation (XQ1), low-temperature Daqu stored for 90 days (XQ2), and traditional low-temperature Daqu (CQ), HS-SPME-GC-MS technology was used to semi-quantitatively analyze the flavor components and their relative contents using the internal standard method. A total of 43 flavor components were identified, including 39 in XQ1 and XQ2, and 33 in CQ, with 28 common to all three types of Daqu (Table 4). This indicated that XQ2 reproduced 84.85% of the flavor substances in CQ, suggesting that the flavor characteristics of CQ could be reproduced in XQ2.

In XQ1 and XQ2, alcohols and esters were significantly higher than CQ. Among these, alcohols served as the primary source of mellow and sweet flavors, contributing to the formation of the flavor profile and enhancing the overall fullness of Daqu (Jia Q H, *et al.*, 2008). Esters were important flavor components, exhibiting a pleasant fruity aroma and enhancing the overall aroma to varying degrees, playing a crucial role in determining the type of Baijiu flavor (Li W Q, 2007).

Table 4: GC-MS Results of Different Daqu(ug/kg)

Component	**CAS**	**XQ1**	**XQ2**	**CQ**
Phenethyl alcohol	60-12-8	7738.53±168.21^{b}	9005.87±279.21^{a}	6261.2±101.88^{c}
Pentanol	71-41-0	1635.6±139.32^{c}	2665.93±53.07^{b}	3171.83±116.79^{a}
Linalool	78-70-6	854.73±27.93^{b}	1266.47±32.2^{a}	ND
Benzyl alcohol	100-51-6	4772.13±456.21^{b}	7478.07±1001.16^{a}	5336±120.34^{b}
1-Hexanol	111-27-3	16171.13±1035.37^{c}	26967.3±1121.38^{a}	20933.67±867.55^{b}
1-Octanol	111-87-5	1974.7±313.27^{b}	3448.33±68.29^{a}	3575.17±453.17^{a}
3-Methyl-1-butanol	123-51-3	2623.43±165.71^{b}	3515.13±63.12^{a}	3671.97±206.51^{a}
Oct-1-en-3-ol	3391-86-4	5738±634.94^{b}	7050.47±46.17^{a}	5102.13±171.92^{b}

2-undecen-1-ol	37617-03-1	ND	ND	2335.03±28.63
Apricolin	104-61-0	1664.53±85.4^{a}	583.47±41.41^{c}	1171.67±121.39^{b}
Phenylacetaldehyde	122-78-1	878.13±32.77^{a}	991.53±85.6^{a}	677.7±61.26^{b}
Nonanal	124-19-6	836.67±21.43^{b}	1847.3±3.85^{a}	1702.47±221.45^{a}
(2E)-2-Octenal	2548-87-0	749±82.12^{a}	633.67±25.57^{b}	ND
Trans-2-Nonenal-D2	213595-54-1	615.8±35.78^{a}	633.47±25.68^{a}	ND
Palmitic acid-13C	287100-87-2	927.07±3.77^{ab}	920.17±0.97^{b}	932.8±7.51^{a}
1-Hexanoic acid	142-62-1	1512.97±110.9c	2572.97±173.17^{b}	7619.2±125.28^{a}
Lauric acid	143-07-7	94.63±2.31^{a}	63.1±0.98^{c}	85.9±1.44^{b}
3-Methylbutanoic acid	503-74-2	ND	ND	625.7±6.42
2-dimethylaminoethyl tetradecanoate	43016-78-0	ND	ND	364.9±0.89
Ethyl butanoate	105-54-4	2473±277.69c	3570.27±243.66^{b}	7199.47±491.51^{a}
Ethyl caprylate	106-32-1	1247.37±128.81^{b}	1365.33±112.31^{b}	1781.2±77.46^{a}
Methyl palmitate	112-39-0	1570.27±163.61^{b}	2044.9±14.95^{a}	ND
Methyl linoleate	112-63-0	1076.57±18.49^{b}	1263.07±83.28^{a}	1218.03±50.59^{a}
Ethyl nonanoate	123-29-5	504.07±6.05c	755.63±8.87^{a}	635.57±4.38^{b}
Methyl myristate	124-10-7	748.77±18.77^{b}	950.87±27.98^{a}	ND
Ethyl Oleate	111-62-6	1170.83±147.72^{b}	1317.1±165.24^{b}	1642.33±106.92^{a}
Ethyl valerate	539-82-2	1521.53±173.14c	2834.03±188.95^{b}	6735.03±971.76^{a}
Ethyl lactate	97-64-3	3547.27±206.54^{b}	3463.73±112.14^{b}	4269.27±122.36^{a}
2-Heptanone	110-43-0	1236.27±201.07^{b}	1702.6±23.66^{a}	1197.37±25.33^{b}
6-Methylhept-5-en-2-one	110-93-0	1611.07±108.55^{a}	1613.27±71.84^{a}	1187.33±16.8^{b}
Methyl nonyl ketone	112-12-9	646.87±35.98^{a}	647.7±19.83^{a}	ND
4,6-Dimethyl-2-Heptanone	19549-80-5	556.17±12.51^{b}	699.07±9.93^{a}	ND
Geranylacetone	3796-70-1	855.4±19.07^{a}	651.37±23.34^{b}	ND
Acetophenone	98-86-2	1405.67±86.02^{b}	1877.93±90.71^{a}	1214.4±42.79^{b}
Phenol	108-95-2	1367.8±8.36^{b}	1868.1±142.04^{a}	1575.7±108.71^{b}
4-Vinylphenol	2628-17-3	521.1±8.23^{b}	762.07±124.04^{a}	ND
Guaiacol	90-05-1	2701.73±115.75^{a}	2297.9±229.44^{b}	1952.27±42.13c
2,6-Dimethylpyrazine	108-50-9	1075.23±79.13^{b}	1476.33±19.5^{a}	ND
2,3,5-Trimethylpyrazine	14667-55-1	9029.9±260.6^{a}	8347.63±216.07^{b}	1794.97±195.08c
Styrene	100-42-5	565.07±3.28^{b}	740.4±35.25^{a}	599.5±26.88^{b}
Myrcene	123-35-3	364.83±49.42^{b}	606.03±79.91^{a}	ND
Component	CAS	XQ1	XQ2	CQ
(+)-Limonene	5989-27-5	ND	ND	2086.73±53.87
1-nitrohexane	646-14-0	1334.63±33.66^{ab}	1548.37±39.71^{a}	1194.57±235.8^{b}

Note: XQ1: clean low-temperature Daqu fermented 26 days, XQ2: clean low-temperature Daqu stored for 90days, CQ: traditional low-temperature Daqu

As illustrated in the heat map depicted in Figure 2. With respect to flavor compositions, XQ exhibited flavor profiles analogous to those of CQ. In terms of concentration, CQ demonstrated relatively higher levels of ethyl butanoate, ethyl valerate, 1-hexanoic acid, phenylethyl alcohol, benzyl alcohol, and ethyl lactate. Conversely, XQ contained higher concentrations of 1-hexanol, 2,3,5-trimethylpyrazine, phenylethyl alcohol, oct-1-en-3-ol, benzyl alcohol, ethyl lactate, guaiacol, and 3-methyl-1-butanol. Most of these components are aroma-enhancing substances that contribute significantly to the overall fragrance of Baijiu. For instance, 1-hexanol imparts a vigorous, exquisite, and floral fragrance (Wang C X, *et al.*, 2019); 2,3,5-trimethylpyrazine bestows a distinct and intense roasted peanut or potato aroma (Wei J, *et al.*, 2014); phenylethyl alcohol contributes aromatic notes reminiscent of sweet bread and roses (Fan H Y, *et al.*, 2015); oct-1-en-3-ol provides a mushroom flavor with strong earthy and herbal undertones (Zhang L L, *et al.*, 2017); benzyl alcohol functions not only as a fragrance component but also as a precursor for acids and esters (You L, *et al.*, 2016); ethyl lactate, characterized by its tender and fruity notes, can smooth and balance the wine, resulting in a more supple, harmonious, and pleasant flavor with a prolonged aftertaste (Wang G N, *et al.*, 2022); 3-methyl-1-butanol contributes a mellow and elegant aroma (Fan J Y, *et al.*, 2023); guaiacol confers woody, smoky, spicy, and sweet vanillin flavors, which can help mitigate saltiness (Wang C J, *et al.*, 2023).

Based on the results from GC-MS analyses, it is revealed that XQ and CQ exhibit similar flavor profiles, and then provides a foundation for the production of Baijiu with a light flavor profile using XQ.

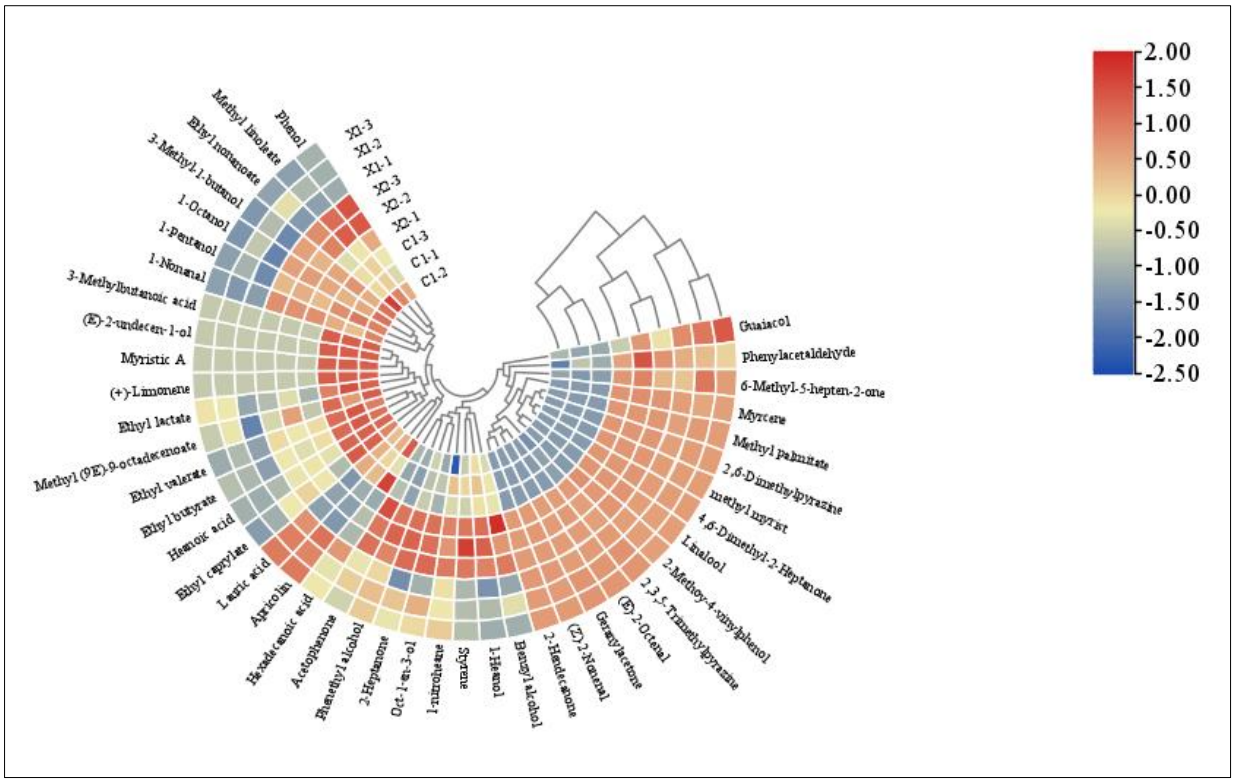

Figure 2: Relative abundance of Daqu flavor components

3.3. Microbial Community Analysis for XQ and CQ

The relative abundance of bacterial and fungal genera in both XQ and CQ was evaluated using high-throughput sequencing, with the results presented in Figure 3.

In CQ, 15 bacterial and fungal genera exhibited abundances exceeding 1%, including *Pediococcus*, *Pantoea*, *Leuconostoc*, *Levilactobacillus*, *Lactiplantibacillus*, *Acetobacter*, *Kosakonia*, *Bacillus*, and *Clostridium sensu stricto 18*, *Pichia*, *Lichtheimia*, *Rhizomucor*, *Rhizopus*, *Wickerhamomyces*, and *Dipodascus*. In XQ, 8 bacterial and fungal genera exhibited abundances exceeding 1%, namely *Pediococcus*, *Bacillus*, *Acetobacter*, *Enterococcus*, *Pichia*, *Lichtheimia*, *Aspergillus*, and *Monascus*.

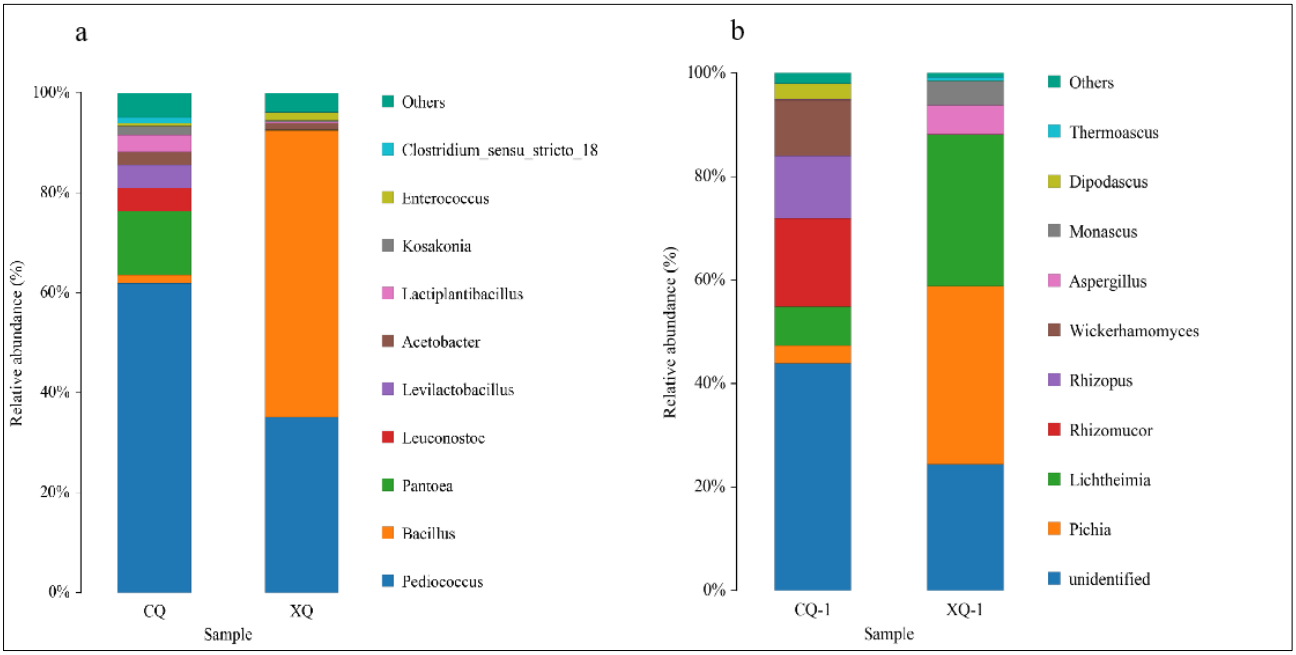

Figure 3: The composition of microbial community abundance at the genus level of two types of Daqu
Note: a: bacterial genera; b: fungal genera

In bacterial genera, two preponderant genera were discerned in CQ: *Pediococcus* and *Pantoea*, both manifesting abundances surpassing 10%. In XQ, these two genera can also be identified, but their relative abundances were attenuated. Concurrently, *Bacillus* emerged as the dominant genus, exhibiting a relative abundance exceeding 50%. This dominance of Bacillus can likely be attributed to its purposeful introduction during the fermentation process. *Bacillus* constitutes the most salient microbial assemblage among diverse types of Daqu and assumes a vital role in Baijiu production (Zhang L Q, *et al.*, 2014). *Bacillus* excretes a multiplicity of extracellular enzymes, preponderantly amylase, and possesses a highly efficacious hydrolytic enzyme system (Jin Y, *et al.*, 2019). *Bacillus* has the ability to metabolize and produce diverse flavor components, such as pyrazine and pyridine. When *Bacillus* becomes the dominant genus, it can inhibit the growth of *Lactobacillus* (He G Q, *et al.*, 2019, Chen W P, *et al.*, 2015). The species and quantity of *Bacillus* directly influence the quality of Daqu, thereby determining the characteristics and style of the resulting Baijiu (Li D N, *et al.*, 2017). Most *Pediococcus* species may possess some potential benefits; however, they are generally considered undesirable due to the peculiar odors and off-flavors they produce. *Pantoea* was negatively correlated with the typical flavor components in Daqu (Zhu Q, *et al.*, 2022). Therefore, reducing its abundance may enhance the quality of Baijiu.

Among fungal genera, *Rhizomucor*, *Rhizopus*, and *Wickerhamomyces* were the dominant genera in CQ, exhibiting a relative abundance exceeding 10%. These genera were also identified in XQ. Meanwhile, the abundance of *Pichia* and *Lichtheimia* exceeded 10% in XQ. Furthermore, the abundance of *Monascus* in XQ was over 300 times higher than that in CQ. *Monascus* not only acts as a natural pigment with high chemical stability in various food products but also produces a wide range of beneficial enzymes during its growth and metabolic processes, including amylase, glucoamylase, protease, and esterification enzymes (Hu N, *et al.*, 2017). These enzymes play a pivotal and indispensable role in Baijiu manufacturing. Esterification enzymes expedite the formation of organic acids and esters, thereby enhancing the aroma bouquet of Baijiu and endowing it with a more mellow and harmonious flavor profile. (Liu L J, *et al.*, 2020, Xu Y Q, *et al.*, 2021). *Pichia* predominantly participates in esterification during Baijiu brewing and is widely regarded as a primary determinant responsible for the elevated concentrations of esters and phenylethanol in Daqu's flavor profile (Wang H Y, *et al.*, 2011). Therefore, appropriately increasing the abundance of these fungi in Daqu might enhance the quality of Baijiu.

3.4. Predictive Analysis of Daqu COG Function

The Clusters of Orthologous Groups of Proteins (COG) database, which classifies homologous protein clusters, provides a functional classification of proteins. Figure 4 illustrated the analysis results, reflecting the functional distribution of sequences in the samples. In this figure, blue-gray trabecula/bubble represented XQ, while yellow trabecula (bubble) represented CQ.

The bubble diagram illustrating the COG function prediction of the two types of Daqu bacteria reveals that they possess distinct functional advantages. Compared to CQ, XQ exhibited enrichment in several COG functional categories, including Energy production and conversion, Cell cycle control, cell division, chromosome partitioning, Amino acid transport and metabolism, Coenzyme transport and metabolism, Cell motility, Posttranslational modification, protein turnover, chaperones, Inorganic ion transport and metabolism, Secondary metabolites biosynthesis, transport, and catabolism, Function unknown, Signal transduction mechanisms, Chromatin structure and dynamics, and Defense mechanisms ($p < 0.01$). Conversely, XQ exhibits significantly reduced enrichment in several COG functional categories compared to CQ, including Cell wall/membrane/envelope biogenesis, Nucleotide transport and metabolism, Carbohydrate transport and metabolism, Lipid transport and metabolism, Translation, ribosomal structure and biogenesis, Replication, recombination and repair, *RNA* processing and modification, General function prediction only, Intracellular trafficking, secretion, vesicular transport, and Cytoskeleton ($p < 0.01$). CQ demonstrated superior capabilities in Translation, ribosomal structure, and biosynthesis, whereas XQ exhibited enhanced efficacy in the Transport and metabolism of essential amino acids. This disparity may be attributed to the differential abundance of lactic acid bacteria present in the two types of Daqu. Certain lactic acid bacteria may inhibit the growth and proliferation of microorganisms capable of synthesizing amino acids (Terrade N, *et al.*, 2009). Therefore, XQ demonstrated a strong capability in amino acid transport and metabolism, which could be attributed to its lower abundance of lactic acid bacteria.

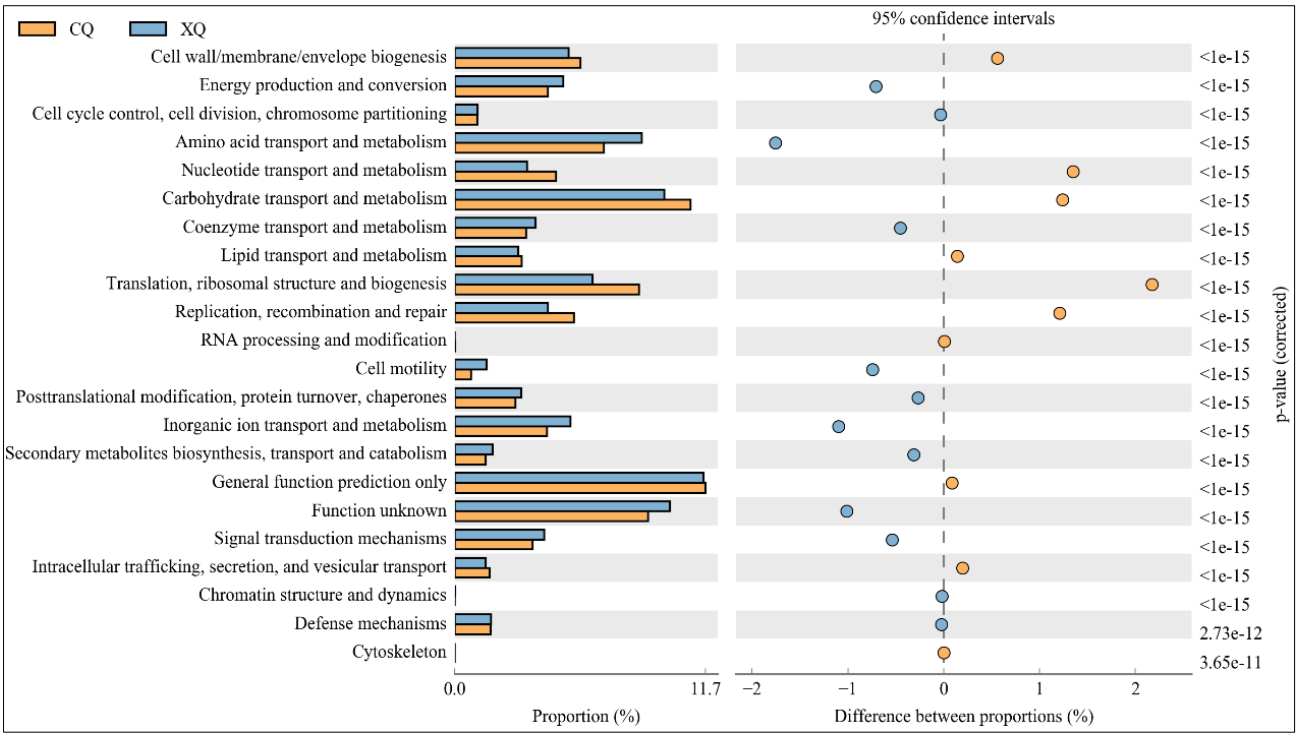

Figure 4: COG function prediction of two types of Daqu

4. CONCLUSION

In this study, we conducted a comparative analysis of physicochemical properties, flavor components, and microbial community of XQ and CQ. The results indicated that the physicochemical properties of XQ met production standards, with its fermenting activity and liquefying activity were superior to those of CQ. Although the flavor components of both types of Daqu were largely similar, there were some differences in the concentrations of certain flavor components. The analysis of the microbial community revealed variations in the relative abundance and function of microorganisms between the two types of Daqu. The study has demonstrated that XQ satisfies the requirements for Baijiu production and exhibits similar function to CQ. This research provided an experimental foundation for the preparation of clean Daqu and presented an idea for the production of safer microbial fermented foods.

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