Effects of mixed cultures of *Candida tropicalis* and aromatizing yeast in alcoholic fermentation on the quality of apple vinegar

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Abstract

There are two steps (alcoholic fermentation and acetic acid fermentation) in the production of fruit vinegar by liquid fermentation. The yeast alcoholic fermentation step plays an important role in the quality of apple vinegar. In this work, *Candida tropicalis* and aromatizing yeast were used in mixed alcoholic fermentation to improve the flavor of the apple vinegar. The total organic acid contents of apple cider and vinegar in mixed cultures were all higher than those in pure culture (*Candida tropicalis*). Umami and sweet free amino acid levels in mixed-culture apple vinegar (MCAV; 1236.71 and 858.25 mg/L, respectively) were significantly higher than those in pure-culture apple vinegar (PCAV; 1214.69 and 820.37 mg/L, respectively). The total esters, total alcohols, and total phenolics were also significantly increased in MCAV (282.36 g/L, 254.22 g/L and 47.49 g/L, respectively), fruit flavor and floral aromas in MCAV were higher than that in PCAV. In the principal component analysis (PCA), the integrative score for MCAV was higher than that for PCAV. Therefore, mixed cultures of *Candida tropicalis* and aromatizing yeast in alcoholic fermentation can effectively improve the flavor and quality of apple cider vinegar; more details about the mixed culture need to be investigated in the future.

Keywords Mixed cultures · Organic acid · Free amino acids · Volatile flavor · Odor activity value

Introduction

Apple vinegar is produced from apples or apple-processing byproducts by two-step fermentation processes which are alcoholic fermentation and acetic acid fermentation. Therefore, it is rich in nutrient and has unique flavor of apple cider and vinegar (Štornik et al. 2016). In addition, apple cider vinegar is abundant in amino acids, esters, and other nutrients (Chen et al. 2017). It also has a variety of functional substances which have beneficial effects on antioxidant capacity, beauty, and immunity (Wang et al. 2017; Xie et al. 2017; Wu et al. 2017). In recent years, researchers have been

focused on the flavor and functional substances of fruit vinegar, because more and more people are interested in using fruit vinegar to keep healthy. Aroma components are important indicators of the quality of apple vinegar. The flavor characteristics are determined by the type, quantity, flavor threshold, and interactions between components (Callejon et al. 2008; Xiao et al. 2011).

As known, the aroma of apple cider fermented by a single strain is weak, whereas mixed fermentation of various strains can increase the complexity of the aroma and increase the variety and concentration of volatile aroma components (Ye 2015). *Saccharomyces cerevisiae* and non-*S. cerevisiae* strains were usually used in mixed fermentation to enhance the flavor of apple cider. Non-*S. cerevisiae* secretes a variety of enzymes, such as glycosidase, pectinase, protease, and lipolytic enzymes, and a variety of volatile aromatic substances is produced, such as esters and alcohols; as a result, the flavor of apple wine will be varied and the quality of the final product apple cider vinegar will be improved (Ciani and Comitini 2011; Bedriñana et al. 2012).

In our previous work, we found that the *Candida tropicalis* (used to produce alcohol in Suzhou Wanshen Flour

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Products Co., LTD) could use the apple and apple byproducts such as peel and pomace to produce high concentration of alcohol, so we tried to use *C. tropicalis* in the alcoholic fermentation during the apple vinegar production. We found that the flavor of apple vinegar was poor with the pure culture of *C. tropicalis* in the alcoholic fermentation and *Acetobacter pasteurianus* JST-S in the acetic acid fermentation. So we wanted to use aromatizing yeast to improve the flavor of the apple vinegar. In this study, to clarify the effects of the mixed alcoholic fermentation on the quality of apple vinegar, *Candida tropicalis* and aromatizing yeast were used in mixed alcoholic fermentation. And the differences in physicochemical and sensory characteristics of apple vinegar in mixed cultures and pure culture were determined.

Materials and methods

Materials and medium

Apples were purchased from the supermarket at Hefei University of Technology. Aromatizing yeast (dry yeast powder) was purchased from Angle Yeast Company Ltd. (China). *C. tropicalis* (CICC17779) and *Acetobacter pasteurianus* JST-S were obtained from the Key Laboratory for Agricultural Products Processing of Anhui Province (China).

YPD medium (yeast extract: 10 g/L, glucose: 20 g/L, peptone: 20 g/L) was used for *C. tropicalis* and aromatizing yeast. The culture medium (YM) contained glucose (11 g/L), yeast extract (11 g/L), KH_2PO_4 (3.3 g/L), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.1 g/L) which were used for *A. pasteurianus* JST-S. The medium was sterilized at 1×10^5 Pa for 20 min, and 2% (v/v) ethanol was added to YM before culture.

Production of apple cider and apple vinegar

Fresh apples were selected, cleaned, cut into small pieces, immersed in a citric acid solution with a pH of 4.0 for 5 min and mashed to apple pulp. 250 mg of pectinase was added in 1 L mashed apple pulp. The mixture named apple juice was enzymatic hydrolysed at 45 °C for 2 h and inactivated at 85 °C for 10 min. The sugar concentration in the apple juice was adjusted to 16 Brix (pH 4.0) using glucose. In the alcoholic fermentation, 100 mL *C. tropicalis* ($\text{OD}_{600}=0.8$, cultured for about 24 h in YPD medium at 28 °C) for 1 L apple juice was used as the inoculation for pure culture, while 67 mL *C. tropicalis* and 33 mL aromatizing yeast ($\text{OD}_{600}=0.8$, cultured for about 24 h in YPD medium at 28 °C, respectively, at a ratio of 2:1) for 1 L apple juice were used for mixed cultures. The alcoholic fermentation lasted for 5 days at 28 °C. Then, the supernatants were obtained by centrifugation at $8000 \times g$ for 10 min, named mixed-culture apple cider (MCAC) and pure-culture apple cider

(PCAC), respectively. The alcohol concentration in apple cider was adjusted to 6% (v/v) using ethanol or apple juice. In the next stage, 100 mL *Acetobacter pasteurianus* JST-S ($\text{OD}_{600}=0.8$, cultured for about 24 h in YM medium at 32 °C and 180 rpm) for 1 L apple cider (MCAC or PCAC) was inoculated at 32 °C in acetic acid fermentation. After culturing at 180 rpm and 32 °C for 5 days, the broths were centrifuged at $8000 \times g$ for 10 min, and supernatants were collected as mixed- or pure-culture apple vinegar (MCAV and PCAV, respectively).

Determination of the pH, alcohol, reducing sugar, and cell growth

The pH was measured using a pH meter (Dapu Instrument Co., Ltd., Shanghai, China). Alcohol concentration was measured by gas chromatography (GC) as previously described (Wang et al. 2013a, b). The reducing sugar concentration was determined by 3,5-dinitrosalicylic acid assays (Cao et al. 2013). The number of colony forming units (CFU) per mL was counted according to the dilution factor and the number of colonies on the plates (range 30–300 colonies) after incubation for 3 days at 28 °C for aromatizing yeast and *C. tropicalis*.

Determination of organic acid contents

The modified method (Gao et al. 2004) was used to determine the concentration of organic acids, in which high-performance liquid chromatography (Waters 2695, USA) with an Ultimate LP-C18 column (4.6 mm $\times$ 250 mm, 5 μm) was used. Different concentrations of oxalic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid, fumaric acid, and succinic acid were prepared. The samples (including apple juice, apple cider and apple vinegar) were centrifuged at $8000 \times g$ for 10 min, and the supernatants were collected, diluted fivefold with ddH₂O, and passed through a 0.45- μm aqueous-phase filter. The chromatographic conditions were as follows: column temperature, 30 °C; flow rate, 0.6 mL/min; injection volume, 15 μL ; wavelength, 210 nm. The mobile phase was 0.01 M K_2HPO_4 buffer solution (pH adjusted to 2.50 with phosphoric acid): methanol (97:3). All organic acids were recorded on a computer-based data system. Compounds were quantified by comparing the peak area to the standard solutions.

Determination of free amino acids

Free amino acid contents in vinegar samples were determined by Thermo Fisher U3000 liquid chromatography (Shanghai Lijing Scientific Instrument Co., Ltd., China). The following chromatographic conditions were used for the determination of PCAV and MCAV: mobile phase A, 0.1 M

sodium acetate solution:acetonitrile (93:7); mobile phase B, acetonitrile:water (8:2); column, octadecylsilane-bonded silica as a filler (4.6×250 mm, $5 \mu\text{m}$); flow rate, 1.0 mL/min ; column temperature, 40°C ; injection volume, $10 \mu\text{L}$; wavelength, 254 nm . Amino acids were quantified using a calibration curve of authentic standards and expressed as mg/L .

Determination of volatile flavor substances

Determination of volatile flavor substances in apple cider vinegar was performed using GC/mass spectrometry (MS) combined with headspace solid-phase microextraction. 5 mL of sample, $20 \mu\text{L}$ internal standard of 3-octanol (8.22 mg/L), and 1 g NaCl were placed in a 15-mL headspace vial, it was then agitated and equilibrated at 30°C for 30 min to extract the volatiles using a $75\text{-}\mu\text{m}$ divinylbenzene/carboxen/polydimethylsiloxane fiber (Beijing Kanglin Technology Co., Ltd., China). The analyses were performed with a Shimadzu gas chromatograph (Shimadzu Corporation, Japan) on a DB-5MS column ($30 \text{ m} \times 250 \mu\text{m}$, $0.25 \mu\text{m}$). The chromatographic conditions were as follows: inlet temperature, 250°C ; temperature program was 50°C for 1 min , increased to 80°C at a rate of 5°C , then further increased from 80°C to 220°C at a rate of 10°C/min , and maintained at 220°C for 2 min ; carrier gas, high-purity He; column temperature, 50°C ; gas flow rate, 10 mL/min ; no split injection. Mass spectra were generated in the electron impact mode (230°C) at 70 eV . The scanning range was $40\text{--}400 \text{ amu}$. Each aroma compound was quantified using the area relative to that of the internal standard of each compound. The relative concentration of an odorant was calculated as the peak area of the compound multiplied by the concentration of internal standard peak area. Each sample was analyzed in triplicates.

Sensory evaluation

To evaluate the sensory quality of apple vinegar, a 9-point pleasure scale was used, and 20 trained experts (10 women and 10 men, ages 20–40) recruited from Hefei University of Technology and formed a sensory evaluation group to test taste and acidity. Sensory evaluation of vinegar samples was performed to assess taste, aroma, flavor, color, and overall acceptability. The specific method was described in a previous study (Chen et al. 2017b). The panelists scored each attribute on a linear scale from 0 (lowest intensity) to 9 (highest intensity).

Data analysis

Origin 8.5 and Excel 2007 were used to complete data analysis. Analysis of variance was performed using SPSS software version 20.0 (SPSS-IBM Chicago, IL, USA). One-way

analysis of variance and Duncan's multiple range tests were used to determine significant difference ($p < 0.05$).

Results and discussion

Effects of mixed cultures on alcoholic fermentation

As shown in Fig. 1a, the reducing sugar of the mixed fermentation was reduced faster than that the pure culture. In the end of alcoholic fermentation, the pH values of the mixed cultures and pure culture reached 3.74 and 3.82, respectively, Fig. 1b. In the beginning of alcoholic fermentation, the alcohol concentration in mixed cultures was lower than that in pure culture. However, on the fifth day, the alcohol concentration in mixed cultures was higher (6.56%) than that in pure culture, Fig. 1c; the conversion rate of ethanol is 93.71% according to the formulation in the recent report (Sun 2015). *C. tropicalis* and aromatizing yeast were inoculated in apple juice at a dose of 10^6 CFU/mL for mixed cultures, and pure culture was carried out with *C. tropicalis* as a control, Fig. 1d. Figure 1 also shows the growth of yeast in two fermentation modes. Notably, the concentration of *C. tropicalis* in pure culture broth reached the highest ($1.3 \times 10^8 \text{ CFU/mL}$) on the second day, and it was remained above $3.25 \times 10^7 \text{ CFU/mL}$ during alcoholic fermentation. However, in mixed cultures, the highest concentration of the bacteria was $1.0 \times 10^8 \text{ CFU/mL}$, which was lower than that in pure culture, indicating that the presence of aromatizing yeast had a slight inhibitory effect on the growth of *C. tropicalis*. Yeast including *S. cerevisiae* has been reported to inhibit non-*S. cerevisiae* via nutritional competition, oxygen, quorum sensing, and inhibition of harmful substances (Ye et al. 2014). This result was the same according to that report.

Effects of mixed cultures on organic acids

The taste of vinegar is formed by many flavor substances and organic acids, including volatile and nonvolatile acids. Changes in eight main organic acids during alcoholic and acetic acid fermentation are presented in Table 1. The total acid concentration of MCAC (9.03 g/L) was 1.16 times higher than that of PCAC (7.78 g/L). The concentration of malic acid was the highest in apple juice and it was significantly decreased during the acetic acid fermentation. Malic acid has a strong acidity, and high malic acid concentration will result in a sour taste (Wang 2008). Moreover, the aromatizing yeast present in mixed cultures could metabolize malic acid to an accessible carbon source or lactic acid (Ye 2015).

Succinic acid is an important metabolite of yeast. The concentration of succinic acid in MCAV (0.60 g/L) was 1.4 times higher than that in PCAV (0.43 g/L). There are

Fig. 1 Reducing sugar concentration (a), pH (b), alcohol yield (c), and growth curves (d) during alcoholic fermentation by pure culture and mixed cultures. Filled diamond and filled triangle represent pure culture and mixed cultures, respectively

Table 1 Contents of organic acids in apple juice, apple cider, and apple cider vinegar

	Apple juice (g/L)	Apple cider (g/L)		Apple cider vinegar (g/L)	
		Pure culture	Mixed cultures	Pure culture	Mixed cultures
Oxalic acid	0.14 ± 0.08 ^a	0.04 ± 0.01 ^b	0.06 ± 0.03 ^b	0.03 ± 0.01 ^b	0.05 ± 0.02 ^b
Tartaric acid	0.08 ± 0.01 ^a	0.25 ± 0.01 ^b	0.29 ± 0.01 ^b	0.33 ± 0.01 ^b	0.68 ± 0.01 ^b
Malic acid	3.96 ± 0.18 ^a	3.49 ± 0.07 ^b	3.53 ± 0.02 ^b	2.67 ± 0.01 ^b	2.60 ± 0.05 ^b
Lactic acid	– ^a	3.17 ± 0.03 ^b	3.50 ± 0.01 ^b	2.77 ± 0.02 ^b	2.56 ± 0.01 ^b
Acetic acid	0.07 ± 0.01 ^a	0.23 ± 0.01 ^a	0.34 ± 0.01 ^a	45.69 ± 1.30 ^b	46.63 ± 1.30 ^b
Citric acid	0.46 ± 0.15 ^a	0.58 ± 0.01 ^b	0.59 ± 0.02 ^b	0.45 ± 0.02 ^b	0.59 ± 0.01 ^b
Succinic acid	0.24 ± 0.01 ^a	0.22 ± 0.01 ^a	0.26 ± 0.12 ^a	0.43 ± 0.01 ^b	0.60 ± 0.01 ^b
Fumaric acid	0.05 ± 0.01 ^a	0.25 ± 0.01 ^b	0.46 ± 0.01 ^b	0.61 ± 0.02 ^b	0.59 ± 0.01 ^b
Total acidity	5.00 ± 0.44 ^a	7.78 ± 0.15 ^b	9.03 ± 0.22 ^b	52.98 ± 1.41 ^b	54.30 ± 1.42 ^b

Values are expressed as averages ($n=3$) ± standard deviations. Different letters in the same row indicate significant differences ($p < 0.05$)

– Not detected

no significant differences between acetic acid concentrations in MCAV (46.63 g/L) and that in PCAV (45.69 g/L); these results were the same with other reports (Qi et al. 2017; Kong et al. 2017). Lactic acid is produced in the end of glycolysis in yeast, and the soft sour flavor of lactic acid enhances the taste of apple vinegar. There are no data for lactic acid, which means no lactic acid was detected in apple juice. For alcoholic fermentation, the apple juice

turned into apple cider by *C. tropicalis* and aromatizing yeast. In mixed cultures, the lactic acid concentration increased from 0 to 3.50 g/L, while it was 3.17 g/L in pure culture during alcoholic fermentation. Lactic acid was used by acetic acid bacteria, so the lactic acid concentration reduced to 2.56 g/L in mixed cultures, while it was 2.77 g/L in pure culture in acetic acid fermentation.

Effects of mixed cultures on free amino acids

Free amino acids are important contributors to the unique taste of vinegar (Wang et al. 2013a, b). According to taste characteristics, free amino acids can be classified as umami, sweet, bitter, and tasteless. In this study, 17 free amino acids were quantitatively analyzed in MCAV and PCAV (Table 2). The total content of amino acids in MCAV was higher than that in PCAV, which reached 3198.68 and 3127.02 mg/L, respectively. The levels of Asp and Glu were significantly higher in MCAV (880 and 356.71 mg/L) than those in PCAV ($p < 0.05$). The concentration of Ala, Thr, Ser, and Pro, which were associated with sweet flavor, was significantly higher in MCAV than in PCAV ($p < 0.05$). However, there were no significant differences in the total bitter taste of free amino acids and total essential free amino acids in the two vinegars (Table 2). Therefore, the mixed cultures increased the total content of free amino acids in apple cider vinegar, particularly umami and sweet free amino acids; as a result, the taste of apple vinegar was improved.

Effects of mixed cultures on volatile compounds

In total, 31 and 34 kinds of volatile compounds (Table 3), including alcohols, acids, aldehydes, ketones, esters, heterocycles, and phenols, were found in PCAV and MCAV. During the acetic acid fermentation process, most of the flavor compounds in MCAV increased with different degrees, and no obviously unpleasant volatile substances were found.

As a marker for the flavor contribution of vinegar, esters play an essential role, particularly ethyl acetate (Câmara et al. 2006). The total ester concentration of MCAV (282.36 mg/L) was 1.1× higher than that of PCAV (255.06 mg/L). In addition, the contents of ethyl acetate and hexyl acetate were significantly higher in MCAV than that in PCAV ($p < 0.05$). The concentration of isoamyl acetate in MCAV was 13.36 mg/L, which was significantly higher than that in PCAV ($p < 0.05$). Isoamyl acetate mainly provides sweetness, fruitiness, and a banana-like odor. Synthetic esters in *C. tropicalis* are produced by the reaction of alcohol and acetyl-CoA under the catalysis of alcohol acetyltransferase (Li et al. 2011); the pathway for the synthesis of

Table 2 Effects of apple cider vinegar on free amino acids

Free amino acids	Taste attributes	Concentration (mg/L)	
		Pure culture	Mixed cultures
Glu	Umami	344.69 ± 0.30 ^a	356.71 ± 1.84 ^b
Asp	Umami	870.00 ± 1.00 ^a	880.00 ± 1.00 ^b
	Total umami	1214.69 ± 1.30 ^a	1236.71 ± 2.84 ^b
Gly	Sweet	74.30 ± 0.53 ^a	76.83 ± 0.26 ^b
Thr ^a	Sweet	82.88 ± 0.41 ^a	94.36 ± 0.45 ^b
Ala	Sweet	160.50 ± 0.83 ^a	165.90 ± 0.58 ^b
Ser	Sweet	189.33 ± 0.70 ^a	200.60 ± 0.80 ^b
Pro	Sweet	313.36 ± 0.50 ^a	320.56 ± 1.10 ^a
	Total sweet	820.37 ± 2.97 ^a	858.25 ± 3.19 ^b
Met ^a	Bitter	48.56 ± 1.00 ^a	49.01 ± 1.00 ^b
Lys ^a	Bitter	105.66 ± 1.20 ^a	100.76 ± 1.00 ^b
Leu ^a	Bitter	120.55 ± 1.00 ^a	125.63 ± 0.90 ^b
Ile ^a	Bitter	60.25 ± 1.50 ^a	56.38 ± 0.90 ^b
Phe ^a	Bitter	69.56 ± 1.00 ^a	65.28 ± 1.20 ^b
His ^a	Bitter	51.38 ± 1.30 ^a	50.46 ± 1.00 ^a
Arg ^a	Bitter	489.66 ± 2.60 ^a	500.16 ± 2.10 ^b
Tyr	Bitter	52.89 ± 1.20 ^a	54.63 ± 0.90 ^b
Val ^a	Bitter	78.56 ± 0.99 ^a	81.33 ± 0.80 ^b
	Total bitter	1077.07 ± 11.79 ^a	1083.64 ± 9.80 ^a
Cys	Tasteless	14.89 ± 0.80 ^a	20.08 ± 0.90 ^b
	Total tasteless	14.89 ± 0.80 ^a	20.08 ± 0.90 ^b
	Total essential amino acids	1028.50 ± 11.00 ^a	1042.04 ± 9.35 ^a
	Total free amino acid	3127.02 ± 16.86 ^a	3198.68 ± 16.73 ^b

Values are expressed as averages ($n=3$) ± standard deviations. Different letters in the same row indicate significant differences ($p < 0.05$)

– Not detected

Table 3 Concentrations of volatile compounds in PCAV and MCAV

Compounds	Concentration (mg/L) ^A		Method of identification ^B
	Pure culture	Mixed cultures	
Esters			
Ethyl acetate	119.00 ± 2.50 ^a	136.00 ± 3.10 ^b	ABC
Phenethyl acetate	55.39 ± 2.64 ^a	54.39 ± 3.11 ^a	ABC
Ethyl decanoate	1.87 ± 0.01 ^a	1.65 ± 0.01 ^b	AB
Hexyl acetate	14.30 ± 0.15 ^a	17.70 ± 0.20 ^b	AB
Ethyl benzoate	0.56 ± 0.02 ^a	1.37 ± 0.07 ^b	ABC
Isoamyl acetate	10.25 ± 0.04 ^a	13.36 ± 0.20 ^b	ABC
Ethyl butyrate	5.01 ± 0.10 ^a	5.80 ± 0.44 ^a	AB
Ethyl caproate	0.35 ± 0.01 ^a	0.15 ± 0.01 ^b	AB
Ethyl isovalerate	— ^a	2.99 ± 0.01 ^b	ABC
Isobutyl acetate	48.33 ± 1.17 ^a	49.10 ± 1.93 ^b	AB
Total content	255.06 ± 6.64 ^a	282.36 ± 9.08 ^b	
Alcohol			
Isobutyl alcohol	0.32 ± 0.01 ^a	0.40 ± 0.01 ^b	ABC
2-Methyl-1-butanol	1.55 ± 0.15 ^a	1.18 ± 0.10 ^b	ABC
2,3-Butanediol	5.88 ± 0.17 ^a	6.78 ± 0.12 ^b	AB
3-Methyl-1-butanol	4.88 ± 0.06 ^a	4.49 ± 0.25 ^b	AB
Phenethyl alcohol	165.55 ± 2.30 ^a	239.99 ± 2.80 ^b	ABC
Nerolidol	— ^a	0.60 ± 0.05 ^b	ABC
Dodecanol	0.59 ± 0.02 ^a	0.78 ± 0.01 ^a	ABC
Total content	178.77 ± 2.71 ^a	254.22 ± 3.34 ^b	
Acids			
Acetic acid	175.28 ± 2.21 ^a	191.44 ± 4.25 ^b	ABC
2-Methylpropionic acid	0.19 ± 0.01 ^a	0.28 ± 0.03 ^b	ABC
Isovaleric acid	0.29 ± 0.01 ^a	0.32 ± 0.01 ^a	AB
3-Methylbutanoic acid	13.60 ± 0.15 ^a	12.05 ± 0.08 ^b	ABC
Decanoic acid	9.89 ± 0.44 ^a	8.95 ± 0.38 ^b	ABC
Benzoic acid	16.20 ± 0.24 ^a	28.37 ± 0.37 ^b	ABC
Caproic acid	4.16 ± 0.15 ^a	4.88 ± 0.21 ^b	AB
Octanoic acid	3.29 ± 0.10 ^a	2.88 ± 0.30 ^b	AB
Total content	222.90 ± 3.31 ^a	249.17 ± 5.63 ^b	
Aldehydes			
Benzaldehyde	19.38 ± 1.31 ^a	17.28 ± 0.99 ^b	AB
Phenylacetaldehyde	4.67 ± 0.28 ^a	5.89 ± 0.22 ^b	ABC
Nonanal	— ^a	1.49 ^b	AB
Furfural	22.36 ± 0.18 ^a	25.38 ± 0.23 ^b	ABC
Total content	46.41 ± 1.77 ^a	50.04 ± 1.44 ^a	
Ketones			
Acetoin	15.56 ± 0.22 ^a	24.39 ± 0.33 ^b	ABC
Total content	15.56 ± 0.22 ^a	24.39 ± 0.33 ^b	
Phenols			
3,5-Ditert butyl phenol	18.90 ± 0.23 ^a	25.39 ± 0.50 ^b	ABC
2,4-Ditert butyl phenol	19.36 ± 0.57 ^a	22.10 ± 0.50 ^b	ABC

Table 3 (continued)

Compounds	Concentration (mg/L) ^A		Method of identification ^B
	Pure culture	Mixed cultures	
Total content	38.26 ± 0.80 ^a	47.49 ± 1.00 ^b	
Pyrazines			
2,3-Dimethylpyrazines	— ^a	3.39 ± 0.23 ^b	AB
2,3,5-Trimethylpyrazine	3.11 ± 0.06 ^a	5.23 ± 0.16 ^b	ABC
Total content	3.11 ± 0.06 ^a	8.62 ± 0.39 ^b	

— not detected

^AValues are expressed as averages ($n=3$) ± standard deviations. Different letters in the same row indicate significant differences ($p < 0.05$)

^BMethod of identification: (A) by comparison of the mass spectrum with the NIST/Wiley mass spectral library; (B) by comparison of RI (Kovats indices) with RI of an authentic compound; and (C), by comparison of retention time and spectrum of an authentic compound

esters from aromatizing yeast may be different from that of *C. tropicalis*; it needed more research in the future.

Higher alcohol compounds are another important type of volatile aroma substance in apple vinegar. The total alcohol and phenylethyl alcohol concentrations in MCAV were significantly higher than those in PCAV ($p < 0.05$). Phenylethanol, 2,3-butanediol, and 3-methyl-1-propanol are the main alcohols detected in apple vinegar. Among these compounds, phenylethyl alcohol is an important aroma component in apple vinegar and is associated with a rosy scent (Duan et al. 2017).

Moreover, this compound had the highest concentration (239.99 mg/L) in MCAV, which was significantly higher than that in PCAV ($p < 0.05$). This indicated that the aromatizing yeast affected the synthesis of phenylethyl alcohol. This was consistent with the previous report (Comitini et al. 2011). Nerolidol is a terpene alcohol compound that imparts floral, grassy, and woody notes to apple cider vinegar (Zhang et al. 2015). The concentration of nerolidol in MCAV was 0.6 mg/L, but this compound was not detected in PCAV.

The acids in MCAV were increased by varying degrees compared with those in PCAV, except for 3-methylbutyric acid, citric acid, and octanoic acid. In this experiment, acetic acid produced by MCAV (191.44 mg/L) was obviously higher than that in PCAV (175.28 mg/L) and this did not cause a poor flavor to the apple vinegar. In addition to acetic acid and caproic acid, some medium-chain fatty acids, such as caprylic acid and capric acid, which are products of fat oxidation during alcoholic fermentation (Dittrich et al. 2010), were also identified. Excess fatty acids can impart a fatty, soapy, sour, or even bad odor to the fermented vinegar (Ye et al. 2014). The concentration of octanoic acid in MCAV and PCAV reached 3.29 and 2.88 mg/L, respectively.

Thus, the concentration of octanoic acid in MCAV increased compared with that in PCAV.

Numerous other volatile compounds were detected after acetic acid fermentation, including aldehydes, ketones, and phenolic substances; although these compounds are typically present at low levels, they are important for determining the aroma of apple vinegar and for giving vinegar its characteristic flavor. No furfural was produced in PCAV, and the furfural concentration in MCAV was only 1.49 mg/L. Furfural has a strong fruity and floral aroma, which can have a positive effect on the aroma of apple cider vinegar. These findings supported the interaction between *C. tropicalis* and aromatizing yeast again, demonstrating that the mixed cultures produced new substances that not found in pure culture. That is to say, mixed cultures in alcoholic fermentation were considered as an effective method to enhance volatile compounds, particularly alcohols and esters.

Odor activity value (OAV) calculation and aroma wheel

To assess the contribution of each compound to the aroma of apple vinegar, we assessed OAVs (Table 4), which were obtained by dividing the content of the compound in the matrix by the odor threshold (Birch et al. 2013). In MCAV,

phenylacetaldehyde (floral) had the highest OAV (1472.5), followed by ethyl isovalerate (fruity, 747.5), phenylethyl acetate (fruity, 618.07), phenylethyl alcohol (floral, 615.36), and benzaldehyde (nutty, 109.36). In PCAV, phenylacetaldehyde had the highest OAV (floral, 1167.5), followed by phenylethyl acetate (fruity, 629.43), phenylethyl alcohol (floral, 424.5), isoamyl acetate (fruity, 341.66), and benzaldehyde (nutty, 122.66). In summary, fruitiness was the main aroma component in MCAV and was more intense than that in PCAV.

To associate volatile components with aroma descriptors, we grouped aromatic compounds with similar sensory descriptors into classes (Zhu et al. 2016). The aromatic series used in this study included the standard sensory descriptors used in the aromatic wheel. The wheel breaks down vinegar aromas into six basic categories (floral, nutty, fruity, roasty, fatty, and woody). Then, with reference to the six defined standardized sensory descriptor categories, the OAV of each type of sensory descriptor was calculated by adding the OAVs of all the compounds forming the class. This also included compounds, which OAV was less than 1, because subthreshold compounds may also contribute to aroma characteristics through additive effects with compounds having similar structures or odors (Francis and Newton 2005). Finally, we plotted the results in a radar chart

Table 4 Odor activity values (OAVs) of aroma-active compounds in apple vinegar

Compounds	Odor quality	OT ^a (mg/L)	Ref.	OAV ^b		Sensory descriptors ^c
				Pure culture	Mixed cultures	
Ethyl acetate	Fruity, sweet	33	f	3.60	4.10	3
Phenylethyl acetate	Fruity, sweet	0.09	e	629.40	618.07	3
Ethyl benzoate	Floral	1.43	d	0.35	0.96	3
Isoamyl acetate	Fruity, sweet	0.03	f	341.66	445.30	3
Ethyl isovalerate	Fruity, sweet	0.004	e	–	747.50	3
Ethyl caproate	Fruity, wine	0.014	f	24.78	10.71	3
Acetic acid	Vinegar, fatty	34	d	5.15	5.63	5
Decanoic acid	Rancid	1.1	e	8.99	8.13	5
Octanoic acid	Rancid	0.50	g	6.58	5.76	5
Phenethyl alcohol	Floral, sweet	0.39	e	424.50	615.36	1
2-Methylbutanoic acid	Rancid	0.4	d	34.00	30.13	5
Benzaldehyde	Almond	0.158	d	122.66	109.36	2
Phenylacetaldehyde	Floral, sweet	0.004	d	1167.50	1472.50	1
Acetoin	Mushroom	8.8	d	1.77	2.77	5
2,3-Dimethyl-pyrazines	Roasted	7.7	d	–	0.44	4

^aOdor threshold as reported in the literature reference (Ref.)

^bOdor activity value was defined as a ratio between odor concentration and odor threshold

^cEach compound was attributed to 1 or more of the following six classes of sensory descriptors: (1) floral, (2) nutty, (3) fruity, (4) roasty, (5) fatty, (6) woody

^dZhu et al. (2016)

^eChen et al. (2017a, b)

^fVilanova et al. (2007)

^gSun et al. (2014)

called the aromatic wheel, which is based on mixed chemical/sensory parameters. As shown in Fig. 2, fruity, floral, and fatty were considered the most important aroma attributes in the sensory evaluation of apple vinegar. Compared with PCAV, MCAV showed significantly higher floral and fruit characteristics ($p < 0.05$).

Principal component analysis (PCA)

PCA was applied to investigate the compounds and to investigate possible correlations between PACV and MCAV. PCA was performed on 16 key volatile compounds detected in PCAV and MCAV. Two groups A and B are clearly distinguished for mixed and pure fermentation vinegar, respectively (Fig. 3 PC1 and PC2). PC1 was positively correlated with acetic acid, ethyl acetate, phenylethyl alcohol, and acetoin, whereas PC2 was positively correlated with phenylacetate and other volatile compounds, such as citric acid. The total of PC1 and PC2 explained a total variance of 98.61% (83.21% and 15.4%, respectively) for 16 key volatile compounds detected in PCAV and MCAV. In contrast, ethyl hexanoate, caprylic acid, and benzaldehyde showed negative correlations (Fig. 3). The highest score for MCAV was the integrative score of 1.06, whereas PCAV had the lowest score (data not shown). These results indicated that mixed cultures improved the quality and type of apple vinegar aroma.

Sensory evaluation

Sensory evaluation is often used to estimate the quality of vinegar. The scores for color, aroma, taste, mouth-feel, flavor, acidity, and overall acceptance of PCAV and MCAV are shown in Fig. 4. Notably, MCAV scores were higher than PCAV scores. With regard to flavor acceptance and mouth-feel, MCAV scored higher than PCAV, and MCAV had a

Fig. 2 Aroma wheel of MCAV and PCAV based on the odor activity values of each class of sensory descriptors (* $p < 0.05$)

Fig. 3 PCA of PCAV and MCAV

softer palate and stronger aroma, which may be attributed to more esters produced during mixed cultures. The taste and overall acceptance of MCAV was significantly higher than that of PCAV, which was rich in organic and free amino acids, affecting the sourness and sweetness of apple cider vinegar.

Conclusion

In this study, *C. tropicalis* and aromatizing yeast were used to investigate the effects of mixed cultures on the quality of apple vinegar. The results showed that mixed cultures increased the flavor and quality of aroma substances. Our findings provide a scientific basis and effective method for the production of high-quality apple vinegar, thereby improving economic benefits. This study was performed at laboratory scale; therefore, further studies are needed to determine how to carry on in the pilot scale and industrial-scale production.

Fig. 4 Sensory evaluation of PCAV and MCAV (* $p < 0.05$)

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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