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The Effect of Adding Different Amounts of *Lachancea thermotolerans* together with *Saccharomyces cerevisiae* on Simultaneous Fermentation in Emir Wine Production

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ABSTRACT

Our current research aimed to investigate the impacts of the use of indigenous yeast *Saccharomyces cerevisiae* with different amounts of *Lachancea thermotolerans* yeasts in mixed culture on the general composition, aroma compounds, sensory analysis and yeast growth of cv. Emir wine. The utilization of *L. thermotolerans* in mixed cultures reduced the total acidity of wines from 5.40 to 5.19 g/L (as tartaric acid). The acidity of high acid grapes musts obtained from various viticulture areas can be relatively decreased in wine production. In addition, there may be a slight decrease in the amount of ethyl alcohol. On the other hand, increasing the inoculum level of *L. thermotolerans* led to an increase in the amount of higher alcohols. However, the concentration of esters declined with the higher inoculum levels. According to the sensory evaluation, the most preferred wine was the one obtained with co-inoculation of *S. cerevisiae* and *L. thermotolerans* strains at the level of 5×10^6 and 1×10^8 cells/mL, respectively. As a result, it can be said that the use of *L. thermotolerans* yeast in different inoculum levels has a positive effect on wine fermentation.

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Introduction

Grapes (*Vitis* spp.) are commercially cultivated all over the world for table grapes and wine-making. Yeast cells, Saccharomyces spp. and/or non-Saccharomyces spp., originating from the grape surface and inoculated as starters play a major act in vinification (Marzano et al., 2016). Traditionally, wines are spontaneously obtained by the action of indigenous yeast cells present on the surface of raw materials and winery types of equipment. Currently, the majority of wines are manufactured by commercial yeasts, primarily *Saccharomyces*, (S.), *cerevisiae* species. These yeasts lead to the control of alcoholic fermentation and result in a wine with a pleasant taste and flavour (Querol et al., 1992; Pretorius, 2000; Hirst and Richter, 2016).

Türkiye has the fourth largest vineyard in the world. It is the sixth largest grape producer with 3,933 million tonnes. Up to 1-2% of Türkiye's total grape harvest is used for winemaking. Turkish types of wine, considered one of the peak wine manufacturers in the world, was produced in 2014 with a total amount of 44.707,000 L (FAO, 2020).

Cv. Emir is one of the main varieties of grapes grown in Türkiye. It is known as a major variety in local wine-growing in Türkiye. It accounts for about 25% of the region's total vineyards (Cabaroglu et al., 1997; Unal and Sener, 2016). The Nevşehir-Ürgüp region is the most widely used viticulture region in Türkiye. The Emir grape grown in this region is medium in size; yellowish, green and thin crustacean. It is a variety that is used to make excellent white wine (Cabaroglu, 1995).

S. cerevisiae (*SC*) is the main wine yeast for the conversion of grape or other fruit juices/musts into wine, although numerous other varieties grow during fermentation and can do extremely dynamic steps in the process (Romano et al., 2019). *SC* is the most common strain in winery equipment, accounting for maybe just 30-40 per cent of the yeast population. However other strains of yeast that are non-*Saccharomyces* can occupy the surface and equipment of wineries (Bokulich et al., 2013). In addition to wine yeast, non-Saccharomyces yeasts are an important new practice in different types of winemaking (Roudil et al., 2020). These are some non-Sacharomyces yeast such as *Torulaspora delbrueckii, Metschnikowia pulcherrima* and *Lachancea thermotolerans* (*LT*). These yeasts are commercially available on the market as a starter culture. These yeasts can contribute to improving the characteristics of wines (Jolly et al., 2014). *LT* can improve wines by the partial transformation of sugar into lactic acid throughout ethyl alcohol fermentation (Hranilovic et al., 2021). During winemaking, LT yeast can form up to 10% (v/v) alcohol. *L. thermotolerans* produces high amounts of lactic acid from fermentable sugars, as well as low amounts of volatile acid. Therefore, *LT* can be used to increase acidity in low acid musts (Kapsopoulou et al., 2007; Hranilovic et al., 2018). Acidity has a significant effect on the taste and durability of wines. The acidity of grape varieties can be low, especially in regions with climatic conditions above the average air temperature (Ribéreau-Gayon et al., 2000). It is reported that the total amount of acid (as tartaric acid) in wines should be over 3.5 g/L (46.6 meq/L) according to the Turkish Food Codex (2008). For this reason, the acidity of the wine can be increased by using *LT*, especially in wines obtained from low-acid wine grapes.

There are limited studies to enhance the aroma potential of local strains of *SC* and the commercial strains of *LT*. Our present study aimed to investigate the effects of the use of *SC* with different amounts of *LT* yeasts in mixed culture on the general composition, aroma compounds, sensory analysis and yeast growth of cv. Emir fermentation.

Materials and Methods

Yeast Cultures Used in the Fermentation

In this study, *SC* isolated from cv. Emir wine fermentation was used (Nurgel et al., 2005). *Lachancea thermotolerans* CBS 2860 was purchased from a culture Netherlands collection (CBS Yeast Culture Collection, Utrecht/Netherland). This yeast was held on malt extract agar. Two different types of agars called Malt extract and L-lysine were supplied from "Merck" (Germany) and "Difco" (Germany) companies, respectively. *LT* yeast obtained from culture collections in lyophilized form was activated in malt extract broth and grown on malt extract agar.

Wine Production

The white grape Emir (density, 1.084 g/mL; sugar, 20.52°Brix) used in the experiments was collected in Türkiye, Nevşehir-Ürgüp region. The experiments were carried out in the Faculty of Engineering, Çukurova University. The Emir grapes were crushed by the mill. Then, it was pressed and left to keep at 15°C for 24 hours. Fermentations were accomplished in 1L sterile Erlenmeyer flasks covered with cotton. 800 mL grape must be poured into the flasks and heat-treated in the autoclave at 105°C for 5 minutes. Fermentations were achieved at 20°C in duplicate. The fermentation process was controlled by density measurements. Yeasts were inoculated in the grape must with the orbit shaken for 48 hours at 160 rpm at 25°C. After this process, strains were separated with the help of centrifuges for 10 minutes at +4°C at 5000 rpm and counted under a microscope using methylene blue solution. Different amounts of yeast culture were added to the fermentation media (Erten and Campbell, 2001).

Inoculation strategies for types of yeast were as follows: L1; *SC* and *LT* yeasts were added into grape must at 5×10^6 and 1×10^7 cells/mL, respectively. L2; *SC* and *LT* yeasts were added into grape must at 5×10^6 and 5×10^7 cells/mL, respectively. L3; *SC* and *LT* yeasts were added into grape must at 5×10^6 and 1×10^8 cells/mL, respectively.

Enumeration of Yeast

Samples for yeast enumeration were collected under sterilized terms during fermentation. The samples in the flask were mixed before 1 mL of the sample was taken. The specimens were diluted properly in 0.25% of brine and dispersed on agar by spreading. L-lysine agar was used to count *LT* strain. The incubation of two different types of agars namely, Malt extract and L-lysine agar were done at 25°C for 4-5 days and the colony of yeasts was counted in triplicate (Fleet, 1993).

Chemical Analysis

The density determination of the samples was made with an automatic density measurement device (Mettler Toledo, Switzerland). On the other hand, the value of pH and titration acidity were analyzed as reported by Ough and Amerine (1988).

Analysis of Volatile Compounds

The determination of aroma in samples was made using GC (Gas chromatography, HP5890, Hewlett Packard and Stockport/UK). After alcohol fermentation, the alcohol content of the centrifuged wines to abolish cell residues was first reduced to four percent (v/v). Then, 5 mL of wine and 2 g of NaCl were inserted into vials that were closed. Internal standard (3-heptanon) was also used. Then, one mL liquid mix was inserted into a 60m long and 0.4m thick column (Chrompac CPWax 57CB, Netherland). The injection was carried out at 160°C. After waiting 2 minutes at 43°C, the column temperature was raised to 30°C per minute and kept on hold at this condition for 4 minutes. The column stream was split 1:1 to an FID (Flame ionization detector). The carrier gas was helium at a flow rate of 2.2 mL/min. The results of the determination of aroma compounds were made automatically with the help of the computer from the retention times with the aid of the internal standard (Erten and Campbell, 2001). Two parallel analyzes were also made for each of the wines produced in two replicates.

Sensory Analysis

Sensory analyses of produced wine were performed by 13 panellists according to the preference test. Panellists who participated in the sensory analysis were asked to rank wine samples from best to worst (Barillère and Bénard, 1986).

Statistical Analysis

The chemical composition and aroma compounds of the wines were subjected to one-way variance analysis (ANOVA). The results of the sensory analysis of wines were evaluated according to the Friedman test in the preference test. Means were compared by Duncan test statistical analysis (P<0.05) (IBM-SPSS Statistic).

Results and Discussion

Fermentation Kinetics

Sugar consumption measured by the drop in density was given in Figure 1. The use of different combinations of yeasts in wine production affected the fermentation conditions (P<0.05). The density value at the beginning of fermentation was determined as 1.084 g/mL and this value decreased throughout the fermentation. It was determined that the addition of *LT* in different amounts during fermentation increased the fermentation rate, so density results decreased faster. At the end of the 7 days of fermentation, the density value was measured as 0.9928 g/mL in the experiment L1, while the density was found as 0.9943 g/mL in experiment performed with the addition of $5x10^6$ *SC* + $1x10^8$ *LT* (L3). Morales et al. (2019) investigated the effects of co-inoculation *SC* and *LT* on grape must of the Pedro Ximénez genus on the volatile compounds of wine. They reported that when the fermentation of alcohol ended on the 11th day, the inoculation of *LT*: *SC*, at the ratio of 50:1 had the highest density, followed by *LT*: *SC*, 20:1 inoculation.

Titration Acidity

The changes in titration acidity and pH value during ethyl alcohol fermentation were given in Figure 2. As can be seen from Figure 2, the titration acidity value at the beginning of fermentation was found to be 4.22 g/L as tartaric acid. During the fermentation of different wine samples, L3 wine had the highest titration acidity value on the first day (5.84 g/L), while L3 wine had the lowest pH value on the 2nd day (pH 3.07) (P<0.05). The titration acidities of the wine samples decreased in the following fermentation days. On the last day of fermentation, the titration acidity value (as tartaric acid) of L1, L2 and L3 wine samples was found to be between 5.19 and 5.40. On the first day of fermentation, the addition of *LT* culture to the wine samples increased the total acidity. Among the three samples, L3 wine reached the highest value on the first day (P>0.05). However, in the days following the fermentation, a decrease was observed in the total acidity of the wine samples. When the reductions in the total acidity in the wine samples are compared with the initial values, it is concluded that the addition of *LT* culture increases the total acidity of the wine samples. Considering this result, it is important to add the optimum amounts of *LT* strain to the grape musts. Wine samples had pH values of between 3.27 and 3.32 on the last day of fermentation. Conversely, Petruzzi et al. (2017) suggested that the inoculation of *LT* increases the acidity and overall flavour of wines compared to pure SC yeast, and therefore recommended its use. Similarly, it is known that *LT* yeast has a positive impact on the overall quality of wine and improves acidic properties, as well as gives spicy flavours to wines (Gobbi et al., 2013; Balikci et al., 2016).

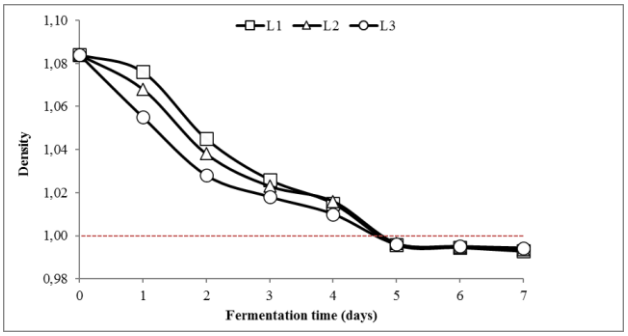

Figure 1. Change in density in wine samples during fermentation. Addition of *SC* and *LT* (L1; *SC*=5×10^6 + *LT*=1×10^7 cells/mL); addition of *SC* and *LT* (L2; *SC*=5×10^6 + *LT*=5×10^7 cells/mL); addition of *SC* and *LT* (L3; *SC*=5×10^6 + *LT*=1×10^8 cells/mL).

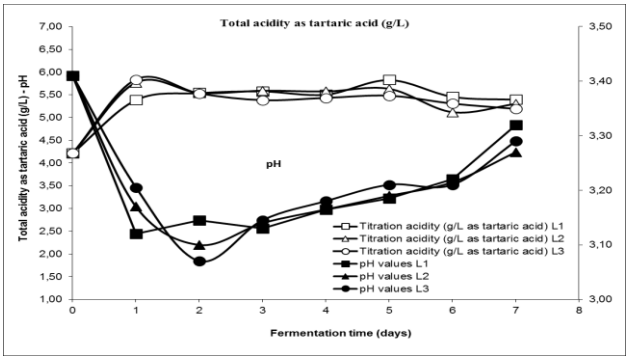

Figure 2. Change in titration acidity and pH value during ethyl alcohol fermentation. Addition of *SC* and *LT* (L1; *SC*=5×10^6 + *LT*=1×10^7 cells/mL); addition of *SC* and *LT* (L2; *SC*=5×10^6 + *LT*=5×10^7 cells/mL); addition of *SC* and *LT* (L3; *SC*=5×10^6 + *LT*=1×10^8 cells/mL).

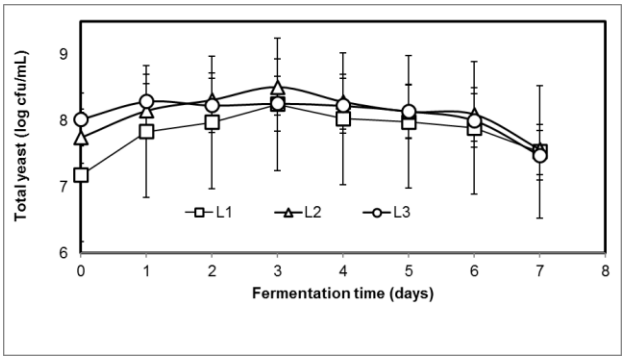

Figure 3. Total yeast growth during co-inoculation fermentation. Addition of *SC* and *LT* (L1; *SC*=5×10^6 + *LT*=1×10^7 cells/mL); addition of *SC* and *LT* (L2; *SC*=5×10^6 + *LT*=5×10^7 cells/mL); addition of *SC* and *LT* (L3; *SC*=5×10^6 + *LT*=1×10^8 cells/mL).

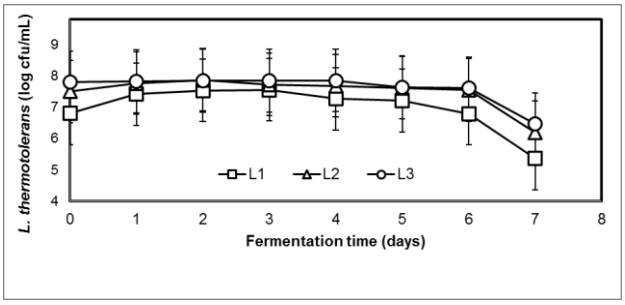

Figure 4. *Lachancea thermotolerans* yeast growth during co-inoculation fermentation. Addition of *SC* and *LT* (L1; *SC*=$5x10^6$ + *LT*=$1x10^7$ cells/mL); addition of *SC* and *LT* (L2; *SC*=$5x10^6$ + *LT*=$5x10^7$ cells/mL); addition of *SC* and *LT* (L3; *SC*=$5x10^6$ + *LT*=$1x10^8$ cells/mL).

Table 1. The main oenological properties of Emir wines fermented with distinctive yeast at the end of alcoholic fermentation

Oenological properties	L1	L2	L3
Ethanol (%v/v)	11.31±0.10^{b}	11.05±0.15^{ab}	10.60±0.19^{a}
pH	3.32±0.01^{b}	3.27±0.01^{a}	3.29±0.01^{a}
Density	*0.992	*0.994	*0.994
Total acidity as tartaric acid (g/L)	5.40±0.03^{b}	5.30±0.02^{ab}	5.19±0.07^{a}

Addition of *SC* and *LT* (L1; *SC*=5×10^{6} + *LT*=1×10^{7} cells/mL); addition of *SC* and *LT* (L2; *SC*=5×10^{6} + *LT*=5×10^{7} cells/mL); addition of *SC* and *LT* (L3; *SC*=5×10^{6} + *LT*=1×10^{8} cells/mL). The values given in the table are the mean ± standard deviations of two parallel samples. According to the statistical analysis performed, the results with different superscript letters (a, c) in each row are statistically significant (P<0.05). *: not significant.

Table 2. Volatile composition of the wine samples (mg/L)

*Aroma Compounds	L1	L2	L3
Higher alcohols			
2-Methyl butanol	32.3±0.1^{a}	34.9±0.7^{a}	39.5±4.4^{a}
3-Methyl butanol	129±0.5^{a}	133±1.8^{a}	132±10.7^{a}
Isobutanol	45.2±6.9^{a}	37.1±0.7^{a}	38±2.4^{a}
Propanol	42±2.05^{a}	57.3±0.65^{b}	60.3±2.53^{b}
Total	249±9.5	263±3.8	270±19.9
Esters			
Isobutyl acetate	0.095±0.02^{b}	0.049±0.01^{a}	0.024±0.02^{a}
Ethyl butyrate	0.22±0.05^{b}	0.158±0.03^{ab}	0.08±0.01^{a}
Ethyl hexanoate	0.348±0.11^{b}	0.228±0.02^{ab}	0.11±0.012^{a}
Ethyl octanoate	0.106±0.04^{b}	0.06±0.03^{ab}	ND
Isoamyl acetate	2.89±1.08^{b}	0.894±0.12^{a}	0.35±0.012^{a}
Ethyl acetate	42.1±6.2^{a}	42.62±2.8^{a}	42.25±2.7^{a}
Total	45.8±7.5	44.0±2.9	42.8±2.8
Aldehydes			
Acetaldehyde	29.6±27.4^{a}	13.5±4.4^{a}	14.2±7.6^{a}
2,3-Pentanedione	0.015±0.02^{a}	0.013±0.01^{a}	0.015±0.01^{a}
2,3-Butanedione	0.086±0.01^{a}	0.114±0.01^{a}	0.117±0.03^{a}
Total	29.7±27.4	13.6±4.4	14.3±7.6
General Total	324±44.4	321±11.2	327±30.3

Addition of *SC* and *LT* (L1; *SC*=5×10^{6} + *LT*=1×10^{7} cells/mL); addition of *SC* and *LT* (L2; *SC*=5×10^{6} + *LT*=5×10^{7} cells/mL); addition of *SC* and *LT* (L3; *SC*=5×10^{6} + *LT*=1×10^{8} cells/mL). * The values given in the table are the mean ± standard deviations of two parallel samples. According to the statistical analysis performed, the results with different superscript letters (a, d) in each row are statistically significant (P<0.05). ND: not detected.

Yeast Growth During Ethyl Alcohol Fermentation

The total yeast growth as a result of simultaneous inoculation of L1, L2 and L3 wine samples with *SC* and *LT* yeast cultures is shown in Figure 3. In the light of simultaneous inoculation of different amounts of *LT* yeasts with *SC*, the highest total yeast count was reached on the third day in the L1 and L2 treatments (8.25 log CFU/mL and 8.51 log CFU/mL, respectively) and on the first day in the L3 trial (8.29 log CFU/mL). In the continuation of the fermentation, the total yeast counts decreased in all experiments and were determined to be between 7.47 (L3) and 7.56 (L2) on the last day of fermentation. Benito et al. (2016) reported that *SC* and *LT* cells began to decrease rapidly with the progression of fermentation in wine samples.

The changes in the total levels of *LT* strains during alcoholic fermentation were given in Figure 4. As can be seen from Figure 4, an increment was detected in the number of *LT* yeast with the start of fermentation. Among the three wines, L3 wine reached the highest population on day 4 (8,05 log CFU/mL) compared to other wines (P<0.05). Then, a decrease was observed in *LT* numbers. At the end of 7 days of fermentation, *LT* strains were determined between 5.56 and 6.66 log CFU/mL, while the lowest value was determined in the L1 wine sample and the highest value in the L3 wine sample (P<0.05). Similarly, Mills et al. (2002) reported that although the SC strain domi×nates the fermentation, the medium *LT* strain has very good persistence.

Zohre and Erten (2002) used *SC* as pure culture in their experiments with the addition of 1x10^{6} cells/mL and reached the highest yeast population value on the 5th day during fermentation. Erten et al. (2006) in a study conducted by adding different amounts of yeast to Emir grape, the highest yeast population was reached on the 3rd day with the addition of 1×10^{6} and 1×10^{7} cells/mL yeast. Hranilovic et al. (2021) investigated the effect of *LT* yeast on the chemical composition of Merlot wines. Morales et al. (2019) investigated the effects of the addition of SC and *LT* on the grape must of the Pedro Ximénez genus on the volatile compounds of wine. They reported that during the entire fermentation process, *LT* yeast was the dominant strain. So, it could be stated that our findings are consistent with the literature.

General Properties of Wines

The main oenological properties of cv. Emir wines obtained from the treatments were given in Table 1.

As can be seen from Table 1, density was found in the general composition of wines between 0.992-0.994. Ethyl alcohol % (v/v) 10.60-11.31, titration acidity (g/L as

tartaric acid) 5.19-5.40 and pH 3.27-3.32 analysis results were determined. As can be seen from Table 1, among the three samples, While L1 wine had the highest amount of ethanol at 11.31% (v/v), this ratio reached the lowest level in L3 wine (10.60 % v/v) ($P<0.05$). As a result, ethanol content diminished as the amount of *LT* rose. Similar to our study, some studies have notified that the *LT* strain has effects on the reduction of alcohol grades of wines (Ciani et al., 2016; Benito et al., 2015; Binati et al., 2020; Benito et al., 2016). Similarly, Morales et al. (2019) found that the alcohol content of pure SC culture was 11.1 (%v/v), while the alcohol content of pure *LT* culture was 8.3 (%v/v). Similarly, Balikci et al. (2016) reported that the addition of *LT* caused a decrease in the amount of ethyl alcohol compared to SC yeast. As a result of co-inoculation fermentation, the increase in the addition of *LT* strains decreased the amount of ethanol.

Volatile Compounds of Wine

The concentrations and the compositions of the volatile compounds determined in three different wines are shown in Table 2. As a result of the GC-MS analysis, a total of 13 volatiles with different classes such as higher alcohols, esters and aldehydes were analyzed in wine samples. In the present study, *LT* yeast inoculated simultaneously with wine yeast (*SC*) at different rates partially increased the number of aroma compounds in wine samples. The total amounts were found between 324 and 327 mg/L in simultaneous inoculation samples of *SC* and *LT* yeasts in different quantities. In addition, the total volatile compounds of L3 wine were found as 327 mg/L. Similar results were stated by Morales et al. (2019) who emphasized that the addition of *LT* and *SC* strain mixture preserved the aroma better in Pedro Ximénez wine as compared to the pure culture.

Among the volatile compounds, higher alcohols obtained through both catabolic (Ehrlich pathway) and anabolic (with biosynthesis) pathways are important aroma compounds. (Etiévant, 1991; Stewart and Russell, 1998). The concentration of higher alcohols partially rose with an increase in the amount of *LT*. Oppositely, the number of esters decreased as the ratio of the *LT* yeast increased. Higher alcohols representing the most present group in wines contribute positively to the volatiles of wine at concentrations under 300 mg/L, while it can negatively affect aroma above 400 mg/L (Padilla et al., 2016). When these results are evaluated, it can be said that the total higher alcohol content of three different wine samples is in the range of 249 and 270 mg/L, which positively affects the aroma. It has been formerly stated that there are strains of *Metschnikowia pulcherrima*, *LT* and *S. bacillaris*, which can produce large amounts of higher alcohol (Padilla et al., 2016). In the study carried out, the most dominant compound was found to be 3-methyl butanol and its amount was determined between 129-133 mg/L.

Total esters in three different wines, which include isobutyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, isoamyl acetate and ethyl acetate are shown in Table 2. Ethyl acetate was the most prominent volatile compound among esters and generally, their amount was determined at 42 mg/L levels. Vaquero et al. (2021) stated that ethyl acetate produced the highest amount (38.35 mg/L) on the 17th-day fermentation in wine, in which *LT* and *M. pulcherrima* yeasts were inoculated together. Isoamyl acetate, isobutyl acetate, ethyl butyrate, ethyl hexanoate and ethyl octanoate were identified in very small amounts in all wine samples, while the amount of all decreased with increasing *LT* levels. Ethyl octanoate could not be detected in the L3 sample wine.

Only three aldehyde aroma compounds were detected in wine samples. Among the wine samples, aldehyde had a smaller amount of aroma compounds than other volatile groups. Among the aldehydes, the acetaldehyde compound had the largest amount in all three trials. Aldehyde amounts were determined between 13.5 (L2) and 29.6 mg/L (L1). In addition, the quantitative change of the aldehydes found in three different samples was not found significant ($p>0.05$). In a study, while acetaldehyde compound was determined as 14.3 mg/L in the pure *SC* strain, it was reported that as a result of the addition of *LT* and *SC* strains, the amount of acetaldehyde increased and was obtained as 15 mg/L. They also stated that sequential *LT* inoculation contained more acetaldehyde compounds than co-inoculation (Hranilovic et al., 2021). It has been reported that the high amount of acetaldehyde compounds found in wines both negatively affect the aroma and causes many diseases that affect negatively human health (Zea et al., 2015). The amount of acetaldehyde found in the wines we obtained is consistent with the results in the literature and is not at a level that threatens health. So, it is very important to determine strains that produce low amounts of acetaldehyde to ultimately produce healthy wine (Binati et al., 2020). On the other hand, Cheraiti et al. (2005) reported that redox interactions can occur between yeasts in co-culture and that acetaldehyde produced by any strain is metabolized by others.

Sensory Evaluation

Though the sensory assessment is specific, the human nose has the ability to discern aroma, taste, and other nuances that cannot be identified by instrumental techniques (Jolly et al., 2003). Although there are many studies on non-Saccharomyces in oenology, there are still deficiencies regarding the sensory evaluation of wines (Tempere et al., 2018). In our research, sensory profiles of wine obtained by co-inoculation of three different amounts of non-*Saccharomyces* yeasts by 13 experienced panellists are also investigated. As a result of the sensory evaluation of the wines according to the sorting test, the preferences increased with the addition of increased *LT* amount and the most preferred wine was L3 (*SC* $=5\times10^6$ + *LT* $=1\times10^8$ cells/mL).

Conclusions

In this study, the effect of mixed cultures of *SC* and different amounts of *LT* yeasts on Emir grape wine was investigated. In the experiments carried out, it was observed that *LT* yeast was present in the medium until alcoholic fermentation was completed. With the addition of an increased amount of *LT*, ethanol, titratable acidity, and total ester amounts decreased. In contrast, with the addition of increasing *LT*, the amounts of 2-methyl butanol, propanol and total higher alcohol increased. The total number of aroma compounds of wines inoculated with increasing amounts of *LT* yeast (L1, L2 and L3) was found to be

between 321 and 327 mg/L. Concerning sensory analysis, the most preferred wine was the L3 sample. As a result, it can be said that the use of *LT* yeast in different amounts has a positive effect on wine fermentation. However, further research needs to be accomplished on the use of these strains of yeast on a larger scale in wine-making.

Conflict of Interest

The authors declare no conflict of interest.

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