ROLE OF *LACTOBACILLUS FERMENTUM* AS A STARTER CULTURE FOR MALOLACTIC FERMENTATION TO IMPROVE QUALITY OF WHITE WINES

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ABSTRACT

Fermentation of fruit pulp and fruit juices is a relative and simple avenue for production of wine. In the present investigation apple, banana, orange and mead wines were prepared using bakers yeast. After primary fermentation and clarification, secondary or malolactic fermentation of apple and orange wine was carried out using *Lactobacillus fermentum* MTCC 10770. During fermentation and after malolactic fermentation, a detailed analysis of various biochemical and microbiological parameters, antioxidant activity and sensory attributes of wines were comparatively investigated. Results obtained in the study indicated an important role of *L. fermentum* in the secondary fermentation and in the enhancement of antioxidant, nutritional and sensory characteristics of the fruit wines.

Keywords: Apple cider; Malolactic fermentation; *Lactobacillus fermentum*; Physicochemical properties; Antioxidant.

INTRODUCTION

Fruits provide several vital nutrients that maintain our health. From ancient time, fruits are used for the production of different alcoholic beverages like wine. Wine is perhaps the oldest fermented drink known to man. However, though it had been prepared somewhere in 3500 BC but still the actual birthplace of wine is unknown. Wine generally refers to the fermented
by products of grapes (*Vitisvinifera*) but may include any undistilled alcoholic fermented fruit product. Production, importance and nutritive value of different fruit wines has been reported earlier [1].

Wines have been considered as safe and healthy drinks, besides an important adjunct to the diet. The recent years have witnessed several reports on the consumption of wine in moderation and beneficial effect on the cardiovascular system as well as the general well being of the consumers [2]. In wines, alcohol is a macro nutrient and is an energy source, capable of providing calories for all essential biological activities of the human cells, energy for physical work and thermogenesis [1].

Although grapes are the primary raw material used for wine production, there is an increasing interest in the use of other fruits, such as apricot, apple and palm sap etc. suitable for wine making. In countries where grapes are not abundantly available, local fruits that are cheap and readily available are used as an alternative [3, 4]. In India, wine industry is in its infant stage [5]. The high heat and humidity of the far eastern half of the country limits viticultural activity [6]. There are only few industries in our country which produce wine but fruit wine production at this time is insignificant in spite of tremendous increase in the fruit production. Amount of wine production from other fruits is very small as compared to grapes [1].

The basic process of wine making involves the fermentation of grape juice or other fruits by *Saccharomyces cerevisiae* followed by maturation. The quality of wine is known to depend upon number of factors like cultivars and their characteristics such as adequate sugar level, acid content, colour and aroma of the fruits. Other process variables which affect the quality of wine include initial sugar concentration, addition of enzyme, yeast strains and water [1].

In the literature, preparation of wine from orange [7], apple cider [5], banana [8] and mead wine [9] has been described but there is only a limited work carried out on the malolactic fermentation of orange wine and apple cider wine especially using *Lactobacillus fermentum* which is earlier reported to cause spoilage in wine [10]. Therefore, a need was felt to investigate the changes in wine after MLF with *L. fermentum* and to evaluate its potential as a starter culture for secondary i.e. MLF.
MATERIALS AND METHODS

Preparation and Clarification of fruit wines
Different wines were prepared using standard method of Robinson [11]. Fruits (Apple, Banana and Orange) were procured locally and cut into small pieces. Water was then added to the fruit pieces and boiled for half an hour. The degree brix was set at 22° by adding appropriate amount of concentrated sugar solution after cooling. Resins, cloves, cinnamon, cardamom and lemon juice were added to the fruit must to flavor the wine. KMS i.e. potassium metabisulphite was added at a concentration of 100 ppm and must was kept for overnight before inoculation. Baker’s yeast (1% w/v) was added by activating it in small amount of hot water. Different samples were then kept for fermentation in cool and dark place for 7 days. Similarly, mead wine was prepared by diluting Dabur honey to 23° brix and by addition of other flavoring agents as described earlier.

Clarification of all the wines was performed using bentonite (0.01% w/v). The clarified wines were siphoned off, filtered and sealed in bottles. Wine bottles were stored at 25-30°C temperature for maturation [11].

Malo-lactic fermentation
Apple cider and Orange wine were processed further and subjected to secondary i.e. malo-lactic fermentation to reduce acidity, sourness and off flavours of these two wines. TSS was adjusted to 12° brix before MLF. Lactobacillus fermentum MTCC 10770 was used as a starter culture for MLF. 1% (v/v) of the overnight grown bacterial culture was inoculated in different wine samples and incubated at 25°C for 24 h.

Physical-chemical characterization of wines
Wines from different treatments were analyzed for various physico-chemical and sensory quality characteristics. The pH of all the wine samples was determined after every 24 h by using digital pH meter (Eutech Instruments, pH Tutor Singapore) by the method of Moore and Jaselskis [12]. TSS of wine samples was determined using a hand refractometer after every 24 h and the readings were expressed as °Brix [13]. For TSS, standard curve was prepared by checking °Brix of sucrose at different concentrations ranging from 5-50%. Total reducing sugars were determined spectrophotometrically using dinitrosalicylic acid (DNS) method [14]. For the analysis of ethanol content, potassium dichromate method of Caputi et al. [15] was followed and calibration curve of ethyl alcohol was used as standard. Protein content in wine samples was estimated by Lowry’s method using bovine serum albumin as
standard [16]. Total titratable acidity (TTA) was determined by titrating wine samples with 0.1N NaOH in the presence of phenolphthalein indicator as described by Mahindru [17]. TTA results were expressed as % acidity according to the main organic acid present in different fruits. For the measurement of color intensity, absorbance of wines was directly measured at 420 nm (yellow or brown pigments mainly flavanoids, tannins and some anthocyanins), 520 nm (red pigments, mostly anthocyanins) and 620 nm (blue pigments, mostly anthocyanins) using 2 mm optical path. Color intensity (CI) was measured as a function of tint (redness in %) and brilliance (hue) of wine (dA) which were calculated by the formula given by Glories [18].

Tint of wine (Redness of wine): Defined by ratio: \( \frac{A_{420}}{A_{520}} \)
Brilliance of wine (hue): \( dA \) (%) = \( 1- \left( \frac{A_{420} + A_{620}}{A_{520}} \right) \times 100 \)

**Analysis of vitamin content of wines**

**Vitamin-E Content:** The vitamin-E (mainly tocopherols) content was estimated using Emmerie-Engel reaction, based on the reduction of ferric to ferrous ions by tocopherols, which form a red color with 2, 2-dipyridyl. Following formula was used for the calculation of Vitamin-E content in various wine samples [19, 20, 21].

Vitamin-E (mg/l) = \( A_{520} - A_{460} / StdA_{520} \times 0.29 \times 15 \)

**Ascorbic acid Content:** The content of ascorbic acid in wine samples was determined using 2, 6-dichlorophenol-indolphenol dye by applying following formula [22].

Ascorbic acid (mg/l) = \( \frac{\text{Titre value} \times \text{dye factor} \times \text{Volume make up} \times 100}{\text{Vol. of sample extract} \times \text{Vol. of wine sample taken for estimation}} \)

Where, Dye factor (mg of ascorbic acid/ml of dye) = 0.5/ titre value of standard dye

**Analysis of Pigments**

**Total anthocyanin content:** Total anthocyanin content (TA) in extracts was estimated by method of Lee *et al.* [23] using methanol solution and the amount of anthocyanins present in wine samples was calculated as

\[
\text{Anthocyanin (mg/l)} = A \times M.W \times D.F \times 10^3 / \epsilon \times I
\]

Where, \( A = \text{Absorbance (A520nm – A 657nm)} \), \( \epsilon = \text{Molar extinction coefficient (26,} \)
900=molar extinction coefficient, in L and mol$^{-1}$& cm$^{-1}$, for cyanidin-3-glucoside), I = Path length (1cm), D.F = Dilution factor, MW (molecular weight) = 449.2 g/mol for cydanidin-3-glucoside and $10^3 = \text{factor for conversion from g to mg.}$

**Total flavanoid content:** Total flavonoids were determined using the aluminium chloride colorimetric method described by Zhishen *et al.* [24]. The absorbance was measured at 510 nm and results were expressed as mg of catechin equivalents for wines.

**Total Carotenoid content:** The clarified wine samples were centrifuged and suspended in 2 ml of 80% acetone (1:1). The optical density was observed at 663 nm and 643 nm for chlorophyll a and b respectively and at 470 nm for carotene. The content of total carotenoids present in wine sample was calculated in terms of mg/l by using Roldan equation [25]

\[
\text{Carotenoids} = \frac{100A_{470} - 1.80 \text{Chl}_a - 85.02 \text{Chl}_b}{198}
\]

**β-carotene and Lycopene content:** After centrifugation 2 ml of acetone and hexane solution (1:1) was added to 2 ml of clarified wine sample. Then the absorbance of samples was measured at 453 nm, 505 nm, 645 nm and 663 nm for estimation of β-carotene and lycopene content using the following formula [26].

\[
\begin{align*}
\text{Lycopene (mg/l)} &= -0.0458A_{663} + 0.372A_{505} - 0.0806A_{453} \\
\text{β-Carotene (mg/l)} &= 0.216A_{663} - 0.304A_{505} + 0.452A_{453}
\end{align*}
\]

**Antioxidant activity (DPPH Scavenging Activity)**

The antioxidant activity of wine samples was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). 0.1 mM DPPH solution was freshly prepared in methanol. Then, 1.5 ml of wine samples were taken and 1.5 ml of DPPH was added to the wine samples. The samples were incubated at room temperature for 15 min and the absorbance was noted at 517 nm [27]. The percentage antioxidant property of wine samples can be calculated using following formula.

Percentage antioxidant activity (scavenging effect of DPPH) = \[\frac{A_0 - A_1}{A_0}\] X 100

Where, $A_0 = \text{Absorbance of control and } A_1 = \text{Absorbance of test sample}$
Microbiological Analysis

The evolution of undesired micro-organisms during different stages of winemaking can result in the production of volatile acidity, off-flavors and polysaccharide hazes, all of which can diminish the quality and acceptability of the final product [28]. So all the prepared wine samples (100µl each) were analyzed after regular intervals up to 3 months of storage at 25°C by a standard plate count method on different culture media such as EMB agar, Listeria Enrichment agar, Luria agar, MacConkey agar, MRS agar, Nutrient agar, Potato Dextrose Agar (PDA) media, Reinforced Clostridial (RCM) agar and Salmonella ShigellaHiVeg Agar to check microbial spoilage. Agar plates were incubated for 24 h at 37°C and analyzed for any kind of microbial contamination. The test was performed to ensure the stability of the wine before bottling and during storage [29].

Sensory evaluation of wines

Wines were organoleptically evaluated according to 9-point hedonic scale by group of 10 volunteers. Sensory examination and evaluation of wine was performed considering different attributes which included color, odor, mouth feel, after taste and overall acceptability [30]. The comparison between the different attributes of prepared wines which include apple cider before and after MLF, orange wine before and after MLF, banana wine and mead were evaluated for sensory attributes they possess. The judges were asked to rate the extent of particular attribute in a product and give the score, accordingly. During the sensory evaluation the judges rinsed their mouth with water in-between the testing of the products. The samples of wines were given as the coded samples to judges.

Statistical analysis

All the tests were run in triplicates and averaged to determine the mean and standard deviation. Standard deviation (S.D) is given by: \( \text{S.D} = \sqrt{\frac{\sum (x-x')^2}{n-1}} \). Where \( \Sigma x \) is the sum of the sample, \( x \) is sample mean, \( x' \) is population mean and \( n \) is the number of sample in the population. Significance was accepted at \( p \leq 0.05 \).

RESULTS AND DISCUSSION

During course of fermentation pH of the wine decreased due to two main reasons i.e. presence of organic acids (citric acid, malic acid and tartaric acid) in fruits and production of alcohol [31, 32]. The pH of different fruit musts was recorded regularly during course of fermentation (apple cider, banana wine, orange wine and mead) which was found to decrease as the fermentation proceeded as shown in tables 1, 2, 3 and 4. There was a maximum
decrease in the pH of apple cider as compared to other wines. This may be due to the fact that apple cider contained maximum amount of tartaric and malic acid as compared to other wines, which resulted in maximum fall in pH during fermentation. Fall in pH is directly correlated to acidity of the wines.

Acidity of all the wines increased as the fermentation progressed which also resulted in decrease in pH of the wine (tables 1, 2, 3 and 4). Maximum amount of titrable acidity as % tartaric acid (4.70%) was observed in Apple cider as compared to other wines, whereas Orange wine acidity was rated highest in terms of % citric acid content (3.63%) and least titratable acidity (2.90% MAE) was observed in banana wine. Higher acidic content results in sourness of wine especially malic acid and tartaric acid in apple cider and citric acid and tartaric acid in orange wine and it also have impact on fruit flavor, acid taste and sugar/acid balance of wines. The pH of juice or wine results from the balance between anionic forms of organic acids (mainly malic acid and tartaric acid) and the major cations (mainly potassium) [33]. Any alteration of the concentration of organic acids (malic acid, tartaric acid and other acids) in fruit juice eventually affects the final pH of wine and its acceptability. A wine with too much acidity will taste excessively sour and sharp. A wine with too little acidity will taste flabby and flat, with less defined flavors [6].

Generally, total sugar content (°B) during fermentation of fruit wines is an indicator for activity of yeasts to use sugars in fermented mixture [34]. TSS content of each wine was found to decrease as the fermentation proceeded towards completion. Among all the wines prepared the TSS of apple cider was highest (12° B) and corroborated with the rate of fermentation, the lowest TSS was recorded in orange wine (9° B) as observed from tables 1, 2, 3 and 4. As the °Brix decreased, the level of sugar in the wine samples also decreases and acidity increased due to production of alcohol which resulted in sourness of wine. Higher the TSS, the better is the taste, mouthfeel and acceptability of wine [35].

A similar trend was observed for the reducing sugars content of the wines. The greater the amount of reducing sugar present in the wine sample, sweeter the wine will be. Apple cider (1.477 g/l) was better to taste among other wines as it contained some amount of residual sugars which made it sweeter whereas orange wine had least amount of sugar content. The allowed amount of sugar in wine is 4 g/L for dry wines and 4-12 g/L for semi-dry ones [36]. Considering the results obtained all the prepared wines (apple cider, banana wine, mead and
orange wine) can be classified in the category of dry wines which are good for diabetics and calorie conscious people.

The conversion of sugar to alcohol is a vital step in the process of making wine, during fermentation [6]. Ethanol content of each wine sample was estimated (tables 1, 2, 3 and 4) and comparison between alcohol content of all the wines was made (Fig. 1). Maximum alcohol content was recorded in apple cider (9.6%) followed by mead and least content of alcohol was present in orange wine. It was also noticed that ethanol content of each wine increased at the end of primary fermentation. During fermentation decrease in total sugar content results in increase in the level of alcohol content because of the conversion of sugar to alcohol. The ethanol content in strong white wines usually ranges from 10-14% [37]. Except for apple cider all other wines were comparatively mild in terms of alcohol content.

**Determination of protein content**

The protein content in wines is usually very small and ranges from 0.001-0.5%. Hsu and Heatherbell [38] found protein in the range of 19–44 mg/l in four different un-fined white wines, while a very large variation (20–260 mg/l) was noted by Bayly and Berg [39]. White wines prepared in this study have protein content in the range from 31.0 to 83.4 mg/l i.e. 0.003 to 0.008% as shown in fig 2. Maximum amount of proteins were reported in mead wine which is 83.32 mg/l. Presence of proteins in wines adds to their nutritional quality and therefore, increases their caloric value.

**Determination of color intensity**

Color intensity of the wines enhanced as the fermentation proceeded and more and more phenolics were extracted from the fruit mash (Fig 6, 7, 8 and 9). Tables 1, 2, 3 and 4 gives the comparison between tint (redness) and brilliance (hue) of all the prepared wine samples. Redness of mead and brilliance of apple cider was found to be maximum as compared to other wines as fruits along with skin were used for the production of wine. Reddish yellow color was recorded in orange wine, which may be due to the colour of the raw material used for fermentation. Color intensity of wines showed variation from 1.30% to 0.94% with respect to tint or redness and from 36.9% to 1.30% with respect to brilliance/hue due to different pigments present in wines. The phenolic compounds mainly anthocyanins and their polymeric products present in skin of apples are responsible for the colour of apple cider (fig. 11). The color density and hue are substantially dependent on pH and the presence of sulfur dioxide [40]. However, the intensity of red color decreases with increasing pH value.
Table 1. Variation in physico-chemical characteristics of Apple cider

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH</th>
<th>Acidity (%)</th>
<th>TSS ('B)</th>
<th>Reducing sugars (g/l)</th>
<th>Alcohol content (%)</th>
<th>Proteins content (mg/l)</th>
<th>Color intensity</th>
<th>Vitamins-E content (mg/l)</th>
<th>Ascorbic acid content (mg/l)</th>
<th>Antioxidant activity (mg/l)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Malic acid</td>
<td>Tartaric acid</td>
<td>Citric acid</td>
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<td></td>
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Table 2. Variation in physico-chemical properties of Orange wine

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<tr>
<th>Time(h)</th>
<th>pH</th>
<th>Malic acid (g/l)</th>
<th>Tartaric acid (%)</th>
<th>Citric acid (%)</th>
<th>TSS (°B)</th>
<th>Reducing sugars (g/l)</th>
<th>Ehanol content (%)</th>
<th>Protein content (mg/l)</th>
<th>Color intensity</th>
<th>Tint of wine (redness in %)</th>
<th>Brilliant (hue) of wine (%)</th>
<th>Vitamin E content (mg/l)</th>
<th>Ascorbic acid content (mg/l)</th>
<th>Anthocyanin (mg/l)</th>
<th>Total flavanoids (mg/l)</th>
<th>Total carotenoids (mg/l)</th>
<th>β-carotene (mg/l)</th>
<th>Lycopene (mg/l)</th>
<th>Antioxidant activity (%)</th>
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<td>0.37±0.00</td>
<td>0.11±0.00</td>
<td>80.1±0.07</td>
</tr>
<tr>
<td>144</td>
<td>3.4±0.03</td>
<td>3.24±0.05</td>
<td>4.64±0.00</td>
<td>3.6±0.05</td>
<td>9.0±0.2</td>
<td>0.54±0.020</td>
<td>8.5±0.47</td>
<td>33.19±0.03</td>
<td>1.33±0.02</td>
<td>64.40±0.01</td>
<td>310±0.71</td>
<td>379±1.40</td>
<td>80.0±0.41</td>
<td>8.74±0.00</td>
<td>50.00±0.00</td>
<td>1.18±0.01</td>
<td>0.43±0.01</td>
<td>0.17±0.00</td>
<td>82.2±0.05</td>
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</tbody>
</table>
Table 3. Variation in physico-chemical properties of Banana wine

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH</th>
<th>Acidity (%)</th>
<th>TSS (°B)</th>
<th>Reducing sugars (g/l)</th>
<th>Ethanol content (%)</th>
<th>Proteins content (mg/l)</th>
<th>Color intensity</th>
<th>Vitamins-E content (mg/l)</th>
<th>Ascorbic acid content (mg/l)</th>
<th>Anthocyanins (mg/l)</th>
<th>Total flavonoids (mg/l)</th>
<th>Total carotenoids (mg/l)</th>
<th>β-Carotene (mg/l)</th>
<th>Lyco-pene (mg/l)</th>
<th>Antioxidant activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.1±0.02</td>
<td>0.46±0.02</td>
<td>0.52±0.02</td>
<td>21.0±0.02</td>
<td>0.862±0.009</td>
<td>2.7±0.3</td>
<td>18.21±0.02</td>
<td>1.30±0.05</td>
<td>36.9±0.02</td>
<td>90±0.71</td>
<td>26±0.85</td>
<td>9.29±0.03</td>
<td>4.09±0.03</td>
<td>0.29±0.01</td>
<td>0.04±0.002</td>
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<tr>
<td>24</td>
<td>5.0±0.10</td>
<td>1.30±0.01</td>
<td>1.50±0.01</td>
<td>19.5±0.01</td>
<td>0.844±0.02</td>
<td>2.8±0.14</td>
<td>22.47±0.05</td>
<td>1.34±0.01</td>
<td>40.0±0.01</td>
<td>120±0.67</td>
<td>30±0.91</td>
<td>14.04±0.01</td>
<td>5.21±0.02</td>
<td>0.36±0.03</td>
<td>0.05±0.001</td>
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<tr>
<td>48</td>
<td>4.8±0.03</td>
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<td>-</td>
<td>18.0±0.03</td>
<td>0.719±0.01</td>
<td>3.5±0.2</td>
<td>23.61±0.01</td>
<td>1.40±0.02</td>
<td>42.7±0.01</td>
<td>150±0.92</td>
<td>61±1.20</td>
<td>19.32±0.02</td>
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<td>72</td>
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<td>-</td>
<td>16.0±0.01</td>
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<td>24.97±0.03</td>
<td>1.67±0.04</td>
<td>43.5±0.01</td>
<td>200±0.4</td>
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<td>0.71±0.07</td>
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<td>96</td>
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<td>2.20±0.05</td>
<td>-</td>
<td>14.5±0.05</td>
<td>0.570±0.07</td>
<td>4.9±0.1</td>
<td>28.33±0.06</td>
<td>1.70±0.10</td>
<td>47.6±0.01</td>
<td>260±0.69</td>
<td>139±0.99</td>
<td>39.02±0.02</td>
<td>6.69±0.01</td>
<td>0.96±0.04</td>
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<tr>
<td>120</td>
<td>3.9±0.01</td>
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<td>2.60±0.02</td>
<td>-</td>
<td>12.0±0.10</td>
<td>0.482±0.05</td>
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<td>31.48±0.01</td>
<td>1.90±0.03</td>
<td>51.0±0.03</td>
<td>290±0.97</td>
<td>177±0.56</td>
<td>43.70±0.04</td>
<td>7.97±0.03</td>
<td>1.03±0.03</td>
</tr>
<tr>
<td>144</td>
<td>3.5±0.01</td>
<td>2.90±0.05</td>
<td>3.30±0.05</td>
<td>-</td>
<td>9.5±0.01</td>
<td>0.364±0.02</td>
<td>6.9±0.1</td>
<td>35.99±0.02</td>
<td>2.00±0.01</td>
<td>52.3±0.1</td>
<td>330±0.94</td>
<td>208±0.72</td>
<td>50.10±0.09</td>
<td>8.91±0.07</td>
<td>1.09±0.04</td>
</tr>
</tbody>
</table>
Table 4. Variation in physico-chemical properties of Mead wine

| Time (h) | pH    | Acidity (%) | Malic acid (°B) | Tartaric acid (°B) | Citric acid (°B) | TSS (°B) | Reducing sugars (g/l) | Ethanol content (%) | Protein content (mg/l) | Color intensity | Tint of wine (redness in %) | Brilliancy (hue) of wine (%) | Vitamin-E content (mg/l) | Ascorbic acid content (mg/l) | Anthocyanins (mg/l) | Total flavonoids (mg/l) | Total carotenoids (mg/l) | β-Carotene (mg/l) | Lycope ne (mg/l) | Antioxidant activity (%) |
|----------|-------|-------------|-----------------|-------------------|------------------|--------|---------------------|-------------------|----------------------|---------------|----------------------------|---------------------------|--------------------------|-----------------------------|-----------------------------|--------------------------|--------------------------|-----------------------------|
| 0        | 4.7 ±0.01 | 0.13±0.01   | 0.15±0.01 | - | 19.0±0.05 | 0.856±0.03 | 0.6±0.4 | 27.19±0.07 | 1.00±0.02 | 31.00±0.05 | 60±0.56 | 21±0.52 | 24.15±0.07 | 1.37±0.09 | 0.52±0.03 | 0.06±0.001 | 0.01±0.001 | 33.7±0.14 |
| 24       | 4.6 ±0.02 | 0.25±0.02   | 0.20±0.02 | - | 17.5±0.01 | 0.812±0.05 | 1.7±0.1 | 34.48±0.04 | 1.07±0.01 | 32.00±0.02 | 80±0.71 | 49±0.64 | 26.01±0.03 | 1.53±0.07 | 0.63±0.01 | 0.08±0.002 | 0.03±0.002 | 36.2±0.01 |
| 48       | 4.0 ±0.02 | 0.39±0.05   | 0.46±0.05 | - | 16.0±0.02 | 0.787±0.07 | 2.6±0.1 | 37.10±0.03 | 1.33±0.05 | 37.40±0.01 | 76±0.88 | 31.90±0.05 | 1.78±0.010 | 1.07±0.04 | 0.11±0.001 | 0.05±0.001 | 41.1±0.05 |
| 72       | 3.7 ±0.01 | 0.44±0.10   | 0.59±0.10 | - | 15.0±0.05 | 0.691±0.01 | 3.8±0.2 | 49.25±0.01 | 1.40±0.02 | 40.00±0.12 | 130±0.47 | 88±0.94 | 35.02±0.07 | 2.52±0.005 | 1.21±0.02 | 0.13±0.004 | 0.09±0.005 | 47.9±0.02 |
| 96       | 3.5 ±0.03 | 0.67±0.01   | 0.75±0.01 | - | 13.5±0.02 | 0.669±0.06 | 5.3±0.1 | 54.33±0.05 | 1.67±0.07 | 45.80±0.10 | 160±0.82 | 129±0.69 | 40.80±0.09 | 3.44±0.017 | 1.79±0.05 | 0.14±0.002 | 0.12±0.005 | 52.0±0.06 |
| 120      | 3.4 ±0.12 | 0.81±0.02   | 0.82±0.02 | - | 12.0±0.07 | 0.604±0.004 | 7.2±0.1 | 67.51±0.02 | 2.20±0.02 | 48.00±0.02 | 210±0.93 | 150±0.72 | 49.07±0.04 | 4.91±0.03 | 2.16±0.03 | 0.15±0.002 | 0.13±0.003 | 57.6±0.03 |
| 144      | 3.1 ±0.01 | 0.87±0.05   | 0.97±0.05 | - | 10.0±0.05 | 0.570±0.007 | 8.6±0.3 | 83.32±0.03 | 2.40±0.03 | 52.00±0.02 | 240±0.81 | 230±1.10 | 55.00±0.10 | 5.39±0.020 | 2.29±0.07 | 0.17±0.003 | 0.15±0.001 | 61.7±0.03 |
Table 5: Malolactic fermentation of apple and orange wine

<table>
<thead>
<tr>
<th>Physiological testing</th>
<th>Apple cider before MLF</th>
<th>Apple cider after MLF</th>
<th>Orange wine before MLF</th>
<th>Orange wine after MLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.0±0.02</td>
<td>4.0±0.01</td>
<td>3.4±0.03</td>
<td>4.0±0.01</td>
</tr>
<tr>
<td>Acidity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malic acid</td>
<td>3.3±0.05</td>
<td>2.84±0.05</td>
<td>3.2±0.05</td>
<td>2.94±0.05</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>4.7±0.05</td>
<td>4.08±0.02</td>
<td>4.6±0.05</td>
<td>3.75±0.02</td>
</tr>
<tr>
<td>Citric acid</td>
<td>-</td>
<td>-</td>
<td>3.63±0.05</td>
<td>4.80±0.03</td>
</tr>
<tr>
<td>TSS as °Brix</td>
<td>12.0±0.05</td>
<td>9.5±0.02</td>
<td>9.0±0.2</td>
<td>9.5±0.01</td>
</tr>
<tr>
<td>Reducing sugars (g/l)</td>
<td>1.477±0.04</td>
<td>2.713±0.07</td>
<td>0.542±0.02</td>
<td>3.02±0.05</td>
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<tr>
<td>Ethanol (%)</td>
<td>9.6±0.21</td>
<td>9.1±0.07</td>
<td>8.5±0.47</td>
<td>9.5±0.12</td>
</tr>
<tr>
<td>Protein content (mg/l)</td>
<td>3.10±0.04</td>
<td>3.21±0.07</td>
<td>2.79±0.03</td>
<td>2.81±0.02</td>
</tr>
<tr>
<td>Color intensity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tint of wine</td>
<td>2.20±0.02</td>
<td>2.20±0.01</td>
<td>1.33±0.02</td>
<td>2.25±0.01</td>
</tr>
<tr>
<td>Brilliance of wine</td>
<td>69.00±0.12</td>
<td>59.90±0.02</td>
<td>64.40±0.01</td>
<td>63.00±0.04</td>
</tr>
<tr>
<td>Vitamin content (mg/l)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin-E</td>
<td>210±0.81</td>
<td>218±0.73</td>
<td>310±0.68</td>
<td>299±0.71</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>312±1.90</td>
<td>319±1.38</td>
<td>379±1.70</td>
<td>354±1.49</td>
</tr>
<tr>
<td>Pigments (mg/l)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>69.3±0.07</td>
<td>107.6±0.02</td>
<td>80.0±0.11</td>
<td>91.0±0.02</td>
</tr>
<tr>
<td>Total Flavanoids</td>
<td>5.47±0.02</td>
<td>11.83±0.02</td>
<td>8.74±0.07</td>
<td>9.33±0.07</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>2.33±0.04</td>
<td>7.0±0.05</td>
<td>1.18±0.01</td>
<td>1.0±0.03</td>
</tr>
<tr>
<td>β-carotene</td>
<td>0.27±0.003</td>
<td>1.6±0.03</td>
<td>0.43±0.01</td>
<td>0.3±0.01</td>
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<tr>
<td>α-lycopene</td>
<td>0.21±0.004</td>
<td>1.01±0.07</td>
<td>0.17±0.003</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>Antioxidant activity (%)</td>
<td>52.6±0.02</td>
<td>76.0±0.23</td>
<td>82.2±0.05</td>
<td>81.3±0.47</td>
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<td>Microbiological analysis</td>
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<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

(-ve : free of microbial contamination)

Table 6: Sensory evaluation of different wines using 9-hedonic scale

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Apple cider before MLF</th>
<th>Apple cider after MLF</th>
<th>Orange wine before MLF</th>
<th>Orange wine after MLF</th>
<th>Banana wine</th>
<th>Mead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>7.0±0.92</td>
<td>7.7±0.92</td>
<td>5.5±1.41</td>
<td>5.8±0.79</td>
<td>6.2±0.91</td>
<td>7.7±0.97</td>
</tr>
<tr>
<td>Odor</td>
<td>6.8±0.69</td>
<td>7.8±1.10</td>
<td>5.1±1.17</td>
<td>5.2±0.87</td>
<td>6.0±0.75</td>
<td>7.5±0.77</td>
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<tr>
<td>Mouth feel</td>
<td>6.5±0.90</td>
<td>7.5±0.81</td>
<td>5.4±1.08</td>
<td>5.7±1.19</td>
<td>6.2±1.24</td>
<td>7.2±0.82</td>
</tr>
<tr>
<td>After taste</td>
<td>6.7±0.70</td>
<td>7.8±0.69</td>
<td>5.4±1.52</td>
<td>5.5±1.42</td>
<td>6.5±1.29</td>
<td>7.5±1.50</td>
</tr>
</tbody>
</table>
Figure 1: Ethanol content of different wine samples

Figure 2: Protein content of different wines

Figure 3: Vitamin-E content in different wine samples
Figure 4: Ascorbic acid content in different wine samples

Figure 5: Antioxidant activity of different wine samples

Figure 6: Changes in color intensity of apple cider during fermentation as function of anthocyanin content and total antioxidants
Figure 7: Changes in color intensity of Orange wine during fermentation as function of anthocyanin content and total antioxidants

Figure 8: Changes in color intensity of Banana wine during fermentation as function of anthocyanin content and total antioxidants

Figure 9: Changes in color intensity of Mead wine during fermentation as function of anthocyanin content and total antioxidants
ANALYSIS OF VITAMIN CONTENT OF WINES

Determination of vitamin-E

Vitamin-E play vital role in the antioxidant properties of the wines. Vitamin-E was estimated after the completion of primary fermentation for each wine sample as shown in fig 3. Banana wine contained maximum amount of vitamin-E and least amount was present in apple cider. Banana wine (330 mg/l) contained maximum amount of vitamin-E followed by orange wine. Both banana wine and orange wine also showed good antioxidant activity as compared to apple cider and mead wine. Alpha-tocopherol (vitamin-E) and flavanoids contain chemical structural elements that may be responsible for their antioxidant activities [41].

Determination of vitamin-C i.e. ascorbic acid

Citrus fruits contain high value of vitamin-C which acts as antioxidant and also increases the non-haem iron, inorganic iron from plant foods [42]. Vitamin-C plays a vital role in imparting antioxidant properties to orange wine. Ascorbic acid content was calculated in different samples of prepared wines and the content was found to increase during the process of fermentation as shown in fig. 4. After evaluation of results it was found that the ascorbic acid content of orange wine was maximum (379 mg/l) as compared to other prepared wine samples followed by mead. Least ascorbic acid content was given by banana wine (i.e. 208 mg/l). Figure 4 gives the comparison between ascorbic acid content of apple cider, banana wine, mead and orange wine.

ANALYSIS OF PIGMENTS

Anthocyanins

Anthocyanins are the predominant pigments present in wine which are derived from fruit to
wine in maceration process [43]. The anthocyanin content is one of the major factors responsible for antioxidant and antimicrobial activity of the wine. Anthocyanins are mainly red colored pigments present in wines. Orange wine (80 mg/l) contained maximum amount of anthocyanins followed by apple cider (tables 1, 2, 3 and 4). The lower is the pH of the wine, there will be higher proportion of flavyliumcation and higher will be contribution of red-colored anthocyanins [44].

**Total flavanoids**

The flavonoid composition of white wines depends on the composition of fruit, its extraction into the juice, and also, on the subsequent reactions occurring during the, post fermentation treatments, and wine aging [45]. Flavanoids are mainly yellow colored pigments which play an important role in antioxidant properties of wines. High intake of flavanoids result in reduction of cardiovascular and carcinogenic risk [46]. Fermented fruit juices i.e. wines are considered beneficial if taken in moderation. Flavanoid content (8.91 mg/l) was maximum in banana wine and least in mead, when compared with all the prepared wines (tables 1, 2, 3 & 4).

**Total carotenoids**

Carotenoids are responsible for the red color of wine. Apple cider (2.33 mg/l) was found to contain maximum amount of total carotenoid content as compared to other prepared wines with banana wine showing least amount of total carotenoids (tables 1, 2, 3 & 4.). Vitamins A, C and E and carotenoids are antioxidants derived from the fruits. The contribution of total carotenoid content to the antioxidant activity of various fruit juices was negligible as indicated by their poor contribution in total pigments [47].

**β-carotene and lycopenes**

Carotenoids play an important role in human nutrition through their provitamin A activity. They also acts as antioxidants, prevents age related macular degeneration and protects skin against UV radiation [48]. Their high content in wine indicates utility of wine for reducing incidence of certain diseases, so they were measured at regular intervals. β-carotene and lycopene content of wine samples was evaluated (tables 1, 2, 3 and 4). Lycopene content was quite less in all the prepared wines as compared to β-carotene content. Orange wine (0.43 mg/l) contained maximum amount of β-carotene where as apple cider had more lycopene (0.21 mg/l) when compared to other wines. However, there is no reference in the literature to levels of carotenoids in musts and wines. The evaluation that these compounds are present in
wines might be important since it is possible that during aging these molecules are degraded into aromatic compounds such as nor-isoprenoids, which can impact wine flavor [49]. Recent studies have found that diets rich in lycopene containing foods results in reductions in risks of cardiovascular disease. These reductions in risk have been attributed to the antioxidant properties of lycopene [50].

**Antioxidant activity (DPPH SCAVENGING ACTIVITY ASSAY)**

Wine is a rich source of polyphenol compounds, mainly anthocyanins, catechins, proanthocyanidins, flavonols, and stilbens, which show potent antioxidant activity and protect cardiovascular system [51]. Several assays have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables and their products and foods for clinical studies which includes 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and the oxygen radical absorption capacity (ORAC) [52]. Antioxidant activity of the prepared wines was determined using DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (table 1, 2, 3 and 4). The DPPH radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay like ABTS. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH. The test is simple and rapid and needs only a UV-vis spectrophotometer to perform, which probably explains its widespread use in antioxidant screening [53].

The percentage inhibition was 39.0–60.2% for white wines as reported earlier [54]. Macerated wines were much greater DPPH scavengers, with inhibition rates of about 90% (which is comparable to red wines). Non macerated wines have shown poor antiradical properties, inhibition lower than 50% [55]. The antioxidant potential of wine depends mainly on polyphenols compounds [50]. Results obtained in present study indicates that the antioxidant activity of white wine ranges from 52.6-85.0%, maximum in banana wine (85%) due to high content of vitamin-E followed by orange wine(82.2%) having high content of vitamin-C and anthocyanin and least activity was shown in apple cider (52.6%).

**MICROBIOLOGICAL ANALYSIS**

Microbiological analysis of wines is necessary because even prior to the start of fermentation, the fruits themselves can be infected with molds, yeasts and bacteria that can enter and alter the fermentation in a negative fashion. Improper wine storage and handling post-fermentation
can also encourage microbiological defects, which can negatively impact wine quality. As a result the basic physical, chemical, sensory and microbiological analysis of musts and wines to assure wine quality is must [56].

Microbiological analysis of each wine sample before and after malolactic fermentation was carried out on 8 different enrichment media to check for microbial spoilage. No microbial growth was found in any of the wine sample (table 6). It shows that wine samples were free of any type of contaminating microorganism such as *Escherichia coli*, *Bacillus* sp., *Clostridium* sp., *Lactobacilli* sp., *Leuconostoc* sp., *Oenococcus* sp., *Pediococcus* sp., *Salmonella* sp., *Shigella* sp. even after 3 months of storage which reflects good hygiene level of wines maintained during fermentation of fruits.

**Effect Of Malolactic Fermentation On Physico-Chemical And Sensory Attributes Of Wines**

MLF was carried out for the purpose of biodeacidification since it improves wine stability and quality [57]. The malolactic fermentation (MLF), or secondary fermentation, results from the metabolism of certain lactic acid bacteria in wine and results in the conversion of l-malate to l-lactate with the evolution of CO2. Basically, the two acidic groups of malate are replaced with only one acidic group present in lactate which results in a decrease in acidity of the wine. MLF usually carried out in wine after the alcoholic fermentation when the bacterial population is about $10^6$ CFU/ml. MLF in wine is desirable for three main reasons i.e. to decrease the acidity, to enhance the organoleptic characteristic and to increase the microbiological stability of wine. However, MLF is not favorable for all wines [58]. Clarified wines (apple cider and orange) were kept for MLF and evaluated for various sensory and physiological parameters before and after MLF as summarized in table 5. MLF of apple and orange wine resulted in increase in pH and decrease in % acidity of the wines as evidenced earlier by Zoecklein [59]. After MLF malic acid content of apple and orange wine was decreased while total carbohydrate and reducing sugar content increased due to addition of sugar just before MLF. Alcohol content of apple cider was almost same before and after MLF whereas there was 1% increase in orange wine. There was slight increase in the protein content of both the wines. As for the color intensity, the tint (redness) of apple cider remain same whereas there was increase in tint of orange wine and there was decrease in the brilliance of both the wines (for apple cider it decreases to 59.90% and for orange wine little decrease i.e. 63.0% from 64% was observed). On the other hand, pigments like anthocyanins
and flavonoids increased in apple cider from 69.3 mg/l to 107.6 mg/l respectively. In orange wine, anthocyanin content increased from 80 mg/l to 91 mg/l whereas, total carotenoids, carotene and lycopene content showed only a slight increase. There was no marked effect seen in vitamin content (Vitamin-E and vitamin-C) and it was almost same in both apple cider and orange wine. There was increase in antioxidant activity of apple cider from 52.6% to 76% whereas that of orange wine remained almost same.

Sensory Analysis of White Wines

Sensory evaluation and analysis of wine plays a huge role in the way people perceive wine. In order to produce wines that will be successfully sold in the wine market it is important to understand consumer preferences. For the evaluation, all the wines were tasted and examined for acceptance by considering different flavor attributes which included color, odor, mouth feel, after tasting and overall acceptability as shown in table 6 and fig. 10. Different studies have focused on the biosynthesis of aroma compounds during MLF and the concomitant organoleptic consequences [60]. Earlier, Maicas et al. [61] demonstrated that MLF noticeably changes major and minor volatile compounds which are beneficial to wine flavour. Phenolic acids can act as anthocyanin copigments, stabilizing the colour of wine, higher contents of these compounds will have a positive effect on the colour [62]. In sensory evaluation apple cider after MLF recorded a score of 8/10 for its sensory qualities and was most acceptable among all the wines under study because of its color, odor, mouth feel and taste.

CONCLUSION

All the seasonal fruits used in the study are suitable for wine production, but apple cider was most accepted wine before and after MLF. In the last few years, the interest of the scientists in wine Lactic acid bacteria (LAB) has increased. Currently, the enologist has more and better ways to control the activity of lactic acid bacteria and to counter their effect on the quality of the wine through a multidisciplinary and more extensive vision. A considerable amount of research has been conducted to determine the enzymatic properties of LAB specifically isolated from wine. *Lactobacillus fermentum* which is usually associated with mousiness in wine was used as starter culture for MLF and it gave satisfactory results without any spoilage. Thus the study is first of its kind highlighting role of *L. fermentum* in MLF. MLF with *L. fermentum* increased the fruity and butty aromas and reduced vegetable or grassy aromas. Formation and hydrolysis of esters during MLF may also lead to an increase in the fruity aroma and it is, probably, due to the action of LAB esterases responsible for the
synthesis and degradation of these compounds. However, to date there are no studies that demonstrate these changes. The reduction in vegetable or grassy aromas could be due to the catabolism of aldehydes by lactic acid bacteria. However, further studies are required to be able to relate the wine characteristics that are modified during MLF with the production and/or degradation of a specific chemical compound by wine lactic acid bacteria. With this information, the winemaker can choose the best strain of lactic acid bacteria to obtain wine with a specific aroma or flavour. A good understanding of different strains of LAB and their role in MLF is important in the manufacture of wine and offers a great potential for improving the quality of wine. Mead wine was highest in protein content among all other wines prepared. Inspite of lot of research on the production and nutritive quality of mead wine it is still not available commercially in the market.

REFERENCES


