

## Influence of wine components on pellicle formation by pellicle-forming yeasts

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### ABSTRACT

This study aimed to clarify differences in susceptibility to red wine pellicle formation by pellicle-forming yeasts between two wine grape cultivars and to investigate wine components affecting pellicle formation. Twenty wines each of Muscat Bailey A (MBA) and Merlot (MR), the major grape cultivars of Japanese red wine, were used. Pellicle formation occurred more often in MBA wines than in MR wines, and almost all MBA wine surfaces were covered with pellicle after incubation for five days. Principal component analysis revealed the relationships between pellicle formation and the concentrations of ethanol, phenolics and tannins. The mean concentration of tannins in the pellicle MR wines (436 mg/L) was significantly lower than that in the non-pellicle MR wines (660 mg/L). Furthermore, the mean concentration of tannins in MBA wines (139 mg/L) was also significantly lower than that in MR wines (570 mg/L). Wine grape cultivar having a low concentration of tannins may be highly susceptible to pellicle formation by pellicle-forming yeasts during winemaking.

### KEYWORDS

Flor yeasts, Microbial contamination, Muscat Bailey A, Phenolics, Tannins

## INTRODUCTION

After alcohol fermentation, the formation of a pellicle, or ‘flor velum’, on the wine surface sometimes occurs. Several types of wines are deliberately subjected to biological ageing (flor ageing) with flor-forming yeasts (flor yeasts) to develop a characteristic aroma (Cortes *et al.*, 1998; Moreno *et al.*, 2005; Moreno *et al.*, 2016). Sherry wine mainly produced in Southern Spain is the most widely known of this type of wine. Similar types include Vin Jaune (France), Vernaccia di Oristano (Italy) and Szamorodni (Hungary). On the other hand, the unexpected formation of pellicle by pellicle-forming yeasts (hereinafter, pellicle yeasts) is a problem in wine production. When pellicle is formed on the wine surface, a pungent odour is generated through the oxidative metabolism of pellicle yeasts, resulting in inferior products (Moyano *et al.*, 2002; Zea *et al.*, 2015).

The genera *Saccharomyces*, *Pichia*, *Candida* and *Hansenula* are well-known pellicle yeasts (Martínez *et al.*, 1997; Alexandre, 2013; Csoma *et al.*, 2021). Of these, genus *Saccharomyces* adapts best to harsh conditions of low oxygen concentration, high ethanol concentration, low pH and the presence of sulphur dioxide (SO<sub>2</sub>). More than 95 % of flor yeasts isolated from flor velum after alcohol fermentation are the species *Saccharomyces cerevisiae* (Martínez *et al.*, 1997). When fermentable sugars are present in wine, pellicle yeasts (pellicle *S. cerevisiae*) assimilate the sugars as a carbon source in the same way as non-pellicle *S. cerevisiae*, and does not form a pellicle under this condition (Nakagawa *et al.*, 2017). On the other hand, pellicle yeasts have a diauxic growth pattern; it shifts from anaerobic to aerobic metabolism to use ethanol as a carbon source after the sugars are depleted (Alexandre, 2013). In this process, *FLO11* expression, which encodes for a highly hydrophobic cell-wall glycoprotein, and cell surface hydrophobicity increase, and yeast cell aggregation occurs (Legras *et al.*, 2016). The yeast cell aggregates trap CO<sub>2</sub> bubbles and float to the wine surface where oxygen is available. Once they float to the wine surface, oxidative metabolism is enabled, and pellicle yeast growth and yeast cell aggregation are repeated, resulting in pellicle formation on the wine surface (David-Vaizant and Alexandre, 2018).

To prevent microbial contamination, the use of antimicrobial agents has been proposed. SO<sub>2</sub> is the most well-known antimicrobial agent (Waterhouse *et al.*, 2016).

When SO<sub>2</sub> is added to wine, the majority of SO<sub>2</sub> binds with wine components to form the bound form of SO<sub>2</sub> (bound SO<sub>2</sub>). The remaining SO<sub>2</sub> (free SO<sub>2</sub>) exists as molecular SO<sub>2</sub>, bisulphite and sulphite. Among these forms, only molecular SO<sub>2</sub> has antimicrobial activity, and its concentration increases with decreasing wine pH (Jackson, 2014). The molecular SO<sub>2</sub> concentration required to inhibit the growth of spoilage microorganisms ranges from 0.5 to 0.8 mg/L. It was reported that the use of high doses of SO<sub>2</sub> (>100 mg/L) in Sherry wine delays pellicle formation (Roldán *et al.*, 2012). Yajima *et al.* (1998) studied the inhibitory effects of SO<sub>2</sub> and sorbic acid on the growth of pellicle yeasts, such as *Saccharomyces* and *Candida* genera. The minimum inhibitory concentrations (MICs) of SO<sub>2</sub> and sorbic acid were low against *Candida* but high against *Saccharomyces*. Roldán *et al.* (2012) investigated the effect of lysozyme on flor yeast growth and flor velum formation when lysozyme was added to Sherry wine to prevent contamination by lactic acid bacteria. They found that lysozyme affected cell multiplication and cell surface hydrophobicity of flor yeasts when the yeast inoculation was carried out under submerged culture conditions during specific biological ageing. Consequently, the aggregation and flotation of flor yeasts and the subsequent development of flor velum were inhibited.

Phenolic compounds (hereinafter, phenolics) are one of the most important compounds in wine, and anthocyanins, flavonols, nonflavonoids and other flavonoids are present in red wine as phenolics. Red wines contain total phenolics (TPs) at concentrations of 1000 to 3000 mg/L, with a typical average of ~1800 mg/L (Waterhouse, 2002). These compounds exist in monomeric and polymeric forms. Tannins, which account for 50% of the concentration of TPs in red wine, are polymeric phenolics that have an affinity for proteins (Sacchi *et al.*, 2005). Wine tannins are divided into hydrolysable and condensed tannins based on their structures, and these tannins are derived from grape and oak, respectively (Smith *et al.*, 2015). Condensed tannins are extracted from the skin, the seeds and, to a lesser extent, the flesh of grapes during winemaking, and thus the concentration of tannins in wine grape affects that in the final wine (Waterhouse, 2002).

Phenolics contribute to the sensory properties of wine (particularly red wine) because these compounds have a bitter and astringent taste (Vidal *et al.*, 2004; Hufnagel and Hofmann, 2008; Soares *et al.*, 2017).

Furthermore, phenolics exhibit important biological activities, including antioxidant, anti-inflammatory and antimicrobial activities (Arnous *et al.*, 2001; Majo *et al.*, 2008; Joseph *et al.*, 2016; Vaquero *et al.*, 2007). Sabel *et al.* (2017) showed that ferulic acid, resveratrol and syringaldehyde exhibit antimicrobial activities against wine spoilage microorganisms, such as lactic acid and acetic acid bacteria, at concentrations of 250 to 1000 mg/L. Yajima *et al.* (1998) measured the antimicrobial activities of monomer phenolics, such as protocatechuic acid, gallic acid, *p*-coumaric acid, caffeic acid, ferulic acid, catechin and cinnamic acid, against pellicle yeasts. Among these compounds, only cinnamic acid showed high antimicrobial activity, its MIC being 50 to 100 mg/L. They explained that phenolics in wine may not be able to inhibit pellicle yeast growth because the monomer phenolics have high MICs. However, they did not investigate the antimicrobial activity of tannins, the major phenolics in red wine, against pellicle yeasts, nor did they study the influence of phenolics on the stage of pellicle formation. It has been shown that phenolics affect cell surface hydrophobicity, cellular auto-aggregation and biofilm formation of *Enterococcus faecalis* strains or *Streptococcus* strains (Wojnicz *et al.*, 2016; Wang *et al.*, 2021) even though bacterial biofilm is different compared to the pellicle. Therefore, phenolics in red wine may also alter pellicle formation by pellicle yeasts.

Muscat Bailey A (MBA) is a hybrid grape variety [*Vitis labrusca* (Bailey) x *Vitis vinifera* (Muscat Hamburg)] and its red wine is one of the most popular wines in Japan. MBA wine has unique characteristics such as a light mouthfeel and a strawberry-like aroma (Sasaki *et al.*, 2015). It was reported that TPs and tannins concentrations of MBA wine are extremely lower than Cabernet-Sauvignon and Merlot (MR) wines (Ichikawa *et al.*, 2011). It is considered that the low phenolic concentration of MBA wine contributes to its light mouthfeel. In addition, MBA was recognised as a wine grape cultivar by the International Organization for Vin and Wine in 2013. Because of these, Japanese winemakers have been working tirelessly to further improve the quality of MBA wine. However, some winemakers have noticed that pellicle formation occurs more often in the winemaking process of MBA than that in other grape cultivars. Watanabe *et al.* (1982) reported that 17 of the 30 isolated pellicle yeast strains were from MBA wines contaminated with a pellicle. However, there are few studies on the causes of pellicle formation and experimental data

of differences in pellicle formation among wine grape cultivars are sparse.

This study aimed to clarify differences in susceptibility to pellicle formation by pellicle yeasts between wine grape cultivars MBA and MR (another major grape cultivar used in Japanese red winemaking) and to investigate wine components affecting pellicle formation. We speculated that there are relationships between phenolics concentration in red wine and susceptibility to pellicle formation because some phenolics have an antimicrobial activity or inhibition activity of biofilm formation. Therefore, the effect of wine components including phenolics on pellicle formation were investigated.

## MATERIALS AND METHODS

### 1. Yeast strain and media

#### 1.1. *S. cerevisiae* and media

*S. cerevisiae* strain YFY-1 was provided by the applied microbiology laboratory of the University of Yamanashi, Japan. The strain was previously isolated from the pellicle of a wine made in Yamanashi Prefecture and confirmed to have the ability to form a pellicle (Nakagawa *et al.*, 2011). The strain was cultured on yeast extract-polypeptone-dextrose (YPD) solid medium (1 % yeast extract, 2 % polypeptone, 2 % glucose and 2 % agar) and then further cultured synthetic dextrose (SD) solid medium (0.67 % yeast nitrogen base without amino acids, 2 % glucose and 2 % agar).

#### 1.2. Preparation of cell suspension

The yeast cells were cultured overnight in 10 mL of SD liquid medium (without agar) at 30 °C with shaking. The culture was transferred to 50 mL of the same medium and then incubated overnight with shaking for scale-up. The cells were collected by centrifugation (740 g, 5 min), washed once with sterile distilled water, and re-suspended in flor medium (0.67% yeast nitrogen base without amino acids and 6% (v/v) ethanol as the sole carbon source) which was adjusted to pH 3.5 with hydrochloric acid (Nakagawa *et al.*, 2017). The optical density at 600 nm (OD<sub>600</sub>) of the cell suspension was adjusted to 10 ( $6.5 \times 10^7$  cells/mL) with flor medium. During pre-culture, the yeast was cultured in the medium without ethanol. As the yeast cells firmly adhered to the test tube and were not suspended well in the medium, when precultured in the presence of ethanol.

However, pellicle yeasts have ethanol resistance property and pellicle formation in the flor medium containing 6 % ethanol was also good.

## 2. Wine

A total of 40 commercial red wines (20 MBA wines and 20 MR wines) produced by twenty-eight Japanese wineries were used in this study. All the wines were made of grapes harvested in Japan and were stored in the dark at 15 °C until analysis. The vintages of these wines were from 2014 to 2018.

## 3. Chemical analysis

pH measurement was conducted using a pH meter (model F-71, Horiba, Co., Ltd., Kyoto, Japan). Titratable acidity (TA) was determined by titration with 0.1 M NaOH and is expressed as tartaric acid equivalent (g/L). Ethanol concentration was analysed on a gas chromatograph-flame ionization detector (GC 2014, Shimadzu, Co., Ltd., Kyoto, Japan) equipped with a packed column (polyethylene glycol 600, Shinwa Chemical Industries, Co., Ltd., Kyoto, Japan). The concentrations of free and bound SO<sub>2</sub> were determined by the aspiration method as described by Rankine and Pocock (1970). Molecular SO<sub>2</sub> was calculated using the following equation: molecular SO<sub>2</sub> = free SO<sub>2</sub>/(1+10<sup>(pH-1.83)</sup>) (Butzke, 2002). Glucose and fructose were determined by a Biosystems Y15 Enology Automatic Analysis System (Biosystems, S.A., Barcelona, Spain). The concentration of TPs was measured by the Folin-Ciocalteu method (Singleton and Rossi, 1965), and is expressed as gallic acid equivalent (mg/L). The analysis of tannins was carried out by the method of Harbertson *et al.* (2002) with some modifications. A protein solution (1 mg/mL bovine serum albumin in buffer containing 200 mM acetic acid and 170 mM NaCl adjusted to pH 4.9 with NaOH) for tannin precipitation was prepared according to the described method. A 1 mL aliquot of the protein solution was dispensed into a 2.0 mL microfuge tube, and then 0.5 mL of wine was added. After incubation for 15 min at room temperature, the mixture was centrifuged for 5 min at 13,500 g and the supernatant was obtained. The concentration of phenolics in the supernatant was measured by the Folin-Ciocalteu method. The concentration of tannins was calculated by subtracting the concentration of phenolics in the supernatant from the concentration of TPs and is expressed as gallic acid equivalent (mg/L). All measurements were replicated three times.

## 4. Pellicle formation and measurement

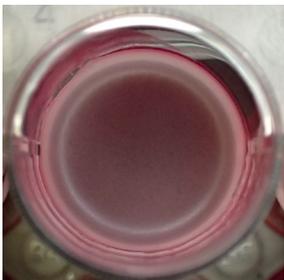
The wine samples were subjected to filter sterilisation with a 0.2 µm PTFE membrane filter (Advantec Toyo, Co., Ltd., Tokyo, Japan). Pellicle formation or other microorganism growth were not observed in the wine samples without the addition of pellicle yeast. Furthermore, it was confirmed that the concentrations of phenolics in the wine samples did not change after the filtration treatment. The pellicle formation test was performed as described by Moreno-García *et al.* (2018). A 0.2 mL aliquot of cell suspension was added into a well of a 24-well polystyrene microplate and then mixed with 1.8 mL of wine sample. Instead of the wine sample, an equal amount of flor medium was added as a comparison medium. In addition, the flor medium containing cinnamic acid at the final concentration of 60 mg/L was also used. The plates were photographed after static incubation for three days at 30 °C. The degree of pellicle formation was judged based on the criteria shown in Table 1. In our preliminary experiment, the amount of pellicle statistically differed between ±, + and ++ by Kruskal-Wallis test ( $p < 0.05$ ) (data not shown). The amount of ++ pellicle was significantly larger than that of + pellicle (data not shown); for this reason, the wine samples that had ++ pellicle were grouped into ‘pellicle wine’, and the rest into ‘non-pellicle wine’. After static incubation for five days at 30 °C, the content in each well was harvested into a 2.0 mL microcentrifuge tube by pipetting. The biomass was collected by centrifugation (14,200 g, 10 min), washed once with sterile distilled water and dried overnight at 50 °C. Then, total biomass (dry weight) was determined. This experiment was carried out three times independently.

A 0.2 mL aliquot of pellicle yeast cell suspension was added to 1.8 mL of MBA wine after filter sterilisation. After static incubation for three days at 30 °C, typical pellicles in each degree of pellicle formation were photographed and the amount of each pellicle was determined. The amount of pellicle statistically differed between ±, + and ++ by Kruskal-Wallis test ( $p < 0.05$ ) (data not shown).

## 5. Statistical analysis

Correlation analysis between wine components or between wine components and total biomass was performed by Pearson’s correlation test. Principal component analysis (PCA) was performed to analyse the relationships between wine components and pellicle formation.

**TABLE 1.** Criteria for pellicle formation degree

Judge	Pellicle	The degree of pellicle formation
–		No pellicle formation
±		a very thin pellicle was formed on some portion of the wine surface
+		a very thin pellicle was formed on the entire wine surface
++		a very thick and well-defined pellicle was formed on the entire wine surface

One-way ANOVA was used to investigate differences in wine components based on grape cultivar and pellicle formation. All statistical analyses of experimental data were carried out by using JMP 13 statistical software (SAS Institute, Inc., Cary, NC, USA).

## RESULTS AND DISCUSSION

### 1. Pellicle formation in wines

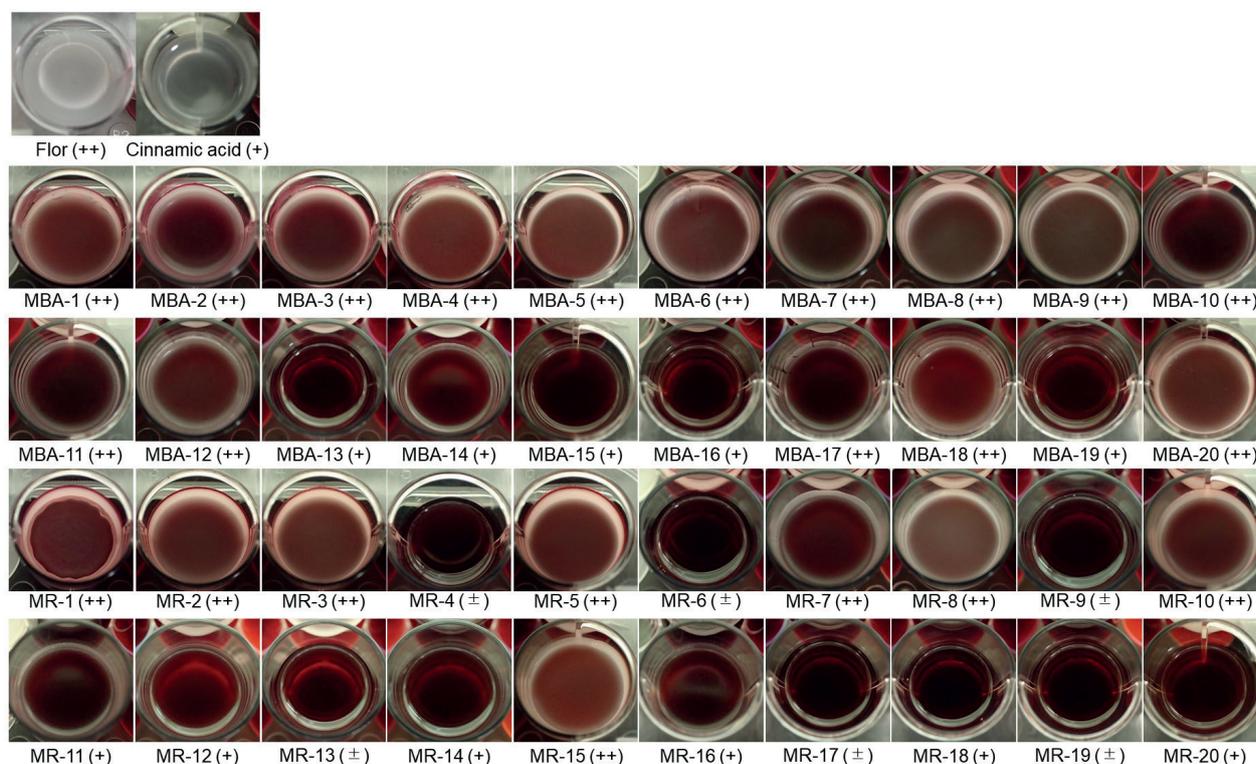
Immediately after the yeast cells were added to the medium, it was observed that the entire medium was turbid and the yeast cells were diffused. After static incubation for one day, a very thin pellicle

was observed on the surface of some wine samples. The photographs of pellicles formed on MBA or MR wine surfaces after incubation for three days are shown in Figure 1. Very thick and well-defined pellicles (shown by symbol ++ ) were formed on the surfaces of 15 of the 20 MBA wines. The ++ pellicle was also formed on the flor medium surface. The pellicle on the flor medium surface was slightly thin, and the bottom of the well could be seen by looking through the pellicle. The pellicle on the MBA wine surfaces was thicker than that on the flor medium surface, and the bottom of the well could not be seen by looking through the pellicle.

Thin pellicles (shown by symbol +) were formed on the entire surface of the remaining five MBA wines. On the other hand, ++ pellicle was noted on the surfaces of eight of the 20 MR wines. Of the remaining 12 MR wines, + pellicles were formed on the entire surfaces of six MR wines and some portions of the surfaces of the other six MR wines (shown by symbol ±). After incubation for five days, pellicle growth from + to ++ progressed and ++ pellicles were formed on the surfaces of all MBA wines except one (MBA-13). For MBA-13, the + pellicle observed on day 3 could not be observed on day 5. Pellicle growth was also noted and ++ pellicles were formed on the surfaces of 14 of 20 MR wines on day 5. Of the remaining six MR wines, the ± pellicles observed on day 3 did not change on the surfaces of two MR wines (MR-4 and 9) and pellicle growth was not noted. Whereas the ± or + pellicles were observed on day 3 were not observed on day 5 for the other four MR wines (MR-6, 13, 18 and 19). It was reported that the MIC range of cinnamic acid against pellicle yeasts was from 50 to 100 mg/L (Yajima *et al.*, 1998). In the presence of cinnamic acid, + pellicle was formed on day 3, and no pellicle growth was apparent on day 5. A very small amount of cell sediment was observed on the bottom of the well on day 5. Cinnamic acid could inhibit the pellicle yeast proliferation or

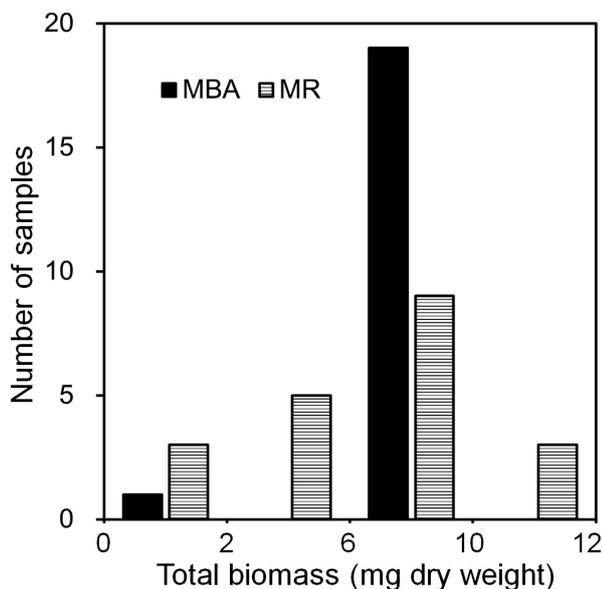
the pellicle development at the concentration of 60 mg/L. It was considered that inoculated pellicle yeast precipitated without forming a pellicle in the presence of cinnamic acid. After incubation for 5 days, total biomass (dry weight) was determined. In this study, the biomass was collected without separating the pellicle and the cell sediment (non-pellicle) because it was difficult to separate these. However, we considered that pellicle would account for a large portion of total biomass because the amount of cell sediment was very small. For instance, the amount of cell sediment in the cinnamic acid medium was 0.97 mg. Except for one MBA wine, total biomass in MBA wines ranged from 6.0 to 10 mg (Figure 2) and was two to three times greater than that in the flor medium (3.23 mg). Total biomass in MR wines ranged from 1.01 to 11.2 mg, and there was variation among the MR wines. The means of total biomass in ‘non-pellicle wines’ and ‘pellicle wines’ were  $1.56 \pm 0.80$  mg and  $8.07 \pm 1.42$  mg, respectively.

A 0.2 mL aliquot of pellicle yeast cell suspension was added to 1.8 mL each of MBA and MR wines after filter sterilisation. The flor media containing either 60 mg/L cinnamic acid or not were also used. After static incubation for three days at 30 °C, the wells were photographed and the degree of pellicle formation was judged.



**FIGURE 1.** Pellicle formation in MBA and MR wines after incubation for three days.

The symbols in parentheses show the degree of pellicle formation ( $\pm$ : a very thin pellicle was formed on some portion of the wine surface; +: a very thin pellicle was formed on the entire wine surface, and ++: a very thick and well-defined pellicle was formed on the entire wine surface).



**FIGURE 2.** Histogram of total biomass after incubation for five days at 30 °C.

Pellicle yeast cells were resuspended in 2 mL each of MBA and MR wines after filter sterilisation. After static incubation for five days at 30 °C, the content in each well was harvested and then total biomass (mg dry weight) was determined.

As far as we know, there are no reports of differences in pellicle formation among wine grape cultivars. In this study, we found that the number of pellicle MBA wines is significantly larger than that of pellicle MR wines. Almost all MBA wines formed a pellicle. The fact that MBA wine more easily forms a pellicle than MR wine is supported by this experimental finding. Such differences indicate that the risk of pellicle formation during winemaking may differ among wine grape cultivars. Furthermore, the difference in pellicle formation may be attributed to differences in chemical components between wine grape cultivars.

## 2. Wine components and their influences on pellicle formation

Table 2 shows the mean pH and concentrations of chemical components in MBA and MR wines. It is known that molecular SO<sub>2</sub> has antimicrobial

activity, and it is proposed that the concentration of molecular SO<sub>2</sub> needed to inhibit microbial spoilage ranges from 0.5 to 0.8 mg/L. To confirm whether SO<sub>2</sub> is involved in pellicle formation, the concentration of molecular SO<sub>2</sub> in the wine samples was determined. The mean concentration values of molecular SO<sub>2</sub> in MBA and MR wines are 0.126 and 0.091 mg/L, respectively (Table 2). The maximum concentration of molecular SO<sub>2</sub> is 0.381 mg/L and is lower than 0.5 mg/L. This result suggests that SO<sub>2</sub> does not influence pellicle formation.

Correlation analysis was performed to uncover the relationship between wine components and/or total biomass, and the correlation coefficients are shown in Table 3. Among the wine components, a strong positive correlation was found between TPs and tannins ( $R = 0.835$ ). A positive correlation between the concentrations of TPs and tannins have been reported in some grape cultivars, including MBA and MR (Ichikawa *et al.*, 2011), consistent with our result. In addition, significant positive correlations were noted between ethanol concentration and the concentrations of TPs and tannins ( $R = 0.515$  and  $0.527$ , respectively). The extraction of phenolics in grape tissues into wine is improved by ethanol produced during alcohol fermentation. Singleton and Draper (1964) reported that the concentration of extractable tannins was increased by increasing ethanol concentration in wine. On the other hand, a weak correlation between pH and Glc + Fru concentration was observed ( $R = 0.367$ ). As the concentrations of Glc + Fru in two MBA wines were significantly higher than the mean glucose and fructose concentrations, these concentration data were considered outliers. When these two concentrations data were excluded from the regression analysis, the correlation coefficient became 0.014. Therefore, it was considered that no correlation exists between pH and Glc + Fru concentration. Free SO<sub>2</sub> had weak negative correlations with the concentrations of ethanol and tannins. Free SO<sub>2</sub> may inhibit alcohol fermentation by yeasts with its antibacterial ability. It is known that tannins react with SO<sub>2</sub> (Ma *et al.*, 2018), and the concentration of free SO<sub>2</sub> may have decreased by reacting with tannins in the wines.

In general, microbial proliferation is inhibited in acidic pH. Iimura *et al.* (1980) measured pellicle formation in flor medium at the pH range of 2.5 to 6.5. The pellicle formation was most promoted in the pH range of 3.0 to 3.5 but was suppressed by further increases of pH. Although the pH of almost all of the wine samples used in this study

**TABLE 2.** Mean pH and concentrations of chemical components in MBA (20) and MR (20) wines.

	MBA	MR
pH	3.76 (3.39–4.01)	3.66 (3.41–4.01)
TA (g/L) <sup>a</sup>	5.92 (4.40–7.42)	5.32 (4.30–6.32)
Free SO <sub>2</sub> (mg/L)	10.7 (2.93–16.8)	6.27 (0.80–13.6)
Bound SO <sub>2</sub> (mg/L)	60.6 (9.07–123)	45.2 (6.93–113)
Molecular SO <sub>2</sub> (mg/L) <sup>b</sup>	0.126 (0.041–0.386)	0.091 (0.009–0.236)
Ethanol (% v/v)	12.4 (11.3–13.6)	13.0 (12.1–14.2)
Glc + Fru (g/L)	0.636 (0.044–4.40)	0.349 (0.062–0.800)
TPs (mg/L) <sup>c</sup>	1470 (959–2045)	2084 (1189–3170)
Tannins (mg/L) <sup>d</sup>	139 (ND–383)	570 (120–893)

Values in parentheses are minimum and maximum values.

<sup>a</sup>: Titratable acidity (TA) is expressed as tartaric acid equivalent.

<sup>b</sup>: Molecular SO<sub>2</sub> concentration was calculated using the following equation: molecular SO<sub>2</sub> = free SO<sub>2</sub>/(1+10<sup>(pH-1.83)</sup>)

<sup>c</sup> and <sup>d</sup>: TPs and tannins are expressed as gallic acid equivalent.

**TABLE 3.** Correlation coefficients between wine components and/or total biomass.

	pH	TA	Free SO <sub>2</sub>	Bound SO <sub>2</sub>	Molecular SO <sub>2</sub>	Ethanol	Glc + Fru	TPs	Tannins
pH	1								
TA	-0.195	1							
Free SO <sub>2</sub>	-0.131	0.284	1						
Bound SO <sub>2</sub>	-0.202	0.340*	0.634***	1					
Molecular SO <sub>2</sub>	-0.252	0.249	0.759***	0.702***	1				
Ethanol	-0.019	-0.034	-0.375*	-0.194	-0.267	1			
Glc + Fru	0.367*	-0.135	-0.013	-0.025	-0.032	-0.092	1		
TPs	-0.076	-0.029	-0.130	0.007	-0.049	0.515**	-0.107	1	
Tannins	-0.004	-0.126	-0.405**	-0.164	-0.250	0.527**	-0.170	0.835**	1
Total biomass	-0.036	0.061	-0.084	-0.103	-0.070	-0.327*	0.023	-0.170	-0.253

Correlation analysis between wine components for both MBA and MR and/or total biomass was performed by Pearson's correlation test. Total biomass was determined after static incubation for five days. A single asterisk (\*) and double asterisks (\*\*) indicate significant correlations at the levels of 5 % and 1 %, respectively.

was in the range of 3.5 to 4.0, the correlation between pH and total biomass was not evident in this pH range. Zara *et al.* (2005) described that the increase of cell surface hydrophobicity and the cell aggregation of pellicle yeasts began when sugar concentration decreased to approximately 2 g/L. When sugar concentration in the medium was higher than 2 g/L, pellicle formation was inhibited because pellicle yeasts used glucose primarily as the carbon source. The total glucose and fructose concentrations in the wine samples were very low except for two MBA wines (2.45 and 4.4 g/L). Therefore, it was considered that sugar did not affect the pellicle formation in this concentration range.

On the other hand, a weak negative correlation was noted between ethanol and total biomass. It is known that pellicle yeasts assimilate ethanol as the carbon source in the absence of sugar and proliferate. Alexandre *et al.* (1999) discussed that the higher the ethanol concentration, the greater the growth of pellicle, and this was manifested in cases of up to 10 % ethanol concentration. On the other hand, Jiménez and Benítez (1986) reported that pellicle yeast proliferation was decreased in the range of 6 % to 10 % ethanol concentration. In our preliminary experiments, we found that pellicle yeast proliferation was significantly inhibited in flor medium containing 12 % ethanol

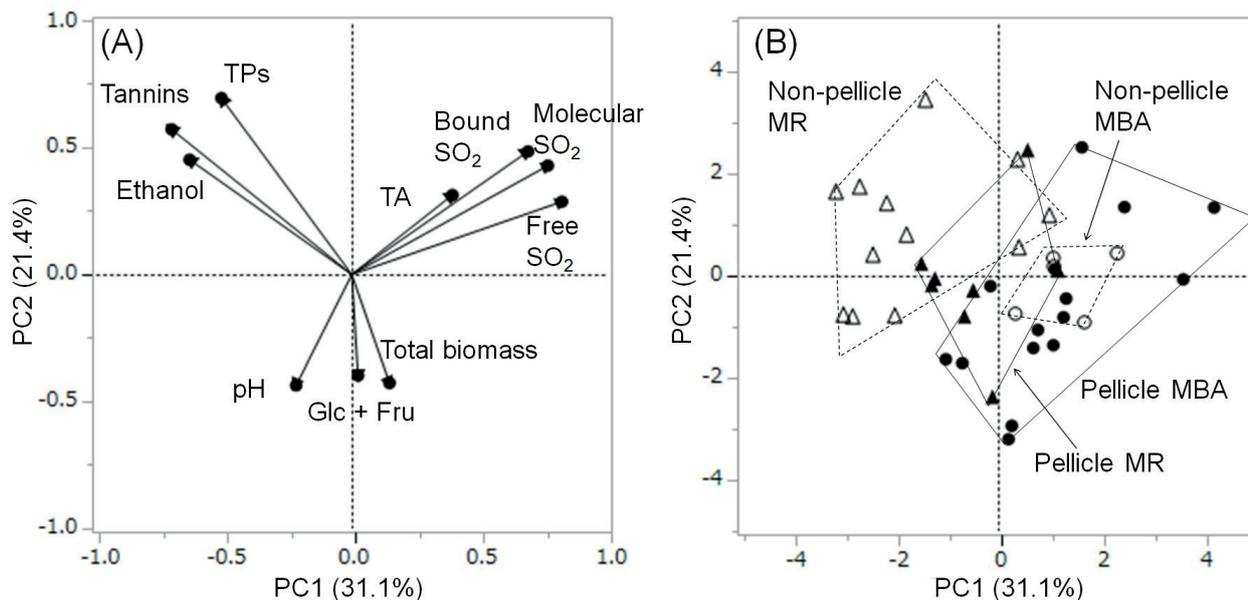
compared with flor medium containing 6 % ethanol (data not shown). Ethanol may prevent pellicle formation by inhibiting pellicle yeast proliferation. In the correlation analysis of wine components and total biomass, wine components having a strong influence on pellicle formation were not noted. This correlation analysis revealed only the influence of individual wine components on pellicle formation. Wine is a complex matrix and several wine components may affect pellicle formation in a complicated manner. To investigate the multiple influences of wine components on pellicle formation, other statistical analyses were performed.

### 3. Principal component analysis (PCA)

PCA analysis was performed on data generated from the chemical analysis of the wines. The first three principal components (PCs) with eigenvalues above 1 accounted for 31.1 %, 21.4% and 13.0 % of the variance, respectively, for the total variance of 65.5 %. The loading plot of the first and second components is shown in Figure 3A. The concentration of molecular SO<sub>2</sub> is increased at lower wine pH. The higher the TA, the lower the pH. The vectors of SO<sub>2</sub> (free, bound and molecular forms) and TA are pointed toward the upper right direction, and the vector of pH is pointed toward the opposite (lower left) direction.

This relationship of SO<sub>2</sub> and TA with pH was consistent with the theory. On other hand, the vector of total biomass is pointed toward the lower right direction, and the vectors of ethanol, TPs and tannins are pointed toward the opposite (upper left) direction. From these results, we speculate that the concentrations of ethanol, TPs and tannins may be related to total biomass, that is, pellicle formation.

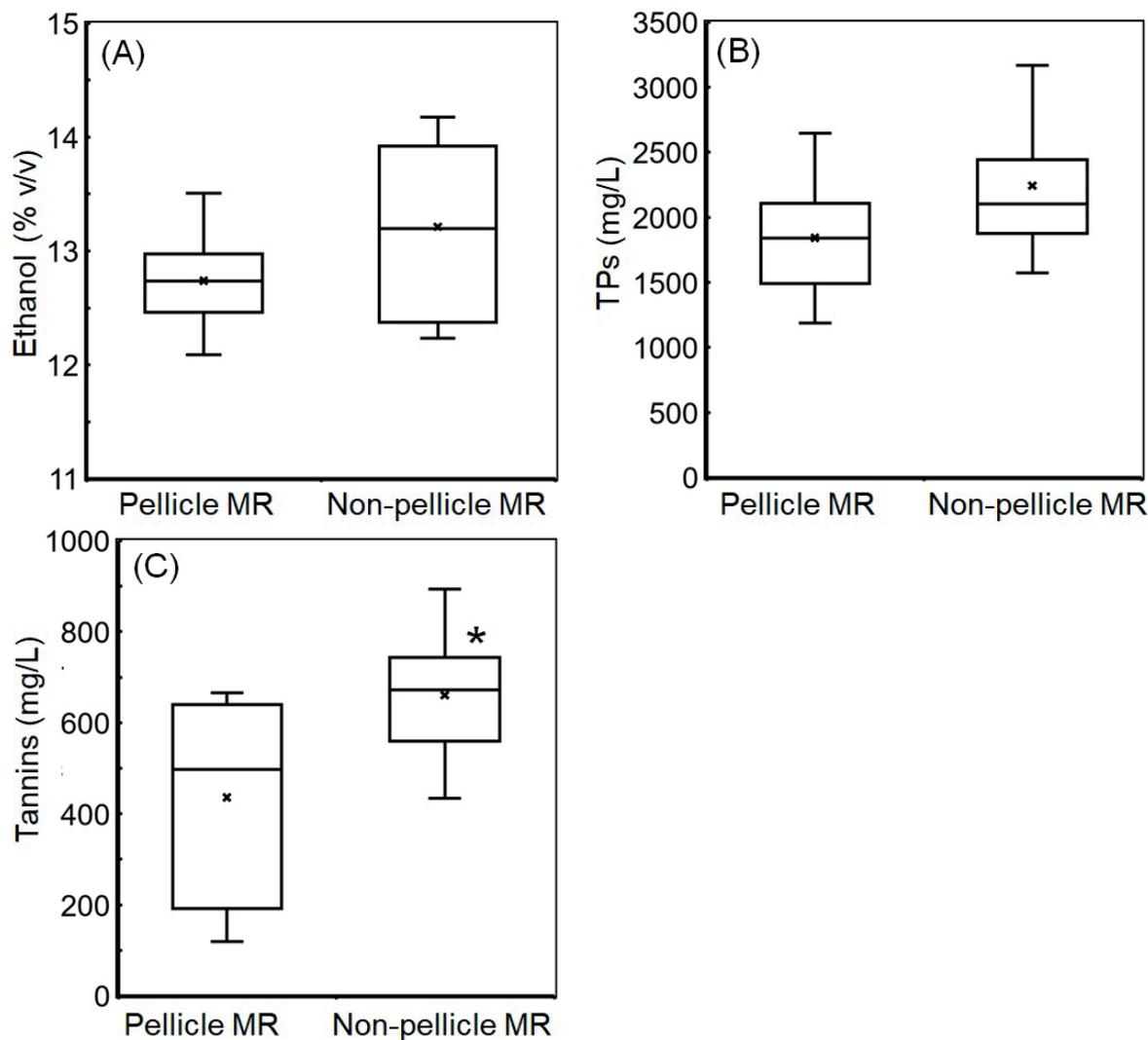
In Figure 3B, wines classified into ‘pellicle wine’ and ‘non-pellicle wine’ after incubation for three days are represented by closed and open symbols, respectively. MBA and MR wines are represented by circles and triangles, respectively. When the scores for each wine sample were examined in a two-dimensional plot of the first two PCs, some clustering of wine samples based on pellicle formation and grape cultivar was observed. On the whole, the scores for non-pellicle wines and pellicle wines were distributed in the upper left and lower right directions, respectively. This means that the concentrations of ethanol, TPs, and tannins may inhibit pellicle formation. Furthermore, the MR wines were clustered loosely in the non-pellicle and pellicle groups. Figure 4 shows the box plots of the concentrations of ethanol, TPs, and tannins in the non-pellicle and pellicle MR wines. The concentrations of ethanol and TPs in the non-pellicle MR wines



**FIGURE 3.** Principal component analysis (PCA) of MBA and MR wines.

(A) Plot of loadings of PC1 versus PC2.

(B) Plot of scores of PC1 versus PC2. Wines classified into ‘pellicle wine’ and ‘non-pellicle wine’ after incubation for three days are represented by closed and open symbols, respectively. MBA and MR wines are shown by circles and triangles, respectively.



**FIGURE 4.** Box plots of concentrations of ethanol (A), TPs (B) and tannins (C) in pellicle and non-pellicle MR wines.

Asterisk (\*) indicates a significant correlation at the level of 5 %.

**TABLE 4.** *P*-Values for one-way ANOVA between MBA/MR wines and non-pellicle and pellicle wines.

	MBA/MR	Non-pellicle/pellicle
pH	0.44	0.96
TA (g/L)	0.0027**	0.43
Free SO <sub>2</sub> (mg/L)	0.0016**	0.21
Bound SO <sub>2</sub> (mg/L)	0.11	0.62
Molecular SO <sub>2</sub> (mg/L)	0.15	0.40
Ethanol (% v/v)	0.0058**	0.039*
Glc + Fru (g/L)	0.23	0.50
TPs (mg/L)	<0.001***	0.026*
Tannins (mg/L)	<0.001***	0.0019**

Single asterisk (\*), double asterisks (\*\*), and triple asterisks (\*\*\*) indicate significant correlations at the levels of 5 %, 1 % and 0.1 %, respectively.

tended to be higher than those in the pellicle MR wines (Figures 4A and 4B). Furthermore, the concentration of tannins in the non-pellicle MR wines (660 mg/L) was significantly higher than that in the pellicle MR wines (436 mg/L) (Figure 4C). The concentration of tannins in MBA wine was lower than 660 mg/L (Table 2). From the result that pellicle formation occurred in almost all MBA wines after incubation for five days, pellicle formation might have occurred in wines having lower than 660 mg/L tannin concentration. Ethanol exerts an inhibitory effect on pellicle yeast proliferation. Moreover, ethanol enhances the extraction of phenolics and tannins into wine. Ethanol may inhibit pellicle formation not only directly but also indirectly by increasing the concentrations of phenolics and tannins in wine.

Differences in pH and chemical components between MBA and MR wines and between non-pellicle and pellicle wines were analysed using one-way ANOVA (Table 4). Significant differences in the concentrations of ethanol, TPs, and tannins were found between MBA and MR wines as well as between non-pellicle and pellicle wines. In particular, the *p*-values of tannins were significantly low ( $p < 0.001$  and  $0.0019$ , respectively). The ease of pellicle formation in MBA wine may be attributed to the low concentration of tannins. One reason for the low concentration of tannins in MBA wines is the low concentration of tannins in MBA grapes. It was reported that the concentrations of tannins in seed and skin in MBA grape are roughly half of those in Cabernet-Sauvignon grape. Another reason is the extraction behaviour of tannins during the maceration process. Ichikawa *et al.* (2012) showed that the concentration of tannins in Cabernet-Sauvignon must increase with increasing ethanol concentration and become constant after reaching a maximum, whereas that in MBA must decrease rapidly after reaching a maximum, resulting in the low concentration of tannins in MBA wine. Avoiding pellicle yeast contamination and controlling pH and the concentrations of SO<sub>2</sub> and tannins are important to prevent pellicle formation.

There are many studies on the biological activities of wine phenolics, such as anti-oxidant, anti-inflammatory, and antimicrobial activities. On the other hand, there are few studies on the relationship between pellicle formation and phenolics and tannins in wine. Our results underscored the importance of phenolics and tannins in preventing pellicle formation by pellicle yeasts.

Wine contains various monomer phenolics, the concentrations of which differ depending on the kind of grape cultivar and the type of wine. Tannins, which are polymeric phenolics, have complex structures, and their compositions, molecular weights, and sizes also vary with the kind of grape cultivar and the type of wine (Jackson, 2014). Mekoue Nguela *et al.* (2019) reported that red wine polyphenols and tannins adsorbed yeast cells. Inoue *et al.* (2019) indicated that the adsorption properties of tannins changed with pH and ethanol concentration. Interestingly, the adsorption ability of tannins (Inoue *et al.*, 2019) and the cell surface hydrophobicity of pellicle yeasts (Iimura *et al.*, 1980) are high in the same pH range (pH 3.0 to 3.5). Tannins may have the ability to adsorb pellicle yeast cells and alter the pellicle yeast cells through adsorption. Further studies on the inhibition mechanism of phenolics and tannins and the multiple influences of wine components on pellicle formation should be pursued.

## CONCLUSIONS

We found out that MBA wine has a higher susceptibility to pellicle formation by a pellicle yeast than MR wine. This may be attributed to the low concentrations of ethanol, TPs, and tannins in MBA wine. We are currently investigating the inhibition mechanism of ethanol, TPs, and tannins on pellicle formation in detail.

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