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New ways of grape pomaces valorization: production of functional beverages or nutraceuticals

Patricia Taillandier[1], Nathalie Barakat[1], Sandra Beaufort[1], Isaura Caceres[1], Jalloul Bouajila[1], Youssef El Rayess[2]

[1] LGC, Laboratoire de Génie Chimique, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France
[2] Department of Agriculture and Food Engineering, School of Engineering, Holy Spirit University of Kaslik, Jounieh, Lebanon

Abstract. The wine industry generates each year 20 million tons of by-products. Among them grape pomaces represent a big part that can be considered as a source of potentially bioactive molecules such as polyphenols. Kombucha fermentation is an ancestral process which allow to increase the biological properties of tea by the action of a microbial consortium formed by yeasts and bacteria called SCOBY. The obtained beverage belongs to the field of healthy food for which the interest of consumers is growing. The objective of this work was to develop new products made from grape pomaces fermented by a Kombucha SCOBY such as functional beverage, nutraceuticals or cosmetic ingredients. The work was focused on grape pomaces originated from red winemaking. In a first step several parameters were varied during the fermentation process of pomace infusions in order to optimise it: temperature, pomace concentration, sugar concentration, temperature, duration... The fermentation kinetics and final composition of grape Kombucha were monitored. From this fermented grape pomace infusion 2 alternatives have then been considered in a second step. The product can be directly consumed as functional beverage; an ethanolic extract can be prepared in order to use it as nutraceutical or cosmetic ingredient. In both cases the following biological activities were assessed *in vitro* at the beginning andat the end of fermentation: anti-oxydant, anti-diabetic and anti-inflammatory. For the use as beverage a sensoryevaluation was performed. For all fermentation conditions the biological activities were increased, at least by a factor 2, at the end of fermentation compared to the non-fermented grape pomaces infusions. This work demonstrated the feasibility of valorisation of grape pomace by Kombucha fermentation and the robustness of the process. Indeed it is addressing the challenge of transforming wastes into useful products that can be re- used in a circular economy perspective necessary for the wine industry.

1. Introduction

The valorization of agro-industrial waste has seen growing interest in recent decades, largely due to environmental and economic concerns. The wine industry, in particular, produces substantial amounts of waste each year, including grape pomace, vine shoots, stalks, wine lees, and filtration cakes. It is estimated that producing a liter of wine generates between 0.3 and 0.5 kg of by-products (Nanni *et al.*, 2021). These by-products generally have an acidic pH, are rich in polyphenols, and have low concentrations of micronutrients and heavy metals. Given their chemical composition, wine by-products can be processed and repurposed for a variety of uses, such as composting, animal feed, extracting bioactive compounds, and producing biodegradable packaging, as well as additives for the food and cosmetic industries (Bordiga *et al.*, 2019).

Kombucha, a traditional fermented beverage made from sweetened tea and a symbiotic culture of bacteria and yeast (SCOBY), has gained significant popularity in recent years. During the fermentation process, yeasts convert sugars into ethanol, which is subsequentlytransformed into acetic acid by acetic acid bacteria. The resulting biofilm, often referred to as "tea fungus," is a byproduct of cellulose synthesis by the microorganisms involved (Villarreal-Soto *et al.*, 2018). Kombucha is credited with a variety of health benefits, including antioxidant, antimicrobial, antihypertensive, and anti- carcinogenic effects, due to its rich content of bioactive compounds. The therapeutic potential of the beverage, and the need to valorize agro-industrial residues motivated scientists to explore the fermentation of non- tea substrates with the Kombucha inoculum. The rawmaterial used to produce Kombucha analogues can be categorized into fruits and vegetables,

plants and herbs, dairy, bakery, and industrial by-products. The valorization of agro-industrial residues by the Kombucha inoculum was explored on different items such as leaves, peels, cascara, kernels, broth, and wastewater. An improvement of the phenolic profile and enhancement of some biological activities was obtained as a result of Kombucha fermentation (Barakat *et al.*, 2022). Despite these developments, the use of grape pomace, a key by-product of winemaking, as a substrate for Kombucha fermentation remains unexplored.

Given the lack of research on using grape pomace as a Kombucha substrate, our study aimed to explore the potential of fermenting grape pomace with Kombucha cultures to create a healthy beverage or active extracts. We experimented with various fermentation parameters, including sucrose levels, fermentation duration, and temperature, to optimize the process.

2. Materials and methods

2.1. Preparation of grape pomace kombucha

Grape pomace was collected from a local winery in Lebanon. It was made of different grape varietiesincluding Cabernet sauvignon, Cabernet franc, Syrah and Merlot. The material was dried using air dryer at low temperature (30°C), grinded using Robot Coupe R5 plus and stored in polyethylene bags at -18°C. The Kombucha inoculum was obtained from the website je.mange- vivant.com and reproduced by the backslopping method as described by Villarreal-Soto et al., (2019). Different batches of grape pomace Kombucha were prepared. The same procedure followed in the preparation of tea Kombucha was followed to produce grape pomace Kombucha, with a few modifications. Pomace (100 g/L dry weight) was added to the heated and sweetened water (20 or 35 g/L sucrose) and was infused at 80°C for 15-20 minutes. After cooling to 30°C, Ammonium sulfate (0.20-0.25 g/L), Kombucha biofilm (SCOBY) and Kombucha tea were added to the infusion. Fermentation was carried at 20 or 25°C and for 7 or 10 days depending on the sample.

2.2. Quantification of sugars, acetic acid and ethanol

High performance liquid chromatography (HPLC, ThermoScientific, France) analysis was used to study the kinetics grape pomace Kombucha fermentation. Samples were centrifuged at 10000 rpm for 5 minutes(Fisherbrand Centrifuge, Illinois, USA). The supernatant was then filtered through a 0.45 µm membrane filter (Fisherbrand, PTFE) into vials and diluted with deionizedwater (1:10).

Quantitative analysis of glucose, fructose, sucrose, acetic acid, and ethanol was performed using Hpx87h aminex biorad column (300 x 7.8 mm) thermostated at 50°C; in addition to a refractive index detector at 210 nm and regulated at 40°C. The elution was done with a constant flow rate of 0.6 mL/min and using a 10 mM sulfuric acid solution as a solvent (pH 2.2). 20 µL of sample were automatically injected into the equipement for analysis. Compounds were quantified through standard curves (expressed in g/L). Standard solutions were prepared with de-ionized water at concentrations ranging between 0 and 15 g/L. All measurements were done in triplicates.

2.3. Quantification of polyphenols

The concentration of total polyphenols was determined by following the Folin-Ciocalteu colorimetric method (Ribéreau-Gayon *et al*., 2006). Phenolic compounds oxidized by the Folin-Ciocalteu reagent composed of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PW_{12}O_{40}$).

Samples were diluted (1:10) with distilled water. Standards solutions of gallic acid were prepared at concentrations of (0, 10, 25, 50 et 80 mg/L). The analysis was carried in a 96-well microplate.

2.4. Antioxidant activity of grape pomace kombucha

The antioxidant potential was evaluated by measuring the scavenging activity against 1, 1-diphenyl- 2-picrylhydrazyl free radicals (DPPH) as described by (Pure & Pure, 2016) with a few modifications. The principle of the DPPH assay is based on the neutralization of free radicals, causing a discoloration in the dark violet color. A 96-well microplate was used to conduct the experiment. Liquid samples (grape Kombucha) were diluted with distilled water (1:10). Ascorbic acid was used as a positive control. Different volumes of the sample were analyzed to determine the IC_{50} value (half maximal inhibitory concentration).

2.5. Anti-inflammatory and antidiabetic activities of grape pomace kombucha ethanolic extracts

Grape pomace Kombucha samples were evaporated through a vacuum rotary evaporator at 35 °C to obtain a dry extract. The dry extract was collected, weighed, and stored at ambient temperature. An ethanolic extraction by dissolving 1 g of dry extract in 20 mL ethanol and placing the mixture once again in the vacuum rotary evaporator (35°C) until dryness.

2.5.1. Anti-inflammatory activity

The anti-inflammatory potential was evaluated by the method described by Villarreal-Soto *et al*. (2019), where the capacity of inhibiting the activity of 15-lipoxygenase enzyme (15-LOX). The test was conducted in a 96-well plate containing 150 µL of 100 mM phosphate buffer (pH 7.4), 20 µL of the extract solution, 60 µL of linoleic acid (3.5 mmol/L), and 20 µL of 15-LOX (soybean). The mixture was then incubated at 25°C for 10 minutes in a Multiskan Go spectrophotometer and the absorbance was measured at 234 nm. A blank experiment was performed using the same procedure without the extract. The anti-

inflammatory activity was defined as the percentage of inhibition of the 15-LOX enzyme. Nordihydroguaiaretic acid (NDGA) was used as a control, due to its high ability to obstruct the enzyme.

2.5.2. Antidiabetic activity

The antidiabetic activity of grape pomace Kombucha was evaluated by the inhibitory assay of α-amylase and α-glucosidase enzymes. The α-amylase inhibitory activity was determined as per the method described by (Mccue *et al*., 2005), with some modifications. The α- glucosidase inhibitory assay protocol followed was described by Kim et al. (2008). Acarbose was used as a standard inhibitor.

3. Results and discussion

The quantity of total sugars includes the sum of saccharose, glucose and fructose. As exposed in figure 1, sugars are gradually consumed throughout fermentation.

During the first 3 days, or what is commonly known as the lag phase of fermentation, the consumption rate was slower than the remaining days (Lopes *et al*., 2020). On average, the momentum for the utilization of sugars in grape pomace Kombucha ranged between 1.68 and 4.14 g. L^{-1}. day $^{-1}$, depending on the recipe. Samples fermented at 20ºC scored the slowest rates. During this period, carbon sources are typically used for microbial cell formation. A higher pace for sugar consumption is noticed between days 3 and 7. During this stage, carbon sources are typically used for energy instead of microbial cell formation (De-Filippis *et al*., 2018). In the last stage of fermentation (days 7-10), a drop in the utilization rate was observed in all samples. A similar observation in the decrease of sugar consumption after a certain time in tea Kombucha fermentation was noted by Lončar *et al*. (2014) and Sreeramulu *et al*. (2000) who attributed this finding to an acid shock that might impact the activity of microorganisms. Fermentation temperature seemed to impact the overall consumption rate of sugars .According to Lončar *et al*. (2014), higher fermentation temperatures would have a positive effect on reaction rates, and therefore would cause a higher consumption of sugars in Kombucha production.

Samples made with 20 g/L added sucrose reach final concentration of total sugars of 12 g/L (day 7) and 6 g/L (day 10) at 25 ºC, and 17 g/L (day 7) and 12 g/L (day 10) at 20 ºC. On the other hand, when 35 g/L of sucrose are added, the remaining total sugars account about 21 g/L and 15 g/L at 25 ºC (days 7 and 10 respectively), and about 32 g/L and 18 g/L (days 7 and 10 respectively) at20 ºC. On average, at 25 ºC, 60-68% of sugars are consumed after 7 days and 74-83% after 10 days. At 20 ºC, the consumption was relatively slower, ranging between 44 and 56 % on day 7 and 66-67% on day 10. Those findings are somehow not surprising considering the effect of fermentation parameters on the activity of microorganisms and consequently on sugar consumption (Lonc˘ar *et al*., 2006).

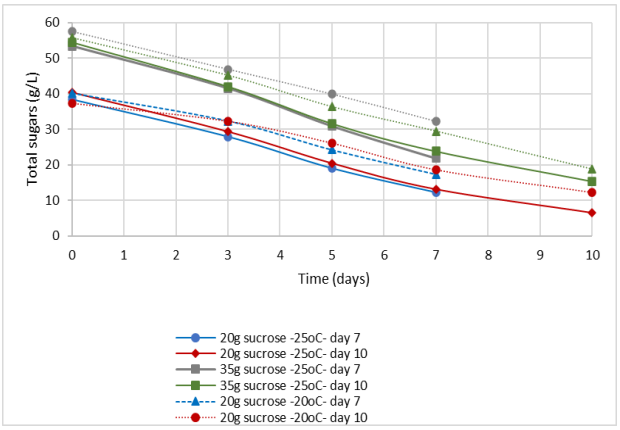

Figure 1. Consumption of sugars during fermentation.

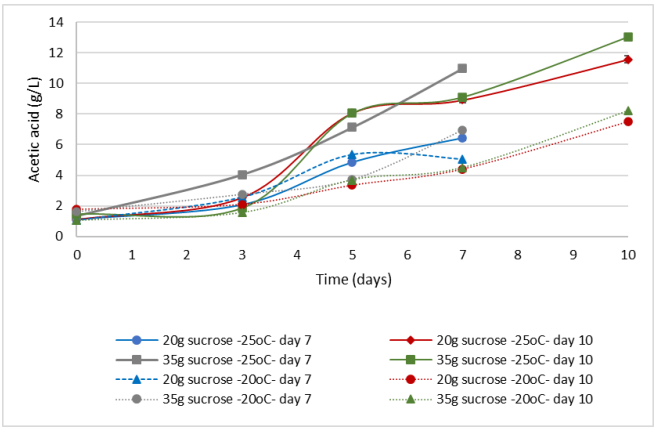

Figure 2. Acetic acid production during fermentation.

The production rate of acetic acid exhibited a similar pattern to sugar consumption. As shown in figure 2, during the first 3 days of fermentation, a very slow generation of acetic acid was observed. Acetic acid output increased between days 3 and 7. According to De- Filippis *et al*. (2018), this phase also known as the exponential growth period, is characterized by an increase in the number of acetic acid bacteria; resulting in more substrate consumption and, thus, more formation of microbial metabolism compounds. During the last stage of fermentation (days 7-10), the production rate of acetic acids decreased. This observation was in accordance with the drop noted in sugar utilization and is probably due to the disruption in the activity of microorganisms.

The production of acetic acid during Kombucha fermentation is usually attributed to the acetic acid bacteria found in the SCOBY. Acetic acid concentration increased in all samples regardless of the fermentation parameters; in fact, the augmentation ranged between 324% and 933%. Samples fermented for a longer period (10 days) and prepared with the highest concentration of sucrose (35g/L) were the highest in acetic acid. Sample GPK-3-L-100/35-10/25 contained around 13 g/L acetic acid by the end of fermentation.

This finding is also in accordance with what was observed by other authors; although there is some disparity in the values of acetic acid, the production pattern is somehow the same (Chakravorty *et al*., 2016; Jayabalan *et al*., 2007). Different production rates ofacetic acid might

be linked to more than one factor such as type of substrate used, production parameters, and microbial diversity of the inoculum.

Ethanol is produced during Kombucha fermentation by the yeasts present in the SCOBY. Traditionally, the generation of ethanol is characterized by an increase (until reaching a peak) followed by a decrease. As shown in figure 3, the concentration of ethanol rises gradually until the end of fermentation in all samples apart from the sample prepared with 35 g/L sucrose and fermented for 7 days at 25 ºC. In that specimen, ethanol content started dropping after reaching a maximal value of 9.3 g/L approximately on day 7. The absence of the ethanol decline might be attributed to the relatively short fermentation period (Chen & Liu, 2000; Neffe-Skocińska et al., 2017). Being a highly volatile compound, the interpretation of ethanol production rates throughout fermentation can be a bit complex. However, similarly to sugar consumption and acetic acid generation, a slow momentum was observed in the first 3 days of fermentation. Between days 3 and 7, a more intense production was detected in the majority of the samples . The decrease in ethanol concentration in some samples and its bioconversion into acetic acid were occurring at a higher rate than its formation.

Table 1. Concentration of total polyphenols andantioxidant activity of grape pomace kombucha.

Sample	Total polyphenols (GAE mg/L)			Antioxidant activity (IC50 mL/L)	
	Start	End	% Change	Start	End
20g sucrose -25ºC- day 7	198.75±6.54^{c}	357.19±8.25^{d}	78.72	2.54±0.06^{b}	1.15±0.02^{b}
20g sucrose -25ºC- day 10	187.08±7.21^{b}	245.95±5.13^{b}	31.47	2.45±0.07^{a}	1.33±0.02^{f}
35g sucrose -25ºC- day 7	246.19±5.21^{h}	360.1±0.51^{d}	46.27	2.68±0.03^{g}	1.26±0.02^{de}
35g sucrose -25ºC- day 10	175.62±4.32^{a}	183.92±7.51^{a}	4.73	2.65±0.08^{e}	1.23±0.01^{d}
20g sucrose -20ºC- day 7	211.28±9.21^{e}	507.14±9.21^{e}	140.03	2.62±0.19^{d}	1.08±0.03^{a}
20g sucrose -20ºC- day 10	213.41±4.28^{f}	340.11±4.21^{d}	59.38	2.58±0.09^{c}	1.19±0.1^{c}
35g sucrose -20ºC- day 7	201.62±3.23^{d}	483.17±3.18^{c}	139.64	2.55±0.02^{b}	1.25±0.05^{de}
35g sucrose -20ºC- day 10	234.88±5.27^{g}	335.71±1.33^{d}	42.93	2.67±0.05^{g}	1.26±0.07^{e}

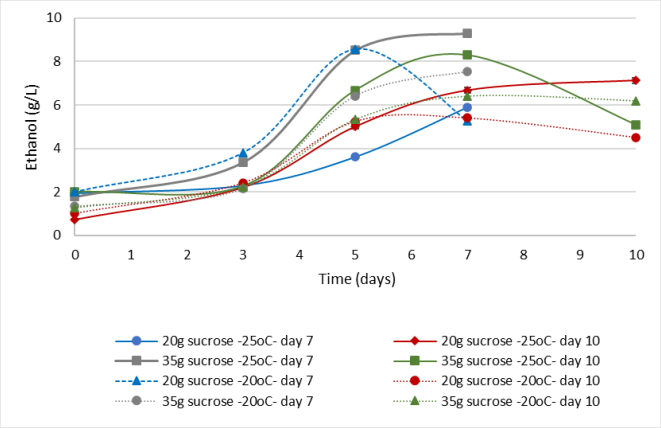

Figure 3. Ethanol production during fermentation.

The concentration of total polyphenols and the antioxidant activity are presented in table 1.

With regards to the quantification of total polyphenols, the richest sample was the sample containing 20 g/L ofadded sucrose and fermented for 7 days at 20ºC. This sample was also significantly different from all others ($p < 0.05$). Grape pomace Kombucha made with 35 g/Ladded sugar and fermented for 7 days at 20ºC scored the 2nd highest concentration of polyphenols and was statistically different as well ($p < 0.05$). Samples fermented for a longer duration (10 days) were poorer in polyphenols. Different studies have reported that ashorter fermentation period would result in a richer concentration of polyphenols in Kombucha products (Ayed *et al*., 2017; Muzaifa *et al*., 2021). The highest antioxidant potential (lowest IC_{50} value) was noted in the sample prepared with 20 g/L sucrose and fermented for 7 days at 20 ºC (which was also the richest sample in polyphenols). The same recipe fermented at 25 ºC ranked second, which suggests that time, temperature and added sucrose had the most impact on the inhibition of DPPH radical. According to Aspiyanto *et al*. (2016), Jakubczyk *et al*. (2020) and Muzaifa *et al*. (2021), fermentation time is the most influential parameter on the antioxidant potential; However, Zubaidah *et al*. (2018) found thatbrix levels impacted the scavenging potential of DPPH radicals.

The biological activities of ethanolic extracts were then evaluated. As shown in table figure 4, a significant increase (55-77%) in the inhibition of α-amylase was detected in all extracts. All grape pomace samples were statistically similar however they were different from unfermented grape pomace in terms of α-amylase inhibition. Moreover, the improvement of the antidiabetic potential of the beverage was accompanied with a drop in IC_{50} values. A similar pattern was obtained for α-glucosidase; however, the increase was higher (62-91%). Additionally, more resemblances were found among the samples. For example, extract obtained from the sample prepared with 35g/L of sucrose and fermented for 10days at 25 ºC (who scored the highest inhibition rate) was statistically similar to some samples while differing from others. The improvement of the antidiabetic potential of Kombucha products has been investigated by different authors. Watawana, *et al*. (2015) examined the effect of fermentation with SCOBY on the activity of starch

hydrolase enzyme in coffee Kombucha. The α-amylase and α-glucosidase inhibitory activities increased after the 5th day of fermentation; and a better inhibition was noticed for α-amylase. Furthermore, the antidiabetic activity was enhanced after inoculation with tea fungus in soymilk Kombucha Xia *et al*. (2019) and green andblack tea Kombucha (Kallel *et al*., 2012).

The inhibition rate of 15-lox was originally around 36%in unfermented grape pomace. The anti-inflammatory activity increased significantly in all grape pomace Kombucha samples (by 55-74%) reaching an inhibition up to 63% in some cases. Kombucha fermentation has been proven to improve the anti-inflammatory potential of different substrates. A two-time increase in the inhibition of 15-lox was obtained after inoculating yerba mate with SCOBY (Ziemlewska *et al*., 2021). In tea Kombucha extracts, a notable improvement was obtained as well. The inhibition rate of 15-lox was resembling to the effect Nordihydroguaiaretic acid (NDGA), a natural anti-inflammatory molecule (Villarreal-Soto *et al*., 2019).The values obtained are close to one another; however, we notice that the samples fermented at 20°C scored a better anti-inflammatory activity.

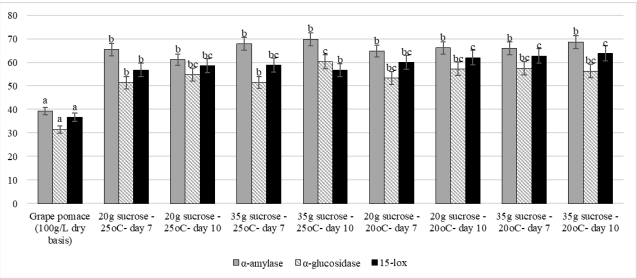

Figure 4. Antidiabetic and anti-inflammatoryactivities of grape pomace kombucha extracts.

4. Conclusion

The production of grape pomace kombucha can be considered as a successful solution to the valorization of wine by-products. Fermentation parameters can be optimized to yield a product with desired characteristics or an extract with biological activities. In terms of kinetics, grape pomace kombucha fermentation is characterized by a consumption of sugars, and a production of acetic acid and ethanol. An increase in the concentration of total polyphenols was observed. Moreover, an improvement in the antioxidant, antidiabetic and anti-inflammatory potential was found inthe beverage and in the ethanolic extracts compared to thenon fermented pomace infusion. A sensory analysis ofthe beverages has been performed showing that the aromatic profile should be improved with the use of flavor additives in order to ensure that the product isappealing to consumers.

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