Characterization of major volatile compounds in whey spirits produced by different distillation stages of fermented lactose-supplemented whey

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ABSTRACT

This research aimed to advance the understanding of acceptable sensory qualities of potable whey-based spirit from nonsupplemented, mid-supplemented, and high-supplemented whey samples by analyzing major volatile compounds during different stages of distillation (head, heart, and tail). The results demonstrated that commercial *Saccharomyces cerevisiae* strain in lactase-hydrolyzed whey showed rapid and complete sugar hydrolysis and efficient ethanol production in 24, 30, and 36 h on average, producing up to 29.5, 42.1, and 56.4 g/L of ethanol, respectively. The variations in titratable acidity, specific gravity, pH value, residual protein, sugar content, and alcohol yield were investigated during the fermentation. The total amount of volatile compound concentrations significantly decreased from the head (2,087–2,549 mg/L) to the tail whey spirits (890–1,407 mg/L). In the whey spirit, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methyl-1-propanol, 1-propanol, acetaldehyde, and ethyl acetate were the most prevalent dominant compounds, accounting for the largest proportion of total volatile compounds. The volatile compounds detected were far below the acceptable legal limit. The results suggest that high sensory qualities of potable whey-based spirits can be produced by fermentation of lactose-supplemented whey with *S. cerevisiae* cells.

Key words: whey, fermentation, whey spirit, volatile compound

INTRODUCTION

A significant proportion of whey is generated by the manufacture of cheese and casein, which contains valuable nutrients, but also poses a significant risk to the environment due to its high biological and chemical oxygen demand (Siso, 1996; Das et al., 2016; Ganju and Gogate, 2017). Particularly among small- and medium-sized cheesemakers in developing countries, whey remains an important waste product that is disposed either directly into water resources or into landfill sites. Several studies on alcohol production from fermented whey have provided strong evidence of environmental and economic benefits from this process. Although most of the nutritious proteins from whey are recovered, the lactose portion is mostly unused, and its disposal represents a severe environmental concern. The fermentation process reduces the biochemical oxygen demand by approximately 75% (Risner et al., 2018). Therefore, there is still scope for further work in terms of optimization value-added products, as well as the development of more economical and cost-effective strategies. The main uses of lactose include food ingredients, infant formula ingredients, alcohol and acid production, or tablet fillers. Numerous studies have attempted to develop new technologies to use whey as value-added chemicals and products such as oligosaccharides, enzymes, propionic acids, citric, lactosyl urea, lactic acid, and single-cell proteins (Athanasiadis et al., 2004; Li, et al., 2021; Shi, et al., 2021). At the industrial level, special attention has been paid to the production of starter culture, novel probiotic foods with improved characteristics, health-promoting whey drinks, biopolymers, and vinegar (Koutinas, et al., 2009; Dimitrellou et al., 2017; Zotta et al., 2020). The majority of the research papers have focused on its use as a substrate for large-scale production of supplementary fuel. Only a few studies have applied fermented whey to produce potable whey spirits for human consumption. Risner

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et al. (2019) reported that volatile aroma composition of potable distilled spirit from sweet and acid whey by *Kluyveromyces marxianus*. The craft spirit market has been expanding steadily for the last 10 yr (Lozier, 2017). Currently, several craft distilleries are producing commercial examples of milk vodka, such as Black Cow vodka (Black Cow, 2017), Vermont White vodka (Vermont White, 2016), and Dairy Distillery vodka (Dairy Distillery, 2021). This market is small but growing, and research into the aroma chemistry of whey spirit is needed to improve the quality and acceptability of these new products. The findings of current research have helped us to better understand the aroma chemistry of whey spirits and benefit the distilleries by improving the quality.

Alcohol fermentation is a complex metabolic process in which sugar is converted into energy, producing ethanol, carbon dioxide, and other byproducts that affect the chemical composition and sensory characteristics of fermented beverage (Buratti and Benedetti, 2016). Direct use of whey or whey permeate to produce ethanol is not considered economically viable. The lower sugar concentration leads to a lower ethanol yield (2–3%, vol/vol), making it a relatively expensive approach. Therefore, to maximize the final ethanol concentration, it is necessary to use high lactose concentration at the beginning of the fermentation process. The use of cheese whey powder, a concentrated form of cheese whey, and high concentration of lactose make it possible to give a better yield and a higher ethanol content before distillation (Oda and Nakamura, 2009; Das et al., 2016). *Saccharomyces cerevisiae* was proposed as the best option for industrial fermentation because its ability to accomplish complete fermentation of solutions with high-sugar contents produces ethanol yield on the high gravity level (20%, vol/vol) and rapid growth under anaerobic conditions (Guimarães et al., 2010; Das et al., 2016).

Vacuum distillation has been used for years in the manufacture of spirit production (McGee, 2009). Vacuum distillation provides the possibility to avoid protein denaturation of whey by lowering the boiling point, allowing for the possibility of recovering the residual soluble protein after distillation. The lower temperature means that the fermented whey is essentially heated less than it would be in a normal still, resulting in a fresher flavored whey spirit and less denatured protein at the end of the process. The distilled spirits obtained by distillation from fermented whey contain many major and minor compounds such as carbonyl compounds, esters, aldehydes, ethyl acetate, acetic acid, fatty acids, higher alcohols, and methanol. The major volatile compounds and their corresponding

contents in raw spirits depend on the yeast species, the raw materials fermented, and the distillation conditions. The limits of major volatile compounds found in distilled spirits (higher alcohols, carbonyl compounds, esters, and acetals) are imposed by the European Community Regulations 1576/89 (EEC, 1989; Geroyiannaki, et al., 2007). The majority of whey-use studies focus on maximizing ethanol production, and there is little literature on the volatile constituents found in distilled whey-based spirits.

Therefore, the primary aim of the current research was to advance the understanding of acceptable sensory qualities of potable whey-based spirits by analyzing the major volatile compounds from different distillation stages. This study also examined the effect of lactose levels, fermentation conditions, and distillation processes for the product of a whey-based spirit. Lactase was used to enable sugar utilization by *S. cerevisiae*, and we monitored titratable acidity (TA), pH, specific gravity (SG), protein content, and sugar concentration throughout the fermentation.

MATERIALS AND METHODS

Chemicals and Reagents

Whey produced during Swiss cheese production was obtained from the Key Laboratory of Dairy Science, Northeast Agricultural University, Harbin, China, and all whey samples were stored at -20°C until processing. Sweet whey was made using liquid bovine rennet (containing 95% chymosin and 5% pepsin) obtained from New England Cheesemaking Supply Company, as earlier described by Timón et al. (2014). Industrial alcohol active dry yeast *S. cerevisiae* (containing rhizopus, α -amylase, glucoamylase, phytase) was purchased from Angel Yeast Co. Ltd. Lactase (10,000 acid lactase unit/g, 37°C , pH 6) was bought from the Nanning Pangbo Bioengineering Co. Ltd. We purchased α -D-lactose monohydrate, D-galactose, and D-(+)-glucose compounds from Shanghai Gold Wheat Biological Technology Company, prepared from commercially available standards, purity grade $\geq 98\%$. Ethyl acetate, acetaldehyde, acetic acid, methanol, n-butanol, 1-propanol, 2-butanol, amyl alcohol, isobutyl alcohol, isoamyl alcohol, and 4-nanonol (GC standard purity) were purchased from Sigma-Aldrich as internal standards.

Whey Fermentation

Sweet whey was pasteurized at 63°C (145°F) for 30 min to avoid microbial contamination during fermenta-

tion. The pasteurized whey was defatted by centrifugation at $2,236 \times g$ for 10 min at room temperature. The skimmed whey was heated to 38°C to dissolve added lactose. Three different sugar concentrations were prepared by adding 0 (nonsupplemented whey; **NS**), 12 (mid-supplemented whey; **MS**), and 24 (high-supplemented whey; **HS**) grams of lactose up to 400 mL of skimmed whey to achieve lactose concentrations of 4.5, 7.5, and 10%, respectively. Subsequently, all of the samples prepared for incubation by adding 0.15 g of *S. cerevisiae* yeast (7×10^6 cells/mL) and lactase 360 acid lactase unit/g and placed in an incubator shaker (Duzhuang, Minhang) to optimize fermentation conditions for the production of ethanol. It was decided to first establish the incubation temperature, which was carried out at various temperatures (32, 34, 36, 38, and 40°C), without any agitation, and then at different agitation speeds (0, 50, 100, 200, and 300, rpm) performed at the chosen temperature. Finally, the determination of the optimal fermentation time for each sample was performed. During the fermentation process, TA, pH value, sugar analysis, SG, and alcohol yield were regularly monitored. After fermentation, the whey was centrifuged at $8,000 \times g$ for 10 min at 4°C, and the supernatants were distilled.

Sugar Analysis by Ion Chromatography

The lactose, glucose, and galactose contents of non-supplemented and lactose-supplemented whey samples were quantified by ion chromatography (**IC**) using a Metrohm 850 Professional IC (Ionenstrasse) equipped with an IC Amperometric Detector (Agilent Technologies). Metrohm 850 Professional IC was fitted with a Hamilton RCX-30-250/4.6 carbohydrate analysis column (Hamilton Company Headquarters). All the nonsupplemented and lactose-supplemented whey samples were centrifuged at $2,236 \times g$ at 4°C for 15 min to remove suspended solids before analysis. The whey samples from centrifuged supernatant were diluted 1:100 (vol/vol) with ultrapure water and filtered through a 0.22- μ m filter. The filtrates were placed on the sample processing rack and then directly injected into the IC system. Sodium hydroxide (10 mmol/L) and sodium acetate (0.5 mmol/L) were the eluents at a flow rate of 1.5 mL/min in the system. The injection volume was 20 μ L, the column temperature was 32°C, and the recording time was 45 min. Different concentration levels (0.25, 1, 5, 15, 25, 50, and 100 mg/L) of the standard sugars (glucose, galactose, and lactose) were used for each external calibration. Finally, sugar contents were quantified 3 times during fermentation at the beginning, during the process, and end of each

fermentation. Instrument control, data acquisition, and processing were performed by MagIC Net 3.3 software.

Protein Analysis

The total protein concentration of nonfermented and fermented whey samples was determined as earlier described (Smith et al., 1985) using BSA as a standard. Each sample was measured at 562 nm on a plate reader using the micro bicinchoninic protein assay kit. All samples were measured in triplicate. After distillation, the residual protein content in the fermented whey was recovered by 10-kDa UF for further analysis.

Determination of TA, pH, and Soluble Solids

The TA values of each whey sample were evaluated by titration with 0.1 *M* sodium hydroxide using phenolphthalein as an indicator expressed as % lactic acid. Twenty milliliters of distilled water and 10 mL of whey samples were added into a 50-mL beaker. Then, 3 drops of phenolphthalein were added to the sample and titrated with 0.1 *M* sodium hydroxide until the first color change persisted for 30 s. We calculated the TA as follows:

$$\text{TA (\%)} = \frac{100 \times 90 \times N \times mL}{1,000 \times v},$$

where *mL* represents the volume of 0.1 *M* NaOH used, *N* represents normality of 0.1 *M* NaOH, and *v* represents the volume of whey samples.

Analysis of pH monitoring values in whey samples was recorded with a pH meter (Sartorius-PB-10). Each experiment was carried out without adjusting the pH, and in all experiments, the pH was left to evolve freely from the initial value. The DM content of whey is relatively low, approximately 6.0 to 7.5%. Among the total solids in whey, over 75% is lactose. A digital density meter can provide a quick approach for measuring the SG or density of the whey. The SG of the solutions were measured using the densitometer DMA 4500 (Anton Paar).

Distillation

The fermented whey distillation was carried out using vacuum distillation according to the method of Zheng et al. (2014a,b). A total of 27 fresh whey spirits (9 head, 9 heart, 9 tail) were collected from 3 distillation stages of 3 different fermented whey. Distillation of each whey replicate was conducted using a 1-L rotary evaporator with a vacuum system using water

as the refrigerant (Shanghai Yuanhuai Industrial Co., Ltd.). The evaporator flask was filled with 300 mL of fermented whey to be distilled, and vapor temperature was kept below 52°C in the heating water bath. During the distillations, distillate fractions were collected in a 100-mL receiving flask.

The first distilled fraction (corresponding to the head whey spirit; **HdWS**) was collected for 6 min. Subsequently, the second distillation continued to collect the next 12 to 16 min; this fraction of the whey spirit was named heart whey spirit (**HtWS**). Finally, the last distillation continued for the next 12 to 16 min and was named tail whey spirit (**TWS**). The HtWS fraction was collected to avoid the presence of toxic compounds and undesired flavorants. Each distillate's ethanol content was measured using a hand-held ATC-RZ116 alcohol meter (Beijing Century Euron Co., Ltd.), which determined alcohol content based on the temperature-corrected densities of samples. The distillations were performed in triplicate. The collected distillate was stored in nonreactive, screw-top, amber vials crowned with a solid cap with a polytetrafluoroethylene liner and kept at -18°C until further analysis. The residue fraction (distilled fermented whey) was also kept for the recovery of residual soluble proteins.

Characterization of Volatile Compounds

In the distillation process, the same 3 fractions were separated from each fermented whey and analyzed by GC. Major volatile compounds of the spirits were analyzed by GC on an Agilent 7890A/5975C GC system fitted with a flame ionization detector (Agilent). A CP-Wax 57 CB capillary column (50 m × 0.25 mm, i.d. = 0.2 µm) from Agilent Technologies was used for the separation. The injector temperature was set at 230°C. The temperature of the detector was set to 250°C. The carrier gas was helium at a rate of 3 mL/min. We used 4-nonanol as the internal standard to calculate the quantity of the other compounds. The retention time of standards was used to identify the compounds, and the calibration curve for each compound was 10 to 1,000 mg/L. All samples were analyzed in triplicates.

Statistical Analysis

Data obtained were determined from 3 independent experiments. Analyses, including the quantification of sugar concentrations and volatile components, were run in at least 3 replicates. Data were analyzed using SPSS software (ver. 26.0, 2019), and the mean comparisons were conducted using the Tukey test in one-way ANOVA. Regression values were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Whey Fermentation

The fermentation process is a complex technological process that depends on many factors. It is necessary to optimize the incubation temperature, agitation rate, fermentation time, pH value, SG, pH value, and acidity to maximize the profits from the fermentation process. To determine the effects of incubation temperature, agitation speed, and fermentation time on ethanol production, 3 different sugar concentration whey samples were incubated at 32, 34, 36, and 38°C for 36 h. The different incubation temperatures showed a considerable difference between the samples (Figure 1a). Among them, fermented whey incubated at a temperature of 36°C showed a faster sugar-utilization rate compared with others. Subsequently, samples were fermented in an incubator shaker with shaking at 50, 100, 200, and 300 rpm at 36°C (Figure 1b). For the optimization of the agitation rate, a significant difference was observed at 200 rpm of batch fermentation ($P < 0.05$), which showed the shortest fermentation time. Finally, the optimum fermentation time to maximize ethanol content was determined under the selected conditions. Fermentation process continued until all the sugars were exhausted (Figure 1c). The most optimal conditions for alcohol production in 3 whey samples were found to be at a temperature of 36°C, at 200 rpm for 24, 30, and 36 h.

pH Changes

The change in pH during fermentation can be found in Figure 2b. The initial pH value of whey was 6.13, and the pH dropped to about 4.3 as a result of organic acid production and basic AA absorption during the fermentation. It has been demonstrated that *S. cerevisiae* produces acetic, glycerol, pyruvic, succinic, and citric acid (Coote and Kirsop, 1976; Chidi et al., 2018; Risner et al., 2019). The drop in pH was relatively sharp within the first 6 h, which stabilized around pH 4.7. The pH changes of fermented whey rapidly declined until 12 h and reached a stable level at pH 4.3 due to the increase in organic acid from whey production. The whey pH decreased slightly during the 12- to 24-h fermentation period and then stayed in the range of 4.2 to 4.3 in MS and HS whey samples. In contrast, pH variation in NS whey slightly increased after sharp drop to around 4.4 during 12 to 24 h, which was as a result of *S. cerevisiae* consuming the nutrients and producing organic acids released into the whey. Accordingly, microorganisms faced a shortage of nutrients and began to consume the organic acids as nutrient sources, where

Figure 1. The effect of incubation temperature (a), agitation speed (b), and fermentation time (c) on ethanol production by *Saccharomyces cerevisiae* with lactase. NS = nonsupplemented whey; MS = mid-supplemented whey; HS = high-supplemented whey. Error bars represent SD of 3 independent experiments.

cells utilized AA as a carbon source, which raised the pH slightly. Thus, the pH value of NS whey was lower than that of lactose-supplemented whey samples during the fermentation. The pH showed no decrease, and the hydrolysis was almost complete after 24 h fermentation (Figure 3). During the fermentation, these pH styles agreed with those published by Qiao et al. (2010) and Risner et al. (2019).

Titrateable Acidity

Batch experiments were performed in the presence of different lactose concentrations for 24 to 36 h with an initial pH of 6.13, 6.13, and 6.12 in NS, MS, and HS whey, respectively. The TA levels of 3 whey samples significantly increased ($P \leq 0.05$) from 0.18 to 0.40, 0.50, and 0.53%, respectively, which was due to the production of organic acids and absorption of basic AA that were formed by the yeast during fermentation (Figure 2a). In *S. cerevisiae*, a direct correlation has been demonstrated between acetic acid and glycerol production during alcoholic fermentation (Erasmus et al., 2004; Goold et al., 2017). Pyruvic acid is generally present during alcoholic fermentation, which is produced at the beginning of fermentation and drops toward the end of fermentation (Usseglio-Tomasset, 1995). Aragon et al. (1998) also pointed out that the influence of *S. cerevisiae* on the organic acid production such as acetic, citric, and succinic acid. As expected, higher titers of TA were detected from supplemented whey samples. In very high-sugar products, alcoholic fermentation by *S. cerevisiae* tends to end up low in ethanol and high in organic acid production (Chidi et al., 2018). In other words, the increased osmotic stress caused by a higher initial sugar concentration leads to a higher initial glycerol formation, and thus indirectly to acetic acid formation as a result of redox balancing (Erasmus et al., 2004). Pigeau et al. (2007) also demonstrated that enhancing sugar concentration showed reduced growth performance and sugar consumption rate and increased acetic acid levels. Moreover, organic acids were produced continuously until the peak concentration was reached at 12 h of the cultivation time. Subsequently, the titers of organic acids were decreased slightly. Consequently, the values of TA continued to decline slightly until the end of the fermentation, which was due to the decrease of organic acid production fluxes from pyruvate, caused by the yeast introducing a pyruvate to ethanol route. The TA influences the perception of acidity and the physical stability of alcoholic beverages (Heymann and Ebeler, 2016). Generally, whey produced from rennet-coagulated cheeses has a low acid content; however, lactic acid might be produced by sugar fermentation, namely by *S. cere-*

visiae (Panesar et al., 2007). As a result, increasing ethanol production at high-sugar concentrations by supplementing an additional source of lactose led to lower pH and increased acidity.

Whey SG

The results of SG analysis on 3 different sugar concentrations are shown in Figure 2d. The primary soluble solids in whey were mono- and disaccharides, including lactose, glucose, and galactose. The SG of 3 whey samples decreased gradually during the fermentation, whereas the total acidity increased as the fermentation progressed. The concentration of lactose was converted to ethanol, leading to a decrease in the SG of the whey (Demott et al., 1981). Initial SG values of 3 whey samples were 1.024, 1.034, and 1.045 g/cm³, respectively.

The final average SG values (1.001–1.002 g/cm³) of 3 samples were observed at the end of the fermentation, which was independent of overall fermentation duration. At the end of the fermentation, differences in the SG values among the different supplemented samples were not found to be significantly different.

Protein Analysis

The total protein changes of NS, MS, and HS whey samples are shown in Figure 2c during the 24, 30, and 36 h of the fermentation. The initial total protein contents in NS, MS, and HS whey were approximately 8.26, 8.71, and 9.22 mg/mL, respectively. After fermentation, protein concentrations of 3 whey samples decreased to 6.72, 6.23, and 6.03 mg/mL, respectively. A decrease in the soluble protein concentration during

Figure 2. Changes in titratable acidity (a), changes in pH value (b), protein consumption (c), and changes of specific gravity values (d) during fermentation. NS = nonsupplemented whey; MS = mid-supplemented whey; HS = high-supplemented whey. Error bars represent SD of 3 independent experiments. L.A. = lactic acid.

Figure 3. Overlaid anion chromatograms of galactose, glucose, and lactose of NS, MS, and HS whey during the fermentation. NS = non-supplemented whey; MS = mid-supplemented whey; HS = high-supplemented whey. R = retention; nA = peak current height.

fermentation due to its consumption by the yeast as a nitrogen source (Yadav, et al., 2014). Moreover, the increased ethanol production from higher initial sugar contents lead to higher demands of nitrogen sources in the whey. During the fermentation, protein consumption of supplemented whey samples was higher than NS whey due to the additional lactose. Klupsaite et al. (2017) showed that 24, 48, and 72 h of fermentation process resulted in a lower 3%, 8%, and 19% protein content, respectively, which indicates that there is a direct relationship between protein content and fermentation time. The total whey protein content decreased until the end of the fermentation, but major proteins remain unutilized. Klupsaite et al. (2017) also showed that changes in protein content during fermentation

could be caused by the complex of different factors (e.g., pH value, acidification, microorganisms, and enzyme activities).

Sugar Analysis by IC

An effective and accurate approach using IC was developed to quantify mono- and disaccharides (Sukson et al., 2015). Overlaid anion chromatograms of galactose, glucose, and lactose in NS, MS, and HS whey were presented in Figure 3. The retention times of the 3 sugars on the column were determined as galactose (7.42 min), glucose (8.24 min), and lactose (9.47 min), respectively. Lactose was completely converted by lactase into glucose and galactose, respectively. Although

both sugars were utilized almost entirely by the yeast, a very small amount of galactose remained at the end of the fermentation, as shown in Figures 3. In the yeast *S. cerevisiae*, the flux through the glucose utilization pathway is 3 times higher than the galactose utilization rate (Bro et al., 2005). The purpose of adding lactose to whey was to maximize the ethanol yield coefficient. The 3 samples' initial soluble sugar concentrations were 46.24, 74.72, and 100.84 g/L. The sugar utilization in the first 6 h of nonsupplemented and supplemented whey samples were almost the same, followed by a rapid consumption period up to 24 to 36 h in all experiments (Figure 2d). Additionally, the total sugar concentration of NS, MS, and HS whey samples decreased with time. The lactose and glucose content in whey samples were exhausted within 24, 30, and 36 h of fermentation. The final residual sugar concentrations were less than 0.72, 0.85, and 1.42 g/L, respectively, at the aforementioned fermentation times. This result indicated that more than 98% of sugar was hydrolyzed during fermentation.

Alcohol Yield Determination

The ethanol yields of NS, MS, and HS whey during whey fermentation were reported in Figure 4. The addition of lactose to whey increased the chances of ethanol production. A significant increase in the ethanol yield was obtained when the sugar concentration increased from 46.2 to 74.7 and 100.8 g/L ($P \leq 0.05$). Under optimum conditions, the ethanol concentration reached its highest level after 24, 30, and 36 h, respectively. The lowest ethanol yield production was obtained with the NS whey. The highest final ethanol concentration was obtained with the HS whey. As expected, the total concentration of volatile compounds in NS whey-based spirits from different distilling stages was far less than in supplemented whey-based spirits, which was strongly associated with the ethanol concentration. The findings of earlier studies suggested that high-level alcohol production had both positive and negative effects on the spirit aroma (Wang et al., 2015). The highest total level of volatile compounds was found in HS whey-based spirits, followed by MS whey-based spirits (Table 1). Moreover, results of the batch fermentation showed that the final ethanol concentration was 29.5 g/L with NS whey, which was the lowest ethanol yield coefficient. Ethanol production reached 42.1 g/L with MS whey. The maximum yield of ethanol production was obtained from 29.5 to 56.4 g/L, when the lactose concentration increased from 46.2 g/L to 100.8 g/L. Ethanol production efficiency level was calculated assuming a theoretical ethanol production, which was 0.56 g of ethanol per gram of lactose consumed by the yeast. A similar efficiency level in ethanol production

from lactose-added whey by *Kluyveromyces marxianus* has been reported by Parrondo et al. (2009), which was 0.54 g of ethanol per gram of lactose. In general, lactose is hydrolyzed by lactase to glucose and galactose. Subsequently, 95% of these monosaccharides are converted by *S. cerevisiae* to ethanol and carbon dioxide, 1% is converted into cellular material, and the remaining 4% is converted to the other end products such as higher alcohols, organic acids, aldehydes, and esters. (Chidi et al., 2018). The final ethanol production of NS fermented whey was above the expected published range of 15 to 25 g/L (Ozmihci and Kargi, 2007; Guimarães et al., 2010; Koushki et al., 2012). These efficiency differences could be attributed to the use of sweet whey, produced by addition of rennet to the milk, as the fermentation substrate (Sansonettil et al., 2009). In the articles cited above acid, whey, whey permeate, and reconstituted whey cheese powder were investigated as the substrate. In addition, the optimal operating conditions could also account for this observation. However, as mentioned previously, this is a major obstacle for whey-based alcohol because the yields are much lower due to low sugar concentration compared with other fermentation of a sugar-rich substrate, followed by the distillation of the fermented solution, such as sugarcane, sugar beet, sweet sorghum, corn, cassava, and barley (Dincer, 2018). Therefore, the use of supplemented whey solutions for alcohol fermentation yields high ethanol concentrations and reduces distillation costs. The TA, pH value, SG, yield of alcoholic, protein, and sugar content of pre- and postfermentation whey samples are summarized in Table 2.

Volatile Compounds in Whey Spirit

Chromatographic techniques are generally used to analyze spirit-based beverages because such drinks have complex matrices, and the specific groups of compounds play different roles (Wiśniewska et al., 2015). Distilled whey is an alcoholic liquid produced by the distillation process of fermented whey after alcohol fermentation (Dragone et al., 2009). The concentrations of volatiles, as well as their changes during distillation, were measured. In this study, 10 main compounds with different chemical families including higher alcohols, aldehyde, ester, and acid in whey spirit, were identified and quantified, with concentration values higher than 11.2 mg/L (Table 1). Although the concentration values were different, the majority of these compounds were similar to the most well-known distilled alcoholic beverages including vodka, whiskey, rum, brandy, gin, and Chinese liquor. The volatile fraction of these distilled spirits is primarily represented by esters, higher alcohols, aldehydes, ketones, acetals, and acids. These

Figure 4. Yield of alcohol of NS, MS, and HS whey during whey fermentation. Each value is the mean of 3 independent replicates $\pm$ SD. Means that do not share the same letter (a–c) are significantly different at $P \leq 0.05$. NS = nonsupplemented whey; MS = mid-supplemented whey; HS = high-supplemented whey.

distilled spirits have similar major volatile constitutions, such as acetaldehyde (0.9–186 mg/L), ethyl acetate (13.8–213 mg/L), methanol (8.7–1,318 mg/L), 2-butanol (0.02–80.3 mg/L), 1-propanol (35.66–400.53 mg/L), 1-butanol (0.01–26), 2-methyl-1-propanol (0.14–180.73 mg/L), 2-methyl-1-butanol (119.29–326.74 mg/L), and acetic acid (0.05–224 mg/L), which are considered to be the main compounds due to their high concentrations (De León-Rodríguez et al., 2006; Madrera and Valles, 2007; Savchuk et al., 2007; Zhao et al., 2013; Zheng et al., 2014b). These compounds are mainly responsible for the aroma and taste of the whey spirit. The most quantitatively significant volatile compounds in these spirits corresponded to yeast-derived fermentative alcohols and ester. A typical chromatogram of other whey spirits followed the same trend as those in Figure 5, although the peak heights were different for each case. The HS whey had the highest concentrations for most of the analyzed compounds but did not include

ethyl acetate. Total volatile compound concentration significantly decreased ($P \leq 0.05$) from the HdWS (2,087–2,549 mg/L) to the TWS (890–1,308 mg/L). The highest concentration of 2,549 mg/L was presented in a head spirit of HS whey, which was 1.23- and 1.95-fold higher than those in the HtWS (2,065 mg/L) and TWS (1,308 mg/L). Various compounds with high levels such as isoamyl alcohol, 2-methyl-1-butanol, 1-propanol, ethyl acetate, acetaldehyde, and 2-butanol were found to decrease during distillation.

The concentrations of alcohols, ethyl acetate, and acetaldehyde significantly decreased from the HdWS to TWS. The concentrations of ethyl acetate, acetaldehyde, and high alcohols might negatively affect alcoholic beverages. It should also be noted that the volatile compounds detected are far below the acceptable legal limit (EU regulation 1576/89; EEC, 1989). The ethanol concentration of NS, MS, and HS whey-based spirits ranged from 12.5, 19.8 and 28.5%, respectively,

Table 1. Concentration of 10 volatile compounds present in whey spirit and distillation stages (expressed in mg/L)¹

Peak number	Volatile compound	Retention time (min)	NS		MS		HS	
			HdWS	HtWS	HdWS	HtWS	HdWS	HtWS
1	Acetaldehyde	3.51	125.51 ± 19.42 ^{bcd}	98.25 ± 23.26 ^{bcd}	242.67 ± 43.33 ^a	152.64 ± 41.69 ^b	263.43 ± 53.05 ^a	151.17 ± 50.50 ^b
2	Ethyl acetate	4.64	98.20 ± 31.77 ^a	82.13 ± 26.79 ^a	109.52 ± 11.48 ^a	90.50 ± 30.52 ^a	107.34 ± 37.66 ^a	89.44 ± 21.35 ^a
4	2-Butanol	7.84	79.34 ± 15.42 ^{ab}	33.83 ± 6.34 ^d	41.58 ± 11.58 ^d	57.21 ± 17.21 ^{cd}	89.40 ± 20.60 ^a	68.72 ± 13.7 ^{abc}
5	1-Propanol	9.31	232.31 ± 72.31 ^{ab}	190.03 ± 39.95 ^{bc}	93.23 ± 21.95 ^d	205.20 ± 64.75 ^{abc}	293.87 ± 48.87 ^a	275.02 ± 45.02 ^{ab}
6	1-Butanol	10.73	15.79 ± 11.35 ^a	11.20 ± 6.15 ^a	ND	14.53 ± 5.02 ^a	17.91 ± 5.73 ^a	15.36 ± 2.48 ^a
7	2-Methyl-1-propanol	12.21	489.81 ± 151.40 ^{abc}	301.30 ± 80.91 ^{cd}	523.14 ± 156.10 ^{ab}	401.84 ± 94.23 ^{abc}	548.67 ± 132.09 ^a	407.92 ± 88.81 ^{abc}
8	2-Methyl-1-butanol	14.46	141.35 ± 57.49 ^{ab}	120.87 ± 18.94 ^{ab}	129.39 ± 38.83 ^{ab}	138.28 ± 38.77 ^{ab}	201.48 ± 42.63 ^a	146.78 ± 26.6 ^{ab}
9	3-Methyl-1-butanol	14.69	879.32 ± 226.63 ^{ab}	751.44 ± 240.47 ^{abc}	425.19 ± 130.74 ^c	821.48 ± 210.05 ^{ab}	1,003.15 ± 267.13 ^a	890.41 ± 164.94 ^{ab}
10	Acetic acid	23.07	25.65 ± 13.81 ^a	21.78 ± 5.46 ^a	20.01 ± 3.58 ^a	25.52 ± 11.33 ^a	23.85 ± 10.01 ^a	20.32 ± 3.24 ^a
Total			2,087.28 ± 599.60 ^{abc}	1,610.83 ± 448.27 ^{cd}	889.64 ± 225.62 ^e	1,907.20 ± 513.57 ^{bc}	2,549.10 ± 617.77 ^a	2,065.14 ± 416.64 ^{abc}

^{a-c}Different letters in the same row indicate significant differences ($P \leq 0.05$).¹Each value is expressed as mean $\pm$ SD ($n = 3$); NS = nonsupplemented whey; MS = mid-supplemented whey; HS = high-supplemented whey; HdWS = head whey spirit; HtWS = heart whey spirit; TWS = tail whey spirit; ND = not detected.

Table 2. pH, titratable acidity, protein, sugar, and ethanol content of pre- and postfermentation whey samples¹

Sample ²	Pre-fermentation				Postfermentation				
	pH	Titratable acidity (% L.A.)	Protein content (g/L)	Sugar content (g/L)	pH	Titratable acidity (% L.A.)	Protein content (g/L)	Sugar content (g/L)	Ethanol content (g/L)
NS	6.13	0.18	8.26 ± 0.14 ^c	46.24 ± 0.44 ^c	4.43 ± 0.05 ^a	0.40 ± 0.02 ^b	6.72 ± 0.15 ^a	0.72 ± 0.07 ^c	29.53 ± 0.65 ^c
MS	6.13	0.18	8.71 ± 0.11 ^b	74.72 ± 1.35 ^b	4.28 ± 0.07 ^b	0.50 ± 0.01 ^a	6.23 ± 0.23 ^b	0.85 ± 0.05 ^b	42.10 ± 0.41 ^b
HS	6.12	0.18	9.22 ± 0.12 ^a	100.84 ± 0.85 ^a	4.29 ± 0.03 ^b	0.53 ± 0.02 ^a	6.03 ± 0.12 ^b	1.42 ± 0.06 ^a	56.43 ± 0.81 ^a

^{a-c}Values followed by different superscript letters in the same row differ significantly ($P \leq 0.05$).¹Results are presented as mean values ± SD (n = 3).²NS = nonsupplemented whey; MS = mid-supplemented whey; HS = high-supplemented whey.

in the HdWS to 11.3, 17.4 and 25.8%, respectively, in the HtWS, and the final ethanol concentration levels in the TWS were 6.4, 9.1, and 11.2%, respectively. The distillation process is performed at a low temperature to prevent whey protein denaturation to recover the residual soluble protein after distillation, which was directly related to the relatively low alcohol content.

Alcohols are among the most important fermentation process products (Silva et al., 1996; Tufariello et al., 2012). Higher alcohols are usually the predominant concentration group in spirit, regarded as essential flavor compounds and character (Ferreira et al., 1999; Silva and Malcata, 1999). In whey spirit, a total of 6 higher alcohols were identified. Among the 6 alcohols detected, 2-methyl-1-propanol, 3-methyl-1-butanol, 1-propanol, and 2-methyl-1-butanol were the most abundant and are considered the main alcohols. Among the 3 whey spirits, the highest alcohol concentrations were detected in HS whey, whereas the MS whey had the highest level for ester. Generally, a minimum concentration level of 400 mg/L is required to manifest positive features (Gómez-Míguez et al., 2007). In this study, alcohols represented approximately 84 to 88% of the total volatile composition, followed by aldehyde (5–8%). In particular, the alcohol fractions, 2-methyl-1-butanol, 1-propanol, 1-butanol, and 2-butanol, were all below the critical negative threshold of 400 mg/L; additionally, they might have similar positive effects. Furthermore, the isobutyl alcohol and isoamyl alcohol concentrations were higher than their odor thresholds, and they were expected to make an increased contribution to the overall aroma of the spirit. Attention should be drawn to the fact that methanol was not found in whey-based spirit, which was one of the interesting characteristics. This indicated that the absence of methanol due to the lack of pectin in whey was a very good identifier for the whey-based beverage (the maximum permitted content limit 1,000 g per hectoliter of pure alcohol, as per council regulation no. 1576/89; EEC, 1989), as opposed to plant-based alcoholic products, which provide a safe and excellent end product (Apostolopoulou et al., 2005). Methanol is formed from pectin by pectolytic enzymes, which hydrolyze the methoxyl group during fermentation, and can cause serious health difficulties such as metabolic problems, blindness or even death when ingested or inhaled (Mangas et al., 1996; Geroyiannaki, et al., 2007). The concentration of 2-methyl-1-propanol and 1-propanol significantly decreased from the HdWS to TWS. As one of the volatile compounds in spirits, bacterial metabolites formed various aldehydes (Lachenmeier and Sohnius, 2008). According to the EU regulation 1576/89 (EEC, 1989), standard analytical concentration limit of acetaldehyde is 73 to 500 g per hectoliter.

Figure 5. Typical chromatogram of volatile compounds found in a nonsupplemented whey heart whey spirit as follows: (1) acetaldehyde; (2) ethyl acetate; (3) ethanol; (4) 2-butanol; (5) 1-propanol; (6) 1-butanol (7) 2-methyl-1-propanol; (8) 2-methyl-1-butanol; (9) 3-methyl-1-butanol, (10) acetic acid, (11) 4-nanonal (IS). AU = absorbance units.

Aldehyde concentrations in alcoholic drinks have been previously reviewed (Liu, and Pilone, 2000; Park et al., 2006; Paiano, et al., 2014). In comparison, a decreased trend in acetaldehyde concentration was observed due to its high volatility. The acetaldehyde content in whey spirits ranged from 46.23 (mg/L) to 263.43 (mg/L), representing the second-largest group detected in this study. Another major compound also found in the whey spirit was ethyl acetate. The ester formation may be attributed to the fatty acid metabolism of yeast. Ethyl acetate identified in the whey distillates provides fruity and floral aromas and is usually a desirable aroma in distilled spirits (Risner et al., 2019). The sample's total concentration ranged from 13.70 to 109.52 mg/L, which provided a positive sensory quality to the distillate based on the supplementation, leading to a spirit with a stronger fruity-floral character. Similar acetic acid levels, about 20 to 29 mg/L, were found in all samples, which could lead to a spirit with a distinctive sour taste.

CONCLUSIONS

Ten major volatile compounds with different chemical families were quantified in the nonsupplemented and lactose-supplemented whey-based spirits obtained during fermentation with *S. cerevisiae* with lactase.

The results demonstrated that the commercial *S. cerevisiae* strain in lactase-hydrolyzed whey displayed efficient ethanol production and fast sugar hydrolysis in the fermentation of NS, MS, and HS whey samples in 24, 30, and 36 h on average, producing up to 29.5, 42.1, and 56.4 g/L ethanol, respectively. The HS whey contained 100.81 g/L lactose, yielding 56.4 g of ethanol/L. The total concentration of volatile compounds was reduced from the HdWS (2,087–2,549 mg/L) to the TWS (890–1,308 mg/L). The highest concentration was detected in the head of HS whey (2,549 mg/L), which was 1.23- and 1.95-fold higher than that in the HtWS (2,065 mg/L) and TWS (1,308 mg/L), respectively. Furthermore, the key dominant compounds obtainable in whey spirit included alcohols isoamyl, isobutyl alcohol, 2-methyl-1-butanol, 1-propanol, acetaldehyde, and ethyl acetate; these compounds contributed to the maximum significant proportion of the total aroma. The volatile compounds detected were within the legal limit. The results suggested that the whey-based spirit had good quality in terms of the volatile composition and the absence of methanol. Further research is required to advance the understanding of novel technologies for processing spirits from whey and to optimize the technological process, quality control, and safety evaluation of distilled spirits.

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