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Effect of fermentation time on the physicochemical, microbiological and sensory properties of the indigenous yellow wine from Mandara Mountains in the Sudano Sahelian part of Cameroon

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Abstract
The yellow wine is an indigenous pale to dark yellow coloured beverage resulting from natural long-term fermentation of the pomelo (*Citrus maxima*) must. Given that fermentation could have a great impact on the beverage quality, this study seeks to assess whether changes occurring during long term-fermentation could affect the quality of this drink. To achieve this, the wine was sampled twice after six and nine months of fermentation at the producers' level, and the physicochemical, microbiological, and sensory evaluations were performed according to referenced methods. From the physicochemical results, it has been observed that most of the measured parameters were significantly different ($p<$ 0.05). The pH, total acidity, sugar content, dry matter, and electrical conductivity values were within the range of 4.35 and 3.84, 8.16 g/L and 7.58 g/L, 74.4 g/L and 23.95 g/L, 9.53% and 7.59%, 612.58 µS/cm and 767.11 µS/cm for the wine samples collected after six and nine months of fermentation respectively. However, any significant decrease hasn't been detected in the alcohol content which varied from 13.77% to 13.58% for six and nine months aged samples. The microbial analysis results showed that the total aerobic bacteria, spore-forming bacteria, and coliforms counts were 7.6±0.52 log cfu/mL, 7.96±4.1 log cfu/mL and 3.95±0.76 log cfu/mL respectively in the six months aged wine samples. In contrast, none of these microorganisms wasn't observed in the nine months aged wine samples. As for the sensory profiles, both fermented wines received almost equal acceptance, even if the colour and odour significantly changed during the fermentation.

Keywords: Indigenous beverage, long-term fermentation, pomelo wine, quality, safety

1. Introduction
One of the most widely artisanal food processing and preservation techniques used for more than a millennium is fermentation [1]. Across the globe, wine processing are popular, and the fermented alcoholic beverages produced are culturally and socially accepted products for consumption, entertainment, customary practices, and religious purposes [2]. Wine, an alcoholic beverage is produced from the fermentation of fruit juices especially grape, but many tropical and subtropical fruits, including apples, sugar cane, oranges, mangoes, and bananas yield good quantity of juice upon fermentation and can be changed into indigenous wine [2]. Across Africa, many indigenous fermented alcoholic beverages have been described, including honey wine also known as Tej and Borda in Ethiopia, palm wine locally called Matango in Cameroon [3], Nsafufuo in Ghana, Emu in Nigeria [4], and Bandji in Ivory Coast [5]. In Cameroon, especially in the arid northern regions, a spectrum of fermented alcoholic beverage are traditionally processed from local resources, and the most studied have been the honey-based wine known as Kuri [6], sorghum-based beers such as Amgba [7], Té and Mpedli [8, 9]. The yellow wine also called Téa lémi is another example of a traditional alcoholic drink obtained after fermenting the extracted juice of pomelo (*Citrus maxima*) fruits in the Mandara Mountains, located in the Far north region of Cameroon. The yellow wine is rudimentary processed from spontaneous fermentation. Like many other traditional fermented foods in Africa, the production of yellow wine is by ancient method of chance inoculation and uncontrolled fermentation. Therefore, the usual variations in the quality and stability of the product are not unexpected. Indeed, the fermentation process which takes place spontaneously thanks to the development of the epiphytic microflora can lead to products of an undesirable organoleptic, microbiological or toxicological quality [10]. This wine considered as primitive type of wine is home-processed, but also commercially available as pomelo wine.

For commercial purpose, mixture of juice extract from pomelo and sugar is usually used for its preparation. Some producers also blend different ingredients such as banana extract, honey or egg white to improve the sensory attributes and to entice the consumers. However, according to the regular producers, the main point which determines the acceptance of the ready-to-serve product is the fermentation time. Indeed, the longer the fermentation, the better will be the final product. During the preliminary surveys, local producers indicated that natural fermentation and maturation lasts from three to twelve months. They also reported that for the drink to be accepted by most consumers, the yellow wine must be dark yellow in appearance, sweet taste, and exhibit effervescence. To date, the only study carried out on yellow wine has been devoted to the technological processing and quality characterisation of that traditional beverage. It has been shown that the properties of the marketed wine varied from one site of production to another. Given that fermentation time in the production process of indigenous fermented alcoholic beverages is a crucial point and must be well mastered due to either the production of unpleasant or enhancing compounds responsible for altering food quality therefore, it emerges from this study that the duration of fermentation and maturation could be critical stages, and may be the key determinants for the properties of the final product. For a better understanding of the fermentation process of the beverage and to improve the quality of the ready-to-sell product, this study aimed to evaluate the impact of fermentation time on the physicochemical, microbiological and sensory qualities of the artisanal yellow pomelo wine.

2. Materials and Methods

2.1. Sampling of the yellow wine

The main biological material was yellow pomelo-based kapsiki wine sampled from three experienced producers located in the "Mandara" Mountains of the Far-North region of Cameroon. Sampling was performed in that area because the beverage is highly produced (Figure 1). Regarding the long-term fermentation involved in the production of this indigenous wine, the sampling was done twice at 3 months of interval. The first sampling was carried out in June 2019 and the second one was performed in September 2019. According to the producers, fermentation of the must started in January 2019. This means that we collected traditional wine samples after 6 and 9 months of fermentation. According to the same producers, traditional wine is considered ready-to-serve after 6 months of fermentation, and this operation can last up to 12 months (a year). To avoid bias, samples were collected and divided into two lots. The first set was immediately harvested (June 2019) and the second set was kept at the producer level to be collected 3 months later (September 2019). At the end, a total of eighteen samples equivalent to six samples from each producer (after 6 and 9 months of fermentation), because sampling was carried out in triplicate. The collected samples were packaged in sterile 1000 mL bottles, labelled and were transported aseptically under cold regime to the laboratory for physicochemical, microbiological and sensory analyses.

Fig 1: Some of the traditional wine samples collected from the three main producers

2.2. Samples preparation

Prior to the analyses, the commercial samples were centrifuged for 10 min at 1000×*g* and the supernatant was successively filtered through filter paper (Whatman #1) and a membrane filter (0.62 µm diameter). The filtrates collected were used for analysis of the collected samples.

2.3. Physicochemical analysis of wine

The pH of the wine samples (10 mL) was determined directly using a pH meter (Eco Testr, Singapore) after calibration with pH 4 and pH 7 buffer solutions at 25 °C [11]. The total titratable acidity was determined by the alkaline potentiometric technique using 0.1N NaOH and 1% phenolphthalein as an indicator as previously described by [12]. The modified heating method was used to determine the proximate alcohol content [13]. Fifty (50 mL) milliliters of samples were put into cylinder and heated under specific conditions (78 °C), and the weight loss was used to calculate the alcohol content because of evaporation of ethanol at 78 °C. The soluble solids content of the samples (TSS) expressed in °Brix was determined using a portable ATC refractometer (RHB 90, Shenzhen, China) after calibration with distilled water and total sugar was evaluated by the modified phenol-sulfuric method proposed by [14]. The optical density values recorded at 490 nm wavelength were measured using UV-Vis spectrophotometer (Jenway 7305, Bibby Scientific, Group HQ, UK) and the total sugar content was expressed as g of glucose equivalent per litre (g/L). Dry matter was determined according to the standard ISO 11465 [15] by desiccation at 105 °C until a constant weight was obtained. Electrical conductivity was evaluated directly using a portable conductimeter (Eco Testr, Singapore).

2.4. Microbial analysis

One milliliter aliquot of each yellow wine was taken aseptically from samples both fermented for 6 and 9 months. These samples were serially diluted 10-fold in sterile saline water (NaCl 8.5%). After dilutions, microbiological enumerations were done using referenced methods. Enumeration of total aerobic mesophilic bacteria was determined according to ISO 4833 [16] standard in Plate

Count Agar (PCA, Difco, BD, Sparks, MD) after 24 hours of incubation at 30 °C. Total fungi were enumerated according to ISO 21527-2 [17] standard in Potatoes Dextrose chloramphenicol Agar (PDA, Difco, BD) after 72 hours of incubation at 25 °C. Counting of total and fecal coliforms were carried out according to the standards ISO 4832 [18] and ISO 9308-1 [19] respectively. After spreading of dilutions in Eosine Methylene Blue agar (EMB, oxoid), plates were incubated for 48 hours at 35 °C for total coliforms and 44 °C for faecal coliforms before enumeration. After 10 minutes of heat activation at 80 °C of wine samples, the wine samples and dilutions were spread in Bromocresol Purple Glucose agar (BPA) and mesophilic spore-forming bacteria were counted after incubation at 35 °C for 24 hours [20]. The same samples were used for counting sulphite-reducing clostridia by sowing in Trypticase Sulphite Neomycin agar (TSN, Difco, Sparks, MD) followed by incubation in an anaerobic jar at 46 °C for 48 hours [21]. The enumeration of faecal streptococci was done in 0.5 % cycloheximide supplemented Slanetz and Bartley agar after 24 hours of incubation at 37 °C [22]. The enumeration of *Salmonella* and *Shigella* was performed according to the method described by [23]. Samples (50 mL) were pre-enriched in 200 mL of sterile and buffered peptone water for 24 hours at 37 °C. Then, 1 mL of pre-enriched samples was introduced in enrichment Muller-Kauffann Broth for 24 hours at 37 °C. Enumeration was carried out on *Salmonella-Shigella* agar after incubation for 24 hours at 37 °C.

2.5. Sensory analysis

Sensory evaluation was made using indigenous wine fermented for six and nine months. This evaluation was carried out with sixteen naive panelists recruited among the regular wine consumers, using an adapted five-point (5) hedonic scale (1= dislike extremely, 2 = dislike moderately, 3 = neither like nor dislike, 4 = like moderately and 5 = like extremely), to assess changes in the sensory attributes such as taste, colour, mouth feel and overall acceptability during fermentation of the traditional wine [24]. A separate sensory evaluation sheet was developed for the taste to specify the preference of panelists. The taste had four categories namely alcoholic, bitterness, sourness, and sweetness. The panel was the same for sensory evaluation of both fermented samples for 6 and 9 months. This panel was made up of by twenty members aged between twenty and thirty-five years.

2.6. Statistical analysis

The various data obtained were recorded in a designed Excel database. Statgraphics Centurion 16.1 software (Technologies Inc., Virginia, USA) was used for the statistical analysis. One-way analysis of variance (ANOVA) was performed and the differences between means were determined by Tukey's Honest Significant Differences (HSD) multiple comparison test at 5% level of significance.

3. Results

3.1. Changes in physicochemical property of yellow wine during fermentation

Table 1 below shows the variation in the physicochemical parameters of kapsiki wine after six and nine months of fermentation. In general, most of the physicochemical parameters of kapsiki wine varied significantly ($p < 0.05$) during fermentation for 6 and 9 months. Regarding the parameters such as pH, titratable acidity, total sugar, soluble solids, dry matter and conductivity, there was a remarkable decrease with the fermentation time. The mean pH of kapsiki wine samples varies between 4.35±0.38 and 3.84±0.59 for samples fermented after 6 and 9 months respectively. Traditional wine samples fermented for six months had the lowest total acidity (7.58±1.1 g/L) compared to the nine-month fermented samples (8.16±1.29 g/L). Total soluble solids (12.01 ± 1.70 °Brix and 11.76 ± 0.46 °Brix); total sugar (74.4 ± 12.6 g/L and 23.95 ± 4.08 g/L) dry matter (9.53 ± 1.87% and 7.59 ± 0.78%) and conductivity (767.11 ± 60.92 µS/cm and 612.58 ± 63.74 µS/cm) were greatly reduced for the nine-month fermented wine samples compare to the six-month samples. All these data showed that fermentation was on going up to the 9th month at least. However, the proximate alcohol content was the only parameter that didn't significantly change ($p < 0.05$) between wine samples collected after 6 months (13.77±1.39%) and nine months (13.58±0.54%). The stability of this parameter over the fermentation time suggests that the alcoholic fermentation tends to slow down or stop from the 6th month.

Table 1: Main analytical characteristics of the yellow "tea lémi" wine samples collected after six and nine months of fermentation

Parameters	Yellow wine samples (fermentation time)	
	6 months	9 months
pH	4.35±0.38[a]	3.84±0.59[b]
Titratable acidity (g/L)	7.58±1.1[a]	8.16±1.29[b]
Alcohol content (%, v/v)	13.77±1.39[a]	13.58±0.54[a]
Soluble solids (°Brix)	12.01±1.70[a]	11.76±0.46[b]
Total sugar (g/L)	74.4±12.6[a]	23.95±4.08[b]
Dry matter (%)	9.53±1.87[a]	7.59±0.78[b]
Conductivity (µS/cm)	612.58±63.74[a]	767.11±60.92[b]

Values are means of three determinations. In a line, means values followed by the same superscript are not statistically different (Tukey multiple test range at $p<0.05$).

3.2. Evolution of the microbiological quality of the yellow wine with fermentation time

The microbial evolution of fermented kapsiki wine is presented in Table 2 below. There was a significant microbial loads ($p < 0.05$) for the total mesophilic bacteria (7.6±0.52 log cfu/mL), spore-forming bacteria (7.96±4.1 log cfu/mL) and total coliforms (3.95±0.76 log cfu/mL) for the six months old fermented kapsiki wine. According to the French standard agency (AFNOR), all these values were higher than the maximum values recommended which should be < 6 log cfu/mL (total plate count), < 4 log cfu/mL (spore-forming bacteria) and < 3 log cfu/mL (total coliforms). However, neither fungi, nor pathogens (fecal coliforms, fecal streptococci, anaerobic-sulphite reductase, and *Salmonella/Shigella*) were detected in both six and nine months fermented kapsiki wine samples. Furthermore, the nine months fermented kapsiki wine didn't reveal any total plate count, spore-forming bacteria and total coliforms. So, the fermentation time has significantly ($p < 0.05$) improved the microbiological quality of the indigenous kapsiki wine and the beverage samples obtained after 9 months of fermentation seems more safe than those collected after 6 months of fermentation.

Table 2: Changes in microbial population of the pomelos must samples collected after six and nine months of fermentation during the production of the kapsiki wine.

Parameters (log cfu/mL) Yellow wine samples (fermentation time)			
	6 months	**9 months**	**AFNOR (log cfu/mL)**
Total plate count	7.6±0.52	NG	< 6
Total fungi	NG	NG	< 5
Total coliforms	3.95±0.76	NG	< 3
Faecal coliforms	NG	NG	< 2
Mesophilic spore- forming bacteria	7.96±4.1	NG	< 4
Salmonella and *Shigella*	NG	NG	0/20 g
Faecal streptococci	NG	NG	< 3
Anaerobic-sulphite reductase (ARS)	NG	NG	< 1

Values are mean of three determinations. NG: no growth.

3.3. Assessment of the sensory quality of fermented kapsiki wine over time

The sensory quality scores of the kapsiki wine fermented for six and nine months are summarized in table 3. There was a significant difference ($p< 0.05$) in the colour and appearance of fermented kapsiki wine after six (3.08±1.19) and nine (2.89±1.46) months of fermentation. Similarly, there was a significant difference in the mouthfeel and flavour of fermented kapsiki wine but, the beverage samples collected after nine months of fermentation were the most graded (3.18 ±1.06). On the contrary, no significant change ($p<0.05$) was observed in the alcoholic taste, sourness, bitterness, sweetness and overall acceptability of both fermented beverages. But, wine samples fermented for 9 months (3.14±1.10) were slightly more accepted than those obtained after 6 six months of fermentation (3.09±1.10).

Table 3: Changes in sensory quality of the pomelos must during production of the yellow wine.

Attributes		Yellow wine samples (fermentation time)	
		6 months	**9 months**
Taste descriptors	Alcoholic	3.5±1.05[a]	3.29±0.84[a]
	Acidity	3.6±1.04[a]	3.10±1.01[a]
	Bitterness	2.83±1.1[a]	2.79±1.21[a]
	Sweetness	3.08±1.04[a]	3.18±1.04[a]
Mouth feel and flavour		2.5±1.2[a]	3.18±1.06[b]
Colour and appearance		3.08±1.19[a]	2.89±1.46[b]
Overall acceptability		3.09±1.10[a]	3.14±1.10[a]

In a line, means values followed by the same superscript are not statistically different (Tukey multiple test range at $p<0.05$).

4. Discussion

Globally, it has been observed that the fermentation time significantly influenced the physicochemical and microbiological properties of the kapsiki wine. Except for the alcohol content, all the other physicochemical parameters varied significantly ($p<0.05$) with fermentation time. The significant decrease in pH and sugar between both yellow wine fermented for 6 and 9 months could be a sign that the fermentation process was still ongoing. Similarly, according to [5], the tapped palm wine samples collected at the fourth week of tapping had pH and sugar values (3.4 and 54 g/L) greatly lower than those of the samples harvested on the first day of tapping (5.2 and 502 g/L). The same changes have been reported in some tropical fruit wines such as mango wine produced from overripe fruit [25], banana wine [26], and coconut wine made from coconut water, honey and Roselle calyces [2]. The decrease in pH and sugar content overtime has accounted for a significant increase in total acidity witnessed during the fermentation period of kapsiki wine. The increase in acidity coupled with the decrease in alcohol content observed in the pomelos "must" sampled after 9 months of fermentation could be the result of the apparent increase of some acid-producing bacteria such as *Acetobacter*, *Citrobacter*, and *Lactobacillus* which might convert alcohols into various organic acids [26], and probably explain why the beverage collected after 9 months of fermentation exhibited strong acid pH values with no significant decrease in alcohol content compared to those fermented for 6 six months. This reduction in the alcohol content of beverage samples collected after nine months of fermentation is also explained by the absence of fungal growth observed in the wine sampled after 6 months and consequently the absence of species such as *Saccharomyces* spp. associated with alcohol production during fermentation of sugar. This means that the alcohol content of the wine sampled after 9 months of fermentation remains alcoholic after a slight conversion of this into organic acids. However, the alcohol present after the sixth month of fermentation might be the result of natural fermentation of sugars contained in the pulp of ripe fruits by the wild yeasts and bacteria. Given that any exogenous starter hasn't been added during the production of the kapsiki wine, fermentation is likely triggered by autochthonous flora. Indeed, fungi with high fermentation potential have been isolated from the pulp and "must" of certain fruits and fermentation equipment [26, 27]. Furthermore, bacteria like *Zygomonas mobilis* has also been associated with alcohol production during palm wine fermentation [28]. The significant decrease in soluble solids and dry matter observed in kapsiki wine after nine months of fermentation confirms the fact that carbohydrates are the main substrates for fermenting microorganisms and the loss of matter by the production of volatile compounds (carbon dioxide and organic acids) during fermentation.

Statistically, there was a significant difference ($p< 0.05$) between the hygienic quality of six and nine months fermented kapsiki wine. Microbiological analysis showed the presence of total plate count, total coliforms and aerobic spore-forming bacteria in the wine samples collected after 6 months of fermentation. Their presence could be due to inappropriate hygienic practices during the production, in particular poor handling and treatment of raw material, equipment used for pressing and more so, suspicious water quality used. It is known that the ingredients of various fermented beverages contain spores of *Bacillus* [29]. *Bacillus* spp. are also reported to participate in the initiation of fermentation by hydrolyzing starch and thus making sugars available for acid formation [30]. However, as kapsiki wine has a very low pH, spore of various *Bacillus* spp.

might not be able to germinate and play an active role in the kapsiki wine fermentation [31]. Furthermore, the quality of water used in the production of artisanal drinks is always a limiting factor. This water which comes from springs, wells and rain is used not only for household activities but also for consumption by livestock. The absence of bacteria and fungi in the yellow wine at 9 months of fermentation showed that the product reaches the suitable wine's characteristics. This absence could be related to the acid environment (acid pH and high total acidity) of the wine which doesn't favor the proliferation of any spoilage and pathogenic microorganisms. It has been reported that the antimicrobial activity of organic acids is due to their dissociating capacity. Biological membranes of most microorganisms are permeable to the non-dissociated form of these molecules which when permeate into the cell's cytoplasm dissociate and partake in reducing strongly the intracellular pH within the microbial cell. These changes can go a long way to inhibit energy production which may induce the death of the cell.

The fermented kapsiki wine after six months scored the highest organoleptic properties in terms of taste and colour compared to the nine months fermented wine. However, nine months fermented kapsiki wine had the best mouth feel and flavour compared to that of six months of age. This may be related to the high organic acids content in the yellow wine sampled after nine months of fermentation. This acidification of the must during the fermentation has been reported as been crucial for the wine quality [2]. Indeed, acidity plays a vital role in determining wine quality as it enhances the overall characteristics and balance of the wine. No significant difference was observed in the overall acceptability. This could be due to the processing homogeneity and on the other hand the mastering of some processing stages of the fermented drinks. However, the wine samples fermented for nine months were more accepted than those obtained after six months of fermentation. This is explained by the absence of undesired microbes after nine months of fermentation as shown by the results of table 2. Their presence can lead to the production of volatile acidic compounds, off-flavours and polysaccharide hazes that can be responsible for reducing the quality and acceptability of the ready-to-serve beverage [2].

5. Conclusion

Changes in the physicochemical parameters, the microbiological and sensory qualities of the indigenous kapsiki wine have been assessed during a long-time fermentation of the pomelos "must". The pH value below 4.5 complies with the standard required, and the mouth feel (odour) and colour were the main sensory attributes of the traditional wine that were significantly affected during fermentation. Furthermore, the microbiological quality of the indigenous wine was significantly improved with the fermentation time. At six months of age, the beverage was of insufficient quality compared to the drink fermented for nine months which had an excellent hygienic quality. Regarding the long fermentation time required to reach a good hygienic quality, further studies on the technological aspect could help to elucidate autochthonous micro-organisms involved in the natural fermentation of this traditional wine, and their use in the optimum conditions as starter in order to both reduce the time of fermentation and preserve the good quality displayed by aged wine.

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Competing interests

The authors have no competing interests. The beverage used for this research are commonly and predominantly use products in our research area. We do not intend to use this product as an avenue for any litigation but for the advancement of knowledge.

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