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INVESTIGATING THROUGH MICROORGANISMS INVOLVED IN THE RAPHIA WINE FERMENTATION: HIGHLIGHT ON SUBSTRATES IN THE NDE DIVISION WEST-CAMEROON

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ABSTRACT

The present survey focussed on detection, identification and enumeration of microbial populations implicated in the fermentation of the raphia sap. The substrate collected in the Ndé Division (West-Cameroon) was incubated for seven days post-collection, tested for their pH and plated on convenient isolation media every day according to standard protocols recommended by BioMerieux® and REMIC. Incubation was thereafter, conducted at 20°C, 30°C and 37°C for 24 h, 48 h and 72 h. For accurate identifications, API galleries used included API 20 NE for *non-Enterobacteriaceae*; API 20 E for *Enterobacteriaceae*; API 50 CHL for *Lactobacillus* and API 20 C AUX for yeast. Overall findings indicated that the specimens contained mixed microbial populations that underwent selections with increasing acidity. Common non-fermentative contaminants were eliminated while *Lactobacillus* spp., and to a lesser extent, *Saccharomyces cerevisae* underwent optimal growth. Optimal ferment growth was recorded on specimens after four days of fermentation. Suitable conditions for this growth were 30°C

and 72 h plate incubation. From this investigation, isolation of specific microorganisms from unexpected culture media was yet to understand clearly (yeast on McConkey, and Mannitol salt, for instance), though the chemical composition of the sap and fermentation by-products might exert putative influences on the original chemical composition of each culture medium. These pieces of information might guide isolation of naturally developing ferments that could be used in local contexts. Based on the above findings anticipating that the sap from raphia plants could serve as a source for microorganisms endowed with key roles in the production of alcohol and acetic acid could be welcome. These by-products could also serve as alternative sources of energy.

KEYWORDS: Fermentation, Microbial populations, Ferments, Raphia sap.

INTRODUCTION

Throughout food chains, microorganisms perform hosts of biological activities that provide energy and regulate both the functions and the equilibrium of all ecological systems.[1] Accordingly, they can play very important roles at specific stages along the production chain of many food items at natural or industrial scales. More specifically, members are involved in fermentation processes that ultimately sustain production and storage of foodstuffs like milk, cheese, and alcohol, for instance. Fermentation are oxidation-reduction chemical reactions which develop in poorly oxygenated environments and generate reduced amount of energy when substrates are catabolized; compared to oxidation from which larger amounts of energy are released in the presence of oxygen.[2] Fermentation may develop naturally or, be initiated to promote human activities for improved output. Ferments involved in natural transformation are environmental hosts, part of the ecological system that self regulates while introduction and activity of specific microorganism are rigorously controlled in artificial processes for intended goals. Raphia wine for instance, is a liquid natural substrate from plant origin. Fermentation of the sap from different species of raphia palm trees results in the raphia wine, also referred to as palm wine.[3] Vitamin, energy and socio-cultural characteristics confer to palm wine the properties that make of it a favourite drink for rural populations in many parts of the world.[2,4] Some people take it fresh and sweet while others generally use it after it has fermented.[4] Plant species that produce the substrate are diverse and include *Raphia hookeri. R. vinifera; R. mambillensis.*[1,2] This sap is opaline. First sweet, the sap ferments gradually and loses its sweet taste to become acidic with the production of alcohol.[5,6]

Although the methods for extracting related wines differ from one palm species to the other, they are all destructive (cutting down the palm tree, incising the flowering end of the trunk) then unlikely to be used sustainably. This bears important ecological, nutritional and economic consequences.[1,2,5]

Palm tree sap contains important chemicals like amino acids, proteins, vitamins and carbohydrates.[1,7] This richness in nutrients may provide suitable growth environment for wide ranges of microorganisms. The ferments that instigate fermentation process carry out metabolic processes responsible for the physical and chemical changes in the raw substrate. These changes provide in turn a selection pressure on resident microorganisms and naturally give rise to competition and gradual ecological reorganizations in the microbial community[1,4,5] through positive selection that provides fitness or negative selection that causes extinction. By-products throughout fermentation include alcohol and organic acids, which play relevant role in the biological system reorganization. Nowadays, energy production is a key variable, which deserves attention in projecting the economic and social development of human communities. Otherwise, access to energy is a paramount target for poverty alleviation. That is why biofuels from plant sugar are increasingly becoming reliable alternatives to conventional fuel built on underground fossil[6], essentially limited.

Thus, the rise in the price of the barrel of oil on the one hand and the harmful effects of greenhouse gas on the other have justified the unbridled search for new forms of energy that are "less costly and more environmentally friendly". It is in this context that biofuel like bioethanol represents one alternative to current and future energy provision.[8]

Research on ethanol production and related goods from palm sap by fermentation has intensified in developed countries in recent decades because of these various energy crises. Solutions are investigated in various countries around the world with sugar-rich agricultural substrates, but very few advanced processes or technologies are used in this field.[5] Despite the high demand of raphia wine, its abundance in rural areas throughout Cameroon and its relatively high contents of carbohydrates and other nutrients, its use as a substrate (or source) for ferments that could serve in the production of ethanol, acetic acid or lactic acid has not yet been developed and standardized. Taking into consideration these aspects could promote public health and economic welfare in many resource-limited areas in Africa and in other developing human communities. Otherwise, it would be important to master the processes throughout fermentation in order to implement these at semi-industrial scale while ensuring

consumer safety with little resources. The present investigation focussed on metabolic processes that occur during the fermentation of the palm sap and on the microorganisms involved. Special focus was on the microbial flora in the palm sap and ecological succession that develops in it. Kinetics of evolution for the most important microorganisms as well as some of their optimal growth conditions were also investigated.

MATERIAL AND METHODS

Study Type and site, specimen collection

The present experimental study was conducted in the Ndé Division, West-Cameroon. This division consists of four sub-divisions (Bangangté, Bazou, Bassamba and Tonga). The climate in the locality is characterized by two seasons (a dry and a rainy season). Typically semi-urban Bangangté hosts several income generating activities conducted by mixed populations with different traditional values, dissimilar education background and purchasing power, favoured by development several higher learning institutions. One of these activities is the small business with raphia wine, a traditional beverage that is particularly relished locally. Samples collection was done from wine producers and retailers in the above three villages of Bangangté sub-division. Overall, 15 specimens were obtained and submitted to laboratory analyses that were conducted in the Laboratory of Chemistry of the "Université des Montagnes" and the Laboratory of Microbiology at the "Université des Montagnes" Teaching Hospital. The fresh sap was collected by pruning the phloem of the raphia palm after what it flew through a funnel into plastic bottles (≈ 100 mL /h in young plant).

These raphia wine samples collected according to routine traditional protocols in small sterile plastic containers were conveyed to the Laboratories for investigations. Each sample was diluted (1/5000th) to facilitate isolation and counting (enumeration) of microorganisms. Then, 100 µl of each solution was platted three times (three repeats) on five culture agars (Liofilchem®) in Petri dishes. All cultures, isolations and identifications were conducted according to standard guidelines.[9] The isolation culture media used included Sabouraud-Chloramphenicol (5%), Mannitol salt, McConkey and Man Rogosa Sharpe (MRS) agars. Subsequent to plate inoculation, the preparations were incubated on the work stand at room temperature (≈ 20-22°C) for 1 h upside-down for absorption then, at 25°C 30°C and 37°C for 24h; 48h and 72 h additional hours. Further identifications procedures were completed according to standard morphological, biochemical, and enzymatic guidelines as recommended by the reference repository (REMIC, 2017). Gram staining and bio-enzymatic

tests followed with API galleries (API 20NE for *non-Enterobacteriaceae*; API 20 E for *Enterobacteriaceae*, API 50 CHL for *Lactobacillus* and API 20 C AUX for yeast). BioMérieux®-related protocols guided all bio-enzymatic identifications.

The count (enumeration) made with naked eye and a magnifying glass was based on the number of colonies found in the Petri dishes. The value obtained was expressed in terms of "CFU/mL". The number of microorganisms was plotted as a curve according to the incubation length and temperature.

Ecological dynamics in the Raphia Wine

For all specimens, the dilution, platting and counting protocols described above were repeated throughout a week (seven consecutive days).

Time-related pH values were recorded with a pH meter calibrated with pH4 and pH6 buffer solutions (Tapsaoba *et al.*, 2011).[3] These were conducted on the total of specimens and the average values were subsequently recorded and plotted. Special emphasis was laid on most important ferments identified in the sap throughout the experiment, with attention on incubation temperature (in °C) and time (in days/hours).

RESULTS

Culture, isolation and identification of raphia sap's microorganisms

All investigations were conducted on fifteen (15) specimens from three villages (Bangangté, Bangoulap and Bahouoc) in the Ndé Division. Upon completion of incubation that followed overall procedural steps, the results summary of macroscopic examinations of microorganisms from culture grown on specific culture media were expressed as shown in table I.

Table I: Gram staining from in term of culture media and incubation time.

Cultures media	Incubation time (Hour)		
	24	48	72
Sabouraud-Chloramphénicol	Yeast	Yeast	Yeast
Mac Conkey	GNR	GNR, Yeast	GNR, Yeast
Chapman	GPR, GPC	GPR GNR	GPR GNR, Yeast
Mueller Hinton	GPR, GNR, Yeast	GPR, GNR, Yeast	GNR, GPR, Yeast
MRS	GPR, GPC	GPR, GPC	GPR, GPC

GNR: Gram-negative rods; **GPR**: Gram-positive rods; **GPC**: Gram-positive Cocci.

Its highlighted diversity of microorganisms, which was more or less media-specific, but generally, they develop in mix cultures. In addition, some culture media tend to have reduced specificity and allowed the growth of unexpected microorganisms. Those that retained highest specificity were Sabouraud/Chloramphenicol and MRS.

Subsequent to macroscopic identification, isolates further underwent refined bio-enzymatic traits screening. Results thereof were table as summarized in table II that namely provided the list of the microbes.

Table II: Microscopic and bio-enzymatic feature-dependent isolates' identification.

GPR	Yeast	GNR	GPC
- *Lactobacillus fermentum* - *Lactobacillus casei casei* - *Lactobacillus plantarum* - *Lactobacillus casei rhamnosus* - *Leuconostoc cremeris* - *Bacillus* spp - *Clostridium* spp	- *Saccharomyces cerevisae 1* - *Candida guillermondi* - *Trichosporon cutaneum* - *Candida inconspicua* - *Candida pelliculosa* - *Rhodotorula rubra 1*	- *Actinomyces* spp - *Acetobacter* spp - *Pantoa* spp 1 - *Sphingobacterium multivorium* - *Aeromonas sobria* - *Enterobacter cloacae*	- *Staphylococcus aureus* - *Streptococcus lactis* - *Streptococcus diacetylactis* - *Enterococcus* spp

Microbial diversity was high amongst and within morphological types in the substrate. Most diversified were Gram-positive rods and Gram-negative rods; followed by yeasts and Gram-positive cocci. Further consideration based on the API 50 CHL galleries identified *Lactobacillus fermentum*, *Lactobacillus casei casei*, *Lactobacillus casei rhamnosus* and *Lactobacillus plantarum*. Other bacterial types were lactic acid ferments (*Actinomyces* and *Acetobacter*).

A subtle look on yeast indicated that they belonged to four genera, namely *Saccharomyces, Candida, Trichosporon* and *Rhodotorula.*

Once assessed, the relative rates of isolates from the specimens under study provided pieces of information expressed in terms of percentage of isolation/growth (table III).

Table III: Rates of isolation from the specimens.

Fermenting microorganisms	%	Non fermenting microorganisms	%
Lactobacillus spp.	100	*Staphylococcus aureus*	93
Bacillus spp.	100	*Candida inconspicua*	66
Saccharomyces cerevisae 1	100	*Enterococcus* spp.	66
Streptococcus lactis	93	*Sphingobacterium multivorium*	47
Acetobacter spp.	87	*Clostidium* spp.	40

Streptococcus diacetylactis	80	*Trichosporon cutaneum*	20
Candida guillermondi	73	*Rhodotorula rubra 1*	20
Actinomyces spp.	33	*Pantoa* spp. *1*	20
-	-	*Enterobacter cloacae*	13
-	-	*Candida pelliculosa*	13
-	-	*Aeromonas sobria*	07

This table indicated seven most common fermentative categories in the raphia palm sap. Namely these were *Lactobacillus* spp., *Bacillus* spp., *Acetobacter* spp., *Saccharomyces cerevisae* 1, *Candida guillermondi*, *Streptococcus lactis* and *Streptococcus diacetylactis*.

Other categories included *Staphylococcus aureus*, *Enterococcus* spp., *Sphingobacterium multivorium*, *Candida inconspicua*, *Clostridium* spp., and faecal coliforms (*Pantoa* spp. 1, *Sphingobacterium multivorium*, *Aeromonas sobria* and *Enterobacter cloacae*).

Table IV provided the list of microorganisms endowed with known fermenting traits.

Table IV: Microorganisms endowed with fermentation properties.

Bacteria	**Fungi**
- *Lactobacillus fermentum* - *Lactobacillus casei casei* - *Lactobacillus plantarum* - *Lactobacillus casei rhamnosus* - *Leuconostoc cremeris* - *Bacillus* spp - *Acetobacter* spp - *Streptococcus lactis* - *Streptococcus diacetylactis*	- *Saccharomyces cerevisae 1* - *Candida guillermondi*

It came out that raphia palm sap contained eleven microbial types that are likely implicated in one or several steps in the fermentation processes. These include nine bacteria and two fungi species.

Evolution of the pH of Raphia Wine with incubation time

In connection with microbial metabolic processes and outcomes, time- and fermenting organism-related pH values variations were recorded on regular basis, then plotted as presented in figure 1.

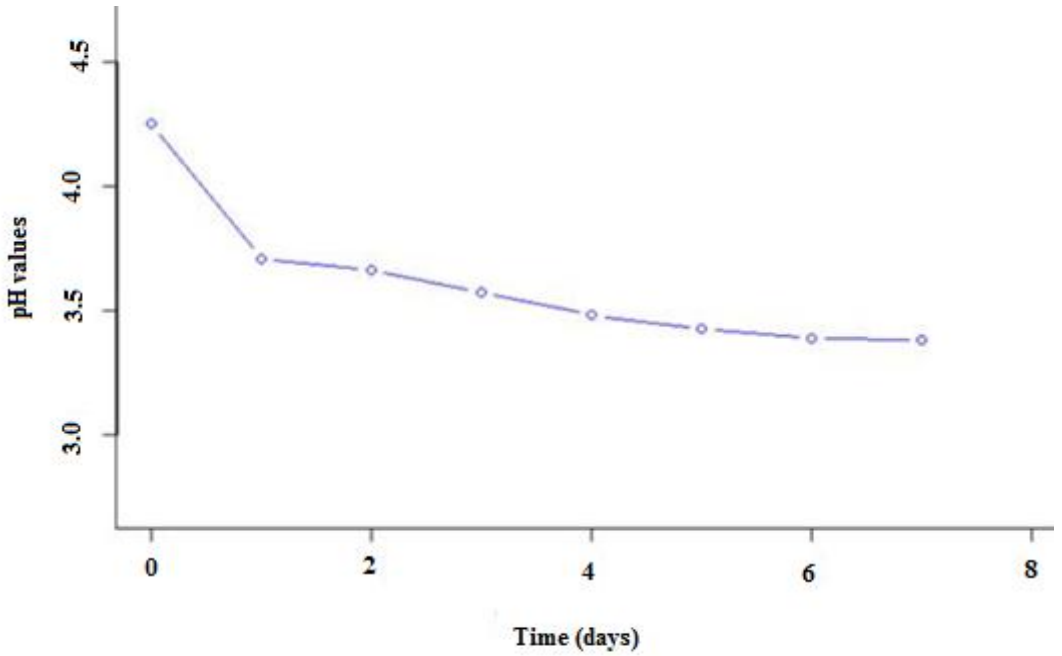

Figure 1: pH variations with time (in days).

It revealed gradual decrease of the pH values from day zero through day seven. The drop was abrupt at the beginning but subsequently tended to stabilize day after day.

Ecological dynamics in Raphia Wine

Growing microorganism from the original substrate revealed mix microbial populations. About the non-fermenters flora, their variations were recorded and plotted as displayed in figure 2.

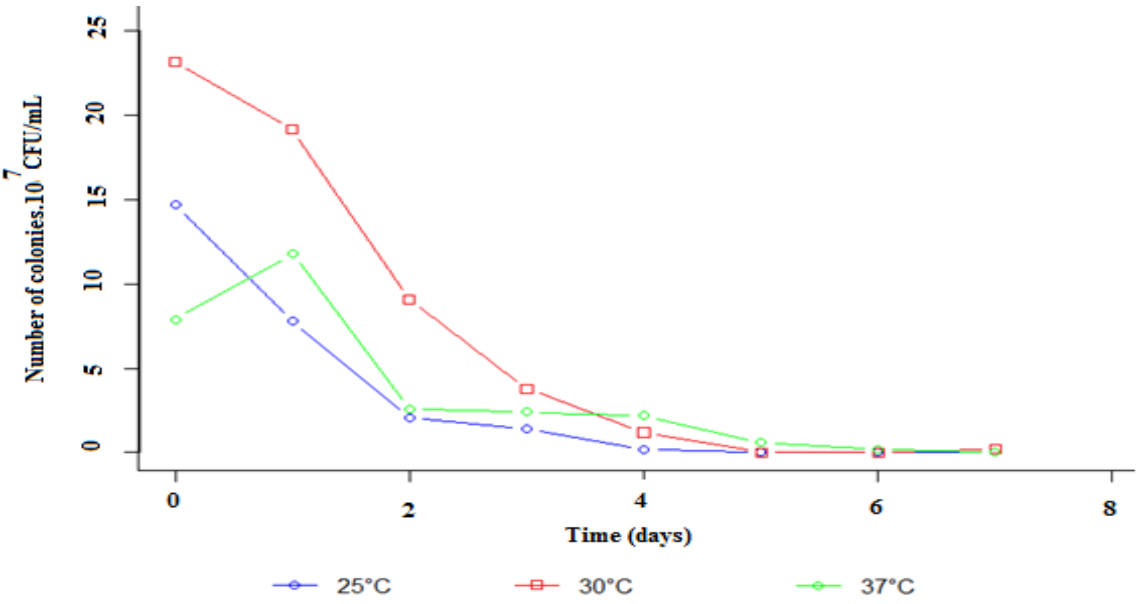

Figure 2: Evolution of non-fermenters' populations with time.

This figure indicated overall populations decreased over time, with no regards to prevailing temperatures. The abrupt population drops were recorded from day zero through day four; then tended to stabilize.

Likewise and throughout the seven incubation days, investigation steps focused on evolution of key fermenting microbial populations (*Lactobacillus* spp and *Saccharomyces cerevisae* 1). Figure 7 displays the findings' summary.

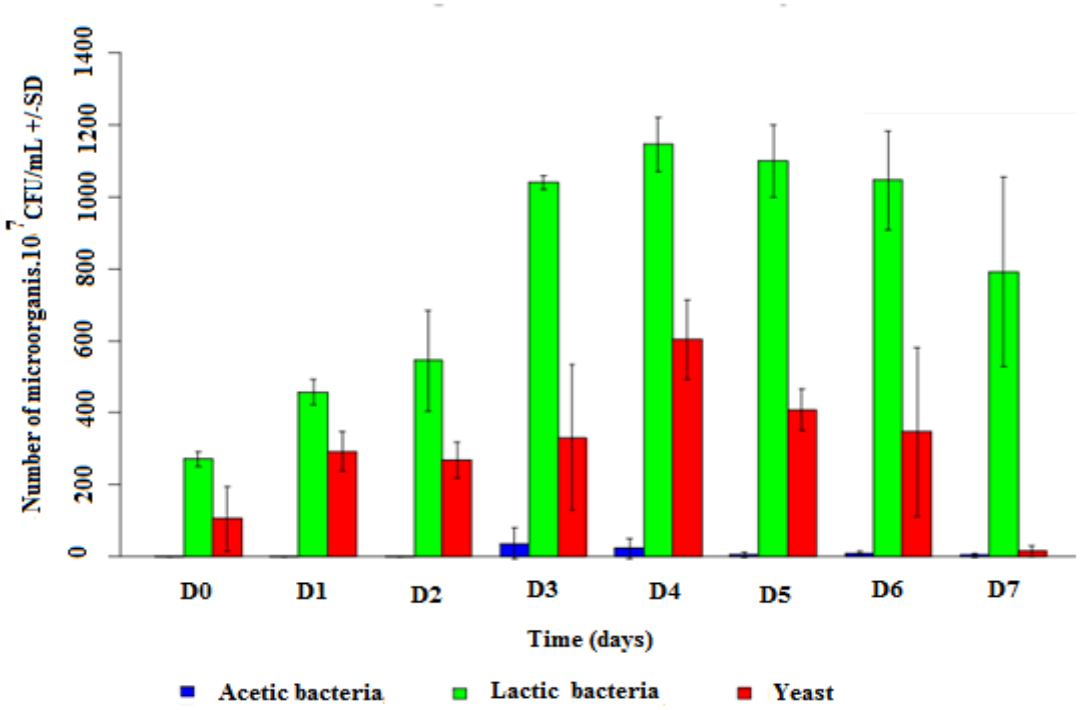

Figure 3: Evolution of fermenters' populations after 72 h at 30°C.

Overall, lactic acid bacteria predominated microbial populations from day zero through day seven. Yeast population followed similar trend, but was at least twice less frequent than lactic acid bacteria, while acetic acid fermenters were extremely rare. The maximum values were recorded on the fourth day after what, population decrease was observed. On day seven, lactic acid bacteria overwhelmed the isolations (≈ 800.10^7 CFU/mL).

Enumeration of microorganisms in raphia palm sap

With further focus on major groups of fermenters, day-to-day temperature-dependent enumeration was performed. Accordingly, assessment of yeast population variations in terms of incubation temperature and duration throughout the seven days yielded their tendencies shown in table V.

Table V: Effect of temperature and incubation time on *Saccharomyces cerevisae*.

Temperature	Specimen	24 h	48 h	72 h
25°C	D_0	64.4 ± 32.3	94.4 ± 33.3	106.4 ± 35.5
	D_1	133.6 ±17.8	239.2 ± 77.4	292 ± 74.9
	D2	125 ± 21.5	198.5 ± 47.5	212 ± 49.1
	D_3	183.2 ± 76.5	256 ± 121.7	305.6 ± 159.1
	D_4	122 ± 27.9	376.4 ± 74.5	429.2 ± 90.7
	D_5	104.6 ± 23.1	300.4 ± 49.5	349.6 ± 101.4
	D_6	110.8 ± 41.0	188.4 ± 77.7	219.4 ± 104.9
	D_7	1 ± 1.4	18.6 ± 15.03	19.6 ± 16.15
30°C	D_0	82.4 ± 65.4	88.4 ± 66.6	105.6 ± 89.2
	D_1	190 ± 67.7	220.8 ± 63.6	292.5 ± 54.6
	D_2	206.3 ± 36.1	248 ± 45.4	269 ± 51.2
	D_3	231.2 ± 110.9	280.4 ± 145.0	331.4 ± 202.4
	D_4	308 ± 80.4	512 ± 65.35	604 ± 110.6
	D_5	267.6 ± 31.9	339.6 ± 49.3	408.4 ± 56.5
	D_6	216.6 ± 124.6	281 ± 157.1	347 ± 233.2
	D_7	12.8 ± 11.78	14.8 ± 12.91	15.4 ± 13.58
37°C	D_0	63 ± 54.9	70.1 ± 57.8	72.2 ± 59.0
	D_1	116.8 ± 74.8	163.3 ± 102.1	195 ± 102.
	D_2	108.9 ± 66.5	178 ± 103.6	191.1 ± 106.0
	D_3	181.6 ± 89.7	244.2 ± 137.0	296.2 ± 182.5
	D_4	251 ± 26.6	337.6 ± 120.4	350.2 ± 128.2
	D_5	167.2 ± 56.5	222.2 ± 57.5	348 ± 89.3
	D_6	105 ± 51.0	224.6 ± 176.1	256.6 ± 183.5
	D_7	9.8 ± 8.87	15 ± 10.46	15.6 ± 10.90

Based on the yeast populations, it came out that the fermentation peak was recorded on the fourth day. Additional details indicated optimal growth at 30°C (regardless of the incubation time) and around 72 hours (regardless of the incubation temperature**).**

Likewise, a glance on lactic acid bacteria resulted in the summarized pictures displayed in table VI.

Table VI: Effect of temperature and incubation time on *Lactobacillus* spp.

Temperature	Specimen	24 h	48 h	72 h
25°C	D_0	204 ± 19.2	194.8 ± 31.5	232.1 ± 14.6
	D_1	249.2 ± 35.1	311 ± 42.5	425 ± 25.0
	D2	364 ± 50.4	445 ± 32.6	455 ± 41.1
	D_3	678 ± 106.2	830 ± 78.8	934 ± 45.1
	D_4	771 ± 73.9	902 ± 47.6	1160 ± 96.2
	D_5	750 ± 61.2	850 ± 100.0	1000 ± 80.9
	D_6	426 ± 104.5	800 ± 156.0	934 ± 92.4
	D_7	188.4 ± 107.4	445.2 ± 226.0	546 ± 257.2
30°C	D_0	224 ± 27.0	230.4 ± 32.2	270.8 ± 20.2
	D_1	235.6 ± 27.1	363 ± 32.1	457 ± 34.4
	D_2	311 ± 67.9	472 ± 29.3	545 ± 137.0
	D_3	770 ± 152.5	982 ± 25.9	1040 ± 20.0
	D_4	850 ± 94.6	1034 ± 43.9	1146 ± 74.7
	D_5	852 ± 96.8	994.6 ± 84.4	1100 ± 100.0
	D_6	640 ± 108.4	870 ± 135.1	1046 ± 136.3
	D_7	340.6 ± 243.9	604 ± 249.7	792 ± 263.7
37°C	D_0	124.7 ± 24.4	163.5 ± 41.2	209.7 ± 78.8
	D_1	146.5 ± 90.5	251.6 ± 96.9	335.5 ± 93.8
	D_2	193.1 ± 84.0	280 ± 161.4	280 ± 160.5
	D_3	492 ± 57.8	734 ± 161.6	816 ± 155.0
	D_4	256.4 ± 39.8	547 ± 60.2	651 ± 75.9
	D_5	302.4 ± 48.9	506 ± 70.2	780 ± 115.1
	D_6	195.8 ± 44.2	514 ± 199.2	776 ± 273.7
	D_7	34.75 ± 18.9	133.75 ± 107.4	212.5 ± 125.3

About lactic acid bacteria, the fermentation peak was recorded on the fourth day of fermentation. A subtle glance at other variables indicated optimal growth at 30°C (regardless of the incubation time) and around 72 hours (regardless of the incubation temperature).

DISCUSSION

The present study revealed large numbers of microorganisms in the raphia sap, with slight differences in population diversity from one specimen to the other. Acknowledging a couple of considerations: First, the sap is originally sterile and, Second, spontaneous "generations is a chimera", it is reasonable to anticipate that these microbial populations are environmental hosts that got into contact with the product. Contact with the germs probably occurred during one or several of the steps that include harvesting, transporting or storing the liquid. Likely sources of these germs are primarily animals, but also humans, harvesting equipment like container, or ambient air. Previous works in Cameroon, Nigeria, Burkina Faso, Ghana and

Tunisia[1,3,10–12] documented overall similar conclusions. Bees for instance can carry extreme varieties of microorganisms and serve as effective vectors for contaminations. Otherwise, diversity of ferments can change based on specific environmental specificities like the availability of other germs' reservoirs, which provide permanent or transient shelters for the vectors responsible for cross-contamination. This development is consistent with the time of sap collection (100 mL/h) that might allow large ranges of contaminants in 24 h (the frequency of sap collection by the winegrower). Some of the identified microorganisms known for their fermentation properties included yeasts that belonged to the genera *Saccharomyces* and *Candida* whose anaerobic metabolic activities in raphia sap result in the production of alcohol. Likewise, metabolic processes by *Bacillus*, *Streptococcus* and *Leuconostoc* in this sap can generate lactic acid, while *Acetobacter* is an acetic acid ferment and *Clostridium butyricum,* a ferment for butyric transformation.

In addition to known ferments, other microorganisms without known intrinsic fermentative properties were identified: faecal coliforms (*Pantoa* spp. 1; *Sphingobacterium multivorium*; *Aeromonas sobria*; *Enterobacter cloacae*); *Enterococcus*, and *Staphylococcus aureus.* Under conditions like high microbial load, reduced immunity or both, they could become opportunistic and cause serious harm to the consumers. As microbial flora of human and animal faeces, *Enterococcus* is a reliable indicator of faecal adulteration. These results may reflect the known behaviour of some populations in the areas where the samples were collected, for instance, the use of latrine and animals breeding in the wild like cows, beside the role of wild animals, which feed on this sap like squirrels. Previous investigators[3,6,11,13,14] reported indicators of faecal pollution in raphia sap.

Staphylococcus and more precisely, *S. aureus* is a marker in food hygiene quality in food toxicology, for its pathogenic potential that is often multifactorial. *Staphylococcus* is a facultative aerobe like the above faecal coliforms. The oxygen affinity provides explanation to their ubiquity, then, their isolation from the material investigated. Their presence in raphia wine alongside, faecal contaminants reduces the microbiological quality of the product as discussed above.

Ferments identified in the course of the present investigation included lactic acid bacteria (*Lactobacillus* spp., *Bacillus* spp. and *Leuconostoc* spp.), acetic fermenters (*Acetobacter* spp) and yeast (*Saccharomyces cerevisae* and *Candida guillermondi*). Similar reports were done by several previous investigators.[1,3,6,10,11,13] Unlike the current results, however, none of

these authors distended on *Streptococcus lactis* and *Streptococcus diacetylactis*. This contrast might be consistent with earlier discussions on the role that the holistic ecosystem specificities might have on the selection of the microbial populations in saps.

The pH values ranged from 4.25 at day zero (D0) through 3.25 at day 7 (D7). This decrease was likely related to concentration' increase of derivatives as consequence of metabolic processes carried out by the resident microorganisms. In connection with their inherent fermentation potential, this might be due to ethanol, organic acids and lactic acid or combined actions. Others authors reported this pH drop with in Burkina Faso (4.50 to 3.60), in Ghana (4.05 to 3.94) and in Nigeria (4.08 and 3.21).[3,11,15] Gaps in the original pH values (that is the first one) might be in agreement with the microbial loads and, once again, consistent with the types of resident microbial flora, their loads within the collection ecosystem and/or the sap collection frequency.[1,3,15,16]

A subtle glance on pH variations further revealed association with growth of some microbes. Higher pH values appeared to favour the growth of contaminants, while subsequent decrease became selective, reducing fitness in some. Ferments also expressed variable potentials with increased acidity. Most fitted were *Lactobacillus* and to some extent, *Saccharomyces cerevisae.*

Fermentation germs (lactic acid bacteria, acetic acid bacteria and yeasts) developed and persisted in the medium for more than five days, while those of the adulterating flora tended to disappear from the fourth day. This could imply that contaminant flora germs did not survive from the fourth day onwards at lower pH in the medium and/or, in the presence antimicrobial substances like ethanol, bacteriocins or hydrogen peroxide (H_2O_2) generated by fermentative microbial flora activities. Similar observations were made by Tapsoba *et al.*[3] who observed that after four days of fermentation no faecal contamination indicators remained in the palm wine. This disappearance seemed to relate to the peak of the most fitted ferment's growth that was observed around the fourth day after what, population decrease was recorded. Convenient ferment isolation would therefore, be done around the fourth day (D3-D6) of fermentation after 72 h of incubation, based on the finding from the present survey. At least three hypotheses could be used to justify the overall population drop that was observed after four days of incubation. 1-acidity of the medium had a lethal effect or inhibits the growth of certain microbial populations (i.e. products derived from their metabolism became toxic); 2-substrates and nutrients were depleted and 3-spaces were reduced (i.e.

spatial congestion that developed resulted in growth inhibition by contact), in line with the standard microbial growth curve analyses. Combinations of these three events may also justify the overall picture. Fermentation increases acidity and makes the product safer to consumers, consistent with the role of acid and other fermentation by-products known in food protection.[14,17]

More detailed data screening indicated that acetic bacteria express optimal growth in raphia wines from the third day onwards. This could be explained by the fact that the main nutrient for these germs is ethanol[10,18], suggesting that acetic bacteria do used the ethanol produced by yeast during alcoholic fermentation. Accordingly, around day 3 (D3), its concentration reached the optimal values for acetic bacteria growth, in line with Amoa-Awua *et al.*[10] It would however, be reasonable to anticipate the presence of other microorganisms in the sap and other biochemical phenomena that the present survey could not elucidate.

Further considerations displayed parallelism of ferments' growth trends, suggesting a correlation between the evolutions of microbial types. It could also be observed that this correlation was positive ($P= 0.001$, $\alpha =0.05$).

Optimal growth was recorded at 30°C after 72 h. This optimal growth was highest on day four with peak values approximating $604x10^7$ CFU/mL for *Saccharomyces* and $1146x10^7$ CFU/mL for *Lactobacillus*. The yeast population could be compared to that reported by Tapsoba *et al.* ($3.4x10^6$ and $2.85x10^6$ CFU/mL); Amoa-Awua *et al.* (10^5 and 10^7 CFU/mL) and Manel *et al.* ($\approx10^6$ UFC/mL).[3,10,16] These differences resist justification but might be related to stochastic variations of microbial flora from one environment to the other, as alleged above. Fermenters populations with specific characteristics are very likely crucial in the overall fermentation process. Whether or not raphia wine might be more suitable for the growth of lactic acid bacteria than for acetic bacteria and yeasts is yet to address accurately with further researches.

Overall, above development imply that it would be rational to inoculate raphia sap on the fourth day if the option is to detect and isolate ferments, certainly due to shortage of nutrient and /or higher environmental stressing conditions that develop after this time. Incubation's temperature and length would be crucial in the process. However, why specific microorganisms microorganisms were isolated from unexpected culture media is yet to understand (yeast on McConkey, and Mannitol salt, for instance) beyond the knowledge that

the chemical composition of the sap and fermentation by-products might have somewhat putative influences on the original composition of the culture medium.

CONCLUSION

The raphia palm sap hosted wide ranges of microorganisms that were post-production contaminants. Some amongst these organisms took advantage into the growing acidity that developed in the course of fermentation and eliminated the least fitted, which included primarily common ones that are not involved in fermentation. *Lactobacillus* spp., and, to a lesser extent *Saccharomyces cerevisae* were identified as the most potent ferments. They could optimally be isolated on the fourth day at 30°C after 72 h of incubation.

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