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Producing probiotic beverage based on raffia sap fermented by *Lactobacillus fermentum* and *Bifidobacterium bifidum*

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ABSTRACT

In order to contribute to the diversification of the range of probiotic products, limited until now to the dairy matrices, and to valorize the local raw materials of Cameroon, we set ourselves the objective of finding the conditions of fermentation of the fresh sap of *Rafia farinifera* by two probiotic ferments, namely, *L. fermentum* and *B. bifidum*. We first characterized raffia sap physico-chemically and studied the ability of ferments and their viability in the sap. The physico-chemical characteristics of the sap (3.801%±0.037 dry matter, 7.044±0.172 g of ash per 100 g of dry matter, 10.789±1.388 g of reducing sugars per liter, titratable acidity of 4.44 equivalent grams of lactic acid per liter, pH of 4.12, 2.91±0.94 g of total phenol compounds per liter, density of 1.158 g/l and a Brix of 4.2) and the viability of the ferments in the latter showed the feasibility of this work. Moreover, the optimization of physico-chemical parameters thanks to the Box-Behnken model after maximizing Brix, reducing sugars and proteins and setting the pH at 4, lactic acidity at 9 g/l, the amount of probiotics at 1.00 E+10CFU/ml led to the following operating conditions : *B. bifidum* and *L. fermentum* seeding rates of 10 and 2.82%, respectively, an incubation temperature of 37°C and an incubation time of 14 h 2 min. Thus, these conditions made it possible to obtain a drink having a titratable acidity of 8 g of lactic acid/l, a pH of 3.87, a protein content of 574.6 (mg/l), a Brix of 5.47 and a quantity of probiotics of 1.13 E+8 CFU/ml. Beyond this optimization, a sensory analysis performed on the optimized product showed that it was organoleptically acceptable.

Key words : *Bifidobacterium bifidum*, fermentation, *Lactobacillus fermentum*, probiotics

INTRODUCTION

The beneficial properties to health and longevity resulting from the consumption of fermented milk are known for thousands of years (Schrezenmeir and Vrese, 2001). Many microbial strains have since found their probiotic potential and clinical studies in human patients have demonstrated the effectiveness of some of them in the prevention or attenuation of several health disorders (Reid and Hammond, 2005; Sullivan and Nordh, 2005).

The probiotic market is continually expanding and has significant economic interests. Indeed, during the last decade, a constant increase in demand for products fortified with probiotics was found in the United States, Japan and Europe where consumption increased up to 150% (Heller, 2001). Although dairy products are now the main suppliers of probiotics (Heller, 2001; Zargar and Pakina, 2014), more and more non-dairy products containing probiotics are developed. The market is diversified and it shows in, among others, cereals, chocolate bars, cookies, granola bars and juice fortified with probiotics (USProbiotic, 2006; Mafakheri *et al.,* 2012; Zargar *et al.,* 2018). Despite its growing diversification, the probiotic market still appears too limited to ensure sufficient consumption of probiotics necessary to obtain the benefits they confer. In addition, it is still mainly limited to dairy products, making probiotics inaccessible to

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non-dairy probiotic consumers (Heller, 2001).

The raffia sap seems to be an interesting matrix because it usually undergoes fermentation leading to sudden raffia wine. This fermentation is an indicator as it implies that it can allow the incorporation of probiotics (*L. fermentum* and *B. bifidum*). This work not only intervenes in a spirit of appreciation of raffia sap that is a local product but especially in a dynamic diversification of the range of probiotic products. Thus, the main objective of this work was to determine the optimum fermentation conditions of raffia sap by *L. fermentum* and *B. bifidum* as part of the production of a probiotic drink. The aim of the study was characterizing the sap physicochemically, to model the impact of each fermentation factor on the physico-chemical and microbiological characteristics of the beverage obtained, and finally to optimize these responses due to determine the optimal operating conditions, region of Cameroon.

MATERIALS AND METHODS

The physico-chemical part of this work was carried out at the food microbiology laboratory of the IUT of Ngaoundere (LAMBA) and the microbiology part was carried out in the Laboratory of Genius and Enzymatic Technology of ENSAI of Ngaoundere (LAGETA), in the Adamaoua region of Cameroon. The fresh sap of less than eight hours was harvested and transferred to the laboratory in a 25 l container, then immediately dispensed into one l bottles to be pasteurized in a water bath at 65°C for 30 min. The bottles were cooled and stored at 4°C. The physico-chemical analyses performed during this study are described in following para.

Determination of dry matter by the method AFNOR (1982), determination of ash content by the method described in AFNOR (1981), determination of Brix by using an optical refractometer (HANNA HI 96801), determination of pH by immersing the electrode of the pH meter in the samples (Al-Otaibi, 2009; Zargar *et al.,* 2017), pH was measured after 0, 2, 4 , 24 and 48 h for studying of acidifying properties, determination of sugar content by the method of Fischer and Stein (1961) with DNS and using maltose for the calibration curve, determination of protein content by the method of Lowry *et al.* (1951), determination of phenolic compounds content by the method of Marigo (1973), determination of titratable acidity was carried out according to the standardized method AFNOR (1982).

To revitalize and multiply probiotic cells contained in the freeze-dried products, 1 g of lyophilisate of each strain was rehydrated as mentioned by the manufacturer. First, the powder was rehydrated in 10 ml of saline dilution solution (SD) to maximum recovery (0.85% NaCl and 0.1% peptone in distilled water stirred for 10 min. The solution was then transferred into one l of MRS broth previously prepared and sterilized. After incubation for 48 h at 42°C MRS broth containing multiplicity were centrifuged at 6500 xg for 15 min at 4°C. The supernatant subsequently removed, the pellet washed in the SD without being resuspended and then recentrifuged as above. The supernatant was discarded and the pellet was finally resuspended in 10 ml of SD. These 10 ml were transferred to 250 ml of SD for a total of 250 ml. The probiotic concentration of this solution was obtained by dilution serial factor 10 tubes containing 9 ml of SD. The dilutions were plated on MRS Petri dishes and incubated for 24 h at 42°C prior to colony count.

Experimental Design Sap Fermentation Process

The model of Box-Benhken was used. Four factors considered in the study were as follows : the seeding rate of *B. bifidum*, the seeding rate of *L. fermentum*, temperature and time of incubation. Each factor descriptions are shown in Table 1.

Table 1. Range of variation and factor levels

Variable	Variable code	Level factor		
		-1	0	+1
Seeding rate Lb (%)	X_1	37.0	39.5	42.0
Bif seeding rates (%)	X_2	2.0	25.0	48.0
Temperature (°C)	X_3	0.0	5.0	10.0
Incubation time (h)	X_4	0.0	5.0	10.0

Lb : *L. fermentum* and Bif : *B. bifidum.*

The Box-Benhken model used allowed us to generate an experimental matrix with 28 experiments. Each experiment was the combination of four factors ranging from X_1 to X_4, where : X_1 represented the seeding rate of *Bifidobacterium,* X_2 the seeding rate of

Lactobacillus fermentum, X_3 and X_4 were, respectively, incubation temperature and incubation time. Each experiment was repeated three times and the average result was considered as the final result.

The specifications that were to be considered when optimizing the mathematical models obtained using the MINITAB 16 software for beverage with optimum characteristics are given in Table 2.

Table 2. Physico-chemical and microbiological specifications for optimization

Terms	Maximize	Fixed
Settings	Soluble proteins Reducing sugars Brix	pH=4 Lactic acid=9 g/l Probiotics load : 1.00E+10UFC/ml

RESULTS AND DISCUSSION

Physico-chemical Characteristics of the Raffia Sap

The dry matter content (3.801 ± 0.037%) high for a liquid beverage showed that the raffia sap contained more than 3% of organic elements; compared to the fresh sap of palm wine, the dry matter content was relatively low, fresh palm wine contained about 10.82% of dry matter (Lupien-Meilleur, 2012). The density of the sap (1.158±0.000 g/l) seemed to confirm the presence of elements other than water in the sap as if it was made only water, its density would have been closed to 1.

The sugar content (10.789±1.388 g/l) and the Brix (4.2) are both indicators of the amount of sugars present in the sap. Both parameters indicated that the amount of sugars present in the sap was quite low for the production of a fermented beverage and the Brix was adjusted to 6.5 equivalent of a sugar content of 30.24 g/l by addition of fructose to perform fermentation. A similar study conducted by Lupien-Meilleur (2012) on the feasibility of a probiotic drink made from maple sap showed Quebec that probiotics used were better developed in the maple sap whose Brix was between 6 to 8. All the results of the physico-chemical characterization of the raffia fresh sap are shown in Table 3.

Given all these physico-chemical characteristics, sap raffia appeared as a good matrix for receiving probiotics, but very little work had been done on this sap, it was sorely

Table 3. Physico-chemical characteristics of the sap of raffia

Characteristics	Values
Dry matter content (%)	3.80±0.037
Ash content (%)	7.04±0.172
Sugar concentration (g/l)	10.789±1.388
Soluble protein content (mg/ml)	8.70±0.07
Brix	4.2±0.0
Titratable acidity (lactic acid g eq/l)	4.440±0.104
pH	4.12±0.0
Total phenolic compounds (mg/ml)	2.91±0.94
Density (g/l)	1.158±0.000
Protein (mg/l)	870±0.07

lacking information on the possibility presence of elements that could affect the growth of the ferments. Thus, failing to make further characterization, it was imperative to study in advance the viability of ferments on the sap of raffia.

Curve of Growth and Viability of the Ferments in Raffia Sap

Fig. 1 shows the growth curves of ferments in the sap of raffia. This curve shows that both enzymes are viable in sap of Raphia. However, a prolonged incubation period helped reduce the amount of *B. bifidum* as *L. fermentum* growth continued. Indeed, *L. fermentum* was more resistant to fermentation-related acidification than *B. bifidum* because of its different genetic make-up. This decrease could also be explained by the production of antimicrobial compounds.

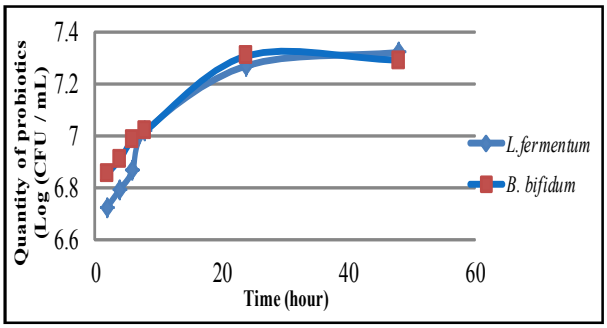

Fig. 1. Bacteria growth curve in the sap of raffia.

Experimental data showed the results of physico-chemical analyzes of probiotic drinks after experimentation (Table 4). These results were the subject of statistical mathematical modelling. The physico-chemical characteristics such as pH, Brix, the content of reducing sugars (g/l), the soluble protein content (mg/l) and titratable acidity were followed.

Table 5 presents the analysis of the

Table 4. Results of physico-chemical analysis

Trial	Factors				Physico-chemical characteristics				
	X_1	X_2	X_3	X_4	pH	Brix	Lactic acid (g/l)	Sugars (g/l)	Protein (mg/l)
1	-1	-1	0	0	4.2	6.5	4.44	30.0523	868.3560
2	1	-1	0	0	3.79	5.3	5.79	20.6841	504.3950
3	-1	1	0	0	3.98	5.6	5.43	19.4641	518.1294
4	1	1	0	0	3.79	5.2	5.73	14.8453	391.0864
5	0	0	-1	-1	4.11	6.1	5.22	23.5599	562.7661
6	0	0	1	-1	4.18	6.4	4.74	23.3856	586.8013
7	0	0	-1	1	3.63	4.2	7.53	8.0044	336.1489
8	0	0	1	1	3.7	4.8	7.65	8.7451	339.5825
9	-1	0	0	-1	4.16	6.3	5.01	29.0937	782.5161
10	1	0	0	-1	4.1	5.9	5.475	21.2070	545.5981
11	-1	0	0	1	3.8	5.3	6.36	9.9651	562.7661
12	1	0	0	1	3.58	4.0	7.92	6.8715	212.5395
13	0	-1	-1	0	3.78	5.3	5.79	18.4183	562.7661
14	0	1	-1	0	3.74	5.2	5.94	15.7168	411.6880
15	0	-1	1	0	3.92	5.5	5.55	20.6841	586.8013
16	0	1	1	0	3.73	5.1	6.21	16.1525	459.7583
17	-1	0	-1	0	3.89	5.5	5.7	18.3747	545.5981
18	1	0	-1	0	3.72	5.0	6.3	14.8453	607.4028
19	-1	0	1	0	3.96	5.9	5.49	18.5490	607.4028
20	1	0	1	0	3.7	4.8	6.45	16.4575	439.1567
21	0	-1	0	-1	4.18	6.5	4.74	29.6166	607.4028
22	0	1	0	-1	4.1	6.0	5.31	20.6841	562.7661
23	0	-1	0	1	3.87	5.4	6.33	10.2266	377.3520
24	0	1	0	1	3.68	4.6	7.17	6.4357	315.5473
25	0	0	0	0	3.75	5.1	5.82	13.8431	518.1294
26	0	0	0	0	3.8	5.2	5.79	14.1046	494.0942
27	0	0	0	0	3.79	5.4	5.76	14.3660	490.6606
28	0	0	0	0	3.85	5.2	5.97	13.7560	487.2270

variance followed the physico-chemical characteristics of the probiotic drink. Red spaces indicated significant actions and interactions at the 10% probability level. The bias factor (Bf), the absolute average deviation analysis (AADM) and the R2 were used to validate each model. Apart from the lactic acidity that increased significantly, the analysis of the variance of the physico-chemical characteristics (Table 5) showed that *L. fermentum* and *B. bifidum* had a significant effect on all the physico-chemical characteristics and contributed to decrease them. This decrease in physico-chemical parameters was also observed at the level of the interactions between the ferments and the incubation time. However, the seeding rate of *B. bifidum* further decreased the pH compared to *L. fermentum*. This difference was due to the fact that *B. bifidum* in addition to being able to perform glycolysis as *L. fermentum*, the almost specific degradation pathway to Bifido bacteria

Table 5. Values of the optimal characteristics of the probiotic drink

Responses	Theoretical values	Experimental values
Acidité titrable (g d'acide lactique/l)	6.87	8.0
pH	3.84	3.87
Protein (mg/l)	586.6	574.2
Sugar (mg/ml)	19.48	21.06
Brix	5.47	5.2
Quantity of probiotics	3.13E+7	1.13E+8

commonly called bifidus pathway (Tannock, 1999) which provided more ATP than the usual fermentation voices of other bacteria and allowed it to better adapt and multiply more quickly in circles and thus to produce more acid.

Acid production is of great importance in the production of probiotic foods. Indeed, Callaway *et al.* (2008) and Garcia *et al.* (2010) reported that beneficial bacteria, mainly lactic acid bacteria and bifidobacteria, could be a useful and effective strategy for preventing or reducing the incidence of pathogens, thereby improving food safety and protecting consumer health (Thomsen, 2006). The production of lactic acid lowered the pH by creating an unfavourable environment for the development of pathogenic microorganisms (Aslim *et al.,* 2004). Otherwise, lactic acid bacteria exerted a strong antagonistic activity against microorganisms, including those of the deterioration of food and pathogenic microbes. In addition, the antimicrobial effect of some strains extended the shelf life of food (Haller, 2001, O'Sullivan *et al.,* 2002).

Optimization has led to the following optimal experimental conditions :

- Seeding rate of *B. bifidum* : 10%;
- Seeding rate of *L. fermentum* : 2.82%;
- Incubation temperature : 37°C; and
- Incubation time : 14 h 2 min.

After modelling and optimization, we produced the optimum beverage from factor values obtained during optimization. We have, therefore, been able to compare the theoretical values given by the mathematical models of the physico-chemical characteristics and the experimental values resulting from the physico-chemical analyses of the optimum beverage as recorded in Table 5. We can observe that the experimental values are almost similar to the theoretical ones, which confirm the adequacy of the models used in this study.

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