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Production and Characterization of a Probiotic Sorghum Beverage Fermented with Lactic Acid Bacteria (*Lactobacillus fermentum* and *Bifidobacterium bifidum*) and Bil-bil

Brai Olivier[1], Wilson Agwanande Ambindei[1,*], Ngwasiri Pride Ndasi[2], Makebe Calister Wingang[3], Wiyeh Claudette Bakisu Muala[2], Desobgo Zangue Steve Carly[4], Nso Emmanuel Jong[1]

[1]ENSAI, University of Ngaoundere, P.O. box 454, Ngaoundere, Cameroon
[2]College of Technology, University of Bamenda, P.O. box 39, Bamenda, Cameroon
[3]National Higher Polytechnic Institute, University of Bamenda, P.O. box 39, Bamenda, Cameroon
[4]University Institute of Technology, University of Ngaoundere, P.O. box 454, Ngaoundere, Cameroon
*Corresponding author: agwanande@gmail.com

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Abstract The main objective of this study was to determine optimum conditions for aerobic fermentation for the production of a probiotic sorghum beverage using as ferment *Lactobacillus fermentum*, *Bifidobacterium bifidum* and bil-bil (a sorghum based traditional beer). Microbiological analyses on bil-bil showed $5x10^4$ CFU/mL total mesophilic flora and $3.5x10^3$ CFU/mL lactic acid bacteria. Physicochemical characterization on sorghum grains gave a water content, thousand corn weight, germinative energy and germinative capacity of 7.02±0.16%, 19.2±0.1g 99.82±0.11%, 100% respectively. Optimization of physicochemical and microbiological parameters of the beverage through a D-optimal plan after maximizing reducing sugars, polyphenols, vitamin C, total soluble sugars, antioxidant activity, probiotic load and minimizing titratable acidity, pH, turbidity and viscosity, resulted in an inoculation rate of 10 % *L. fermentum* and *B. bifidum* and 80 % bil-bil, a fermentation temperature of 37°C and fermentation time of 3 days. These operating conditions resulted in a beverage with a titratable acidity of 3.15 mEqg ac.mal/mL, pH of 3.05, vitamin C content of 74.28 mg/L, polyphenol content of 0.46 mg/mL, reducing sugar content of 0.86 mg/mL, TSS of 4.88°Brix, a probiotic load of $25.11x10^6$ CFU/mL, turbidity of 409.38 EBC, and a viscosity of 5.18 mPa.s. Mix fermentation could be exploited in the production of a probiotic sorghum beer.

Keywords: *Fermentation, Lactic Acid Bacteria, Sorghum, Beverage, Probiotic, beer, bil-bil*

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1. Introduction

The artisanal production of sorghum beer (locally called bil-bil in the northern regions of Cameroon) is of a remarkable socio-economic pertinence as it is widely used in traditional ceremonies and is an important source of income for producers [1]. The relative success of bil-bil among consumers could be due to the therapeutic virtues attributed to it and the diet improvement of millions of people [2] partly due to the presence of lactic acid bacteria. The interest needed for the recognition of probiotic microorganisms as important health agents was renewed by the characterization of specific probiotic cultures and by the scientific demonstration of their positive influence on health [3]. The Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) joint Working Group defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4,5,6].

Over the past decade there has been a steady increase in demand for probiotic-enriched products where consumption increase of up to 150% has been recorded [7]. The probiotic market currently accounts for 10% of the functional food market [8]. Although dairy products are currently the main suppliers of probiotics [9,10], more and more non-dairy products containing probiotics are being developed from cereals, chocolate bars, cookies, soft bars and probiotic-enriched juices [11,12,13]. There is a growing emphasis on healthy and beneficial eating and a positive public perception of the positive effects of "good bacteria".

Despite its increasing diversification, the probiotic market still appears to be too limited to ensure sufficient

consumption of probiotics necessary to achieve the beneficial effects they provide [14,15]. Moreover, it remains mainly limited to dairy products, which makes it not easily accessible to low consumers of this range of products [10]. Indeed, habits, food tastes and behaviors, such as the increasingly important emergence of vegetarianism and veganism, keep consumers away from dairy probiotic products [16]. Moreover, a good percentage of the world's population is limited in their access to dairy probiotics due to lactose intolerance [17].

Whole grain cereals and cereal components offer a route of having probiotic vehicles with a dual advantage of providing beneficial bioactive constituents and fibers [18,19,20]. Such constituents comprise soluble fiber, non-digestible carbohydrates and phytochemicals such as antioxidants (phenolic compounds, vitamin C, carotenoids), phytoestrogens and phytic acids [18,19].

Despite being the principal source of dietary nutrients, cereal grains are deficient in some basic food constituents such as amino acids [6,21]. Fermentation may improve the nutritional value, sensory attributes and functional qualities of cereals [21,22], and their products such as beer.

In order to maximize consumption and diversify the sources of probiotics, and to give an added value to beer, the concept of developing a probiotic sorghum beer-like beverage fits. The main objective of this study is to determine the optimal aerobic fermentation conditions for the production of a probiotic sorghum beverage using *Lactobacillus fermentum*, *Bifidobacterium bifidum* and bil-bil. Specifically, the fermentation temperature, duration and the proportion of each component in the ferment – mix has to be determined.

2. Material and Methods

2.1. Material

The plant material used for the production of the beer was a sorghum cultivar, *S.35*, which was purchased from the Institute of Agricultural Research for Development (IRAD) Maroua in April 2020.

One of the ferment, a traditional beer (bil-bil), produced from a random mixture of two sorghum cultivars: dark red ndjigari and pale red mbayéri was obtained from local vendor in the Ngaoundere locality. The lactic ferments used, whose probiotic potential were prior determined, were *Lactobacillus fermentum* and *Bifidobacterium bifidum*, obtained from the Food Microbiology Laboratory of ENSAI, University of Ngaoundere.

2.2. Methods

2.2.1. Microbiological characterization of traditional beer

- ***Revival and multiplication of strains***

The probiotic strains, *Lactobacillus fermentum* and *Bifidobacterium bifidum* were initially lyophilized and therefore necessitates revival before it could be applied. Into 10 mL of maximum recovery dilution saline (DS; 0.85% NaCl and 0.1% peptone in distilled water) was added 1 g of lyophilizate of each strain and the resulting suspension well agitated for 10 min. The solution was then transferred to 1 L of previously prepared and sterilized MRS (De Man, Rogosa and Sharpe) broth. After incubation for 48 h at 42 °C, the MRS broth containing the multiplicates was centrifuged at 6500 *g* for 15 min at 4 °C. The supernatant was then removed, the pellet washed in DS without being re-suspended and then re-centrifuged as before. The supernatant was discarded and the pellet finally re-suspended in 10 mL of DS, and the volume later made up to 250 mL with DS. The probiotic concentration of this solution was obtained by serial dilutions of factor 10 in tubes containing 9 mL of SD. The dilutions were spread on MRS Petri dishes and incubated for 24 h at 42 °C and the colonies counted.

- ***Determination of the cell concentration of multiplicates***

Serial dilutions of factor 10 were carried out in tubes containing 9 mL of normal saline solution. Dilutions were inoculated by the spreading method on MRS plated Petri dishes and incubated at 42 °C for 24 h before colony counts were conducted.

2.2.2. Physicochemical characterization of sorghum grains

In order to determine if the sorghum grains cultivar *S.35* could be malted, several preliminary analyses were carried out: water content (about 5 g of crushed grains were dried at 105 ^{o}C for 24 hrs. The difference in mass before and after drying was expressed as the percentage water content per the massing before drying), germinative capacity (hydrogen peroxide method), germinative energy (4 mL and 8 mL test) and the weight of a thousand grains (to predict density). All tests were carried out according to the European Brewing Convention (EBC) methods [23].

2.2.3. Malting

Sorghum grains were sorted to be cleared of bad grains and foreign matter and then washed with distilled water. The grains were then steeped in distilled water at ambient temperature (22-25 °C) for 48 hrs. The steep liquor was changed after every 18 hours with 30 minutes of air-rest before re-steeping in a fresh liquor. At the end of steeping, the grains were put in a germination chamber at an initial temperature of 25 oC to allow for germination. Germination is a traditional and well-known technique to ameliorate the nutrient composition of grains. Furthermore, according to Gunenc *et al.* [24], germination has continuously been applied in softening grain structure and reducing anti-nutritional factors. Germination lasted for 48 hours upon development of shoots and rootlets. The germinated grains were kilned in a ventilated oven at a temperature of 45 °C for two days in order to arrest germination, conserve enzymes and stop further degradation of the grains' starchy endosperm [25].

2.2.4. Mashing

Decoction mashing was adopted for sweet wort production. The malted sorghum grains were ground to a coarse flour using a Polymix PX-MFC 90D grinder. Using a PAKWA DAHONGYING brand electronic scale, 4 kg of coarse flour were weighed and added to 25 L of distilled water (at 45 °C) contained in the brewing tank (BRAUMEISTER). The pump was turned on to agitate

the media to avoid floc formation and the vessel was held at 45°C (optimal temperature for protein hydrolyses) for one hour. Upon resting to allow for decantation, part of the supernatant (21 L) was removed. The rest, containing that starch granules, was brought to boiling while stirring intermittently at regular intervals, in a separate vessel to allow for starch gelatinization. After gelatinization, the resulting paste and supernatant were returned to the Braumeister and the temperature raised to 65 °C, marking the start of saccharification (amylase activity). After one and a half hour, the temperature was raised to 72°C and agitation continued at this temperature for 30 min. The spent grains were then filtered off and the resulting wort was boiled for one hour. Cooling immediately followed to prepare for fermentation.

2.2.5. Fermentation

A 5-factor D-optimal mixing plan (three components of the mixture and two process factors) was used for the fermentation of cultivar S.35. The five factors were the quantity of L. fermentum, the quantity of B. bifidium, the amount of bil-bil, the fermentation temperature and fermentation time. For the seeding rates of L. fermentum and B. bifidium, the choice of levels (1-10%) took account of exploratory studies. For the bil-bil rate, the interval (80-98 %) was chosen, taking into account the fundamental stress after setting the rate of L. fermentum and B. bifidium. The interval (37-42°C) was considered for temperature based on the optimal growth temperature of the two lactic ferments. Exploratory studies showed a considerable contamination after three days of fermentation, thus, 1-3 days was considered. Fermentation was carried out in an anaerobic condition. The fermentation operation was repeated trice and each fermentation sample analyzed.

2.2.6. Physicochemical analyses

- ***Probiotic load***

The probiotic loading was determined by the seeding dilution method.

- ***Total Soluble Solids (Brix)***

Total soluble solid was measured with the help of an optical refractometer (Hanna Instruments HI-96801). Distilled water was used to calibrate the instrument before reading of samples.

- ***pH***

The pH of samples were measured directly using a CONSORT C830 pH meter.

- ***Titratable acidity***

The AFNOR [26] method was used to determine titratable acidity using 0.1 N NaOH with phenolphthalein as an indicator.

In a conical flask, 5 mL of each beverage sample was introduced, and three drops of phenolphthalein was added. The sample was titrated with a 0.1 N NaOH solution till a persistent pink coloration was attained. The volume of NaOH consumed, was noted in mL.

The total titratable acidity was calculated thus:

$$Titratable\ \text{Acidity} = \frac{75*V*N}{T} \qquad Eq\ 1$$

V = volume of NaOH used,

N = concentration of NaOH T = sample volume

- ***Antiradical activity***

Antiradical activity at DPPH (2.2 diphenyl-1-picrylhydrazine) was evaluated using the method described by Muanda et *al.* [27].

- ***Turbidity***

Beverage sample was inserted in an HACH 2100N turbidimeter and the turbidity read directly.

- ***Viscosity***

It was evaluated using a NDJ-5S rotary viscometer.

- ***Vitamin C content***

The iodine titration method was employed in the determination of vitamin C as described by Helmenstine [28]. In the form of triiodide, iodine oxidizes vitamin C to form dehydroascorbic acid.

$$C_6H_8O_6 + I_3^- + H_2O \rightarrow C_6H_6O_6 + 3I^- + 2H^+$$

After complete oxidation of vitamin C, excess iodine and triiodide will react with starch to form a blue-black complex, marking the endpoint of the titration.

A 1 % starch solution was prepared by adding 0.5 g of soluble starch in 50 mL of distilled water at 90 ^oC. The solution was well agitated to enable complete dissolution.

Iodine solution was prepared by dissolving 5 g of KI and 0.268 g of KIO_3 in 200 mL of distilled water. 30 mL of 3 M sulfuric acid was then added. The volume was then completed to 500 mL with distilled water.

A standard solution of vitamin C was prepared by dissolving 0.25 g of vitamin C in 100 mL of distilled water. After complete dissolution, distilled water was used to make up 250 mL.

The vitamin C content of the standard solution was determined using 25 mL in a 250 mL conical flask and 5 drops of 1 % starch solution added to it. The resulting solution was titrated with iodine until the endpoint and the volume noted. A similar procedure was carried out for the probiotic beer samples.

The vitamin C content was calculated as the ratio of the volume of iodine used for the standard solution compared to beer samples.

- ***Polyphenol Content***

Phenolic compounds were extracted using 70% ethanol, then determined by the Folin-Ciocalteu reagent method [29].

Beer samples were diluted at a ratio of 1:5 with distilled water and placed in a volumetric flask. To prepare 5000 mg/L mother solution, 50 mg of gallic acid was added in a 10 mL volumetric flask and dissolved in 2 mL of methanol, and distilled water was used to complete the volume. From the mother solution, 1 mL was added to a 50 mL volumetric flask and the volume completed with distilled water. A standard was prepared with 1, 2, 3, 8, 12, and 24 mg/L and distilled water was used as blank. The calibration standard curve was achieved by adding 3, 6, 12, 24, 36, and 72 µL of 100 ppm gallic acid to a final volume of 208 µL with distilled water. For the beer samples, 24 µL were mixed with 184 µL distilled water in a thermos-microtiter 96-well plate (TM Roskilde), adding 12 µL of Folin-Ciocalteu reagent and 30 µL of sodium carbonate (200 g/L). The mixtures were incubated in the dark for 1 hr at ambient temperature. After the incubation period, 50

µL of distilled water was added, and absorbance was read at 765 nm.

The results were expressed as milligram equivalent of gallic acid in 100 g of dry product from equation 2 obtained from the gallic acid standard curve.

Optical density = aQ + b …… Eq 2

Q: the amount of phenolic compounds; a, b constants to be determined.

- ***Reducing Sugars***

The method described by Alejandro *et al*. **[30]** for the determination of reducing sugar was modified and used. In a 50 mL flask, 0.5 g of DNS was weighed and dissolved in 10 mL of 10 % NaOH. Then 15 g of Na and K double tartrate was dissolved in 25 mL of distilled water. The two solutions were mixed and made up to 50 mL with distilled water.

A standard solution, S_1, of maltose with a concentration of 2 mg/mL was prepared by mixing 0.1 g of maltose in 50 mL of distilled water. Standard solutions S_2, S_3, S_4, and S_5, of concentrations 0.25, 0.5, 1, and 1.5 mg/mL, were prepared by serial dilution of solution S_1. Using standard solutions S_1, S_2, S_3, S_4, and S_5 of maltose, the calibration range was prepared, and the test of the samples was carried out as indicated. The quantity of reducing sugars in each test sample was determined by referring to the regression equation calibration curve as in equation 2.

2.2.7. Modeling

The factors chosen were those which would have a significant influence on the microbiological and physicochemical characteristics of the beverage. These include the seeding rate of *L. fermentum* (A), the seeding rate of *B. bifidum* (B), the seeding rate of bil-bil (C), the fermentation temperature (D) and the fermentation time (E). The D-optimal mixing plan was used to define the different tests.

The mathematical models obtained took into account the coded variables. These were polynomial mathematical models of the quadratic type taking into account the elements of the first degree (A, B, C, D, and E), the second degree (A^2, B^2, C^2, D^2 and E^2) and interactions (AB, AC, AD, AE, BC, BD, BE, CD, CE and DE). These factors were considered statistically significant if the probability (p) was ≤ 0.05.

The responses that were measured were: pH, Brix, probiotic load, reducing sugar content, Vitamin C content, total polyphenols, antioxidant activity, turbidity, viscosity and titratable acidity.

2.2.8. Optimization

Digital optimization was carried out with the software Design-Expert 11. To optimize a response or all of the responses, it was a question of setting the optimal conditions for each response and then using the software for practical modalities. The latter made it possible to obtain the theoretical optimum (maximum or minimum) of all the responses.

3. RESULTS AND DISCUSSION

3.1. Physico-chemical characteristics of sorghum grains

Prior to malting, sorghum grains were tested for their malting potential and aptitude for beer production. The physico-chemical characteristics of the grains are presented in Table 1.

Table 1. Physico-chemical characteristics of S.35 sorghum grains

Characteristics	Experimental values	Reference value
Water content (%)	7.02 ± 0.16	≤ 13 [31]
Thousand corn weight (g)	19.2 ± 0.1	7 – 61 [31]
Germinative energy (4 ml) (%)	99 ± 0.0	60 – 100 [23]
Germinative energy (8 ml) (%)	97 ± 0.0	40 – 100 [23]
Germinative capacity (%)	100	92–100 [23]

The water content of the sorghum S35 cultivar was 7.02 ± 0.16 %, a value that is less than the recommended 13 % [31] for long term storage of cereal grains. This implies that the grains could be stored for a long period of time. More so, appropriate steeping duration will be required to increase the water content to facilitate germination during malting.

The thousand corn weight is an indication on grain size. For cereals destined for beer making, this parameter gives an estimate of the yield of wort density if starch is totally hydrolyzed [31]. The thousand corn weight also had a value within the recommended range.

The germinative energy gives the percentage of grains which can be expected to germinate fully if the sample is malted normally at the time of the test. The values obtained were 99 ± 0.0 and 97 ± 0.0 % for the 4 and 8 mL tests respectively, giving a water sensitivity value of 2 %. The germinative capacity gives a measure of living grains in the sample. A value of 100 % was obtained, implying that the sample was entire viable.

According to Analytica-EBC [23] and Briggs *et al*. [31], the sorghum S35 grains met with malting expectations owing to the analyzed characteristics.

3.2. Physico-chemical characteristics of sorghum wort and bil-bil

After malting of sorghum grains and subsequent mashing of the malt, wort to be fermented was obtained. The wort, and one of the ferment, the traditional beer bil-bil, were characterized. The results of the physico-chemical analyses are shown in Table 2.

The reducing sugar content and brix are indications of wort to undergo fermentation as they indicate the nutrient source (fermentable sugar) to be converted by specific microorganisms during the fermentation process [31]. The relative high values of these parameters makes the wort suitable as a good fermentation medium.

The pH of the wort was found to be 6.12 ± 0.55, a pH value suitable for the growth of *B. bifidum*, whose optimal pH range is 6.5 – 7.0, and does not grow below pH 4.5 – 5.0 [32, 33]. *L. fermentum*, on the other hand, is known to tolerate pH values down to 4.5 [34].

Vitamin C content was lower in the sorghum wort compared to that of the bil-bil. This could be due to the fact that the bil-bil has undergone fermentation and is therefore richer in vitamin C. Similarly, polyphenols were lower in the sorghum wort compared to that in the bil-bil. On the other hand, the titratable acidity, reducing sugar content, total soluble sugars and pH were relatively lower in bil-bil, due to fermentation metabolism [35].

Table 2. Physico-chemical characteristics of sorghum wort and bil-bil

Parameters	Sorghum wort	Bil-bil
Titratable acidity (mEqg Ac.gal/mL)	5.84 ± 0.36	3.72 ± 0.13
Reducing sugar content (mg/mL)	15.12 ± 0.18	0.02 ± 0.11
Polyphenol content (mg/mL)	0.2 ± 0.01	0.3 ± 0.02
Brix (°Brix)	11.43 ± 0.20	5.90 ± 1.10
pH	6.12 ± 0.55	3.26 ± 0.16
Viscosity (mPa.s)	3.50 ± 0.50	8.50 ± 0.50
Turbidity (EBC)	98.57 ± 1.04	612.68 ± 0.10
Vitamin C (mg/L)	7.61 ± 0.08	16.82 ± 0.24

3.3. Microbiological Characteristics of bil-bil

Microbiological analyses showed that bil-bil contained 5×10^4 CFU/mL of total mesophilic bacteria and 3.5×10^3 CFU/mL of lactic acid bacteria. These values were relatively low, enabling the bil-bil to be a non-pathogenic source. Worthy of note is the fact that bil-bil is a consortium of different microorganism, among which are lactic acid bacteria which are of probiotic interest [36]. Though bil-bil contains probiotics, it is far from being considered a probiotic drink because of its relatively low concentration of probiotic bacteria compared to the limits (10^6 to 10^9 CFU/mL) stipulated be WHO. If used as a starter culture, upon cell multiplication during fermentation, the eventual product obtain could have the recommended dose of probiotic concentration.

Yeast cells were identified but the concentration was not determined since the point of interest was on probiotic strains.

3.4. Modelling and Evolution of Parameters during Fermentation

3.4.1. Probiotic Load Evolution

The mathematical model obtained following the evolution of probiotic load took into consideration coded and real variables, as presented in equation 2:

$$Y_{CP}\ (A,B,C,D,E) = +44.43A +16.96B +0.9765C -38.39AB -39.77AC +2.77AD +13.60AE -10.03BC -15.22BD +14.32BE + 0.0470CD +0.4498CE + 20.75ABD -51.17ABE -7.79ACD -23.06ACE +21.19BCD -25.72BCE \quad \textbf{Eq 3}$$

With Y_{CP}: Probiotic load

- ***Influence of single factors on probiotic load***

Figure 1a presents the evolution of the probiotic load as a function of the inoculation rates of L. fermentum (A) and bil-bil (C). Taken individually, the two ferments contribute significantly in increasing the probiotic load, as shown by their positive coefficients in the model equation. A similar tendency was obtained with L. fermentum (A) and B. bifidum (B) as shown in Figure 1. Effectively, the probiotic load increased from 2 to 23×106 CFU/mL. This is due to microbial multiplication during the fermentation process [37].

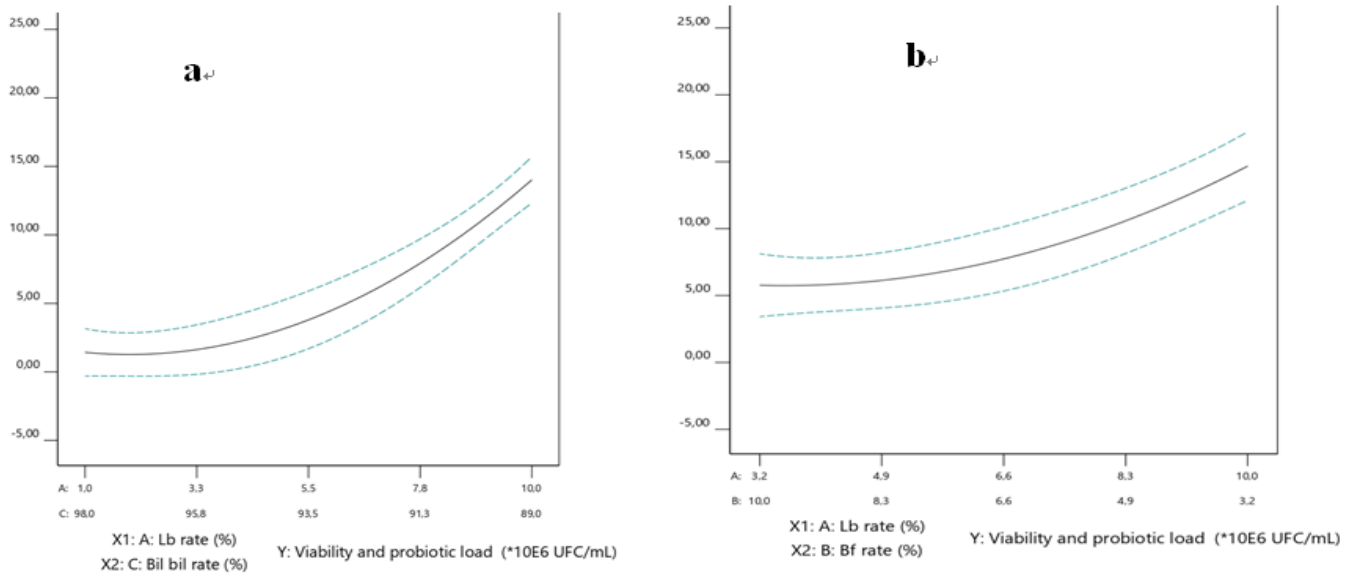

Figure 1. Evolution of probiotic load as a function of (a) L. fermentum and bil bil and (b) L. fermentum and B. bifidum seeding rates

- ***Influence of ferment seeding rate interactions on probiotic load***

Evolution of the probiotic load as a function of the seeding rates of L. fermentum (A), B. bifidum (B) and bil-bil (C) is as shown in Figure 2. The three ferments, taken

individually, contribute significantly in increasing the probiotic load as the concentration of the ferment increases. This is shown by the coefficients of the model which are positive for all three factors. Indeed, the probiotic load increases from 2 to 23x106 CFU/mL. This could be due to the multiplication of microorganisms in the medium as a function of fermentation time [37].

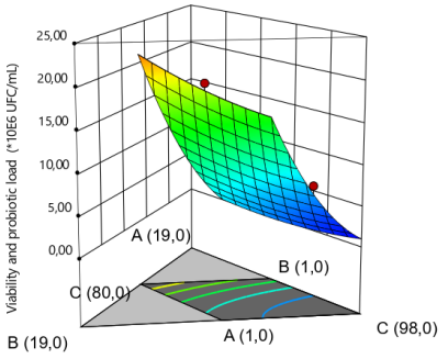

Figure 2. Evolution of probiotic load as a function of seeding rates of *L. fermentum*, *B. bifidum* and bil-bil

3.4.2. Monitoring Titratable Acidity

The mathematical model obtained for titratable acidity is shown in equation 4:

$$Y(A,B,C,D,E) = +3.64\times A +3.634\times B +3.59\times C +0.0268\times A\times D -0.3694\times A\times E + 0.0477 \times B \times D - 0.2705\times B\times E +0.0050\times C\times D -0.5421\times C\times E - 0.0410\times A\times D\times E + 0.0036 \times C\times D\times E + 0.0042 \times C\times D\times E + 0.2918\times A\times D^2 -0.4541\times A\times E^2 +0.1511\times A\times C\times D - 0.3485\times B\times E^2 - 0.2590 \times C\times D^2 +0.6876\times C\times E^2$$

Eq 4

Evolution of titratable acidity of the beers as a function of fermentation time (E) and the seeding rates of *L. fermentum* (A) and *B. bifidum* (B) is shown in Figure 3a, while Figure 3b shows the evolution of the titratable acidity of the beers as a function of the fermentation time (E), L. fermentum seeding rate (A) and bil-bil rate (C).

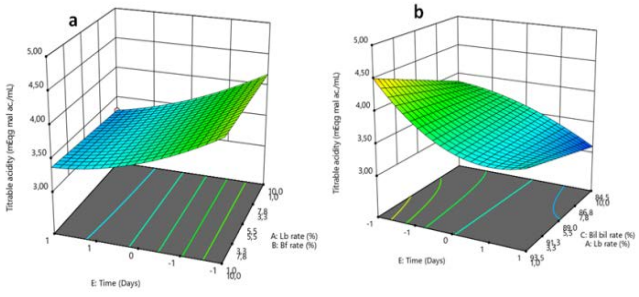

Figure 3.: Evolution of titratable acidity of beers as a function of **(a)** fermentation time, seeding rates of L. *fermentum and B. bifidum* and **(b)** fermentation time, seeding rate of L. *fermentum and bil-bil*

In each case, all three factors combined contribute significantly in increasing the titratable acidity. Indeed, the acidity decreased from 4.3 to 3.4 mEqg of malic acid/mL for fermentation times ranging from 1 to 3 days and for the seeding rates of L. fementum and B. bifidum varying from 1 to 10%. During aerobic fermentation, organic acids are released over time by the microorganisms present, thus acidifying the medium [38]. This is done either by genetic mutation of the microorganisms or by co-culture (bacteria-yeast).

3.4.3. Monitoring Brix

The sugar saturation model is depicted by equation 5:

$$Y(A,B,C,D,E) = +3.80\times A +7.04\times B +4.60\times C +1.37\times A\times B +6.06\times A\times C -0.3232\times A\times D -0.8585 \times A\times E -0.2682\times B\times C +0.2493\times B\times D -1.42\times B\times E +0.1761\times C\times D -1.23\times C\times E + 0.0987 \times A\times B\times D +0.0998\times A\times B\times E +0.4151\times A\times C\times D +0.2924\times A\times C\times E +0.3237\times A\times D\times E -0.7112 \times B\times C\times D +1.30\times B\times C\times E -0.5855\times B\times D\times E +0.1292\times C\times D\times E - 0.3027\times A\times D^2 + 1.85 \times A\times E^2 +1.20\times B\times D^2 -2.05\times B\times E^2 +0.4459\times C\times D^2 +0.3363\times C\times E^2 + 0.5623 \times A \times B \times D \times E - 0.3180 \times A\times C\times D\times E +1.25\times B\times C\times D\times E -1.32\times A\times B\times D^2 + 0.8051 \times A \times B\times E^2 -0.3697 \times A \times C\times D^2 -5.25\times A\times C\times E^2 - 3.39\times B\times C\times D^2 +2.32\times B\times C\times E^2$$

Eq 5

Figure 4 shows the evolution of the Brix of beers as a function of the *L. fermentum* seeding rate (A) and the bil-bil (C). The Brix increases when the *L. fermentum* seeding rate increases in the beer and a decrease in the bil-bil rate. Furthermore, the Brix value increases from 4.6 to 5.72 ºBrix for *L. fermentum* seeding rates ranging from 1 to 10% and for bil-bil seeding rates ranging from 98 to 89%. This could be due to the fact that bil-bil contains a consortium of microorganisms capable of transforming the sugars contained in the wort.

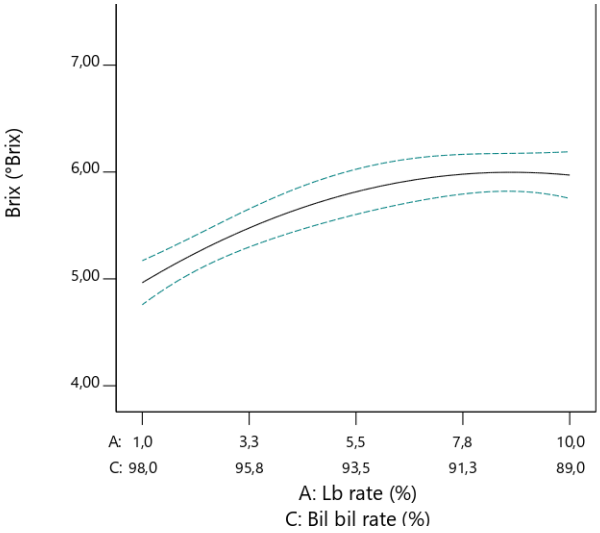

Figure 4. Evolution of the Brix of beers per the seeding rate of *L. fermentum* and bil-bil

Figure 5a shows Brix evolution in beers as a function of the *B. bifidum* seeding rate (B) and the fermentation time (E). The *B. bifidum* seeding rate alone does not significantly influence the Brix. However, combined with the fermentation time, they have a significant impact on Brix. They contribute significantly to lowering the Brix. For any value of *B. bifidum* rate and for a long fermentation time (E = 3 days), the Brix was the lowest possible (4.5 °Brix). This could be explained by the multiplicative metabolism of *B. bifidum* over time, although it was in small quantities compared to bil-bil.

The Brix evolution of the beers as a function of the bil-bil seeding rate (C) and the fermentation time (E) is shown in Figure 5b.

The interaction of these two factors significantly influences the Brix. They contribute significantly in lowering the Brix. For a high value of bil-bil (C = 98%) and for a long fermentation time (E = 3 days), the Brix is the lowest possible (4.5 °Brix). This could be as a result of the fermentative metabolism of the microorganisms

contained in bil-bil. Indeed, these microorganisms need nutrients (mainly sugars) to ensure their survival over time. It is the phenomenon of glycolysis that is involved.

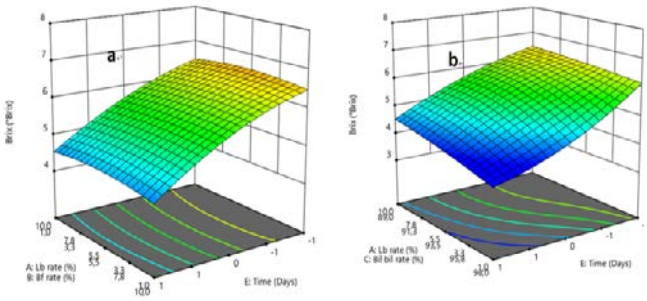

Figure 5. Evolution of the Brix of beers according to **(a)** seeding rate of B. bifidum and fermentation time, and **(b)** seeding rate of bil-bil and fermentation time.

3.4.4. pH monitoring

Equation 6 represents the mathematical model for pH

$$Y(A,B,C,D,E) = 3.17\times A + 3.30\times B + 3.11\times C - 0.5817\times A\times B - 0.2470\times A\times C - 0.2112\times A\times D + 0.3646\times A\times E - 0.4436\times B\times C + 0.2694\times B\times D + 0.2287\times B\times E + 0.0715\times C\times D - 0.0831\times C\times E + 0.3071\times A\times B\times D - 1.75\times A\times B\times E + 0.2017\times A\times C\times D - 1.02\times A\times C\times E + 1.03\times A\times D\times E - 0.9669\times B\times C\times D - 0.6183\times B\times C\times E - 0.6137\times B\times D\times E - 0.0706\times C\times D\times E - 0.5078\times A\times D^2 + 0.0053\times A\times E^2 + 1.02\times B\times D^2 - 1.77\times B\times E^2 - 0.1636\times C\times D^2 + 0.1028\times C\times E^2 - 1.22\times A\times B\times D\times E - 1.68\times A\times C\times D\times E + 1.53\times B\times C\times D\times E - 0.5873\times A\times B\times D^2 + 3.53\times A\times B\times E^2 + 1.52\times A\times C\times D^2 - 0.2343\times A\times C\times E^2 - 1.28\times B\times C\times D^2 + 3.04\times B\times C\times E^2 \quad \textbf{Eq 6}$$

The evolution of the pH of the beers as a function of the bil-bil seeding rate (C), the *L. fermentum* seeding rate (A) and the fermentation time (E) is shown in Figure 6.

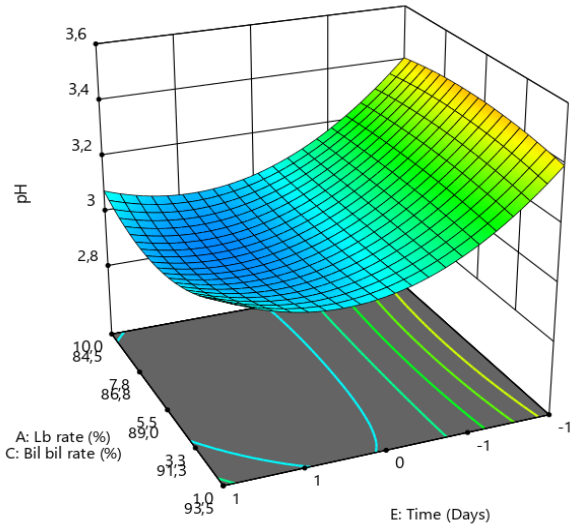

Figure 6. Evolution of pH of beers as a function of the bil-bil and *L. fermentum* seeding rates and the fermentation time.

The interaction of these factors significantly decreases the pH. This decrease in pH could be due to the fact that the microorganisms consume the sugars present in the medium to produce ethanol. The latter is then oxidized into organic acids by lactic acid bacteria and yeasts, releasing organic agents. The lactic acids and organic agents released contribute to the formation of free H+ ions in the medium, resulting in acidification of the medium [39,40].

3.4.5. Monitoring antioxidant activity

The mathematical model for antioxidant activity is depicted in equation 7:

$$Y(A,B,C,D,E) = +53.85 + 2.52\times D + 20.35\times E \quad \text{Eq 7}$$

The evolution of the antioxidant activity of beers as a function of fermentation time is shown in Figure 7.

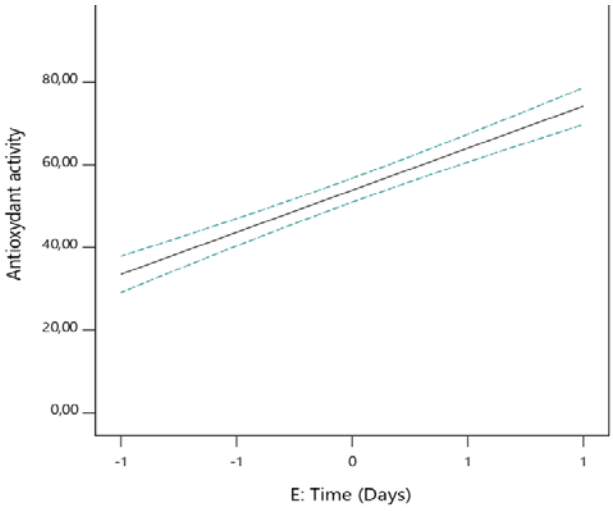

Figure 7. Evolution of the antioxidant activity of beers as a function of fermentation time

Time contributes significantly to the increase in antioxidant activity. This increase could be due to the secretion of free radical scavenging compounds such as vitamin C and phenolic compounds from the medium with time, which over time act as primary antioxidants. The action of these antioxidants is thought to be due to their ability to donate hydrogen atoms or electrons derived mainly from the hydroxyl ring of flavonoids [41].

3.4.6. Monitoring reducing sugars

Equation 8 shows the mathematical model for reducing sugars evolution during fermentation.

$$Y(A,B,C,D,E) = +1.37\times A + 1.26\times B + 0.0.8334\times C + 0.2214\times A\times D - 0.7687\times A\times E - 0.0212 \times A \times D - 0.6016\times B\times E + 0.0628\times C\times D - 0.5547\times C\times E \quad \text{Eq 8}$$

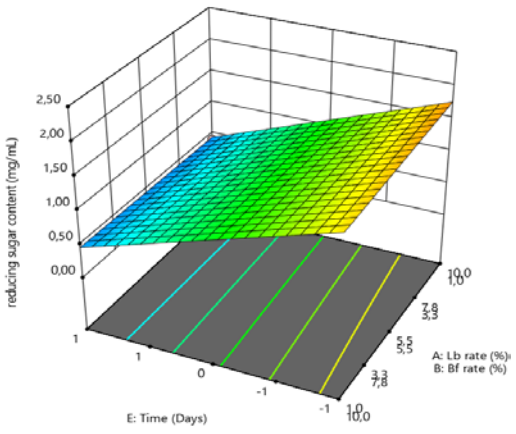

Figure 8. Evolution of reducing sugars as a function of fermentation time and the seeding rate of bil-bil

Figure 8 shows the evolution of reducing sugars as a function of fermentation time and bil-bil seeding rate.

Reducing sugars decreases significantly with increase in fermentation time combined with the bil-bil seeding rate. Indeed, the values range from 1.5 mg/mL to 0.0 mg/mL for fermentation time values ranging from 1 to 3 days and for ferment rate values varying between 1 and 10%. The bil-bil used is a consortium of microorganisms including yeasts. The yeast will consume the sugar present in the medium, producing alcohol. The higher the bil-bil seeding rate for a fix volume of wort, the greater the yeast concentration and the more reducing sugar is being consumed.

3.4.7. Monitoring phenolic content

Evolution of the phenolic content of beers during fermentation is shown in the mathematical model of equation 9:

$$Y(A,B,C,D,E) = +0.2860\times A +0.2676\times B +0.3261\times C -0.0160\times A\times D ++0.2038\times A\times E +0.0080\times C\times D\times E +0.0362\times A\times D^2 -0.0861\times A\times E^2 +0.2172\times B\times D^2 -0.0598\times B\times E^2 +0.0152\times C\times D^2 +0.0973\times C\times E^2 \quad \textbf{Eq 9}$$

The evolution of phenolic content as a function of fermentation time and bil-bil seeding rate is shown in Figure 9. The phenolic compound content increases significantly with increase in fermentation time combined with the bil-bil seeding rate. Indeed, it goes from 0.00 mg/mL to a value of 5.59 mg/mL for fermentation time values ranging from 1 to 3 days and for bil-bil values varying between 84.5 and 93.5%. This growth could be due to the release of polyphenols during fermentation. Indeed, microbial metabolism is characterised by the production of antioxidant compounds including polyphenols [42].

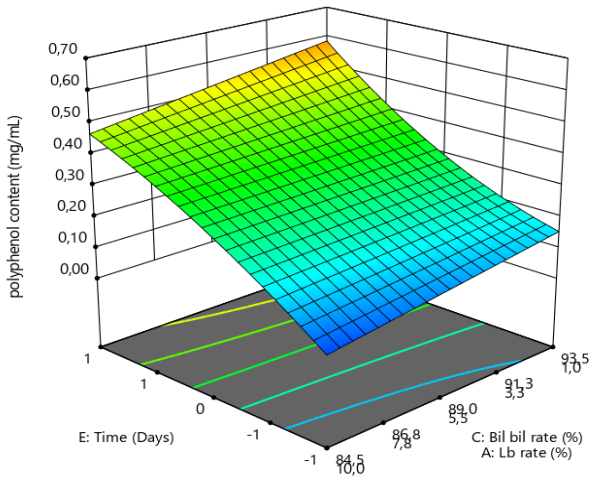

Figure 9. Evolution of the polyphenols of beers according to the rate of sowing of bil-bil, the rate of seeding of L. fermentum and the fermentation time.

3.4.8. Monitoring Vitamin C content

The mathematical model of vitamin C evolution is depicted in equation 10.

$$Y(A,B,C,D,E) = +50.84\times A +36.81\times B +42.14\times C -2.54\times A\times D +26.45\times A\times E -2.28\times B\times D +16.39\times B\times E +3.84\times C\times D +20.01\times C\times E -6.57\times A\times D\times E +1.65\times B\times D\times E +1.13\times C\times D\times E -22.34\times A\times D^2 -26.90\times A\times E^2 -2.48\times B\times D^2 +8.89\times B\times E^2 +5.93\times C\times D^2 +6.47\times C\times E^2 \quad \textbf{Eq 10}$$

Figure 10 shows the evolution of vitamin C as a function of the fermentation time, the seeding rates of bil-bil and *L. fermentum*.

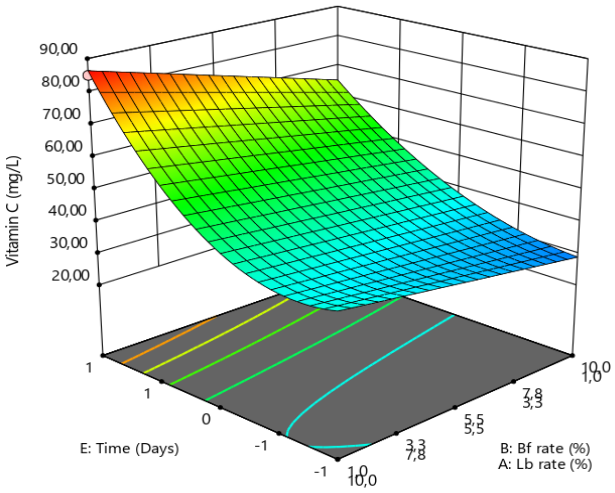

Figure 10. Evolution of vitamin C content as a function of fermentation time, the seeding rates of *B. bifidum* and *L. fermentum*.

The vitamin C content increases significantly with fermentation time combined with the *B. bifidum* rate. In fact, it goes from 26 mg/L to a value of 83 mg/L for values of fermentation time ranging from 1 to 3 days and for values of bil-bil rate varying between 1 and 10% (possibly 1 to 10% *L. fermentum*). This growth could be due to the release of antioxidants during fermentation. Indeed, microbial metabolism is characterized by the production of antioxidant compounds, including vitamin C [42].

3.4.9. Monitoring turbidity

Turbidity evolution was modelled mathematically as shown in equation 11.

$$Y(A,B,C,D,E) = +3184.04\times A -4616.04\times B +865.56\times C +4486.02\times A\times B -5968.64\times A\times C +9636.49\times B\times C -5555.16\times A\times B\times C -9848.94\times A\times B(A\text{-}B) -3943.58\times A\times C(A\text{-}C) + 6762.36 \times B \times C(B\text{-}C) \quad \textbf{Eq 11}$$

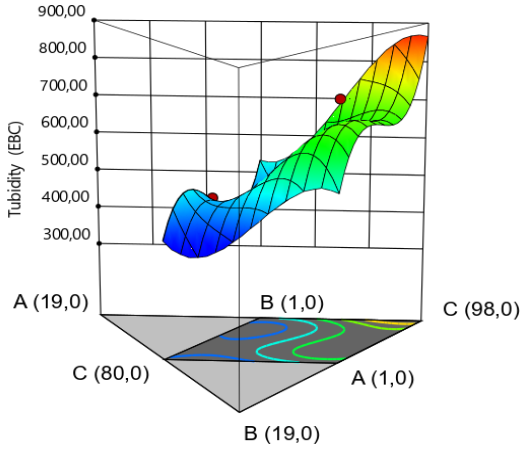

Figure 11. Evolution of the turbidity of beers as a function of the seeding rate of the ferments

Figure 11 shows the evolution of the turbidity of beers as a function of the different ferments. It can be seen that all factors significantly influence turbidity, even when combined. Furthermore, the turbidity value increases between 300 and 800 EBC for A and B values ranging from 1 to 10% and for bil-bil seeding rates varying between 84.5 and 93.5%. This could be due to the effect of microbial metabolism whereby the microorganisms present release biopolymers into the medium thereby increasing turbidity [43].

3.4.10. Monitoring viscosity

The mathematical model for viscosity is shown in equation 12:

$$Y(A,B,C,D,E) = +44.57\times A -15.84\times B +10.24\times C -36.96\times A\times B -81.41\times A\times C +37.60\times B\times C +40.42\times A\times B\times C -75.14\times A\times B(A-B) -55.98\times A\times C(A-C) +24.06\times B\times C(B-C) \quad \text{Eq 12}$$

Figure 12 shows the evolution of beer viscosity as a function of temperature, *B. bifidum* seeding rate (B) and bil-bil rate (C). It was found that the viscosity decreased significantly when these factors were combined. Furthermore, the viscosity value decreases from 8.8 to 5.00 mPa.s for seeding rate values of *L. fermentum* and *B. bifidum* ranging from 1 to 10%. This could be due to the effect of microbial metabolism whereby the microorganisms present release biopolymers into the medium which make it more viscous.

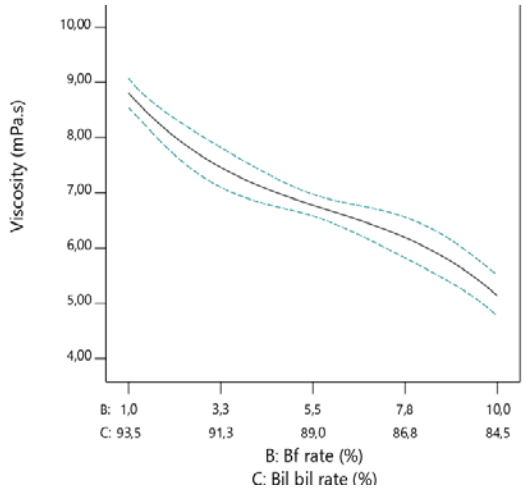

Figure 12. Evolution of viscosity of beers as a function of *B. bifidum* and bil-bil seeding rates

3.5. Fermentation Optimization

In order to find ideal and optimal physicochemical and microbiological characteristics for consumption, an optimization was carried out using the Design Expert 11 software. The aim was to find an optimum for each response. The conditions obtained were: Inoculation rate of L. fermentum (A) = 10%; Inoculation rate of B. bifidum (B) = 7%; Inoculation rate of bil-bil (C) = 83%; Fermentation temperature (D) = 39.5 °C; Fermentation time (E) = 2 days. The optima responses obtained are presented in Table 4.

To perceive a positive health effect from consumption, a least concentration of probiotic microbes is essential. The recommended quantity is between 10^6 – 10^{11} CFU/day [44]. With a probiotic load of 17.9 × 10^6 CFU/mL, the beer produced under optimised conditions could be considered as a source of probiotics.

Table 4. Physico-chemical characteristics of beer produced under optimized conditions.

Characteristics	Optimum values
Titratable acidity (mEqg Mal.Ac./mL)	3.63
pH	3.06
Brix (°Brix)	5.74
Probiotic load (×10^6 CFU/mL)	17.90
Reducing sugars content (mg/mL)	0.95
Phenolic compound content (mg/mL)	0.28
Vitamin C content (mg/L)	44.61
Percentage of inhibition	51.80
Turbidity (mPa.s)	446.98
Viscosity (EBC)	5.43

Conclusion

The outcome of this study showed that mixed fermentation can be successfully carried out on sorghum wort to produce a probiotic beer. Alcohol fermentation was achieved by yeast present in bil-bil, while *L. fermentum*, *B. bifidum* and lactic acid bacteria found in bil-bil were responsible, not only for lactic acid fermentation, but also for the probiotic load.

Conflict of Interest

The authors declare no conflict of interest.

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