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Antagonistic effects of raffia sap with probiotics against pathogenic microorganisms

Manga Joseph Arsene Mbarga[1,2]*, Steve Carly Zangue Desobgo[1], Léopold Ngoune Tatsadjieu[1], Nyasha Kavhiza[2], Louis Kalisa[2]

[1] University of Ngaoundere, Ngaoundere, Cameroon

[2] Peoples' Friendship University of Russia, Moscow, Russia

* e-mail: joseph_mbarga@yahoo.com

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Abstract:
Introduction. Probiotics are known for their beneficial properties. Numerous studies have been conducted to find advantages that probiotics can provide. This study aimed to evaluate the functional properties of raffia sap, a Cameroonian drink, fermented with probiotics by investigating its antagonistic activity against pathogenic bacteria.
Study objects and methods. The study objective was raffia sap fermented by *Lactobacillus fermentum* and *Bifidobacterium bifidum*. Box-Behnken design with four factors (seeding rates of *L. fermentum* and *B. bifidum*, temperature, and incubation time) was used to generate mathematical models. The disc diffusion method was used to evaluate an antagonistic effect of the probiotics against four pathogenic bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp., and *Bacillus cereus*). An optimization of mathematical models of the inhibition diameters allowed to determine the optimal conditions of antagonistic effect.
Results and discussion. The experimental data showed that zones of inhibition were 0–21 mm for *Salmonella* sp., 0–23 mm for *E. coli*, 0–20 mm for *L. monocytogenes*, and 0–22 mm for *B. cereus*. ANOVA results and the mathematical models obtained showed that *L. fermentum* was effective against *B. cereus* and *B. bifidum* against *Salmonella* sp., *E. coli*, and *B. cereus*. The optimization of the models revealed maximum zones of inhibition at the seeding rates of *L. fermentum* and *B. bifidum* of 2 and 10%, respectively, incubation time of 48 h, and temperature of 37°C.
Conclusion. Raffia sap fermented by *L. fermentum* and *B. bifidum* demonstrated antagonistic effect against pathogenic bacteria such as *E. coli*, *L. monocytogenes*, *Salmonella* sp., and *B. cereus*.

Keywords: Probiotics, antagonistic activity, pathogenic bacteria, response surface methodology, mathematical model

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INTRODUCTION

Probiotics are defined as microorganisms that, when ingested in sufficient quantity, effect beneficially the host [1, 2]. The beneficial effects resulting from the consumption of foods enriched with probiotics have been known for millennia [3]. At the beginning of the 20th century, Mechnikov, a winner of the Nobel Prize, suggested replacing the dangerous germs by useful bacteria [4]. Additionally, *Bifidobacterium* spp. was recommended against infantile diarrhea [3, 5]. Despite the scientists' research, the idea of eating certain bacteria to improve the health of the digestive system was ignored. Taking into account the different technical issues related to the production of foods with probiotics, attention must be focused on their beneficial effects on health [6].

The latest studies in this area have shown that probiotic bacteria are able to stimulate the immune system and inhibit the adhesion and multiplication of pathogenic bacteria [7, 8]. Since pathogenic microorganisms are becoming resistant to antibiotics, probiotics are a new alternative to be studied in the search for new molecules and/or antibacterial organisms [9].

Such an antimicrobial or antibacterial effect is generally called an antagonistic effect. Factors responsible for the antagonistic effect of one microorganism against another one are: production

of organic acids or hydrogen peroxide that lower pH, competitive exclusion, immune system modulation, stimulation of defence systems, as well as production of antimicrobials such as bacteriocins and antioxidants [10]. Lactic, acetic, benzoic and other organic acids are the antimicrobial substances generally produced by beneficial microorganisms. The most produced bacteriocins are plantaricin, enterolysin, lacticin, lactocin, reuterin, pisciolin, enterocin, and pediocin [11].

Many probiotics have a broad spectrum of action and can be effective against diseases caused by food contaminated with certain pathogenic strains such as *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus* and *Salmonella*. These four bacteria are the most common pathogens causing food-borne diseases [12]. Generally, there are difficulties in selecting an appropriate strain, substrate, as well as in determining optimal conditions for probiotic effectiveness.

In this context, researchers use local Cameroonian raw materials, including raffia sap. Raffia sap is a widespread drink in sub-Saharan Africa and particularly in Cameroon. Raffia sap undergoes wild fermentation and produces raffia wine that is difficult to keep. In 10 h after the harvest, alcohol produced during the primary fermentation transforms into acid, which seriously compromises the organoleptic characteristics appreciated by consumers.

In our previous research, we developed a probiotic beverage with raffia sap fermented by *Lactobacillus fermentum* and *Bifidobacterium bifidum* [13]. In the current research we studied an antagonistic potential of raffia sap inoculated by probiotics. The study was aimed to use Response Surface Methodology (RSM) to evaluate and optimize the effectiveness of *L. fermentum* and *B. bifidum* against *E. coli*, *L. monocytogenes*, *Salmonella* sp., and *B. cereus*.

STUDY OBJECTS AND METHODS

Raffia sap harvesting. The fresh sap of less than eight hours was harvested in a 25 L container and transferred to the laboratory. The sap then was immediately dispensed into 1 L bottles and sterilized in a water bath at 65°C for 30 min. The bottles were cooled and stored at 4°C.

Bacteria and probiotics. Pathogenic bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp. and *Bacillus cereus*) were provided by the Food Microbiology Laboratory of Ngaoundere University. Probiotics (*Lactobacillus fermentum* and *Bifidobacterium bifidum*) were prepared using KwikStik™ lyophilized microorganism.

Revitalization and multiplication of probiotics. To revitalize and multiply probiotic cells contained in the freeze-dried products, 1 g of lyophilisate of each strain was rehydrated as recommended by the manufacturer. First, the powder was rehydrated in 10 mL of dilute saline solution (DS)consisting of 0.85% NaCl and 0.1% peptone in distilled water and stirred for 10 min until maximum recovery was reached. The solution was then transferred into 1 L of MRS broth previously prepared and sterilized. After incubation at 42°C for 48 h, MRS broth with probiotics was centrifuged at 6500 g and 4°C for 15 min.

The supernatant was removed, the pellet was washed in the saline solution without being resuspended and then recentrifuged as above. The supernatant was discarded and the pellet was finally resuspended in 10 mL of DS first and then transferred into 250 mL of DS. The concentration of probiotics in this solution was obtained by serial dilutions. The dilutions were spread on MRS petri dishes and incubated at 42°C for 24 h, then the colonies were counted [14].

Antagonistic effect of raffia sap fermented. To evaluate the antagonistic effect of the fermented raffia sap, we used the disc method described by Tadesse *et al*., with some modifications [5]. Mueller-Hinton agar was seeded with pathogenic bacteria (*L. monocytogenes*, *B. cereus*, *E. coli* and *Salmonella* sp.) and incubated at 37°C for 30 min. Sterile discs (5 mm) then were placed on the agar surface incubated at 37°C for 24 h. Each disk was impregnated with 100 µL of raffia sap fermented by probiotics according to the experimental design (Table 1). The inhibition of pathogenic bacteria resulted in the formation of clear zones around the discs. The zone of these inhibition zones was measured, which was used as the main response of the trial.

Experimental design for sap fermentation process and data analysis. Fermentation was done following a four factor Box-Behnken design. The factors were seeding rates of *L. fermentum* (X_1) and *B. bifidum* (X_2), temperature (X_3), and incubation time (X_4). The levels of each factor were chosen after prior testing (Table 1).

The Box-Benhken experimental matrix in coded variables (–1; 0; +1) was generated with the Minitab 18 software. This coded variable matrix consisted of 28 trials, four of which enabled a better evaluation of the experimental error; each trial was repeated three times. The experimental matrix applicable to the laboratory was obtained by transforming the matrix into the coded variables with the EXCEL software using the following formula:

Table 1 Range of variation and factor levels

Variable		Level of factor		
		–1	0	+1
Seeding rate of *Lactobacillus fermentum*, % (v/v)	X_1	0	5	10
Seeding rate of *Bifidobacterium bifidum*, % (v/v)	X_2	0	5	10
Temperature, °C	X_3	37.0	39.5	42.0
Incubation time, h	X_4	2	25	48

$$X_j = \frac{U_j - U_j0}{\Delta Uj} \quad (1)$$

where *Xj* is a value of the coded variable *j*; *Uj* is a value of the real variable *j*; *Uj0* is a value of real variable *j* at the center, and *ΔUj* is called a "step" of variation.

Modelling and optimization. The zones of inhibition zones obtained after the application of the various tests of the experimental matrix were analysed on Minitab 18. The obtained models were in the form of:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j \quad (2)$$

where y is the model of the inhibition zones of the strain concerned, $\beta_{(i,j)}$ are model coefficients and $x_{(i,j)}$ are the factors. The data was analysed at the level of 10%, including the maximum of significant factors on each model. Response Surface Methodology was used for the three-dimensional graphical representation of the models of each inhibition zone after setting temperature and incubation time at constant values. Sigmaplot 12 software was used to plot the curves. Optimization was done on Minitab with the specifications for maximizing the inhibition zones of each pathogen.

RESULTS AND DISCUSSION

The results of the measurements of the inhibition zones of pathogenic bacteria (*Salmonella* sp., *Escherichia coli*, *Listeria monocytogenes*, and *Bacillus cereus*) obtained after the implementation of the four factor Box-Behnken experimental matrix showed that the zones of inhibition ranged from 0 to 21 mm for *Salmonella* sp., 0 to 23 mm for *E. coli*, 0 to 20 mm for *L. monocytogenes*, and 0 to 22 mm for *B. cereus*.

Raffia sap without probiotics did not demonstrated an inhibitory activity against pathogenic bacteria (inhibition zone = 0). However, the study conducted by Ojo and Agboola displayed different results [15]. The authors evaluated the antagonistic activity of bacteria isolated from Palm wine (*Raphia vinifera* L.) towards *Salmonella typhi*. The study revealed that raffia sap, due to its own microbial flora, was antagonistic against several pathogenic bacteria, including *Salmonella* sp. This also could be explained by pasteurization of fresh sap to avoid any interaction between the natural microflora of the sap and added probiotics, as well as wild fermentation.

Thus, seeding rates of *Lactobacillus fermentum* and *Bifidobacterium bifidum* played an important role in the antagonistic effect of the drink against the pathogenic bacteria tested, but statistical analysis was performed for a better demonstration of these effects (Table 2).

Effect of factors on microbial inhibition. According the data in Table 2, *B. bifidum* did not show a strong antagonistic effect on *E. coli*, *L. monocytogenes*, and *Salmonella* sp., but it was effective against *B. cereus* ($P \leq 0.1$). *L. fermentum* had a significant antagonistic effect on *Salmonella* sp., *E. coli*, and *B. cereus* with probabilities of 0.060, 0.040 and 0.072, respectively. Moreover, the incubation time significantly increased all the zones of inhibition ($P = 0.000$).

Effect of incubation time on inhibition of pathogenic bacteria. The curves of inhibition zone of *E. coli*, *L. monocytogenes*, *B. cereus*, and *Salmonella* sp. as a function of time were obtained after fixing seeding rates of *B. bifidum* and *L. fermentum* at 0 in coded variables (5% in real variables) and the temperature at 0 in coded variable (39.5°C in real variable). Under these conditions, these curves (Fig. 1) showed that the inhibition zones of *E. coli* ranged from 8 mm (2 h of incubation) to 20 mm (48 h).

Table 2 ANOVA results and coefficients of mathematical model of inhibition zones for *Lactobacillus fermentum* and *Bifidobacterium bifidum* on *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus,* and *Salmonella* sp.

Terms	*Escherichia coli*		*Listeria monocytogenes*		*Bacillus cereus*		*Salomonella sp.*	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Constant	13.750	0	15.750	0	14.250	0	15.000	0
X_1	0.917	0.233	0.333	0.722	1.500	0.058	1.167	0.173
X_2	1.667	0.041	1.083	0.259	1.417	0.072	1.667	0.060
X_3	–0.500	0.507	0.333	0.722	0	1.000	0.417	0.615
X_4	6.417	0	5.750	0	6.083	0	6.583	0
X_1+X_1	–1.290	0.235	–1.580	0.244	–1.750	0.110	–1.040	0.379
X_2+X_2	–1.170	0.281	–3.460	0.019	–0.630	0.551	–2.540	0.045
X_3+X_3	–0.170	0.875	0.170	0.900	0.750	0.476	1.080	0.361
X_4+X_4	0.710	0.506	–0.710	0.594	0.370	0.719	–0.920	0.438
X_1+X_2	–3.750	0.011	–2.750	0.107	–4.000	0.007	–2.750	0.072
X_1+X_3	–0.250	0.847	–1.000	0.540	0.250	0.845	–1.000	0.488
X_1+X_4	0.250	0.847	0.250	0.877	–0.250	0.845	–0.250	0.861
X_2+X_3	0	1.000	0.250	0.877	1.750	0.185	2.250	0.133
X_2+X_4	0.250	0.847	–1.250	0.446	–0.500	0.696	0.500	0.727
X_3+X_4	–0.750	0.565	–0.750	0.645	–1.000	0.438	–1.000	0.488

where X_1 and X_2 are seeding rates of *L. fermentum* and *B. bifidum*, respectively, X_3 is temperature, and X_4 is incubation time

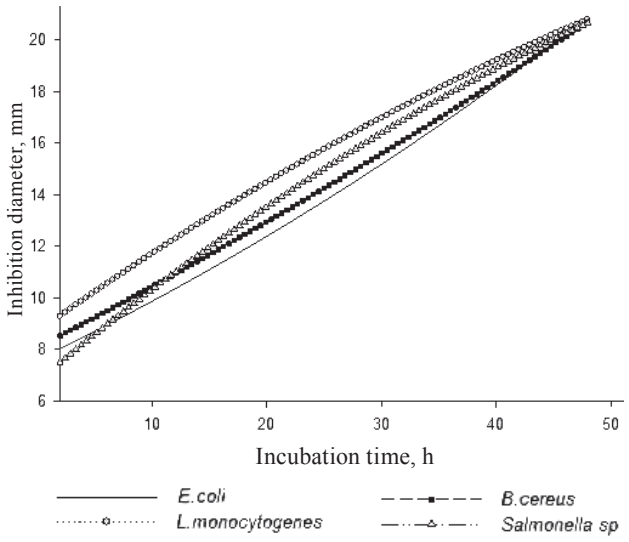

Figure 1 Effect of incubation time on inhibition of pathogenic bacteria

The inhibition zones of *B. cereus*, *L. monocytogenes*, and *Salomonella* sp. varied from 8.5, 9.0 and 7.3 mm in 2 h of incubation, respectively. In 48 h, the zones reached 20 mm in all the samples. The inhibition zones measured for each pathogenic strain as a function of the incubation time demonstrated that time is an essential factor to assess the antagonistic effect of probiotic drink based on raffia sap fermented with *L. fermentum* and *B. bifidum*. In fact, *B. bifidum* and *L. fermentum* need time to synthesize acids and other antimicrobial compounds contributing to antagonist effect against pathogenic bacteria [16, 17].

Individual effect of *B. bifidum* on *E. coli*, *B. cereus* and *Salmonella* sp. To obtain the inhibition curves of *E. coli*, *B. cereus*, and *Salmonella* sp. (Fig. 2) as a function of the seeding rate of *B. bifidum*, the seeding rate, incubation temperature, and incubation time of *L. fermentum* were set at 0 in coded variable – 5%, 39.5°C, and 25 h in real variables, respectively.

The inhibition curve of *Salmonella* sp. as a function of the seeding rate of *B. bifidum* showed that the maximum zone of inhibition of *Salmonella* sp. (15 mm) was obtained when the seeding rate of *B. bifidum* was 6%. The curve of the inhibition zone of *B. cereus* demonstrated that the inhibition zone depended directly on the seeding rate of *B. bifidum*. The inhibition zones of *B. cereus* ranged from 12.1 to 14.2 mm for the seeding rates of 0 and 10%. As for *E. coli*, its curve of the inhibition increased and then decreased, with a peak of 13.3 mm when the seeding rate of *B. bifidum* was 6.6 %.

According to Luquet and Corrieu, bifidobacteria promote better absorption of milk lactose in adults with intestinal lactase deficiency [18]. In our study, these probiotics (in particular *B. bifidum*) in raffia sap also played an important antagonistic role against *E. coli*, *B. cereus*, and *Salmonella* sp. In addition, some invitro studies showed that bifidobacteria and their metabolites stimulated IgA production, phagocytic activity, and growth [19]. These metabolites produced in raffia sap as well as the *B. bifidum* strain itself can therefore be a natural way to stimulate the immune system, to inhibit pathogenic strains such as *E. coli*, *B. cereus* and *Salmonella* sp., and to balance intestinal flora.

Individual effect of *L. fermentum* on *B. cereus*. Figure 3 shows the curve of the inhibition zone of *B. cereus* as a function of the seeding rate of *L. fermentum*. This curve increased then decreased, with the inhibition zone peak of 14.3 mm at the seeding rate of 6.5%. This curve was obtained by setting the seeding rate of *B. bifidum*, incubation temperature, and incubation time at 0 in coded variables – 5%, 39.5°C, and 25 h in real variables, respectively.

Thus, if it were necessary to optimize the antagonistic properties of our probiotic drink by referring only to an ability to inhibit the *B. cereus*

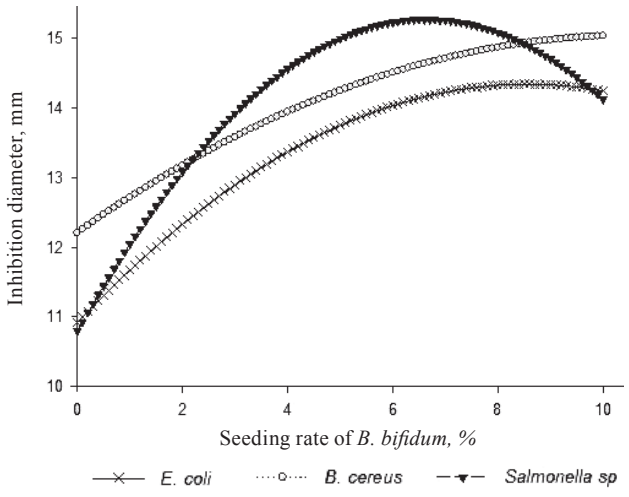

Figure 2 Individual effect of *Bifidobacterium bifidum* on *Escherichia coli*, *Bacillus cereus*, and *Salmonella* sp.

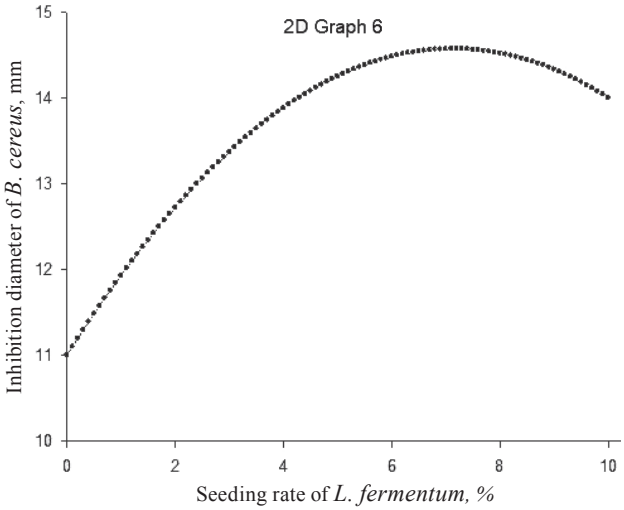

Figure 3 Individual effect of *Lactobacillus fermentum* on *Bacillus cereus*

strain, the seeding rates of *L. fermentum* and *B. bifidum* would be 5% and 5%, respectively, with an incubation temperature of 39.5°C and an incubation time of 25 h. Under these conditions, this probiotic drink could eventually be used as a means of combating infectious diseases which can be caused by *B. cereus*. *B. cereus* is a group of bacteria that can be pathogenic for humans. The infections they can cause are generally infrequent and not serious. However, ingestion of these bacteria, and their toxins in particular, can lead to infections characterized by vomiting or diarrhea [20].

In spite of the fact that our results were obtained *in vitro*, it is clear that *L. fermentum* introduced into raffia sap had a significant antagonistic effect on *B. cereus*. However, further research should be carried out *in vivo* to take into account factors that could affect the drink properties such as its passage through the intestinal tract, the survival of strains and the bioavailability of antibacterial compounds, as well as their direct or indirect effect on the body.

Effects produced by combination of *L. fermentum* and *B. bifidum* in raffia sap on the pathogens tested. The response surface methodology was applied to represent the mathematical models obtained by holding temperature and incubation time at 0 in coded variables –39.5°C and 25 h in real values, respectively.

Figure 4 presents the response surface of the mathematical model of inhibition zone of *L. fermentum* and *B. bifidum* against *Salmonella* sp. An increase in the seeding rate of *B. bifidum* and a simultaneous increase in the seeding rate of *L. fermentum* and *B. bifidum* considerably increased the antagonistic effect, with the inhibition zone of 16 mm.

However, only *B. bifidum* had a significant antagonistic effect on *Salmonella* sp. (P = 0.060, Table 2) at a 10% probability level. Indeed, lactic acid produced by *B. bifidum* lowers the pH by creating an unfavorable conditions for pathogenic microorganisms such as *Salmonella* sp. [21, 22]. Garcia *et al.* and Callaway *et al.* reported that bifidobacteria can prevent or reduce diseases caused by pathogens, protecting thus consumers' health [16, 23]. Based on our study results, raffia sap fermented by *B. bifidum* can be effective against salmonellosis due to *Salmonella* proliferation.

Figure 5 demonstrates the response surface of *L. fermentum* and *B. bifidum* against *E. coli*. As in the case with *Salmonella* sp., only *B. bifidum* showed a significant antagonistic effect on *E. coli* (P = 0.041, Table 2) at a 10% probability level. An increase in the seeding rate of *B. bifidum* considerably increased the antagonistic effect, with the maximum inhibition zone of 18 mm.

Indeed, lactic acid bacteria exert a strong antagonistic activity against several microorganisms, including those causing the deterioration of food and pathogenic microbes such as *E. coli* [4, 24]. In addition, the antimicrobial effect of some probiotic extends the shelf life of food [25]. This effect is mainly due to the production of organic acids (lactic acid) and also the production of antimicrobial compounds such as hydrogen peroxide, diacetyl, acetaldehyde, amino acid isomers and bacteriocins [19, 26].

It is important to remember that *E. coli* is a Gram-negative mammalian intestinal bacterium that makes up about 80% of the aerobic intestinal flora in humans [27, 28]. However, some strains of *E. coli* can be pathogenic, resulting in gastroenteritis, urinary tract infections, meningitis, or sepsis. Therefore, consumption of raffia

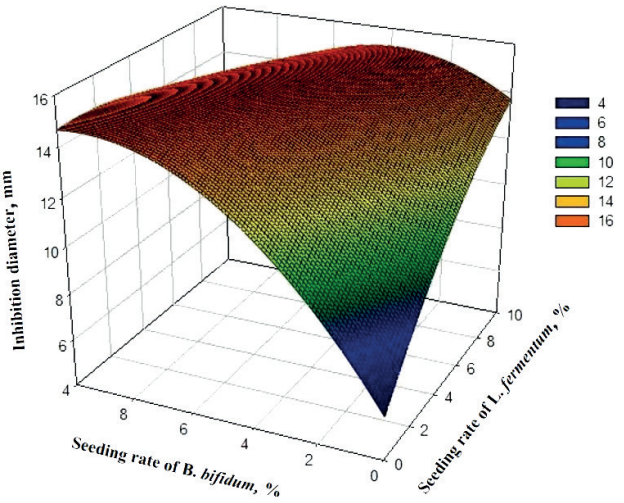

Figure 4 Response surface model of inhibition zone of *Lactobacillus fermentum* and *Bifidobacterium bifidum* against *Salmonella* sp. with seeding rates of *Lactobacillus fermentum* (X_1) and *Bifidobacterium bifidum* (X_2), incubation temperature (X_3), and incubation time (X_4) at temperature 0 (39.5°C) and time 0 (24 h)

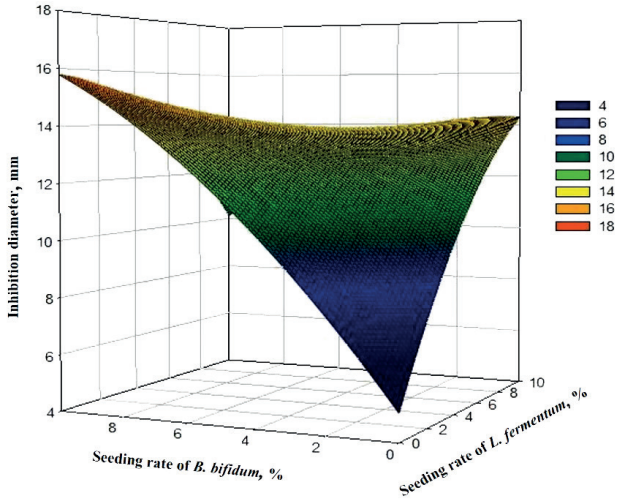

Figure 5 Response surface model of inhibition zone of *Lactobacillus fermentum* and *Bifidobacterium bifidum* against *Escherichia coli* with seeding rates of *Lactobacillus fermentum* (X_1) and *Bifidobacterium bifidum* (X_2), incubation temperature (X_3), and incubation time (X_4) at temperature 0 (39.5°C) and time 0 (24 h)

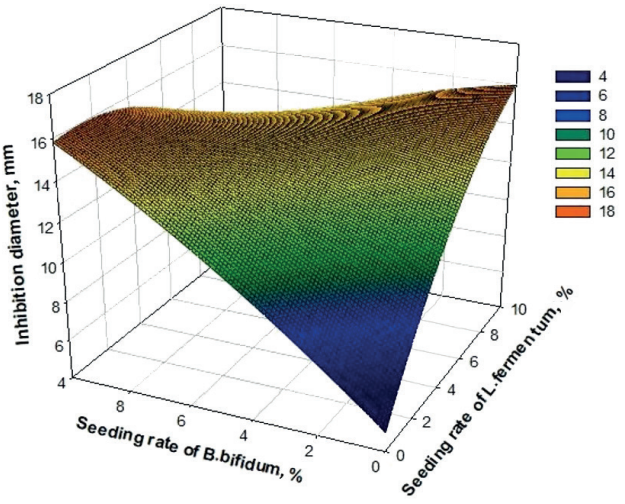

Figure 6 Response surface model of inhibition zone of *Lactobacillus fermentum* and *Bifidobacterium bifidum* against *Bacillus cereus* with seeding rates of *Lactobacillus fermentum* (X_1) and *Bifidobacterium bifidum* (X_2), incubation temperature (X_3), and incubation time (X_4) at temperature 0 (39.5°C) and time 0 (24 h)

sap fermented by *B. bifidum* can prevent and control the pathogenicity of *E. coli*.

Figure 6 presents the response surface of the mathematical model of inhibition zone of *L. fermentum* and *B. bifidum* against *B. cereus*. Both *L. fermentum* and *B. bifidum* individually had a significant antagonistic effect (P = 0.058 and 0.072, respectively, Table 2), whereas their combination was a highly effective (P = 0.007). *B. cereus* had similar sensitivities to both probiotics in raffia sap (Fig. 6). Inhibition zones reached 18 mm when the seeding rates of *L. fermentum* and *B. bifidum* were maximum. The acids and antimicrobial compounds secreted by *L. fermentum* and *B. bifidum* in raffia sap are thus a pathway to be exploited to treat diseases, although rare, due to consumption of *B. cereus*-infected foods. *B. cereus* is a well-known food-borne pathogen that is ubiquitously distributed in nature and is frequently responsible for food poisoning [20].

Effect of *L. fermentum* and *B. bifidum* on *L. monocytogenes* and optimization of the antagonistic effect. In the case of *L. monocytogenes*, response surface curves were not required because neither of the probiotic bacteria in raffia sap had a significant antagonistic effect (P = 0.722 for *L. fermentum* and P = 0.259 for *B. bifidum*, Table 2). This can be explained by the greater resistance of this bacterium to acidity [29]. Probably, the fermentation time should be increased to enhance the antagonistic properties of the raffia sap drink, but it would make the drink more acidic and hence undrinkable. It would be better to exploit this hypothesis in the context of the synthesis, isolation and production of biologically active compounds from raffia sap fermented by *L. fermentum* and *B. bifidum*.

In conclusion, the optimization of the antagonistic effect was done on the basis of specifications that aimed to maximize the inhibition zones. Thus, an optimal antagonistic effect would be given by seeding rates of *L. fermentum* and *B. bifidum* of 2 and 10%, respectively, incubation time of 48 h, and temperature of 37°C.

CONCLUSION

The results obtained in this study revealed that raffia sap fermented by probiotics (*Lactobacillus fermentum* and *Bifidobacterium bifidum*) had antibacterial properties against bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* sp., and *Bacillus cereus* which can sometimes be pathogenic. However, further studies should be carry out to determine the mechanism of action of this finding and to confirm its beneficial effect in animal models.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism. The idea and analysis belongs to S.C.Z. Desobgo. M.J.A. Mbarga and L.N. Tatsadjieu collected the data, performed the analysis and wrote the paper. L. Kalisa and N. Kavhiza translated and edited the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests related to this article.

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ORCID IDs
Manga Joseph Arsene Mbarga https://orcid.org/0000-0001-9626-9247