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# Use of Selected *Bacillus* spp. Strains for Directed Fermentation of *Hibiscus sabdariffa* Seeds into Mbuja

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author BAM designed the study, wrote the protocol, carried out the experiments, performed the statistical analysis and wrote the first draft of the manuscript under the supervision of authors CMM, JM and EC hosted part of the laboratory work and managed the analyses of the study as well as the careful reading of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

This work aimed at developing starter cultures for fermenting *Hibiscus sabdariffa* seeds into the traditional condiment Mbuja. In this context, two *Bacillus amyloliquefaciens* S1 and S5 and one *Bacillus subtilis* S12 strains, which had been selected in previous studies, were used separately and in combination (S5/S12) to produce Mbuja. The microbiological changes in fermenting seeds, as

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well as the physicochemical characteristics of the different obtained Mbuja, were evaluated. A sensory analysis was finally carried out to assess the organoleptic quality of laboratory-scale Mbuja compared to the most and the less appreciated products from traditional and spontaneous fermentation. Variable growth ability was noted for the tested starters. The combination of *B. amyloliquefaciens* S5 and *B. subtilis* S12 grew to the highest cell population of the end-product. Nutrients were globally affected by fermentation and were either increased (proteins, ash, and fats) or reduced (carbohydrates, crude fibers). Phytochemicals with potential positive impact on human health were detected and increased by the fermentation process. From the organoleptic point of view, Mbuja fermented with the combination S5 & S12 was the most appreciated by the hedonic panel, appreciation being driven by the taste and flavour of the product which was very close to that of the reference Mbuja.

*Keywords: Mbuja; hibiscus; Bacillus spp.; starter; fermentation.*

## 1. INTRODUCTION

The traditional fermentation of food condiments in Africa usually relies on spontaneous solid state fermentation and reuse of a fraction of the precedent production for further productions (back-slopping). Thus, the use of starter cultures is not a common practice, yielding products of variable microbiological and physico-chemical characteristics. However, different traditional methods to initiate and control food fermentation processes have been reported by many authors [1,2,3,4,5,6]. Most of the traditional fermented foods from Africa are produced essentially at the household level with two major consequences: the inconsistent product quality on the one hand and the potential occurrence of pathogens due to the general hygienic conditions of the manufacturers.

In recent times, a number of research works focused on developing starter cultures to upgrade the processing of different traditionally fermented foods given giving more value-added products. Hence, indigenous fermented foods from Africa like mawé, kenkey, kivunde, pito, ugba and dawadawa were studied to produce more consistent and foodborne pathogen-free foods [7,8,9,10,11,12]. In the same line, studies aimed at mastering and standardizing the production of Mbuja, a traditional condiment produced by spontaneous fermentation of *Hibiscus sabdariffa* seeds in Cameroon, were published [13,14,15]. Among other things, these previous studies led to the selection of two *Bacillus amyloliquefaciens* and one *Bacillus subtilis* strains exhibiting very good proteolytic and amylolytic properties as well as important antimicrobial activities against most common food spoilage and foodborne pathogens bacteria and fungi.

The aim of this study was therefore to compare the individual or combined abilities of these selected *Bacillus* strains to ferment *H. sabdariffa* seeds into quality Mbuja. The obtained results may help to standardize the sensory and nutritional quality of the product as well as improving the safety of the Mbuja condiment compared to the one made locally.

## 2. MATERIALS AND METHODS

### 2.1 *Bacillus* Cultures

Based on their biochemical properties and their antimicrobial activities [14] three *Bacillus* strains isolated from traditional Mbuja were used as starters. *B. amyloliquefaciens* S1 (JQ410767) and S5 (JQ410771) both exhibited high amylolytic and proteolytic activities. They were active against *Bacillus cereus, Listeria innocua, Staphylococcus aureus* and *Escherichia coli*; and against *B. cereus, L. innocua, E. coli, Aspergillus flavus and Fusarium oxysporum*, respectively. *B. subtilis* S12 (JQ410778) also showed important proteolytic activity and was active against *B. cereus, L. innocua, S. aureus, Pseudomonas aeruginosa,* and *Debaryomyces hansenii* [13].

### 2.2 Inocula Preparation

Cryopreserved *Bacillus* strains (S1, S5 and S12) were cultured on nutrient agar (NA) (AES Chemunex, Bruz, France) plates and incubated for 24 h at 37ºC. The strains were then subcultured for 18 h at 37ºC in 10 mL tryptic soy broth (TSB) (AES Chemunex, Bruz, France). The culture was then centrifuged at 3000xg for 15 min (Jouan CR3i Multifunction, Thermo Scientific, Saint-Herblain, France). Finally, the cells were re-suspended in sterile physiological water (0.85% NaCl) and diluted to a 0.03 absorbance at 600 nm.

## 2.3 Laboratory Scale *Hibiscus sabdariffa* Seed Fermentations

Fermentations were carried out for ten days in sterilized earthenware pots according to the traditional procedure (a two-steps fermentation for ten days: First phase of 7 days, pounding seeds, and second phase of 3 days). Five hundred grams of cooked and sterilized *Hibiscus sabdariffa* seeds were weighed in the pots and inoculated with a measured volume of cell suspension to give an initial concentration of $10^4$cfu/g. Five pots were used: 3 were individually inoculated with either *B. amyloliquefaciens* S1, *B. amyloliquefaciens* S5 or *B. subtilis* S12. One pot was inoculated with a mixture of *B. amyloliquefaciens* (S5) and *B. subtilis* (S12); one sample was not inoculated and served as a control. The seeds were left to ferment for 10 days at 30ºC and each batch was sampled at day 0, day 1, day 3, day 5, day 7 and day 10 for microbiological and physico-chemical analyses.

## 2.4 *Bacillus* Counts in Fermenting Seeds

Samples (10 g) of fermenting *H. sabdariffa* seeds were taken in sterile stomacher bags, diluted in 90 mL of tryptone salt buffer and homogenized with a stomacher (AES laboratoire, France). Then ten-fold dilutions were prepared from the homogenates, heat-treated (80ºC for 10 minutes), plated on Nutrient Agar (AES, France) and incubated at 30ºC for 24 h. Colonies shape, Gram staining and catalase tests were analyzed to confirm strain identity (Gram-positive and catalase-positive rods).

## 2.5 Physico-chemical Analysis

### 2.5.1 pH measurement

Ten grams of fermenting *H. sabdariffa* seeds were weighed and homogenized with 90 ml distilled water and the pH determined with a CyberScan pH/Ion 51 pH-meter (Eutech Instruments, Illkirch, France).

## 2.6 Proximate Analysis

The parameters analyzed were dry matter, ash, crude fibers, fats, carbohydrates and proteins. These parameters were measured for crude seeds and fermented Mbuja. The proximate compositions of these samples were determined using AOAC standard procedure [16]. The crude protein content was calculated by multiplying the total nitrogen by a 6.25 factor, using Kjeldahl method [17]; and crude fibers by AOAC [16]. The amount of lipid was determined, using Soxhlet extraction method; while the ash content was determined by the AOAC method [16]. Reducing sugars were determined as described previously by Terlabie et al. [18].

## 2.7 Phytochemical Qualitative Determination

Extracts were prepared from crude *H. sabdariffa* seeds and from the fermented Mbuja . Extract preparation was carried out according to the procedure described by Obouayeba et al. [19]. These extracts were subjected to qualitative test for the identification of various phytochemical compounds including saponins, alkaloids, flavonoids, tannins, phenolic compounds, triterpenes and sterols, and carotenoids as described by Harborne [20].

## 2.8 Sensory Analysis

Sensory tests were performed in 4 villages of the Far North Region of Cameroon where the production and consumption of Mbuja is very common. A panel of eighty members (20 per village) evaluated the taste, texture, flavor and overall acceptability of 6 Mbuja samples coded M0 to M5 using a 9-point hedonic scale where '1' means dislike extremely and '9' means like extremely. The 6 samples included starter culture produced Mbuja fermented with *B. amyloliquefaciens* (S1) coded 'M1', *B. amyloliquefaciens* (S5) coded 'M2', *B. subtilis* (S12) coded 'M3', a mixture of *B. amyloliquefaciens* (S5) and *B. subtilis* (S12) coded 'M3' and two other samples traditionally fermented and evaluated in earlier studies as the most appreciated (coded 'M5') and the less accepted (coded 'M0') condiments. For starter culture produced Mbuja, fermentation was carried out in the same larger earthen-ware pots (30 L) used by local producers. Five kilograms of cooked seeds were inoculated with 250 g of laboratory starter culture fermented Mbuja to represent 5% (w/w), the average inoculum proportion used by local producers.

## 2.9 Statistical Analysis

Physico-chemical data were subjected to analysis of variance (ANOVA) and the Duncan's test was applied to compare the means (level of significance $P \leq 0.05$) using Statgraphics Plus 5.1. (Manugistic Inc. Software, Rockville, USA).

Sensory evaluation data was subjected to Principal Component Analysis PCA using StatBox 6.6 (Grimmersoft, France).

## 3. RESULTS

### 3.1 Microbial and pH follow-up during *H. sabdariffa* Seed Fermentations

The growth ability of *B. amyloliquefaciens* S1, *B. amyloliquefaciens* S5, *B. subtilis* S12 and the combination of *B. amyloliquefaciens* S5 - *B. subtilis* S12 in *H. sabdariffa* seeds was investigated. The obtained results presented in Fig. 1 showed variable ability of the inoculated strains to grow in the seeds. *B. amyloliquefaciens* S1 counts were the lowest throughout the fermentation process and only gain about 1 log CFU/g. On the contrary, the combination of *B. amyloliquefaciens* S5 – *B. subtilis* S12 yielded the highest counts. Except for the *B. amyloliquefaciens* S1 for which counts were 3.25 x $10^4$ cfu/g, all counts were higher than $10^7$ cfu/g.

The pH values measured varied between 6.5 and more than 8 (Fig. 2) during the fermentation. In all cases, it was quite stable in the first step of the fermentation and varied significantly after the 7$^{th}$ day.

### 3.2 Physico-chemical Characteristics of Mbuja Fermented with Different Starters

The physico-chemical parameters of *H. sabdariffa* seeds were globally affected by fermentation (Table 1). There was a significant increase in the ash content of Mbuja fermented by all inocula compared to the ash content of crude seeds, with increased rates varying from 11% (*B. amyloliquefaciens* S5) to 40% (*B. amyloliquefaciens* S1). Crude fibers significantly increased in Mbuja fermented with S1 but was stable or decreased when the seeds were fermented using S5, S12 or the S5/S12 combination.

For reducing sugars, fermentation with S1 and S5/S12 significantly decreased the sugar

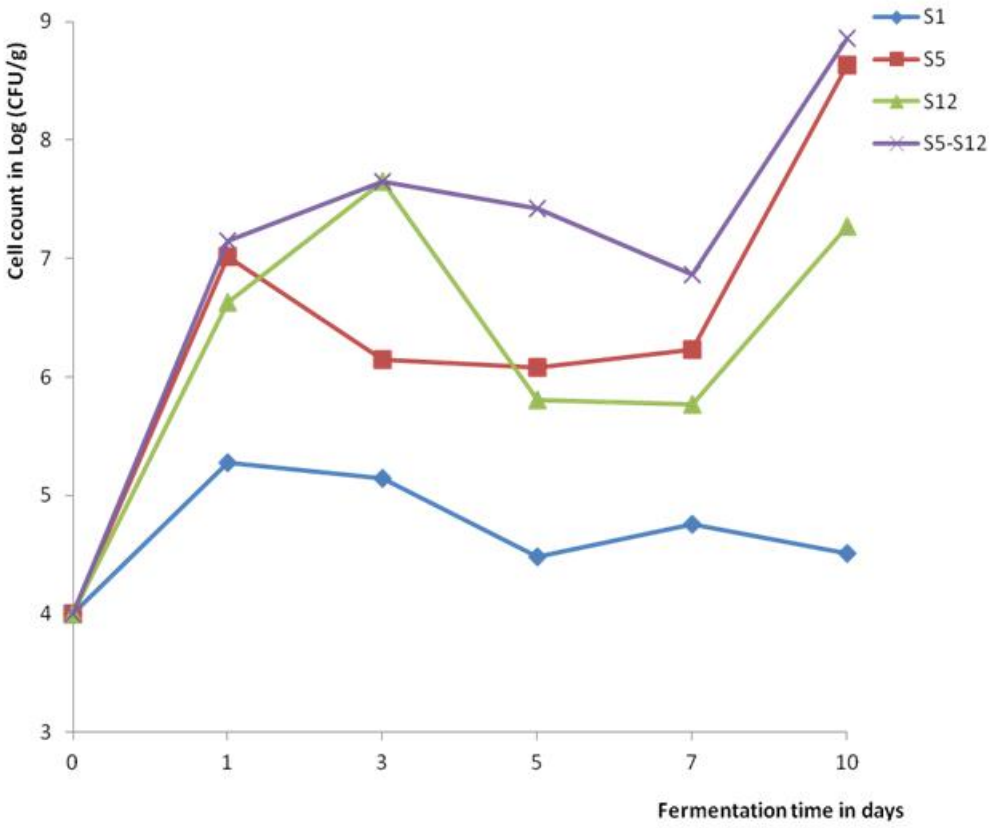


**Fig. 1. Growth of the *Bacillus* starter population in fermenting *Hibiscus sabdariffa* seeds**

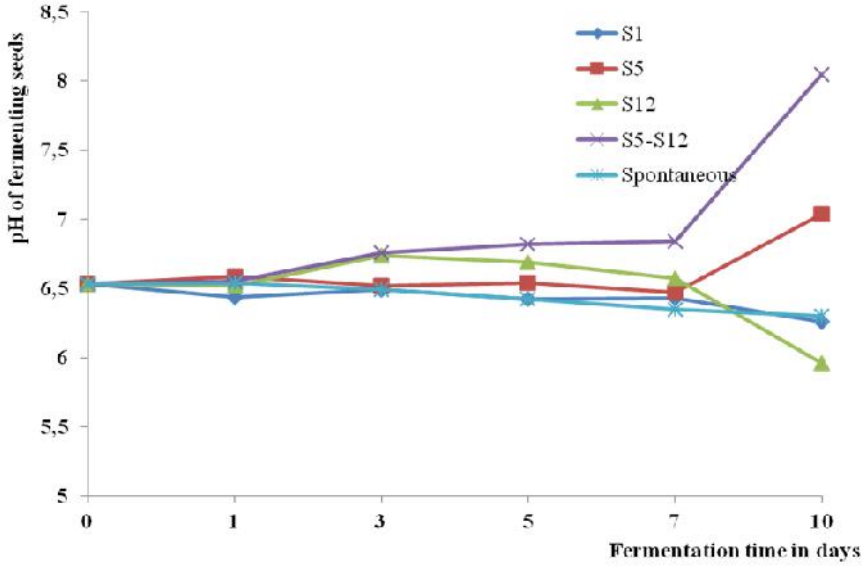


**Fig. 2. Changes in pH during the fermentation of *Hibiscus sabdariffa* seeds with different starter cultures**

**Table 1. Proximate composition of Mbuja fermented with different inocula**

| Analysis | Chemical composition in g/100 g (DM) | | | | |
|---|---|---|---|---|---|
| | **Crude seeds (control)** | **S1** | **S5** | **S12** | **S5 –S12** |
| Dry matter | 36.92 ± 0.19[a] | 57.86 ± 6.37[d] | 42.26 ± 3.21[b] | 53.94 ± 0.85[c] | 53.19 ± 0.28[c] |
| Reducing sugars | 4.68 ± 0.38[a] | 2.66 ± 0.97[b] | 6.19 ± 1.33[d] | 4.81 ± 1.04[e] | 4.18 ± 0.84[c] |
| Proteins | 4.15 ± 0.0[a] | 3.94 ± 0.01[a] | 4.11 ± 0.12[a] | 4.12 ± 0.01[a] | 5.95 ± 0.04[b] |
| Fats | 20.19 ± 1.1[a] | 31.90 ± 6.54[d] | 12.94 ± 1.82[b] | 15.09 ± 0.16[e] | 25.57 ± 0.34[c] |
| Ash | 1.61 ± 0.59[a] | 2.27 ± 0.12[b] | 1.79 ± 1.01[c] | 1.98 ± 0.37[c] | 2.07 ± 0.06[b] |
| Crude Fibers | 20.95 ± 1.24[a] | 32.35 ± 1.06[c] | 13.2 ± 0.52[d] | 21.66 ± 2.37[a] | 18.73 ± 0.47[b] |

*Values on the same line with the same letter as superscript are not significantly different at 5% by ANOVA*

contents of Mbuja. However, sugar contents of products fermented with single strains of S5 and S12 increased.

Proteins decreased or were stable in most cases except for Mbuja fermented with the combination of *B. subtilis* and *B. amyloliquefaciens*. The fermentation also produced higher fats contents for two inocula, combination of S1 and S5/S12, and yielded lesser fats values for samples fermented with singles strains of S5 and S12.

Phytochemicals screened in Mbuja fermented with different inocula varied according to the microorganism used (Table 2). The concentration of saponins in crude seeds remained unchanged by fermentation with *B. amyloliquefaciens* (S1), *B. subtilis* (S12) and the S5/S12 combination). However, saponins were significantly reduced in sample fermented by the single *B. amyloliquefaciens* (S5).

### 3.3 Sensory Evaluation of Mbuja Produced with Selected *Bacillus* spp. Starters

Six Mbuja samples including two controls (one considered in a previous study by Mohamadou et al. [21] as the best product and another evaluated as the less accepted) were assessed by a panel of men and women who assessed the flavor, the texture, the taste and the overall acceptability based on a 9 points hedonic scale. The principal component analysis of their answers (Fig. 3) showed that they clearly separated two groups of products: accepted and

**Table 2. Qualitative analysis of phytochemicals in Mbuja fermented with different inocula**

| Analysis | Phytochemical composition | | | | |
|---|---|---|---|---|---|
| | Crude seeds (Control) | S1 | S5 | S12 | S5 –S12 |
| Saponins | ++ | ++ | ++ | ++ | ++ |
| Alkaloids | - | - | - | - | - |
| Flavonoids | ++ | ++ | ++ | ++ | ++ |
| Tannins | ++ | ++ | ++ | ++ | ++ |
| Phenols | ++ | ++ | ++ | ++ | ++ |
| Triterpenes and sterols | ++ | - | - | ++ | ++ |
| Carotenoids | - | - | - | - | - |

*Absence ++ presence*

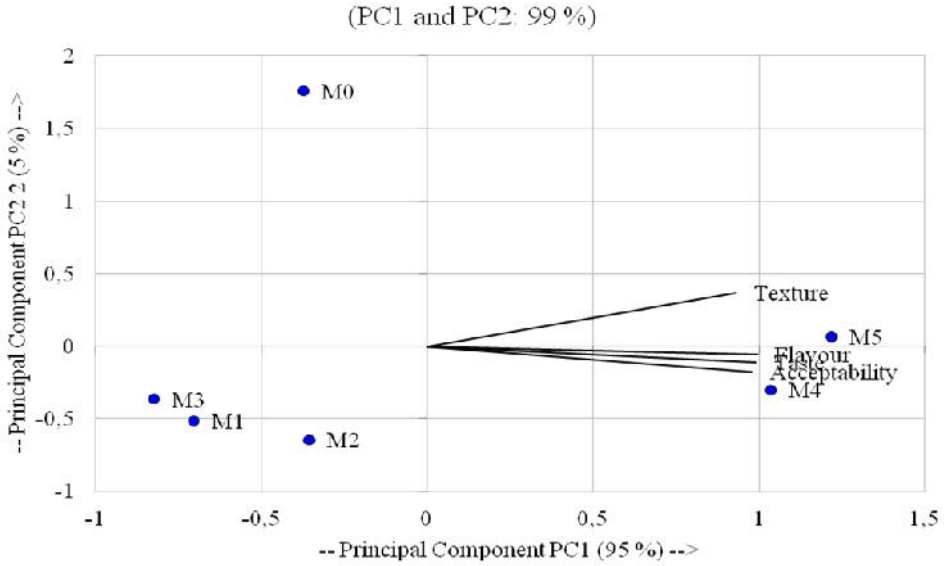


**Fig. 3. Principal Component Analysis on sensory evaluation of six samples of Mbuja including two controls and four samples fermented by selected starters (M0: Rejected Mbuja fermented traditionally-Negative control, M1: Mbuja produced in laboratory with *B. amyloliquefaciens* S1, M2: Mbuja produced in laboratory with *B. amyloliquefaciens* S5, M3: Mbuja produced in laboratory with *B. subtilis* S12, M4: Mbuja produced in laboratory with *B. amyloliquefaciens* S5 and *B. subtilis* S12, M5: Most accepted Mbuja fermented traditionally - Positive control)**

rejected. Seeds fermented individually by *B. amyloliquefaciens* S1 (M1), *B. amyloliquefaciens* S5 (M2) or *B. subtilis* S12 (M3) were scored at rates close to the rejected control (M0) for all the sensory parameters considered. Reversely, Mbuja fermented by the combination of *B. amyloliquefaciens* S5/*B. subtilis* S12 (combination M4) obtained scores close to the most appreciated control (M5).

## 4. DISCUSSION

The fermentation ability of single and combined strains investigated in fermenting seeds of *Hibiscus sabdariffa* revealed that the growth of the combination of *B. amyloliquefaciens* S5 – *B. subtilis* S12 was greater than the individual population increases in each of *Bacillus*, indicating a probable synergistic association. No bacterial growth was noted for the control as the seeds were cooked, therefore sterilized. In all cases, the bacterial growth showed a two-phase kinetic, clearly differentiating the two different steps of fermentation. Indeed, at day 7 of the process, the fermenting seeds were pounded before re-incubation for three extra days. This operation probably made available new substrates resulting in the inflexion of the curve at this time point. In general, the two most important counts were at the same level than the *Bacillus* counts reported in earlier studies of traditionally fermented Mbuja [21]. Similar range

of bacterial counts was also obtained for other fermented *H. sabdariffa* seeds like dawadawan botso and Bi-Kalga [22]. However, no bacterial growth was observed in the control samples.

The change in pH, in association with nutrient release through pounding at day 7, either increased (in the case of *B. amyloliquefaciens* S5 and the combination of *B. amyloliquefaciens* S5 – *B. subtilis* S12) or decreased (for *B. amyloliquefaciens* S1 *and B. subtilis* S12) whilst the control, in which the seeds were left to ferment spontaneously, recorded a steady pH. It can be noted that only the combination of *B. amyloliquefaciens* S5 and *B. subtilis* S12 led to a pH of 8.05. This value is in agreement with those recorded in previous studies by other authors in similar African fermented condiments [23]. One important reason for the choice of the strains used in this study was their biochemical profiles. In this context, a mixture of one amylolytic and proteolytic *B. amyloliquefaciens* strain and one proteolytic *B. subtilis* strain provided the most appropriate pH value of Mbuja. Thus, the increase in pH at the end of the fermentation might be due to the proteolytic activity of both strains while hydrolysis of starch provides more free sugars to both species. This scheme, where proteins are degraded into peptides, amino acids and ammonia, is common in *Bacillus* fermentation and has already been observed in other traditional fermentations in Africa [24,25].

The influence of fermentation by *Bacillus* spp. on some physico-chemical characteristics of seeds is evidenced. Indeed, the increased levels of crude fibers in fermented Mbuja showed that the condiment would provide dietary fibers and may potentially contribute to protect its consumers against cardiovascular diseases as well as against gastro-intestinal cancers and a number of other diseases as suggested by Dhingra et al. [26].

Also, the increase in the concentration of reducing sugars could be due to breakdown of starch and to the amylolytic activities of *B. amyloliquefaciens* S5 and *B. subtilis* S12. Such observations have also been made in the fermentation of soy-dawadawa by *B. subtilis* [18].

With regard to protein, the very important bacterial counts (and microbial mass increase) recorded for the S5/S12 combination could explain this protein content and could be attributed to an extensive hydrolysis of protein molecules into peptides and amino acids. Thus, Mbuja could be a source of protein that can help people with low incomes to meet their required needs in this crucial nutrient. This issue is particularly important to developing countries where meat and fish are not easily affordable. Increase in protein was also noted for other alkaline fermented products and related to *Bacillus* counts [27,18].

For the high amounts of fats measured, the increase could be a result of large lipid molecule degradation into small fatty acids as a probable consequence of lipolytic enzyme activity.

Interesting amounts of phenolic compounds were determined. These compounds that occur naturally in numerous vegetables are believed to be health-promoting due to their ability to lower cholesterol, improve immune function, and prevent cancer, among other virtues [28]. Alkaloids, generally regarded as poisonous, were not detected in any sample. . The flavonoid contents in crude seeds were increased in most fermented samples, probably as a consequence of an increased release of flavonoids by the fermentation process. This phenomenon has already been observed in other fermentation processes and was attributed to the release of bound flavonoids due to the degradation of the cell wall structure by microbial enzymes [29]. Flavonoids from dietary sources have attracted more interest for their potential health benefits against cancer and cardiovascular diseases [30]. As observed for flavonoids, tannins were increased by fermenting *H. sabdariffa* seeds. The effect of these compounds on human health has also been documented as tannins prevent pathogenic bacterial growth and are potent antioxidants already used for treating gastrointestinal disorders [31]. Triterpenes and sterols found in crude seeds were not detected in Mbuja produced by S1 and S5. However, their contents were increased by fermentation with the combination S5 – S12. A number of studies reported their respective effect against chronic diseases such as breast cancer and cholesterol [32,33]. Carotenoids were not detected in both fermented Mbuja and non-fermented crude seeds.

From the panel point of view, Mbuja fermented by the combination of *B. amyloliquefaciens* S5/*B. subtilis* S12 (combination M4) and the most appreciated control (M5) were almost indistinguishable when considering their flavour, taste and overall acceptability. However, the laboratory fermented condiment (M4) obtained a

slightly lower texture score than the reference product (M5). To improve texture, the fermented seeds need to be reduced into smaller particles in the production process. Nevertheless, this reserve did not significantly impact the overall appreciation of Mbuja M4. As a reminder, in this sample, *Bacillus* counts and pH value were the highest. The important bacterial growth of the most appreciated product is probably linked to a higher metabolic activity, notably proteins breakdown, which yielded appreciated flavors and taste. In other condiments, trials on sensory evaluation of dawadawa, *B. subtilis* used alone as starter gave an acceptable product [18] while other work did not find a significant difference between Iru fermented by starters made of single or mixed cultures of *Bacillus and/or Staphylococcus* [34].

## 5. CONCLUSION

Overall, two *Bacillus* strains (*B. amyloliquefaciens* S5 and *B. subtilis* S12) used in combination as a starter were able to ferment *H. sabdariffa* seeds into a Mbuja that exhibited nutritional and organoleptic qualities of interest. The potential of Mbuja in helping to meet the basic nutritional requirements in the analysed nutrients was highlighted and its potential health benefits were mentioned. This aspect should be further investigated in future work. Therefore, controlled fermentations using these two strains could produce a safe, nutritious and potentially health promoting Mbuja.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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