Optimal conditions for production of fermented flour from pumpkin (*Cucurbita pepo* L.) for infant foods

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ABSTRACT

The aim of this study was to determine the optimal conditions of production of a fermented pumpkin flour by lactic fermentation using *Lactobacillus plantarum* and the effect of the fermentation on nutritional potential and functional properties of pumpkin. To achieve this, pumpkin fruit was collected in the Ngaoundere main market, peeled, sliced, and the flesh obtained was grated, pasteurized at 90°C for 5 minutes and placed under lactic fermentation using L. plantarum (108 cfu/mL). The sample obtained was dried at 45°C ± 2°C for 24 hours and crushed to obtain a flour with a diameter of particles ≤ 500 µm. According to Doehlert's plan used, time and temperature of fermentation varied from 24 to 96 hours and 30 to 50°C respectively. Responses sought were the optimal levels of total carotenoids and reducing sugars in the flours obtained. Chemical composition of flour was determined to evaluate the effect of fermentation on food matrix used. Results indicate that to produce a pumpkin flour with highest content in both carotenoids and reducing sugars, optimal conditions of lactic fermentation with *L. plantarum* are 70h at 45°C. Under these conditions, there is a decrease of 72.1% of proteins and 67% of fibers, against an increase of 106% of reducing sugars. Total carotenoids content decreases by 4.6%, but the level is still higher than the threshold recommended for infant food formulation, while mineral content increases with fermentation. A reduction of anti-nutrients (phytates, tannins, phenolic compounds and oxalates) of more than 50% is also observed when fermenting pumpkin. The functional properties of fermented pulp show a decrease of water absorption capacity of 24% and an increase of 134.4% in bulk density. Fermented pumpkin flour could be used in infant food formulation, but need to be associated with other sources of proteins and minerals.

Keywords: Vitamin A Deficiency, Pumpkin, Infant Food Formulation, Lactic Fermentation, Lactobacillus plantarum.

1. INTRODUCTION

Vitamin A Deficiency (VAD) is a public health problem in many developing countries [1], the insufficient dietary intake of this vitamin being the primary cause. Children under 5 years old, pregnant and lactating women are mainly affected by VAD, with consequences like increasing vulnerability to infections, delay of growth, night blindness, anemia and death [2]. In Cameroon, the situation remains worrying with 62.5% children affected in the Northern Regions (Adamawa, North and Far North) [3]. To alleviate this situation, clinical and food strategies were set up by Cameroonian authorities. Concerning food measures, the principal is the fortification in Vitamin A of some commonly consumed foods (vegetable oils, wheat flour, dairy products) [4]. Unfortunately, many of those fortified products are not accessible to the target population, as they do not form part of their food habits, reason why the level of VAD remains high, mainly in children. It thus appears important to think of alternative local resources that are accessible and also rich in vitamin A, a method increasingly recommended by WHO and FAO [5].

In this light, the use of pumpkin (Cucurbita pepo L.), a variety of squash highly consumed in Cameroon [6] can be exploited. Its yellow-orange flesh is a good source of provitamin A with total carotenoids content varying from 234.21 to 404.98 µg/g DM [7]. In addition to its richness in carotenoids provitamin A, pumpkin is also a good source of energy due to its high sugars content [8], what makes it an interesting food matrix for infant food formulation to alleviate VAD. It also has high levels of starch (48.3%) [9], which can cause digestive problems to infants, thus necessitating the use of appropriate treatment before use in infant food formulation. Studies have already been recorded on the fermentation of pumpkin to reduce its starch level in the production of canned fruits, jams and drinks [10] [11], but this fermentation was spontaneous with unspecified bacterial strain, thus with no controlled conditions. Moreover, these studies were not targeted to the formulation of infant foods. Amylolytic properties of *Lactobacillus plantarum* having been proven on other food matrices [12] [13], hence a fermentation of pumpkin by this bacterium could be conceivable, with suitable results.

Lactic fermentation can affect the nutritional quality of the food matrix, as well as its functional properties. It is therefore interesting to study its effect on pumpkin by determining the chemical composition and some functional properties of the flours and compare with non- fermented pumpkin flour. The aim of this study was then to determine the optimal conditions for production of fermented pumpkin flour by lactic fermentation using *L. plantarum* and the effect of this fermentation on the nutritional potential and some functional properties of pumpkin flour.

2. MATERIAL AND METHODS

2.1. Production of pumpkin flour

Pumpkin fruit was collected in August 2017 in the Ngaoundere main market and transported to the Food Biophysics and Nutritional Biochemistry Laboratory of the National School of Agro Industrial Sciences of the University of Ngaoundere. where it was peeled and cut up. The flesh obtained was grated (Combined Kit Censol ®), pasteurized (Autoclave tipo 760_7139) at 90°C for 5 minutes to get rid of other potential fermenting microorganisms and placed under lactic fermentation (L. plantarum: 108 cfu/mL) in optimal conditions of time and temperature to be determined. The bacteria strain was provided by the Food Microbiology and Biotechnology Laboratory of the same school. Sample obtained was dried at 45°C ± 2°C for 24 hours, crushed with a hammer mill (Culatti typ MFC CZ13) to obtain the flour with a diameter of particles ≤ 500 µm, and kept for further analysis.

2.2. Determination of optimal conditions for flour production

Doehlert plan at 2 factors was used to study the effect of time (x1) and temperature (x2) of lactic fermentation of pumpkin flesh on simple sugars and carotenoids by surface response method. The independent variables x1 and x2 varied respectively from 24 to 96 hours (with 5 variations) and from 30°C to 50°C (with 3 variations), according to literature [14]. The responses sought were the highest levels of carotenoids and simple sugars in pumpkin flesh. The model was considered valid, when at least two of the three conditions were satisfied: coefficient of determination R2 ≥ 80% [15], absolute mean deviation analysis (AADM) close to zero [16], bias factor (Bf) between 0.75 and 1 [17].

2.3. Determination of the proximal composition of flours

To determine the effect of fermentation on the nutritional quality of pumpkin, macronutrients composition of flours was determined. Dry matter was determined by drying the samples in a ventilated oven at 105°C during 24h until constant mass [18]. To determine oil content, the extraction of lipids in Soxhlet based on their differential solubility in solvent of extraction (ether of petroleum or hexane) was done hot during approximately 12h [19]. Total protein content was given after mineralization of the samples according to the method of Kjedahl [20], and quantification with spectrophotometric method at 412 nm using the colorimetric reaction of Hantzsch [21]. Total sugars were extracted with sulphuric acid 1.5N, then quantified by spectrophotometric reading of absorbance at 420nm [22]. Crude fiber content was determined by the method of Weende which consists in treating the sample by boiling it in sulphuric acid, and then in sodium hydroxide. The residue obtained is dried, then calcined and weighed [23]. The determination of total ash content was done by complete incineration of the samples in a furnace adjusted at 550°C, until obtaining white ashes [24]. Reducing sugars were quantified [25] to have information on the hydrolysis of starch by *L. plantarum* during fermentation.

2.4. Quantification of micronutrients

Ash obtained was washed with concentrated hydrochloric acid [26], and the filtrates used for the quantification of iron, phosphorus, calcium and magnesium. Iron and phosphorus were determined by colorimetric, respectively with orthophenanthroline [27] and ammonium molybdate [28] in an acid medium. The determination of calcium and magnesium was made by titrimetric with a solution of ethylene diamine tetra-acetic acid (EDTA) disodium salt [28]. Total carotenoids were extracted with a hexane-acetone mixture: 30/70 (v/v), dissolved in hexane, and the optical density was read with the spectrophotometer between 430 and 450 nm to determine the maximal absorbance [29]. The maximal optical density was used to determine the level of total carotenoids in the sample.

2.5. Quantification of anti-nutrients

Phytates were extracted with hydrochloric acid and sodium chloride and quantified using Wade's reagent [30]. Extraction of phenolic compounds and tannins was done with acetone (70%), and quantification with Folin Ciocalteu and polyvinylpyrrolidone (PVPP) [31]. The oxalates were measured by hot titration with potassium permanganate solution [32].

2.6. Functional properties

Water Absorption Capacity (apparent and real) was determined as the quantity of water (in grams) absorbed by 100g of powder after saturation and centrifugation (Centrifuge DL-6000B) by a method previously described [33]. The dried solid mass in supernatant was used to evaluate the Water Solubility Index [34]. Bulk Density (g/mL) was measured in a graduated cylinder by gently adding 2g of pumpkin flour into an empty 10 mL graduated cylinder and holding the cylinder on a vortex vibrator (Vortex RS Lab_6Pro) for 1 min. The volume was read, recorded and the result was expressed as described by the method [35].

2.7. Statistical analysis

The averages of three repetitions and standard deviations were calculated using Excel 2016, the test of Student (T test) was done using XL Stat 2016 to compare nutrient content with and without fermentation. The plot of the curves was done using Sigmaplot 12.5 software.

3. RESULTS AND DISCUSSION

3.1. Optimal conditions of fermentation of pumpkin flesh by *L. plantarum*

3.1.1 Response of total carotenoids content

Figure 1 (a) shows the effect of time and temperature of fermentation with *L plantarum* on carotenoids content of pumpkin. Carotenoids content increases with time and temperature until 70 hours and begin to decrease. From Equation (1), it appears that lactic fermentation time and temperature have positive linear effects on carotenoid content as well as their interaction, but their quadratic effects affect carotenoid content negatively. From Figure 1 (b), the hatched size of the plot represents the satisfactory zone of carotenoid content, assuming 100g of infant flour may contain at least 350 µg of vitamin A [36]. The increase of total carotenoids content is a concentration, due to the loss of other nutrients during fermentation. The decrease in carotenoid content after 70 hours of fermentation could be explained by the use of carotenoids by *L. plantarum* as secondary metabolite, knowing that in certain conditions, in addition of carbon, nitrogen and minerals, bacteria need vitamins for their metabolism [37].

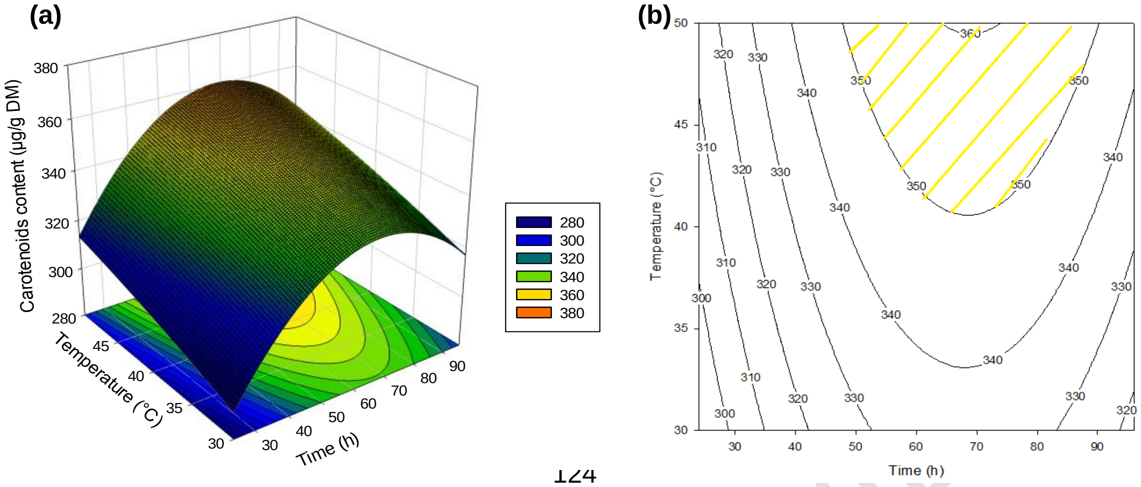

Fig. 1. Total carotenoids (a) Response surface and (b) Contour plot

$$\text{Carotenoids Content } = 176.017 + 3.092x_1 + 2.118x_2 - 0.023x_1^2 + 0.003x_1x_2 - 0.013x_2^2 \quad (1)$$

(R^2 = 98.83%; R^2adjusted = 98.56%)

3.1.2 Response of reducing sugars content

As shown in Figure 2(a) reducing sugars content increases with time and temperature of fermentation until 90 hours. From Equation (2), it appears that time of fermentation alone (x1) has a negative effect on reducing sugars content, while temperature, quadratic effects and interactions of these two variables affect positively though all the effects were negligible. The hatched zone on Figure 2(b) shows the satisfactory zone for reducing sugar content, 100 g of infant flour must contain at least 55 % of free sugars [36]. The increase of reducing sugar content could be attributed to the amylolytic activity of *L. plantarum* which digest the starch of pumpkin into reducing sugars as described earlier [12].

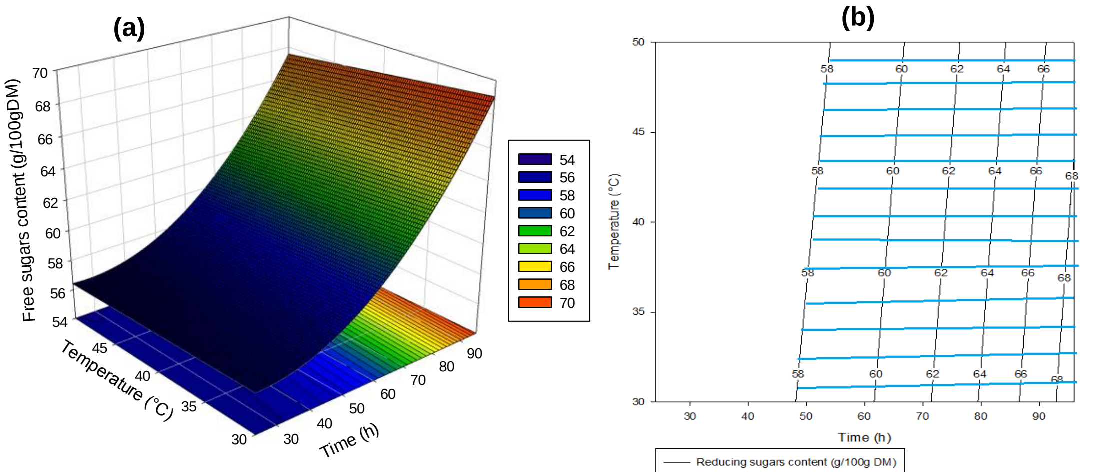

Fig. 2. Reducing sugars (a) Response surface, (b) Contour plot

$$\text{Reducing sugars content} = 57.765 - 0.092x_1 + 0.011x_2 + 0.002x_1^2 + 0.0009x_1x_2 + 0.0006x_2^2 \quad (2)$$

(R^2 = 87.76%; R^2adjusted = 84.85%)

3.1.3. Validation of models and compromise size

Table 1 shows that the values of coefficient of determination, Absolute Mean Deviation Analysis and Bias Factor respect the standards, from which the various models are validated.

Table 1. Validation of models

Elements of validation	Abbreviation	Carotenoids	Reducing sugars	Standards values
Coefficient of determination	R^2	98.83 %	87.76 %	≥ 80 %
Absolute Mean Deviation Analysis	AMDA	0.005	0.013	0
Bias factor	Bf	0.99	0.99	0.75 ≤ Bf ≤ 1

For the determination of the optimal zone, contour plots were superposed (figure 3). A point in this optimal zone was chosen, analyzed, and the results noted in Table 2. It arises from figure 3 that the optimal zone is between 60 and 80 hours for time and from 43 to 48°C for temperature. The compromise size corresponding to the optimal conditions of production of a fermented and digestible pumpkin flour shows that after 70h of fermentation at 45°C, total carotenoids and reducing sugars contents are both at their optimal level.

Table 2. Optimal value of the responses

Responses	Experimental	Multi-response optimisation
Total carotenoids content (µg/g DM)	362.01 ± 2.62^a	365.03 ± 1.01^a
Reducing sugars (g/100g DM)	61.55 ± 1.86^a	63.99 ± 0.01^a

Values on the same line carrying the same letters are not significantly different (p > 0,05). DM = Dry matter

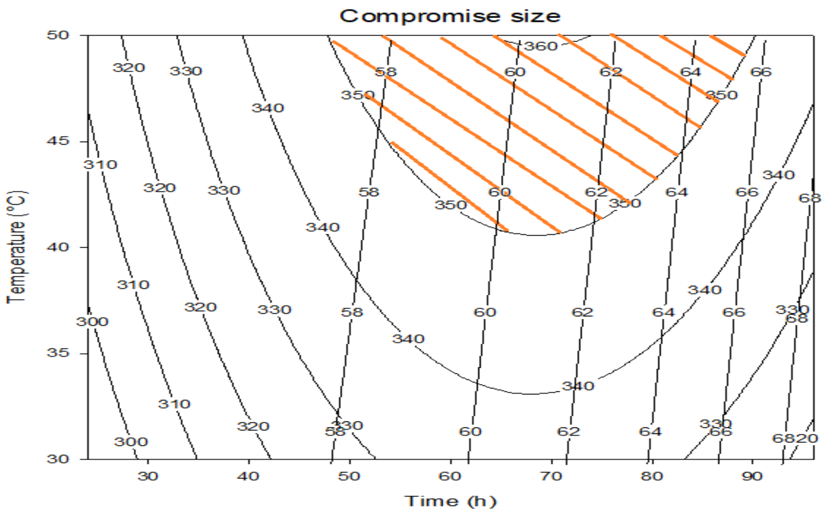

Fig. 3. Compromise between total carotenoids and reducing sugars content.

3.2. Effect of fermentation with *L. plantarum* on proximal composition

Proximate composition of the flours produced with and without fermentation is presented in Table 3. The macronutrients are significantly affected by the fermentation ($p < 0.05$) which increases some and decreases others. Fermentation with *L. plantarum* in optimal conditions leads to a significant reduction of 72.1% of proteins and 67% of fibers, as against an increase of 38.4% of carbohydrates. There is also a significant increase of reducing sugar (106%), fat (19.3%) and total ash (29.4%) with average contents rising from 31.05 to 63.97 g, 2.85 to 3.40g and from 5.71 to 7.39 g respectively when pumpkin flesh is fermented

The increase of carbohydrates could be attributed to *L plantarum* which could have degraded the starch into reducing sugars, making it more soluble and more digestible. The amylolytic properties of *L. plantarum* enable it to breakdown the polymeric structure of starch and decompose it into mono and di saccharides [12]. This is the rational for the increase of 106% of reducing sugars. The same observation was obtained with the fermentation of a precooked sorghum flour by saccharomyces cerevisiae [38]. The degradation of starch into reducing sugars makes flour of fermented pumpkin more digestible, and thus more suitable for infant food formulations.

The reduction of fibers content could be due to the action of *L. plantarum*. Bacteria have enzymes which enable them to digest fibers especially cellulose known to be found in C. pepo, [39]. *L. plantarum* strongly degrade cellulose to cellulobiose, and finally to glucose [40]. This could also be used to explain the increase of reducing sugars after fermentation. This reduction of fibers content in the flour of fermented pumpkin flesh is beneficial for carotenoids bioavailability, knowing that food fibers reduce the bioavailability of carotenoids by trapping them, or by interacting with biliary acids having as result the increase in fecal excretion of lipids and fat-soluble substances like carotenoids [41]. In addition, the digestion of fibers by micro-organisms leads to the generation of short chain fatty acids such as acetate, butyrate, and propionate which are beneficial for the prevention of colon cancer [42]. The reduction in quantity of fibers has also been reported during the fermentation of soybean [43].

The reduction in the protein content could be attributed to their degradation by *L. plantarum* to fulfil its nitrogen needs. The same effect was observed with the spontaneous fermentation of C. pepo pulp for 72 hours [11]. The reduction of protein content in fermented flour is a disadvantage taking into consideration that transportation of retinol from liver to tissue is done by combination with Retinol Binding Protein produced in the liver [44]. Therefore, the ingestion of small quantities of proteins can have a negative influence on transport, and thus the availability and the use of the retinol in the organism. Also knowing that proteins are important for the growth of children, it is then essential to think of a source of proteins to add to fermented flour of pumpkin if it is destined for infant food formulation.

The increase of total ash could be explained by the fact that the digestion by *L. plantarum* could have liberated certain minerals from their link with macronutrients, making them more available in the fermented flour [45].

The increase in fats During fermentation could be attributed to the intense lipolytic activity of *L. plantarum* [46]. The low quantity of total fats in pumpkin is not good for the bioavailability of carotenoids which are fat soluble substances, hence are more absorbed in presence of oil in the same food matrix [47].

The low water content (about 5%) of flour obtained after fermentation indicates that it has a good conservation ability.

Table 3. Macronutrients (g/100g) content of pumpkin flour (Dry Basis)

Flours	**Water**	**Carbohydrates**	**Proteins**	**Fats**	**Fibers**	**Reducing sugars**	**Total Ash**
Raw	5.32 ± 1.13	56.55 ± 2.02	7.27 ± 1.16	2.85 ± 0.24	26.84 ± 1.89	31.05 ± 0.55	5.71 ± 1.11
Fermented	5.03 ± 0.74	78.25 ± 1.11	2.03 ± 0.11	3.40 ± 0.82	8.85 ± 1.14	63.97 ± 1.09	7.39 ± 0,72
***P* value**	.015	.000	.008	.002	.002	.000	.012

P value- <0.05 indicates a significant difference in a column.

3.3. Effect of fermentation with *L. plantarum* on some micronutrients

Effect of fermentation with *L. plantarum* on carotenoids and four bivalent cations is presented in Table 4. Results show that fermentation significantly ($p < 0.05$) reduces carotenoids and increases all minerals studied. The level of reduction is about 4.7% for total carotenoids. For minerals, the increase is about 12.9% for iron, 19.5% for magnesium, 7.4% for calcium, and 15.7% for phosphorus. The pumpkin flour, both fermented and unfermented is poor in iron with 2.31 and 2.05mg per 100g of flour respectively.

The reduction of carotenoids content could be due to the pasteurization before fermentation as studies have reported that carotenoids can be degraded (isomerized) by heat treatment [48]. However, for some authors, the reduction is not significant [47]. Pasteurization applied to clean up pumpkin flesh from any contamination microorganism before fermentation by *L. plantarum* has reduced carotenoids content, and the fermentation itself has increased it to reach the targeted level. Though there is a reduction of carotenoids content in fermented pumpkin flour, the remaining level (356.88 ± 4.11 µg/g) is still higher than 350 µg/100g recommended for infant food formulations, thus a good source of vitamin A.

Concerning minerals, the increase corroborates the increase of total ash after fermentation of pumpkin. However, flour of fermented pumpkin is poor in iron, yet iron deficiency which could lead to anemia is a problem of public health in many developing countries [49]. This low iron content suggests the need to fortify fermented pumpkin flour with food iron source to meet requirements for infant foods.

Table 4. Carotenoids (µg/g) and minerals (mg/100g) content of flour (Dry weight)

Pumpkin flour	**Carotenoids**	**Iron**	**Calcium**	**Phosphorus**	**Magnesium**
Raw	374.29 ± 6.32	2.05 ± 0.21	40.05 ± 0.21	16.02 ± 0.52	78.51 ± 1.05^a
Fermented	356.88 ± 4.11	2.31 ± 0.03	43.02 ± 0.32	18.53 ± 0.12	93.84 ± 0.67^c
***P*-value**	.0001	.003	.0001	.000	.0001

P value < 0.05 indicates a significant difference in a column

3.4. Effect of fermentation with *L. plantarum* on some anti-nutrients

The level of anti-nutrients in pumpkin flours obtained with and without fermentation was quantified, and results reported in Table 5. Generally, anti-nutrients content significantly decreases with fermentation. A decrease of 74.7%, 80.4%, 86.5% and 81.9% for phytates, tannins, phenolic compounds and oxalate content respectively were observed. These reductions of anti-nutrients observed in table 5 could be attributed to the combined effect of sterilization and fermentation. Sterilization which is a thermic treatment allows a reduction in the amount of anti-nutritional factors present in food matrices [50]. On the contrary, fermentation has been shown to significantly reduce phytates level in foods [51]. These anti-nutrients, principally phytates and oxalates can be digested by microorganisms [52], thus liberating the trapped bivalents cations such as iron and calcium thus improving their bioavailability.

Table 5. Anti-nutrient content (mg/100g) of pumpkin flours (Dry weight)

Pumpkin flour	Phytates	Tannins	Phenolic compounds	Oxalates
Raw	0.75 ± 0.02	1.07 ± 0.02	1.85 ± 0.05	1.71 ± 0.06
Fermented	0.19 ± 0.02	0.21 ± 0.02	0.25 ± 0,02	0.31 ± 0.02
***P* value**	.000	.000	.000	.000

P value < .05 indicates a significant difference in a column.

3.5. Effect of fermentation with *L. plantarum* on some functional properties

The effect of fermentation of pumpkin on the rehydration properties of its flour is presented in Table 6. Fermentation of pumpkin causes a significant decrease ($p<0.05$) of about 24% of water absorption capacity, and a significant increase ($p<0.05$) of about 134.4 % of bulk density. Water solubility index do not change significantly with fermentation.

In food materials water solubility index is complementary to water absorption capacity. The first provides indication of which portion of material can get solubilized in water upon soaking, while the second indicates the capacity of material to absorb water. The observation that fermented pumpkin flour is able to hold water up to three and half times its mass, is very appreciable since solid-water interactions constitute a limiting factor in the utilization of food powders [53]. The decrease of water absorption capacity after fermentation indicates a decrease of starch, which is the major component absorbing water in pumpkin. The water absorption capacity of food powders is positively correlated to the reduction of proteins, starch and pentosans [54]. Thus the low values of water absorption capacity in fermented flour indicate that proteins and sugars were made more available by the fermentation process, and therefore more adapted for digestion and absorption in the digestive tract of the young child.

The bulk density provides an indication of the packing and arrangement of the particles, as well as the compaction profile of a material [55]. It is the capacity of the flour to be concentrated in a small volume. The increase in the bulk density by fermentation indicates that for the same volume of water, the quantity of flour necessary to reach saturation is more significant when the pumpkin is fermented, thereby supplying more nutrients. This property is interesting in the formulation of infant flours, because the gruels which are prepared from them must have a higher caloric density.

Table 6. Rehydration properties of pumpkin flour

Pumpkin flour	WAC (g H_2O / 100g DM)		WSI (%)	BD (g/mL)
	aWAC %	rWAC %		
Raw	491.75 ± 7.84	567.65 ± 18.97	11.35 ± 1.34	0.32 ± 0.02
Fermented	369.00 ± 2.12	430.51 ± 15.83	11.55 ± 3.04	0.75 ± 0.02
***P* value**	0.007	0.015	0.053	0.02

P-value < .05 indicates a significant difference in a column; WAC: Water Absorption Capacity; a: apparent; r: real; WSI: Water Solubility Index; BD: Bulk Density

4. CONCLUSION

Lactic fermentation of pumpkin with *L. plantarum* at 45°C for 70 hours produces an energetic flour with high content in reducing sugars and carotenoids. Under these conditions, anti-nutrients content is significantly reduced, and flour obtained has a good water absorption capacity. However, the low level of proteins and minerals (iron) in the fermented flour suggests an enrichment with other food matrix rich in these elements for infant food formulation.

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