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Effect of Pure Culture Fermentation on Biochemical Composition of *Moringa oleifera* Lam Leaves Powders

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ABSTRACT

This study was carried out to determine the effect of the age of the leaves and fermentation on *in vitro* protein digestibility and biochemical properties of leaves powder of *Moringa oleifera*. A 6 × 2 × 2 factorial design with two ages of the leaves (one and seven-month-old leaves), six times of fermentation and two fermentation temperatures was used for this purpose. One and seven-month-old fresh leaves were dried at 45˚C for 24 h, crushed to 1000 µm then fermented at 30˚C and 37˚C for 120 hours with *Lactobacillus plantarum* A6 at 10^8 CFU/g. Samples were withdrawn every 24 hours for physico-chemical analyses. Results showed that 7 month-old leaves were richer in iron, proteins, polyphenols and phytates than one month old leaves. The phytates content dropped from 66.92% and 61.95% in the seven and one month-old leaves powders respectively fermented at 37˚C, and from 54.15% and 67.95% in the seven and one month-old leaves powders respectively fermented at 30˚C. Protein content increased by 26.34% and 24.48% for the 1- and 7-month-old leaves powders respectively fermented at 37˚C, and by 13.06% and 13.97% for the 1- and 7-month-old leaves powders respectively, fermented at 30˚C. Iron availability increased from 35.97% to 40.57% and 20.74% to 30.98% for the 1- and 7-month-old leaves powders respectively, fermented at 37˚C and from 35.97% to 39.79% and 20.76% to 23.72% for the 1- and 7-month-old leaves powders respectively, fermented at 30˚C. There was a negative correlation between pH, total and reducing sugar contents, time as well as fermentation temperature, whereas there was a positive correlation between total protein content and pepsic digestibility of protein and fermentation time. From these results, fermentation of *M. oleifera* leaf powder by *Lactobacillus plantarum* A6 increases protein content, pepsic digestibility of protein and availability of iron and reduces the phytates content of these powders.

Keywords: Moringa Oleifera; Leaf Powder; Fermentation; Lactobacillus Plantarum A6; Availability; Protein Digestibility

1. Introduction

In recent times malnutrition is still one of the major concerns in developing countries [1]. In Africa, in 2012, there were about 200 million under fed with an increase of 20% compared to the year 1990 [2]. The analysis of the causes of malnutrition in Africa showed that the most alarming nutritional problems are those of food deficiencies of proteins and micronutrients [3]. Indeed, the diets of many African populations are mainly made up of products of plant origin and often rest on the transformation and consumption of mainly starch-based species [4]. This lack of food diversity often leads to nutritional deficiencies [5]. The reduction of malnutrition thus requires a design of fast solutions of which one is the research of the local resources of plant proteins [3].

In many countries of Africa and Asia, due to its capacity to resist the dryness and their high amounts of nutriaents, *Moringa oleifera* leaves represent a significant local plant source of nutrients such as proteins, vitamins and minerals [6]. *M. oleifera* is used in several areas of the world as food and to fight food deficiency diseases or to enrich various foods in proteins, iron, calcium, manganese and zinc [7]. However, although rich in nutrients, *M. oleifera* leaves contain anti-nutritional factors (ANF) in particular phytates, tannins, saponins and fibres [8] which reduce the bioavailability of nutrients [9].

The technological processes such as mechanical,

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thermal, chemical and biological processes are used to reduce ANF content and improve the bioavailability of the nutrients. Among these processes, the biological processes such as fermentation, contrary to thermal, chemical and mechanical processes which can deteriorate quality of food, constitutes an effective means to reduce ANF content of foods [4] and in addition to increase the bioavailability of nutrients, also improves organoleptic and sanitary properties [10] and preservation of food [11].

Among the microorganisms used in food fermentation, lactic bacteria represent the principal group and are found on various substrates [12]. In this group *L. plantarum* is found in the fermentation of corn pastes [13], sauerkraut, *ogi* and several other fermented food of vegetable origin [14]. In addition to its abilities to reduce ANF content in food such as phytates due to the production of phytase [15], fibre by the β-galactosidase [16,17], *L. plantarum* used as ferment increases protein content, bioavailability of nutrients, improves shelf life of food [18-20] and increases the energy density of rich starch foods [21].

As part of main study aimed on improving the nutriational qualities of *Moringa oleifera* leaves, the present study was carried out to evaluate the effect of fermentation by *Lactobacillus plantarum* A6 on chemical composition and nutritional properties of *Moringa oleifera* leaves powder.

2. Materials and Methods

2.1. Raw Material

The raw material (fresh *M. oleifera* leaves) was collected at Maroua town (Far-North region of Cameroon) in December 2012. Two groups of leaves were collected: The first group constituted by one (01)-month-old leaves considered in this work as "young leaves". The second group made up with seven (07)-month-old leaves considered as "old leaves".

2.2. Starter

L. plantarum A6 was provided by the Microbiology Laboratory of CIRAD Montpellier, France.

2.3. Production of Leaves Powders

The fresh leaves of *M. oleifera* were sorted to eliminate the impurities, fade and dead leaves then washed with tap water. Thereafter, these leaves were washed with distilled water and were drained on plastic trays. The leaves were then dried at 45˚C ± 2˚C for 24 h in a ventilated hot air dryer (CK2000AUF). The dried leaves were crushed in a hammer mill (standard Culatti, Polymix, Germany) through a sieve mesh of 1000 µm.

2.4. Culture of *L. plantarum* A6

Stock culture of *Lactobacillus plantarum* A6 was obtained from UMR of CIRAD Montpellier, France. This strain was propagated in MRS Broth (pH 5.5) at 37˚C for 16 h. The culture obtained was centrifuged at 6500 rpm for 20 minutes at 4˚C and the bottom used as starter.

2.5. Fermentation

Approximately 200 g of leaves powder of each age were introduced into glass bottles of 1000 ml, and then sterilized at 121˚C for 20 min. After cooling, the powders were inoculated with *L. plantarum* A6 at 10^8 CFU/g. The water content of medium was based on water absorption capacity of powders. The mixtures were homogenized in aseptic condition using a sterile glass rod. Fermentation was carried out at 30˚C and 37˚C for 120 hours. Samples without bacteria were set up at each temperature of fermentation as control.

2.6. Chemicals Analysis

For the analysis, 50 g of samples were withdrawn every 24 h and dried at 45˚C for 24 hours. Dried fermented and non-fermented powders were analyzed for moisture, proteins, ash and lipids content essentially according to standards methods of the Association of Official Analytical Chemists (AOAC) [22,23]. Powders samples were acid-hydrolyzed and the resulting reducing sugar designated as available carbohydrates, was determined by the dinitrosalicylic acid (DNS) method of Fisher and Stein [24]. Total sugars were extracted and measured by spectrophotometry using Dubois *et al*. method [25]. Total protein content was determined by Devani method [26] after sulfuric acid mineralization of samples in presence of selenium catalyst, with a coefficient of conversion of nitrogen into protides of 6.25. Total lipids were quantified using method described by Bourely [27] and the total iron content using Rodier method [28]. The phytic acid content was estimated using a modified method of McCance and Widdowson as described by Abiodun *et al*. [29] while the phenolic compounds were estimated by the method of Marigo [30].

In vitro pepsic digestibility of protein (IVPDP) was determined using modified Mertz *et al.* [31] method. In this procedure 1 g of sample was mixed with 35 ml of 0.1 M phosphate buffer with pepsin (1.5 g pepsin/L pH 2.0), incubated for 2 hrs in a shaking water bath at 37˚C and centrifuged at 10,000 rpm for 15 min. The residue was washed in 10 ml of phosphate buffer and re-centrifuged at 10,000 rpm for 15 minutes. The supernatants were collected and their proteins content's determined by the method of Lowry *et al.* [32]. The IVPDP was determined as a proportion of soluble proteins after pepsic digestion.

Iron availability was determined in 1 mL of supernatant obtained after peptic digestion of one gram of leaf powder according to the method described by Lestienne [4] with some modifications. Practically, 1 mL of the supernatant of peptic digestion was introduced into a test tube in which was added 1 mL of hydrochloric acid (1 N), 0.5 mL of sodium acetate saturated solution, 0.3 mL of ascorbic acid and 1mL of orthophenantrolin (0.1%). The mixture was shaken and heated for 5 min at 100˚C in a water bath. After cooling, the volume was completed to 10 ml and the absorbance read at 540 nm against a blank. The standard curve was carried out using Mohr salt [$(NH4)_2$, Fe $(SO4)_2$, $6H_2O$].

Water absorption capacity (WAC) of leaves powders was determined according to method of American Association of Cereal Chemist (AACC) 44-1A [33].

2.7. Statistical Analysis

The experiment was carried out in triplicate and data obtained were analyzed by analysis of variance in Statistica software [34]. Differences between means were tested using the Duncan Multiple Range Test and correlations between variables were tested using correlation table of Pearson.

3. Results and Discussion

3.1. Chemical Composition and Water Absorption Capacity of *M. oleifera* Leaves Powders

The results for proximate composition and the Water absorption capacity of *M. oleifera* are shown in **Table 1**. The phosphorus, ash, total polyphenol, total lipid and phytates content of *M. oleifera* leaves powders analyzed vary ($p < 0.05$) according to the age of the leaves.

The increase in phosphorus content from 17.18 ± 0.71 g/100g DM to 26.57 ± 1.11 g/100g DM, in total polyphenols from 0.92 ± 0.18 g/100g DM to 3.75 ± 0.84 g/100g DM and in phytates from 699 ± 13 mg/100g DM to 1182.25 ± 21.69 g/100g DM were observed from young to old leaves. The ash, lipids and reducing sugars contents decrease respectively from 13.67 ± 0.33 g/100g DM to 12.68 ± 0.51 g/100g DM; 16.091 ± 0.52 to 9.34 ± 0.67 g/100g DM and from 4.35 ± 0.025 g/100g DM to 3.73 ± 0.32 g/100g DM from young to old leaves. However, dry matter, total sugars and crude fibres contents as well as WAC remain constant in the old and the young leaves powders. Total sugars contents (13.02 ± 0.79 g/100g DM and 13.62 ± 0.54 g/100g DM respectively for young and the old leaves powders) are near to those reported by Ray-Yu *et al.* [35] (14.47 ± 2.68 g/100g MS and 13.20 ± 1.98 g/100g MS). The phosphorus contents are lower than the value reported by Pallavi and Dipika [36] (203 mg/100g) obtained in sun dried leaves of *M. oleifera*. On the other hand, the DM content is greater than the value reported by Moyo *et al.* [37] (90.47%) on *M. oleifera* leaves powder obtained after air-drying and milling green leaves.

Table 1. Chemical composition and water absorption capacity of powders of *M. oleifera* leaves.

	Young (1 month)	Old (7 months)
Dry matter (DM) (%)	94.85 ± 0.35[a]	94.42 ± 0.59[a]
Ash (g/100 g DM)	13.67 ± 0.33[a]	12.68 ± 0.51[b]
Phosphorus (mg/100 g DM)	17.18 ± 0,71[b]	26.57 ± 1.11[a]
Lipids (g/100 g DM)	16.091 ± 0.52[a]	9.34 ± 0.67[b]
Reducing sugar (g/100 g DM)	4.35 ± 0.02[a]	3.73 ± 0.32[b]
Total sugar (g/100 g DM)	13.02 ± 0.79[a]	13.62 ± 0.54[a]
Iron (mg/100 g DM)	20.34 ± 0.65b	33.68 ± 1.22[a]
Polyphenols (g/100 g DM)	0.92 ± 0.18[b]	3.75 ± 0.84[a]
Phytate (mg/100 g DM)	699.00 ± 13[b]	1182.00 ± 22[a]
Crude fibers (g/100 g DM)	11.63 ± 0.84[a]	13.02 ± 0.66[a]
Total protein (g/100 g DM)	31.62 ± 2.84[a]	35.59 ± 2.43[a]
WAC (%)	572.00 ± 21[a]	579.00 ± 17[a]

Each value in the table is a mean of 3 replications; Different superscripts in the same line indicate significant differences ($P < 0.05$).

The crude fibres content varied from 11.63% to 13.02% respectively for young and old leaves powders. These values are close to those reported by Tchiégang and Aïssatou [38] (12.03%), but are lower than that found by Price [39] (19.2%) and Broin [40] (15%) in *M. oleifera* leaves.

The lipid content of the old-leaves powders (9.34 ± 0.67 g/100 g DM) is near to the value reported by Richter *et al.* [41] (10.6 g/100g DM) on the leaves powder of *M. oleifera* of Niger, but higher compared to lipid content reported by Tchiégang and Aïssatou [38] (5.17 ± 0.01g/100g DM) in *M. oleifera* leaves collected in the locality of Bini-Dang (Adamawa-Cameroun).

The ANF (total polyphenols and phytates) were more concentrated in the old-leaves powders as shown by Ray-Yu *et al.* [35]. The young leaves powder has 0.92 ± 0.18 g/100g DM of total polyphenol however the old-leaves powder the polyphenol content is 3.75 ± 0.84 g/100g DM similar to the value reported by Ray-Yu *et al.* [35] (3.06 ± 0.01 g/100g DM). The phytates contents varied from 699 mg/100g DM in young leaves powder to 1182.25 mg/100g DM in old leaves powder; values lower than that found by Foidl [42] (3.1 g/100g DM) in those leaves. Amaglo *et al.* [43] studied the effect of age on chemical composition of *M. oleifera* leaves and showed an increase in protein and phytochemical compounds content in the leaves with the age of the leaves due to

their accumulation during the growth of plants. However, the differences in nutrients and anti-nutrients contents obtained with others authors would be due to the agro-ecological differences of the analyzed species, the seasons of harvests or the ages of these leaves.

3.2. Effect of Fermentation on Physico-Chemical and Nutritional Properties of M. oleifera Leaves Powders

3.2.1. Changes in pH

The changes in the pH during fermentation of *M. oleifera* leaves powders are presented in **Figure 1**. The fermentation conditions have a significant effect ($p < 0.05$) on the pH. Fermentation Time, age of leaves, temperature and their interactions influence significantly the pH. Generally, the pH changes in two phases. In the first phase a slight reduction in the pH during the first 72 hours of fermentation. This fall goes from 5.8 to 5.4 for old leaves powders fermented at 37˚C and from 5.8 to 5.2 for young leaves powders fermented at 30˚C. In a second phase, there is an increase in the pH from the 72nd hour of fermentation. This increase reaches values of 6.4 for the old leaves powder fermented at 37˚C and 7.1 for the young leaves powders fermented at 30˚C. The reduction ($p < 0.05$) in the pH of the powders during the first 72 hours of fermentation, would be due to a production of organic acids in the medium especially lactic acid resulting from the metabolic activity of *L. plantarum* A6 [44]. After 72 hours of fermentation, there is a rise in the pH from the 72nd hour until the end of fermentation (120 Hours). The increase of the pH observed in this second phase would be due either to the increase of proteins content of the leaves powders or to the proteolytic activities of the lactic bacteria [45], releasing peptides, amino acids and ammonia [46] which increases the pH of the medium.

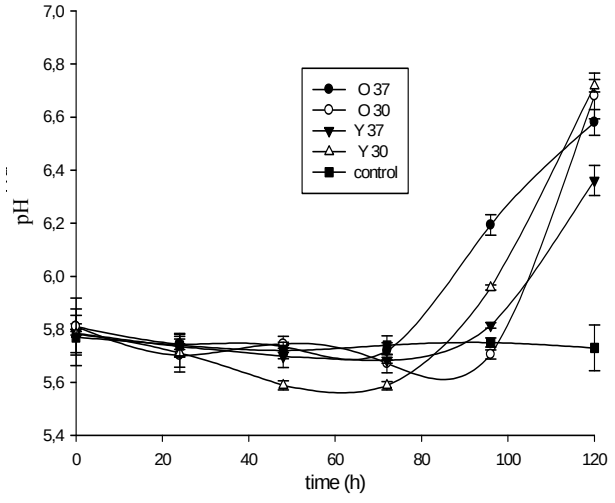

Figure 1. Changes in pH during fermentation (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

3.2.2. Total Sugars

The changes in total sugars during fermentation are shown in **Figure 2**.

There was a drop of total sugars contents from 14.12 g/100g DM to 9.39 g/100g DM and from 14.12 g/100g DM to 10.41 g/100g DM for the young leaves powders respectively fermented at 37˚C and 30˚C. For the old leaves powders, total sugar content varied from 12.73 g/100g DM to 8.52 g/100g DM and from 12.73 g/100g DM to 9.67 g/100g DM when they were fermented respectively at 37˚C and 30˚C.

The drop of the total sugars contents would be due to their metabolism by *L. plantarum* A6 during fermentation. Indeed the lactic bacteria use sugars as energy sources during fermentation with production of organic acids [47-49]. Moreover *L. plantarum* have pectinases [49,50] and α-amylase. Thus, there are able to metabolized complex sugars.

3.2.3. Total Polyphenols

The changes in total polyphenols during fermentation are shown in **Figure 3**. There is no significant variation of polyphenols contents during fermentation ($p > 0.05$). However, several authors noticed decrease of polyphenol content during fermentation.

Indeed, Medoua [51] obtained 48.4% of total poly-

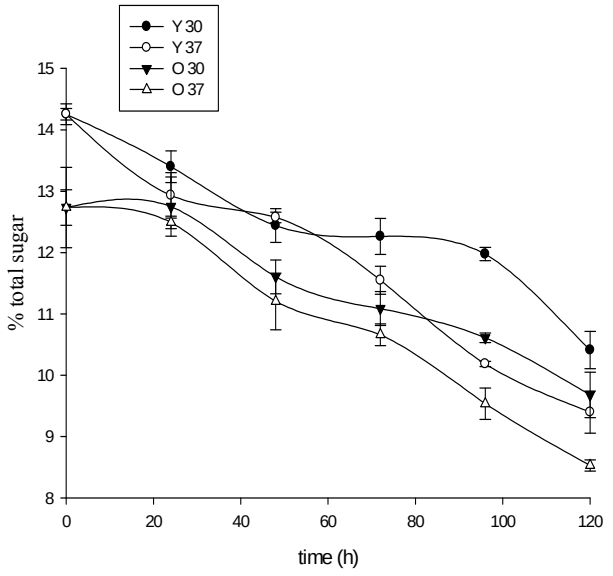

Figure 2. Changes in total sugars during fermentation (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

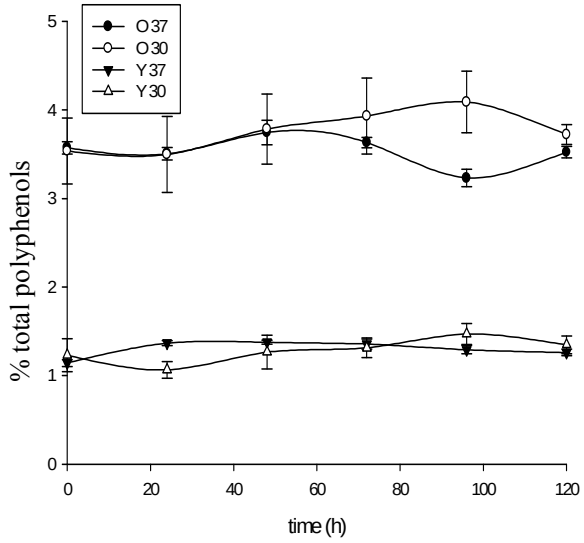

Figure 3. Changes in total polyphenol during fermentation (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

phenol losses after 14 days of fermentation of the hardened tubers of yams (*Dioscorea dumetorum*). These differences may result in absence of enzymes responsible of the phenolic compounds hydrolysis during fermentation such as the polyphenols oxydases, peroxidases, laccases. The reduction of the polyphenols contents during the fermentation of food is due to the presence of these enzymes which may be present in food stuff or produce by the micro-organisms during fermentation [4].

3.2.4. Total Protein

The evolution of total proteins during fermentation is represented by **Figure 4**. There is a general increase in total protein content ($p < 0.05$). However, this increase varies with the age of the leaves and the temperature of fermentation. Increases from 38 g/100g DM to 44 g/100g DM for the old leaves powders fermented at 37˚C and from 33 g/100g DM to 39 g/100g DM for the young leaves powders fermented at 37˚C were noticed.

The improvement of the protein value of food during fermentation has been reported by several authors [52-55].

The increase in the protein content would be due either to the increase in the biomass supported in this case by the pH of the powders during the fermentation which is in the interval of optimal pH of the lactic bacteria [13], or the reduction of the amount of dry matter [56] with consumption of component such as sugars.

3.2.5. Changes in Phytates

The changes in phytates contents of the different powders during fermentation are shown in **Figure 5**. The different factors of leaves ages, temperatures of fermentation and fermentation time affect phytates contents ($p < 0.05$) of powders during fermentation. There is a negative correlation ($p < 0.05$) between the phytates contents, the temperature of fermentation ($r = -0.53$) and the fer-

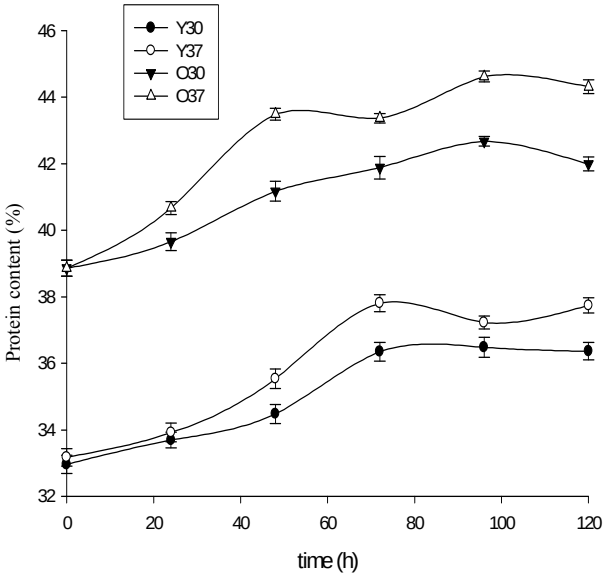

Figure 4. Changes in total protein during fermentation (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

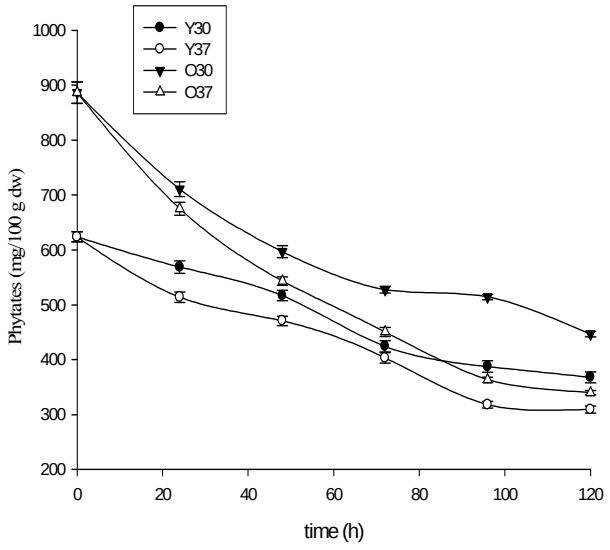

Figure 5. Evolution of phytates contents during fermentation (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

mentation time (r = −0.72). A reduction of 66.92% (from 1182 mg/100g DM to 391 mg/100g DM) of phytates contents in old-leaves powders fermented at 37˚C was noticed whereas a drop was from 1182 mg/100g DM to 542 mg/100g DM when the old-leaves powders were fermented at 30˚C. In the young leaves powders, the decrease from 699 mg/100g DM to 266 mg/100g DM (61.95%) and from 699.42 mg/100g DM to 224.76 mg/100g DM were noticed with fermentation at 37˚C and 30˚C respectively. Several authors also noted a reduction of phytates contents in fermented food [57-59]. The reduction of phytates contents during fermentation could be allotted to the production of phytases during fermentation by *L. plantarum* [60].

3.2.6. *In Vitro* Pepsic Digestibility of Proteins of *M. oleifera* Fermented Leaves Powders

The **Figure 6** shows the *in vitro* peptic digestibility proteins (IVPDP) of *M. oleifera* leaves powders during fermentation. There is a general increase of IVPDP of *M. oleifera* leaves powders during fermentation. The factors age of leaves, temperature and time of fermentation have a significant effect ($p < 0.05$) on IVPDP. The increases of IVPDP from 34.72% to 55.46% and 53.08% were observed for the young leaves powders respectively fermented at 37˚C and 30˚C. In the old-leaves powders, the increases were from 39.97% to 63.97% and 55.57% when they were fermented respectively at 37˚C and 30˚C.

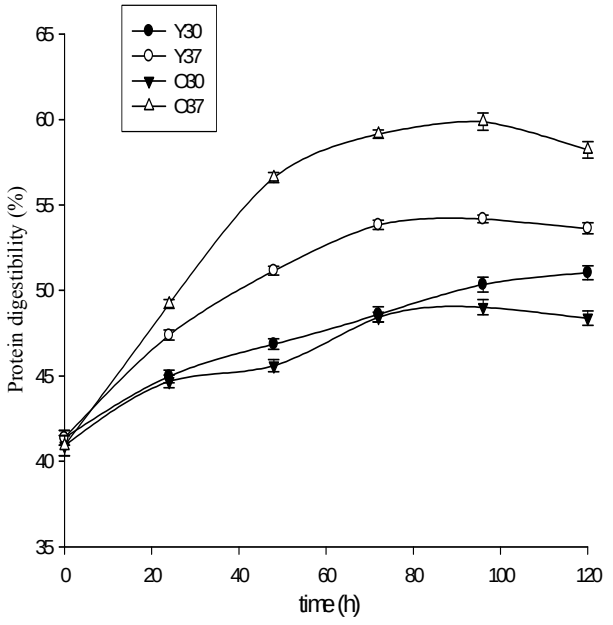

Figure 6. Protein pepsic digestibility during fermentation (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

The increase in IVPDP express an improvement of protein quality of *M. oleifera* leaves powder during the fermentation. The increase in IVPDP would be due to the drop in ANF contents [4] in particular phytates and to the increase in the protein content. The phytates are responsible to formation of the insoluble complexes with the nutrients such as proteins and minerals [61]. The hydrolysis of the phytates releases proteins from complex and increases their bioavailability.

Indeed, there is a positive correlation ($p < 0.05$) between the protein content (r = 0.42) and IVPDP, but negative correlation between the phytates content (r = −0.46) and IVPDP.

The increase in IVPDP can also result to hydrolysis of proteins to peptide and amino acids by *L. plantarum* A6 used as starter.

3.2.7. Iron Availability

The fluctuation of iron availability in the *M. oleifera* leaves powders during fermentation is presented by **Figure 7**. There is a general increase of iron availability during fermentation. This increase varies from 21.80% to 31.12% and 28.28% in the young leaves powders respectively fermented at 37˚C and 30˚C. In old-leaves powders, availability of iron increase from 31.86% to 52.14% and

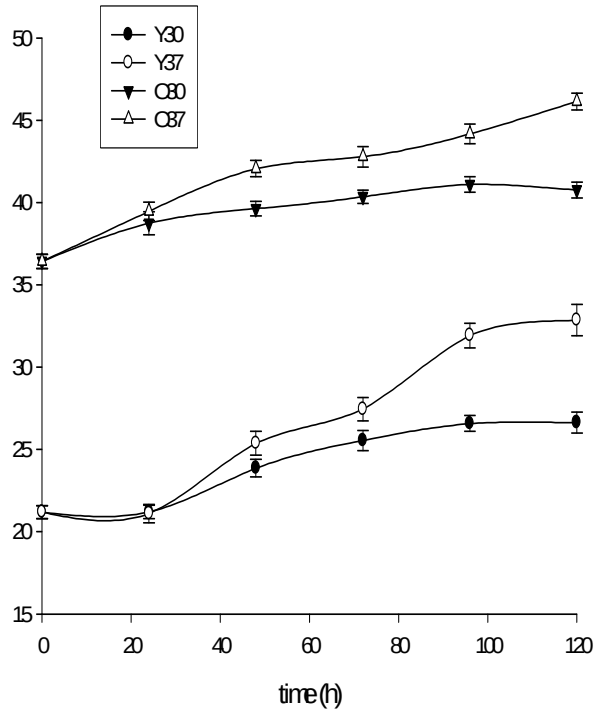

Figure 7. Ion availability of *M. oleifera* fermented leaves powders (O37 = powders of 7-month-old leaves (old) fermented with 37˚C; O30 = powders of 7-month-old leaves (old) fermented with 30˚C, Y37 = powders of leaves of 1 month (young leaves) fermented with 37˚C; Y30 = powders of leaves of 1 month (young leaves) fermented with 30˚C).

47.94% for fermentations at 37˚C and 30˚C respectively. There is a positive correlation ($p < 0.05$) between time ($r = 0.68$) the temperature of fermentation ($r = 0.52$) and iron availability. The increase of iron availability can result to the reduction of phytates content. Indeed, phytic acid is responsible for the reduction of the availability of cations in food, forming insoluble complexes [4]. The hydrolysis of phytic acid release ions thus, allows an increase of minerals availability [59]. There is a negative correlation ($p < 0.05$) between phytates content ($r = -0.73$) and iron availability.

Fermentation is known as a process improving iron bioavailability in food of vegetable origin [59,61]. Several authors reported the effect of the reduction of phytates on the increase in the bioavailability of iron. Icard-Vernière *et al.* [60] noticed an increase of 31% of the soluble iron rate in the fermented millet with a reduction of almost 95% of phytates content. In the same way, an increase of iron bioavailable in fermented sorghum of 3.1% was reported by Towo *et al.* [58] during fermentation with a reduction of 88% of the content of phytates.

4. Conclusion

The fermentation of the *M. oleifera* leaves powders by *Lactobacillus plantarum* A6 reduce the phytates content to 66.92%, increase protein content and their peptic digestibility to a value of 63.97%. Fermentation also increases iron availability in these powders. The fermentation of *M. oleifera* leaves powders allows the improvement of its nutritional qualities. From this work, for the best improvement of nutritional qualities of *M. oleifera* leaf powder, the old leaves must be fermented during 120 hours at 37˚C. These fermented powders thus could be used to fight deficiency malnutrition, a public endemic health problem in the developing countries.

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