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The figure on the front cover is from the article published in Food and Nutrition Sciences, 2014, Vol. 5, No. 21, pp. 2053-2062 by Florinda Fratianni, Rosa Pepe and Filomena Nazzaro.

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Comparison of Direct Microbial Count Procedures for Planktonics and Sessiles Enumeration

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Abstract

In the present investigation, the sensitivity of different direct microbial count procedures applied on systems containing both planktonics and sessiles was tested. The direct count pour plate was compared with direct epifluorescent microscopic enumerations in order to evaluate the efficiency of the studied techniques in giving information about microbial activity or viability. Our results indicate that the standard plate count procedure is the most sensitive method to estimate viable and cultivable planktonic cells. On the other hand, direct enumeration by epifluorescent microscopy may become an interesting alternative to count sessile cells.

Keywords

Direct Viable Count, Biofilm, Epifluorescence Microscopy, Fluorochromes

1. Introduction

Accurate quantitative evaluations of bacterial populations, of biomass and of community structure are critical prerequisites for assessing the roles of bacteria in natural and technical systems (*i.e.* sewage or food processing plants). There is no scientific univocality on which procedure gives the best results in representing a viable microbial population. Plate count methods are often employed for microbiological quantitative analyses, but they are time consuming (because of required lengthy incubations) and typically do not provide useful information concerning microbial activity, or viability. Bacteria are generally physically removed (by filtration or dilution) from the native sample and are therefore no longer subject to possible inhibitory substances or conditions that

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may limit their metabolic activity *in situ*. Moreover, plate count procedures cannot be used to directly observe active cells *in situ*, especially when the cells are attached to suspended particulate matter or other solid surfaces (*i.e.* biofilm). In this case, a fundamental difficulty in efficient separating bacteria from their substratum lies in the conflict between using procedures hard enough to achieve both the near-complete detachment, and the cell wholeness. Consequently, opportune conditions need to be carefully chosen in order to maximize the detachment efficiency and minimize the cell damage. A variety of procedures have been proposed to directly count sessile cells *in situ* and these include direct counts by means of epifluorescence techniques [1] [2].

Our objective in the present investigation was to test the sensitivity of different procedures in counting bacteria, both as planktonics and sessiles. In particular, the results of the direct count pour plate were compared with those obtained by direct epifluorescent microscopic enumerations, in order to point out their effectiveness and accuracy in giving information about microbial activity or viability.

2. Materials and Methods

2.1. Preparation of Samples

One strain of *Salmonella* sp. purchased from a public collection (American Type Culture Collection, ATCC 35,664) was used in the present work. Prior to use, the culture was grown aerobically at 37˚C for 24 h in Tryptone Soya Broth (TSB, Oxoid, Milan, Italy), composed as follows: 17 g/l pancreatic digest of casein, 3.0 g/l enzymatic digest of soya bean, 5.0 g/l sodium chloride, 2.5 g/l di-potassium hydrogen phosphate, 2.5 g/l glucose (pH 7.3 ± 0.2).

Planktonic *Salmonella* sp. samples were prepared pouring 20 ml of TSB into sterile Coplin jars and inoculating each of them with 10^3 CFU/ml. Incubation was performed at 37˚C, without shaking, for 5 days.

Glass slides (25.4 mm × 76.2 mm) were used as surfaces to get the biofilm attached. All slides were cleaned with acetone before soaking in 3.5% sodium hypochlorite (V/V) at 75˚C for 5 min. Then they were rinsed and transferred into 7.0 g/l phosphoric acid solution for 5 min. Slides were rinsed in distilled water, air dried and autoclaved at 121 ˚C for 15 min [3]. This cleansing was required to remove fingerprints, oils, grease and other soils that may have been on glass.

Sessile samples were prepared pouring 20 ml of TSB into sterile Coplin jars and vertically dipping sterile slides in; the inoculum with 10^3 CFU/ml was performed in each of them and the samples were incubated at 37˚C, without agitation, for 5 days.

2.2. Bacterial Count Pour Plate

After 6, 24, 48, 72 and 96 hours since the inoculum, populations in planktonic state were determined by a standard plate count procedure with Tryptone Soya Agar (TSA), incubated at 37˚C for 48 h [4]. At the same times, slides were aseptically removed from the medium and rinsed with sterile distilled water to remove the unattached cells. Then each slide was placed into a test-tube containing 20 ml of sterile saline and sonicated with a 20 Hz "Vibra Cell" sonicator (SONICS, Newcastle, CT, USA) for 3 min in order to detach and collect the sessile cells. Opportune serial dilutions of the resulting suspensions were enumerated by plating on TSA and incubating at 37˚C for 48 h.

2.3. Bacterial Count by Epifluorescence Microscopy

The methods used were those suggested by Rodriguez *et al.* [1]. In particular, after 6, 24, 48, 72 and 96 hours since the inoculum, 1 mL aliquot of *Salmonella* sp. TSB culture was transferred into a 1.5 ml micro centrifuge tube. The surnatant was discarded after the suspension was centrifuged at 12.000 rpm/min for 60 s. The pellet was stained in 1 ml of stain solution. Three different stain dyes were used:

- 4',6-diamidino-2-phenylindole (DAPI), 100 µg/ml in sterile deionized water for 1 h;
- 3,6-bis-dimethylamine-acridine (acridine orange), 0.025% (w/V) in sterile deionized water for 1 h;
- 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), 0.15 nM in diluted TSB (1:5) for 1 h in the dark.

All fluorochromes were purchased from Sigma-Aldrich (Milan, Italy); working solutions were prepared immediately before using and sterilized by membrane (0.2 µm pore size) filtration.

After staining incubation time, the cell suspension was centrifuged and the pellet was washed three times in the same solution used for the staining. Finally the pellet was resuspended in 1 mL of stain solution and 10 µl of

the cell suspension was placed on a glass microscopic slide and air dried at room temperature.

As regard to sessile cells, slides were aseptically removed from the medium, rinsed twice with sterile deionized water and stained with the three different fluorochromes. After 1 h, the slides were rinsed and air dried.

Preparations were examined with the ×100 oil immersion fluorescence objective of a Nikon microscope (NIKON Instruments Inc., NY, USA), equipped with a 50W mercury burner. The filter set combination found to be most effective for successfully viewing all the different stained preparations, consisted of a 365 nm excitation filter, an emission filter and a 400 nm cutoff filter. Bacterial cells were counted in a minimum of 10 microscopic fields. Counts were performed in triplicate.

2.4. Statistical Analysis

All the experiments were replicated at least twice and the obtained results were expressed as Log CFU/ml and Log CFU/cm^2 for planktonic and sessile cells enumerations, respectively.

The planktonic *Salmonella* sp. cell load data (average of the repetitions), obtained by the different count methods, were modeled according to the Gompertz equation as modified by Zwietering *et al.* [5]:

$$y = k + A \times \exp\left\{-\exp\left[\left(\mu_{\max} \times e/A\right) \times \left(\lambda - \text{time}\right) + 1\right]\right\}$$

where k is the initial cell load (LogCFU/ml); A, the maximum bacteria growth attained at the stationary phase (LogCFU/ml); $\mu_{\max}$, the maximal growth rate (Δ LogCFU/ml/ h); λ, the lag phase (h).

Differences in sensitivity of the tested methods were examined by an analysis of variance and Tukey test. Only P-values < 0.05 were considered significant.

"Statistica per Windows" software (Statsoft Inc., release 6.0, Tulsa, USA) was used to perform statistical analyses.

3. Results and Discussion

After testing several concentrations of fluorochromes (DAPI, acridine orange and CTC) and several incubation times, the highest numbers of bacteria were obtained in the chosen staining conditions. Moreover, in preliminary experiments we optimized conditions for CTC assay detecting that the addition of TSB to the sample intensified the formation of formazane crystals (data not shown).

Figure 1 reports the best fits of the model to experimental data, referring to planktonic *Salmonella* sp. cell load, estimated by the different count methods. The absolute sensitivity (*i.e.* the number of planktonic cells counted) significantly differed among the tested methods. It is clear that the standard plate count procedure was the most sensitive method to estimate viable and cultivable planktonic cells, while microscopy enumerations gave significantly lower bacterial counts (P < 0.05). After 24 h, the pour plate method performed a cell load about 8.83 LogCFU/ml, instead of 5.83 Log CFU/ml and 7.03 Log CFU/ml, counted using DAPI and acridine orange, respectively. The number of formazan-containing bacteria (CTC) even was significantly lower with respect to all other methods.

Figure 2 shows sessile *Salmonella* sp. cell loads estimated by the different count methods. After 6 h, microscopy enumerations with DAPI and acridine orange were significantly more sensitive than the other two count methods (P < 0.05). Results recorded after 24 hours by microscopy techniques were similar (P > 0.05), but significantly differed from plate count ones. After 48 h the number of counted cells was relatively similar over time in every tested method.

The enumeration of active bacteria by epifluorescence microscopy is a rapid, simple, low-cost, and high-sensitivity procedure. In this work, we decided to use CTC, a tetrazolium dye, beside DAPI and acridine orange, generally used as fluorochromes. Tetrazolium salts are used as artificial electron acceptors which are reduced within the respiratory chain. This results in the intracellular formation of colored formazans, equivalent to the respiratory activity of cells. In some studies, CTC was introduced to determine the number of metabolically active bacteria in studies of several aquatic environments, including biofilms [1] [6]-[8], but it is still poorly used. Our preliminary experiments demonstrated that the enrichment of samples with TSB intensified the formation of formazane crystals; this is in agreement with the results of Rodriguez *et al.* [1], who proposed to use CTC for the enumeration of viable bacteria and incubated their samples of marine, ground and waste waters with a nutrient R2A broth added at a ratio 1:2.

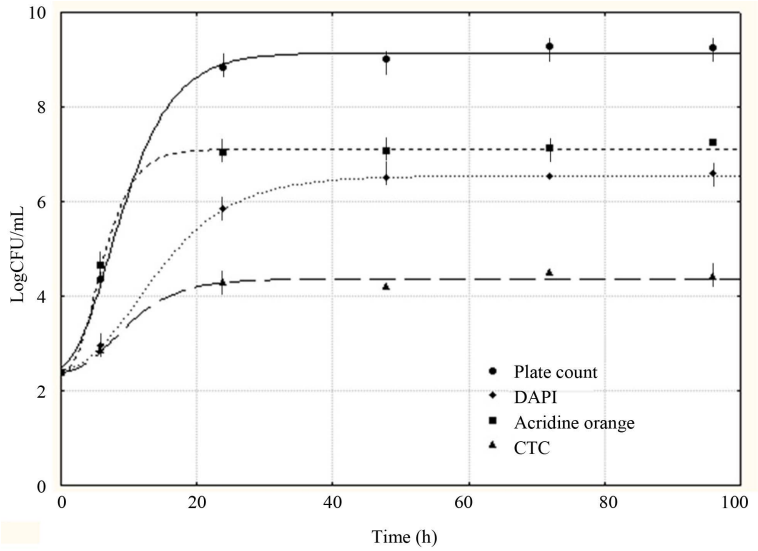

Figure 1. Best fit of modified Gompertz equation to experimental data referring to planktonic *Salmonella* sp. cell load, estimated by the different tested count methods. The error bars represent the standard deviation of the experimental data.

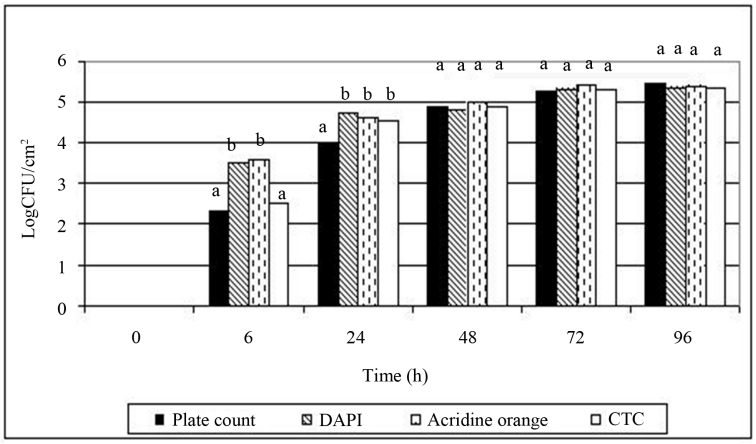

Figure 2. Sessile cell loads estimated by the different tested count methods. Bars with similar superscript do not significantly differ ($P > 0.05$).

The results obtained in our study showed that standard plate count procedure is the most sensitive method to estimate planktonic cells. In fact, all microscopy counts underestimated the planktonics number. The underestimation of microscopy enumerations with DAPI and acridine orange was probably due to the several centrifugation stages used in our procedures. It is possible that during this step, recurring four times, planktonic cells were mechanically damnaged or lost in the surnatant discharges. Nevertheless this hyphotesis cannot explain the results obtained by the CTC assay, because no centrifugation was provided for this procedure. There are other possible explanations for this method result. 1) The assay was not sensitive enough to detect low-rate respiring microorganisms, which might especially be a problem for the detection of small bacteria. 2) Formazane granules are soluble in oil, which is used in immersion microscopy. 3) Bacterial metabolic activity is directly suppressed by the chemical CTC. There are already some indications for a toxic effect of CTC on bacterial cell metabolism in literature, in spite CTC has generally been assumed to be non toxic [2]. In our experiments the detected inhibitory effect of CTC on bacterial metabolism contradicts the usefulness of the CTC assay, and it can be assumed that this method is not suitable for the evaluation of *Salmonella* sp. planktonic cells.

As regard to sessile *Salmonella* sp. population, microscopy enumerations proved more useful than plate count

procedure, showing several advantages: 1) Rapidity (1 - 2 h). 2) Simplicity. 3) Ability to provide useful information concerning microbial activity (or viability) of sessile cells. 4) Possibility to be used to directly observe cells *in situ*. 5) High sensitivity. Moreover, the CTC assay appeared clearly a useful tool to count cells into a biofilm, without exerting any toxic effect on bacterial cell metabolism. It is well established that bacterial biofilms exhibit an increased resistance to toxic agents than the individual cells grown as planktonics [7] [9]. Probably this recorded resistance to CTC may be attributed to the varied properties associated with the biofilm, including a reduced diffusion of the compound into the structure and/or the production of enzymes degrading toxic substances.

4. Conclusion

We have investigated the sensitivity of different procedures in counting bacteria, both as planktonics and sessiles. Our results indicate that standard plate count procedure is the most sensitive method to estimate viable and cultivable planktonic cells. On the other hand, the direct enumeration by epifluorescent microscopy may become an interesting alternative to the traditional techniques in order to count sessile cells.

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Pasting Properties of White Corn Flours of *Anoman* 1 and *Pulut Harapan* Varieties as Affected by Fementation Process

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Abstract

This research was aimed to evaluate the pasting properties of white corn flour made from *Anoman* 1 and *Pulut Harapan* varieties as affected by the fermentation process of the corn grits. The fermentation process studied were 1) spontaneous fermentation (SF); 2) fermentation with the addition of a complete starter culture at 0 hour (CC fermentation) and 3) fermentation of (CC) with additional inoculation of starter culture containing amylolytic microorganisms at 16 hours (AC fermentation). The evaluation of pasting properties was done on the flour made from corn grits fermented for 0, 36, 48, and 72 hours. Our results showed that pasting properties of corn flour of *Anoman* 1 and *Pulut Harapan* varieties were affected by fermentation process. Addition of starter culture in the fermentation showed more complex effect on the pasting properties and was a function of the fermentation time. Fermentation process of corn grits affected the pasting properties of the resulted flour, both for Anoman I and *Pulut Harapan* corn varieties. The differences in the effect of fermentation process on the pasting properties were due to the different amylose/amylopectin content. AC fermentation of corn grits could increase the stability of paste for flour containing higher amylose content but decrease the stability of paste for flour containing high amylopectin. Specifically, CC fermentation caused significant increase in the peak viscosity value especially for corn flour of *Pulut Harapan*. Fermentation for up to 48 h had resulted in corn flour of *Pulut Harapan* variety having a higher PV value, but it did not affect the tendency to retrograde.

Keywords

Pasting, Corn Flour, White Corn, Fermentation

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1. Introduction

Corn is an important carbohydrate source after rice in Indonesia. However, the utilization of corn flours and/or starches in native form is limited due to its physical properties, especially with regard to the retrogradation properties, syneresis of pasta, and low stability of pasta at high temperature and at low pH [1]. Consequently, there is a need to modify flour properties to improve its pasting properties.

Traditionally, corn flour is made by soaking corn kernels in water followed by the process of draining, drying and milling. Aini *et al.* (2010) [2] showed that changes in the physicochemical properties of white corn flour produced was attributable to the spontaneous fermentation occurring during soaking. Several other studies of spontaneous fermentation of corn have been published, such as in the production of ogi [3] and pozol [4] which are African traditional foods.

Previously, we have identified that microorganisms responsible for the spontaneous fermentation of corn were *Penicillium chrysogenum*, *Penicillium citrinum*, *Aspergillus flavus*, *A. niger*, *Rhizopus stolonifer*, *R.oryzae*, *Fusarium oxysporum*, *Acremonium strictum*, *Candida famata*, *Kodamaea ohmeri*, *Candida krusei/incospicua*, *Lactobacillus plantarum*1*a*, *Pediococcus pentosaceus*, *Lactobacillus brevis*1, *Lactobacillus plantarum*1*b*, and *Lactobacillus paracasei* ssp. *paracasei*3 [5]. Of all microorganisms identified; four molds (*Penicillium citrinum*, *Aspergillus flavus*, *Aspergillus niger*, *Acremonium strictum*) and one yeast (*Candida famata*) were found to be amylolytic, while none of the LAB was capable of starch hydrolysis. The amylolytic activity is thought to be important for physicochemical changes of flour due to its high carbohydrate content.

Since microorganisms were involved in the spontaneous fermentation (SF) of corn, the influence of addition of starter culture to the fermentation process on the pasting property of the resulted corn flour was evaluated. A complete starter culture (CC) was made using all microorganisms indentified responsible for the spontaneous fermentation of corn [5], except for *Aspergillus flavus*, due to its ability to produce aflatoxin in corn. Microorganisme used for preparation of complete culture were *Penicillium chrysogenum*, *Penicillium citrinum*, *Aspergillus niger*, *Rhizopus stolonifer*, *Rhizopus oryzae*, *Fusarium oxysporum*, *Acremonium strictum*, *Candida famata*, *Kodamaea ohmeri*, *Candida krusei/incospicua*, *Lactobacillus plantarum*1*a*, *Pediococcus pentosaceus*, *L. brevis*1, *L. plantarum*1*b*, and *L. paracasei* ssp. *paracasei*3. In addition, an amylolytic starter culture (AC) was made using the three amylolytic molds (without *Aspergillus flavus*) and one amylolytic yeast.

Three experiments of fermentations process of corn grits were conducted, *i.e.* spontaneous fermentation (SF) by water soaking of corn grits as a control, fermentation with addition of complete starter culture (CC) from the start of fermentation, and treatment of (CC) with additional amylolytic starter culture (AC) after 16 hours of fermentation. Observations were done on the flour made from corn grits after 0 (unfermented flour, U), 36, 48, and 72 hours of fermentation.

2. Materials and Methods

2.1. Corn

Corn types used in this research were local white maize *Anoman* 1 and waxy maize *Pulut Harapan* varieties obtained from the Cereal Crops Research Institute, Maros, Sulawesi, Indonesia. *Anoman* 1 maize kernel contains high amylose (29.92%), while the waxy maize kernel local Pulut has low amylose content (4.25%) [6].

Corn was made into grits for a more standardized fermentation process. Kernels of corn were washed with drinking water (corn: water = 1:4 w/v) and drained on a siever. Drained and clean corn kernels were then ground using pin disc mill and sieved to produce grits with diameter of $\geq$4 mm. The grits were washed with drinking water (corn grits: water = 1:4 w/v) for 30 minutes and then drained and ready for fermentation.

2.2. Microorganisms

Microorganisms used for starter culture preparation were amylolytic *Penicillium citrinum*, *Aspergillus niger*, *Acremonium strictum*, and *Candida famata*, as well as non amylolytic *Penicillium chrysogenum*, *Rhizopus stolonifer*, *Rhizopus oryzae*, *Fusarium oxysporum*, *Kodamaea ohmeri* and *Candida krusei/incospicua*, *Lactobacillus plantarum*1*a*, *Pediococcus pentosaceus*, *Lactobacillus brevis*1, and *Lactobacillus paracasei* ssp. *paracasei*3. The microorganisms used were previously isolated and identified from a spontaneous fermentation of corn grits [5], at which for lactic acid bacteria were identified using API kits program API CH50 (biochemical rapid kits, bioMérieux).

2.3. Culture Preparation and Enumeration

One loop of each mold was streaked onto fresh Potato Dextrose Agar (PDA) slant and then incubated at 30˚C for 5 days. After 5 days molds were harvested by scrapping, suspended in 10 mL sterile water and appropriately diluted for enumeration using hemacytometer. Yeast culture was prepared as above but incubation was carried out at 30˚C for 2 days. Yeast enumeration was also carried out using hemacytometer. Meanwhile Lactic Acid Bacteria (LAB) cultures were prepared by transferring one loop of each LAB growth into de Man Rogosa Sharpe (MRS) Broth for 24 hours at 30˚C using shaking incubator. After 24 hours, the culture was centrifuged aseptically for 15 mins, 3500 rpm at 4˚C and the cell pellets were resuspended in phosphate buffer. The 24 h culture was also enumerated by plating on MRS agar.

2.4. Fermentation with Added Starter Culture

Five days old molds and two days old yeast in sterile water as well as 24 hours LAB in phosphate buffer made up the complete starter culture. For amylolytic starter culture, only *Penicillium citrinum*, *Aspergillus niger*, *Acremonium strictum*, and *Candida famata* were used. Each microorganism was inoculated aseptically into container (15 L) containing of maize grits and drinking water (1:2 w/v) such that each microorganism has an initial load of ca. 10^6 CFU/mL.

The fermentation studied included spontaneous fermentation, *i.e.* water soaking of corn grits as a control (SF) and fermentation with added starter cultures. Two treatments of fermentation with added starter were (CC) a complete starter culture containing 15 microbes previously isolated from the spontaneous fermentation added at the beginning of fermentation (0 hours), and (AC) fermentation of (CC) with additional inoculation of amylolytic starter culture at 16 hours of fermentation. Observations were done on flour made from corn grits after 0, 36, 48, and 72 hours fermentation.

2.5. Pasting Properties of Corn Flours Measured Using Rapid Visco Analyzer TecMaster Newport Scientific Pty Limited Australia (RVA Standard 2)

Corn flour samples of 3.5 g (14% moisture content) were added to 25 mL of distilled water in an aluminum can. Sample was spinned (160 rpm) at 50˚C for 1 min, and heated to reach 95˚C within 7.5 min, and held at 95˚C for 5 min, and then cooled back to 50˚C within 7.5 min and held at 50˚C for additional 2 min. A typical complete RVA curve obtained is presented in **Figure 1**.

Parameters derived from the RVA curve were peak viscosity (PV); trough viscosity (TV; also called as hot

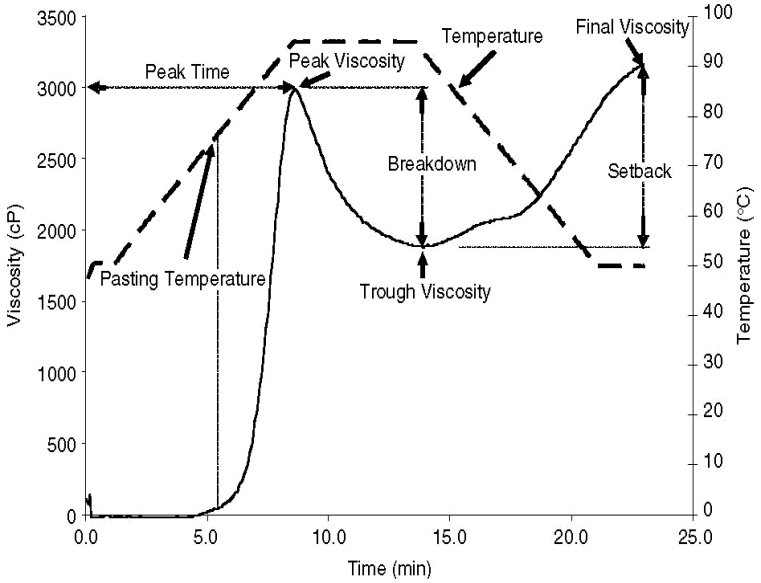


Figure 1. Typical pasting profile obtained by RVA, showing the main parameters used to described pasting properties.

viscosity), and final viscosity (FV). The breakdown (BV) and setback (SV) viscosities were calculated from the differences between (PV and TV) and (FV and TV), respectively.

2.6. Statistical Analysis

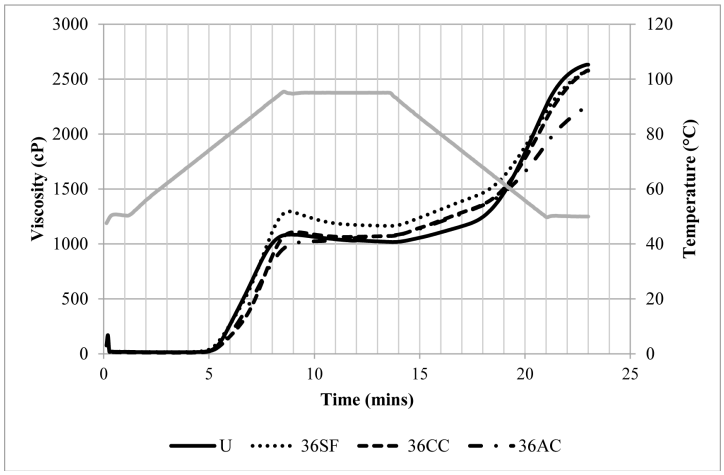
Statistical signifcance of differences between sample means was determined using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test at 95% confidence level.

3. Results and Discussion

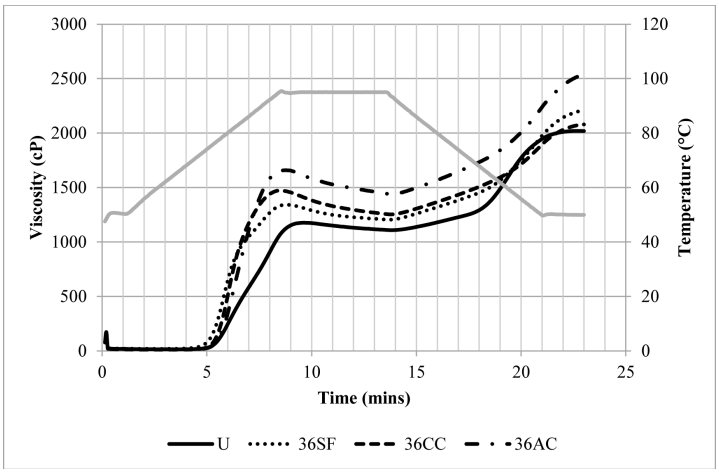
Our results showed that pasting properties of corn flour of *Anoman* 1 and *Pulut Harapan* varieties were affected by fermentation process. Addition of starter culture for the fermentation showed more complex effect on the pasting properties and was a function of the progress of fermentation reaction.

3.1. Pasting Properties of Corn Flours after 36 Hours of Fermentation

The pasting profiles (**Figure 2**) and parameters (**Table 1**) of corn flours made from corn grits after 36 hours of



(a)



(b)

Figure 2. Pasting profile of corn flour of *Anoman* 1 (a) and *Pulut Harapan* (b) varieties made from corn grits after 36 hours of fermentation. U: Unfermented flours; 36SF: flour made from corn grits after 36 hours of spontaneous fermentation, 36CC: flour made from corn grits after 36 hours of fermentation with addition of a complete starter culture; 36AC: flour made from corn grits after 36 hours of fermentation with complete culture and additional amylolytic starter (AC) culture at 16 hours of fermentation.

Table 1. Pasting properties profile of maize flour *Anoman* 1and *Pulut Harapan* varieties during 36 hours fermentation.

Treatments	Pasting temperature (˚C)	Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)
Anoman 1 flour						
U	81.5 ± 30.9^{a}	1276.9 ± 24.4^{a}	1012.6 ± 27.6^{a}	264.4 ± 36.4^{b}	2630.8 ± 0.1^{c}	1618.3 ± 0.4^{c}
36SF	83.4 ± 28.7^{a}	1451.9 ± 14.8^{b}	1156.3 ± 29.7^{b}	295.6 ± 35.7^{c}	2579.4 ± 0.0^{b}	1423.1 ± 0.3^{b}
36CC	85.0 ± 24.5^{a}	1290.4 ± 8.8^{b}	1062.6 ± 23.3^{b}	227.8 ± 21.7^{bc}	2575.1 ± 0.0^{bc}	1512.5 ± 0.3^{b}
36AC	82.2 ± 48.8^{a}	1193.8 ± 22.6^{a}	1065.8 ± 64.3^{b}	128.0 ± 62.6^{a}	2249.0 ± 0.1^{a}	1183.3 ± 0.5^{a}
Pulut Harapan flour						
U	82.1 ± 25.9^{a}	1237.7 ± 20.7^{a}	1090.3 ± 29.5^{a}	147.4 ± 15.8^{a}	2017.4 ± 0.7^{a}	927.1 ± 0.8^{a}
36SF	75.6 ± 37.2^{a}	1680.2 ± 33.3^{b}	1248.6 ± 24.0^{b}	431.6 ± 22.3^{b}	2443.4 ± 0.0^{bc}	1194.8 ± 0.8^{a}
36CC	78.4 ± 23.1^{a}	1638.4 ± 15.4^{b}	1251.9 ± 21.1^{bc}	386.6 ± 18.2^{b}	2079.4 ± 0.1^{b}	827.6 ± 0.2^{a}
36AC	78.5 ± 9.9^{a}	1790.0 ± 48.1^{c}	1439.3 ± 52.7^{c}	350.8 ± 32.5^{b}	2537.1 ± 1.0^{c}	1097.9 ± 2.4^{a}

Samples means with different superscripts in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.

fermentation both for Anoman I and *Pulut Harapan* varieties obtained from the RVA curve were evaluated. Unfermented corn flour of Anoman I and *Pulut Harapan* varieties has peak viscosities (PV) of 1276.9 ± 24.4 cP and 1237.7 ± 20.7 cP, respectively (**Table 1**). Spontaneous fermentation for 36 hours (36SF) increased PV of flours from both corn varieties to1451.9 ± 14.8 cP and 1680.2 ± 33.3 cP, respectively for *Anoman* 1 and *Pulut Harapan*. Increase of PV is associated with enzymatic activity during fermentation process. *Rhizopus oryzae has been shown to* produce cellulase, hemicellulase, pectinase, tannase, phytase, lipase and protease [7], while *Rhizopus stolonifer* produces cellulase [8]. Beside its amylolytic activity, *Aspergillus niger* also has pectinolytic activity [5] [9]. Panagiotou *et al.* [10] reported that *Fusarium oxysporum* has cellulolytic and xylanolytic activity. *Kodamae ohmeri* produces phytase in cereals [11] and lipase [12]. *Candida famata* produced glucoamylase [13], as well as has lypolytic and proteolytic activity [14]. *Candida krusei* has lypolytic and esterase activity which contribute on the flavor of end product [15]. Amylase hydrolyzes α-1,4-D-glycosidic bond of starch [16] thus the structure of starch granule becomes more porous facilitating water absorption to granules. This will increase the swelling of granules [17] and increase PV. PV illustrates the capacity of starch in absorbing water and swelling of granules when it is heated [18].

Inoculation of a complete starter culture (CC) however reduced the PV of the corn flour of Anoman I variety to 1290.4 ± 8.8 cP. This is due to excessive amylolytic activity leading to more amylose breakdown. Different phenomenon was observed for corn flour of *Pulut Harapan* variety; which showed that its PV was as high as that of flour obtained from corn grits after 36 hours of spontaneous fermentation (36SF; 1680.2 ± 33.3 cP). Addition of amylolytic culture, however, caused further increase of PV for corn flour of *Pulut Harapan* variety. This is due to the lower content of amylose (higher content of amylopectin) of corn flour of *Pulut Harapan* variety. Structurally, the higher amylopectin content of *Pulut Harapan* corn support granule to be more resistant to amylolytic activity during fermentation. Since the flour has stronger granule integrity, it will swell bigger and has increased peak viscosity.

The viscosity of flour paste decreased after heating process for certain period due the granule breakdown followed by leaching of amylose into the solution [19]. The degree of viscosity reduction during heating process is termed as breakdown viscosity (BV); and can be used as an indicator for pasting stability during heating and stirring [20] [21].

As we can see from **Table 1**, the breakdown (BV), final (FV) and setback (SV) viscosities of *Anoman* 1 corn flour prepared from corn grits after 36 hours spontaneous fermentation were relatively lower than that of unfermented flour. This suggests that high amlyose content of corn of Anoman variety is more sensitive toward amylolytic activity during the 36 hours of fermentation. This also apparent with reduced final viscosity (FV) observed; especially those with amylolytic culture added (AC). FV is a viscosity at 50˚C at the end of analysis. FV indicates the stability of paste during cooling process. At the end of cooling process of 95 to 50˚C, the viscosity increased. This is due to the alignment and rearrangement the amylose chain [18] [21]. During cooling process,

amylose polymers start to aggregate through hydrogen bond forming a junction bond and generates a gel network, and is called retrogradation [22]. Reduction or breakdown of amylose at Anoman I corn is also indicated by the final viscosity drop from 2630.8 ± 0.1 cP for the unfermented flour to 2579.4 ± 0.0 cP due to spontaneous fermentation (36SF) and 2575.1 ± 0.0 cP due to fermentation with addition of complete culture (36CC). This may be associated with more amylolytic microbes causing more breakdown of amylose into simple sugar, thus reduced the final amylose level as indicated by the lower FV value. Further decrease in FV up to 2249.0 ± 0.1 cP observed due to additional amylolytic culture (36AC) also support this explanation, because further breakdown of amylose molecules occured (**Table 1**).

In general, the FV of unfermented flour of *Pulut Harapan* (2017.4 ± 0.7 cP) was lower than that of Anoman I (2630.8 ± 0.1 cP). This is associated with its higher amylopectin content of corn of *Pulut Harapan*. Flour containing higher amylopectin has shown to produce highly gelatinous dispersions when cooked and form soft and runny gels [22]. This is because amylopectin has more ability to absorb water than that of amylose [23]. Furthermore; for flour with low to medium amylose content, the present of higher percentage of amylopectin molecules may hamper aggregation of free amylose chains during aggregation [22]; as indicated by lower value of FV. After 36 hours of fermentation, however, flour of *Pulut Harapan* showed increased FV value (**Figure 2(b)**; **Table 1**), suggesting more free amylose was produced to allow the formation of more viscous network. The least increase of FV (of 2079.4 ± 0.1 cP) was observed for corn flour of *Pulut Harapan* obtained from 36 hour of fermentation with addition of complete culture starter (36CC). This suggests that addition of CC starter would hydrolyze amylose molecule to form shorter chain; reducing FV value. Further addition of amylolytic culture (36AC) however, will increase the FV value (2537.1 ± 1.0 cP). This might be associated with more amylose molecules produced by hydrolysis reaction of amylopectin molecules.

The stability of paste during cooling and storage is indicated by SV value; *i.e.* the differences between FV and TV values. The higher SV value indicates the higher tendency of amylose to retrograde [21] forming a gel structure when the polymer molecules, especially amylose chains, realign themselves. Our results shows that in general, fermentation has caused corn flour of Anoman I variety to have less tendency to retrograde (SV value of 1183.3 to 1512.5 cP) as compared to that of the unfermented one (SV value of 1618.3 ± 0.4 cP). Corn flour of Anoman I with the lowest tendency to retrograde was the one produced from AC fermentation (SV value of 1183.3 ± 0.5 cP); indicating a more intensive hydrolysis of amylose molecule occurred during the fermentation.

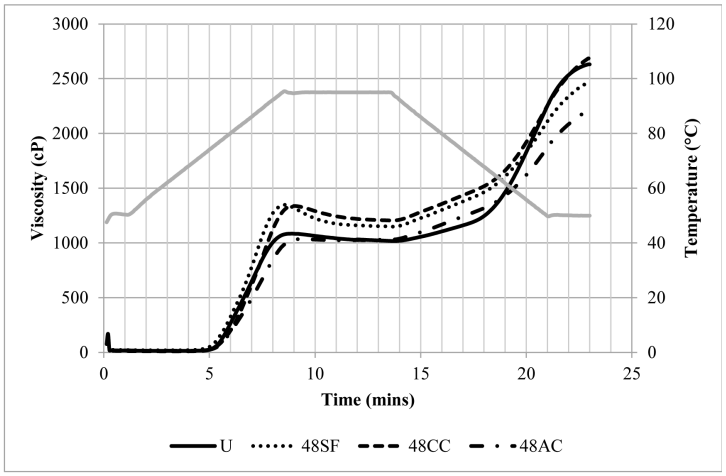
For *Pulut Harapan* corn, unfermented flour has an SV value of 927.1 ± 0.8 cP (**Table 1**). Our results showed that fermentation treatments increased tendency to retrograde, except for fermentation with addition of complete culture starter (SV value of 827.6 ± 0.2 cP). Corn flour obtained after 36 hour of spontaneous fermentation (36SF) has an SV value of 1194.8 ± 0.8 cP, and flour of 36AC has an SV value of 1097.9 ± 2.4 cP. This phenomenon is related with their respective FV; as explained in the previous paragraph.

3.2. Pasting Properties of Corn Flours after 48 Hours of Fermentation

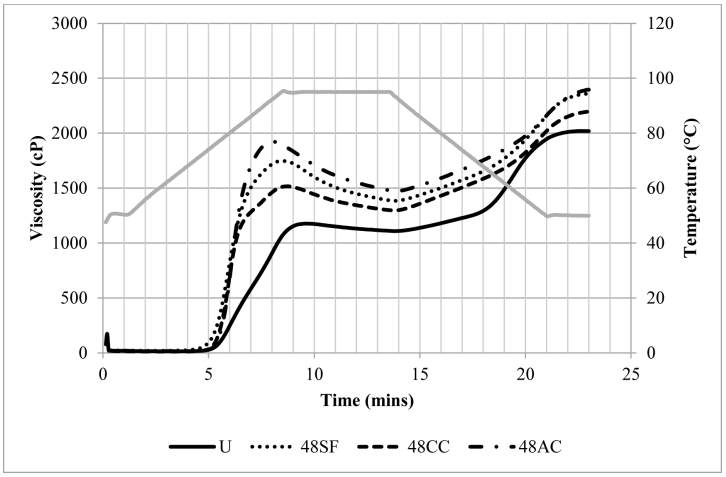
Continued fermentation process of corn grits up to 48 hours of fermentation, resulted in corn flour with pasting profile as presented in **Figure 3(a)** (for Anoman I) and 3B (for *Pulut Harapan*), with their respective pasting parameter presented at **Table 2**. In general, the PV value of corn flour of both *Anoman* 1 obtained from fer mented corn grits for 48 hours (48SF, 48 CC and 48AC; **Figure 3(a)**) is very similar to that of flour obtained from fermented corn grits for 36 hours (36SF, 36 CC and 36AC; **Figure 2(a)**). For corn of *Pulut Harapan*, the peak viscosity continued to increase to 1825.4 ± 26.4 (for 48SF) and 2134.5 ± 29.7 (for 48 AC) cP. Again, this phenomenon is associated with higher amylopectin content of *Pulut Harapan* corn flour because the flour is more resistant to enzymes and acid thus it was able to swell bigger. Interesting results were observed that all fermentation treatments has increased PV values of corn flour of *Pulut Harapan* variety; but it did not affect the tendency to retrograde; as indicated by the relatively similar SV value (927.1 ± 0.8 cP for unfermented flour, 989.1 ± 1.1 cP for SF, 935.1 ± 0.2 cP for CC and 924.4 ± 0.2 cP for AC flours, **Table 2**).

3.3. Pasting Properties of Corn Flours after 72 Hours of Fermentation

The pasting profile and properties of corn flour of both *Anoman* 1 and *Pulut Harapan* obtained after 72 h of fermentation of corn grits was presented at **Figure 4** and **Table 3**. Longer fermentation time (72 hour), the corn flour of *Anoman* 1 from fermented corn grits had higher value of PV; with the highest value (1487.1 ± 12.6 cP)



(a)



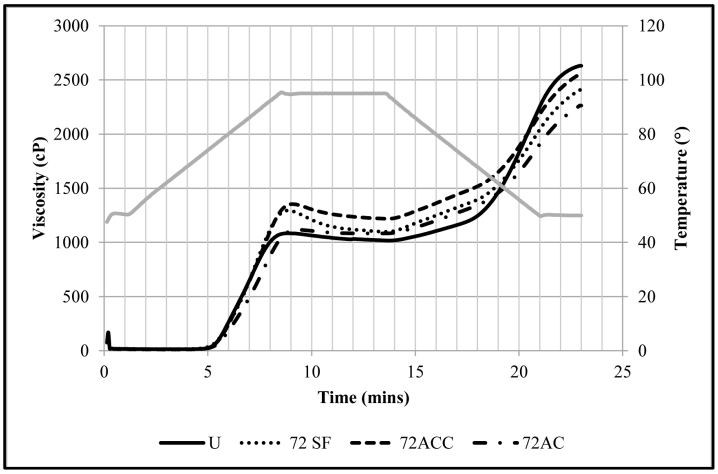
(b)

Figure 3. Pasting profile of corn flour of *Anoman* 1 (a) and *Pulut Harapan* (b) varieties made from corn grits after 48 hours of fermentation. U: Unfermented flours; 48SF: flour made from corn grits after 48 hours of spontaneous fermentation, 48CC: flour made from corn grits after 48 hours of fermentation with addition of a complete starter culture; 48AC: flour made from corn grits after 48 hours of fermentation with complete culture and additional amylolytic starter (AC) culture at 16 hours of fermentation.

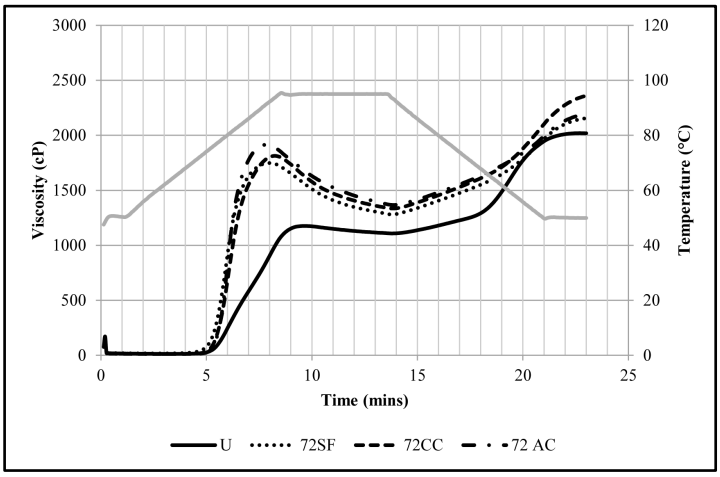
Table 2. Pasting properties profile of maize flour *Anoman* 1and *Pulut Harapan* varieties during 48 hours fermentation.

Treatments	Pasting temperature (˚C)	Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)
Anoman 1 flour						
U	81.5 ± 30.9^{a}	1276.9 ± 24.4^{a}	1012.6 ± 27.6^{a}	264.4 ± 36.4^{b}	2630.8 ± 0.1^{c}	1618.3 ± 0.4^{c}
48SF	80.8 ± 17.8^{a}	1432.6 ± 4.9^{b}	1121.7 ± 23.4^{b}	310.9 ± 17.1^{c}	2382.3 ± 0.0^{b}	1260.6 ± 1.3^{b}
48CC	83.8 ± 11.7^{a}	1489.0 ± 18.0^{b}	1175.2 ± 29.8^{b}	313.8 ± 30.9^{bc}	2645.9 ± 0.0^{bc}	1470.8 ± 0.3^{b}
48AC	81.8 ± 32.5^{a}	1189.8 ± 13.8^{a}	1021.3 ± 47.1^{b}	168.5 ± 36.8^{a}	2213.0 ± 0.0^{a}	1191.8 ± 0.4^{a}
Pulut Harapan flour						
U	82.1 ± 25.9^{a}	1237.7 ± 20.7^{a}	1090.3 ± 29.5^{a}	147.4 ± 15.8^{a}	2017.4 ± 0.7^{a}	927.1 ± 0.8^{a}
48SF	76.9 ± 24.0^{a}	1901.8 ± 29.6^{b}	1326.6 ± 48.3^{b}	575.2 ± 37.3^{b}	2315.7 ± 0.0^{bc}	989.1 ± 1.1^{a}
48CC	79.7 ± 17.2^{a}	1825.4 ± 26.4^{b}	1298.9 ± 38.4^{bc}	526.5 ± 21.9^{b}	2234.0 ± 0.1^{b}	935.1 ± 0.2^{a}
48AC	78.1 ± 49.7^{a}	2134.5 ± 29.7^{c}	1471.4 ± 62.2^{b}	663.1 ± 57.1^{b}	2395.8 ± 0.4^{c}	924.4 ± 0.2^{a}

Samples means with different superscripts in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.



(a)



(b)

Figure 4. Pasting profile of corn flour of *Anoman* 1 (a) and *Pulut Harapan* (b) varieties made from corn grits after 72 hours of fermentation. U: Unfermented flours; 72SF: flour made from corn grits after 72 hours of spontaneous fermentation, 72CC: flour made from corn grits after 72 hours of fermentation with addition of a complete starter culture; 72AC: flour made from corn grits after 72 hours of fermentation with complete culture and additional amylolytic starter (AC) culture at 16 hours of fermentation.

Table 3. Pasting properties profile of maize flour *Anoman* 1 and *Pulut Harapan* varieties during 72 hours fermentation.

Treatments	Pasting temperature (˚C)	Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)
Anoman 1 flour						
U	81.5 ± 30.9^{a}	1276.9 ± 24.4^{a}	1012.6 ± 27.6^{a}	264.4 ± 36.4^{b}	2630.8 ± 0.1^{c}	1618.3 ± 0.4^{c}
72SF	83.3 ± 14.0^{a}	1470.5 ± 6.9^{b}	1113.6 ± 26.9^{b}	356.9 ± 26.6^{c}	2459.5 ± 0.0^{b}	1345.9 ± 0.2^{b}
72CC	82.7 ± 10.3^{a}	1487.1 ± 12.6^{b}	1179.6 ± 12.5^{b}	307.4 ± 13.9^{bc}	2427.9 ± 0.1^{bc}	1248.3 ± 0.1^{b}
72AC	82.0 ± 23.3^{a}	1251.8 ± 44.0^{a}	1238.3 ± 43.6^{b}	176.6 ± 33.7^{a}	2263.3 ± 2.3^{a}	1188.1 ± 4.1^{a}
Pulut Harapan flour						
U	82.1 ± 25.9^{a}	1237.7 ± 20.7^{a}	1090.3 ± 29.5^{a}	147.4 ± 15.8^{a}	2017.4 ± 0.7^{a}	927.1 ± 0.8^{a}
72SF	77.4 ± 23.0^{a}	1701.0 ± 37.7^{b}	1214.9 ± 11.2^{b}	486.1 ± 26.6^{b}	2091.8 ± 0.1^{bc}	876.9 ± 0.5^{a}
72CC	78.7 ± 19.2^{a}	2038.0 ± 4.4^{b}	1333.2 ± 16.3^{bc}	704.8 ± 9.2^{b}	2357.3 ± 0.1^{b}	1024.1 ± 0.2^{a}
72AC	77.9 ± 47.8^{a}	2148.3 ± 40.3^{c}	1203.1 ± 40.9^{c}	782.0 ± 21.9^{b}	2190.4 ± 2.0^{c}	824.1 ± 4.7^{a}

Samples means with different superscripts in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.

observed in flour obtained from fermentation process with additional complete culture (72CC). Corn flour of *Pulut Harapan* obtained from fermentations of corn grits with additional complete culture (72CC) showed further increase in in PV (2038.0 ± 4.4 cP) much higher that that of corn flour obtained from 36 hours of fermentation (36 CC, 1825.4 ± 26.4 cP).

4. Conclusion

Fermentation process of corn grits affected the pasting properties of the resulting flour, both for Anoman I and *Pulut Harapan* corn varieties. The differences in the effect of fermentation process on pasting properties were due to the different amylose content of the corn varieties; as observed in Anoman I (high amylose content) and *Pulut Harapan* (low amlyose content) varieties. The addition of starter culture containing amylolytic microorganisms at 16 h of fermentation (AC) resulted in corn flour of Anoman I with lower values of PV, BV, FV, and SV. Meanwhile for corn flour of *Pulut Harapan* variety, CC fermentation resulted in flour with higher value of PV, BV, FV, and SV. In general, AC fermentation of corn grits could increase the stability of paste for flour containing higher amylose content but decrease the stability of paste for flour containing high amylopectin. Specifically, fermentation with complete starter culture (CC) caused significant increase in the peak viscosity value especially for corn flour of *Pulut Harapan*. Fermentation for up to 48 h had resulted in corn flour of *Pulut Harapan* variety having higher value of PV; but it did not affect the tendency to retrograde.

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List of Abbreviations

SF = Spontaneous Fermentation
CC = Complete Culture
AC = Amylolytic Culture
RVA = Rapid Visco Analyzer
PV = Peak Viscosity
TV = Trough Viscosity (also called as hot viscosity)
FV = Final Viscosity
BV = Breakdown Viscosity
SV = Setback Viscosity

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Validation of a Food Frequency Questionnaire for Vitamin D and Calcium Intake in Healthy Female College Students

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Abstract

***Objective*: The objective of this study was to examine the reproducibility and validity of a Food Frequency Questionnaire (FFQ) and assess calcium and vitamin D intake in health female college students. *Methods*: Thirty-five healthy female students were conveniently selected to participate in the study. None of the subjects were taking any supplements. The FFQ was validated against intakes from a three-day diet food record report (FR). *Results*: Positive correlations were observed of daily vitamin D ($r = 0.82$, $p < 0.001$) and calcium intake ($r = 0.74$, $p < 0.001$) derived from FFQ compared with the FR. The mean intake of vitamin D and calcium derived from FFQ and FR were (233 ± 90, 654 ± 391) and (231 ± 79, 611 ± 352), respectively. The FFQ overestimated vitamin D and Calcium by 2 IU /day (95% CI: −8, 9, $p < 0.676$ and 43 mg/d (95% CI: 20, 65, $p < 0.01$). *Conclusions*: The FFQ used in this study shows promising validation evidence to be used in the future for assessing vitamin D and calcium intakes in female students.**

Keywords

Vitamin D, Calcium, Food Frequency Questionnaire, Osteoporosis, Diabetes Type 2, Female Students

1. Introduction

Vitamin D deficiency and related diseases such as osteoporosis and type 2 Diabetes are common problems in countries where the sun is limited [1] but is also seen in Arabic countries [2] [3] where cultural or religious reasons play important role even though they may enjoy abundant of sunshine all twelve months of a year. Dietary

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intake of calcium and vitamin D may play significant role in the development of diabetes type 2 and osteoporosis and early assessment and prevention of a possible deficiency of these nutrients may seem beneficial later on their life [4] [5].

Nutrient intakes can be estimated by using standard methods such as food frequency questionnaire (FFQ) and food records (FR). FFQ are administrate in a clinical setting by a registered dietitian and it is usually used to asses relation of dietary intake to a development of a disease [6], while FR assess dietary intake of a short time of period usually three days which will include two weekdays and one weekend day [7].

The FFQ should be validated under a specific population taking in consideration country, age, list of food items that all will capture the study population's eating behaviors and customs [7] [8]. The FFQ has been also validated based on socioeconomic class, adolescents and also in college students [8]-[10]. A pilot validation study of FFQ has been also reported by Pritchard and his collogues [11] in order to assess calcium, vitamin D and vitamin K in obese subjects.

Until now, there are no studies in Arabic countries that they have validated a FFQ for the assessment of vitamin D and calcium intake in adolescents. The purpose of this study was to examine the reproducibility and validity of a FFQ and to assess calcium and vitamin D intake in healthy female adolescents from a university of Abu Dhabi.

2. Methodology

Forty-five female students were conveniently recruited from January 2014 to March 2014. Ten of them were taking medication or supplements and excluded from the study. The rest 35 subjects (self reported) were free of any known diseases such as diabetes 1 and 2, hyperlipidemia, renal and liver problems, etc. The subjects completed a FFQ and a FR by a registered dietitian two different periods that were one month apart. The study was approved by the ethical committee of Zayed University of Abu Dhabi and a written consent form was signed by all subjects.

Subjects were self-reported on age, weight and height. Body mass index was calculated based on the following formula, BMI: weight (kg)/height $(m)^2$.

Vitamin D and Calcium was assessed using a FFQ and a FR (3 days-two weekdays and one weekend). The FFQ used was a modified version of a previous validated questionnaire [9], adapted to Arabic eating and culture habits by including specific foods eaten in these areas. The FFQ and FD administered twice by the same person with one-month interval period. An average daily intake of vitamin D and calcium was calculated for the FFQ, while in the FQ the subjects were asked to describe portions sizes and preparation methods for the similar food items were in FFQ.

3. Statistics

Statistical analysis was performed using IBM SPSS for Mac (version 21) package. Descriptive statistics measures and box-plots are provided. Testing of the Normality assumption for the two continuous variables was performed by using the Kolmogorov-Smirnov non-parametric test. The comparison of continuous variables' means of the Ca and vitamin D for the two questionnaires was performed by using a paired samples t-test. For all tests, a significance level of 5% was used.

4. Results

The mean age of the subjects were 20 - 22 years old with a mean weight (kg) of 65 ± 5.8 and a mean height (cm) of 162 ± 6.3.

Table 1 represents the mean values ± SD of calcium and vitamin D intake using the FFQ and FR over one month period apart. Calcium Intake was found statistically significantly lower ($p < 00.5$) in FR compared with FFQ in the base period. Correlation between FFQ and FR is also indicated. Strong and moderate positive correlations were observed of daily vitamin D ($r = 0.82$, $p < 0.001$) and calcium intake ($r = 0.74$, $p < 0.001$), respectively, derived from FFQ compared with the FR. Similar results were found also after the one-month period.

Figure 1 and **Figure 2** plots the differences in both time periods between the FFQ and FR for calcium and vitamin D, respectively.

The mean differences between FFQ and FR for calcium and vitamin D in baseline and after one month are presented in **Table 2**. The FFQ overestimated calcium by 43 mg/d (95% CI: 20, 65, $p < 0.01$).

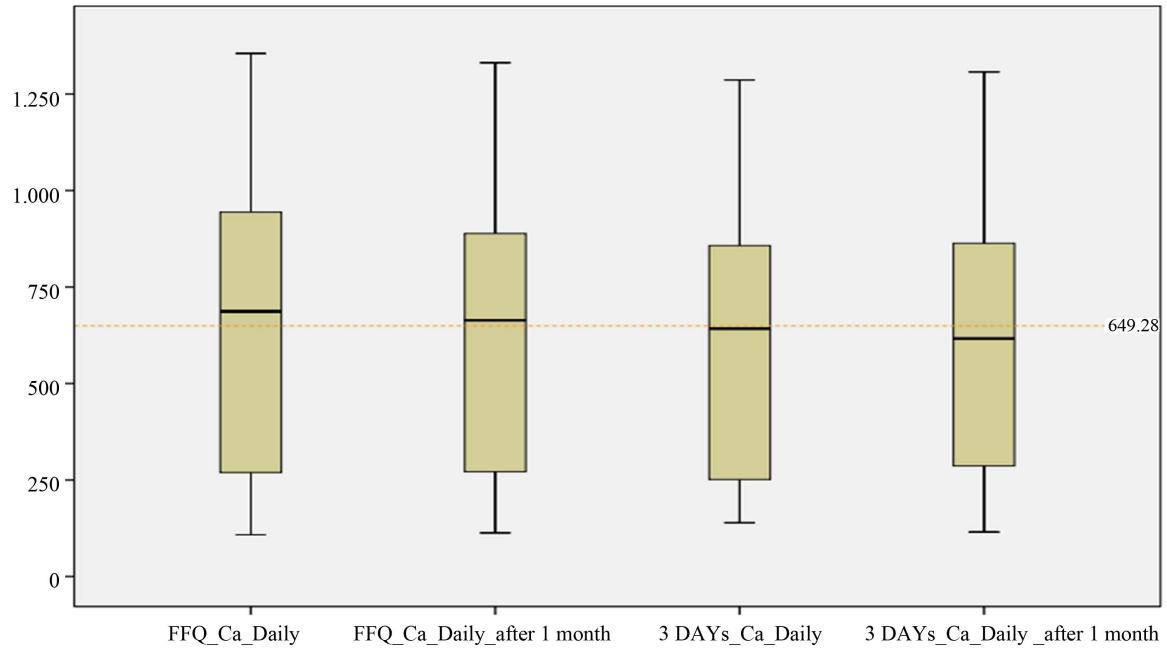

Figure 1. Box plots showing the differences of calcium intake between FFQ and FR (3 days) in baseline and after one month. Abbreviations: FFQ: food frequency questionnaire; 3 day: food record; Ca: calcium.

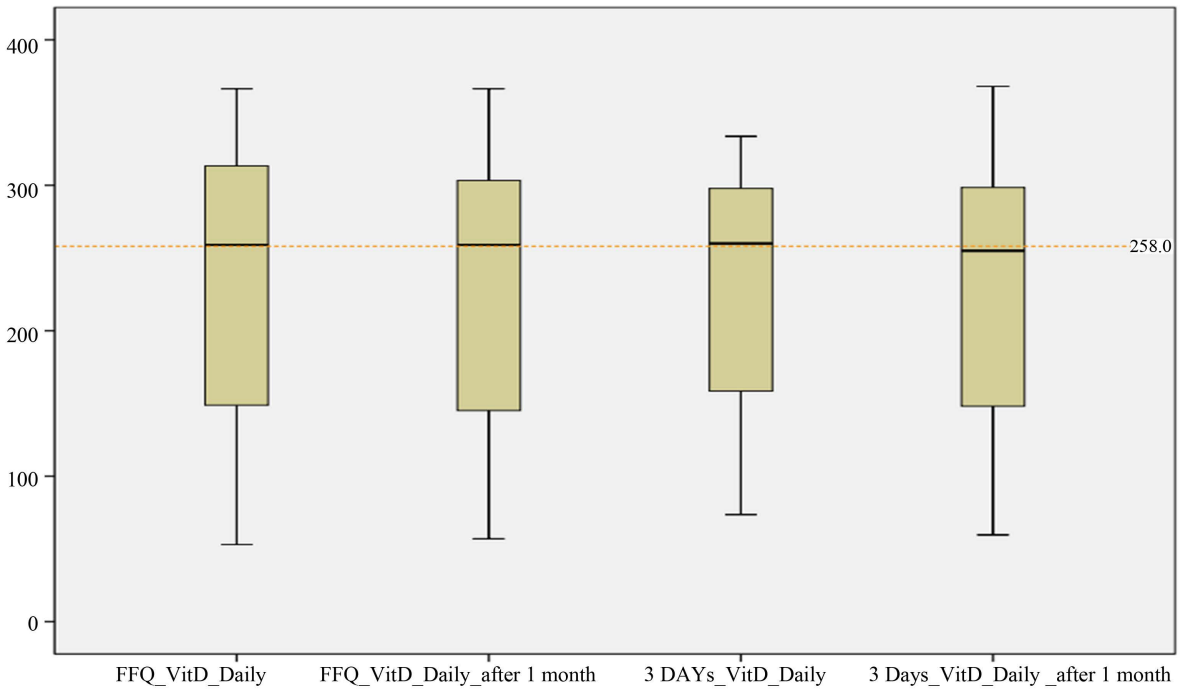

Figure 2. Box plots showing the differences of vitamin D intake between FFQ and FR (3 days) in baseline and after one month. Abbreviations: FFQ: food frequency questionnaire; 3 day: food record; Ca: calcium.

5. Discussion

It is well known that in order to assess intakes of key nutrients such as calcium and vitamin D for bone health as well for diabetes type 2, a valid tool is required [12].

The Calcium intake derived from the FFQ was significantly higher compared with the FR. The difference

Table 1. Dietary intakes of calcium and vitamin D.

	Mean ± SD[1]	r
FFQ_Ca_Daily	654 ± 391*	0.74**
FFQ_VitD_Daily	233 ± 90	0.82**
FFQ_Ca_Daily_after 1 month	637 ± 373	0.73**
FFQ_VitD_Daily_after 1 month	229 ± 90	0.81**
FR_Ca_Daily	611 ± 352*	0.74**
FR_VitD_Daily	231 ± 79	0.82**
FR_Ca_Daily_after 1 month	624 ± 356	0.73**
FR_VitD_Daily_after 1 month	226 ± 88	0.81**

*Statistically significantly difference $p < 0.05$, **$p < 0.001$. [1]t-test, [2]Pearson correlation test, Abbreviations: FFQ: food frequency questionnaire; FR: food record; Ca: calcium intake; vitD: vitamin D intake.

Table 2. Mean differences of Calcium and vitamin D for FFQ and FR in baseline and after one month.

	Mean ± SD	95% CI	p
FFQ_Ca_Daily vs FR_Ca_Daily	43 ± 62	20, 65	**0.01***
FFQ_Ca Daily after 1 month vs FR_Ca daily after 1 month	13 ± 108	−24, 50	0.493
FFQ_VitD_Daily vs FR_VitD_Daily	2 ± 26	−8, 9	0.834
FFQ_VitD_Daily_after 1 month vs FR_VitD daily after 1 month	3 ± 6	1, 5	0.512
FFQ_Ca_Daily vs FFQ_Ca_after 1 month	17 ± 35	5, 28	0.239
FFQ_VitD_Daily vs FFQ_vit_D_after 1 month	4 ± 17	−2, 9	0.235
FR_Ca_Daily_ vs FR_ Ca_ Daily after 1 month	−13 ± 120	−55, 28	0.509
FR_VitD_Daily_ vs FR_vitD_after 1 month	5 ± 30	−5, 15	0.307

*Statistically significantly difference, $p < 0.05$. Abbreviations: CI: confidence intervals; FFQ: food frequency questionnaire; FR: 3 days food record; Ca: calcium.

however is so small that clinically would have no significant effect to the patients. The results could be possibly be affected by the small number of subjects. However the reproducibility of the two methods was similar after the result of one month indicating that both methods can be accurately measure calcium intake. Similar results have been found in a recent study [13].

Other dietary assessment tools have been reported in the literature with their own weaknesses and strengths. FR has been found to overestimated vitamin D intake [14] while the 24-hour recall is more often preferred because it is less demanding on a study subject; however it relies basically on the memory of the person [6]. In our study we demonstrated that the vitamin D intake reported with negligible differences in both FFQ and FR methods. In addition to that the validity of the method was also high after the results of one month. This is very important since it is the first time we validate a FFQ and a FR in a specific population in an Arabic city. Similar data for other population have been identified in the literature such as in Italy [15], Brazil [16] and Malaysia [17].

Our study indicated that 67% of participants were classified into the same quartile of intake of calcium and vitamin D (data not shown). These finding are in accordance with other studies [11].

Our study has its strength that is the inclusion of local foods of Abu Dhabi and also that nature of data that was interviewer administrated.

However, the study also has its limitations. First, it is the small sample sizes, which limit the conclusions, and second the general results to the female adult population. Nevertheless, this the first time to publish data of a validation tool in an Arabica city and it can be used as a pilot study for the future since vitamin D deficiency and

type 2 diabetes are very common in United Arab of Emirates.

6. Conclusion

The FFQ used in this study shows promising validation evidence to be used in the future for assessing vitamin D and calcium intakes in female students. Additional studies with larger samples are needed to validate this FFQ so it can be used as a dietary screening tool for prevention of osteoporosis and diabetes type 2 later on.

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Polyphenol Composition, Antioxidant, Antimicrobial and Quorum Quenching Activity of the "Carciofo di Montoro" (*Cynara cardunculus* var. *scolymus*) Global Artichoke of the Campania Region, Southern Italy

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Abstract

Biochemical characteristics, antimicrobial and quorum quenching activity of the extract of the "Carciofo di Montoro", a typical ecotype of *Cynara cardunculus* var. *scolymus* of the Campania region (Southern Italy) were studied, to consider it as potential reserve of bioactive constituents useful for food industry and beneficial for managing and preventing several chronic illnesses in humans. The extract exhibited a good polyphenol content (528 μg GAE/g) and antioxidant activity (EC_{50} less than 5 mg). Ultra pressure liquid chromatography (UPLC) revealed high amount of chlorogenic acid, cynarin and epicatechin. The extract showed antimicrobial activity against *Escherichia coli*, *Staphylocccus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Bacillus cereus* pathogen strains. Finally, quorum quenching activity was demonstrated. The variety Carciofo di Montoro could represent a good source of health-promoting polyphenols, encouraging a nutraceutical use of such ecotype, for several phyto-pharmaceutical applications.

Keywords

Artichoke, Polyphenols, Antioxidant, Antimicrobial, Quorum Sensing

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1. Introduction

Polyphenols and antioxidants, present in plant-based foods, offer several health benefits further than basic nutrition and are positively implicated in the prevention of chronic diseases. Many studies discovered several interesting biological properties, such as anti-inflammatory, antioxidant, antimutagenic, antiviral, antimicrobial and quorum quenching activities [1]-[3], and many of them are also used in the preservation of food [4]. Globe artichoke (*Cynara cardunculus* L. var. *scolymus* (L.) Fiori), belonging to the family of *Asteraceae* (*Compositae*), is an herbaceous perennial crop, widely cultivated in the Mediterranean area. Heads, represented by the large immature inflorescences with edible fleshy leaves (bracts) and receptacle, are used worldwide and represent a basic element of the Mediterranean diet. Leaves are used as herbal medicine and are appreciated for their beneficial and therapeutic effects, including promotion of blood circulation, mobilization of energy reserves, induction of choleresis, inhibition of cholesterol biosynthesis, hepatoprotective effects and LDL oxidation, as well as antibacterial, antifungal and antioxidant activities [5] [6]. Many studies converged on the artichoke health and antioxidant properties, assuming that these actions could be strictly related to the polyphenolic fraction, mainly composed of mono- and dicaffeoylquinic acids, and flavonoids. Such properties are consistent with the well-known double role of phenolic compounds as antioxidants and as substrates for oxidative browning reactions, primarily in the presence of high iron concentrations [7]. Chemical activity of polyphenols in terms of their reducing properties, as hydrogen or electron-donating agents, predicts their potential effect as free-radical scavengers. In Italy, artichoke is considered the most important horticultural crop together with tomato and potato, with a surface of about 33,296 ha and a production of about 372,378 tons in the year 2013, mainly giving rise from Apulia, Sicily, Campania, Lazio and Tuscany [8]. Italy is also the richest source of artichoke genetic resource, with numerous local varieties; these last, in the course of the centuries, were capable to adapt themselves to the different environments, and which now can differ in chemical composition, especially of the polyphenolic fraction, hence revealing diverse nutraceutical and pharmacological properties. Many ecotypes of artichoke present in the Campania region of Southern Italy reveal excellent results in terms of polyphenols content and antioxidant activity [9]. The ecotype "Carciofo of Montoro", cultivated mainly in the province of Avellino, is a product with exceptional organoleptic characteristics. The techniques of cultivation for such species include the transplanting of the plant, recurrent irrigations and a very low use of synthetic chemicals. A particular aspect of the cultivation of this ecotype is the practice of covering its little heads with a terracotta cup to defend them from the damaging chill (**Figure 1**). The traditional Campania vegetal panorama is well recognized as

Figure 1. A typical "Carciofo di Montoro" artichoke.

environment characterized by a rich genetic biodiversity. The protection of such vegetables, as well as the study of their biochemical and nutritional aspects is an essential instrument also to protect the local economy. Such aspects are of noticeable significance, taking into account that the actual trend of the global food market is devoted to its standardization, with a concurrent drastic decrease in the number of traditional species and varieties (often with high functional properties) and a dramatic decline of the genetic variability. Vegetables belonging to the family *Asteraceae* represent an important font of natural antioxidants with high capability to manage against oxidative stress and, thus, with high potential to act as strong anticancer as well as anti-degenerative foods. Furthermore, they have been recently also recognized as natural antimicrobial compounds capable to reduce outbreaks of food-borne and human pathogenic microorganisms [10] [11]. The well-known capability of these phytochemicals to contribute for the maintenance of health and for the protection against heart disease and cancer [12], as well as to act against microbial attacks, is also raising interest among scientists and food manufacturers to identify foods, also among autochthonous ecotypes, with specific health effects for consumers. Therefore, the aim of our work was to study the polyphenols composition, the antioxidant activity and antimicrobial potential of the extract from the "Carciofo di Montoro" globe artichoke, to evaluate the possibility to consider this ecotype as reserve of bioactive constituents and as a resource, due to its antimicrobial and quorum quenching properties, for food preservation and human health.

2. Materials and Methods

2.1. Standards and Reagents

Luteolin was provided from Extrasynthese (Genay, France). All other standards were obtained from Sigma (Milan, Italy). Acetoni-trile and trifluoroacetic acid were obtained from Carlo Erba Reagenti (Milan, Italy). Acetone, methanol, ethanol and ethyl acetate were purchased from Sigma (Milan, Italy). All reagents were of analytical grade.

2.2. Extraction of Polyphenols

Samples of artichoke "Carciofo di Montoro" were obtained from the experimental fields of CRA, located in Montoro (AV), Italy. Polyphenols were extracted following the method of Fratianni *et al.* [9] with some modifications. Briefly, bracts were subjected to a two-time extractive process, the first one to allow the extraction mainly of phenolic acids, the other to permit the extraction of flavonoids. Samples of frozen bracts were incubated for 1 h at 4˚C in 3 volumes of acetone:ethanol:meth-anol (70:15:15). After the recovery of the first supernatant, the residues of bracts were treated with ethyl acetate (1:3 w/vol) and kept at 4˚C for 1 hour. The two supernatants were separately dried under air flow, re-dissolved in ethanol, pooled and stored at −30˚C in the dark until analysis was performed.

2.3. Colorimetric Analysis of Polyphenols

Total polyphenols were determined following the method of Singleton and Rossi [13] using the Folin-Ciocalteu reagent. The absorbance was determined at room temperature at λ = 760 nm using a Cary Uv/Vis spectrophotometer (Varian, Palo Alto, CA, USA). Quantification was based on a standard curve generated with gallic acid. Results were expressed as micrograms gallic acid equivalent (GAE)/g of fresh weight product ± Standard Deviation (SD).

2.4. Free Radical Scavenging Capacity

The free radical scavenging capability of the extract was determined using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [14]. The analysis was performed in microplates by adding 7.5 µL of extract to 303 µL of a methanol DPPH solution (153 mM). Next, the absorbance at λ = 517 nm was measured (Cary 50 MPR, Varian, Palo Alto, USA). Absorbance of DPPH without antioxidant (control sample) was used for baseline measurement. Scavenging activity was expressed as the 50% effective concentration (EC_{50}), which is defined as the sample concentration (mg) necessary to inhibit the 1 mL DPPH radical activity by 50% during a 60 min incubation. Ascorbic acid (Fluka Buchs, Switzerland) was dissolved in methanol. The solution was used for a calibration curve of DPPH reduction and as a chemical reference in comparison to the antioxidant capacity of the extracts. These experiments were performed in triplicate, and the results are expressed as the mean values ±

standard.

2.5. Chromatographic Analysis

All standards utilized in the experiments were accurately weighed, dissolved in methanol, treated with ultrasonics for 10 min and filtered (0.45 μm, Waters, Milford, MA, USA). The calibration curves were generated with concentrations ranging from 0.001 to 0.5 mM of chlorogenic acid (5-O-caffeoyl-D-quinic acid), p-coumaric acid (p-hydroxycinnamic acid), ferulic acid, gallic acid, epicatechin, apigenin, luteolin, cynarin (1-3-dicaffeolylquinic acid), and rutin. The extracts of the globe artichoke Carciofo di Montoro (previously dissolved in methanol) were filtered (0.45 μm, Waters, Milford, MA, USA) before analysis. The analysis of polyphenols was performed by using an ACQUITY^{TM} ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) linked to a PDA 2996 Photodiode Array Detector (Waters). Empower software (Waters) was used to control the instruments and for data acquisition and processing. Analysis was carried out at 30˚C using a reversed phase column (BEH C18, 1.7 μm, 2.1 × 100 mm, Waters) [15]. Mobile phase consisted of solvent A (7.5 mM acetic acid) and solvent B (acetonitrile) at a flow rate of 250 $\mu L \cdot min^{-1}$. Gradient elution was employed, starting with 5% B for 0.8 min, then 5% - 20% B over 5.2 min, isocratic 20% B for 0.5 min, 20% - 30% B for 1 min, isocratic 30% B for 0.2 min, 30% - 50% B over 2.3 min, 50% - 100% B over 1 min, isocratic 100% B for 1 min, and finally 100% - 5% B over 0.5 min. At the end of this sequence, the column was equilibrated under the initial conditions for 2.5 min. Pressure ranged from 6000 to 8000 psi during the chromatographic run. The effluent was introduced into an LC detector (scanning range: 210 - 400 nm, resolution: 1.2 nm). The injection volume was 5 μL.

2.6. Antimicrobial Assays

To screen the antimicrobial activity, a filter paper disc method was used [16]. The bacteria used in this study included Gram-positive *Bacillus cereus* (strains DSM 4313 and DSM 4384), *Enterococcus faecalis*, and *Staphylococcus aureus* DSM 25923 and Gram-negative *Escherichia coli* DSM 8579 and *Pseudomonas aeruginosa* ATCC 50071 strains. All strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The strains were incubated in a Nutrient broth (Oxoid) at 37˚C for 18 h. The optical densities of all cultures were adjusted to match a 0.5 McFarland standard of 1×10^8 colony forming units (cfu)/mL; then 2.625 mg - 5.25 mg and 10.5 mg of the extracts were added to sterile filter paper discs (5 mm) previously placed in Nutrient agar Petri dishes inoculated with the above mentioned pathogen strains. A disc treated with Dimethyl sulfoxide (DMSO, Sigma) alone was used as negative control; tetracycline (7 μg/disc; Sigma) served as positive control. Plates were left for 30 min at room temperature under sterile conditions and then incubated at 37˚C for 24 h, and the inhibition halo around the disc was measured. The experiments were performed in triplicate and averaged. All of the experiments were carried out in triplicate. The results are expressed as means ± standard deviation. Means followed by different letters in each column differ significantly to Dunnett's multiple comparisons test, at the significance level of $p < 0.05$.

2.7. Quorum Sensing Activity

The *Chromobacterium violaceum* quorum sensing system was used for this assay. Quorum sensing (QS) in this wild-type strain of bacterium controls the production of the purple pigment violacein [3] in response to autoinducer molecules, such as C6-acyl and C4-acyl homoserine lactones. The disc diffusion method was employed to detect the anti-QS activity of the extract. In this test, bacterial growth inhibition would result in a clear halo around the disc, whereas a positive result of quorum sensing inhibition (quorum quenching activity) would result in a turbid halo harbouring the pigmentless bacterial cells of *C. violaceum* DSM 30191 (purchased from DSMZ). The test strain was incubated in Lab Lemco broth (Oxoid, Milano, Italy) for 16 - 18 h at 26˚C. The culture was adjusted to the 0.5 McFarland standard (1×10^8 CFU /mL). Different doses of the extract, prepared as described above, were added to *C. violaceum* inoculated Lab Lemco agar plates (0.1 mL/plate), followed by incubation at 26˚C for 24 - 48 h.

2.8. Statistical Analysis

All of the experiments were carried out in triplicate. The results are expressed as means ± standard deviation.

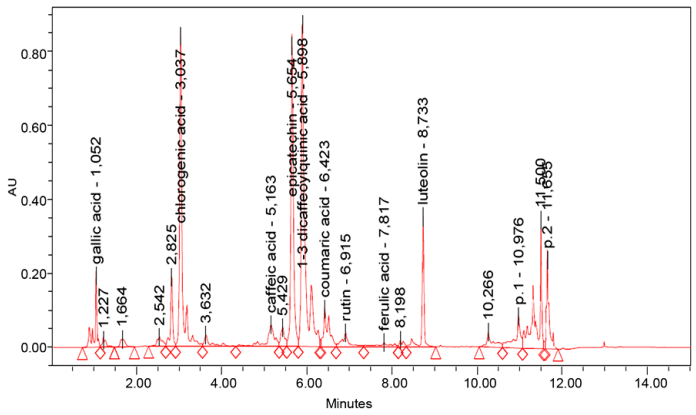
3. Results and Discussion

3.1. Total Polyphenols and Antioxidant Activity

Total polyphenols and the antioxidant activity of the globe artichoke variety Montoro are shown in **Table 1**. The amount of polyphenols was 525 GAEμg/g of product. This result are in agreement with the range observed for other cultivars, such as Green Globe or Violetto di Toscana [7], and higher than other typical Italian artichokes, such as Violetto di Sicilia, Violetto di Provenza, and Tema [17] indicating that "Carciofo di Montoro" could represent another important source of phytochemicals, capable to exhibit therapeutic activity [18]. The content of polyphenols present in such ecotype affected, with all probability, also its antioxidant activity: in fact, the amount of extract needed to inhibit 1 ml of DPPH at 50% was just 4.24 mg, more effective than other artichokes studied, for example, by Velez *et al.* (EC_{50} = 9.16 mg) [19] or Menghini (EC_{50} of about 15.95 mg) [20], Ferracane *et al.* [21] observed that the cooking process of artichokes in boiling water increased their antioxidant content and activity with respect to raw artichokes, independently of the assay used, stating that the spatial arrangement of the phenolic groups can deeply affect the antioxidant activity of the molecules. Thus, we could hypothesize that the antioxidant activity of the Carciofo di Montoro might increase as cooking process is improving their extraction out of the plant tissue. Thus, a wider utilization of this typical variety Montoro should be strongly encouraged both for the fresh market and for the food industry, in view of its capability to supply a high level of these important biomolecules.

3.2. Chromatographic Analysis

Chromatographic analysis, herein performed for the first time on this typical Italian globe artichoke through UPLC, is shown in **Figure 2**. Panel A of the **Figure 2** reports the amount (as μg GAE/g) of the polyphenols found in the extract of the Carciofo di Montoro globe artichoke. We identified different polyphenols, basically gallic, chlorogenic, caffeic, p-coumaric, ferulic acid, 1 - 3 dicaffeoylquinic acid (cynarin), as well as epicatechin, rutin and luteolin. These results are consistent with those obtained on other *Cynara cardunculus* var. *scolymus* [7] [8]. Epicatechin, cynarin and chlorogenic acid were very abundant in the Carciofo di Montoro globe artichoke, representing the 33.1%, 32.7% and 26%, respectively. The presence of epicatechin as the most abundant polyphenol (173.79 μg GAE/g) in this ecotype is noteworthy. Epicatechin can be easily absorbed [22] and reach



Polyphenols (μgGAE/g)	
gallic acid	2,80
chlorogenic acid	136,75
caffeic acid	4,18
epicatechin	173,79
1-3 dicaffeoylquinic acid	171,58
p-coumaric acid	3,40
rutin	30,48
ferulic acid	0,50
luteolin	2,19

Panel A

Figure 2. UPLC profile of polyphenols present in Carciofo di Montoro artichoke. On the right (Panel A) is shown (as μg GAE/g of sample ± SD) the amount of each polyphenol known detecte.

Table 1. Phenolic content and antioxidant activity of the artichoke Carciofo di Montoro. Concentration of phenolic compounds are expressed as μg of gallic acid equivalents (GAE)/g sample (dry matter basis). The scavenging activity was expressed as the 50% effective concentration (EC_{50}), which was defined as the sample concentration (mg) necessary to inhibit activity of the radical DPPH by 50% after a 60-min incubation.

Polyphenol content (gGAE/g of product) ± SD	Antioxidant activity (mg EC_{50})
525.49 ± 0.11	4.24 ± 0.87

different tissues, plasma, and the gut, where it not only inhibits the growth of some pathogens, as observed by an in vitro study by Parkar *et al.* [23], but also can act as potent metal chelator and free radical scavenger, thereby significantly influencing the function of various mammalian cellular systems [24]. Flavanols like epicatechin represent an important part of the human diet and have different biological activities including antioxidant and anti-inflammatory properties. Like the other polyphenols, flavanols concur to the beneficial effects of a diet rich in fruits and vegetables and play a beneficial impact against a wide range of diseases from cardiovascular pathologies to cancer and degenerative conditions. The intake of epicatechin has been demonstrated inversely associated with coronary heart disease [25]. Carciofo di Montoro exhibited high amount of cynarin, suggesting that such ecotype could be of particular relevance from a health point of view. Cynarin, isolated from artichoke and characterized for the first time by Panizzi and Scarpati [26], has a strong effect in stimulating bile secretion and cholesterol metabolism, as well as in protecting liver [27], although its effect can be certainly reinforced (as in all fruit and vegetables provided by nature) by the concurrent presence of other active components present in the artichoke extract. Preziosi *et al.* [28] observed that a dose of 15 - 30 mg/kg of body weight of cynarin could increase the secretion of bile similarly to equimolar doses of Na-dehydrocholate (>130%), and stimulate the elimination of the biliary cholesterol; in addition, the diuretic activity could be enhanced by increasing the doses of cynarin until 100 mg/kg. Artichoke is a rich source of polyphenolic compounds, with mono- and dicaffeoylquinic acids as the major chemical components [7]. It accumulates various hydroxycinnamic acids, for which formation different mechanisms are described [29], allowing also to the production of chlorogenic acid. These biomolecules may act as an acyl donor molecule for caffeoyltransferase [30] and constitute the sckeleton of other biomolecules. The content of chlorogenic acid in the artichoke Carciofo di Montoro is also in accordance with the range of such biomolecules (0.013 mg - 1.3 mg GAE/g) observed in the unique other study performed on artichoke through UPLC [31], even if this study was performed in different chromatografic conditions. *In vivo* studies demonstrated the antioxidant and anticarcinogenic properties of chlorogenic acid [32]: it is usually poorly absorbed in the small intestine; however, the absorbed fraction of such bio-molecules enters into the blood system and has posi-tive effects on the cardiovascular system; the not absorbed fraction arrives to the colon and has biological effect on microbiome, which provides high levels of important microbial metabolites, active compounds responsible for the biological properties attributed to dietary polyphenols, such as an indirect action of cancer colon prevention [33] [34]. Therefore, both chlorogenic acid and cynarin have important effects, for instance acting in beneficial manner on the cardiovascular system and on the colon, and diminishing the risk of type II diabetes [35]. The presence of rutin, (30.48 µg GAE/g corresponding to 6% of total polyphenols, Panel A **Figure 1**) is of particular importance. Rutin is a well known flavonoid which can be metabolized to quercetin, another powerful antioxidant. The team rutin/quercetin can decrease allergic and inflammatory reactions in different parts of the body, affecting cytokine pattern, decreasing the level of Th2 cytokine, and down regulating neutrophilic inflammation; it is also capable to affect the IgE mediated mast-cell activation [36]. Rutin/quercetin may inhibit the oxidation of high-density lipo-protein (HDL) and cholesterol [37] [38], with an indirect effect in the prevention of the risk for arteriosclerosis. Rutin has also dilating effect on insulated rat aorta, and may affect the nitric oxide (NO) synthesis [39]. Flavonoids can be absorbed in the gastrointestinal tract and excreted as whole form or like metabolites. At intestinal level, microbiome splits the heterocyclic ring of flavonoids which can be subsequently be decomposed to phenolic acids; these, on the other hand, can be absorbed, conjugated, excreted or further metabolized by bacteria, and supply the body with other defense mechanisms against inflammation, allergic reactions or in the prevention of cancer. From a quantitative viewpoint, luteolin confirmed to be a minor constituent of the total polyphenols present in the extract of Carciofo di Montoro artichoke (2.19 µg GAE/g). Nevertheless, the presence of luteolin is very important, taking into account that such flavonoid has a strong antioxidant activity and, in this role, it has the capability to protect low density lipoproteins against oxidation. Screening of individual phenolic constituents of artichoke extracts revealed that luteolin is mainly responsible for the inhibition of cholesterol biosynthesis, and high luteolin concentrations are capable to efficiently block the insulin effect on cholesterol biosynthesis, as well as to improve aortic relaxation [40].

3.3. Antimicrobial Activity

The inhibition halo test, as shown in **Table 2**, indicated that the extract was capable to exhibit antimicrobial activities against all tested microorganisms. Generally, the degree of the extract activity is revealed by the size of inhibition zone that is expressed by the diameter of the referred inhibition zone. Due to the simple nature of thisassay and the reduced amount of extract required, use of such technique is generally recommended. The test,

Table 2. Antimicrobial activity exhibited by the extract of Carciofo di Montoro globe artichoke against different Gram + and Gram-pathogen strains used as tester. Tetracycline (7 µg) and DMSO were used as positive and negative control, respectively. Results are shown as mean (in mm) (±standard deviation) (n = 3). Means followed by different letters in each column differ significantly to Dunnett's multiple comparisons test, at the significance level of $p < 0.05$. *PA = Pseudomonas aeruginosa*; *EC = Escherichia coli*; *BC= Bacillus cereus*; *EF = Enterococcus faecalis*; *SA = Staphylococcus aureus.*

	Gram (+) tester strains		Gram (−) tester strains			
	PA	EC	BC DSM 4313	BC DSM 4384	EF	SA
2.625 mg	10 (0^{a})	13.3 (2.9^{a})	10.4 (0.6^{a})	10 (0^{a})	0	13 (1.7^{a})
5.25 mg	10.7 (1.1^{a})	15.7 (2.9^{a})	11.3 (2.1^{a})	16.7 (2.9^{e})	2.2 (0^{e})	14.3 (2.0^{b})
10.5 mg	12.1 (2.9^{a})	16.8 (2.9^{a})	15.3 (2.8^{b})	16.5 (1.1^{e})	3.7 (1.4^{e})	15.5 (2.9^{c})
Tetracycline	9.8 (1.6^{a})	12.6 (1.1^{a})	9.6 (1.1^{a})	8.4 (1.2^{a})	9.7 (1.3^{a})	11.5 (0.5^{a})
DMSO	0	0	0	0	0	0

although less suitable for more precise quantification purposes, such as the determination of the MIC values, is also used to determine the susceptibility of a range of microbial species to a particular compound or mixture [41]. On the whole, the most resistant strain was *E. faecalis*, (3.7 mm of zone of inhibition using 10.5 mg of extract). The other strains showed higher susceptibility against the artichoke extract, with zones of inhibition ranging from 10 mm (with 2.625 mg GAE/g of polyphenols, against *P. aeruginosa*, and *B. cereus*) to 16.8 mm (observed using 10.5 mg against the Gram negative *E. coli*); interestingly, *E. coli* was the most sensitive strain, and a zone of inhibition of 13.3 mm was observed using just 2.625 mg GAE/g of polyphenols. Such results are consistent to Zhu and others [10] [11] but are in contrast with Ionescu and others [42], which observed a resistance of *S. aureus* against the antimicrobial activity of artichoke extracts, confirming the capability of the extracts of *Cynara cardunculus* var. *scolymus* to act as antimicrobial agents, but in different manner, probably due to a different method of extraction as well as to the different ecotype and strains used in the experiments. In our study, we used two different strains (DSM 4313 and DSM 4384) of the *B. cereus*, which exhibited a different sensitivity/resistance against the extract, mainly when they were exposed to 5.25 mg GAE/g of polyphenols, confirming that natural extract may act in different way also within the same species [16]. The antimicrobial activity exhibited by the extract against both Gram positive and negative bacteria may indicate the presence of a broad spectrum of compounds with antibiotic activity [43]. Usually, natural extracts have antimicrobial activity which supplies a natural barrier against the invasion of microorganisms and, when possible, block the systems of communication among pathogens. The antimicrobial activity exhibited by this extract might be ascribable to the presence of high amounts of chlorogenic acid, cynarin and epicatechin, which, with all probability, affected in synergistic way its antimicrobial activity.

3.4. Quorum Sensing Activity

Phenolic compounds bond strongly to bacterial cell walls and are generally classified as surface-active compounds that provoke leakage of cytoplasmic constituents, disruption of cell peptidoglycan, and injure to the cell membrane. Phenolics can also act as strong protein cross-linkers and protein-denaturing agents. The presence of different polyphenols, such as epicatechin, and cynarin present in high concentrations in Carciofo di Montoro artichoke probably affected in synergistic way the quorum sensing activity of *C. violaceum*. Due to their acidic side chains, phenolic acids, such as chlorogenic acid, the most abundant in our extract, can be easily transported across the cell membrane; this property may explain their stronger inhibitory effect. Alternatively, they can interact with membrane lipids to neutralize the membrane's electric potential subsequent to the penetration of the molecule. A similar effect may occur in the bacterial cell membrane to affect energy metabolism and perhaps also the production of some molecules, such as violacein, expressed by *C. violaceum* in response to the QS inducers C6-acyl- and C4-acyl-homoserine lactones [3]. In our experiment, quorum quenching activity, the capability to inhibit the quorum sensing action of the strain, was observed with 5.25 mg GAE of extract. Such dose did not cause the block of the growth of *C. violaceum*; however it was sufficient to negatively change the bacterial metabolism, and to stop the production of violacein (**Figure 3**). Castillo and others [44] reported an effect

Figure 3. Quorum quenching activity expressed on plate by the extract of Carciofo di Montoro global artichoke, using 10.3 mg GAE of polyphenols.

tive activity of *Cynara scolymus* to inhibit adherence and cytotoxic activity of *Campylobacter* to host mucosal surfaces, which are well known critical steps in pathogenesis. Our results strengthen case for the "functional" significance of the artichoke, in this case Carciofo di Montoro, which can constitute a precious source of antimicrobial and quorum quenching molecules that might be applied in many fields, including medicine or food technology, safety and food preservation. In summary, our data suggest that the variety Carciofo di Montoro globe artichoke may represent a good source of health-promoting polyphenols; they encourage a nutraceutical use of this ecotype, in addition to the other *Cynara cardunculus* var. *scolymus* species, as an alternative to the more traditional phyto-pharmaceutical applications.

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Enhancing the Yields of Phenolic Compounds during Fermentation Using *Saccharomyces cerevisiae* Strain 96581

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Abstract

Phenylethanol, tyrosol, and tryptophol are phenolic compounds or fusel alcohols formed via the Ehrlich pathway by yeast metabolism. These compounds can yield health benefits as well as contribute to the flavors and aromas of fermented food and beverages. This research shows that *Saccharomyces cerevisiae* Strain 96581 is capable of producing significantly higher levels of these three compounds when the precursor amino acids were supplemented into either the Chardonnay concentrate for wine-making or the malt concentrate for brewing English Ale. Strain 96581 can produce phenylethanol, tyrosol, and tryptophol as high as 434 mg/kg, 365 mg/kg, and 129 mg/kg, respectively, in the beer fermentation. The performance of Ale yeast WLP002 from White Labs Inc. was also analyzed for comparison. Strain 96581 outperformed WLP002 in the control beer, the amino acids supplemented beer, and the kiwi-beer background. This shows that Strain 96581 is more effective than WLP002 in converting the malt and the kiwi fruit supplements via its endogenous enzymes.

Keywords

Phenolic Antioxidants, Tyrosol, Tryptophol, Phenylethanol, Fermentation Technology

1. Introduction

Saccharomyces cerevisiae Strain 96581 isolated from spent liquor sulfite drums of pulp-making process was found to produce high amounts of fusel alcohols such as tyrosol, tryptophol, phenylethanol [1] [2]. These phenolic compounds have significant applications in food and beverage manufacturing including wine-making. Ty-

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rosol, found abundantly in olive oil, is a natural phenolic compound known to have high anti-oxidative properties [3] [4]. Samuel *et al.* [5] observed that tyrosol treatment in animals significantly reduced heart cell death as a result of protective signaling from Akt, eNOS, FOXO3a, and induced expression of the longevity protein SIRT1 in the heart. This suggests that tyrosol induces myocardial protection against ischemia-related stress by inducing survival and longevity proteins that may be considered as anti-aging therapy for the heart. Similarly, white wine has been reported as having cardioprotective benefits due to the presence of components such as tyrosol and caffeic acids that are believed to not only modulate oxidative stress and inflammation but also activate the cell survival signaling pathway and the FOXO3a longevity associated gene [6] [7].

It is known that tryptophol has a sleep-inducing property similar to the effects of serotonin or melatonin. Tryptophol present in wine and beer as byproducts of fermentation can also be produced in the human liver after disulfiram treatment for chronic alcoholism [8]. The synthesis of tryptophol by yeast was first described by Felix Ehrlich in 1912 [9] as the metabolic conversion of amino acids via the successive steps of transamination, decarboxylation, and reduction [10]. Tryptophol is also used as a precursor in the synthesis of the drug Indoramin, which is used commonly in many applications as an alpha blocker for cardiovascular diseases and high blood pressure treatment [11]. Phenylethanol is an aromatic compound that is commonly found in plants such as rose, carnation and orange blossom. It has a pleasant floral odor which makes it desirable in flavors and perfumes, particularly when the rose smell is desired. Due to its preservative properties, phenylethanol is often used in soap because of its stability in basic conditions. This can allow phenylethanol to act as a natural preservative in wine and beer to prevent spoilage. Phenylethanol has biological applications due to its antimicrobial properties. For example, it is produced by the fungus *Candida albicans* as an autoantibiotic [12].

Like wine, beer contains carbohydrates, amino acids, minerals, vitamins, and phenolic compounds. Many of these phenolic components have been identified as chemopreventive agents, antioxidants, cytochrome P450 1A inhibitors, detoxification enhancers, and anti-inflammatory agents through inhibition of the inducible nitric oxide synthase (iNOS) and the cyclooxygenase I (Cox-1) enzymes. Other potential benefits include estrogenic/anti-estrogenic properties, anti-proliferative and differentiation-inducing mechanisms, anti-angiogenic and anti-viral activities [13]-[16].

Wine and beer also contains flavonoids or polyphenolic compounds, which have potential health benefits such as antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities. The flavonoid xanthohumol in beer is from the hop whereas the flavonoids in wine are mainly from the fruit. The fruit and the malt are also sources of amino acids. Natural fruit supplements were evaluated for possible application in beer production. Kiwi fruit and banana were among the fruits with the highest amounts of reported tyrosine, while grapes and apples were among the lowest [17]. The purpose of this study is to investigate the possibility of increasing the yield of tyrosol, tryptophol, and phenylethanol in fermentable beverages using two different yeast strains and supplementing the fermentable substrate with the relevant amino acid precursors or fruits high in these amino acids. In addition, the *S. cerevisiae* Strain 96581, which has never been classified as a wine or a beer strain, was tested for its ability to enhance the yields of the three phenolic compounds in both types of the fermentable media.

2. Materials and Methods

2.1. Yeast Strains

The yeast strains used in this study were the *Saccharomyces cerevisiae* ATCC Number 96581 from the American Type Culture Collection, [1] [2] and the English Ale yeast WLP-002 from the White Labs (Nashville, TN).

2.2. Wine Fermentation

The wine fermentation media was prepared with Alexander's Pinot Chardonnay grape juice concentrate (Grape and Granary, Akron, OH, USA) containing sulfite diluted to 16% (w/v) sugar content, minimal media of 0.67% Difco yeast nitrogen base without amino acids (Voigt Global Distribution, Lawrence, KS, USA), and with either one amino acid supplement *i.e.* tyrosine, tryptophan, phenylalanine added to each flask or all three amino acids (TTPaa) added at final concentrations of 0.05 mg/mL, 0.02 mg/mL, 0.05 mg/mL respectively into 50 mL final volume. Also included was the TTPaa NH4 which contained the fermentation media with 3 amino acid supplements plus 0.89 mg/mL yeast-nutrient-ammonium phosphate salt (Grape and Granary, Akron, OH, USA). The

two controls were the fermentation media without amino acid supplements and the fermentation media with no amino acid supplements but contained 0.89 mg/mL yeast-nutrient-ammonium phosphate salt.

Overnight pre-inoculum cultures in YEPD (1% yeast extract, 2% peptone, 2% dextrose) were incubated in a Barnstead Lab-line MaxQ 4000 incubator at 28˚C with shaking at 200 rpm and the optical density (OD) of the culture was determined at the wavelength of 600 nm using a Hitachi U-2000 UV-Vis spectrophotometer. Each aliquot of the 50 mL fermentable media was inoculated with 6.0×10^8 cells based on the conversion factor of 0.50 OD being equal to 1.0×10^7 cells. The flasks were topped with an air lock filled with sterile water to the point where no exchange with ambient air occurred. The cultures were kept in a Barnstead Lab-line MaxQ 4000 incubator at 18˚C for 16 days (stirring twice daily). The fermentation was carried out in triplicate sets of experiments.

2.3. Beer Fermentation

The beer fermentation media was prepared using a modification of the English Ale recipe composed of chocolate malt barley grain, dried malt extract or DME and liquid malt extract or LME, supplemented with either the equivalent volume of Malt-kiwi purée or with amino acids. The chocolate malt barley grain (Dimgemams, Belgium), plain light Sparymalt dried extract (Muntons, England) and light barley malt liquid extract (Muntons, England) were purchased from All Seasons Brewing Supply (Nashville, TN). The Malt-DME-LME stock concentrate was prepared by steeping 17.97 grams of crushed chocolate malt barley grain in 600 mL water at 50˚C for 1 hour, after which 119.8 grams DME powder and 395.5 grams of LME was added and the mixture boiled for 1 hour with continuous stirring. The entire mixture was reconstituted into a final volume of 1 liter. The "Malt only" stock, which contained only 2.995 grams of crushed barley grain in 500 mL water, was prepared the same way. The Malt-kiwi mixture was prepared using the 2.995 grams of crushed barley grain plus 446 mL of kiwi purée from 8 fruits in a final volume of 500 mL. This mixture was also steeped at 50˚C for 1 hour before boiling for 1 hour with stirring.

The fermentation samples consisted of a flask with the 5-fold diluted Malt-DME-LME stock medium supplemented with tyrosine, tryptophan, and phenylalanine at final concentrations of 0.05 mg/mL, 0.02 mg/mL, 0.05 mg/mL, respectively, in a final volume of 50 mL and a flask containing 1:1 volume ratio of the 5-fold diluted Malt-DME-LME stock to Malt-kiwi mixture. The controls were 1) a flask containing only the 5-fold diluted Malt-DME-LME stock medium without the amino acids supplement, and 2) a flask containing 1:1 volume ratio of the 5-fold diluted Malt-DME-LME and "Malt only" stock (*i.e.* No Kiwi). Each aliquot of the 50 mL fermentable media was inoculated with 6.0×10^8 cells from an overnight pre-inoculum. The cultures were kept under an anaerobic condition in a Barnstead Lab-line MaxQ 4000 incubator at 18˚C for 14 days (stirring twice daily). The fermentation was carried out in triplicate sets of experiments.

2.4. Sample Preparation and GC-MS Analysis

The amount of glucose left in the fermentation was determined using the Clinitest kit (Fisher Scientific, Fairlawn, NJ, USA). The fermentation was considered complete if the glucose level in the culture dropped to less than 1%, which then required the removal of cells by centrifugation. Exactly 20 mL aliquots of the supernatant were extracted with Merck Li Chrolut EN cartridges with 200 mg resin each (purchased from VWR International, West Chester, PA, USA) using a modified solid-phase extraction procedure described by Ooi *et al.* [2]. For the quantitation of selected fermentation components and for monitoring the consistency of the SPE extraction, a 60 µL standard solution containing 1000 ppm (w/w) of d5-deuterated-phenethyl alcohol (Isotec, Sigma-Aldrich, St. Louis, MO, USA) in ethanol (Pharmaco Inc., Brookfield, CT, USA) was added to 20 mL of the fermentation samples as an internal standard before being loaded onto the SPE cartridges for elution. Sample extracts were eluted from the LiChrolut EN twice with 1.0 mL of 1:1 volume mixture of dichloromethane (Sigma-Aldrich, St. Louis, MO, USA) and methanol (HPLC grade, Fisher Scientific, Fairlawn, NJ, USA). The eluate was filtered through a syringe filter (Pall Life Sciences, Ann Arbor, MI, USA) of 0.22 µm pore size and 13 mm diameter and diluted 1 to 10 fold or 1 to 5 fold in a dichloromethane: methanol (1:1) solvent mixture for GC-MS analysis. Analysis of fermentation components were carried out using the Shimadzu QP2010 S GC-MS equipped with a Phenomenex ZB5-HT, 30-m column with 0.25 mm i.d. and 0.25 µm film thickness. The temperature program used had an initial temperature of 40˚C for 2 minutes followed by a heating rate of 12˚C /min to reach to 140˚C, which was held for 1 min and then increased at 20˚C/min to 300˚C with a final hold period of 4 minutes. A he-

lium carrier gas flow rate of 1.23 mL/min and split injection mode with split ratio of 1:10 was used for GC analysis of a sample size of 1 µL. GC-MS analyses were repeated three times for triplicate batches of fermentation from each strain.

2.5. Calibration and Statistical Analysis

The concentrations ($mg \cdot kg^{-1}$ or ppm) reported in this paper were calculated using external standard calibration. A standard stock solution containing 1000 ppm (w/w) each of 2-phenethyl alcohol, 4-hydroxyphenethyl alcohol (4-hydroxy-benzeneethanol or tyrosol), 3-indoleethanol (tryptophol) and d5(ring)-phenethyl alcohol (D5) purchased from Sigma-Aldrich, St. Louis, MO, USA were prepared in the dichloromethane-methanol solvent mixture. Serial dilutions of this multicomponent standard were carried out for quantitative analysis. The mean values with standard deviations (SD) are reported. The beer fermentation data from this study was statistically analyzed using the general linear model (GLM) Multivariate Analysis of Variance (MANOVA; SAS 9.3 from the SAS Institute Inc.). The GLM-MANOVA approach was used to test the hypothesis of the significance association between a set of interrelated dependent variables (concentrations of phenolic compounds) and two grouping variables. A univariate analysis was performed to establish whether the supplements and yeast strains were associated significantly with any of the phenolic yields. The Wilks' Lambda technique was used to test the impact of each variable included in the multivariate model on the phenolics. The null hypotheses of no overall statistical significance of yeast strain influence on the yield of the three phenolic compounds and of no overall difference in yields from the amino acids and kiwi supplemented beer fermentation among the two yeast strains was carried out using the MANOVA test criteria at the significance level or "α value" of $P < 0.05$. The subsequent one-way ANOVA test criteria at the significance levels or $P < 0.05$ was performed to determine whether the yeast strain has a significant influence on tyrosol, tryptophol, and phenylethanol production and whether the sample media have a significant effect on the production of each type of phenolic compound [18].

3. Results and Discussion

There was a significant increase in phenylethanol, tyrosol, and tryptophol, when wine fermentation was supplemented with phenylalanine, tyrosine, or tryptophan respectively. The increase in the production of tryptophol by the 96581 strain when tryptophan was supplemented in the media was in the order of 7 to 8-fold and 2-fold for phenylethanol and tyrosol. If all three amino acids were supplemented, then there was increase in all three fusel alcohols as in the fermentation samples labeled as TTPaa (**Figure 1**). The TTPaa sample plus the yeast nutrient ammonium phosphate salt (TTPaa NH4) showed about the same level of increase in yield over the TTPaa sample without added ammonium phosphate salts, suggesting that the additional ammonium phosphate salts were not essential for enhancing the synthesis of fusel alcohols from their amino acid precursors.

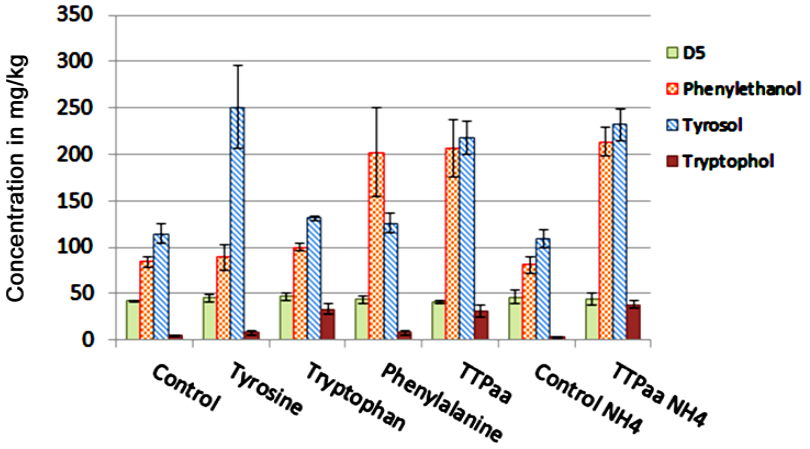

Figure 1. Concentration levels of phenylethanol, tyrosol, and tryptophol produced in wine fermentation. Fermentation was carried out using yeast Stain 96581. Amino acids supplements are indicated on the x-axis. TTPaa stands for the amino acids tyrosine, tryptophan, and phenylalanine supplements in the media. NH_4 indicates that 0.89 mg/mL of yeast-nutrient-ammonium phosphate salt had been added to the media. D5 is the d5-deuterated-phenylethanol.

In the control beer fermentation, Strain 96581 produced more fusel alcohol than the English Ale yeast strain WLP002. When supplemented with all three amino acids precursors both strains were able to increase production of all three fusel alcohols. The total amounts of phenylethanol, tyrosol, and tryptophol produced by Strain 96581 were 434 ± 90 mg/kg, 365 ± 67 mg/kg, and 129 ± 32 mg/kg, respectively. Strain WLP002 under the same fermentation conditions produced 307 ± 2 mg/kg, 305 ± 16 mg/kg, and 110 ± 6 mg/kg, respectively (**Figure 2** and **Table 1**). The higher yields of the three fusel alcohols in the control sample fermented by the 96581 strain

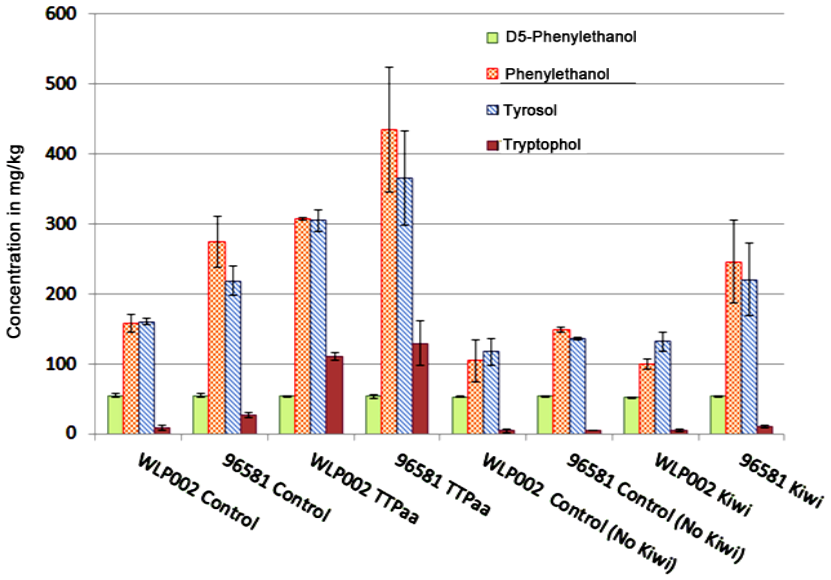

Figure 2. Concentration levels of phenylethanol, tyrosol, and tryptophol produced in beer fermentation. Fermentation was conducted using an ale yeast Strain WLP002 versus a non-beer yeast Strain 96581 for comparison. TTPaa stands for the amino acids tyrosine, tryptophan, and phenylalanine supplements in the media.

Table 1. Concentration of D5-phenylethanol, phenylethanol, tyrosol, and tryptophol in beer sample.

Strain	[a]Sample			
	D5	Phenylethanol	Tyrosol	Tryptophol
WLP002 Control	54 ± 3	158 ± 13	160 ± 5	9.0 ± 3.5
96581 Control	54 ± 3	275 ± 37	219 ± 21	27 ± 3
WLP002 TTPaa	53 ± 1	307 ± 2	305 ± 16	110 ± 6
96581 TTPaa	54 ± 3	434 ± 90	365 ± 67	129 ± 32
WLP002 Control (No Kiwi)	52 ± 1	104 ± 30	117 ± 20	4.1 ± 2.2
96581 Control (No Kiwi)	53 ± 1	149 ± 4	136 ± 2	5.0 ± 0.6
WLP002 Kiwi	51 ± 1	100 ± 7	132 ± 13	4.1 ± 2.0
96581 Kiwi	53 ± 1	245 ± 59	220 ± 52	10 ± 2
ANOVA F Value				
[b]Strain Effect	-	40.26	18.28	5.15
[c]Media Effect	-	38.49	46.05	132.92

[a]All sample concentrations are given in mg/kg units including their corresponding standard deviation values. Statistical calculation was performed for 0.05 level of statistical significance using the one-way ANOVA test and F values for the hypothesis of no effect of yeast strains and no effects of sample media on the concentration of phenylethanol, tyrosol, and tryptophol produced. F values are tabulated in the bottom two rows. [b]The significant effects of yeast strains are expressed as ($F_{(1,16)}$ = 40.26, $P < 0.05$, R^2 = 0.91, MSE = 1753.5); ($F_{(1,16)}$ = 18.28, $P < 0.05$, R^2 = 0.91, MSE = 1049.4); ($F_{(1,16)}$ = 5.15, $P < 0.05$, R^2 = 0.96, MSE = 138.1) for phenylethanol, tyrosol, and tryptophol, respectively. [c]The significant effects of sample media are expressed as ($F_{(3,16)}$ = 38.49, $P < 0.05$, R^2 = 0.91, MSE = 1753.5); ($F_{(3,16)}$ = 46.05, $P < 0.05$, R^2 = 0.91, MSE = 1049.4); ($F_{(3,16)}$ = 132.92, $P < 0.05$, R^2 = 0.96, MSE = 138.1) for phenylethanol, tyrosol, and tryptophol, respectively.

suggests that this strain is more effective than the WLP002 strain in utilizing the fermentable malt and malt sugar substrates. This could be attributed to the greater efficiency and/or higher levels of endogenous enzymes to help break down the malt precursors to produce the fusel alcohols in Strain 96581. The WLP002 strain, however, relies more on the supplemented amino acids.

The increased fusel alcohol production in beer by Strain 96581 can be attributed to its highly active and efficient Ehrlich pathway for utilizing nutrients from the fermentable substrate and allowing the yeast to grow and propagate more rapidly. The release of nutrients occurs during the malting process of the barley grain when the partially germinating grain produces specialized enzymes that break down starches and proteins into simple sugars and amino acids, respectively [19]. Researchers at the United States Agricultural Research Service found that the enzymes called serine-class proteases serve the dual purpose of digesting proteins in the grain into amino acids as well as degrading the beta-amylases, which are involved in the conversion of carbohydrates into simple sugars [20], creating an optimal balance between the amount of amino acids and sugars that would influence the malted barley flavor [21]. The availability of additional amino acids and basic sugars in the yeast culture medium allows for faster growth of the fermenting yeast as well as more efficient fusel alcohol production.

The rationale for using kiwi fruit as a fermentation substrate is related to the desirable aroma profile of the aqueous essence and fresh puree. About thirty aroma active components consisting of predominantly alcohols, ketones, and esters have been characterized by the multidimensional gas chromatography-olfactometry technique [22]. In this study, the addition of kiwi fruit purée resulted in about 1.6 to 2-fold increase in the levels of phenylethanol, tyrosol, and tryptophol produced by Strain 96581. However, there was no appreciable increase in the production of these alcohols by the English Ale WLP002 strain (**Figure 2**, **Table 1**). The 96581 strain apparently was able to utilize amino acid precursors and simple sugars derived from the kiwi fruits and produce more fusel alcohols. This characteristic was not observed for the English Ale yeast. The F values from the one-way ANOVA statistics for the formation of tyrosol, tryptophol, and phenylethanol shown on **Table 1** further support the significance of nutrient supplements as well as the significant influence of yeast strains on the production of the three phenolic compounds. The multivariate analysis of variance also revealed that there is an overall significant influence of yeast strain on the production of tyrosol, tryptophol, and phenylethanol (Wilks' λ = 0.1377, $F_{(3,14)} = 29.23$, $P < 0.05$) and a significant difference between control, TTPaa, No Kiwi control, and Kiwi samples on the production of the phenolic components (Wilks' $\lambda = 0.0075$, $F_{(9,34)} = 24.54$, $P < 0.05$).

Although phenylethanol may be considered a desirable constituent contributing to wine or beer flavor, high levels of tyrosol and tryptophol may not be desirable due to their bitter yeast bite and bitter almond taste, respectively. Yet, threshold levels as high as 200, 100, and 75 mg/liter for tryptophol, tyrosol, and phenylethanol have been reported in the literature [23] [24] and it is known that ale drinkers prefer lager samples containing 8 times the original level of tryptophol [25]. Szlavko [25] has also shown that inherent differences in metabolic process among yeast strains, raw materials, fermentation media composition, and fermentation conditions can influence the level of each fusel alcohol formed. Despite the inhibitory effects of high levels of phenylethanol to yeast growth, the thermotolerant and multi-stress resistant strains of *S. cerevisiae* have been shown to produce levels of phenylethanol as high as 4.5 g/liter [26].

4. Conclusion

Wine and beer have been known to provide multiple health benefits when consumed in moderation. The goal of improving wine and beer quality with boosted amounts of fusel alcohols such as tyrosol, tryptophol and phenylethanol was to increase the antioxidant levels present in these alcoholic beverages for health benefits as well as enhancing the flavor of these alcoholic beverages. This study showed that the outcomes of flavor enhancement and enrichment of antioxidants could be achieved through supplementing the fermentation media with precursor amino acids as well as careful choices of the appropriate yeast strain. The 96581 strain outperformed the English Ale yeast WLP002 because it could utilize additional nutrients from the malt and kiwi for the production of fusel alcohols. Strain 96581 performed well in all three fermentable substrates for making wine, beer, and fruit-supplemented beer, making it an attractive candidate for commercial applications.

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Dietary Intake of Vitamin A and Macronutrients among Pregnant Women in Ngaoundere Town, Adamawa Region, Cameroon

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Abstract

This paper examines the risk of Vitamin A deficiency among selected pregnant women in Ngaoundere, Adamawa Region, Cameroon. A total of one hundred (100) pregnant women attending ante natal visits at the Regional Hospital in Ngaoundere were involved in a survey conducted in 2012 which used a 24-hour dietary recall to assess their dietary intake. Food models were used to assist memory and portion sizes of food taken. Carotenoids were quantified in the meals commonly consumed, as well as proteins, sugar, fibers and oil, nutrients intervening in carotenoids absorption and Vitamin A metabolism. The results indicated that the dietary intake of Vitamin A was inadequate with 37.5% of the selected pregnant women exposed to acute Vitamin A deficiency, while 20% of them were exposed to the severe form. The daily consumption of Vitamin A was about 539.09 ± 43.09 µg, corresponding to a contribution of 67.39% to the Vitamin A requirement of the pregnant women. The dietary intake of macronutrients was also inadequate with a daily consumption of 57.28 ± 12 g for oils, 177.88 ± 29 g for sugars, 47.34 ± 11 g for proteins and 11.28 ± 3 g for fibers corresponding respectively to a contribution of 63.6%, 70.8%, 78.90% and 45.1% of these nutrients requirement in these women. Given that over 50% of the women surveyed were exposed to Vitamin A deficiency, there is a need for urgent intervention programs in the area to alleviate this situation.

Keywords

Vitamin A Intake, Dietary Intake, Vitamin A Deficiency, Pregnant Women, Ngaoundere

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1. Introduction

Vitamin A deficiency (VAD) is a major public health problem in many developing countries, and malnutrition during pregnancy has been recognized as one of the major factors for high maternal and infant mortality in these countries [1]. This is because maternal VAD aggravates the other causes of maternal morbidity such as anemia in pregnancy, birth defects, blindness, increased infections and decreases of cellular differentiation [2]. VAD in children usually follows from a deficiency in the mother. Therefore, eliminating this deficiency in pregnant women will go a long way towards alleviating it in children. Several studies have shown that poor dietary patterns before and during pregnancy are among the major causes of malnutrition and VAD in most populations [3]-[5]. In addition, Vitamin A (VA) content of human breast milk is strongly affected by maternal nutrition during pregnancy and lactation [6] [7].

During pregnancy, adequate supply in Vitamin A is necessary for the growth of the fetus. Unfortunately, in many developing countries, the average daily consumption of VA by non-supplemented pregnant women is lower than the recommended daily intake [8]. The WHO report [9] reveals that approximately 54% of pregnant women suffer from VAD in the CEMAC sub-region. A survey of available literature on the studies of VAD in Cameroon indicates that they are mostly national prevalence studies on children less than 5 years old, lacking detailed information on pregnant and lactating women, who equally constitute a vulnerable group. However, existing data shows that the Northern Regions (Adamawa, North and Far-North) are mostly affected with a prevalence rate of 62.5% in children less than 5 years old compared to the prevalence rate of 17.5% in the Southern Regions, and the national prevalence of 40% [10]. Vitamin A deficiency is also a cause for concern amongst women of child bearing age (15 - 49 years) affecting about 45% of them [10].

Addressing the problem of VAD in pregnant women will certainly go a long way towards reducing the prevalence of VAD, not only in pregnant women but also in their children. Hence, the need for this study whose objective was to evaluate the dietary intake of energy and VA among pregnant women in the town of Ngaoundere, Adamawa Region, which appears to be amongst the at risk population for VAD with a view to assisting decision makers and stakeholders design appropriate and targeted nutrition intervention programs.

2. Material and Methods

2.1. Study Area

The study took place in Ngaoundere, located in the Adamawa region of Cameroon. The Region is limited to the North by the North region, to the South by the East, Center and West regions, to the East by Central African Republic and to the West by Nigeria. There are three types of climate in the Region: an equatorial climate of Guinean type in the southern part of the Region, a tropical climate of Sudanese type on the Adamawa plateau, and an equatorial climate of Cameroonian type in the southwestern part. The high altitude gives a relatively fresh climate between 22 and 25 degrees and the annual average precipitation varies from 900 to 2000 mm, decreasing towards the northern part of the Region.

The soil is poor and the Region sparsely populated. The principal economic activity is cattle rearing (Zebu type). However, the cultivation of tubers (*Ipomea batata*, *Dioscorea rotundata*), vegetables (*Moringa oleifera*, *Solanum nigrum*, *Hibiscus sabdarifa*) and cereals (*Zea mays*, *Panicum miliaceum* L.) is also practiced. Islam is the principal religion, but there are also Christians in the area. Peuls form the principal ethnic group of the Region, but there are Tikar and Gbaya strong minorities, as well as other smaller ethnic groups.

2.2. Subjects and Sampling

The study group comprised women at different stages of pregnancy, attending the maternity service at the Ngaoundere Regional Hospital (NRH). Patients attending this hospital come from various socioeconomic groups and from urban, semi-urban and rural areas in its catchment areas, which includes the town of Ngaoundere and some surrounding villages. Women attending this hospital are, therefore, of mixed background. Our sample population was drawn from among this group.

Pregnant women aged 40 years and below, not suffering from any infectious disease, not undergoing treatment for any disease, not on any diet, who did not use contraceptive pills prior to pregnancy and who gave their consent to be admitted in the study were recruited. Those who didn't respond to these criteria were excluded from the study. The recruitment stage lasted for two weeks at the NRH, from 17th to 31st March 2012. Informed

consent was obtained from the women and the study period on the field itself lasted for one month at their residence. Ethical approval for this study was obtained from the ethical committee of the Regional Hospital in Ngaoundere.

Of the 125 subjects interviewed who had initially given their consent to participate in the study, only 100 effectively took part. Socio-demographic information was collected and anthropometric data measured for every pregnant woman. These included age, weight, height, level of education, professional status, marital status, number of children and age of pregnancy.

2.3. Evaluation of Dietary Intake of Participants

Subjects were interviewed at home and nutrition information was obtained using the 24 hour dietary recall method for four weeks, from 2nd April to 3rd May 2012. Dietary recall was done every 72 hours given that the women mostly ate the same food for about 2 days. Information on type, quantity and frequency of meals consumed was collected. A survey was also made of the culinary practices and samples of meals commonly consumed were retained for carotenoid analysis.

2.4. Collection of Meal Samples

Meal samples were collected in clean, tightly closed glass containers, labeled and transported in dark containers to the Food Biophysics and Nutritional Biochemistry Laboratory of the National School of Agro Industrial Sciences of the University of Ngaoundere. They were then dried at 45˚C ± 2˚C and frozen at –20˚C. Sixteen samples were collected representing the dishes most consumed by pregnant women in Ngaoundere town.

2.5. Extraction and Quantification of Carotenoids from the Meal Samples

To 1 g of dried sample, 30 ml of hexane-acetone mixture 30/70 (v/v) was added, and the mixture heated under reflux for one hour, cooled and filtered [11]. The filtrate was washed with distilled water in a separating funnel, the lipid phase was then decanted into a 25 ml graduated flask and the volume adjusted to the mark with hexane. The solution obtained was diluted 1/10 with hexane and the optical density read using a spectrophotometer RAYLEIGH (VIS-723N) between 430 and 450 nm in order to determine the maximum optical density. Carotenoids were then quantified using of relationship:

$$C = \frac{\left(DO_{\max} \times f\right)}{\left(196 \times m\right)}$$

where, $DO_{\max}$ is optical density where max absorption was obtained; f, dilution factor; and m, mass of the sample.

2.6. Estimation of Vitamin A Activity in the Meal Samples

The VA content (Retinol Equivalent or RE) of the meals consumed was computed using the values of carotenoids determined earlier on and based on the assumption that 12 µg of dietary carotenoid yields 1 µg of VA activity or 1 µg of dietary carotenoids equals 0.083 µg RE [12] [13]. Vitamin A and proteins from animal products were obtained from Food Composition Tables [14].

2.7. Quantification of Macronutrients in the Meal Samples

Dry matter [15], oil [16], total proteins [17] [18], total sugars [19] and crude fibers [20] contents of meals were determined to establish their effect on VA absorption and metabolism.

2.8. Calculation of Dietary Intakes of Pregnant Women

Vitamin A, protein, fats, fibers and sugar intake were obtained by summing intakes coming from vegetable and animal fractions from various dishes consumed during the study period. This was obtained from the laboratory analyses of the meal samples and from food composition tables [14]. Thus, the daily VA and nutrient intake was the mean consumption per woman, per day during the period of study, and the general intake for all the studied population was the mean consumption for all the women.

2.9. Statistical Analysis

The data obtained in this study was subjected to analysis of variance (ANOVA) and means separated using the least significant difference test with the statistical software Stat graphics centurion at the 5% level of significance. Microsoft Excel 2010 was used to generate the graphs. Where appropriate, percentages (%) were calculated. Results are presented as means and standard deviation of three determinations.

3. Results

Table 1 shows the distribution of the studied population with respect to measured socio-demographic and anthropometric parameters. About 93% of the women were under 30 years of age. Of these 41% were teenagers between the ages of 14 and 20 years, whereas 53% were between 21 and 30 years. In addition to their young age 37% of them had never gone to school, and only 33% had completed primary education. A small proportion (9%) had university education. A total of 87% of these women were married with only 13% being single. Most of them however, were housewives (57%) while 29% practiced some income generating activity (employed, petit traders, farmers). Their Body Mass Index (BMI) was generally in the normal range between 18.5 and 24.9 kg/m^2 (84%). A small proportion (9%) was undernourished with a BMI below 18.5 kg/m^2, and 7% were overweight with BMI above 30. About half of the women were in the first trimester of pregnancy, with 25% in the third trimester. A total of 79% of women had two or more children compared to only 21% who had at most one child.

The nutritional compositions of the sixteen dishes commonly consumed by pregnant women in Ngaoundere are presented on **Table 2**. Results show that vegetable dishes had high carotenoid contents with Eru (*G. buchol*

Table 1. Socio-demographic and anthropometric parameters of the studied population.

Parameter	Range	Percentage of women (%)
Age distribution (years)	14 - 20	41
	[21 - 30]	52
	>30	07
Level of education	None	37
	Primary education	33
	Secondary education	21
	University education	09
Socio-professional status	Housewife	57
	Worker	29
	Student	14
Body Mass Index (kg/m^2)	<18.5	09
	[18.5 - 24.9]	84
	[25 - 29.9]	07
Number of children	0	10
	1	11
	2	39
	3	32
	≥4	08
Marital status	Married	87
	Single	13
Age of pregnancy	1st trimester	49
	2nd trimester	26
	3rd trimester	25

Table 2. Nutritional composition of dishes commonly consumed by pregnant women in Ngaoundere per 100 g (dry basis)[*°].

Dishes	Water (%)	Oils (g)	Protein (g)	Sugars (g)	Fibers (g)	Carotenoid (mg)	VA equivalent (mg)
Rice[1] + tomato sauce[2]	76.64 ± 2.12^{de}	15.05 ± 0.94^{b}	11.26 ± 0.21^{b}	63.56 ± 2.62^{h}	7.87 ± 1.03^{cd}	11.63 ± 0.85^{c}	0.97 ± 0.07^{c}
Rice + roasted groundnut soup[3]	77.85 ± 2.81d^{ef}	18.21 ± 0.37^{b}	12.02 ± 0.91^{b}	60.01 ± 3.12^{g}	7.98 ± 0.74^{cd}	1.88 ± 0.18^{ab}	0.16 ± 0.02^{ab}
White sweet potato[4] (boiled with flesh)	60.38 ± 0.83^{a}	2.72 ± 0.14^{a}	5.01 ± 0.38^{a}	81.31 ± 2.45^{i}	8.23 ± 1.04^{cd}	1.64 ± 0.17^{ab}	0.14 ± 0.01^{ab}
Cassava fufu[5]	63.61 ± 1.96^{abc}	2.00 ± 0.11^{a}	4.15 ± 0.55^{a}	82.78 ± 2.84^{i}	8.64 ± 1.37^{cd}	0.12 ± 0.09^{a}	0.01 ± 0.01^{a}
Rice fufu[1]	65.29 ± 1.37^{bc}	1.79 ± 0.17^{a}	3.84 ± 0.39^{a}	81.68 ± 3.04^{i}	9.69 ± 0.85^{de}	0.14 ± 0.05^{a}	0.01 ± 0.00^{a}
White corn fufu[6]	67.15 ± 1.14^{c}	3.46 ± 0.33^{a}	5.56 ± 0.74^{a}	81.52 ± 3.11^{i}	6.88 ± 1.01^{bc}	0.37 ± 0.12^{a}	0.03 ± 0.01^{a}
Koki[7] (with palm oil)	64.28 ± 3.67^{abc}	31.69 ± 2.34^{de}	48.89 ± 3.05^{i}	13.05 ± 1.49^{d}	4.17 ± 0.77^{a}	35.94 ± 1.47^{de}	3.00 ± 0.12^{de}
Beans[8] (in cotton seed oil)	61.74 ± 1.98^{ab}	29.02 ± 1.89^{cd}	44.41 ± 2.43^{h}	18.15 ± 1.21^{f}	5.05 ± 0.71^{ab}	4.92 ± 0.89^{b}	0.41 ± 0.07^{b}
Baobab leaves[9] (with local ingredients)	87.15 ± 3.22^{g}	29.72 ± 1.04^{cd}	32.21 ± 1.84^{d}	17.35 ± 1.25^{f}	15.41 ± 2.29^{g}	37.59 ± 1.25^{e}	3.13 ± 0.02^{e}
Eru[10] (with palm oil)	75.12 ± 1.53^{d}	48.31 ± 2.24^{g}	28.79 ± 1.71^{c}	6.51 ± 0.63^{a}	12.13 ± 0.79^{ef}	81.59 ± 3.07^{i}	6.80 ± 0.26^{i}
Zom[11] (with groundnuts)	81.72 ± 2.05^{f}	35.82 ± 2.76^{f}	35.70 ± 1.34^{ef}	9.74 ± 0.43^{bc}	16.14 ± 0.81^{ghi}	40.98 ± 2.09^{f}	3.42 ± 0.17^{f}
Folong[13] (with groundnuts)	81.53 ± 3.16^{f}	34.02 ± 2.39^{ef}	36.12 ± 1.75^{f}	8.93 ± 0.74^{ab}	15.76 ± 1.32^{gh}	33.19 ± 3.93^{d}	2.77 ± 0.33^{d}
Ndole[12] (with groundnuts)	79.35 ± 1.79^{ef}	35.98 ± 3.17^{f}	40.33 ± 2.98^{f}	7.45 ± 0.87^{ab}	12.45 ± 2.58^{f}	34.94 ± 3.14^{de}	2.91 ± 0.26^{de}
Fried zom[11] (with tomatoes)	78.72 ± 2.44^{def}	29.96 ± 3.01^{cd}	31.19 ± 1.69^{cd}	16.40 ± 0.83^{ef}	18.07 ± 2.37^{hi}	63.30 ± 1.04^{h}	5.28 ± 0.09^{h}
Fried folong[13] (with tomatoes)	78.56 ± 3.09^{def}	29.76 ± 2.39^{cd}	32.98 ± 1.45^{de}	13.99 ± 1.24^{de}	18.69 ± 1.99^{i}	55.39 ± 1.51^{g}	4.62 ± 0.13^{g}
Fried ndole[12] (with tomatoes)	76.34 ± 2.31^{de}	28.38 ± 2.46^{c}	36.81 ± 1.71^{g}	12.63 ± 0.91^{cd}	18.73 ± 2.69^{i}	57.95 ± 2.88^{g}	4.83 ± 0.24^{g}

[*]Figures in the same column followed by the same letter are not significantly different at ($p > 0.05$) [°]Nomenclature of principal ingredients in meals: [1]*Oryza sativa*; [2]*Lycopersicum esculentum*; [3]*Arachis hypogea*; [4]*Ipomea batata*; [5]*Manihot utilissima*; [6]*Zea mays*; [7]*Vigna unguiculata*; [8]*Phaseolus vulgaris*; [9]*Adansonia digitata*; [10]*Gnetum bucholzianum*; [11]*Solanum nigrum*; [12]*Vernonia amygdalina*; [13]*Amaranthus hybridus*.

zianum) (81.59 ± 3.07 mg/100 g DM) having the highest content followed by fried Zom (*S. nigrum*) with 63.30 ± 1.04 mg/100g DM. Addition of tomatoes to vegetables improved carotenoid content of dishes, while addition of groundnuts improved protein content. Dishes in which palm oil was incorporated had high carotenoid content as was the case with Eru and koki. Cereals and tubers had low carotenoid contents with cassava fufu (*M. utilissima*) being the lowest (0.11 ± 0.09 mg/100 g). Oil content in dishes varied from 2.0 ± 0.11 g in cassava fufu to 48.13 ± 2.24 g in Eru, proteins from 4.15 ± 0.55 g in cassava fufu to 47.89 ± 3.05 g in koki (*V. unguiculata*), fibers from 4.17 ± 0.77 g in koki to 18.73 ± 0.61 g in fried Ndole (*V. amygdalina*) and finally, sugars from 6.51 ± 0.63 g in Eru to 82.78 ± 2.84 g in cassava fufu.

The quantity of animal products consumed, their VA and protein contents, were also measured (**Table 3**). Results show that pregnant women consumed on average 550.7g of animal proteins during the period of study, hence a daily consumption of 18.36 g. From these foods, they obtained an estimated 1722.5 µg of VA, giving a daily consumption of 57.42 µg VA per pregnant woman from animal sources. This coupled with the 481.67 µg from plant sources gives a total daily VA intake estimated at 539.09 µg per pregnant woman per day.

Table 4 shows the average daily macronutrient, VA and fiber intake of pregnant women in Ngaoundere. These women consume on average 539.09 ± 43 µg of VA daily, corresponding to 67.4% of their daily requirements. Apart from fibers whose daily requirements are met at only 45%, the requirements for the other nutrients are met at over 60%. Their total energy consumption from these nutrients stands at 1416.4 Kcal per pregnant woman per day.

Figure 1 shows the distribution of Vitamin A intake among the studied population and hence the percentage of pregnant women at risk for VAD. A total of 12.5% barely covered daily requirements in VA (>750 µg), 30% were exposed to slight VAD (650 - 749 µg), 37.5% to an acute VAD (500 - 649 µg) and 20% to severe VAD (<500 µg).

Table 3. Types and quantities of animal products consumed by pregnant women in Ngaoundere.

Foods	Average consumption per woman/month (g)	Proteins (g)*	Vitamin A (µg)*
Smoked fish	450	198	-
Fresh fish	250	55	-
Beef	300	54	75
Lamb mutton	200	34	20
Goat meat	175	31.5	-
Chicken	150	30	127.5
Pork	110	13.2	-
Chicken eggs	750	135	1500

*Source: [14].

Table 4. Average daily intakes of nutrients and energy per pregnant woman in the studied population.

Nutrients	Intake/day	RDA*	% of need covered	Energy (Kcal)
Sugars (g)	177.88 ± 29	250 - 300	70.8	711.52
Oils (g)	57.28 ± 12	90 - 105	63.6	515.52
Proteins (g)	47.34 ± 11	60 - 70	78.9	189.36
Fibers (g)	11.28 ± 3	25 - 30	45.1	-
Vitamin A (µg)	539.09 ± 43	800	67.4	-

*Recommended daily allowance [21].

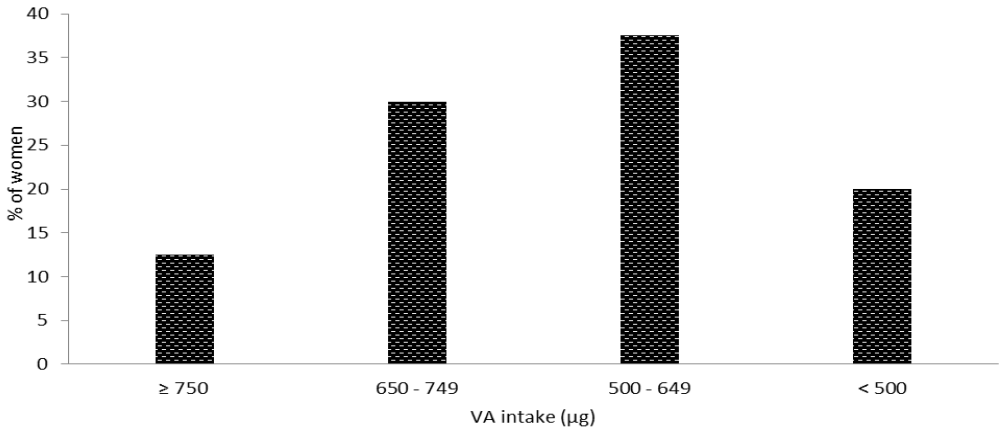

Figure 1. Classification of pregnant women according to their risk level of VAD.

4. Discussion

Pregnant women interviewed in this study were generally 30 years old and below (93%), with 41% of them being teenagers 14 to 20 years old. This could be related to the fact that women in this part of the country marry early as is customary. Recent studies in the Adamawa Region [22] report that the average age of first marriage for women is 16.3 years, with 18.5 years as the mean age of first delivery. This high prevalence of teenage pregnant mothers is a cause for concern as teenage pregnancy is considered high risk given that the mother herself is still growing and has high nutrient needs, coupled with the usual pregnancy-induced changes such as hemodilution, increased urinary excretion and fetal utilization of VA, as well as a changes in the equilibrium be-

tween the liver and blood VA [5].

The fact that these women marry early affects their education as most of them are unable to complete school. In addition, the education of the boy child is privileged over that of the girl child, reasons for the high percentage of women who never attended school (37%) or who only succeeded in completing primary education (33%) for those who attempted (**Table 1**). Previous studies had reported that in the Adamawa Region only 14.3% of girls attempt secondary education as against 24.8% for [22]). The poor educational background of these women is a hindrance to them understanding and applying nutritional policies and interventions. As a result of poor education, most of them cannot get employment and are housewives (57%) with no income of their own, but dependent on their husbands for money to buy food and on what comes from the farms for those who do farm. Added to this, is the fact that 40% of the women studied had three or more children, who could imply reduced food intake for the mother and the children. These are risk factors for malnutrition and micronutrient deficiencies and could in part be the reason why the Adamawa Region is amongst those with a high prevalence of chronic malnutrition (40%) [22].

In addition to cereals and tubers which are staple foods, pregnant women in Ngaoundere do consume green leafy vegetables which are rich in carotenoids; and to a minor extent animal product, some with considerable Vitamin A activity. As expected, carotenoid content was highest in green leafy vegetables dishes with values ranging from 33.19 mg for Folong (*Amaranthus hybridus*) to 81.49 mg for Eru (*Gnetum bucholzianum*). Variations were observed with the type of vegetable as well as the method of preparation (**Table 2**). Vegetables of a similar variety prepared with tomatoes had higher carotenoid content than their counterparts cooked with groundnuts. Tomatoes are a good source of β-carotene [23] and this explains the increase in carotenoid content of dishes cooked with them. Groundnuts are poor in carotenoids and in addition could have a dilution effect on the quantities of carotenoids initially present in the vegetables. The use of palm oil in the preparation of certain dishes equally improved their carotenoid content. This is the case of Eru, fried Zom and Koki. Palm oil is a rich source of β-carotene [23].

Previous studies [24] had shown that ingredients added during preparation of vegetables (oil, tomatoes, carrots) enhanced carotenoid content. These authors equally demonstrated that treatments applied to vegetables such as solar drying, blanching in water or blanching with calcium carbonate salt, significantly reduced carotenoid content of the dishes. Thus, a meal cooked directly with fresh raw vegetables should have a higher carotenoid content than a dish prepared with vegetables that have been previously treated. These findings were supported in this study as could be observed in the differences in carotenoid content of different dishes prepared with a similar type of vegetable. This could also explain in part why the results obtained in this study are higher than those reported in Burkina-Faso [25] for "Yinkum" (*Solanum nigrum*) and Baobab leaves (*Adansonia digitata*), but lower than those found by in Ivory Coast [26] for "Morelle noire" (*S. nigrum*) *and* "Amarante" (*Amaranthus hybridus*). In addition Vitamin A analysis in this study was carried out on the cooked dishes while in Ivory Coast, β-carotene content was determined in the raw leaf samples [26].

Pregnant women in Ngaoundere consume on average 539.09 ± 43 µg of VA daily, corresponding to 67.4% of the recommended 800 µg. This suboptimal consumptions shows that pregnant women of Ngaoundere are exposed to Vitamin A deficiency, and consequently to other complications like night blindness, anemia, and birth defects. This level of coverage of VA needs is similar to that of 62% found in Burkina-Faso [25] with a sample of 150 households in a village of approximately 2000 inhabitants, which is an under developed country like Cameroon. These finding attest to the high prevalence of VAD in Africa reported by WHO [9].

The high carotenoid content in the dishes containing green leafy vegetables (**Table 2**) could let believe that their consumption would be enough to meet the Vitamin A requirements for these pregnant women, but these values are based on dry matter. The dishes being diluted during preparation and cooking, these contents are reduced in the cooked and consumed dishes, thus unable to meet their VA requirements. For example, 100 g of folong cooked with groundnuts as consumed by women will bring only 511.6 µg of VA. The women consume on average 65g of this soup per day, thus 332.54 µg of VA, which is far from the recommended 800 µg per day for pregnant women.

The average daily consumption of fats, proteins, fibers and sugar by the women were respectively 63.6%, 78.9%, 45.1% and 70.8% of the recommended daily intake for pregnant women. The low consumption of fats could result to a reduction in the absorption of β-carotene which is fat soluble, and to a reduction in its bioconversion to VA. Studies have shown that pure β-carotene dissolved in an oily dispersion was absorbed more efficiently (>50%) compared to carotenoids in raw fruits and vegetables like β-carotene of carrot or lycopene of to-

mato which were slightly absorbed (<3%) [27].

Food fibers reduce the bioavailability of carotenoids by trapping them, or by interacting with biliary acids having as result increase in the fecal excretion of lipids and fat-soluble substances like carotenoids [28]. Therefore, the low consumption of fibers by these women could be beneficial for their pro-Vitamin A absorption. Proteins are very important in VA metabolism. Plant proteins equally contributed to enhancing the Vitamin A status of these women. In fact retinoic acid binds to albumin at the level of intestinal mucosa to be transported in the blood [29], and VA is transported from the liver to the tissues by Retinol Binding Proteins [30]. Thus, small quantities of proteins in meals could negatively affect protein status and consequently VA absorption and transport within the body.

A total of 57.5% of the pregnant women studied were at risk for acute and severe VAD (**Figure 1**). This implies the majority of these women have limited access to VA sources. This rate is higher than that reported by Razafiarisoa [31] who carried out a nutritional study on VA deficiency in mothers (15 - 49 years) in Madagascar and found a prevalence rate of 29%. This difference could be due to the diet that is significantly different from one country to another, as well as to climate, soils, socio-economic conditions, nutritional behaviors and food patterns. In the Adamawa Region, consumption of liver and its products, red palm oil and carrots, which are very rich in Vitamin And pro-Vitamin A, is low, due to the fact that they do not enter in the food habits of most of the population. This, therefore, could contribute to the high risk of VAD (87.5%) among pregnant women in this region.

It is possible that the low VA and nutrient intake in this study could be related to the fact that, this study was carried out during the dry season when food crops and vegetables were scarce and expensive on the market, and as such quantities consumed in the household are reduced. Secondly, the majority of women in this study were in the first trimester of pregnancy, corresponding to a period when the amounts of cooked foods consumed may be reduced due to bouts of nausea resulting from hormonal changes in their body [32]. It is therefore necessary that this study be repeated during the rainy season when food crops and vegetables are in abundant supply and affordable, to establish seasonal variations.

5. Conclusion

This study reveals suboptimal dietary intake of Vitamin A, oils, proteins, fibers, sugars and energy, insufficient to cover the needs of pregnant women in Ngaoundere town. More than half of the pregnant women interviewed were exposed to acute and severe VAD and malnutrition. Thus, nutritional intervention programs should be envisaged in this area to remedy this situation as Vitamin A deficiency in the mother could lead to Vitamin A deficiency in the infant. The fact is that more than a third (41%) of the pregnant women teenagers further aggravate the problem and call for urgent solutions.

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Scientific Research

Chemical and Functional Properties of Hard-to-Cook Bean (*Phaseolus vulgaris*) Protein Concentrate

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Abstract

The objective of this research was to evaluate the chemical and functional properties of hard-to-cook (HTC) bean (*Phaseolus vulgaris*) protein concentrate to determine their potential practical applications. The respective protein concentrate was obtained from the flour using isoelectric precipitation and the protein content was 73.03%. Proximate composition and *in vitro* digestibility were measured to evaluate the chemical properties, and nitrogen solubility, emulsifying capacity, emulsion stability, foaming capacity, foam stability and viscosity were measured to evaluate its functional properties. The proximate composition of the HTC bean (*P. vulgaris*) flour and protein concentrate registered values of moisture, ash, protein, fat, fiber and NFE of 8.92, 4.52, 21.71%, 4.41%, 4.11% and 65.25% for flour and of 2.68%, 2.54%, 73.03%, 2.77%, 1.31% and 20.35% for protein concentrate. The *in vitro* digestibility was of 76.7%. The hard-to-cook bean protein concentrate exhibited good functional properties suggesting its use as additive. This concentrate registered solubility values that are ranging from 2.5% to 71.81%. The emulsifying (EC) and foaming capacity (FC) registered values of 89% - 97% and of 7% - 53% at different pH levels, respectively as well as an emulsion (ES) and foaming stability (FS) pH- and time-dependent. The HTC bean (*P. vulgaris*) protein concentrate registered a viscosity profile dependent of shear rate. The results suggest that HTC bean (*P. vulgaris*) protein concentrate is a valuable food ingredient or additive.

Keywords

Hard-to-Cook Bean, *Phaseolus vulgaris*, Protein Concentrate, Chemical and Functional Properties

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1. Introduction

Inadequate postharvest handling and storage techniques (*i.e.* temperature > 25˚C and relative humidity > 65%) produce the HTC defect in beans. This defect is the result of physical and chemical changes at the intercellular level during storage, which causes increased stability of the middle lamella during cooking. Insolubilization of pectic substances by enzyme phytase is the most widely accepted explanation. Although other possible enzymatic reactions may contribute to hardening, such as removal of methyl groups from pectins by pectinesterases, hydrolysis of storage proteins byproteases, polyphenol oxidation assisted by peroxidases or polyphenolases and, less likely, lipids oxidation by lipoxygenases. The HTC defect in beans and other legumes is considered a fundamental textural quality, and occurs when they absorb sufficient water during cooking but fail to soften. The longer required cooking time and consequently higher energy requirements for preparation of HTC seeds negatively affect their nutritional quality and make them less acceptable and marketable [1].

The rapidly growing food industry demands new ingredients. This has drawn the attention of researchers to legume components suited for wet-fractionation. At the moment, there is a strong public interest in food ingredients from natural sources. Some technological alternatives have been proposed for use of hardened beans (HTC), including extrusion [2] dry fractionation, alkaline heat treatment and soaking in saline solutions [3]. In response, the wet-fractionation process has been proposed as a means of detoxification of beans, but has also been proven as a viable technology for integral use of this seeds. Wet-fractionation produces protein concentrates, fiber rich fractions and starch fractions [4]. Alkaline extraction is a technological alternative for protein isolation from HTC beans. The concentrated protein has low trypsin inhibitor activity and meets Food and Agriculture Organization recommendations for essential amino acids content in diets for adults, meaning they are potentially useful as a supplementary vegetable protein source in food manufacturing [5].

Given the demand of the food industry for new functional ingredients, it is worthwhile to characterize the hard-to-cook bean (*Phaseolus vulgaris*) protein concentrate with a view toward establishing its possible uses and adding values to this legume seed. For the above mentioned, the objective of this research is to evaluate the chemical and functional properties of hard-to-cook bean (*Phaseolus vulgaris*) protein concentrate.

2. Materials and Methods

2.1. Seeds and Chemicals

Common black beans (*Phaseolus vulgaris* L.) var. Jamapa used in this study were obtained from local market in Mérida, Yucatán, México. Two lots of 1 kg each were used to determine cooking time and hardness following methods in applicable Mexican regulations [6]. All chemicals were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Flour Preparation

Selected seeds were processed in a disk mill (model 4-E Quaker, Mill Straub Co., Philadelphia, PA) until producing flour. The flour was then sifted through 4.76 and 2.38 mm screens and the hulls removed with a fluidizing air-bed. It was then milled in a Cyclotec mill (Tecator, Hoganas, Sweden) until passing through a 0.841-mm screen.

2.3. Protein Concentrate

A single extraction was done with 10 kg of HTC bean flour using a wet fractionation method [7], with some modifications. Briefly, whole flour was suspended in distilled water at a 1:10 (w/v) ratio, pH adjusted to 8.0 with 1.0 M NaOH, and the dispersion stirred at room temperature for 1 h at 400 rpm with a mechanical agitator (Caframo Rz-1, Heidolph Schwabach, Germany). This suspension was wet-milled with a Kitchen-Aid mill and the fiber solids separated from the starch and protein mix by straining through 0.177-mm screens and washing the residue five times with distilled water. The protein-starch suspension was allowed to sediment for 30 min at room temperature to recover the starch and protein fractions. The pH of the separated solubilized proteins was adjusted to the isoelectric point (4.3) with 1.0 N HCl. The suspension was then centrifuged at 1317 × g for 12 min (Mistral 3000i, Curtin Matheson Sci., Houston, TX), the supernatants discarded and the precipitates freeze-dried at −47C and 13×10^{-3} mbar until use (Free Zone 4.5, Labconco. Kansas City, MO).

2.4. Chemical Properties of Hard-to-Cook Bean (*Phaseolus vulgaris*) Protein Concentrate

2.4.1. Proximate Composition

Proximate composition of the HTC bean flour and the protein concentrate was calculated using official Association of Official Analytical Chemists (AOAC) procedures [8]: nitrogen (method 954.01), fat (920.39), ash (923.03), fiber (962.09) and moisture (925.09). Protein content was calculated as nitrogen × 6.25 and carbohydrate content was estimated as nitrogen-free extract.

2.4.2. *In Vitro* Protein Digestibility

This characteristic was determined according to Hsu *et al*. method [9], using a multi enzymatic solution containing 1.6 mg trypsin (Type IX Sigma T-0303 with 13,000 - 20,000 BAEE units/mg protein), 3.1 mg chymotrypsin (Type II Sigma C-4129 with ≥40 units/mg powder) and 1.3 mg peptidase (III grade Sigma P-7500 with 50 - 100 units/g powder) per millilitre. Changes in pH were measured with a potentiometer after 10 min. Apparent *in vitro* digestibility (*Y*) was measured with the follow Equation:

$$Y = 210.464 - 8.103X$$

where, X = protein suspension pH immediately after digestion with multi-enzymatic solution for 10 min.

2.5. Functional Properties of Hard-to-Cook Bean (*Phaseolus vulgaris*) Protein Concentrate

2.5.1. Nitrogen Solubility

The nitrogen solubility was determined following the procedure of Were *et al.* [10]. A total of 125 mg of protein concentrate was dispersed in 25 mL of distilled. The pH was adjusted at values of 2, 4, 6, 8 and 10 with NaOH 0.1 M or HCl 0.1 M; the solutions were stirred for 30 min at room temperature and centrifuged at 4320 × *g* for 30 min. The supernatant was analyzed for nitrogen using the AOAC (1997) method 954.01. The solubility defined as the amount of soluble nitrogen from the total nitrogen, was calculated as follows:

$$\text{Nitrogen solubility}(\%) = \frac{\text{Supernatant nitrogen concentration}}{\text{Sample nitrogen concentration}} \times 100$$

2.5.2. Emulsifying Capacity (EC) and Emulsion Stability (ES)

The EC was measured by an oil titration method similar to that of Jiménez-Colmenero and García-Matamoros [11]. A total of 300 mg of protein concentrate was dissolved in 300 mL of distilled water with 3.0% of NaCl. The pH was adjusted at values of 2, 4, 6, 8 and 10 with NaOH 0.1 M or HCl 0.1 M. The solutions were placed in the baker of a blender. A burette filled with 100% pure maize oil was placed above the beaker. A pair of electrodes connected to a multimeter was fixed to the baker to measure the electrical resistance (in ohms) of the emulsion. The solution was first stirred at 60% output of a 120 V rheostat for 45 s to make a homogenized solution and to get a constant resistance reading. The output was then increased to 100%, and the oil was immediately dispensed from the burette into the beaker at 0.5 mL/s, generating an oil-in-water emulsion at room temperature. A sudden increase in resistance was observed when the oil capacity of the sample emulsion reached a maximum value and the emulsion collapsed to form a water-in-oil emulsion. At that point, oil delivery was stopped. EC was measured in control solution 3% NaCl to subtract the value of the sample. The EC was expressed in mL of oil per mL of protein dispersion according to the following relationship:

$$\%\ \text{EC} = \frac{\text{mL of oil expended in the test sample}}{\text{mL of dispersion employed}} \times 100$$

ES was determined according Dagorn-Scaviner *et al.* [12]; to a 30 mL dispersion of protein (3 mg/mL) in water with 3.0% of NaCl were added 10 mL of 100% pure maize oil. The pH was adjusted at 5.5 with NaOH 0.1 M. The solution was stirred for 30 s to make a homogenized solution. The emulsion was placed in a graduated test tube. Simultaneously were recorded the time and the phase separation. The volume (mL) of the aqueous phase was determined at 30 s, 5, 30 and 120 min.

2.5.3. Foam Capacity (FC) and Foaming Stability (FS)

The FC and FS were determined according to the method described by Chau *et al.* [13]. 100 mL of a suspension of 1.5% hydrolyzed was prepared; the pH was adjusted at values of 2, 4, 6, 8, and 10. The suspension was stirred

at low speed in a blender for 5 min. The suspension was transferred to a 250 mL graduated test tube and recorded the volume of foam after 30 s; the FC was expressed as the percentage increase in foam volume after 30 s. The foam was allowed to stand and the volume (mL) was measured after 5, 30 and 120 minutes. The FS was determined as the volume of foam remaining after 5, 30 and 120 min.

2.5.4. Viscosity

Viscosity was evaluated using an adaptation of the Li and Chang (1997) method [14]. The protein concentrate was dispersed in water to 4% (w/v, db) and it was homogenized during 30 min at 25˚C. The viscosity was measured in a digital rheometer (AR2000, TA Instruments, New Castle, DE) with concentric cylinders geometry using a scanning speed from 0 to 1000 Hertz at 25˚C. The results were reported in Pa·s.

2.6. Statistical Analysis

All results were analyzed using descriptive statistics with a central tendency and dispersion measures. One-way ANOVAs were run to evaluate chemical properties. A LSD multiple range test was used to determine differences between treatments. All analyses were done according to Montgomery [15] and processed with the Statgraphics Plus version 5.1 software.

3. Results and Discussion

3.1. Chemical Properties of Hard-to-Book Bean (*Phaseolus vulgaris*) Protein Concentrate

The moisture, ash, protein, fat, fiber and NFE contents are shown in **Table 1**. Crude protein content in the HTC bean flour (21.71%)was similar to those reported for HTC of *P. vulgaris* (21.7%) and *P. sativum* (21.4%), but lower than this reported for HTC of *Vigna unguiculta* (25.64%) [16]. Using alkaline extraction and isoelectric precipitation, HTC bean protein concentrate crude protein content (73.03%) was similar to the 71.9% reported by Morales de León *et al.* [5]. However, protein recovery under the studied conditions was 13.65%, much less than the 36.15% reported for HTC bean [5]. Recovery was probably low due to the more neutral pH used in the present study. Values between pH 10.0 and 12.0 are more efficient, but run the risk of protein denaturation, structure modifications and destroying some relevant amino acids.

The *in vitro* digestibility of the HTC bean protein concentrate (76.7%) was notably better than that reported for raw HTC bean varieties (25% - 29%) [17], and similar to HTC *V. unguiculata* protein concentrate (75.25%) [16]. However, it was lower than for freshly harvested *P. lunatus* (79.8%) [18] or *V. unguiculata* (78.5%) [19]. Protein digestibility in common beans is inhibited by changes in the protein structure and formation complexes between the protein and starch, hemicelluloses, minerals and other proteins during storage. This implies that the factors, which control protein digestibility, are similar to those responsible for increased cooking time in HTC beans. Storage at high temperature and relative humidity increases endogenous protease activity and consequent hydrolysis of bean storage protein. This would apparently increase overall protein digestibility but this also causes increased interactions with digestibility-limiting agents such as high molecular weight tannins, which are 1.64 times greater in HTC beans [16].

Table 1. Proximate composition (% d.b.) of hard-to-cook bean flour and protein concentrate.

Component	Flour	Protein concentrate
Moisture	8.92 ± 0.47^{b}	2.68 ± 0.29^{a}
Protein	21.71 ± 0.13^{a}	73.03 ± 0.21^{b}
Fat	4.41 ± 0.21^{b}	2.77 ± 0.12^{a}
Crude fiber	4.11 ± 0.44^{b}	1.31 ± 0.12^{a}
Ash	4.52 ± 0.05^{b}	2.54 ± 0.01^{a}
NFE	65.25 ± 0.48^{b}	20.35 ± 0.20^{a}

$^{a-b}$Different superscript letters in the same row indicate statistical difference ($p < 0.05$). Data are the mean, n = 3.

3.2. Functional Properties of Hard-to-Cook Bean (*P. vulgaris*) Protein Concentrate

Solubility is one of the most important functional properties of proteins. Many of the other functional properties such as emulsification and foaming are affected by solubility. The solubility for HTC bean (*P. vulgaris*) protein concentrate ranging from 2.5% to 71.81% is shown in **Figure 1**. The nitrogen pH-solubility profile of HTC bean (*P. vulgaris*) protein concentrate showed three general regions: one of minimum solubility (pH5-6) essentially the isoelectric pH range and two of solubility maxima at pH 2 and 8. Minimum solubility in the HTC bean (*P. vulgaris*) protein concentrate was around pH 5, a level similar to that reported for minimum solubility in the protein isolates of *P. calcaratus* (5%), *Dolichoslablab* (5.08%) and *Glycine max* (5.26%) [13]. HTC bean (*P. vulgaris*) protein concentrate had good nitrogen solubility at both extremes of the pH range (acid and alkaline). A similar behavior has been reported for Chel-Guerrero *et al.* [4] in *P. lunatus* and *C. ensiformis* protein isolates. This makes the HTC bean (*P. vulgaris*) protein concentrate potentially useful in applications where high solubility profiles could have a widespread application in formulation of food systems. Possible uses as ingredient in baby food, baked products or additive in carbonated drinks, diet drinks, and desserts [4].

Emulsion capacity measures the ability of a protein to help dispersion of an oil phase into an aqueous medium [20]. The HTC bean (*P. vulgaris*) protein concentrate exhibited good EC values (89% - 97%) at different pH levels (**Figure 2**), with values higher to those of *P. lunatus* and *C. ensiformis* protein isolates (41.78% - 56.46%) [4]. The results obtained in the current study and previous studies tend to indicate that the responses of the emulsification functionality to extraction technique and conditions are dependent on the botanical source of the proteins. According to Mwasaru *et al.* [21] differences in the emulsifying activity of protein may be related to their solubility and conformational stability. Paredes-Lopez *et al.* [22] observed that the sample with the lowest

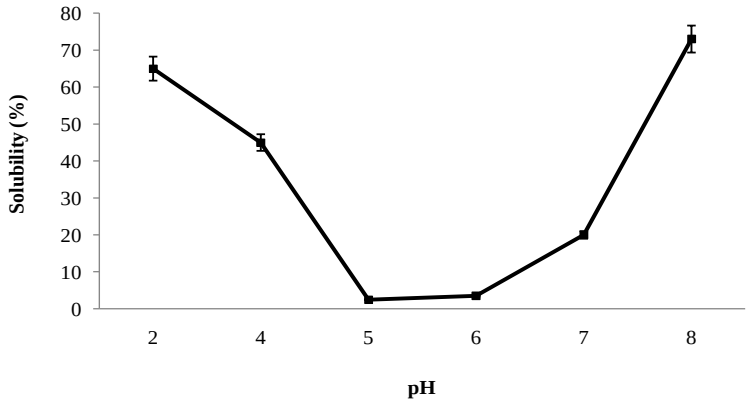

Figure 1. Solubility curve of hard-to-cook bean (*P. vulgaris*) protein concentrate. Protein solubility is expressed as percentage of soluble nitrogen at various pH values. Results are means of 3 replicates.

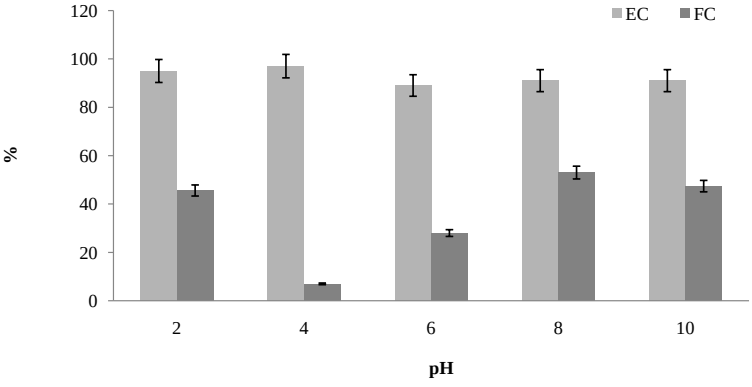

Figure 2. Emulsifying and foaming capacity of hard-to-cook bean protein (*P. vulgaris*) concentrate at different pH values. Results are means of 3 replicates.

solubility exhibited the lowest emulsifying activity and the highest emulsion stability, an observation partly consistent with the results obtained in the present study sinceHTC bean (*P. vulgaris*) protein concentrate at pH 5-6 exhibited the lowest solubility and the lowest emulsifying activity but was not the highest in emulsion stability. According to Mwasaru *et al.* [21], hydrophobicity of proteins has also been reported to influence their emulsifying properties The results obtained here may partially result of the high hydrophobic amino acids content in the HTC bean (*P. vulgaris*) protein concentrate, which allows the protein-protein interaction in the interface. Emulsion stability for HTC bean (*P. vulgaris*) protein concentrate was pH- and time-dependent reaching values of near 100% at acid pH (**Figure 3**). Interactions between proteins and lipids are common in many food systems, and thus, the ability of proteins to form stable emulsions is important. Thus, considering these emulsifying properties, HTC *P. vulgaris* protein concentrate could be used as ingredient and stabilizer in emulsion-based food formulations such as salad dressing and mayonnaise.

Foaming reflects the capacity of proteins to form stable layers surrounding gas droplets in a liquid phase. Proteins with good foaming properties should be soluble in the aqueous phase, diffuse and concentrate at the air/water interface, partially unfold to form a cohesive layer around the gas bubbles, and possess sufficient viscosity and mechanical strength to prevent rupture and coalescence of the droplets [20]. Some food proteins are capable of forming good foams, and their capacity to form and stabilize foams depends on the type of protein, degree of denaturation, pH, temperature and whipping methods. Foaming properties for HTC bean (*P. vulgaris*) protein concentrate were measured based on their whippability at pH 2.0, 4.0, 6.0, 8.0 and 10.0. Foaming capacity (FC) was pH-dependent, with the lowest value at pH 4 and the highest at pH 8. The high FC at alkaline pHs may be due to an increase in the net charge of the protein which weakens hydrophobic interactions and increases protein flexibility, allowing them to spread to the air-water interface more quickly, encapsulating air particles, and increasing foam formation. Foaming stability (FS) diminished through time (30 s, 5, 30 and 120 min) (**Figure 4**). This property was lowest at neutral pH for the established times, but higher at acid and alkaline pHs. Given these results, the relationship of hydrophilic versus hydrophobic properties is a key factor in balancing FC and FS. The poor foaming stability of HTC bean (*P. vulgaris*) protein concentrate was probably due to its protein denaturation, which would hinder formation of a stable film around the gas bubbles. Although the HTC bean (*P. vulgaris*) protein concentrate was capable of forming films with the air interface, the films were not strong enough to maintain their integrity.

The viscosity profile of HTC bean protein concentrate is shown in **Figure 5**. The results showed that to higher shear rate a higher viscosity was registered. The HTC bean (*P. vulagris*) protein concentrate can be used in food systems as thickening agents, such as in dry foods and in soup mixes, to obtain a certain viscosity when reconstituted with water.

The use *Phaseolus vulgaris* beans and protein derivatives such as flour, concentrates and isolates depends on their capacity to absorb water and soften sufficiently during soaking and cooking. Products derived from fresh beans are widely used in industry, however hard-to-cook bean they are poorly used food processors. The good

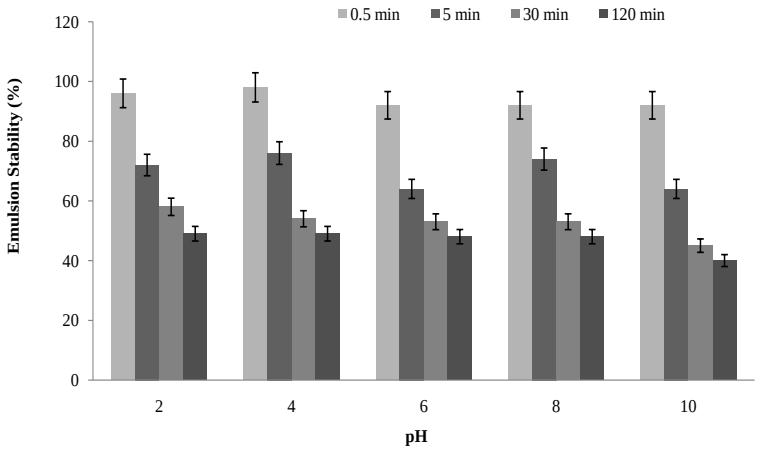

Figure 3. Emulsion stability of hard-to-cook bean protein (*P. vulgaris*) concentrate at different pH values (2 - 10).

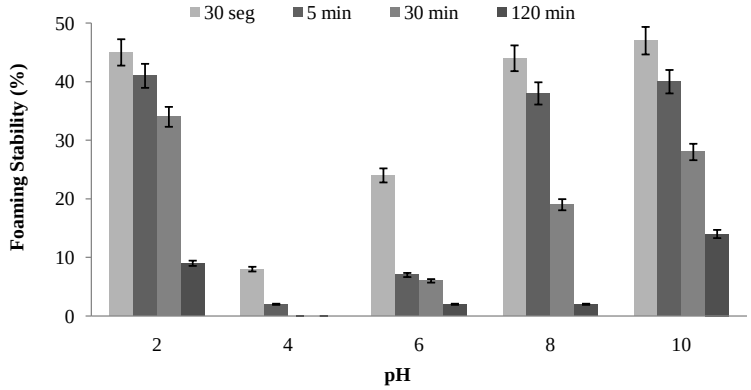

Figure 4. Foaming stability of hard-to-cook bean protein (*P. vulgaris*) concentrate at different pH values (2 - 10).

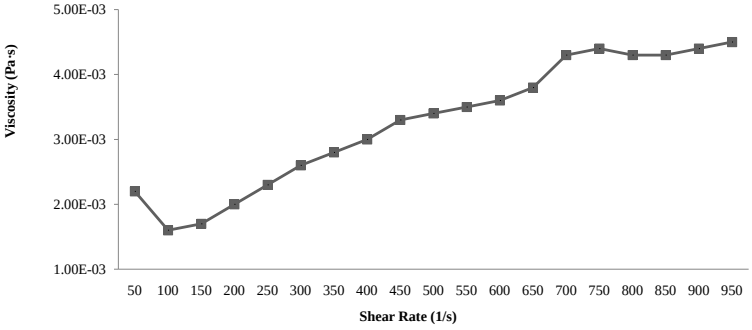

Figure 5. Viscosity profiles of HTC bean (*P. vulgaris*) protein concentrate.

properties of water interactions by the protein concentrate on study permit its use in the development of food systems with nutritional and functional quality.

4. Conclusion

The HTC bean (*P. vulgaris*) protein concentrate exhibits good functional properties. The low cost of HTC *P. vulgaris* as a substrate represents the revalorization of an agricultural product with reduced acceptability and marketability that may be transformed into a highly valuable food ingredient or additive. Because of its functional properties, the protein concentrate of HTC bean is very attractive as the functional ingredient in food systems. They can be incorporated into products such as bakery products, seasonings, and sausages among others. But sensory and texture analyses of the products are necessary.

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Isolated Soy Protein-Based Diet Ameliorates Glycemia and Antioxidants Enzyme Activities in Streptozotocin-Induced Diabetes

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Abstract

The objective of this study was to evaluate the changes induced by isolated soy protein (ISP)-based diet on glycemia and oxidative stress biomarkers in diabetic rats. Fifteen male Wistar rats (35 ± 4 g, aged 21 days) were assigned to three groups: Casein (C group), which received casein-based diet during experimental protocol; Diabetic treated with Casein (D + C group) that received casein-based diet before and after diabetes induction; Diabetic treated with ISP (D + S group) that received casein-based diet before diabetes induction and after received ISP-based diet for the experimental protocol. Diabetes was induced by a single dose of streptozotocin (50 mg/kg body weight i.v.). After three weeks of dietary treatment, total nitrates, lipid peroxidation, antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) were measured in heart homogenates. ISP-based diet promoted an improvement in the glycemic levels of diabetic rats compared with casein-based diet (362 ± 25 *vs* 461 ± 30 mg/dL). CAT activity demonstrated a significant decrease in D + C and D + S groups. D + S group presented a significant in-

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crease in SOD and GST activities. Lipid peroxidation was not different among experimental groups. The overall results suggested the potential benefits of ISP-based diet consumption to improve the life quality of diabetic patients.

Keywords

Isolated Soy Protein, Diabetes, Oxidative Stress

1. Introduction

Diabetes mellitus (DM) is a chronic disease of epidemic proportion being a major public health problem in the world. In the United States, patients with diabetes spend an average of 6.000US$ on medical costs to treat this disease. It is very important the inclusion of strategies using natural products to prevent and treat DM [1]. DM is characterized by relative or absolute deficiency of insulin secretion and/or insulin resistance that causes chronic hyperglycemia and impaired carbohydrates, lipids and proteins metabolism [2]. Hyperglycemia is an important predictor of metabolic abnormalities, it is may be caused by polyol formation, activation of kinase protein C, advanced glycation end products, and hexosamine formation [3] [4].

During hyperglycemia and its metabolic abnormalities, the diet balance, mainly about protein quality to protect against diabetes complications. Moreover, nutritional counseling is important for protecting diabetics patients against reactive oxygen species (ROS) production and modulate antioxidants defenses, such as superoxide radical (NO_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$), preventing oxidative stress and the development and progression of diabetes [5].

Antioxidants have been investigated in relation to the treatment of diabetes and its complications, such as the cardiovascular disease [6] [7]. The generation of ROS and reactive nitrogen species (RNS) inducing oxidative and nitrosative stress is related to cellular dysfunction and death in cardiomyopathy [8]. Flavonoids are one of the group of compounds that has been highly considered in recent years as polyphenolic compounds that are able to prevent oxidative injury by several mechanisms, such as ROS scavenging, protecting against chelating metal ions [9]-[11].

Soybean contains phytoestrogen (isoflavones), which are plant components that interact with mammalian endocrine systems. Furthermore, soybean products are good source of high-quality amino acids, contains no cholesterol, is low in saturated fat and it has been shown to reduce glycemia, to ameliorate glucose tolerance and insulin tests. In 2000, the American Heart Association (AHA) Nutrition Committee concluded that it is prudent to recommend including soy protein foods in a diet low in saturated fat and cholesterol [12]. Beneficial effects of soybean were demonstrated in many studies where soybean consumption prevented against some types of cancer, reduced risk of osteoporosis, ameliorated chronic renal disease, exhibited anti-atherosclerotic activity, and decreased the risk of coronary disease [13] [14].

The soybean mechanisms of action for cardiovascular system protection are associated with the antioxidant properties of genistein, daidzein, and the synergetic action of both mentioned molecules [15]. Genistein is the most abundant isoflavone and may exert its effects via mechanism such as modulation of cell signalling pathways and effects on gene expression [16]. Moreover, *in vitro* studies have suggested the hydrogen-donating ability and the inhibition of lipid peroxidation by isoflavones and their metabolites [15] [17].

Nowadays, there are a wide variety of soy products available for consumption, therefore is necessary to known about differences among the products and their effects. Isolated soy is 92.2% protein, while the soybean hypocotyl (SH) is 42.6% protein, 43.4% carbohydrates and 11.4% fat [18]. The soy products with more fat content cannot be stored for much time. By the way, this ingredient is cheaper for food industry. In relation to isoflavones, it was possibly observed that SH has greater amounts of glycoside isoflavone (daidzin), where ISP daidzein and genistein are the main isoflavones [19].

This work was conducted to evaluate the effects of an isolate soy protein (ISP) diet on glycemia and oxidative stress biomarkers in heart of streptozotocin-induced diabetes rats. We tested the hypothesis that the ISP diet would promote better glycemic control in diabetic rats and provide cardioprotective effects by improving anti-

oxidant enzymes activities in myocardium.

2. Materials and Methods

2.1. Animals, Induction of Diabetes and Experimental Protocol

Fifteen male Wistar rats with twenty one days old, weighing 35 ± 4 g were obtained from the Instituto de Ciências Básicas da Saúde (ICBS) of the Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil. All animals were housed in individual cages, received water and rat chow *ad libitum*, and were maintained under standard laboratory conditions (controlled temperature 21˚C, 12-hour light/dark cycle). Before the injection, rats were fasted for 10 hours [20]. Induction of diabetes was administrated to animals four weeks after the beginning of dietby a single injection of STZ (Sigma Chemical Co., St. Louis, MO, USA), 50 mg/kg, in tail vein. STZ was dissolved in citrate buffer (pH 4.5) and injected slowly. One week after injection, glycaemia was analyzed by the glucose oxidase method with a glycosimeter (test strips, Advantage, Roche, Brazil) in blood collected from the venous retro-orbital plexus. Rats that showed a glycemic concentration ≥ 200 mg/dL were randomized to one of the diabetic groups.

2.2. Diets

The diets were prepared according to AIN-G 93 [21] [22] and contained similar amounts of protein (adjusted according values of AIN-G 93 for protein using cornstarch), fat, carbohydrates, vitamins, minerals, except for the protein source: casein or ISP. The isoflavone content of ISP (analysis was performed at the Physical Chemistry Laboratory of the Empresa Brasileira de Pesquisa Agropecuária—EMBRAPA, Paraná, Brazil) was reported to be 189 mg/100g of ISP; 67% of isoflavones are in aglycone form, being more easily absorbed [19].

All animals were fed with the experimental diets for three weeks and were weighed weekly throughout the study. Food intake was measured three times a week. Twenty rats were allocated into three groups (n = 5/group): 1) Casein (C), euglycemic and fed with casein-based diet; 2) Diabetic Casein (D + C), streptozotocin-diabetic rat (STZ) and fed with casein-based diet; and 3) Diabetic Soy (D + S), fed with casein-based diet before diabetic induction by STZ. Seven days after diabetes induction, glycaemia was measured, if it was ≥200 mg/ dL, rats were fed ISP. All animal procedures used in this study were in accordance with the Principles of Animal Care (COBEA-Brazilian College of Animal Experimentation.

2.3. Tissue Homogenate Preparation

After 3 weeks of dietary treatment, immediately following euthanasia, the hearts were removed, cleaned, and washed in ice-cold normal saline. The hearts was homogenized in 1.15% (w/v) potassium chloride containing 1 mM phenylmethylsulfonyl fluoride in Ultra-Turrax. The homogenates were centrifuged at 1000 g for 10 min at 4˚C to discard nuclei and cell debris, and the supernatant fraction obtained was frozen at −70˚C for further measurements [23].

2.4. Nitric Oxide Metabolites

Total nitrates (NO_3^-) were determined as total nitrites (initial nitrite plus nitrite reduced from nitrate) after its reduction using nitrate reductase, from *Aspergillus* species. Nitrites (NO_2^-) were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by the reaction of nitrite with a mixture of naphthyl-ethylenediamine (0.1%) and sulphanilalamide (1%). A standard curve was established with a set of serial dilutions (10^{-8} - 10^{-3} mol/L) of sodium nitrite. Results were expressed as mmol/mg protein of nitrates plus nitrites [24].

2.5. Determination of Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of superoxide radical reaction with pyrogallol [25]. Catalase (CAT) activity was determined by following the decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm. It was expressed as nanomol of H_2O_2 reduced per minute per milligram of protein [26]. Glutathione-S-transferase activity, expressed as nanomols per minute per milligram of protein, was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm [27].

2.6. Tert-Butyl Hydroperoxide-Initiated Chemiluminescence

Chemiluminescence was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB-Produkter AB, Sweden). Homogenates were placed in low-potassium vials at a protein concentration of 0.5 - 1.0 mg/mL in a reaction medium consisting of 120 mmol/L KCl, 30 mmol/L phosphate buffer (pH = 7.4). Measurements were started by the addition of 3 mmol/L tert-butyl hydroperoxide, and data are expressed as counts per second per milligram of protein of the homogenates (cps/mg protein).

2.7. Determination of Protein Concentration

Protein was measured by the method of Lowry *et al.* [28], using bovine serum albumin as the standard.

2.8. Statistical Analysis

Data were expressed as mean values and standard error of mean (SEM) and were compared by using two-way analysis of variance. The post-hoc Bonferroni test was used to determine significant differences among individual groups. Values were considered significant when $P < 0.05$. Statistical analysis was performed using the SPSS 17.6 software.

3. Results

Typical signs of diabetes were achieved as can be seen in **Table 1**, where glycemia was increased in diabetic groups compared with their controls. The ISP diet was able to reduce glycemia in the D + S group compared with the D + C (reduction of 21%), but this value was still higher than control treatment. The average food consumption in diabetic groups was significantly higher than the control (43% and 47%, D + C and D + S respectively). Diabetic rats have also shown a decreased body weight compared with the control (38% and 44%). The heart weight was decreased in the D + C and D + S groups compared with control Heart to body weight ratio was no different among diabetic groups compared with the control group.

Table 2 presents the antioxidant enzyme activities in the heart homogenates. SOD activity was significantly increased in D + S when compared to D + C (48%). A significantly decrease was found in CAT activity when D + C (57%) and D + S (65%) was compared with control. GST activity, while the D + S group had a significant increase (52%) compared with D + C.

Figure 1 demonstrates total nitrates and lipid peroxidation. Myocardial concentration of nitrates (μmol/mg prot) was increased in D + S (1.40 ± 0.24) compared with C group (0.62 ± 0.10), being no statistically significant when comparing with D + C group (0.91 ± 0.15). The lipid peroxidation, evaluated by tert-butyl hydroperoxide-initiated chemiluminescence, was not different (207 ± 55; 212 ± 55 and 255 ± 14) among the experimental groups.

4. Discussion and Conclusions

The major findings of the present study were that glycemia decreased in diabetics rats, NO metabolites incr-

Table 1. Glycemia, Chow consumption, Body weight, Heart weight, Heart/body Weight ratio in experimental groups.

Parameters groups	C	D + C	D + S	*P*-value
Glycemia (mg/dL)	102 ± 3	461 ± 30^{b}	362 ± 25^{d}	0.002
Chow consumption (g/week)	241 ± 16	344 ± 24^{c}	355 ± 20^{b}	0.002
BW (g)	345 ± 19	212 ± 18^{b}	192 ± 8^{c}	<0.0001
HW (mg)	1153 ± 37	924 ± 59^{d}	783 ± 50^{b}	0.0007
HW/BW (mg/g)	3.50 ± 0.20	4.06 ± 0.30	4.20 ± 0.10	0.09

Body weight (BW) and heart weight (HW). The data appear as the mean ± SEM (n = 5) ($P < 0.05$, two-way ANOVA, followed by the Tukey post hoc test). C = casein; D + C = diabetic + casein; D + S = diabetic + soy. ^{a,b,c}Means with the same letter in the same raw into each parameter are not significantly.

Table 2. Antioxidant defenses in heart homogenates of experimental groups.

Parameters	C	D + C	D + S	*P*
SOD (U/mg prot)	22.2 ± 4.2	13.3 ± 1.0	19.7 ± 2.2[b]	0.02
CAT (nmol/mg prot)	170 ± 34	72 ± 14[a]	60 ± 9[b]	0.007
GST (nmol/mg prot)	10.0 ± 0.5	23.0 ± 0.2[a]	35.0 ± 1.1[b]	<0.0001

Superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST). The data appear as the mean ± SEM (n = 5) two-way ANOVA, followed by the Tukey post hoc test). [a,b,c]Means with the same letter in the same raw into each parameter are not significantly

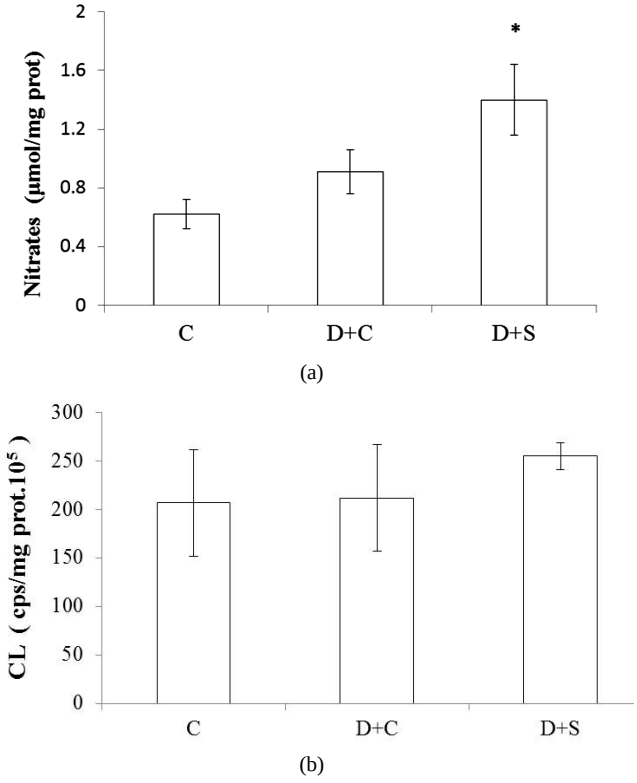

Figure 1. (a) Effects of 3 weeks of dietary treatment on the concentration of nitrates in heart homogenates (*P* = 0.02). (b) Levels of lipid peroxidation in heart homogenates after treatment. Values are expressed as mean ± SEM (5 animals/group) (*P* = 0.72), two-way ANOVA, followed by the Bonferroni post hoc test. C = casein; D + C = diabetic + casein; D + S = diabetic + soy; *vs C; **vs D + C.

eased in diabetic rats and diabetic rats presented antioxidant profile amelioration measured by antioxidant enzymes activities after ISP-based diet administration. These findings imply that the ISP –based diet might ameliorate the life quality of diabetic patients during treatment.

Initially, the induction of diabetes by STZ is the most used model for induce diabetes in mice or rats because show similar signs of diabetes in humans. This model of diabetes is frequently utilized to study diabetes and its complications. The usually symptoms of this model are elevation in plasma glucose levels, reduction in body weight, and increase in food intake. In our study we found hyperglycemia, weight loss and increased of food intake in diabetic groups. The results about the STZ model were confirm in preliminary findings of our group [29]-[31].

It's well described that type 1 diabetes results from destruction of the majority of the pancreatic beta cells, therefore expansion of the β cell mass is possible approach to its treatment [32]. Our data indicated a therapeutic effect of the ISP-based diet in the reducing glycemia in the D + S group compared with D + C, suggesting an ISP diet hypoglycemic effect. The phytoestrogen genistein and your beneficial effects on glycemic control are still unclear. The isoflavones might directly intervene in the activity of some enzymes involved on glycemic

control such as α-glucosidase and tyrosine kinase [33].

ISP-based diet begun 7 days after diabetes induction, because in the soy composition a high L-arginine amino acid concentration is found that may promote increased insulin secretion [34]. This hypothesis was demonstrated by Lu *et al.* [35] who investigated diabetic male Sprague-Dawley rats with a diet supplemented with isoflavones and observed a significant increase in insulin secretion. In fact, this effect could be due to the insulinotropic substances present in soy fractions, which induce protection of the functional β cells from further deterioration.

In terms of antioxidant enzyme activities, our results show that SOD activity was significantly increased in D + S. In agreement with our findings, another study of our group showed that SOD activity increased in rats sham-infarcted fed with ISP [21]. A preliminary study demonstrated a decrease in SOD activity in the liver of STZ-diabetic animals compared to the controls, but when genistein and ISP was administrated, the enzyme activity increased [36]. The SOD activity might be induced by ISP diet and the inhibition of the ROS generation and important regulatory effect on nitric oxide provoked by genistein [37].

GST is one of the enzymes that help the body to detoxify from products of oxidative metabolism. Another study investigated the effect of genistein supplementation in Nrf2 that in oxidative stress state is separated of keap1 and translocated to the nucleus. It activates enzymes such as heme-oxigenase-1 (HO-1), NADH (H), quinolone oxidoreductase-1(NQO-1) and GSH-Px (glutathione peroxidase). The results are reverted by genistein supplementation, supporting the hypothesis that genistein reestablish the cell homeostasis [38] [39]. This result is in agreement with us, because the increase of GST activity in D + S group may be reflecting the detoxify process in this experimental group.

Our results indicate that CAT activity, a specific enzyme to decompose H_2O_2, is decreased in D + C and D + S compared with control, showing that ISP-based diet might not altered CAT metabolism. In disagreement with our results, another study using soybean supplementation for 4 weeks in diabetics 2 subjects showed that CAT activity, evaluated in blood, was higher after supplementation period [40]. This result may suggest that ISP-based diet not influence CAT metabolism in this experimental protocol.

Besides ROS, we evaluated the metabolism of nitric oxide by nitrates concentration. A previous study demonstrated that in menopausal women the soy isoflavone had no effect on nitric oxide metabolism [40]. Another study showed that soy protein seemed to be effective improving NO generation in renal system of obese Zucker rats [40]. However, it is important to highlight that NO bioavaibility is diminished in insulin resistance. We observed that the NO metabolites were increased after ISP administration [39]. This result suggested the role of ISP-based diet ameliorating the NO bioavaibility and protecting the cardiovascular system.

In relation to lipid peroxidation, we did not observe differences among experimental groups. Diabetic animals generally present increased production and liberation into the circulation of lipid peroxides due to pathological changes [36]. This result may indicate that the antioxidant enzymes modulation in this experimental protocol is resulting in a transient compensatory mechanism by the body in an attempt to neutralize the ROS production.

In summary, the findings of the present study demonstrate that an ISP diet ameliorates the glycemia associated with modulation of the SOD and GST profile, and NO bioavailability. Collectively, our results reinforce the role of ISP as an antioxidant and a useful strategy to help the treatment of diabetes.

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Scientific Research

Inhibition of Proliferation of Breast Cancer Cells MCF7 and MDA-MB-231 by Lipophilic Extracts of Papaya (*Carica papaya* L. var. Maradol) Fruit

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Abstract

Several epidemiological studies have suggested that carotenoids have antineoplasic activities. The objective of this study was to determine the antiproliferative effect of rich carotenoid lipophilic extracts of papaya fruit pulp (*Carica papaya* L., cv Maradol) in breast cancer cells, MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative), and in non-tumoral mammary epithelial cells MCF-12F. Antiproliferative effect was evaluated using the methylthiazolydiphenyl-tetrazolium bromide (MTT) assay and testing lipophilic extracts from different papaya fruit ripening stages (RS1, RS2, RS3, RS4), at different times (24, 48 and 72 h). Papaya lipophilic extracts do not inhibit cell proliferation of MCF-12F and MDA-MB-231 cells. However, MCF-7 cells showed a significant reduction in proliferation at 72 h with the RS4 papaya extract. Results suggested that lipophilic extracts had different action mechanisms on each type of cells and therefore, more studies were required to elucidate such mechanisms.

Keywords

***Carica papaya*, Antiproliferative Activity, Breast Cancer, Carotenoids**

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1. Introduction

Cancer caused 8.2 million deaths in 2012, according to the Global Cancer Statistics, from which 521,000 were breast cancer related, and it is forecasted that by 2030 this number will increase to 22 million [1]. Recent research studies established that if oxidative stress (generated by an overproduction of free radicals) is excessive, and DNA repairing systems are surpassed, a mutagenesis and carcinogenesis could be promoted [2].

Several studies have suggested that the consumption of fruits and vegetables could reduce the risk of many chronic diseases, and have a protective effect against certain types of cancer [3]. In response to this, the US Department of Health and Human Services [4] is has recommended to increase the consumption of fruits and vegetables from 5 to 13 portions a day. The beneficial effect of diets rich in fruits and vegetables is attributed mainly to bioactive components (carotenoids, phenolic compounds, flavonoids, vitamins C and E) that provide antioxidant, antimicrobial and antiproliferative properties [5]-[7]. A high consumption of carotenoids could be associated with the reduction of the risk of breast cancer, because these types of compounds show several biological activities, from which the promotion of apoptosis in transformed cells stands out [8]. These antioxidants induce cell differentiation, repair damaged DNA, inhibit gene mutation, and activate tumor-suppressive genes [9]. Additionally, a panel of experts concluded that an inverse association of carotenoids with the risk of breast cancer is possible due to their antioxidant properties [10].

Mexico is the biggest exporter of papaya [11], a climacteric tropical fruit that has diverse bioactive components (BC) that give the fruit several antioxidant properties [12] [13]. This fruit is especially rich in carotenoids, such as lycopene, β-cryptoxantine and β-carotene [12] [14]. Most of the research done in different cell lines have focused on the utilization of isolated doses of several BCs, and have not considered the importance of employing a mix of these, like those naturally occuring in the fruit, in order to determine the beneficial effect on health of a diet rich in fruits and vegetable. The objective of this study was to evaluate the antiproliferative effect of lipophilic extract (carotenoids) of "Maradol" papaya fruit on breast cancer cell MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) and non-tumoral breast epithelial cells MCF-12F for possible use as chemopreventive agent.

2. Materials and Methods

2.1. Plant Material

Fresh papaya fruit (*Carica papaya* L., cv. Maradol) was obtained from a local market in Hermosillo, Sonora, Mexico. Fruit were selected for uniformity in size, color, level of external ripeness, and were divided into 4 lots based on four ripeness stages: RS1 (fruit with 0% - 25% skin yellow color); RS2 > 25% to 50%; RS3 > 50% to 75%; and RS4 > 75% to 100%.

2.2. Papaya Extracts Preparation

Lipophilic extracts were prepared as described previously by Yahia *et al.* [15] and Gayosso García-Sancho *et al.* [16]. Papaya flesh dry sample (0.5 g) was briefly homogenized in 10 mL of hexane:dichloromethane (1:1, v/v), using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC); then it was centrifuged at 9000 g for 10 minutes at 5˚C. The organic phase was separated and the procedure was repeated three times. For alkaline hydrolysis, 10 mL of methanolic KOH 40% (1:1, v/v) were added to extracts for 1 hour at 50˚C, and stirred at 100 rpm in a stirring bath set. After saponification, 10 mL of 10% sodium sulfate were added for phase separation, and the extracts were left for 1 hour in the dark at room temperature. Extracts were evaporated in a Rotovapor® (Büchi Labortechnik AG, Flawil, Switzerland), at 30˚C in a Buchi low pressure evaporator. Samples were re-suspended in 2 mL acetone, filtered through nylon membrane of 0.45 µm of pore size (Millipore Corp., Bedford, MA), and stored at −78˚C until their utilization in cell culture.

2.3. Cell Culture

The non-tumoral breast epithelial cell line MCF-12F and breast cancer cell lines MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) were kindly supplied by Dr C. Aceves (Instituto de Neurobiología, UNAM, Queretaro, Mexico). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin,

and 100 mg/ml streptomycin (basal medium), and then were incubated at 37˚C in a 95% humidified atmosphere containing 5% CO_2.

2.4. Determination of the Antiproliferative Activity

The antiproliferative activity of papaya extracts at four different stages of ripeness (RS) in MCF-12F and in MCF-7 and MDA-MB 231 cells, was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, as previously described [13], with some modifications. Cells were seeded at a density of 5000 cells/well, in 96-well flat-bottomed plates, in a final volume of 100 µl, and incubated for 24 hours prior to the addition of lipophilic extracts of papaya. Then, 100 µl of fresh medium were added at carotenoid concentrations of 0.92, 1.61, 2.08 and 3.27 mg/mL, which correspond to RS1, RS2, RS3, and RS4, respectively. The kind and concentrations of carotenoids in each papaya extract are shown in **Table 1**. Acetone at non-toxic level (0.5%) was used in the medium as a control. The kind and concentrations of carotenoids in the extracts were determined previously according to García-Solís *et al.* method [17], using concentrations of lipophilic extracts of 0.5%, 1% and 2% (v/v), then cells were incubated for 24, 48 and 72 h. As a positive control in each essay a treatment with 500 nM thapsigargin was included, which is considered to be a strong apoptotic inducer and inhibitor of cell proliferation [18]. Thapsigargin was dissolved in dimethyl sulfoxide (DMSO), which represents 0.1% (v/v) of the culture medium, and solvent controls were also included. MTT solution at 5 mg/ml was dissolved in 1 mL of phosphate-buffered saline (PBS); 20 µl of it was added to each of the 96 wells at 24, 48 and 72 hours of incubation, at 37˚C for 1 hour. The solution in each well containing MTT, media and dead cells was removed by suction, and formazan crystals were dissolved with 100 µl DMSO in each well. DMSO was used as solvent control. The plates were then shaken and the absorbance was measured using a micro plate reader (Multiskan Ascent®, Thermo electron corporation), at 610 nm. Cell proliferation was determined using the average of absorbance units reading from the wells and was expressed as percentage with respect to the control (untreated cells). At least three replications for each sample were used to determine cell proliferation. All experiments were performed at least in duplicate.

2.5. Statistical Analysis

Comparisons of mean values of control and treatment cells were made using ANOVA, followed by Duncan's test, between control and each treatment group. The statistical significance of difference ($P < 0.05$) for the treatment groups was determined relative to their respective control group, using the statistical software SAS version 8.0 (SAS Inst. Inc. Cary, NC, USA). At least three replications for each sample were used to determine cell proliferation, and all experiments were performed at least in duplicate.

3. Results and Discussion

The effect of carotenoids from lipophilic extracts of "Maradol" papaya, at four stages of ripeness (RS1, RS2, RS3 and RS4), on the proliferation of non-tumorogenic MCF-12F breast epithelial cells, as well as the effect on MCF-7 and MDA-MB-231 breast cancer cells is shown in **Figure 1**. In MCF-12F cells it was observed that papaya lipophilic extracts did not inhibit cell proliferation after 72h of treatment, which suggests that the main component of extracts, carotenoids, did not affect cell growth in a normal breast epithelium. Other studies observed that when using *β*-carotene a similar behavior was obtained; suggesting that carotenoids have a protective effect on normal cells by facilitating their growth [19]. This result suggests that MCF-12F cells could be a

Table 1. Contents of the main carotenoid compounds identified in the pulp of "Maradol" papaya.

Ripeness Stage (RS)	Lycopene (mg/100 g DW)	*β*-Cryptoxanthin (mg/100g DW)	*β*-Carotene (mg/100 g DW)
1	0.524^{a}	0.331^{a}	0.387^{a}
2	2.48^{b}	0.558^{b}	0.409^{a}
3	2.78^{b}	0.794^{c}	0.441^{a}
4	4.23^{c}	1.295^{d}	0.752^{b}

Different letter in the same column indicate significant differences ($P \leq 0.05$).

good model to explore the effect of papaya fruit carotenoids on initiating carcinogenesis of breast cancer.

We have reported on the physiological and biochemical changes that occur during the ripening of "Maradol" papaya [12]; but the effect of this process on the potential antiproliferative activity of the mix of carotenoid compounds in the fruit is not known; which is why we have decided to investigate the effect of carotenoids from lipophilic extracts of "Maradol" papaya, at four stages of ripeness on the proliferation of non-tumorogenic MCF-12F breast epithelial cells, as well as the effect on MCF-7 and MDA-MB-231 breast cancer cells. Cancer, one of the main causes of mortality, originates as the result of the interaction of various genetic, physical, chemical and biological factors that transform cells until they become malignant tumors [20]. Tumors are usually localized, but can disseminate to other organs, originating metastasis [1]. According to the Mexican Ministry of Health, breast cancer has become the main cause of death in Mexican women 25 years of age and older since 2006, replacing uterine cancer [21].

The effects of papaya extracts on MCF-7 cell line, which is the most utilized for studies of estrogen receptor-positive breast cancer [22], are shown in **Figure 2**. Cell proliferation levels were lower with RS3 and RS4 (67.3% and 66.8%, respectively), while proliferation has higher with RS1, and RS2 (77.3% and 75.5%, respectively), showing that lipophilic extracts of papaya reduced significantly the proliferation of MCF-7 cells, being generally more effective at 72 h of incubation . These results, which ranged from 22% and 33% in the inhibition of cell proliferation, could be related with the concentration of carotenoids contained in the extracts. In a preliminary study [12] we identified and quantified the three main carotenoids found in "Maradol" papaya at different stages of ripeness (**Table 1**), been lycopene, β-criptoxantine and β-carotene. This might indicate that bioactive

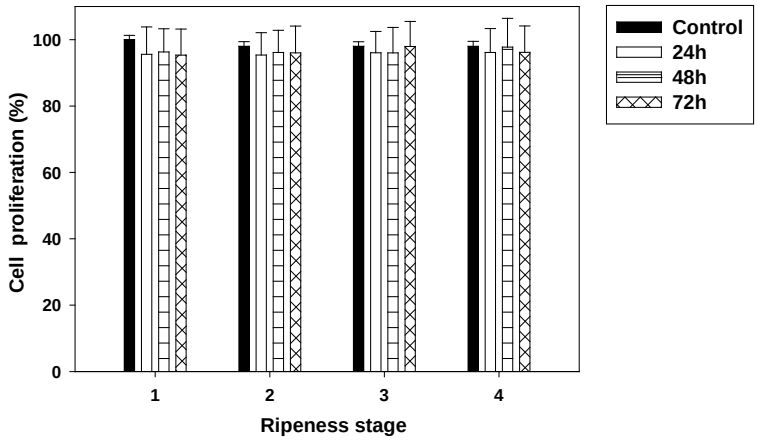

Figure 1. Effect of papaya extracts on cell proliferation in MCF-12F cells after 24, 48 and 72 h.

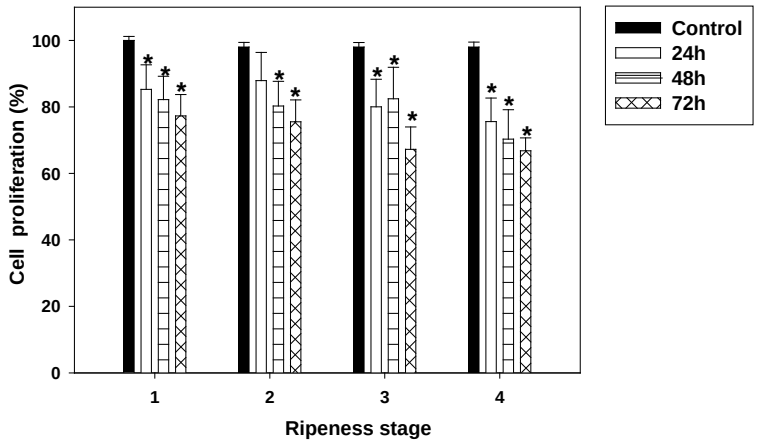

Figure 2. Effect of papaya extracts on cell proliferation in MCF-7 cells after 24, 48 and 72 h.

compounds of the extracts could act in a synergetic manner with other anticarcinogenic compounds, giving as a result an enhanced antiproliferative activity, hence, a greater health benefit [23]. Previous studies have shown the inhibition effect of carotenoids on this type of cancer cells: García Solís *et al*. [17] found that papaya extract had a significant inhibitory effect on proliferation of MCF-7 cells and Cui *et al.* [24] observed that *β*-Carotene induced apoptosis in MCF-7 cells.

Carotenoids regulate cell growth, inhibit the growth of malignant cells, and promote apoptosis in transformed cells [24]. Additionally, some individual carotenoids, such as *β*-carotene and lycopene, have inhibited the growth of MCF-7 cells *in vitro* [25]. The antiproliferative effect of aqueous extracts of 14 plant foods on the MCF-7 breast cancer cell line was evaluated [17], and only papaya extract, rich in *β*-carotene (dose of 0.85 mg/mL) after 72 h of treatment, significantly decreased cell proliferation. On the other hand, a study performed in a group of women who followed a diet rich in carotenoids, observed that the survival probability increased during the first stages of breast cancer [26]. Other studies have shown the inhibiting effect of various compounds on MCF-7 cancer cells, especially during the early stages of carcinogenesis, through antioxidant and antinflammatory routes; the induction of apoptosis; the halting of cell cycle in early tumors, and in tumors with greater development; and the potential blocking of progression and metastasis, through the expression of genes that suppress tumors [19]. Though, carotenoids present in fruits and vegetables show several beneficial properties in humans (especially lycopene and *β*-carotene), such as antioxidant activity, increased intercellular communications, modulation of insulin-like growth factor-1 (IGF1), inhibition of cell proliferation, and enhancement of immune system function [27].

We have also explored the effect papaya extracts in the proliferation of estrogen receptor–negative breast cancer cell line MDA-MB-231 (**Figure 3**). MDA-MB-231 cells did not show any significant inhibitory effect in their proliferation in frank contrast with the effect on MCF-7 cells. Moreover, a slight non-significant cell proliferation increment was observed with extract RS1 after 48 and 72 h of treatment and this behavior changed in RS2 to RS4 extracts MDA-MB-231 cells with negative response to estrogens are considered a more de-differentiated form than MCF-7 cells, therefore they are considered more aggressive [28], and this could explain why their inhibition was not significant. Our results coincided with those obtained by other researchers [29], where the use of retinoids and *β*-carotene had no inhibitory effect on this type of cells. In a study performed with anoikis-resistant MDA-MB-231 cells [30], it was found that this type of cells can turn refractory to several chemotherapeutic agents, due to a gene overexpression related to nuclear factor kappa B (*NF-κB*), which is one of the vital transcriptional cell factors, giving as a result greater aggressive behavior and greater cell resistance. Nevertheless, a possible way to reduce cell proliferation MDA-MB-231 could be the use of a mix of carotenoids, which have biological mechanisms that still have not been completely elucidated; then it would be necessary to focus on determining their molecular mechanism on this type of cell lines.

4. Conclusion

Papaya (*Carica papaya* L. var Maradol) is a tropical fruit that has significant antioxidative properties. Our re-

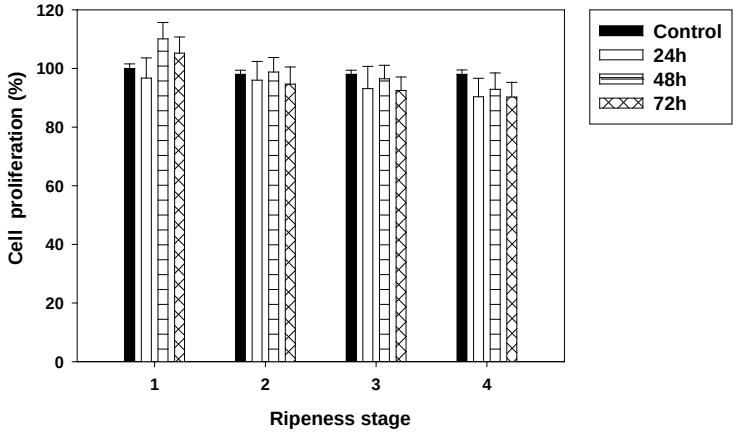

Figure 3. Effect of papaya extracts on cell proliferation in MDA-MB-231 cells after 24, 48 and 72 h.

sults showed that carotenoids present in ripened papaya could contribute to the reduction of the proliferation of MCF-7 breast cancer cells. Although the relationship between the consumption of carotenoids and breast cancer is not completely elucidated, it is necessary to continue with this type of research to be able to determine the mechanism by which these types of compounds are able to provide a beneficial effect to human health.

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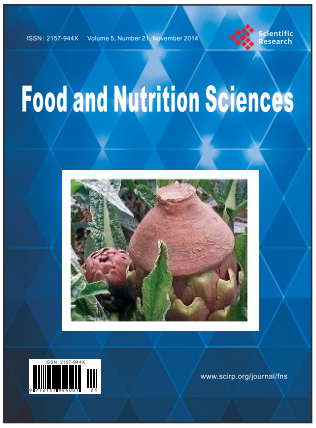

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