The 1st International Conference on Local Resource Exploitation
www.lorexp.org / info@lorexp.org
REF: LOREXP_2021_A1198 Pages: 679–696

LOREXP
LOCAL RESOURCE EXPLOITATION

Effect of Defatted *Cucurbita maxima* Duchesne seed flour Paste as Fat Replacer on Biochemical and Microbial Qualities of Beef Patty During Cold Storage

Effet de la pâte de farine délipidée des graines de Cucurbita maxima Duchesne comme substituant du gras sur les qualités biochimiques et microbiennes du pâté de bœuf conservé à froid

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ABSTRACT:

The objective of this study was to evaluate the shelf life of low-fat beef patty formulated using defatted *Cucurbita maxima* Duchesne seed flour (DCMxSF) paste as fat replacers during cold storage. The samples formulated using DCMxSF paste containing 60 % of water to replace fat at 75 (CMx360) and 100 % (CMx460) as well as the control processed using kidney's beef fat (P0) were stored at 4 and 8 °C. The markers of lipid oxidation (TBA index) and protein degradation (TVBN) as well as different groups of microorganisms were analysed during the storage to determine the shelf life. The storage study revealed that the substitution of fat at 75 and 100 % using DCMxSF paste of 60 % moisture content does not affect the stability of beef patty during cold storage. Thus, the defatted *C. maxima* seeds flour paste can serve as fat replacers in beef patty to reduce the negative effect of animal fat consumption without detrimental effect on its stability during cold storage.

Keywords: Beef patty, *Cucurbita maxima*, Defatted seeds flours, Fat replacer, Shelf-life.

RÉSUMÉ :

Le but de cette étude été d'évaluer la durée de conservation du pâté à faible teneur en gras formulé en utilisant la pâte de la farine délipidée des amandes de *Cucurbita maxima* (DCMxSF) Duchesne comme substituant du gras conservé au frais. Les échantillons formulés en utilisant la pâte de DCMxSF contenant 60 % d'eau pour remplacer le gras à 75 (CMx360) et 100 % (CMx460) ainsi que le control formulé en utilisant le gras de rognon de bœuf (P0) ont été conservés à et 8 °C. les marqueurs de l'oxydation des lipides (Indice ATB) et de dégradation des protéine (ABVT) ainsi que les différents groupes de microorganismes ont été analysés pendant le stockage pour déterminer la durée de conservation. Les résultats ont révélé que la substitution du gras à 75 et 100 % avec la pate de DCMxSF contenant 60 % d'eau n'affecte pas significativement la durée de la stabilité du pâté de bœuf conservé au frais. Ainsi, la pâte de la farine délipidée des amandes de *C. maxima* peut servir de substituant de gras dans le pâté de bœuf sans pour réduire les effets néfastes de la consommation du gras animal sans affecter négativement sa stabilité lors de la conservation.

Mots clés : Pâté de bœuf, *Cucurbita maxima*, Farine délipidée des amandes, Substituant du gras, Durée de vie.

1. INTRODUCTION

Meat and meat products qualitatively and quantitatively good sources of all macro and micronutrients (Özlem and Kemal, 2003). However, many processed meats are high fat foods, with high contents of cholesterol and saturated fatty acids and have been associated to some chronic diseases (cardiovascular disease, cancers, obesity, etc.) (Wan et al., 2011). To improve the nutritional quality of meat products, various strategies have been employed to reduce the fat content, mainly the replacement of fat with fat mimetic ingredients (Cengiz and Gokoglu, 2007). Protein rich ingredients are the most used for this purpose, soy proteins being the widely used in the form of soy flour, soy protein concentrate (Gehan and Emara, 2010). Despite of its good technological and nutritional properties, the use of soy products is limited due to their negative flavour. As an attempt to propose new alternative for fat replacement, defatted *C. maxima* seeds flour (DCMxSF) was used as fat replacer in beef patty in the previous study (Noumo et al. 2016), and results showed that replacement of fat at 75 and 100 % with hydrated DCMxSF paste containing 60 % of water improves the technological, nutritive and sensory properties of beef patty. However, as meat products, patty is highly prone to microbial contamination during processing, handling and storage and lipid oxidation during processing and storage. Microbial contamination and lipids oxidation in foods during processing and storage are the major causes of food-borne illnesses and loss of shelf life (Sachindra et al., 2005).

Patty is considered as "non-stabilised food" with a short shelf live (Fredot, 2005). Moreover, the modification of chemical composition of ready to eat meat products (patty, sausage, burger…) by incorporating plants proteins during formulation could affect their stability during storage (Arun et al., 2008). As a ready to eat food, one of the keys parameters determining the quality of beef patty is his shelf life which may be affected by the modification of the formulation thus the physicochemical properties of the final product. Spoilage is commonly detected by sensory and/or microbial analysis. Alternative methods involve the measurement of chemical changes associated with the growth of specific spoilage organisms in meat and meat products. Lipid oxidation is also one of the main factors used to estimate meat quality due to the susceptibility of meat and meat products to oxidative degeneration. The aim of this study was to evaluate the effect of fat reduction on stability of beef patty during cold storage. Thus, microbial and physico-chemical parameters of the samples were evaluated during storage at 4 °C and 8 °C.

2. MATERIALS AND METHODS

2.1. Preparation of samples

The patty samples were produced according to Noumo et al., (2016) method using DCMxSF paste to substitute fat substituted at 75 % (CMx360) and 100 % (CMx460). The control (P0) was formulated using kidney fat from beef (table 1). The defatted flour used contains mainly protein (78/100 g DM) and carbohydrates (13.82/100 g DM) and some bioactive compounds (45.66 mg/100 g DM of Vitamin C and 246.85 mg/100 g DM of polyphenols (Noumo et al., 2016). For storage study, samples were packed in metallised polyester pouches (oxygen transmission rate: 20 ml/m^2/24 h at 27 °C; water vapour transmission rate: 1.2 g/m^2/24 h at 27 °C, 65 % RH) and stored at two refrigeration temperatures, standard and common

home fridge temperatures, 4 ± 1 °C and 8 ± 1 °C respectively. The samples were withdrawn at 0, 4, 10 and every 6 days till 34 and at 0, 3, 7 and every 4 days till 28 days for samples stored at 4°C and 8°C respectively for physicochemical and microbiological analysis.

Table 1: Formulation of different samples of patty

	Main constituents (%)				**% fat**
Samples	Lean meat	Liver	Kidney fat	DCMxSF with 60 % of moisture	
P0	60	20	20	0	0
CMx360	60	20	5	15	75
CMx60	60	20	0	20	100

DCMxSF= Defatted Cucurbita maxima Duchesne seed flour

2.2. Measurement of pH of samples during storage

The pH of samples was determined after griding 10 g of sample and mixing with 90 mL of double-distilled water using a lab mortar. The pH of the suspension was recorded by dipping combined glass electrode of pHmeter (EUTECH Instruments pH Tutor, Cyberscan Oakton Instruments, Vernon Hills, USA) and the value noted from the display panel.

2.3. Total Volatile Basic Nitrogen assay by steam distillation

TVB-N represents the sum of ammonia, dimethylamine, trimethylamine and others basic nitrogenous compounds volatile under the analysis conditions (alkaline media). TVB-N was measured by steam-distillation of the TCA-patty extract, using the method of Malle and Poumeyrol (1989). Briefly, 200 ml of a 7.5 % aqueous trichloroacetic acid solution was added to 100 g sample; homogenized and centrifuged at 2500 rpm for 5 min. The supernatant containing the volatile nitrogen was filtered through a Whatman n°3 filter paper. Twenty-five ml of filtrate were loaded into the 300 ml distillation tube followed by 5 ml of 10 % (w/v) aqueous NaOH solution and few drops of paraffine oil as antifoaming agent. The mixture was distilled using a Kjeldahl-type distillator (Vapodest 2 Gerhardt). The distillate (40 mL) was collected into a beaker containing 10 ml of 4 % (w/v) aqueous boric acid solution and 3 drops of indicator (mixture of 2 parts 0.2 % alcohol methyl red solution with 1 part 0.2 % alcohol methylene blue solution). The boric acid solution turned green when alkalinized by the distilled TVB-N which was titrated with aqueous 0.025 N sulphuric acid solution using a 0.01 ml graduated micro burette. Complete neutralization was obtained when the colour turned purple on the addition of a further drop of sulphuric acid. A blank test was performed in the same conditions but without sample extract. The TVB-N content of the samples was calculated (equation 1)

$$\text{TVB-N (mg N/100 g sample)} = \frac{(V - V_0) \times N \times 14.007 \times 200}{w \times 25} \times 100 \quad (1)$$

Where: V is the volume of 0.025 N sulphuric acid solution for test sample (mL); V_0 is the volume of 0.025 N sulphuric acid solution for blank (mL); W is the weight of sample (g); N is the molarity of sulfuric acid solution (0.05 mol/L).

2.4. Thiobarbituric Acid Reactive Substances Value

The extent of lipid oxidation in patty during storage was measured in terms of Thiobarbituric Acid Reactive Substances (TBARS) value using Buege and Aust (1978) method with slight modification. TBARS were extracted from sample (2 g) after blending using 50 ml of 20 % Tri-chloroacetic acid (TCA) (containing 0.1 % tertio-hydroxylbutylquinon (TBHQ) as antioxidant and 0.1 % ethylene-diamine-tetra-acetic acid (EDTA) as metal chelator) and 50 ml of distilled water. The mixture was left undisturbed for 10 minutes for decantation, then filtered through Whatman No.1 filter paper. The filtrate (5 ml) was pipetted out in screw cap test tube and mixed with 5 ml of 0.02 M 2-Thiobarbituric acid. Colour was developed by incubating the tubes in boiling water bath for 30 minutes. The content was cooled to room temperature, mixed with 4ml of methanol and absorbance was recorded at 532 nm.

Blank tube was made using distilled water in place of sample. The results expressed as mg malondialdehyde/kg of meat were calculated from the standard curve using 2.10^{-5} M 1,1,3,3-tetraethyoxypropane (TEP) solution.

2.5. Microbial analysis

All the media used for the microbial analysis were purchased from Himedia (India) and prepared according to the manufacturer recommendations.

2.5.1. Samples preparation

Twenty-five grams of cooked patties were aseptically collected, ground in a mortar and mixed with 225 mL of sterile saline solution (8.5 % NaCl). This provides a dilution of 10^{-1}. One mL of the 10^{-1} dilution was added into a 9 mL of diluent and mixed using a vortex. This process was repeated using the successive dilution with a decreasing of the concentration by 10-fold to obtain a serial dilution of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.

2.5.2. Total mesophilic Aerobic microorganism

The pour plate method was used to evaluate the Total mesophilic Aerobic (TMA) microorganism count using Plate Count Agar (PCA) (Herrera, 2001). For the analysis, 1 ml aliquot from the serial dilution was pipetted in a Petri dish of 9 cm of diameter. About15 mL of melted and tempered PCA were promptly pour into Petri dishes. The aliquots were immediately mixed with the agar medium by tilting and rotating the Petri dishes. After agar solidification, the Petri dishes were inverted and incubate at 30 ± 1°C for 48 ± 3 h. The dishes containing 30-300 colonies count were used to calculate microbial count of the sample (N in CFU/g) (equation 2):

$$N = \frac{\mathrm{C}}{\mathrm{D}\times\mathrm{W}} \qquad (2)$$

Where C is the number of colonies count on one Petri dish, D is the dilution factor corresponding to first dilution and W is the weigth of the sample in g.

2.5.3. Coliforms and Escherichia coli

Total coliform and *E. coli* were enumerated using Levine Eosin Methylene Blue (EMB) agar (Herrera, 2001). The coliform group organisms are capable of fermenting lactose with the production of acid and gas

within 48 h at 35°C. Lactose fermenters form colonies with dark centres and transparent, colourless peripheries. The non-lactose fermenters form completely colourless colonies. On EMB agar, *E. coli* colonies are characterized by a metallic green sheen appearance.

For analysis, about 15 mL of sterile and tempered medium were pour into Petri dishes and allow for solidification for 2 to 3 h at room temperature then 0.1 ml of aliquot from each dilution was spread properly on the solidified medium until the surface was dried. The dishes were inverted and incubated at 30 ± 1°C for 48 h. The colonies were counted and used to calculate microbial count of the sample (N in CFU/g) (Eq2)

2.5.4. Lactic acid bacteria

Man, Rogosa and Sharpe (MRS) agar was used for LAB enumeration. About 15 mL of sterile and tempered medium were pour into Petri dishes and allow for solidification for 2 to 3 h at room temperature then 0.1 ml of aliquot from each dilution was spread on the solidified medium, incubated at 30 ± 1°C for 48 h and the colonies counted and used to calculate microbial count of the sample (N in CFU/g) (equation 2)

2.5.5. Staphylococcus

Enumeration of *staphylococcus* was carried out using Baird-Parker Agar (BPA). Prior to analysis, 50 ml of egg yolk tellurite potassium emulsion were mixed with 950 ml of sterile and tempered BPA. About 15 mL of sterile and tempered medium were pour into Petri dishes and allow for solidification for 2 to 3 h at room temperature then 0.1 ml of aliquot from each dilution was spread on the solidified medium and incubated at 30 ± 1°C for 48 h. In addition to their black or grey colour, *S. aureus* colonies are surrounded by a clear zone due to lipolytic activity used to differentiate *S. aureus* from other black or grey Staphylococcus colonies (Zangerl and Becker, 2012).

2.5.6. Psychrotrophic Microorganisms

These microorganisms can cause off flavours and physical defects in foods. Their growth rates are highly temperature dependent. Their presence indicates a high potential for spoilage during extended storage (Herrera, 2001). Psychrotrophic count was carried out using PCA as for Total mesophilic Aerobic microorganism (paragraph 1.5.2) but, the incubation temperature and time were 7 ± 1°C and 7 days respectively.

2.5.7. Yeast and moulds

Acidified potatoes dextrose agar (PDA) with sterile 10 % tartaric acid to pH 3.5 ± 0.1 were used for yeast and moulds analysis with the pour plate method (Herrera, 2001). The sterile and tempered medium was acidified with tartaric acid solution immediately before pouring the agar onto plates. For the analysis, 1 ml aliquot from the serial dilution was pipetted in a Petri dish of 9 cm of diameter. About 15 mL of acidified medium were promptly pour into Petri dishes, mixed with aliquots immediately, incubated at 25 ± 1°C for 3 to 5 days and the colonies counted (N in CFU/g) (equation 2).

2.6. Statistical Analysis

The experiments were carried out in triplicates and data obtained were analysed by Analysis of Variance, differences between means were tested using the Duncan Multiple Range test and correlations between variables were tested using correlation table of Pearson in Statgraphic® Centurion XVI software. Different

polynomial models were used to fit the data from microbial analysis and chemical indicators of patty, using SigmaPlot 12.5 software. For the maximum microbial count acceptable, the European Norm (EC no 94/65 of 14 December 1994 modified on 29 February 1996) (Bonnefoy et al., 2002) was used to determine the storage period whereas the limit of TVBN (20mg/kg) and TBARS (20mgMDA/kg) were fixed according to the Egyptian Organization Standardization (ESS 2005/1114, 2005).

3. RESULTS AND DISCUSSIONS

3.1. Evolution of pH of beef patty during storage

The pH has a significant impact on shelf life of meat and meat products and is therefore one of the most important parameters within the production of meat products and meat itself. The changes in the pH of beef patty during cold storage are presented in figure 1. The fat substitution rate and the storage time have significant effects on the pH ($p < 0.05$).

For the samples stored at 4°C, during the storage, there is a slight decrease in pH whereas for the samples stored at 8°C, the pH changes in two phases. In the first phase a reduction in the pH during the first 7 days of storage was observed, the samples stored at 8°C showing lower values of pH than those stored at 4°C. For both temperatures, the control (P0) recorded the lowest pH value during this period dropping from 6.23 ± 0.06 to 5.73 ± 0.05 at 4°C and from 6.67 ± 0.06 to 5.67 ± 0.06 at 8°C.

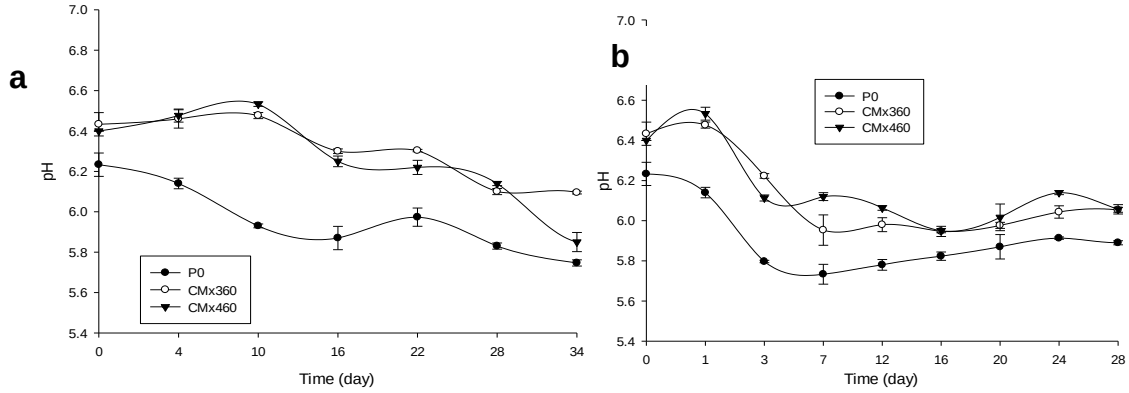

Figure 1: Evolution of pH of samples during storage at 4°C (a) and 8°C (b) (*CMx360 and CMx460: samples containing defatted C. maxima seed flour paste with 60 % of moisture content used to replace fat at 75 and 100 % respectively*).

In a second phase, there is a slight increase in pH from the 7th day till the end of the storage time. This increase reaches values of 5.98 ± 0.03 (CMx360) and 5. 96 ± 0.02 (CMx460). The decrease ($p \leq 0.05$) in the pH of the beef patty during the storage at 4°C and the first phase of storage at 8°C, could be due to the degradation of the sugar (glycogen and starch) present in the patties with the production of organic acids in the medium (Ibrahim, Hassan and Hamed, 2018). Indeed, the organic acids are the principal metabolites produced during bacterial growth (Djoulde et al., 2003). Some authors had also found a decrease in pH of sausage (Dzudie and Okubanjo, 1998) and beef burger (Al-Juhaimi et al., 2016) during cold storage.

The increase in pH observed in the second phase of storage at 8°C could be due to the proteolytic activities of microorganism and deamination of proteins, releasing peptides, amino acids and ammonia (Omafuvbe,

2006), which increases the pH of the medium. Indeed, bacteria, upon exhaustion of stored glucose, utilize amino acids released during protein breakdown and, as a product of amino acid degradation, ammonia accumulates and pH rises. The pH of meat products may rise during ageing owing to the formation of alkaline metabolic by-products (mainly NH_3) of enzyme or microbial activity, which eventually lead to spoilage (Feiner, 2006). The increase in chicken patty pH (Devendra and Tanwar, 2011) during cold storage has also been reported.

3.2. Change in total volatile basic nitrogen during storage

Total volatile basic nitrogen (TVBN) is considered the most commonly used biochemical methods for assessing meat spoilage. It is a storage stability index to microbial decomposition and endogenous enzymes of muscle proteins and non-protein nitrogenous compounds of foods (Pasdar et al., 2016). It is a group of some volatile basic nitrogenous substances (ammonia and primary, secondary and tertiary amines (dimethylamine, trimethylamine)) formed in food products during storage (Horsfall, Kinigoma and Spiff, 2006). TVBN changes of beef patties sample during storage are illustrated in figure 2. At the beginning of storage (zero day) the fat substitution rate significantly affects the TVBN values ($P \leq 0.05$). In general, the substitution of fat using the defatted seed flour paste increased the total volatile basic nitrogen in patty. The control, CMx360 and CMx460 samples exhibited 4.99 ± 0.23, 7.10 ± 0.0.61 and 7.53 ± 0.58mg N/100g respectively. The increase in TVBN might be due to the presence of nitrous non-protein compound in seed flours used as fat replacers, the breakdown of proteins as result endogenous protease activity, or the decrease in lipid/protein ratio, which resulted in more protein oxidation (Fuentes et al., 2014). These low values of TVBN indicate the good quality of raw material used for patty formulation in this study. Similar results were reported using fermented soy protein (natto) in beef burger (Abu-Salem et al., 2014). During storage, TVBN of all patties sample was significantly and positively affected by storage temperature and time. The TVBN gradually increased and proportionally with fat substitution rate and storage time (figure 2). The TVBN values of samples stored at 8°C were higher than those of the samples stored at 4°C. This may be due to the increase of microbial activity from 4°C to 8 °C. Indeed, the production of biogenic amines during the storage of food products is an extremely complex phenomenon depending on several variables, such as the growth of microorganisms, several extrinsic and intrinsic factors during the manufacturing process such as formulation, some physico-chemical parameters and enzymes (proteolytic and decarboxylase) (Makri and Douvi, 2014; Ibrahim et al., 2018) activities which interact with each other.

The sample with 100 % of fat substitution using the DCMxSF paste recorded the highest values of TVBN (23.57 ± 1.07 mg N/100g and 19.89 ± .08 mg N/100g for CMx460 after 28 and 34 days of storage at 8°C and 4°C respectively). This may be due either to the presence of nitrogenous compound easily metabolizable by microorganisms like free amino acids or peptides or the presence of proteases in CMxDSF active after hydration of flour and heating during patty production.

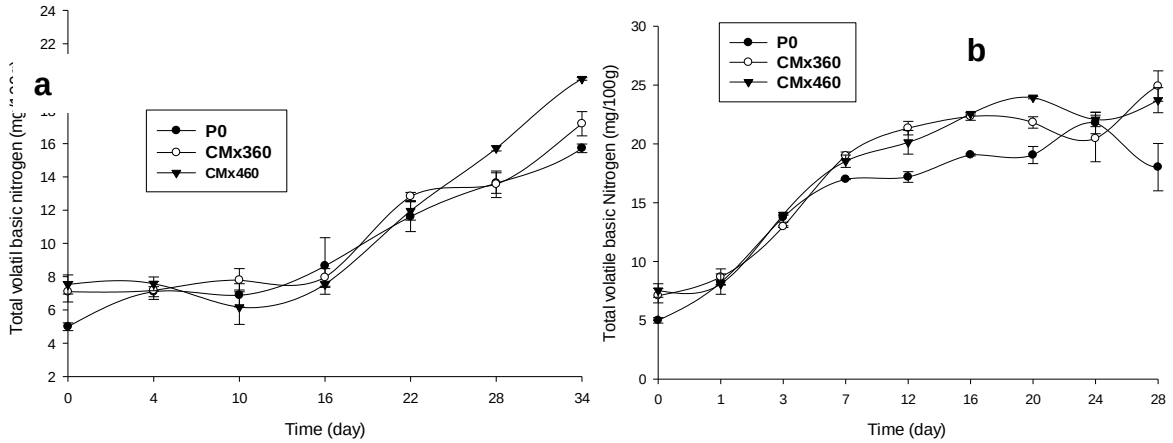

Figure 2: Evolution of TVBN of samples during storage at 4°C (a) and 8°C (b) (*CMx360 and CMx460: samples containing defatted C. maxima seed flour paste with 60 % of moisture content used to replace fat at 75 and 100 % respectively*).

Indeed, TVB-N is a measure of ammonia, and primary, secondary and tertiary amines resulting from the degradation of proteins and non-protein nitrogenous compounds (Panpipat and Chaijan, 2017). Different results were reported by Ibrahim et al. (2018) in patties containing orange peels where negative corelation was observed between TVBN and orange peels proportion. Mohamed et al. (2012) also reported negative correlation between fat substitution rate and TVBN values during storage of low fat ow-fat beef burger using soy flour as fat replacer. These differences could be due to the type of fat replacer used, the orange peels having antimicrobial properties (Ibrahim et al., 2018)

3.3. Evolution of thiobarbituric reactive substances index in beef patty during storage

Lipid oxidation is a serious problem faced by the food industry since it produces off-flavours and also decreases the nutritional quality, safety and shelf life of foods. Therefore, the control of lipid oxidation in food products is desirable and beneficial during processing and storage. TBA values generally used as indicator of lipid oxidation and is correlated with the state of rancidity. The increase of TBA values indicate an advanced state of rancidity and imply quality loss in meat products. The TBA value of different samples during storage are presented in figure 3. TBA index of samples is significantly affected by storage time and temperature. The TBA value drop with fat substitution rate from 0.47 ± 0.02 mg MDA/kg (control) to 0.10 ± 0.01 mg MDA/kg (CMx460). This is due to the reduction of fat content of beef patty as reduction of lipid/protein ratio could result in less lipid oxidation (Fuentes et al., 2014). Moreover, the high value of TBA index obtained with control may be due to the products of Strecker degradation which involves the oxidative deamination and decarboxylation of α-amino acids in the presence of lipid carbonyls, such as volatile aldehydes (Fuentes et al., 2014). The drops in TBA index have also been reported by Arun et al. (2008) using soy paste in goat meat nuggets, and by Al-Juhaimi et al. (2016) using *M. oleifera* seed flour in beef burger as fat replacers. This could be due to the reduction of lipid content of product, or to the antioxidant properties of fat replacers used.

In general, TBA index displayed an upward trend during the storage mainly for control sample from 0.47 ± 0.02 mg malondialdehyde /kg to 1.88 ± 0.03 mg MDA/kg and 2.31 ± 0.07 mg MDA/kg for the samples stored at 4°C and 8°C respectively. This increase during the storage is probably as a result of lipid oxidation

and production of volatile metabolites in the presence of oxygen (Manish et al., 2007) as the samples were packed in aerobic condition.

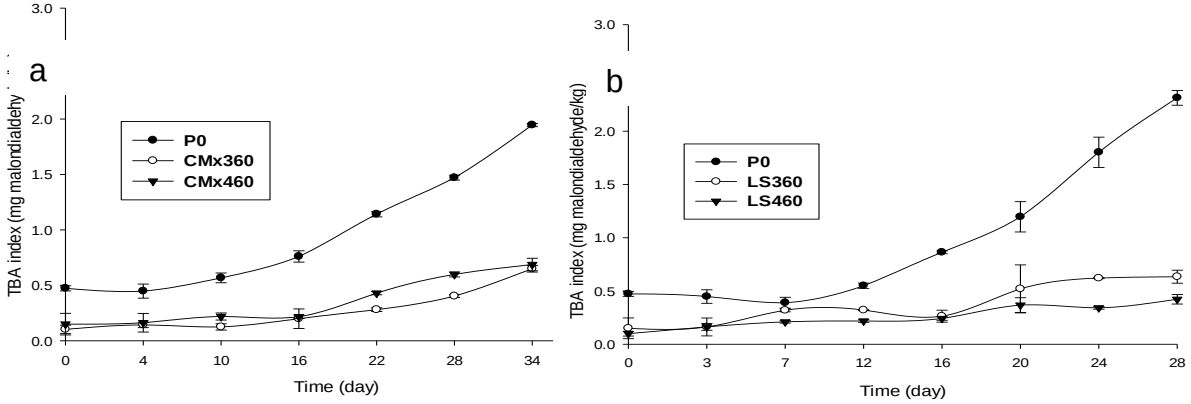

Figure 3: Evolution of TBA index of samples during storage at 4°C and 8°C (*CMx360 and CMx460: samples containing defatted C. maxima seed flour paste with 60 % of moisture content used to replace fat at 75 and 100 % respectively*).

Control samples showed the larger extent of lipid oxidation compared to low fat samples. The low variations of TBA index for the samples containing DCMxSF pastes as fat replacers could be due to the fact that reducing fat content reduced the substrate for oxidation (Kumar and Sharma, 2004) or the antioxidant activity of the flours (Al-Juhaimi et al., 2016). The low TBA value may be also due to further reaction of TBARS with amino groups of seeds proteins (Fuentes et al., 2014). Similar results have been reported during the cold storage pork patties (Manish et al., 2007) and of ham (Fuentes et al., 2014) with the increase of TBA value positively correlate to the fat content. Thus, the reduction of fat content of beef patty using defatted *C. maxima* seed flours as fat replacers reduce the lipids oxidation during cold storage.

3.4. Change in microbial counts in beef patty during storage

3.4.1. Total mesophilic aerobic microorganisms

The total mesophilic aerobic microorganisms based on Total plate count (TPC) is an important indicator for meat quality because of the effect of bacteria in spoilage and is commonly used in shelf-life studies. TPC of beef patties were evaluated and the counts (as $\log_{10}$ CFU/g) are presented in figure 4. The initial TPC of patty is significantly affected by fat substitution rate. In general, TPC ($\log_{10}$ CFU/g) increases from 2.08 ± 0.07 (P0) to 2.79 ± 0.7 (CMx460). These differences could be due to the microbial quality of the flours used as fat replacers or to the modification of the thermal conductivity of the patty by introducing the DCMxSF, resulting in lower thermal treatment and low microbial inactivation during cooking. This could also be to the high which acts as a hurdle for the growth of some microbes (Kumar and Sharma, 2004; Manish et al., 2007). The initial TPC obtained in the present study are lower than those reported in cooked buffalo sausage (3.75 $\log_{10}$ CFU/g) (Sachindra et al., 2005), in chicken sausage (4.41 $\log_{10}$ CFU/g) (Sallam, Ishioroshib and Samejima, 2004). This may be due to the quality of raw material (chemical composition, microbial count) (El-Refai, El-Zeiny and Rabo, 2014), or the cooking conditions (time/temperature). The results indicate that heat treatment used for this beef patty processing was adequate considering the microbiological quality.

During the storage, the TPC was significantly affected by storage time and temperature as well as fat substitution. As expected, the increase in storage time induced significant increase in TPC for all samples. The low TPC in the control (high fat) can be due to lipid oxidation with production of low molecular weight compounds with antimicrobial properties (Kumar and Sharma, 2004; Manish et al., 2007).

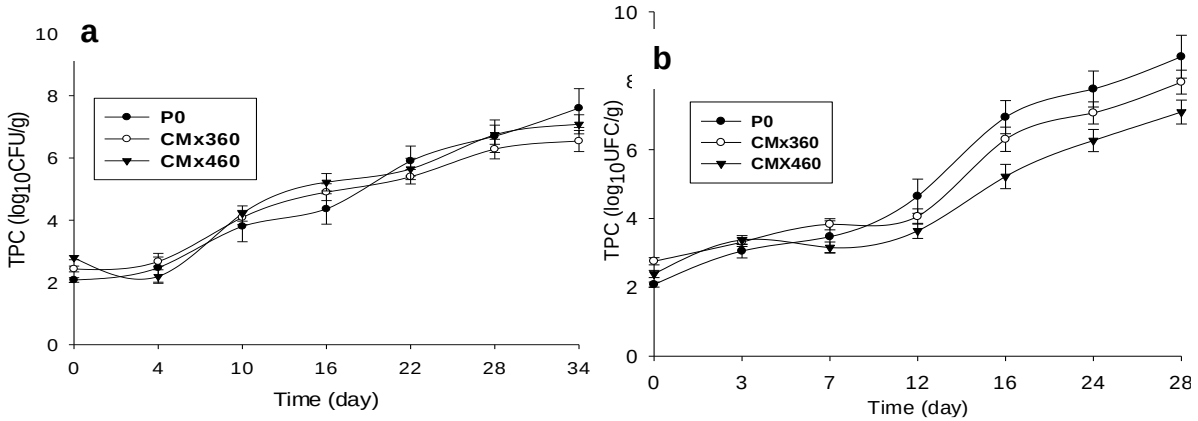

Figure 4: Evolution total viable count (TPC) of samples during storage at 4°C and 8°C (*CMx360 and CMx460, sample with fat substituted respectively at 75 % and 100 % by the paste of defatted C. maxima seeds flour containing 60 % of water; P0: control*).

The TPC increase from 2.08 ± 0.07 to 6.80 ± 0.62 (P0); 2.43 ± 0.09 to 6.54 ± 0.34 (CMx360) and 2.79 ± 0.07 to 7.08 ± 0.31 (CMx460) respectively after 34 days of storage at 4°C, and from 2.08 ± 0.07 to 8.69 ± 0.63 (P0); 2.43 ± 0.09 to 8.53 ± 0.33 (CMx360) and 2.79 ± 0.07 to 9.08 ± 0.64 (CMx460) respectively after 28 days of storage at 8°C. Thus, the samples stored at 8°C recorded higher TPC than samples stored at 4°C. This is due to the activity of microorganisms which increase from 4 to 8°C and the long latency phase at low temperature (Yabrir et al., 2018).

3.4.2. Psychrotrophic bacteria

Psychrotrophic bacteria are one of the most important groups of microorganisms in beef patties as they can alter products during cold storage and include foodborne pathogens and spoilage bacteria (Petruzzi et al., 2017). The evolution of psychrotrophic microorganisms in beef patty stored at 4 °C and 8°C was evaluated and the results are presented (as $\log_{10}$ CFU/g) in figure 5. At the initial time, psychrotrophic bacteria were non-detectable in all samples. This may be due to the microbial destruction during cooking. However, during storage, fat substitution rate, storage time and storage temperature significantly influenced the psychrotrophic bacteria growth ($p \leq 0.05$) and they were detected from the 16th of storage in all samples maintained at 4°C, from 16th day (P0) and 20th day (CMx360 and CMx460) of storage at 8°C.

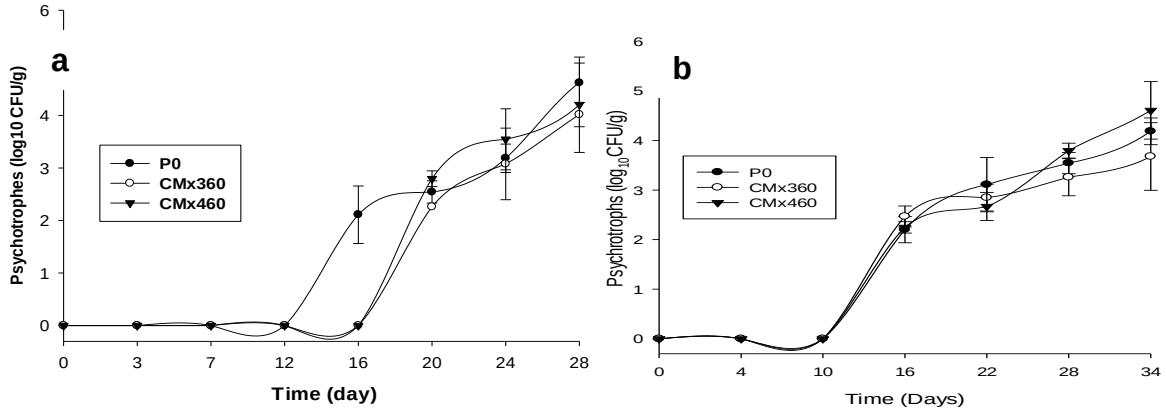

Figure 5: Evolution of psychrotrophs in samples during storage at 4°C (a) and 8°C (b) (*CMx360 and CMx460, sample with fat substituted respectively at 75% and 100 % by the paste of C. maxima defatted seeds flour containing 60 % of water; P0: control*).

This could be due to the low residual count of psychrotrophic bacteria in the samples after cooking and the different count in different samples could be due to difference in chemical composition which affect heat transfer. Psychrotrophic counts increase from (log_{10} CFU/g) 2.20 ± 0.07 to 4.19 ± 0.27 (P0); 2.45 ± 0.21 to 3.68 ± 0.68 (CMx360) and 2.25 ± 0.12 to 4.61 ± 0.58 (CMx460) respectively at the end of storage at 4°C (34 days), and from 2.10 ± 0.55 to 4.62 ± 0.37 (P0); 2.26 ± 0.23 to 4.02 ± 0.23 and 2.80 ± 0.15 to 4.20 ± 0.91 (CMx460) respectively after 28 days of storage at 8°C. At the end of the storage, the phychrotrophic counts for samples stored at 4°C and 8°C were similar while the TPC was higher in samples at stored 8 °C. This may be due to the presence at 8 °C, of strains with antibacterial properties against phychrotrophic or the high acidity of samples stored at 8 °C.

3.4.3. Lactic acid bacteria

Lactic acid bacteria (LAB) are facultative anaerobic bacteria that can grow under both anaerobic and aerobic conditions (Özpolat et al., 2014). The evolution of LAB in patties samples during storage were evaluated and the results are presented on figure **6**.

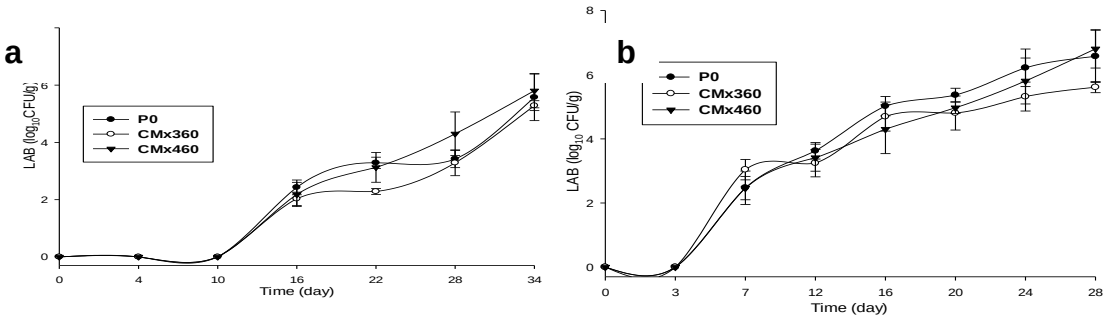

Figure 6: Evolution of lactic acid bacteria on patty samples during storage at 4°C (a) and 8°C (b) (*CMx360 and CMx460, sample with fat substituted respectively at 75 % and 100 % by the paste of C. maxima defatted seeds flour containing 60 % of water; P0: control*).

The latency phase varies from 10 days at 4°C to 3 days at 8°C. This may be due to the initial low LAB count of the raw material used, the heat treatment and the antimicrobial effect of the ingredients (nitrite, spices, DCMxSF) (Kumar and Sharma, 2004) used in the beef patty formulation. After this latency phase, the LAB counts increase are in general higher in samples stored at 8°C than those stored at 4°C. Fat substitution rate, temperature and storage time affected ($p \leq 0.05$) LAB counts. The LAB counts are (log_{10} CFU/g) 5.57 ± 0.82 (P0); 5.28 ± 0.17 (CMx360) and 5.80 ± 0.17 (CMx460) respectively after 34 days of storage at 4°C, and 6.58 ± 0.81 (P0); 5.61 ± 0.17 (CMx360) and 6.81 ± 0.60 (CMx460) respectively after 28 days of storage at 8°C. This increase of LAB count could justify the drop of pH of samples during the storage.

3.4.4. Moulds and yeast

Moulds and yeast counts of the beef patties samples during storage were evaluated and presented in figure 7.

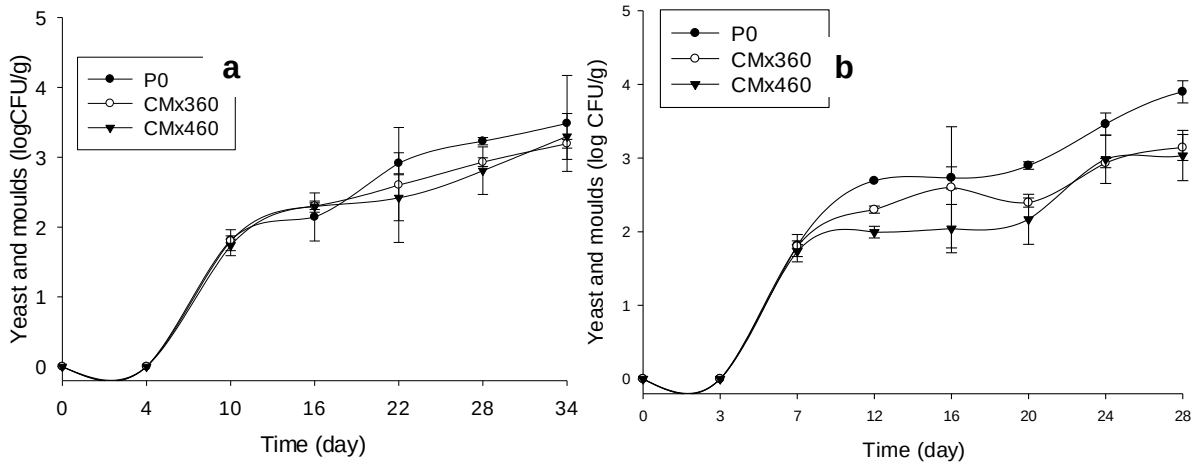

Figure 7: Evolution of yeast and moulds on patty samples during storage at 4°C (a) and 8°C (b) (*CMx360 and CMx460, sample with fat substituted respectively at 75 % and 100 % by the paste of C. maxima defatted seeds flour containing 60 % of water; P0: control*).

As for LAB, yeast and moulds were non-detectable at the initial time of storage and were detected from the 10^{th} and 7^{th} day of storage for samples stored at 4°C and 8°C respectively. It was observed that both storage time and temperature significantly ($p \leq 0.05$) affected yeast and moulds counts during storage. However, the contribution of yeast and moulds to total microbial counts during storage of patties samples is markedly very low. The low counts of this microorganisms could be due to the effect of heat treatment during the cooking of patty, to the low yeast and moulds counts in raw material and ingredients or to microbial interaction on samples during storage. At the end of the storage period, the yeast and moulds counts were (log_{10} CFU/g) 4.90 ± 0.26 (P0); 4.32 ± 0.23 (CMx360) and 4.53 ± 0.21 (CMx460) respectively at the end of storage (34 days) at 4°C, and 3.99 ± 0.25 (P0); 3.31 ± 0.20 (CMx360) and 3.72 ± 0.33 (CMx460) respectively after 28 days of storage at 8 °C. Thus, for both temperatures, the control had the highest mould and yeast counts throughout the storage period due probably to its high fat content (Panpipat and Chaijan, 2017).

3.4.5. Enterobacteriaceae

Enterobacteriaceae, is also part of the microflora of many meat products (Oğuzhan, 2013). They can play a key role in food spoilage due to their ability to metabolize amino acids to malodorous volatile compounds, such as foul-smelling diamines and sulfuric compounds (Petruzzi et al., 2017). The presence of coliforms in cook samples during storage may be attributed to the quality of heat treatment in connection to the microbial quality of the raw material and ingredients or to recontamination during handling of cooked product (Sachindra et al., 2005). Figure 8 represents the evolution of coliforms during storage of beef patties.

Coliforms were non-detectable at the initial time of storage and were detected from the 10^{th} and 16^{th} day of storage at 4 °C and 8 °C respectively. This could be due to good sanitary practices during handling and processing (Panpipat and Chaijan, 2017) or the heat treatment during cooking as core temperature is 72 °C, which is above their thermal death point of 57 °C (Manish et al., 2007). Coliform growth is influenced by storage time and temperature ($p \leq 0.05$) and is positively correlated with the storage temperature. At the end of storage time, the coliforms counts (log_{10} CFU/g) were 2.51 ± 0.32 (P0); 2.54 ± 0.16 (CMx360) and

2.79 ± 0.12 (CMx460) respectively after 34 days at 4 °C, and 4.90 ± 0.26 (P0); 3.53 ± 0.60 (CMx360) and 3.83 ± 0.15 (CMx460) respectively after 28 days at 8 °C.

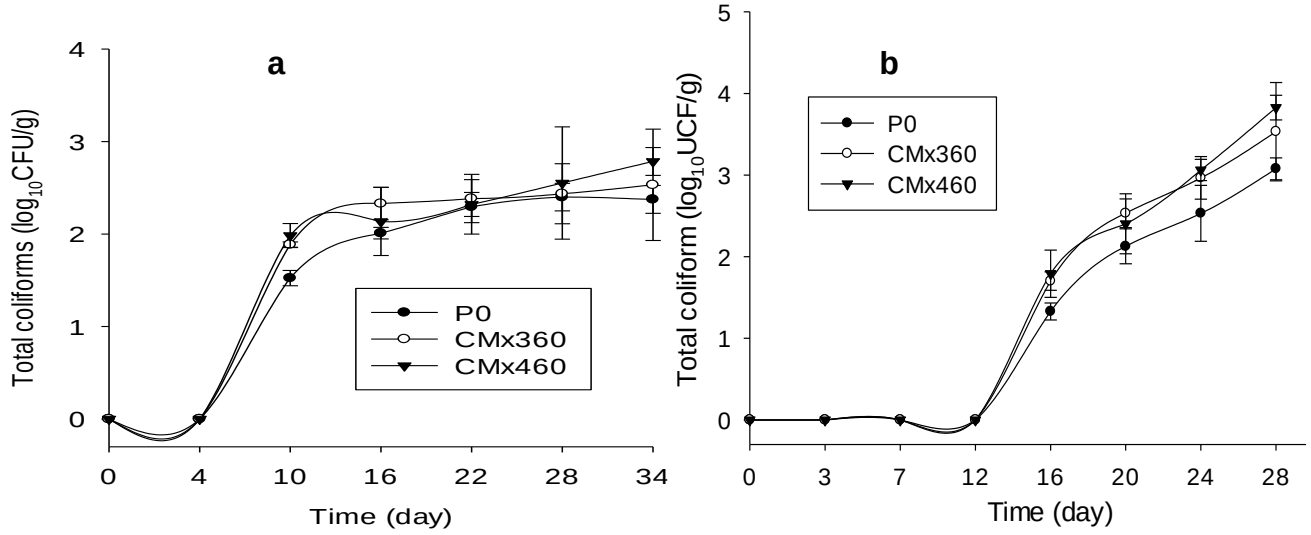

Figure 8: Evolution of coliform on patty samples during storage at 4 °C (a) and 8 °C (b) (*CMx360 and CMx460, sample with fat substituted respectively at 75 % and 100 % by the paste of C. maxima defatted seeds flour containing 60 % of water; P0: control*).

These values are lower than those reported in buffalo patty stored at 4 °C (Sachindra et al., 2005) and may be due to the difference in microbial population diversity, the cooking conditions, or the initial microbial count of raw materials. Indeed, the growth of LAB found in the samples in the present study causes a drop in pH, with possible production of antimicrobial compounds, creating non-favourable conditions for the development of other microorganisms such as Enterobacteriaceae. It has been demonstrated that lactic acid produced by LAB have inhibitory effect on the growth of spoilage bacteria and cold-tolerant pathogens (Pasdar et al., 2016).

3.4.6. Staphylococcus and Escherichia coli

The evolution of *Staphylococcus* counts of the beef patties samples during storage was evaluated and the results presented in figure 9.

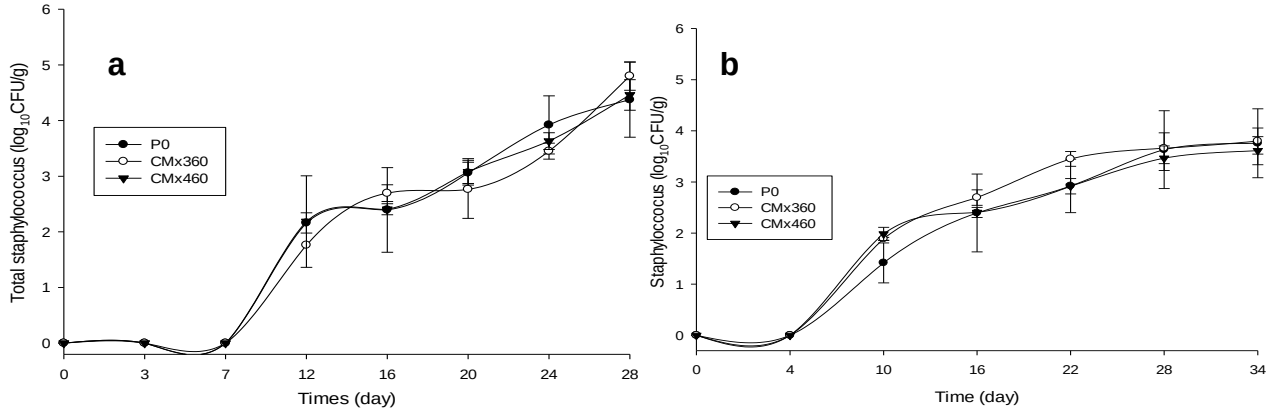

Figure 9: Evolution of *Staphylococcus* on patty samples during storage at 4 °C (a) and 8 °C (b) (*CMx360 and CMx460, sample with fat substituted respectively at 75 % and 100 % by the paste of C. maxima defatted seeds flour containing 60 % of water; P0: control*).

The *Staphylococcus* spp growth during storage was significantly ($p < 0.05$) influenced by storage time and temperature. At the initial storage time, the *Staphylococcus* spp were not detected on samples. This may be due to the low *Staphylococcus* spp count in raw material or to the heat processing. *Staphylococcus* spp were detected from the 10^{th} and 12^{th} day of storage for samples stored at 4 °C and 8 °C respectively and might also be due to good hygienic practices during handling and processing (Panpipat and Chaijan, 2017) or the cooking conditions (core temperature 72 °C).

Staphylococcus growth was influenced by storage time and temperature ($p \leq 0.05$) and was positively correlated with the storage temperature. Thus, even though the latency phase was higher at 8 °C, the growth rate of *Staphylococcus* spp was higher at 8 °C than at 4 °C and at the end of storage time, the *Staphylococcus* spp counts (log_{10} CFU/g) were 3.75 ± 0.67 (P0); 3.80 ± 0.25 (CMx360) and 3.67 ± 0.25 (CMx460) respectively at the end of storage (34 days) at 4 °C, and 4.38 ± 0.43 (P0); 4.80 ± 0.26 (CMx360) and 4.46 ± 0.27 (CMx460) respectively after 28 days of storage at 8 °C. The low *Staphylococcus* count is an indicator of good hygienic practices during handling and processing as microorganisms of *Staphylococcus* group are resistant to the cleaning due to the formation of biofilm on working surface and equipment. Moreover, the growth of LAB causes a reduction in the product pH, with possible production of antimicrobial compounds, creating unfavourable conditions for the development of other microorganisms such as *Staphylococcus*. Indeed, it has been demonstrated that lactic acid produced by LAB have inhibitory effect on the growth of spoilage bacteria and cold-tolerant pathogens (Pasdar et al., 2016). The growth of *Staphylococcus* was also reported during cold storage of smoked Gilthead Seabream (Bilgin, Ünlüsayin, Izci and Günlü, 2008).

E. coli was not detected in samples during the storage time. This attested either the effectiveness of heat processing during cooking (Sachindra et al., 2005) or the good microbial quality of raw material and ingredients. Similar results were reported in meat patties using moringa seeds flour (Al-Juhaimi et al., 2016), in buffalo patty (Sachindra et al., 2005), in *Capoeta umbla* sausages (Özpolat et al., 2014) where *E. coli* and *S. aureus* were non-detectable during the storage periods at 4 °C.

In the samples containing DCMxSF, the low microbial counts could also have been due to antimicrobial properties of DCMxSF. In fact, pumpkin seeds contain peptides and proteins that have been reported to inhibit different microorganisms like *B. cinerea, F. oxysporum* and *M. arachidicola* (Wang and Ng, 2003), *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumonia* (EI-Aziz and EI-Aziz, 2011). The microbial inhibitory effect of DCMxSF used as fat replacer could also be due to the presence of phenolic compounds, altering microbial cell permeability, interfering with membrane functions including electron transport, nutrient uptake, protein and nucleic acid synthesis (Abu-Salem et al., 2014).

3.5. Samples shelf life

Shelf-life of highly perishable food products like patty is limited due to lipid oxidation and the microbial deterioration. The effect of temperature and fat substitution rate on beef patty shelf life was investigated during storage periods. The microbial flora of the patty comprises mainly mesophilic bacteria, psychrotrophic bacteria, lactic acid bacteria, yeast and moulds. The data obtained from these groups of microorganisms and from biochemical parameters of patty spoilage (TVBN and TBARS) during storage were used for model fitting in order to determine the shelf life of different samples. The different empiric

models tested and the model used were selected based on $R^2 \geq 95$ % for all the parameters. Thus, sigmoidal model with four parameters was used for microbial growth fitting (equation 3)

$$y = y_0 + \frac{a}{1+e^{-\frac{(x-x_0)}{b}}} \quad (3)$$

and the cubic model (equation 4)

$$y = y_0 + ax + bx^2 + cx^3 \quad (4)$$

was used for biochemical parameters. The limits for microbial counts were fixed using the acceptable maximum level from French legislation (Bonnefoy et al., 2002) and the thresholds for chemical parameters were fixed using Egyptian legislation (ESS 2005/1114, 2005).

The maximum limits used for microbial criteria were 5×10^5 CFU/g, 50 CFU/g and 10^2 CFU/g for TPC, *E. coli* and *S. aureus* respectively (Bonnefoy et al., 2002). For the biochemicals markers, the maximum permissible limits were 2mg MDA/kg and 20mg TVBN /kg of patty respectively. From the data obtained, only TPC was used to determine the shelf life of samples as at the end of storage, only the values of TPC count were higher than the limit in all samples at 4 °C and 8 °C. The different shelf life obtained from these models are presented in table 2.

The temperature and the fat substitution rate significantly ($p \leq 0.05$) affect the shelf life of the samples. In general, the shelf life of samples stored at 4 °C was higher than those of samples stored at 8 °C. The shelf life of samples varies from 27.5 days (CMx460) to 29.6 days (CMx360) for samples stored at 4 °C and from 14 days (CMx460) to 16 days (P0 and CMx360) for the samples stored at 8 °C. the sample containing the DCMxSF paste used to replace fat at 100 % is the less stable. The decrease in shelf-life with the increase in fat substitution rate could be due to the increase of water activity of the product with fat substitution rate, promoting the microbial activity. The longer shelf-life at 4 °C could be due to the decrease in microbial activity with temperature.

Table 2: Shelf live (in day) of patty samples stored at 4 °C and 8 °C.

Samples	**Microbial indicators**			
	TPC[a]	TPC[b]	*S. aureus* [a]	*S. aureus*[b]
P0	**29**	16	>34	20
CMx360	29.6	16	>34	20
CMx460	27.5	14	>34	20.5
	Chemical indicators			
	TVBN[a]	TVBN[b]	TBA[a]	TBA[b]
P0	31.0	26.6	27	16
CMx360	22.0	18.2	>34	>28
CMx460	23.0	17.8	>34	>28

The symbol > shows the value lower than the limit at the end of storage period, [a] corresponds to the data obtained from samples stored at 4 °C, [b] corresponds to the data obtained from samples stored at 8 °C.

Similar results had been reported with a shelf life of 21 days during the storage of chicken sausage (Sallam, Ishioroshib and Samejima, 2004) and 21 days for low-fat ground pork patties in air permeable films

(Manish et al., 2007) stored at 4 ± 1 °C. However, a shelf life of 8 days has been reported for raw beef patties (Ibrahim et al., 2018), 14 days for air packaged *Capoeta umbla* sausages stored at 4 °C (Özpolat et al., 2014) and 16 days for air packed buffalo sausages (Sachindra et al., 2005). This difference may be due to differences in thermal processing and chemical composition of the formulated patty as many Cucurbitaceae seeds have been proved to exhibit antimicrobial and antioxidant property. This can also be due to the difference in packaging conditions (Arun *at al.*, 2008).

4. CONCLUSION

Defatted *C. maxima* seed flour hydrated at 60 % was used to replace fat at 75 and 100 %. The stability of the samples obtained along with a control during cold storage was studied. From the results obtained, the fat substitution using defatted *C. maxima* seeds flours pastes as fat replacer reduces lipid oxidation proportionally to the fat substitution rate during cold storage. The used of defatted *C. maxima* seeds flours reduced the protein degradation and lipid oxidation during cold storage. From the different groups of microorganisms evaluated during storage, only total aerobic mesophilic bacteria counts were beyond the acceptable limit at the end of the storage period. Thus, using total aerobic mesophilic bacteria counts the shelf life of samples was determined. Samples stored at 4 °C have higher shelf life, the samples in which fat was substituted at 75 % with the paste of *C. maxima* containing 60 % of water was the most stable with a shelf life of 29.6 and 16 days respectively at 4 °C and 8 °C. Thus, defatted *C. maxima* seeds flours have the potential of being used in industrial processing of beef patty as fat replacer without detrimental effect on stability of the product during cold storage.

5. ACKNOWLEDGEMENT

The authors gratefully acknowledge The World Academy of Sciences (TWAS), Italy, and The Council of Scientific and Industrial Research (CSIR), India, for the award of TWAS-CSIR Sandwich Postgraduate Fellowship.

6. CONFLICT OF INTEREST:

None.

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