Effects of Fermentation and Storage on bioactive activities of cow-Milk supplemented with soymilk.

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Previous study showed that E. faecalis DAPTO 512 (E.fc) a proteolytic lactic acid bacteria, possesses an immunomodulatory effect in milk allergy disease. The aim of this study was to evaluate the effect of fermented cow milk (FCM) alone and with added soymilk (SFCM). The fermentation was carried out using starter strains and (E.fc). A comparison of the viable cells count (VCC), changes of post-acidification and antioxidant activity of milks during refrigerated storage have been realised. In order to search an improvement of the systemic response in Balb/c mice after consumption of (SFCM). The level of IgG and IgE was determined. Four groups of mice (n=10/groups) were studied. Group 1 composed of naive mice (negative control), group 2 made of mice sensitized by oral route to cow milk after NaCl supplementation (positive control). Group 3 composed of mice administered with (FCM) before to be sensitized with oral route to cow milk. Group 4 composed of mice administered with (SFCM) before to be sensitized with oral route to cow milk. The addition of soymilk revealed a significant increase of (VCC), changes of post-acidification in fermented milks, an antioxidant activity of (SFCM) and a significant decrease in IgG and IgE in the same group. The addition of fermented soymilk with enhanced the post-acidification and the viability of bacteria during refrigerated storage. Hence, the suggestion to use this combination of soymilk and cow milk in fermentation with Enterococcus faecalis DAPTO 512 for use as functional food and to prevent the allergic risk.

Keywords: Enterococcus, soymilk, fermentation, antioxidant activity, milk allergy disease

INTRODUCTION

Fermented dairy products are the interesting sources of bioactive peptides. They provide many peptides with bioactive properties and form lactic acid and flavor compounds during fermentation and storage (Tamine et Deeth, 1980). It has been reported that active peptides in the protein sequence were defined as fragments that remain inactive in precursor protein sequences, but when released by the action of proteolytic enzymes, they may regulate the body’s physiological functions (Meisel et Bockelmann, 1999). Antioxidant peptides in foods can play an essential role in the stability of antioxidant defense systems by blocking or inhibiting the formation of free radicals (Abd El-Salam and Shibiny, 2013). Among these physiological functions, immunomodulatory (Kitts and Weiler, 2003), antiallergenic (Belkaaloul et al., 2015), antimicrobial, antihypertensive (Vermeirssen et al., 2004) and antitumourigenic properties (Hafeez et al., 2014). Milk proteins have been known to be the most important source of bioactive peptides (El Hatemi et al., 2016). It has been considered that yogurt has been considered as a functional food because it provides therapeutic effect during fermentation (Deeth and Tamime, 1981; Marshal, 1986). Therefore, recently the interest of
working on antioxidant peptides present in natural foods has been long considered, as these peptides can prevent oxidative stress and prevent the evolution of many diseases. Meenakshi et al., (2009) has reported that natural antioxidants from plant ingredients could be consumed to control the increase formation of free radicals and decrease in antioxidant capacity, it has been proven that the bioactive peptides resulting from enzymatic or bacterial hydrolysis during fermentation of soymilk (Halliwell et al.,1995; Aruoma,2003) possess a powerful power oxidative inhibitory due to their ability to scavenge free radicals (Apostolidis et al., 2006; Darmawan et al., 2010 ; Eliaas et al.,2008). The nutritional value of the fermented soy milk differs based according to the lactic acid bacteria used in the fermentation in order to develop new functional food products (Shori, 2013). Authors reported that the fermentation modifies also the protein components of bovine milk in an effort to reduce their allergenic potential (El ghaish et al., 2010; Belkaaloul et al., 2012; Belkaaloul et al., 2015), following the release of peptides which probably have antioxidant activity. Also Muguruma et al., (2012) have showed that a combination of soymilk and skimmed milk reduces osteoporosis in rats, they suggested that the potential use of fermentation to prepare mixtures of indigenous foods and dairy products for use as functional foods could be an effective technique for improving the nutritional value and could substantially improve bone condition of young people and increase the nutritional value of the food in some diseases like osteoporosis. The present study was designed in the first time to achieve a fermentation of cow milk after supplementation of soymilk, to follow the change in acid production, the viability of lactic acid bacteria, and the antioxidant activity during refrigerated storage. In the second time, to determine if the combination of plant and animal products, specifically soymilk with cow-milk would be considered as functional foods, who could probably improve the systemic response of Balb/c mice as a model of milk allergy.

MATERIALS AND METHODS

Milks used in this study for making fermented drink are: cow milk and soymilk. Cow milk was collected from local farms (Oran, Algeria) then skimmed and pasteurised. The soymilk was purchased from a local market. 6

Preparation of Starter

The bacterial strains used in this study were Streptococcus thermophilus (S.t) from feces of breast-fed new born, Lactobacillus plantarum (L.p) from Olives pomace, Enterococcus faecalis DAPTO 512 (E.fc) isolated from cow milk and identified by 16S rDNA sequence analysis. from bovine milk, belonging to the Culture Collection of the Laboratory of Physiology of the Nutrition and Food Safety, Faculty of Science of Nature and Life, University of Oran 1. The freeze-dried stocks are revivified by sowing in liquid media like this: bacteria have been activated in sterile 10 ml aliquots of de Man Rogosa and Sharpe (MRS) broth (Merck) for (L.p), into the (M17) broth (Merck) for (S.t) and into the Citrate Azide Tween Carbonate (CATC) broth (Merck) for (E.fc), at 42°C during 18 h. The cultures have been centrifuged at 5000g for 15 min to separate bacterial cells (biomass) and were washed twice with sterile distilled water. Biomass has been inoculated into skim bovine milk 12% (w/v) then incubated at 37°C for 18h as pre-culture to obtain approximately 108 cfu/mL for (S.t), 106 cfu/mL for (L.p) and 108 cfu/mL for (E.fc).

Preparation of fermented milks

Two types of fermented milks were prepared according to the method described by (Muniandy et al., 2017) with few modifications. They have been named during this study, (FCM) for fermented cow milk and (SFCM) for soymilk fermented cow milk. For the preparation of the fermented milks, we followed this protocol: the strains have been used to get a starter culture. They were grown overnight at 42°C, 18 h by inoculation of 5 mL of the stored culture into 500 mL of pasteurized skimmed cow 7 milk. In the end of the incubation, viable bacteria in the starter culture was at the rate of (108, 106,108) cfu/mL for (S.t, E.fc, L.p) respectively. For SFCM, 40 mL of soymilk was mixed with 10 mL of starter culture and 450 mL of Pasteurized milk. FCM was prepared in the same manner as previously described without soymilk. The fermented milks were then refrigerated 4°C up to 21 days. Samples of each milk type were removed from the fridge, the following day day 0 and on days 7, 14 and 21 of storage for further analysis.

Viable cell counts (VCC) of microorganisms

The change in bacterial counts of (S.t), (E.fc) and (L.p) during refrigeration (4°C) storage in FCM and SFCM was studied. The strains have been enumerated at days 0, 7, 14 and 21 on their
appropriate media agar according to the method of enumeration of bacteria using the petri dishes. Fermented milks samples (1 mL) have been removed at days 0, 7, 14 and 21. They were individually mixed with 9 mL of 0.15% sterilebuffered peptone water. The mixtures were thoroughly stirred and serial dilutions were prepared by using buffered peptone water. For the enumeration, appropriately diluted fermented milks 1 mL were transferred in the liquilified MRS, M17 and CATC agar. The mixture was evenly mixed by tilting the petri dish. Thereafter, the plates were placed in the incubator 37°C during 18 h.

**Buffered peptone water**

Twenty grams of buffered peptone have been mixed with 1L distilled water, the mixture was distributed into final tubes followed by autoclaved at 121 C for 20 min.

**Measurement of pH and titratable acidity (TA)**

The pH of FCM and SFCM have been measured by using a digital pH meter (Kika Laboretechnik, West Germany) calibrated by standard buffer solutions (Merck) at pH 4.0 and 7.0. Titratable acidity (TA) expressed in degree dornic (°D) determined as described by Belkaaloul et al., (2010).

**Preparation of fermented milk water extract FCMW and SFCMW**

Ten ml of Fermented milks FCM and SFCM samples have been mixed with 2.5 mL distilled water and the pH was adjusted to 4.0. Then FCM and SFCM were incubated in water bath (42°C) for 20 min and centrifuged (10,000 rpm, 10 min, 4°C). The pH of the supernatant has been adjusted a second time to 7.0 followed by another centrifugation (10,000 rpm, 10 min, 4°C). In the end the supernatants FCMW and SFCMW were recovered for further work.

**Measurement of antioxidant activity of FCMW and SFCMW**

The antioxidant activity was evaluated by the colorimetric technique using trolox (6-hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid, sigma) (Re et al., 1999; Salami et al., 2011) this test is based on the ability of an antioxidant to stabilize the cationic radical ABTS + (2,2-azino-bis 3-ethyl-benzthiazoline-6-sulfonic acid) provided by sigma-Aldrish. The ABTS is blue coloring with the ability to transform into ABTS + by trapping a proton by the antioxidant. A comparison was made with the trolox’s ability to capture ABTS+ (water soluble structural analogue of vitamin E). The decrease of the absorbance caused by the antioxidant reflects the catching capacity of the free radical. The result gave in μM trolox equivalent antioxidant capacity (TEAC) per (g) of product. The anti-oxidant activity was evaluated by reference to a 9 standard Trolox curve (Trolox Equivalent Antioxidant Capacity) corresponding to the concentration of Trolox having the same activity as the substance to be tested.

**Animals and diets**

This protocol was followed according to belkaaloul et al., (2015) with few modifications. Female Balb/c mice (5-7 weeks old) were purchased from Pasteur Institute (Alger, Algeria). Mice were fed and provided with water ad libitum for 1 week before use. Four groups of mice (n=10/groups) were used. Group 1 composed of naive mice was used as negative control, group 2 made of mice sensitized by oral route to cow milk after 18 days of 0,9% NaCl supplementation was used as positive control. Group 3 was constituted of mice administered for 18 days with FCM before to be sensitized with oral route to cow milk. Group 4 was constituted of mice administered for 18 days with SFCM before to be sensitized with oral route to cow milk. At the end of the experimentation, blood samples were collected for measuring antibody titers.

**Blood Sampling and determinations of antibody levels**

Blood samples were collected a day before sensitization and in the end of the experimentation. The blood was centrifuged at 3,500 rpm for 10 min at 4 °C. The serum was collected and stored in aliquots at -25 °C. Specific ant-b-Lg IgG and IgE were tested in serum samples using an enzyme linked immunosorbent assay (ELISA) according to Engvall et Perlmann, (1971).

**Statistical analysis**

Data are presented as mean ± standard error of mean (SEM). A p value of < 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Viable cell counts (VCC) of microorganisms**

The supplementation of soymilk in cow milk showed increased of VCC of (L.p). However, the
highest growth rate was observed at the beginning of storage. VCC of (L.p) showed significant reduction from (35, 66 × 106) cfu/mL to (15,16 × 106) cfu/mL for soymilk–fermented cow milk and from (8,33 × 106) cfu/mL to (5 × 106) cfu/mL for fermented cow milk during 21 days of storage (Fig.1a). Shimakawa and his collaborators in (2003) have reported that several strains of lactobacillus and Bifidobacterium were classified as potent starter in the soybean fermentation with pleasant flavor and bioactivities (Shimakawa et al., 2003). About (S.t), an increase in growth was observed in the second week (32 x106) cfu/ml. this result is in agreement with several studies. They have reported that the growth of Streptococcus thermophilus continues to grow during the first week of refrigerated storage. On the other hand, the addition of soymilk in fermented cow milk did not affect the VCC of (S.t) except on day 14 of storage, the growth decreased in SFCM but remains significantly comparable to FCM (Fig. 1b). The changes in E. faecalis counts in milks have been shown in (Fig. 1c). The highly significant growth was obtained at day 7 of storage in soymilk-fermented cow milk (29 x 106) cfu/mL, hence the growth was stable in fermented cow milk. A decrease in growth was observed in day 14 and 21. Wessels et al., (1991) have reported that Enterococci can grow in the restrictive environment of high salt content and low pH in dairy products.

**Measurement of pH and titratable acidity (TA)**

Changes in pH and TA values of fermented milks during storage are presented in (Fig. 2). pH values of all fermented milks significantly (p< 0.05) decreased after 7 12 days of storage. However SFCM showed sustained reduction in pH during 21 days of refrigerated storage from pH.5 to pH.3.6 with significant differences compared to FCM on day 7 and 14 of storage (pH. 3.7 and pH. 3.6) respectively (p< 0.05). The total acidity (Fig.2b) showed a significant increase during the storage refrigerated period for FCM and SFCM. Prolonged refrigerated storage for 2 weeks significantly increased the rate of TA to about 28.5% for FCM and 47% for SFCM. However, a reduction of TA for SFCM (p< 0.05) was observed in day 21 of storage at 5°C compared with FCM. It has been reported by authors that the post-acidification of fermented milk may occur during refrigeration due to residual metabolic activity of the bacteria in a fermented product, and the activity of β-galactosidase released by the LAB to cleave lactose at temperatures ranging from (0-5°C) (Kailasapathy et Sultana, 2003).

**Determination of antioxidant activity**

The antioxidant activity of FCMW and SFCMW was determined by an ABTS+ radical cation assay. In this assay, the decrease in absorbance was studied to observe the consumption of the colored ABTS radical. The degree of decolorization induced by a compound was compared with that induced by Trolox. The presence of soymilk during milk fermentation increased (p<0.05) the antioxidant activity in fermented cow milk. Korhonen and Pihlanto, (2003) have defined that milk-derived antioxidative peptides are comprised of several hydrophobic amino acids in sequence which are widely distributed among caseins, soymilk and gelatine in hydrolysis by proteolytic enzymes. The Trolox-equivalent antioxidant capacity (TEAC) values (in μM) in the end of fermentation found for FCMW and SFCMW were 16,33± 0.5 and 30,25±0.4 respectively (Fig.3). Moslehiashad et al. (2013) have reported the presence of hydrophobic residues in peptides that are a determinant factor in antioxidant activity. 13 These results are perfectly in line with sato et al., (2004, 2010), who have demonstrated that an antioxidant such as β-carotene inhibits the production of IgE and IgG1 against ovalbumin. At day 0 of refrigerated storage, the antioxidant activity of SFCMW was significantly higher than FCMW (p<0.05). However the antioxidant activity of SFCM showed significant reduction during 21 days of refrigerated storage 24±0, 19 cfu/mL which remains significantly compared to FCM with a concentration of 18,66±0,1 cfu/mL. Sirilun et al. (2017) mentioned that the fermented soybean is the most acceptable with several desired bioactivities. Also it had been reported that the fermented soymilk products are rich in antioxidants and isoflavones (Sirilun et al., 2017).
Figure 1 a. Changes in bacterial counts of *Lactobacillus plantarum* (10^6 cfu/ml) during refrigerated (4°C) storage. Values are presented as mean ± SEM (n = 3). *p<0.05, P-value represents the comparison of *Lactobacillus plantarum* counts between FCM and SFCM.

Figure 1 b. Changes in bacterial counts of *Streptococcus thermophilus* (10^8 cfu/ml) during refrigerated (4°C) storage milk. Values are presented as mean ± SEM (n = 3). *p<0.05, P-value represents the comparison of *Streptococcus thermophilus* counts between FCM and SFCM.
Figure 1 c. Changes in bacterial counts of *Enterococcus faecalis DAPTO 512* (10^6 cfu/ml) during refrigerated (4-°C) storage. Values are presented as mean ± SEM (n = 3). *p*<0.05, P-value represents the comparison of *Enterococcus faecalis* counts between FCM and SFCM.
Figure 2. Changes in a) pH and b) titratable acidity (TA) during refrigerated (4°C) storage. Fermented cow milk, Soymilk fermented cow milk. Values are presented as mean ± SEM (n = 3). *p<0.05, P-value represents the comparison of pH and TA between FCM and SFCM.

Figure 3. Changes in antioxidant activity in FCM and SFCM during refrigerated (4°C) storage. Values are presented as mean ± SEM (n = 3). *p<0.05, P-value represents the comparison of TEAC between FCM and SFCM.
Antibody responses
Antibody responses were characterised in Balb/c mice sensitised by oral route to cow milk. Results showed that the introduction of cow milk via oral route induced high levels of antibodies anti-β-Lg production (fig. 4). In the same time, no anti-β-Lg antibody responses were detected in negative control group during all time of the experiment. This result is in agreement with Kamer et al., (2010). They showed the existence of an anomaly between the antioxidant/prooxidant balance at the systemic level as well as an alteration of the antioxidant barrier in allergic children. In addition, the results showed a significant decrease in IgG and IgE in the SFCM group compared to the FCM (P < 0.05) (Fig.4a and Fig.4.b). Data suggest that fermented plant and milk-based products improve the indigenous microflora (probiotics) and thus optimisation of the health status by the regulation physiological or metabolic functions (Reza et al., 2013). Authors suggested an antioxidant supplementation to prevent cow’s milk protein allergy (katsuura et al., 2009, Nishida et al., 2014). In 14 addition, the contribution of a nutrition enriched with antioxidants makes it possible to decrease the risk of allergic sensitization as well as the rate of the antibodies (Patel et al., 2009). However, before any public health recommendations were made, these results need to be confirmed in varied settings.

Figure 4. a) Measure of residual antigenicity immunoglobulin G of β-lactoglobulin (β-Lg) (μg/mg) in mice sensitized to cow milk per os. b) Measure of residual allergenicity immunoglobulin E of β-lactoglobulin (β-Lg) (μg/mg) in mice sensitized to cow milk per os. Values are presented as mean ± SEM (n = 10). *p<0,05 P-value represents the comparison of IgG and IgE between FCM group and SFCM group.
CONCLUSION
The current study revealed that the addition of soymilk enhanced the post-acidification in fermented cow milk. The viability of LAB was improved in soymilk fermented cow milk in the end of fermentation and during refrigerated storage. The suggestion to prepare a fermented cow-milk supplemented with soymilk may be interesting. Thus, soymilk may be used to support the survival of LAB and antioxidant activity in fermented cow milk during refrigerated storage. It can also be used in the prevention and reduction of allergic risk. Further studies are needed to investigate the viability of these bacteria in fermented milk after simulated gastrointestinal digestion.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS
KB designed and performed the experiments and also wrote the manuscript. HK, DS and OK reviewed the manuscript. All authors read and approved the final version.

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