Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 12, No. 3, 39-50, 2022
DOI: 10.30495/jfbt.2022.19730 http://dorl.net/dor/20.1001.1.22287086.2022.12.3.4.7

Functional, Sensory and Microbial Properties of Milk Fortified by Bioactive Peptides Derived from Fish Waste Collagen

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Received: 5 September 2021 Accepted: 28 September 2021

ABSTRACT: The bio-peptides additives use for multifunctional purposes of the dairy industry. Functional beverage investigated in this article based on milk and collagen peptides is by evaluation of organoleptic, physicochemical, and microbial properties. Hence confirmed the consumption of milk in the diet, that is a source of vitamins, minerals, and other biologically active substances and valuable nutrients in the prevention and treatment of bone as based are enriched, by collagen peptides with the effect on cartilage diseases, especially the treatment of Osteoarthritis. Sensory properties were evaluated in terms of taste and smell, chemical parameters such as total protein, pH, and texture stability. Experiments were designed to optimal peptide concentration in the range of 1.5 to 3.5% of collagen peptide during the 6-day shelf life of pasteurized milk. The results showed that the optimal concentration of 2.63% (w/v) from peptides to skim milk led to an increase in total protein from 3.022 to 5.77 g / 100 ml. Acidity, density, and Solid Non-Fat (SNF) increased compared to the control sample, while the pH decreased from 6.86 to 6.60. Besides increasing the total protein and nutritional properties of milk, regarding the sensory assessment, the concentrations of 2.63 and 2.86%, respectively had the most acceptable taste and closest flavoring to the control sample without any flavors contrarily source of the extracted peptide. Based on the microbial evaluation, the addition of the minimum concentration of peptide in milk resulted in a decrease in microbial load and an increase in shelf-life.

Keywords: Collagen Peptides, Fish Wastes, Functional Beverage, Fortified Milk, Shelf Life.

Introduction

Proteins, along with carbohydrates and fats, are one of the three main macronutrients in food, which are consisting of amino acids. Over the last century, protein research has investigated the relation between nutritional and health benefits of them and especially the biological value of proteins. The biological function of certain dietary proteins has been investigated in detail. The term "bioactive proteins" refers to dietary proteins with special bioactivities that have the potential to influence health, mainly in a beneficial way (Walther *et al.*, 2011). Collagen peptides contain essential amino acids with health functions (Hays *et al.*, 2009). Considering experimental and clinical studies, and according to published research, orally administered collagen

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peptides are absorbed intestinally and accumulate in cartilage. Collagen peptides ingestion stimulates a statistically significant increase in the synthesis of extracellular matrix macromolecules by chondrocytes. These findings suggest mechanisms that might help patients affected by joint disorders such as Osteoarthritis (OA) (Bello *et al.,* 2006). That using this is a successful health promotion strategy (Dar *et al.,* 2017). For many years, bovine and pigs have been used as the common source for collagens (Jia *et al.,* 2010). But the outbreak of bovine spongiform encephalopathy (BSE) or the foot-and-mouth disease (FMD) in the collagen of animal origins that happened during the last decades has limited the use of collagen from these sources (Tang *et al.,* 2015). Scientists have found an alternative source of collagen with weak immunogenicity, biocompatible, and low chances of causing transmissible diseases. One of the alternative sources of collagen is the production of recombinant collagen using host models such as yeast or plants and marine sources (Tang *et al.,* 2015). Biologically derived peptides from marine waste processing that contain nitrogen-rich sources have potential health benefits, and proteins derived from these marine organisms are used as substrates for the production of biological peptides in functional foods (Harnedy *et al.,* 2012). In recent years, the oral consumption of collagen peptide or collagen hydrolysate investigated in several studies and effectiveness for osteoarthritis and various aspects of it have been reviewed, considering in this disease, oral medicines are not able to repair damaged tissue, and on the other hand, the high rate of patients in the world and high treatment costs along with the side effects of medicine in this complication there is the effectiveness of oral collagen peptide with positive results after three to twelve weeks of uses as a safe solution (Dar *et al.,* 2017). Collagen peptide extracted from a type of salmon, which is the fourth largest agricultural product in the world and as a result of its large volume of waste causes environmental pollution, was used to prepare and derived collagen peptide was effective in cell life as antioxidant and anti-inflammatory. Collagen peptide has potential applications in functional and fortified foods (Chen *et al.,* 2018). The proteins and special attention to their biological properties in recent years are due to the role of bioactive peptides that may be considered for non-pharmaceutical alternatives for purposes such as cosmetic, prevention, and control of diseases associated with collagen deficiency (Felician *et al.,* 2018). On the other hand, there is a strong need to search for new natural preservatives for the preservation of food. The major benefit of using antimicrobial peptides is that it preserves the food without changing its quality and it is not harmful (Wang *et al.,* 2016). Considering the problem of food spoilage, food products can be preserved by using microbes and their antimicrobial products as bio-preservatives, which improve the shelf-life of food and enhance food safety (Galvez *et al.,* 2014; Song *et al.,* 2014). The objective of this study was to investigate the production of functional milk-based drink, that is the most important source of calcium useful from the bone, and the possibility of their development with bioactive peptides containing hydrolyzed collagen.

Materials and Methods

- *Materials*

Collagen peptide (Peptan™, F/2000 Da) was enzymatically extracted from fish skin. The Peptide with a purity of more than 90 percent determined by HPLC was purchased from Rousselot Co., Ltd (Rousselot France). Pasteurized skimmed bovine milk, with total fat less than 0.05 ± 0.01 was provided from Pak Co., Ltd. (Pak Iran). All other chemicals used in this study were of analytical grade and commercially available.

- ***Preparation***

Peptide powder was dissolved in pasteurizing skimmed bovine milk at a temperature of 15ºC to form a 1.5-3.5 mg/ml solution. The mixture was maintained for 10 minutes, stirred under the conditions of room temperature, and packed in sterile plastic bottles and milk samples (control and the fortified) were stored at 4-7 °C (refrigerated) for 6 days. Parameters such as sensory acceptability, pH and acidity, SNF, alcohol stability, and microbial load were evaluated throughout the storage period.

- ***Determination of physicochemical and microbial characteristics of fortified milk***

- ***Acidity***

Acidity described according to the Handbook of Food Analysis, Dairy Products, (Dornic degree) and was calculated using Eq. 1 (ISIRI, 2001).

$$\text{Acidity (\%)} = \frac{N*0.009*100}{V} \qquad \textbf{Eq. 1}$$

Where N represents the consumed volume (ml) of 0.1N sodium hydroxide and V refers to sample volume.

- ***pH***

Measurement of the pH was carried out on the Handbook of Food Analysis, Dairy Products the estimation of pH and acidity of milk samples (control and peptide treated) and method (ISIRI, 2001).

- ***Density***

Density was measured as described by Lewis (1990) and Iranian standards (ISIRI, 1993).

- ***Alcohol stability***

Alcohol stability of milk samples (control and peptide treated) was estimated using the Handbook of Food Analysis Dairy Products standard (ISIRI, 2017).

- ***Total protein***

The Kjeldahl method was used to determine the total protein of the samples (control and peptide treated) (ISIRI, 2018).

- ***Bacterial count***

The total bacterial count measured for the samples according to the Iranian standard handbook (ISIRI, 2015).

- ***Sensory analysis***

A panel of 15 semi-trained judges was designed and asked to grade the fortified milk (fresh as well as stored milk) using the 9-point hedonic scale (1=weak and 9=strong) concerned with odor, color, texture, and taste. Further, sensory analyses of treatments were conducted on the production day (Clark *et al.*, 2009).

- ***Statistical analysis***

In this study, the response surface method in the central compound plan model was used to optimize of peptide effect. Analysis of data and drawing the graph was performed by Design-Expert version 8 (Stat-Ease Inc., Dulles, Washington, USA) to produce surface plots and to estimate the responses of the dependent variables, the Friedman's and Duncan using the SPSS (ver. 20), Duncan test was used to observe the differences among the sensory evaluation samples and Friedman's for significance level bacterial load in raw milk. Designed treatments have been presented in Table 1.

Results and Discussion

- *Acidity of peptide fortified milk*

This study showed the acidity in control skim milk was 14.4ºD and increased by adding collagen peptide. The different concentrations of collagen peptide influenced significantly acidity ($p<0.01$). Further, treatments with 1.50 and 3.50% peptide achieved minimum and maximum acidity, from 20.50 ºD - 32 ºD (on the sixth day) respectively. The skim milk was 14.4ºD and increased by the addition of collagen peptide. By increasing the concentration of peptides in the treatments, the amount of acidity increased significantly (Figure 1). The equation for acidity content obtained by the Response Surface Methodology (RSM) data indicated the effect.

Table 1. the treatment designed for preparing the peptide concentration

Row	Time (day)	Peptide concentration (%)
0	1	0
1	1	2.16
2	1	2.16
3	1	3.50
4	2	1.50
5	2	1.50
6	2	2.86
7	4	1.85
8	4	2.63
9	4	2.63
10	4	2.63
11	4	3.50
12	4	3.50
13	5	2.86
14	6	1.50
15	6	2.20
16	6	3.50

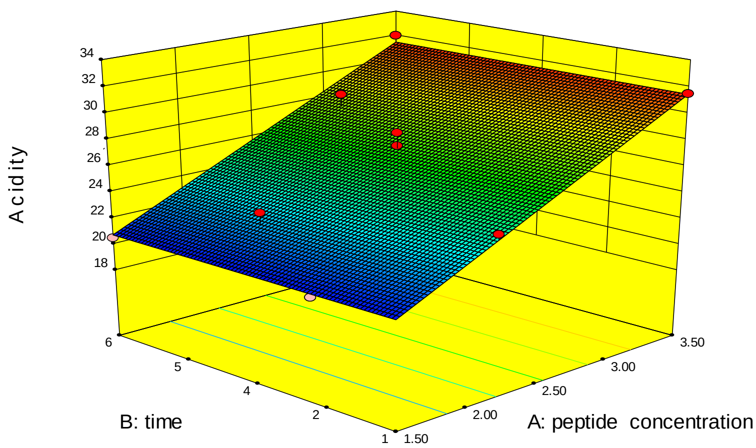

Fig. 1. Acidity increased in the fortified milk.

Acidity =+26.11+5.36 ×A **Eq. 2**

Where (A) is the concentration rate of peptide

Fortified milk with complex whey protein-Zn showed a significant ($P<0.05$) change in acidity only on the 7th day (Pralhadrao *et al*., 2021). In the Bilek study, the addition of collagen peptide significantly increased the acidity of the treatments of juice-based beverages (Bilek *et al*., 2015). Sharma et al also reported that the addition of Zn sulfate to milk significantly ($P<0.05$) affects the pH and acidity from the 3rd and 5th day of storage respectively (Sharma *et al*., 2015). However, lactose-Zn complex fortified milk and control remained comparable till the 7th day of storage (Gamal El-Din *et al*., 2012). A significant decrease of acidity by collagen peptide may cause the lower size of peptide (2000 Da) with the prolonged hydrolysis, more and more free amino acids were produced (Wang *et al*., 2012).

- ***The pH of peptide fortified milk***

The effects of the different peptide concentrations on pH have been shown during 6-day shelf life with sample skim-milk non-significant. The pH was one of the important characteristics of milk that varied between 6.6-6.9 and the model maximum and minimum pH was following maximum concentration on the first day and minimum concentration on the sixth day, also pH in the control sample was 6.6. Collagen peptide fortified milk showed a non-significant ($P<0.05$) difference in pH with control, although the maximum pH belongs to the treatment of maximum concentration on the first day (pH 6.86) and minimum pH refer to treatment with a minimum concentration on the sixth day (pH 6.60) (Figure 2). pH variation non-significant in research of Pralhadrao *et al* (2021) as fortified milk with complex whey protein-Zn, nevertheless in the investigation, of the juice-based beverage the addition of collagen hydrolyzate increased the pH (Bilek *et al*., 2015). This could be due to the pH changes of milk and might be generally affected by different factors including protein compounds, enzymes activity, differentiation of the acids existing in milk, and buffer state of milk, which is mainly developed by the existence of colloidal phosphates and acids such as lactic acid, citric acid, phosphoric acid and their salts (Walstra *et al*., 1999).

- ***Density of peptide fortified milk***

The density of 1.5% peptide-treated milk was 1.039 (g/ml) (on the second day). The density of skim milk is higher than the complete milk as it was in the control sample (1.035 g/ml). The added peptide has increased the milk density and the results had a significant effect at different concentrations during storage time. The lowest density (1.038) (g/ml) was for the treatment of 1.5% peptide (on the sixth day) and the highest density was for the treatment with 3.5% peptide (1.044) (g/ml) on the fourth day. The density of milk is affected by the densities of its various components in milk (Walstra *et al***.,** 1999). The density of milk fortified with peptide was significantly ($P<0.01$) increased during storage. The effect of density increasing has first-order by peptide concentration and second-order by time (Figure 3). The equation for density showing the effects of time and concentration in the Response Surface Methodology data is presented;

$$\text{Density}=+1.03198+4.15855\times10^{-003}\times A-7.33469\times10^{-004}\times B-3.79992\times10^{-005}\times A\times B-2.60213\times10^{-004}\times A^2-1.02633\times10^{-004}\times B^2$$ **Eq. 3**

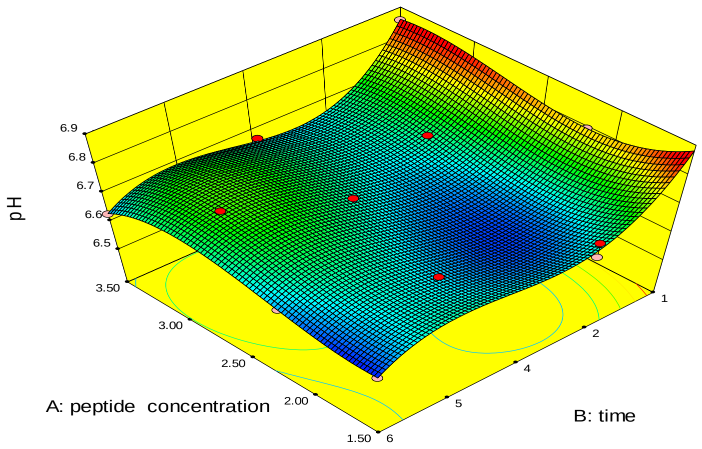

Fig. 2. pH in the fortified milk

Where (A) is the concentration rate of peptide

(B) is the Time of storage

Pralhadrao et al show Viscosity of milk fortified with Zn sulfate increased on the 5th day of storage whereas whey protein-Zn fortified milk showed change on the 7th day of storage (Pralhadrao *et al.*, 2021). Salt addition to milk and milk products increases viscosity, which might not be due to change in pH of milk and the physical nature of fat, but changes in hydration of milk protein especially casein (Dhaka, 1982). Any change in the size of the proteins led to a change in viscosity (Pralhadrao *et al.*, 2021). Therefore, it could be concluded that the size and concentrate of collagen peptides influence milk density by the time of storage.

- ***Alcohol stability test of peptide fortified milk***

Considering the acidity results, casein micelle stabilization was proposed for fortified milk. The treatments showed that peptides concentration by 3.50% on different days were not stable, but below this concentrate were stable and the alcohol stability test was negative. Alcohol stability test (AST) is generally used as an indicator of the freshness of milk and its suitability for processing temperature (Pralhadrao *et al.*, 2021). AST results showed increased peptide concentration till the range of 3% was negative during 6 days of storage while more concentrated 3.50% casein micelles were not stable. ($p<0.01$) (Figure 4). The quadratic equation for AST content obtained by the Response Surface Methodology data indicated the effect;

$$1/\mathrm{Sqrt}\ (\text{Alcohol test} + 0.00) = +32.93-13.77\times A-16.99\times A^2 \qquad \textbf{Eq. 4}$$

Where (A) is the concentration rate of peptide.

AST is the stabilization of casein micelles and is considered as a condition for being test negative. To maintain this structure, carboxylic groups and some phosphate groups create the negative charge on the surface of casein micelle, leading to the electrostatic stabilization of micelles in milk (Holt *et al.,* 1996). The alcohol test is one of the most significant that shows the stability of milk against temperature. In Pralhadrao research complex whey protein-Zn fortified milk samples were alcohol negative (absence of flakes or clot denotes a negative test) on the addition of ethanol (75% or 68%) till the 7th day of storage (Pralhadrao *et al.*, 2021). The research on Zn fortification (20 ppm) did not alter the heat stability of milk through COB (clot on boiling) and dry sediment test (Rana *et al*., 2018).

- ***Total protein of peptide fortified milk***

Total protein content was enhanced by different concentrations of collagen peptide, from 3.02 % in skim milk sample to 6.24% in 3.5% peptide treated sample. Collagen peptide fortified milk showed a significant (P<0.01) increase in total protein, (Figure 5). The equation for increasing the total protein by the Response Surface Methodology data is presented;

$$\text{Total Protein} = +4.06590-0.26211\times A+0.24935\times A^2 \quad \textbf{Eq. 5}$$

Where (A) is the concentration rate of peptide

Also in Bilek investigation, the addition of collagen hydrolyzate increased the total protein of the juice-based beverage (Bilek *et al.*, 2015).

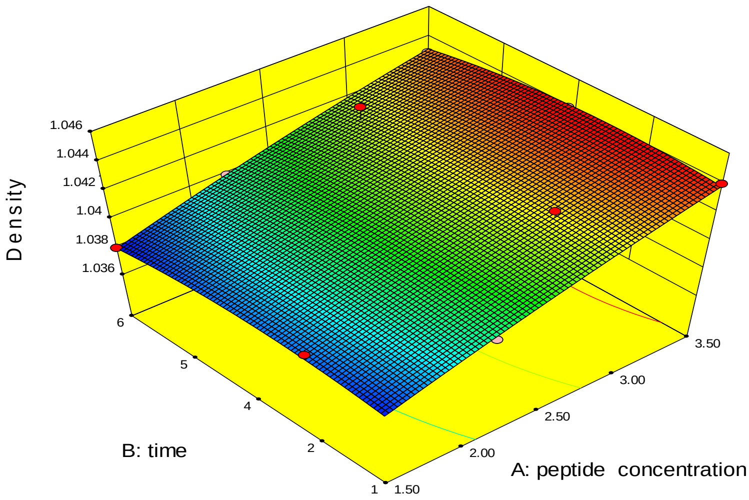

Fig. 3. Density increased in the enriched milk.

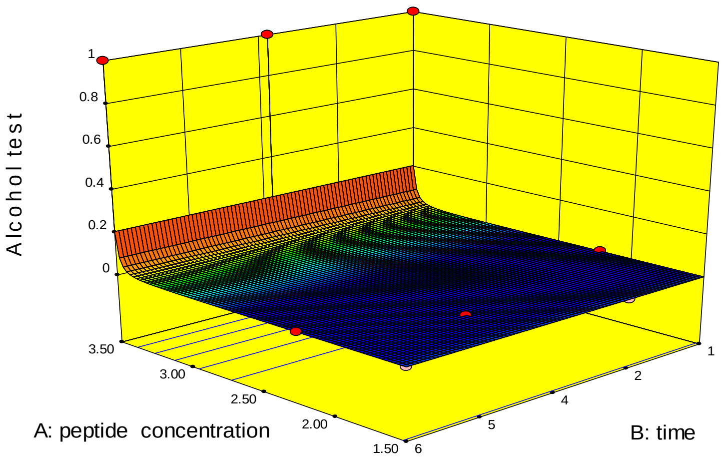

Fig. 4. Changes of alcohol test in the enriched milk.

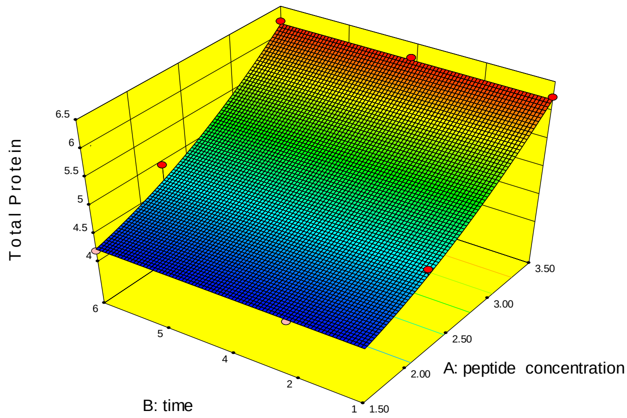

Fig. 5. increase of total protein content in the enriched milk.

- ***Microbial load of peptide***

- ***Microbial load of peptide fortified milk***

Bacterial total count was decreased during the stored time. Bacterial load on the fourth day showed a minimum (1×10^2 CFU/ml), while the maximum total count was measured on the sixth day of stored time (5×10^2 CFU/ml), which was equal to the pasteurized skim- milk sample on the first day. In the storage of pasteurized milk at refrigeration temperature, the microbial count increases slowly. According to an investigation with Sadhu, this increasing gradient of microbial load became fast after the fifth day, otherwise temperature influences positively on it, and at the higher temperature, the increase of microbes count accelerates (Sadhu, 2018). Fortified milk with collagen peptide showed all treatments (concentration non-significant) on the fourth day had a minimum bacterial load, the effect of peptide addition on the microbial load of pasteurized milk was significant during storage time ($p<0.05$) (Figure 6), The equation for decreasing by the Response Surface Methodology data is presented;

$$\text{Sqrt (Total bacterial count)} = +5.92 + 0.52\times B + 12.20\times B^2 \qquad \textbf{Eq. 6}$$

Where (B) is the Time of storage

The microbial load on the sixth day reach the highest level, but it was equal to the load on the first day (5×10^2 CFU/ml). It could be concluded that collagen peptide influences the total count of microorganisms during the time of storage, and increase shelf life.

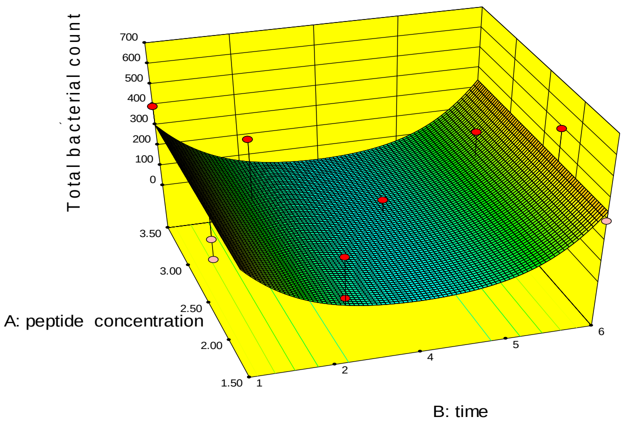

Fig. 6. Changes of microbial load in the enriched milk.

- ***The effect of peptide on raw milk microbial load***

The effect of collagen peptide on bacterial load was significant (P<0.05) and the total count decreased during 14 days of storage, (Figure 7) Control sample had a bacterial load of 5×10^5 CFU and there was a no-significant difference between the different concentrations. The yang et al study showed antimicrobial peptide (LCWAP) derived from whey acidic protein (WAP) of croaker to possess good antimicrobial activity against S. aureus and could inhibit the growth of S. aureus in milk during storage. Therefore, LCWAP has broad application prospects as a preservative in the food industry (Yang *et al*., 2020). Collagen peptides showed antibacterial effects in the total count of raw milk, and need to be assessed in more studies.

- ***Sensory evaluation of peptide fortified milk***

Treatments were conducted on the sensory characteristics flavor; odor, color, and texture of fortified milk obtained results were evaluated by using the Friedman test. Sensory evaluation was showed the taste and smell of fish waste collagen peptide decreased in skim-milk versus complete milk but not affected on color and texture, best acceptance relegates 2.63% and 2.86% and the next concentration was for 3% of the peptide. The flavor of skim-milk fortified with peptide was significantly (P<0.05) decreased at 1.5% (collagen peptide concentration) whereas, treatments at 2.63% and 2.86% concentrations were selected as optimal concentration and were selected for further studies (Figure 8). Different concentrations of peptide influenced significantly on milk odor and flavor. the closest odor to the control sample and the maximum score was at 2.63% and 2.86% collagen peptide, while the treatment with 1.5% collagen peptide attained a minimum score (Figure 9). The color and texture of skim milk fortified with peptide were not significant (P<0.05). Research on sensory acceptability in beverage fortification with collagen peptides is limited. Bilek et al (2015) studied the use of natural mint flavor (0.02–0.03%) to mask collagen taste and an odor due to fish origin. Pralhadrao et al (2021) fortified milk with WP-Zn the sensory attributes decrease in low level and accepted at 35 ppm then decrease by more concentrate.

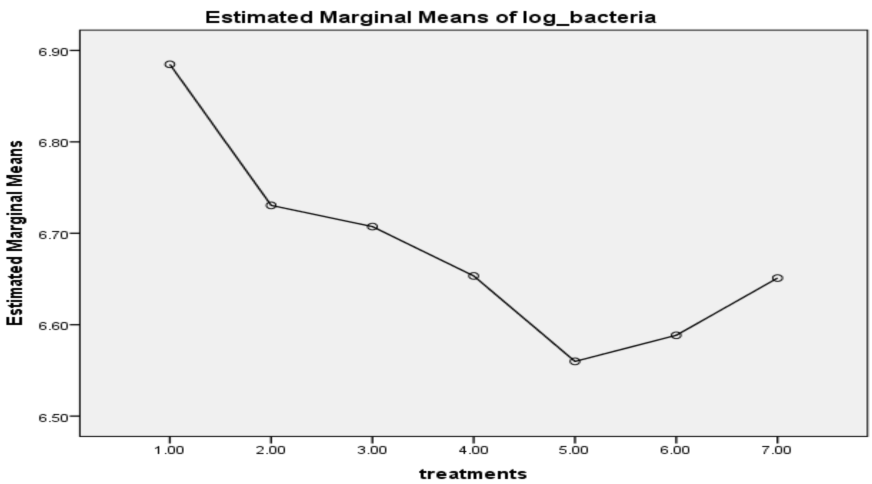

Fig. 7. Effect of collagen peptide on the microbial load of raw-milk.

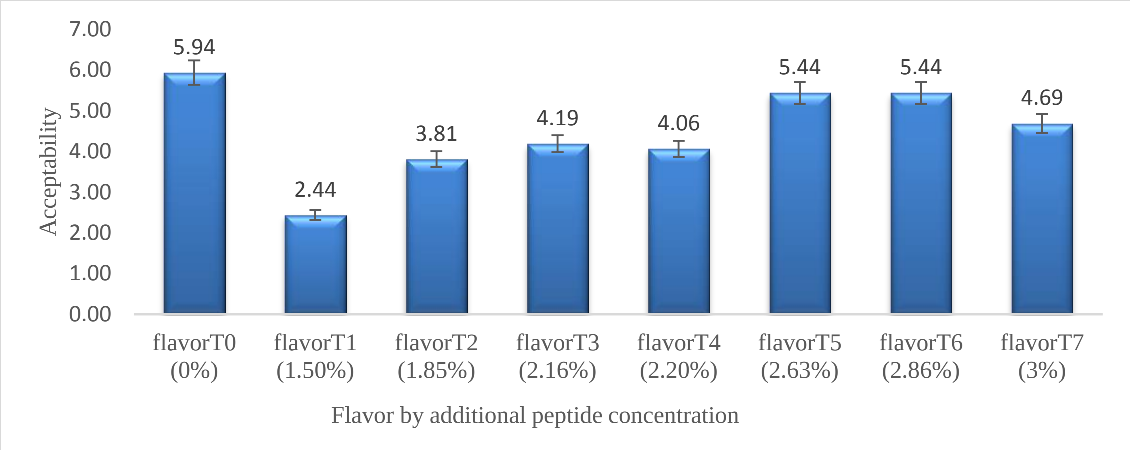

Fig. 8. Statistical comparison of Friedman grading related to effect of collagen peptide concentration on milk flavor.

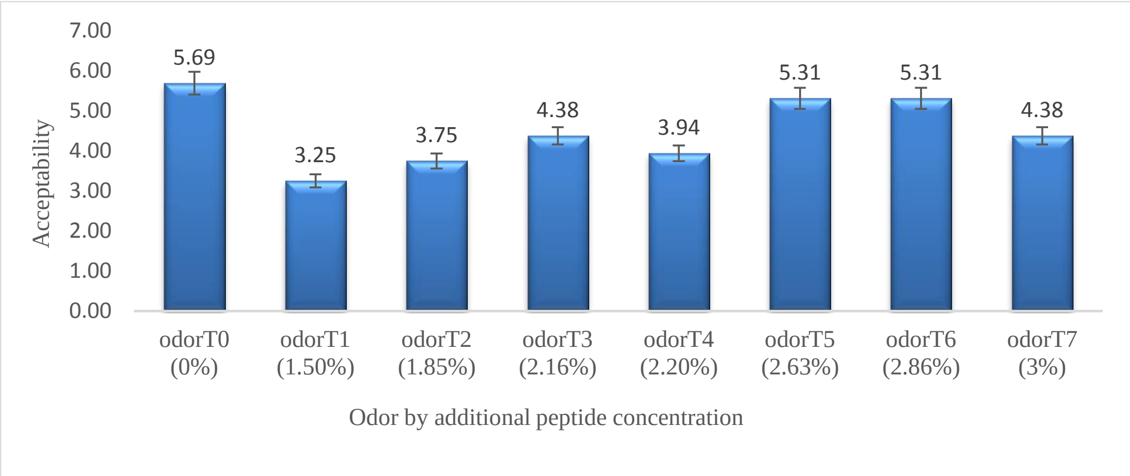

Fig. 9. Statistical comparison of Friedman grading of the effect of collagen peptide concentration on milk odor.

Conclusion

Milk is perceived as one of the main foods in the diet, that provides the essential body nutrient ingredients with high calcium bioavailable. Fortified milk, with collagen bio-peptide, provided nutritional needed besides functional properties, based on clinical studies, the daily use of 5-10gr collagen peptide for a 3-6-month period is regarded as a proper solution for cartilage, bone, and skin health. The finding of this research revealed that the optimal concentration by responses physicochemical and sensory characteristics with a produced functional drink is 2.63-2.86 gr/100ml collagen peptide, these concentrations can cover the peptide flavor without any additive and increase the intake of protein and be successfully used as a commercial product. Moreover, it decreased the bacterial load and developed shelf life.

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