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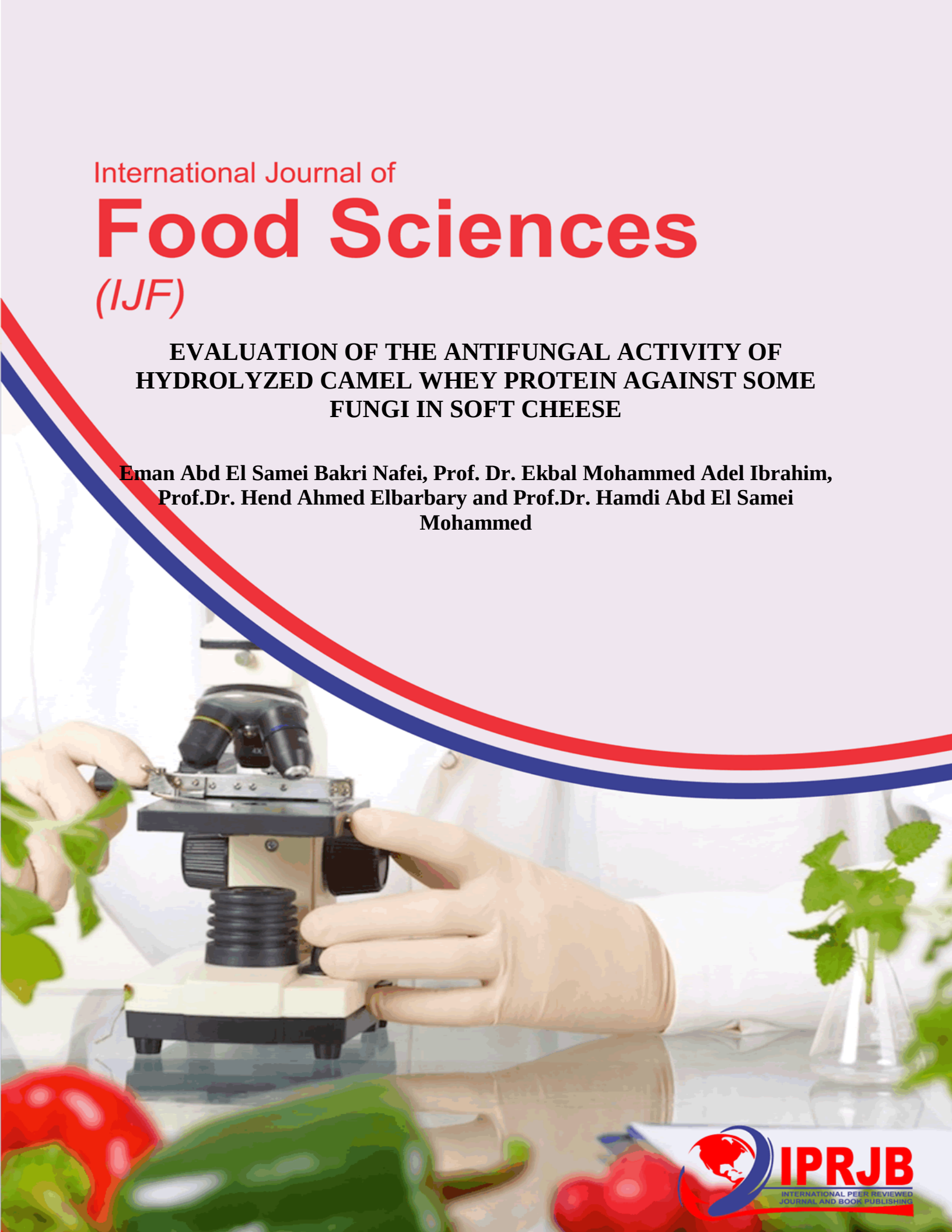

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EVALUATION OF THE ANTIFUNGAL ACTIVITY OF HYDROLYZED CAMEL WHEY PROTEIN AGAINST SOME FUNGI IN SOFT CHEESE

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Abstract

Purpose: The current study's aim is to investigate the effect of hydrolyzed whey protein concentrate (WPC) derived from camel's milk on growth of some fungi inoculated in soft cheese during refrigerated storage.

Methodology: The pepsin-trypsin (P-T) camel´s WPC hydrolysate (20 mg/g) was incorborated in to soft cheese and their effects on the survival of *Candida albicans*, *Asperigillus fumigatus Asperigillus flavus and Asperigillus niger* (10^3-10^4cfu/g) were examined till cheese deterioration.

Findings: The results revealed that P-T hydrolysate had the ability to decrease the viability of *C.albicans, A.fumigatus A.flavus and A.niger* in soft cheese. *C.albicans* was the most sensitive strain.

Unique contribution to theory, practice and policy: Thus, camel´s WPC hydrolysates can be exploited as. This study was conducted to elaborate antifungals from camel´s WPC after enzymatic hydrolysis which could serve as a safe alternative of natural antifungal agents in soft cheese

Keywords: *Camel´s WPC, Enzymatic Hydrolysis, Antifungal Activity, Soft Cheese, Refrigerated Storage*

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INTRODUCTION

Soft cheese repesent a great part of dairy industry throughout the world and their large acceptability by consumers can be attributed to their pleasant organoleptic properties and good nutritional properties (Miller, 2006). Moreover, the microbial growth in soft cheese is improved through high miosture, unsafe handling and inadequate storage practices which may distributed in dairy environment (Kure *et al*., 2004). Despite multiple trials in the dairies to reduce mould growth, fungal spoilage of cheese still causes significant economic losses in soft cheese due to reduction of their quality (Garnier *et al*., 2017).

Fungal cotamination is responsible for production of undesirable appearance, offensive flavors and other metabolic products which rendering cheese unsuitable for consumption (Banjara *et al*., 2015). *Candida spp.* and *Aspergillus spp.* are most common fungal isolates obtained from several soft cheese varieties (Abd- El Tawab *et al*., 2020). Some *Asperigillus spp* mainly *Aspergillus flavus* is able to produce toxic metabolities adversely affect consumers causing health hazard effects as mutagenic, carcinogenic, cytotoxic, neurotoxic and immunogenic effects (Hymery *et al*. 2014).

Camel milk is a rich source of high quality whey proteins with high nutritional, therapeutic and biological activities for people in dessert areas (Konuspayeva *et al*., 2009). The combination of several antimicrobial proteins in camel whey induce a longer and a stronger antimicrobial activity of camel milk compared with that of other species (Ramet, 2001). The bioactive peptides of whey proteins are inactive in the native protein and can be easily released and enhanced by enzymatic hydrolysis (Fatchiyah *et al*., 2015).The antimicrobial activity of these peptides occur through perturbation of DNA or RNA synthesis and increase membrane permeability or by direct hydrolytic activity on peptidoglycan of the microbial cell wall (Zdybicka-Barabas *et al.*, 2013).

Most bioactive hydrolysates obtained from whey proteins can be successfully utilized as harmless and inexpensive bio-preservatives for application in food products (Kumar *et al*., 2017). The present study aimed to prepare the potent antifungal hydrolysate from camel´s WPC and to evaluate its effectivness to control fungal growth in soft cheese.

MATERIALS AND METHODS

Materials

Pepsin and trypsin enzymes were obtained from Sigma-Aldrich Chemical Company, Nasr City, Egypt). Chemicals (Sodium phosphate buffer and HCL) were obtained from Loba Chemical Company, Kafer El Sheikh, Egypt.

Preparation of the camel´s WPC

Camel´s WPC was prepared from fresh camel's milk according to Wang (2020). Camel milk was skimmed by centrifugation at 12,000 rpm at 4°C for 30 min. Acetic acid solution (1M) was added skimmed milk till pH reached to 4.5 (isoelectric point). After centrifugation at 13000 rpm at 4˚C for 30 min, the supernatant whey solution was dialyzed for 72 hr at 4°C, then the retentate was freeze-dried at -60°C for 24hr and the obtained WPC powder was kept refrigerated at 4°C.

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Enzymatic hydrolysis of the camel´s WPC

According to the method of Wang (2020), the hydrolysis process of camel´s WPC was carried out by both pepsin and trypsin enzymes together under the specific conditions of each enzyme**.** Camel´s WPC suspension of 3.0% (wt/vol) was firstly acidified to pH 2 using HCl (1M) and pepsin was added and hydrolysis started at 37^{o}C for 3hr, consequently pH of pepsin hydrolysate was increased to 7.72 using sodium phosphate buffer (0.1 M) and trypsin was added at 42^{o}C for another 3hr.

Both enzymes were used at the same enzyme to substrate ratio of 2%(w/w). At the end of the hydrolysis, the obtained hydrolysate was heat treated at 85°C for 5 min, then was centrifuged at 10,000 rpm for 30 minutes and the supernatant was freeze dried. The obtained powder was designed as the P-T hydrolysate (pepsin - trypsin hydrolysate).

The antifungal assay in soft cheese

Seventeen liters of Full cream buffalo's milk (4.30% total protein, 7.50% fat) was obtained from the herd of Faculty of Veterinary Medicine, Benha University.Milk was heat treated at 63 °C for 30 min, then cooled to 40°C and divided in to 3 equal portions as follows:

1- Control (C): The batch of milk was thoroughly mixed and equally subdivided in two portions and distributed in 4sterile flasks (2 liter of each). The four flasks were inoculated with 10^3-10^4 cfu/ml dof *C.albicans,A.fumigatus, A.Niger* and *A.Flavus* in 1st,2nd, 3rd and 4th flasks, respectively without any additives (act as control positive samples). However, were prepared without pathogens.

2-Examined (E): The batch of milk was treated with 20 mg/ml of P-T hydrolysate, then thoroughly mixed and equally subdivided in four portions and distributed in 4 sterile flasks (2 liter of each).The four flasks were inoculated with dose 3 $\log_{10}$ cfu/ml of *C.albicans, A.fumigatus, A.niger* and *A.flavus.* The batches were prepared separately for each fungal pathogen tested.

3-The last batch 2 liter of milk was maintained without any further inoculation (act as control negative samples).

Low salt soft cheese (3%) was prepared as described by Denis *et al*. (1997). Calcium chloride, sodium chloride and rennet powder were added at levels of 0.02%, 3% and 0.05% (w/v), respectively then stirred with milk for 5 min then the mixtures were poured in previously sterilized stainless-steel containers (500ml) and incubated at 42°C 2-3 hours till complete coagulation. After curd formation and drainage of the whey, the cheese groups were stored at the refrigerator temperature (4°C) and were subjected to mycological assessment at day zero and weekly till sample deterioration. From the already prepared serial dilutions, 1 ml was transferred and mixed with Sabaraud Dextrose Agar (SDA) medium. The plates were incubated at 25°C for 5-7days, then colonies were counted Oxoid (2006).

Statistical analysis

All the experiments were conducted in triplicate and the results were expressed using one-way ANOVA analysis. Differences among means were tested for significance ($P<0.05$) as described

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by Hill and Lewicki (2007). Statistical analysis of the data was carried out employing analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Antifungal activity in soft cheese

Camel milk proteins have a greater antifungal activity through the massive fungal cells destruction and the inhibition of mycotoxins production (Al-Abdalall, 2010).Camel milk possesses a specific protective system of antimicrobial proteins with a powerful activity and concentration represent about three to four times higher than these present in milk from other species (Jrad *et al*., 2013). So, the P-T camel´s WPC this hydrolysate was applied at concentration of 20 mg/g to control growth of fungi (*C.albicans, A.fumigatus, A.niger and A.flavus*) inoculated in soft cheese during refrigerated storage at 4°C.

Figure (1) showed the effect of camel WPC hydrolysate on *C.albicans* growth in soft cheese, the mean value of *C.albicans* in control group was 3.30±0.08 Log_{10}cfu/g at zero day and gradually increased till reached to 6.29±0.09 Log_{10}cfu/g at 28th day of storage. While, in the treated group, the mean values of *C.albicans* were decreased from 3.26± 0.36 to 2.10 ± 1.05, 2.57± 0.13, 2.67 ± 1.33 and 3.63± 0.15 Log_{10}cfu/g at the 7th, 14th , 21th and 28th day of refrigerated storage, respectively. This result agreed with Seifu *et al*. (2005).

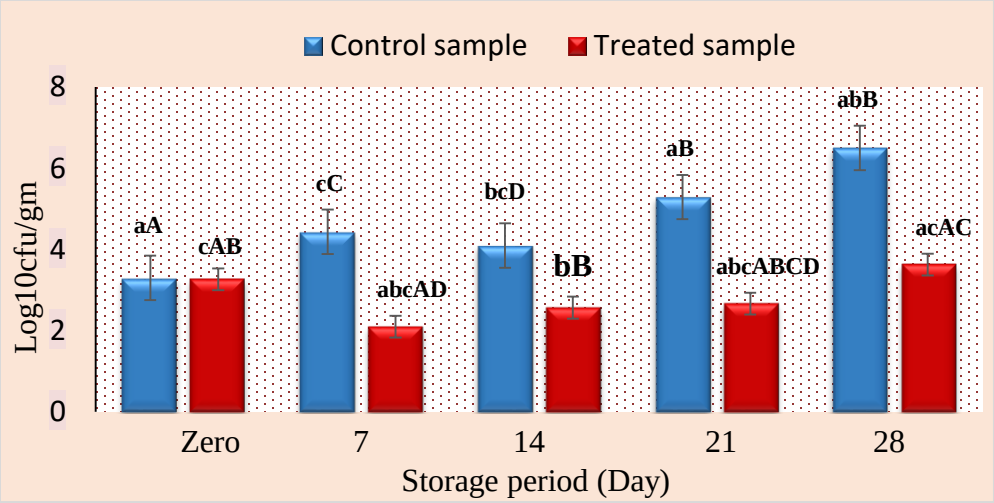

Figure (1): The viability of *Candida albicans*(10^3 - 10^4cfu/ml) in soft cheese preserved by 20 mg/g of P-T camel WPC hydrolysate (pepsin and trypsin generated camel WPC hydrolysate).^{abcd} the differences between the values in the same cheese samples are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage peroid; ^{ABCD} the differences between the values in the control cheese samples and those treated with CWP hydrolysates are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage period.

Concerning to mould growth, as shown in Figure (2), the mean value of *A. fumigatus* in control group was increased from 3.45±0.05Log_{10}cfu/g at zero day till reached to 5.22±0.38 and 6.60±0.16

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Log_{10}cfu/g at 21th and 28th day of storage, respectively. However, in treated group, the mean values of *A.fumigatus* slightly decreased from 3.58±0.15 Log_{10}cfu/g at zero day to 2.85±0.21 Log_{10}cfu/g at 7th day, then gradually increased till reached to 4.21±0.13 Log_{10}cfu/g at 28th day of refrigerated storage.

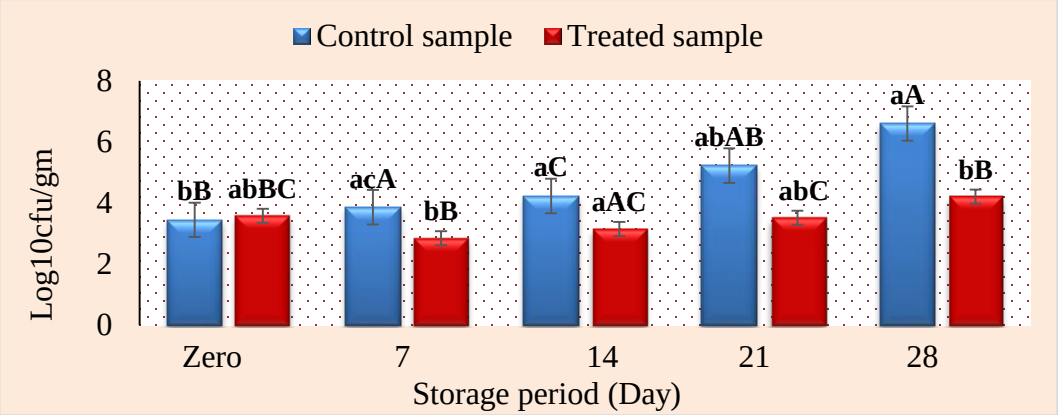

Figure (2): The viability of *Asperigillus fumigatus* (10^3 - 10^4cfu/ml) in soft cheese preserved by 20 mg/g of P-T camel WPC hydrolysate (pepsin and trypsin generated camel WPC hydrolysate). abcd the differences between the values in the same cheese samples are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage peroid; ABCD the differences between the values in the control cheese samples and those treated with CWP hydrolysates are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage peroid.

Figure (3) showed the mean values of *A.niger* in treated group which increased from 3.15±0.15 Log_{10}cfu/g at zero day till reached to 4.69±0.34 and 6.88±0.23 Log_{10}cfu/g at 21th and 28th day of storage, respectively. These mean values were lower compared with these revealed in control group that increased from 3.13 ±0.09 Log_{10}cfu/g at zero day till reached to 6.44±0.30 and 7.35±0.38 Log_{10}cfu/g at 21th and 28th day of refrigerated storage, respectively.

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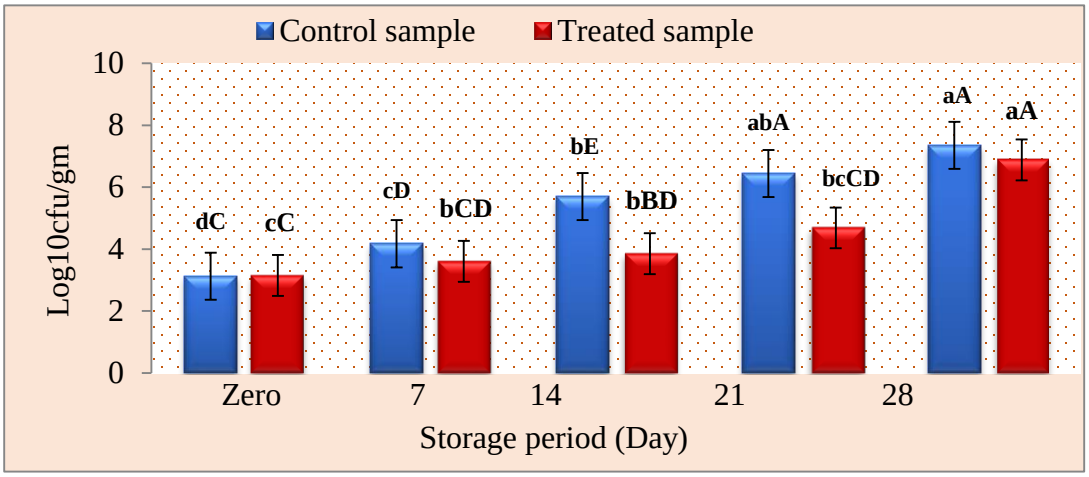

Figure (3): The viability of *Asperigillus niger* (10^3 - 10^4cfu/ml) in soft cheese preserved by 20 mg/g of P-T camel WPC hydrolysate (pepsin and trypsin generated camel WPC hydrolysate). ^{abcd} the differences between the values in the same cheese samples are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage peroid; ^{ABCD} the differences between the values in the control cheese samples and those treated with CWP hydrolysates are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage period.

The growth rate of *A. flavus* has been shown in Figure (4), the mean value of *A.flavus* in control group was 3.20±0.1 Log_{10}cfu/g at zero day, then increased to 3.87±0.43 , 4.49±0.55 and 6.04±0.14 Log_{10}cfu/g at 7^{th} , 21^{th} and 28^{th} day of storage, respectively. However, in treated group, the mean values of *A. flavus* were ranged from 3.25±0.25 Log_{10}cfu/g at zero day till reached to 3.53±0.77, 3.73±0.25 and 4.52 ±0.32 Log_{10}cfu/g at 7^{th}, 21^{th} and 28^{th} day of refrigerated storage, respectively.

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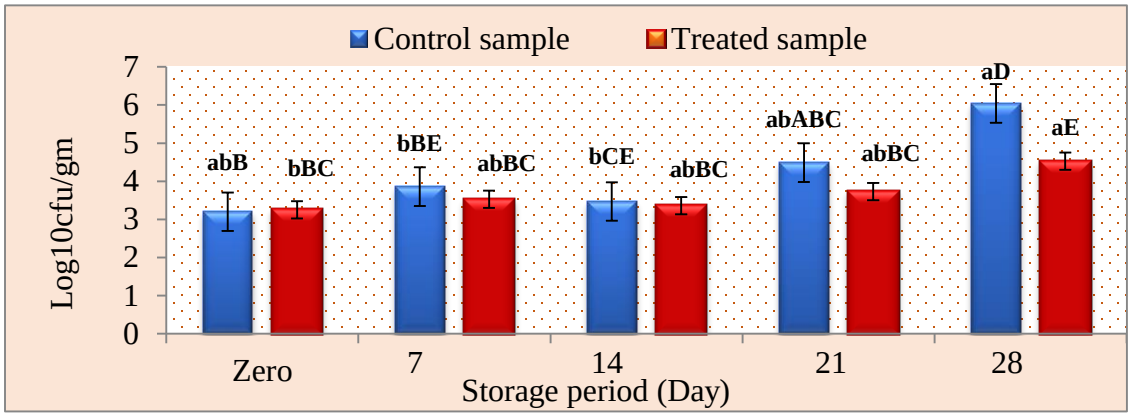

Figure (**4**): The viability of *Asperigillus flavus* (10^3 - 10^4cfu/ml) in soft cheese preserved by 20 mg/g of P-T camel WPC hydrolysate (pepsin and trypsin generated camel WPC hydrolysate). [abcd] the differences between the values in the same cheese samples are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage peroid; [ABCD] the differences between the values in the control cheese samples and those treated with CWP hydrolysates are statistically significant ($p < 0.05$) from each other during prolonged refrigerated storage peroid.

DISCUSSION

The fungal spoilage of cheese act as a source of mycotoxins with public health hazard and as a main cause of massive economic losses in cheese industry (Garnier *et al*., 2017). The emerging threats of fungal contamination in food industry is especially associated with the extreme resistance of certain spoilage fungi for applied preservatives and factory hygiene (Benedict *et al.*, 2016). The current results revealed that 20 mg/g of camel WPC hydrolysate reduced the fungal growth and proliferation although it was not able to totally inhibit their growth in soft cheese during storage for 28 days at 4°C. The growth inhibitory activity was storage period and strain dependent with the greater effectivness against *C.albicans* . These results may be attributed to greater antifungal activity of camel whey proteins and their derived peptides (El-Desoukey *et al*., 2020). These proteins exerted potent antifungal activity either by the potent iron chelating activity of their hydrolysates or through more hydrophobic amino acids of their peptide fractions (Abd El-Fattah *et al*., 2017). These results agreed with Silva *et al*.(2013b) who revealed greater antifungal activity of camel whey proteins mainly lactoferrin and its generated peptides against *A. fumigatus*. This was in harmony with Alsteens *et al*. (2008) who stated the greater antifungal activity of camel lysozyme through generation of chitinase enzyme causing a strong lysis of chitin which is a major component in fungal cell wall.

The camel lactoferrin peptides in P-T hydrolysate mainly lactoferampin, lactoferricin, and lactoferrin chimera have a potent antifungal activity (Pirkhezranian *et al.*, 2020). These peptides may lead to damage of fungal cell membranes and alter their permeability (Al-Majali *et al*., 2007). However, the inability of P-T hydrolysate for the complete inhibition of fungi in soft cheese may be due to slower transmission of antimicrobial agents in food system (Cagri *et al*., 2002).These results agreed with Guimarães *et al.* (2020) who revealed the delayed development of *Penicillium*

nordicum on cheese surface coated with WPC-edible film. These findings are similar to those stated by Shashikumar and Puranik (2012) who used lactoferrin as one of potent antifungal whey protein increased shelf life of paneer cheese.This results agreed with Vogel *et al*.(2002) who reported that potent antimicrobial effect of lactofericcin peptide may be attributed to their richness with tryptophan and Arginine amino acids in their sequences.Other studies carried out by Medeiros *et al.* (2014) revealed the complete absence of fungal proliferation up to 20 days in cheese coated lysozyme solution.

CONCLUSION AND RECOMMENDATION

The current results revealed that the P-T camel´s WPC hydrolysate showed its ability to decrease growth of *C.albicans, A.fumigatus A.flavus and A.niger* in soft cheese compared to progressive growth of these fungi in control group up to 28 days at refrigerated storage. The P-T hydrolysate exhibited a greater activity against *C.albicans* than other tested strains. Consequently, further studies should be applied for further purification and characterization of peptides from this hydrolysate to be suitable as effective natural antifungal in food system**.**

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