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Application of *Lactobacillus helveticus* CNRZ 32 to Control the Microbial Contaminants in Cheese by Addition of Bacterial Extracts or by *In Situ* Production of Bioactive Metabolites

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Abstract

Microbial spoilage of cheese represents a major concern from both health and economic views. The addition of food preservatives is considered the most applied strategy to ensure food quality and to control microbial contamination. It well established that natural preservatives such as Nisin and Natamycin are of great effectiveness against a wide range of microbial concerns, but the lack of wide spectrum effect induces looking for more efficient alternatives. This research suggests alternative treatments to be evaluated side by side with both Nisin and Natamycin within real cheese models to control microbial contamination during the storage period. To evaluate this, two varieties of cheese were manufactured and inoculated with a set of pathogen and cheese spoiling microorganisms. Talaga cheese batches were separately treated with extract of MRS that previously fermented by Lactobacillus helveticus CNRZ 32, Nisin and Natamycin at Free State and Chitosan Nanoparticles-loaded state to become 6 treatments (T1 – T6) other than the control (C). The same treatments were applied to Karish cheese batches, in addition to inoculation of Lactobacillus helveticus CNRZ 32 1% (v/v) in the seventh batch. Upon microbiological analyses, results show that T2; the extract loaded on Chitosan Nanoparticles completely reduced the count of all pathogens and spoiling populations after two weeks of cold storage (2 – 6oC) in Talaga cheese. In the case of Karish cheese, the 7th batch treated with Lactobacillus helveticus CNRZ 32 inoculation had no pathogenic nor spoiler growth after one week of cold storage (2 – 6oC). These results suggest that Lactic acid bacteria especially Lactobacillus helveticus CNRZ 32 can efficiently ensure the safety and quality of cheese if applied in appropriate form.

Keywords: Green food production; *Lactobacillus helveticus* CNRZ 32; Cheese bio-preservation; Talaga cheese; Karish cheese.

1. Introduction

The high nutritional value of dairy products and the popularity of cheese led to their wide spread among world populations. Inclusion of multi-nutrients makes dairy products a suitable substrate for microbial contamination along production chain. Microbial concerns may be pathogenic that affect the product safety causing food-based outbreaks or spoilage that lowered the food quality, or may be the both of them [1]. The bad impact of food spoilage appears mainly as economical loss of product and packaging material, as well as, other resources during different stages of production and distribution chains.

Food safety organizations which regulate all steps and operators along food production chain, such as the European Food Safety Authority (EFSA) have been created to ensure the safety of foods within food markets and to prevent the National spread of food-based diseases what so-called “cross-species contamination” [2]. Also, knowledge and training programs such as Hazard analysis and critical control points (HACCP), Good hygienic practices (GHPs) and Good agricultural practices (GAPs) need to be upgraded in dairy industries chain [3].

Incidence of the microbial contamination can be controlled via following hygienic rules during crop collection and food processing [4], and by addition of preservative substances that inhibit microbial growth and development during storage [5].

Other than bio-preservation, there are several methods or techniques have been applied to extend the shelf life of cheese. The main methods include modified atmosphere, high pressure, active coating and edible coating either separately or in combinations [6]. The trend of green-food includes the application of natural compounds that replace chemical preservatives to ensure food safety and quality without causing health issues [5]. The prospective use of

bio-preservatives in food production has the benefit of leading towards the production of safer and healthier foods, not ignoring their contribution as ecofriendly [7]. In the field of bio-preservation researches, there are some contributions to preservation of cheese using natural, but non-microbial substances such as propolis and essential oils [8]. Natural bio-preservation has utilized both Nisin and Natamycin as microbial preservatives. The former is a bacteriocin that has a considerable inhibitory activity against most gram-positive bacteria [9], but less or not effective against gram-negatives [10, 11]. According to EFSA panel on food additives and nutrient sources, the extension use of Nisin as unripened cheese additive would not have any safety concern [12]. The latter is an antimycotic polyene [13] with effective concentrations between < 1 and 10 ppm [14]. Natamycin has been permitted as a surface preservative in some cheese types in just 32 countries and due to its poor absorption, there is no safety concern according to the EFSA panel on food additives and nutrient sources [15]. Improving the application of nisin as food bio-preservatives with broad spectrum is ensured by producing of nano-based formulas [16].

The current work aims to promote the safety and quality of Talaga and Karish cheeses by applying a lactic acid bacterium; *Lactobacillus helveticus* CNRZ 32 and its metabolic extract in comparison with Nisin and Natamycin both in free state and loaded on chitosan nanoparticles.

2. Materials and Methods

2.1. Microbial Strains

Pathogenic and food spoilage microorganisms; *Escherichia coli* strain E11 (accession number KY780346.1), *Salmonella enterica* strain SA19992307 (accession number CP030207.1), *Pseudomonas aeruginosa* strain Kasamber5 (accession number KY549641.1), *Bacillus cereus* strain 151007-R3-K09-40-27F (accession number KY820914.1), and *Staphylococcus sciuri* strain 2-6 (accession number MH491952.1), *Penicillium chrysogenum* strain J127 (KF572447.1) and *Candida parapsilosis* strain F2-17 (KP852497.1) were isolated and identified by Al-Gamal, *et al.* [17] from Egyptian cheese at Dairy Microbiological Lab., National Research Centre, Egypt.

The lactic acid bacterium; *Lactobacillus helveticus* CNRZ 32 were supplemented with Centre National de Recherche Zootechnique, Jouy-en-Josas, France.

2.2. Fermentation and Preparation of the Extract

About 250 ml of MRS bottles were inoculated with 2% of *Lactobacillus helveticus* CNRZ 32 [17], and incubated at 30^{o}C for 72 hours. CFS was prepared by centrifugation of fermented media for 20 min at 7000 rpm. For the recovery and concentration of bioactive compounds in CFS, aliquot (20 ml) of the CFS of selected LAB were initially extracted (1:1, v/v) with diethyl ether. CFS was extracted three times with diethyl ether and then dried over anhydrous Na_2SO4 and concentrated by rotary evaporator under a vacuum at a temperature lower than 40^{o}C.

2.3. Cheese Manufacturing Procedures

Two varieties of Egyptian cheese; Talaga cheese and Karish were manufactured to apply the preservative treatments and record the protective behavior during proper storage periods. **Talaga cheese** was manufactured by conventional method as described by Mehaia [18]. In details, four kilograms of whole buffalo milk were subjected to pasteurization at 65^{o}C for 30 minutes, then cooled to 42 ^{o}C. After addition of table salt at 4%, yoghurt starter (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus* 1:1) was added as 1% (w/w) and let incubated for 10 minutes. Rennet (CHY-MAX Powder Extra NB, Chr. Hansen; Denmark) solution was added as 45 IMCU/Litre and let incubated for 3 hours till full coagulation. Prior to the addition of rennet, pathogenic inoculation was performed to finally give 10^3 CFU/ml of *Escherichia coli*; *Staphylococcus sciuri*; while 10^2 cfu/ml of *Penicillium chrysogenum*; *Candida parapsilosis*.

Karish cheese was manufactured according to Hamad [19]. In details, four kilograms of skimmed milk that kindly obtained from Egyptian farmers were subjected to heat treatment 65^{o}C for 30 minutes, then cooled to 32 ^{o}C. Prior to pathogenic inoculation, Traditional yoghurt starter was added at 3% and let standing at room temperature overnight. After curdling, whey was filtered out, and cheese preparations were cut and stored at 2 – 6 ^{o}C after application of treatments.

2.4. Preparation and Addition of Treatments

Treatments; either in free state or as loaded on chitosan nanoparticles, were prepared and characterized as described by our previous work [20]. In details, treatment-loaded Chitosan nanoparticles (Ch. NPs) were prepared by dissolving 2 Grams of chitosan in 1% acetic acid solution. After complete dissolution, the chitosan solution was added drop wisely to the vigorously stirred Sodium Tripolyphosphate (TPP) solution (0.03%). The resulted suspension was then subjected to sonication (DAIGGER Sonicator Model GEX 750, USA; sonication power, 750 Watts, frequency, 20 kHz and amplitude 50%, in Marine Toxins Lab., National Research Centre) for 30 minutes at 25°C. Nanoparticles were stabilized by the addition of 0.4% Cetyltrimethylammonium bromide (CTAB) as a cationic surfactant.

After manufacturing, Talaga cheese was divided into three batches; **(1)** Addition of different treatments (**T1**, **T3** and **T5**) in free state to salting solution, **(2)** Addition of the treatments after loading on chitosan nanoparticles (**T2, T4** and **T6**), and **(3)** Untreated group (Control). In case of Karish cheese, **T7** was also applied (Table 1).

Table-1. Applied treatments with their codes

Code	Treatment	Code	Treatment
T1	Diethyl ether extract	**T5**	free Natamycin
T2	Diethyl ether extract/Ch. NPs.	**T6**	Natamycin/Ch. NPs.
T3	free Nisin	**T7**	1% v/v of *Lactobacillus helveticus* (10^8cfu/ml)
T4	Nisin/Ch. NPs.	**C**	Untreated group (Control)

2.5. Microbiological Analyses

According to American Public Health Association APHA and Association of Official Agricultural Chemists US [21] **and** Food and Drug Administration FDA [22], analyses were performed during storage periods at 2 – 6 ^{o}C to evaluate the effect of different treatments on growth and survival of the inoculated microorganisms (*Staphylococcus sciuri*, *Escherichia coli*, *Penicillium chrysogenum*, and *Candida parapsilosis*). Total viable count also was estimated.

2.6. Statistical Analysis

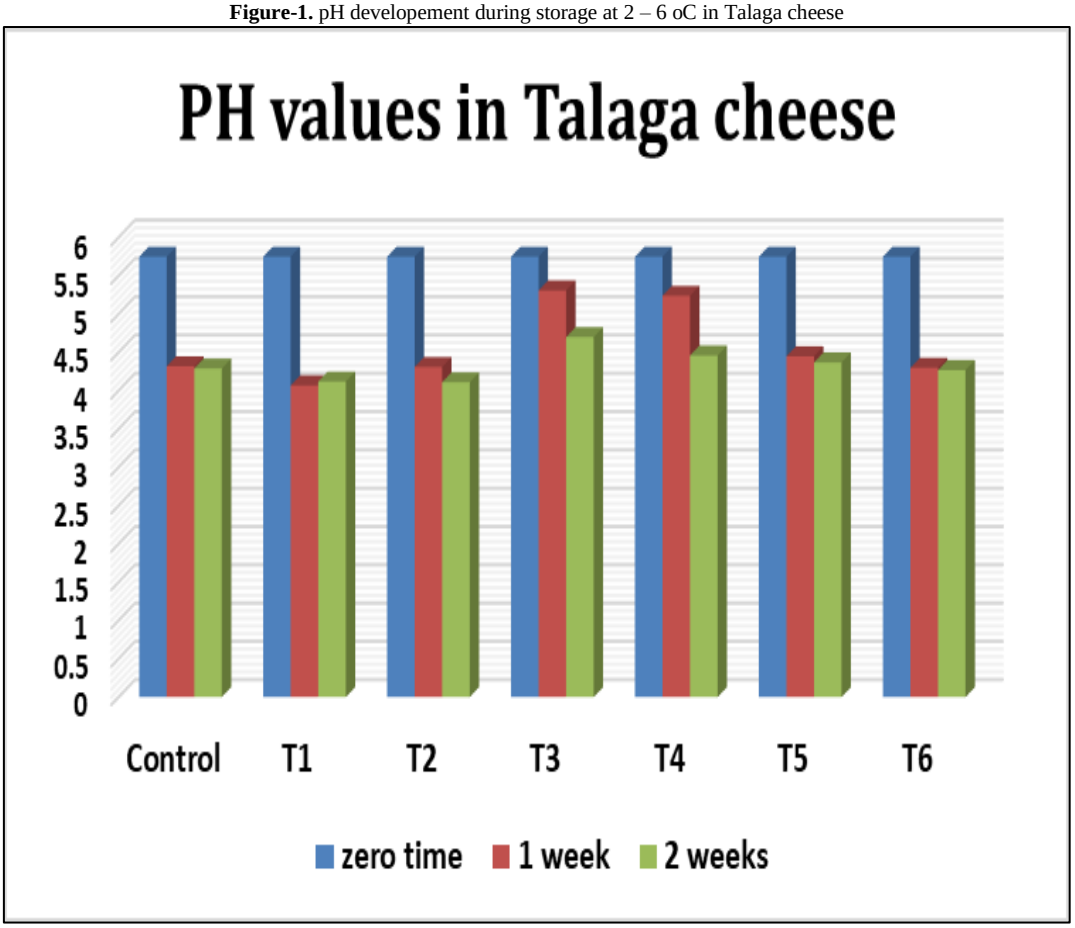
Statistical significance was determined using Statistica Version 9 (StateSoft, Tulsa, Okla., USA). The means were determined by analysis of variance test (ANOVA, two way analysis) ($p<0.05$). Fisher's LSD (Least Significant Difference) Method ($\alpha = 0.05$) was applied to compare significant differences between treatments.

3. Results

In the way to get an effective cheese bio-preservative, this research evaluated the antimicrobial activity of seven treatments within Talaga cheese and Karish cheese models. The findings in this part symbolize the application of the all tested antimicrobial treatments, on Talaga and Karish cheese models. This application targeted to use two models of cheese to evaluate the preservation potentials, and to select the successful treatment inside a real dairy product during storage period. Such assessment enables the judgment of the treatment efficiency to be introduced to industrial application. Two types of Egyptian cheese; Talaga and Karish were manufactured under full aseptic laboratory conditions. Each type of cheese was inoculated with 10^3 cfu/g for bacteria, and 10^2 for both yeasts and fungus. After addition of the treatments, evaluation of the consequence of different preservative treatments, on the count of inoculated pathogenic and spoilage microbial populations, in both Talaga and Karish cheeses were shown.

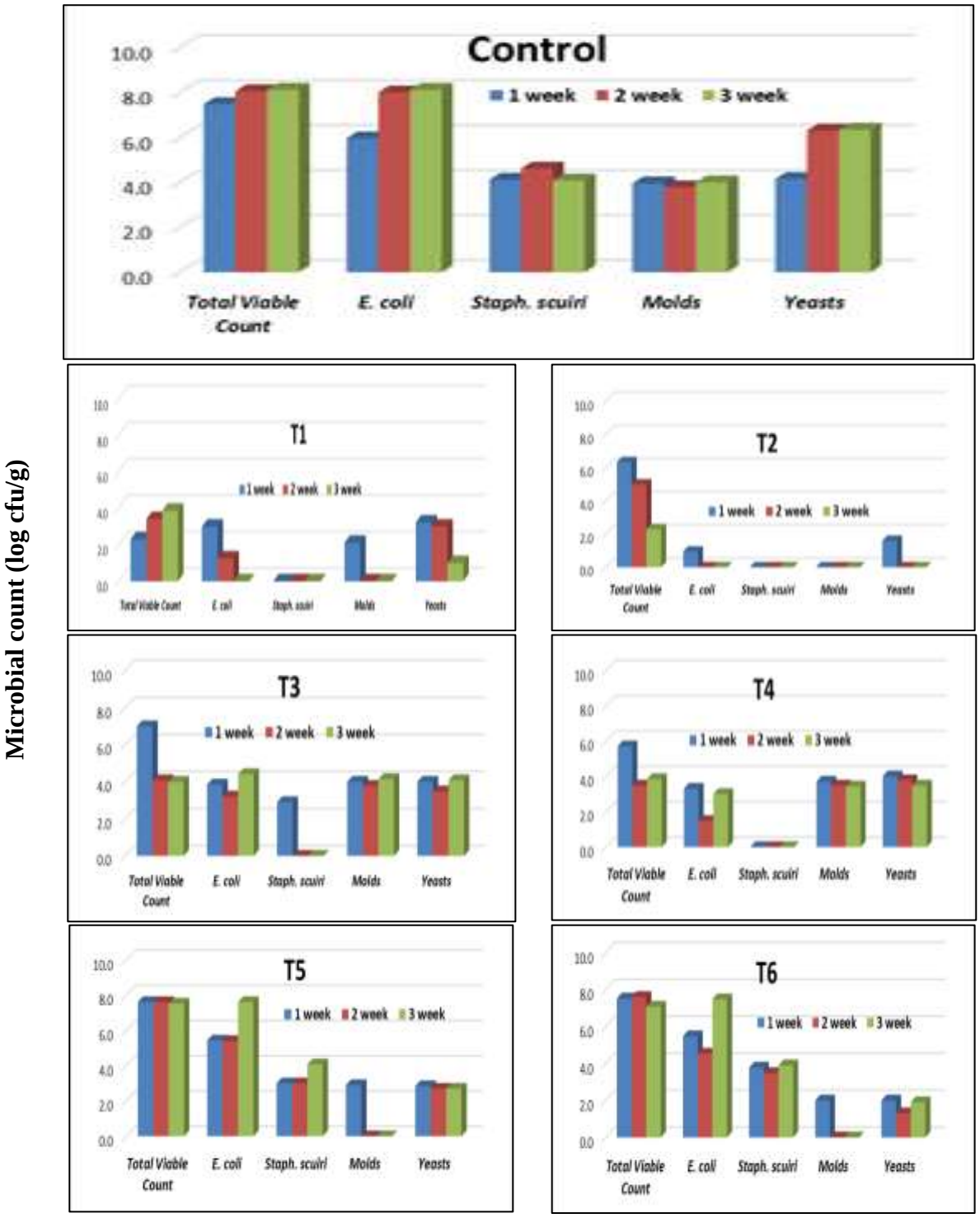
Figure 1 shows the effect of added treatments on the development of pH values during storage.

Figure-1. pH developement during storage at 2 – 6 oC in Talaga cheese



These observed change in pH during cold storage reflects the situation of microbial community inside cheese which represented in Figure 2.

Figure-2. Consequence of the treatments on microbial growth during storage at 2 – 6 °C in Talaga cheese



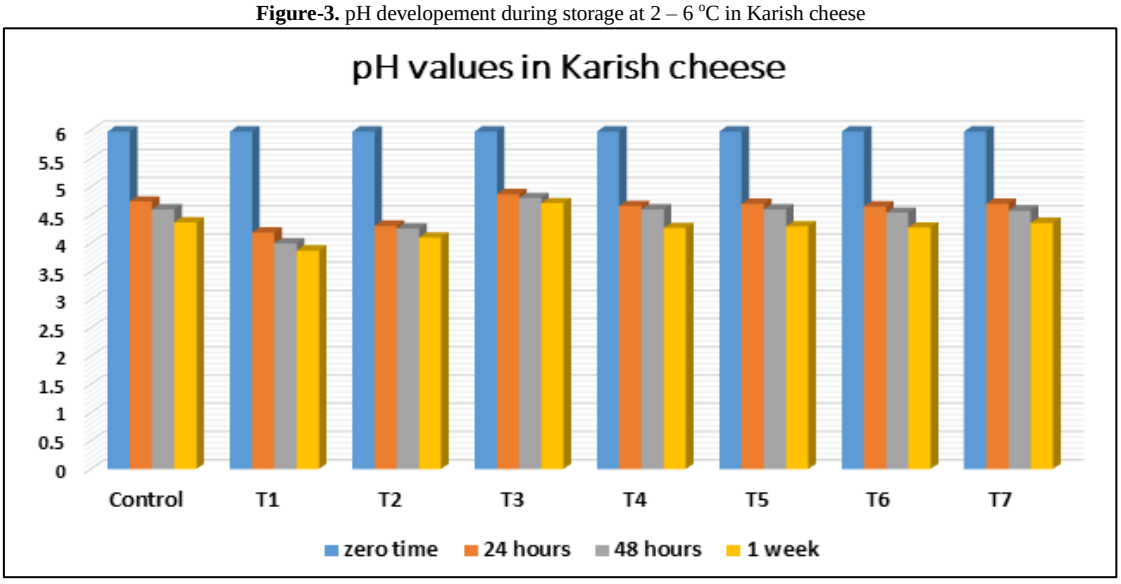
T1: the crude extract of Diethyl ether; **T2**: the extract loaded on Ch. NPs.; **T3**: the free Nisin; **T4**: Nisin loaded on Ch. NPs.; **T5**: the free Natamycin; **T6**: Natamycin loaded on Ch. NPs.

After the first week of cold storage (2 – 6 °C), the highest reduction in all microbial populations was seen in the treatment **T2** (Extract-Ch. NPs). This treatment successfully reduced *Escherichia coli* count to 1 log cfu/g, while still at 6 log cfu/g in control. In the treatments **T1** (Extract), **T3** (Free Nisin), **T4** (Nisin-Ch. NPs), **T5** (Free Natamycin), and **T6** (Natamycin-Ch. NPs), the *Escherichia coli* count reduced by 3 log cfu/g, 2.2 log, 2.7 log, 0.6 log, and 0.5 log respectively than control. By the treatments T1, T2, and T4, the growth of *Staphylococcus sciuri* was not detected any more. There was no significant difference between free Nisin and free Natamycin on the growth of *Staphylococcus sciuri*. The observed reduction in fungal growth was 100% in case of Extract-Ch. NPs., followed by 50%, 47.5%, and 27.5% for Natamycin-Ch. NPs., Free Extract, and Free Natamycin respectively, while both free Nisin and Nisin-Ch. NPs. did not changed significantly from the control.

After two weeks, except the control, there was a clear declining in all microbial populations at all treatments, especially the extract-Ch. NPs. that attained the "No detected growth" stage in all populations; bacteria, molds and yeast. The growth of *Staphylococcus sciuri* was completely disappeared in case of T3, and T4, as well as, fungal count in T1, T5, and T6.

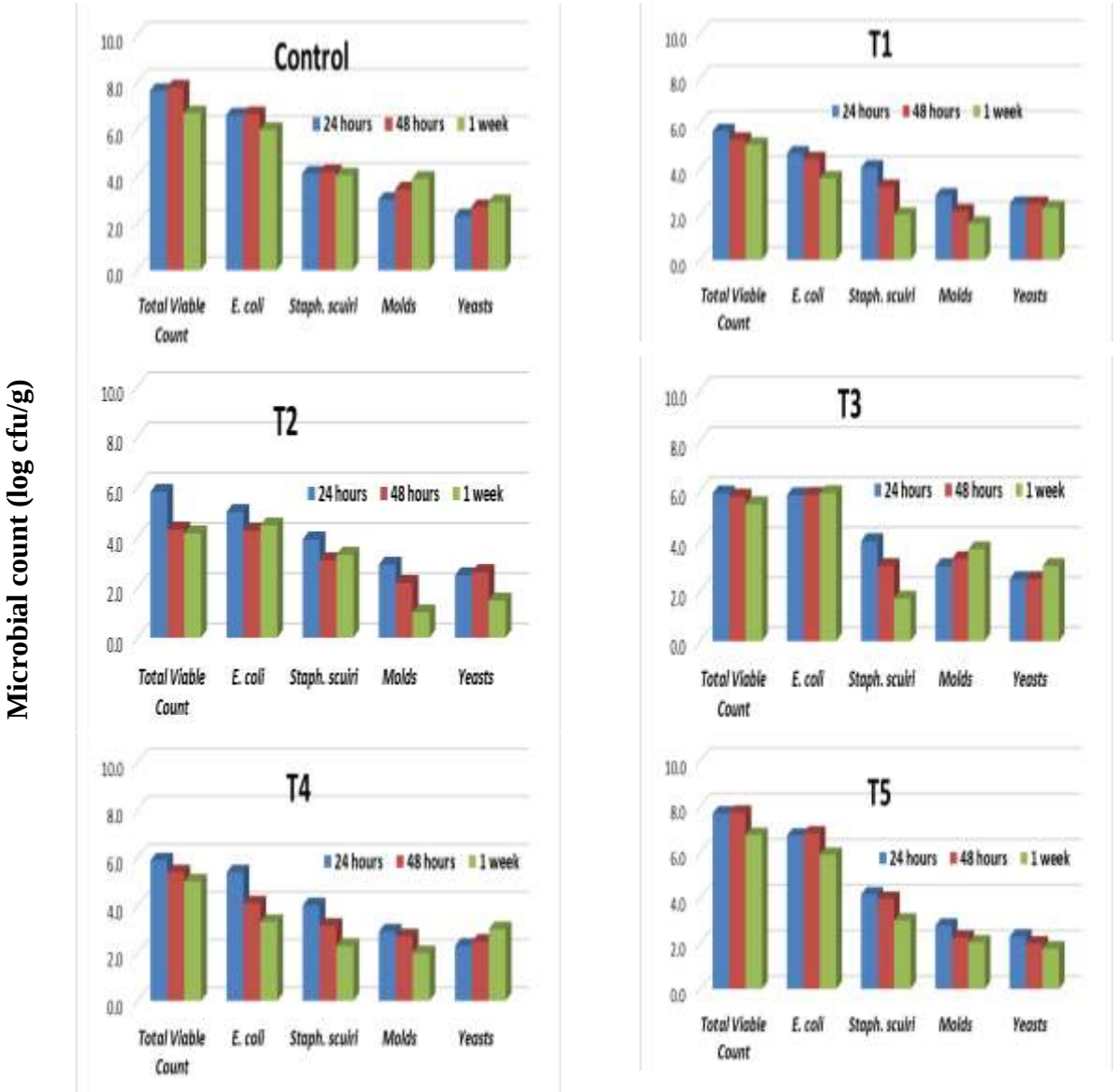
By the end of the 3rd week, the remaining yeasts growth left by T2, T5, and T6 was 1, 2.7, and 1.9 log cfu/g, which represents 15.8%, 42.9%, and 30.2% respectively of the control.

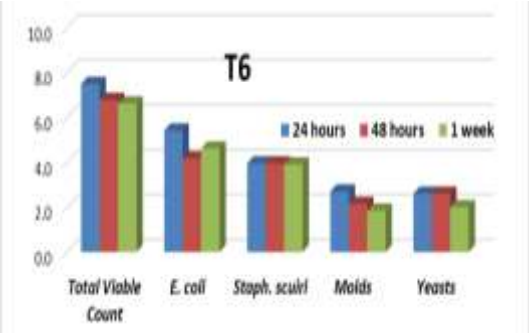
Figure-3. pH developement during storage at 2 – 6 °C in Karish cheese

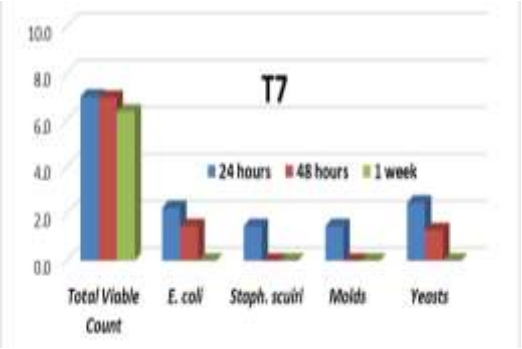


In the studied model of Karish cheese, the influence of the same studied preservative treatments, in addition to the role of *Lactobacillus helveticus* an adjunct starter, was illustrated in Figures 3 and 4. As guided by preliminary studies, microbiological analysis was conducted at 24, 48 hours, and one week of cold storage period.

Figure-4. Influence of the treatments on microbial growth during storage at 2 – 6 °C in Karish cheese







T1: the crude extract of Diethyl ether; **T2**: the extract loaded on Ch. NPs.; **T3**: the free Nisin; **T4**: Nisin loaded on Ch. NPs.; **T5**: the free Natamycin; **T6**: Natamycin loaded on Ch. NPs.; and **T7**: 1% w/v of *Lactobacillus helveticus*

After 24 hours of storage period, the maximum overall microbial count reduction was achieved in treatment **T7** (viable cells of *Lb. helveticus* CNRZ 32). By T7, the count of *Escherichia coli* decreased to 2.3 log cfu/g, while not less than 6.6 log cfu/g in control; meaning that the reduction in *Escherichia coli* population reached 66% higher than the control, followed by *Staphylococcus sciuri*, and molds whose counts were 1.5 log cfu/g that dropped by 64% and 50%, respectively, more than the control. Other than *Escherichia coli*, there was no significant difference among Treatments from T1 to T6 in the detected counts.

After 48 hours' time passed, the count decreasing of microbial populations was considerable. The inoculation with *Lb. helveticus* (1% v/v) successfully reduced the *Escherichia coli* and yeasts counts to 1.5, and 1.3 log cfu/g, respectively, to become 78%, and 52% lower than the control. At the same time, the residual growth of *Staphylococcus sciuri*, and molds were completely eliminated.

At the end of one week of storage, none of the inoculated microbial populations was detected in the case of T7, while T3; the free Nisin lowered the growth of *Staphylococcus sciuri* to 1.7 log cfu/g reducing than control by 57.5%. In the case of T2; the extract-Ch. NPs., molds and yeasts counts were reduced to 1, and 1.5 log cfu/g, respectively, meaning raising down than control by 74.3% and 48.3% respectively.

4. Discussion

The current work was designed to understand the impact of different preservative treatments on behavior of microbial contaminants within a cheese model, simulating the real production and storage conditions. This application was conducted as a step to introduce an effective preservative to the field of cheese industries.

As illustrated in Figure 2 that shows the status of Talaga cheese with different preservative treatments, the greatest preservative potential and the fastest pathogenic reduction were reached in the case of Extract-Ch. NPs. that completely reduced the counts of all inoculated pathogens after 2 weeks of storage at 2 – 6 ^{o}C. It is important to comparably interpret the findings presented by Ibrahim, *et al.* [20]. They found that the greatest inhibition zones were obtained in case of gram positive pathogens, the multi-active bio-autographic bands in gram positive bacterial pathogens, and their fastest inhibition among all microbial concerns; bacteria, fungi and yeasts. Inclusion of bacteriocins in all extracts may come in the front of all suggestions as confirmed by Lv, *et al.* [23] who successfully extracted a bacteriocin from *Lactobacillus plantarum* using Ethyl acetate solvent. Recently, Hassan, *et al.* [24] recorded that *Staphylococcus aureus* and *Acinetobacter baumannii* were significantly inhibited by *Lactobacillus helveticus* and *Lactobacillus plantarum* bacteriocins. In addition, the considerable content of organic acids, as confirmed by pH measurement results in Figure 1, supports the antimicrobial activity. To understand the inhibitory effect of *Lb. helveticus* extract toward bacteria and fungi, Ibrahim, *et al.* [20] established the metabolic profile by GC-MS analysis, and they detect many compounds of great antimicrobial activities e.g. Butanoic acid and its methyl esters which has been applied in the disinfection of Enterobacteriaceae in broiler chicken production stations [25]. In addition, 9,12- Octadecadienoic acid (Z,Z)-, methyl ester that has good antimicrobial and health promoting properties [26]. Also, the arising effect of Extract-Ch. NPs. has resulted by the inhibitory effect of chitosan nanoparticles toward all microbial concerns.

In relation to Karish cheese, it is clearly noted that both free Nisin and Nisin-Ch. NPs. decreased the count of the gram positive *Staphylococcus sciuri*, while *Escherichia* coli, molds and yeasts were increased. The great activity of this bacteriocin; Nisin was recorded by many researchers [27-31]. The arising count of gram negative bacteria and mycotic growth may be organized by the nutritional competition among microbial populations [32]. In contrary to Talaga cheese, the inefficient role of Diethyl ether extract, in either in free or loaded states, in Karish cheese can be elucidated by the inclusion of Talaga in salting solution along storage period that enables effective exposure to the treatments. Also, the higher viscosity of chitosan somewhat retards the diffusion of Ch. NPs. through Karish cheese, especially in absence of salting solution.

In both T5 and T6, the count of *Staphylococcus sciuri* was noted to increase and decrease along with yeast count. This behavior can be simplified by the report of Falsetta, *et al.* [33] that recorded a symbiotic relationship between *Candida sp.* and *Staphylococcus sp.* reporting that the *Staphylococcus* colonization even infection virulence were enhanced in the presence of *Candida* than if absent. Also, Carolus, *et al.* [34] co-isolated *staphylococcus spp.* as the common partners of the yeast *Candida albicans* in his niche.

The viewed promising results belonging to T7; *Lactobacillus helveticus* and its role in controlling pathogen growth and development, was widely reviewed by many researches [35]. Specifically, Bian, *et al.* [36] proved the successful control of *Penicillium sp.* especially *Penicillium chrysogenum* growth [37] by *Lactobacillus helveticus*.

5. Conclusion

The narrow antimicrobial spectrum of the conventional bio-preservatives makes them not efficient to apply as cheese preservatives. The present research recommends the DEE extract that loaded on Ch. NPs. to be applied as a preservative of Talaga cheese, while favored inoculation of milk with *Lactobacillus helveticus* 1% (w/v) to ensure the quality of Karish cheese.

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