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Prevalence of Fungal Spoilage in Local Tallaga Cheese and Applicate Novel Antimicrobial "PHR" to Extend Its Shelf-Life

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Abstract

Twenty five random samples of Tallaga cheese purchased from various markets in Cairo Governorate, Egypt were analyzed for molds and yeasts. Also, to control fungal growth in Tallaga cheese during manufacturing and storage by adding modified mixed antimicrobials was prepared according to our previous research. Our results revealed that, the molds and yeasts were detected in 36 % and 80%, respectively of examined samples. The yeast counts ranged between 7.0 and 1.0 log cfu/g, with an average 6.6 log cfu/g. Data also show that the numbers of mold ranged between 4.0 to 1.0 log cfu/g with an average of 3.7 log cfu/g. The preparation of a mix bio-preservatives powder is described. Depending on the producing mixed cultures; Lactobacillus plantarum DSA 20174, Lactobacillus helveticus CNRZ 32 and Lactobacillus rhamnosus GG, we suggested the name "PHR" for the new product. The powder is shown to have effective anti-microbial activity in a Tallaga cheese. PHR was applied during manufacturing of Tallaga cheese to prevent the growth of mold and yeast during storage period. The bio-preservative showed considerable effectiveness, and significantly eliminated mold and yeast growth by the end of shelf life.
Keywords: Tallaga cheese, mold, yeast, bio-preservative, shelf life, PHR.

Introduction

Cheese is a good source of protein, minerals particularly calcium and phosphorus which are essential components in most highly consumed foods. Tallaga cheese is usually made from heated milk with addition of low concentration of salt and cold stored cheese [**1, 2**]. Contamination of this cheese with yeast and molds may occur from a variety of sources, including the starter culture, ambient air, brine, processing equipment, and workers [**3, 4**].

Addition of preservatives is one of the simple and oldest ways to extension shelf life of cheese. Preservatives in cheese processing may help to retard alterations caused by growth of microorganisms. Green pepper, sorbic acid, sodium benzoate plus benzoic acid and hydrogen peroxide, nisin, natamycin and chitosan as additives have been used for prolong cheese shelf life [**5-8**]. The current work aims to evaluate the mycological quality of Tallaga cheese sold under market conditions at Cairo area, then to study the efficiency of modified bio-preservative mix powder to control growth of cheese contaminant; yeasts and molds to extend its shelf-life.

Materials and Methods

Microbial Strains

Lactobacillus plantarum DSA 20174 was provided by Cairo MIRCEN, Faculty of Agriculture, Ain Shams University, Egypt; *Lactobacillus helveticus* CNRZ 32 was obtained from Centre National de Recherché Zootechnique, Jouy-en-Josas, France). *Lactobacillus rhamnosus* GG was supplemented by Afify *et al*., [**9**] from the collection of the Food sciences & Nutrition dept., NRC.
Aspergillus flavus 3357 and *Saccharomyces cerviceae* Y-2223 were provided by the Northern Regional Research Laboratory Illinois, USA (NRRL).

Samples Collection

Twenty five samples of Tallaga cheese were randomly collected as sold to consumers from dairy shops in Cairo governorate.

Materials

UF milk concentrate (retentate) was obtained from Dairy Processing Unit, Faculty of Agriculture, Cairo University Egypt. Rennet powder

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(Hanelase) was obtained from Chr. Hansen's Lab., Denmark.

Production of modified mixed antimicrobials (PHR powder)

Medium described in our previous research (El-Ssayad *et al*. [**10**] was used with some modifications in this study.

Media and production of the fermentate

The new production media as listed in Table (1) was prepared and sterilized at 121 ºC / 15 min. Fermentation was conducted at 30 ºC for 72 h using all of *Lactobacillus plantarum* DSA 20174 , *Lactobacillus helveticus* CNRZ 32 and *Lactobacillus rhamnosus* GG .By the end of fermentation, the product was introduced to spray drying.

Table (1): Ingredients of modified fermentation media

Ingredients	Concentration g/l
Yeast extract	5
Meat extract	8
Peptone	10
Tri-ammonium citrate	2
Sodium acetate	5
Mg SO4. 7 H2O	0.2
Mn SO4. 4 H2O	0.05
K2HPO4	2
Glucose	20
Tween 80	1
Skimmed milk powder	11
Distilled water	1 litre

Determination of the Minimal Inhibitory Concentration (MIC).

MIC (mg/ml) of PHR-fermented milk-MRS powder was tested for MIC value. MIC was performed by the agar dilution method according to EUCAST protocol, [**11**]. Briefly, molten agar tubes were allowed to cool in the water bath at 50ºC, and then supplemented with the accurately prepared dilution series of the tested substance, vortexed well, and poured in sterile pre-labeled Petri dishes. After complete dryness of the agar surface at room temperature, 1µl of 10^7 CFU/ml of microbial suspension was inoculated. As recommended for disc diffusion plates were incubated, and results were recorded.

Spray drying of the fermentate

The produced fermentate was dried using a B-290 Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland) at temperature $_{inlet}$ 130ºC and feeding rate 50% as programmed on the device. The produced powder was collected by means of vacuum at an aspirator rate of 50%.

Cheese Manufacturing Procedures

Tallaga cheese manufacturing was carried out as follow, fresh ultra filtered (UF) milk retentate was heated at 80 °C for 15 sec, then immediately cooled to 37 °C. Enough broth culture of mold or yeast added to the warmed (37 °C) heat treated milk to provide approximately 10^3 cfu/ml. Further, Calcium chloride, sodium chloride and rennet were added at the ratios of 0.02, 3 and 0.05% (w/v), respectively. The milk was divided for two groups. The first group was untreated control, while the other group was treated, directly before addition of rennet, by antimicrobial PHR. At day zero, control only, then either control or treatment stored at refrigerator temperature, and periodically checked after 3, 7, 14, 21 and 28 days to mycological assessment.

Determination of mold and yeast count:

Mold and yeast counts were determined by pour plate technique using the media of acidified potato dextrose agar (Oxoid)**.** The inoculated plates were incubated at 25 ºC for 5-7 days. During the incubation period, the inoculated plates were examined daily for mold or yeast counts/ g and were calculated and recorded [**12**].

Chemical Analysis:

Total solids of UF-soft cheese samples were determined according to AOAC [**13**]. The pH values were measured by a digital pH meter (HANNA, Instrument, Portugal) with a glass electrode.

Organoleptic properties

The organoleptic properties of Tallaga cheese samples were evaluated ,when 1, 2, 7,14 ,21 and 28 days of cold storage by 10 expert panelists of members of Dairy Department, National Research Center. Panelists evaluated cheese for flavor (35 points), color & appearance (20 points) and body & texture (45 points) to be 100 points for the total scores.

Statistical analyses

Statistical significance was determined using Statistica Version 9 (State Soft, Tulsa, Okla., USA). The means were determined by analysis of variance test (ANOVA, two-way analysis) ($p<0.05$) [**14**].

Results and Discussion

The data in Table (2) shows that yeasts were implicated in 80% and molds were found in 36% of Tallaga cheese with mean values of 6.6 and 3.7 log cfu/g respectively.

Table (2): Incidence and counts of yeasts and molds in the examined samples. (25 samples)

Tested microorganism	Positive samples		Log count (c.f.u./gm)	
	No	%	Min.	Max. Av.
Yeast	80	20	7.1	6.6 1.0
Mold	36	9	3.7	4.0 1.0

Table 3: Frequency distribution of mould and yeast of examined Tallaga cheese

Frequency		Mould		Yeast
%	No		%	No.
0	5	20	16	**64**
10>	20	80	9	36
10^2>	15	60	6	12
10^3>	12	48	5	20
10^4>	9	36	1	4
10^5>	3	12	0	0
10^6>	1	4	0	0
10^7>	1	4	0	0

It is evident that the highest frequency distribution of all examined samples for yeast and mold count lies within the range 10^4 – 10^5 and 10^3-10^4 cfu/g respectively (Table 3). Similar results were detected by Hassan *et al.*, [**15**], who reported that the yeast and mold count of cheese samples lies within the range <10 – 9 x10^3 cfu/g. Also, EL-Shinawy *et al.*, [**16**] and El-Bagoury *et al.*, [**17**] detected higher results for total yeast and mold count in locally produced cheese. These results in contrast with results in Egyptian soft cheese obtained by Sharaf *et al.*, [**18**]. The high total yeast counts have resulted from inadequate processing [**19**]. The main defects caused by spoilage yeasts are fruity, bitter or yeasty off flavors, gas production, discoloration changes and texture [**19-21**]. Mould growth on cheese causes economic losses from discoloration, poor appearance, flavor and production of mycotoxins [**22**]. Results of mould and yeast in Tallaga cheese were similar to that found by El Kholy *et al.*, [**23**], since the rejected 80% of the vended Tallaga cheese were not meet the Egyptian standard because of high mycological counts. In the same concern, Abou Dawood *et al.*,[**24**] and Lotfy *et al.*, [**25**] found that many of different cheese samples collected from Giza and Cairo Governorates contained higher counts of yeasts and molds than the Egyptian Standard Specifications .

The minimum inhibitory concentration (MIC)

All statistical analysis was done using EXCEL-2010 software. All measurements were done for at least three replicates. Results are presented as means ± standard error of means (SEM).

Table (4): (MIC) of the dried antimicrobial PHR

Tested Organism	MIC (mg/ml)
Aspergillus flavus	11.3 ± 0.55
Saccharomyces cerviceae	6 ± 0.00

All statistical analysis was done using EXCEL-2010 software. All measurements were done for at least three replicates. Results are presented as means ± standard error of means (SEM).

MIC of the dried antimicrobial PHR was evaluated to optimize the dose that could fully inhibit the growth of the tested spoilage yeast and fungi. The PHR inhibited target fungi *Aspergillus flavus* at a concentration of 11.3± 0.55 mg/mL, and this was the lowest MIC observed in this study. However, the MIC for the yeast strain S*accharomyces cerviceae* was 6 ± 0.00 mg/mL.

The effect of antimicrobial PHR in Tallaga cheese

1-Chemical Analysis

Total solids (TS) of UF- soft cheese samples as fresh and during storage period were illustrated in Table (5). The total solids values were (37.34%) as fresh sample and were slightly increased to (38.94%) after one month of storage period. It was observed that the range of TS values were in similar with that found by El-Kholy *et al.*, [**26**] who fortified UF-soft cheese with different ratios of mushroom. Also, Farrag *et al.*, [**27**] showed the same range of total solids for probiotic UF-soft cheese fortified with various ratios of garlic extract. The rise of TS values was slightly pronounced after 30 days and it could be due to the aqueous phase that covered the cheese surface during the storage period. This phase is characteristic with antimicrobial effect and can boost the shelf life of the product. Same results were found by Abbas *et al.*, [**28**] who displayed a significant increase in UF-soft cheese total solids during storage period for 30 days. On the other hand, the pH values of UF-soft cheese samples were explained in Table (5). It was noticed that the pH values were decreased as storage period increased.

Table (5): Total solids (%) and pH of Tallaga cheese during storage period.

Storage period	Fresh	7 d	14 d	21 d	28 d
Total Solids (TS)	37.34	37.70	37.81	38.23	38.94
pH	6.29	5.89	5.85	5.71	5.57

It was 6.29 at fresh sample and reached to 5.57 after one month of storage period. This reduction was comparable with the pH values of UF-cheese samples that displayed by Abbas *et al*., [**29**] and Abbas *et al*., [**28**]. On the same side, Abbas *et al*., [**2**] represented a homogenous data for pH values of UF-soft white cheese after 30 days of storage period.

2- Organoleptic assessment

Organoleptic properties are one of the most important factors in desirable product for the consumer. New product will only succeed when the consumer agree with it and vice versa. The results of the organoleptic properties of Tallaga cheese stored at 7ºC for 28 days are given in Table (6).

Addition of PHR powder as preservative during the manufacturing of cheese had no significant changes in both texture and flavor after the first two days. After 7 days till the end of 28 days, there a significant enhancement of all sensory items. Flavor parameter there were significant differences ($p \leq 0.05$) between control compared to treatments sample when cold storage period. So, Table (6) illustrated the acceptability of our new product Tallaga cheese to the panelist according to some parameters such as flavor, body, texture and appearance.

3- The inhibitory effect of PHR on yeast and mold in Tallaga cheese

In continuation to our previous research, selecting the "PHR" mixture of antifungal was the most effective on yeast and fungi, modifications and dried form were made to the product for ease of handling in preparation for testing it on food products. It was applied to food products, and the Tallaga cheese was selected to test the inhibitory effect of dried "PHR". The results of antifungal effect of "PHR" against yeast and mold in Tallaga cheese are shown in Tables (7) & (8). The initial mold log count of Tallaga cheese, at zero time, was 2.0 ± 0.0 cfu/ gm. The log count of mold after 3days of refrigerated storage started to increase gradually till reach 3.1 ± 0.03 cfu/ gm by the end of the 28 days, (Table 7) while in the cheese samples treated with PHR, after the first 3 days, a significant reduction and disappeared in mold count over the storage time.

From the results gained and summarized in Table 8, the initial yeast log count of Tallaga cheese, at zero time, was 3.0 ± 0.0 cfu/ gm. Then, the count of yeast of refrigerated storage started to increase gradually till reach 6.5 ± 1.7 cfu/ gm. by the end of the 7 days, while in the cheese samples treated with PHR, the yeast count was much lower than the count of control sample stored for the same period. The yeast could not be detected after 14 days of storage in treated samples, while log counts recorded 5.4 ± 0.01 cfu/ gm in PHR free samples. Wang *et al*., [**30**] and Knight *et al*., [**31**] used Lactic acid bacteria (LAB) to inhibit spoilage microorganisms. Other antimicrobial substances, such as organic acids, cyclic dipeptides, short-chain fatty acids, hydrogen peroxide and bacteriocins, might play an important role in the preservation capabilities of LAB [**32, 33**].

Nisin is a bacteriocin widely used as a food preservative particularly in cheese [**7, 34-35**]. Nisin is active against most Gram-positive bacteria. Fermentation of *Streptomyces natalensis* and related species commonly employed for the prevention of molds and yeasts contamination. Chitosan is an antimicrobial agent and can use to prolong the shelf life of cheese [**6**]. Fernandez *et al*., [**36**], found that *Lb. rhamnosus* A238 alone or in combination with *Bifidobacterium animalis* subsp. *lactis* A026 inhibited mold growth in cottage cheese. The results obtained by Ombarak & Shelaby [**37**], showed that natamycin and potassium sorbate have an inhibitory effect on survival of mold in Tallaga cheese kept at refrigeration temperature.

Table (6): Organoleptic properties of Tallaga cheese during storage period.

Storage periods	Control& Treatments	Color & appearance (20)	Body & texture (45)	Flavor (35)	Total score (100)
1day	C	19.7±0.70	43.3±3.60	33.5±3.02	96.5
	T	17.3±1.58	43.2±2.32	32.3±2.66	92.8
2 days	C	18.9±1.46	42.3±3.37	33.0±2.56	94.2
	T	16.1±1.25	42.7±3.41	32.6±2.77	91.4
7 days	C	18.6±1.4	40.3±3.71	30.3±2.68	89.2
	T	18.8±0.83	42.5±3.08	33.8±0.97	95.1
14 days	C	19.5±0.50	42.0±0.00	30.5±0.50	92
	T	18±0.00	43±0.00	33±0.00	94
21 days	C	19.5±0.50	42.0±0.00	30.5±0.50	92
	T	19±0.00	44±0.00	34±0.00	97
28 days	C	15.5±0.50	42.0±0.00	28.5±0.50	86
	T	20±0.00	44±0.00	34.5±0.50	98.5

Table (7): Inhibition in mold counts in treated Tallaga cheese by PHR powder as compared with untreated ones during refrigerated storage

Storage periods	Untreated samples	Treated samples
Zero time	±0.0 2.0	----------
3 days	±0.03 3.1	±0.0 2.0
7 days	±0.04 3,8	Nil
14 days	±0.0 2.0	±0.0 1.0
21 days	±0.0 2.0	Nil
28 days	±0.03 3.1	Nil

Data expressed as Mean ± Standard error, all columns or rows of the different letter are significantly different (P< 0.05)

Table (8): Inhibition in yeast growth in treated Tallaga cheese by PHR powder as compared with untreated ones during refrigerator storage

Storage periods	Untreated	Treated samples
Control(Zero time)	3.0±0.0	
3 days	3.7±0.02	2.3±0.0
7 days	6.5±1.7	2.8±0.03
14 days	5.4±0.01	Nil
21 days	5.4±0.02	Nil
28 days	5.1±0.02	Nil

Data expressed as Mean ± Standard error , all columns or rows of the different letter are significantly different (P< 0.05)

The present work was conducted as a step to introduce an effective preservative to the field of cheese industries. This application is in the way to improve tools of food preservation that based on nature, utilizing mixture consisting of *Lb. plantarum*, *Lb. helveticus* and *Lb. rhamnosus* GG. The considerable potential of *Lb. plantarum* as both antibacterial and antifungal can be attributed to the co-production of 2-Hydroxyisocaproic acid and derivatives that are considered a great bactericidal and fungicidal compound [**38**]. Also, *Lb. plantarum* is commonly used to inhibit fungal growth especially that invade cereals-based products. The results obtained by Arena *et al.*, [**39**] reported the promising applications of *Lb. plantarum* is a probiotic strain to be used in different fields. Russo *et al.*, [**40**] used the *Lb. plantarum* as antifungal especially against the cereals based products. Bian *et al.*, [**41**] found that the use of *Lb. helveticus* KLDS 1.8701 as a bio-protective culture, could inhibit *Penicillium* sp. and other spoilage microorganisms.

Conclusion

Overall, results of the study clearly indicated that the bacteriological quality of the examined Tallaga cheese was inferior. Most of the examined samples especially those obtained from small scale are highly contaminated with large number of yeasts and molds. Therefore, the use of natural products such as antimicrobial "PHR" during cheese manufacture reduced the growth of yeasts and molds and prolonged the shelf-life of cheese.

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