

IMPACT OF SOME INHIBITORS ON FUNGI GROWTH DURING RAS CHEESE STORAGE

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ABSTRACT

The growth of fungi on cheese surface causes significant economic losses for producers in addition to the negative impact on the consumers health as a result of their exposure to mycotoxins, especially that produced by *Aspergillus spp.* The present study is a trail to protect Ras cheese from fungal attack. The cheese wheels was coated with a water suspension of 50% of bentonite clay, sprayed with 5% of sodium bicarbonate (NaHCO_3) solution or wrapped with plastic membranes treated with 5% solution of NaHCO_3 and compared with control cheese wheels (without treatment) during 6 months of ripening and cold storage. The results showed *in vitro* growth inhibition effect of of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium requeforti* cultivated in PDA medium amended with bentonite clay (50%) or NaHCO_3 (5%) solutions. These two treatments were found to prevent fungal growth on cheese surface without affecting the chemical and the sensory properties of cheese.

INTRODUCTION

Ras cheese is a popular dairy product in Egypt and the Arab world (Abou-Donia, 2002). As any hard cheese, Ras cheese usually ripened for several months in relatively low temperature and high humidity rooms. Under such conditions moulds may grow on the cheese surface and may penetrate the cheese leading to sever economic losses and health hazard to the customers because some moulds are capable of producing toxic metabolites in cheese (Morquard and Frolich, 1992; and Tournas, 1994). The moulds are widely spread in the air, walls and shelves surfaces of cheese ripening and storage rooms and may cause spoilage of Ras cheese, especially when general good cheese manufacturing practices are not fully followed (Zommara and Rahed, 2004). Most molds commonly found on cheese belong to Genera *Penicillium* and *Aspergillus*. *Penicillium* species are predominating in temperate climates, while *Aspergillus* species are predominate in tropical and subtropical countries (Taniwaki and Dender, 1992; and Ominski *et al.*, 1996). Therefore, prevention of moulds growth on cheese surface is of great importance. Currently, many methods based in antimicrobial packaging (Brody *et al.*, 2001), synthetic and natural mycostatic agents are widely used to control growth of moulds on Ras cheese (Hassan and El-Deeb, 1988, Brul and Coote, 1997, Abou Dawood, 1996 and 1999, Abdel-Kader, *et al.*, 2001 and Zommara and Rashed, 2005). However, these methods are expensive and increase the production cost. Therefore, the small cheese factories use some traditional methods such as washing the cheese

wheels after ripening or storage with salted water or scraping the cheese wheel surface for removing mould growth. Superficial removal of the visible mold on cheese surface may enhance its appearance for marketing but does not affect the metabolites inside the cheese rind, and therefore, gives no guarantee of safety to the consumer. The aim of the present study was to investigate 3 treatments to control fungal growth on Ras cheese surface. The cheese wheels was coated with a water suspension of 50% of bentonite clay, sprayed with 5% of sodium bicarbonate (NaHCO_3) solution or wrapped with plastic membranes treated with 5% solution of NaHCO_3 and compared with untreated cheese wheels during 6 months of ripening and cold storage.

MATERIALS AND METHODS

Materials

Bentonite was purchased from the Egyptian Foundation for Mining and Stone Grinding, Burg El-Arab city, Egypt. It was used as a 50% suspension for cheese coating. The ideal half-cell chemical formula for Bentonite was as a 2:1 expanding smectite clay minerals is; $\text{M}_0.33\text{H}_2\text{OAl}_1.67(\text{Fe}^{+2}, \text{Mg}^{+2})_{0.33}\text{Si}_4\text{O}_{10}(\text{OH})_2$. where M refers to a metal cation in the interlayer space between sheets and its cation exchange capacity (CEC) is 52 mef/100g Bentonite (Sparks, 1995). Sodium bicarbonate NaHCO_3 (Algomohriya Company, Cairo, Egypt) was dissolved in distilled water to give 5% (W/V) Commercial plastic sheet roll for food rapping was purchased from the local market at Tanta city, Gharbia governorate, Egypt. The sheets were immersed in 5% solution of NaHCO_3 and used for cheese rapping.

Ras Cheese Treatments

Twelve samples of green cheese wheels (3kg) were purchased from a local Ras cheese plant at Dakhlia governorates. The cheese was divided into four groups with 3 wheels in each group: Group (1) without treatment (control), group (2) were coated by 50% suspension of Bentonite clay, group (3) were wrapped using plastic sheet (PI) immersed in 5% sodium bicarbonate (NaHCO_3) solution, group (4) were sprayed with 5% NaHCO_3 solution.

Microbiological Analysis

In Vitro Antifungal Tests Of NaHCO_3

The antifungal effect of NaHCO_3 was tested using PDA medium supplemented with 1, 2, 3, 4 and 5% of NaHCO_3 . Thirty Petri dishes (6 dishes for each NaHCO_3 concentration) were poured with the medium and then a 1 cm^2 disc from a previously cultivated *P. requeforti*, *A. niger* or *A. flavous* in PDA medium were placed in the middle of the dishes (2 plates/mould). The fungal growth was visually observed.

In vitro antifungal tests of Bentonite

The antifungal effect of Bentonite clay was tasted using PDA medium supplemented with 10, 20, 30, 40 and 50% of Bentonite. Thirty Petri dishes (6 dishes for each Bentonite concentration) were poured with the medium and then a 1 cm^2 disc from a previously cultivated *P. requeforti*, *A. niger* or *A.*

flavous in PDA medium were placed in the middle of the dishes (2 plates/mould). The fungal growth was visually observed.

Microbiological analysis of cheese

Surface samples were collected by scribing cheese wheels surface by using a sharp knife, then the collected samples were analyzed for their yeast and mold content as described by Clark *et al.*, (1978). Total bacterial count (CFU/g cheese) was counted on nutrient agar medium according to Difco, (1974). Mold and yeast in the cheese, Yeasts and moulds were counted on Potato dextrose agar medium (PDA) according to Lück and Gavorn, (1990). Coliform bacterial count was counted on MacConkey agar medium according to Difco, (1974).

Chemical analysis of cheese

Cheese samples were analyzed when fresh and after 30, 60 and 90 days of ripening period at refrigerator temperature (about 7 °C). The moisture content of cheese was determined according to the British Standard Institution B.S.I., (1952). Cheese fat was determined using the conventional Gerber's method using the special butyrometer for cheese according to Ling, (1963). Cheese titratable acidity was estimated as percentage of lactic acid according to Ling, (1963). Total nitrogen content of cheese samples was determined by Kjeldahl method as described by A.O.A.C. (1984). The soluble nitrogen content of cheese samples were measured by blending 5 g of cheese sample in an auto-mixer with 100 ml distilled water for 5 minutes. The mixture was decanted to 250 ml volumetric flask and the nitrogen was determined in 25 ml aliquots of filtrate by the kjeldahl method (Ling, 1963). The Shilovich ripening index (SRI) and formol ripening index (FRI) were carried out according to Abd El-Tawab and Hofi, (1966).

Statistical Analysis

Data are expressed as mean \pm SE for 3 replicates and statistical analysis was performed using Duncan's multiple range test according to Duncan, (1955) and T-test according to Fisher (1970).

RESULTS AND DISCUSSION

***In vitro* studies**

The effect of different concentrations of sodium bicarbonate (NaHCO_3) or Bentonite clay on some fungi strains commonly isolated from Ras cheese (Saleh, 2003, Hassan *et al.*, 2004, Zommara & Rashed, 2004) and other hard cheeses (Hocking and Faedo 1992, and Torkar & Vengušt, 2008). were investigated *in vitro*.

***In vitro* effect of NaHCO_3**

Effect of different concentrations of NaHCO_3 on the growth of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium requeforti* cultivated in PDA medium amended with 1, 2, 3, 4, 5% of NaHCO_3 are shown in Fig. (1). There was an inverse relationship between the concentration of NaHCO_3 in the media and the degree of fungal growth however, *Aspergillus flavus* was more sensitive for the medium pH than the other two fungi strains. The fungal growth was markedly suppressed by increasing the concentration of NaHCO_3 .

to 5% in the cultivated PDA medium. Bandelin (1958) stated a progressive loss of antifungal activity of spore suspensions planted on sterile slants of a modified Sabouraud's agar medium with increasing pH values to the point that some were totally ineffective in the neutral and alkaline range. Also, Wheeler, *et al.*, (1990) found that *Aspergillus* species were more tolerant of alkaline pH while *Penicillium* species appeared to be more tolerant of acidic pH.

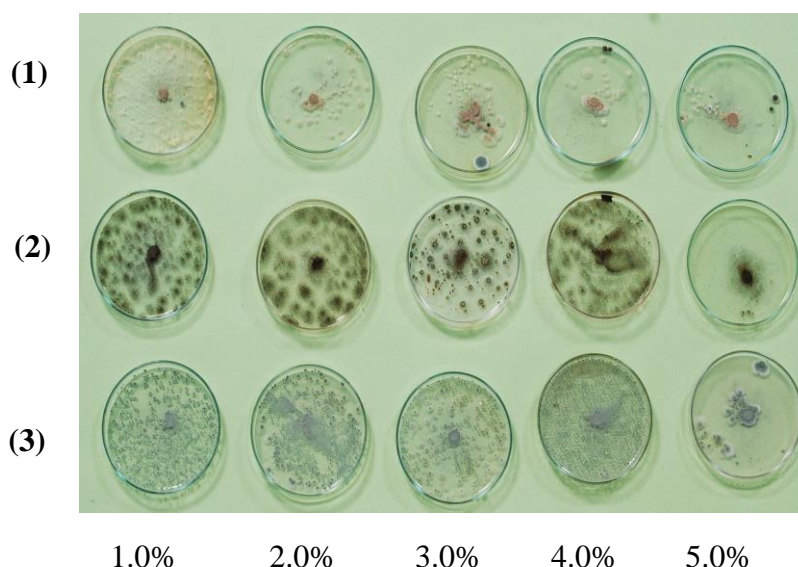
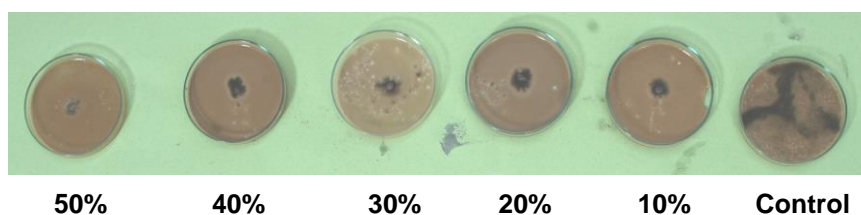


Fig. (1): The antifungal effect of different concentrations of NaHCO₃ in PDA medium cultivated with (1) *A. flavous*, (2) *A. niger* or (3) *P. requeforti*.

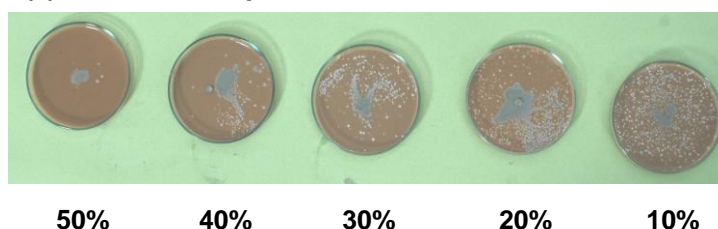
***In vitro* effect of bentonite clay**

Bentonite is a kind of clay, which is distributed in many places in Nile valley. It is used in the industry purpose or for preparing animal and poultry diets and food staffs. Fungi-Tox Forte used in poultry feeding is a high technological products from Grand vet international, llc U.S.A composed of about 25% Bentonite. The aim of using Bentonite in poultry feeding is to protect chickens from fungi and enhancement of poultry biological performance (Fairchild *et al.*, 2008 and Shi *et al.*, 2009). Fig. (2) shows the effect of different concentrations of Bentonite clay on growth of *Aspergillus niger*, *Penicillium requeforti* and *Aspergillus flavus* cultivated in PDA medium. The results showed that *Aspergillus spp.* was more sensitive than *Penicillium requeforti* as the Bentonite clay completely suppressed the growth of *Aspergillus niger* and *Aspergillus flavus* at concentration of 10% in the PDA medium. However, the growth of *Penicillium requeforti* was inhibited at 50%.

(1) *Aspergillus niger*



(2) *Penicillium requeforti*



(3) *Aspergillus flavus*

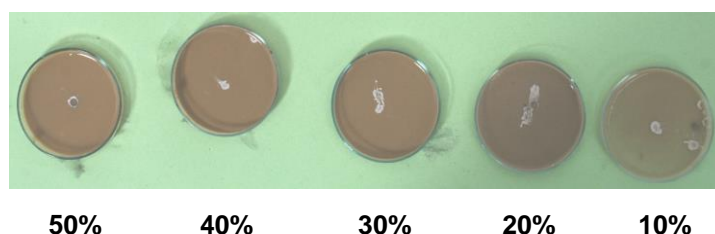


Fig. (2).The antifungal effect of different concentrations of Bentonite clay in PDA medium cultivated with *A. niger* (1), *P. requeforti* (2) or *A. flavus* (3).

Mould and yeast count on Ras cheese surface

Data in Fig. (3) show moulds and yeasts counts (CFU) on Ras cheese surface. Compared to control, a remarkable reduction of fungi numbers was found on surface of cheese coated with Bentonite (50%), sprayed with NaHCO₃ (5%) or wrapped with 5% NaHCO₃ treated plastic membrane during the storage period. Molds can grow well on the surfaces of cheeses when oxygen is present, with the low pH being selective for them. In packaged cheeses, mold growth is limited by oxygen availability, but some molds can grow under low oxygen tension. Molds commonly found growing in vacuum-packaged cheeses include *Penicillium* spp. (Hocking & Faedo, 1992). Coating of cheese with Bentonite clay or spraying it with NaHCO₃ resulted in a unsuitable growth condition for fungi on cheese surface either by increasing the pH or prevention of air from cheese in addition to protection cheese surface from contacting the surrounding environment (Manzon & Merlo, 2004 and Zommara.& Rashed, 2005). Galli & Vezzuli, (1982) found

that coating semi hard and hard cheese with Bentonite clay delayed the appearance of moulds on the cheese compared to control and completely inhibited if the treatment with was repeated every 15 days. Also, Saleh, (2003) stated that coating Ras cheese with Bentonite prevented the growth of molds on cheese surface up to 60 days of cold storage.

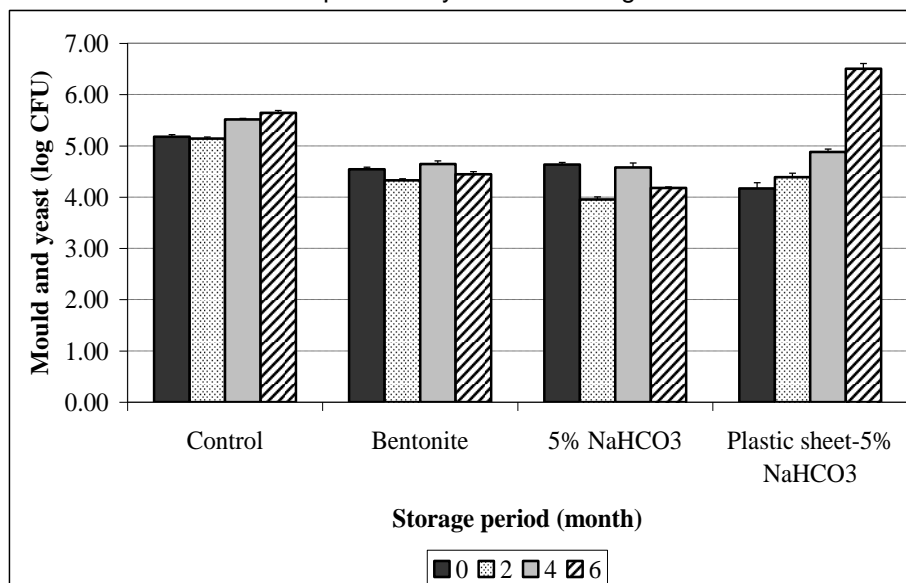


Fig. (3): Mould and yeast count on Ras cheese surface during 6 months of experimental period.

^{abc}Means with unlike superscript letters within groups are significantly different ($p < 0.05$).

Cheese mould and yeast count

The results of determining the number of moulds and yeasts in cheese are illustrated in Fig. (4). The data showed lack of differences among all treatments at the beginning of the experiment, however, there was a marked reduction of these numbers after two months of storage in the NaHCO₃ treated cheese. On the other hand, these numbers markedly decreased after four months of storage in all treatments compared to control. There were no significant differences among all treatments at the end of the experimental period, which could be attributed to low water activity (a_w) of cheese because of water evaporation from cheese during ripening and storage. Also, it is known that several antifungal active components derived from the hydrolysis of casein, fat and lactose are produced during ripening of cheese. Magnusson (2003) studied the antifungal effect of lactic acid bacteria and identified several antifungal compounds. The majority was low molecular weight compounds, e.g. hydroxy fatty acids, 3-hydroxypropionaldehyde and cyclic dipeptides and a proteinaceous compound.

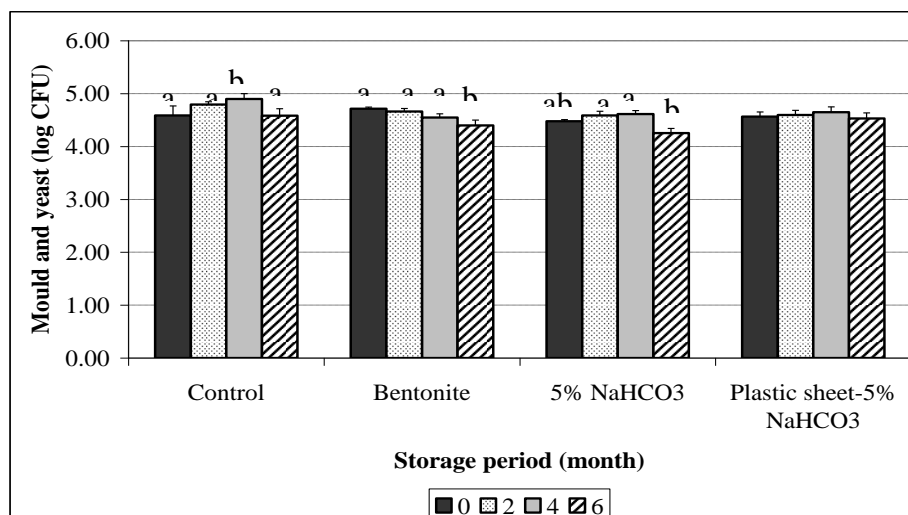


Fig. (4): Mould and yeast count in Ras cheese during 6 months of experimental period.

^{ab}Means with unlike superscript letters within groups are significantly different ($p < 0.05$).

Cheese total bacterial count

The results of total microbial count in cheese are shown in Fig. (5). The results showed considerable variation in numbers among samples at the beginning of the experiment. After two months of storage the analysis showed significant increase of these numbers for all treatments with no differences among them except for the cheese wrapped in the NaHCO₃ treated plastic membrane that increased significantly compared to the other treatments. While the total bacterial count continued to increase after four months of storage only in the control cheese it declined at the end of the experimental period in all cheese treatments. On the other hand, cheese treated with Bentonite or NaHCO₃ characterized with a marked low bacterial count compared to other treatments. The growth of the natural cheese flora resulted in a significant increase in total bacterial counts after 2 months of ripening and storage. However, the subsequent reduction of total bacterial counts may be attributed to the decrease of cheese water activity (a_w) and production of antimicrobial components during fermentation process as previously mentioned by Magnusson (2003).

Cheese coliform bacterial count

The results of estimating the numbers of coliform bacteria in cheese are shown in Fig. (6). The data showed low incidence of this bacteria in all cheese treatments (50-70 cfu) with no significant differences among all treatments at the beginning of the experiment. However, these bacteria were disappeared from all treatments after two months of storage until the end of the experimental period. These may reflect relatively good hygienic condition for the cheese production process and the growth inhibition effect of some lactic acid bacteria (Dabiza & El-Deib, 2007). These results are in agreement with the results obtained by Saleh, (2003) who found a significant gradual

reduction in coliform bacterial count in Ras cheese treated superficially with different plant extracts, Bentonite or Aswan clay to protect it from fungal attack during 90 days of cold storage.

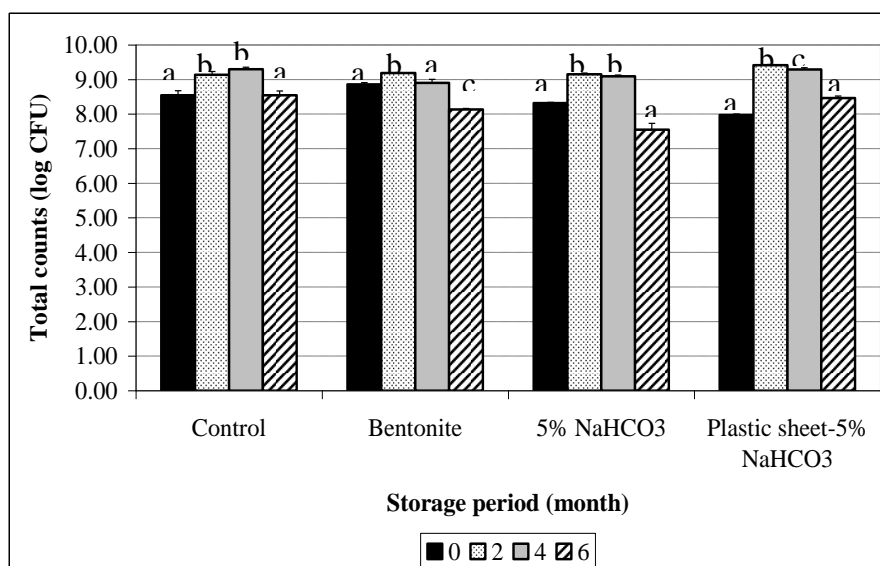


Fig. (5): Total microbial count in Ras cheese during 6 months of experimental period.

^{abc}Means with unlike superscript letters within groups are significantly different ($p < 0.05$).

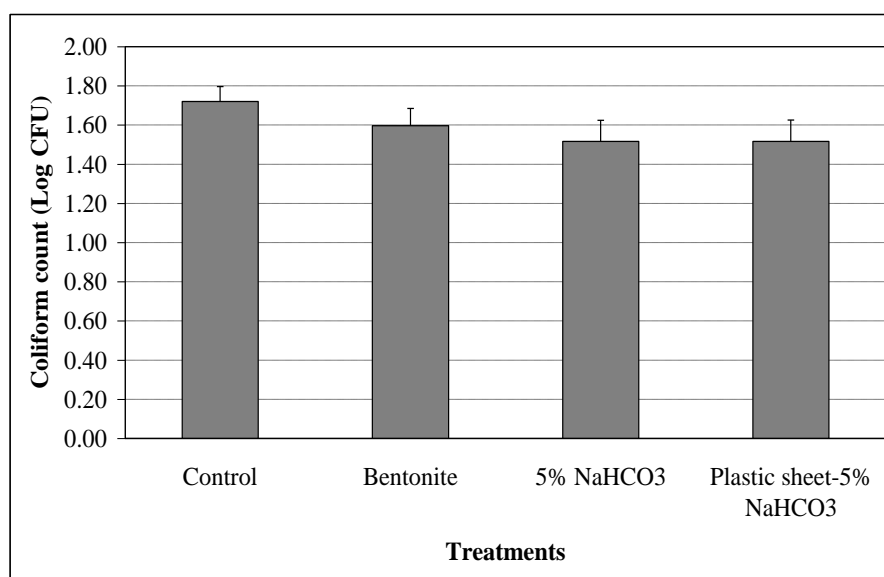


Fig. (6): Coliform bacterial count in Ras cheese at zero time of experimental period.

Chemical analysis of cheese

Cheese moisture

Data illustrated in Fig. (7) shows the moisture content of Ras cheese treatments during the experimental period. Moisture content of cheese showed no significant differences among all treatments during the storage period. However, a gradual decrease in cheese moisture was found in all treatments during storage period but to a lesser extent in the cheese coated with Bentonite or wrapped with the plastic membrane. These materials, to some extent, prevented evaporation of water from cheese during storage. Ras cheese is a typical hard cheese, so that the loss of water from cheese during manufacture and storage to meet the official standards at sale is of great important to the producers. Ras cheese used in this study was from the mini wheel type (~ 3k Kg / wheel) and it was expected to loss its moisture faster than the original type weighing about 13-15 Kg / wheel.

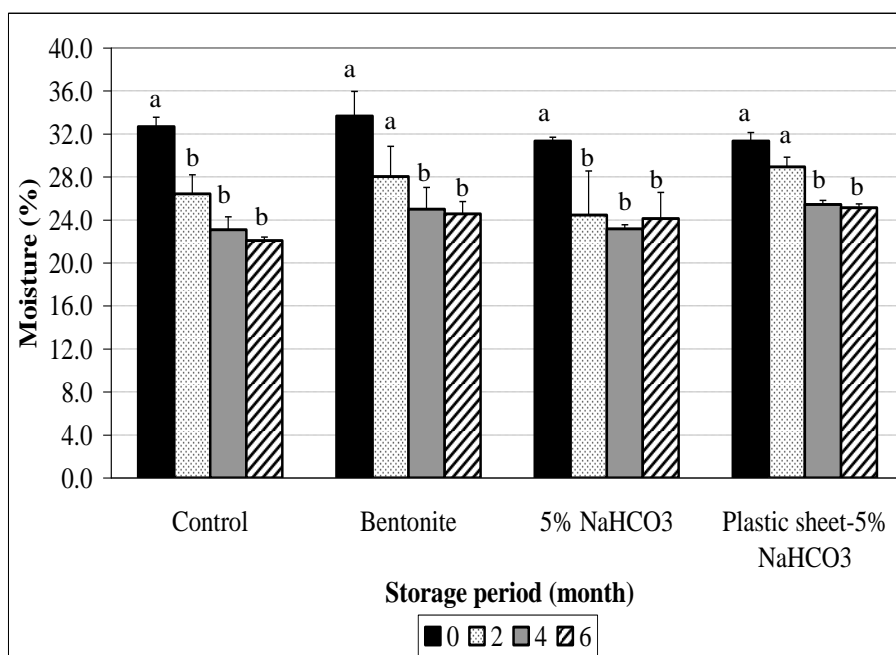


Fig. (7): Moisture content of Ras cheese during 6 months of experimental period.

^{a, b} Means with unlike superscript letters within groups are significantly different ($p < 0.05$).

Cheese acidity

Data presented Fig. (8) shows the effect of Ras cheese treatments on cheese acidity during storage period 6 months of ripening and cold storage. The data show no significant differences among all treatments at the beginning of the experiments (zero time). Increasing storage period resulted in a slight increase in cheese acidity which was more prominent in cheese treated with 5% solution of sodium bicarbonate after 4 months of storage. Sodium bicarbonate (NaHCO_3) solution did not penetrate the cheese as it

can be noticed from the acidity development data for this treatment. The elevation of cheese acidity may play a significant rule in the inhibition of microbial growth in cheese by offering unsuitable condition for the growth of many microbes, namely coliforms and other acid sensitive bacteria as shown in Fig. (6).

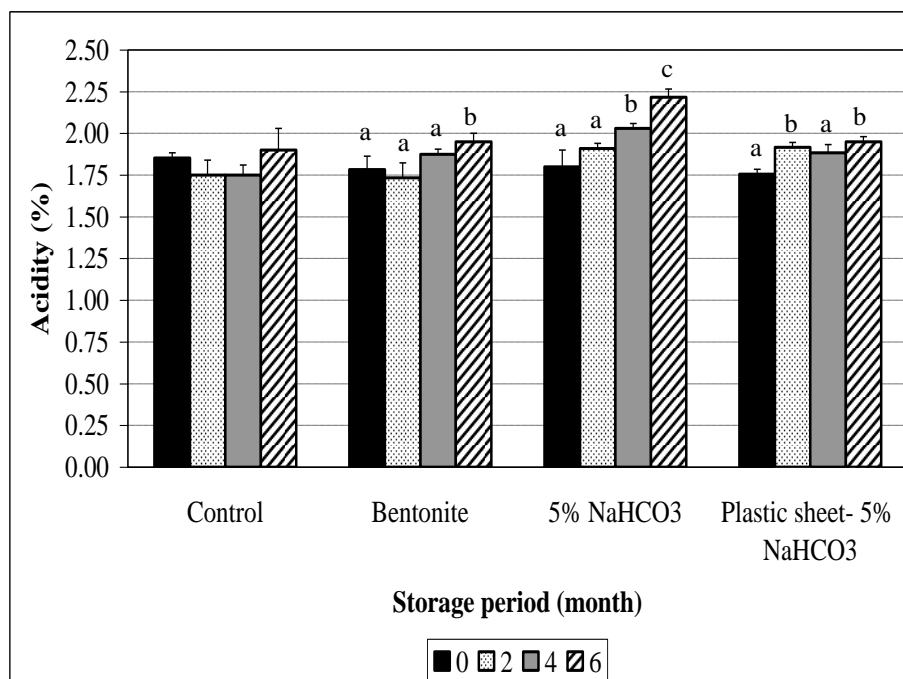


Fig. (8): Acidity content of Ras cheese during 6 months of experimental period.

^{a, b, c} Means with unlike superscript letters within groups are significantly different ($p < 0.05$).

Cheese fat content

Data for cheese fat content are shown in Fig (9) There were no significant differences in the fat content of cheese among all treatments during the storage period. However, the decrease of moisture in cheese inflected on its fat content. The data show a slight gradual increase in cheese fat content during the storage period which may be attributed to the gradual decrease in cheese moisture during storage. Our results are in agreement with that obtained by Genied (1990), Saleh (2003), El-Fadley (2010) and Kebary *et al.*, (2011).

Shilovich ripening index (SRI)

Data for Shilovich ripening index (SRI) of cheese are shown in Table (9) and illustrated in Fig. (10). The obtained data showed no significant differences among all cheese treatments which gradually increased during the storage period. It was observed Shilovich Ripening Index (SRI) increased with the advancement of storage period. It was observed that (SRI) increased with the advancement of storage period from 41.6 ± 1.43 at the beginning to

80.5 ± 2.0 at the end of storage period ($P < 0.05$). Increasing ripening index with time is in agreement with the reports of Abdalla & Abdel Razig (1997), Abdel Razig (2000), Tarakci & Kucukoner (2006), and Kebary *et al.*, (2011). The increase was mainly due to breakdown of proteins with the advancement of storage period (Abdu & Dawood, 1977, Saleem & Abdel Salam, 1979).

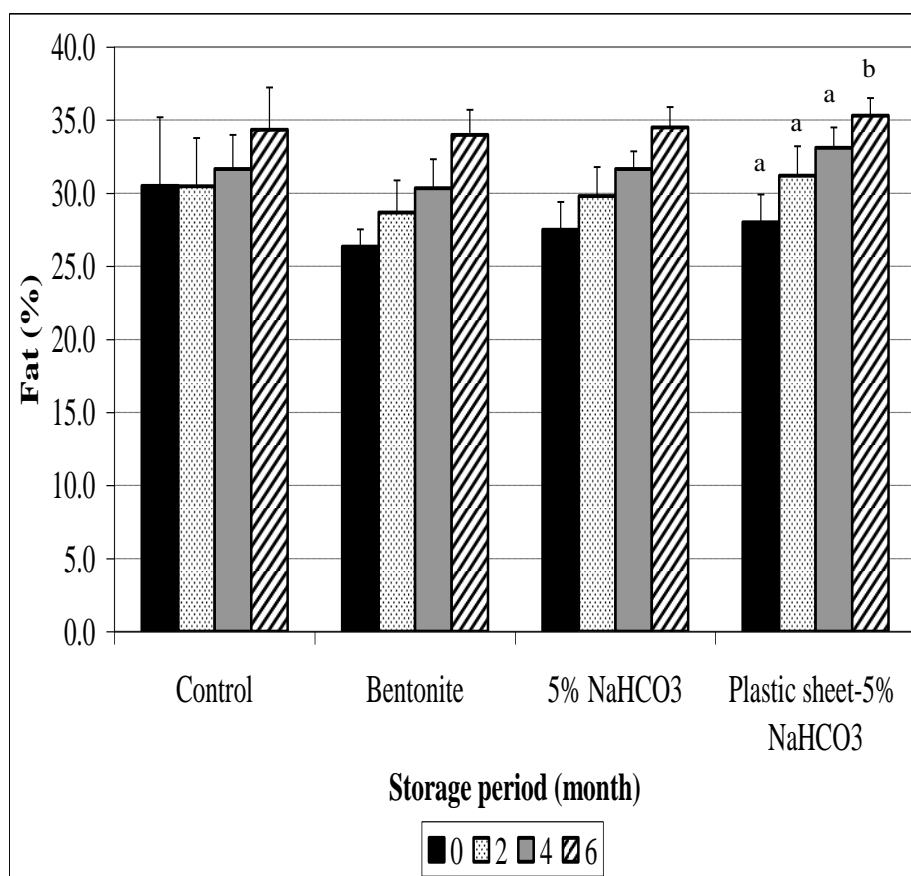


Fig. (9): Fat content of Ras cheese during 6 months of experimental period.

^{a, b}Means with unlike superscript letters within groups are significantly different at $p < 0.05$.

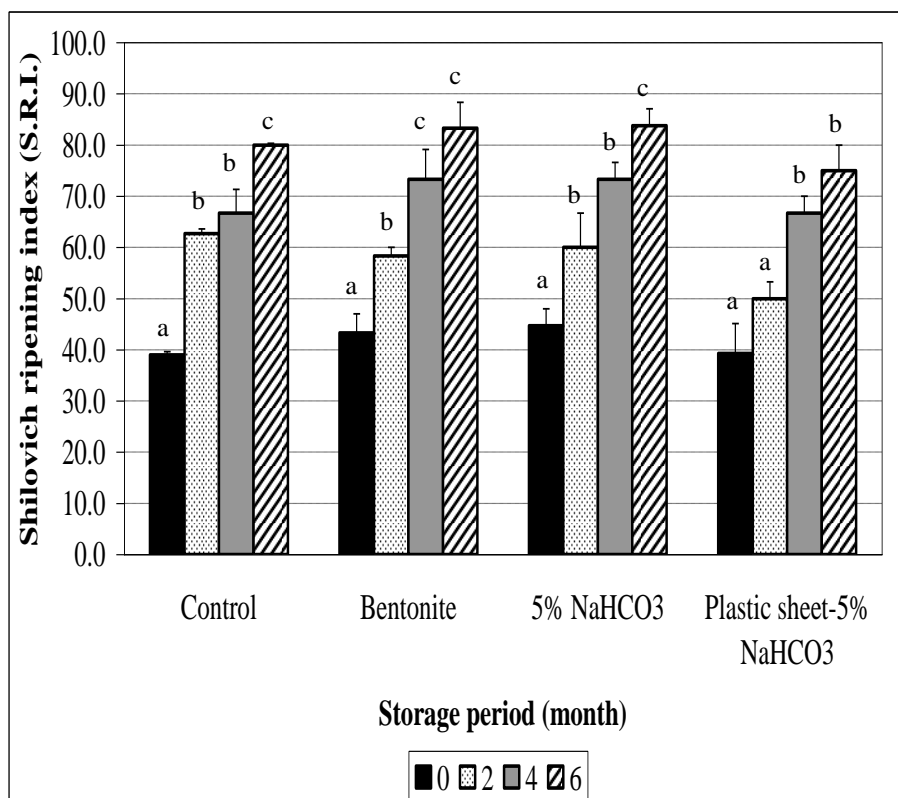


Fig (10): Shilovich ripening index (SRI) of Ras cheese during 6 months of experimental period.

a, b, c Means with unlike superscript letters within groups are significantly different at $p < 0.05$.

Formol ripening index (FRI)

Data for formol ripening indices (FRI) of cheese treatments are shown in Fig. (11). Formol ripening index have been developed for testing the ripening degree of cheeses and appears to be a useful indicator, the formol titer increasing steadily with increasing age of cheese (Abd El-Tawab & Hofi, 1966). The formol number is an indication for protein hydrolysis by milk and microbial proteolytic enzymes during cheese ripening. In consistence with the data obtained for SRI, no significant differences were found among all cheese treatments, however, FRI gradually increased during the storage period.

Cheese soluble nitrogen (SN)/total nitrogen (TN) ratio

Data illustrated in Fig. (12) shows the cheese SN/TN ratio as another measurement for cheese ripening. Cheese coated with Bentonite was characterized by high SN/TN ratio compared to the control. However, the rest of cheese treatments were located in a midway between it. These results are in agreement with those reported by Mehanna *et al.*, (2002); Fayed *et al.*, (2006) and Chen *et al.*, (2009).

In conclusion, the present study showed the possibility of using the Bentonite clay suspension (50% w/w) in coating Ras cheese to prevent and

discourage the growth of moulds and yeasts on cheese surface. Also, cheese wheels can also be sprayed with NaHCO_3 solution (5% w/w) to change the conditions of cheese surface and make it unsuitable for fungal growth. The results of wrapping cheese with NaHCO_3 (5%) treated plastic membranes was not appropriate and useless as it provide a moist environment on the surface of cheese that encourage the fungal growth. The results showed no significant deleterious effect of all treatments on the chemical composition and ripening properties of cheese.

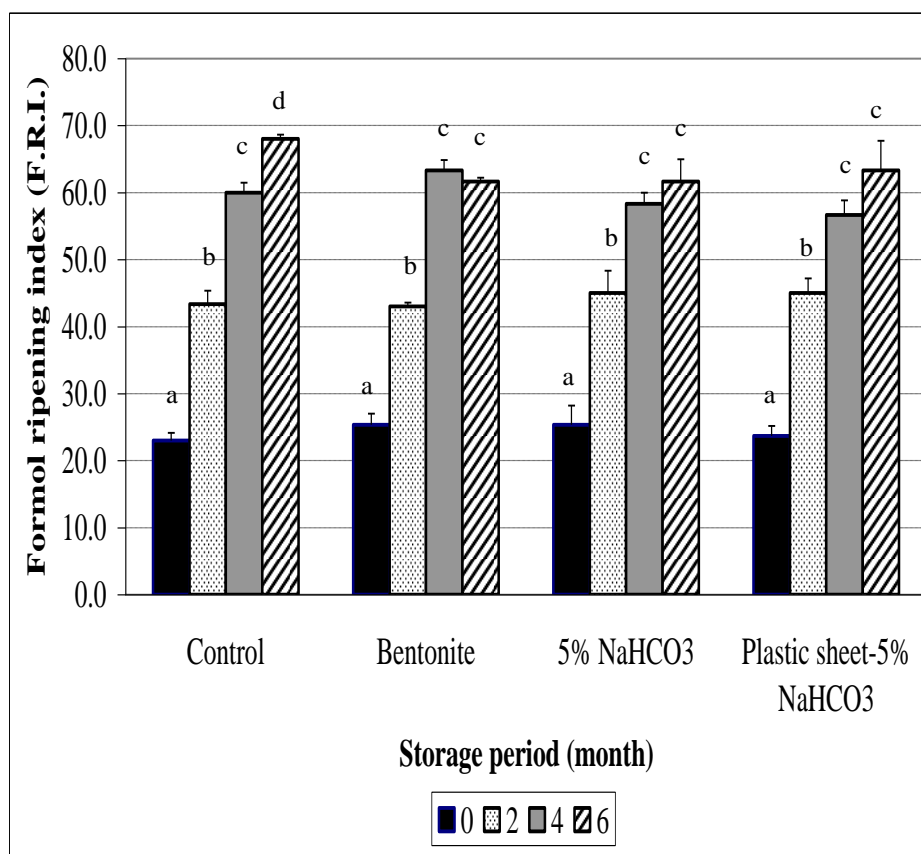


Fig (11): Formol ripening index (FRI) of Ras cheese during 6 months of experimental period.

a, b, c, d Means with unlike superscript letters within groups are significantly different at $p < 0.05$.

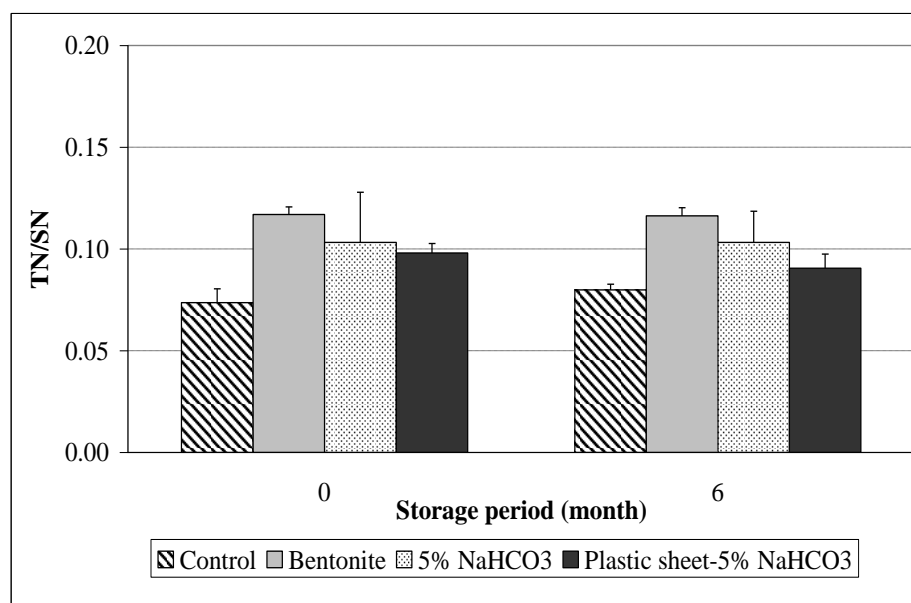


Fig. (12): Soluble nitrogen (SN) / total nitrogen (TN) of Ras cheese after 6 months of experimental period.

^{a, b} Means with unlike superscript letters within groups are significantly different at $p < 0.05$.

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تأثير بعض المثبطات على نمو الفطريات خلال تخزين الجبن الراس
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تعتبر جبن الراس هو الجبن الجاف التقليدى والرئيسى فى جمهورية مصر العربية ويطلق عليه ايضا الجبن الرومى او التركى وفقا للمنطقة التى يباع بها . ومن اهم المشاكل التى تواجه الجبن اثناء عمليات التسوية والتخزين هو نمو الفطريات على سطحها لتوافر الرطوبة مما يسبب خسائر اقتصادية فادحة للمنتجين . كما ان العديد من الفطريات تفرز انواع من السموم الفطرية مثل مادة الافلاتوكسين الذى تفرزها بعد سلالات فطر الاسبراجلس والتى اثبتت العديد من الابحاث العلمية تسببها فى كثير من المشاكل الصحية للانسان . ويمكن تلخيص نتائج الدراسة فى امكانية استخدام مادة البنتونيت بتركيز ٥٠ % فى طلاء اقراص الجبن الراس لمنع وتنشيط نمو الفطريات والخمائر على سطحها وكذلك يمكن رش اقراص الجبن بمحلول قلوئى مخفف (٥ %) من بيكربونات الصوديوم وكذلك تغليف الجبن بغشاء من البلاستيك المعامل من البيكربونات (٥ %) لتغير الظروف السطحية على الجبن وجعلها غير ملائمة لنمو الفطريات والخمائر عليها .

قام بتحكيم البحث

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