

CONTROL OF BRUCELLA ORGANISMS DURING MANUFACTURING OF ACID CHEESE USING SOME ORGANIC ACIDS

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ABSTRACT

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Kariesh cheese is a highly nutritive soft acid cheese made from naturally fermented skimmed milk, mainly manufactured at home by small holders and sold at local markets. Due to unhygienic condition during manufacturing and low quality raw milk, many outbreaks of human brucellosis were reported. This study was done to evaluate the use of acetic and citric acids, as natural organic acids, to produce kariesh cheese instead of the traditional method to control Brucella infection. Sensory evaluation revealed that all treatments didn't differ ($P>0.05$) than control except 2% citric acid which characterized to a little extent by lemon flavor, crudeness body and texture and less whiter color than other treatments. *B. melitensis* strains didn't affected by acetic (1%) or citric (1.5%) acids, but both of them were completely inhibited by acetic (1.5%), or have 4 log reduction by citric (2%) acids. For *B. abortus* the field and S19 strains were completely inhibited by 1% acetic acid, but citric acid 2% significantly ($P<0.05$) reduced the count of S19 and 544 strains by 4.46 and 3.32 log cfu/g, respectively. These results suggested that treatment of raw skimmed milk with acetic acid 1.5% or citric acid 2% is a good alternative for production of hygienic kariesh cheese to control brucellosis in human.

Key words: Kariesh cheese; acid cheese; organic acids; brucellosis; Brucella strains.

INTRODUCTION

Kariesh cheese is a soft acid cheese commonly made and consumed in Egypt. This cheese is an excellent source of protein, amino acids, calcium, phosphorus, vitamins and many micronutrients. It is an acid coagulated fresh cheese, made from skimmed milk (cow milk, buffalo milk or buttermilk from sour cream) with soft composition, white curd and slightly salty (Francois *et al.*, 2004). Environmental conditions prevailing during processing and storage, combined with the composition of the cheese often, which reduces considerably its quality (Reps *et al.*, 2002). As this cheese is mainly manufactured at home by small holders and sold at local markets, so it is exposed to contamination with several types of microorganisms. All microbiological quality parameters indicated low milk quality just before the milk is transferred into cheese factories. These results suggest that milk is heavily contaminated right from the farm level so kariesh cheese is unhygienically produced and pose public health risks (Hofi, 2011).

According to Center for Disease Control (CDC), important factors in reported cheese-associated outbreaks were manufacturing cheese with raw or improperly pasteurized milk and post-pasteurization contamination (Altekruse *et al.*, 1998). Raw or

inadequately pasteurized milk from infected animals and cheese made with such milk are a frequent vehicle for human brucellosis infection (Lusk, *et al.*, 2013). Incidence of *B. melitensis* isolated from fresh cheese samples made from non boiled or non-pasteurized ewes' milk vary from area to other. Ongor *et al.* (2006) isolated *B. melitensis* from 5% of the samples, meanwhile Alim and Tomul (2005) isolated it from 8% of the samples and Kasimoglu (2002) isolated it from 14.2% of ewes' milk cheese samples at the level of 1.5-4 log MPN/g.

Brucellosis is a widespread zoonosis which is transmitted to man through direct contact with infected animals or through consumption of contaminated raw animal products, especially non-boiled or non-pasteurized milk and milk products such as cheese, cream and butter (Palmer *et al.*, 1998; Alim and Tomul, 2005 and Seleem *et al.*, 2010). Therefore, in countries where eradication of the disease in animals is not feasible, prevention of human brucellosis is primarily based on control of animal brucellosis, food-safety measures and raising the awareness of people on the impact of the disease on public health. Presenting complaints were variable, but a constant feature was persistent fevers that frequently were present for months. Evidence of liver cell dysfunction was present and diffuse

hepatitis with focal necrosis (Young, and Suvannoparrat, 1975). Consumption of raw milk products by household members is a common risk factor for brucellosis outbreak among family members (Celebi *et al.*, 2007). Independent risk factors for brucellosis found were younger age, male gender, profession and contact with animals and consumption of contaminated fresh cheese. Brucellosis were more prevalent in rural areas associated with cheese consumption (Asencio *et al.*, 2014). Rates of *Brucella* in human in the province that makes cheese with raw milk is the double as that use pasteurized milk (Celebi *et al.*, 2013). This may be due to the lack of appropriate education of the public regarding the risks associated with raw, non-heat treated cheese consumption (Karagiannis *et al.*, 2012).

Likewise, distribution of brucellosis, as one of the most common zoonosis in the world, has changed in recent years due to socioeconomic factors and international travel. Brucellosis should be considered as a differential diagnosis of fever of unknown origin even in countries where it is not endemic; it is necessary not only to rule out travels to endemic regions through clinical history, but also the consumption of infected foods imported from such areas (Farina *et al.*, 2008). *B. melitensis* outbreaks due to consumption of cheese made from unpasteurized milk were recorded all over the world, In London (Galbraith *et al.*, 1969), In Houston, USA (Thapar and Young, 1986), In Argentine (Wallach *et al.*, 1997), In Spain (Mendez Martinez *et al.*, 2003), In Greece (Minas *et al.*, 2007 and Karagiannis *et al.*, 2012), and In France (Mailles *et al.*, 2012).

There is an increasing interest in applying natural antimicrobial compounds in the food industry as consumers are increasingly avoiding the consumption of foods treated with chemicals. Natural alternatives are needed to achieve a high level of safety with respect to foodborne pathogenic microorganisms (Rauha *et al.*, 2000). Organic acids offer several advantages as antimicrobials because they are generally recognized as safe (GRAS), have no limited acceptable daily intake, are low-cost, easy to manipulate, and effect minor sensory changes on the product. However, it is important to use these acids according to good manufacture practices in order to avoid the development of strains resistant to acidic conditions (Mani-López *et al.*, 2012).

Organic acids such as citric and acetic acids have been used for years for decontamination of bacteria on beef, pork, and poultry (Mani-Lopez *et al.*, 2012). Using of lemon cheese as alternative method for home traditional method without affecting the traditional taste. Juice or vinegar in food (as salads) provide a harsh environment for foodborne pathogens to survive because of the acetic or citric acids (Beuchat *et al.*, 2006). Also, El-Khawas and Hassaan

(2015) indicate that adding of acetic acid (vinegar) to milk in the rate of 1% or citric acid 3% are effective antimicrobial intervention for kariesh cheese making. Given the significant microbial reductions observed it is an attractive and effective alternative for traditional process.

A very diffident obstacle in studying the survival of pathogenic bacteria under practical conditions in commercial cheese is the striving effort put forth for attaining cheese known to be infected with the bacteria under inspection. Generally, it is impossible under commercial conditions to predict in advance that the milk is infected with brucella and to determine the extent of the contamination that naturally occurs in the milk. Under such circumstances, it is recommended under laboratory conditions to workout artificially fortify milk then manufacture this milk into cheese.

Therefore, the aim of this study was designed to investigate the effect of citric and acetic acids (from natural sources) on *Brucella* species during manufacturing of kariesh cheese made from artificially fortified milk as alternative to the long-standing conventional home method without affecting the traditional customary taste.

MATERIALS and METHODS

Cow's skimmed milk was divided into 5 groups; the first (control for sensory analysis) was used for making of kariesh cheese as described by Fahmi (1950). Milk was pasteurized at 80°C for 15 s, thereafter cooled to 40°C then inoculated with 3% (v/v) of yoghurt starter culture. The other 4 groups, were used for making kariesh cheese by adding citric acid (Merck) (1.5% and 2%) and vinegar (as acetic acid 1% and 1.5%) as final concentration. Each one of the four groups were divided into 5 subgroup, each subgroup was inoculated with freshly grown culture of *Brucella* strain. While adding of acids, milk was shaken. All treatments were incubated at 37 °C up to curding (about 2-3 hours). Salt at 1% was added between cheese layers and the curd was left to whey drain into small cheese molds at room temperature overnight. For consistency of results three replications were performed.

Sensory analyses: Organoleptic evaluation was carried out according to the scheme of Bodyfelt and Potter (2009). The sensory attributes evaluated were the flavour (1-10 points), body and texture (1-5 points) and appearance and colour (1-5 points).

Bacterial strains: *Brucella melitensis* biovar 3 field strain (B. mel. Field), *B. melitensis* biovar 3 Ether reference strain (B. mel. Ether), *B. abortus* biovar1 strain 544 reference strain (B. ab. 544), *B. abortus* S19 vaccinal strain (B. ab. S19) and *B. abortus* biovar1 field strain (B. ab. Field). All these strains

were attained from the Department of Brucellosis, Animal Health Research Institute, Dokki, Giza, Egypt.

Culture preparation: Cultures were activated with two successive passes in 9 ml of tryptic soy broth (TSB) (Oxoid) and incubated at 37° C for 3 days. Cultures of *B. ab.* 544 and *B. ab.* Field strains were incubated in 5% CO₂ atmosphere as these strains are CO₂ dependent Brucellae. For each individual strain, 1 ml of the stock inoculum was added to 100 ml of TSB and incubated with shaking at 37° C for 18 - 24 h to reach a final concentration of approximately 10⁸ CFU/ml (determined by McFarland standard). The stock inoculum was added to milk to give final concentration of approximately 10⁶ CFU/ml. The inoculated milk was serially diluted and plated on tryptone soya agar media supplemented with antibiotic supplement (Oxoid) in duplicate to determine the initial count before treatment with the organic acids.

Bacterial count in cheese: For enumeration of *Brucella* organisms in cheese for each treatment, 25 g of cheese were placed in a stomacher bag with 225 ml of 0.1% peptone water and stomached for 1 min. Serial ten-fold dilutions were made in such a way that one or more dilutions when plated on solid medium produce a countable number of colonies and 0.1ml of

the suspension of each treatment dilutions were plated on duplicate on Brucella selective medium (tryptone soya agar media supplemented with antibiotic supplement (Oxoid)) and incubated at 37 °C either in air or in 5-10% CO₂ atmosphere according to the type of strain. Colonies were enumerated, and the CFU/g for each treatment was determined. Sample plates were kept in incubation for at least 2 weeks before ruling out.

Furtherly cheese samples in which bacterial count was less than the detection limit (Negative plates), attempts for isolation of the inoculated strain were tried as the remaining portion of each cheese was emulsified in the least amount of peptone water and all the suspension was inoculated on medium plates and incubated in appropriate conditions as previously stated. Multiple plates were used to insure accuracy and maximum opportunity for isolation of *Brucella* organisms exist if any.

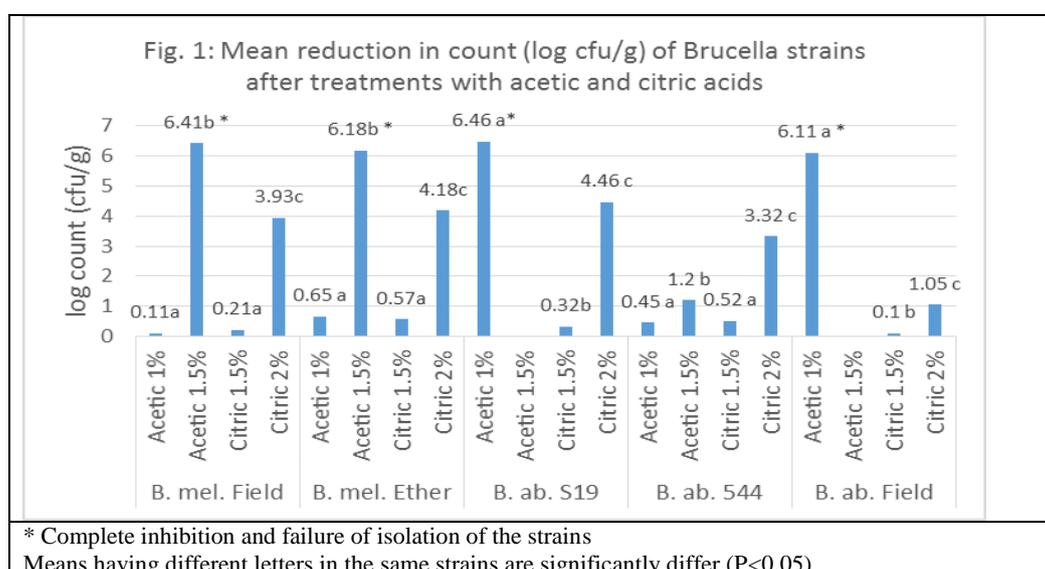
Statistical analysis: A completely randomized design was selected. The experiment was conducted in three repetitions. Data were analyzed by using the mixed procedure from SPSS software (release 20, IBM CO) after logarithmic transformation for bacteriological count. Means were separated by Fisher's least significant difference test, and significance was tested at $\alpha = 0.05$.

RESULTS

Table 1: Sensory evaluation scores of kariesh cheese with different treatments.

Cheese treatments	Flavor (1-10 points)	Body and texture (1-5 points)	Appearance and color (1-5 points)	Total (20 points)
Control	10 ^a	4.9 ^a	4.7 ^a	19.6 ^a
Citric 1.5%	9.8 ^a	4.8 ^a	4.8 ^a	19.4 ^a
Citric 2%	7.5 ^b	3.2 ^b	3.3 ^b	14 ^b
Acetic 1%	9.8 ^a	4.9 ^a	4.8 ^a	19.5 ^a
Acetic 1.5%	9.6 ^a	4.7 ^a	4.5 ^a	18.8 ^a

Means having different letters are significantly differ (P<0.01)



Concerning the yield, in previous study (El-Khawas and Hassan, 2015) there was no significant difference ($P>0.05$) between the yield between control and citric acid treatments. On the contrary, acetic acid treatments were significantly ($P<0.01$) the lowest. For sensory characteristics control, the citric acid (1.5%) and the acetic acid (1% and 1.5%) were more accepted ($P<0.01$) by the test panel (flavor and creamy body and texture) as compared with that made by the citric acid (2%), which characterized by lemon flavor, crudeness body and texture and less whiter color than other treatments (Table, 1).

Fig. (1) represents the reduction in counts of *Brucella* strains for different treatments during manufacturing of kariesh cheese. Both strains of *B. melitensis* (ether and field) didn't affected by treatment with acetic acid 1% and citric acid 1.5% meanwhile, both of them were completely inhibited (failed to be isolated) by treatments with acetic acid 1.5%, and citric acid 2% reduced the count by about 4 log cfu/g.

Whatever, Both of *B. abortus* field strain and *B. abortus* S19 vaccinal strain were completely inhibited (failed to be isolated) by treatment with 1% acetic acid, *B. abortus* 544 strain didn't affect by this concentration and was reduced to lesser extent (1.2 log cfu/g) by 1.5% acetic acid. Citric acid 2% significantly ($P<0.05$) reduced the count of *B. abortus* S19 strain (4.46 log cfu/g) and *B. abortus* 544 strain (3.32 log cfu/g) and to a lesser extent *B. abortus* field strain (1.05 log cfu/g).

Although all strains were significantly ($P<0.05$) affected by treatment with acetic acid, *B. abortus* 544 strain was the more resistant strain. On the other hand, all strains were significantly ($P>0.05$) resistant to 1.5% citric acid, but treatment with 2% citric acid significantly ($P<0.05$) reduced the count of all strains, nevertheless *B. abortus* field strain was the more resistant strain.

DISCUSSION

Significant proportion of cheeses in Egypt are made from raw milk due to the desired flavor and aroma of cheese which much more favorable in raw milk cheeses than in the ones made from pasteurized milk. Due to the immense demand nowadays on such type of food, producing cheese from raw milk has resuscitate again. Cheeses made from raw milk are in the category of the dangerous food in recent years (West, 2008). Despite the in-adequate hygienic quality used in processing such kind of raw milk processed cheese, yet remains widespread.

Sensory properties: Gobbetti *et al.* (1998) remind that the flavour intensity score of cheese was probably due to the combination of the higher concentration of lactic, citric and acetic acids and free

amino acids and soluble peptides. Very few sensory differences between the control samples and the treated samples were detected by a consumer panel. Thus, acetic and citric acids treatments in the used concentrations did not cause serious adverse sensory changes. Use of these antimicrobial treatments can be a promising intervention (Harris *et al.*, 2006).

Bacteriological analysis:

Although kariesh cheese is a good source of nutritive materials, there is a risk of contracting brucellosis due to consumption of non-pasteurized dairy products, when these are not subjected to the normal sanitary and health controls (Castell Monsalve *et al.* 1996). Meanwhile, the survival of *Brucella* strains in different dairy products is inversely proportional to the pH (El-Daher *et al.*, 1990 and Estrada *et al.*, 2005), these strains continue to increase due to elevated temperature during curdling. Mendez-Gonzalez *et al.* (2011) and Santiago-Rodriguez Mdel *et al.* (2015) recovered *B. melitensis* and *B. abortus* at pH 4 and a_w 0.89 in significantly high count in cheese elaborated with raw milk and ripened at 24° C. Nearly most previous researches studied the effect of pH on brucella organisms and they stated that pH of the product plays a critical role in the survival and growth of *Brucella* spp. in dairy products. The authors suggested a direct correlation between the survival of the microorganism and the pH and to argue as well that the role of dairy products as a vehicle for transmitting several pathogens could be predicted by determining the pH values of the products (Davies and Casey, 1973; Eloy and Lacrosse, 1976; Northold, 1984 and El-Daher *et al.*, 1990).

Organic acids are considered weak acids meaning the antimicrobial effect of organic acids is mainly caused by its undissociated forms (Malicki *et al.*, 2004). They passively diffuse through the bacteria cell wall and internalizing into neutral pH dissociating into anions and protons. Release of the protons causes the internal pH to decrease which exert inhibitory effects on the bacteria (Ricke, 2003). Moreover, the pH at which the acid is half dissociated (pKa) of citric and acetic acids varies between 3 and 4 (Dibner and Buttin, 2002) which is near to pH of cheese making (4.6).

Likewise, rapid acidification in the early stages of the process of raw milk cheeses manufacture is a key factor to effectively control the development of pathogens with low core contamination in raw-milk (Millet *et al.*, 2006).

In comparison between acetic and citric acids in the same concentration, acetic acid was more effective than citric acid, the result which agree with that obtained by Seoknam *et al.* (2003) and El-Khawas and Hassan (2015). Difference between the effect of acetic and citric acids may be referred to that, lethal

effects of these weak acids depend on concentration, pH of the environment and the dissociation constant of each acid beside adapted or resistant strains due to sub-lethal conditions (Foster and Hall, 1990). Acetic acid is widely used as preservative substances and pH-adjusting ingredients in various foods. As preservatives, acetic acid gain their antimicrobial effect by lowering the pH-value below the growth range, and their undissociated forms of acid inhibit the metabolic mechanisms of organisms (Jay, 1978).

The attained results assures the serviceable advantageous and usable outcomes of the study and the beneficial influences on the public although the resistance of field strains of *B. abortus*, as this organism die much more rapidly in commercial cheese than in the fortified laboratory samples. This could be attributed to the great influence of some factors, such as bacteriocins, hydrogen peroxide, volatile compounds, and other metabolites produced by lactic acid bacteria which possess an inhibitory effect on the growth of pathogens (Northold, 1984 and Ming, *et al.*, 1989). Also, extraction of cream layer before manufacturing of cheese may wane the probability of contracting brucellosis as huge number of the organism is removed with fat globules to which the *Brucella* microorganisms adhered (Hamdy and Abdel-Hakim, 1994).

CONCLUSION

Results of this study indicated that adding of vinegar to milk in the rate of 1.5% or citric acid 2% are effective antimicrobial intervention for kariesh cheese making that could control most of *Brucella* strains, while retain the favorable sensory properties of traditional non-heat treated product. Given the significant microbial reductions observed it is an attractive and effective alternative for traditional process.

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السيطرة على ميكروب البروسيلات أثناء تصنيع الجبن الحمضي باستخدام بعض الأحماض العضوية

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الجبن القريش جبن حمضي طرى وبالرغم من قيمته الغذائية العالية إلا انه يصنع عادة في المنازل من لبن خام مما يجعله مصدرا للعدوى حيث تم رصد عدة اوبئة بمرض البروسيلات كان السبب فيها الجبن المصنوع بهذه الطريقة، لذا قامت هذه الدراسة بمحاولة استخدام الأحماض العضوية من مصادر طبيعية متمثلة في الخل (كمصدر لحمض الخليك) وملح الليمون (كمصدر لحمض الستريك) بديلا للطريقة التقليدية لتصنيع الجبن القريش وتأثير ذلك على السيطرة على ميكروب البروسيلات لمنع انتقاله عن طريق تناول هذا الجبن المصنوع من اللبن الخام غير المعالج بالحرارة. وقد دلت النتائج على ان الخواص الحسية للجبن المنتج بالأحماض العضوية لا تختلف معنويا عن تلك المنتجة بالطريقة التقليدية، إلا ان الجبن المعالج بحمض ستريك ٢ ٪ تميز بنكهة الليمون وكانت الخثرة والملمس واللون الابيض اقل من باقى المعاملات. كما اظهرت النتائج ان عترتي البروسيلات مليتنسس لم تتأثرا بأى من المعاملة بحمض الخليك ١ ٪ او حمض الستريك ١.٥ ٪ ولكن تم القضاء عليهما تماما بالمعاملة بحمض الخليك ١.٥ ٪ او انخفاض العد البكتيرى لهما (٤ لو خلية/جم) بالمعاملة بحمض الستريك ٢ ٪. وبالنسبة لعترتي البروسيلات ابورتس الحقلية والتحصينية (S19) فقد تم القضاء عليهما تماما بالمعاملة بحمض الخليك ١ ٪ بينما احدث حمض الستريك ٢ ٪ انخفاضا معنويا فى أعداد العترة التحصينية وعترة ٥٤٤ بمقدار ٤.٤٦ و ٣.٣٢ لو خلية/ جم على التوالى. وقد دلت نتائج هذه الدراسة على ان معالجة اللبن الخام منزوع الدسم بالخل (كمصدر لحمض الخليك) بتركيز ١.٥ ٪ او ملح الليمون او عصير الليمون (كمصدر لحمض الستريك) بتركيز ٢ ٪ هو بديل مناسب وفعال لانتاج الجبن القريش والسيطرة على ميكروب البروسيلات بدون التأثير على الطعم المميز له.

الكلمات الدالة: الجبن القريش ، البروسيلات ، الأحماض العضوية ، الخل ، الليمون