GROWTH INHIBITION OF MICROORGANISMS IN REFRIGERATED MILK BY THE ADDITION OF MAILLARD REACTION PRODUCTS OBTAINED FROM A GLUCOSE-HISTIDINE MIXTURE

By

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Scope of Study: The influence of the addition of different amounts (3, 6, and 9%) of a "crude" mixture of Maillard reaction products on growth of microorganisms was determined in raw milk during storage (with or without agitation) at 5°C. Similar experiments were conducted in autoclaved (121°C for 15 min.) reconstituted 10% non-fat milk solids (NFMS) using a Gram negative psychrotroph as the test organism.

Findings and Conclusions: Growth inhibition of microorganisms was evident in both raw and autoclaved milk during four days of storage. The inhibition was more pronounced in autoclaved milk than in the raw milk. It was also more effective in samples maintained statically than in those stored with agitation. An apparent germicidal action was observed at the higher rates of added MRP in autoclaved milk; however, such an effect was not evident in raw milk.

Present studies suggest the feasibility of the use of MRP as food preservatives. However, further study is needed in this field. Purification of the inhibitory agent(s), for instance, and assurance that its use will be safe are subjects of primary interest. Association, if any, of the inhibitor with brown-colored components needs to be investigated since discoloration would be a problem in a product such as milk.

ADVISER'S APPROVAL Stanley E. Gilliland

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CHAPTER I

INTRODUCTION

The Maillard reaction is a non-enzymatic type of browning (Nickerson and Ronsivalli, 1978) common in many processed foods (Lingnert, 1980a, 1980b). The reaction is initiated by a combination of an amino acid and a sugar (Ellis, 1959). The amino acid may be present in the food in a free stage, or as part of a polypeptide or protein; the sugar must contain a reactive carbonyl group. Once the reaction is initiated, it proceeds through a series of chemical changes resulting in the formation of complex substances (Nickerson and Ronsivalli, 1978) frequently called Maillard reaction products (MRP).

These substances are flavorful and brown to black in color, and are desirable for some foods while undesirable for others (Shallenberger and Birch, 1975; Nickerson and Ronsivalli, 1978; McWeeny, 1981). An important characteristic that has been attributed to MRP is their efficacy as antioxidants (Eneirson and Reineccius, 1977; Eneirson and Reineccius, 1978; Lingnert and Eriksson, 1980; Lingnert and Lundgren, 1980; Eichner, 1981). Besides, some evidence indicates they may play a role as inhibitors of microbial growth (McKeen, 1956; Zabrodskii and Tikhomirova, 1956; Viswanathan and Sarna, 1957; Rosen et al., 1970; Andrade et al., 1979). From a food preservation standpoint, these last two factors open the possibility of using such products as antimicrobial food additives.

Despite considerable discussion in recent years about the

possibility of hazards from the use of food additives, the world wide crisis in the food supply demands that losses be reduced to a minimum. Consequently, the use of chemical preservatives alone, or as a supplement to other means of preservation is essential. The fact that the Maillard reaction is common in processed foods, and people have been exposed for many years to the consumption of these reaction products, encouraged us to assay such substances for antimicrobial activity in milk.

The primary objectives of the present work were: to study the effect of added MRP on growth of psychrotrophs in refrigerated raw milk; and the influence of agitation on the antimicrobial effectiveness.

The results from this study could be of use in improving the shelf-life of refrigerated milk with possible extrapolation to other foods.

CHAPTER II

REVIEW OF LITERATURE

The Maillard reaction in foods can have both positive and negative aspects. The positive ones comprise the production of desirable color, aromas, and flavors; anti-oxidative properties; and, probably, antimicrobial effect. The negative ones include amino acid loss or unavailability; loss of protein nutritive value; and, sometimes, production of undesirable color, aromas, and flavors (Zabrodskii and Tikhomirova, 1956; McKeen, 1956; Viswanathan and Sarna, 1957; Nickerson and Ronsivalli, 1978; Mauron, 1981; Cheftel et al., 1981).

Since in terms of food preservation the antioxidative properties and the possible antimicrobial effect of MRP are very important attributes, these two aspects will be considered in this chapter.

Antioxidative Properties of MRP

The formation of Maillard-type reductone-like compounds, characterized as having antioxidative properties, has been described by Hodge and Rist (1953). According to Cheftel et al. (1981), two classes of MRP have been shown to possess such properties: (1) low molecular weight colorless compounds (Premelanoidins), and (2) higher molecular weight pigmented substances (melanoidins).

Lingnert and Eriksson (1980a, 1980b) recently evaluated the antioxidative effect of MRP (synthesized by refluxing sugar with free amino

acids as well as sugars with dipeptides, or protein hydrolysates) employing model systems containing linoleic acid. The antioxidative effect was found to be dependent on pH, nature and proportion of reactants, and amino acid sequence in the proteins.

The antioxidative effect of MRP in cookies was demonstrated by Lingnert (1980). Such effect was obtained as a consequence of baking the doughs to which sugars and free amino acids had been added previously.

Apparently, the first attempt in using preformed MRP to prevent lipid oxidation in food was made by Sato et al. (1973). They observed that either water extracts from retorted (121°C for 50 min.) beef, or retorted solutions of sugar with amino acids inhibited the production of warmed-over flavor (WOF), an oxidized flavor developed in refrigerated cooked meat. Similar experiments were carried out by Eneirson and Reineccius (1977, 1978). These researchers demonstrated that retorted (121°C for 50 min.) turkey meat was significantly more resistant to the development of WOF than turkey meat cooked at lower temperatures. Based on the results from tests and chemical analyses, the authors concluded that reductone-like compounds, formed from the interaction between sugars and amines during the retorting process, were responsible for the resistance to the formation of WOF.

Recently, Lingnert and Lundgren (1980) incorporated preformed MRP as a sausage ingredient in an effort to improve the oxidative stability of the product during frozen storage. The development of rancid flavor, as determined by sensory evaluation, was found to be retarded as a consequence of the addition of MRP.

According to Yamaguchi et al. (1981), browning reaction products

from sugars and amino acids acted synergistically with tocopherol in preventing oxidation in margarine. These authors further stated that MRP compared favorably with other food antioxidants, such as butylated hydroxyanisole (BHA) and propyl gallate, at the same level of reducing power.

Antimicrobial Effect of MRP

Whereas the antioxidative properties of Maillard-type browning reaction products are now relatively well established and the feasibility of their use in foods as antioxidants is being considered, studies concerning the use of these substances as antimicrobials in foods have not been reported.

Despite the lack of information regarding the use of MRP as food additives to control microbial growth, several research papers have focused on the influence of these products on the growth of microorganisms in media other than foods. Nevertheless, there is not general agreement about the behavior of microorganisms in the presence of such substances. While a stimulatory effect has been reported in some research papers, an inhibitory action has been described in others. Yet others have reported no effect on microorganisms.

Growth inhibition of Streptococcus fecalis, due to prolonged heating of the medium (containing glucose and tryptophan) in which the microorganism was cultured, was reported by Patton and Hill (1948). The inhibition was attributed to damage suffered in both amino acids and vitamins during autoclaving in the presence of glucose. Similar results were reported for L. arabinous, L. casei and S. fecalis by Rose and Peterson (1949). It was concluded that the Maillard reaction affected

the growth of such organisms when an essential amino acid (or other nutrient), present in limiting quantities, was destroyed by the reaction.

Peterson et al. (1949) studied the effect of MRP (formed from the interaction of dextrose with the peptide constituents of either a casein hydrolysate medium, or a yeast extract medium during autoclaving) on growth of Bacillus polymyxa. No significant effect on growth was noticed over a period of 72 hours. When preformed MRP (obtained by heating a dextrose-casein hydrolysate mixture) were incorporated into the casein medium, a slight inhibition was evident during the first 24 hours; but it disappeared within 48 hours.

Rogers et al. (1953) cultured Lactobacillus galloni in a medium to which N-D-glucosylglycine (a compound formed in the first step of the Maillard reaction) had been added. Although stimulatory action was found at low levels of N-D-glucosylglycine, toxicity was observed at higher levels of such compound. In 1956, Zabrodskii and Tikhomirova studied the effect of melanoidins on the microflora of malt. The addition of these substances (prepared by condensing either a glucose-glycine mixture, or a corn wash) to sweet mash inhibited the growth of microorganisms responsible for souring of the raw material used in alcohol fermentation. The inhibition was attributed to the killing effect of melanoidins on microorganisms. These results were said to be in accordance with those previously reported in which the inhibitory action of melanoidins toward yeast had been noticed (Zabrodskii and Tikhomirova, 1956).

McKeen (1956) stated that Pytophthora fragariae, a fungus causing red stele of strawberries, was sensitive to the products of the Maillard reaction. The reaction was induced in situ by autoclaving oatmeal and

Difco lima-bean agar containing dextrose. The addition of an autoclaved dextrose-glycine mixture to the culture medium did not permit the growth of P. fragariae. This mixture was also effective against other Pytophthora species. The author postulated that a toxic or fungistatic substance had been formed through the interaction of amino acids and carbohydrates.

The presence of a substance inhibitory to the growth of Lactobacillus bulgarius was detected while assaying skim milk powder samples which had been stored at 60°C for six months (Viswanathan and Sarna, 1957). The antimicrobial substance was also formed when skim milk powder was heated at 100°C for five hours, or autoclaved at 121°C for 30 min. Concentrated solutions of the inhibitory compound were prepared, and chemically analyzed. The researchers concluded that the inhibitory compound was a peptide.

Inhibition of Bacillus megaterium by a trimethylamine oxide-associated browning reaction product was observed by Rosen et al. (1970). The inhibition was expressed primarily as an increase of the lag phase of growth.

Recently, the incorporation of melanoidins in the growth medium of Staphylococcus aureus has been reported to account for a 46 percent decrease in the growth rate of this microorganism (Andrade et al., 1979).

Jemmali (1969) stated that the products of the Maillard reaction have been presumed to influence the cellular metabolism of some compounds by interfering with, among other things, the pyruvate descarboxylase inhibitors.

CHAPTER III

MATERIALS AND METHODS

Preparation of Maillard Reaction Products

Anhydrous D-glucose (Fisher Scientific Co.) and monohydrochloride D-L-Histidine (Sigma Chemical Co.) were dissolved in sufficient distilled water to make 100 ml of solution that was 0.2 M for both compounds. The sugar-amino acid mixture was adjusted to pH 6.5 with 5 N NaOH. Thereafter, the mixture was equally divided in 10 ml portions into each of ten 10-ml glass ampules (Wheaton Scientific Lab.); then the ampules were sealed with an oxygen-natural gas open flame. The sealed containers were autoclaved at 121°C for two hours, and allowed to cool at 20-22°C. All ampules, except one whose content was used to determine the pH after autoclaving, were maintained under refrigeration (5±1°C) until used.

Disc Assay to Screen Cultures for Inhibition by MRP

Source and Maintenance of Cultures

Six species of bacteria and one yeast were used in the disc assay technique as test organisms in checking for inhibition by freeze-dried MRP. The cultures were obtained from the stock culture collection in the Dairy Food Microbiology Research Laboratory at Oklahoma State

University, and maintained by daily transferring 1% (volume per volume) inoculum of the test culture into Trypticase Soy Broth (Baltimore Biol. Lab.). Pseudomonas fragi, P. fluorescens and Streptococcus lactis were incubated at 21°C for 20 hours while Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Kluyveromyces fragilis were incubated at 37°C for 20 hours. All the cultures were stored under refrigeration (5±1°C) between transfers.

Preparation of Freeze-dried MRP

Twenty ml of MRP, made according to the same procedure described earlier in this chapter, were placed into each of 12, 50-ml erlenmeyer flasks. The contents of the flasks were fast-frozen by rotating the flasks by hand in a mixture of dry ice and acetone. The frozen samples were dried in an Unitrap II lyophilizer (Vitris Co.). After which, all flasks were tightly covered with Parafilm "M" laboratory film (American Can Co.) and stored in a desiccator at 20-22°C until utilized.

Disc Assay Procedure

Each of seven screw cap test tubes containing ten ml of sterile, molten (45°C) Plate Count Agar (PCA; Difco Lab.) was inoculated at a rate of 1% with a 20-h broth culture of the test organism (one tube per culture). The inoculated medium in each tube was mixed by inverting six times, after which five ml from it was dispensed into a sterile, 100X15 mm petri dish. While the agar was allowed to solidify, the dried MRP in one flask was rehydrated with two ml distilled water and passed through a sterile membrane filter (45 μ) into a sterile, five-ml beaker. Once the agar solidified, sterile filter paper discs (0.127 cm) were submerged

individually into the rehydrated MRP and one was put onto the surface of the agar in each plate. A second sterile disc (used as control) was dipped into sterile distilled water and placed parallel to the other disc on each plate. Thereafter, all the plates were incubated at 32°C for 24 hours.

The diameter of the clear inhibitory zone around each disc was measured after the incubation period to determine the intensity of the inhibition. This was accomplished, with the aid of a vernier, by taking two perpendicular measurements of the diameter of the clear zone surrounding each disc, and the result expressed as the average diameter (cm).

Assay for Inhibition of *Pseudomonas fragi* in Autoclaved Milk by MRP

Source of Maintenance of the Culture

Pseudomonas fragi, the psychrotrophic culture used as the test organism in these experiments, was from the culture collection in the Dairy Food Microbiology Research Laboratory at Oklahoma State University. It was maintained by two-weekly subculture (1% inoculum) in sterile, reconstituted 10% nonfat milk solids (NFMS). Each subculture was incubated at 21°C for 20 hours, and stored under refrigeration (5±1°C) between subcultures. Monthly, the culture was checked for purity by streaking on PCA (Difco Lab.) and incubating the plates at 21°C overnight. The plates were observed to insure that only one colony type was present. The culture was additionally tested by microscopically examining slides of the culture stained by the Gram procedure (modified by Burke, 1922).

Preparation and Inoculation of the Nonfat Milk Medium

Eighty grams of NFMS (Carnation Co.) were rehydrated with distilled water to make 800 ml reconstituted milk. It was rehydrated with the aid of a magnetic stirrer, autoclaved as 121°C for 15 min., allowed to cool to room temperature then placed in a refrigerator at 5±1°C to chill. The milk was prepared the day before the assay and maintained under refrigeration until utilized.

The nonfat milk medium was inoculated with $1X10^3$ to $1X10^4$ cells of P. fragi from a 20-hr milk culture. This was performed by aseptically pipeting eight ml of a 1:1000 dilution of the culture into the flask containing 800 ml autoclaved reconstituted milk. The milk was mixed with the aid of a magnetic stirrer for 30 sec., and placed in an ice-water bath until utilized (within an hour).

Preparation, Treatment, and Storage of Test Sub-samples

In order to assess the effect of different MRP concentrations and the influence of agitation on bacterial growth, two sets of four subsamples each were obtained from the original batch of autoclaved milk after thorough mixing (Figure 1). For each set, the subsamples were prepared by aseptically delivering a 90-ml portion of milk into each of four sterile 250 erlenmeyer flasks containing, respectively, the following: (1) 10 ml sterile distilled water (control); (2) three ml sterile MRP plus seven ml sterile distilled water; (3) six ml sterile MRP plus four ml sterile distilled water; and (4) nine ml sterile MRP plus one ml

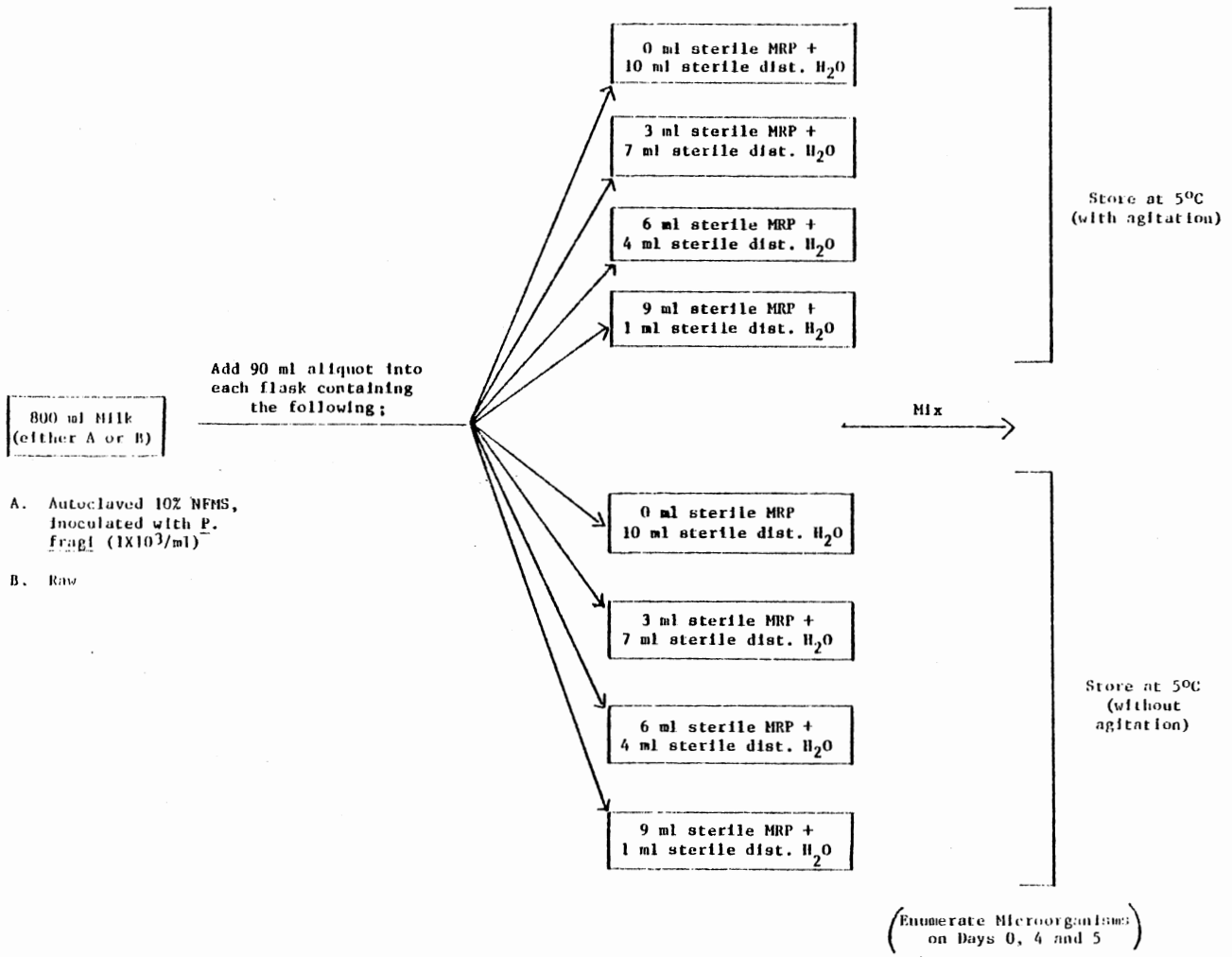

Figure 1. Flow Chart for Evaluating the Influence of the Addition of MRP on Numbers of Microorganisms in Refrigerated Milk

sterile distilled water. For convenience, the MRP concentration in these sub-samples were considered as being, respectively, 0 (control), 3, 6, and 9%. The two sets of flasks (eight total) were placed in an ice-water bath and swirled until a uniform suspension was attained. One set of four sub-samples was stored at 5±1°C in a gyrotory water bath shaker model G76 (New Brunswick Scientific Co.) adjusted to agitate at 120 RPM, the other set was stored statically at 5±1°C in a Freas 815 low temperature incubator (GCA Precision Scientific).

Microbiological Evaluation

A ten ml aliquot was aseptically removed from each test sub-sample, delivered aseptically into a previously labeled sterile screw cap test tube, and placed in an ice-water bath. These aliquots were taken prior to storage (day 0) and after four and five days of storage at 5±1°C.

Appropriate dilutions of the samples were made employing 99 ml dilution blanks according to the procedures indicated in Compendium of Methods for Microbiological Examination of Foods (Speck, 1976). Such dilutions were plated in duplicate with PCA using the pour plate technique. All the plates were incubated at 32°C for 48 hours. Colonies on the plates were counted after the incubation period with the aid of a Quebec colony counter in accordance with the Aerobic Plate Count (APC) procedures, also described previously (Speck, 1976).

Assay for Inhibition of Psychrotrophs in Refrigerated Raw Milk

Source of Milk

Raw milk was obtained from the Oklahoma State University Dairy

Cattle Center, where the milk was stored in refrigerated (5±1°C) tanks immediately after the milking process, and mechanically mixed at regular time intervals.

Samples were collected once a week on a regular schedule (the same day, at the same hour). A total of eight samples, each consisting of 800 ml of milk, was tested. The milk was aseptically placed into a sterile 2000 ml flask, placed in an ice water bath, and transported to the Dairy Food Microbiology laboratory for its immediate use.

Preparation, Treatment, and Storage of Test Sub-samples

The procedure employed to prepare the test sub-samples was similar to that utilized for the autoclaved milk experiment, the exception being the fact that raw milk was not inoculated. A diagram of such procedure is outlined in Figure 1.

Once again, two sets of four sub-samples were obtained from the original batch of milk. Within each set, the sub-samples were prepared by aseptically delivering a 90-ml volume of milk into each of four sterile, 250 erlenmeyer flasks containing, respectively, the following: (1) ten ml sterile distilled water (control), (2) three ml sterile MRP plus seven ml sterile distilled water, (3) six ml sterile MRP plus four ml sterile distilled water, and (4) nine ml sterile MRP plus one ml sterile distilled water. As in the autoclaved milk experiment, the MRP concentrations in these flasks are referred, respectively, as 0 (control), 3, 6, and 9%. The two sets of flasks (eight total) were placed in an ice water bath, and each flask was swirled until a homogeneous suspension was obtained. After which, a set was placed at 5±1°C in the gyrotory water

bath shaker adjusted to agitate at 120 RPM, the other set of sub-samples was placed statically at 5±1°C in the low temperature incubator.

Microbiological Evaluation

The enumeration of microorganisms in raw milk was carried out by following the same procedures utilized in the autoclaved milk assay.

Again, a ten-ml aliquot was aseptically removed from each test sub-sample, placed into a previously labeled sterile screw cap test tube, and submerged in an ice-water bath. These aliquots were taken prior to storage (day 0) and after four and five days of storage at 5±1°C. They were appropriately diluted and plated in duplicate with PCA by means of the pour plate procedure, and incubated at 32°C for 48 hours. After incubation, colonies on the plates were counted with the aid of a Quebec colony counter by using the ACP procedures (Speck, 1976).

Statistical Analyses

The effect of different MRP concentrations, and the influence of agitation on the growth of microorganisms in either autoclaved or raw milk was statistically analyzed by means of an analysis of variance for a 2X4 factorial arrangement of treatment factors in a randomized complete block design. Further analysis was carried out using the Duncan's Multiple Range test (Snedecor and Cochran, 1967).

CHAPTER IV

RESULTS

Susceptibility of Various Microorganisms to Inhibitory Action of MRP

The results obtained from screening seven species of microorganisms for susceptibility to inhibition by MRP (using the disc assay procedure) are presented in Table I. Whereas no inhibition of growth was observed around the control discs, the formation of a clear zone surrounding the discs impregnated with MRP was evident for all the species tested. The average diameter (AD) of the inhibitory zone varied from 1.79 to 2.61 cm. *P. fragi*, *P. fluorescens* and *S. lactis* (2.61, 2.41 and 2.21 AD, respectively) seemed to be more sensitive to the effect of MRP than *S. aureus*, *E. coli*, *K. fragilis* and *B. subtilis* (2.08, 1.96, 1.81 and 1.79 cm AD, respectively).

Effect of the Addition of MRP on Microbial Growth in Refrigerated Milk

The data obtained from eight trials showing the influence of added MRP on the growth of bacteria in both autoclaved reconstituted 10% NFMS, and raw milk stored (agitated and static) at 5°C are summarized respectively in Tables IV and V (Appendix A). Bacterial counts are expressed as $\log_{10}$ count/ml of milk. The analysis of variance (ANOVA) Tables for the data as well as the corresponding Duncan Tables are in Appendix B.

TABLE I

GROWTH INHIBITION OF SEVEN SPECIES OF MICROORGANISMS BY MRP AS DETERMINED BY THE DISC ASSAY PROCEDURE

Species	Average Diameter[a] of the Inhibitory Zones (cm)	
	MRP	Dist. Water (control)
Pseudomonas fragi	2.61	0.00
P. fluorescens	2.41	0.00
Streptococcus lactis	2.21	0.00
Staphylococcus aureus	2.08	0.00
Escherichia coli	1.96	0.00
Kluyveromyces fragilis	1.81	0.00
Bacillus subtilis	1.79	0.00

[a]Average of twelve assay discs.

The fourth day of storage was selected for comparing treatment effects among the eight trials because, by that time, the population in none of the samples had reached the stationary phase of growth.

Autoclaved Milk Experiment

Significant ($P<0.001$) variations in the numbers of P. fragi were noticed from batch to batch (among trials) of autoclaved reconstituted milk in which MRP were assessed as antimicrobials. The variation was most apparent among the counts determined after four days of storage at 5°C (Table IV).

On day 0, the mean $\log_{10}$ counts for the controls (both agitated and static) were significantly ($P<0.001$) higher than the means for the samples treated with MRP (Table II). If one compares the arithmetic counts (i.e., antilog of the $\log_{10}$ count/ml) obtained on day zero from the control with those from each of the different MRP treatment samples (3, 6 and 9% added MRP), it may be seen that the differences among these counts (Table IV) as well as the differences among their means are small (Table II). Although the differences were small, it indicates an immediate slight bactericidal action of the MRP.

After four days of storage at 5°C, the growth of P. fragi was not significantly ($P>0.05$) affected by storing the milk with or without agitation (Table II). However, under both conditions of storage significantly ($P<0.001$) lower numbers of microorganisms were observed after four days in all samples containing MRP. As the MRP concentration increased the bacterial counts decreased (Table II). The mean $\log_{10}$ count/ml of milk samples stored with agitation were 6.30 for the control; and 4.75, 3.84 and 3.45 for those samples containing 3, 6 and 9% MRP,

TABLE II

INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF PSEUDOMONAS FRAGI IN AUTOCLAVED RECONSTITUTED MILK BEFORE AND AFTER FOUR DAYS OF STORAGE AT 5°C

Storage Condition	Day	Means of Log_{10} Counts/ml[A]			
		Added MRP (%)			
		0 (control)	3	6	9
Agitated[C]	0	3.61 (a)[B]	3.49 (b)	3.49 (b)	3.44 (b)
	4	6.30 (a)	4.75 (b)	3.84 (c)	3.45 (c)
Static[C]	0	3.62 (a)	3.52 (b)	3.46 (b)	3.45 (b)
	4	6.62 (a)	4.81 (b)	2.90 (c)	2.82 (c)

[A]Each value represents the mean log_{10} count/ml from 8 trials.

[B]Numbers in same row followed by different letters are significantly different ($P<0.05$).

[C]No significant difference ($P>0.05$) between counts from agitated and static samples.

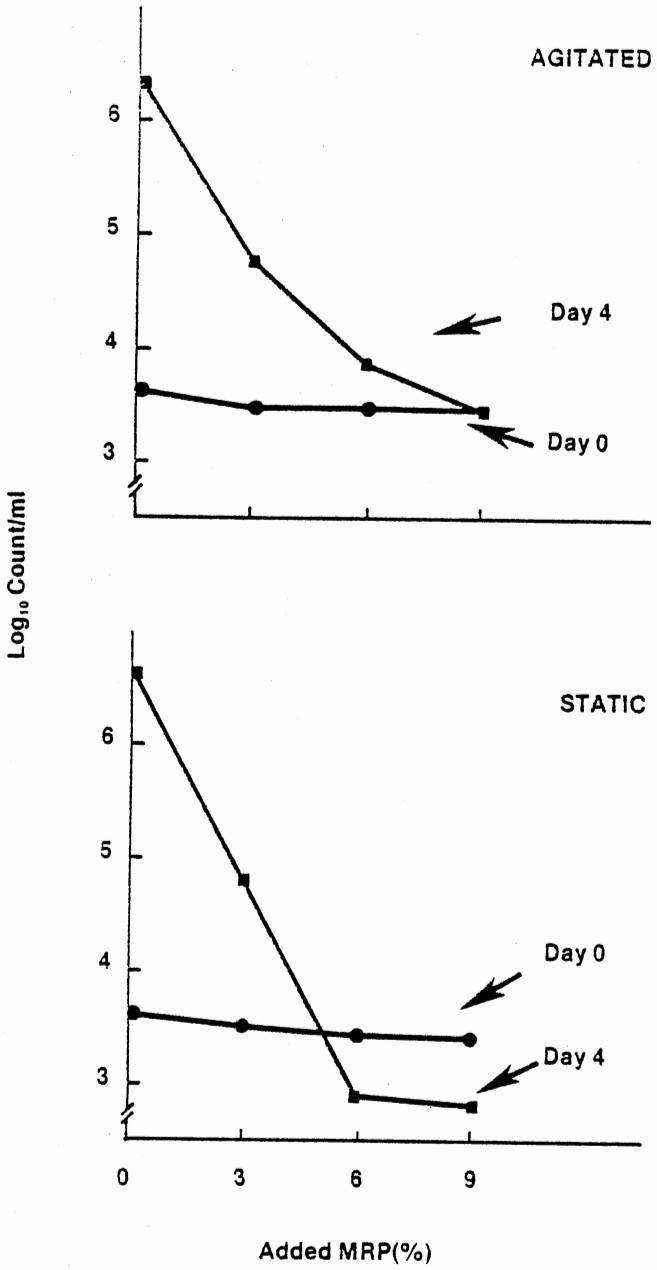

Figure 2. Influence of the Addition of MRP on Numbers of Pseudomonas fragi in Autoclaved Reconstituted Milk Before and After 4 Days of Storage at 5°C (Each Point Represents an Average Value From 8 Trials)

respectively (Table II). For the corresponding static samples (0, 3, 6 and 9% added MRP), the mean $\log_{10}$ counts/ml were 6.62, 4.81, 2.90 and 2.82, respectively (Table II). The Duncan's test (Table VII) indicated that the addition of 6% MRP was significantly ($P<0.05$) more inhibitory than 3% MRP, but similar ($P>0.05$) to 9% MRP in effectiveness in milk stored statically or agitated.

There was no significant ($P>0.05$) interaction between storage condition (agitated or static) and amount (0, 3, 6 and 9%) of MRP as to influence the growth of P. fragi.

The results from these experiments are presented graphically in Figure 1. The $\log_{10}$ of populations at day 0 and day 4 are plotted against the concentration of MRP. The consistency of the day 0 counts is easily seen. The magnitude of the inhibitory action of the increasing concentrations of MRP is also easily observed. Although statistical analyses indicated no significant treatment interactions, it is interesting to note that for the static cultures the day 4 counts for the samples containing 6 and 9% MRP were lower than the day 0 counts. This was not observed for the samples stored with agitation, although the day 0 and day 4 were approximately the same for the sample containing 9% MRP.

Raw Milk Experiment

Significant ($P<0.001$) variations with respect to the numbers of microorganisms were found from batch to batch of raw milk where MRP were tested as inhibitors of bacterial growth. The variations were more noticeable among the counts obtained after four days of storage at 5°C (Table V).

On day 0, the Duncan's multiple range test showed no significant

TABLE III

INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF BACTERIA IN RAW MILK BEFORE AND AFTER FOUR DAYS OF STORAGE AT 5°C

Storage Condition	Day	Mean of Log_{10} Counts/ml[A]			
		Added MRP (%)			
		0 (Control)	3	6	9
Agitated[C]	0	3.37 (a)[B]	3.38 (a)	3.34 (a)	3.34 (a)
	4	5.86 (a)	5.57 (a)	5.37 (b)	5.11 (c)
Static[C]	0	3.43 (a)	3.45 (a)	3.45 (a)	3.44 (a)
	4	6.34 (a)	6.10 (a)	5.63 (b)	5.04 (c)

[A]Each value represents the mean log_{10} count/ml from 8 trials.

[B]Numbers in same row followed by different letters are significantly different ($P<0.05$).

[C]Significant difference ($P<0.05$) between counts from agitated and static samples.

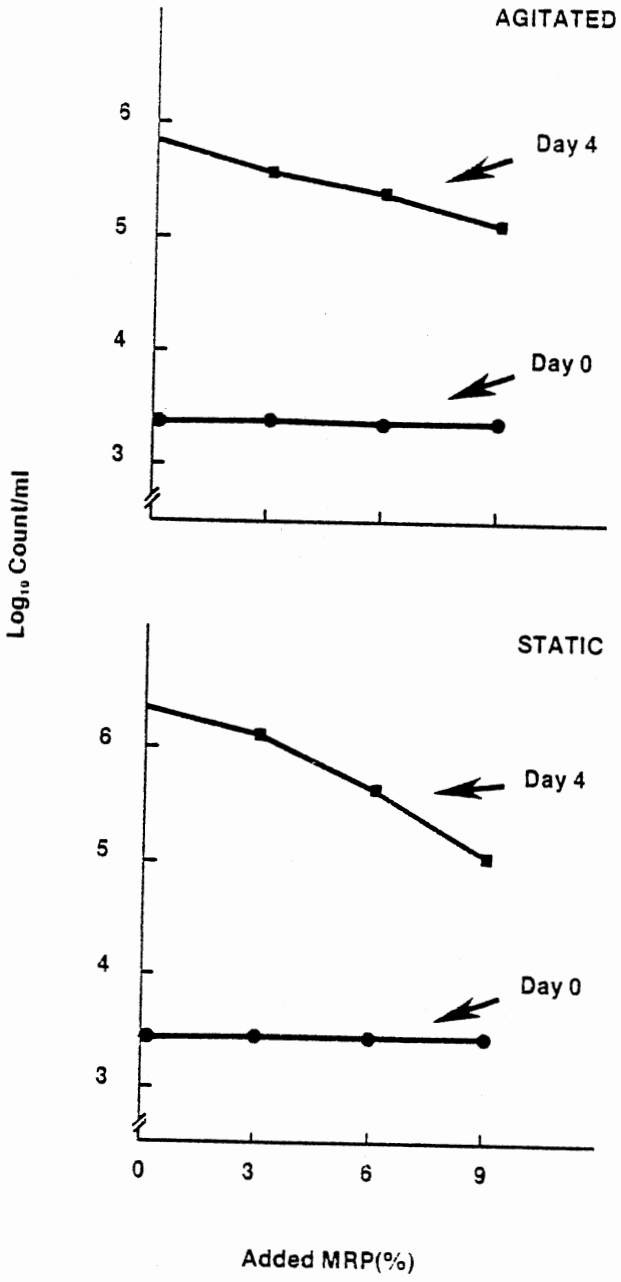

Figure 3. Influence of the Addition of MRP on Numbers of Bacteria in Raw Milk Before and After 4 Days of Storage at 5°C (Each Point Represents an Average Value From 8 Trials)

($P>0.05$) difference among means for the samples treated with different concentrations (0, 3, 6 and 9%) of MRP (Table III). This indicates that these products did not have an immediate germicidal effect on microorganisms in raw milk.

Based on the counts observed after four days of storage at $5^{o}C$, the growth of microorganisms was significantly ($P<0.001$) affected by storing the milk with or without agitation (Table III). The Duncan's test (Table IX) indicated that bacteria were significantly ($P<0.05$) less numerous in agitated than in static milk samples (with the exception of the samples containing 9% MRP). With the significant difference observed in the amount of growth obtained in the static and agitated control samples, it is difficult to ascertain whether or not the different storage conditions influenced the intensity of inhibition caused by the MRP. However, there appeared to be greater inhibition in the statically stored samples than in the agitated ones, especially in the 6 and 9% MRP.

The numbers of bacteria detected at the fourth day of storage were significantly ($P<0.001$) influenced by treating the milk with different concentrations of MRP. Overall, a reduction in the bacterial count was observed as the percentage added MRP was increased for both the agitated and static samples (Table IV). However, the inhibitory effect appeared to be less pronounced than that observed in autoclaved milk (Table V). The means of the $\log_{10}$ counts/ml of milk samples stored with or without agitation were 5.86 for the control; and 5.57, 5.37 and 5.11 for those samples containing 3, 6 and 9% MRP, respectively (Table III). For the corresponding static samples (0, 3, 6 and 9% added MRP), the means $\log_{10}$ counts/ml were 6.34, 6.10, 5.63 and 5.04, respectively (Table III). The Duncan's test (Table IX) showed that only 6 and 9% MRP significantly

($P<0.05$) inhibited growth compared to the control. Nine percent MRP was significantly ($P<0.05$) more inhibitory than 6% MRP.

The interaction between storage condition (agitated or static) and amount (0, 3, 6 and 9%) of added MRP did not have a significant ($P>0.05$) effect on the growth of microorganisms in raw milk.

The data from these experiments are presented graphically in Figure 3. The $\log_{10}$ of populations are plotted against the concentration of MRP. The consistency in the day 0 counts as well as the inhibitory effect of the increasing concentrations of MRP may be easily observed in samples stored either with or without agitation. Despite of the lack of significant treatment interaction (storage condition vs. MRP concentration), it is interesting to observe that; once again, the growth of microorganisms was lower in samples maintained agitated than in samples maintained statically.

CHAPTER V

DISCUSSION

Organisms such as yeast, molds, and bacteria are believed to be influenced by the Maillard reactions. Whereas certain species have been adversely affected by these substances (Zabrodskii and Tikhomirova, 1956; McKeen, 1956; Viswanathan and Sarna, 1957; Rosen et al., 1970; Andrade et al., 1979), other microorganisms have exhibited either no response or a stimulatory one when inoculated into culture media containing such substances (Peterson et al., 1949; Rogers et al., 1953; Jemmali, 1969; Horikoshi et al., 1981). According to Jemmali and Petit (1966), the type and intensity of the microbial response depends on the kind of organism, the strain used, composition of culture medium, heat treatment applied to the medium prior to inoculation, the concentration and/or the compounds involved in formation of MRP.

In the present work, growth inhibition due to MRP was observed for all the species tested by the disc assay procedure. Although P. fragi, P. fluorescens, and S. lactis appeared to be more sensitive to these products than S. aureus, E. coli, K. fragilis and B. subtilis, additional microbiological assays are needed in order to draw definite conclusions regarding any variations in susceptibility of these species to such products. However, the fact that the inhibition was observed for a yeast, three Gram positive and three Gram negative bacteria indicates that the inhibitory substance has a wide antimicrobial spectrum.

Experiments showing the antimicrobial activity of MRP in foods have not been reported previously. This paper describes the inhibition of microorganisms by MRP in refrigerated milk (both autoclaved reconstituted and raw). The inhibitory effect was more pronounced in autoclaved milk than in raw milk. This was not surprising because these media are known to differ in some aspects. The milk composition, for instance, should be considered. The following two factors could have been involved: (1) the existence of higher concentration of MRP in autoclaved milk (as a result of the MRP already present in the powdered milk that was reconstituted plus the amount of these products formed during the autoclaving process), and (2) the fat contained in the raw milk could have influenced the degree of inhibition observed. The difference in the intensity of the inhibition may also have been influenced by a higher resistance of the natural microflora in the raw milk than the pure culture of the psychrotroph in the autoclaved milk to the action of the tested products. Furthermore, the existence of enzymes or other substances naturally occurring in raw milk could have reduced the intensity of the inhibitory agent.

An interesting observation was that the MRP were more effective as antimicrobials in the raw milk samples stored statically than in those stored with continuous agitation. The increased aeration caused by the agitation could have increased the amount of oxygen in the milk during storage. Perhaps the oxygen reacted with the MRP in such a way that the inhibitory action was lessened.

The exact mechanism(s) by which the MRP exert antimicrobial action is not yet clear. Patton and Hill (1948), and Rose and Peterson (1949) attributed the failure of microorganisms to grow in culture media

submitted to prolonged heating to the destruction of amino acids by the Maillard reaction rather than to the formation of inhibitors in this reaction. Other authors have suggested that the products of the Maillard reaction are inhibitory because the amine reactant is essential for growth and when combined with a reducing sugar the amino-compound formed becomes less available for the microorganisms (Sheikh et al., 1961; Horn et al., 1968; Hagan et al., 1970). In the present study, neither destruction nor unavailability of nutrients appeared to be the cause of growth inhibition. The reason for such an assumption is the fact that the MRP used in these experiments were preformed materials incorporated in milk which should be nutritionally adequate to support growth of the microorganisms as indicated by growth in the control samples. Furthermore, the milk was not exposed to any heat treatment after addition of the MRP.

The higher antimicrobial effect observed in raw milk samples containing 9% MRP compared with that observed in raw milk samples containing 6% MRP, and the lack of influence on growth determined for the samples to which 3% MRP was added indicates that there is a limiting concentration level below which the inhibitory substance loses its effectiveness in raw milk. On the other hand, it might be possible that above a determined concentration these substances become sufficiently toxic to cause total inhibition of growth, or to kill part of the microorganisms. With the experimental results obtained from raw milk, it is very difficult to ascertain whether or not the incorporated MRP exerted a germicidal effect on any of the microflora. However, such an effect could have been involved in both the total growth inhibition observed for the agitated autoclaved milk samples containing 9% added MRP and in

the apparent killing effect observed in the static autoclaved milk samples containing 6 and 9% added MRP. Evidence concerning the killing or fungistatic effect of added MRP on several Pytophthora species was found by McKeen (1956). Killing action at high levels of added N-D-glucosylglycine (a MRP) had been previously reported for L. galloni by Rogers et al. (1953). They also reported that this compound was stimulatory to the same organisms at low concentrations.

Other mechanisms to explain the inhibitory action of the MRP have been proposed by Jemmali and Petit (1966), and Andrade et al. (1979). The former workers indicated that premelanoidins could adversely influence certain enzyme systems in microorganisms. The latter group reported that melanoidins could bind ferrous ions in the media used to culture S. aureus. However, this chelating action accounted for only 10% of the total of 46% inhibition observed for such organisms as a consequence of the incorporation of MRP into the medium.

The objective of the present study was not to elucidate the mechanism of the inhibitory action of the MRP. A "crude" solution of these substances was used in each of the experiments. No attempt was made to isolate specific antimicrobial agents from the mixture. However, the conditions under which the Maillard reaction was allowed to occur as well as the formation of dark brown, aromatic substances suggested that the reaction had proceded to the advanced stage. This is a very important point to consider since, according to Sheikh et al. (1961), growth stimulants are formed in the earlier steps of the reaction while the inhibitors predominate as the reaction becomes more advanced. This assumption is, however, in disagreement with the results reported by Horikoshi et al (1981) in which both inhibitory and stimulatory substances were

found in the lower molecular weight MRP fraction (i.e., those formed in earlier stages of the reaction).

It has been generally recognized that the Maillard reaction involves a series of events in which the chemical changes that occur as well as the compounds formed are influenced by parameters such as temperature, pH, nature, and concentration of reactants, and moisture content (Ellis, 1959; Reynolds, 1963; Shallenberger and Birch, 1975). Thus, another possible explanation for the variation of the microbial behavior in the presence of MRP reported in different papers may be related with the environmental conditions under which these substances were produced.

Additional research is needed concerning the antimicrobial effect of the Maillard reaction products. The isolation and further purification of the inhibitory substance(s), for instance, as well as its antimicrobial spectrum are important subjects of investigation. One problem with using MRP, such as the ones in the present study, are preservatives in raw milk is the slight discoloration (light brown) of the milk. If purification of the antimicrobial fraction of the MRP revealed that non-pigmented components were inhibitory, then perhaps these would be useful in milk. Additional experiments are needed in order to assess the possible antimicrobial action of MRP that have already been shown to be effective as food antioxidants. The applicability of these substances to other foods need to be investigated. With regard to safety of MRP as food additives, more research has to be done because of the controversial opinions that have developed in recent years with respect to the involvement of the Maillard reaction in imparting mutigenic properties to heated foods. All this information is important when considering the feasibility of the use of such products as food preservatives.

CHAPTER VI

SUMMARY AND CONCLUSIONS

A "crude" mixture of MRP (prepared by heating a 0.2 M glucose-histidine mixture) was incorporated into raw milk at different proportions (3, 6, and 9%) to determine its effect on growth of the microflora naturally present in raw milk during storage with or without agitation at 5°C. Similar experiments were carried out in autoclaved reconstituted (10% NFMS) milk using P. fragi as the test organism. In addition, the disc assay procedure was conducted in order to test seven species of microorganisms for inhibition by MRP.

The "crude" MRP mixture was effective against all the organisms (1 yeast, 3 Gram positive, and 3 Gram negative bacteria) tested by the disc assay procedure. Although the inhibitory substance seemed to have a wide antimicrobial spectrum, some species appeared to be more sensitive to these products than others.

Growth inhibition due to the added MRP was observed for the organisms in both raw, and autoclaved refrigerated milk during four days of storage at 5°C. The inhibition was more pronounced in autoclaved milk than in raw milk, probably because of the difference in composition between these media and/or the higher resistance of the natural microflora in raw milk to MRP compared with that of the pure culture of P. fragi in the autoclaved milk. The higher antimicrobial effect of the 9% added MRP with respect to that of the 6% added MRP, and the lack of

influence on growth of microorganisms in the milk samples to which 3% MRP was incorporated indicated that there is a limiting concentration below which the inhibitor loses its effectiveness. In the autoclaved milk, there appeared to be a germicidal action involved in the agitated samples to which 9% MRP was added. A similar effect was observed for the static samples to which 6 or 9% MRP was incorporated. However, such an effect was not evident for raw milk.

Overall, MRP were more effective as antimicrobials in the milk samples stored statically than in the ones stored with agitation, probably due to a decrease in action of the inhibitor as a result of its interaction with the oxygen incorporated by continuous agitation.

The use of MRP as preservatives in refrigerated milk, or other foods may be of great importance in the future. However, for these chemicals to be permissible as food additives, they must meet certain characteristics and be the object of a deeper study. The following aspects are some of the more obvious gaps in our knowledge concerning the antimicrobial effect of such products: (1) Purification and antimicrobial spectrum of the isolated or purified inhibitory agent(s); (2) Applicability of this coumpound(s) to other types of foods; (3) Relationship between brown-colored pigmentation and effectiveness of the inhibitor; (4) Possible two-fold function of the purified substance: as antimicrobial and as antioxidant; (5) Safety for use of this substance(s) as a food additive.

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APPENDIXES

APPENDIX A

DATA OBTAINED FROM EACH TRIAL SHOWING THE INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF BACTERIA IN REFRIGERATED MILK

TABLE IV

INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF PSEUDOMONAS FRAGI IN AUTOCLAVED RECONSTITUTED MILK BEFORE AND AFTER FOUR DAYS OF STORAGE AT 5°C

Treatments			Log_{10} Counts/ml								
Storage Condition	MRP Conc. (%)	Days	I[a]	II	III	IV	V	VI	VII	VIII	Average
Agitated	0 (control)	0	3.72	3.61	3.58	3.60	3.52	3.71	3.59	3.57	3.61
		4	4.64	4.76	7.69	6.99	7.30	6.11	6.04	6.86	6.30
	3	0	3.65	3.49	3.48	3.32	3.34	3.60	3.53	3.51	3.49
		4	4.66	2.18	5.11	5.51	5.46	3.54	5.45	6.08	4.75
	6	0	3.65	3.48	3.49	3.32	3.34	3.60	3.53	3.51	3.49
		4	4.51	2.18	3.11	4.43	4.43	3.34	4.08	4.66	3.84
	9	0	3.67	3.46	3.48	3.30	3.34	3.46	3.49	3.34	3.44
		4	4.41	2.18	2.97	3.63	3.61	3.28	3.76	3.72	3.45
Static	0 (control)	0	3.80	3.52	3.66	3.59	3.58	3.63	3.56	3.60	3.62
		4	5.48	6.65	7.60	7.40	7.38	4.52	6.95	6.99	6.62
	3	0	3.72	3.49	3.43	3.38	3.59	3.53	3.40	3.59	3.52
		4	3.43	2.60	3.69	6.20	6.36	4.08	6.00	6.08	4.81
	6	0	3.63	3.43	3.34	3.30	3.57	3.49	3.22	3.58	3.46
		4	3.20	2.54	2.89	2.84	2.86	3.28	2.74	2.85	2.90
	9	0	3.65	3.46	3.43	3.23	3.56	3.48	3.26	3.52	3.45
		4	3.23	2.41	2.77	2.68	2.78	3.23	2.65	2.83	2.82

[a]Roman numeral refers to trial.

TABLE V

INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF BACTERIA IN RAW MILK BEFORE AND AFTER FOUR DAYS OF STORAGE AT 5°C

Treatments			Log_{10} Counts/ml								
Storage Condition	Added MRP (%)	Days	I[a]	II	III	IV	V	VI	VII	VIII	Average
Agitated	0	0	3.97	3.52	3.26	3.08	3.08	3.41	3.53	3.15	3.37
	(control)	4	5.74	4.58	6.89	6.04	5.96	6.04	5.58	6.04	5.86
	3	0	3.90	3.62	3.26	3.11	3.08	3.38	3.53	3.15	3.38
		4	5.76	4.32	6.57	5.48	5.45	5.66	5.83	5.86	5.57
	6	0	3.84	3.54	3.23	3.08	3.04	3.36	3.51	3.11	3.34
		4	4.83	4.26	6.51	5.49	5.36	5.46	5.30	5.79	5.37
	9	0	3.87	3.54	3.26	3.08	3.04	3.34	3.46	3.11	3.34
		4	4.45	4.23	6.08	5.11	5.04	5.18	5.18	5.64	5.11
Static	0	0	3.75	3.61	3.34	3.20	3.04	3.65	3.56	3.28	3.43
	(control)	4	4.76	5.90	7.11	6.51	6.45	6.79	5.98	7.23	6.34
	3	0	3.72	3.65	3.34	3.20	3.20	3.63	3.56	3.30	3.45
		4	4.41	5.34	6.65	6.45	6.43	6.72	6.83	6.93	6.10
	6	0	3.76	3.64	3.34	3.23	3.20	3.58	3.56	3.28	3.45
		4	3.91	5.18	6.23	5.91	5.82	5.76	5.40	6.86	5.63
	9	0	3.76	3.70	3.34	3.20	3.18	3.58	3.48	3.26	3.44
		4	3.49	4.41	5.69	5.56	4.49	5.70	5.26	5.70	5.04

[a]Roman numeral refers to trial number.

APPENDIX B

STATISTICAL ANALYSES

TABLE VI

ANALYSIS OF VARIANCE AND DUNCAN'S MULTIPLE RANGE TEST FOR SIGNIFICANT DIFFERENCES AMONG MEANS FOR DATA[a] TO EVALUATE THE INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF PSEUDOMONAS FRAGI IN AUTOCLAVED RECONSTITUTED MILK BEFORE STORAGE AT 5°C

Source	DF	SS	MS	F	PR>F
Corrected Total	63	0.9947	0.0256		
Trials	7	0.4424	0.0632	11.34	0.0001
Treatments					
Storage	1	0.0000	0.0000	0.00	0.9588
Concentration	3	0.2624	0.0975	15.70	0.0001
Storage*Concentration	3	0.0069	0.0023	0.41	0.7444
Error	49	0.2730	0.0056		

Concentration (% added MRP)	Means[b]	Grouping
0	3.614955	A
3	3.503470	B
6	3.474680	B
9	3.445993	B

[a]Data: see Table IV (Appendix A).

[b]Means obtained from Duncan's Multiple Range Test (0.05 level). Those with the same grouping letter are not significantly different.

TABLE VII

ANALYSIS OF VARIANCE AND DUNCAN'S MULTIPLE RANGE TEST FOR SIGNIFICANT DIFFERENCES AMONG MEANS FOR DATA[a] TO EVALUATE THE INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF PSEUDOMONAS FRAGI IN AUTOCLAVED RECONSTITUTED MILK AFTER FOUR DAYS OF STORAGE AT 5°C

Source	DF	SS	MS	F	PR>F
Corrected Total	63	172.19	2.73		
Trials	7	23.24	3.32	5.29	0.0002
Treatments					
Storage	1	1.41	1.41	2.24	0.1408
Concentration	3	112.68	37.56	59.88	0.0001
Storage*Concentration	3	4.12	1.37	2.19	0.1010
Error	49	30.74	0.63		

Concentration (% added MRP)	Means[b]	Grouping
0	6.460935	A
3	4.777411	B
6	3.371469	C
9	3.135029	C

[a]Data: see Table IV (Appendix A).

[b]Means obtained from Duncan's Multiple Range Test (0.05 level). Those with the same grouping letter are not significantly different.

TABLE VIII

ANALYSIS OF VARIANCE AND DUNCAN'S MULTIPLE RANGE TEST FOR SIGNIFICANT DIFFERENCES AMONG MEANS FOR DATA[a] TO EVALUATE THE INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF BACTERIA IN RAW MILK BEFORE STORAGE AT 5°C

Source	DF	SS	MS	F	PR>F
Corrected Total	63	3.8655	0.0614		
Trials	7	3.5218	0.5031	114.21	0.0001
Treatment					
Storage	1	0.1136	0.1136	25.78	0.0001
Concentration	3	0.0070	0.0023	0.53	0.6620
Storate*Concentration	3	0.0073	0.0024	0.55	0.6506
Error	49	0.2159	0.0044		

Storage Condition	Means[b]	Grouping
Agitated	3.357764	A
Static	3.442015	B

[a]Data: see Table V (Appendix A).

[b]Means obtained from Duncan's Multiple Range Test (0.05 level). Those with the same grouping letter are not significantly different.

TABLE IX

ANALYSIS OF VARIANCE AND DUNCAN'S MULTIPLE RANGE TEST FOR SIGNIFICANT DIFFERENCES AMONG MEANS FOR DATA[a] TO EVALUATE THE INFLUENCE OF THE ADDITION OF MRP ON NUMBERS OF BACTERIA IN RAW MILK AFTER FOUR DAYS OF STORAGE AT 5°C

Source	DF	SS	MS	F	PR>F
Corrected Total	63	42.28	0.67		
Trials	7	23.01	3.29	21.30	0.0001
Treatments					
Storage	1	1.43	1.43	9.27	0.0037
Concentration	3	9.37	3.12	20.25	0.0001
Storage*Concentration	3	0.91	0.30	1.97	0.1301
Error	49	7.56	0.15		

Treatments		Means[b]	Grouping
Storage Condition	Agitated	5.48	A
	Static	5.78	B
Concentration (% added MRP)	0	6.10	A
	3	5.83	A
	6	5.50	B
	9	5.08	C

[a]Data: see Table V (Appendix A).

[b]Means obtained from Duncan's Multiple Range Test (0.05 level). Those with the same grouping letter are not significantly different.

VITA 2

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