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EFFECTS OF PROCESSING TEMPERATURE AND ADDED ANTIMICROBIAL
AGENTS ON THE KEEPING QUALITY OF MEXICAN-STYLE SAUCE

THE UNIVERSITY OF ARIZONA

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EFFECTS OF PROCESSING TEMPERATURE AND ADDED
ANTIMICROBIAL AGENTS ON THE KEEPING
QUALITY OF MEXICAN-STYLE SAUCE

by
Siew Lian Chung

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For the Degree of

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In the Graduate College
THE UNIVERSITY OF ARIZONA

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Dec. 13, 1984
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Dedicated

to my parents and H. Seng with all my love

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ABSTRACT

Mexican-style hot sauce, salsa, was prepared and heated to 62.8°C, 71.1°C and 79.4°C (145°F, 160°F and 175°F). Seven combinations of antimicrobial agents (potassium sorbate, 900 ppm, sodium benzoate, 700 ppm, and ethylenediamine tetraacetate acid, 50 ppm) were incorporated into the sauce. Samples, taken initially and monthly for three months, stored at summer room temperature 31.1°C (88°F), were taken for analysis of microbial growth using five culture media. Yeasts and coliforms were completely absent, but a few green and yellowish-black molds were found initially. The population of bacteria decreased over the storage period. Results indicated that either increasing the temperature of processing or adding antimicrobial agent(s) alone or their synergistic action reduced the numbers of microorganisms. However, the seven combinations of antimicrobial agents did not show obvious differences in efficacy among one another in decreasing microbial growth. After three months, a slight darkening of all samples was evident with that salsa receiving no heat treatment nor preservative being the most affected. In addition, the color of all samples was satisfactory and the flavor was indistinguishable from that of the fresh samples after the three-month storage period.

INTRODUCTION

Mexican-style hot sauce, salsa, is a very important part of the Mexican cuisine as many dishes are considered incomplete without the addition of the sauce. It is a highly acidic sauce with tomatoes, onions, chilies, vinegar and certain combination of spices as its chief ingredients. It is said that the Mexican eating habits are greatly influenced by the ancient Aztec Indians (31, 52).

In recent years, Mexican-style foods have increased in popularity in the United States. As a result, the nutritional value and quality improvement of Mexican foods as well as potential health problem which may arise have been studied. In 1981, Draughon et al. showed that there was no potential health problem associated with Mexican-style sauces, enchilada and taco sauces, because the low pH value of the sauces prevented the growth of foodborne pathogens (16).

Draughon et al. found that spoiled taco and enchilada sauces contributed various types of microorganisms to the sauces, Bacillus in particular (16). In fact, most of the spices harbor large number of microorganisms, including spoilage types, to a food product (24). On the contrary, onions, garlic and oregano were reported to have certain antimicrobial activities (23, 24, 51).

The objective of this study was to determine effects of processing temperatures and antimicrobial agents on keeping the quality of salsa, a commercial product from Miguel's of Tucson. Different

degrees of processing temperature were adopted: no heating (all ingredients were mixed without heat treatment), 62.8°C, 71.1°C and 79.4°C. Potassium sorbate (900 ppm), sodium benzoate (700 ppm) and ethylenediamine tetraacetic acid (EDTA, 50 ppm) were used as antimicrobial agents. Their inhibitory action against colony formation was studied in seven combinations which were mixed among one another.

This investigation took a period of about three months. The results of this study should indicate the degree of heat processing necessary and preservative or combinations of preservatives which would result in superior sauces after storage.

LITERATURE REVIEW

Mexican-style sauce, salsa, is one type of hot sauces. Its chief ingredients are tomatoes, onions, chilies, vinegar and certain combination of spices. Salsa is indeed a very important part of the Mexican cuisine as many dishes depend upon the addition of the sauce for their completion (52). It may serve as an excellent dip for tortillas and potato chips, and as a seasoning on other Mexican foods.

Mexican foods today are influenced by the ancient Aztec Indians. Before the arrival of the conqueror, Cortez, this group of Indians mostly depend upon beans, corns, chilies, avocados, onions and chocolate. Their eating habits later provided the mainstay and influential background for modern Mexican cooking (31, 52).

In order to improve the quality as well as to prolong the shelf-life of salsa, from Miguel's Food Products, Tucson, hence investigation was carried out to determine the effects of processing temperature and added antimicrobial agents, potassium sorbate, sodium benzoate, and EDTA either alone or in combinations among one another on the keeping quality of the sauce. This literature review first focuses on potassium sorbate, sodium benzoate, EDTA, vinegar, the effect of temperature on microbial growth, followed by the microbiological review of various types of spices; Mexican-style sauces investigated by Draughon et al. are discussed lastly.

Sorbate

Sorbic acid was first isolated from the berries of the mountain ash in 1859 by A. W. von Hofmann. In 1939, its antimicrobial action was discovered in Germany by E. Muller and a few months later by C. M. Gooding in the U. S. A. (29).

Sorbic acid, a six-carbon alpha-beta unsaturated fatty acid, has the molecular formula $\text{CH}_3\text{-CH=CH-CH=CH-COOH}$. The carboxyl group of sorbic acid reacts readily to form salts and esters. Regarding biochemical behavior, sorbic acid is metabolized by the body to carbon dioxide and water in the same manner as fatty acid are degraded. The salts usually employed as food preservatives are calcium, sodium or potassium salt. Potassium sorbate has greater solubility in water than others, thus it has a wide application as an effective food preservative.

Potassium sorbate ($\text{C}_6\text{H}_7\text{KO}_2$) or 2,4-hexadienoic acid is a white powder with molecular mass of 150.22. At 25°C, the solubility of potassium sorbate in water is over 50 gm/100 ml; whereas, the solubility of sorbic acid is only 0.16 gm/100 ml water. The solubility of potassium sorbate in water increases with pH and temperature (48). Sorbate is more effective at pH values approaching its dissociation constant of 4.75, which implies that a shift of pH value of a foodstuff to the acid range will improve its antimicrobial action. It is in fact the undissociated molecules which play the chief role in controlling the microbial growth by migrating through the semipermeable cell membranes of the microorganisms and exerting their antimicrobial action

by enzyme inhibition (29). In order to prevent off-flavors in more acidic foods, sorbate can partially or totally replace benzoate (48).

Generally, both sorbic acid and potassium sorbate are recognised as GRAS (generally recognized as safe), and are permitted as preservatives in all countries of the world. The maximum permissible range is 0.1-0.2%. Because of the low toxicity factor, sorbate is now increasingly more preferable as a preservative than other less well investigated chemicals. Shtenberg and Ignat'ev reported that sorbic acid fed to rats and mice in quantities of 40 to 80 mg per Kg body weight daily for 3, 17 or 18 months showed no deleterious effects (47).

The antimicrobial action of sorbate is directed mainly against yeasts and molds. Bacteria are only partially inhibited, with the aerobic bacteria being the most affected. Because of the presence of the alpha-beta unsaturation of the sorbate molecule, its antimicrobial action is greatly enhanced. Cellular enzymes are inactivated when their SH groups form covalent bonds with the double bonds of sorbate. Desrosier reported that dehydrogenase system in molds was inhibited by the antimicrobial action of sorbic acid (15).

The actual mechanism of action of sorbate toward bacteria is not very well known. However, some investigations showed that sorbate inhibited the growth of Bacillus cereus, Bacillus subtilis (34), Staphylococcus aureus (32) and Pseudomonas fragi (30). The optimum pH at which bacteria are highly inhibited is about 3.5. Bell found that 0.1% sorbic acid inhibited lactic acid bacteria at

pH 3.5 (4). On the whole, sorbate is less effective against lactic acid bacteria and clostridia.

There are several factors which influence the antimicrobial effectiveness of sorbate: pH of product, product moisture, product ingredients, other additives present, product contamination, processing, packaging, storage temperature, storage length and sanitation (48).

Sorbate has a synergistic effect with temperature to prolong the shelf-life of fruit products (39). The combination of preservatives with one another had been proved to show a broader spectrum of antimicrobial action. Beuchat reported that potassium sorbate and sodium benzoate have a synergistic effect with heat on inactivation of conidia of Aspergillus flavus and Penicillium puberulum and vegetative cells of Geotrichum candidum. He described that a smaller amount of potassium sorbate and sodium benzoate was required to inactivate the microorganisms with heat (7). The author further reported that when both potassium sorbate and sodium benzoate were present at the same concentration, the degree of effectiveness varied, depending on the type of solute in the heating menstrue (6).

Robach and Stateler reported that potassium sorbate in combination with sodium benzoate, tertiary butylhydroquinone, butylated hydroxanisole or ethylenediamine tetraacetic acid showed synergistic inhibition on the growth of Staphylococcus aureus (38).

The additive effect of sorbate and benzoate on inactivation of yeasts was shown by Rushing and Senn (40).

Sorbate is used commercially in the preservations of mayonnaise, cheese, sausages, sauces, jellies, fruit juices, etc.

Benzoate

Benzoic acid occurs naturally in certain fruits such as cranberries, foxberries, raspberries and apricots. In 1875, the preservative action of benzoic acid was investigated by H. Fleck. Nevertheless, it was not until the turn of the century that benzoic acid was first introduced as food preservative. In fact, benzoic acid was the first chemical permitted in food preservation by the U. S. Food and Drug Administration (22).

Benzoic acid occurs in several forms of salt; they are sodium, potassium and calcium salts. It has the molecular formula C_6H_5COOH . Sodium benzoate ($C_6H_5NaO_2$) is a white crystalline powder and has a molecular mass of 144.11. The solubility of sodium benzoate in water at room temperature is 63 gm/100 ml, whereas, benzoic acid is only 0.34 gm/100 ml of water.

Although benzoic acid and sodium benzoate are generally considered as GRAS, in which the maximum permissible quantity is 0.1%, there is a certain tendency to replace benzoate by another well-investigated chemical, sorbate, because of the toxicological considerations. Shtenberg and Ignat'ev showed that a daily dosage of 40 to 80 mg benzoic acid per Kg body weight fed to rats and mice over

three months resulted in the mortality of both species of animals (47). Although the addition of benzoate in foodstuffs such as soft drinks is in permissible amount, it is frequently perceptible in the flavor (29).

The antimicrobial action of benzoate is highly influenced by the pH value. Its inhibitory action resides in the undissociated molecules, and since that benzoates are relatively weak acids, these compounds are most active in high acid foods. The optimum pH range at which benzoates act actively is at 2.5-4.0 (49). Benzoic acid and its sodium salt are employed in the preservation of high acid foods, such as fruit juices, soft drinks, mayonnaise, pickles, margarine, sauces and others.

Like sorbate, the spectrum of action of benzoate is chiefly against yeasts and molds, but bacteria are only partially inhibited. Because benzoate exerts antimicrobial action in the high acid foods, the growth of bacteria is mostly depressed. The inhibitory action of benzoate is also based on the enzyme inhibition. Bosund reported that those enzymes which take part in oxidative phosphorylation and acetic acid metabolism were inactivated by benzoic acid (9). In biochemical metabolism, after the absorption of benzoic acid from the intestine, it conjugate with glycine to form hippuric acid and is excreted in the urine; the relatively small quantities of benzoic acid are conjugated to glucuronic acid and are excreted in the urine as well.

It has been found that benzoate has certain antimicrobial action against the *Pseudomonas* species, *Micrococcus* species, *Streptococcus* species, *Lactobacillus* species, *Escherichia coli* and

Bacillus cereus (29). Its weak effect on lactic acid bacteria and clostridia is similar to that of sorbate.

Since no preservative is effective against all types of microorganisms, the spectrum of action of benzoate is enhanced when combined with various preservatives (7, 40). The combined action of benzoate with sorbate was discussed in the previous section of sorbate.

Ethylenediamine tetraacetic acid (EDTA)

EDTA is used as a sequestrant in food systems. The sodium and calcium salts of EDTA are used as a synergist for antioxidants. In most oxidation-sensitive food substrates such as fats and oils, trace metals, copper and iron, present in oils serve as pro-oxidant catalysts which enhance oxidation resulting in rancidity, off-flavor and reversion. But in the presence of a sequestrant and antioxidant mixture, metals are chelated by sequestrant and pro-oxidant catalytic effects were inhibited. As a result, antioxidant can perform its function properly. In addition, EDTA functions as stabilizer for vitamins, color and flavor (19).

Disodium EDTA has the molecular formula

$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$. It is a white crystalline powder and is soluble in water. The maximum permissible amount as a preservative in sauces is 75 ppm. The efficacy of EDTA increases as pH rises because as more molecules dissociate, the quantity of metal complexed increases as well (19).

Leive found that only the sodium salts possess antibacterial action (26), and sensitivity is particularly directed against gram-negative bacteria (43).

In 1956 and 1958, an important finding was made by Repaske, who discovered that when EDTA was introduced in a tris buffer (2-amino-2-hydroxymethyl-propane-1,3-diol), certain gram-negative bacteria became sensitive to the enzyme lysozyme, which in turn caused lysis of the cells. The bacteria involved in these experiments were Escherichia coli, Pseudomonas aeruginosa, Pseudomonas fluorescens and Azotobacter vinelandii. Repaske further reported that the lysis of cells in the presence of lysozyme-tris EDTA took place in some gram-positive bacteria like Bacillus megaterium, Bacillus subtilis and Sarcina lutea, the yeast cell Saccharomyces cerevisiae and the mold Toluropsis utilis (35, 36). Lysis of E. coli was almost completed within the first minute when the pH of tris buffer was raised from 7.2 to 8.6 (36).

The outer membrane of the gram-negative bacteria is composed of a lipopolysaccharide-phospholipid-lipoprotein layer. Bobo et al. found that large amounts of calcium and magnesium, and traces of zinc were associated with the phospholipid components of the cell walls (8). The binding of divalent metals consequently produce cross-linkages which is essential for the integrity of the cell walls. Thus any chemical preservatives which are capable of removing the lipopolysaccharide (IPS) layer of the cell walls, improve the efficacy of the antimicrobial agents.

EDTA, a chelating agent, has the ability to react with the essential metals of the cell wall, which consequently disrupt the impermeable layer responsible for resistance. Under this condition, an increased sensitivity to a wide variety of antimicrobial agents resulted. In P. aeruginosa, EDTA was found to chelate the divalent metal ions, Ca, Mg and Zn from the cell walls, with Ca^{2+} cation the most strongly chelated (17). Brown and Melling showed that Mg-depleted cells were more resistant to EDTA resulting in less death of bacteria (10).

In gram-negative bacteria cell, it was reported that the removal of a portion of the IPS layer by EDTA treatment rendered the organisms sensitive to long-chain fatty acids (12).

EDTA was said to be effective primarily against bacteria, but not against yeasts and molds. However, Indge found that EDTA increased lysis of yeast protoplasts when the protoplasts were undergoing osmotic stress; this is due to the removal of Mg^{2+} by EDTA, which is responsible in maintaining the structure of the cell membrane (21).

EDTA has been found to be an effective preservative for fish fillets (27) and some other seafoods such as shrimp.

Vinegar (Acetic acid)

Vinegar has been used for food preservation purposes at least as early as the first century A. D. All vinegar must contain a minimum of 4% acetic acid to be legal vinegar. It can be manufactured from fruits, cereals, starchy vegetables, sugars and spirit or alcohol. In

the United States, vinegar is mainly made from apples. Besides the constituent acetic acid, vinegars also contain small quantities of glycerol, organic phosphates, pyruvic, formic, propionic, and butyric acids (3).

The manufacture of vinegar from sugars involves two steps:

(a) Anaerobic fermentation of sugar to ethyl alcohol

(b) Aerobic oxidation of alcohol to acetic acid

The yeast, Saccharomyces cerevisiae, performs the anaerobic process; oxidation of ethanol by vinegar bacteria, Acetobacter species, yields acetic acid (3).

Acetic acid is considered GRAS. Due to the fact that acetic acid can dissolve lipoids, it is a powerful degenerative agent which may attack the body cells when its concentration is above 30% (29).

In food preservations, the general action of acetic acid is based on lowering the pH value of the foods to be preserved. Owing to its pH-reducing effect, and the fact that the growth of bacteria is highly inhibited at pH below 3.5, the action of acetic acid is primarily directed against bacteria. However, lactobacilli, which

possess considerable acid tolerance, are less sensitive to acetic acid. In order to provide protection from the attacks of yeasts and molds, it is advisable that when vinegar is used as food preservative, it be combined with other strong fungistatic agents like sorbate or benzoate.

Levine and Fellers reported that acetic acid has an inhibitory action against bacteria as well as yeasts and molds, according to different inhibitory pH. Their results are summarized in Table 1 (28):

Table 1. Inhibitory action of acetic acid on various microorganisms (28).

Microorganism	Inhibiting pH	Inhibiting acidity(%)	Lethal acidity(%)
<u>Salmonella aertrycke</u>	4.9	0.04	0.09
<u>Staphylococcus aureus</u>	5.0	0.03	0.04
<u>Phytomonas phaseoli</u>	5.2	0.02	0.02
<u>Bacillus cereus</u>	4.9	0.04	0.04
<u>Bacillus mesentericus</u>	4.9	0.04	0.04
<u>Saccharomyces cereviseae</u>	3.9	0.59	0.59
<u>Aspergillus niger</u>	4.1	0.27	0.59

According to these investigators, the inhibitory action of acetic acid against microbial growth is proportional to the amount present.

Because of its lethal activity at comparatively high pH values, the toxicity of acetic acid for various microorganisms is not confined to

the hydrogen-ion concentration alone but also seems to be a function of the undissociated acetic acid molecules (28).

That the mode of action of acetic acid on bacteria depends principally on the undissociated molecules was further confirmed by Reynolds (37).

The effect of acetate on sugar and amino acid uptake of Bacillus subtilis was shown by Sheu et al., who found that acetate and other short chain fatty acids inhibited the uptake of amino acids and other compounds, resulting in the inhibition of growth (46).

Vinegar has a wide application in food preservations. It is employed in fruits and vegetable picklings, in mayonnaise, salad dressings and sauces to provide flavor as well as to keep good qualities, in flour to prevent the growth of rope, and in the preservation of meat, fish products and others.

Effect of temperature on microorganisms

The killing of microorganisms or their spores by heat varies with the kind of microorganisms as well as the environment during heating. During heat treatment, the killing of microorganisms is supposed to be due to coagulation of the proteins and inactivation of enzymes required for metabolism (18). From the aspects of inhibition and destruction of cells by heat, there is no doubt that every part of the cells will be damaged provided sufficient heat is applied.

Russell and Harries concluded that the primary site of heat damage was the cytoplasmic membrane (41), and they later found that

ribonucleic acid degradation was probably responsible for the death of the heat-treated E. coli cells (42).

Allwood and Russell described that there was a leakage of free amino acids and protein from heated cells of Staphylococcus aureus (1).

According to Arrhenius's equation, the first order of kinetics of decay is expressed as:

$$X = X_0 e^{-kt}$$

$$\log_e X = \log_e X_0 - kt$$

When a graph of $\log_e X$ is plotted against t , a linear relationship between the logarithm of the number of survivors and exposure time is obtained. This implies that in any given time interval, a constant proportion of the survivors lose viability (11).

Microbiology of spices

Spices are the roots, bark, buds, seeds, or fruits of aromatic plants which grow throughout the world, in the tropics and the temperate zones in particular. Due to the lack of microbiological knowledge in the early days, many people thought that spices had germicidal properties and used them as food preservatives. In fact, with a few exceptions, such as onions, garlic and oregano, most of the spices contribute large numbers of microorganisms, including spoilage types, to food products (24).

Onion, garlic and oregano

The antibacterial activities of onion, garlic and oregano are still not well known. However, Vaughn concluded from his investigation that juice of fresh onions showed bactericidal effects upon Bacillus subtilis, Escherichia coli, Candida krusei, and Saccharomyces cerevisiae (51).

There are six volatile disulfides present in onions which are believed to have certain bacterial action: di-n-propyl disulfide (Pr_2S_2), n-propyl allyl disulfide (PrAlS_2), methyl-n-propyl disulfide (MePrS_2), methyl allyl disulfide (MeAlS_2), dimethyl disulfide (Me_2S_2), and diallyl disulfide (Al_2S_2) (5).

An experiment was carried out to examine the bactericidal activity of garlic and onion against Salmonella typhimurium and Escherichia coli. Results showed that as the concentrations of onion increased from 1% to 5%, and garlic increased from 5% to 10%, maximum death rates resulted. On the other hand, the major volatile disulfide compounds of onions, n-propyl allyl and di-n-propyl, only exhibited a bacteriostatic effect (23).

The results of Julseth and Deibel showed that both onion and oregano had toxic effects upon Salmonella (24).

Microorganisms in spices that cause spoilage

The spore-forming Bacillus were found to dominant on whole spices, herbs, and spice blends (20). Most members of the genus Bacillus are normally present in soil and in decaying animals and

vegetable matters (13). It was found that the growth of Bacillus stearothermophilus and Bacillus coagulans was common on black pepper, ginger and imported paprika (3). Vajdi and Pereira found that Bacillus stearothermophilus and Bacillus mycoides were present in considerable numbers in raw black pepper (50).

Pepper, paprika and celery flakes often had bacteria counts which ranged from two million to one hundred million per gram of spice (25).

Black pepper often has high counts of microorganisms, especially aerobic sporeformers. Draughon et al. found that black pepper and red pepper had microbial numbers approaching log 7 per gram (16). Plate counts up to about log 7.7 microorganisms per gram of black pepper were found by Julseth and Deibel (24).

Since spices are grown in warm and humid areas, not only a wide variety of bacteria are found, but fungi may grow favorably especially during post-harvest deterioration. Contamination by these microorganisms may also occur during handling of the commodities. Samples of either whole or ground black pepper from various sources yielded numerous colonies of the genus Aspergillus (3).

Chili powder was reported to contain bacterial numbers ranging from log 2 to log 5.7 per gram; however, yeast and mold counts were less than one hundred per gram in nearly all samples tested (33). Draughon et al. reported that chili powder had bacteria counts of log 4.1 per gram and yeast and mold counts of log 3.64 and log 3.82 per gram respectively (16).

Furthermore, the bacteria counts of garlic powder ranged from log 3.6 to log 6.0 per gram; however, yeast and mold counts were between log 2.0 to log 2.7 per gram (33).

There is little information available regarding the incidence and number of potentially harmful microorganisms, such as Salmonella, Shigella, Escherichia coli, and coagulase-positive staphylococci (24). This indicates that spices may not contribute to public health problems since foodborne pathogens are rarely encountered.

Mexican-style sauces

Many different types of sauces are boiled with various spices to produce palatable condiments. Mexican-style sauce, salsa, is one of the examples.

Although in recent years, Mexican-style foods have increased in popularity in the United States, very few investigations regarding the spoilage and quality improvement of Mexican-style sauces have been done. In 1981, Draughon, Elahi and McCarty identified different types of microorganisms present in the spoiled Mexican-style sauces, enchilada and taco sauces. Various microorganisms isolated from the spoiled sauces are reported as shown in Table 2 (16):

Table 2. Microorganisms isolated from spoiled enchilada and taco sauces (16)

Microorganism	Number of isolates
Bacteria	
<u>Bacillus</u>	83
<u>Corynebacterium</u>	8
<u>Arthrobacter</u>	3
<u>Streptococcus</u>	23
<u>Leuconostoc</u>	14
<u>Micrococcus</u>	10
<u>Staphylococcus</u> (coagulase -)	8
<u>S. aureus</u> (coagulase +)	5
<u>Salmonella</u>	0
<u>C. perfringens</u>	11
Yeast	
<u>Rhodotorula</u>	6
<u>Torulopsis</u>	3
<u>Zygosaccharomyces</u>	7
<u>Candida</u>	14
<u>Saccharomyces</u>	28
Mold	
<u>Aspergillus</u>	21
<u>Penicillium</u>	3
<u>Alternaria</u>	2
<u>Rhizopus</u>	3

Microbial populations in spices has been effectively reduced by fumigation practices such as with ethylene oxide (16). Nevertheless, the treatment is costly and time consuming. In addition, ethylene oxide reacts with chloride ions in foods to produce the carcinogenic chlorhydrin (3). In fact, gamma irradiation of spices is a more effective sterilant (50).

Because of the low pH value of the Mexican-style sauces, the growth of most foodborn pathogens is greatly depressed; as a result, there is no indication of a potential health problem associated with these sauces (16).

In order to increase the quality and shelf-life of Mexican-style sauces, the importance of using good sanitary practices was emphasized during preparation using good quality spices with low microbial growth (16).

MATERIALS AND METHODS

Samples

Salsa was manufactured at a commercial factory in Tucson, Arizona, in a large batch of 48 gallons with the following ingredients: parsley, oregano, garlic, salt, pepper, crushed red pepper, jalapenos, green chilies, tomatoes, tomatoes puree, vinegar, and fresh onions.

Due to fast and convenient operation, four-fluid ounce bottles with solutions of antimicrobial agents were prepared beforehand. The bottles were sanitized by soaking in chlorine water, 50 ppm, and drained before each combination of chemical solution was added into the bottles respectively. The sterilized chemical solutions of potassium sorbate, sodium benzoate and EDTA were employed in the following combinations:

- (1) No chemical added
- (2) Potassium sorbate alone
- (3) Sodium benzoate alone
- (4) EDTA alone
- (5) The mixture of potassium sorbate and sodium benzoate
- (6) The mixture of potassium sorbate and EDTA
- (7) The mixture of sodium benzoate and EDTA
- (8) The mixture of potassium benzoate, sodium benzoate and EDTA

Different processing temperatures were adopted: no heating, 62.8°C, 71.1°C, 79.4°C. Sauces were first made with the necessary ingredients by mixing with an electric stirrer without heat treatment

in a closed stainless tank. Sauces were then placed into each bottle using a commercial filler and shaken thoroughly for complete mixing of the chemical solution with sauce.

The temperature was then raised gradually to 62.8°C; and the second batch of samples were again placed into each bottle respectively immediately followed by complete shaking.

The third and fourth batches of samples processed at temperature 71.1°C and 79.4°C were produced in the same manner.

Sauces were taken for microbiological analysis at four intervals: zero-time (immediately after sauces were made), after one month, after two months, and after three months. At each interval, 64 bottles of sample were tested for total counts of microorganisms, and the rest of them were kept at summer room temperature 31.1°C.

Total plate counts

The pH of each sample was first recorded. Eleven gm of sauce was added to 99 ml of 0.1% sterilized peptone water. Decimal dilutions were made in peptone water, and plate counts were made in duplicate from 10^{-2} to 10^{-5} on the following media:

- (1) Plate count agar, by pour plate method (PCA, Difco)
- (2) Tomato juice agar, by pour plate method (TJA, Difco)
- (3) Violet red bile agar, by pour plate method (VRBA, Difco)
- (4) Malt extract agar, by pour plate method (MEA, Difco)
- (5) Potato dextrose agar, by spread plate method (PDA, Difco)

PCA and TJA are generally used for aerobic and acidophilic plate count bacteria respectively, were incubated at 32°C for 48 hours. VRBA, used mainly to determine the existence of coliforms, were incubated at 32°C for within 24 hours. On the other hand, MEA and acidified PDA were both incubated at 24°C for 5 days to plate count the growth of yeasts and molds.

Chemicals like tartaric acid crystals (for acidifying PDA) were purchased from Matheson Coleman and Bell (Norwood, Ohio), potassium sorbate, sodium benzoate and disodium salt of EDTA were purchased from Sigma (St. Louis, Missouri), peptone was bought from Difco (Detroit, Michigan).

Total plate counts were done using colony counter.

Statistical analysis

Analysis of variance was done by the VAX-11/780 system, using the SAS (Statistical Analysis System) program, at The University of Arizona Computer Center. Duncan's multiple range tests were used to test for statistically significant differences at 5% level.

RESULTS AND DISCUSSION

Mexican-style sauce, salsa, under the effects of processing temperature or added antimicrobial agent(s) either alone or acting synergistically, showed a reduction in colony recovery.

Altogether, two hundred and fifty six samples were analyzed at four intervals in a period of about three months. The total counts per gram of sauce on PCA and TJA are presented in Tables 3-10.

Because of the knowledge gained from the preliminary experiment, decimal dilutions of the sauce were made only to dilution 10^{-5} .

Five media were used to detect bacteria, yeasts and molds. There were no coliforms found using VRBA. In fact, " coliforms are not a necessary ingredient of spices " (33); their occurrence is sporadic and rare (24). On the other hand, a few green and yellowish black molds were found in three treatment samples initially. Two molds were found in samples under the conditions of no heat treatment and no added antimicrobial agent(s). One mold was obtained in samples containing EDTA alone and no heat treatment. Another mold was found in the samples to which no antimicrobial agent was added but under the processing temperature 62.8°C; this phenomenon was reasonable since certain molds are quite heat resistant. Draughon et al. (16) reported from their Mexican-style sauces investigation that Aspergillus was the most numerous genus of mold detected. Apart from those samples mentioned above, molds were completely absent in all the samples

Table 3. Total counts using PCA with no heat treatment

Anti-microbial agent	First interval (0 to 1 day)		Second interval (34 to 36 days)		Third interval (70 to 71 days)		Fourth interval (96 to 97 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	850 * 10 ³ 900 * 10 ³	3.75	44 * 10 ³ 35 * 10 ³	3.78	218 * 10 ² 235 * 10 ²	3.75	298 * 10 ² 258 * 10 ²
OP	3.85	98 * 10 ³ 161 * 10 ³	3.80	36 * 10 ³ 51 * 10 ³	3.85	280 * 10 ² 31 * 10 ³	3.81	320 * 10 ² 279 * 10 ²
OS	3.75	85 * 10 ³ 80 * 10 ³	3.80	41 * 10 ³ 42 * 10 ³	3.82	33 * 10 ³ 35 * 10 ³	3.79	223 * 10 ² 199 * 10 ²
OE	3.70	74 * 10 ³ 71 * 10 ³	3.75	37 * 10 ³ 21 * 10 ³	3.78	263 * 10 ² 225 * 10 ²	3.75	254 * 10 ² 180 * 10 ²
PS	3.80	82 * 10 ³ 71 * 10 ³	3.85	50 * 10 ³ 32 * 10 ³	3.88	329 * 10 ² 36 * 10 ³	3.84	44 * 10 ³ 30 * 10 ³
PE	3.80	168 * 10 ³ 69 * 10 ³	3.85	44 * 10 ³ 37 * 10 ³	3.85	276 * 10 ² 244 * 10 ²	3.80	234 * 10 ² 244 * 10 ²
SE	3.80	64 * 10 ³ 80 * 10 ³	3.80	295 * 10 ² 294 * 10 ²	3.83	234 * 10 ² 247 * 10 ²	3.79	214 * 10 ² 321 * 10 ²
PSE	3.80	52 * 10 ³ 71 * 10 ³	3.85	34 * 10 ³ 270 * 10 ²	3.88	220 * 10 ² 196 * 10 ²	3.82	245 * 10 ² 268 * 10 ²

Table 4. Total counts using PCA under processing temperature 62.8°C

Anti-microbial agent	First interval (2 to 3 days)		Second interval (36 to 38 days)		Third interval (71 to 72 days)		Fourth interval (97 to 98 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	50 * 10 ³ 56 * 10 ³	3.80	214 * 10 ² 280 * 10 ²	3.78	243 * 10 ² 269 * 10 ²	3.72	236 * 10 ² 201 * 10 ²
OP	3.85	43 * 10 ³ 56 * 10 ³	3.85	155 * 10 ² 283 * 10 ²	3.85	246 * 10 ² 298 * 10 ²	3.81	260 * 10 ² 292 * 10 ²
OS	3.85	41 * 10 ³ 40 * 10 ³	3.80	220 * 10 ² 227 * 10 ²	3.82	228 * 10 ² 171 * 10 ²	3.78	204 * 10 ² 204 * 10 ²
OE	3.80	44 * 10 ³ 42 * 10 ³	3.77	237 * 10 ² 188 * 10 ²	3.78	209 * 10 ² 206 * 10 ²	3.75	187 * 10 ² 229 * 10 ²
PS	3.90	42 * 10 ³ 38 * 10 ³	3.85	285 * 10 ² 172 * 10 ²	3.90	222 * 10 ² 238 * 10 ²	3.85	278 * 10 ² 206 * 10 ²
PE	3.80	54 * 10 ³ 41 * 10 ³	3.80	261 * 10 ² 299 * 10 ²	3.85	267 * 10 ² 238 * 10 ²	3.81	250 * 10 ² 188 * 10 ²
SE	3.80	81 * 10 ³ 42 * 10 ³	3.80	352 * 10 ² 235 * 10 ²	3.82	206 * 10 ² 216 * 10 ²	3.78	203 * 10 ² 208 * 10 ²
PSE	3.85	34 * 10 ³ 32 * 10 ³	3.85	350 * 10 ² 216 * 10 ²	3.90	189 * 10 ² 229 * 10 ²	3.85	243 * 10 ² 190 * 10 ²

Table 5. Total counts using PCA under processing temperature 71.1°C

Anti-microbial agent	First interval (3 to 4 days)		Second interval (38 to 39 days)		Third interval (72 to 73 days)		Fourth interval (98 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	198 * 10 ² 239 * 10 ²	3.80	218 * 10 ² 189 * 10 ²	3.90	214 * 10 ² 184 * 10 ²	3.85	230 * 10 ² 247 * 10 ²
OP	3.85	194 * 10 ² 253 * 10 ²	3.85	150 * 10 ² 174 * 10 ²	3.85	241 * 10 ² 170 * 10 ²	3.81	163 * 10 ² 179 * 10 ²
OS	3.80	221 * 10 ² 215 * 10 ²	3.80	167 * 10 ² 150 * 10 ²	3.82	213 * 10 ² 205 * 10 ²	3.78	140 * 10 ² 148 * 10 ²
OE	3.80	172 * 10 ² 204 * 10 ²	3.79	169 * 10 ² 151 * 10 ²	3.78	200 * 10 ² 168 * 10 ²	3.72	140 * 10 ² 160 * 10 ²
PS	3.85	145 * 10 ² 150 * 10 ²	3.85	210 * 10 ² 154 * 10 ²	3.90	170 * 10 ² 162 * 10 ²	3.85	177 * 10 ² 104 * 10 ²
PE	3.80	176 * 10 ² 160 * 10 ²	3.80	144 * 10 ² 158 * 10 ²	3.85	138 * 10 ² 154 * 10 ²	3.80	172 * 10 ² 124 * 10 ²
SE	3.80	163 * 10 ² 172 * 10 ²	3.80	172 * 10 ² 153 * 10 ²	3.80	190 * 10 ² 139 * 10 ²	3.78	107 * 10 ² 120 * 10 ²
PSE	3.85	185 * 10 ² 116 * 10 ²	3.85	139 * 10 ² 178 * 10 ²	3.88	148 * 10 ² 151 * 10 ²	3.85	153 * 10 ² 123 * 10 ²

Table 6. Total counts using PCA under processing temperature 79.4°C

Anti-microbial agent	First interval (4 to 5 days)		Second interval (40 days)		Third interval (73 to 74 days)		Fourth interval (98 to 99 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	135 * 10 ² 162 * 10 ²	3.80	131 * 10 ² 106 * 10 ²	3.80	75 * 10 ² 102 * 10 ²	3.75	64 * 10 ² 90 * 10 ²
OP	3.85	110 * 10 ² 102 * 10 ²	3.85	116 * 10 ² 90 * 10 ²	3.88	95 * 10 ² 80 * 10 ²	3.81	78 * 10 ² 76 * 10 ²
OS	3.85	129 * 10 ² 130 * 10 ²	3.85	123 * 10 ² 108 * 10 ²	3.85	70 * 10 ² 99 * 10 ²	3.80	60 * 10 ² 62 * 10 ²
OE	3.80	103 * 10 ² 99 * 10 ²	3.80	98 * 10 ² 85 * 10 ²	3.82	83 * 10 ² 69 * 10 ²	3.79	44 * 10 ² 56 * 10 ²
PS	3.90	125 * 10 ² 130 * 10 ²	3.90	98 * 10 ² 83 * 10 ²	3.95	71 * 10 ² 61 * 10 ²	3.90	60 * 10 ² 59 * 10 ²
PE	3.90	123 * 10 ² 121 * 10 ²	3.90	69 * 10 ² 72 * 10 ²	3.95	77 * 10 ² 52 * 10 ²	3.90	42 * 10 ² 47 * 10 ²
SE	3.80	122 * 10 ² 95 * 10 ²	3.80	71 * 10 ² 74 * 10 ²	3.80	58 * 10 ² 49 * 10 ²	3.80	69 * 10 ² 56 * 10 ²
PSE	3.90	76 * 10 ² 110 * 10 ²	3.91	56 * 10 ² 65 * 10 ²	3.95	70 * 10 ² 50 * 10 ²	3.91	45 * 10 ² 37 * 10 ²

Table 7. Total counts using TJA with no heat treatment

Anti-microbial agent	First interval (0 to 1 day)		Second interval (34 to 36 days)		Third interval (70 to 71 days)		Fourth interval (96 to 97 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	101 * 10 ³ 149 * 10 ³	3.75	26 * 10 ³ 48 * 10 ³	3.78	341 * 10 ² 30 * 10 ³	3.75	156 * 10 ² 147 * 10 ²
OP	3.85	67 * 10 ³ 70 * 10 ³	3.80	48 * 10 ³ 34 * 10 ³	3.85	102 * 10 ² 117 * 10 ²	3.81	134 * 10 ² 133 * 10 ²
OS	3.75	71 * 10 ³ 56 * 10 ³	3.80	35 * 10 ³ 30 * 10 ³	3.82	169 * 10 ² 104 * 10 ²	3.79	113 * 10 ² 99 * 10 ²
OE	3.70	56 * 10 ³ 46 * 10 ³	3.75	127 * 10 ² 124 * 10 ²	3.78	98 * 10 ² 99 * 10 ²	3.75	120 * 10 ² 113 * 10 ²
PS	3.80	55 * 10 ³ 58 * 10 ³	3.85	43 * 10 ³ 142 * 10 ²	3.88	272 * 10 ² 138 * 10 ²	3.84	105 * 10 ² 117 * 10 ²
PE	3.80	150 * 10 ³ 80 * 10 ³	3.85	36 * 10 ³ 30 * 10 ³	3.85	110 * 10 ² 124 * 10 ²	3.80	140 * 10 ² 125 * 10 ²
SE	3.80	58 * 10 ³ 63 * 10 ³	3.80	87 * 10 ² 105 * 10 ²	3.83	86 * 10 ² 119 * 10 ²	3.79	43 * 10 ² 120 * 10 ²
PSE	3.80	63 * 10 ³ 64 * 10 ³	3.85	211 * 10 ² 118 * 10 ²	3.88	51 * 10 ² 44 * 10 ²	3.82	69 * 10 ² 68 * 10 ²

Table 8. Total counts using TJA under processing temperature 62.8°C

Anti-microbial agent	First interval (2 to 3 days)		Second interval (36 to 38 days)		Third interval (71 to 72 days)		Fourth interval (96 to 98 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	45 * 10 ³ 77 * 10 ³	3.80	161 * 10 ² 223 * 10 ²	3.78	167 * 10 ² 142 * 10 ²	3.72	70 * 10 ² 83 * 10 ²
OP	3.85	50 * 10 ³ 54 * 10 ³	3.85	105 * 10 ² 140 * 10 ²	3.85	132 * 10 ² 129 * 10 ²	3.81	84 * 10 ² 79 * 10 ²
OS	3.85	55 * 10 ³ 54 * 10 ³	3.80	178 * 10 ² 166 * 10 ²	3.82	111 * 10 ² 126 * 10 ²	3.78	104 * 10 ² 131 * 10 ²
OE	3.80	165 * 10 ² 149 * 10 ²	3.77	100 * 10 ² 101 * 10 ²	3.78	93 * 10 ² 74 * 10 ²	3.73	131 * 10 ² 120 * 10 ²
PS	3.90	150 * 10 ² 152 * 10 ²	3.85	145 * 10 ² 108 * 10 ²	3.90	84 * 10 ² 104 * 10 ²	3.85	82 * 10 ² 62 * 10 ²
PE	3.80	169 * 10 ² 114 * 10 ²	3.80	133 * 10 ² 131 * 10 ²	3.85	140 * 10 ² 132 * 10 ²	3.81	90 * 10 ² 80 * 10 ²
SE	3.80	70 * 10 ³ 56 * 10 ³	3.80	110 * 10 ² 92 * 10 ²	3.82	87 * 10 ² 99 * 10 ²	3.78	50 * 10 ² 61 * 10 ²
PSE	3.85	139 * 10 ² 115 * 10 ²	3.85	108 * 10 ² 80 * 10 ²	3.90	103 * 10 ² 53 * 10 ²	3.85	75 * 10 ² 89 * 10 ²

Table 9. Total counts using TJA under processing temperature 71.1°C

Anti-microbial agent	First interval (3 to 4 days)		Second interval (38 to 39 days)		Third interval (72 to 73 days)		Fourth interval (98 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	94 * 10 ² 113 * 10 ²	3.80	118 * 10 ² 110 * 10 ²	3.90	61 * 10 ² 59 * 10 ²	3.85	44 * 10 ² 71 * 10 ²
OP	3.85	89 * 10 ² 74 * 10 ²	3.82	79 * 10 ² 80 * 10 ²	3.85	85 * 10 ² 95 * 10 ²	3.81	44 * 10 ² 76 * 10 ²
OS	3.80	103 * 10 ² 105 * 10 ²	3.80	86 * 10 ² 101 * 10 ²	3.82	72 * 10 ² 95 * 10 ²	3.78	45 * 10 ² 55 * 10 ²
OE	3.75	87 * 10 ² 69 * 10 ²	3.79	85 * 10 ² 80 * 10 ²	3.78	45 * 10 ² 71 * 10 ²	3.72	58 * 10 ² 69 * 10 ²
PS	3.85	86 * 10 ² 94 * 10 ²	3.85	101 * 10 ² 91 * 10 ²	3.90	72 * 10 ² 82 * 10 ²	3.85	46 * 10 ² 40 * 10 ²
PE	3.80	78 * 10 ² 71 * 10 ²	3.80	74 * 10 ² 73 * 10 ²	3.85	30 * 10 ² 70 * 10 ²	3.80	30 * 10 ² 42 * 10 ²
SE	3.80	85 * 10 ² 90 * 10 ²	3.80	125 * 10 ² 89 * 10 ²	3.80	92 * 10 ² 82 * 10 ²	3.78	38 * 10 ² 58 * 10 ²
PSE	3.85	97 * 10 ² 77 * 10 ²	3.85	58 * 10 ² 79 * 10 ²	3.88	72 * 10 ² 71 * 10 ²	3.85	44 * 10 ² 45 * 10 ²

Table 10. Total counts using TJA under processing temperature 79.4°C

Anti-microbial agent	First interval (4 to 5 days)		Second interval (40 days)		Third interval (73 to 74 days)		Fourth interval (98 to 99 days)	
	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm	pH	Organisms per gm
OO	3.80	77 * 10 ² 63 * 10 ²	3.80	53 * 10 ² 50 * 10 ²	3.80	63 * 10 ² 56 * 10 ²	3.75	36 * 10 ² 51 * 10 ²
OP	3.85	70 * 10 ² 66 * 10 ²	3.85	50 * 10 ² 41 * 10 ²	3.88	54 * 10 ² 45 * 10 ²	3.81	31 * 10 ² 33 * 10 ²
OS	3.85	68 * 10 ² 60 * 10 ²	3.85	46 * 10 ² 62 * 10 ²	3.85	44 * 10 ² 45 * 10 ²	3.80	36 * 10 ² 37 * 10 ²
OE	3.80	69 * 10 ² 57 * 10 ²	3.80	59 * 10 ² 35 * 10 ²	3.82	44 * 10 ² 31 * 10 ²	3.79	28 * 10 ² 23 * 10 ²
PS	3.90	55 * 10 ² 50 * 10 ²	3.90	44 * 10 ² 42 * 10 ²	3.95	43 * 10 ² 46 * 10 ²	3.90	32 * 10 ² 29 * 10 ²
PE	3.90	45 * 10 ² 42 * 10 ²	3.90	27 * 10 ² 29 * 10 ²	3.95	35 * 10 ² 32 * 10 ²	3.90	38 * 10 ² 33 * 10 ²
SE	3.80	40 * 10 ² 32 * 10 ²	3.80	35 * 10 ² 41 * 10 ²	3.80	36 * 10 ² 26 * 10 ²	3.80	28 * 10 ² 32 * 10 ²
PSE	3.90	41 * 10 ² 40 * 10 ²	3.85	34 * 10 ² 32 * 10 ²	3.95	40 * 10 ² 41 * 10 ²	3.91	31 * 10 ² 30 * 10 ²

tested. Since the presence of molds was so few, no table is shown. Yeasts were not found in any of the samples analyzed. Yeasts were reported to be found infrequently in spices (2). In fact, yeasts and molds were found in low numbers in both spices and sauces (16, 33).

Tables 3-10 shows the population of microorganisms recovered from PCA and TJA. Tables 3 and 7 which show results at the beginning of the experiment, indicate that those samples without heat treatment and without added antimicrobial agent had the highest plate counts. But when antimicrobial agent(s) was added, there were a great drop in total counts. The results indicated that all of the antimicrobial agents exerted inhibitory action against microbial growth. However, from the results obtained, it is difficult to discern the most effective antimicrobial agent among the seven combinations.

When increasing heat treatments were applied, great decreases in total counts were observed as can be seen from Tables 4-6 and Tables 8-10. However, there was no obvious distinction in effect among various combinations of antimicrobial agents acting synergistically with heat.

After one month of storage at temperature 31.1°C, decline in microbial growth was seen, which appeared to be exponential (see also Figures 1-8). But after storage for one month, the rate of decay started to level off. An explanation for this is that the "tail" of the exponential curve had been reached. At this stage, the decline of microorganisms had reached a steady stage because of the more resistant cells. From Figure 1, those curves under no heat treatment

Figure 1. Effect of processing temperature on inactivation of microorganisms when no antimicrobial agent(s) was added

Figure 2. Effects of processing temperature and potassium sorbate on inactivation of microorganisms

Figure 3. Effects of processing temperature and sodium benzoate on inactivation of microorganisms

Figure 4. Effects of processing temperature and ethylenediamine tetraacetic acid on inactivation of microorganisms

Figure 5. Effects of processing temperature and the combination of potassium sorbate and sodium benzoate on inactivation of microorganisms

Figure 6. Effects of processing temperature and the combination of potassium sorbate and ethylenediamine tetraacetic acid on inactivation of microorganisms

Figure 7. Effects of processing temperature and the combination of sodium benzoate and ethylenediamine tetraacetic acid on inactivation of microorganisms

Figure 8. Effects of processing temperature and the combination of potassium sorbate, sodium benzoate and ethylenediamine tetraacetic acid on inactivation of microorganisms

and under processing temperature of 62.8°C and 71.1°C, the numbers of bacteria were reduced to almost the same level after storage for three months.

In order to determine more precisely the effects of temperature and antimicrobial agent(s) on the rate of inhibition of microorganisms, analysis of variance (ANOVA) was performed; Duncan's multiple range test was applied to determine the significant differences among various treatment means.

From ANOVA, the overall results obtained using PCA and TJA (Table 11 and Table 17) indicated that both increasing temperatures and antimicrobial agent (additive was used in the tables instead) cause significant effects on inactivation of microorganisms. However, increasing temperature was more effective than the addition of antimicrobial agent since the experimental F value has a greater deviation from the critical one. Storage time also played an important role on the death rate of microorganisms especially during the first month. The interaction effects of time and temperature, time and antimicrobial agent, temperature and antimicrobial agent, and time, temperature and antimicrobial agent were significant as well.

Duncan's multiple range test on overall results (Table 12 and Table 18) showed that each level of processing temperature had a highly significant effect on heat inactivation of microorganisms. There were significant differences between the first two intervals of time. However after the second time interval, decay curves (as shown in Figures 1-8) started to level off, which indicated that the steady

Table 11. ANOVA for overall result using PCA

ANOVA

Source	DF	SS	F	Critical F ($\alpha = 0.05$)
Error	127	10,850,691,836		
Time	3	102,788,666,055	401.02*	2.68
Temperature	3	138,129,979,492	538.91*	2.68
Additive	7	76,566,802,461	128.02*	2.08
Replication	1	64,903,164	0.76	3.92
Time * Temperature	9	196,156,387,852	255.10*	1.95
Time * Additive	21	209,178,544,258	116.59*	1.66
Temperature * Additive	21	198,694,045,820	110.74*	1.66
Time * Temperature *Additive	63	611,967,438,086	113.69*	1.42

* Significantly different

DF = Degree of freedom

SS = Sum of square

Table 12. Duncan's multiple range test for overall result using PCA

Duncan's multiple range test for variable: population
 $\alpha = 0.05$ $DF = 127$ $MSE = 85,438,518$

Time	Mean	Grouping
1	65,555	A
2	21,866	B
3	18,750	BC
4	17,638	C
Temperature		
1	69,145	A
2	29,044	B
3	17,145	C
4	8,473	D
Additive		
1	76,206	A
2	29,513	B
6	26,438	BC
5	24,809	BCD
3	24,644	BCD
7	23,453	CD
4	22,091	CD
8	20,463	D

Means with the same letter are not significantly different.

MSE = Mean squares of errors

Table 13. ANOVA and Duncan's multiple range test for the first interval using PCA

ANOVA

Source	DF	SS	F	Critical F
Error	31	9,551,107,344		
Temperature	3	320,034,654,219	346.25*	2.91
Additive	7	285,037,732,344	132.16*	2.33
Replication	1	237,656	0.00	4.16
Temperature * Additive	21	809,343,327,031	125.09*	1.92

* Significantly different

Duncan's multiple range test
MSE = 308,100,237

Temperature	Mean	Grouping
1	186,000	A
2	46,000	B
3	18,519	C
4	11,700	C
Additive		
1	241,175	A
2	52,988	B
6	48,750	BC
7	40,275	BC
3	39,438	BC
4	36,100	BC
5	36,000	BC
8	29,713	C

Means with the same letter are not significantly different.

Table 14. ANOVA and Duncan's multiple range test for the second interval using PCA

ANOVA

Source	DF	SS	F	Critical F
Error	31	801,704,375		
Temperature	3	6,801,168,125	87.66*	2.91
Additive	7	177,596,875	0.98	2.33
Replication	1	82,355,625	3.18	4.16
Temperature * Additive	21	595,479,375	1.10	1.92

* Significantly different

Duncan's multiple range test
MSE = 25,861,431

Temperature	Mean	Grouping
1	36,869	A
2	24,838	B
3	16,725	C
4	9,031	D
Additive		
1	24,100	A
2	22,975	A
3	22,813	A
5	22,775	A
6	22,663	A
7	20,575	A
8	20,175	A
4	18,850	A

Means with the same letter are not significantly different.

Table 15. ANOVA and Duncan's multiple range test for the third interval using PCA

ANOVA

Source	DF	SS	F	Critical F
Error	31	138,439,375		
Temperature	3	3,497,152,500	261.03*	2.91
Additive	7	232,625,000	7.44*	2.33
Replication	1	3,330,625	0.75	4.16
Temperature * Additive	21	352,552,500	3.76*	1.92

* Significantly different

Duncan's multiple range test
MSE = 4,465,786

Temperature	Mean	Grouping
1	26,981	A
2	22,969	B
3	17,794	C
4	7,256	D
Additive		
2	21,500	A
3	20,825	A
5	20,163	AB
1	19,250	ABC
6	18,075	BCD
4	17,788	CDE
7	16,738	DE
8	15,663	E

Means with the same letter are not significantly different.

Table 16. ANOVA and Duncan's multiple range test for the fourth interval using PCA

ANOVA

Source	DF	SS	F	Critical F
Error	31	316,094,375		
Temperature	3	3,953,392,500	129.24*	2.91
Additive	7	297,392,500	4.17*	2.33
Replication	1	22,325,625	2.19	4.16
Temperature * Additive	21	370,125,000	1.73	1.92

* Significantly different

Duncan's multiple range test
MSE = 10,196,593

Temperature	Mean	Grouping
1	26,731	A
2	22,369	B
3	15,544	C
4	5,906	D
Additive		
2	20,588	A
1	20,300	A
5	20,300	A
8	16,300	B
6	16,263	B
7	16,225	B
4	15,625	B
3	15,500	B

Means with the same letter are not significantly different.

Table 17. ANOVA for overall result using TJA

ANOVA

Source	DF	SS	F	Critical F ($\alpha = 0.05$)
Error	127	5,519,248,398		
Time	3	23,683,743,867	181.66*	2.68
Temperature	3	29,291,853,242	224.67*	2.68
Additive	7	3,556,575,898	11.69*	2.08
Replication	1	22,266,602	0.51	3.92
Time * Temperature	9	26,857,509,102	68.67*	1.95
Time * Additive	21	3,504,515,820	3.84*	1.66
Temperature * Additive	21	5,775,688,945	6.33*	1.66
Time * Temperature *Additive	63	9,452,587,461	3.45*	1.42

* Significantly different

Table 18. Duncan's multiple range test for overall result using TJA

Duncan's multiple range test for variable: population
 $\alpha = 0.05$ $DF = 127$ $MSE = 43,458,649$

Time	Mean	Grouping
1	31,438	A
2	13,131	B
3	9,195	C
4	7,072	C
Temperature		
1	31,813	A
2	17,205	B
3	7,500	C
4	4,319	D
Additive		
1	23,028	A
2	16,866	B
3	16,784	B
6	16,241	BC
7	13,931	BCD
5	13,044	CD
4	11,075	D
8	10,703	D

Means with the same letter are not significantly different.

state had been reached for further degradation of bacteria. On the other hand, although seven combinations of antimicrobial agents were used, the differences among the agents were not significant since overlappings were shown in groupings.

Futhermore, analysis of variance and Duncan's multiple range tests were done on each individual interval (Tables 13-16 and Tables 19-22). The statistical analysis again showed similar results to the overall ones except that Duncan's multiple range test on temperature for the first interval (Table 13 and Table 19) indicated that there was no difference in effect between 71.1°C and 79.4°C; moreover, from the second interval using PCA (Table 14), ANOVA showed that the effect of antimicrobial agent was not significant. Furthermore, the interaction effect between temperature and antimicrobial agent(s) at the fourth interval using PCA was not significant (Table 16). These variations may due to random sampling fluctuation and do not affect the overall results.

In order to have a better idea about the effects of processing temperatures on colony formations in the presence of different combinations of antimicrobial agents, let us look at Figures 1-8 again. The related natural logarithm data were presented in Tables A1-A4 (in Appendix B). From Figure 1, when heat treatments were employed alone, the inhibitory action of temperature was clearly shown by a sudden drop in total counts. Temperature 79.4°C has the strongest action against microbial growth. From Figures 2-8, in the presence of antimicrobial agent(s), the initial population of

Table 19. ANOVA and Duncan's multiple range test for the first interval using TJA

ANOVA

Source	DF	SS	F	Critical F
Error	31	4,418,754,375		
Temperature	3	50,283,023,750	117.59*	2.91
Additive	7	5,748,777,500	5.76*	2.33
Replication	1	15,405,625	0.11	4.16
Temperature * Additive	21	13,003,048,750	4.34*	1.92

* Significantly different

Duncan's multiple range test
MSE = 142,540,464

Temperature	Mean	Grouping
1	75,438	A
2	36,019	B
3	8,825	C
4	5,469	C
Additive		
1	50,838	A
6	35,238	B
7	33,963	BC
2	33,863	BC
3	33,700	BC
8	22,238	BCD
5	21,463	CD
4	20,200	D

Means with the same letter are not significantly different.

Table 20. ANOVA and Duncan's multiple range test for the second interval using TJA

ANOVA

Error	DF	SS	F	Critical F
Error	31	854,789,375		
Temperature	3	4,334,996,250	52.40*	2.91
Additive	7	822,355,000	4.26*	2.33
Replication	1	29,430,625	1.07	4.16
Temperature * Additive	21	1,367,206,250	2.36*	1.92

* Significantly different

Duncan's multiple range test
MSE = 27,573,851

Temperature	Mean	Grouping
1	26,338	A
2	13,006	B
3	8,931	C
4	4,250	D
Additive		
1	18,188	A
2	16,438	A
3	16,113	A
6	14,088	AB
5	13,788	AB
8	9,000	B
4	8,888	B
7	8,550	B

Means with the same letter are not significantly different.

Table 21. ANOVA and Duncan's multiple range test for the third interval using TJA

ANOVA

Source	DF	SS	F	Critical F
Error	31	161,162,344		
Temperature	3	914,014,219	58.60*	2.91
Additive	7	417,992,344	11.49*	2.33
Replication	1	5,232,656	1.01	4.16
Temperature * Additive	21	731,127,031	6.70*	1.92

* Significantly different

Duncan's multiple range test
MSE = 5,198,785

Temperature	Mean	Grouping
1	14,213	A
2	11,100	B
3	7,213	C
4	4,256	D
Additive		
1	14,863	A
5	10,513	B
3	9,575	BC
2	9,488	BC
6	8,413	BCD
7	7,838	CD
4	6,938	D
8	5,938	D

Means with the same letter are not significantly different.

Table 22. ANOVA and Duncan's multiple range test for the fourth interval using TJA

ANOVA

Source	DF	SS	F	Critical F
Error	31	51,900,000		
Temperature	3	617,328,125	122.91*	2.91
Additive	7	71,966,875	6.14*	2.33
Replication	1	4,840,000	2.89	4.16
Temperature * Additive	21	126,894,375	3.61*	1.92

* Significantly different

Duncan's multiple range test
MSE = 1,674,194

Temperature	Mean	Grouping
1	11,263	A
2	8,694	B
3	5,031	C
4	3,300	D
Additive		
4	8,275	A
1	8,225	A
3	7,750	AB
2	7,675	AB
6	7,225	AB
5	6,413	BC
8	5,638	C
7	5,375	C

Means with the same letter are not significantly different.

microorganisms was greatly reduced; it can be seen clearly when compared to Figure 1, the curve under no heat treatment. Moreover, antimicrobial agent(s) acted synergistically with temperature to further depress the microbial growth. During heat treatment, microorganisms are heat-injured, whereby their cytoplasmic membrane are damaged (42), resulting in inactivation of enzymes (18) and degradation of ribonucleic acid (42). On the other hand, because of the expansion of cell walls and cytoplasmic membranes at elevated temperature, antimicrobial agents are permitted to penetrate more easily through the permeable cell walls and exert their action resulting in the death of cells.

Inhibitory action of antimicrobial agents depends principally on their structural configuration. In fact for potassium sorbate and sodium benzoate, their undissociated molecules are considered to exert detrimental action toward cells of different microorganisms. Because of the high acidity of the sauces, around pH 3.8, this condition is favorable to the action of both potassium sorbate and sodium benzoate.

Salsa is considered as an acid food, due chiefly to one of its ingredients, vinegar. Vinegar (acetic acid) itself indeed is a preservative, and its general action is based on lowering the pH value. Because of its pH-reducing effect, the growth of bacteria is highly inhibited. However, according to Levine and Fellers, acetic acid has an inhibitory action against bacteria as well as yeasts and molds (28). Due to the high acidity of sauces, most of the microbial growth was inhibited. Under such conditions, there are no potential health

problems involved since the occurrence of foodborne pathogens is rare (16). Onions, garlic and oregano present in the sauces may have contributed certain antimicrobial action to the sauces (23, 24, 51). It is believed that the volatile disulfide compounds present in onions are capable of inactivating the growth of microorganisms.

The pH of sauces was recorded at each interval, and a slight increase and decrease in pH during the three-month investigation was found. The slight rise and fall in pH may be explained by two factors: (1) due to the action of bacteria, and (2) due to the inaccuracy of the pH meter.

During the experiment, certain microorganisms grew on TJA tended to produce sticky levan and spread over the whole plate. This caused difficulty in counting the colonies. The spore-forming Bacillus has the characteristic mentioned above (13). Bacillus was present in highest numbers among others in the Mexican-style sauces (16). Sticky spreading was seldom encountered in using PCA. Decay curves were plotted using the results from PCA because of the counting difficulties with TJA.

The color and flavor of the sauces were observed at each interval. In the second interval (i. e. after the first month of storage), a slight darkening appeared in each bottle with the most darkening occurring near the surface of the sauce. Nevertheless, the degree of darkening was hardly distinguishable between each bottle at each interval, except those samples with neither heat treatment nor antimicrobial agent added in which the darkening was somewhat more

apparent. Those samples at the end of the three-month study showed only a slightly darker color than those samples at the first month. The flavor of the sauces at all the intervals was indistinguishable from that of the fresh samples. In short, the sauces were of excellent quality after storage for three months.

Although antimicrobial agents added to salsa will help to inactivate the microbial growth, they have two disadvantages when incorporated into sauces: (1) The processing cost will be increased. (2) There may be some toxicological implications. At present, there is certain tendency to replace benzoate by other preservative such as sorbate. In addition, the fact that EDTA is a chelating agent should not be ignored, since EDTA may disturb the metabolism of the body by reacting with bivalent and multivalent metals in the body (29).

CONCLUSIONS

The results of this study indicated that increasing heat treatment during processing resulted in lower microbial numbers in Mexican-style sauce, salsa; moreover, addition of antimicrobial agent(s) alone or in a synergistic relation with heat, resulted in lower total counts of microorganisms too. There is a significant reduction in microbial growth in salsa during the first month of storage at temperature 31.1°C. Although the number of microorganisms in salsa can be reduced by the independent or combined effects of processing temperature and antimicrobial agent(s), increasing processing temperature was more inhibitory to microorganisms than various combinations of preservatives. All sauces processed at a temperature of 79.4°C had lower total counts of microorganisms than sauces processed at other temperatures. It was difficult to discern the efficacy of the seven combinations of potassium sorbate, sodium benzoate and EDTA because they showed close effectiveness on inactivation of microbial growth.

Yeasts and coliforms were absent in the salsa. Only a few green and yellowish black molds were found at the beginning of the investigation in sauces under conditions with neither heat treated nor antimicrobial agent(s) added, with EDTA treated alone, and with no antimicrobial agent(s) added but under the processing temperature 62.8°C. Otherwise, there was no mold growth.

After three months of storage, slight darkening appeared in all the samples. It was more noticeable in those samples with no heat treatment and added antimicrobial agent(s). However, the color of all samples was acceptable, and the flavor was satisfactory and indistinguishable from that of the fresh samples at the end of the three-month storage at 31.1°C.

Since salsa has low pH value around 3.8, the occurrence of pathogenic microorganisms in the sauce is unlikely. From Figure 1, it shows that the curve representing bacteria from salsa with no heat treatment, and those with processing temperature of 62.8°C and 71.1°C, all almost converged to the same point at the end of the three-month storage. This phenomenon indicated that during storage, the numbers of bacteria were reduced to nearly the same level. In conclusion, the commercially manufactured salsa can be produced under conditions with no heat treatment nor antimicrobial agent(s) added economically, though the darkening will appear darker in color after storage.

APPENDIX A

MEDIA PREPARATION

Plate Count Agar

To rehydrate the medium, suspend 23.5 gm in 1000 ml of cold distilled water. Heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 min. at 15 pounds pressure (121°C).

Tomato Juice Agar

To rehydrate, suspend 51 gm in 1000 ml distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C).

Violet Red Bile Agar

To rehydrate the medium, suspend 41.5 gm in 1000 ml cold distilled water and heat to boiling to dissolve the medium completely. Cool to about 45°C and pour 15 ml of medium into petri dishes containing the inoculum. After the inoculated medium has solidified, 4 ml of the medium are poured over the surface to form a thin covering film.

Potato Dextrose Agar

To rehydrate the medium, suspend 39 gm in 1000 ml cold distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C)

To obtain a reaction of pH 3.5 add 0.95 ml of a sterile 1 to 10 dilution of tartaric acid per 100 ml of sterile liquid medium

immediately before pouring of plates. Never heat medium after addition of acid.

Malt Extract Agar

To rehydrate the medium, suspend 33.6 gm in 1000 ml distilled water and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121°C).

In as much as this medium has an acid reaction, care should be taken to avoid overheating which will result in a softer medium.

APPENDIX B

TABLES OF THE GRAPHS

Table A1. Ln numbers of total counts using PCA with no heat treatment

Anti-microbial agent	First interval	Second interval	Third interval	Fourth interval
Ln numbers of microorganisms per gm				
OO	13.65	10.69	9.99	10.30
	13.71	10.46	10.06	10.16
OP	11.49	10.49	10.24	10.37
	11.99	10.84	10.34	10.24
OS	11.35	10.62	10.40	10.01
	11.29	10.64	10.46	9.90
OE	11.21	10.52	10.18	10.14
	11.17	9.95	10.02	9.80
PS	11.31	10.82	10.40	10.69
	11.17	10.37	10.49	10.31
PE	12.03	10.69	10.22	10.06
	11.14	10.52	10.10	10.10
SE	11.07	10.29	10.06	9.97
	11.29	10.29	10.11	10.38
PSE	10.86	10.43	10.00	10.11
	11.17	10.20	9.88	10.20

Table A2. Ln numbers of total counts using PCA under processing temperature 62.8°C

Anti-microbial agent	First interval	Second interval	Third interval	Fourth interval
Ln numbers of microorganisms per gm				
OO	10.82	9.97	10.10	10.07
	10.93	10.24	10.20	9.91
OP	10.67	9.65	10.11	10.17
	10.93	10.25	10.30	10.28
OS	10.62	10.00	10.03	9.92
	10.60	10.03	9.75	9.92
OE	10.69	10.07	9.95	9.84
	10.65	9.84	9.93	10.04
PS	10.65	10.26	10.00	10.23
	10.55	9.75	10.08	9.93
PE	10.90	10.17	10.19	10.13
	10.62	10.31	10.08	9.84
SE	11.30	10.47	9.93	9.92
	10.65	10.06	9.98	9.94
PSE	10.43	10.46	9.85	10.10
	10.37	9.98	10.04	9.85

Table A3. Ln number of total counts using PCA under processing temperature 71.1°C

Anti-microbial agent	First interval	Second interval	Third interval	Fourth interval
	Ln numbers of microorganisms per gm			
OO	9.89	9.99	9.97	10.04
	10.08	9.85	9.82	10.11
OP	9.87	9.62	10.09	9.70
	10.14	9.76	9.74	9.79
OS	10.00	9.72	9.97	9.55
	9.98	9.62	9.93	9.60
OE	9.75	9.74	9.90	9.55
	9.92	9.62	9.73	9.68
PS	9.58	9.95	9.74	9.78
	9.62	9.64	9.69	9.25
PE	9.78	9.57	9.53	9.75
	9.68	9.67	9.64	9.43
SE	9.70	9.75	9.85	9.28
	9.75	9.64	9.54	9.39
PSE	9.83	9.54	9.60	9.64
	9.36	9.79	9.62	9.42

Table A4. Ln numbers of total counts using PCA under processing temperature 79.4°C

Anti-microbial agent	First interval	Second interval	Third interval	Fourth interval
	Ln numbers of microorganisms per gm			
OO	9.51	9.48	8.92	8.76
	9.69	9.27	9.23	9.10
OP	9.31	9.36	9.16	8.96
	9.23	9.10	8.99	8.94
OS	9.46	9.42	8.85	8.70
	9.47	9.29	9.20	8.73
OE	9.24	9.19	9.02	8.39
	9.20	9.05	8.84	8.63
PS	9.43	9.19	8.87	8.70
	9.47	9.02	8.72	8.68
PE	9.42	8.84	8.95	8.34
	9.40	8.88	8.56	8.46
SE	9.41	8.87	8.67	8.84
	9.16	8.91	8.50	8.63
PSE	8.94	8.63	8.85	8.41
	9.31	8.78	8.52	8.22

APPENDIX C

SAS

SAS which stands for Statistical Analysis System, is a computer software system for organizing data. SAS was developed by the SAS Institute Inc. of Cary, North Carolina and was originally designed for statistical research needs. Today SAS is used in many different areas from general problem solving with matrices to complex multivariate techniques. In addition, the SAS system provides adaptability for data analysis in areas such as information storage and retrieval, data modification and programming, report generation and file handling.

Like any language, SAS has its own vocabulary and syntax; in which each of its words has a specific meaning and there are definite yet simple rules for putting them together. A SAS job is divided into DATA steps and PROC steps. DATA steps are used to create SAS data sets, to change data values, for report writing and for file management. On the other hand, PROC steps are used to analyze SAS data sets, i.e. PROC steps ask SAS to call and execute a procedure(s) from its library. DATA and PROC steps may appear in any order and may be used in any number in a particular job.

Every SAS statement begin with a SAS keyword and ends with a semicolon. Some examples of keywords with an indication of the step involved and a brief explanation of the function of each keyword are given below:

Keyword	Step	Function
INPUT	DATA	describes the arrangement of variables on the input data lines.
CARDS	DATA	signals SAS that input data follows immediately after this statement.
DELETE	DATA	performs observation deletion for a data set at the creation stage.
LIST	DATA	lists input data lines corresponding to the observation being processed.
DELETE	PROC	deletes SAS data sets.
PRINT	PROC	prints SAS data sets.

The SAS DATA step always begins with a DATA statement. The DATA statement is followed by "program" statements, which is used to describe input or output data; to produce reports; or to manipulate data sets.

The PROC step begins with a PROC statement and this is followed by a variable number of descriptor statements defining exactly what work you want the procedure to perform.

ANOVA

ANOVA is one of several procedures in SAS for analysis of variance for balanced data. This technique is designed for testing the homogeneity of a set of n treatment means.

The variables in SAS analysis-of-variance procedures that identify levels of the classifications are declared in the CLASS statement. The specifications which are used for writing statement for ANOVA are given below:

```

PROC ANOVA option;

CLASS variable;

MODEL dependents = effects/option;

MEANS effects/options;

ABSORB variables;

FREQ variable;

TEST H = effects E = effect;

MANOVA H = effects E = effect/option;

BY variables;

```

Duncan's Multiple Range Test

When the homogeneity hypothesis in the analysis of variance is rejected, it gives no decisions as to which of the differences among the treatment means may be considered significant and which may not. Under this condition, Duncan's multiple range test is used to group the means, based on equal sample sizes, n , into subgroups of not significantly distinguishable means.

Supposing there are k sample treatment means in the test, all combinations of the k ranked means taken p (where $p = 2, \dots, k$) at a time are tested. We say that X_1 and X_k are k steps apart; X_2 and X_k are $k-1$ steps apart, etc. In each case we compare the value of the range of the set of compared means with the statistic R_p , where

$$R_p = r_p \sqrt{\frac{MSE}{n}}$$

and the value of r_p is obtained from Table. The degree of freedom in the table refer to the degree of freedom associated with MSE. Duncan's test uses a significance level depending on the number of means being tested, if the "nominal level" is α , then it is expressed as:

$$\alpha_p = 1 - (1 - \alpha)^{p - 1}$$

When the difference between two means is less than or equal to the value of the appropriate R_p , the respective means are grouped together, and no further testing need be made of intermediate means. On the other hand, when the value of the difference is greater than R_p , the means are not grouped together.

Given below is one of SAS program that was used in this study for statistical analysis:

DATA OVERALL;

INPUT TIME TEMP ADD REP POP;

CARDS;

DATA

;

PROC ANOVA;

CLASS TIME TEMP ADD REP;

MODEL POP = TIME TEMP ADD REP TIME*TEMP TIME*ADD TEMP*ADD;

TIME*TEMP*ADD;

MEANS TIME TEMP ADD REP/DUNCAN;

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