
Antimicrobial Agents

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I. INTRODUCTION

Humans have attempted to preserve food products from the detrimental effects of microorganisms since prehistoric times. Processes such as heating, drying, fermentation, and refrigeration have been used to prolong the shelf-life of food products. Some chemical food preservatives, such as salt, nitrites, and sulfites, have been in use for many years, however some have seen extensive use only recently. One of the reasons for the increased use of chemical preservatives has been the changes in the ways foods are produced and marketed. Today, consumers expect foods to be available year-round, to be free of food-borne pathogens, and to have a reasonably long shelf-life. While some improvements have been made using packaging and processing systems to preserve foods without chemicals, today antimicrobial food preservatives still play a significant role in protecting the food supply.

In selecting a food antimicrobial agent, several factors must be taken into consideration (Branen, 1993). First, the antimicrobial spectrum of the compound to be used must be known. This, along with knowledge of the bioburden of the food product, will allow the use of correct antimicrobial agent for the microorganism(s) of concern. Second, the chemical and physical properties of both the antimicrobial and the food product must be known. Such factors as pK_a and solubility of the antimicrobial and the pH of the food will facilitate the most efficient use of an antimicrobial. Third, the conditions of storage

of the product and interactions with other processes must be evaluated to ensure that the antimicrobial will remain functional over time. Fourth, a food must be of the highest microbiological quality initially if an antimicrobial is to be expected to contribute to its shelf-life. None of the antimicrobials discussed in this section is able to preserve a product that is grossly contaminated. In most cases, while food antimicrobials will extend the lag phase or inactivate low numbers of microorganisms, their effects can be overcome. With rare exceptions, food antimicrobials are not able to conceal spoilage of a food product, i.e., the food remains wholesome during its extended shelf-life. Because food antimicrobials are generally bacteriostatic or fungistatic, they will not preserve a food indefinitely. Depending upon storage conditions, the food product will eventually spoil or become hazardous. Finally, the toxicological safety and regulatory status of the selected compound must be known.

This chapter will focus on food antimicrobials approved by regulatory agencies for use in foods as direct additives. For each antimicrobial or class of antimicrobials, characteristics of the compound(s), the antimicrobial spectrum, the antimicrobial effectiveness in foods, the mechanism of action, applications, regulations, and toxicology are discussed. The detail of the discussions varies depending upon research available and importance of the compounds.

II. DIMETHYL DICARBONATE

Dimethyl dicarbonate ($\text{CH}_3\text{-O-O-C-O-C-O-O-CH}_3$) is a colorless liquid which is slightly soluble in water. The compound is very reactive with substances including water, ethanol, alkyl and aromatic amines, and sulfhydryl groups (Ough, 1993a). The use of dimethyl dicarbonate (DMDC) as an antimicrobial was reviewed by Ough (1993a). The primary target microorganisms for DMDC are yeasts including *Saccharomyces*, *Zygosaccharomyces*, *Rhodotorula*, *Candida*, *Pichia*, *Torulopsis*, *Torula*, *Endomyces*, *Kloeckera*, and *Hansenula*. Terrell et al. (1993) evaluated sulfur dioxide, sorbic acid, and DMDC for ability to act as preservatives against yeast spoilage in grape juice. Grape juice was inoculated with yeast at 2200 and 20,000 CFU/mL and fermentation monitored at 21 or 31°C. DMDC at 0.8 mM was most effective in suppressing fermentation for all inoculation levels and temperatures. When added to tomato juice stored at 5 or 20°C, DMDC was highly effective in inactivating molds and yeasts (Bizri and Wahem, 1994). It was more effective than sorbate/benzoate in controlling aerobic plate counts in tomato juice acidified to pH 3.7 and stored at 5 or 20°C. The compound is also bactericidal at 30–400 µg/mL to a number of species including *Acetobacter pasteurianus*, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, several *Lactobacillus* species, and *Pediococcus cerevisiae* (Ough, 1993a). DMDC has been shown to be bactericidal against *E. coli* O157:H7 in apple cider (Fisher and Golden, 1998). Molds are generally more resistant to DMDC than yeasts or bacteria. van der Riet and Pinches (1991) evaluated DMDC against the heat resistant mold, *Byssoschlamys fulva*, in apple and strawberry juices. They found that DMDC decreased viable *Byssoschlamys fulva* ascospores when it was added at 24-h intervals during storage of the fruit juices at 25–30°C. They described the treatment as a modified tyndallization process using DMDC rather than heat as the lethal agent.

DMDC may be used in wine, teas, carbonated and noncarbonated nonjuice beverages (e.g., sports drinks), carbonated and noncarbonated fruit-flavored or juice beverages. It is approved by the United States Food and Drug Administration (FDA) (21 CFR 172.133) as an inhibitor of yeast for the following: (1) in wine, dealcoholized wine, and

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low alcohol wine that has less than 500 yeast CFU/mL at ≤ 200 ppm ($\mu\text{g/mL}$); (2) in ready-to-drink teas (< 500 yeast CFU/mL) at ≤ 250 ppm; (3) in carbonated or noncarbonated, nonjuice-containing ($\leq 1\%$), flavored or unflavored beverages containing added electrolytes at ≤ 250 ppm; and (4) in carbonated, dilute beverages containing juice, fruit flavor, or both, with juice content $\leq 50\%$ at ≤ 250 ppm. The label of the product to which compound is added must indicate that "dimethyl dicarbonate" is added.

III. LYSOZYME

Lysozyme (1,4- β -N-acetylmuramidase; EC 3.2.1.17) is a 14,600-Da enzyme present in avian eggs, mammalian milk, tears and other secretions, insects, and fish. While tears contain the greatest concentration of lysozyme, dried egg white (3.5%) is the commercial source (Tranter, 1994). The enzyme catalyzes hydrolysis of the β -1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan of bacterial cell walls. This causes cell wall degradation and lysis in hypotonic solutions. Lysozyme is stable to heat (80°C for 2 min). It is inactivated at lower temperatures when the pH is increased. It has an optimum temperature for activity of 55–60°C but has ca. 50% activity at 10–25°C (Inovatech, 2000). Yang and Cunningham (1993) studied the effect of pH, ionic strength, and other antimicrobial substances on the lytic ability of lysozyme on *Micrococcus lysodeikticus*. The compound remained stable for over 30 days at pH 7.0 or ionic strength < 0.1 . Activity was reduced at pH 9.0 and ionic strength > 0.14 . The compound was stable or relatively stable ($> 70\%$ activity retained) for 30 days to 1.0% NaCl, 100 $\mu\text{g/mL}$ sodium nitrite, 4.0% ethanol, 0.1% sodium benzoate, 0.3% calcium propionate, 0.1% potassium sorbate, or 0.1% propyl paraben. In 0.5% EDTA, 50% of the activity was lost and no activity was detected in the presence of 0.5% lactic acid, 4% acetic acid, or 100 $\mu\text{g/mL}$ chlorine.

Lysozyme is most active against gram positive bacteria most likely because the peptidoglycan of the cell wall is more exposed than in gram negative bacteria. The enzyme has been shown to be inhibitory to *Clostridium botulinum*, *Clostridium thermosaccharolyticum*, *Clostridium tyrobutyricum*, *Bacillus stearothermophilus*, *Bacillus cereus*, *Micrococcus lysodeikticus*, and *Listeria monocytogenes* (Vakil et al., 1969; Carminiti et al., 1985; Duhaiman, 1988; Hughey and Johnson, 1987; Hughey et al., 1989). The enzyme has shown potential for use as an antimicrobial with EDTA to control the growth of *Listeria monocytogenes* in vegetables, but was less effective in refrigerated meat and soft cheese products (Hughey et al., 1989). At 1000 $\mu\text{g/mL}$, lysozyme alone was effective against only two of four strains of *Listeria monocytogenes* in milk and gave less than 1 log reductions for both (Carminati and Carini, 1989). Wang and Shelef (1991) showed that lysozyme was the primary antimicrobial compound in egg albumen, but that activity was enhanced by ovotransferrin, ovomucoid, and alkaline pH. Johansen et al. (1994) suggested that low pH (5.5) caused increased inhibition of *Listeria monocytogenes* by lysozyme because the organism had a slower growth rate allowing enzymatic hydrolysis of the cell wall to exceed the cell proliferation rate. Variation in susceptibility of gram positive bacteria is likely due to the presence of teichoic acids and other materials that bind the enzyme and the fact that certain species have greater proportions of 1,6 or 1,3 glycosidic linkages in the peptidoglycan which are more resistant than the 1,4 linkage (Tranter, 1994). For example, four strains of *Listeria monocytogenes* were not inhibited by lysozyme alone, but when EDTA was added growth inhibition resulted (Hughey and Johnson, 1987; Payne et al., 1994). Hughey and Johnson (1987) hypothesized that the peptidoglycan of

the microorganism may be partially masked by other cell wall components and EDTA enhanced penetration of the lysozyme to the peptidoglycan.

Lysozyme is less effective against gram negative bacteria due to their reduced peptidoglycan content (5–10%) and presence of the outer membrane of lipopolysaccharide (LPS) and lipoprotein (Wilkins and Board, 1989). Gram negative cell susceptibility can be increased by pretreatment with chelators (e.g., EDTA). In addition, gram negative cells may be sensitized to lysozyme if the cells are subjected to pH shock, heat shock, osmotic shock, drying, freeze–thaw cycling, and trisodium phosphate (Wilkins and Board, 1989; Ray et al., 1984; Tranter, 1994; Carneiro de Melo et al., 1998). Samuelson et al. (1985) found that EDTA plus lysozyme was inhibitory to *Salmonella* Typhimurium on poultry. In contrast, no inhibition was demonstrated with up to 2.5 mg/mL EDTA and 200 µg/mL lysozyme in milk against either *Salmonella* Typhimurium or *Pseudomonas fluorescens* in a study by Payne et al., 1994. It was theorized that there may be a significant influence of the food product on activity of lysozyme and EDTA. According to Inovatech (2000), lysozyme is effective against spoilage and pathogenic bacteria in beer/wine, bread, canned foods, cheeses, meat, and rice. Ibrahim et al. (1996) found that lysozyme with reduced enzymatic activity through heating at 80°C, pH 6.0 exhibited strong bactericidal activity against gram negative and gram positive bacteria suggesting action independent of catalytic function.

The minimum inhibitory concentration of lysozyme against most fungi tested, including *Candida*, *Sporothrix*, *Penicillium*, *Paecilomyces*, and *Aspergillus*, was >9,530 µg/mL in potato dextrose agar at pH 5.6 (Razavi-Rohani and Griffiths, 1999a). Only *Fusarium graminearum* PM162 (1,600 µg/mL) and *A. ochraceus* MM184 (3260 µg/mL) were less than the maximum concentration evaluated. However, when combined with an equivalent concentration EDTA, lysozyme was inhibitory to all species of fungi except *Candida lipolytica* 1591 (MIC > 9530 µg/mL) and *Candida parapsilosis* NCPF 3207 (MIC = 960 µg/mL) at ≤500 µg/mL.

Lysozyme is one of the few naturally occurring antimicrobials approved by regulatory agencies for use in foods. In Europe, lysozyme is used to prevent gas formation (“blowing”) in cheeses such as edam and gouda by *Clostridium tyrobutyricum* (Wasserfall et al., 1976; Carini and Lodi, 1982). Cheese manufacturers using egg white lysozyme for this purpose add a maximum of 400 mg/L. A tentative final rule (FR 1998, 63:12421–12426) listing egg white lysozyme as a “direct food substance affirmed as generally recognized as safe” (21 CFR 184.1550) was published by FDA in 1998. The enzyme is allowed to be used in cheeses to prevent gas formation. Lysozyme is used to a great extent in Japan to preserve seafood, vegetables, pasta, and salads. Lysozyme has been evaluated for use as a component of antimicrobial packaging (Padgett et al., 1998).

Since egg whites have been used for food since the beginning of recorded history, there is little concern by regulatory agencies about the toxicity of lysozyme. However, there exists the potential for allergenicity to the protein.

IV. NATAMYCIN

Natamycin was first isolated in 1955 from a culture of *Streptomyces natalensis*, a microorganism found in soil from Natal, South Africa (Anonymous, 1991). The generic name “natamycin,” which is approved by the World Health Organization, is synonymous with “pimaricin,” a name used in earlier literature.

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Natamycin ($C_{33}H_{47}NO_{13}$; MW, 665.7 Da) is a polyene macrolide antibiotic. Like many polyene antibiotics, natamycin is amphoteric, possessing one basic and one acidic group. Natamycin has low solubility in water (30–100 mg/L) and polar organic solvents and is practically insoluble in nonpolar solvents (Anonymous, 1991). Solubility ranges in other solvents include (mg/L): methanol, 2–15; ethanol, 0.04–1.2; n-butanol, 0.05–0.12; chloroform, 0.01–0.013 (Brik, 1981). Raab (1967) reported the isoelectric point of natamycin as 6.5.

Natamycin is active against nearly all molds and yeasts, but has no effect on bacteria or viruses. Most molds are inhibited at concentrations of natamycin from 0.5 to 6 $\mu\text{g}/\text{mL}$ while some species require 10–25 $\mu\text{g}/\text{mL}$ for inhibition. Most yeasts are inhibited at natamycin concentrations from 1.0 to 5.0 $\mu\text{g}/\text{mL}$. Ray and Bullerman (1982) reported that 10 $\mu\text{g}/\text{mL}$ natamycin inhibited aflatoxin B₁ production of *Aspergillus flavus* by 62.0% and eliminated ochratoxin production by *A. ochraceus*. The same level of natamycin inhibited penicillic acid production by *Penicillium cyclopium* by 98.8% and eliminated patulin production of *P. patulum*. The inhibitory effect of natamycin was reported to be greater against mycotoxin production than mycelial growth. Gourama and Bullerman (1988) studied the effect of natamycin on growth and mycotoxin production (penicillic acid) by *Aspergillus ochraceus* OL24 in yeast extract sucrose (YES) medium and olive paste. Natamycin at 20 $\mu\text{g}/\text{mL}$ delayed onset of growth, inhibited sporulation and reduced mycelial weight of *A. ochraceus* at 15, 25, and 35°C. Penicillic acid production by *A. ochraceus* at all temperatures was inhibited by 10 $\mu\text{g}/\text{mL}$ natamycin. This was in contrast to potassium sorbate which, at sublethal concentrations, caused stimulation of penicillic acid production. Growth initiation was delayed and penicillic acid production inhibited by natamycin in olive paste. The authors concluded that natamycin could provide protection against fungal growth and mycotoxin formation in olives.

Several factors affect the stability and resulting antimycotic activity of natamycin, including pH, temperature, light, oxidants, and heavy metals. While pH has no apparent effect on antifungal activity, it does influence stability of the compound. After 3-week storage at 30°C, 100% of natamycin activity is retained at pH 5–7, while ca. 85% remains at pH 3.6 and only about 75% at pH 9.0 (Anonymous, 1991). In the pH range of most food products, natamycin is very stable. Under normal storage conditions, temperature has little effect on natamycin activity when in neutral aqueous suspension. Little or no decrease in activity occurs after several days at 50°C or a short time at 100°C (Brik, 1981). In contrast, dilute solutions of natamycin are less stable and susceptible hydrolysis (Anonymous, 1991). Irradiation due to sunlight, contact with certain oxidants (e.g., organic peroxides and sulfhydryl groups), and heavy metals all adversely affect stability of natamycin solutions or suspensions (Brik, 1981; Anonymous, 1991). van Rijn et al. (1999) reported that complexing natamycin to one or more proteins, such as whey, or amino acids increased that antifungal activity of the compound. Further, they theorized that this increased activity was due to improved availability due to improved solubility. The complexed natamycin is also less susceptible to hydrolysis.

All microorganisms which are susceptible to polyene macrolide antibiotics contain sterols, while resistant microorganisms do not (Hamilton-Miller, 1973). The mode of action of polyene macrolide antibiotics therefore is binding to ergosterol and other sterol groups of the fungal cell membrane. Ergosterol is a naturally occurring sterol that can be found at concentrations of up to 5% (dry weight) in strains of *Saccharomyces* (Hamilton-Miller, 1974). Other sterols found associated with the fungal cell membrane include 24,28 dehydroergosterol and cholesterol (Hamilton-Miller, 1974). Generally, binding of nata-

mycin by sterols causes inhibition of ergosterol biosynthesis and distortion of the cell membrane with resultant leakage (Hamilton-Miller, 1973). While inhibition of glycolysis and respiration by polyene macrolide antibiotics can be demonstrated, they are considered to be secondary to cell membrane effects (Hamilton-Miller, 1973). Ziogas et al. (1983) studied 17 natamycin-resistant mutants of *Aspergillus nidulans*. Some mutants contained no ergosterol while others had reduced levels of the compound compared to the wild type. Ergosterol-deficient mutants were most resistant to natamycin (ca. 14–16 µg/mL) compared to the wild type (ca. 2 µg/mL), but grew at much slower rate than the wild type.

Nilson et al. (1975) determined the effect of natamycin in comparison with mycostatin on the shelf-life of cottage cheese stored at 4.4, 10.0, or 15.6°C. The compounds were added through curd wash water or in the cheese dressing. The cottage cheese was inoculated with *Aspergillus niger* or *Saccharomyces cerevisiae* or was uninoculated. Natamycin (100 µg/mL) added in the wash water was effective in increasing the days to spoilage of uninoculated cottage cheese by 13.6, 7.7, and 6.3 days over the control when stored at 4.4, 10.0, and 15.6°C, respectively. Cottage cheese inoculated with *A. niger* stored at the same temperatures had increased days to spoilage of 12.7, 6.0, and 4.3 days, and samples inoculated with *S. cerevisiae* had increased shelf-life of 10.3, 6.3, and 3.7 days, respectively. Adding natamycin to the cottage cheese dressing was even more effective in extending shelf-life. At 4.4, 10.0, and 15.6°C, the inoculated and uninoculated cottage cheese had increased days to spoilage ranges of 20.4–26.7, 9.7–12.3, and 2.6–5.0, respectively. Natamycin was found to be slightly more effective than mycostatin. *Aspergillus niger* was found to be the most sensitive of the two microorganisms used in the inoculation studies. Lück and Cheeseman (1978) found that 500 or 1000 µg/mL concentration of natamycin delayed mold growth on cheese for up to 6 months but did not prevent it completely. Verma et al. (1988) evaluated the effectiveness of natamycin against sorbic acid, benzoic acid, and nystatin in inoculated and uninoculated butter and cheese. All four preservatives were effective in reducing fungal growth on uninoculated butter and cheese samples stored 30 days at 7°C compared to the controls. Against *Aspergillus terreus*, *Trichoderma harzianum*, *Penicillium janthinellum*, or *Saccharomyces* sp. inoculated on butter or cheese and stored at 7°C for up to 30 days, nystatin was the most effective antifungal agent followed by natamycin. Lodi et al. (1989) found that natamycin was effective in preserving seven types of Italian cheeses with no detrimental effect on ripening. The antimycotic has been used to some extent as a butter preservative by being applied to the wrapper in small concentrations (Anonymous, 1991).

In addition to cheese, early work with natamycin suggested its use to inhibit fungal growth on fruits and meats. Ayres and Denisen (1958) investigated several antifungal agents including nystatin, rimocidin, ascocin, candidan, and natamycin (Myprozine®) for their potential in extending the shelf-life of berries. Strawberries, raspberries, and cranberries were dipped in solutions containing 0, 5, 10, 20, 50, and 100 µg/mL of each of the antibiotics. The berries were then stored at 5 ± 3°C for various periods. Natamycin and rimocidin were the most effective of the antifungal agents tested. Natamycin at 10–50 µg/mL decreased fungi on strawberries after 3–5 days storage and 50 µg/mL maintained fungal counts at equal to or less than initial count for 9 days. Natamycin (100 µg/mL) was also effective in prolonging the shelf-life of raspberries by 4 days. On cranberries, 10 µg/mL decreased the viable yeast count for 14 days of storage. When sprayed on raspberries and strawberries in the field, 50 µg/mL lowered the percentage of deterioration of the fruits during storage compared to controls. Shirk and Clark (1963) investigated the

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effectiveness of natamycin against yeast spoilage of orange juice. At 20 µg/mL, natamycin immediately reduced viable yeasts in uninoculated and inoculated (*Saccharomyces cerevisiae*) samples and eliminated viable yeast cells within 1 week of storage at 2.5–4°C. No spoilage was detected in inoculated or uninoculated natamycin-treated samples after 8 weeks storage, whereas after 1 week storage inoculated control samples were spoiled. The uninoculated control was judged to be palatable after 7 weeks. In a second study, natamycin at 5 µg/mL and sorbic acid at 1000 µg/mL eliminated viable yeast cells in orange juice inoculated with natural contaminants and stored for up to 12 weeks at 2.5–4°C. The uninoculated control sample spoiled in approximately 4 weeks and the inoculated control in 1 week. The authors concluded that natamycin was of potential use in orange juice because it was an effective antimycotic and did not cause off-flavors, while sorbic acid did.

Ayres et al. (1956) evaluated several antibiotics including chlortetracycline, oxytetracycline, tetracycline, streptomycin, neomycin, mycostatin, aerosporin, ascocin, rimocidin, and natamycin against spoilage microflora of raw cut-up chicken. They added the antibiotics at 10 ppm to chill water (1.7°C) and dipped the chicken for 2 h. Fungi were enumerated on acidified malt agar incubated at 30°C for 4 days. Rimocidin and natamycin, alone and in combination with chlortetracycline (all at 10 ppm), were the most effective antifungal agents and inhibited yeast growth on chicken stored 12–15 days at 4.4°C. There was a 2 log reduction in yeast counts at day 12 compared to the control (untreated). In contrast, there was little effect of the two antifungal compounds on total microflora (nutrient agar) when used in combination with chlortetracycline as a dip. Natamycin is an effective inhibitor of fungi on the surface of sausage (van Rijn et al., 1999).

Ticha (1975) investigated natamycin for use in the baking industry. Natamycin at 100 ppm was found to be effective against 5 molds, including *Aspergillus flavus* isolated from bakery products. The compound was effective in preventing growth of molds and yeasts in quarg fillings and icings at 0.05% and cream fillings at 0.01%. Natamycin at 50 ppm was effective in inhibiting *A. parasiticus* growth and toxin production on raw peanuts by 99% after 11 days (Gelda et al., 1974).

In the United States, natamycin is approved for use in cheese making as a mold spoilage inhibitor (21 CFR 172.155). Natamycin may be applied to the surface of cuts and slices of cheese by dipping or spraying an aqueous solution containing 200–300 ppm. The regulation concerning natamycin specifies that it may be applied to cuts and slices of cheese only if the cheese standard allows for use of “safe and suitable” antimycotics.

Regulations in the Netherlands limit natamycin concentrations on cheese surfaces to ≤ 2 mg/dm² (surface) and ≤ 1 mm in depth (Daamen and Van den Berg, 1985). The acceptable daily intake allowed for natamycin (FAO/WHO Expert Committee on Food Additives) is 0.3 mg/kg body weight/day (Smith and Moss, 1985).

The intravenous route is the path by which polyene macrolide antibiotics are most toxic and oral administration is least toxic. There is apparently no absorption of up to 500 mg/day natamycin from the human intestinal tract after 7 days administration (Brik, 1981). Levinskas et al. (1966) carried out a study to determine the acute and chronic toxicity of natamycin. The single oral dose LD₅₀ for natamycin in the male rat was found to be 2.73 g/kg (1.99–3.73 g/kg) and 4.67 g/kg (3.0–7.23 g/kg) for female rats. The oral LD₅₀ for fasted male albino rabbits was 1.42 g/kg (0.46–4.39 g/kg). The single dermal dose LD₅₀ was estimated at >1.25 g/kg. For rats, no signs of toxicity occurred after large single doses and no gross lesions could be detected related to natamycin ingestion. Rabbits which died had congested and hemorrhagic gastric mucosa. In a 3 month feeding study with

rats, Levinskas et al. (1966) found that animals fed 8000 ppm natamycin had body weights which averaged 54–67% of the control group (no natamycin). At 2000 ppm, animal weight averaged 85% of the control group. Natamycin had no apparent effect on body organs nor did it produce any lesions.

Oral administration of natamycin to rats for 2 years at up to 1000 ppm did not have an effect on survival of the animals (Levinskas et al., 1966). The diet containing natamycin was judged to have had no adverse effects on food utilization, reproductive performance, neoplasms, or other lesions above that encountered in controls. Dogs were fed diets containing 125, 250, or 500 ppm natamycin for 2 years (Levinskas et al., 1966). Oral administration of 500 ppm resulted in a slight decrease in body weight. No significant hematologic abnormalities nor significant lesions could be attributed to the consumption of natamycin (Levinskas et al., 1966). Nausea, vomiting, and diarrhea in humans given natamycin orally in doses exceeding 1000 mg/day (Brik, 1981), and vomiting and diarrhea in dogs fed 5000 ppm natamycin (Levinskas et al., 1966), have been reported.

V. NISIN

Nisin was first recognized in 1928 by Rogers and Whittier and was later isolated, characterized, and named by Mattick and Hirsh (1947). The compound is a peptide produced by a strain of the dairy starter culture *Lactococcus lactis* ssp. *lactis*. The structure and amino acid content of nisin was determined by Gross and Morell (1971). The molecular weight of nisin is 3500 (Gross and Morell, 1967); however, it usually occurs as a dimer with a molecular weight of 7000 (Jarvis et al., 1968). Nisin is a 34 amino acid peptide which contains the unusual amino acids dehydroalanine, dehydrobutyrine, lanthionine, and β -methyl-lanthionine (Delves-Broughton and Gasson, 1994). The latter amino acids are found in other inhibitory peptides produced by gram positive bacteria. The group of antimicrobial peptides containing these amino acids are called "lantibiotics" (Delves-Broughton and Gasson, 1994). A variant of nisin, called nisin Z, is produced by some strains of *L. lactis* ssp. *lactis* and contains an asparagine in place of a histidine at residue 27.

The solubility of the compound is dependent upon the pH of the solution. The solubility at pH 2.2 is 56 mg/mL, at pH 5.0 the solubility is 3 mg/mL, and the solubility at pH 11 is 1 mg/mL (Liu and Hansen, 1990). Nisin solution at pH 3 retains the greatest activity (97.5%) following heating at 115°C for 20 min (Davies et al., 1998). At pHs above and below that level, activity is reduced. At pH 7.0, inactivation occurs at room temperature. Nisin stability during storage is variable. Hirsch et al. (1951) noted rapid degradation of nisin in Swiss cheese after 8 days, and after 20 days very little was detected. Nisin degradation has also been reported in meat slurries and pasteurized process cheese spread at elevated temperature storage (Rayman et al., 1981; Delves-Broughton, 1990). Nisin is readily inactivated by proteolytic enzymes at pH 8. Nisinase from *Streptococcus thermophilus*, *Lactobacillus plantarum*, other lactic acid bacteria, and certain *Bacillus* species inactivate nisin (Koop, 1952; Alifax and Chevalier, 1962; Jarvis, 1967).

Nisin by itself has a narrow spectrum affecting only gram positive bacteria, including *Alicyclobacillus*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Staphylococcus*, and *Sporolactobacillus* (Table 1) (Hawley, 1957; Aplin & Barrett Ltd. no date). It does not generally inhibit gram negative bacteria, yeasts, or molds. *Clostridium botulinum* types A, B, and E were evaluated for their nisin susceptibility in brain heart infusion (BHI) broth and cooked meat medium (CMM) (Scott and Taylor, 1981a). Nisin concentrations necessary to inhibit the organism

Table 1 Susceptibility of Various Bacteria to Nisin

Microorganism	Susceptible strains/no. strains tested	MIC ($\mu\text{g/mL}$)	Reference	Method
Streptococci groups A-M exc. C,D,L	17/17	0.00625-0.1	Mattick and Hirsch, 1947	1
<i>Pneumococcus</i>	4/4	0.00625-2.5	Mattick and Hirsch, 1947	1
<i>Staphylococcus</i>	33/33	ND ^a	Laukova, 1995	2
<i>Staphylococcus pyogenes</i>	6/6	2.5	Mattick and Hirsch, 1947	1
<i>Enterococcus</i>	26/26	ND	Laukova, 1995	2
<i>Neisseria</i>	3/3	0.05-1.25	Mattick and Hirsch, 1947	1
<i>Bacillus</i>	6/6	0.05-0.1	Mattick and Hirsch, 1947	1
<i>Clostridium</i>	8/8	0.00625-2	Mattick and Hirsch, 1947	1
<i>Corynebacterium</i>	12/12	0.1-3	Mattick and Hirsch, 1947	1
<i>Actinomyces</i>	6/6	0.025-25	Mattick and Hirsch, 1947	1
<i>Mycobacterium tuberculosis</i>	6/6	2.5-12.5	Mattick and Hirsch, 1947	1
<i>Erysipelothrix monocytogenes</i>	3/3	0.05	Mattick and Hirsch, 1947	1
<i>Listeria monocytogenes</i>	9/9 (TSA)	18.5-2950	Benkerroum and Sandine, 1988	3
<i>Pedococcus</i>	30/30 (GMA and TJA)	ND	Radler, 1990a	4
<i>Leuconostoc</i>	18/18 (GMA and TJA)	ND	Radler, 1990a	4
<i>Lactobacillus</i>	31/35 (GMA)	ND	Radler, 1990a	4

^a ND, not determined. Author established susceptibility to nisin but did not specifically determine the MIC.

Methods:

1. Determined by broth dilution assay in 1% dextrose-0.3% Lemco-0.5% peptone broth. Units are estimated because experiments were done before units for nisin were standardized.
2. Determined by well diffusion assay on nutrient agar with wells containing 250 μg nisin. Susceptible strains showed a zone of inhibition around wells.
3. Determined by agar dilution assay on tryptic soy agar (TSA) or MRS agar.
4. Determined by well diffusion assay on Grape Must Agar (GMA), pH 4.5, and Tomato Juice Agar (TJA), pH 6.0. Wells contained 0.25, 2.5, and 10 $\mu\text{g/mL}$ nisin. Resistant strains had no zone of inhibition around wells with 10 $\mu\text{g/mL}$ nisin.

in BHI were 200, 80, and 20 µg/mL for types A, B, and E, respectively. The concentration required to inhibit *C. botulinum* in CMM was not determined, as it was beyond the highest concentrations tested for types A (>200 µg/mL) and B (>80 µg/mL). It was theorized that the higher levels required in CMM were due to binding of the nisin by meat particles. Rose et al. (1999) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry demonstrated that nisin is likely inactivated in raw meat by an enzymatic reaction with glutathione. In contrast, *Clostridium sporogenes* was inhibited in a meat system (pork) by 5–75 µg/mL nisin at pH 6.5–6.6 (Rayman et al., 1981). Strains of psychrotrophic *Bacillus cereus* were evaluated for their resistance to nisin (up to 50 µg/mL) in BHI broth or trypticase soy broth at 8 or 15°C (Jaquette and Beuchat, 1998). Tolerance of *Bacillus cereus* vegetative cells and spores was dependent upon temperature and pH. As the temperature was increased from 8 to 15°C and the pH from 5.53 to 6.57, more strains were tolerant to up to 50 µg/mL nisin. At pH 5.53 and 15°C, all vegetative cells of all 8 strains tested were inhibited by 50 µg/mL and only 1 grew at 10 µg/mL. At pH 6.07 and 15°C, spores of all 6 strains tested were inhibited by 10 µg/mL. Laukova (1995) found that several strains of *Staphylococcus* (including *S. aureus*) and *Enterococcus* were sensitive to nisin. In general, *Enterococcus* was more susceptible than *Staphylococcus*. Radler (1990a) reported that the majority of 83 strains of lactic acid bacteria, including *Pediococcus*, *Leuconostoc*, and *Lactobacillus*, were sensitive to 0.25–10 µg/mL nisin in grape must agar and tomato juice agar. Nisin has been shown by a number of researchers to be inhibitory to *Listeria monocytogenes* (Benkerroum and Sandine, 1988; Harris et al., 1991; Ukuku and Shelef, 1997).

The spectrum of activity of nisin can be expanded to include gram negative bacteria when it is used in combination with chelating agents, such as ethylenediamine tetraacetic acid (EDTA) or trisodium phosphate, heat, and freezing (Delves-Broughton and Gasson, 1994; Carneiro de Melo et al., 1998). Stevens et al. (1992) reported that 20 mM of chelators such as ethylenebis tetraacetic acid (EGTA), citrate, and phosphate enhanced activity of nisin against gram negative bacteria, but none were as effective as EDTA. Schved et al. (1996) found that 20 mM maltol and ethyl maltol, which are flavor enhancers and have chelating properties, enhanced the activity of nisin (0.2–0.8 µg/mL) against two *E. coli* strains in cell buffer. Carneiro de Melo et al. (1998) demonstrated that *Campylobacter jejuni*, *Escherichia coli*, and *Salmonella* Enteritidis exposed to sublethal concentrations of trisodium phosphate (0.5–5 mM) had increased susceptibility to 1 µM nisin in deionized water and dried on the surface of chicken skin.

Nisin activity is affected by a number of environmental factors, including pH, inoculum size, and interaction with food components (Somers and Taylor, 1972; Scott and Taylor, 1981a,b; Benkerroum and Sandine, 1988; Rayman et al., 1984; Harris et al., 1991; Ukuku and Shelef, 1997). Activity generally increases with decreasing pH and decreased initial numbers of microorganisms. The presence of food components such as lipids and protein influence nisin activity (Scott and Taylor, 1981a). Nisin was less active against *L. monocytogenes* in milk (Jung et al., 1992) and ice cream (Dean and Zottola, 1996) with increasing fat concentrations. This was probably due to binding of nisin to fat globules (Jung et al., 1992); this binding was overcome by adding emulsifiers (e.g., Tween 80). Thomas et al. (1998) demonstrated that sucrose fatty acid esters (sucrose palmitate and sucrose stearate) enhanced the activity of nisin against *Bacillus cereus*, *Listeria monocytogenes*, *Lactobacillus plantarum*, and *Staphylococcus aureus*, but had no effect on the activity of nisin against any gram negative bacteria.

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The cytoplasmic membrane of vegetative cells is the primary site of action of nisin. The primary mechanism of nisin is believed to be the formation of pores in the cytoplasmic membrane which result in depletion of proton motive force and loss of cellular ions, amino acids, and ATP (Crandall and Montville, 1998). Ruhr and Sahl (1985) reported that the addition of nisin to susceptible cells (*Staphylococcus cohnii*, *Bacillus subtilis*, *Micrococcus luteus*, *Streptococcus zymogenes*) resulted in a rapid loss of intracellular amino acids and Rb^+ , a K^+ analog. In addition, nisin inhibited proline and glutamine uptake by membrane vesicles of susceptible cells. The loss of ions resulted in a rapid decrease in the membrane potential, $\Delta\Psi$. The effects of nisin in depleting proton motive force components, $\Delta\Psi$ and ΔpH , were confirmed in *L. monocytogenes* (Bruno et al., 1992) and *C. sporogenes* (Okereke and Montville, 1992). Okereke and Montville (1992) reported that nisin treatment also results in a decrease in intracellular reserves of ATP. Winkowski et al. (1994) also reported efflux of ATP from nisin-treated *L. monocytogenes* cells but it only accounted for 20% of the loss of intracellular ATP. Therefore these researchers suggest that ATP hydrolysis also occurs within cells, perhaps as an attempt to maintain the proton motive force.

The effects of nisin on energized cells is consistent with a pore-forming mechanism (Winkowski et al., 1994). Sahl et al. (1987) detected nisin-induced pores of 0.2–1 nm diameter in black lipid membranes (artificial membranes with induced $\Delta\Psi$), which would allow the passage of solutes as big as 500 Da but would exclude larger compounds. Garcera et al. (1993) found that nisin caused leakage of fluorescein from liposomes indicating that pores allowing passage of low molecular weight compounds were formed. Garcera et al. (1993) suggested that nisin may induce pores in membranes by a “barrel-stave” mechanism consisting of three steps: (1) nisin monomers bind to a target membrane, (2) nisin inserts into the membrane, (3) nisin molecules aggregate to form a barrel-like hole around a water-filled pore. The nisin molecules may also aggregate before inserting into the membrane.

Several studies have shown that nisin-induced dissipation of the proton motive force is more effective against energized cells rather than starved or low energy cells (Ruhr and Sahl, 1985; Gao et al., 1991), indicating that nisin activity may be dependent upon a membrane potential. Kordel et al. (1989) also reported the need for a membrane potential in order for nisin to span liposome membranes. In further studies of nisin mechanism, Gao et al. (1991) reported that either a membrane potential and/or a pH gradient (with a more alkaline interior pH) is necessary in order for nisin to increase permeability of model liposomes made from various phospholipids. The $\Delta\Psi$ needed for dissipation of proton motive force was lower at lower external pH. Therefore it is possible that as the pH gradient increases, the dependence of nisin activity on $\Delta\Psi$ may be lower.

In most cases nisin is sporostatic rather than sporocidal (Delves-Broughton et al., 1996). Hitchens et al. (1963) determined that nisin inhibits postgermination swelling to prevent spore outgrowth. Morris et al. (1984) reported the existence of sulfhydryl groups in the membrane of germinated *B. cereus* spores. Sulfhydryl agents such as S-nitrosothiols inhibit outgrowth of germinated spores by interacting with the sulfhydryl groups in the membrane. At low concentrations, nisin inhibited spore outgrowth and competed with sulfhydryl agents for membrane sulfhydryl groups, indicating that the membrane sulfhydryl groups may be the target for nisin. Morris et al. (1984) hypothesized that the dehydroalanine (DHA) groups on nisin may react with the membrane sulfhydryl groups. In light of the results of Hansen (1994), where alterations of the 5-dehydroalanine group of subtilin

eliminated activity against spores, it is becoming clearer that the DHA groups may in fact play a very important role in the sporostatic activity of nisin.

The application of nisin as a food preservative has been studied extensively (Marth, 1966; Lipinska, 1977; Hurst, 1981; Hurst and Hoover, 1993). Nisin was first used as a food preservative by Hirsch et al. (1951). Nisin-producing starter cultures were used to prevent "blowing" of Swiss-type cheese caused by *Clostridium tyrobutyricum* and *Clostridium butyricum*. This was followed by a similar application for the preservation of processed Swiss-type cheese (McClintock et al., 1952). Later, Hawley (1957) recommended the addition of a nisin-containing skim milk powder. Somers and Taylor (1987) studied the use of nisin to prevent *C. botulinum* outgrowth in process cheese spread formulated to have higher than normal moisture content and/or lower salt content. Nisin was an effective antibotulinal agent at 12.5–250 µg/g. The higher nisin levels allowed for the safe formulation of cheese spreads with higher moisture content and lower salt concentration. Delves-Broughton (1990) reported that nisin levels of 6 to 12.5 µg/g controlled non-*Clostridium botulinum* spoilage in process cheese.

The use of nisin to prevent growth of spoilage and pathogenic bacteria in fluid milk has also been investigated. Heinemann et al. (1965) reported that the time required for heating canned chocolate milk at 250°F could be reduced from 12.0 to 3.3 min and still prevent growth of added *Bacillus stearothermophilus* or *Clostridium sporogenes* PA 3679 spores with 2.5 µg/mL nisin. Jung et al. (1992) studied the efficacy of nisin against *L. monocytogenes* growth and the effect of fat and emulsifiers. They reported that nisin (0.25 and 1.25 µg/mL) reduced *L. monocytogenes* counts by 4.0 log CFU/mL after 24 h in skim milk, but, as fat content was increased, activity of nisin decreased. A similar study by Dean and Zottola (1996) found that nisin decreased *L. monocytogenes* cells to undetectable levels in 3% and 10% fat ice cream stored at -18°C. Nisin did not kill cells as quickly in the 10% fat ice cream, but after 3 months the effect of nisin was the same in both the 3% and 10% fat samples.

Nisin has been recommended for use in canned vegetable products to prevent the outgrowth of *Clostridium botulinum* when less severe sterilization conditions are desired or required (Campbell et al., 1959; Denny et al., 1961). In addition, Delves-Broughton et al. (1992) demonstrated that nisin may be used to increase the shelf-life of pasteurized liquid whole eggs. They found that 5 µg/mL nisin added to eggs prior to pasteurization increased the refrigerated shelf-life by 11–14 days. In addition, nisin prevented growth of the pathogen *B. cereus*, which grew well in pasteurized eggs with no added nisin.

Nisin has been shown to have potential benefit in some meat products. Jarvis and Burke (1976) demonstrated that 400 mg/kg nisin, in conjunction with 0.1% sorbic acid and 2.5% (w/w) polyphosphate retarded spoilage of fresh sausage at 5°C. Chung et al. (1989) dipped meat in a nisin solution (250 µg/mL) for 10 min at room temperature, then inoculated the surface of the meat and allowed 10 min for attachment of bacteria. Nisin significantly ($p < 0.05$) lowered the number of *Lactococcus lactis*, *S. aureus* and *L. monocytogenes* attached to the meat. However nisin had no effect on attachment by gram negative bacteria including *S. Typhimurium*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. Scannel et al. (1997) investigated the use of nisin combined with sodium lactate on *S. aureus* and *Salmonella* Kentucky in fresh pork sausage. They found that 2% lactate combined with 12.5 µg/g nisin was superior to nisin alone at controlling growth of total aerobes, *S. aureus*, and *S. Kentucky* in sausage stored at 4°C for 10 days. Budu-Amoaka et al. (1999) found that heating canned lobster in brine at 60 or 65°C for 5 or 2 min, respectively, in combination with 25 µg/g nisin reduced *Listeria monocytogenes* by 3–5

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logs. They proposed that using nisin could reduce the commercial thermal process for this product (13–18 min at 65.5°C) with equivalent lethality and reduced drained weight loss.

Nisin has been suggested as an adjunct to nitrite in cured meats for the purpose of preventing the growth of clostridia (Caserio et al., 1979; Holley, 1981). Calderon et al. (1985) found that 12–24 µg/g nisin added to bacon containing 50 µg/g nitrite increased the shelf-life by one day compared to bacon containing 50 and 150 µg/g nitrite only. They predicted that 18 µg/g nisin would increase the shelf-life of bacon at 5°C by one week. Rayman et al. (1981) showed that 75 µg/g nisin was more effective than 150 µg/g nitrite at inhibiting outgrowth of *C. sporogenes* spores (3.0 log CFU/g) in pork, beef, and turkey meat slurries. Although nisin appears to be more effective than nitrites at preventing the growth of some pathogenic and spoilage microorganisms in cured meats, it is yet to be shown to prevent *C. botulinum* growth in cured meats. Nisin is effective against *C. botulinum* in TPYG broth, but it is ineffective in cooked meat medium (Scott and Taylor, 1981a; Somers and Taylor, 1972). Therefore it would not be wise to replace some or all of the nitrites in cured meat with nisin until studies confirm that nisin will prevent *C. botulinum* growth in meat systems.

Lactic acid bacteria are common spoilage microorganisms in beer and wine. Since nisin is effective against most lactic acid bacteria but is inactive against yeasts, there is potential use for nisin in alcoholic beverages to prevent growth of spoilage lactic acid bacteria. Ogden et al. (1988) recommended nisin for preservation of beer and ale against spoilage by lactic acid bacteria. Radler (1990b) added nisin (2.5 and 25 µg/mL) to grape musts artificially contaminated with lactic acid bacteria. He reported that 25 µg/mL nisin inhibited growth of the lactic acid bacteria and had no effect on the fermentation of the musts, or on the composition and tastes of the wines. Choi and Park (2000) used nisin at 100 IU/mL to inhibit lactobacilli responsible for spoilage of kimchi, traditional Korean fermented vegetables. Nisin has been evaluated for use as a component of antimicrobial packaging (Ming, et al., 1997; Padgett et al., 1998).

Nisin is approved in many countries. In the United States, nisin is approved as a “nisin preparation” (21 CFR 184.1538) with a content of not less than 900 IU/mg. It is approved to inhibit outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads and pasteurized process cheese spreads (21 CFR 133.175); pasteurized cheese spread with fruits, vegetables, or meats (21 CFR 133.176); pasteurized process cheese spread (21 CFR 133.179); and pasteurized process cheese spread with fruits, vegetables, or meats (21 CFR 133.180). It has a maximum use level of 250 ppm of nisin in the finished product. In addition, nisin is approved for use in liquid eggs, salad dressings, and sauces.

Nisin has generally been considered nontoxic. The oral LD₅₀ in mice is 6950 mg/kg body weight, which is similar to that of common salt (Hara et al., 1962). Nisin was found to have no effect on animals in studies of subchronic or chronic toxicity, reproduction, or sensitization (FR 1988, 53:11247). It has also been shown that nisin does not produce cross-resistance in microorganisms to therapeutic antibiotics (FR 1988, 53:11247).

Bacteriocins with potential for use in foods have been demonstrated to be produced by strains of *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Propionibacterium*. Many of these compounds could potentially be used as food antimicrobials, but at the present time none are approved in the United States to be added to foods in their purified form. One approach to using these compounds has been to grow bacteriocin-producing starter cultures in a medium such as whey, nonfat dry milk, or dextrose. The

fermentation medium is then pasteurized and spray-dried, which kills the starter culture but retains the active antimicrobial. These products act as antimicrobial additives but are generally considered GRAS and may be listed as ‘‘cultured whey’’ or ‘‘cultured nonfat dry milk’’ on the food label. Examples of such products are Microgard®, Alta™, and Perlac™. Alta at 0.1–1.0% was shown to decrease the growth rate of *Listeria monocytogenes* on vacuum-packaged smoked salmon stored at 4 or 10°C (Szabo and Cahill, 1999). Degnan et al. (1994) inoculated fresh blue crab (*Callinectes sapidus*) with a three strain mixture of *L. monocytogenes* (ca. 5.5 log CFU/g) and washed with various fermentation products (2000–20,000 arbitrary units [AU]/mL of wash) and stored at 4°C. Counts of *Listeria monocytogenes* decreased 0.5–1.0 log with Perlac or MicroGard and 1.5–2.7 logs with Alta.

VI. NITRITES

Nitrite salts (KNO_2 and NaNO_2) have been used in meat curing for many centuries. Meat curing utilizes salt, sugar, spices, and ascorbate or erythorbate in addition to nitrite. The reported contributions of nitrite to meat curing include characteristic color development, flavor production, texture improvement, and antimicrobial effects (IFT, 1987). The specific contribution of nitrite to the antimicrobial effects of curing salt was not recognized until the late 1920s (NAS/NRC, 1981), and evidence that nitrite was an effective antimicrobial agent came even later (Steinke and Foster, 1951). Nitrites are white to pale yellow hygroscopic crystals that are quite soluble in water and liquid ammonia but much less so in alcohol and other solvents.

The primary use for sodium nitrite as an antimicrobial is to inhibit the growth and toxin production of *Clostridium botulinum* in cured meats. In association with other components in the curing mix—such as salt, ascorbate, and erythorbate—and pH, nitrite exerts a concentration-dependent antimicrobial effect on the outgrowth of spores from *C. botulinum* and other clostridia. The use of nitrites in cured meat products to control *C. botulinum* has been studied extensively. An in-depth review of these studies is beyond the scope of this discussion, and the reader is referred to Holley (1981), Roberts and Gibson (1986), and Tompkin (1993).

The effectiveness of nitrite is dependent upon several environmental factors. Nitrite is most inhibitory to bacteria at an acidic pH. A tenfold increase in the inhibitory effect of nitrite against *C. botulinum* was found when the pH was reduced from 7.0 to 6.0 (Roberts and Ingram, 1966). Nitrite is more inhibitory under anaerobic conditions (Castellani and Niven, 1955; Lechowich et al., 1956; Buchanan and Solberg, 1972). Temperature, salt concentration, and initial inoculum size also significantly influence the antimicrobial role of nitrite (Riemann et al., 1972; Roberts, 1975; Roberts et al., 1966, 1976; Genigeorgis and Riemann, 1979). Ascorbate and isoascorbate enhance the antibotulinal action of nitrite most likely by acting as reducing agents (Roberts et al., 1991).

Nitrite has been shown to have variable effects on microorganisms other than *C. botulinum*. *Clostridium perfringens* growth in laboratory medium at 20°C was inhibited by 200 µg/mL nitrite and 3% salt or 50 µg/mL nitrite and 4% salt at pH 6.2 (Gibson and Roberts, 1986b). *Listeria monocytogenes* growth was inhibited for 40 days at 5°C by 200 ppm sodium nitrite with 5% NaCl in vacuum packaged and film wrapped smoked salmon (Pelroy et al., 1994b). Fecal streptococci showed growth in the same medium at 20°C with 400 µg/mL and 6% salt present (Gibson and Roberts, 1986b). In a similar study,

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Gibson and Roberts (1986a) tested nitrite and salt against *Salmonella* sp. and Enteropathogenic *E. coli*. *Salmonella* showed visible growth within a week at 20°C in the presence of 400 µg/mL nitrite and 4% salt. Significant inhibition by salt and nitrite was achieved only at lower temperatures (10 or 15°C) and at pH 5.6 or 6.2. *Escherichia coli* was more resistant than *Salmonella*. Inhibition was demonstrated only at the extremes of pH (5.6), salt (6%), nitrite (400 µg/mL), and temperature (10°C). Other researchers have also demonstrated microbial resistance to nitrite among species of *Salmonella* (Castellani and Niven, 1955; Rice and Pierson, 1982), *Lactobacillus* (Castellini and Niven, 1955; Spencer, 1971), *Clostridium (perfringens)* and *Bacillus* (Grever, 1974). In TSB at pH 5.0, 4 strains of *Escherichia coli* O157:H7 were completely inhibited in the presence of 200 µg/mL nitrite at 37°C (Tsai and Chou, 1996). At higher pH values or lower temperatures, inhibition and/or inactivation of the microorganism was diminished. Korenekova et al. (1997) found that 100 mg/kg of nitrite added to milk inhibited the growth of yogurt culture.

The mechanism of nitrite inhibition of microorganisms has been studied for 50 years (Roberts and Gibson, 1986; Woods et al., 1989; Tompkin, 1993). The inhibitory effect of nitrite on bacterial sporeformers is apparently due to inhibition of outgrowth and during cell division (Cook and Pierson, 1983; Tompkin, 1978; Genigeorgis and Riemann, 1979). Nitrite does not inhibit spore germination to a significant extent. Ingram (1939) first postulated that nitrite inactivated enzymes associated with respiration. Since that time, nitrate has been found to affect a variety of enzymes and enzyme systems. Nitrite was shown to inhibit active transport, oxygen uptake, and oxidative phosphorylation of *Pseudomonas aeruginosa* by oxidizing ferrous iron of an electron carrier, such as cytochrome oxidase, to the ferric form (Rowe et al., 1979). Nitrite inhibited the active transport of proline in *Escherichia coli* but not group translocation by the phosphoenol-pyruvate:phosphotransferase system (Yarbrough et al., 1980). The growth of *Clostridium sporogenes* and *C. botulinum* was inhibited by nitrite through interference with the phosphoroclastic system (Woods et al., 1981; Woods and Wood, 1982). Inhibition is due to the reaction of nitric oxide with the nonheme iron of pyruvate-ferredoxin oxidoreductase (Woods et al., 1981). Nitrite also inhibits the iron-sulfur enzyme, ferredoxin, of *C. botulinum* and *C. pasteurianum* (Carpenter et al., 1987). McMindes and Siedler (1988) reported that nitric oxide was the active antimicrobial principle of nitrite and that pyruvate decarboxylase may be an additional target for growth inhibition by nitrite. Roberts et al. (1991) also confirmed inhibition of the phosphoroclastic system and found that ascorbate enhanced inhibition. In addition, they showed that other iron-containing enzymes of *Clostridium botulinum* were inhibited including other oxidoreductases and the iron-sulfur protein, hydrogenase. It has been suggested that inhibition of clostridial ferredoxin and/or pyruvate-ferredoxin oxidoreductase is the ultimate mechanism of growth inhibition for clostridia (Carpenter et al., 1987; Tompkin, 1993). These observations are substantiated by the fact that the addition of iron to meats containing nitrite reduces the inhibitory effect of the compound (Tompkin et al., 1978). Chelating agents like sodium ascorbate, EDTA, and polyphosphate enhance the antibotulinal action of nitrite.

The mechanism of inhibition against nonsporeforming microorganisms may be different than for sporeformers. Nitrite was shown to inhibit active transport, oxygen uptake, and oxidative phosphorylation of *Pseudomonas aeruginosa* by oxidizing ferrous iron of an electron carrier, such as cytochrome oxidase, to the ferric form (Rowe et al., 1979). Muhoberac and Wharton (1980) and Yang (1985) also found inhibition of cytochrome oxidase of *Pseudomonas aeruginosa*. *Streptococcus faecalis* and *S. lactis*, bacteria that

do not depend on active transport or cytochromes, were not inhibited by nitrite (Rowe et al., 1979). Woods et al. (1989) theorized that nitrites inhibited aerobic bacteria by binding the heme iron of cytochrome oxidase.

In a bacteriological medium, the inhibitory effect of nitrite is enhanced tenfold after heating due to a substance known as "Perigo inhibitor" (Perigo et al., 1967). The Perigo inhibitor is formed at 105°C or higher, which exceeds the temperatures normally used in the processing of cured meats. Its antibacterial activity is also neutralized by meat particles. Perigo inhibitor is formed in culture medium only when sulfhydryl groups and iron are present (Holley, 1981).

As stated, the use of nitrites in meats produces characteristic cured meat color, contributes to flavor development, inhibits *C. botulinum* growth and neurotoxin production, and retards rancidity. Meat products that may contain nitrites include bacon, bologna, corned beef, frankfurters, luncheon meats, ham, fermented sausages, shelf-stable canned cured meats, and perishable canned cured meat (e.g., ham). Nitrite is also used in a variety of fish and poultry products. The concentration of nitrite used in these products is specified by the U.S. FDA and USDA regulations. Sodium nitrate is used in certain European cheeses to prevent spoilage by *Clostridium tyrobutyricum* or *C. butyricum* (Tompkin, 1993).

Regulations for nitrite in (9 CFR 318.7) in bacon allow one of the following: (1) 120 ppm sodium nitrite or 148 ppm potassium nitrite plus 550 ppm sodium erythorbate or isoascorbate, (2) 100 ppm sodium nitrite or 123 ppm potassium nitrite plus 550 ppm sodium erythorbate or isoascorbate if a demonstration of adequate process control is met, or (3) 40–80 ppm sodium nitrite or 49–99 ppm potassium nitrite plus 550 ppm sodium erythorbate or isoascorbate plus 0.7% sucrose and a lactic acid bacterial culture (*Pediococcus*). The use of nitrite in other products is limited to a maximum residual level of 200 ppm. The input levels for nitrite are as follows: 2 lb/100 gal of pickle, 1 oz/100 lb (625 ppm) of meat for dry cured products, and 0.25 oz/100 lb (156 ppm) of chopped meat.

Use of nitrites in certain types of smoked and cured fish is specified in 21 CFR 172.160; 21 CFR 172.1170; 21 CFR 172.175; and 21 CFR 172.177. Presently, the level of nitrites allowed is a maximum of 10 ppm in smoked cured tuna fish and 200 ppm (input not to exceed 500 ppm) in smoked cured sablefish, salmon, shad, cod roe, and in home-curing mixtures. The level in smoked chub has been fixed at 100–200 ppm.

The lethal dose of nitrites in humans is 32 mg/kg body weight or 2 g (Burden, 1961) and 4–6 g (Wagner, 1956). Prolonged ingestion of sodium nitrite or sodium nitrate has been shown to cause methemoglobinemia, that is, excessive production of abnormal hemoglobin, especially in infants (NAS/NRC, 1981). Exposure to nitrites has been implicated as a causative agent of a variety of diseases in human beings and in animals. The major adverse effect is the possible induction of cancer. Studies conducted at the Massachusetts Institute of Technology have indicated that nitrites increase the incidence of lymphoma in rats fed 250–2000 ppm nitrite in their food or water (Newberne, 1979). The induction of cancer was considered to be a direct effect of nitrite on the lymphocytes and was independent of nitrosamine formation. A study of Newberne's data by the FDA (1980), however, disputed his histopathological findings and concluded that no demonstration of nitrite-induced tumors could be found. At one time there was concern over the reaction of nitrites with secondary amines to form nitrosamines or with substituted amides to form nitrosamides, both of which are known to cause cancer in many animal species (NAS/NRC, 1981; IFT, 1987). Epidemiological studies indicated a possible link

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between exposure to high levels of nitrites and a high incidence of stomach and esophageal cancer. However, in none of these studies was the exposure of nitrites or *N*-nitroso compounds actually measured in the individuals who developed cancer (NAS/NRC, 1981).

VII. ORGANIC ACIDS

Organic acids are commonly used by food manufacturers as antimicrobial preservatives or acidulants in a variety of food products. This is due to their solubility, flavor, and low toxicity.

Many factors influence the effectiveness of organic acids as antimicrobials, including hydrophobicity. However, the most important factor in the use of these compounds is undoubtedly the pH of the food. Early research demonstrated that the activity of organic acids was related to pH and that the undissociated form of the acid was primarily responsible for its antimicrobial activity (Cruess and Irish, 1932; Fabian and Wadsworth, 1939; Levine and Fellers, 1940). Many subsequent studies on organic acids have yielded similar findings concerning the effects of pH on activity (Ingram et al., 1956; Doores, 1993). In selecting an organic acid for use as an antimicrobial food additive, both the use pH and the pK_a of the acid must be taken into account. The use of organic acids is generally limited to foods with $pH < 5.5$, since most have a pK_a in the range of 3–5 (Doores, 1993).

The mechanism(s) by which organic acids inhibit microorganisms has been studied extensively. There is little evidence that the organic acids influence cell wall synthesis in prokaryotes or that they significantly interfere with protein synthesis or genetic mechanisms. Instead, organic acids more likely act at the cytoplasmic membrane level. The undissociated form of the organic acid penetrates the cell membrane lipid bilayer, and once inside the cell it dissociates because the cell interior has a higher pH than the exterior. Since bacteria must be able to maintain an internal pH near neutrality, protons generated from dissociation of the organic acid must be extruded to the exterior. Therefore, since protons generated by the organic acid inside the cell must be extruded using energy in the form of ATP, a constant influx of these protons will eventually deplete cellular energy. It must be noted that this same phenomenon could take place due to interference with membrane permeability as well. Sheu and Freese (1972) observed that short-chain organic acids altered the structure of the cell membrane. They hypothesized that this interfered with the regeneration of ATP by uncoupling the electron transport system or inhibiting the transport of metabolites into the cell. Sheu et al. (1972) determined that short-chain organic acids acted as uncouplers of amino acid carrier proteins from the electron transport system. As proof, they showed that transport of L-serine and other L-amino acids was inhibited in membrane vesicles of *Bacillus subtilis* when exposed to acetate and other fatty acids. Sheu et al. (1975) determined that organic acids inhibited active transport by interfering with the proton motive force (PMF) of the cell membrane. Eklund (1985) evaluated the effect of sorbic acid and parabens on the ΔpH and $\Delta \Psi$ components of the PMF in *E. coli* membrane vesicles. Both compounds eliminated the ΔpH but did not significantly affect the $\Delta \Psi$ component of the PMF so that active transport of amino acids continued. Eklund (1989) concluded that neutralization of the PMF and subsequent transport inhibition was not the sole mechanism of action of the parabens. To summarize, the organic acids and their esters have a significant effect on bacterial cyto-

plasmic membrane, interfering with the transport of metabolites and maintenance of membrane potential.

A. Acetic Acid and Acetate Salts

Acetic acid (CH_3COOH ; $\text{p}K_a = 4.75$; MW, 60.05 Da), the major component of vinegar, and its salts are widely used in foods as acidulants and antimicrobials. Acetic acid is more effective against yeasts and bacteria than against molds (Ingram et al., 1956). Only acetic, lactic, and butyric acid-producing bacteria are markedly tolerant to the acid (Baird-Parker, 1980; Doores, 1993).

The activity of acetic acid varies with food product, environment, and microorganism. Woolford (1975a) studied the antimicrobial role of acetic acid at pH 4, 5, and 6. At pH 6, it was observed that *Bacillus*, *Clostridium*, and gram negative bacteria were inhibited more than lactic acid bacteria, yeast, molds, and other gram positive bacteria. When the pH was reduced to 5, gram positive bacteria were more inhibited than lactic acid bacteria, yeast, and molds. At pH 4, the concentration of acetic acid required for inhibition was considerably reduced. The foodborne pathogen, *Staphylococcus aureus* was inactivated 90 and 99% in 12 h at pH 5.2 and 5.0, respectively, by acetic acid (Minor and Marth, 1970). Against *Listeria monocytogenes*, Sorrells et al. (1989) demonstrated that, at an equivalent pH and temperature, acetic acid was a more effective antimicrobial than lactic acid, malic acid, or citric acid. However, on an equimolar basis, malic and citric acids were more effective. One percent acetic acid inactivated *Pseudomonas aeruginosa* by > 99.9% in 1 h (Hedberg and Miller, 1969). Brackett (1987) found that acetic acid was one of the least effective organic acids against the growth of *Yersinia enterocolitica*. In contrast, Karapinar and Gonul (1992a) showed that acetic acid completely inhibited the growth of *Yersinia enterocolitica* at 0.156% (v/v) in microbiological media at 22°C for 48 h. Entani et al. (1998) found that 0.1% (w/v) acetic acid from vinegar was bacteriostatic to multiple strains of *E. coli* O157:H7, *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Aeromonas hydrophila* after 4 days at 30°C on the surface of nutrient agar. The same concentration of acetic acid did not inhibit growth of *Bacillus cereus* or *S. aureus*. Enterohemorrhagic *E. coli* strains were less susceptible to bactericidal concentrations (2.5%) of acetic acid than an enteropathogenic strain. Finally, Entani et al. (1998) showed that stationary phase cells of *E. coli* O157:H7 were more resistant to acetic acid than log phase cells.

Acetic acid was inhibitory at pH 3.5 to *A. niger* and *Rhizopus nigricans* (Kirby et al., 1937). At 0.8–1% and pH 3.5, acetic acid inhibited growth of *S. cerevisiae* var. *ellipsoideus* and *Penicillium glaucum* (Crues and Irish, 1932). A concentration greater than 4% was required to inhibit their growth at pH 7.0.

Acetic acid and its salts have shown variable success as antimicrobials in food applications. Acetic acid was used to increase the shelf-life of poultry when added to cut-up chicken parts in cold water at pH 2.5 (Mountney and O'Malley, 1965). Addition of acetic acid at 0.1% to scald tank water used in poultry processing decreased the D_{52} of *Salmonella* Newport, *Salmonella* Typhimurium, and *Campylobacter jejuni* five- to tenfold (Okrend et al., 1986). Increasing the acetic acid to 1.0% caused inactivation of all three microorganisms. In contrast, Lillard et al. (1987) found that 0.5% acetic acid in the scald water had no significant effect on *Salmonella*, total aerobic bacteria, or *Enterobacteriaceae* on unpicked poultry carcasses. At 1.2% as a 10-s dip for beef, acetic acid reduced microflora such as *S. Typhimurium*, *Shigella sonnei*, *Y. enterocolitica*, *E. coli*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis* by an average of 65% (Bell et al., 1986). The compound

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has been used as a spray sanitizer at 1.5–2.5% on meat carcasses and as an effective antimicrobial dip for beef, lamb, and catfish fillets (Anderson et al., 1988; Bala and Marshall, 1998). Use of 2% acetic acid resulted in reductions in viable *E. coli* O157:H7 on beef after 7 days at 5°C (Siragusa and Dickson, 1993). Acetic acid was the most effective antimicrobial in ground roasted beef slurries against *E. coli* O157:H7 growth in comparison with citric or lactic acid (Abdul-Raouf et al., 1993). Acetic acid added at 0.1% to bread dough inhibited growth of 6 log CFU/g rope-forming *Bacillus subtilis* in wheat bread (pH 5.14) stored at 30°C for >6 days (Rosenquist and Hansen, 1998). In brain heart infusion broth (BHI), 0.2% acetic acid at pH 5.1 or 0.1% at pH 4.8 inhibited rope-forming strains of *B. subtilis* and *Bacillus licheniformis* for >6 days at 30°C (Rosenquist and Hansen, 1998). In a study by Post et al. (1985), shrimp, shrimp puree, tomato puree, and shrimp and tomato puree were acidified to 4.2 and 4.6 with acetic acid. No significant *Clostridium botulinum* growth or toxin production occurred in the products after 8 weeks at 26°C. In a frankfurter emulsion slurry, acetic acid (0.3–1.16%) caused increased inactivation rates of *Bacillus stearothermophilus* and *B. coagulans* at pH 4.6, 121°C and pH 4.2, 105–110°C, respectively (Lynch and Potter, 1988). Karapinar and Gonul (1992b) demonstrated that dipping parsley contaminated with 7.0 log CFU/g of *Y. enterocolitica* into the 2% (v/v) acetic acid or 40% (v/v) vinegar solution for 15 min was bactericidal against the microorganism. Delaquis et al. (1999) utilized gaseous acetic acid at 242 µL/L of air to inactivate up to 3–5 log CFU/g *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium inoculated on mung bean seeds for sprouting. The procedure did not effect germination of the seeds.

Sodium acetate is an inhibitor of rope-forming *Bacillus* in baked goods and of the molds, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. glaucus*, *Penicillium expansum*, and *Mucor pusillus* at pH 3.5–4.5 (Glabe and Maryanski, 1981). It is useful in the baking industry because it has little effect on the yeast used in baking. Al-Dagal and Bazaraa (1999) found that whole or peeled shrimp dipped in a 10% sodium acetate (w/w) solution for 2 min had extended microbiological and sensory shelflife compared to controls.

Sodium diacetate ($pK_a = 4.75$) is effective at 0.1–2.0% in inhibiting mold growth in cheese spread (Doores, 1993). In BHI broth adjusted to pH 5.4, 0.45% (32 mM) sodium diacetate was shown to be inhibitory to *L. monocytogenes*, *E. coli*, *Pseudomonas fluorescens*, *Salmonella* Enteritidis, and *Shewanella putrefaciens*, but not *S. aureus*, *Yersinia enterocolitica*, *Pseudomonas fragi*, *Enterococcus faecalis*, or *Lactobacillus fermentis* after 48 h at 35°C (Shelef and Addala, 1994). In addition, 0.2% or 0.3–0.4% (21–28 mM) sodium diacetate suppressed growth by the natural microflora of ground beef after storage at 2°C for 14 days or 5°C for up to 8 days, respectively (Ajjarapu and Shelef, 1999; Shelef and Addala, 1994). Degnan et al. (1994) showed a 2.6 log decrease in viable *L. monocytogenes* in blue crab meat washed with 2M sodium diacetate after 6 days at 4°C. In contrast, Ajjarapu and Shelef (1999) found no effect of sodium diacetate at 0.2% on survival of *Escherichia coli* O157:H7 in ground beef stored at 2 or 10°C.

Dehydroacetic acid has a high pK_a of 5.27 and is therefore active at higher pH values. It is inhibitory to bacteria at 0.1–0.4% and fungi at 0.005–0.1% (Doores, 1993).

In the United States, acetic acid is GRAS (21 CFR 184.1005) for use as a pickling agent in (maximum amounts shown in parentheses) baked goods (0.25%), cheeses (0.8%), condiments and relishes (9.0%), dairy product analogs (0.8%), fats and oils (0.5%), gravies and sauces (3.0%), and meats (0.6%). Other foods may contain up to 0.15%. Sodium acetate (21 CFR 184.1721) is available for use in breakfast cereals (0.007%), fats and oils (0.1%), hard candy (0.15%), jams and jellies (0.12%), meats (0.12%), soft candy (0.2%),

and snack foods, soup mixes, and sweet sauces (all 0.05%). Calcium acetate (21 CFR 184.1185) is approved for cheese (0.02%), gelatin (0.02%), snack foods (0.06%), and sweet sauces (0.15%). Sodium diacetate is also GRAS (21 CFR 184.1754) for use in baked goods (0.4%); cheese spreads, gravies, and sauces (0.25%); meats (0.1%); candy (0.1%); and soup mixes (0.05%). Acetic acid may also be added to meat products (9 CFR 318.7).

B. Benzoic Acid and Benzoates

Benzoic acid is found naturally in apples, cinnamon, cloves, cranberries, plums, prunes, strawberries, and other berries. Sodium benzoate (144.1 Da) is a stable, odorless, white granular or crystalline powder that is soluble in water (66.0 g/100 mL at 20°C) and ethanol (0.81 g/100 mL at 15°C). Benzoic acid (122.1 Da), also called phenylformic acid, occurs as colorless needles or leaflets and is much less soluble in water (0.27% at 18°C) than sodium benzoate. For the latter reason the salt is preferred for use in most foods.

The primary uses of benzoic acid and sodium benzoate are as antimycotic agents. Most yeasts and molds are inhibited by 20–2000 µg benzoic acid per mL at pH 5.0 (Baird-Parker, 1980; Chipley, 1993). One yeast that it is particularly resistant is *Zygosaccharomyces bailii*. Wind and Restaino (1995) found that up to 0.3% sodium benzoate did not prevent spoilage of a salsa mayonnaise by *Z. bailii*. While some bacteria associated with food poisoning are inhibited by 1000–2000 µg/mL undissociated acid, the control of many spoilage bacteria requires much higher concentrations (Chipley, 1993). Benzoates are most effective at pH 2.5–4.0 and significantly lose effectiveness at > pH 4.5 (Lloyd and Drake, 1975; Chichester and Tanner, 1981). The antimicrobial spectrum of benzoic acid against selected microorganisms is shown in Table 2. Rajashekhara et al. (1998) demonstrated that sodium benzoate reduced the $D_{85^{\circ}\text{C}}$ value of the heat resistant mold *Neosartorya fischeri* from 69.3 and 63.5 min to 50.1 and 27.6 min in grape and mango juices, respectively.

The undissociated form of benzoic acid ($\text{p}K_a = 4.19$) is the most effective antimicrobial agent. Rahn and Conn (1994) reported that the compound was 100 times as effective in acid solutions as in neutral solutions and that only the undissociated acid had antimicrobial activity. Macris (1975) studied the effect of benzoic acid on *Saccharomyces cerevisiae*. A rapid uptake of benzoic acid by the yeast was observed, reaching saturation in about 2 min. Only the undissociated form was taken up by the cells. When cells were subjected to elevated temperatures, a decrease in the rate of uptake was observed above 60°C. This indicated an irreversible heat inactivation of the uptake process, similar to that observed for enzymatic inactivation. Therefore, protein compounds may be involved in the uptake of this preservative.

The target of benzoic acid in the microbial cell has not been completely elucidated but is likely similar to other organic acids. For example, Freese (1978) suggested that benzoic acid destroyed the proton motive force of the cytoplasmic membrane by continuous transport of protons into the cell causing disruption of the transport system. Benzoates also inhibit enzymes in bacterial cells such as those controlling acetic acid metabolism and oxidative phosphorylation (Bosund, 1962), α -ketoglutarate and succinate dehydrogenases in the citric acid cycle (Bosund, 1962), lipase production by *Pseudomonas fluorescens* (Anderson et al., 1980) and trimethylamine-*N*-oxide reductase activity (57% inhibition at 2.5 mM, pH 6.0) of *Escherichia coli* (Kruk and Lee, 1982). In fungi, aflatoxin production by a toxigenic strain of *Aspergillus flavus* (Uraih and Chipley, 1976; Uraih et

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Table 2 Minimum Inhibitory Concentrations of Benzoic Acid Against Growth of Selected Microorganisms

Microorganism	pH	Minimum inhibitory concentration ($\mu\text{g/mL}$)
Bacteria, Gram Positive		
<i>Bacillus cereus</i>	6.3	500
<i>Lactobacillus</i>	4.3–6.0	300–1800
<i>Listeria monocytogenes</i>	5.6, 4°C; 5.6, 21°C	2000; 3000
<i>Micrococcus</i>	5.5–5.6	50–100
<i>Streptococcus</i>	5.2–5.6	200–400
Bacteria, Gram Negative		
<i>Escherichia coli</i>	5.2–5.6	50–120
<i>Pseudomonas</i>	6.0	200–480
Molds		
<i>Aspergillus</i>	3.0–5.0	20–300
<i>Aspergillus parasiticus</i>	5.5	>4000
<i>Aspergillus niger</i>	5.0	2000
<i>Byssosclamyces nivea</i>	3.3	500
<i>Cladosporium herbarum</i>	5.1	100
<i>Mucor racemosus</i>	5.0	30–120
<i>Penicillium</i>	2.6–5.0	30–280
<i>Penicillium citrinum</i>	5.0	2000
<i>Penicillium glaucum</i>	5.0	400–500
<i>Rhizopus nigricans</i>	5.0	30–120
Yeasts		
<i>Candida krusei</i>	—	300–700
<i>Debaryomyces hansenii</i>	4.8	500
<i>Hansenula</i>	4.0	180
<i>Pichia membranefaciens</i>	—	700
<i>Rhodotorula</i>	—	100–200
<i>Saccharomyces bayanus</i>	4.0	330
<i>Zygosaccharomyces bailii</i>	4.8	4500
<i>Zygosaccharomyces rouxii</i>	4.8	1000

Sources: Chipley, 1993; El-Gazzar and Marth, 1987; El-Shenawy and Marth, 1988; Jermini and Schmidt-Lorenz, 1987; Marwan and Nagel, 1986; Roland et al., 1984.

al., 1977; Chipley and Uraih, 1980) and 6-phosphofructokinase activity (Francois et al., 1986) were inhibited.

Sodium benzoate is used as an antimicrobial in carbonated and still beverages (0.03–0.05%), syrups (0.1%), cider (0.05–0.1%), margarine (0.1%), olives (0.1%), pickles (0.1%), relishes (0.1%), soy sauce (0.1%), jams (0.1%), jellies (0.1%), preserves (0.1%), pie and pastry fillings (0.1%), fruit salads (0.1%), and salad dressings (0.1%), and in the storage of vegetables (Chipley, 1993). It is also used in pharmaceutical preparations, toiletries, and cosmetics. Kimble (1977) reviewed the use of these compounds for the preservation of fish by means of dips or germicidal ices. Zhao et al. (1993) showed that 0.1% benzoic acid was effective in reducing viable *E. coli* O157:H7 in apple cider (pH 3.6–4.0) by 3–5 logs in 7 days at 8°C. Kasrazadeh and Genigeorgis (1994,1995) found

that 0.3% sodium benzoate added to queso fresco cheese had an inhibitory effect on *Escherichia coli* O157:H7 and *Salmonella*.

Benzoic acid and sodium benzoate were the first antimicrobial compounds permitted in foods by the FDA (Jay, 1992). Benzoates are GRAS preservatives (21 CFR 184.1021; 21 CFR 184.1733; 9 CFR 318.7) up to a maximum of 0.1%. In most countries of the world, the maximum permissible use concentration is 0.15–0.25%.

Evidence has shown that benzoates have a low order of toxicity for animals and humans (FAO/WHO, 1962). In humans, toxic doses of 6 mg/kg were reported upon intradermal administration (Sax, 1979). However, oral administration of sodium benzoate at rates of 5–10 g for several days had no adverse effect on health (Dakin, 1909). The reason for low toxicity is that humans and animals have an efficient detoxification mechanism for benzoate. The compounds are conjugated with glycine in the liver to form hippuric acid, which is then excreted in the urine (Chipley, 1993). Griffith (1929) reported that this mechanism removed 66–95% of the benzoic acid from individuals ingesting large quantities. It was also suggested that the remaining benzoate was detoxified through conjugation with glucuronic acid. Sensitization reactions to benzoate have been reported in some individuals (Michaelsson and Juhlin, 1973).

Sodium benzoate had no teratogenic activity when administered orally (Minor and Becker, 1971). No carcinogenic effect was observed when rats were given 1–2% (Sodemoto and Enomoto, 1980), or 5% (Hartwell, 1951) sodium benzoate, orally. Sodium benzoate has been found to be nonmutagenic (Njagi and Gopalan, 1980).

C. Lactic Acid and Lactates

Lactic acid ($pK_a = 3.79$) is a primary end-product of the lactic acid bacteria and serves to assist in preservation of many fermented dairy, vegetable, and meat products. It is used as a food additive primarily for pH control and flavoring. The antimicrobial activity of the compound is variable. For example, in cold pack cheese formulated with lactic and acetic acid and inoculated with *Salmonella* Typhimurium, no increase in destruction of the organism was found (Park et al., 1970). In contrast, lactic acid was found to be four times as effective as malic, citric, propionic, and acetic acids in inhibiting growth of *Bacillus coagulans* in tomato juice (Rice and Pederson, 1954). Lactic acid inhibited sporeforming bacteria at pH 5.0 but was much less effective against yeasts and molds (Woolford, 1975b). *Staphylococcus aureus* was inactivated by 90 and 99% in 12 h at pH 4.9 and 4.6, respectively, by lactic acid (Minor and Marth, 1970). Based upon molar concentration, pH, and activity of undissociated acid, lactic acid was one of the most effective organic acids against the growth of *Yersinia enterocolitica* (Brackett, 1987). Addition of 5.0% lactic acid in ground beef patties stored at 4°C for 10 days reduced coliforms by 3.9 logs (Podolak et al., 1996b). Oh and Marshall (1993) reported that there was little interaction between lactic acid and ethanol against *L. monocytogenes*. The MIC value of lactic acid alone was 0.5%, but was lower when 1.25% ethanol was combined with 0.25% lactic acid. When 2.5% ethanol was combined with 0.25% lactic acid, the extent of inhibition was not more than that of the most active single compound alone.

Smulders et al. (1986) and Snijders et al. (1985) advocated the use of lactic acid for surface decontamination of fresh meats, slaughter byproducts, and poultry. Lactic acid at 1–2% was reported to reduce *Enterobacteriaceae* and aerobic plate counts by 0.3–2.7 log on beef, veal, pork, and poultry. The compound also delayed growth of spoilage microflora during long-term storage of products. Visser et al. (1988) confirmed the antimicro-

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bial effectiveness of 2.0% (v/v) L-lactic acid on fresh veal tongues and demonstrated that the compound decreased the growth rate of spoilage organisms during vacuum-packaged storage at 3°C.

Brewer et al. (1991) reported that 2 or 3% sodium lactate added to fresh pork sausage delayed microbial deterioration, pH decline, development of sour and off-flavors by 7–10 days at 4°C and appeared to protect red color of products. Lamkey et al. (1991) reported that 3% (w/w) sodium lactate was effective in maintaining low microbial numbers and the shelf-life was extended by more than 2 weeks when fresh pork sausage was stored at 4°C. Sodium lactate at 3 or 4% prolonged shelf-life of refrigerated beef roasts up to 84 days (Papadopoulos et al., 1991). Blom et al. (1997) added 2.5% sodium lactate plus 0.5% sodium acetate to vacuum-packaged sliced ham or sausage inoculated with *Listeria monocytogenes* and stored at 4 or 9°C for 5 weeks. The antimicrobials inhibited the microorganism for the 5 week period at 4°C.

Psychrotrophic and coliform populations in 40% fat fresh pork sausage patties were retarded with 2% potassium lactate (Bradford et al., 1993). Shelef and Yang (1991) showed that 4% (w/w) potassium lactate in beef corresponding to a concentration of 416 mM in the water phase, was more effective in controlling growth of *L. monocytogenes* than was 3% (w/w) NaCl, corresponding to a concentration of 684 mM in the water phase.

Sodium lactate (1.8–5.0%) inhibits *Clostridium botulinum*, *Clostridium sporogenes*, and *Listeria monocytogenes* in various meat products (Anders et al., 1989; Maas et al., 1989; Shelef and Yang, 1991; Unda et al., 1991; Chen and Shelef, 1992; Stillmunkes et al., 1993; Weaver and Shelef, 1993; Pelroy et al., 1994a; Hu and Shelef, 1996). The antimicrobial effect of sodium lactate against *L. monocytogenes*, *Salmonella spp.*, *Yersinia enterocolitica* increases with decreasing pH values (5.7–7.0). However, for *S. aureus* the MIC value of sodium lactate did not change with pH (Houtsma et al., 1996). In contrast, 1.8% sodium lactate alone or with 0.2% sodium diacetate had no effect on survival of *Escherichia coli* O157:H7 in ground beef at 2 or 10°C (Ajjarapu and Shelef, 1999).

Presumably lactic acid applied as antimicrobial to foods functions similarly to other organic acids and has a primary mechanism involving disruption of the cytoplasmic membrane proton motive force (Eklund, 1989). In contrast, there has been some controversy concerning the mechanism of lactate salts used at high concentrations. These salts have been shown to have little effect on product pH. Therefore, most of the lactate remains in the less effective anionic form. Initially it was thought that the high concentration of the salts may reduce water activity sufficiently to inhibit microorganisms (Loncin, 1975; Debevere, 1989). However, Chen and Shelef (1992) and Weaver and Shelef (1993) using cooked meat model systems and liver sausage, respectively, containing lactate salts up to 4% concluded that water activity reduction was not sufficient to inhibit *Listeria monocytogenes*. In addition, Papadopoulos et al. (1991) reported that water activity was not lowered in cooked beef top rounds injected with various levels of sodium lactate. Inhibition may be due to the presence of sufficient undissociated lactic acid, possibly in combination with a slightly reduced pH and water activity, to cause inhibition of some microorganisms.

Lactic acid was probably one of the first acids used in food due to its wide distribution in nature as a product of fermentation. The U.S. FDA has approved lactic acid as GRAS for miscellaneous and general purpose usage (21 CFR 184.1061) with no limitation upon the concentration used. It may not be used in infant foods and formulas. The U.S. Department of Agriculture allows lactic acid in meat products at the lowest concentration necessary for the intended purpose (9 CFR 318.7).

D. Propionic Acid and Propionates

Up to 1% propionic acid ($pK_a = 4.87$) is produced naturally in Swiss cheese by *Propionibacterium freudenreichii* ssp. *shermanii*. The use of propionic acid and propionates has been directed primarily against molds. Some yeasts and bacteria, particularly gram negative strains, may also be inhibited. The activity of propionates depends upon the pH of the substance to be preserved. In an early study, O'Leary and Kralovec (1941) demonstrated that the microorganism in bread dough that causes rope formation, *Bacillus subtilis* (*mesentericus*), was inhibited at 0.19% at pH 5.8 and 0.16% at pH 5.6. In a similar study, another strain of *B. subtilis* was inhibited by propionates at pH 6.0 (Woolford, 1975b). Propionates (0.1–5.0%) have been found to retard the growth of *S. aureus*, *Sarcina lutea*, *Proteus vulgaris*, *Lactobacillus plantarum*, *Torula* (*Candida*), and *Saccharomyces cerevisiae* var. *ellipsoideus* for up to 5 days (Woolford and Anderson, 1945). The minimum inhibitory concentration of undissociated propionic acid against three *Bacillus* species, *E. coli*, and *Staphylococcus aureus* ranged from 0.13% to 0.52% and for the yeast *Candida albicans*, 0.29%. Chung and Goepfert (1970) found that propionic acid was the most effective inhibitor of *Salmonella* serotypes Anatum, Senftenberg, and Tennessee among acetic, adipic, citric, lactic, propionic, and tartaric acids. *Listeria monocytogenes* growth was inhibited at 13, 21, and 35°C by 0.3% propionic acid at pH 5.0 and totally inhibited at 4°C (El-Shenawy and Marth, 1989). Propionic acid and propionates at 8–12% retard mold growth on the surface of cheese and butter (Ingle, 1940; Deane and Downs, 1951). The activity of propionic acid against *Aspergillus* and *Fusarium* was enhanced by the presence of EDTA (Razavi-Rohani and Griffiths, 1999b).

Early research on the mechanism of inhibition of microorganisms by propionic acid showed that sodium propionate inhibition of *E. coli* was overcome by the addition of β -alanine (Wright and Skeggs, 1946). However, this potential interference with β -alanine synthesis is probably not a universal mechanism as a reduction in inhibitory effect was not observed with *B. subtilis*, *Pseudomonas*, or *Aspergillus clavatus*. Some strains of *Penicillium*, however, may grow in nutrient media containing over 5% propionic acid (Heseltine, 1952). The primary mode of action of propionic acid is likely similar to that of the other short chain organic acids discussed, i.e., acidification of the cytoplasm and inhibition of an unspecified function by the undissociated acid.

Propionic acid and propionates are used as antimicrobials in baked goods and cheeses. Propionates may be added directly to bread dough because they have no effect on the activity of baker's yeast. There is no limit to the concentration of propionates allowed in foods but amounts used are generally less than 0.4% (Robach, 1980).

In the United States, propionic acid and calcium and sodium propionates are approved as GRAS (21 CFR 184.1081; 21 CFR 184.1221; and 21 CFR 184.1784). No upper limits are imposed except for products which come under standards of identity. A limit of 0.32% is allowed in flour in white bread and rolls, 0.38% in whole wheat products, and 0.3% in cheese products (Robach, 1980).

E. Sorbic Acid and Sorbates

Sorbic acid and its potassium, calcium, or sodium salts are collectively known as sorbates. Sorbic acid was first isolated in 1859 by A. W. Hoffman, a German chemist, from the berries of the mountain ash tree (rowanberry) (Sofos and Busta, 1993). The structure of sorbic acid was determined around 1880, and it was first synthesized by O. Doebner in 1900. It was not until ca. 1940, however, that the antimicrobial preservative power of

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sorbic acid was discovered. In 1945, a U.S. patent was awarded to C. M. Gooding and Best Foods, Inc. (Gooding, 1945). They recognized sorbic acid as an effective fungistatic agent for food. Since that time, sorbates have been used in foods as effective inhibitors of fungi, including those that produce mycotoxins, and certain bacteria (Robach, 1980; Sofos and Busta, 1981, 1993). Sorbic acid ($pK_a = 4.75$) is a trans-trans, unsaturated monocarboxylic fatty acid ($\text{CH}_3\text{-CH=CH-CH=CH-COOH}$), which is slightly soluble in water (0.15 g/100mL at 20°C). The potassium salt of sorbic acid is highly soluble in water (58.2 g/100 mL at 20°C).

As with other organic acids, the antimicrobial activity of sorbic acid is greatest in the undissociated state. The effectiveness of the compound is greatest as the pH decreases below 6.5 (Anonymous, 1999). Eklund (1983) determined the minimum inhibitory concentrations of dissociated and undissociated sorbic acid against several strains of bacteria and a yeast. Both forms were shown to cause inhibition, but the undissociated acid was 10–600 times more effective than the dissociated acid. At $\text{pH} > 6$, however, the dissociated acid was responsible for >50% of the inhibition observed. Sorbates are reported to be more effective against spoilage microorganisms than propionates or benzoate at the same pH (Anonymous, 1999).

Food-related yeasts inhibited by sorbates include species of *Brettanomyces*, *Byssoschlamys*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Endomycopsis*, *Hansenula*, *Oospora*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulasporea*, *Torulopsis*, and *Zygosaccharomyces* (Anonymous, 1999; Sofos and Busta, 1993). Food-related mold species inhibited by sorbates belong to the genera *Alternaria*, *Ascochyta*, *Aspergillus*, *Botrytis*, *Cephalosporium*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Helminthosporium*, *Humicola*, *Mucor*, *Penicillium*, *Phoma*, *Pullularia* (*Auerobasidium*), *Rhizopus*, *Sporotrichum*, and *Trichoderma* (Anonymous, 1999; Sofos and Busta, 1993). Sorbates have been found to inhibit the growth of yeasts and molds in cucumber fermentations, high-moisture dried prunes (Nury et al., 1960), and cheeses (Smith and Rollin, 1954), on the surface of country-style hams (Baldock et al., 1979), in broth culture (Deak et al., 1970), and in Mexican hot sauce (Flores et al., 1988). Rajashekhara et al. (1998) demonstrated that potassium sorbate reduced the $D_{85^\circ\text{C}}$ value of the heat-resistant mold *Neosartorya fischeri* from 69.3 and 63.5 min to 25.1 and 29.4 min in grape and mango juices, respectively.

Certain species of yeast are more resistant, and some acquire a resistance to sorbates. Pitt (1974) reported that *Zygosaccharomyces* (*Saccharomyces*) *baillii*, a preservative-tolerant yeast, was not inhibited by sorbic acid at 0.06% in 10% glucose. Deak and Novak (1972) reported that sorbic acid suppressed yeast metabolism and growth at high concentrations but was metabolized by yeast at low intracellular concentrations. Warth (1977) further studied the ability of *Z. baillii* to resist the antimicrobial activity of sorbic acid. Data indicated that, while the yeast accumulated the acid at a rate expected from conditions of concentration and pH, it was capable of transporting the sorbic acid from the cells. This transport system required energy as was demonstrated by the increased efflux in the presence of glucose. Further, Warth (1977) suggested that metabolism of sorbic acid played little, if any, part in resistance. Bills et al. (1982) showed that *Saccharomyces rouxii* that were exposed to 0.1% sorbate became resistant to subsequent exposure to the compound and that this resistance was not influenced by the presence of sucrose. Certain mold species are also resistant to sorbic acid, which results in occasional spoilage of foods. Melnick et al. (1954) reported that high initial mold populations were able to degrade sorbic acid in cheese. Liewen and Marth (1985) found that a sorbic-resistant *Penicillium*

roqueforti strain grew in yeast extract sucrose broth and yeast extract/malt extract broth with 6000 and 9000 µg/mL, respectively. Degradation of sorbates is through a decarboxylation reaction carried out in the fungal mycelium and is accompanied by the formation of 1,3-pentadiene, which may produce a kerosenelike or hydrocarbonlike odor (Marth et al., 1966; Liewen and Marth, 1985).

The inhibitory effect of sorbates may be lethal as well as static (Melnick et al., 1954; Costilow et al., 1955). Pederson et al., (1961) showed that yeasts die slowly in fruit juices treated with sorbates, while Harada et al. (1968) found only fungistatic effects. Przybylaski and Bullerman (1980) reported that conidia of *Aspergillus parasiticus* lost viability in the presence of sorbate. Bullerman (1984) studied the effect of potassium sorbate on growth and patulin production by strains of *Penicillium patulum* and *P. roqueforti* isolated from cheese. Potassium sorbate at 0.05, 0.10, and 0.15% delayed initiation of growth, prevented spore germination, and decreased the rate of growth of *P. patulum* in potato dextrose broth at 12°C. *Penicillium roqueforti* was affected less. Similar results were noted with *A. parasiticus* and *Aspergillus flavus* in yeast extract sucrose broth (Bullerman, 1983). Potassium sorbate reduced or prevented the production of the mycotoxins patulin by *P. patulum* and of aflatoxin B₁ by *A. parasiticus* and *A. flavus* for up to 70 days at 12°C (Bullerman, 1983,1984). Potassium sorbate has been found to inhibit markedly the growth and patulin production of *P. expansum* and *Byssoschlamys nivea* in grape and apple juice (Lennox and McElroy, 1984; Roland et al., 1984; Roland and Beuchat, 1984). In contrast, Yousef and Marth (1981) found that sorbate delayed mold growth but did not inhibit biosynthesis of aflatoxin by *A. parasiticus*. The ability to synthesize aflatoxin was greater in the early stages of growth and then decreased as mold growth progressed. In a subsequent study, Rusul and Marth (1987) showed that 0.2% (pH 5.5) or 0.05% (pH 4.5) potassium sorbate completely inhibited *A. parasiticus* growth and toxin production for 3 days in a glucose-yeast extract salts medium. However, growth and toxin production were nearly normal by day 7 of incubation. Tsai et al. (1984) reported that potassium sorbate was more effective against injured spores of *A. parasiticus* and that inhibition was dependent on the concentration of sorbate and pH. The activity of potassium sorbate as an antifungal agent has been shown to be enhanced by the presence of EDTA and vanillin (Razavi-Rohani and Griffiths, 1999b; Matamoros-León et al., 1999).

Sorbate has been found to inhibit the growth of *Salmonella*, *Clostridium botulinum*, and *Staphylococcus aureus* in cooked, uncured sausage (Tompkin et al., 1974); *S. aureus* in bacon (Pierson et al., 1979); *Pseudomonas putrefaciens* and *P. fluorescens* in TSB (Robach, 1978,1979); *Vibrio parahaemolyticus* in crab meat and flounder homogenates (Robach and Hickey, 1978); *S. aureus* and *E. coli* in poultry (Robach, 1980; Robach and Sofos, 1982); *Yersinia enterocolitica* in pork (Myers et al., 1983); and *Escherichia coli* O157:H7 and *Salmonella* in queso fresco cheese (Kasrazadeh and Genigeorgis, 1994,1995). Doell (1962) reported that sorbate at concentrations as low as 0.075% inhibited *Salmonella* Typhimurium and *E. coli*. Sorbates also inhibited *S. Typhimurium* in laboratory media and in milk and cheese (Park et al., 1970; Park and Marth, 1972). Uljas and Ingham (1999) developed processes that were effective in reducing *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 by 5 logs in apple cider at pH 3.3–4.1. Sorbic acid (0.1%) in combination with 12 h at 25°C plus freeze-thawing (FT = 48 h at –20°C, 4 h at 4°C), 6 h at 35°C, or 4 h at 35°C plus FT all resulted in a 5 log reduction of the two pathogens in apple cider at pH 4.1. Potassium sorbate at 0.5% inhibited growth and histamine production by *Proteus morgani* and *K. pneumoniae* strains in a TSB fortified with histidine. The inhibition was effective for 215 h and 120 h at 10°C and 32°C, respec-

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tively (Taylor and Speckhard, 1984). Potassium sorbate inhibited anaerobic growth of *S. aureus* more than growth under aerobic conditions in an agar–meat model system and was more inhibitory when lactic acid was added (Smith and Palumbo, 1980). Larocco and Martin (1987) demonstrated no inhibition or injury of *S. aureus* MF-31 by potassium sorbate (0.3%) or sorbate in combination with salt at pH 6.3. Microorganisms isolated from seafood show varying degrees of sensitivity to sorbate (Chung and Lee, 1982). Potassium sorbate (0.1%) added to scallops (pH 6.3–6.5) resulted in more rapid *Clostridium botulinum* toxin development than in controls (Fletcher et al., 1988). It was postulated that this might be due to inhibition of microflora competitive to *C. botulinum*.

Curran and Knaysi (1961) found that 0.01–0.1% sorbic acid did not affect the germination of *Bacillus subtilis* spores. In contrast, Gould (1964) demonstrated that germination and outgrowth of six *Bacillus* species was decreased by sorbate at pH 6.0. Smoot and Pierson (1981) reported that potassium sorbate was a strong inhibitor of bacterial spore germination at pH 5.7 but much less so at pH 6.7.

Reports in the 1950s concluded that sorbates had either stimulatory or no effect on clostridia (York and Vaughn, 1954; Hansen and Appleman, 1955). Later studies concluded that sorbates acted as an anticlostridial agent in cured meat products (Sofos et al., 1979b; Robach and Sofos, 1982; Sofos and Busta, 1983). The contradictory results of the studies are most likely due to the fact that in the 1950s, media with pH values approaching 7.0 were used. This was optimal for growth of clostridia but not optimal for the activity of sorbates, since only about 0.6% of sorbic acid is in the undissociated form at pH 7.0. Potassium sorbate, sufficient to give an undissociated sorbic acid concentration of 250 mg/L in culture medium at pH 5.5–7.0, retarded the growth of proteolytic strains of *Clostridium botulinum* from spores and vegetative cells (Blocher et al., 1982; Blocher and Busta, 1983). Sorbate has been shown to prevent spores of *C. botulinum* from germinating and forming toxin in poultry frankfurters and emulsions (Sofos et al., 1979c,d,1980a; Huhtanen and Fienberg, 1980) as well as beef, pork, and soy protein frankfurter emulsions and bacon (Ivey et al., 1978; Sofos et al., 1979a,1980b). Roberts et al. (1982) found that 0.26% (w/v) potassium sorbate significantly decreased *C. botulinum* toxin production in a model cured meat system. The effect of the sorbate was greatest at 3.5% sodium chloride, a pH less than 6.0, and low storage temperatures. Lund et al. (1987) did a systematic study to determine the concentration of potassium sorbate necessary to inhibit a single *C. botulinum* cell in culture medium. They reported that 1000 mg/L (0.1%) at pH 5.0 would reduce the probability of growth of *C. botulinum* at 30°C in 14 days to 1 in 10⁸. They also concluded that 0.2% sorbic acid alone in meat products at pH 6.5 would not significantly inhibit *C. botulinum* vegetative cells.

Research on the effect of sorbic acid on catalase-producing microorganisms is contradictory. Several studies showed that sorbic acid inhibited catalase-positive microorganisms and could be used as a selective agent for lactic acid bacteria and clostridia (Phillips and Mundt, 1950; York and Vaughn, 1954,1955). However, Emard and Vaughn (1952) reported that some catalase-positive strains of *S. aureus* grew as well in the presence of sorbate as catalase-negative *Lactobacillus*. Sorbic acid at 0.05–0.10% inhibited both growth and acid production of catalase-negative organisms such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Hamden et al., 1971). Costilow et al. (1955) reported that two strains of *Pediococcus cerevisiae* (weakly catalase-positive) were found to be as tolerant to sorbic acid as catalase-negative strains.

The mechanism by which sorbic acid inhibits microbial growth may partially be due to its effect on enzymes. Melnick et al. (1954) postulated that sorbic acid inhibited

dehydrogenases involved in fatty acid oxidation. Addition of sorbic acid resulted in the accumulation of β -unsaturated fatty acids that are intermediate products in the oxidation of fatty acids by fungi. This prevented the function of dehydrogenases and inhibited metabolism and growth. Sorbic acid has also been shown to be a sulfhydryl enzyme inhibitor. These enzymes are very important in microorganisms and include fumarase, aspartase, succinic dehydrogenase, and yeast alcohol dehydrogenase (Whitaker, 1959; Martoadiprawito and Whitaker, 1963; York and Vaughn, 1964). York and Vaughn (1964) suggested that sorbate reacted with sulfhydryl enzymes through an addition reaction with the thiol groups of cysteine. Rehm (1963) indicated that the activity of sorbic acid against *Aspergillus niger* was increased by cysteine. Whitaker (1959) suggested that sorbate activity is due to the formation of stable complexes with sulfhydryl-containing enzymes through a thiohexenoic acid derivative. Sorbate therefore inhibits enzymes either by formation of a covalent bond between sulfur of the essential sulfhydryl group or the ZnOH of the enzyme and the (α and/or β) carbons of the sorbate ion. Other suggested mechanisms for sorbate have involved interference with enolase, proteinase, and catalase, or through inhibition of respiration by competitive action with acetate in acetyl CoA formation (Azukas et al., 1961; Troller, 1965; Dahl and Nordal, 1972). Kouassi and Shelef (1995) found that sorbate inhibited activation of the hemolytic activity of listeriolysin O of *Listeria monocytogenes* by reacting with cysteine. Freese (1978) suggested that lipophilic acids, such as sorbic acid, interfere with transport across the cytoplasmic membrane. Eklund (1985) demonstrated that sorbic acid eliminated the Δ -pH component of the proton motive force (PMF) in *E. coli* membrane vesicles but did not affect the Δ - ψ component. Ronning and Frank (1987) also showed that sorbic acid reduced the cytoplasmic membrane proton gradient and consequently the PMF. They concluded that the loss of PMF inhibited amino acid transport, which could eventually result in the inhibition of many cellular enzyme systems. The varied proposed mechanisms indicate that researchers disagree regarding the exact mechanism(s) of sorbate action on microorganisms. It might be concluded that no single mechanism holds true under all conditions.

The methods of sorbate addition available include direct addition into the product, dipping, spraying, dusting, or incorporation into packaging. Sorbates are used in many food products including artificially sweetened confections, cakes and cake mixes, cheeses, diet drinks, doughnuts, dried fruits, fruit drinks, fudges, icing, jams, jellies, mayonnaise, olives, orange juice, packaged fresh salads, pet foods, pickles, pies and pie fillings, relishes, salad dressings, sausage casings, sour cream, wine, and yogurt. Use concentrations for sorbates vary considerably (Table 3). In the United States, sorbic acid and potassium sorbate are considered GRAS (21 CFR 182.3089; 21 CFR 182.3225; 21 CFR 182.3640; and 21 CFR 182.3795). They may be used in more than 90 food products having standards of identity, and their use may be requested in any food product that allows preservatives. The maximum concentration of sorbic acid is set at 0.2% in pasteurized blended cheese; pasteurized process cheese, cheese food, and cheese spread; and cold pack cheese, cheese food, and cheese spread (21 CFR 133). A maximum of 0.3% is allowed in other cheeses (21 CFR 133). Potassium sorbate at 0.05–0.08% is used in olives, sauerkraut, pickled cucumbers, and sweet relish. It does not interfere with the lactic acid fermentation necessary for the production of these products. In wines it may be used as a replacement or adjunct to sulfur dioxide as a sterilizing and preservative agent to prevent the occurrence of secondary fermentation. The maximum level of sorbic acid or its salt has been set at 0.1% in wine or in materials for the production of wine, and it is not necessary to declare its presence on the label. United States standards of identity (21 CFR 150.141 and 21

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Table 3 Food Products in Which Sorbates Are Used and Typical Use Concentrations

Food product	Use concentration (%)
Beverage syrups	0.1
Cakes and icings	0.05–0.1
Cheese and cheese products	0.2–0.3
Cider	0.05–0.1
Fruits, dried	0.02–0.05
Fruit drinks	0.025–0.075
Margarine	0.1
Pie fillings	0.05–0.1
Pet food, semimoist	0.1–0.3
Salad dressings	0.05–0.1
Salads, prepared vegetable	0.05–0.1
Wine	0.02–0.04

Source: Anonymous, 1999.

CFR 150.161) for artificially sweetened jams, jellies, and preserves permit the use of sorbic acid and its salts to maximum levels of 0.1%. The compound is generally added during the cooling cycle. Potassium sorbate may be used to protect dry sausages from mold spoilage by dipping the casing, either before or after stuffing (9 CFR 318.7). The use of sorbic acid and sorbates are permitted in all countries of the world for the preservation of a variety of foods.

Sorbic acid is considered one of the least harmful antimicrobial preservatives, even at levels exceeding those normally used in foods (Lueck, 1980; Sofos and Busta, 1981). The LD₅₀ for sorbates in rats ranges from 7.4 to 10.5 g/kg body weight. Rats fed sorbic acid at 10% in feed for 40 days had no ill effects (Lueck, 1980). When the feeding period was increased to 120 days, the growth rate and liver weight increased (Demaree et al., 1955). Sorbic acid at 5% in the diet of rats did not affect health after 1000 days (except for two tumors in 100 rats) (Lang, 1960b). When rats were given sorbic acid (10 mg/100 mL) or potassium sorbate (0.3%) in drinking water or 0.1% levels in the diet, no tumors were observed after 100 weeks. The growth of tumors was not seen when mice were fed 40 mg sorbic acid per kilogram body weight (Shtenberg and Ignat'ev, 1970). Repeated subcutaneous administration of sorbic acid in peanut oil or water in rats produced sarcomas at the site of injection. Sarcomas produced locally in this manner were not considered a valid index of carcinogenicity (Gangolli et al., 1971). When sorbic acid is used for cosmetic and pharmaceutical products, it may irritate the mucous membranes, and in highly sensitive individuals it may cause skin irritation (Lueck, 1980).

F. Other Organic Acids

While citric acid generally is not used as an antimicrobial, it has been shown to possess activity against some molds and bacteria. Reiss (1976) found that 0.75% citric acid slightly reduced growth and greatly reduced toxin production by *Aspergillus parasiticus*. With *Aspergillus versicolor*, growth was inhibited at the same level, but toxin production was prevented by 0.25% citric acid. In contrast, 0.75% citric acid did not influence the growth

or toxin production of *Penicillium expansum* (Reiss, 1976). Citric acid was observed to be more inhibitory to *Salmonella* than lactic or hydrochloric acids (Subramanian and Marth, 1968). In a related study, Thomson et al. (1967) found that as little as 0.3% citric acid could reduce the level of viable *Salmonella* on poultry carcasses. Shrimp, shrimp puree, tomato puree, and shrimp and tomato puree acidified to pH 4.2 and 4.6 with citric acid showed no significant growth or toxin production by *C. botulinum* after 8 weeks at 26°C (Post et al., 1985). Minor and Marth (1970) showed that *Staphylococcus aureus* was inhibited 90% and 99% in 12 h at pH 4.7 and 4.5, respectively, by citric acid. Citric acid was found to be particularly inhibitory to flat-sour organisms isolated from tomato juice, but the inhibition was pH-dependent (Murdock, 1950). Xiong et al. (1999) reported that citric acid in lemon juice could be used to prepare homemade mayonnaise with raw eggs that was free of *Salmonella enteritidis* PT4. Both the concentration of lemon juice and time of storage influenced inactivation of the *Salmonella* serovar.

The mechanism of inhibition by citrate has been theorized to be related to its ability to chelate metal ions. Branen and Keenan (1970) were the first to suggest that inhibition may be due to chelation, in studies with citrate against *Lactobacillus casei*. Chelation was also indicated as the reason for inhibition of *S. aureus* (Rammell, 1962) and *Arthrobacter pascens* (Imai et al., 1970) by citrate. In contrast, Buchanan and Golden (1994) found that while undissociated citric acid was inhibitory against *Listeria monocytogenes*, the dissociated molecule protected the microorganism. They theorized that this protection was due to chelation by the anion. The U.S. FDA has classified citric acid as GRAS for miscellaneous and general purpose use (21 CFR 182.1033).

Fumaric acid has been used to prevent the occurrence of malolactic fermentation in wines (Ough and Kunkee, 1974) and as an antimicrobial agent in wines (Pilone, 1975). Esters of fumaric acid (monomethyl, dimethyl, and ethyl) at 0.15–0.2% have been tested as a substitute or adjunct for nitrate in bacon. Fumaric acid esters (0.125%) retarded swelling and toxin formation in canned bacon inoculated with *Clostridium botulinum* for up to 56 days at 30 °C (Huhtanen, 1983). Dymicky et al. (1987) studied the structure–activity relationships of *n*-mono-alkyl, di-alkyl, and methyl *n*-alkyl fumarates in a microbiological medium against *C. botulinum* 62A. The *n*-mono-alkyl fumarates (C₁–C₁₈) were found to have the greatest activity, with minimum inhibitory concentrations ranging from 6.2 to 400 µg/mL. Fungal growth in tomato juice was inhibited with the use of 0.2% methyl and ethyl fumarates or 0.05% dimethyl and diethyl fumarates. Fumaric acid esters were also found to inhibit mold growth on bread (Huhtanen et al., 1981; Huhtanen and Guy, 1984). At 1% as a sanitizer on lean beef, fumaric acid reduced *L. monocytogenes* and *E. coli* O157:H7 by approximately 1 log after 5 s at 55°C (Podolak et al., 1996a). Addition of 5.0% fumaric acid in ground beef patties stored at 4°C for 10 days resulted in less than a 1.5 log increase in aerobic and psychrotrophic microorganisms and fecal coliforms (Podolak et al., 1996b).

Many other organic acids, including adipic, caprylic, malic, succinic, and tartaric have been evaluated for their antimicrobial properties. The antimicrobial activity of adipic, malic, and tartaric acids may be attributed only to their ability to reduce pH. Malic, citric, and tartaric acids did not have a statistically significant effect on the thermal resistance of spores of *Alicyclobacillus acidoterrestris* spores in a model fruit juice system (Pontius et al., 1998). Caprylic acid has been shown to have variable effects on microorganisms (Kabara et al., 1972). The compound has been found to be inhibitory to *Vibrio parahaemolyticus* (Beuchat, 1980), *Escherichia coli*, and *Shigella* spp. (Nakamura and Zangar, 1968). In contrast to other organic acid antimicrobials, Woolford (1975b) found that as the pH

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was decreased, the concentration of caprylic acid required for inhibition was not markedly lowered. Succinic acid at 3% or 5% was found to be effective in reducing the microbial level on chicken carcasses; however, the appearance of the product was adversely effected (Cox et al., 1974).

VIII. PARABENS

Alkyl (methyl, ethyl, propyl, butyl, and heptyl) esters of *p*-hydroxybenzoic acid are collectively known as the "parabens." Sabalitschka and coworkers (Prindle, 1983) first described antimicrobial action of parabens in the 1920s. Esterification of the carboxyl group of benzoic acid allows the molecule to remain undissociated up to pH 8.5 versus benzoic acid with a pK_a of 4.2. While the pH optimum for antimicrobial activity of benzoic acid is 2.5–4.0, the parabens are effective at pH 3–8 (Aalto et al., 1953; Chichester and Tanner, 1972). They are effective at both acidic and alkaline pH levels. The molecular weights of various esters are: methyl, 152.14; ethyl, 166.17; propyl, 180.21; butyl, 196.23; and heptyl, 236.21. The solubility of parabens in ethanol increases from methyl to heptyl ester, but the water solubility is inversely related to the alkyl chain length.

The antimicrobial activity of *p*-hydroxybenzoic acid esters is, in general, directly proportional to the chain length of the alkyl component (Table 4). Parabens are generally more active against molds and yeast than against bacteria. Against bacteria, they are more effective against gram positive than gram negative bacteria.

Moir and Eyles (1992) compared the effectiveness of methyl paraben and potassium sorbate on the growth of four psychrotrophic foodborne bacteria: *A. hydrophila*, *Listeria monocytogenes*, *Pseudomonas putida*, and *Yersinia enterocolitica*. At pH 5, there was little difference in inhibition between the antimicrobials. At pH 6 however, methyl paraben was effective at a lower concentration than potassium sorbate for all pathogens except *A. hydrophila*, where the two were equal. Robach and Pierson (1978) investigated the effect of methyl and propyl paraben on toxin production of *Clostridium botulinum* NCTC 2021. At 100 $\mu\text{g}/\text{mL}$ of methyl and 100 $\mu\text{g}/\text{mL}$ of propyl paraben, toxin formation was prevented, while 1200 $\mu\text{g}/\text{mL}$ methyl and 200 $\mu\text{g}/\text{mL}$ propyl were necessary for growth inhibition. Reddy and Pierson (1982) and Reddy et al. (1982) determined the effect of methyl, ethyl, propyl and butyl parabens on growth and toxin production of ten *Clostridium botulinum* strains (5 Type A, 5 Type B). In TYG medium at pH 7.0 and 37°C, 1000 $\mu\text{g}/\text{mL}$ methyl paraben blocked growth and toxin formation for only 1 day. Ethyl and propyl paraben, at the same concentration, prevented growth and toxin production for the maximum incubation time of 7 days. Butyl paraben, as might be expected, was most effective and prevented growth and toxin production for 7 days at 200 $\mu\text{g}/\text{mL}$. Inhibition of *C. botulinum* by the parabens in food systems is less than in laboratory media (Davidson, 1993). Propyl paraben added to vacuum-packaged sliced ham or sausage inoculated with *Listeria monocytogenes* and stored at 4 or 9°C for 5 weeks was ineffective in controlling the microorganism (Blom et al., 1997).

As with bacteria, inhibition of fungi increases as the alkyl chain length of the parabens increases. Jermini and Schmidt-Lorenz (1987) evaluated ethyl paraben against osmotolerant yeasts at various water activities and pH levels. They found that the concentration of ethyl paraben necessary for inhibition was a function of initial number of yeast cells present. At 600 $\mu\text{g}/\text{mL}$ ethyl paraben, the time to initiate growth was approximately 15, 12, 5, and 2–3 days for 10^2 , 10^3 , 10^4 , and 10^5 cells at a_w 0.900 and pH 4.8. Inhibition by parabens was also a function of pH, with more acid environments requiring less ethyl

Table 4 Minimum Inhibitory Concentrations of Methyl, Propyl, and Heptyl Esters of *p*-Hydroxybenzoic Acid Against Growth and End-Product Production of Selected Microorganisms

Microorganism	Minimum inhibitory concentration ($\mu\text{g/mL}$)		
	Methyl	Propyl	Heptyl
Bacteria, gram positive			
<i>Bacillus cereus</i>	2000	125–400	12
<i>Bacillus subtilis</i>	2000	250	—
<i>Clostridium botulinum</i> Type A	1000–1200	200–400	—
<i>Clostridium botulinum</i> toxin production	100	100	—
<i>Clostridium perfringens</i>	500 ^a	—	—
<i>Lactococcus lactis</i>	—	400	12
<i>Listeria monocytogenes</i>	>512	512	—
<i>Staphylococcus aureus</i>	4000	350–500	12
Bacteria, gram negative			
<i>Aeromonas hydrophila</i> , protease secretion	—	>200	—
<i>Enterobacter aerogenes</i>	2000	1000	—
<i>Escherichia coli</i>	2000	400–1000	—
<i>Klebsiella pneumoniae</i>	1000	250	—
<i>Pseudomonas aeruginosa</i>	4000	8000	—
<i>Pseudomonas fragi</i>	—	4000	—
<i>Pseudomonas fluorescens</i>	2000	1000	—
<i>Salmonella</i> Typhi	2000	1000	—
<i>Salmonella</i> Typhimurium	—	>300	—
<i>Vibrio parahaemolyticus</i>	—	50–100	—
Fungi			
<i>Aspergillus flavus</i>	—	200	—
<i>Aspergillus niger</i>	1000	200–250	—
<i>Byssosclamyces fulva</i>	—	200	—
<i>Candida albicans</i>	1000	125–250	—
<i>Penicillium chrysogenum</i>	500	125–250	—
<i>Rhizopus nigricans</i>	500	125	—
<i>Saccharomyces bayanus</i>	930	220	—
<i>Saccharomyces cerevisiae</i>	1000	125–200	100

^a 3:1 methyl:propyl.

Sources: Aalto et al., 1953; Bargiota et al., 1987; Davidson, 1993; Dymicky and Huhtanen, 1979; Jurd et al., 1977; Klindworth et al., 1979; Lee, 1973; Marwan and Nagel, 1986; Moustafa and Collins, 1969; Robach and Pierson, 1978; Veugopal, 1984.

paraben for inhibition. Little effect of water activity or type of humectant was observed. Of the genera tested, *Zygosaccharomyces bailii* was most resistant, requiring 900 $\mu\text{g/mL}$ at 25°C, a_w 0.900 and pH 4.8. Other yeasts evaluated included *Torulasporea delbrueckii*, *Z. rouxii*, *Z. bisporus*, and *Debaryomyces hansenii* with minimum inhibitory concentrations of 700 $\mu\text{g/mL}$, 700 $\mu\text{g/mL}$, 400 $\mu\text{g/mL}$, and 400 $\mu\text{g/mL}$, respectively. They concluded that the concentration of ethyl paraben required to preserve a product from the effect of osmotolerant yeast for 30 days at 25°C and a_w 0.795–0.980 was 900 $\mu\text{g/mL}$ or 400 $\mu\text{g/mL}$ at a pH of 4.8 or ≤ 4.0 , respectively. Thompson (1994) evaluated butyl, propyl, ethyl and methyl parabens, alone and in combination, against multiple strains of mycotoxigenic *Aspergillus*, *Penicillium*, and *Fusarium*. The most effective parabens were the propyl

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and butyl esters with minimum inhibitory concentrations of 1.0–2.0 mM in potato dextrose agar. Combinations of the various parabens were determined to have synergistic activity against the mold species.

The mechanism by which the parabens inhibit microorganisms is most likely related to their effects on the cytoplasmic membrane. Freese et al. (1973) demonstrated that parabens inhibited serine uptake and ATP production in *Bacillus subtilis*. They concluded that the parabens were capable of inhibiting both membrane transport and the electron transport system. Eklund (1985) found that parabens eliminated the ΔpH of the cytoplasmic membrane of *E. coli*. The compounds did not significantly affect the $\Delta\Psi$ component of the proton motive force. He concluded that neutralization of the proton motive force and subsequent transport inhibition was not the sole mechanism of action of the parabens. Bargiota et al. (1987) postulated that mediation of the effectiveness of parabens as antimicrobials was due to cellular lipid components. Juneja and Davidson (1992) altered the lipid composition of *L. monocytogenes* by growth in the presence of added fatty acids (C14:0, C18:0 or C18:1). Growth of *L. monocytogenes* in the presence of exogenously added C14:0 or C18:0 fatty acids increased the resistance of the cells to TBHQ and parabens. However, growth in the presence of C18:1 led to increased sensitivity to the antimicrobial agents. Results indicated that, for *L. monocytogenes*, a correlation existed between lipid composition of the cell membrane and susceptibility to antimicrobial compounds.

To take advantage of their respective solubility and increased activity, methyl and propyl parabens are normally used in a combination of 2–3:1 (methyl:propyl). The compounds may be incorporated into foods by dissolving in water, ethanol, propylene glycol, or the food product itself. Methyl and propyl paraben (3:1) at the 0.03–0.06% level may be used to increase the shelf-life of fruit cakes, pie crusts, pastries (nonyeast), icing, toppings, and fillings such as fruit, jellies, and creams. Methyl and propyl paraben (2:1) at 0.03–0.05% may be used in soft drinks. A combination of esters at 0.03–0.06% is recommended for marinated, smoked, or jellied fish products. A combination of esters at 0.05–0.1% is used in flavor extracts. Approximately 0.05% methyl and propyl paraben (2:1) are used to preserve fruit salads, juice drinks, sauces, and fillings. Methyl paraben at 0.05–0.1% or a combination of methyl and propyl paraben has been used for gelatinous coatings or jellied foods. Methyl and propyl paraben (2:1) may be used as preservatives in jams and jellies (0.07%), in salad dressings (0.1–0.13%), and in wines (0.1%) (Davidson, 1993).

The FDA considers methyl (21 CFR 184.1490) and propyl (21 CFR 184.1670) parabens as GRAS, with a total addition limit of 0.1%. Both methyl and propyl parabens are permitted as antimycotics in food packaging material (21 CFR 121.2001). Heptyl paraben is permitted in fermented malt beverages (beer), noncarbonated soft drinks, and fruit-based beverages at a maximum of 12 ppm (21 CFR 172.145). In the United Kingdom, methyl, ethyl, and propyl parabens are permitted in food. In many countries, the butyl ester is allowed for use in foods.

Parabens are known to have low toxicity. They are rapidly hydrolyzed, conjugated in the body, and excreted in the urine (Jones et al., 1956; FAO/WHO, 1967). Methyl paraben has been shown to be noncarcinogenic in rats fed 2–8% in the diet (Matthews et al., 1956). Methyl paraben at 1% administered intraperitoneally to mice was shown to be noncarcinogenic. Similar results were observed by subcutaneous administration of 1.0 mL/week through the life span (WHO, 1974). Ethyl paraben at 2% levels in feed also has been shown to be noncarcinogenic in rats (WHO, 1974).

Parabens have been observed to have a local anesthetic effect. The action increases with the number of carbon atoms in the alkyl group (Adler-Hradecky and Kelentey, 1960; WHO, 1974). The effect of a 0.1% solution of methyl paraben is similar to that of a 0.05% procaine solution. Propyl or ethyl paraben at 0.05% concentration also have been reported to have a local anesthetic effect on the buccal mucosa (Bubnoff et al., 1957).

Parabens in foods have been reported to cause dermatitis of unknown etiology (Epstein, 1968). Cross-sensitization phenomenon among the parabens has been observed in individuals sensitive to *p*-hydroxybenzoate. Matthews et al. (1956) reported that 0.1% methyl or propyl paraben solution applied to skin produced no skin reaction.

IX. PHOSPHATES

Phosphates are used extensively in food processing. Some phosphate compounds, including sodium acid pyrophosphate (SAPP), tetrasodium pyrophosphate (TSPP), sodium triphosphate (STPP), sodium tetraphosphate, sodium hexametaphosphate (SHMP), and trisodium phosphate (TSP), have demonstrated variable levels of antimicrobial activity in foods. There are over 30 phosphate salts used in food products and their functions include buffering or pH stabilization, acidification, alkalization, sequestration or precipitation of metals, formation of complexes with organic polyelectrolytes (e.g., proteins, pectin, and starch), deflocculation, dispersion, peptization, emulsification, nutrient supplementation, anticaking, antimicrobial preservation, and leavening (Ellinger, 1981).

Gram positive bacteria appear to be generally more susceptible to phosphates than gram negative bacteria. Post et al. (1968) found that 0.1% sodium hexametaphosphate (SHMP) was effective against many gram positive bacteria, while gram negative bacteria grew at 10% SHMP. Kelch (1958) tested commercial mixtures of tetrasodium pyrophosphate (TSPP), sodium acid pyrophosphate (SAPP), sodium triphosphate (STPP), and SHMP with and without heat against gram positive bacteria. *Staphylococcus aureus* and *Streptococcus faecalis* were completely inhibited in nutrient medium plus heat (50°C). Without heat, susceptibility was variable. The MICs of food grade phosphates added to early exponential phase cells of *Staphylococcus aureus* ISP40 8325 in a synthetic medium were determined to be 0.1% for sodium ultraphosphate and sodium polyphosphate glassy and 0.5% for sodium acid pyrophosphate, sodium triphosphate, and tetrasodium pyrophosphate (Lee et al., 1994a). A mixture of TSPP (15%)–STPP (70%)–SHMP (15%) was an effective inhibitor of *Bacillus subtilis*, *Clostridium sporogenes*, and *Clostridium bifermentans* at 0.5%. In a similar study, Jen and Shelef (1986) tested seven phosphate derivatives against the growth of *Staphylococcus aureus* 196E. Only 0.3% SHMP (with a phosphate chain length *n* of 21) and 0.5% STPP or SHMP (*n* = 13; 15) were effective growth inhibitors. Magnesium reversed the growth-inhibiting effect. Wagner and Busta (1985) found that while 0.4% SAPP had no effect on the growth of *Clostridium botulinum* 52A, the compound delayed or prevented toxicity to mice. It was theorized that toxin inhibition was due to binding of the toxin molecule or inactivation of the protease responsible for protoxin activation. Gould (1964) showed that 0.2–1.0% SHMP permitted germination of *Bacillus* spores but prevented outgrowth. Zaika and Kim (1993) found that 1% sodium polyphosphates inhibited lag and generation times of *Listeria monocytogenes* in BHI broth, especially in the presence of NaCl.

Phosphate derivatives also have antimicrobial activity in food products. In several studies, SAPP, SHMP, or polyphosphates have been shown to increase the effectiveness of the curing system (nitrite–pH–salt) against *Clostridium botulinum* (Ivey and Robach,

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1978; Nelson et al., 1980; Roberts et al., 1981; Wagner and Busta, 1983). Other studies have reported variable results. Post et al. (1968) found that a 10% sodium tetrapolyphosphate dip was effective in preserving cherries against the growth of several mold species including *Penicillium*, *Rhizopus*, and *Botrytis*. SHMP, STPP, and tetrasodium pyrophosphate were less effective. In cheese and pasteurized process cheese products, phosphates serve several roles. Tanaka (1982) showed that phosphate along with sodium chloride, water activity, water content, pH, and lactic acid interacted to prevent the outgrowth of *Clostridium botulinum* in pasteurized process cheese. Ebel et al. (1965) reported preservative action of condensed phosphates on fish and inhibitory activity against *Staphylococcus aureus* and *Bacillus subtilis* in a broth medium. Phosphate salts have also been shown to have varying antimicrobial activity against rope-forming *Bacillus* in bread and *Salmonella* in pasteurized egg whites (Tompkin, 1983).

Trisodium phosphate (TSP) at levels of 8–12% has been reported to reduce pathogens, especially *Salmonella*, on poultry (Giese, 1992). Kim et al. (1994) found a 1.6–1.8 log reduction in *S. Typhimurium* on postchill chicken carcasses using a 10% TSP dipping treatment. Wang et al. (1997) reported that 10% TSP reduced *S. typhimurium* attached to 38.5 cm² chicken skin by 1.6 to 2.3 log. In addition, Slavik et al. (1994) evaluated a 10% TSP dipping of chicken carcasses inoculated with *Campylobacter jejuni* and reported a 1.5 log reduction. A treatment of beef surfaces with TSP reduced the level of attached *E. coli* O157:H7 and *S. Typhimurium* by 0.51–1.39 logs, respectively (Kim and Slavik, 1994). Lillard (1994) determined that while 10% TSP appeared to reduce viable *Salmonella* by 2 logs, it was a function of the high pH of the (11–12) of the system. Waldroup (1995) and Waldroup et al. (1995) concluded that TSP had no effect on pathogens or indicator microorganisms on poultry and may actually allow greater survival of *Listeria monocytogenes* than non-TSP processes. A trisodium phosphate (10 or 15%) dip (37°C) of 15 s inactivated >5 log CFU/cm² *Salmonella* Montevideo inoculated on the surface of tomatoes, but was only capable of reducing the microorganism by 2 logs in the tomato core tissue (Zhuang and Beuchat, 1996). The pH of the dip solutions was 11.8–12.6.

Several mechanisms for bacterial inhibition by polyphosphates (Sofos, 1986). The ability of polyphosphates to chelate metal ions appears to play an important role in the antimicrobial activity of these compounds. The presence of magnesium has been shown to reverse inhibition of gram positive bacteria by antimicrobial phosphates (Post et al., 1968; Jen and Shelef, 1986; Lee et al., 1994b). With some phosphates, calcium and iron are also effective in reversing inhibition (Jen and Shelef, 1986; Lee et al., 1994b). Knabel et al. (1991) stated that the chelating ability of polyphosphates was responsible for growth inhibition of *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Lactobacillus* and *Aspergillus flavus*. The target metal chelated was dependent upon the microorganism. Orthophosphates had no inhibitory activity against any of the microorganisms and have no chelating ability. Further, Knabel et al. (1991) reported that inhibition was reduced at lower pH due to protonation of the chelating sites on the polyphosphates. They concluded that polyphosphates inhibited gram positive bacteria and fungi by removal of essential cations from binding sites on the cell walls of these microorganisms. It has been suggested that polyphosphates may also interfere with RNA function or metabolic activities of cells (Ellinger, 1981; Sofos, 1986).

Excessive intake of phosphates may decrease the availability of calcium, iron, and other minerals; however, no adverse effects have been reported with moderate doses (Lauerson, 1953; Lang, 1959). Nephrocalcinosis was reported when rats were fed a diet containing 1% of a mixture of diphosphates and polyphosphates (Van Esch et al., 1957).

At 2.5% of the diet, the mixture caused anemia characterized by a decrease in erythrocytes, and at 5% retarded growth rate and decreased fertility resulted (Van Esch et al., 1957). Life span was not significantly altered with up to 2.5% phosphates (Van Esch et al., 1957). Problems in humans are likely to occur when high levels of phosphates are consumed, as in large quantities of soft drinks (Jacobs, 1959).

X. SULFITES

Sulfur dioxide and its various salts claim a long history of use dating back to times of the ancient Greeks (Ough, 1993b). They have been used extensively as antimicrobials and to prevent enzymatic and nonenzymatic discoloration in a variety of foods (Wedzicha, 1981). The earliest recorded food use of sulfur dioxide was the treating of wines in ancient Rome (IFT, 1975). The use of sulfur dioxide as a food preservative was reported in the literature in the 17th century by Evelyn (1664), who suggested that carts should be filled with cider that contained sulfur dioxide (produced by burning sulfur).

The salts of sulfur dioxide include (formula; solubility in g/L at temperature specified): potassium sulfite (K_2SO_3 ; 250, 20°C), sodium sulfite (Na_2SO_3 ; 280, 40°C), potassium bisulfite ($KHSO_3$; 1000, 20°C), sodium bisulfite ($NaHSO_3$; 3000, 20°C), potassium metabisulfite ($K_2S_2O_5$; 250, 0°C), and sodium metabisulfite ($Na_2S_2O_5$; 540, 20°C) (Ough, 1993b).

The most important factor impacting the antimicrobial activity of sulfites is pH. Sulfur dioxide and its salts set up a pH-dependent equilibrium mixture when dissolved in water:



Aqueous solutions of sulfur dioxide theoretically yield sulfurous acid (H_2SO_3), however evidence indicates that the actual form is more likely $SO_2 \cdot H_2O$ (Gould and Russell, 1991). As the pH decreases, the proportion of $SO_2 \cdot H_2O$ increases and the bisulfite (HSO_3^-) ion concentration decreases. The pK_a values for sulfur dioxide, depending upon temperature, are 1.76–1.90 and 7.18–7.20 (Rose and Pilkington, 1989; Gould and Russell, 1991; Ough, 1993b). The inhibitory effect of sulfites is most pronounced when the acid or $SO_2 \cdot H_2O$ is in the undissociated form (Hailer, 1911). Therefore the most effective pH range is <4.0. King et al. (1981) proved this when they found that undissociated $H_2SO_3(SO_2 \cdot H_2O)$ was the only form active against yeast and that neither HSO_3^- nor SO_3^{2-} had antimicrobial activity. Similarly, $SO_2 \cdot H_2O$ was shown to be 1000, 500, and 100 times more active than HSO_3^- or SO_3^{2-} against *E. coli*, yeast, and *Aspergillus niger*, respectively (Rehm and Wittman, 1962). Increased effectiveness at low pH likely is due to the fact that unionized sulfur dioxide can pass across the cell membrane in this form (Rahn and Conn, 1944; Ingram et al., 1956; Rose and Pilkington, 1989).

Sulfites, especially as the bisulfite ion, are very reactive. These reactions not only determine the mechanism of action of the compounds, they also influence antimicrobial activity. For example, sulfites form addition compounds (α -hydroxysulfonates) with aldehydes and ketones. These addition compounds are in equilibrium in solution with free sulfite ions, resulting in the formation of a thiol (R-SH) and S-substituted thiosulfates (R-SSO₃) (Means and Feeney, 1971). It is generally agreed that these bound forms have much less or no antimicrobial activity compared to the free forms. For example, Ough (1993b) reported that addition compounds with sugars completely neutralized the anti-

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crobial activity of sulfites against yeast. However, Stratford and Rose (1985) did demonstrate that pyruvate-sulfite complexes retained some activity against *Saccharomyces cerevisiae*.

Sulfurous acid inhibits yeast, molds, and bacteria; however, yeasts and molds are generally less sensitive to sulfur dioxide than bacteria (Hailer, 1911). As antimicrobials, sulfites are used primarily in fruit and vegetable products to control three groups of microorganisms: spoilage and fermentative yeasts and molds on fruits and fruit products (e.g., wine), acetic acid bacteria, and malolactic bacteria (Ough, 1993b).

Sulfur dioxide is fungicidal even in low concentrations against yeast and mold. The inhibitory concentration range of sulfur dioxide against yeasts is as follows ($\mu\text{g/mL}$): *Saccharomyces*, 0.1–20.2; *Zygosaccharomyces*, 7.2–8.7; *Pichia*, 0.2; *Hansenula*, 0.6; *Candida*, 0.4–0.6 (Rehm and Wittmann, 1962). Goto (1980) determined that wild yeasts of the general *Saccharomyces* and *Torulopsis* were the most tolerant to sulfur dioxide in grape juice. Roland et al. (1984) and Roland and Beuchat (1984) compared the effectiveness of sodium benzoate, potassium sorbate, and sulfur dioxide against *Byssoschlamys nivea* growth and patulin production in grape and apple juices. Sulfur dioxide at 25–100 $\mu\text{g/mL}$ was the most effective inhibitor (based upon concentration) of both growth and toxin production. Parish and Carroll (1988) found that sulfur dioxide had greater antimicrobial effectiveness alone than in combination with benzoate or sorbate against *Saccharomyces cerevisiae* Montrachet 522. Similarly, Knox et al. (1984) showed no synergistic antimicrobial interactions with combinations of sulfur dioxide, potassium sorbate, and butylated hydroxyanisole against *Saccharomyces cerevisiae* in grape or apple juice.

Against some bacteria, low concentrations of sulfur dioxide (1–2 $\mu\text{g/mL}$) are bacteriostatic, while only high concentrations are bactericidal. This may be due to differential uptake of sulfur dioxide by fungi and bacteria. Sulfur dioxide at 1–10 $\mu\text{g/mL}$ is capable of inhibiting most lactic acid bacteria in fruit products at pH 3.5 or less (Wibowo et al., 1985). Tompkin et al. (1980) found that addition of sodium metabisulfite as a source of sulfur dioxide delayed *C. botulinum* outgrowth in perishable canned comminuted pork when it was temperature-abused at 27°C. Sulfurous acid and an excess of aldehyde in a medium inhibited growth of *Lactobacillus hilgardii* and *Leuconostoc mesenteroides* isolated from wine (Fornachon, 1963). Sulfur dioxide is reported to be more inhibitory to gram negative bacteria such as *E. coli* and *Pseudomonas* than to gram positive bacteria (Roberts and McWeeny, 1972). Banks and Board (1982) tested several genera of *Enterobacteriaceae* isolated from sausage for their metabisulfite sensitivity. The microorganisms tested and the concentration of free sulfite ($\mu\text{g/mL}$) necessary to inhibit their growth at pH 7.0 were as follows: *Salmonella* sp., 15–109; *E. coli*, 50–195; *Citrobacter freundii*, 65–136; *Yersinia enterocolitica*, 67–98; *Enterobacter agglomerans*, 83–142; *Serratia marcescens*, 190–241; and *Hafnia alvei*, 200–241.

The most likely targets for inhibition by sulfites include disruption of the cytoplasmic membrane, inactivation of DNA replication, protein synthesis, inactivation of membrane-bound or cytoplasmic enzymes, or reaction with individual components in metabolic pathways. Cell damage may result from interaction with SH groups in structural proteins and interactions with enzymes with SH groups in structural proteins and interactions with enzymes, cofactors, vitamins, nucleic acids, and lipids. The sensitivity of enzymes with SH groups is a primary inhibitory effect against NAD-dependent reactions (Pfleiderer et al., 1956). Sulfur dioxide also reacts with end products or intermediate products and inhibits enzyme chain reactions. Sulfur dioxide cleaves essential disulfide linkage

in proteins and induces changes in the molecular confirmation of enzymes. This modifies the enzyme active site or destroys the coenzymes. It destroys the activity of thiamine and thiamine-dependent enzymes by cleavage and produces cytotoxic effects by cross-linking individual nucleic acid residues or nucleic acid residues and proteins. It also damages cell metabolism and membrane function by peroxidizing lipids. One or more of these factors may result in microbial death or inhibition (Hammond and Carr, 1976).

Sulfur dioxide is used to control the growth of undesirable microorganisms in soft fruits, fruit juices, wines, sausages, fresh shrimp, and acid pickles, and during extraction of starches. It is added to expressed grape juices used for making wines to inhibit molds, bacteria, and undesirable yeasts. The concentration of sulfur dioxide used depends on the cleanliness, maturity, and general condition of the grapes, but 50–100 ppm ($\mu\text{g}/\text{mL}$) is generally used (Amerine and Joslyn, 1960). At appropriate concentrations, sulfur dioxide does not interfere with wine yeasts or with the flavor of wine. During fermentation, sulfur dioxide also serves as an antioxidant, clarifier, and dissolving agent. The optimum level of sulfur dioxide (50–75 ppm) is maintained to prevent post fermentation changes by microorganisms. Sulfur dioxide, at 0.01–0.2%, is used as a temporary preservative in fruit products. Residues from the final products are removed by heat or vacuum (Lueck, 1980). Sulfur dioxide is not only used as an antimicrobial, but also has other functions such as protection against oxidative, enzymatic, and nonenzymatic browning reactions and inhibition of chemically induced color losses. Sulfur dioxide used as a solution in water is very effective and controls the growth of *Botrytis*, *Cladosporium*, and other molds on soft fruits (Roberts and McWeeny, 1972). It is used extensively in preserving strawberries, raspberries, and gooseberries after picking for jam production. In this way, jam production may be spread over the year rather than concentrated in the harvesting season. Sulfur dioxide solution in water is used to sanitize equipment.

In some countries, sulfites may be used to inhibit the growth of microorganisms on fresh meat and meat products (Kidney, 1974). Sulfur dioxide restores a bright color, but may give a false impression of freshness. Sulfite or metabisulfite added in sausages is effective in delaying the growth of molds, yeast, and salmonellae during storage at refrigerated or room temperature (Ingram et al., 1956). Banks and Board (1982) showed that 600 $\mu\text{g}/\text{g}$ sodium metabisulfite prevented the growth of species of *Enterobacteriaceae* at 4, 10, and 15°C, but not at 22°C in fresh sausage. *Salmonella* did not grow in sulfited sausage except at 25°C.

The U.S. FDA considers sulfur dioxide and several sulfite salts as GRAS (21 CFR 182). However, sulfites cannot be used in meats, food recognized as a source of thiamine, or on fruits or vegetables intended to be served raw to consumers, sold raw to consumers, or presented to consumers as fresh. They are allowed in fruit juices and concentrates, dehydrated fruits and vegetables, and in wine. In other countries, sulfites may be permitted in meats, meat products, poultry, poultry products, and seafood (Chichester and Tanner, 1981). The maximum level of sulfur dioxide allowed in wine was set at 350 mg/L by the regulating body for the U.S. alcoholic beverage industry, the Bureau of Alcohol, Tobacco and Firearms of the Department of the Treasury. The amount of sulfites used in food products is dictated by good manufacturing practice.

The oral LD_{50} for rats is 1000–2000 mg sulfur dioxide per kilogram body weight. The LD_{50} for rabbits and cats was determined as 600–700 and 450 mg sulfur dioxide/kg body weight, respectively. In dogs and human beings, fatal poisoning is not possible because sulfur dioxide induces vomiting (Lang, 1960a). The toxic effect of sulfur dioxide in humans is variable. Some persons may tolerate up to 50 mg/kg body weight, while

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others have headache, nausea, and diarrhea (Schroeter, 1966). Sodium bisulfite fed at 0.5–2% in feed to rats had an injurious effect on the nervous system, reproductive organs, bone tissue, kidneys, and other visceral organs within 12 months (Fitzhugh et al., 1946). Inhalation of sulfur dioxide at concentrations higher than 33 mg/L in air may cause death due to pulmonary dysfunction (Amadur, 1980). Postmortem lesions include pulmonary edema, lung hemorrhage, and visceral congestion. Symptoms observed before death include coughing, lacrimation, and sneezing. Sulfites binding with certain nucleotides may cause point mutations. Such mutagenicity was observed in a lambda phage of *E. coli* with a dose of 3 M sodium bisulfite at pH 5.6 for 37°C for 1.5 h (Hayatsu and Miura, 1970). Mutation was induced in cytosine–guanine pairs at specific sites (Mukai et al., 1970). That sulfites destroy thiamine has been known since 1935 (Williams et al., 1935). Symptoms of thiamine deficiency accompanied by decreased urinary excretion of thiamine are observed (Til et al., 1972a,b). Growth inhibition in rats fed 0.1% or more of sulfite was due to thiamine destruction (Fitzhugh et al., 1946). Bhagat and Lockett (1964) observed that feeds containing 0.6% sodium metabisulfite produced two types of toxic effects in rats. Feeds stored for 7 weeks resulted in vitamin B₁ (thiamine) deficiency symptoms. However, feeds stored for 3–4 months produced diarrhea and growth retardation that were not reversed by thiamine administration. Sulfites elicit allergic responses in certain individuals, especially steroid-dependent asthmatics (Stevenson and Simon, 1981). This led to the ban on the use of sulfites on raw fruits and vegetables to be consumed fresh.

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