ANTIMICROBIAL EFFECTS OF SELECTED ANTIOXIDANTS IN LABORATORY MEDIA AND IN GROUND PORK

by

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This thesis is dedicated

to my brother, Hassan.

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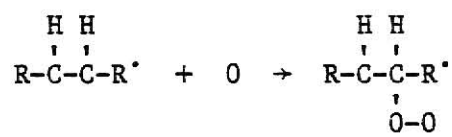
INTRODUCTION

Oxidative rancidity of lipids is a major cause of food deterioration. It results from the reaction of oxygen with unsaturated fatty acids in lipids or lipid-containing foods. Sometimes it is referred to as autoxidation because the rate of oxidation increases as the reaction proceeds. Unless mediated by other oxidants or enzyme systems, oxidation proceeds through a free-radical chain reaction mechanism involving three states: 1) initiation, formation of free radicals, 2) propagation, free radical chain reaction, and 3) termination, formation of non-radical products. In the initiation stage an unsaturated hydrocarbon loses a hydrogen to form a radical, $R\ H \rightarrow R^{\cdot} + H^{\cdot}$, and oxygen is added at the double bond to form a di-radical:



During propagation, the chain reaction is continued by $R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$ and $ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$ to form peroxy radicals, hydroperoxides, and new hydrocarbon radicals. The new radical formed then contributes to the chain by reacting with another oxygen molecule.

When two radicals react, termination occurs:

$$R^{\bullet} + R^{\bullet} \rightarrow RR$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$$

$$RO^{\bullet} + R^{\bullet} \rightarrow ROR$$

$$ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$$

$$2RO^{\bullet} + 2ROO^{\bullet} \rightarrow 2ROOR + O_2$$

Autoxidation can be retarded by vacuum packaging, refrigeration, or addition of antioxidants to the food product. An antioxidant is a substance that is added to fats or fat-containing foods to retard oxidation and thereby prolong their wholesomeness and palatability (2,10,15,32).

Ideally an antioxidant should:

1. have no harmful physiological effects,
2. not contribute objectionable flavor, odor, or color to the food in which it is added,
3. be effective in small concentrations,
4. be fat soluble,
5. persist following processing to provide effective protection to the food in which it exists,
6. be readily available,
7. be economical.

In food systems, the most effective antioxidants function by interrupting the free radical chain mechanism, while those used in non-food products may function as peroxide decomposers.

An antioxidant AH apparently reacts with radicals produced during autoxidation according to the scheme:

$$R^{\bullet} + AH \rightarrow RH + A^{\bullet}$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$R^{\bullet} + A^{\bullet} \rightarrow RA$$

$$RO^{\bullet} + A^{\bullet} \rightarrow ROA$$

According to Ranney (44), antioxidants are used in many products including:

1. Polyolefin resins
2. Polyvinyl chloride resins
3. Polyester, polycarbonate and polyurethane plastics
4. Elastomers
5. Petroleum products and synthetic lubricants
6. Food products

Antioxidants used in food products include:

a. Nonabsorbable antioxidants, those of a molecular size so as to not appreciably be absorbed from gastrointestinal tract. They include polystyrylphenols, phenolicdivinylbenzene polymers, polymeric N-substituted maleimides, polymeric hydroquinones, and covalently bonded antioxidants.

b. General antioxidants including phenolic and degraded protein, substituted hydroquinone, tertiary butyl hydroquinone, butylated hydroxyanisole, chroman compounds, derivatives of 3, 4-dihydroxyphenylalanine, substituted hydroxylamines, substituted aminophenol compounds, dilauryl thiodipropionate, methional, dialkyl

pentaerythritol diphosphite, polymeric triarylphosphine compounds, coriander shoots and leaves.

c. Ascorbic acid, halogen derivates of ascorbic acid, and granular sorbic acid.

d. Others; carotene, esterified synthetic polysaccharides, and non chemically inhibited starch.

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) are commonly added to foods to prevent autoxidation. They comply with safety regulations of the Food and Drug Administration of the United States. However, these regulations limit the concentrations of antioxidants applied to foods to 0.01%, based on the fat content of the food when one antioxidant is used, and to less than 0.02% of a combined total of which no one antioxidant exceeds 0.01% when more than one antioxidant is used (13).

Antioxidants, like other food additives, are evaluated basically upon the benefits and risks that result from their addition to a food product. Food additives that have dual or multiple functions in food products are more beneficial and more economical. It is thus essential to define the risks and benefits of these antioxidants in order to evaluate them. In addition to their antioxidant properties, BHA, BHT, PG, and TBHQ were found to have antimicrobial properties against bacteria, fungi, protozoa, and viruses (11,14,22,24,26,27,28,57,70).

Meat is one of the most perishable foods. The fact that most meats are very good culture media - high in moisture, nearly neutral in pH, and high in nutrients - coupled with the fact that some microorganisms may be in the lymph nodes, bones, and muscle and contamination with spoilage

organisms is almost unavoidable makes the preservation of meats more difficult than that of most kinds of food. Contamination of the meat comes from external sources during bleeding, handling, and processing.

Meat can be contaminated with many kinds of microorganisms including molds, especially species of genera Cladosporium, Sporotrichum, Geotrichum, Thamnidium, Mucor, Penicillium, Alternaria, and Monilia, yeasts, and bacteria of many kinds among which some of the important are Pseudomonas, Lactobacillus Proteus, Flavobacterium, Bacillus, Alcaligenes, Micrococcus, Streptococcus, Sarcina, Leuconostoc, Clostridium, Escherichia, Salmonella, and Streptomyces. Many of these bacteria are psychrotrophic and can grow at chilling temperatures (16, 29). Raw meat is subject to change by its own enzymes, by microbial actions, and by oxidation of its fat. Autolytic changes include some proteolytic action on muscle and connective tissues and slight hydrolysis of fats. The defect caused by excessive autolysis has been called "souring." Souring due to autolysis is difficult to separate or distinguish from defects caused by microbial action, especially from simple proteolysis. However, this preliminary hydrolysis by the meat enzymes helps microorganisms start growing in the meat by furnishing the simpler nitrogen compounds needed by many microorganisms that cannot attack complete native proteins.

Growth of the spoilage microorganisms in meat is determined by the numbers and kinds of contaminating microbes, the spread of these microorganisms in the meat, by the intrinsic (water activity, pH, relative humidity, nutritive value, antimicrobial constituants and biological structures), and by extrinsic parameters (storage temperature, relative

humidity of the environment, and presence and concentration of gases) of meat. Grinding the meat encourages microbial growth because it increases the surface area, releases moisture, and distributes bacteria throughout the meat.

Effective methods of meat preservation have been the subject of great interest by man since the start of civilization. The continuing crises in our world food supply dictate that spoilage of any food product be reduced as much as possible. The purposes of this study were: 1) To evaluate the antimicrobial effects of different concentrations of BHA, BHT, PG, and TBHQ alone or in combinations, against 24 bacteria species (16 Gram-negative, and 8 Gram-positive) growing in laboratory media, and 2) Determine the antimicrobial efficacy of these antioxidants in preventing the growth of psychrotrophs, coliforms, and fecal coliforms in ground pork stored at 4C.

LITERATURE REVIEW

Antimicrobial Activities of Antioxidants

Ward and Ward (71) were the first to report the antimicrobial activity of antioxidants. Studying the effect of butylated hydroxytoluene (BHT) on *Salmonella senftenberg* on commercial fish meal, they found that a concentration of 1.0% BHT was inhibitory to the bacteria. They concluded that the inhibitory effects of BHT upon cultures of *Salmonella senftenberg* was slight. Inhibition beyond 24 hours would require the impractically high concentration of at least 1.0% BHT. The control of *Salmonella* in fish meal by BHT therefore has little practical value.

Chang and Branen (8) found BHT to be inhibitory to *Aspergillus parasiticus* at 250 ppm, *Staphylococcus aureus* at 150 ppm and *Escherichia coli* at 400 ppm. They proposed that this antimicrobial pattern was similar to that reported for another group of phenolic compounds, the alkyl esters of parahydroxybenzoate. They also reported that these antioxidants were more effective against fungi and Gram-positive than against Gram-negative bacteria.

Shih and Harris (55) tested the antimicrobial effects of butylated hydroxyanisole (BHA), nordihydroguaiaretic acid (NDGA), propyl gallate (PG), and a combination of BHA-PG against *Escherichia coli* and *Staphylococcus aureus* in trypticase soy broth. They found that both

PG and NDGA at 400 ppm had a strong lethal effect against E. coli. BHA and BHA-PG combination was more effective against S. aureus than against E. coli. They found that NDGA had the strongest antimicrobial activity. Their results were in accordance with the concept that phenols have more antimicrobial activity against Gram-positive than against Gram-negative cells.

Robach et al. (45) working to determine concentration of BHA required to inhibit the growth of Vibrio parahaemolyticus 04:K11 in laboratory media and a crab meat homogenate, reported that 50 ppm of BHA inhibited the growth of V. parahaemolyticus in soy broth containing 2.5% added NaCl, whereas 400 ppm of BHA was required to inhibit the growth of a crab meat homogenate. BHA had more antimicrobial activity in laboratory media than in a food product. They attributed this reduction in activity to partial inactivation of the antioxidant properties of BHA by the presence of oxidized crab meat lipids; therefore, the antimicrobial activity of BHA may depend on the lipid content and degree of lipid oxidation in the food product.

Vardaman et al. (69) tested the effect of BHT on serological responses and its chemoprophylactic effects on chicken exposed to Mycoplasma synoviae. They reported that in vitro application of 10 ppm BHT in mycoplasma medium prevented growth of M. synoviae. However, BHT when added at 100, 200, or 400 ppm in feed did not have any in vivo effects on M. synoviae serological responses or show any chemoprophylactic effect on birds infected with the organism. BHT at the 50-200 ppm level, normally used in animal feeds, would not prevent serological diagnoses of M. synoviae infection in a flock.

Stern et al. (60) studied the influence of BHA, pH and NaCl, in concert, on Staphylococcus aureus S-6 growth. They found that trypticase soy broth containing 100 ppm BHA and 5 or 7% NaCl at pH 5.0, 6.0, or 7.0 has a strong bactericidal effect against S. aureus S-6. They also found that at any pH, the presence of BHA in the medium resulted in the inhibition of S. aureus S-6 with lower concentrations of NaCl. Apparently BHA has potential as an antistaphylococcal agent.

Robach and Pierson (47) studied the effects of phenolic antioxidants on outgrowth and toxin formation in a laboratory medium by spores of three strains of Clostridium botulinum. They found that C. botulinum 10755A was inhibited by 50 ppm BHA in a prereduced thiotone yeast-extract glucose medium, whereas strains 62A and 213B were inhibited by 25 ppm BHA. Spore growth and toxin production was inhibited by 200 ppm, but no inhibition of growth or toxin production was caused by 25-200 ppm PG. They proposed that antioxidants inhibit or compete with electron donor system within the cells and thereby inhibit growth. Their results amplify the suggestion made by Wyss (72) that inhibition of microorganisms by antioxidants could be due to interference of the phenolic ring of BHA with the cell membrane, or its competition with coenzymes for proteins. They also concluded that since BHT is less effective than BHA as an antioxidant and as an antimicrobial agent, the more effective the compound as an antioxidant, the more effective it will be as an antimicrobial agent.

Klindworth et al. (36) found that Clostridium perfringens strains NCTC 10239, 8798, and 8239 were inhibited by 150 ppm BHA in fluid thioglycolate medium. Inhibition with 100 ppm increased at the pH extremes of 5.5 to 8.5. BHA at 100-200 ppm is bactericidal rather

than bacteriostatic. They proposed that BHA has a synergistic inhibitory effect when used in conjunction with nitrite, sorbic acid, or esters of parahydroxybenzoic acid. They also found that in the presence of lipids and surfactants, the antimicrobial activity of BHA is greatly reduced. They attributed this to the fact that the reaction between BHA and a lipid molecule in effect "uses up" the BHA molecules making it no longer effective as an antimicrobial agent. Similarly, the hydrophobic nature of BHA and its solubility in lipophilic compounds might result in it being localized within the lipid portion of the medium, rendering it unavailable to act against those microorganisms which grow in the aqueous phase of the medium. They concluded that several factors appear to be important in the extent of inhibition of C. perfringens by BHA. These include the age and physiological state of culture, temperature, the pH of the medium, and the presence of lipids. This last factor may limit the usefulness of BHA as an antimicrobial agent in lipid-containing food products unless other antioxidation agents such as vacuum packaging play the antioxidative role and free BHA to act against microorganisms.

Pierson et al. (43) studying the effect of combinations of BHA and propylparaben on growth of Salmonella typhimurium and Staphylococcus aureus in trypticase soy broth, found that 200 ppm BHA was bactericidal to S. aureus, while up to 400 ppm was only restrictive to growth of S. typhimurium. They found that in combination, BHA and propylparaben had no additional inhibition against S. aureus in comparison to BHA alone. However, an additive effect of the two compounds was noticeable with S. typhimurium at 150 ppm of each.

Robach and Statler (49) tested the synergistic effects of potassium sorbate when combined with BHA, tertiary butylhydroquinone (TBHQ), NaCl or ethylenediamine tetraacetic acid (EDTA) to inhibit the growth of Staphylococcus aureus in laboratory medium. The combination of sorbate and TBHQ resulted in a synergistic inhibition of growth of S. aureus 12600 on trypticase soy broth, but 25 ppm of TBHQ alone inhibited growth of strain S-6. Combinations of potassium sorbate with BHA were synergistic against both strains.

Ayaz et al. (1) studying the effects of BHT and BHA on Staphylococcus aureus in brain heart infusion, found that both antioxidants have marked antimicrobial effects on growth of 3 strains of S. aureus, and on enterotoxin production of one. The combination of the two antioxidants provided more effective inhibition than either alone.

Fung et al. (22) studied the effects of BHA and BHT on growth and aflatoxin production of Aspergillus flavus. They applied the two antioxidants to six strains of toxigenic and six strains of nontoxigenic A. flavus in a method similar to antibiotic susceptibility testing. They found that BHA (.005 - .020g per plate) inhibited the growth and toxigenesis of the test microorganisms, while BHT (.005 - .020g per plate) had no visible inhibitory effects. They also found that sporulation of the cultures had no direct relationship with toxigenesis in the presence of BHA. Their study indicated that commercial foods with BHA and/or BHT will not show inhibitory effects on all 12 cultures they tested. BHA exerted an "environmental effect" rather than a genetic effect on growth and toxigenesis of A. flavus.

Lahellec et al. (37) studied the effects of sorbate in combination with BHA, BHT, PG and TBHQ in media having pH values of 5, 6, or 7 against four toxigenic strains of Staphylococcus aureus. They found that potassium sorbate (0, 1, 3, 5, 7, 9, and 10%) inhibited the growth of S. aureus 196, S-6, 137, and 326 in liquid systems starting with 3% level. Potassium sorbate (1, 3, and 5%) in combination with BHA, BHT and TBHQ (50 and 100 ppm) exert greater bacteriostatic and bactericidal effects at pH 5 than at pH 6 and 7. They also found that S. aureus S-6 was more resistant to sorbate with or without the presence of antioxidants compared to other strains of S. aureus they tested.

Surack (63) studied the molecular toxicity of TBHQ using Tetrahymena pyriformis as a model cell system. TBHQ at 26 ppm in the media inhibited cell growth by 50% and inhibited the oxidation of ^{14}C-acetate to $^{14}CO_2$. Also, increasing the concentration of TBHQ decreased the incorporation of ^{14}C-acetate into lipids and proteins, ^{14}C-amino acids into proteins, ^{3}H-uridine into RNA, and ^{3}H-thymidine into DNA. The incorporation of ^{14}C-acetate into glycogen increased with concentration up to 20 ppm TBHQ in the media while glycogen synthesis decreased with 40 ppm TBHQ.

Surak and Singh (66) studied the cellular mode of action of BHA on cell membranes of Tetrahymena pyriformis perturbed by the antioxidant. The addition of BHA to cultures of T. pyriformis causes the organism to alter its cellular lipids composition. The initial effect of BHA on T. pyriformis may be the insertion of the molecule into the lipid bilayer thus altering membrane fluidity. When BHA was added to cultures of T. pyriformis at concentrations up to 12.5 μg/ml, it inhibited the synthesis of polar lipids. Increasing concentrations of BHA decreased the

percentage of Na-2- [^{14}C] - acetate incorporated into lysophosphatidylcholine, 2-aminoethyl-phosphonolipids, and an unknown polar lipid I while increasing the incorporation of radiolabel into phosphotidylethanolamine and an unknown polar lipid II. BHA increased the cellular level of tetrahymanol and increased the molar ratios of tetrahymanol to lipid phosphorus.

Surak (65) studied the changes in synthesis of the major lipids by Tetrahymena pyriformis when the cell is perturbed by BHA. Addition of BHA at a concentration of up to 12.5 μg/ml inhibited the synthesis of lipid fraction. He also found that the carrier used to add the antioxidant to the test culture had an effect on the cellular toxicity of the antioxidant. Dimethyl-sulfoxide reduced the toxicity of BHA when compared to ethanol. The results of his study suggest that BHA will alter the relative rates of synthesis of various lipids in T. pyriformis, thus altering the lipid composition of the cell.

Snipes et al. (57) studied the effects of BHT on the infectivity of several viruses, some that have a membrane as part of their structure and some that do not. Viruses that contain lipids were readily inactivated by BHT, whereas viruses that contain no lipids were less sensitive to BHT.

Kim et al. (34) studied the effects of BHT on human and murine cytomegalovirus (CMV), Semliki forest virus (SFV), and vaccina virus. BHT at 40 μg inactivated human and murine CMV, and SFV virus, but had no effect on poliovirus, which does not contain a lipid membrane as a part of its structure. BHT may have potential as a broad spectrum antiviral agent.

Sankaran (52) using the disc diffusion and plate count method, studied the antimicrobial effects of BHA, BHT, PG, calcium gluconate, sodium sorbate, nisin, sorbic acid, and orange oil against Bacillus subtilis, Saccharomyces cerevisiae, Aspergillus flavus, and Aspergillus fumigatus. At 2500 ppm, the inhibitory effect is highest with orange oil and sorbic acid. Of the antioxidants tested, only BHA is effective in completely inhibiting growth. Lower concentrations delay germination of Aspergillus flavus by 6-12 days and appearance is different from normal colonies. Hence, BHA may aid in delaying the germination and growth of spoilage microorganisms at lower concentrations, and inhibiting them at higher concentrations. Its activity against sporulating toxigenic fungi such as Aspergillus flavus and Aspergillus fumigatus is of particular significance in products such as cured meat or fish.

Drake (12) evaluated the effects of antioxidant or polyphosphate treatment, storage temperature and packaging methods on the keeping quality of purified frozen channel catfish. He found that TBA (Thiobarbituric Acid) values increased during the first 8 months of storage, regardless of treatment; and remained within acceptable limits for 12 months. The bacteriological quality of the precooked fish remained excellent throughout storage for 12 months at 0 or 10 F. Polyphosphates, BHA, and BHT had no effects on quality. Treatment with PG was beneficial, especially for samples fried in lard or hydrogenated corn oil.

Pelroy et al. (41) described a process for the preparation of press cake that is stable at ambient temperatures to microbiological and oxidative deterioration. Fish protein concentrate (FPC) made from a press cake stabilized with antioxidants and sodium metabisulfate or

isopropanol had the same organoleptic and nutritional properties (protein efficiency ratio) as FPC made by conventional processing.

Sahoo (51) studied the formulation of intermediate moisture pork, and its stability during long term storage under various conditions. Addition of antioxidants to the product prevented increases in TBA values and off-flavor development during storage.

Oluski et al. (40) evaluated the antimicrobial activity of selected antioxidants in comminuted meat. BHT inhibited the growth of *Staphylococcus aureus*, but was less effective against *Escherichia coli*, and did not affect the growth of *Streptococcus faecalis*.

Vantassell et al. (68) studied the inhibition of *Salmonella typhimurium* and *Staphylococcus aureus* in tryticase soy broth by the propyl ester of P-hydroxybenzoic acid and BHA alone and in combination. At 100 ppm BHA - 100 ppm propyl paraben combination was bacteriostatic whereas at 150 ppm BHA - 150 ppm propyl paraben was bactericidal to *S. aureus*. Growth of *S. typhimurium* was restricted by 250 ppm propyl paraben, 200 ppm BHA or a 75 ppm BHA - 75 ppm propyl paraben combination. In the presence of either 300 ppm propyl paraben, 400 ppm BHA or a combination of 100 ppm BHA - 100 ppm propyl paraben, a chemical injury recovery phenomenon occurred.

Research done thus far indicates a potential use of the antioxidants, in food products, to retard oxidative rancidity and to control microbial growth.

Safety of Their Use in Food

Johnson and Hewgill (30) studied the effect of BHA, BHT, and PG at different dietary levels in the rat. Their results confirmed the findings by Brown et al. (6) that there are greater hazards in the use of BHT than in that of BHA as antioxidants for use in human foodstuffs. Their results showed BHA and PG to meet the requirements for safety as food additives.

Branen (3) studied the lipid and enzyme changes in organs of monkeys fed BHA and BHT. With the exception of cholesterol levels, neither high nor low levels of BHA or BHT caused any alterations in the lipid levels of juvenile monkeys. BHA and BHT prevented many of the changes caused by feeding corn oil, i.e. increased liver triglyceride and lipid phosphorus levels and increased plasma acid phosphatase. This prevention of fatty liver formation by the antioxidants was related to prevention of lipid peroxidation and maintenance of normal enzymatic activities.

Brown et al. (6) studied the effect of BHA and BHT with variation in the type and quantity of dietary fat, on growth, food consumption, reproduction, mortality, organ weights, and post-mortem pathology of the rat. Using the normal cellular behavior of experimental animals fed diets containing 100 times the amounts of antioxidant normally expected to be present in human foods (Normal Testing Dose [NTD]) as the conventional yardstick of non-toxicity, BHA fulfilled the requirements of non-toxicity. BHT, at the NTD, on the other hand, produced deleterious deviations from normal cellular behavior.

Gad et al. (23) investigated the inhibitory actions of BHT on isolated ileal, atrial, and perfused heart preparations. BHT at 500, 100 and 10 ppm depressed methacholine-induced contractions of isolated rat and rabbit ileal preparation. BHT at the same concentrations depressed the frequency and amplitude of contractions of isolated atrial preparations as compared to controls. Their investigations provided the first evidence that BHT directly depresses contractility of isolated atria, heart, and ileum, and that cellular damage occurs in the isolated heart during this process, and that BHT may affect cardiovascular functions.

Branen (4) reviewed some biochemical effects of subacute levels of BHA and BHT. He estimated that man consumes ca. 0.1 mg/Kg body weight daily of these antioxidants. At levels 500 times this level (50 mg/Kg/day), both BHA and BHT appear to be free of any injurious effects. However, at larger doses (500 mg/Kg/day), both BHA and BHT result in certain pathological, enzyme and lipid alterations in both rodents and monkeys, and BHT in some cases has been reported to have certain teratogenic and carcinogenic effects upon rodents. Alterations appear to differ markedly between rodents and monkeys, apparently as a result of differences which exist in the metabolism and excretion of BHA and BHT by these two species. However, in both animal species, the alterations appear to be physiological responses which are reversible upon removal of BHA and BHT from the diet. Long term chronic ingestion of BHA and BHT may be beneficial in sparing vitamin E and in modifying the acute toxicity of a number of mutagenic and carcinogenic chemicals.

Surak et al. (64) studied the effects of BHA and BHT on serum and liver lipid levels in chicken. They found that BHA and BHT did not affect chicken growth, blood lipid levels or liver lipid levels when the birds were fed a normal ration. But, when a high cholesterol ration was fed, chickens had increased levels of blood and liver lipids, and experienced decreased growth rates. They reported that dietary BHA and BHT play some role in lipid metabolism and transport.

Fulton et al. (17) tested the effect of BHT on rats. No significant differences occurred in body weight, liver weight, ileum histology, or bone marrow histology between the controls and the animals fed 0.02% BHT. However, rats fed BHT at concentrations greater than 0.02% of the diet had a reduction in body weight, villi height, crypt depth, goblet cell count, and a significant increase in liver to body weight ratio.

To evaluate antioxidant cardiotoxicity at the cellular level, Leslie et al. (39) examined the effects of BHT and BHA on lactic dehydrogenase leakage, and depression of beating rates of primary cultures of neonatal rat heart cells. BHT and BHA at 100 ppm produced marked leakage of lactic dehydrogenase from cultured myocardial and endothelial cells in the culture medium. At this concentration both antioxidants depressed the beating rate of cultured heart cells. When the concentrations increased to 100 ppm, marked cell lysis occurred. Their results suggest that both BHT and BHA, in relatively large concentrations, produce injury to myocardial cells in culture.

Singer and Jeffery (56) examined the interaction between BHT and a model phospholipid membrane. They monitored the interaction of BHT with phospholipid bilayer by measuring changes in ^{22}Na transport and

hydrocarbon chain motion (vesicles composed of saturated phospholipids display a marked increase in ^{22}Na permeability in the temperature region of the phase transition temperature of the component phospholipids). BHT greatly reduced the permeability increase, and also lowered the temperature at which the lipid chains display significant motional freedom, i.e. melt. (The phase transition temperature of a phospholipid is associated with a marked increase in fatty acyl chain motion). They proposed the following model as a working hypothesis for the interaction between BHT and phospholipid bilayer membranes. As a result of its high oil solubility, BHT will partition strongly into the lipid phase. However, rather than being 'buried' deep within the bilayer, BHT, by virtue of its hydroxyl group would properly be localized close to the interface. Presumably, this molecule would be intercalated between phospholipid molecules with its hydroxyl group anchored to the membrane-water interface by polar interactions. In this position, BHT could easily disrupt phospholipid packing resulting in the alterations in fatty acyl chain motion.

Johnson (31) reviewed the literature regarding the safety of phenolic antioxidants in foods. He concluded that at the levels of use fixed by present day legislation there is no evidence of any danger to the consumer arising out of the use of BHT, BHA or the gallate esters.

Other Antimicrobial Agents

Lin et al. (38) tested the effect of several additives, sodium chloride, glucose, sorbitol, spice extracts; oleoresins of cardamon, coriender, paprika, BHA, BHT, PG, citric acid, 5'-nucleotides (GMP,

IMP), monosodiumglutamate (MSG), nicotinamide, saccharin, and sorbic acid, on the growth of microorganisms in ground beef. They found that NaCl inhibited growth at 1.5% and 3% by weight, while none of the other additives, either alone or in combinations, affected the growth of the microorganisms as determined by total counts, micrococci, or lactic acid bacteria. The antioxidants at 0.005% by weight, individually or in combination, were effective in preserving the meat color, but had no effect on microbial growth.

Kanner and Juren (33) studied the effects of S-nitrosocysteine (RSNO) as an inhibitor of lipid oxidation, as a color developing, and as anticlostridial agent in a comminuted turkey meat product. Similar effects were obtained by 25 ppm of nitrite or when the corresponding molar concentration of RSNO in color development and inhibition of lipid oxidation in cooked cured meat stored under anaerobic conditions. However, in a microbiological medium or in a meat product, RSNO had less anticlostridial activity than nitrite.

Pierson et al. (42) tested the ability of potassium sorbate to suppress growth of Staphylococcus aureus in vacuum packaged bacon containing various amounts of sodium nitrite and potassium sorbate. Levels of 0.13 and 0.26% potassium sorbate and no nitrite were most effective in suppressing growth of S. aureus through 14 days of storage at 27 C, but when stored at 13 C, bacon containing nitrite and potassium sorbate exhibited lower number of S. aureus after 7 days than did the bacon containing potassium sorbate alone.

Robach (46) investigated the effect of potassium sorbate on growth of two strains of Pseudomonas putrifaciens in broth at pH near that of

fresh poultry. Addition of 0.2% sorbate to trypticase soy broth (pH 6.0) inactivated strain P19X and resulted in a 3-log cycle reduction in number of viable cells of strain P5L1N through 6 days of incubation at 24 C.

Sofos et al. (58) studied the effects of nitrite and/or sorbic acid on Clostridium botulinum growth and toxin production in Frankfurter-type mixtures at elevated temperature (27 C). Sorbic acid alone (0.2%) or in combination with nitrite (80 μg/g) retarded spore germination and outgrowth, and delayed toxin production. The effects were greater in mixtures formulated with both nitrite and sorbic acid, and were more profound in beef, pork, and soy proteins compared to chicken.

Pork Characteristics

Due to its relatively high unsaturated fats, pork is more susceptible to oxidative rancidity than beef or lamb (25).

In studying the microbiology of pork packaged in various gas atmospheres, Christopher et al. (9) found that differences, both in psychrotrophic and lactobacillus counts, between roasts stored in modified gas atmosphere (100% O_2, 20% CO_2 + 80% N_2, 50% CO_2 + 50% O_2, 20% CO_2 + 80% O_2, 25% CO_2 + 25% O_2 + 50% N_2, or 51% CO_2 + 30% O_2 + 18% N_2 + 1% CO) and those stored in vacuum-packages were rarely statistically significant. The initial microbial flora of the roasts consisted of Pseudomonas spp. and Lactobacillus spp. Pseudomonas spp. remained a significant part of the microflora of roasts stored in 100% O_2 whereas lactic acid bacteria (Lactobacillus and Leuconostoc spp) predominated in roasts after 1 week in all other atmospheres tested.

Pierson et al. (42) evaluated the ability of potassium sorbate to suppress growth of Staphylococcus aureus in vacuum packaged bacon containing various amounts of sodium nitrite and potassium sorbate. Bacon with 0.13 and 0.26% potassium sorbate and no nitrite was most effective in suppressing growth of S. aureus through 14 days of storage at 27 C. When stored at 13 C, bacon containing nitrite and potassium sorbate exhibited lower numbers of staphylococci after 7 days than did the bacon containing potassium sorbate alone. Use of 40 ppm sodium nitrite with or without sorbate, or 120 ppm sodium nitrite in bacon should provide adequate protection against S. aureus for at least 14 days when the product is properly refrigerated.

Stiles and Ng (61) studied the fate of pathogens inoculated on to vacuum-packaged ham slices to simulate contamination during packaging. They contaminated the ham with five pathogens: Bacillus cereus, Clostridium perfringens, Escherichia coli, Salmonella typhimurium, and Staphylococcus aureus. All pathogens, except C. perfringens, grew actively in fresh ham and chopped ham with abused holding at 30 C or 21 C. After storage at 4 C or 10 C for 30 days, B. cereus and C. perfringens were no longer detected, even after subsequent holding at 30 or 21 C for 24 h. S. typhimurium survived well, and S. aureus was generally inhibited at high levels of competition. Contamination of ham with low levels of pathogens represented little hazard from the enterotoxigenic pathogens, which must grow to cause food poisoning, providing conditions of storage and handling are reasonable. In contrast, infective pathogenic bacteria survived even prolonged period of product storage. Hence, contamination of product at manufacturers level

represents a potential hazard, especially if the product is subsequently abused by poor consumer handling.

A bacteriological survey of fresh pork sausage was made by Surkiewicz et al. (67). At the time of manufacture, 75% of 67 sets of finished fresh pork sausage collected in 44 plants had aerobic plate counts in the range of 500,000 or fewer/g; 88% contained 100 or fewer *Escherichia coli*/g; and 75% contained 100 or fewer *Staphylococcus aureus*/g. *Salmonellae* were isolated from 28% of 529 samples of pork trimmings used for sausage, and from 28% of 560 finished sausage samples. Semiquantitative analysis revealed that *Salmonellae* were at low levels; more than 80% of *Salmonellae*-positive samples were positive only in 25-g portions (negative in 1.0 and 0.1-g portions).

MATERIALS AND METHODS

Effect of Antioxidants on Selected Microorganisms in Laboratory Media

Organisms Tested

The following microorganisms were used to ascertain the effects of the four selected antioxidants on a wide range of pathogenic and non-pathogenic bacteria.

a) Gram-negative bacteria

1. *Arizonia sp*
2. *Citrobacter freundii*
3. *Edwardsiella tarda*
4. *Enterobacter aerogenes*
5. *Enterobacter cloacae*
6. *Escherichia coli*
7. *Klebsiella oxytoca*
8. *Klebsiella pneumoniae*
9. *Proteus vulgaris*
10. *Pseudomonas aeruginosa*
11. *Pseudomonas fluorescens*
12. *Pseudomonas fragi*
13. *Salmonella sp 20*
14. *Serratia marcescens*
15. *Vibrio angularum*
16. *Yersinia enterocolitica*

b) Gram-positive bacteria

1. *Agrobacterium tumefaciens*
2. *Arthrobacter sp*
3. *Lactobacillus brevis*
4. *Pediococcus sp*
5. *Sarcina lutea*
6. *Staphylococcus aureus* 100
7. *Staphylococcus aureus* 137
8. *Streptococcus faecalis*

All the cultures were obtained from the Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas. Identities of the bacterial cultures were confirmed by using morphology, Gram-reaction, and the Minitek system (BBL microbiology system, Cockeysville). The characteristics of the named bacteria corresponded well with their descriptions in Bergey's manual (7).

Working cultures were prepared by aseptically transferring one loopful of culture from stock culture into 25 ml brain heart infusion, and incubating for 24 hours at either 37C or 32C (for *Agrobacterium tumefaciens*, *Arthrobacter* sp, *Lactobacillus brevis*, *Pediococcus* sp, and *Pseudomonas fluorescens*).

Media Used

The named cultures were studied in brain heart infusion (Difco) as a liquid system, and nutrient agar (Difco) as a solid system. Both liquid and solid systems were tested because antioxidants are being used in liquid foods such as oil and milk, and solid foods such as meat, cheese, and bread. There were some conflicting opinions on using liquid versus solid system in studying effects of antioxidants in food. On one hand, some workers (8,36, and 55) used liquid systems and claimed that the system performed well, while Fung et al. (22) considered a liquid system not as effective as a solid system. Use of both systems in this study may elucidate the effectiveness of each system for studies of this type.

Antioxidants Tested

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) were selected for this study because they are commonly used in foods. Some information on the antimicrobial effects of BHA and BHT is available, which will provide a base for comparison of the effects of these antioxidants on some organisms. The effects of PG and TBHQ on microorganisms has not been extensively studied.

BHA, BHT, PG, and TBHQ were obtained from Eastman Chemical Products Inc., Tennessee. A stock solution (10,000 ppm) of each antioxidant was prepared by dissolving 0.1g of the antioxidant in 10ml of 95% ethyl alcohol. Appropriate amounts were then pipetted from the stock solutions into the growth media (v/v) to give the required concentrations in parts per million (ppm). Concentrations tested were 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 ppm for each antioxidant alone, and 100, 200, and 400 ppm for combinations of the four antioxidants. These concentrations covered a reasonable range for practical application in foods as well as inhibition of microorganisms. The antioxidants were added to the growth media before autoclaving. Preliminary experiments indicated that the antioxidants are stable after autoclaving at 121C for 15 minutes.

Miniaturized Microbiological Testing Procedures for Solid and Liquid Media

All tests were conducted using the "miniaturized microbiological method" developed by Fung (18,19,20,21). Tests were made in the wells

of sterile microtiter plates (8 x 12 wells/plate) for the liquid systems, and rectangular plates (8.5 x 12.5 x 1.0 cm) for the solid systems. The sterile plates and sterile covers were obtained from Dyna-tech Lab group, Alexandria, Virginia. For the liquid system, 0.2ml of sterile media, with or without antioxidants, were added to each well. The plates were covered with sterile plate covers, which prevented contamination and evaporation during storage and incubation of the plates.

A "master plate" was prepared from the individual bacterial cultures by aseptically transferring 2 drops (0.2ml) from a culture into one of the 96 wells of the sterile microtiter plate. An inoculation device having 96 pins, with their heads pointing outwards, was used for inoculation. The pins were spaced so that each one of them fits into one of the wells in the microtiter plate. Sterilization of the inoculation device was achieved by dipping the pins into alcohol for 20-30 seconds, and then flaming, according to Fung and Hartman (20). The sterile inoculation device was then charged with bacterial cultures by dipping it into the "master plate." Organisms were then transferred into either sterile solid (Nutrient Agar) or liquid (Brain Heart Infusion) plates by introducing the charged inoculator into the liquid or solid media. Each pin head transferred about 2×10^5 Colony Forming Units (CFU) of bacteria from a 24 hour old culture having 5×10^8 CFU/ml.

Brain heart infusion plates, with or without antioxidants were prepared by aseptically pipetting 0.2ml of the sterile liquid media into each well in the sterile microtiter plate. Nutrient agar plates, with or without antioxidants were prepared by pouring the sterile agar media into sterile rectangular plates, and allowing them to solidify. After

inoculation and incubation for 24 h at either 32C or 37C, the plates were examined visually for turbidity in the brain heart infusion, or the appearance of the colonies in nutrient agar. Results were recorded as positive (+) or negative (-) for growth or no growth of the cultures, respectively. Differentiation between bacteriostatic and bactericidal effects of the antioxidants, in the liquid media, was made by subculturing liquid from wells that showed no growth, on to sterile nutrient agar. The agar plates were incubated at 32C for 24 hours, before checking for bacterial growth. Positive growth at this last stage indicated bacteriostatic activity, in liquid media with antioxidants, whereas no growth indicated bactericidal activity of the antioxidant or combination of antioxidants. All tests were done in duplicate.

Effects of Antioxidants on Psychrotrophs, Coliforms, and Fecal Coliforms of Ground Pork During Cold Storage

Ground pork was chosen as a meat model system for studying the effects of antioxidants on psychrotrophs, coliforms, and fecal coliforms because it may have autoxidation potential and antioxidants may therefore serve two functions.

Preparation of Samples

Five fresh pork samples, 20 lb each, taken from different carcasses, were obtained from the Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas. Fat was removed from the cuts, and the lean and the fat were ground separately (grinding was done in two

steps, first coarse grinding then fine grinding). The percentage of fat in the lean meat was determined by Hobart fat percentage indicator for ground beef (Hobart Corp., Troy, Ohio). The percent fat was then adjusted to 25% by adding ground fat (ca. 95% fat) to the lean trim. The fat and lean were mixed, reground, and divided into 100g portions in sterile "Stomacher" bags. These sterile bags were manufactured for use along with a machine called the "Stomacher" designed to massage food samples (54). The bags serve as a vessel for blending pork, as well as a storage container for the pork, and a dilution vessel for viable cell counts. The "Stomacher" was used because it eliminates efforts involved in cleaning and sterilizing reusable blenders, and it releases significantly larger numbers of viable microorganisms than most other methods of homogenization.

Addition of Antioxidants

Preparation of antioxidant solutions of BHA, BHT, PG, and TBHQ was previously described. Appropriate amounts, from stock solutions, were pipetted into the 100g meat samples to give the required concentrations (0, 100, 200, and 400 ppm). The control contained no antioxidants. All treatments were done in duplicate. The antioxidants were added to the meat and were massaged in the "Stomacher" bags for 2 minutes in the "Stomacher." Each bag was then rolled, and tied with a rubber band. For each treatment, 6 identical bags were prepared. Microbiological analysis was made on each of two bags after 1, 2, and 4 weeks of storage at 4C. Eight bags were prepared from the control samples and analyzed at zero time, 1, 2, and 4 weeks of storage at 4C.

Microbiological Analysis

Ground pork (100g) was massaged for 2 minutes in the "Stomacher," after the addition of 300ml of sterile buffer. The slurry was again diluted in 99ml of sterile buffer. Pour plates of Standard Plate Count Agar (Difco) were made in duplicate and incubated for 10 days at 7C, for the aerobic psychrotrophs counts. Enumeration of the coliforms was performed as follows: Pour plates of Violet Red Bile Agar (Difco) were prepared and allowed to solidify, then another layer of the media was poured on top to prevent spreaders. Incubation was for 24 hours at 32C for the coliforms, and at 45C for the fecal coliforms according to the procedure developed by Klein and Fung (35).

Only psychrotrophs, coliforms, and fecal coliform populations in ground pork were monitored in this study. Monitoring the effects of antioxidants on these three groups of bacteria in ground pork, during cold storage, will give an indication of the practical applicability of antioxidants as microbial suppressors.

Statistical analysis was done by analysis of variance, and Duncan's multiple range test at 0.05 significance level was used for determining differences between multiple means (70).

RESULTS

Effects of Individual Antioxidants on Selected Microorganisms in Laboratory Media

Tables 1, 2, 3, and 4 show data for individual antioxidants against all test organisms. Figures 1 and 2 show combined data expressed in percentages of cultures inhibited by each antioxidant, in nutrient agar and in brain heart infusion.

Effects of BHA on Bacterial Cultures

Table 1 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of BHA. At all concentrations tested, BHA was more effective in inhibiting the cultures in nutrient agar than in brain heart infusion. In both systems, it inhibited Gram-positive bacteria more than Gram-negative bacteria. Expressed in percentages of cultures inhibited by each antioxidant, in nutrient agar or in brain heart infusion, the percentage of cultures inhibited increased as the concentration of the antioxidant increased. At 400 ppm, BHA inhibited all the cultures in nutrient agar, except *Serratia marcescens*. This bacterium appears to be resistant to BHA even at 500 ppm. At 100 ppm, the concentration allowed for use in foods, BHA inhibited 20% of the cultures in nutrient agar (Fig. 1) and only 4.2% of the cultures in brain heart infusion (Fig. 2). *Staphylococcus aureus*

Table 1

Effect of BHA on Selected Bacteria at 32 C*

		BHA in ppm																	
		Nutrient Agar									Brain Heart Infusion								
		100	150	200	250	300	350	400	450	500	100	150	200	250	300	350	400	450	500
GRAM NEGATIVE	Arizonia sp	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Citrobacter freundii	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
	Edwardsiella tarda	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Enterobacter aerogenes	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-
	Escherichia coli	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Pseudomonas fragi	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	Salmonella sp20	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Vibrio angularum	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Yersinia enterocolitica	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Arthrobacter sp	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
	Lactobacillus brevis	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Pediococcus sp	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Sarcina lutea	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Staphylococcus aureus 100	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	Staphylococcus aureus 137	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	Streptococcus faecalis	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 2

Effect of BHT on Selected Bacteria at 32 C*

		BHT in ppm																	
		Nutrient Agar									Brain Heart Infusion								
		100	150	200	250	300	350	400	450	500	100	150	200	250	300	350	400	450	500
GRAM NEGATIVE	Arizonia sp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Citrobacter freundii	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Edwardsiella tarda	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
	Enterobacter aerogenes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Escherichia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
	Pseudomonas fragi	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-
	Salmonella sp20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Serratia mercescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Vibrio angularum	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
	Yersinia enterocolitica	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Arthrobacter sp	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
	Lactobacillus brevis	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Pediococcus sp	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Sarcina lutea	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Staphylococcus aureus 100	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
	Staphylococcus aureus 137	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Streptococcus faecalis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 3

Effect of TBHQ on Selected Bacteria at 32 C*

		TBHQ in ppm																	
		Nutrient Agar									Brain Heart Infusion								
		100	150	200	250	300	350	400	450	500	100	150	200	250	300	350	400	450	500
GRAM NEGATIVE	Arizonia sp	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	Citrobacter freundii	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Edwardsiella tarda	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
	Enterobacter aerogenes	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
	Escherichia coli	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-
	Pseudomonas fragi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Salmonella sp20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
	Vibrio angularum	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	Yersinia enterocolitica	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
	Arthrobacter sp	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
	Lactobacillus brevis	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Pediococcus sp	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Sarcina lutea	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	Staphylococcus aureus 100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Staphylococcus aureus 137	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	Streptococcus faecalis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

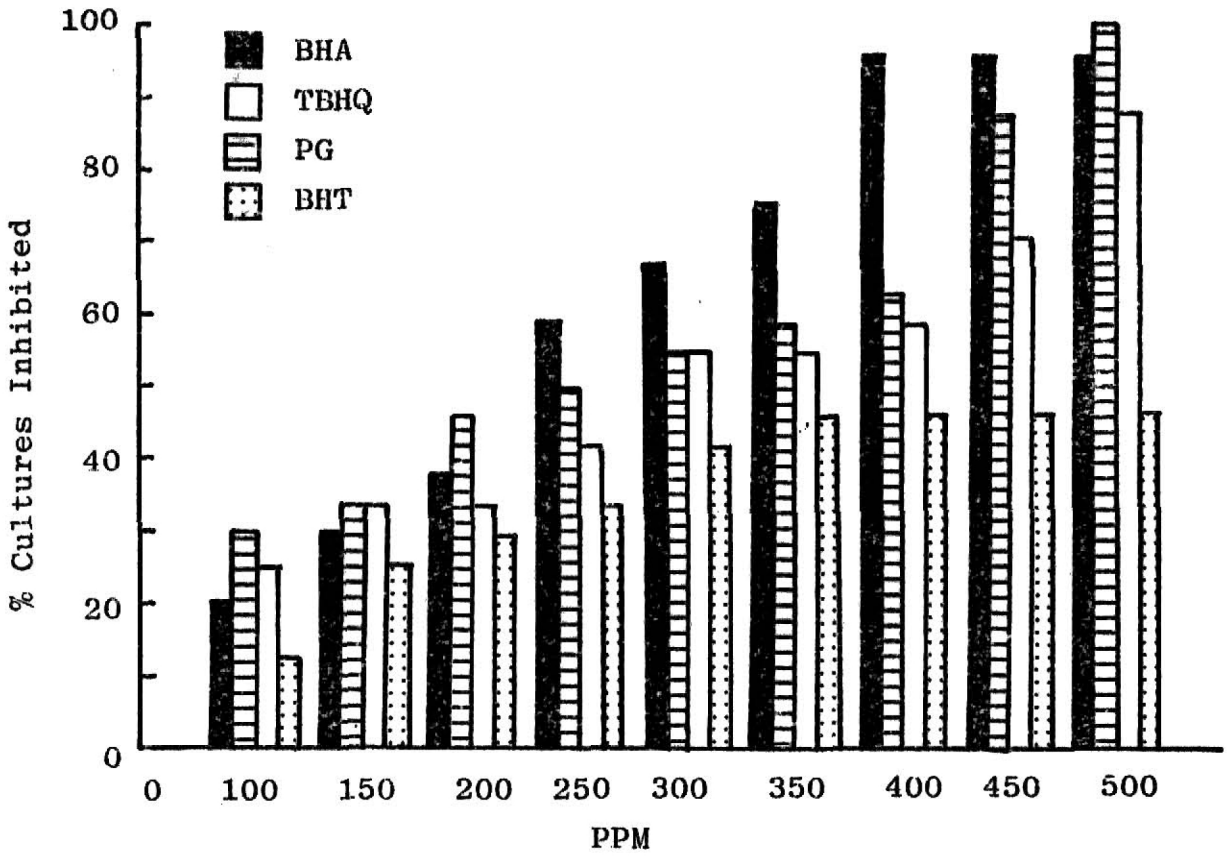
*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 4

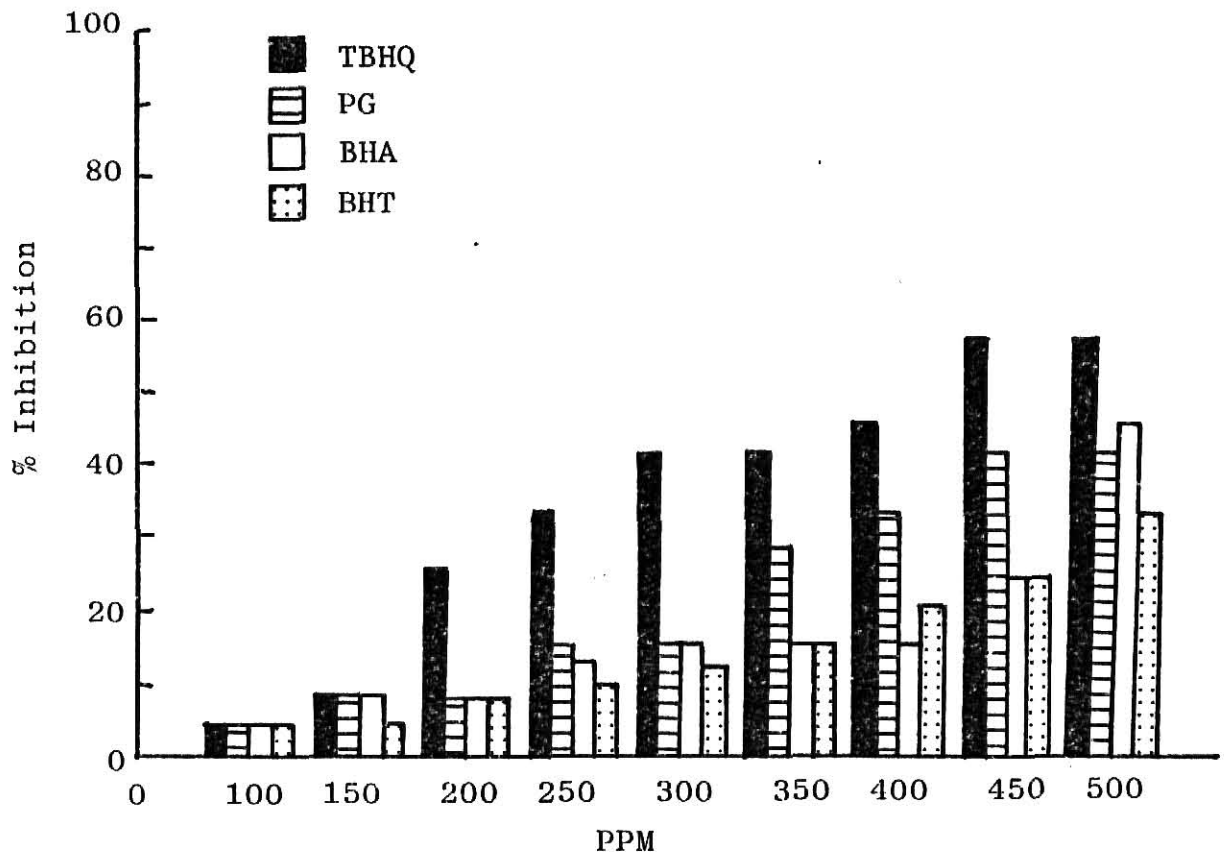
Effect of PG on Selected Bacteria at 32 C*

		PG in ppm																	
		Nutrient Agar									Brain Heart Infusion								
		100	150	200	250	300	350	400	450	500	100	150	200	250	300	350	400	450	500
GRAM NEGATIVE	Arizonia sp	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	Citrobacter freundii	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Edwardsiella tarda	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	Enterobacter aerogenes	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-
	Escherichia coli	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-
	Pseudomonas fragi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Salmonella sp20	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
	Vibrio angularum	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Yarsinia enterocolitica	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
GRAM POSITIVE	Agrobacterium tumefaciens	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	Arthrobacter sp	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-
	Lactobacillus brevis	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Pediococcus sp	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Sarcina lutea	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-
	Staphylococcus aureus 100	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Staphylococcus aureus 137	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-
	Streptococcus faecalis	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.



(Fig. 1) % Cultures inhibited in Nutrient Agar by the Antioxidants



(Fig. 2) % Cultures inhibited in Brain Heart Infusion by Antioxidants

100 was inhibited by the 100 ppm BHA. Lactobacillus brevis and Pediococcus sp were inhibited in nutrient agar by 100 ppm of BHA. Both organisms are used in food industry as starter cultures. BHA had the highest antimicrobial activity in nutrient agar, compared with the other antioxidants tested. In either nutrient agar or brain heart infusion, 50 ppm of BHA did not inhibit any of the bacteria.

Effects of BHT on Bacterial Cultures

Table 2 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of BHT. BHT inhibited Gram-positive bacteria more than Gram-negative bacteria. BHT was more inhibitory in nutrient agar than in brain heart infusion. BHT was the least effective antimicrobial agent of the four antioxidants tested. At 500 ppm, BHT inhibited less than 50% of the cultures growing in nutrient agar, and 33.3% of the cultures growing in brain heart infusion. At 100 ppm of BHT, 12.5% of the cultures were inhibited in nutrient agar, whereas in brain heart infusion, Agrobacterium tumefaciens was the only species inhibited. This organism is a plant pathogen, seldom encountered in foods. In either nutrient agar or brain heart infusion, 50 ppm of BHT did not inhibit any of the bacteria.

Effects of TBHQ on Bacterial Cultures

Table 3 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion under different concentrations of TBHQ. TBHQ inhibited Gram-positive bacteria more than Gram-negative bacteria and was more inhibitory in solid media than in liquid media. Figures 1 and

2 show the percentages of cultures inhibited by the antioxidants at different concentrations, in nutrient agar and in brain heart infusion. At 500 ppm, TBHQ inhibited 87.5% of the cultures in nutrient agar, including all the Gram-positive species tested. At 100 ppm, TBHQ inhibited 25% of the cultures growing in nutrient agar and 4.2% of the cultures in brain heart infusion. Among the 4 antioxidants tested in brain heart infusion, TBHQ showed the highest antimicrobial activity. In either nutrient agar or brain heart infusion, 50 ppm of TBHQ did not inhibit any of the bacteria.

Effects of PG on Bacterial Cultures

Table 4 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of PG. PG inhibited Gram-positive bacteria more than Gram-negative bacteria and was more inhibitory in nutrient agar than in brain heart infusion. PG was the only antioxidant that inhibited all cultures, when used at 500 ppm, in nutrient agar. Although PG at 500 ppm was the most inhibiting in nutrient agar, its effect was surpassed in brain heart infusion by BHA and TBHQ. This indicates that the activity of PG is greatly reduced in liquid systems. At 100 ppm, PG was the most inhibiting in nutrient agar, of the four antioxidants tested. However, in brain heart infusion, *Pseudomonas fragi* was the only species inhibited. In either nutrient agar or brain heart infusion, 50 ppm of PG did not inhibit any of the bacteria.

Effects of Individual Antioxidants on Bacterial Cultures Expressed in Percentages of Cultures Inhibited

Figures 1 and 2 show percentages of cultures inhibited by individual antioxidants, in nutrient agar and in brain heart infusion. The four antioxidants are more effective in inhibiting the bacterial cultures in nutrient agar than in brain heart infusion. Figure 1 shows histograms of percentages of cultures inhibited by different concentrations of individual antioxidants, in nutrient agar. At 100 ppm, the concentration allowed for use in foods, PG inhibited 29.2% of the cultures, TBHQ inhibited 25% of the cultures, BHA inhibited 20% of the cultures, and BHT inhibited 12.5% of the cultures. As the concentrations of the antioxidants increased, the percentage of cultures inhibited also increased. Complete inhibition of all the cultures was achieved by 500 ppm of PG. At the same concentration, BHA inhibited 95.8% of the cultures, TBHQ inhibited 87.5% of the cultures, and BHT inhibited 45.8% of the cultures. Figure 2 shows histograms of the percentages of cultures inhibited, in brain heart infusion, by different concentrations of antioxidants. At 100 ppm, all antioxidants inhibited only 4.2% of the cultures. As the concentration of antioxidants increased, the percentage of cultures inhibited also increased. At 500 ppm, TBHQ inhibited 58.3% of the cultures, BHA inhibited 45.8% of the cultures, PG inhibited 41.6% of the cultures, and BHT inhibited 33.3% of the cultures. Figure 2 shows that the differences in the antimicrobial activities of the antioxidants, in brain heart infusion, are not as great as they were in nutrient agar.

Effects of Combinations of Antioxidants on Bacterial Cultures in Laboratory Media

Chemical compounds may exert synergistic or antagonistic effects on microorganisms when used together in a system. This section presents data of combinations of the four antioxidants used in the study. Tables 5, 6, 7, 8, 9, and 10 show effects of combinations of antioxidants on bacterial cultures. Figures 3 and 4 show data expressed in percentage of cultures inhibited by combinations of antioxidants.

Effects of BHA-BHT on Bacterial Cultures

Table 5 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of a BHA-BHT combination. The BHA-BHT combinations inhibited Gram-positive bacteria more than Gram-negative bacteria and were more inhibiting in nutrient agar than in brain heart infusion. Figures 3 and 4 show percentages of cultures inhibited in nutrient agar and in brain heart infusion. There were no synergistic effects of a BHA-BHT combination. BHA-BHT combinations have been reported to have synergistic antioxidant effect (13). At similar concentrations, the BHA-BHT combinations gave higher percentages of inhibition than BHT, but similar to BHA when used singly. The BHA-BHT combinations had the least antimicrobial effects than any of the combinations tested.

Effects of BHA-TBHQ on Bacterial Cultures

Table 6 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of a BHA-TBHQ

Table 5

Effect of BHA-BHT Combinations on Selected Bacteria at 32 C*

		BHA-BHT (1:1) in ppm							
		Nutrient Agar				Brain Heart Infusion			
		100	200	300	400	100	200	300	400
GRAM NEGATIVE	Arizonia sp	+	+	+	+	+	+	+	+
	Citrobacter freundii	+	+	+	+	+	+	+	+
	Edwardsiella tarda	-	-	-	-	+	+	+	-
	Enterobacter aerogenes	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	+
	Escherichia coli	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	+	-	-	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	-	-	-
	Pseudomonas fragi	-	-	-	-	+	+	-	-
	Salmonella sp20	+	+	+	+	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	+	+
	Vibrio angularum	+	+	+	+	+	+	+	+
	Yersinia enterocolitica	+	+	-	-	+	+	+	+
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	-	-	-	-
	Arthrobacter sp	-	-	-	-	-	-	-	-
	Lactobacillus brevis	+	-	-	-	+	+	+	+
	Pediococcus sp	-	-	-	-	+	+	+	+
	Sarcina lutea	+	+	+	+	-	-	-	-
	Staphylococcus aureus 100	+	-	-	-	+	+	+	+
	Staphylococcus aureus 137	+	+	-	-	+	+	+	+
	Streptococcus faecalis	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 6

Effect of BHA-TBHQ Combinations on Selected Bacteria at 32 C*

		BHA-TBHQ (1:1) in ppm							
		Nutrient Agar				Brain Heart Infusion			
		100	200	300	400	100	200	300	400
GRAM NEGATIVE	Arizonia sp	+	+	+	-	+	+	+	+
	Citrobacter freundii	+	+	+	+	+	+	+	+
	Edwardsiella tarda	-	-	-	-	+	+	+	-
	Enterobacter aerogenes	+	-	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	+
	Escherichia coli	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	-	-	-	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	+	-
	Pseudomonas fragi	-	-	-	-	+	-	-	-
	Salmonella sp20	+	+	+	+	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	+	+
	Vibrio angularum	-	-	-	-	+	+	+	-
	Yersinia enterocolitica	-	-	-	-	+	+	+	-
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	-	-	-	-
	Arthrobacter sp	-	-	-	-	+	+	-	-
	Lactobacillus brevis	-	-	-	-	+	+	+	+
	Pediococcus sp	-	-	-	-	+	+	+	+
	Sarcina lutea	-	-	-	-	-	-	-	-
	Staphylococcus aureus 100	-	-	-	-	+	+	-	-
	Staphylococcus aureus 137	-	-	-	-	+	+	-	-
	Streptococcus faecalis	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 7

Effect of BHA-PG Combinations on Selected Bacteria at 32 C*

		BHA-PG (1:1) in ppm							
		Nutrient Agar				Brain Heart Infusion			
		100	200	300	400	100	200	300	400
GRAM NEGATIVE	Arizonia sp	+	+	-	-	+	+	+	+
	Citrobacter freundii	+	+	-	-	+	+	+	+
	Edwardsiella tarda	-	-	-	-	-	-	-	-
	Enterobacter aerogenes	+	+	-	-	+	+	+	+
	Escherichia coli	+	+	-	-	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	-	-	-	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	+	+
	Pseudomonas fragi	-	-	-	-	+	-	-	-
	Salmonella sp20	+	+	-	-	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	+	+
	Vibrio angularum	-	-	-	-	+	+	+	+
	Yersinia enterocolitica	-	-	-	-	+	+	+	+
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	-	-	-	-
	Arthrobacter sp	-	-	-	-	+	-	-	-
	Lactobacillus brevis	-	-	-	-	+	+	+	+
	Pediococcus sp	-	-	-	-	+	+	+	+
	Sarcina lutea	-	-	-	-	+	+	+	-
	Staphylococcus aureus 100	-	-	-	-	-	-	-	-
	Staphylococcus aureus 137	+	-	-	-	+	+	+	+
	Streptococcus faecalis	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 8

Effect of BHT-TBHQ Combinations on Selected Bacteria at 32 C*

		BHT-TBHQ (1:1) in ppm							
		Nutrient Agar				Brain Heart Infusion			
		100	200	300	400	100	200	300	400
GRAM NEGATIVE	Arizonia sp	+	+	+	-	+	+	+	+
	Citrobacter freundii	+	+	+	+	+	+	+	+
	Edwardsiella tarda	-	-	-	-	+	+	+	+
	Enterobacter aerogenes	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	+
	Escherichia coli	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	-	-	-	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	-	-
	Pseudomonas fragi	-	-	-	-	+	+	-	-
	Salmonella sp20	+	+	+	+	+	+	+	+
	Serratia marcescens	+	+	+	-	+	+	+	+
	Vibrio angularum	-	-	-	-	+	+	+	+
	Yersinia enterocolitica	-	-	-	-	+	+	+	+
GRAM POSITIVE	Agrobacterium tumefaciens	-	-	-	-	+	-	-	-
	Arthrobacter sp	-	-	-	-	+	+	+	+
	Lactobacillus brevis	-	-	-	-	+	+	+	+
	Pediococcus sp	-	-	-	-	+	+	+	+
	Sarcina lutea	-	-	-	-	-	-	-	-
	Staphylococcus aureus 100	-	-	-	-	+	+	+	-
	Staphylococcus aureus 137	-	-	-	-	+	-	-	-
	Streptococcus faecalis	+	+	+	+	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 9

Effect of BHT-PG Combinations on Selected Bacteria at 32 C*

		BHT-PG (1:1) in ppm							
		Nutrient Agar				Brain Heart Infusion			
		100	200	300	400	100	200	300	400
GRAM NEGATIVE	*Arizonia sp*	+	+	-	-	+	+	+	+
	Citrobacter freundii	+	+	+	+	+	+	+	+
	Edwardsiella tarda	+	+	-	-	-	-	-	-
	Enterobacter aerogenes	+	+	+	+	+	+	+	+
	Enterobacter cloacae	+	+	+	+	+	+	+	+
	Escherichia coli	+	+	+	+	+	+	+	+
	Klebsiella oxytoca	+	+	+	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+	+	+	+
	Proteus vulgaris	+	-	-	-	+	+	+	+
	Pseudomonas aeruginosa	+	+	+	+	+	+	+	+
	Pseudomonas fluorescens	+	+	+	+	+	+	+	+
	Pseudomonas fragi	+	+	+	+	+	+	-	-
	Salmonella sp20	+	+	+	-	+	+	+	+
	Serratia marcescens	+	+	+	+	+	+	+	+
	Vibrio angularum	-	-	-	-	+	+	+	+
	Yersinia enterocolitica	-	-	-	-	-	-	+	+
GRAM POSITIVE	*Agrobacterium tumefaciens*	-	-	-	-	-	-	-	-
	Arthrobacter sp	-	-	-	-	+	+	+	+
	Lactobacillus brevis	-	-	-	-	+	+	+	+
	Pediococcus sp	+	+	+	+	+	+	+	+
	Sarcina lutea	-	-	-	-	+	+	+	-
	Staphylococcus aureus 100	-	-	-	-	+	+	+	-
	Staphylococcus aureus 137	+	+	-	-	+	+	+	+
	Streptococcus faecalis	+	+	+	-	+	+	+	+

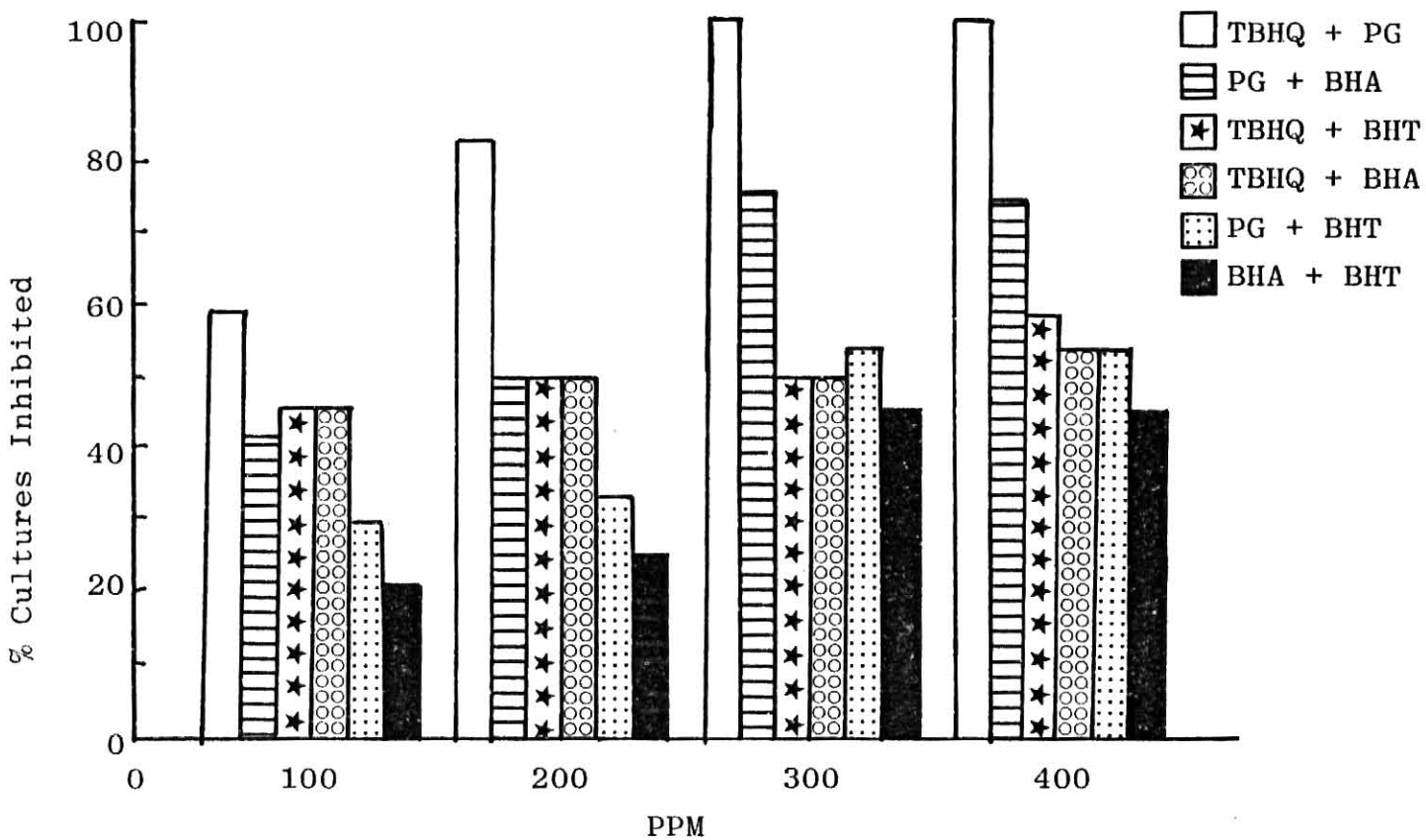
*Experiments were done in duplicates.
+ = Growth.
- = No growth.

Table 10

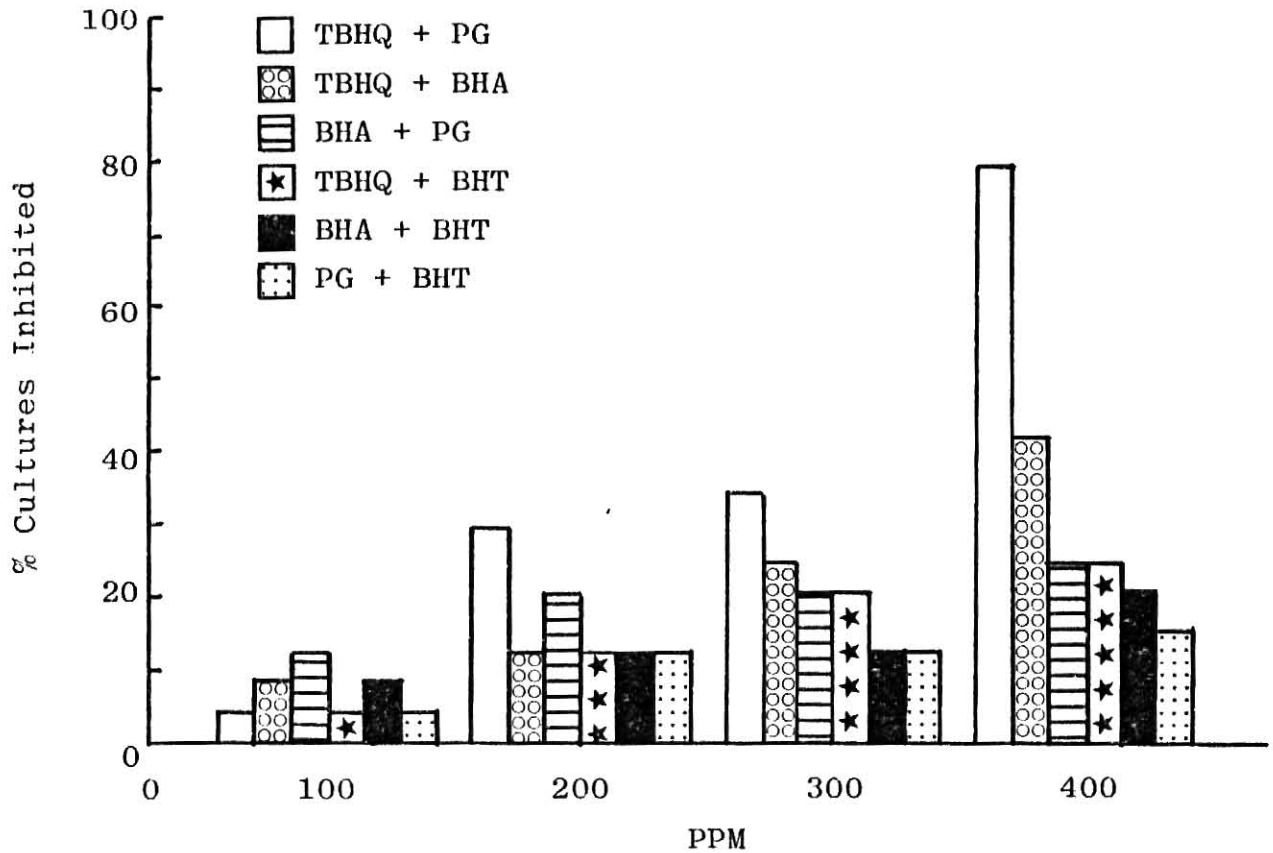
Effect of TBHQ-PG Combinations on Selected Bacteria at 32 C*

		TBHQ-PG (1:1) in ppm							
		Nutrient Agar				Brain Heart Infusion			
		100	200	300	400	100	200	300	400
GRAM NEGATIVE	*Arizonia sp*	-	-	-	-	+	+	+	-
	Citrobacter freundii	+	-	-	-	+	+	+	-
	Edwardsiella tarda	-	-	-	-	+	+	+	-
	Enterobacter aerogenes	+	-	-	-	+	+	+	+
	Enterobacter cloacae	+	-	-	-	+	+	-	-
	Escherichia coli	+	+	-	-	+	+	+	-
	Klebsiella oxytoca	+	+	-	-	+	+	+	+
	Klebsiella pneumoniae	+	+	-	-	+	+	+	+
	Proteus vulgaris	-	-	-	-	+	+	+	-
	Pseudomonas aeruginosa	+	+	-	-	+	+	+	+
	Pseudomonas fluorescens	+	-	-	-	+	-	-	-
	Pseudomonas fragi	-	-	-	-	+	-	-	-
	Salmonella sp20	+	-	-	-	+	+	+	-
	Serratia mercescens	+	-	-	-	+	+	+	-
	Vibrio angularum	-	-	-	-	+	+	+	-
	Yersinia enterocolitica	-	-	-	-	+	+	+	-
GRAM POSITIVE	*Agrobacterium tumefaciens*	-	-	-	-	+	-	-	-
	Arthrobacter sp	-	-	-	-	+	-	-	-
	Lactobacillus brevis	-	-	-	-	+	+	+	-
	Pediococcus sp	-	-	-	-	+	+	+	-
	Sarcina lutea	-	-	-	-	+	-	-	-
	Staphylococcus aureus 100	-	-	-	-	-	-	-	-
	Staphylococcus aureus 137	-	-	-	-	+	-	-	-
	Streptococcus faecalis	+	-	-	-	+	+	+	+

*Experiments were done in duplicates.
+ = Growth.
- = No growth.



(Fig. 3) % Inhibition in Nutrient Agar by combinations



(Fig. 4) % Inhibition in Brain Heart Infusion by combinations

combination. BHA-TBHQ gave higher percentage inhibition, at the same concentrations, than TBHQ when used alone. However, BHA gave higher inhibition, at the same concentrations, than the BHA-TBHQ combination, indicating that the BHA-TBHQ combination did not have synergistic effects. Figures 3 and 4 show percentages of cultures inhibited by different combinations of antioxidants. Increasing the concentration from 100 to 400 ppm did not result in any increase in the percentage of cultures inhibited in nutrient agar, by the BHA-TBHQ combination.

Effects of BHA-PG on Bacterial Cultures

Table 7 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of a BHA-PG combination. BHA-PG combinations gave similar inhibition as BHA, but more than PG, at the same concentrations, when each of them was used alone.

Effects of BHT-TBHQ on Bacterial Cultures

Table 8 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of a BHT-TBHQ combination. BHT-TBHQ combinations gave more inhibition, at similar concentrations, than either BHT or TBHQ when used alone, indicating that the BHT-TBHQ combination may have synergistic effects. At 200 ppm, the concentration allowed for use in food, the BHT-TBHQ inhibited 50% of the cultures in nutrient agar and 12.5% in brain heart infusion.

Effects of BHT-PG on Bacterial Cultures

Table 9 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of BHT-PG combination. The BHT-PG combinations were less effective, as an antimicrobial agent than PG, but more effective than BHT when each of them was used alone. The BHT-PG combination is known to have a negative synergistic effect on the antioxidant ability of the two compounds (13). Of all the antioxidant combinations tested in brain heart infusion, the BHT-PG combination gave the least percentage of inhibition. Only 16.6% of the cultures were inhibited, in brain heart infusion, by 400 ppm of the BHT-PG combination. BHT-PG at 200 ppm inhibited 33.3% of the cultures in nutrient agar, and 12.5% of cultures in brain heart infusion.

Effects of TBHQ-PG on Bacterial Cultures

Table 10 shows growth of the cultures, at 32C, in nutrient agar and in brain heart infusion, under different concentrations of a TBHQ-PG combination. The TBHQ-PG combination had the highest percentage of cultures inhibited, in nutrient agar and in brain heart infusion, than any of the antioxidants tested, alone or in combinations. The TBHQ-PG combination showed sound synergistic effects. 200 ppm of a TBHQ-PG combination in nutrient agar inhibited 83.3% of the cultures tested, including all the Gram-positive species. In brain heart infusion, 29.2% of the cultures were inhibited by 200 ppm TBHQ-PG.

Effects of Combined Antioxidants on Bacterial Cultures Expressed as Percentage Inhibition

Figure 3 shows histograms of percentages of cultures inhibited by different concentrations of antioxidant combinations, in nutrient agar. TBHQ-PG combination gave the highest percentage of inhibition, followed by PG-BHA, TBHQ-BHT, TBHQ-BHA, PG-BHT, and BHA-BHT, respectively. Complete inhibition of all cultures was achieved by 300 ppm of TBHQ-PG. At 200 ppm, the concentration allowed for use in foods, TBHQ-PG inhibited 83.3% of the cultures, each of PG-BHA, TBHQ-BHT, and TBHQ-BHA inhibited 50% of the cultures, PG-BHT inhibited 33.3% of the cultures, and BHA-BHT inhibited 25% of the cultures. As the concentrations increased from 300 to 400 ppm, percentage of cultures inhibited remained the same for all combinations, except TBHQ-BHT and TBHQ-BHA, which gave higher percentages of inhibition at 400 ppm than at 300 ppm.

Figure 4 shows histograms of percentages of cultures inhibited by different concentrations of antioxidants combinations, in brain heart infusion. TBHQ-PG had the highest percentage of inhibition, followed by BHA-PG, TBHQ-BHA, TBHQ-BHT, BHA-BHT, and PG-BHT, respectively. At 200 ppm, TBHQ-PG inhibited 29.2% of the cultures, BHA-PG 20.8% of the cultures, and any of the remaining combinations inhibited 12.5% of the cultures.

Bactericidal Effects of Antioxidants

Compounds that kill bacteria are known as bactericidal, whereas compounds that delay or suppress the growth of the bacteria are known as bacteriostatic compounds. A compound may exhibit bacteriostatic

activities at low concentrations, and bactericidal activities at high concentrations. Results from this study showed that the four antioxidants and their combinations, at the concentrations tested, had bactericidal activities. None of the subcultures, from microtiter wells with negative growth, had any regrowth on agar. The possibility exists that the number of viable cells transferred may be too few for further outgrowth.

Effects of Incubation Temperature on Antimicrobial Activity of Antioxidants

A number of environmental factors affect the inhibitory effectiveness of antioxidants. Klindworth et al. (36) found that all cultures of *Clostridium perfringens* tested were inhibited by 150 ppm at 37C. At 45C, however, this concentration was not always completely inhibitory. The reason for this was not clear, but they theorized that the reduced inhibition was related to other external factors such as culture age or pH of the media. Some research workers (5) found no effect of temperature on the inhibition of *Aspergillus flavus* by BHA. In this study, the test organisms were incubated at 32C and at 37C. Temperature did not have any effect on the inhibitory effectiveness of the antioxidants. Growth results were the same, at 32C and 37C, for the cultures that can grow at both temperatures. The cultures which cannot grow at 37C (*Agrobacterium tumefaciens*, *Arthrobacter sp*, *Lactobacillus brevis*, *Pediococcus sp*, and *Pseudomonas fluorescens*), were not inhibited at 32C. Their inhibition at 37C is thus attributed to temperature effects rather than antioxidants effects.

Effects of Antioxidants on Psychrotrophs in Ground Pork Stored at 4C

Psychrotrophs are a group of bacteria which can grow at temperatures below 10C and above freezing. They are important in refrigerated foods. Psychrotrophic bacteria are found chiefly in genera *Pseudomonas*, *Flavobacterium*, and *Alcaligenes*, although genera *Micrococcus*, *Lactobacillus*, *Enterobacter*, *Arthrobacter* and others may contain psychrotrophic species. Since ground pork samples in this study were stored at 4C, monitoring effects of antioxidants in this group is important.

Effect of BHA

Figure 5 shows that ground pork treated with BHA at 100, 200, or 400 ppm had lower psychrotroph counts than the control throughout storage period. Averages of psychrotroph counts (Table 11) from the 5 pork samples treated with different concentrations of the antioxidants indicate that addition of 100, 200, or 400 ppm of BHA to ground pork did not reduce ($P > .05$) the psychrotroph counts during the first and second weeks of storage, but lowered psychrotroph counts at 4 weeks of storage.

Effect of BHT

Figure 6 shows growth curves of psychrotrophs in ground pork treated with different concentrations of BHT. BHT at 100, 200, or 400 ppm resulted in lower psychrotroph counts than the control. Addition of up to 400 ppm of BHT to ground pork did not reduce ($P > .05$) psychrotroph counts during the first and second week of storage (Table 11). Increasing the concentration of BHT in ground pork resulted in more

FIG. 5

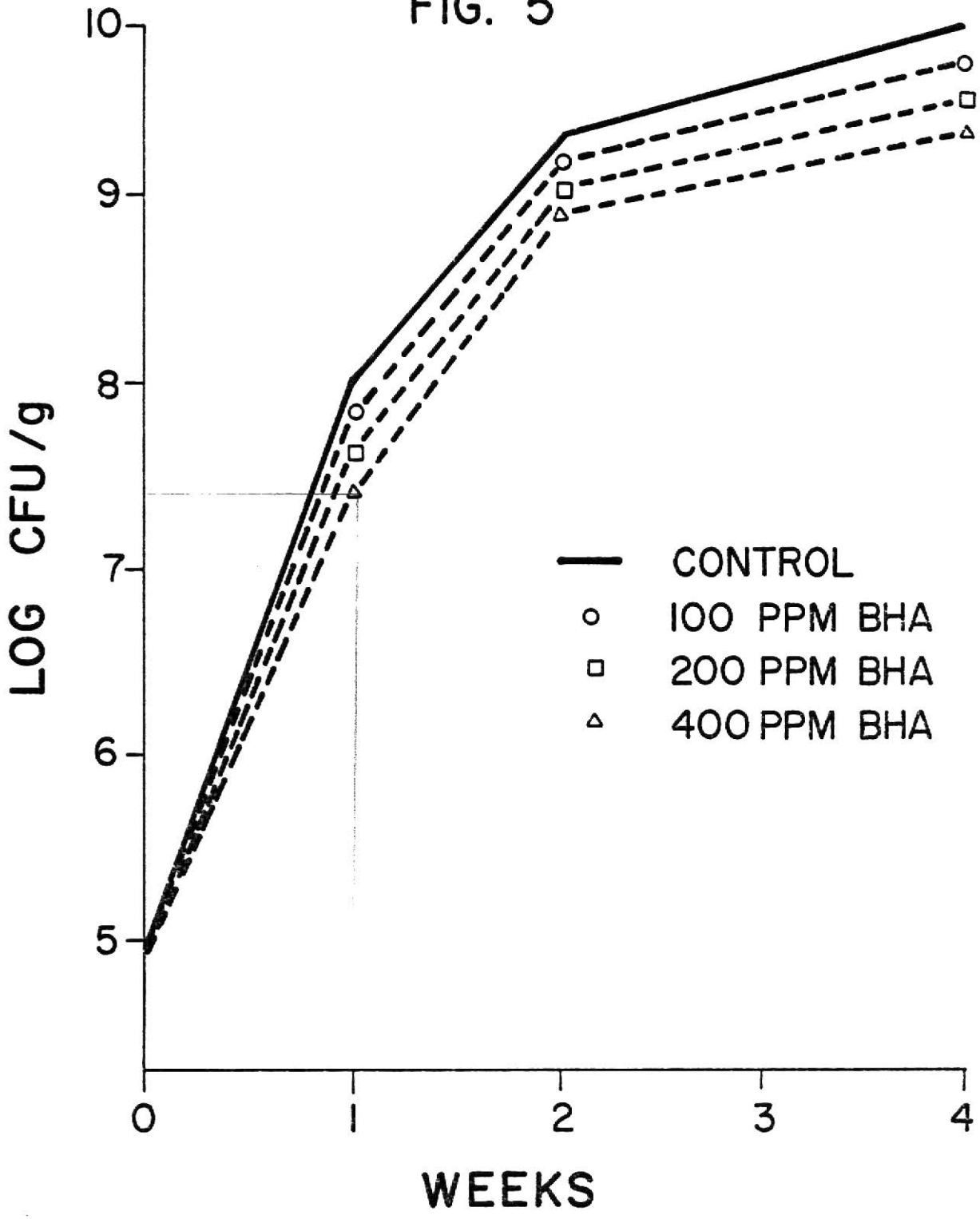

10
9
8
7
6
5
LOG CFU/g
0
1
2
3
4
WEEKS
CONTROL
100 PPM BHA
200 PPM BHA
400 PPM BHA

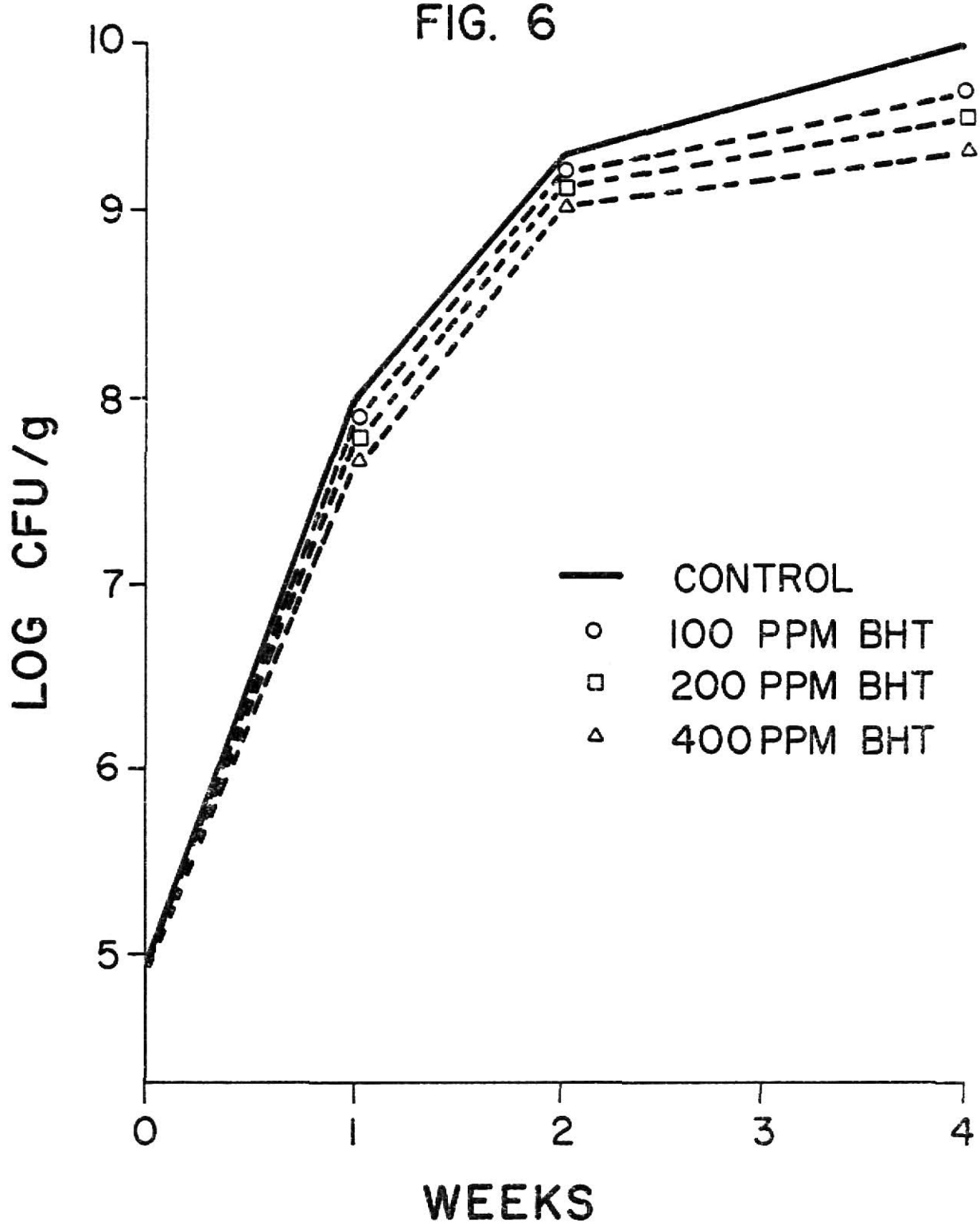
Table 11

Average Psychrotrophic Counts (Colony Forming Units/g) from 5 Pork Samples

	Initially	1 week	2 weeks	4 weeks
Control	$8.6x10^{4}$	$6.7x10^{7a}$	$2.1x10^{9b}$	$9.6x10^{9c}$
PPM				
BHA				
100		$5.0x10^{7a}$	$1.8x10^{9b}$	$7.1x10^{9d}$
200		$4.6x10^{7a}$	$1.6x10^{9b}$	$5.0x10^{9e}$
400		$3.4x10^{7a}$	$8.1x10^{8b}$	$3.9x10^{9e}$
BHT				
100		$6.6x10^{7a}$	$1.9x10^{9b}$	$6.4x10^{9e}$
200		$5.1x10^{7a}$	$1.4x10^{9b}$	$3.6x10^{9e}$
400		$4.4x10^{7a}$	$1.1x10^{9b}$	$3.6x10^{9e}$
TBHQ				
100		$6.7x10^{7a}$	$1.9x10^{9b}$	$6.2x10^{9d}$
200		$6.0x10^{7a}$	$1.3x10^{9b}$	$5.1x10^{9e}$
400		$3.8x10^{7a}$	$9.3x10^{8b}$	$4.7x10^{9e}$
PG				
100		$6.3x10^{7a}$	$1.7x10^{9b}$	$5.9x10^{9e}$
200		$5.4x10^{7a}$	$1.5x10^{9b}$	$4.7x10^{9e}$
400		$3.9x10^{7a}$	$9.9x10^{8b}$	$3.2x10^{9e}$

Means with the same letter are not different ($P > .05$).

FIG. 6


10
9
8
7
6
5
LOG CFU/g
0
1
2
3
4
WEEKS
CONTROL
100 PPM BHT
200 PPM BHT
400 PPM BHT

reduction of psychrotroph counts. Significant reduction ($P < .05$) of psychrotrophs in ground pork by BHT occurred after 4 weeks of storage.

Effect of TBHQ

Figure 7 shows that TBHQ at 100, 200, or 400 ppm resulted in lower psychrotroph counts than the control at all times tested. Increasing the concentration of TBHQ further decreased the psychrotroph counts. Addition of up to 400 ppm TBHQ to ground pork did not reduce ($P > .05$) psychrotroph counts during the first and second weeks of storage (Table 11). Significant reduction ($P < .05$) of psychrotrophs by TBHQ occurred after 4 weeks of storage.

Effect of PG

PG at 100, 200, or 400 ppm resulted in lower psychrotroph counts in ground pork than the control (Figure 8). Increasing the concentration of PG resulted in further reduction of the psychrotroph counts. Addition of up to 400 ppm of PG to ground pork did not reduce ($P > .05$) psychrotroph counts during the first and second weeks of storage. Significant reduction of psychrotrophs in ground pork by PG occurred after 4 weeks of storage.

Effects of Antioxidants on Coliforms in Ground Pork Stored at 4C

Coliforms are short rods that are defined as "all aerobic and facultative anaerobic, Gram-negative, non-spore forming bacteria which ferment lactose with gas formation" (59). They are generally undesirable

FIG. 7

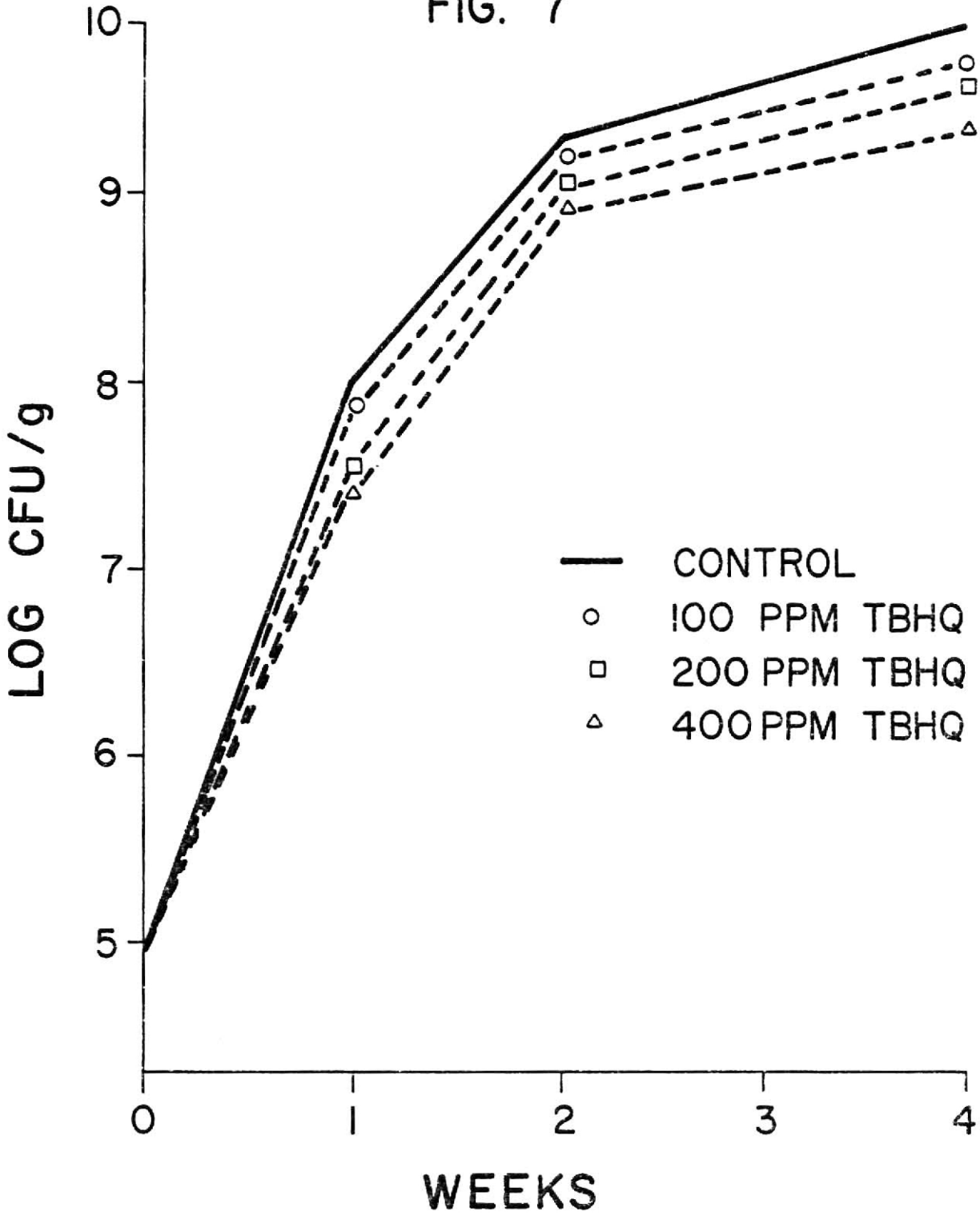
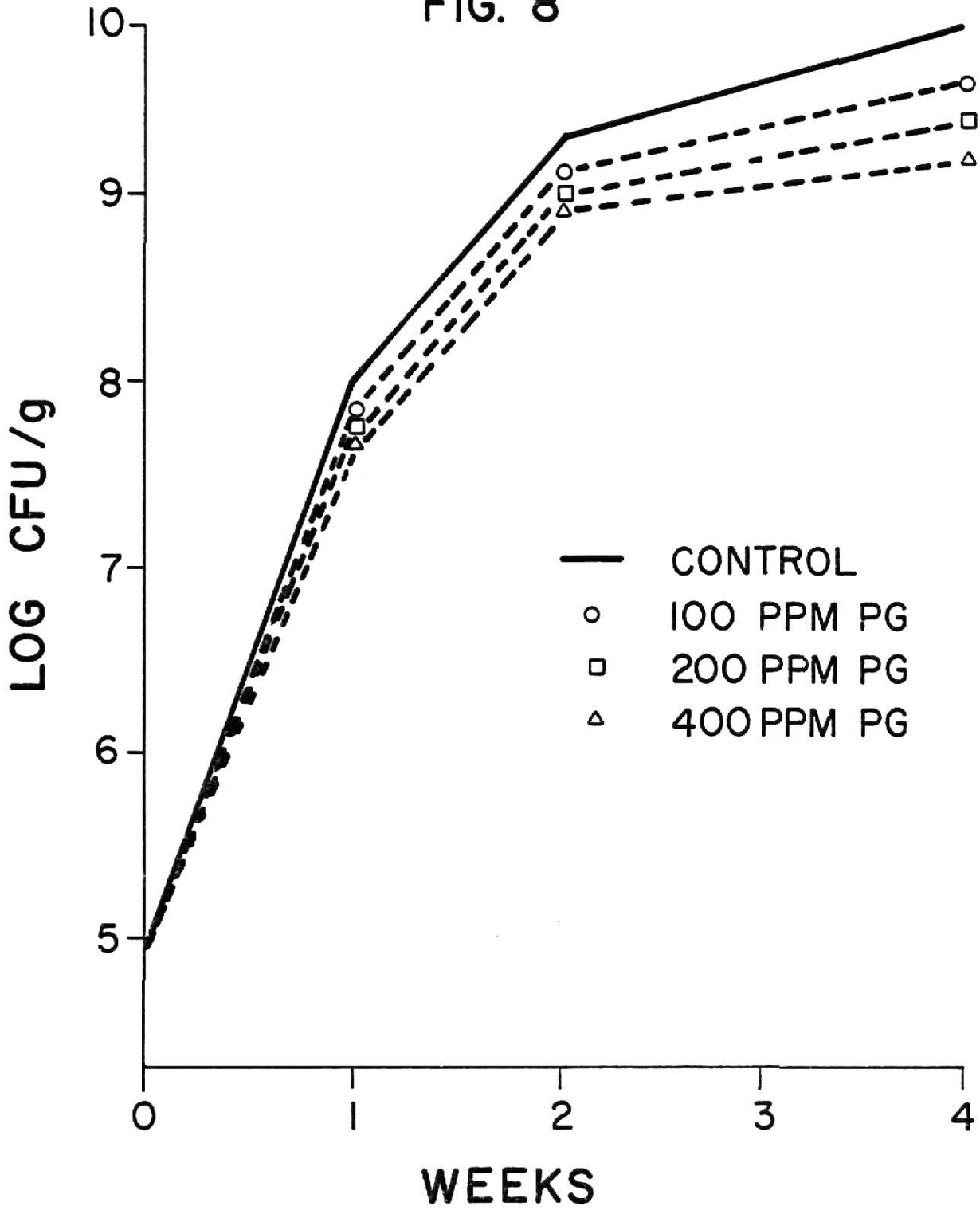

10
9
8
7
6
5
LOG CFU/g
0
1
2
3
4
WEEKS
CONTROL
100 PPM TBHQ
200 PPM TBHQ
400 PPM TBHQ

FIG. 8


10
9
8
7
6
5
LOG CFU/g
0
1
2
3
4
WEEKS
CONTROL
100 PPM PG
200 PPM PG
400 PPM PG

in foods, for their presence is considered to be indicative of sewage contamination and hence of possible presence of enteric pathogens.

Effect of BHA

Table 12 shows the coliforms counts from 4 pork samples, treated with different concentrations of antioxidants. Concentrations of 100, 200, or 400 ppm of BHA in ground pork did not reduce ($P > .05$) the coliform counts in the sample during 2 weeks of storage. However, reduction ($P < .05$) in the coliforms, by BHA, occurred after 4 weeks of storage. Of the 4 antioxidants tested, BHA was the least effective in reducing the coliform counts in ground pork. Data for psychrotrophs and coliforms in the ground pork samples indicated that BHA was more effective against the former group than against the latter.

Effect of BHT

BHT in ground pork at 100, 200, or 400 ppm did not reduce ($P > .05$) coliform counts during 2 weeks of storage, but reduction ($P < .05$) by BHT occurred after 4 weeks of storage (Table 12).

Effect of TBHQ

Addition of up to 400 ppm of TBHQ did not reduce ($P > .05$) the coliform counts in ground pork during 2 weeks of storage. Reduction ($P < .05$) in coliform counts by TBHQ occurred after 4 weeks of storage.

Table 12

Average Coliforms Counts (Colony Forming Units/g) from Samples A, B, D, and E

	Initially	1 week*	2 weeks	4 weeks
Control	N.D.	--	$1.8x10^{5a}$	$6.9x10^{7b}$
PPM				
BHA				
100		--	$2.2x10^{5a}$	$3.3x10^{7c}$
200		--	$1.7x10^{5a}$	$2.2x10^{7c}$
400		--	$3.6x10^{4a}$	$1.1x10^{7c}$
BHT				
100		--	$1.3x10^{5a}$	$3.5x10^{7c}$
200		--	$9.4x10^{4a}$	$2.4x10^{7c}$
400		--	$1.1x10^{4a}$	$1.5x10^{7c}$
TBHQ				
100		--	$1.2x10^{5a}$	$2.3x10^{7c}$
200		--	$8.6x10^{4a}$	$1.4x10^{7c}$
400		--	$1.4x10^{4a}$	$7.3x10^{6d}$
PG				
100		--	$1.1x10^{5a}$	$3.1x10^{7c}$
200		--	$9.6x10^{4a}$	$1.7x10^{7c}$
400		--	$3.5x10^{4a}$	$1.1x10^{7c}$

*No readings were taken.

Means with the same letter are not different ($P > .05$).

N.D. = Non Detectable.

Effect of PG

Like the other antioxidants tested, PG at 100, 200, or 400 ppm in ground pork did not reduce the coliform counts during 2 weeks of storage. Reduction ($P < .05$) in coliform counts by PG occurred after 4 weeks of storage.

Effects of Antioxidants on Fecal Coliforms in Ground Pork Stored at 4C

Fecal coliforms are coliforms of fecal origin. Their presence in foods is also undesirable. Monitoring the effects of effects of antioxidants on coliforms and fecal coliforms is of great importance.

Effect of BHA

Table 13 shows fecal coliform counts from 4 ground pork samples. No fecal coliforms were recovered at the end of first and second week from samples treated with 100, 200, or 400 ppm of BHA. BHA may have delayed the growth of fecal coliforms in ground pork. After 4 weeks of storage, BHA reduced ($P < .05$) fecal coliform counts in ground pork.

Effect of BHT

No fecal coliforms were recovered after 2 weeks of storage from samples treated with 100, 200, or 400 ppm of BHT. BHT may have delayed the growth of fecal coliforms in ground pork. BHT reduced ($P < .05$) fecal coliforms in ground pork after 4 weeks of storage (Table 13).

Table 13

Average Fecal Coliforms Counts (Colony Forming Units/g) from Samples A, B, D, and E

	Initially	1 week	2 weeks	4 weeks
Control	N.D.	$1.4x10^{1}$	$3.0x10^{3a}$	$3.7x10^{4d}$
PPM				
BHA				
100	N.D.	N.D.	N.D.[c]	$1.2x10^{4d}$
200	N.D.	N.D.	N.D.[c]	$3.5x10^{3e}$
400	N.D.	N.D.	N.D.[c]	$1.8x10^{3e}$
BHT				
100	N.D.	N.D.	N.D.[c]	$6.4x10^{3e}$
200	N.D.	N.D.	N.D.[c]	$2.7x10^{3e}$
400	N.D.	N.D.	N.D.[c]	$3.6x10^{3e}$
TBHQ				
100	N.D.	N.D.	$3.9x10^{2a}$	$1.5x10^{4d}$
200	N.D.	N.D.	$1.6x10^{2c}$	$4.8x10^{3e}$
400	N.D.	N.D.	N.D.[c]	$2.1x10^{3e}$
PG				
100	N.D.	4.8x10	$1.6x10^{2c}$	$5.2x10^{3e}$
200	N.D.	N.D.	$9.0x10^{1c}$	$1.2x10^{3e}$
400	N.D.	N.D.	$2.8x10^{1c}$	$8.5x10^{2e}$

Means with the same letter are not different ($P > .05$).

N.D. = Non Detectable.

Effect of TBHQ

TBHQ at 100 ppm did not reduce ($P < .05$) the fecal coliform counts (Table 13). 200 and 400 ppm of TBHQ reduced ($P < .05$) the fecal coliform counts in ground pork at 2 and 4 weeks of storage. TBHQ was the least effective of the 4 antioxidants in reducing the fecal coliform counts in ground pork.

Effect of PG

Ground pork samples treated with PG at 100, 200, or 400 ppm had lower fecal coliform counts than the control at 2 and 4 weeks of storage (Table 13).

Effects of Antioxidants on Coliforms and Fecal Coliforms Counts from Sample C

Table 14 shows the coliforms and fecal coliforms from Sample C at different concentrations of the antioxidants. Sample C contained higher coliform and fecal coliform counts than the other samples. Apparently, all 4 antioxidants, at concentrations tested, did not effectively reduce coliform and fecal coliform counts on Sample C, the deliberately contaminated sample. This indicates that use of antioxidants will not improve the wholesomeness of an abused product. To be effective in prolonging the shelf life of a food product, application of antioxidants should be accompanied by careful handling and proper hygienic procedures.

Table 14

Average Coliforms and Fecal Coliforms Counts
(Colony Forming Units/g) from Sample C*

	Coliforms			Fecal Coliforms		
Weeks	1**	2	4	1	2	4
Control	--	$7.1x10^5$	$1.8x10^8$	$1.9x10^3$	$3.2x10^4$	$4.6x10^4$
BHA						
100	--	$1.2x10^5$	$1.5x10^8$	$1.4x10^3$	$1.6x10^4$	$2.5x10^4$
200	--	$1.6x10^5$	$1.2x10^8$	$1.0x10^3$	$1.2x10^3$	$1.6x10^4$
400	--	$4.0x10^4$	$4.8x10^7$	$1.0x10^3$	$7.4x10^2$	$1.5x10^4$
BHT						
100	--	$1.9x10^5$	$1.2x10^8$	$4.4x10^2$	$4.2x10^3$	$3.6x10^4$
200	--	$1.2x10^5$	$1.0x10^8$	$1.3x10^2$	$1.3x10^3$	$2.5x10^4$
400	--	$8.2x10^4$	$6.2x10^7$	8.4x10	$8.9x10^2$	$1.8x10^4$
TBHQ						
100	--	$4.9x10^5$	$1.5x10^8$	$1.3x10^3$	$6.4x10^4$	$3.8x10^4$
200	--	$4.8x10^5$	$1.1x10^8$	$1.6x10^3$	$6.2x10^4$	$3.3x10^4$
400	--	$3.4x10^5$	$5.6x10^7$	$1.1x10^3$	$3.4x10^4$	$1.6x10^4$
PG						
100	--	$5.2x10^5$	$1.5x10^8$	$1.4x10^3$	$5.5x10^3$	$4.9x10^4$
200	--	$3.5x10^5$	$9.9x10^7$	$1.0x10^3$	$3.2x10^3$	$3.3x10^4$
400	--	$3.0x10^5$	$4.9x10^7$	$7.4x10^2$	$1.2x10^3$	$2.5x10^4$

*The sample showed no coliforms or fecal coliforms at zero time.

**No readings were taken.

DISCUSSION

Effects of the Antioxidants on Bacterial Cultures, in Laboratory Media

Antioxidants, alone or in combinations, had significant antimicrobial activities. Figures 1, 2, 3, and 4 show the growth of the cultures at 32C under different concentrations of the 4 antioxidants and their selected combinations, in nutrient agar (solid system) and in brain heart infusion (liquid system). As expected, inhibition increased with increased antioxidant concentration. Inhibition was greater in nutrient agar than in brain heart infusion. This could be attributed to the fact that being water-insoluble, the antioxidants are unavailable in solution, in the liquid brain heart infusion, to act against the bacteria, whereas in the solid nutrient agar medium, the molecules of the antioxidants are trapped between the particles of the solid media, which restricts their free movement and brings them in direct contact with the bacteria. In agreement with Shih and Harris (55), Pierson et al. (43), and Oluski et al. (40), in both media, Gram-positive bacteria are more sensitive to the antioxidants than were Gram-negative bacteria. However, responses within each group were not identical. For example, *Pseudomonas fragi* and *Edwardsiella tarda*, unlike most of the Gram-negative species tested, were very sensitive to the antioxidants, whereas *Streptococcus faecalis*, among the Gram-positive species tested, was exceptionally resistant to the antioxidants.

Antioxidants tested differed in antimicrobial activity. In nutrient agar, BHA had the highest percentage of inhibition followed by PG, TBHQ, and BHT, respectively. In brain heart infusion, TBHQ had the highest percentage of inhibition followed by PG, BHA, and BHT, respectively. The differences between the activities of the antioxidants in the two media may be attributed to differences in the degrees of their solubility in brain heart infusion (the liquid system).

Robach and Pierson (47) suggested that there is a relationship between the antioxidant ability of a compound and its antimicrobial activity. Their results showed that BHA is not only a stronger antioxidant, but also a better antimicrobial agent than BHT.

Antioxidants are known to have synergistic antioxidant effects when used in combinations with one another and with other antioxidants (13). However, a combination of PG and BHT was found to have a negative antioxidative synergism, in which the keeping quality of a fat is less than the expected from the sum of each one used alone. The TBHQ-PG and TBHQ-BHT combinations had synergistic antimicrobial effects. Of the six combinations tested, the TBHQ-PG combination gave the highest percentage of inhibition followed by the PG-BHA, TBHQ-BHT, TBHQ-BHA, PG-BHT, and BHA-BHT combinations, respectively. Figures 1 and 4 show that 100% inhibition was achieved only by the use of 500 ppm PG or 300 ppm of a PG-TBHQ combination in the solid medium. At the concentration allowed for use in food (100 ppm), only 20% of the cultures were inhibited by BHA, 29.2% by PG, 25% by TBHQ, and 12.5% by BHT in nutrient agar, whereas in brain heart infusion only 4% of the cultures were inhibited by each of the antioxidants. This indicates that wide spectrum antimicrobial

activity is not attainable under the low (100 ppm) concentration permitted for use in food. However, the 4% of the cultures inhibited by 100 ppm of the antioxidants in the liquid system, and the 12.5% inhibited in the solid system included important species such as Staphylococcus aureus, Pediococcus sp, Lactobacillus brevis, and Agrobacterium tumefaciens. At the 200 ppm permitted in food when more than one antioxidant is used, 83.3% of the cultures were inhibited by a TBHQ-PG combination, 50% by PG-BHA, TBHQ-BHT, or TBHQ-BHA, 33.3% by PG-BHT, and 25% by BHA-BHT combinations, in solid media. In brain heart infusion, only 29.2% of the cultures were inhibited by TBHQ-PG, 20.8% by PG-BHA, and 12.5% by each of TBHQ-BHT, TBHQ-BHA, BHA-BHT, and PG-BHT combinations. This suggests that antioxidants could better be used in combinations in order to be effective as an antioxidation-antimicrobial agent. The potential for their use is greater in solid systems than in liquid systems.

This study indicated that the 4 antioxidants tested are effective in inhibiting microbial growth in laboratory media. Effectiveness of the antioxidants likely depends on the type of the system, whether liquid or solid, the solubility of the antioxidants in the media, the concentration of the antioxidant, and the bacterial species. It should be emphasized that the incubation systems were optimum for growth of test organisms; a situation not likely to occur in food systems. This however provides a model system for experiments.

Effects of Antioxidants on Psychrotrophs, Coliforms, and Fecal Coliforms in Ground Pork

Antioxidants are known to have less antimicrobial activity when applied to foods than when used in laboratory media (45). They are also known to be less effective in high-fat products than in low-fat products (5). This reduction in activity could be due to the partitioning of the antioxidants in the lipid system, thus preventing their action as antimicrobial agents. Other environmental factors that affect the activity of the antioxidants are the pH, temperature, and the presence of lipids (5). There are two theories as to why the presence of lipids decreases the antimicrobial activity of the antioxidants (5). First since the antioxidants have nonpolar character, they might migrate and solubilize in any lipid present in a medium, making them unavailable to act on microorganisms. Second, the antimicrobial properties of an antioxidant may be related to its antioxidant properties; therefore, if the compound is used up in preventing autoxidation, their antimicrobial activity may also be lost.

Table 11 shows the psychrotrophs counts from the 5 meat samples treated with different concentrations of the antioxidants. Initially, all the samples were microbiologically acceptable. However, bacterial counts showed considerable increase with time. Figures 5, 6, 7, and 8 show the logarithmic increase in psychrotrophic counts under different treatments. At any time all treatments gave lower bacterial counts than the control.

No differences occurred ($P > .05$) between the control and the antioxidants, under the concentrations tested, at either 1 or 2 weeks.

However, the differences were significant ($P < .05$) after 4 weeks. The fact that the antioxidants became more effective with time could be attributed to the physiological changes linked with aging of the bacteria, or to enzymic, physical, and chemical changes occurring in the meat, rendering it less suitable for the bacteria. Another reason that could have helped in increasing the susceptibility of the bacteria is the competition due to higher population density, and the depletion of nutrients and essential amino acids from the meat. The 4 antioxidants, at the concentrations tested, did not prolong the shelf life of ground pork as far as microbial growth is concerned. Although the antioxidants exhibited significant inhibition at 4 weeks, yet the psychrotrophic counts were still higher (10^9 CFU/g) than acceptable levels, which are generally about 10^7 CFU/g or lower.

Tables 12 and 13 show coliform and fecal coliform counts from 4 ground pork samples. Initially, all samples had no detectable coliform counts. Growth was recorded after one week of storage. All 4 antioxidants did not reduce ($P > .05$) coliform counts after 2 weeks of storage. BHA, BHT, PG at 100, 200, or 400 ppm and TBHQ at 200 or 400 ppm reduced ($P < .05$) the fecal coliform counts after 2 and 4 weeks of storage, and the coliform counts after 4 weeks of storage.

In conclusion, these 4 antioxidants are effective in inhibiting growth in laboratory media, they are less active in liquid media than in solid media, and they have synergistic activities when used in combinations. Their antimicrobial activities were greatly reduced in foods. Further work needs to be done to define the factors that reduce their activities in foods, and to investigate the effects in foods of the

antioxidants when combined with one another and with other known antimicrobial agents.

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ANTIMICROBIAL EFFECTS OF SELECTED ANTIOXIDANTS IN LABORATORY MEDIA AND IN GROUND PORK

by

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ABSTRACT

Antimicrobial properties of antioxidants in laboratory media and in foods has been the subject of research by food scientists in recent years. Most of the work done, so far, was on butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). This present study included BHA, BHT, tertiarybutylhydroquinone (TBHQ), and propyl gallate (PG) at 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 ppm; and selected combinations at 0, 100, 200, 300, and 400 ppm in nutrient agar and in brain heart infusion were tested for antimicrobial activity on 16 Gram-negative and 8 Gram-positive bacteria. Antioxidants were also tested at 0, 100, 200, and 400 ppm, against psychrotrophs, coliforms, and fecal coliforms naturally occurring in ground pork stored at 4C for 4 weeks.

In laboratory media the antioxidants inhibited Gram-positive bacteria more than Gram-negative bacteria. They were more effective in nutrient agar (solid system) than in brain heart infusion (liquid system), and the effects were bactericidal rather than bacteriostatic. In nutrient agar, BHA gave the highest percentage of inhibition followed by PG, TBHQ, and BHT, respectively. In brain heart infusion, TBHQ gave the highest percentage of inhibition followed by PG, BHA, and BHT, respectively. The differences between activities of antioxidants in the two media may be attributed to differences in the degrees of their solubilities in brain heart infusion.

Of the 6 combinations tested, the TBHQ-PG combination gave the highest percentage of inhibition followed by PG-BHA, TBHQ-BHT, TBHQ-BHA, PG-BHT, and BHA-BHT, respectively. At the concentrations allowed for use in foods (100 ppm), 29.2% of the cultures were inhibited by PG, 25%

by TBHQ, 20% by BHA, and 12.5% by BHT in nutrient agar, whereas in brain heart infusion only 4% of the cultures were inhibited by each of the antioxidants. Apparently wide spectrum antimicrobial activity is not attainable under the low, 100 ppm, concentration. However, the 4% of the cultures inhibited in brain heart infusion and the 12.5% of the cultures inhibited in nutrient agar included important species such as Staphylococcus aureus, Pediococcus sp, Lactobacillus brevis, and Agrobacterium tumefaciens. At the 200 ppm permitted in foods when more than one antioxidant is used, 83.3% of the cultures were inhibited by a TBHQ-PG combination, 50% by PG-BHA, TBHQ-BHT, or TBHQ-BHA, 33.3% by PG-BHT, and 25% by BHA-BHT in nutrient agar. In brain heart infusion, 29.2% of the cultures were inhibited by PG-BHA, and 12.5% by PG-TBHQ, TBHQ-BHT, TBHQ-BHA, PG-BHT, or BHA-BHT at 200 ppm.

Tests in ground pork showed that the 4 antioxidants at 100, 200, or 400 ppm reduced psychrotrophs, coliforms, and fecal coliforms counts after 4 weeks of storage. There were no significant differences between the control and the samples treated with antioxidants after either 1 or 2 weeks of storage. However, significant differences ($P < .05$) occurred after 4 weeks of storage. At the concentrations tested, the 4 antioxidants did not prolong the shelf life of ground pork, as far as microbial growth is concerned. Although the antioxidants exhibited significant microbial inhibition at 4 weeks, psychrotrophic counts were 10^9 CFU/g. Initially, no coliforms or fecal coliforms were recovered from any of the samples. Growth was recorded after one week of storage. The 4 antioxidants at 100, 200, or 400 ppm significantly ($P < .05$) reduced the coliforms and fecal coliforms counts in ground pork after 4 weeks of storage at 4C.