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Antimicrobial Effects of Selected Antioxidants in Laboratory Media and in Ground Pork

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ABSTRACT

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (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 sixteen gram-negative and eight gram-positive bacteria. In general, in laboratory media the antioxidants inhibited gram-positive bacteria more than gram-negative bacteria. The antioxidants were more effective in nutrient agar (solid system) than in brain heart infusion (liquid system). In nutrient agar, BHA inhibited the greatest number of organisms followed by PG, TBHQ and BHT, respectively. In brain heart infusion, TBHQ inhibited the greatest number of organisms followed by PG, BHA and BHT, respectively. Of the six combinations tested, the TBHQ-PG combination inhibited the greatest number of organisms followed by BHA-PG, BHT-TBHQ, BHA-TBHQ, BHT-PG and BHA-BHT, respectively. Tests in ground pork indicated the four antioxidants at 100, 200 or 400 ppm significantly ($P<0.05$) reduced psychrotrophs, coliforms and fecal coliform counts after 4 wk of storage at 4°C. There were no significant differences between the control and the samples treated with antioxidants after either 1 or 2 wk of storage.

An antioxidant is a substance that is added to fats or fat-containing foods to retard oxidation and thereby prolong their wholesomeness and palatability (*3,4,7,11,18,25*). Some antioxidants were found to have antimicrobial properties against bacteria, fungi, protozoa and viruses (*2,10,13-17,19,26,27,30,31,35-39,42*). Most studies on the antibacterial effect of selected antioxidants have been on pathogens and indicator organisms, such as *Salmonella* spp., *Staphylococcus aureus, Escherichia coli, Vibrio parahemolyticus, Clostridium perfringens* and *Clostridium botulinum (6,21,22,29,32,35,37,41).*

Davidson and Branen (*8,9*) conducted a detailed study on inhibition of psychrotrophic *Pseudomonas* by BHA. Effects of antioxidants in foods have been determined for sausage (*23*), process cheese (*1*), cooked rice (*34*) strained chicken (*34*) and turkey meat (*24*). In general the effectiveness of antioxidants against microorganisms is reduced in foods. A screening test for a broad spectrum of bacteria against varying concentrations of common antioxidants has not been done.

The purpose of this study was to: (a) evaluate the antimicrobial effects of different concentrations of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG) alone or in combination against twenty-four bacterial species (sixteen gram-negative and eight gram-positive) growing in laboratory media and (b) determine the microbial efficacy of these antioxidants in preventing the growth of psychrotrophs, coliforms and fecal coliforms in ground pork stored at 4°C.

MATERIALS AND METHODS

Effect of antioxidants on selected microorganisms in laboratory media

Organisms. The following microorganisms were used to ascertain the effects of four selected antioxidants on a wide range of pathogenic and nonpathogenic bacteria. The gram-negative bacteria were *Arizona* sp., *Citrobacter freundii, Edwardsiella tarda, Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas fragi, Salmonella* sp. 20, *Serratia marcescens, Vibrio angularum* and *Yersinia enterocolitica.* The gram-positive bacteria were *Agrobacterium tumefaciens, Arthrobacter* sp., *Lactobacillus brevis, Pediococcus* sp., *Sarcina lutea, Staphylococcus aureus* 100, *S. aureus* 137 and *Streptococcus faecalis.* All cultures were obtained from the Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS. Identities of the bacterial cultures were confirmed by using morphology, Gram stain reaction and the Minitek system (BBL Microbiology System, Cockeysville, MD). The characteristics of the named bacteria corrresponded with their descriptions in Bergey's manual (*5*).

Working cultures were prepared by aseptically transferring one loopful of culture from stock culture into 25 ml of brain heart infusion, and incubating for 24 h at 32°C.

Media. 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.

Antioxidants. BHA, BHT, PG and TBHQ were selected for this study because they are commonly used in foods. BHA, BHT, PG and TBHQ were obtained from Eastman Chemical Products Inc., TN. A stock solution (10,000 ppm) of each antioxidant was prepared by dissolving 0.1 g of the antioxidant in 10 ml of 95% ethyl alcohol. Appropriate amounts were then pipetted from the stock solutions into the growth media (vol/vol) to give the required concentrations in parts per million (ppm). Concentrations tested were 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 ppm for each antioxidant alone, and 0, 100, 200, 300 and 400 ppm for combinations of the four antioxidants. Equal amounts of each antioxidant were mixed to give the final concentration (e.g., 50 ppm BHA + 50 ppm BHT = 100 ppm BHA-BHT combination). These

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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 were stable after autoclaving at 121°C for 15 min.

Miniaturized microbiological testing procedures for solid and liquid media. All tests were conducted using the "miniaturized microbiological method" developed by Fung and Hartman (*12*). Tests were done in wells of sterile microtiter plates (8 × 12 wells/plate) for the liquid system, and in rectangular plates (8.5 × 12.5 × 1.0 cm) for the solid system. Data were recorded as growth (+) or no growth (−). All tests were done in duplicate.

Effect of antioxidants on psychrotrophs, coliforms and fecal coliforms in ground pork during cold storage

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

Preparation of samples. Four separate fresh pork samples (20 lb each) taken from different carcasses were obtained from the Department of Animal Sciences and Industry, Kansas State University, Manhattan. Visible fat was trimmed from the cuts, and the lean and the fat were ground separately (grinding was done in two steps, first coarse grinding and then fine grinding). The percentage of fat in the lean meat was determined by a Hobart fat percentage indicator for ground beef (Hobart Corp., Troy, OH). 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 100-g portions in sterile Stomacher (*33*) bags. The bags served as a vessel for blending pork as well as a storage container for the pork, and a dilution vessel for viable cell counts.

Addition of antioxidants. Preparation of antioxidant solutions of BHA, BHT, PG and TBHQ was described above. Appropriate amounts, from stock solutions, were pipetted into the 100-g meat samples to give the required concentrations (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 min in a Stomacher. Each bag was then rolled and tied with a rubber band. For each treatment, six identical bags were prepared. Microbiological analyses were done on the contents of each of two bags after 1, 2 and 4 wk of storage at 4°C. Eight bags were prepared from the control samples and analyzed at zero time, 1, 2 and 4 wk of storage at 4°C.

Microbiological analyses. Ground pork (100 g) was massaged for 2 min in the Stomacher after the addition of 300 ml of sterile buffer. The slurry was again diluted accordingly in sterile buffer. Pour plates of standard plate count agar (Difco) were made in duplicate and incubated for 10 d at 7°C for determination of psychrotroph counts. Enumeration of coliforms was done by pour plates of violet red bile agar (VRB; Difco) capped with a layer of VRB to prevent spreaders. Incubation was for 24 h at 32°C for coliforms and at 44.5°C for fecal coliforms according to the procedure developed by Klein and Fung (*20*).

Statistical analysis was done by analysis of variance, and Duncan's multiple range test at the 0.05-significance level was used for determining differences between multiple means (*40*) for all experiments.

RESULTS

Effects of individual antioxidants on selected microorganisms in laboratory media

The minimum inhibitory concentrations of BHA, BHT, TBHQ and PG against test cultures in nutrient agar and

TABLE 1. *Effect of BHA, BHT, TBHQ and PG on selected bacteria.*

	Minimum inhibitory concentration (ppm) of antioxidants[a]							
	BHA		BHT		TBHQ		PG	
Bacteria	NA[b]	BHI	NA	BHI	NA	BHI	NA	BHI
Gram-negative								
Arizona sp.	200[c]	500 +[d]	500 +	500 +	300	300	100	250
Citrobacter freundii	350	500 +	500 +	500 +	500 +	500 +	500	500 +
Edwardsiella tarda	150	500 +	200	500	150	200	100	250
Enterobacter aerogenes	350	500 +	500 +	500 +	500	500 +	450	500 +
Enterobacter cloacae	400	500	500 +	500 +	450	500 +	500	350
Escherichia coli	200	500 +	500 +	500 +	450	500 +	400	500 +
Klebsiella oxytoca	400	500 +	500 +	500 +	450	500 +	500	500 +
Klebsiella pneumoniae	400	500 +	500 +	500 +	500	500 +	500	500 +
Proteus vulgaris	250	500 +	150	450	250	450	150	500 +
Pseudomonas aeruginosa	400	500 +	500 +	500 +	500	500 +	500	500 +
Pseudomonas fluorescens	300	450	500 +	300	500	200	500	350
Pseudomonas fragi	100	150	300	350	100	100	100	100
Salmonella sp. 20	300	500 +	500 +	500 +	500 +	500 +	300	500 +
Serratia marcescens	500 +	500 +	500 +	500 +	400	500 +	450	500 +
Vibrio angularum	250	450	500 +	500 +	100	300	100	500 +
Yersinia enterocolitica	250	500 +	100	400	250	400	100	500 +
Gram-positive								
Agrobacterium tumefaciens	100	100	100	100	100	200	200	150
Arthrobacter sp.	100	500	150	500	100	200	200	350
Lactobacillus brevis	100	500 +	250	500 +	300	450	250	500 +
Pediococcus sp.	100	500 +	150	500 +	100	450	100	500 +
Sarcina lutea	150	500 +	300	500 +	100	250	300	400
Staphylococcus aureus 100	200	250	100	200	100	100	200	450
Staphylococcus aureus 137	250	300	500 +	500 +	100	100	200	450
Streptococcus faecalis	400	500 +	500 +	500 +	500 +	500 +	500	500 +

[a]Antioxidants tested: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, tertiary-butylhydroquinone; PG, propyl gallate at 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 ppm. Experiments were done in duplicate.

[b]NA, nutrient agar; BHI, brain heart infusion.

[c]The concentration listed is the minimum concentration inhibiting growth of test cultures.

[d]500 +, indicates that organisms grew at the highest concentration tested.

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TABLE 2. *Effect of combined antioxidants on selected bacteria.*

Bacteria	Minimum inhibitory concentration (ppm) of selected combinations of antioxidants[a]											
	BHA-BHT (1:1)		BHA-TBHQ (1:1)		BHA-PG (1:1)		BHT-TBHQ (1:1)		BHT-PG (1:1)		TBHQ-PG (1:1)	
	NA[b]	BHI	NA	BHI	NA	BHI	NA	BHI	NA	BHI	NA	BHI
Gram-negative												
Arizona sp.	400+[c,d]	400+	300	400+	300	400+	400	400+	300	400+	100	400
Citrobacter freundii	400+	400+	400+	400+	300	400+	400+	400+	400+	400+	200	400
Edwardsiella tarda	100	400	100	400	100	100	100	400+	300	100	100	400
Enterobacter aerogenes	400+	400+	400+	400+	300	400+	400+	400+	400+	400+	200	400+
Enterobacter cloacae	400+	400+	400+	400+	400+	400+	400+	400+	400+	400+	200	300
Escherichia coli	400+	400+	400+	400+	300	400+	400+	400+	400+	400+	300	400
Klebsiella oxytoca	400+	400+	400+	400+	400+	400+	400+	400+	400+	400+	300	400+
Klebsiella pneumoniae	400+	400+	400+	400+	400+	400+	400+	400+	400+	400+	300	400+
Proteus vulgaris	300	400+	200	400+	200	400+	200	400+	200	400+	100	400
Pseudomonas aeruginosa	400+	400+	400+	400+	400+	400+	400+	400+	400+	400+	300	400+
Pseudomonas fluorescens	400+	200	400+	400	400+	400+	400+	300	400+	400+	200	200
Pseudomonas fragi	100	300	100	200	100	200	100	300	400+	300	100	200
Salmonella sp. 20	400+	400+	400+	400+	300	400+	400+	400+	400	400+	200	400
Serratia marcescens	400+	400+	400+	400+	400+	400+	400	400+	400+	400+	200	400
Vibrio angularum	400+	400+	100	400	100	400+	100	400+	100	400	100	400
Yersinia enterocolitica	300	400	100	400	100	400+	100	400+	100	300	100	400
Gram-positive												
Agrobacterium tumefaciens	100	100	100	100	100	100	100	200	100	100	100	200
Arthrobacter sp.	100	100	100	300	100	200	100	400+	100	400+	100	200
Lactobacillus brevis	200	400+	100	400+	100	400+	100	400+	100	400+	100	400
Pediococcus sp.	100	400+	100	400+	100	400+	100	400+	400+	400+	100	400
Sarcina lutea	400+	100	100	100	100	400	100	100	100	400	100	200
Staphylococcus aureus 100	300	400+	100	300	100	100	100	400	300	400	100	100
Staphylococcus aureus 137	300	400+	100	300	200	400+	100	200	300	400+	100	100
Streptococcus faecalis	400+	400+	400+	400+	400+	400+	400+	400+	400+	400+	200	400+

[a]Antioxidants tested: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, tertiary-butylhydroquinone; PG, propyl gallate at combined levels of 0, 100, 200, 300 and 400 ppm. Experiments were done in duplicate.
[b]NA, nutrient agar; BHI, brain heart infusion.
[c]The concentration listed is the minimum concentration inhibiting growth of test cultures.
[d]400+, indicates that organisms grew at the highest concentration tested.

brain heart infusion is shown in Table 1. In either agar or brain heart infusion, 50 ppm of all antioxidants were not inhibitory to any of the bacteria. At comparably higher concentrations, BHA was more effective in inhibiting the cultures in nutrient agar than in brain heart infusion. In general, in both systems BHA inhibited gram-positive bacteria more than gram-negative bacteria. At 400 ppm, BHA in nutrient agar inhibited all cultures, except *S. marcescens,* which was resistant to BHA even at 500 ppm. *P. fragi* was exceptionally sensitive to BHA among the gram-negative bacteria tested. *A. tumefaciens, Arthrobacter* sp., *L. brevis* and *Pediococcus* sp. were inhibited in nutrient agar by 100 ppm BHA. *S. aureus* strains were inhibited by 200 to 250 ppm BHA. *S. faecalis* was exceptionally resistant to BHA among the gram-positive bacteria tested. BHA had the greatest antimicrobial activity in nutrient agar, compared with the other antioxidants tested.

BHT, TBHQ and PG also had similar inhibitory trends against gram-positive and gram-negative bacteria. They were more effective in nutrient agar than in brain heart infusion. BHT was the least effective antimicrobial agent of the four antioxidants tested.

Effects of combinations of antioxidants in bacterial cultures in laboratory media are recorded in Table 2 for the combinations of BHA-BHT, BHA-TBHQ, BHA-PG, BHT-TBHQ, BHT-PG and TBHQ-PG. In general, the combinations inhibited gram-positive more than the gram-negative bacteria. Individual bacteria, however, responded differently to different combination. There were also synergistic effects observed in some combinations of antioxidants compared with individual antioxidants against bacteria tested. For example, the TBHQ-PG combination at 100 ppm inhibited more bacterial species than TBHQ or PG alone at 100 ppm. Similarly, the BHA-TBHQ combination was more effective than either BHA or TBHQ alone at 100 ppm.

These data agree with the observations of Shih and Harris (*35*) and Pierson et al. (*26*) who reported gram-positive bacteria are more sensitive to antioxidants than gram-negative bacteria. However, responses within each group were not identical. For example, *P. fragi* and *E. tarda,* unlike most of the gram-negative bacteria tested, were very sensitive to antioxidants, whereas *S. faecalis,* among the gram-positive species tested, was exceptionally resistant to the antioxidants. Of the six combinations tested, the TBHQ-PG combination inhibited the largest number of organisms followed by the BHA-PG, BHT-

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TBHQ, BHA-TBHQ, BHT-PG and BHA-BHT combination, respectively.

This study indicated that the four antioxidants tested were effective in inhibiting microbial growth in laboratory media. It should be emphasized that the incubation systems were optimal for growth of test organisms, which is a situation not likely to occur in food systems. However, this provides a model system for experiments.

Effects of antioxidants on psychrotrophs, coliforms and fecal coliforms in ground pork

The effect of BHA, BHT, TBHQ and PG on psychrotrophs in pork samples is shown in Table 3. There was no significant difference between control and antioxidant-treated samples for the first 2 wk. On the 4th week, there were significant differences, although all samples had very high bacterial counts (10^9 CFU/g). The effect of the four antioxidants on coliform populations is shown in Table 4. There were no significant differences in the samples in the 2nd week of storage but at the 4th week the control samples had higher levels of coliforms than the antioxidant-treated samples. Fecal coliform results (Table 5) indicated that in the 1st week most samples had non-detectable levels of fecal coliforms. After 4 wk of storage, there were significant differences between the control and the antioxidant-treated samples, except samples treated with 100 ppm BHA and 100 ppm TBHQ.

In conclusion, BHA, BHT, TBHQ and PG were effective in inhibiting bacterial growth in laboratory media; however, the antioxidants were less active in liquid than solid media. The antioxidant's antimicrobial activities were greatly reduced in ground pork. Other researchers, also have reported that food (*1,23,24,34*) and food components (*28*) reduce the effectiveness of antioxidants

TABLE 3. *Average psychrotroph counts (colony-forming units/g) of four pork samples.*

Treatment	Initial	1 wk	2 wk	4 wk
Control	8.6×10^4	6.7×10^{7a}	2.1×10^{9b}	9.6×10^{9c}
BHA				
100 ppm		5.0×10^{7a}	1.8×10^{9b}	7.1×10^{9d}
200 ppm		4.6×10^{7a}	1.6×10^{9b}	5.0×10^{9e}
400 ppm		3.4×10^{7a}	8.1×10^{8b}	3.9×10^{9e}
BHT				
100 ppm		6.6×10^{7a}	1.9×10^{9b}	6.4×10^{9e}
200 ppm		5.1×10^{7a}	1.4×10^{9b}	3.6×10^{9e}
400 ppm		4.4×10^{7a}	1.1×10^{9b}	3.6×10^{9e}
TBHQ				
100 ppm		6.7×10^{7a}	1.9×10^{9b}	6.2×10^{9d}
200 ppm		6.0×10^{7a}	1.3×10^{9b}	5.1×10^{9e}
400 ppm		3.8×10^{7a}	9.3×10^{8b}	4.7×10^{9e}
PG				
100 ppm		6.3×10^{7a}	1.7×10^{9b}	5.9×10^{9e}
200 ppm		5.4×10^{7a}	1.5×10^{9b}	4.7×10^{9e}
400 ppm		3.9×10^{7a}	9.9×10^{8b}	3.2×10^{9e}

[a,b,c,d,e]Means with the same letter are not different (P>0.05).

TABLE 4. *Average coliform counts (colony-forming units/g) of four pork samples.*

Treatment	Initial	1 wk[a]	2 wk	4 wk
Control	ND[b]	----	1.8×10^{5c}	6.9×10^{7d}
BHA				
100 ppm		----	2.2×10^{5c}	3.3×10^{7e}
200 ppm		----	1.7×10^{5c}	2.2×10^{7e}
400 ppm		----	3.6×10^{4c}	1.1×10^{7e}
BHT				
100 ppm		----	1.3×10^{5c}	3.5×10^{7e}
200 ppm		----	9.4×10^{4c}	2.4×10^{7e}
400 ppm		----	1.1×10^{4c}	1.5×10^{7e}
TBHQ				
100 ppm		----	1.2×10^{5c}	2.3×10^{7e}
200 ppm		----	8.6×10^{4c}	1.4×10^{7e}
400 ppm		----	1.4×10^{4c}	7.3×10^{6f}
PG				
100 ppm		----	1.1×10^{5c}	3.1×10^{7e}
200 ppm		----	9.6×10^{4c}	1.7×10^{7e}
400 ppm		----	3.5×10^{4c}	1.1×10^{7e}

[a]No counts were made.
[b]ND, Non-detectable.
[c,d,e,f]Means with the same letter are not different (P>0.05).

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TABLE 5. *Average fecal coliform counts (colony-forming units/g) of four pork samples.*

Treatment	Initial	1 wk	2 wk	4 wk
Control	ND[a]	1.4×10^{1}	3.0×10^{3b}	3.7×10^{4e}
BHA				
100 ppm	ND	ND	ND	1.2×10^{4e}
200 ppm	ND	ND	ND	3.5×10^{3f}
400 ppm	ND	ND	ND	1.8×10^{3f}
BHT				
100 ppm	ND	ND	ND	6.4×10^{3f}
200 ppm	ND	ND	ND	2.7×10^{3f}
400 ppm	ND	ND	ND	3.6×10^{3f}
TBHQ				
100 ppm	ND	ND	3.9×10^{2b}	1.5×10^{4e}
200 ppm	ND	ND	1.6×10^{2d}	4.8×10^{3f}
400 ppm	ND	ND	ND	2.1×10^{3f}
PG				
100 ppm	ND	4.8×10^{1}	1.6×10^{2d}	5.2×10^{3f}
200 ppm	ND	ND	9.0×10^{1d}	1.2×10^{3f}
400 ppm	ND	ND	2.8×10^{1d}	8.5×10^{2f}

[a]ND, Non-detectable.
[b,c,d,e,f]Means with the same letter are not different ($P>0.05$).

against test cultures. Further work needs to be done to define the factors that reduce the antimicrobial activity of antioxidants in foods, and to investigate in foods the synergistic effects of antioxidants when combined with one another and with other known antimicrobial agents.

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