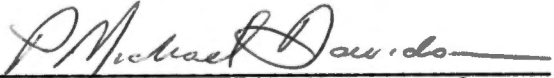


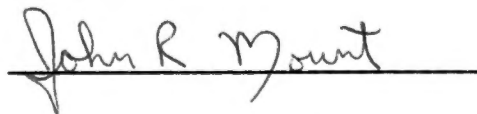
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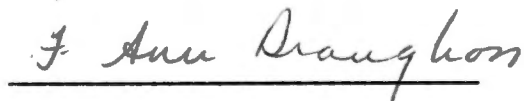
I am submitting herewith a thesis written by Emilia Rico-Munoz entitled "Effect of Food Components on the Antimicrobial Activity of Phenolic Antioxidants." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.


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Vice Chancellor
Graduate Studies and Research

EFFECT OF FOOD COMPONENTS ON THE
ANTIMICROBIAL ACTIVITY OF
PHENOLIC ANTIOXIDANTS

A Thesis
Presented for the
Master of Science
Degree
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Emilia Rico-Munoz

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3062980

DEDICATION

To my parents, Juan and Josefina, who gave me love, support and encouragement to achieve my scholastic goals.

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ABSTRACT

This study was conducted to elucidate exactly what effects various food components, such as lipids and proteins, have on the antimicrobial activity of butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ).

Five microorganisms were used in this study: the molds, Penicillium citrinum NRRL 5727 and Aspergillus niger; a yeast, Saccharomyces cerevisiae; and the bacteria, Pseudomonas fluorescens ATCC 15456 and Staphylococcus aureus ATCC 12600. All cultures were incubated under optimum conditions. Inhibition studies were conducted as follows: (1) bacteria and yeast -- samples were removed from each flask at selected time intervals and enumerated by the pour plate technique and (2) molds -- the mycelial mat weight was used as an indication of growth.

Concentrations of BHA and TBHQ used in this experiment were as follows: P. citrinum, 200 ppm BHA, 400 ppm TBHQ; A. niger, 200 ppm BHA, 600 ppm TBHQ; S. cerevisiae, 200 ppm BHA, 400 ppm TBHQ; S. aureus, 300 ppm BHA, 50 ppm TBHQ; P. fluorescens, 200 ppm BHA, 300 ppm TBHQ. The antimicrobial effectiveness of these compounds was tested in the presence of 0, 1.5, 3.0 and 4.5%(v/v) of a lipid (corn oil) or 0, 3, 6 and 9%(w/v) of a protein (casein).

In every case, the presence of casein in the growth media diminished the antimicrobial activity of BHA and TBHQ. The overall effects were dependent upon species of microorganism tested as well

as the type of antioxidant. BHA was affected more by casein than TBHQ. Decreased antimicrobial activity was probably due to binding of the antioxidants by the casein.

As with casein, corn oil also depressed the antimicrobial activity of both antioxidants. BHA was more affected in all cases by the lipid than by the protein. In most cases, even the presence of the emulsifier, Tween 80, eliminated much of the effectiveness of BHA. In contrast, TBHQ was less affected by the lipid than BHA, except in the case of S. aureus where only 50 ppm TBHQ were used.

In summary, the antimicrobial effectiveness of BHA and TBHQ against a variety of microorganisms was significantly diminished by both casein and corn oil. However, in most cases, TBHQ was affected less by the food components than BHA. The reason for this was most likely a combination of the higher concentrations used and polarity differences of the compound. Finally, the primary cause for the loss in activity of the antioxidants in the presence of the lipid was due to their solubilization into the lipid phase.

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INTRODUCTION

Butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) are phenolic compounds commonly added to many lipids and lipid-containing food products to serve as antioxidants. Their phenolic nature suggests that they might also act as antimicrobial agents since other phenolic compounds are used for this purpose (Aalto et al., 1953). Ward and Ward (1967) were the first to report the antimicrobial activity of phenolic antioxidants. It is now known that these compounds have antimicrobial activity against a variety of bacteria, molds, viruses and protozoa (Branen et al., 1980). Some authors, however, have reported that these compounds are not as effective in food systems as they are in broth or agar systems. Robach et al. (1977) were the first to report a dramatic decrease in the antimicrobial activity of BHA in a food system. They found that the growth of the microorganism Vibrio parahaemolyticus was inhibited by 50 ppm BHA in Trypticase Soy Broth (TSB) while 400 ppm BHA was required to inhibit growth of the bacterium in a crab meat homogenate. Therefore, eight times the concentration of BHA was required in the crab meat as in the media for the same lethal effect. Shelef and Liang (1982) recently reported that while only 100-200 ppm BHA was required to inhibit several Bacillus species in media, 500 ppm or 0.5% was required for any preservative action in comminuted chicken meat. Similar results have been reported by other researches (Klindworth et al., 1979; Ahmad, 1979).

These findings have resulted in the proposal of two theories as to why BHA and TBHQ lose antimicrobial activity in food systems. The first theory states that antioxidants are "used up" by being preferentially oxidized during autoxidation which causes them to lose their antimicrobial activity. The second theory is based on the fact that these compounds are non-polar or lipid soluble and it states that antioxidants are solubilized in the lipid phase of the food which makes them unavailable to act on microorganisms which grow in the aqueous phase of the food.

In studies with non-fat dry milk, Cornell et al. (1971) also found that BHA was bound to casein most probably through hydrophobic interactions. Therefore, loss of the antimicrobial activity of BHA and TBHQ in food systems could be also due, in part, to the binding of the antioxidants to the proteins of the food.

This study was undertaken with two main objectives. The first was to evaluate the effect of the presence of a lipid on the antimicrobial activity of both BHA and TBHQ. The second objective was to determine if other food components, such as proteins, may also have an effect on inhibition by BHA and TBHQ.

CHAPTER 1

REVIEW OF THE LITERATURE

I. INTRODUCTION

Butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) are phenolic compounds commonly added to many lipids and lipid-containing food products to serve as antioxidants. It has been known for many years that phenolic compounds, such as para-hydroxybenzoic acid esters (parabens), possess antimicrobial properties (Aalto et al., 1953). Therefore, the phenolic nature of these antioxidants suggests that they might also act as antimicrobial agents. Ward and Ward (1967) were the first to report the antimicrobial activity of phenolic antioxidants. They found that 10,000 ppm of butylated hydroxytoluene (BHT) was only slightly inhibitory to Salmonella senftenberg. Chang and Branen (1975) were the first to report the antimicrobial activity of BHA. They found that 150 ppm BHA was necessary for inhibition of Staphylococcus aureus in nutrient broth and that Salmonella typhimurium was only slightly inhibited by 400 ppm BHA. Since then, many authors have reported on the antimicrobial activity of BHA against a variety of bacteria, molds, viruses and protozoa (Branen et al., 1980).

Tertiary butylhydroquinone (TBHQ), as an antimicrobial, has been studied less than BHA. Ericksen and Tompkin (1977) were the

first to report that TBHQ was an effective antimicrobial agent against several bacteria. They were able to delay spoilage of a 30% gelatin solution using 0.01% TBHQ and prevent the growth of normal spoilage bacteria in milk at 2°C with 0.005% TBHQ.

Therefore, these phenolic antioxidants may possess dual functions in food products: as antioxidants preventing autoxidation of lipids and as antimicrobials preventing growth of spoilage microorganisms in the food product. This would represent a clear advantage of these phenolic antioxidants over other food additives having a single function as antioxidants or as preservatives. However, some authors have reported a dramatic decrease in antimicrobial activity of the antioxidants due to the presence of added lipid in a laboratory medium (Klindworth et al., 1979; Ahmad, 1979) or the incorporation of the compounds into lipid-containing foods (Robach et al., 1977; Shelef and Liang, 1983).

In this chapter, a review of the studies done on the antimicrobial activity of BHA and TBHQ in laboratory media will be presented followed by a discussion of the environmental factors and food components that may affect that activity. A brief discussion of the mechanism of action and safety of BHA and TBHQ will also be presented.

II. TYPES OF PHENOLIC ANTIOXIDANTS

Phenolic antioxidants are divided depending on their chemical structures as follows:

1. Monohydric phenols -- antioxidants with only one hydroxylic group on their molecule. The standard pair of monohydric synthetic antioxidants which are in common use are the lipophilic compounds BHA and BHT (Figure 1). Neither of these can form a semiquinone or a quinone as such, but a resonance hybrid somewhat analogous to a semiquinone is possible.

2. Dihydric phenols -- antioxidants with two hydroxylic groups, thus, with two rapid, reversible hydrogen donations. The well-delocalized electron of their semiquinone radical makes them also excellent chain stoppers, if solubility and compatibility are met, which is sometimes difficult. Indeed, given the optimum amphiphilic characteristics (Table 1) some of the most effective vegetable oil antioxidants are the paradihydric hydroquinones, particularly since they are not plagued by metal chelation and resultant coloring. One of the most effective antioxidants in this group is TBHQ (Figure 1).

3. Trihydric phenols -- antioxidants with three hydroxylic groups, such as the alkyl esters of gallic acid. One of the most commonly used is propyl gallate (PG) (Figure 1). Its amphiphilic nature (Table 1) results in a very effective antioxidant for dry vegetable oils. The larger alkyl group, the greater the lipid solubility, and after a point, the lower the surface activity and Active Oxygen Method (AOM) effectiveness in oils.

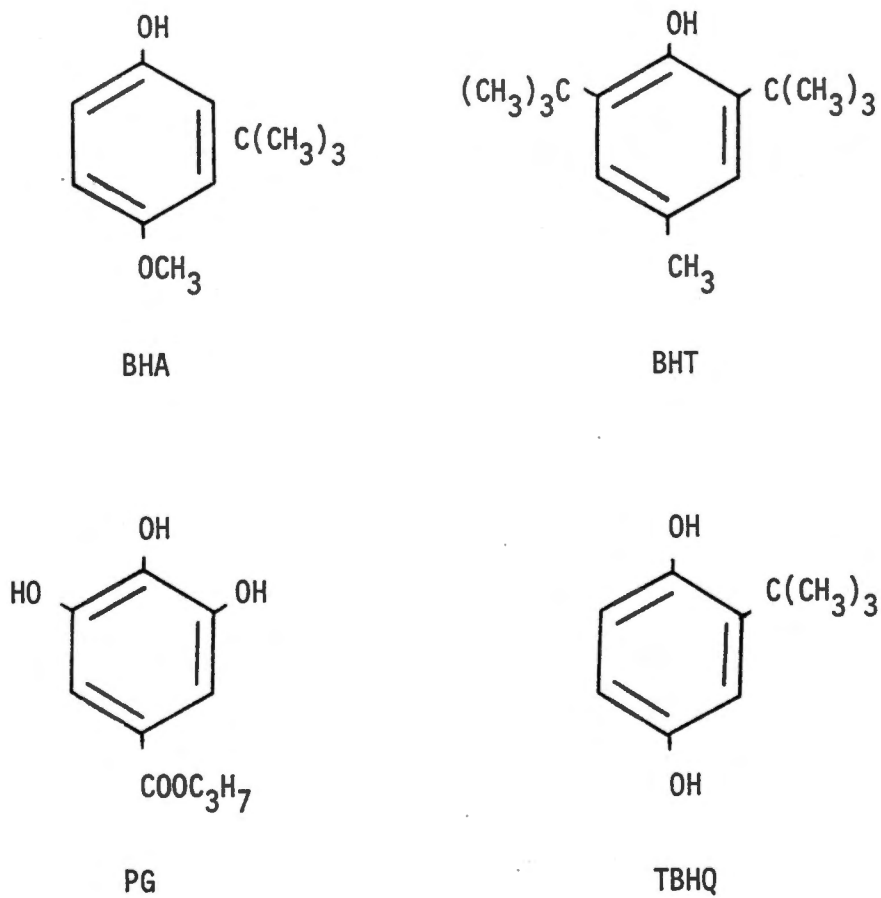


Figure 1. The major synthetic antioxidants in common food use.

TABLE 1
SOLUBILITY OF ANTIOXIDANTS

Solvent	Temperature (°C)	Antioxidant Solubility (%)			
		BHT	BHA	TBHQ	PG
Water	25	Insol.	Insol.	<1	<1
Glycerol	25	Insol.	1	ca. 5	25
Propylene Glycol	25	Insol.	70	30	55
Methyl Linoleate	25	V. sol.	V. sol.	10	1
Corn oil	25	40	30	10	Insol.
Lard	50	50	40	5	1

Source: J. O. Ragnarsson. 1977. "Accelerated temperature shelf life testing of antioxidants in dehydrated and intermediate moisture systems," Ph. D. thesis, Univ. of Minnesota, Minneapolis, MN, 1976, University Microfilms International, Ann Arbor, MI.

III. ANTIMICROBIAL ACTIVITY OF BHA AND TBHQ

A. ACTIVITY AGAINST BACTERIA

BHA and TBHQ have been reported to be inhibitory to a variety of Gram-positive and Gram-negative bacteria. Antimicrobial studies have also shown that these compounds are more effective against fungi and Gram-positive bacteria than they are against Gram-negative bacteria. This antimicrobial pattern is quite similar to that reported for another group of phenolic compounds, the alkyl esters of para-hydroxybenzoic acid or parabens (Aalto et al., 1953).

Among the Gram-positive bacteria, one of the most studied has been Staphylococcus aureus. In either nutrient or Trypticase Soy Broth (TSB), 200 ppm BHA (Chang and Branen, 1975; Pierson et al., 1980) or 400 ppm (Shih and Harris, 1977) resulted in no viable cells after 8-9 hr incubation of different strains of S. aureus. Ayaz (1975) and Ayaz et al. (1980) reported that 150-200 ppm BHA resulted in 93-100% growth inhibition of three strains of S. aureus in brain heart infusion (BHI) broth and that 100 ppm or greater inhibited the formation of enterotoxin. Differences in growth inhibition of S. aureus by BHA have been theorized to be the result of differences in culture media and strain of this microorganism (Sands and Bennet, 1966; Shih and Harris, 1977).

Ericksen and Tompkin (1977) found that 30 ppm TBHQ was totally inhibitory to the growth of S. aureus in an agar medium. Raccach and Henningsen (1981) demonstrated that, while 30-50 ppm TBHQ was inhibitory to S. aureus 100 in BHI broth, more than 50 ppm was needed to inhibit growth of S. aureus z88 under the same conditions. Therefore, it seems that strain differences play a major role in inhibition differences of this microorganism.

Stern et al. (1979) found 100 ppm BHA caused total inhibition of S. aureus S-6 in the presence of 5 or 7% NaCl. Davidson et al. (1981) reported that both BHA and TBHQ had synergistic antimicrobial activity with potassium sorbate against S. aureus at 0.05% sorbate/50 ppm BHA, 0.05% sorbate/10 ppm TBHQ and 0.1% sorbate/10 ppm TBHQ. In a similar study, 25 ppm TBHQ was found to inhibit growth of the strain S-6 while 0.1% sorbate together with 25 ppm TBHQ was needed to inhibit strain 12600 in the same medium (Robach and Stateler, 1980).

Klindworth et al. (1979) showed that three strains of Clostridium perfringens were totally inhibited by 150 ppm BHA in fluid thioglycollate medium (FTM) at 37°C. Growth was delayed or inhibited to a lesser degree at 50 and 100 ppm BHA whereas 200 and 400 ppm BHA killed all C. perfringens cells. At 45°C, 150 ppm BHA was not totally inhibitory for an unknown reason. Some possible causes for variation in recovery were reported to be the age and health of the culture used (von Schelhorn, 1953) or differences in

handling of samples, oxidation-reduction potential, pH or temperature. The results also indicated that at higher concentrations, BHA was bactericidal rather than bacteriostatic.

BHA has also been found to be inhibitory to the growth of three proteolytic strains (10755A, 62A, 213B) of Clostridium botulinum in prereduced thiotone yeast-extract glucose medium (TYG) at pH 7.0 and 37°C (Robach and Pierson, 1979). Outgrowth and toxin production occurred with spores of strain 10755A when 25 ppm BHA was present in the medium but only growth occurred with 50 ppm BHA. Strains 62A and 213B were inhibited by 25 ppm BHA for an incubation period of seven days. Recently, Reddy et al. (1982) reported that 200 ppm BHA or 400 ppm TBHQ were needed to inhibit growth and toxin production of a mixture of equal numbers of ten strains of C. botulinum spores in TYG medium. Variations with the results reported by Robach and Pierson (1979) were reported to be the result of inoculum differences since Reddy et al. (1982) used a mixed culture of type A and type B spores of C. botulinum.

The minimum inhibitory concentration (MIC) of TBHQ against Pediococcus pentosaceus, a meat starter culture, in quarter strength lactobacilli MRS broth (QSMRS) was 15 ppm for 10^3 - 10^5 cells/ml and 20 ppm for 10^6 cells/ml (Raccach and Henningsen, 1981). Inhibition by TBHQ was increased significantly when cells were heat stressed or in the presence of sodium chloride. However, sodium chloride did not enhance the activity of BHA when tested against the same

microorganism (Raccach, 1980). These findings had practical implications concerning the fermentation of meat mixtures. Sodium chloride and TBHQ might extend the normal fermentation period required by P. pentosaceus, thus allowing the growth of pathogenic microorganisms such as Staphylococcus aureus.

Among the Gram-negative bacteria, Salmonella typhimurium and Escherichia coli have been the most extensively studied. Chang and Branen (1975) reported that 200 ppm BHA decreased the initial 10^7 inoculum of S. typhimurium by 90% after 12 hr while 400 ppm decreased the inoculum by 99% after 4 hours. Pierson et al. (1980) found that 400 ppm BHA caused rapid decrease in the viable cell count of S. typhimurium after 3 hr of incubation, followed by an increase in cell numbers to a final count that was two log cycles higher than the initial inoculum. Another strain of S. typhimurium was totally inhibited by 150-200 ppm BHA in Trypticase Soy Broth (TSB) at 32°C (Davidson et al., 1981). The differences in lethal concentrations were reported to be result of the strain used, media, or cultural conditions since these factors have been shown to influence BHA activity in other studies (Branen et al., 1981). Davidson et al. (1981) also reported that there was a synergistic antimicrobial effect against this microorganism by BHA with potassium sorbate at levels of 0.05% sorbate/50 ppm BHA, 0.05% sorbate/100 ppm BHA and 0.1% sorbate/50 ppm BHA.

Chang and Branen (1975) reported that 200 ppm BHA reduced 10^6 cells of Escherichia coli to 10^4 after 2 hr of incubation and that 400 ppm BHA totally inactivated the cells in less than 1 hr in nutrient broth at 37°C. Shih and Harris (1977), however, found that at the 400 ppm level, BHA alone and combined with propyl gallate were not as effective in killing E. coli as was 400 ppm nor-dihydroguaiaretic acid (NDGA) in TSB at 35°C.

Robach et al. (1977) reported that 50 ppm BHA totally inhibited Vibrio parahaemolyticus in TSB containing 2.5% NaCl. In a crab meat homogenate, however, 400 ppm was required to completely inhibit V. parahaemolyticus growth.

Davidson and Branen (1980a) found inhibition of the spoilage microorganism Pseudomonas to be species-dependent. While P. fluorescens was inhibited by 150 ppm BHA, more than 400 ppm BHA was necessary for total inhibition of P. fragi.

B. ACTIVITY AGAINST FUNGI

Chang and Branen (1975) were the first to report on the anti-fungal activity of BHA. They found that 1,000 ppm BHA was necessary to inhibit growth and the production of aflatoxins B₁, B₂, G₁ and G₂ by Aspergillus parasiticus spores in glucose/salts medium at room temperature. However, 50 ppm BHA slightly inhibited growth and aflatoxin production by mycelium and 250 ppm BHA or greater prevented all growth and aflatoxin production by A. parasiticus mycelium.

Sankaran (1976) tested three antioxidants, BHA, BHT and propyl gallate (PG), against two molds, Aspergillus flavus and A. fumigatus, and one yeast, Saccharomyces cerevisiae. At 2,500 ppm, BHA was the most effective of all the antioxidants completely inhibiting the growth of all microorganisms. At lower concentrations however, BHA only delayed the germination of the mold spores by 6-12 days. The resultant colony appearance was reported to be very different from normal colonies in that they were white buttony structures with no color or conidia formation. An explanation of this effect was involved with a lowered oxygen availability.

The effect of BHA and BHT against six toxigenic and six non-toxigenic strains of A. flavus was also studied by Fung et al. (1977). In solid agar, BHT at all levels tested (0.005, 0.010, 0.015 and 0.020 g per plate) did not inhibit growth, sporulation, pigmentation or toxigenesis of any culture tested. However, BHA was found to inhibit A. flavus growth at 0.005-0.020 g per plate of solid media (60 or 100 mm in diameter) at 28°C. At 0.01 g per plate, BHA also inhibited the production of aflatoxins B₂, G₁ and G₂ in the 60-mm-diameter plate of solid medium. Aflatoxin B₁ production was inhibited to a variable degree. BHA inhibited pigment formation which was found to be directly related to toxin production.

Ahmad (1979) reported that BHA, at 200 ppm, caused 100% inhibition of species of Aspergillus, Penicillium and Geotrichum in

glucose/salts broth at 21°C. Addition of Tween 80, an emulsifier, and a lipid, corn oil, depressed the activity of BHA against A. flavus. BHA showed no synergistic antimicrobial activity with sorbate against this microorganism in the same medium.

C. ACTIVITY AGAINST VIRUSES

Wanda et al. (1976) were the first to report on the anti-viral properties of BHA. They studied the effect of BHA and BHT on the enveloped bacteriophage $\phi 6$. They found this virus to be totally inactivated by 0.03 mM BHT while 0.1 mM BHA was needed for the same effect. These authors found the $\phi 6$ envelope to be intact in the BHT-treated viruses but that in the presence of BHT, the phage lost its ability to attach to the host cell. They suggested a similar inactivation mechanism of the virus by BHA.

D. ACTIVITY AGAINST PROTOZOA

Surak et al. (1976) found that 20 ppm BHA inhibited growth and the synthesis of DNA, RNA and protein of the protozoan Tetrahymena pyriformis by 50%. No changes were observed in morphology or in the size distribution of the cells. The primary effect appeared to be at the level of DNA, RNA and protein synthesis. These authors also reported that BHA did not seem to have any effect on the cell membrane of T. pyriformis.

Surak (1977) also studied the effect of TBHQ on Tetrahymena pyriformis and found that 26 ppm in the growth media inhibited cell growth by 50% which was similar to that reported for BHA (Surak et al., 1976). Inhibition by both antioxidants was dose dependent in inhibiting the generation time, synthesis of macromolecules and cell growth during the log phase.

III. MECHANISM OF ACTION

Bacteria and related forms are essentially masses of protoplasm consisting largely of enzymes organized so as to permit the orderly progress of metabolic processes. This organization involves the following factors: (1) a controlling genetic mechanism regulating cell division and insuring that reproduction results in new cells essentially identical with the parent, (2) the cell contents separated from direct contact with environment by means of a living membrane, and (3) the contents consisting of enzymes and enzyme systems with definite spacial orientation in regard both to each other and to the geography of the cell. Severe disturbances of any of these factors results in death of the cell while trivial disturbances result in partial or complete inhibition of cellular activity (Wyss, 1948).

Few authors have reported on the mechanism of action of BHA and TBHQ. It is most likely that they act on the cytoplasmic membrane of the microorganism in a similar way of action of other phenolic

compounds (Vas, 1953; Bernheim, 1972; Woldringh, 1973; DeSmet et al., 1978). Hugo and Bloomfield (1971) studied the mode of action of the phenolic antibacterial agent Fentichlor (2,2'-thiobis (4-dichlorophenol)) against Staphylococcus aureus and Escherichia coli. They found that Fentichlor was absorbed in fairly large amounts by S. aureus and E. coli and, according to the quantity absorbed, it was either bacteriostatic or bactericidal. They observed a pattern of intake that involved reversible adsorption of the neutral molecule on to the cell. The drug was taken up both by the cell wall and the cell membrane while the latter was reported to be the probable site of action. They also found that, even though whole cells and cell walls of E. coli had higher affinity for the drug than those of S. aureus, the former was less susceptible to the antibacterial action of this compound. The same pattern has been reported, in many cases, for BHA and TBHQ (Chang and Branen, 1975; Pierson et al., 1980). The results of Hugo and Bloomfield indicated that the lipid-rich cell wall of E. coli could act as an absorbing barrier preventing the access of the drug to its site of action. Recently, Davidson and Branen (1980b) found that BHA caused leakage of intracellular protein from Pseudomonas fluorescens, apparently as a result of reaction with the cell membrane. Previous evidence has shown that BHA and BHT cause structural modifications in biomembranes (Sgaragli et al., 1977). These modifications were the result of perturbations of the lipid alkyl chains in the membranes caused by

the compounds. When the lipid alkyl chains are perturbed, they lose their ordered state and become more fluid (Eletr et al., 1974; Singer and Wan, 1977). Therefore, it might be expected that microorganisms in the presence of BHA would synthesize and add increased amounts of saturated fatty acids to their membranes to maintain the proper fluid state. However, Davidson and Branen (1980b) found this was not always true when working with Pseudomonas species. Pseudomonas fragi cultures grown in BHA showed an increase in unsaturated to saturated fatty acid ratios. In the case of P. fluorescens, these ratios decreased. Ingram (1977) concluded that small changes in the phospholipid content of membranes could alter membrane organization. Eletr et al. (1974) found that compounds which perturb the lipids of membranes cause decreased activity of membrane-bound enzymes or put in contact some enzymes with substrates from which they are ordinarily restricted by means of cell geography and the resulting action can be disastrous to the cell (Wyss, 1948). Surak et al. (1976) and Surak (1977) stated that the primary effect of BHA and TBHQ on Tetrahymena pyriformis appeared to be inhibition of DNA, RNA and protein synthesis. Therefore, the mechanism of action by which BHA and TBHQ inhibit the growth of microorganisms is not totally understood but is probably involving several actions.

IV. INFLUENCE OF ENVIRONMENTAL FACTORS

The antimicrobial activity of BHA and TBHQ can be decreased or increased depending upon several environmental factors such as the pH of the medium, the culture age, incubation temperature and others.

A. pH EFFECT

Klindworth et al. (1979) tested BHA against Clostridium perfringens at pH values ranging from 5.5 to 8.5. They found 100 ppm BHA to be partially inhibitory to this microorganism at all the pH values. However, inhibition was greater at the pH extremes. They postulated that the increased susceptibility was due to a sensitivity to the pH rather than to the BHA. Ahmad (1979) reported that the activity of BHA against Aspergillus flavus was decreased on either side of the pH 6.3 (control pH). At a concentration of 200 ppm of BHA however, the inhibition was nearly 100% in all cases. This author concluded that although the activity of BHA was affected by extreme pH values, 200 ppm BHA or greater would be useful over a wide range of pH values. Stern et al. (1979) also evaluated the antimicrobial activity of BHA, at 50 or 100 ppm, against S. aureus in TSB with sodium chloride (3.5 or 7%) at pH 5, 6 or 7. BHA at the levels tested became more effective in preventing growth as the pH of the medium was decreased and the NaCl concentration

was increased. In contrast, Ayaz et al. (1980) studying the effect of BHA and BHT against S. aureus at pH values ranging from 3 to 9, found that the least amount of inhibition occurred when pH was between 5 and 8 with 80% inhibition occurring with 100 ppm BHT at pH 7. Therefore, the inhibition decreased as the medium was made more acidic or basic.

B. CULTURE AGE EFFECT

Klindworth et al. (1979) studied the effect of culture age on Clostridium perfringens growth inhibition by BHA. Seed cultures of this microorganism were grown in fluid thioglycollate medium (FTM) at 45°C for 13, 15, 17, 19 and 21 hr and inoculated into flasks containing 0, 100 and 150 ppm BHA in FTM. Results indicated that susceptibility of C. perfringens to BHA was age dependent with 14-18 hr cultures the most resistant. Apparently the variability in inhibition that occurred with cultures of different ages was due to differences in metabolic rates (i.e., a fast metabolism in a young culture rendering it more susceptible to BHA inhibition) or to the presence of toxic metabolic end products in the medium.

C. INCUBATION TEMPERATURE EFFECT

Klindworth et al. (1979) found that all three strains of C. perfringens were completely inhibited by 150 ppm BHA in FTM when grown at 37°C. At 45°C, however, this concentration was not always

completely inhibitory. The reasons for this variability were not known, but the authors postulated that reduced inhibition was related to other external factors such as culture age or medium pH. Ahmad (1979) found that the inhibition of Aspergillus flavus growth by 200 ppm BHA in glucose/salts broth (GSB) was not influenced by incubation temperatures of 7, 22, 32 and 37°C.

D. INCUBATION TIME EFFECT

Ahmad (1979) studied the effect of the incubation time on the inhibition by BHA of A. flavus growth in GSB at 32°C. The weight of the mycelium was determined at 12 hr intervals from 0 to 120 hours. Growth of this fungi was detected after 72 hr in the presence of 200 ppm BHA. The increase in mycelial weight was less from 72-108 hr than from 108-120 hr. The mold continued growing reaching a maximum mycelial weight after 120 hr, although the weight never reached that of the control.

E. AUTOCLAVING EFFECT

Klindworth et al. (1979) conducted a series of tests to determine whether any changes in BHA activity occurred as a result of autoclaving. BHA was either added directly to the medium and then autoclaved or filter-sterilized using a 0.45 µm membrane filter and added aseptically to the sterile cooled medium. The results demonstrated little difference in inhibition of C. perfringens

growth by autoclaved versus filter-sterilized BHA, indicating that the compound was not being volatilized and lost during the autoclaving process. Ahmad (1979) found that autoclaving decreased the antimicrobial properties of BHA against A. flavus very slightly. This author concluded that addition of BHA to food before or after cooking/baking would probably not make a difference in its antimicrobial activity.

V. INFLUENCE OF FOOD COMPONENTS

A. EFFECT OF LIPIDS

Robach and his co-workers (1977) were probably the first to show that the antimicrobial activity of BHA was decreased in the presence of lipid. Growth of Vibrio parahaemolyticus in TSB containing additional 2.5% NaCl was totally inhibited by 50 ppm BHA while 400 ppm BHA was required to inhibit growth in a crab meat homogenate. The BHA concentration needed for total inhibition in the crab meat homogenate was eight times higher than that needed for total inhibition in nutrient broth. These authors postulated that marked decrease in inhibition of growth of V. parahaemolyticus by BHA in the crab meat homogenate might have been due to partial inactivation of the antioxidant properties of BHA by the presence of oxidized crab meat lipids. They theorized that the reaction of BHA in preventing lipid autoxidation "used up" the molecule making it

ineffective as an antioxidant and that the same reaction could eliminate the antimicrobial activity of BHA. Klindworth et al. (1979) found that the presence of corn oil in the growth medium caused a decrease in the antimicrobial activity of BHA against C. perfringens. Antimicrobial activity still remained, however, as greater inhibition was observed for lipid samples containing BHA than those without it. They postulated that the hydrophobic nature of BHA may have resulted in the compound being localized within the lipid portion of the medium rendering it unavailable to act against microorganisms which grow in the aqueous phase of the medium. Ahmad (1979) found that addition of corn oil and Tween 80 depressed the inhibitory effect of BHA against A. flavus. Addition of as little as 0.25% lipid decreased inhibition by 200 ppm BHA to approximately 30% from 100%. This author also concluded that a reduction in the inhibition by BHA may have been due to the solubilization of the BHA in the lipid phase making it unavailable for inhibition of the mold. BHA and BHT have also been found to increase the fermentation time of a lactic starter in a Genoa type sausage mix by only 9.1-16.7% compared to 65% inhibition of the same microorganism in APT broth (Raccach, 1980). Recently, Shelef and Liang (1982) reported that, while the growth of Bacillus species was inhibited by 75 ppm BHA in nutrient broth, 1,000 ppm of this antioxidant was required for inhibition of the vegetative cells of these microorganisms in cooked rice and 5,000 ppm in strained chicken.

To overcome the effect of lipids in foods on the activity of phenolic antioxidants, new methods of applications of these compounds to high fat foods should be tested. For example, Ahmad (1979) showed that in a processed cheese spread, only 100-200 ppm BHA in a propylene glycol-water emulsion was necessary for complete growth inhibition of A. flavus when the emulsion was sprayed on the surface of the cheese, compared to 400 ppm if the compound was mixed into the cheese. Therefore, spraying appeared to provide a useful means of applying BHA as an antimicrobial on food products where surface growth of microorganisms was a problem. In this case, the amount of BHA applied could actually be decreased below the legal limit depending upon the ratio of total surface area of food to total final weight of food.

B. EFFECT OF PROTEINS

In studies done with non-fat dry milk (Cornell et al., 1971), BHA was found to bind with casein, most probably through non-polar or hydrophobic interactions. At low concentrations of BHA, a linear relationship between the amount bound and the concentration of the unbound (free) species existed. The binding was shown to be reversible. Therefore, in milk the protein could compete with the butterfat for the antioxidant, resulting in less antioxidant in the fat phase of milk than expected from its distribution or partition coefficient in protein-free model systems. Cornell (1979) studied

the distribution of some antioxidants in dairy products and derived a mathematical model for the prediction of antioxidant distribution in whole milk. The percent antioxidant found in the fat was smaller than the previously postulated butteroil-water partition coefficients (Cornell et al, 1970). This was reported to be the result of the antioxidant bound by the protein. No other study has been done on the effect of proteins on the antimicrobial activity of phenolic antioxidants. It would seem logical that this binding could be partially responsible for the large decrease in activity of BHA in products such as crab meat homogenates (Robach et al., 1977), cheese (Ahmad, 1979) or strained chicken (Shelef and Liang, 1982).

VI. SAFETY EVALUATIONS

Regulations issued by the U. S. Food and Drug Administration (FDA) permit the use of BHA (21 CFR 182.3169), BHT (21 CFR 182.3173), propyl gallate (21 CFR 184.1660) and TBHQ (21 CFR 172.185), singly or in combinations of two or more, in food products at a maximum concentration of 0.02% based upon the fat content of the food (Code of Federal Regulations, 1980). The exception is that TBHQ is permitted for use in combination only with BHA and/or BHT.

The Food, Safety and Quality Service (FSQS), U. S. Department of Agriculture (USDA) permits the use of BHA, BHT, propyl gallate and TBHQ in meat and poultry food products as follows (USDA Meat Inspection Regulation 9 CFR 318.7 and USDA Poultry Inspection

Regulation 9 CFR 381.147): (1) rendered animal fat or combinations of such fat and vegetable fat, fresh pork sausage and various poultry products -- 0.01% by weight, singly, or 0.02% in combination, based on fat content of the food, (2) dry sausage -- 0.003% singly, or 0.006% in combination, based on the total weight of the food, and (3) dried meats -- 0.01%, singly or in combination, based on the total weight of the food. TBHQ is also permitted for use in combination only with BHA and/or BHT (Code of Federal Regulations, 1980).

The U. S. Food and Drug Administration has estimated that the current use of phenolic antioxidants in the American diet leads to a daily intake of ca. 0.1 mg/Kg body weight. At levels 500 times this level (50 mg/Kg/day) BHA and BHT appeared to be free of any obvious injurious effects (Branen, 1975). However, at larger doses (500 mg/Kg/day) both antioxidants resulted in certain pathological, enzyme and lipid alterations in both rodents and monkeys. However, the alterations appeared to be physiological responses which are reversible upon removal of these antioxidants from the diet. BHA and BHT have toxic effects at extremely high doses, both having an LD₅₀ of ca. 2000 mg/Kg in most animals (Johnson, 1971). Long term chronic ingestion of these antioxidants might be beneficial in sparing vitamin E and in modifying the acute toxicity of a number of mutagenic and carcinogenic chemicals (Branen, 1975). It has been shown that BHA and BHT inhibit chemical carcinogenesis when

administered either before or with the carcinogen (Shamberger, 1980). BHA is probably the most versatile and least toxic inhibitor of chemical carcinogenesis that has been identified.

In the case of TBHQ, the feeding and metabolic studies indicated that the level at which untoward effects are produced by these antioxidants in rats and dogs exceeded 0.5% in the diet (over 300 mg/Kg/day). Astill et al. (1975) estimated the probable daily intake of TBHQ to be 0.02-0.07 mg/kg body weight which provides safety margins of 1,000 to 10,000 times. The LD₅₀ of TBHQ in rats was between 700-1000 mg/Kg.

In summary, BHA and TBHQ have been shown to be safe compounds at the levels used in food products. They are food additives that may have dual functions in food products, i.e. as antioxidants and antimicrobials. However, several authors have suggested that, at the levels allowed in food products, these antioxidants would not be as effective in food systems as they are in broth or agar systems since the antioxidants would solubilize in the lipid phase of the food products thereby eliminating their antimicrobial activity. New methods of incorporation of these antioxidants to high-fat and/or high-protein food products such as spraying should be tested to improve their antimicrobial activity against spoilage microorganisms.

CHAPTER II

MATERIALS AND METHODS

I. TEST ORGANISMS

Five microorganisms were used in this study: two molds, Penicilium citrinum NRRL 5727 and Aspergillus niger, a yeast, Saccharomyces cerevisiae and two bacteria, Pseudomonas fluorescens ATCC 15456 and Staphylococcus aureus ATCC 12600. P. citrinum was obtained from the culture collection of the Northern Regional Research Laboratory, Agricultural Research Service, Peoria, Ill.; P. fluorescens and S. aureus from the American Type Culture Collection, Rockville, MD., and A. niger and S. cerevisiae from the University of Tennessee, Department of Food Technology and Science culture collection. Bacteria were grown on Trypticase Soy Agar slants (BBL, Cockeysville, MD) for 24 hr at 37°C, in the case of S. aureus, or 26°C for P. fluorescens. Yeast extract sucrose agar slants were used to grow the yeast at 32°C for 48 hours. Both molds were grown in potato dextrose agar (PDA) slants (Difco, Detroit, MI) at 25°C for seven days. All stock cultures were maintained at 4°C and transferred weekly.

II. INOCULA PREPARATION

Inocula of the bacteria and yeast were prepared by transferring one loop from the stock culture to 250 ml Erlenmeyer flask containing 125 ml medium and incubating at the appropriate temperature for 12 hours. A 1% inoculum from the first flask (2.5 ml) was then transferred into 250 ml of medium and incubated for 12 hr at the same temperature. For all bacterial and yeast growth studies, 1 ml of the final 12 hr culture was used per 100 ml of test medium. In the case of the molds, spores were harvested into sterilized collection tubes from the PDA slants with 5 ml of a sterile 0.005% Triton-X100 solution. Spore counts were adjusted to approximately 1.0×10^6 spores/ml using a Petroff-Hausser counting chamber. Molds were inoculated at a rate of 1.0×10^6 spores per 50 ml medium.

III. GROWTH MEDIA

Molds and the yeast were grown in a modified yeast extract sucrose broth consisting of 2% yeast extract and 4% sucrose. The 4% sucrose was used instead of the normal 20% to prevent adverse effects of the sugar on the dispersion of the casein. Bacteria were grown in Trypticase Soy Broth (BBL). All media were adjusted to pH 7.3 to facilitate the dispersion of the casein into the media. The media were dispensed into 250 ml Erlenmeyer flasks at a rate of 100 ml medium per flask, in the case of the bacteria and yeast, or

50 ml per flask for the molds. The protein, casein , and the emulsifier, Tween 80, for the lipid studies were added prior to sterilization. Media were then autoclaved at 121°C for 15 minutes. Flasks were allowed to cool at room temperature before addition of antioxidants and the lipid, corn oil.

IV. TEST COMPOUNDS

Butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) were obtained from Eastman Chemical Products, Inc., Kingsport, TN. Both antioxidants were dissolved in 95% ethanol to give a final concentration of 2% (w/v). The stock solutions were filter-sterilized with a 0.45 μ m Millipore filter (Millipore Corp., Bedford, MA) and stored in a sterile stoppered flask at 4°C for the duration of the testing period.

V. FOOD COMPONENTS

The antimicrobial effectiveness of BHA and TBHQ was challenged by the addition of 0, 1.5, 3.0 and 4.5% (v/v) of a lipid, corn oil (Mazola) or 0, 3, 6 and 9% (w/v) of a protein, casein (Fisher Scientific Co., Fair Lawn, NY) to the growth media. To prevent autoxidation, the corn oil was purchased fresh and held at 4°C under nitrogen throughout the experiment. As stated before, casein was added prior to sterilization while corn oil was added aseptically to the sterile cooled medium. Tween 80 at 1% (v/v) was also added

prior to sterilization to emulsify the lipid except with Pseudomonas fluorescens which was inhibited by the emulsifier. In this case, a shaking water bath was used to disperse the lipid during growth. Tween 80 was used for its high hydrophile-lipophile balance (HLB) which helps to produce an oil-in-water emulsion (Griffin and Lynch, 1968).

VI. TREATMENTS

Appropriate amounts of the 2% stock solutions of the antioxidants were added to the sterile cooled medium to give final concentrations of 200 ppm BHA and 400 ppm TBHQ for S. cerevisiae, 300 ppm BHA and 50 ppm TBHQ for S. aureus, 200 ppm BHA and 300 ppm TBHQ for P. fluorescens, 200 ppm BHA and 400 ppm TBHQ for P. citrinum and 200 ppm BHA and 600 ppm TBHQ for A. niger. Most of the concentrations of BHA and TBHQ used in this study resulted in total growth inhibition following 4 hr incubation. The exceptions were P. fluorescens at 200 ppm BHA and 300 ppm TBHQ and S. aureus at 50 ppm TBHQ (Table 2). Both bacteria showed an initial decrease in numbers followed by growth after 12-24 hr of incubation. Antioxidants were dispersed into the media by manual shaking.

TABLE 2
LETHAL CONCENTRATIONS OF BHA AND TBHQ

MICROORGANISM	BHA (ppm)	TBHQ (ppm)
<u>P. citrinum</u>	200	400
<u>A. niger</u>	200	600
<u>S. cerevisiae</u>	200	400
<u>P. fluorescens</u>	200 ^a	300 ^a
<u>S. aureus</u>	300	50 ^a

^aIn these cases the microorganism was inhibited initially but showed subsequent growth after 12-24 hr of incubation.

VII. INCUBATION TIMES AND TEMPERATURES

After flasks containing both antioxidants and food components had been inoculated, they were incubated at the optimum temperature for each microorganism: 32°C for S. cerevisiae, 37°C for S. aureus, 26°C for P. fluorescens, and 25°C for P. citrinum and A. niger. Bacteria and yeast were incubated for 48 hr and molds for 14 days.

VIII. GROWTH INHIBITION STUDIES

Inhibition studies were conducted as follows: for the bacteria and yeast, 1 ml from each flask was removed after 0, 4, 8, 12, 24, 36 and 48 hr of incubation, serially diluted in sterile 0.1% (w/v) peptone at pH 7.0 and pour-plated with either Trypticase Soy Agar, in the case of the bacteria, or yeast extract sucrose agar for the yeast. The plates were incubated at the appropriate temperatures for 48 hr before counts were recorded. Reported plate counts were the mean of duplicated experiments. In the case of the molds, after the 14 day incubation period, the mycelium was harvested by filtration through a pre-weighted Whatman No. 4 filter paper, washed, dried at 110°C for 14 hr and then weighted. Samples containing corn oil were washed first with a soapy solution to eliminate any remaining lipid and then with hot water. In the case of the samples grown on casein, only hot water was used. The dried weight of each mycelial mat was used as an indication of growth.

IX. EXPERIMENTAL DESIGN

A total of 12 flasks, in the case of the protein, or 15 flasks for the lipid were used in each replicate. Three flasks were included for each protein or lipid level tested. The first flask was the control with no added antioxidant, the second contained BHA and the third contained TBHQ. Each experiment was replicated once using the randomized block experimental design, in the case of the molds, or the split-plot design for the bacteria and yeast. Data were analyzed using an analysis of variance (ANOVA) to detect significant differences in main effects and interactions, while Duncan's Multiple Range Test was used to separate differences in means. Growth inhibition percentages, at 24 hr of incubation for the bacteria and yeast or after the 14 day incubation period for the molds, were calculated using the formula:

Percent Inhibition =

$$\frac{(\text{Control plate count}) - (\text{Treatment plate count})}{(\text{Control plate count})} \times 100$$

Each treatment sample was compared with its respective control grown with the same concentration of lipid or casein.

CHAPTER III

RESULTS

The antimicrobial activity of butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) was tested in the presence of a protein, casein, or a lipid, corn oil, in a non-selective medium. In the first section of this chapter, the results of the inhibition of the different microorganisms by BHA and TBHQ in the presence of the protein are presented. In the second section, the results for the lipid are shown. In every section, the results for each microorganism are presented separately.

I. EFFECT OF CASEIN ON THE ACTIVITY OF BHA AND TBHQ

None of the levels of casein used in this experiment inhibited growth of Saccharomyces cerevisiae in yeast extract sucrose (YES) broth at 32°C (Figure 2). On the contrary, growth of this microorganism was somewhat stimulated by the presence of the protein in the medium (Table 3). BHA, at 200 ppm, and TBHQ, at 400 ppm, completely inhibited the growth of this microorganism when no casein was present in the medium. After 4 hr of incubation, no viable cells could be recovered.

The activity of BHA was reduced dramatically however, in the presence of casein (Figure 3). Inhibition of S. cerevisiae was progressively decreased as the casein concentration was increased,

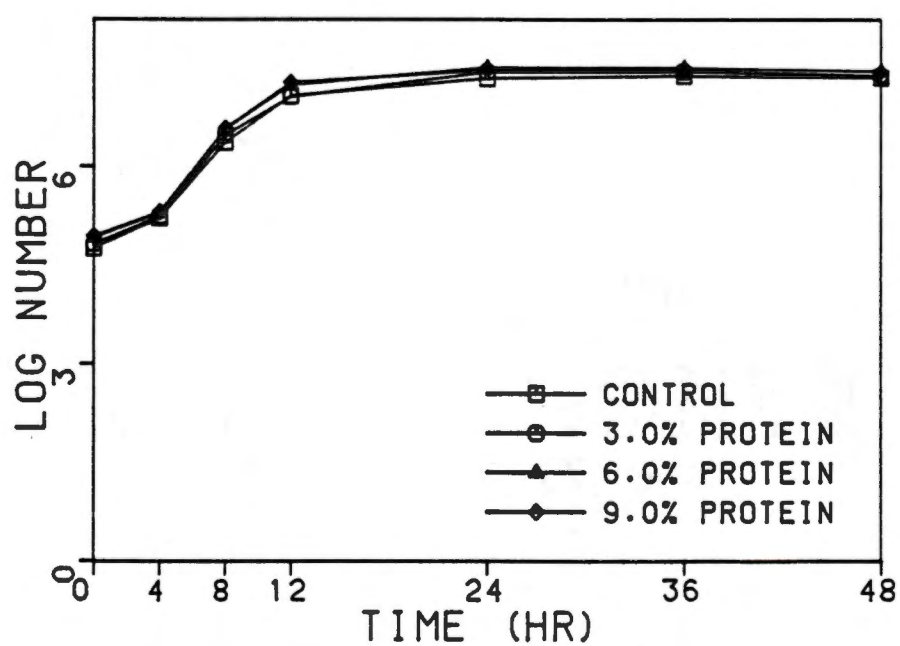


Figure 2. Influence of casein on the growth of *S. cerevisiae* in YES broth.

TABLE 3

EFFECT OF CASEIN ON THE ANTIMICROBIAL ACTIVITY OF BHA AND TBHQ
AGAINST THE BACTERIA AND YEAST

Microorganism	Antioxidant (ppm)	Casein %(w/v)	Average Log Count/ml ¹ + Standard Error
<u>Saccharomyces</u> <u>cerevisiae</u>	0	0	6.5 + 0.3c
	0	3	6.6 - 0.3bc
	0	6	6.6 0.3a
	0	9	6.6 0.3ab
	200 BHA	0	0.7 + 0.5d
	200 BHA	3	5.6 - 0.2c
	200 BHA	6	6.1 0.2b
	200 BHA	9	6.3 0.2a
	400 TBHQ	0	0.7 + 0.5c
	400 TBHQ	3	2.4 - 0.4b
	400 TBHQ	6	4.6 0.4a
	400 TBHQ	9	4.7 0.4a
<u>Staphylococcus</u> <u>aureus</u>	0	0	8.0 + 0.2a
	0	3	8.1 - 0.2a
	0	6	8.1 0.2a
	0	9	8.0 0.2a
	300 BHA	0	0.9 + 0.6c
	300 BHA	3	6.8 - 0.2b
	300 BHA	6	7.6 0.2a
	300 BHA	9	7.8 0.2a
	50 TBHQ	0	6.7 + 0.3c
	50 TBHQ	3	7.8 - 0.2b
	50 TBHQ	6	7.9 0.2a
	50 TBHQ	9	7.9 0.2a

TABLE 3 (Continued)

Microorganism	Antioxidant (ppm)	Casein %(w/v)	Average Log Count/ml ¹ + Standard Error
<u>Pseudomonas</u> <u>fluorescens</u>	0	0	8.2 + 0.3a
	0	3	7.7 - 0.3b
	0	6	7.5 0.3b
	0	9	7.5 0.3b
	200 BHA	0	5.3 + 0.4c
	200 BHA	3	6.7 - 0.2b
	200 BHA	6	7.3 0.3a
	200 BHA	9	7.3 0.3a
	300 TBHQ	0	5.3 + 0.4b
	300 TBHQ	3	6.8 - 0.2a
	300 TBHQ	6	7.0 0.2a
	300 TBHQ	9	7.1 0.2a

¹Mean of two replications averaged over seven time observations; Within an experimental unit, means with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

with each increase resulting in a significant reduction ($P < 0.05$) in the antimicrobial activity of BHA (Table 3). While activity was decreased, it was not eliminated. For example, at 3% casein, the lag phase of the yeast growth curve was increased to more than 12 hr and when growth occurred the rate was much slower than that of the control. Growth curves of the samples containing 6 and 9% casein had smaller increases in lag phase and faster growth rates. Inhibition of S. cerevisiae was slightly higher at 6% than at 9% casein.

TBHQ was much less affected by casein than BHA, even at the 9% level (Figure 4). As with BHA, the effect of protein on growth was related to the concentration present (Table 3). At 3% casein, 400 ppm TBHQ resulted in approximately a 3 log cycle decrease in viable cells after 4 hr of incubation followed by a period of no growth after 36 hr incubation. At 6 and 9% protein, viable cell counts were decreased nearly 1.5 logs after 8 hr incubation and began growing at a slow rate only after 12 hours. There was no significant difference ($P > 0.05$) between the effects of 6 and 9% casein.

An apparent decrease and subsequent increase in viable cells has been reported previously by other authors working with these antioxidants and different microorganisms (Chang and Branen, 1975; Robach et al., 1979; Shih and Harris, 1977; Pierson et al., 1980). Collee et al. (1961) found the same pattern studying Clostridium

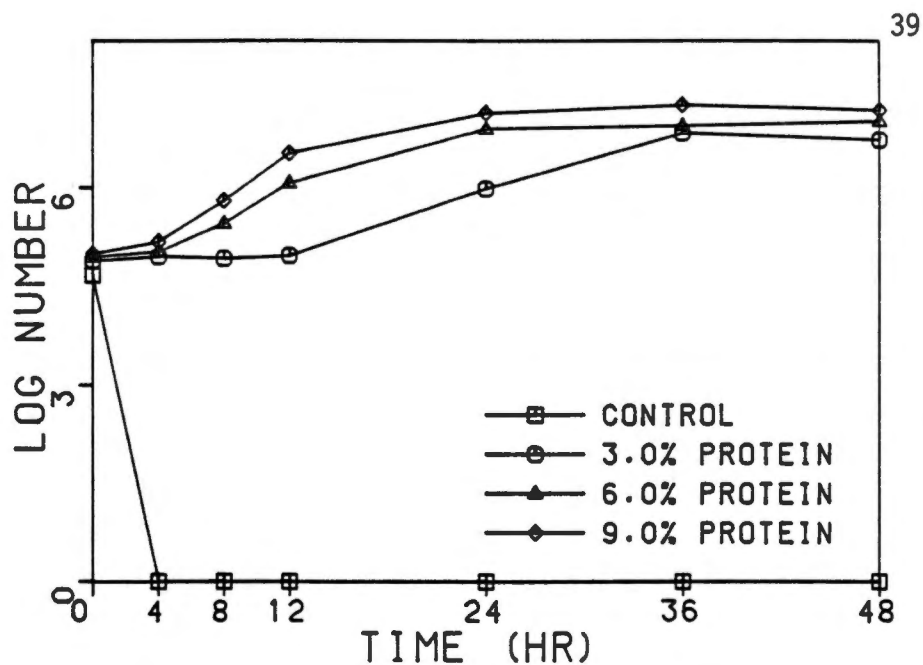


Figure 3. Effect of casein on the antimicrobial activity of 200 ppm BHA against *S. cerevisiae* growth.

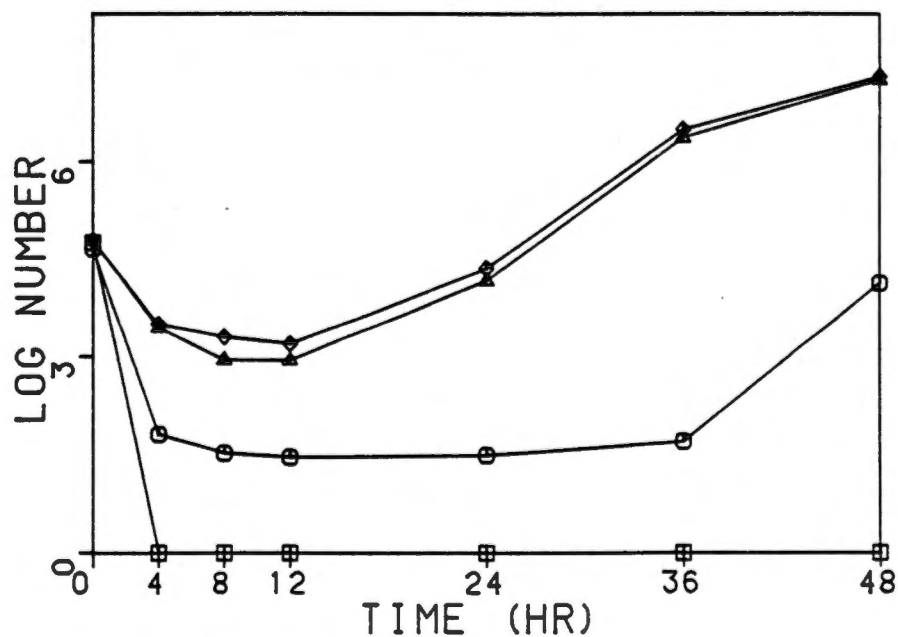


Figure 4. Effect of casein on the antimicrobial activity of 400 ppm TBHQ against *S. cerevisiae* growth.

perfringens growth at 50°C. After inoculation of a broth with C. perfringens they observed an apparent decrease in cell numbers within the first few hours, followed by an increase to a maximum count within 6 hours. Collee et al. (1961) termed this curious pattern the "Phoenix phenomenon," probably since the Phoenix symbolized immortality through "death and resurrection" in ancient times. This phenomenon was reproduced using an inoculum of spores only, vegetative cells only and cells that have been grown at 50°C prior to inoculation. They suggested that an initial heat shock possibly selected heat adapted variants. Thus, this sublethal heat treatment might have had a mutagenic effect since filamentous forms of the microorganism were observed at this temperature. They also reported that normal maximum counts were not attained and that some reduction in viable counts at 50°C might be associated with disfunction of cell division systems. Shoemaker and Pierson (1976) found this phenomenon with C. perfringens to be an injury-recovery process. Therefore, the growth response of S. cerevisiae to the high concentrations of BHA and TBHQ could have been due to an injury-recovery phenomenon. The mechanism of this growth pattern is currently under investigation.

In the case of Staphylococcus aureus, casein did not significantly affect its growth when no antioxidants were present (Figure 5, Table 3). At 300 ppm, BHA completely inhibited S. aureus growth without any casein present in the growth medium. S. aureus growth

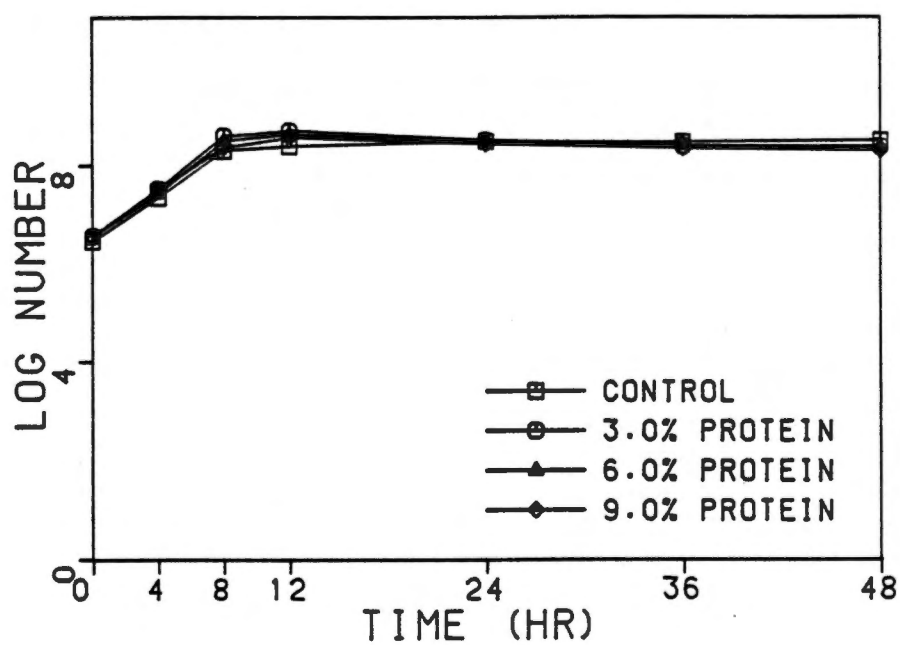


Figure 5. Influence of casein on the growth of *S. aureus* in TSB.

has been reported to be inhibited by concentrations of 100-200 ppm BHA (Chang and Branen, 1975; Ayaz, 1975; Stern et al, 1979; Pierson et al., 1980; Robach and Stateler, 1980; Ayaz et al., 1980; Davidson et al., 1981; Raccach et al., 1981) or 400 ppm BHA (Shih and Harris, 1977). Shih and Harris (1977) reported that differences in growth inhibition of this microorganism by BHA were probably a result of media or culture strain differences. Robach and Stateler (1980) used the same strain as in the present study, S. aureus ATCC 12600, however they found 100 ppm BHA to be nearly 100% inhibitory to the bacterium in the same medium and incubation temperature. In their study, however, growth was not completely inhibited. In the present study, no viable cells of this microorganism were found after 4 hr incubation in the presence of 300 ppm.

As in the case of the yeast, BHA activity was diminished drastically by the presence of casein in the medium (Figure 6). In the presence of 3% casein, BHA caused less than a one log cycle decrease in viable cells and after 12 hr incubation growth resumed, but at a slower rate. At 6 and 9% casein, a slight increase in the lag phase resulted; however, growth reached maximum levels in 12 hours.

In contrast, 50 ppm TBHQ caused an initial decrease in viable cells followed by growth after 8 hr incubation (Figure 7). Robach and Stateler (1980) reported that two strains of this microorganism, S-6 and 12600, were inhibited by 25 ppm TBHQ. However,

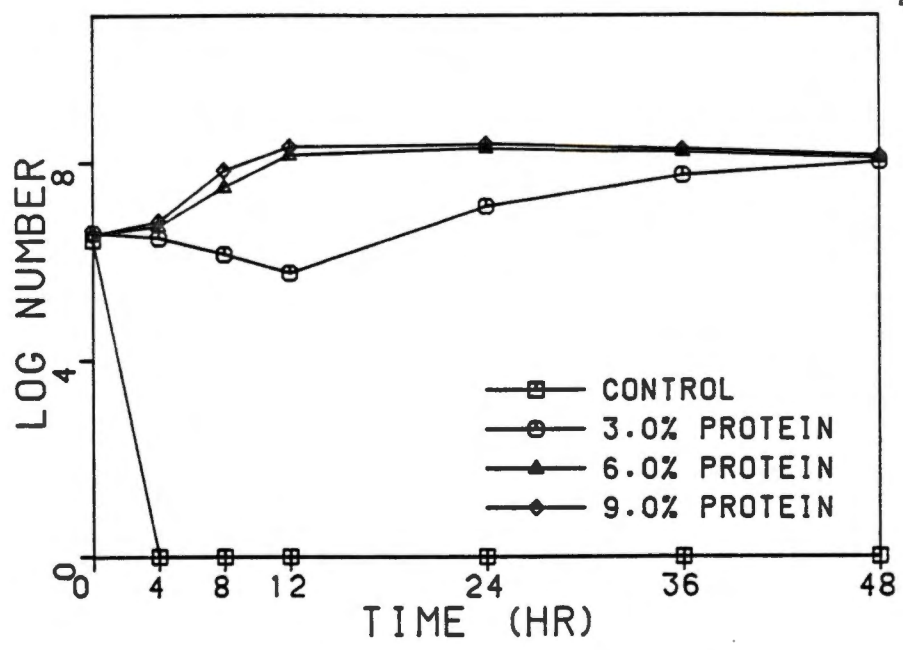


Figure 6. Effect of casein on the antimicrobial activity of 300 ppm BHA against S. aureus growth.

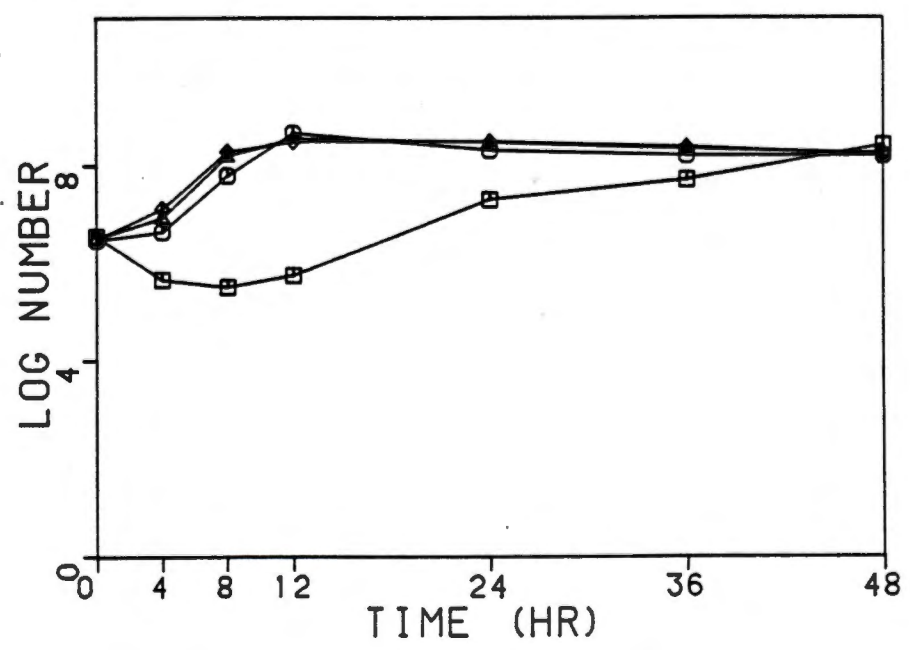


Figure 7. Effect of casein on the antimicrobial activity of 50 ppm TBHQ against S. aureus growth.

Raccach et al. (1981) found that in brain heart infusion (BHI) 30-50 ppm TBHQ inhibited S. aureus 100, while more than 50 ppm were needed to inhibit S. aureus z88. In the presence of any level of casein, the activity of TBHQ was nearly totally eliminated. The lag phase was lengthened slightly in all cases.

The growth of Pseudomonas fluorescens was significantly ($P < 0.05$) depressed in the presence of the protein (Figure 8, Table 3). This could have been due to a reduced water activity (a_w) caused by the absorption of water molecules by the casein micelles (deMan, 1980) or to a lower availability of oxygen for this strict aerobic bacterium. Neither 200 ppm BHA nor 300 ppm TBHQ were completely inhibitory to P. fluorescens. Viable cell counts were reduced nearly 2.5 log cycles after 4 hr of incubation in the presence of both antioxidants (Figure 9 and 10). Counts remained constant up to 12 hr of incubation but growth resumed after this time. Davidson and Branen (1980a) reported that 150 ppm BHA was totally inhibitory to the same strain of P. fluorescens at 7°C and pH 6.0. Differences in incubation conditions and inoculum age could probably explain the greater amount of BHA needed for inhibition of this microorganism in the present study.

As with other microorganisms, the activity of BHA against P. fluorescens was decreased significantly by the presence of the protein (Figure 9). Slight inhibition by BHA was found at 3% casein whereas at higher levels of protein, little activity remained.

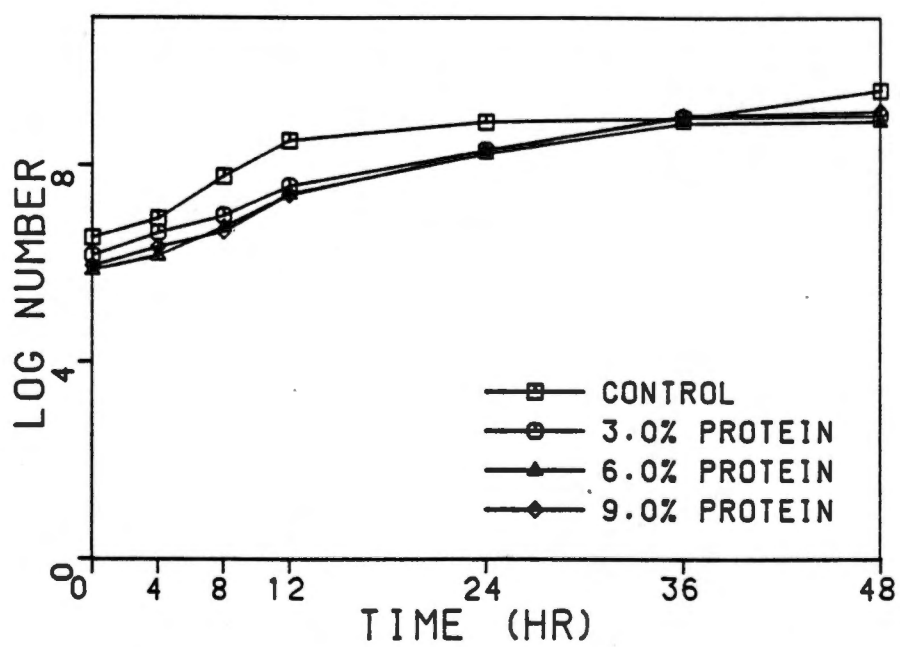


Figure 8. Influence of casein on the growth of P. fluorescens in TSB.

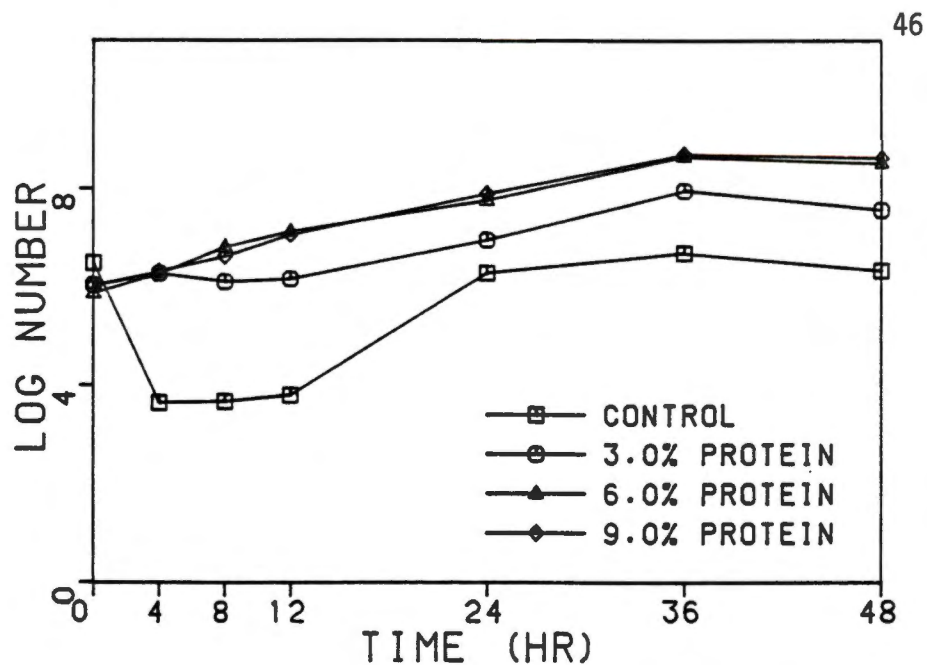


Figure 9. Effect of casein on the antimicrobial activity of 200 ppm BHA against *P. fluorescens* growth.

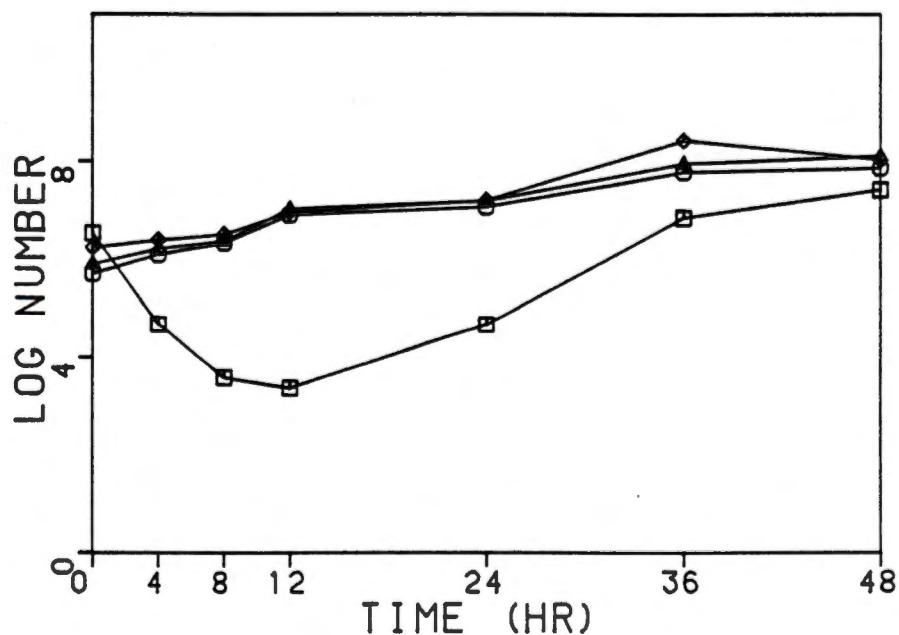


Figure 10. Effect of casein on the antimicrobial activity of 300 ppm TBHQ against *P. fluorescens* growth.

Inhibition was greater by TBHQ than by BHA in the presence of casein (Figure 10). During the entire growing period inhibition by 300 ppm TBHQ was quite similar at all the levels of protein tested ($P>0.05$), i.e. increasing the amount of casein from 3 to 9% did not increased inhibition of P. fluorescens by TBHQ (Table 3).

In the case of the molds, both Penicillium citrinum and Aspergillus niger growth were stimulated in the presence of casein (Table 4). The growth of P. citrinum was inhibited by 200 ppm BHA and 400 ppm TBHQ in absence of casein (Figure 11). The concentration of BHA used was the same that Ahmad (1979) reported to cause 100% inhibition of species of Geotrichum, Penicillium and Aspergillus in glucose/salts broth. There have been no reports as to the antifungal properties of TBHQ.

The antifungal activity of BHA progressively decreased as the concentration of casein increased (Figure 11). Mycelial weight of the antioxidant treatments nearly reached that of the control without antioxidant at 6 and 9% protein. Pigmentation of the mycelium and sporulation also increased significantly at those levels of protein while at 3% casein, the mycelium appeared colorless with no apparent sporulation. The inhibitory effect of BHA on pigmentation and sporulation of several mold species has been reported by several authors (Sankaran, 1976; Fung et al., 1977). Sankaran (1976) postulated that oxygen required for color development or reproduction might not be available.

TABLE 4

EFFECT OF CASEIN ON THE ANTIMICROBIAL ACTIVITY OF BHA AND TBHQ
AGAINST THE MOLDS

Microorganism	Antioxidant (ppm)	Casein %(w/v)	Mycelial Weight ¹ + Standard Error (mg/50 ml broth)	
<u>Penicillium</u> <u>citrinum</u>	0	0	418.0	+ 6.0b
	0	3	763.0	+ 46.0ab
	0	6	1080.0	+ 184.0a
	0	9	1203.0	+ 229.0a
	200 BHA	0	0.0	+ 0.0c
	200 BHA	3	440.5	+ 8.5b
	200 BHA	6	925.0	+ 123.0a
	200 BHA	9	1168.0	+ 153.0a
	400 TBHQ	0	82.5	+ 2.5b
	400 TBHQ	3	521.0	+ 47.0a
	400 TBHQ	6	613.5	+ 157.0a
	400 TBHQ	9	681.0	+ 203.0a
<u>Aspergillus</u> <u>niger</u>	0	0	837.5	+ 16.5c
	0	3	1360.0	+ 49.0b
	0	6	1536.5	+ 27.0ab
	0	9	1671.0	+ 51.0a
	200 BHA	0	0.0	+ 0.0c
	200 BHA	3	1412.0	+ 79.0b
	200 BHA	6	1731.5	+ 153.5a
	200 BHA	9	2022.0	+ 40.0a
	600 TBHQ	0	97.5	+ 23.5b
	600 TBHQ	3	594.5	+ 175.5ab
	600 TBHQ	6	961.0	+ 347.0a
	600 TBHQ	9	1152.5	+ 277.5a

¹Mean of two replications; within an experimental unit, means with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

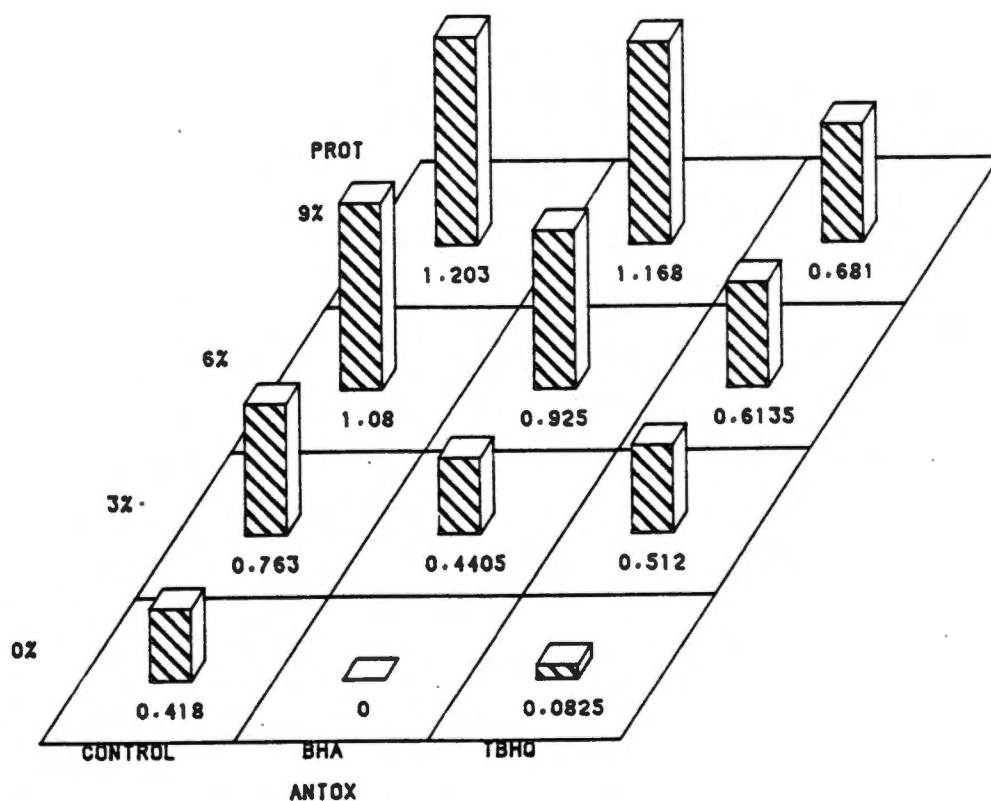


Figure 11. Effect of casein on the antimicrobial activity of 200 ppm BHA and 400 ppm TBHQ against *P. citrinum* growth in YES broth at 25°C. The numbers below each bar represent the mycelial weight of the mold after 14 days of incubation.

Inhibition of P. citrinum by 400 ppm TBHQ was greater than by 200 ppm BHA in most cases (Figure 11). An increase in the casein level did not significantly decrease ($P>0.05$) TBHQ activity. In all cases, a significant decrease in pigmentation and sporulation was noticed.

Aspergillus niger growth was also completely inhibited by 200 ppm BHA; however, BHA activity was eliminated even in the presence of 3% casein in the medium (Figure 12). At all levels of casein tested, the mycelial weight of the samples containing BHA was equal or even greater than that of the control without any antioxidant present. No appreciable effect of the BHA on pigmentation or sporulation was noticed.

TBHQ, at 600 ppm, inhibited growth of this microorganism. Some inhibitory activity of this antioxidant remained at all the levels of casein tested (Figure 12). Increase in the protein concentration slightly decreased inhibition of A. niger by TBHQ. A noticeable decrease in sporulation was found.

II. EFFECT OF CORN OIL ON THE ACTIVITY OF BHA AND TBHQ

The presence of any level of lipid or Tween 80 in the medium significantly increased the growth of Saccharomyces cerevisiae (Table 5, Figure 13). As was shown previously, 200 ppm BHA was lethal to S. cerevisiae. The presence of even the emulsifier, Tween 80, in the growth medium however, dramatically decreased

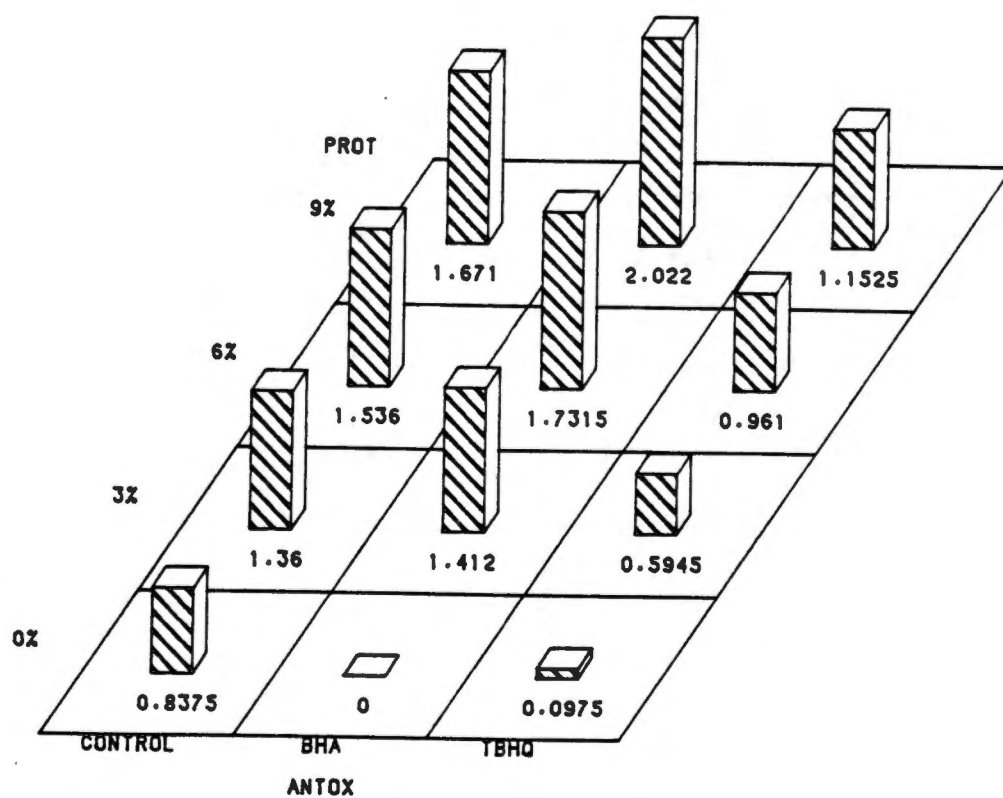


Figure 12. Effect of casein on the antimicrobial activity of 200 ppm BHA and 600 ppm TBHQ against *A. niger* growth in YES broth at 25°C. The numbers below each bar represent the mycelial weight of the mold after 14 days of incubation.

TABLE 5

EFFECT OF CORN OIL ON THE ANTIMICROBIAL ACTIVITY OF BHA AND TBHQ
AGAINST THE BACTERIA AND YEAST

Microorganism	Antioxidant (ppm)	Corn oil %(v/v)	Average Log Count/ml ¹ + Standard Error
<u>Saccharomyces</u> <u>cerevisiae</u>	0	0	6.5 + 0.3b
	0	1.5	6.7 - 0.3a
	0	3.0	6.7 0.3a
	0	4.5	6.7 0.3a
	0	Tween 80	6.6 0.3a
	200 BHA	0	0.7 + 0.5d
	200 BHA	1.5	6.4 - 0.3b
	200 BHA	3.0	6.5 0.3a
	200 BHA	4.5	6.6 0.3a
	200 BHA	Tween 80	6.3 0.3c
	400 TBHQ	0	0.7 + 0.5e
	400 TBHQ	1.5	4.4 - 0.3c
	400 TBHQ	3.0	5.1 0.4b
	400 TBHQ	4.5	5.3 0.4a
	400 TBHQ	Tween 80	3.4 0.3d
	0	0	8.0 + 0.2b
	0	1.5	8.2 - 0.2a
	0	3.0	8.2 0.2a
	0	4.5	8.2 0.2a
	0	Tween 80	8.2 0.2a
<u>Staphylococcus</u> <u>aureus</u>	300 BHA	0	0.9 + 0.6b
	300 BHA	1.5	8.1 - 0.2a
	300 BHA	3.0	8.1 0.2a
	300 BHA	4.5	8.2 0.2a
	300 BHA	Tween 80	8.1 0.2a
	50 TBHQ	0	6.7 + 0.3b
	50 TBHQ	1.5	8.2 - 0.3a
	50 TBHQ	3.0	8.2 0.2a
	50 TBHQ	4.5	8.2 0.2a
	50 TBHQ	Tween 80	8.1 0.2a

TABLE 5 (Continued)

Microorganism	Antioxidant (ppm)	Corn oil %(v/v)	Average Log Count/ml ¹ ± Standard Error
<u>Pseudomonas</u> <u>fluorescens</u>	0	0	8.2 ± 0.3a
	0	1.5	8.3 ± 0.3a
	0	3.0	8.2 ± 0.3a
	0	4.5	8.3 ± 0.3a
	200 BHA	0	5.3 ± 0.4b
	200 BHA	1.5	8.1 ± 0.3a
	200 BHA	3.0	8.1 ± 0.3a
	200 BHA	4.5	8.2 ± 0.3a
	300 TBHQ	0	5.3 ± 0.4b
	300 TBHQ	1.5	7.1 ± 0.3a
	300 TBHQ	3.0	7.6 ± 0.3a
	300 TBHQ	4.5	7.8 ± 0.3a

¹Mean of two replications averaged over seven time observations; Within an experimental unit, means with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

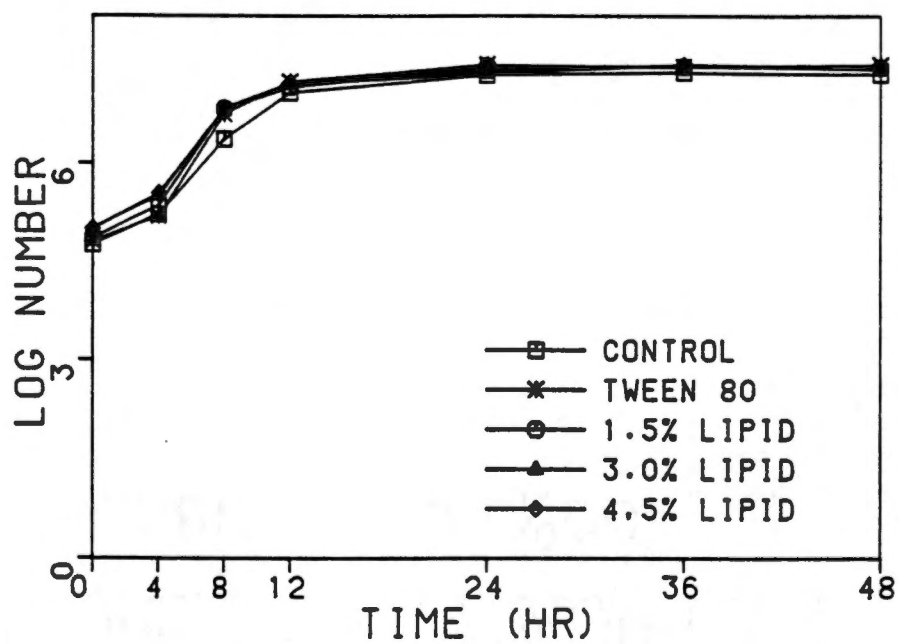


Figure 13. Influence of corn oil on the growth of *S. cerevisiae* in YES broth.

the antimicrobial activity of BHA (Figure 14). Corn oil seemed to depress BHA activity to a greater extent than did casein. BHA was slightly more inhibitory at 1.5% corn oil than it was at the other levels tested. At 3.0 and 4.5% level, the rate of growth was quite similar to that of the controls with the same lipid concentration.

At 400 ppm, TBHQ was less influenced by the corn oil (Figure 15) than was BHA. In the presence of the emulsifier, TBHQ caused nearly a 3 log decrease in viable cells after 24 hr incubation. However, at 1.5, 3.0 and 4.5% corn oil, viable cell counts were decreased 1.5, 1.0 and less than one log cycles, respectively, after 12-24 hours. After that time, growth resumed in all cases but at slower rate. The rate of growth progressively increased as concentration increased.

Staphylococcus aureus growth was also increased in the presence of corn oil or the emulsifier (Figure 16, Table 5). The activity of the previously lethal 300 ppm BHA however, was completely lost even in the presence of the emulsifier (Figure 17). An increase in lipid concentration did not cause additional reduction in BHA activity.

As was shown previously, 50 ppm TBHQ caused an initial decrease in viable cell counts of less than one cycle followed by growth after 8 hr of incubation. However, in the presence of any of the lipid levels or even the emulsifier, any activity of TBHQ against S. aureus was lost (Figure 18).

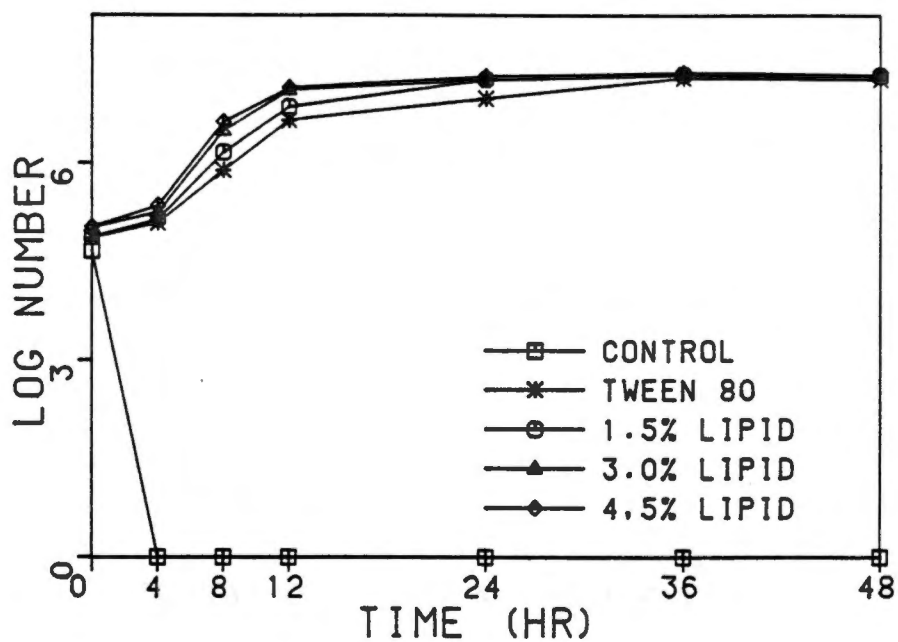


Figure 14. Effect of corn oil on the antimicrobial activity of 200 ppm against *S. cerevisiae* growth.

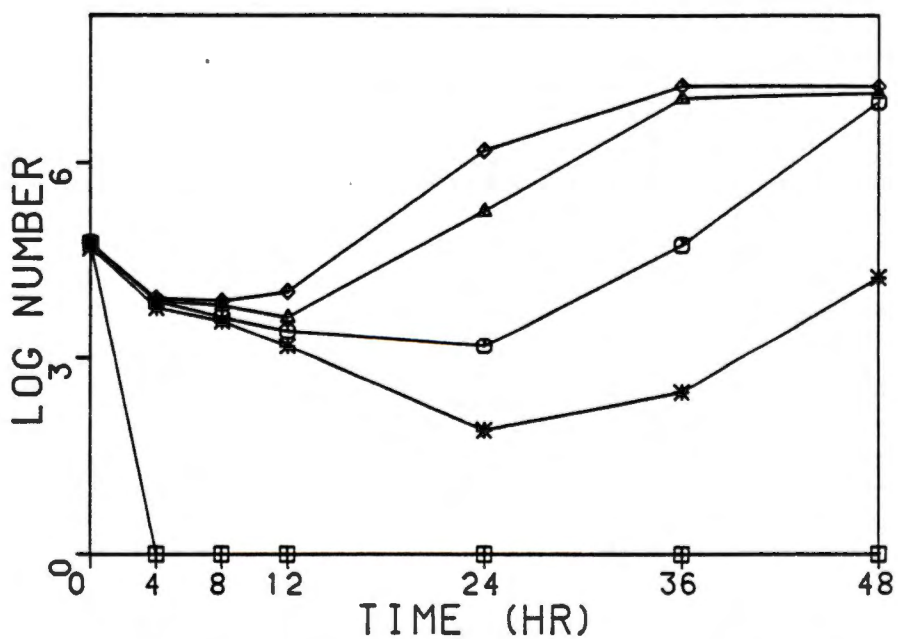


Figure 15. Effect of corn oil on the antimicrobial activity of 400 ppm TBHQ against *S. cerevisiae* growth.

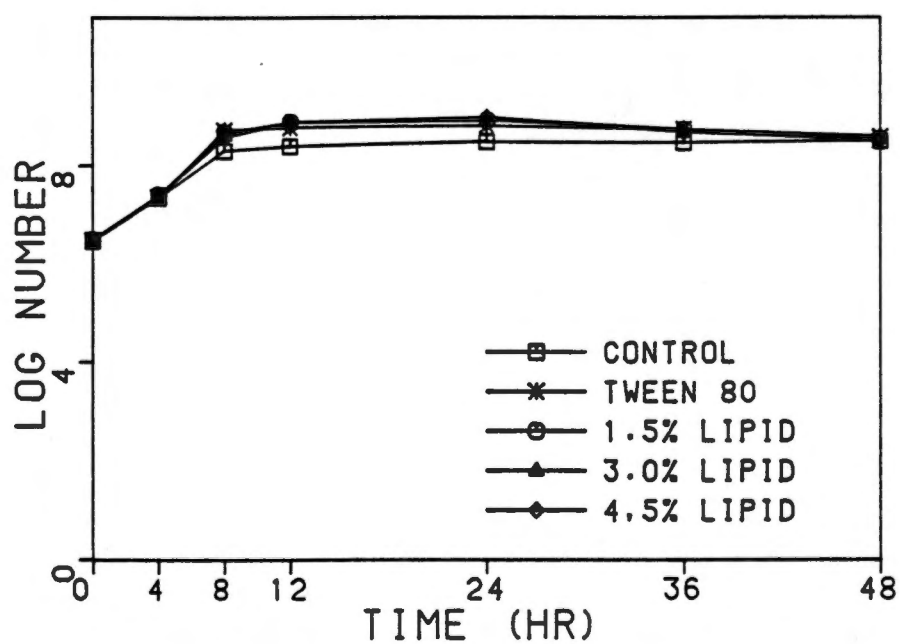


Figure 16. Influence of corn oil on the growth of *S. aureus* in TSB.

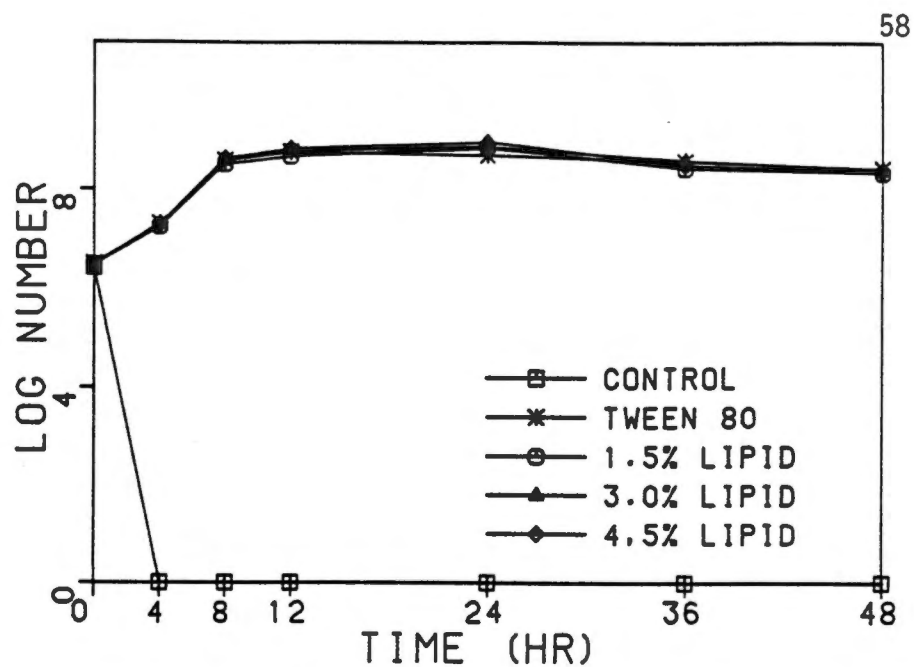


Figure 17. Effect of corn oil on the antimicrobial activity of 300 ppm BHA against S. aureus growth.

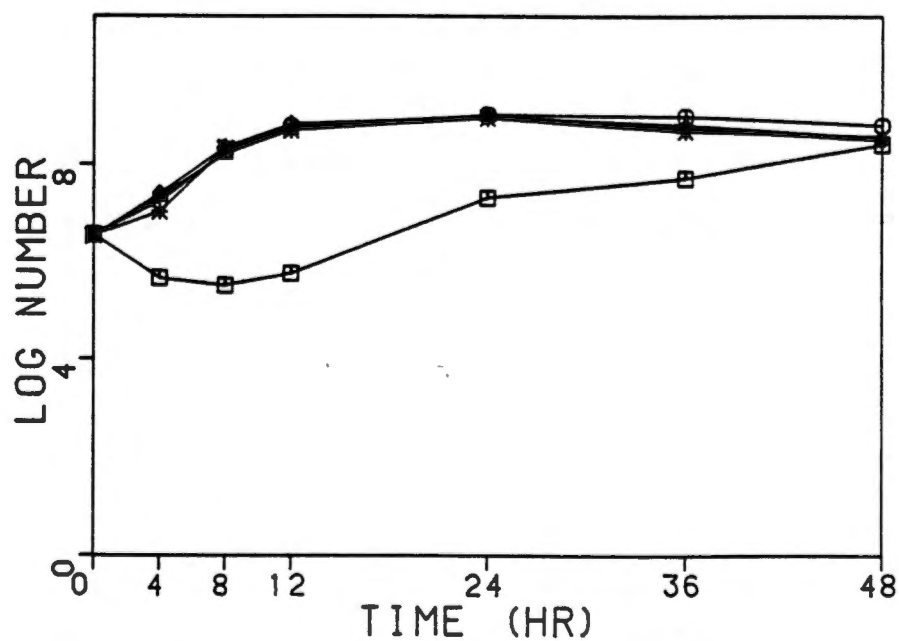


Figure 18. Effect of corn oil on the antimicrobial activity of 50 ppm TBHQ against S. aureus growth.

Lipid had no effect on the growth of Pseudomonas fluorescens (Figure 19, Table 5). As already stated, a shaking water bath was used to disperse the lipid during growth since this microorganism was inhibited by the emulsifier, Tween 80. This method proved to be an effective way of dispersing the lipid into the medium, preventing inhibition of this strict aerobic microorganism due to a reduced availability of oxygen by the presence of the lipid layer.

The activity of 200 ppm BHA against P. fluorescens was dramatically decreased in the presence of any level of lipid. Viable cell counts at any time were similar to those of the controls (Figure 20). With 300 ppm TBHQ in the presence of corn oil, more activity was retained than with BHA against this microorganism (Figure 21). The lag phase was extended in all cases to 8 hours. Growth resumed after 12 hr of incubation but at a slower rate than the control. Greater inhibition was observed at the 1.5% level of corn oil.

The presence of corn oil stimulated the growth of both Penicillium citrinum and Aspergillus niger (Figure 22 and 23). This growth stimulation was greater than that found with the casein (Figure 11 and 12). Since fungi possess both proteases and lipases, corn oil could have supplied the molds with additional essential nutrients. One might expect a decrease in growth of these strict aerobic microorganisms due to the reduced availability of oxygen caused by lipid droplets that accumulated at the surface of the medium. While growth was not decreased, sporulation and pigmentation of the mycelium was decreased by the presence of the lipid in both cases.

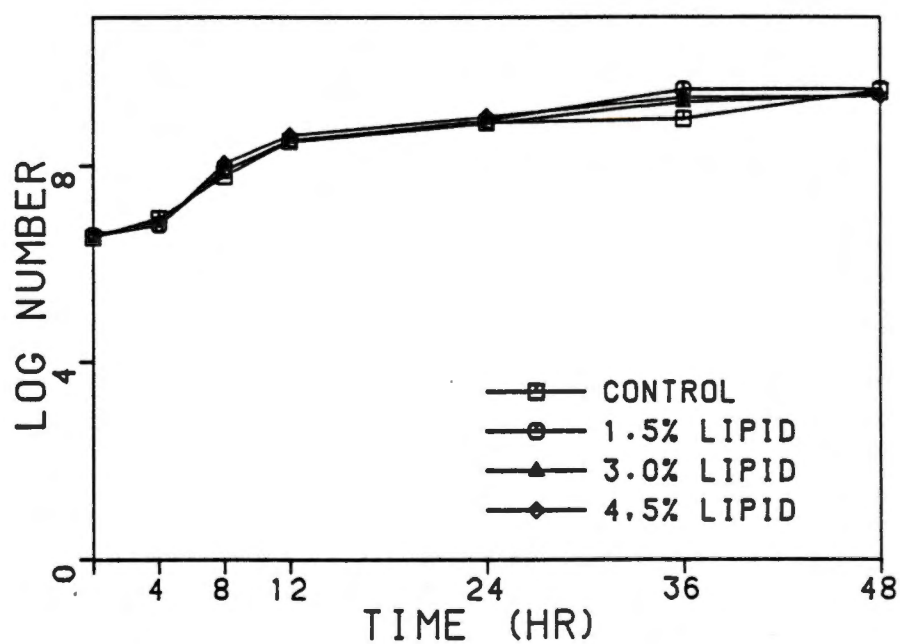


Figure 19. Influence of corn oil on the growth of *P. fluorescens* in TSB.

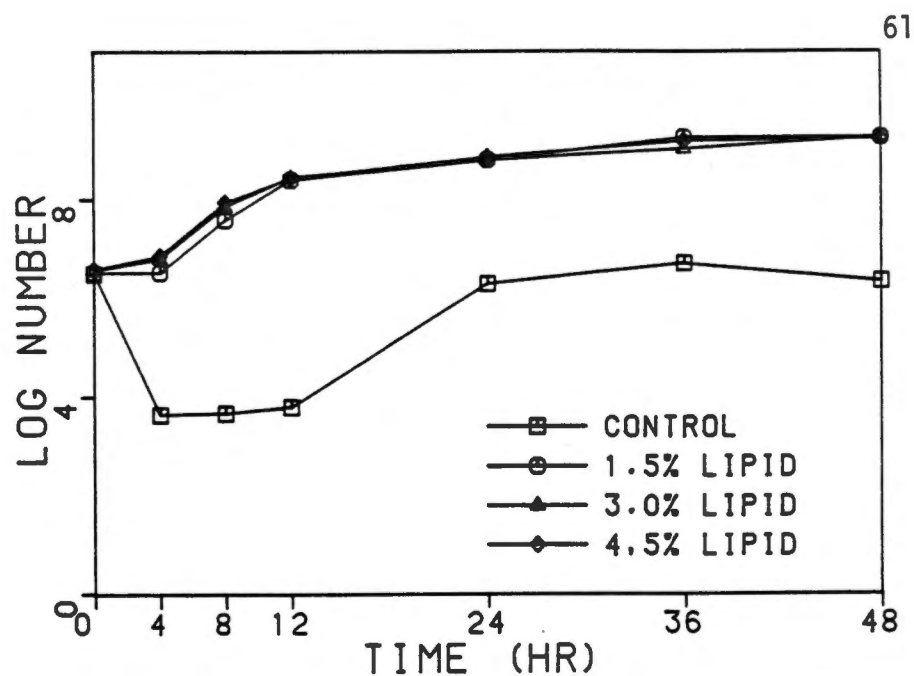


Figure 20. Effect of corn oil on the antimicrobial activity of 200 ppm BHA against *P. fluorescens* growth.

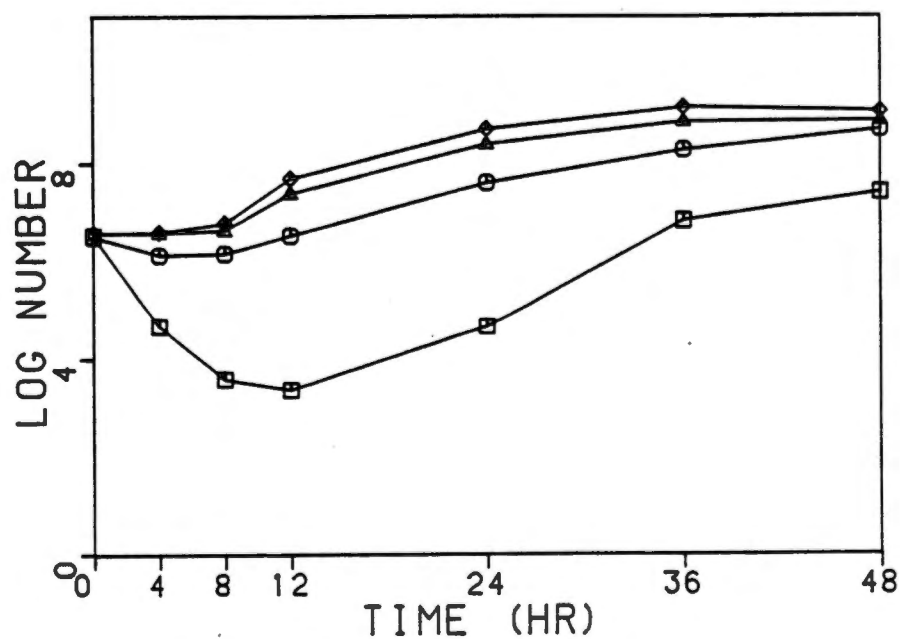


Figure 21. Effect of corn oil on the antimicrobial activity of 300 ppm TBHQ against *P. fluorescens* growth.

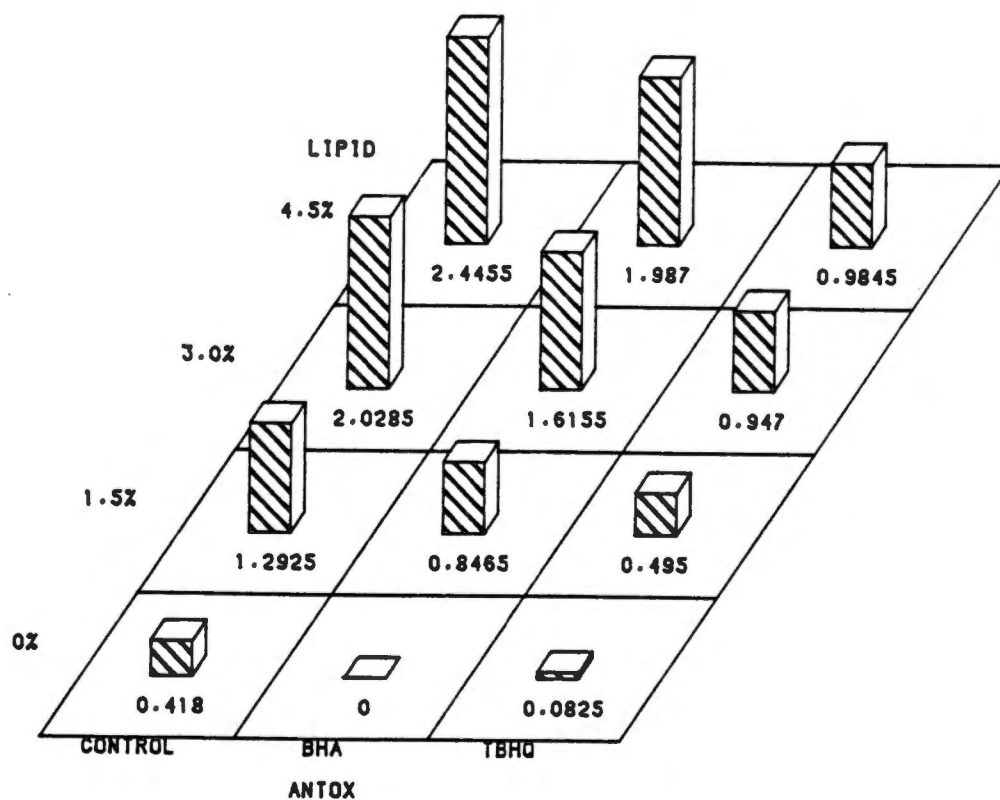


Figure 22. Effect of corn oil on the antimicrobial activity of 200 ppm BHA and 400 ppm TBHQ against *P. citrinum* growth in YES broth at 25°C. The numbers below each bar represent the mycelial weight of the mold after 14 days of incubation.

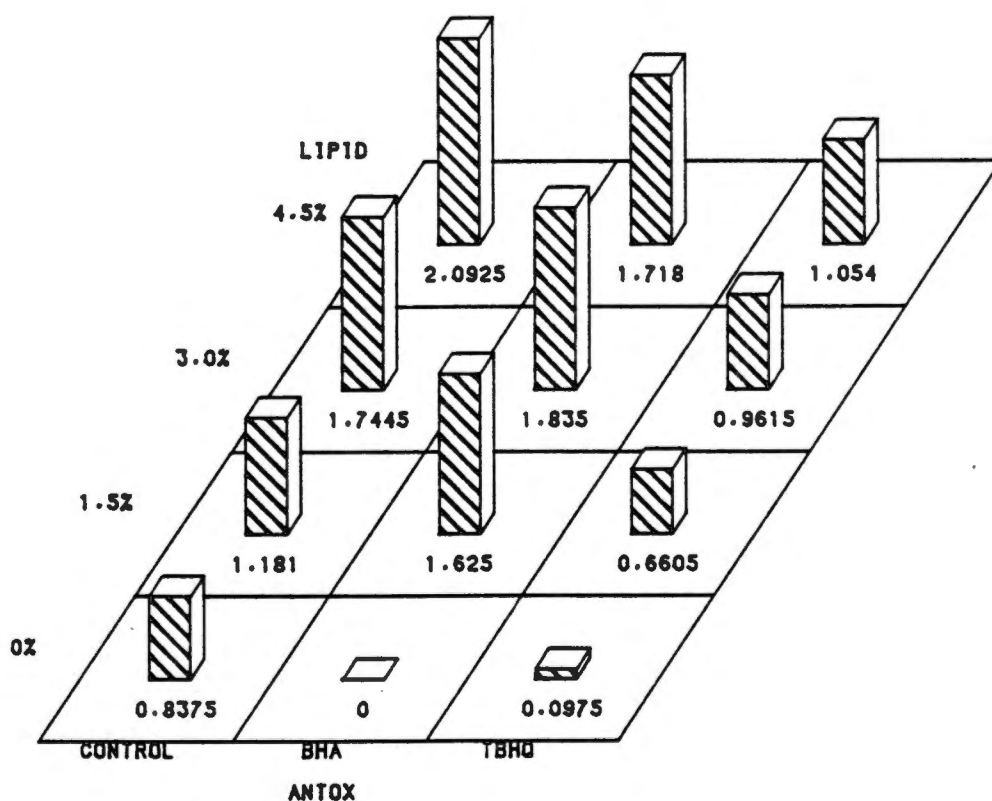


Figure 23. Effect of corn oil on the antimicrobial activity of 200 ppm BHA and 600 ppm TBHQ against *A. niger* growth in YES broth at 25°C. The numbers below each bar represent the mycelial weight of the mold after 14 days of incubation.

In the case of P. citrinum, BHA at 200 ppm was more influenced by the lipid than it was by the protein (Table 6, Figure 22). However, since the respective controls grew more than with casein, a greater inhibition was shown in every case. Therefore, the mycelial weight was always smaller in the samples containing BHA than that of the controls. Pigmentation of the mycelium and sporulation were affected more at 1.5% lipid than they were at the other level tested. A slight increase in the mycelial weight was also found when lipid level was increased from 3.0 to 4.5% (Table 6). TBHQ, at 400 ppm, was less influenced by the lipid than was BHA (Figure 22). In every case, the mycelial weight of the samples containing TBHQ was less than half the weight of the respective control grown with the same concentration of lipid. Pigmentation of the mycelium and sporulation were decreased noticeably in all cases. At 3.0 and 4.5% lipid, the mycelial weight was quite similar.

The activity of 200 ppm BHA against Aspergillus niger was virtually eliminated at any lipid level as shown in Figure 23 and Table 6. At 1.5 and 3.0% lipid, the mycelial weight was even greater in the presence of BHA than without it. At 4.5% corn oil, the mycelial weight was slightly decreased in the presence of the antioxidant. In contrast, TBHQ at 600 ppm inhibited this microorganism to some extent at all the levels of lipid tested. The mycelial weight was slightly greater at the 3.0 and 4.5% levels than it was at the 1.5% level.

TABLE 6

EFFECT OF CORN OIL ON THE ANTIMICROBIAL ACTIVITY OF BHA AND TBHQ
AGAINST THE MOLDS

Microorganism	Antioxidant (ppm)	Corn oil %(v/v)	Mycelial Weight ¹ ± Standard Error (mg/50 ml broth)	
<u>Penicillium</u> <u>citrinum</u>	0	0	418.0	+ 6.0c
	0	1.5	1292.5	- 137.5b
	0	3.0	2028.5	199.5a
	0	4.5	2445.5	280.5a
	0	Tween 80	934.0	66.0b
	200 BHA	0	0.0	+ 0.0c
	200 BHA	1.5	846.5	- 52.5b
	200 BHA	3.0	1615.5	169.5a
	200 BHA	4.5	1987.0	471.0a
	200 BHA	Tween 80	391.0	39.0bc
	400 TBHQ	0	82.5	+ 2.5c
	400 TBHQ	1.5	495.0	- 70.0b
	400 TBHQ	3.0	947.0	185.0a
	400 TBHQ	4.5	984.5	229.5a
	400 TBHQ	Tween 80	221.0	70.0bc
<u>Aspergillus</u> <u>niger</u>	0	0	837.5	+ 16.5d
	0	1.5	1181.0	- 159.0c
	0	3.0	1744.5	61.5b
	0	4.5	2092.5	9.5a
	0	Tween 80	1048.5	25.5cd
	200 BHA	0	0.0	+ 0.0c
	200 BHA	1.5	1625.0	- 75.0a
	200 BHA	3.0	1835.0	77.0a
	200 BHA	4.5	1718.0	5.0a
	200 BHA	Tween 80	912.0	199.0b

TABLE 6 (Continued)

EFFECT OF CORN OIL ON THE ANTIMICROBIAL ACTIVITY OF BHA AND TBHQ
AGAINST THE MOLDS

Microorganism	Antioxidant (ppm)	Corn oil %(v/v)	Mycelial Weight ¹ ± Standard Error (mg/50 ml broth)	
<u>Aspergillus</u> <u>niger</u>	600 TBHQ	0	97.5 ±	23.5b
	600 TBHQ	1.5	660.5 ±	22.5a
	600 TBHQ	3.0	961.5	246.5a
	600 TBHQ	4.5	1054.0	31.0a
	600 TBHQ	Tween 80	169.5	37.5b

¹Mean of two replications; Within an experimental unit, means with the same letter are not significantly different at 0.05 level using Duncan's Multiple Range Test.

CHAPTER IV

DISCUSSION

A discussion of the effects of the protein and the lipid on the antimicrobial activity of butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) is presented in this chapter. Inhibition data were summarized as percent inhibition either at 24 hr of incubation, in the case of the bacteria and yeast, or after the 14 day incubation period for the molds. As already stated in Chapter II, these percentages were obtained by comparison of each treatment sample with its respective control grown with the same concentration of lipid or protein.

The relative effect of casein on the inhibition at 24 hr of incubation by BHA and TBHQ of the bacteria and yeast is shown in Figure 24. The numbers below each bar indicate the sums of the percentages of both antioxidants. The exact percentages for each antioxidant are shown in Table 7. In the presence of 200 ppm BHA, Saccharomyces cerevisiae was totally inhibited when no casein was present whereas inhibition progressively decreased to 96.5, 75.6 and 53.3% at 3, 6 and 9% casein, respectively. In contrast, TBHQ at 400 ppm inhibited this microorganism nearly 100% (99.9%) even in the presence of 9% casein. With Staphylococcus aureus, 3% casein reduced the effect of 300 ppm BHA but inhibition was still above 90%. At 6 and 9% casein however, percent inhibition dropped to

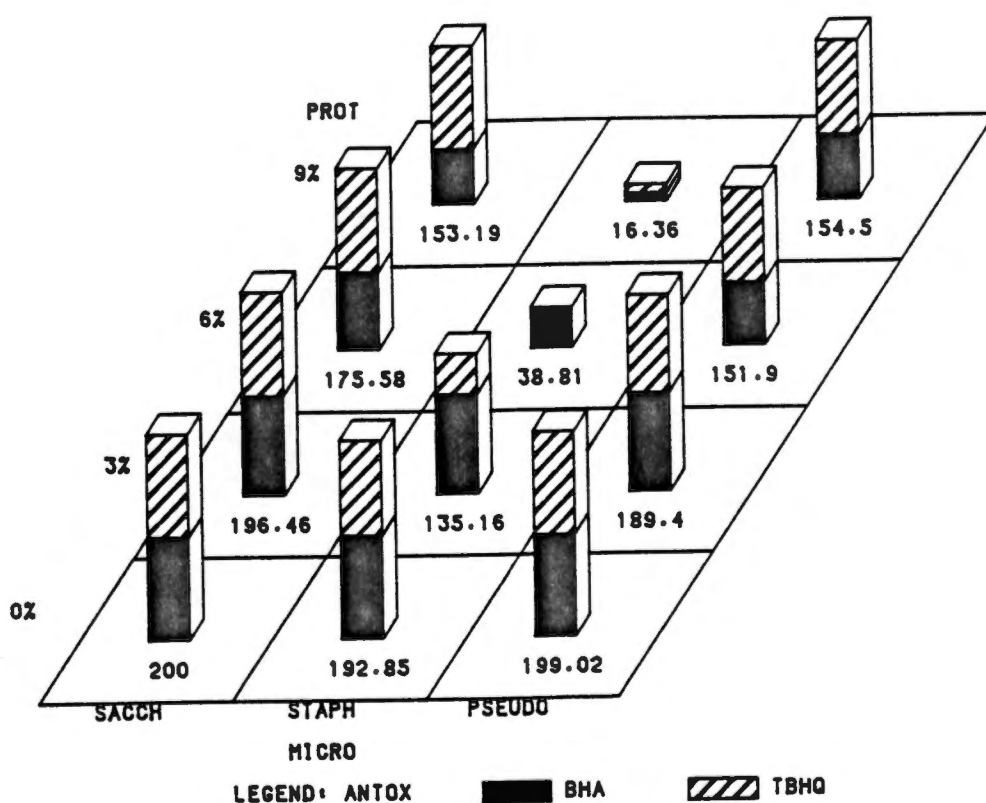


Figure 24. Percent growth inhibition at 24 hr incubation by BHA and TBHQ against the bacteria and yeast in the presence of casein. The numbers below each bar indicate the sums of the percentages of both BHA and TBHQ.

TABLE 7

PERCENTAGE GROWTH INHIBITION BY BHA AND TBHQ AGAINST
THE BACTERIA AND YEAST AT 24 HR INCUBATION
IN THE PRESENCE OF CASEIN

Microorganism	BHA (ppm)	TBHQ (ppm)	Casein %(w/v)	Percent Inhibition
<u>Saccharomyces</u> <u>cerevisiae</u>	200	0	0	100.0
	200	0	3	96.5
	200	0	6	75.6
	200	0	9	53.3
	0	400	0	100.0
	0	400	3	100.0
	0	400	6	99.9
	0	400	9	99.9
<u>Staphylococcus</u> <u>aureus</u>	300	0	0	100.0
	300	0	3	96.2
	300	0	6	38.8
	300	0	9	8.0
	0	50	0	92.8
	0	50	3	39.0
	0	50	6	0.0
	0	50	9	8.4
<u>Pseudomonas</u> <u>fluorescens</u>	200	0	0	99.0
	200	0	3	95.5
	200	0	6	60.8
	200	0	9	62.6
	0	300	0	100.0
	0	300	3	93.9
	0	300	6	91.1
	0	300	9	91.9

38.8 and 8.0%, respectively. Inhibition of S. aureus by 50 ppm TBHQ was dramatically reduced in the presence of the protein to 39.0, 0.0 and 8.4% at 3, 6 and 9% casein. Pseudomonas fluorescens demonstrated a pattern similar to that shown by the yeast. With 6 and 9% casein, the activity of 200 ppm BHA was reduced to 60.8 and 62.6%, respectively, whereas 300 ppm TBHQ retained approximately 92% inhibition even at the 9% casein level.

The relative effect of casein on BHA and TBHQ inhibition of mold growth at 14 days is shown in Figure 25 and the percent inhibition for each case is presented in Table 8. With the molds, the effect of casein on antioxidant inhibition was highly species dependent. With 200 ppm BHA, Penicillium citrinum inhibition was reduced to 42.3, 14.4 and 2.9% at 3, 6 and 9% casein, respectively, while inhibition of Aspergillus niger was eliminated by as little as 3% casein in the growth medium. In contrast, TBHQ at 400 ppm for P. citrinum and 600 ppm for A. niger retained more than 30% of each activity at the 9% casein level.

In a study done with non-fat dry milk, Cornell et al. (1971) reported that BHA could be reversibly bound to casein most probably through hydrophobic interactions. They found that, at low concentrations, the amount of antioxidant bound was a linear function of the concentration of free antioxidant and could be calculated by the equation:

$$M = b C_f$$

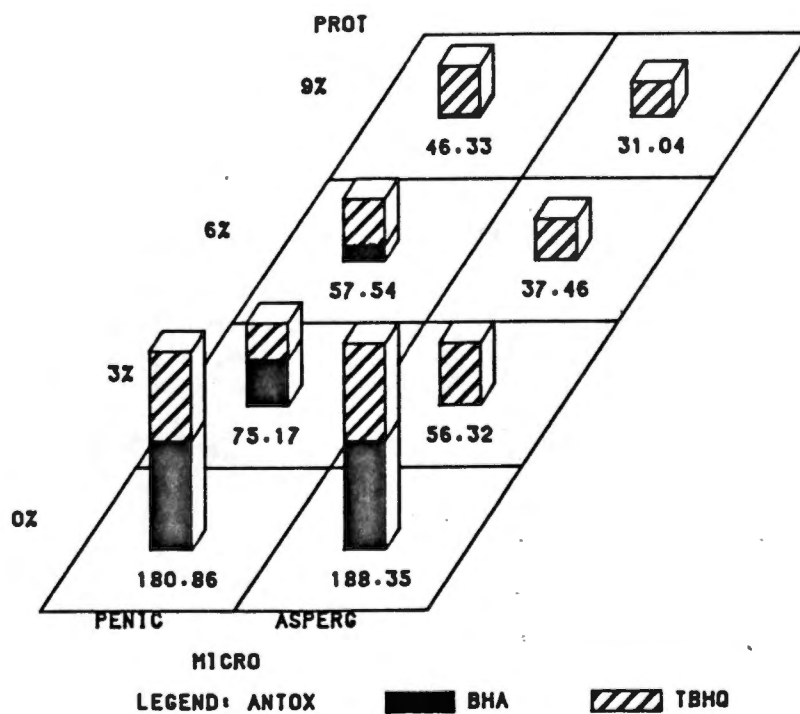


Figure 25. Percent growth inhibition by BHA and TBHQ after 14 days of incubation against the molds in the presence of casein. The numbers below each bar indicate the sums of the percentages of both BHA and TBHQ.

TABLE 8

PERCENTAGE GROWTH INHIBITION BY BHA AND TBHQ AGAINST
THE MOLDS AFTER 14 DAY INCUBATION PERIOD
IN THE PRESENCE OF CASEIN

Microorganism	BHA (ppm)	TBHQ (ppm)	Casein %(w/v)	Percent Inhibition
<u>Penicillium</u>				
<u>citrinum</u>	200	0	0	100.0
	200	0	3	42.3
	200	0	6	14.4
	200	0	9	2.9
	0	400	0	80.9
	0	400	3	32.9
	0	400	6	43.2
	0	400	9	43.4
<u>Aspergillus</u>				
<u>niger</u>	200	0	0	100.0
	200	0	3	0.0
	200	0	6	0.0
	200	0	9	0.0
	0	600	0	88.4
	0	600	3	56.3
	0	600	6	37.5
	0	600	9	31.0

where,

M = moles of antioxidant bound per gram of protein

b = slope = binding coefficient of the antioxidant

C_f = final antioxidant concentration in moles per liter.

Cornell and co-workers calculated binding coefficients, b , for several alkyl gallates and BHA (Table 9). BHA was found to have a binding coefficient of 0.04. These authors also postulated that in milk, the protein would compete with the butteroil for the antioxidant, resulting in less antioxidant in the fat phase of milk than expected from its distribution coefficient in protein-free model systems (Cornell et al., 1970). In 1979, Cornell developed a mathematical model for the prediction of antioxidant distribution in whole milk based both on oil/water partitioning and protein binding of the antioxidant. Protein binding was found to exert a marked influence on the distribution of antioxidants between the aqueous and oil phases. However, comparison between the calculated (94.7%) and the observed (73.5%) antioxidant content of the fat phase of freeze dried milk models suggested that the approximations using this mathematical model were relatively poor for BHA. Therefore, comparing the percentage found, 73.5%, to that previously reported by Cornell et al. (1970), 99%, based on oil/water partitioning alone, shows the effect of protein binding on antioxidant distribution.

TABLE 9
BINDING OF ANTIOXIDANTS TO MILK PROTEINS

Antioxidant	Binding coefficient
Ethyl gallate	0.023
Propyl gallate	0.040 (0.03) ^a
Butyl gallate	0.084
Amyl gallate	0.170
Hexyl gallate	0.360
Isopropyl gallate	0.034
BHA	0.040

^aMean deviation of 5 determinations; the other values are slopes of the respective binding curves.

Source: Cornell et al. (1971). Binding of antioxidants by milk proteins. J. Dairy Sci. 54: 634.

In the present study, binding of the antioxidants by the casein could very well have been the primary cause for the loss in antimicrobial activity of BHA. In all cases except with S. aureus, TBHQ was used at much higher concentrations than BHA to obtain similar lethal effects. With S. aureus, the antimicrobial activity of 50 ppm was decreased dramatically in the presence of the protein. It would seem therefore, that both antioxidants are susceptible to binding by the casein. Since the binding coefficient of TBHQ has not been reported however, no comparison could be made between the binding of BHA and TBHQ by the casein. It would seem logical to assume that the greater inhibition exhibited by TBHQ was due either to a lower binding constant with casein or to the higher concentrations used, in which case, more compound was available for inhibition after binding with the protein.

As with the casein, there was a significant loss of antimicrobial activity with both BHA and TBHQ in the presence of the lipid (Figure 26). The percent growth inhibition by BHA and TBHQ against the bacteria and yeast in the presence of corn oil is shown in Table 10. In the case of S. cerevisiae, as little as 1.5% lipid decreased the effect of the previously lethal 200 ppm BHA to 40.0% inhibition and 3.0 and 4.5% lipid to 20.0 and 22.9%, respectively. TBHQ was influenced less by the lipid, causing nearly 94% inhibition of this microorganism at 4.5% lipid. Inhibition of both S. aureus and P. fluorescens by BHA was reduced significantly in the presence of lipid. At 1.5% corn oil,

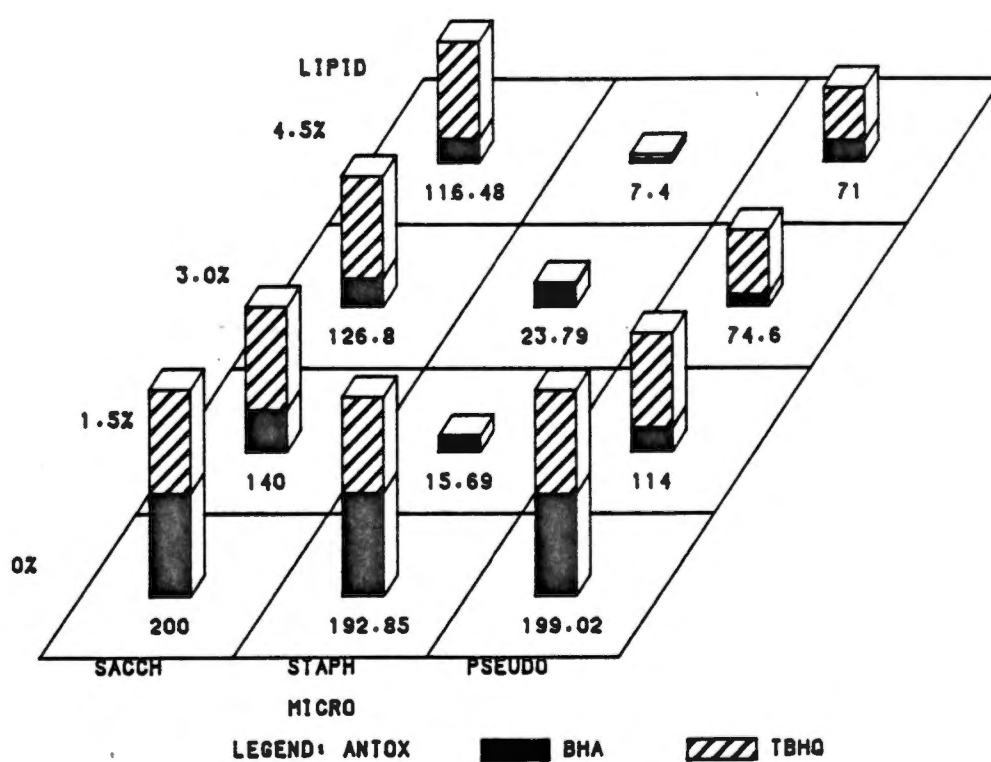


Figure 26. Percent growth inhibition at 24 hr incubation by BHA and TBHQ against the bacteria and yeast in the presence of corn oil. The numbers below each bar indicate the sums of the percentages of both BHA and TBHQ.

TABLE 10

PERCENTAGE GROWTH INHIBITION BY BHA AND TBHQ AGAINST
THE BACTERIA AND YEAST AT 24 HR INCUBATION
IN THE PRESENCE OF CORN OIL

Microorganism	BHA (ppm)	TBHQ (ppm)	Lipid %(v/v)	Percent Inhibition
<u>Saccharomyces</u> <u>cerevisiae</u>	200	0	0	100.0
	200	0	1.5	40.0
	200	0	3.0	28.0
	200	0	4.5	22.9
	200	0	Tween 80	70.6
	0	400	0	100.0
	0	400	1.5	100.0
	0	400	3.0	98.8
	0	400	4.5	93.6
	0	400	Tween 80	100.0
<u>Staphylococcus</u> <u>aureus</u>	300	0	0	100.0
	300	0	1.5	15.7
	300	0	3.0	20.7
	300	0	4.5	3.2
	300	0	Tween 80	21.0
	0	50	0	92.8
	0	50	1.5	0.0
	0	50	3.0	3.0
	0	50	4.5	4.23
	0	50	Tween 80	0.0
<u>Pseudomonas</u> <u>fluorescens</u>	200	0	0	99.0
	200	0	1.5	23.0
	200	0	3.0	11.4
	200	0	4.5	21.1
	0	300	0	100.0
	0	300	1.5	93.9
	0	300	3.0	91.1
	0	300	4.5	91.9

inhibition of S. aureus by 300 ppm BHA and P. fluorescens by 200 ppm BHA was decreased to 15.7 and 23.0%, respectively. In contrast, the effect of lipid on TBHQ activity was very different with the same microorganisms. All antimicrobial activity of 50 ppm TBHQ against S. aureus was lost in the presence of 1.5% lipid while 50% inhibition of P. fluorescens occurred even at 4.5% lipid.

The relative effect of corn oil on the antifungal activity of BHA and TBHQ after 14 days of incubation is shown in Figure 27. The individual percentages in each case are summarized in Table 11. BHA at 200 ppm retained approximately equivalent activity against Penicillium citrinum at all levels of lipid tested having 61.7, 53.3 and 59.8% inhibition at 1.5, 3.0 and 4.5% lipid, respectively. In contrast, 1.5 and 3.0% lipid decreased inhibition of Aspergillus niger by the same concentration of BHA to 0.0%. In most cases, TBHQ was less affected by the presence of corn oil than BHA, exhibiting greater inhibition against P. citrinum than A. niger. In all cases, the addition of the emulsifier, Tween 80, dramatically decreased the activity of BHA but had little effect on TBHQ.

These results have demonstrated that, as with casein, corn oil had a greater influence on BHA activity than on TBHQ. In all cases, BHA was also more affected by the presence of lipid in the growth medium than it was by protein. Since the activity of BHA was lost immediately in the presence of the lipid, the primary cause for the loss in antimicrobial activity of the antioxidant was probably due to its solubilization into the lipid phase of the medium.

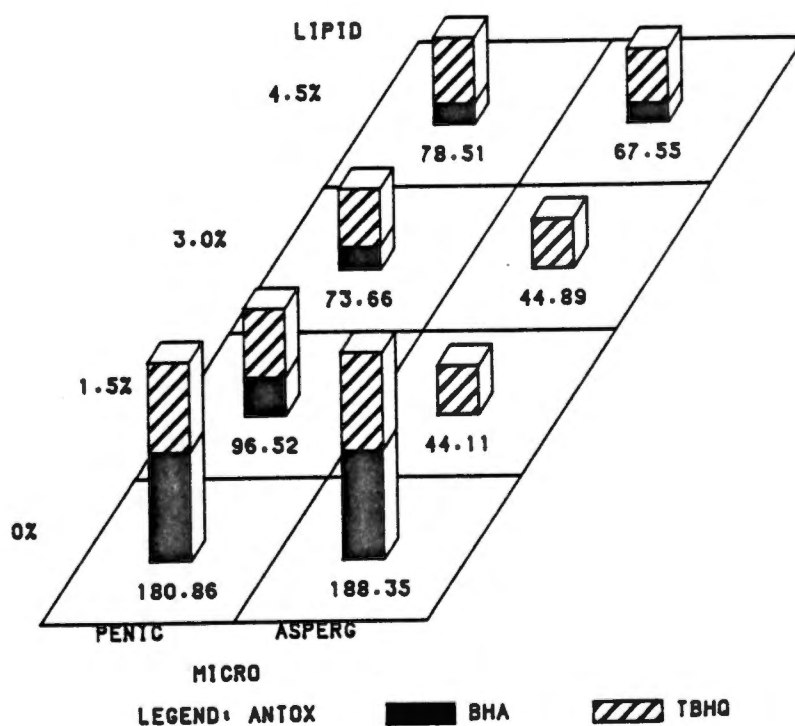


Figure 27. Percent growth inhibition by BHA and TBHQ after 14 days of incubation against the molds in the presence of corn oil. The numbers below each bar indicate the sums of the percentages of both BHA and TBHQ.

TABLE 11

PERCENTAGE GROWTH INHIBITION BY BHA AND TBHQ AGAINST
THE MOLDS AFTER 14 DAY INCUBATION PERIOD
IN THE PRESENCE OF CORN OIL

Microorganism	BHA (ppm)	TBHQ (ppm)	Lipid %(v/v)	Percent Inhibition
<u>Penicillium</u>				
<u>citrinum</u>	200	0	0	100.0
	200	0	1.5	34.8
	200	0	3.0	20.3
	200	0	4.5	18.8
	200	0	Tween 80	58.1
	0	400	0	80.9
	0	400	1.5	61.7
	0	400	3.0	53.3
	0	400	4.5	59.7
	0	400	Tween 80	76.3
<u>Aspergillus</u>				
<u>niger</u>	200	0	0	100.0
	200	0	1.5	0.0
	200	0	3.0	0.0
	200	0	4.5	17.9
	200	0	Tween 80	13.0
	0	600	0	88.4
	0	600	1.5	44.1
	0	600	3.0	44.9
	0	600	4.5	49.6
	0	600	Tween 80	83.8

The lipophilic nature of BHA is well known (Porter, 1980). As already seen in Chapter I, this antioxidant is a monohydric phenol, i.e. with only one hydroxyl group on its molecule. Thus, monohydric phenols such as BHA and butylated hydroxytoluene (BHT), are very lipophilic antioxidants with low hydrophilic-lipophilic balance (HLB) numbers, in contrast to dihydric (TBHQ) and trihydric (propyl gallate) phenols which are more amphipathic molecules with higher HLB numbers. This difference in lipid solubility between BHA and TBHQ could explain the greater decrease in antimicrobial activity suffered by BHA in the presence of the lipid compared to TBHQ. Cornell et al. (1970) calculated the butteroil/water partition coefficient of some esters of gallic acid, BHA and nordihydroguaiaretic acid. The partition coefficient of a compound is approximately the ratio of its solubilities in the oil and water phases. Thus:

$$P_{O/W} = C_O/C_W$$

where,

$P_{O/W}$ = oil/water partition coefficient

C_O = equilibrium concentration of the antioxidant in the oil phase

C_W = equilibrium concentration of the antioxidant in the water phase.

These authors found BHA to exhibit the highest preference for the oil phase having a partition coefficient greater than 800 (Table 12).

TABLE 12

BUTTEROIL/WATER PARTITION COEFFICIENTS OF
PHENOLIC ANTIOXIDANTS AT 40°C

Antioxidant	Water	Milk Salt Solution
Ethyl gallate	0.24 (0.03) ^a	-
Propyl gallate	1.33 (0.05) ^b	0.84 (0.03) ^a
Isopropyl gallate	0.64 (0.02) ^a	-
Butyl gallate	5.8 (0.5) ^a	3.9 (0.1) ^a
Amyl gallate	18.5 (0.2) ^a	9.5 (0.2) ^a
Hexyl gallate	71.0 (1.1) ^a	44.8 (0.7) ^a
Nordihydroguaiaretic acid	21.7 (0.9) ^a	21.5 (1.1) ^a
BHA	834.0 (4) ^a	825.0 (14) ^a

^aNumbers in parentheses are the mean deviations of three or more determinations.

^bStandard deviation of 11 observations.

Source: Cornell et al. (1970). Partition coefficients of some antioxidants in butteroil-water model systems. J. Dairy Sci. 53: 529.

In contrast, the partition coefficients for the esters of the gallic acid ranged from 0.24 for ethyl gallate to 71.0 for hexyl gallate increasing with the length of the alkyl chain.

The way in which the lipid is incorporated into the medium has been shown to have a great influence on the partition of the antioxidant between the oil and water phases (Cornell, 1979; Porter, 1980). In contrast to antioxidants in systems where oil formed a continuous bulk phase, those in emulsions exhibited a higher than predicted preference for the fat phase (Cornell, 1979). The difference between non-homogenized systems and emulsions was reported to be due to the greatly expanded oil/water interface of the latter, which provided for greater absorption of surface active molecules. Since the esters of gallic acid are surface active molecules, it would be expected for them to concentrate at the oil/water interface. However, more lipophilic antioxidants, such as BHA and BHT, would be partitioned into the oil phase to a greater extent. In fact, the same author found the order of preference of the tested antioxidants for the fat phase to be: propyl gallate (PG) < hexyl gallate < BHA. Porter (1980) proposed that two food lipid situations existed which call for two different types of combinations of antioxidants: (1) low surface to volume ratio (LSV) and (2) high surface to volume ratio (HSV) lipid exposure. The LSV type foods are, for example, vegetable oils in bulk storage. For this situation antioxidants with higher HLB numbers, such as PG and TBHQ, were much more effective

than almost total lipophiles like BHA and BHT (Adamson, 1967). This was explained by the fact that the antioxidant action appeared to be more concentrated at the surface of the lipid (Porter, 1980). Foods or situations with HSV ratios include whole tissue foods with polar lipid membranes, intracellular colloidal micelles of neutral lipids and micelles of emulsified oils. Under these conditions, the very lipophilic antioxidants (low HLB numbers), such as BHA and BHT, were more favored.

After taking into account considerations of lipid solubility, partition coefficients, amphiphilic characteristics and HLB numbers, a better evaluation of the results of this study can be made. As already stated above, BHA is a lipophilic antioxidant with a high partition coefficient while TBHQ is an amphiphilic antioxidant with lower lipid solubility. In these experiments, corn oil was emulsified using Tween 80 in all cases, except with Pseudomonas fluorescens. Under these conditions, more BHA would have been solubilized into the oil phase than TBHQ, making BHA less available to act against microorganisms that live in the aqueous phase of the medium. This explains the greater loss in antimicrobial activity of BHA than TBHQ. Another contributory factor could very well have been that TBHQ was used at a much higher concentration than BHA to get similar lethal effects, except in the case of Staphylococcus aureus. Therefore, a higher concentration of TBHQ would have been available for inhibition after reacting with the lipid. In the case of S. aureus, the activity of only 50 ppm TBHQ was nearly completely

eliminated by the presence of the lipid. It was also noticed that, in the case of P. fluorescens where the corn oil was not emulsified, 300 ppm TBHQ was more influenced by the lipid than 400 ppm of the same antioxidant against S. cerevisiae where the corn oil was not emulsified. The difference could also have been due to the greater concentration of TBHQ used in the latter case.

To this point, considerations have been made concerning only lipid solubility and chemical structure of these antioxidants in explaining the loss in antimicrobial activity of both BHA and TBHQ. Another possibility is that since these compounds were antioxidants they could have been "used up" through prevention of the lipid autoxidation which changes their structure and eliminates their antioxidant and antimicrobial power (Robach et al., 1977). The results of the present study indicated that solubility into the oil phase was the primary reason for the loss in antimicrobial activity. More evidence for solubility being the primary factor is that antioxidants already dissolved into the oil are the ones that prevent autoxidation and, since no microorganisms are present in that phase, their being "used up" in the oil phase should make no difference as to their antimicrobial activity in the aqueous phase.

CHAPTER V

CONCLUSION

The antimicrobial activity of two phenolic antioxidants, BHA and TBHQ, was tested in the presence of two food components, a lipid, corn oil, and a protein, casein. Five microorganisms were used in this study: two molds, Penicillium citrinum NRRL 5727 and Aspergillus niger; a yeast, Saccharomyces cerevisiae and two bacteria, Pseudomonas fluorescens ATCC 15456 and Staphylococcus aureus ATCC 12600.

Most of the concentrations of BHA and TBHQ used in this study resulted in total growth inhibition following 4 hr of incubation except in the cases of P. fluorescens at 200 ppm BHA and 300 ppm TBHQ and S. aureus at 50 ppm TBHQ.

In conclusion we can say that first, the presence of a protein, casein, and a lipid, corn oil, significantly reduced but, in most cases, did not eliminate the antimicrobial activity of both BHA and TBHQ. These antioxidants are thought to solubilize into the lipid phase of the medium or to bind to the protein which made them unavailable to act against microorganisms.

A second conclusion was that TBHQ was generally less affected by food components than BHA. The reason for this difference was most likely due to a combination of the concentrations used and polarity differences. In all cases, except with S. aureus, TBHQ

was used at higher concentrations than BHA to obtain equivalent lethal effectiveness. Therefore, more residual TBHQ could have been available for inhibition after reacting with the protein or lipid. TBHQ is also more polar than BHA which means less would have been solubilized into the lipid making it more available for inhibition.

Finally, the results have shown that the primary cause for the loss in antimicrobial activity of BHA and TBHQ in the presence of the lipid was due to their solubility into the lipid phase of the medium. Evidence for this conclusion came from the fact that the loss in activity was immediate in the presence of the lipid. Therefore, the theory based on the formation of stable free radicals through prevention of autoxidation should be discarded since the antioxidants already dissolved into the oil phase are the ones that prevent autoxidation of the lipid; thus, their being "used up" in this phase should make no difference as to their antimicrobial activity in the aqueous phase of the medium were microorganisms are found.

It would seem logical that if these compounds are to be utilized exclusively as preservatives, they should be tested at higher concentrations or used as surface antimicrobials rather than attempting to incorporate the compounds into high fat or protein foods.

LITERATURE CITED

LITERATURE CITED

- Aalto, T. R., Firman, M. C. and Rigler, N. E. 1953. p-Hydroxybenzoic acid esters as preservatives. I. Uses, antibacterial and antifungal studies, properties and determination. J. Amer. Pharm. Assoc. 42:449.
- Adamson, A. W. 1967. "Physical Chemistry of Surfaces," 2nd ed., pp. 520. Interscience Publications, New York.
- Ahmad, S. 1979. Inhibition of mold growth by butylated hydroxyanisole. M. S. thesis, Washington State Univ., Pullman.
- Astill, B. D., Terhaar, C. J., Krasavage, W. J., Wolf, G. L., Roudabush, R. L. and Fassett, D. W. 1975. Safety evaluation and biochemical behavior of monotertiarybutylhydroquinone. J. Amer. Oil Chem. Soc. 52:53.
- Ayaz, M. 1975. Studies on the physiological characterization of staphylococci and the effects of butylated hydroxyanisole and butylated hydroxytoluene on growth and enterotoxin production of Staphylococcus aureus. Ph. D. thesis, Washington State Univ., Pullman.
- Ayaz, M., Luedecke, L. O. and Branen, A. L. 1980. Antimicrobial effect of butylated hydroxyanisole and butylated hydroxytoluene on Staphylococcus aureus. J. Food Prot. 43:4.
- Bernheim, F. 1972. The effect of chloroform, phenols, alcohol and cyanogen iodide on the swelling of Pseudomonas aeruginosa and various salts. Microbios 5:143.
- Branen, A. L. 1975. Toxicology and biochemistry of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). J. Amer. Oil Chem. Soc. 52:59.
- Branen, A. L., Davidson, P. M. and Katz, B. 1980. Antimicrobial properties of phenolic antioxidants and lipids. Food Technol. 34:42.
- Chang, H. C. and Branen, A. L. 1975. Antimicrobial effects of butylated hydroxyanisole (BHA). J. Food Sci. 40:349.
- Code of Federal Regulations. 1980. Title 21 - Food and Drugs, Parts 170-199. Office Fed. Reg., Nat. Arch. Rec. Serv., Gen. Serv. Admin., Washington, D. C.

- Code of Federal Regulations. 1980. Title 21 - Food and Drugs, Parts 170-199. Office Fed. Reg., Nat. Arch. Rec. Serv., Gen. Serv. Admin., Washington, D.C.
- Collee, J. G., Knowlden, J. A. and Hobbs, B. C. 1961. Studies on the growth, sporulation and carriage of Clostridium welchii with special reference to food poisoning strains. J. Appl. Bacteriol. 24:326.
- Cornell, D. G., DeVilbiss, E. D. and Pallansch, M. J. 1970. Partition coefficients of some antioxidants in butteroil-water model systems. J. Dairy Sci. 53:529.
- Cornell, D. G., DeVilbiss, E. D. and Pallansch, M. J. 1971. Binding of antioxidants by milk proteins. J. Dairy Sci. 54: 634.
- Cornell, D. G. 1979. Distribution of some antioxidants in dairy products. J. Dairy Sci. 62:861.
- Davidson, P. M. and Branen, A. L. 1980a. Inhibition of two psychrotrophic Pseudomonas species by butylated hydroxyanisole. J. Food Sci. 45:1603.
- Davidson, P. M. and Branen, A. L. 1980b. Antimicrobial mechanisms of butylated hydroxyanisole against two Pseudomonas species. J. Food Sci. 45:1607.
- Davidson, P. M., Brekke, C. J. and Branen, A. L. 1981. Antimicrobial activity of butylated hydroxyanisole, tertiary butylhydroquinone and potassium sorbate in combination. J. Food Sci. 46:314.
- deMan, J. M. 1980. "Principles of Food Chemistry," 3rd ed. AVI Publishing Co., Inc., Westport, CT.
- De Smet, M. J., Kingma, J. and Witholt, B. 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of Escherichia coli. Biochem. Biophys. Acta 343:64.
- Eletr, S., Williams, M. A., Watkins, T. and Keith, A. D. 1974. Perturbations of the dynamics of lipid alkyl chains in membrane systems: effect on the activity of membrane-bound enzymes. Biochem. Biophys. Acta 339:190.
- Ericksen, D. R. and Tompkin, R. B. 1977. Antimicrobial and antirancidity agent. U. S. Patent 4,044,160.

- Fung, D. Y. C., Taylor, S. and Kahan, J. 1977. Effect of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on growth and aflatoxin production of Aspergillus flavus. J. Food Safety 1:39.
- Griffin, W. C. and Lynch, M. S. 1972. Surface active agents. In "Handbook of Food Additives," ed. Furia, T. E., pp. 113. Chemical Rubber Co., Cleveland, OH.
- Hugo, W. B. and Bloomfield, S. F. 1971. Studies on the mode of action of the phenolic antibacterial agent Fentichlor against Staphylococcus aureus and Escherichia coli. I. The adsorption of Fentichlor by the bacterial cell and its antibacterial activity. J. Appl. Bact. 34:557.
- Ingram, L. O. 1977. Changes in lipid composition of Escherichia coli resulting from growth with organic solvents and with food additives. Appl. Environ. Microbiol. 33:1233.
- Johnson, F. C. 1971. A critical review of the safety of phenolic antioxidants. CRC Crit. Rev. Food Technol. 2:267.
- Klindworth, K. J., Davidson, P. M., Brekke, C. J. and Branen, A. L. 1979. Inhibition of Clostridium perfringens by butylated hydroxyanisole. J. Food Sci. 44:564.
- Pierson, M.D., Smoot, L. A. and Vantassell, K. R. 1980. Inhibition of Salmonella typhimurium and Staphylococcus aureus by butylated hydroxyanisole and the propyl ester of p-hydroxybenzoic acid. J. Food Prot. 43:191.
- Porter, W. L. 1980. Recent trends in food applications of antioxidants. In "Autoxidation in Food and Biological Systems," eds. Simic, M. G. and Karel, M., Plenum Press, New York.
- Raccach, M. 1980. Low temperature lactic acid bacteria for meat fermentation. Proc. 26th European Meeting of Meat Research Workers 2:335.
- Raccach, M. and Henningsen, E. C. 1981. Antibacterial effect of tertiary butylhydroquinone against two genera of Gram positive cocci. J. Food Sci. 47:106.
- Ragnarsson, J. O. 1977. Accelerated temperature shelf life testing of antioxidants in dehydrated and intermediate moisture systems. Ph. D. thesis, Univ. of Minnesota, Minneapolis, MN.

- Reddy, N. R., Pierson, M. D. and Lechowich, R. V. 1982. Inhibition of Clostridium botulinum by antioxidants, phenols and related compounds. Appl. Environ. Microbiol. 43:835.
- Robach, M. C., Smoot, L. A. and Pierson, M. D. 1977. Inhibition of Vibrio parahaemolyticus 04:K11 by butylated hydroxyanisole. J. Food Prot. 40:549.
- Robach, M. C. and Pierson, M. D. 1979. Inhibition of Clostridium botulinum types A and B by phenolic antioxidants. J. Food Prot. 42:858.
- Robach, M. C. and Stateler, C. L. 1980. Inhibition of Staphylococcus aureus by potassium sorbate in combination with sodium chloride, tertiary butylhydroquinone, butylated hydroxyanisole or ethylenediamine tetraacetic acid. J. Food Prot. 43:208.
- Sands, J. G. and Bennett, E. O. 1966. Effect of different commercial agar preparations on the inhibitory activities of phenols. Appl. Microbiol. 14:196.
- Sankaran, R. 1979. Comparative antimicrobial action of certain antioxidants and preservatives. J. Food Sci. 13:203.
- Sgaragli, G., Della Corte, L., Rizzotti Conti, M. and Giotti, A. 1977. Effects of monocyclic compounds on biomembranes. Biochem. Pharmacol. 26:2145.
- Shamberger, R. J. 1980. Is peroxidation important in the cancer process. In "Autoxidation in Food and Biological Systems," eds. Simic, M. G. and Karel, M., Plenum Press, New York.
- Shelef, L. A. and Liang, P. 1982. Antibacterial effects of butylated hydroxyanisole (BHA) against Bacillus species. J. Food Sci. 47:769.
- Shih, A. L. and Harris, N. D. 1977. Antimicrobial activity of selected antioxidants. J. Food Prot. 40:520.
- Shoemaker, S. P. and Pierson, M. D. 1976. Phoenix phenomenon in the growth of Clostridium perfringens. Appl. Environ. Microbiol. 32:803.
- Singer, M. and Wan, J. 1977. Interaction of butylated hydroxytoluene (BHT) with phospholipid bilayer membranes: Effect on ²²Na permeability and membrane fluidity. Biochem. Pharmacol. 26:2259.

- Stern, N. J., Smoot, L. A. and Pierson, M. D. 1979. Inhibition of Staphylococcus aureus growth by combinations of butylated hydroxyanisole, sodium chloride and pH. J. Food Sci. 44:710.
- Surak, J. G., Bradley, R. L., Branen, A. L. and Shrago, E. 1976. Effects of butylated hydroxyanisole on Tetrahymena pyriformis. Food Cosmet. Toxicol. 14:277.
- Surak, J. G. 1977. Monotertiarybutylhydroquinone effects on Tetrahymena pyriformis. Life Sciences 20:1735.
- Vas, K. 1953. Mechanism of antimicrobial action. Interference with the cytoplasmic membrane. Agrokemia es Talajtan 2: 1 (In Chem. Abstracts (1954) 48:94d).
- von Schelhorn, M. 1953. Efficacy and specificity of chemical food preservatives. Food Technol. 7:97.
- Wanda, P., Cupp, J., Snipes, W., Keith, A., Rucinsky, T., Polish, L. and Sands, J. 1976. Inactivation of the enveloped bacteriophage $\phi 6$ by butylated hydroxytoluene and butylated hydroxyanisole. Antimicrob. Agents Chemother. 10:96.
- Ward, M. S. and Ward, B. Q. 1976. Initial evaluation of the effect of butylated hydroxytoluene upon Salmonella senftenberg 775 W. Poultry Sci. 46:1601.
- Woldringh, C. L. 1973. Effects of toluene and phenethyl alcohol on the ultrastructure of Escherichia coli. J. Bacteriol. 114: 1359.
- Wyss, O. 1948. Microbial inhibition by food preservatives. In "Advances in Food Research," vol. 1, pp. 373-393, eds. Mrak, E. M. and Stewart, G. F., Acad. Press, New York.

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