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Heat Stability of the Antimicrobial Activity of Selected Plant Extracts against *Aeromonas hydrophila*

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ABSTRACT - Antimicrobial stability of grape seed extract (ActiVin™), pine bark extract (Pycnogenol®), and oleoresin rosemary (Herbalox®) on the growth of *Aeromonas hydrophila* was investigated in cooked ground beef. When compared to the control, the populations of *A. hydrophila* were most effectively reduced by 4.06 log CFU/g for 1% Pycnogenol® added after cooking at 10 days of refrigerated storage, followed by 3.06 log CFU/g for 1% Pycnogenol® added before cooking and 1.36 log CFU/g for ActiVin™. Bacteriostatic and bactericidal activities were observed for Pycnogenol® added before and after cooking, respectively. Pycnogenol® consists of heat-labile and heat-stable compounds. ActiVin™ and Pycnogenol® could be considered for use as multifunctional preservatives in meat and meat products.

Key words: Grape seed, pine bark, rosemary, bacteriostatic activity, bactericidal activity

Introduction

In recent years, there has been rising concern on the incidence and toxigenicity of *Aeromonas hydrophila*. Since 1984, *A. hydrophila* has been classified as a "new" potential foodborne pathogen by the Food and Drug Administration (FDA)[1,2]. *Aeromonas hydrophila* is a gram-negative, psychrotrophic, aerobic, and facultative anaerobic organism which is ubiquitous in water, meats, seafood, and vegetables[2,3]. It can be an infectious and enterotoxigenic pathogen that grows at a broad range in temperature (1-40°C)[4]. It commonly contaminates meat and poultry products during refrigerated storage and causes gastroenteritis and produces cytotoxin, enterototoxin, aerolysin, and hemolysin[4,5].

Due to the risk of serious health problems, control of microbial contamination has always received a great attention in the meat industry. The shelf-life for ready-to-eat (RTE) meat products is relatively short and generally depends on the extent of microbial cross-contamination during post-processing. Naturally occurring preservatives are used to extend meat shelf-life and improve meat safety and quality. Natural extracts are known to have wide-ranging antimicrobial activities and listed as Generally Recognized As Safe (GRAS)[6]. Polyphenolic compounds are primarily responsible for antimicrobial activity of natural extracts, such as ginger, green tea, pepper, oregano, sage, thyme, grape seed extract, pine bark extract, and rosemary[7-12].

The antimicrobial effect of natural extracts has been well investigated in raw and cooked meat systems[5,8,12-14]. However, relatively fewer studies have been reported on the heat stability of the antimicrobial compounds of natural extracts in precooked meat. Cooking temperature can affect the antimicrobial activity of natural extracts against foodborne pathogens. Therefore, the objective of this research was to examine the antimicrobial stability of selected plant extracts, including grape seed extract (ActiVin™), pine bark extract (Pycnogenol®), and oleoresin rosemary (Herbalox®), against *A. hydrophila* as a function of storage time in cooked ground beef.

Materials and Methods

Plant extracts

The extracts of grape seed extract (ActiVin™), pine bark extract (Pycnogenol®), and oleoresin rosemary (Herbalox®) were kindly provided from Natural Health Science (Hillside, NJ, USA) and InterHealth (Benicia, CA, USA), and Kalsec Inc. (Kalamazoo, MI, USA), respectively.

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Bacterial culture

Aeromonas hydrophila ATCC 7965 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultivated at 35°C in trypticase soy broth with 0.1% yeast extract (TSB; Difco, Detroit, MI, USA) for 18 hr prior to use. The culture was harvested by centrifuging at 10,000 × g for 10 min at 5°C. The pellet was resuspended and serially diluted to approximately 10^6 CFU/mL in 0.1% sterile peptone water for inoculation into the cooked beef.

Experimental design

The experiment was conducted using a 2×3×5 factorial design with the natural extract-adding times, the treatments, and the storage times. The natural extracts, at a level of 1%, were added to meat samples before and after cooking. Treatments included control (no plant extract added), 1% ActiVin™, 1% Pycnogenol®, and 1% Herbalox®. After cooking all treatments, *A. hydrophila* was inoculated at approximately 10^5 CFU/g into cooked ground beef and stored at 5°C for 5 testing periods (0, 1, 3, 5, and 10 days). Microbial enumeration was conducted in duplicate for 3 replicates.

Sample preparation

The 80% lean ground beef used in this study was purchased from a local supermarket. ActiVin™, Pycnogenol®, or Herbalox® was added directly to 500 g portion of the ground beef. The meat mixtures were then thoroughly homogenized for 5 min at speed "2" using a Kitchen Aid mixer (St. Joseph, MI, USA). The mixtures were cooked in a convection oven until the internal temperature reached 75°C. After cooling to room temperature, *A. hydrophila* was inoculated at approximately 10^5 CFU/g into the samples treated with the natural extracts before and after cooking. The inoculated samples and controls were individually packaged in sterilized plastic bags and stored at 5°C for 10 days.

Microbiological analysis

Viable counts were directly determined by the spread plate method. Samples were aseptically mixed with 0.1% sterile peptone water. The mixtures were blended for 2 min (Stomacher Model 400, Tekmar Co., Cincinnati, OH, USA). Mixtures were serially (1:10) diluted with 0.1% sterile peptone water. The sample dilutions (0.1 mL) were plated on the surface of starch-ampicillin (SA) agar consisting red agar base (Difco), soluble starch, and ampicillin[15], and the plates were incubated at 35°C for 48 hr.

Inhibitory kinetics

The kinetic parameters for the bacterial growth were analyzed using the modified Gompertz model[16];

$$G(t) = A + C \cdot \exp[-\exp(-B \cdot (t - M))] \qquad (1)$$

where *G*, *A*, *B*, *C*, and *M* are respectively the viable counts (CFU/g) at time t, inoculum level (CFU/g), the growth rate at the inflection point (1/day), the number of log cycles of microbial growth (CFU/g), and the time required to reach the maximum growth rate (day). The lag phase duration (LPD = $M - 1/B$) and maximum growth rate (GR = $B \cdot C/e$) were calculated.

The inhibitory activity of the natural extract on the growth of *A. hydrophila* was estimated using the Inhibition Index (II)[17];

$$II = 1 - \frac{\log\left(\frac{N_t}{N_0}\right)_{treated}}{\log\left(\frac{N_t}{N_0}\right)_{control}} \qquad (2)$$

where N_0 and N_t are the inoculum level (CFU/g) and the number of microorganism at time t (CFU/g), respectively.

Statistical analysis

The microbial growth curves were analyzed using Nonlinear Curve Fitting Function of Microcal Origin® 7.5 (Microcal Software Inc., Northampton, MA). Data were analyzed using the Statistical Analysis System software (SAS). The General Linear Model (GLM) and least significant difference (LSD) procedures were used to compare means. Significant mean differences were calculated by Fisher's Least Significant Difference (LSD) at $p<0.05$.

Results and Discussion

Antimicrobial effect of plant extracts against *A. hydrophila* on cooked beef

The numbers of *A. hydrophila* for the control, ActiVin™, and Pycnogenol® significantly increased with increasing storage time as shown in Table 1. Compared to the control, the addition of Herbalox® had no inhibitory effect on the growth of *A. hydrophila* throughout 10 days of refrigerated storage. Similar to the control, the numbers of *A. hydrophila* for the Herbalox® added before and after cooking increased from 4.28 to 7.68 log CFU/g and from 4.23 to 7.16 log CFU/g, respectively. Pycnogenol® added after cooking significantly reduced *A. hydrophila* number by 0.78 log CFU/g after 10 days when compared to the inoculum level (4.15 log CFU/g).

Bacteriostatic and bactericidal effects on the growth of *A. hydrophila* were observed for Pycnogenol® added before and after cooking, respectively. Compared to the control, the numbers of *A. hydrophila* were significantly reduced by 1.37, 1.38, 3.06, and 4.06 log CFU/g at 10 days, respectively, by ActiVin™ added before cooking, ActiVin™ added after cooking, Pycnogenol® added before cooking, and Pycnogenol® added after cooking. Pycnogenol® added after cooking showed greater antimicrobial activity than that added before cooking, whereas no significant change in the antimicrobial activity was observed between adding ActiVin™ or Herbalox® before and after cooking. The results suggest that some active antimicrobial compounds can become labile during the cooking of the sample treated with Pycnogenol®.

Changes in pH values in cooked ground treated with plant extracts

No significant changes in pH for all treatments were observed throughout the refrigerated storage (Table 2). The pH values were maintained in cooked ground beef treated with ActiVin™ (pH 5.96-6.10), Pycnogenol® (pH 5.82-5.94), and Herbalox® (pH 6.04-6.16) throughout the refrigerated storage period. However, the pH values of cooked beef treated with Pycnogenol® were significantly lower than those for the other treatments. The result is in agreement with a previous report in which the pH values of ActiVin™, Pycnogenol®, and Herbalox® suspended in water were 4.1, 3.1, and 4.7, respectively[8)]. The initial pH decline for Pycnogenol® added before and after cooking may result from acidic compounds such as gallic acid, vanillic acid, caffeic acid, and ferulic acid[18)].

Inhibitory kinetics of plant extracts against *A. hydrophila* on cooked beef

Growth rates and lag times of *A. hydrophila* were estimated from the modified Gompertz model (Eq. 1) as shown in Table 3. Pycnogenol® most effectively retarded the growth of *A. hydrophila* in cooked ground beef throughout the refrigerated storage. The growth rate of *A. hydrophila* was greater for the ActiVin™ treatment than the control and Herbalox® treatments. This result might be caused by the recovery of injured cells at the late period of refrigerated storage[19,20)]. ActiVin™ treatment showed a longer lag phase duration (LPD) than the Herbolox® treatment, indicating ActiVin™ is more effective in retarding the growth of *A. hydrophila* in cooked ground beef. Inhibition Index (*II*) was used to determine the antimicrobial stability of the natural extracts against *A. hydrophila* during cooking (Eq. 2). The inhibitory index denotes bactericidal activity (*II*>1), bacteriostatic activity (*II*=1), preservative activity (1>*II*>0), or no antimicrobial activity (*II*0)[17)]. Inhibition index values after 10 days of refrigerated storage were shown in Fig. 1. ActiVin™ and Herbalox® produced a preservative action, which is indicated by inhibition index values between 0 and 1. Pycnogenol® added after cooking exerted a bactericidal effect against *A. hydrophila* in cooked ground beef (*II*>1). The antimicrobial activity was the highest for Pycnogenol® added after cooking (*II*=1.24), followed by Pycnogenol® added before cooking (*II*=1.00) and ActiVin™ added before cooking (*II*=0.45). The fact that no significant differences in antimicrobial activity of ActiVin™ and Herbalox® were observed suggests that antimicrobial compounds in these extracts are thermally stable. This is in agreement with the results from Hongpattarakere and Johnson[21)], who reported that natural extract components, such as phenoldienones, epicatechin, epigallocatechin, epigallocatechin gallate, kaempferol, quercetin, and myricetin, are very heat stable. However, the antimicrobial activity of Pycnogenol® against *A. hydrophila* decreased after cooking, suggesting that

Table 1. Effects of 1% plant extracts against *A. hydrophila* on cooked beef inoculated with 10^5 CFU/g during refrigerated storage at 5°C

Treatment		Storage time (day)				
		0	1	3	5	10
	Control	^{z}[3)]4.25_{a}[4)]	$^{z}4.32_{a}$	$^{y}4.71_{a}$	$^{x}5.48_{a}$	$^{w}7.43_{ab}$
Before[1)]	ActiVin™	$^{y}4.31_{a}$	$^{y}4.22_{a}$	$^{y}4.34_{bc}$	$^{x}5.42_{a}$	$^{w}6.06_{c}$
	Pycnogenol®	$^{w}4.27_{a}$	$^{w}4.24_{a}$	$^{w}4.10_{cd}$	$^{x}3.78_{c}$	$^{w}4.28_{d}$
	Herbalox®	$^{z}4.28_{a}$	$^{z}4.35_{a}$	$^{y}4.51_{ab}$	$^{x}5.21_{ab}$	$^{w}7.68_{a}$
After[2)]	ActiVin™	$^{x}4.12_{a}$	$^{x}4.07_{a}$	$^{x}3.96_{d}$	$^{x}4.07_{c}$	$^{w}6.05_{c}$
	Pycnogenol®	$^{w}4.15_{a}$	$^{w}4.04_{a}$	$^{wx}3.83_{d}$	$^{x}3.41_{d}$	$^{x}3.37_{e}$
	Herbalox®	$^{z}4.23_{a}$	$^{z}4.32_{a}$	$^{y}4.57_{ab}$	$^{x}5.01_{b}$	$^{w}7.16_{b}$
	LSD[5)]	0.337	0.357	0.324	0.372	0.442

[1)] Treatment added before cooking.
[2)] Treatment added after cooking.
[3)] Means with different superscript letters within a row are significantly different at $p<0.05$.
[4)] Means with different subscript letters within a column are significantly different at $p<0.05$.
[5)] Least significant difference.

Table 2. pH changes of the cooked ground beef treated with 1% plant extracts during refrigerated storage at 5°C

Treatment		Storage time (day)				
		0	1	3	5	10
	Control	6.07_{a}[3)]	6.09_{a}	6.08_{a}	6.09_{a}	6.12_{b}
Before[1)]	ActiVin™	5.96_{c}	6.04_{b}	6.05_{b}	6.07_{a}	6.10_{bc}
	Pycnogenol®	5.82_{d}	5.87_{c}	5.90_{d}	5.89_{c}	5.94_{d}
	Herbalox®	6.06_{a}	6.08_{a}	6.06_{b}	6.08_{a}	6.12_{b}
After[2)]	ActiVin™	6.01_{b}	6.04_{b}	6.03_{c}	6.03_{b}	6.08_{c}
	Pycnogenol®	5.83_{d}	5.88_{c}	5.86_{e}	5.88_{c}	5.92_{d}
	Herbalox®	6.04_{ab}	6.08_{a}	6.07_{ab}	6.09_{a}	6.16_{a}
	LSD[4)]	0.044	0.018	0.047	0.048	0.034

[1)] Treatment added before cooking
[2)] Treatment added after cooking
[3)] Means with different subscript letters within a column are significantly different at $p<0.05$.
[4)] Least significant difference

Table 3. Growth rate (GR) and lag phase duration (LPD) of *Aeromonas hydrophila* in cooked ground beef treated with 1% plant extracts during refrigerated storage at 5°C

Treatment		GR [log (CFU/g)/day][3]	LPD [day][4]
	Control	$0.51 \pm 0.11_c$ [5]	$2.47 \pm 0.36_d$
Before[1]	ActiVin™	$1.99 \pm 0.47_a$	$4.40 \pm 0.16_b$
	Pycnogenol®	BS[6]	BS
	Herbalox®	$0.55 \pm 0.06_c$	$3.42 \pm 0.53_c$
After[2]	ActiVin™	$1.24 \pm 0.28_b$	$8.32 \pm 0.22_a$
	Pycnogenol®	BC[7]	BC
	Herbalox®	$0.50 \pm 0.08_c$	$3.79 \pm 0.32_{bc}$

[1] Treatment added before cooking
[2] Treatment added after cooking
[3] $GR = B \cdot C/e$
[4] $LPD = M - 1/B$
[5] Means with different subscript letters within a column are significantly different at $p<0.05$.
[6] BS indicates bacteriostatic phase.
[7] BC indicates bactericidal phase.

Pycnogenol® consists both of heat-labile and heat-stable compounds.

Natural extracts still contain many unknown compounds. Therefore, since these specific natural extracts could potentially have commercial use as new antimicrobials applicable to a wide range of meat products, it is necessary to further identify bioactive compounds in them. These results provide not only an interesting insight into the heat stability of natural extracts but also a potential direction for their use.

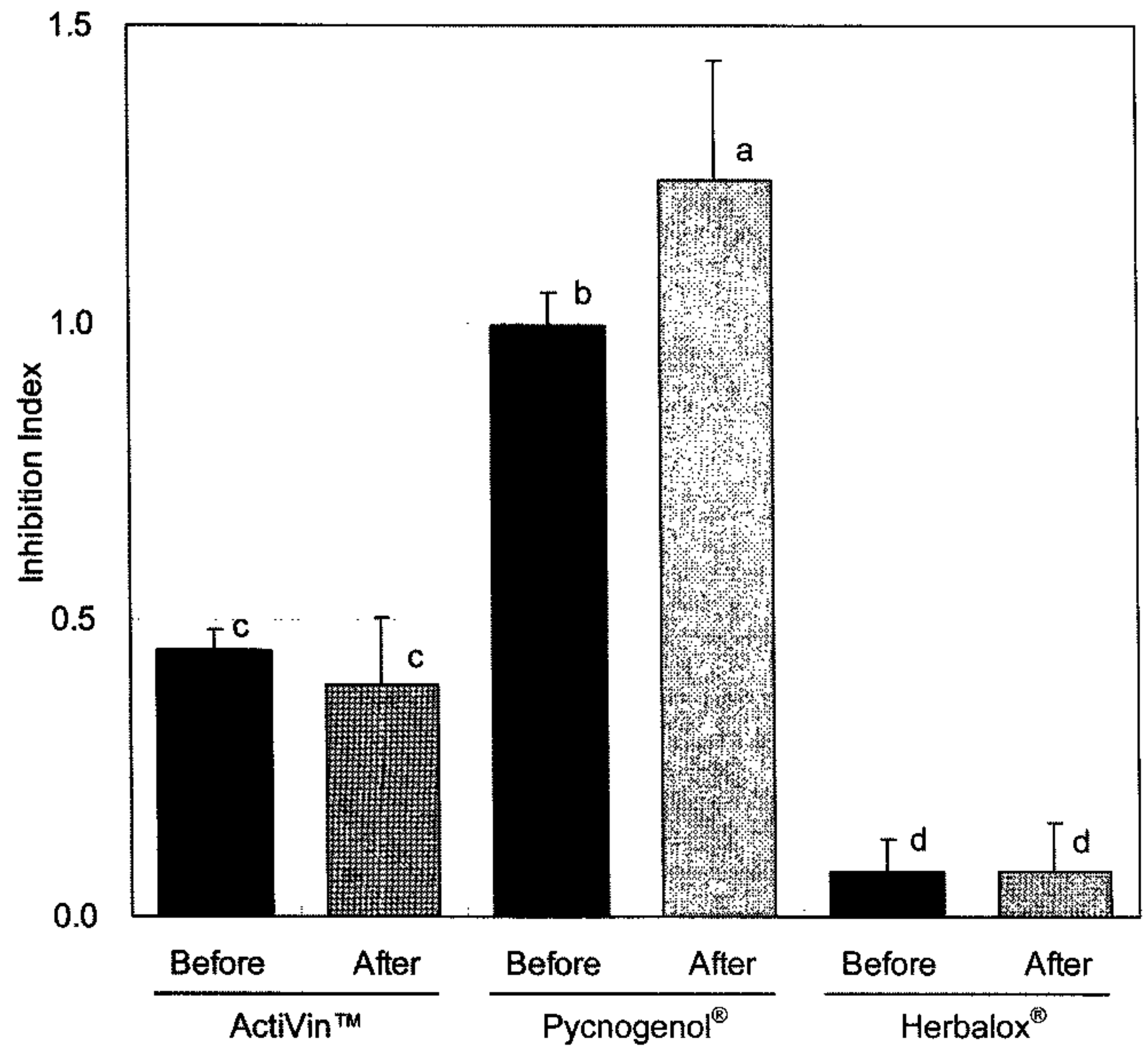

Fig. 1. Inhibitory indices of 1% plant extracts against *Aeromonas hydrophila* in cooked ground beef during refrigerated storage at 5°C.

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