



Sensitivity of *Escherichia coli* to Seaweed (*Ascophyllum nodosum*) Phlorotannins and Terrestrial Tannins

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ABSTRACT : Pure culture experiments were conducted to assess the bacteriostatic and bactericidal effects of phlorotannins (PT) isolated from *Ascophyllum nodosum* (brown seaweed) on *Escherichia coli* O157:H7. In Exp. 1, one non-O157:H7 strain (25922) and three strains of *E. coli* O157:H7 (3081, EDL933 and E318N) were cultured in M9 medium with PT included at 0 (control), 25, 50 or 100 µg/ml ($n = 3$). Bacterial growth was monitored by OD₆₀₀ at 0, 4, 6, 12 and 24 h, and by dilution plating at 0, 4, 6 and 24 h. All strains were inhibited ($p < 0.001$) by PT to varying degrees. At 50 or 100 µg/ml, PT prevented growth of all four strains. At 25 µg PT/ml, growth of 25922, 3081, E318N and EDL933 was inhibited for 6, 12 and 24 h, respectively, but 25922 and 3081 resumed growth by 12 and 24 h. Direct plating confirmed bactericidal effects of PT on all four strains at 100 µg/ml, and on EDL933 and E318N at 50 µg/ml. In Exp. 2, strains 25922 and 3081 were incubated with no tannins or with 50 µg/ml of PT, purified condensed tannins (CT) from Quebracho (*Schinopsis balansae*), or purified tannic acid from *Rhus semialata* (Anacardiaceae) as hydrolysable tannins (HT). Strain 3081 was unaffected by HT or CT, but was completely inhibited ($p < 0.001$) by PT at 4, 6 and 24 h. Strain 25922 was unaffected by HT, slightly inhibited by CT, and almost eradicated by PT at 4 and 6 h. Transmission electron microscopy revealed tannin-mediated alterations to bacterial cell walls. Phlorotannins from *A. nodosum* exhibit growth-inhibiting and bactericidal effects *in vitro* against the strains of *E. coli* O157:H7 investigated. Anti-*E. coli* efficacy of *A. nodosum* PT is superior to that of terrestrial tannins purified from Quebracho and from *Rhus semialata*. (**Key Words :** *Ascophyllum nodosum*, *E. coli* O157:H7, Phlorotannin, Pure Culture, Growth, Cattle)

INTRODUCTION

Food-borne illnesses caused by enterohaemorrhagic *Escherichia coli*, in particular serotype O157:H7 (Wells et al., 1995; Paton and Paton, 1998; Bower, 1999), are a significant health concern in North America, with associated health care costs estimated at over USD 200 million per year. Clinical symptoms caused by this pathogen include diarrhea (Pai et al., 1988), hemorrhagic colitis (Riley, 1987) and occasionally hemolytic-uremic syndrome and thrombocytopenic purpura (Griffin and Tauxe, 1991; Karmali, 1992). Ruminants are the major reservoir of *E. coli* O157:H7 and their fecal shedding of *E. coli* O157:H7 is affected by various factors such as animal health conditions, diet, and season (Stanford et al., 2005; Kobayashi and El-Sawy, 2007). A positive correlation

between fecal and hide prevalence of *E. coli* O157:H7 and adulteration of meat has been reported (Wells et al., 1991; Hancock et al., 1994; Elder et al., 2000). Therefore, methods that focus on reducing *E. coli* O157:H7 populations in ruminants prior to their processing may reduce human illnesses. At present, however, there is no effective method to control the prevalence of *E. coli* O157:H7 in beef cattle (Bach et al., 2002; LeJeune and Wetzel, 2007).

Recent research conducted with seaweeds has shown that brown seaweed (*Ascophyllum nodosum*) reduces the prevalence and shedding of *E. coli* O157:H7 in cattle before harvest (Barham et al., 2001; Braden et al., 2004; Bach et al., 2008). An extract from *A. nodosum* was also shown to reduce the toxicity of endophyte-infected tall fescue, to enhance immune function and to prolong the shelf-life of finished beef (Allen et al., 2001; Fike et al., 2001; Saker et al., 2001). However, the mechanism by which *A. nodosum* may suppress fecal shedding of *E. coli* O157:H7 is not known. Several compounds in marine algae possess biological activity (Mayer and Hamann, 2005). One unique

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group of polyphenols present in brown algae is phlorotannins (PT), which are polymers composed exclusively of phloroglucinol (Ragan and Glombitza, 1986; Arnold and Targett, 1998). Phlorotannins have been reported to have anti-plasmin (Nakayama et al., 1989; Fukuyama et al., 1990), antioxidant (Haug and Larsen, 1958; Nakamura et al., 1996) and anti-bacterial (Targett and Arnold 1998; Horikawa et al., 1999; Nagayama et al., 2002) activities. Wang et al. (2006, 2008) observed that PT from *A. nodosum* exhibited antimicrobial activity against various rumen microbes and affected ruminal fermentation. However, there is no information on specific interactions between PT from *A. nodosum* and *E. coli* O157:H7. The objective of this study was to assess the bacteriostatic and bactericidal effects of PT isolated from *A. nodosum* on *E. coli* O157:H7. The anti-*E. coli* O157:H7 activity of this marine source of PT was also compared to terrestrial tannins from two sources.

MATERIALS AND METHODS

Isolation of phlorotannins

Ascophyllum nodosum collected from the Atlantic coastline of Nova Scotia, Canada was obtained from Acadian Seaplants Limited, Dartmouth, NS. The freeze-dried whole-plant material was ground through a 500- μ m screen and 100 g were mixed with 2,500 ml of aqueous methanol (80 ml/100 ml). After 2 h of stirring at room temperature (22°C), the mixture was filtered through Waterman #1 filter paper. Methanol in the filtrate was evaporated at 40°C, then the remaining aqueous fraction was centrifuged (20 min; 5,000 g; 4°C) and the supernatant was freeze-dried. The freeze-dried powder was used to isolate phlorotannins using a procedure in isolation of tannins similar to that described by Asquith and Butler (1985). Briefly, the freeze-dried material was re-suspended into 80% ethanol (v/v) and added to a Sephadex LH20 column. The column was washed with 95% (v/v) ethanol at the rate of 1.5 ml/min to remove non-tannin materials (absorbance at 280 nm approaches zero) and PT were eluted from the column with 50% (v/v) aqueous acetone. Acetone in the eluate was evaporated at 30°C and the aqueous solution was freeze-dried. This produced a brown powder (the phlorotannins, PT) that was stored in an opaque vial in the dark at -20°C. The PT powder was dissolved in dH₂O and filter-sterilized (0.2 μ m) immediately before use.

Commercially available materials were used for isolation of hydrolysable tannin (HT) and condensed tannin (CT). The HT and CT were isolated and purified from *Rhus semialata* (Anacardiaceae) tannic acid (Sigma Chemical Co., St. Louis, MO, USA) and from Quebracho (*Schinopsis balansaei*) tannin (Unitán, Buenos Aires, Argentina), respectively, using the same as procedure as outlined above.

Bacterial strains and growth conditions for pre-incubation

A non-O157:H7 strain of *E. coli* (25922; ATCC isolate) and three strains of *E. coli* O157:H7: strain 3081 kanR, ampR (i.e., resistant to 100 μ g/ml kanamycin and 100 μ g/ml ampicillin; bovine isolate), strain E318N nalR (resistant to 50 μ g/ml nalidixic acid; bovine isolate, PT14), and strain EDL933 (ATCC isolate) were obtained from the Lethbridge Research Centre culture collection. Strain 3081 was kindly made available by W. C. Cray (National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA, USA), strain E318N by A. Boreczyk (Enteric Reference Laboratory, Ministry of Health, Toronto, ON, Canada) and strain EDL933 by V. P. J. Gannon (Laboratory Centre for Enteric and Zoonotic Diseases, Canadian Food Inspection Agency, Lethbridge, AB, Canada). The strains were maintained separately in tryptic soy broth, supplemented with nalidixic acid at 50 μ g/ml (for E318N) or with kanamycin and ampicillin each at 100 μ g/ml (for 3081). The bacteria were grown at 37°C with shaking (175 rpm) for 16 h prior to being used as inoculants.

Experiment 1

The *in vitro* pure culture experiment was conducted in 150-ml serum vials with M9 medium (M9 Minimal Salts, Sigma Chemical Co.) supplemented with (g/L) casamino acids (5.0), MgSO₄·7H₂O (0.239), glucose (3.0) and CaCl₂ (0.011). Tannins isolated as described above were added to the medium after the bacterial inoculation. Prepared M9 medium (99 ml) was transferred into 150-ml serum vials and sterilized by autoclaving. After cooling, 100 μ l of pre-incubated bacterial culture were added to each vial. The bacterial cultures had been adjusted to identical densities by spectrophotometry (600 nm) across the four strains prior to the inoculation. Immediately after inoculation, 1.0 ml of filter-sterilized solutions of PT in M9 medium were added, which had been prepared to yield final PT concentrations of 0 (CON), 25 (PT25), 50 (PT50) or 100 (PT100) μ g/ml in the inoculated cultures. Triplicate vials were prepared for each bacterial strain and PT concentration. Triplicate vials for each PT concentration, but with no bacterial inoculum, were also prepared as blank controls. All vials were incubated aerobically with shaking (175 rpm) at 37°C. Sub-samples (3.0 ml) from each vial taken after 0, 4, 6, 12 and 24 h of incubation were assessed for bacterial growth as optical density of the whole culture at 600 nm (OD₆₀₀) using an UltraSpec Plus 4054 spectrophotometer (Pharmacia, Baie d'Urfé, QC, Canada). Optical densities were corrected for the blank controls. Culture samples from each vial were also taken at 0, 4, 6 and 24 h of the incubation and enumerated by dilution plating as described by Bach et al. (2008). Bacterial numbers estimated from dilution plating were expressed as colony forming units (CFU) per ml.

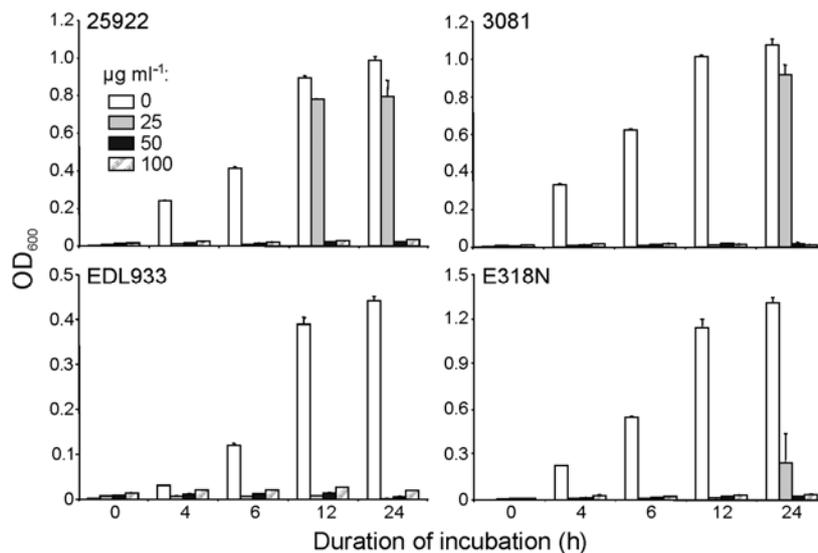


Figure 1. Effects of phlorotannins isolated from the brown seaweed *Ascophyllum nodosum* on growth (OD₆₀₀) of the non-O157:H7 *Escherichia coli* strain 25922 and of *E. coli* O157:H7 strains 3081, EDL933 and E318N. Bars indicate standard error ($n = 3$).

Experiment 2

Strains 25922 and 3081 were incubated in M9 medium with no added tannins (control) or with 50 µg/ml of PT, CT or HT. Procedures for bacterial culture, inoculation and addition of tannins into the culture were the same as described in Exp. 1. Triplicate vials for each tannin type and controls, with and without bacterial inoculation, were prepared. After 0, 4, 6 and 24 h of incubation, vials were sampled and bacteria enumerated as described above. In addition, bacterial cells from 24-h cultures were processed and examined by transmission electron microscopy as described by Bae et al. (1993). Samples were viewed using an H-500 transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

Microbial counts were log₁₀ transformed prior to statistical analysis. All data were subjected to analysis of variance using the MIXED procedure of SAS (SAS, 1999). Data from Exp. 1 were originally analyzed as a 4×4 factorial design with individual vial as a random factor. This revealed a bacterial strain×PT level interaction over the incubation period, therefore the data were re-analyzed as a randomized complete block design for each bacterial strain. Data from Exp. 2 were analyzed as a randomized complete block design with individual vial as a random factor. Time-course data were analyzed using a repeated measures model that included time and treatment×time interactions. When either effect (time or time×treatment interaction) was significant, treatment means were compared at each time point. The significance of differences among treatments was tested using LSMEANS with the PDIF option.

RESULTS

Experiment 1

Significant effects ($p < 0.001$) of treatment, incubation time and treatment×time interaction were evident in OD₆₀₀ readings over the 24-h incubation period for strains 25922, 3081 and E318N, but not for strain EDL933, which exhibited a low OD₆₀₀ reading at all concentrations of PT (Figure 1). The OD₆₀₀ values were similar ($p > 0.05$) among strains at 0 h of the incubation. Strains EDL933 and E318N were inhibited ($p < 0.001$) by all concentrations of PT over 24 h of incubation. Including PT at 25, 50 or 100 µg/ml reduced ($p < 0.001$) bacterial growth of strains 25922 and 3081 at 4, 6 and 12 h of the incubation. At 24 h, however, bacterial growth of 25922 and 3081 were reduced ($p < 0.001$) only by supplementation of PT at 50 or 100 µg/ml; in the PT25 vials, growth of these two strains had recovered to levels similar ($p > 0.05$) to the controls (CON).

Similar to the OD₆₀₀ readings, cell populations of each strain, as measured by dilution plating, were affected ($p < 0.001$) by treatment, incubation time and treatment×time interaction. At all the levels tested, phlorotannins markedly reduced ($p < 0.001$) cell counts of all four strains both at 4 h and at 6 h of incubation (Figure 2). At 24 h, cell populations of all four strains were lower ($p < 0.001$) in PT50 than in CON, and no bacteria were culturable with PT100. In contrast, supplementing PT at 25 µg/ml did not affect strain 25922, tended to reduce ($p = 0.05$) cell populations of strain 3081, and reduced numbers of strains E318N and EDL933 ($p < 0.05$ and $p < 0.001$, respectively), at 24 h of incubation.

Experiment 2

Treatment, incubation time, and treatment×time

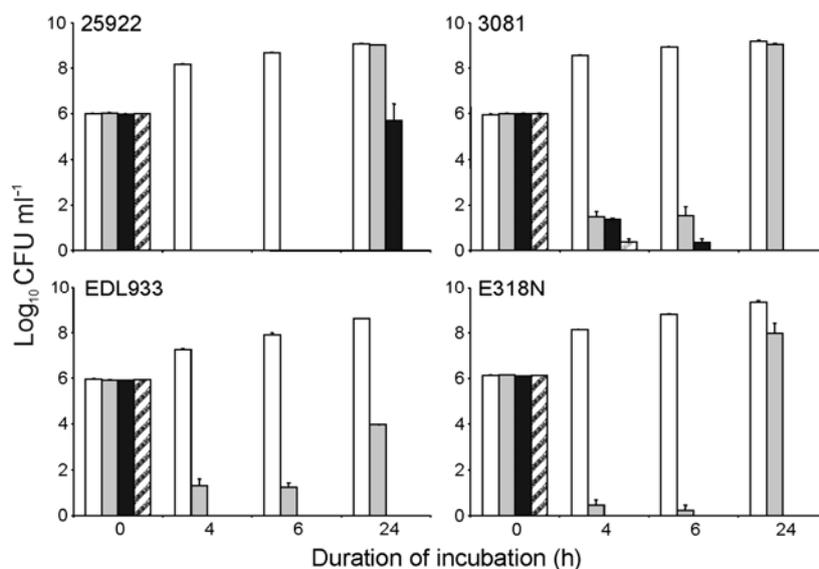


Figure 2. Effects of phlorotannins isolated from the brown seaweed *Ascophyllum nodosum* on cell populations (\log_{10} CFU/ml) in cultures of the non-O157:H7 *Escherichia coli* strain 25922 and of *E. coli* O157:H7 strains 3081, EDL933 and E318N. Phlorotannins were included at 0 (control), 25 (■), 50 (■) or 100 (▨) $\mu\text{g/ml}$. Bars = standard error ($n = 3$).

interactive effects ($p < 0.001$) on growth of *E. coli* 25922 and of *E. coli* 3081 were observed during the 24-h incubation (Table 1). Inhibition of both strains of *E. coli* was much more pronounced ($p < 0.001$) with PT at 50 $\mu\text{g/ml}$, than with CT or HT at the same concentration. As was observed with PT50 in Exp. 1, growth of strain 25922 had resumed to some extent after 24 h of incubation, but *E. coli* O157:H7 strain 3081 did not grow at this concentration of PT. Including CT in the incubation reduced ($p < 0.05$) growth of strain 25922 at 4 h and at 6 h, but not at 24 h, and CT had no effect on growth of strain 3081 over 24 h. Hydrolysable tannins did not affect cell densities of either *E. coli* strain.

Examination by transmission electron microscopy revealed that tannins acted primarily on the bacterial cell wall (Figure 3). A smooth, continuous membrane structure

was clearly observed in the control (untreated) bacteria, whereas the cell walls of those cells exposed to PT or HT were relatively disorganized structures with electron-dense precipitated deposits on the surface. The patterns of cell wall alterations by PT and HT were visually similar, but the alteration associated with PT was more pronounced than that induced by HT. In contrast, the cell wall of the bacteria incubated with CT in the medium became visually thicker than was observed in the control.

DISCUSSION

This study demonstrated that PT isolated from brown seaweed (*A. nodosum*) is bacteriostatic at concentrations of 25 $\mu\text{g/ml}$ or higher and at ≥ 50 $\mu\text{g/ml}$ is bactericidal to the

Table 1. Effects of condensed tannins (CT), hydrolysable tannins (HT) and phlorotannins (PT) on growth of non-O157:H7 (25922) and O157:H7 (3081) strains of *Escherichia coli**

Incubation	Treatment ¹				SEM	p values ²
	Control	CT	HT	PT		
<i>E. coli</i> (strain 25922)						
0 h	5.91	5.86	5.90	5.89	0.019	NS
4 h	7.91 ^c	7.06 ^b	7.76 ^c	0.23 ^a	0.119	<0.001
6 h	8.54 ^c	6.95 ^b	8.37 ^c	0.33 ^a	0.174	<0.001
24 h	8.86 ^b	9.04 ^b	8.65 ^b	4.38 ^a	0.544	<0.001
<i>E. coli</i> O157:H7 (strain 3081)						
0 h	5.68	5.58	5.02	5.88	0.279	NS
4 h	7.99 ^b	7.76 ^b	7.88 ^b	0.39 ^a	0.365	<0.001
6 h	8.46 ^b	8.27 ^b	7.76 ^b	0 ^a	0.359	<0.001
24 h	8.77 ^b	8.72 ^b	8.73 ^b	0 ^a	0.249	<0.001

^{a-c} Within a row, means followed by different letters differ ($p < 0.05$). * Expressed as \log_{10} CFU/ml. Cultures were grown in M9 medium at 37°C.

¹ Tannins (CT, HT or PT) were included at 0 (control) or 50 $\mu\text{g/ml}$.

² Values shown are effect of treatment at each time point. In addition, for each of the two *E. coli* strains, the main effects of treatment and of time, and the interactive effect of treatment \times time were significant ($p < 0.001$).

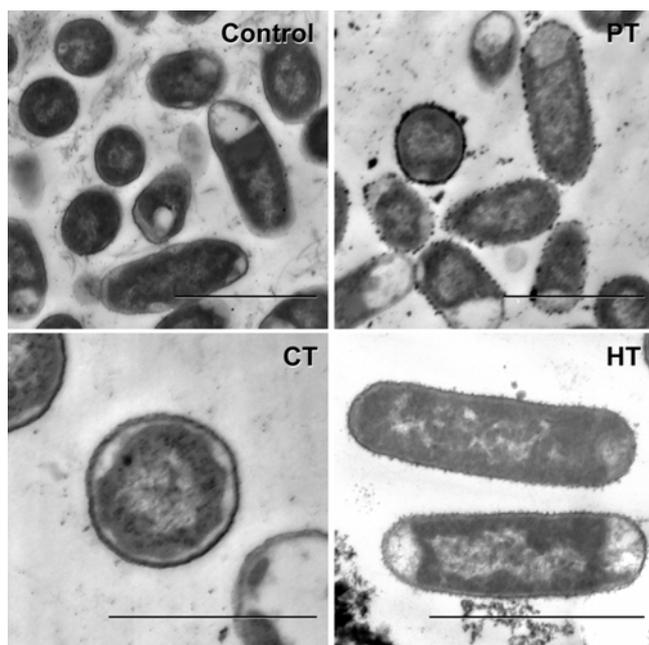


Figure 3. Transmission electron micrographs of *Escherichia coli* O157:H7 strain 3081 incubated for 24 h with no tannins (Control) or with 50 µg/ml of phlorotannins (PT) from *Ascophyllum nodosum*, condensed tannins (from Quebracho, *Schinopsis balansaei*; CT) or hydrolysable tannins (tannic acid, HT). Bars in Control, PT and HT = 0.5 µm; in CT = 0.25 µm.

strains of *E. coli* O157:H7 investigated.

Phlorotannins from brown seaweed have multiple roles in marine ecology, including antimicrobial activity (Targett and Arnold, 1998). Anti-*E. coli* activities of PT from other seaweeds (e.g., *Ecklonia kurome*) have also been reported (Nagayama et al., 2002). However, the mechanism by which the PT inhibits growth of *E. coli* specifically has not yet been defined. The inhibitory effects of terrestrial tannins from different sources on enteric microorganisms have been demonstrated (Chung et al., 1998; Smith and Mackie, 2003; Min et al., 2007). The antibacterial activities of terrestrial tannins are thought to rely on several mechanisms, including inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth, or direct inhibition of oxidative phosphorylation (Scalbert, 1991). Both PT and terrestrial tannins have been shown to form complexes with proteins and with carbohydrates (Ragan and Glombitza, 1986; Haslam, 1989). Given the similarity of terrestrial tannins and PT in their roles in plant evolution, it is likely that the mechanisms of antimicrobial activity are shared between terrestrial and aquatic sources of tannins.

In the present study, the anti-*E. coli* activity of PT isolated from *A. nodosum* was greater than that of CT from Quebracho or HT from *R. semialata*. At a concentration of 50 µg/ml, neither CT nor HT affected growth of strain 25922 or 3081, whereas PT inhibited the growth of strain

25922 and killed strain 3081. Differences in the anti-*E. coli* O157:H7 activities of these differently-sourced tannins may be related to differences in chemical structure. In a study with CT isolated from black wattle (*Acacia mearnsii*), Smith et al. (2003) identified oxidation of tannins and the liberation of hydrogen peroxide as the factor responsible for the antimicrobial properties of these phenolics. It has been demonstrated that polyphenolics from different sources produce different amounts of hydrogen peroxide under aerobic conditions, with the highest production associated with phenolics that are rich in hydroxyl groups (Akagawa et al., 2003). Additionally, the affinity of tannins for protein may also increase with the number of hydroxyl groups available to form hydrogen bonds with proteins (Mueller-Harvey, 2006). Phlorotannins are readily oxidized upon exposure to air (Ragan and Glombitza, 1986) and contain more hydroxyl groups than either HT or CT, a factor that may account for their greater anti-bacterial activity. Other researchers have also observed that the bactericidal activity of terrestrial tannins is related to the number of hydroxyl groups (Smith et al., 2003; Min et al., 2007). Additionally, the greater anti-bacterial properties of aquatic PT appear to be related to the degree of polymerization of phloroglucinol (Nagayama et al., 2002), a property that is not present in terrestrial tannins. We have determined in a related study that rumen bacteria are also more sensitive to PT than to terrestrial tannins (Wang et al., 2006).

This experiment assessed inhibitory effects of PT on one generic strain of *E. coli* and three strains of *E. coli* O157:H7. In particular, the experiment used two antibiotic-resistant strains (3081, resistant to 100 µg/ml kanamycin, and 100 µg/ml ampicillin, and E318N, resistant to 50 µg/ml nalidixic acid). The inhibitory effects of PT on these *E. coli* strains were found to be dose-dependent, irrespective of their resistance phenotypes. Growth of all strains of *E. coli* O157:H7 examined in the present study was inhibited by 25 µg/ml of PT for up to 6 h of incubation. Phlorotannins caused a lag in growth for up to 12 h. Growth was evident after 12 h with strain 25922, and after 24 h with strains 25922, 3081 and E318N. All three *E. coli* O157:H7 strains used in this study were unculturable after 24 h of exposure to PT at concentrations of ≥ 50 µg/ml. Smith et al. (2003) reported that wattle CT at 1,000 µg/ml were bacteriostatic to *E. coli* BW13711, and bactericidal at 2,000 µg/ml. In contrast, Min et al. (2007) reported that chestnut tannins and mimosa tannins were mainly bacteriostatic to several strains of *E. coli* O157:H7, even at concentrations up to 1,200 µg/ml. We have also observed that CT isolated from sainfoin and quebracho are bacteriostatic, as opposed to bactericidal to *E. coli* O157:H7, at concentrations up to 1,500 µg/ml (Wang et al., unpublished data).

The present work suggests that PT may inhibit bacterial growth by altering the cell membrane, an observation

consistent with the effects of condensed and hydrolysable terrestrial tannins (Ikigai et al., 1993; Jones et al., 1994; Hoshino et al., 1999; O'Donovan and Brooker, 2001). Chae et al. (2006) observed that egg yolk antibodies against *E. coli* O157:H7 whole cell had higher activity than those against outer membrane proteins or lipopolysaccharide. Transmission electron micrographs illustrated that the extent to which PT interfere with cellular membrane functions may be even greater than that of HT or CT.

This study showed that strains of *E. coli* differed in their degree of sensitivity to PT, which is consistent with other reports of differential sensitivities of *E. coli* strains to tannins (Yam et al., 1997; Min et al., 2007). *Escherichia coli* cells possess antioxidant enzymes that are induced in response to oxidative stress. Smith et al. (2003) pointed out that the oxidative stress response is necessary for *E. coli* to overcome the inhibitory effect of condensed tannins in the medium and that variation exists among *E. coli* strains in their response to oxidative stress. At higher concentrations of PT, i.e., 50 µg/ml, except for strain 25922 (100 µg/ml), no viable cells of the strains studied were recovered after 24 h. Similarly, Nagayama et al. (2002) showed that PT isolated from *E. kurume* killed all *Vibrio parahaemolyticus* within 0.5 to 2 h of incubation when these phenolics were administered at twice the minimum bactericidal concentration. Taken together, these findings suggest that the susceptibility of *E. coli* O157:H7 to low levels of PT in the medium is likely strain-associated, whereas all strains are killed by higher concentrations of PT. Further study is required to assess the effects of PT on other strains of *E. coli* O157:H7 and on other pathogenic bacteria.

The observed bacteriostatic and bactericidal activities of *A. nodosum* PT against *E. coli* O157:H7 indicate that these phenolic compounds are at least partially responsible for the reduction of *E. coli* O157:H7 in feces of animals supplemented with seaweed extract (Barham et al., 2001; Braden et al., 2004; Bach et al., 2008). Concentrations of PT in brown seaweed vary among species and with growth conditions, and extractable PT at concentrations up to 250 g/kg DM have been reported in some cases (Targett et al., 1992; Van Alstyne et al., 1999). The *A. nodosum* used by Bach et al. (2008) contained approximately 50 g PT/kg DM. In that study, the cattle consumed approximately 200 g of seaweed per day (assuming 10 kg of DM intake). At that level of consumption, ruminal PT concentration would reach approximately 100 µg/ml of ruminal fluid, obviously exceeding the amount of PT required to kill *E. coli* O157:H7 *in vitro*. Given the complexity of the ruminal ecosystem, however, the effects of PT on *E. coli* O157:H7 *in vivo* would be expected to be reduced. Further research is needed to define the optimal dietary concentration of PT necessary to reduce the prevalence of *E. coli* O157:H7 within the GI tract and to define the site of PT reaction with

this bacterium.

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