Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms

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Streptococcus mutans; biofilms; glucans; cranberry; proanthocyanidins; glycolysis.

Abstract
Cranberry fruit is a rich source of polyphenols, and has shown biological activities against Streptococcus mutans. In the present study, we examined the influence of extracts of flavonoids (FLAV), anthocyanins (A) and proanthocyanidins (PAC) from cranberry on virulence factors involved in Streptococcus mutans biofilm development and acidogenicity. PAC and FLAV, alone or in combination, inhibited the surface-adsorbed glucosyltransferases and F-ATPases activities, and the acid production by S. mutans cells. Furthermore, biofilm development and acidogenicity were significantly affected by topical applications of PAC and FLAV (P < 0.05). Anthocyanins were devoid of any significant biological effects. The flavonoids are comprised of mostly quercetin glycosides, and the PAC are largely A-type oligomers of epicatechin. Our data show that proanthocyanidins and flavonoids are the active constituents of cranberry against S. mutans.

Introduction
Streptococcus mutans is generally regarded as a primary microbial agent in the pathogenesis of dental caries although additional acidogenic microorganisms may be involved (Loesche, 1986; Bowen, 2002; Beighton, 2005). This bacterium synthesizes extracellular glucans from sucrose using glucosyltransferases (GTFs) (Loesche, 1986; Bowen, 2002). Glucans promote the accumulation of cariogenic streptococci (and other oral microorganisms) on the tooth surface, and are critical for the formation and structural integrity of biofilms (Dibdin & Shellis, 1988; Schilling & Bowen, 1992; Cury et al., 2000; Marsh, 2004). Furthermore, S. mutans carries out glycosylation of multiple carbohydrates efficiently. However, within the biofilms effective neutralization cannot occur because of limited access by saliva; the low pH values in the plaque matrix contribute to demineralization of tooth enamel and selection of aciduric (acid-tolerant) organisms, such as mutans streptococci. Streptococcus mutans has developed mechanisms to alleviate the influences of acidification by increasing proton-translocating F-ATPase activity in response to low pH (Sturr & Marquis, 1992; Quivey et al., 2000). F-ATPase transports protons (H⁺) out of cells in association with ATP hydrolysis to maintain intracellular pH more alkaline than the extracellular environment pH (Sturr & Marquis, 1992). Therefore, there are several avenues for chemotherapeutic intervention other than attempting to eliminate mutans streptococci selectively, which include inhibition of glucan production by GTFs and F-ATPase activity.

American cranberry (Vaccinium macrocarpon Ait., Ericaceae) is a widely consumed fruit in North America, and has been recognized to have several biological properties which may provide human health benefits (Sobota, 1984; Ofek et al., 1996; Ahuja et al., 1998), including effects on virulence factors of S. mutans involved in the pathogenesis of dental caries. Cranberry juice and a high-molecular weight non-dialyzable material (NDM) extracted from cranberry inhibit the formation of biofilms and coaggregates of oral bacteria, the activity of GTFs, and the bacterial adherence on apatitic surfaces (Weiss et al., 2002; Steinberg et al., 2004; Yamanaka et al., 2004; Duarte et al., 2005). Recently, we have shown that cranberry juice disrupts the accumulation and acidogenicity of S. mutans biofilms in vitro without killing the organisms (Duarte et al., 2005).
Cranberry fruit is a unique and rich source of various classes of potentially bioactive flavonoids (polyphenols). Flavonoids are a large group of polyphenolic natural compounds that are universally distributed in higher plants; they are built upon a C6–C3–C6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. Several classes are differentiated according to the degree of unsaturation and degree of oxidation of the three-carbon segment. Four phenolic classes identified in cranberry include phenolic acids, anthocyanins, flavonols and flavan-3-ols, which consist of monomers and the polymer classes of proanthocyanidins. Anthocyanins, flavonols and proanthocyanidins are among the most abundant flavonoid classes (Vvedenskaya & Vorsa, 2004), and have been associated with the health promoting benefits of cranberry and its products (Cunningham et al., 2004).

Therefore, the aim of the present study was to examine the influence of anthocyanins, flavonols, and proanthocyanidins extracts from cranberry on (i) glucans production by purified glucosyltransferases (GTFs) adsorbed to sHA, (ii) membrane-associated F-ATPase and glycolytic activities, and (iii) viability, development, polysaccharide composition and acidogenicity of S. mutans biofilms.

Materials and methods

Cranberry constituents

The flavonol (FLAV)-, anthocyanin (A)-, and proanthocyanidin (PAC)-rich fractions were prepared from fruit of the cranberry variety 'Stevens' harvested at Rutgers University, PE Marucci Center during September/October 2003 and kept frozen at −20°C. Fractions were eluted from a Sephadex LH-20 column in following sequence: anthocyanins with water, flavonols with 60% methanol, and proanthocyanidins with 80% acetone/water. Fractions were characterized using HPLC-Photodiode/electrochemical detection, LC-MS, and MALDI-TOF.

A cranberry extract was provided by NIH/NCCAM approved contractor, which was diluted to a final concentration of 20 mg of dry-weight mL⁻¹ (CE solution); this is the usual concentration of cranberry extract in most of the products available commercially, such as cranberry cocktail juice. The content of various cranberry polyphenols in CE solution is subjected to variation depending on several factors, such as seasonal and varietal effects; concentrations of the cranberry polyphenols in the CE solution in these experiments were estimated to be 300–500 μg mL⁻¹ (for PAC), 50–200 μg mL⁻¹ (for A), and 30–125 μg mL⁻¹ (for FLAV). We tested each of the extracts alone or in combination as follows: PAC+A, PAC+FLAV, A+FLAV, and A+PAC+FLAV. A 10% ethanol [volume in volume (v/v)] solution was used as a vehicle control; the pH of all solutions was adjusted to 5.5 using 5 mM potassium phosphate buffer. The bioassay data reported here utilized the highest concentration of each of the extracts (125 μg mL⁻¹ of FLAV, 500 μg mL⁻¹ of PAC, and 200 μg mL⁻¹ of A).

Bacterial strains

The bacterial strains used for the production of GTFs were: Streptococcus anginosus KSB88 (Fukushima et al., 1992), which harbors the gtfB gene (for GTF B production) and S. mutans WHB 410 (Wunder & Bowen, 1999), in which the genes for GTF B, D and fructosyltransferase were deleted (for GTF C). The S. mutans UA159, a proven virulent cariogenic pathogen and the strain selected for genomic sequencing (Ajdic et al., 2002), was used for F-ATPase, glycolytic pH drop, and biofilm studies. The cultures were stored at −80°C in low molecular weight medium (Koo et al., 2003) containing 20% glycerol.

GTF B and C assays

The GTF B and C enzymes (EC 2.4.1.5) were prepared from culture supernatants and purified to near homogeneity by hydroxyapatite column chromatography as described by Venkitaraman et al. (1995) and Wunder & Bowen (1999). GTF activity was measured by the incorporation of [14C]glucose from labeled sucrose (NEN Research Products, Boston, MA) into glucans (Venkitaraman et al., 1995; Koo et al., 2000). The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 to 1.5 μmol of glucose over the 4 h reaction (1.0–1.5 U).

The activities of GTFs were determined with the enzymes adsorbed to hydroxyapatite beads (Macro-Prep Ceramic Hydroxyapatite Type I, 80 μm, Bio-Rad®, Hercules, CA) coated with clarified whole saliva (sHA) (free of GTF activity; Koo et al., 2000), in the presence of test agents (CE, PAC, A, FLAV, PAC+A, PAC+FLAV, A+FLAV, PAC+C+FLAV) or to the vehicle control (10% ethanol, v/v) as described elsewhere (Koo et al., 2000). The final pH of the test agents was 5.5. The activities of GTFs in solution were not examined in this study because their enzymatic activities are affected by the acidic pH of the test agents (pH 5.5), which is below the optimum pH for the enzymes in solution (≥ pH 6.5). In contrast, surface-adsorbed GTFs are active over a much broader pH range (pH 4.7–7.5) (Schilling & Bowen, 1988).

F-ATPase and glycolytic pH-drop assays

ATPase assay was performed using permeabilized cells of S. mutans UA159 prepared as described by Belli et al. (1995). F-ATPase activity was assayed in terms of the release of inorganic phosphate in the following reaction mixture:

\[
\text{ATP} + \text{Mg}^2+ + \text{F-ATPase} \rightarrow \text{ADP} + \text{Pi} + \text{F-ATPase} \]

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100 mmol of Tris-maleate buffer (pH 7.0) containing 5 mM ATP, 10 mmol MgCl₂, permeobilized cells, and the test agents (CE, PAC, A, FLAV, PAC+A, PAC+FLAV, A+FLAV, PAC+A+FLAV or the vehicle control). The released phosphate was then determined by the method of Bencini et al. (1983).

The effects of cranberry compounds on glycolysis were measured by standard pH drop with dense cell suspensions as previously described by Belli et al. (1995). Cells of S. mutans UA159 from suspension cultures were harvested, washed once with salt solution (50 mM KCl plus 1 mM MgCl₂), and resuspended in salt solution containing the test agents, alone or in combinations. The pH was adjusted to 7.2 with 0.1 M KOH solution, sufficient glucose was added to give a concentration of 1% [weight in volume (w/v)], and the decrease in pH was assessed by means of a glass electrode over a period of 2 h (Futura Micro Combination pH electrode, 5 mm diameter, Beckman Coulter, Inc, CA) (Belli et al., 1995).

Biofilm assays

Biofilms of S. mutans UA159 were formed on saliva-coated hydroxyapatite discs placed in a vertical position (HAP ceramic – calcium hydroxyapatite, 0.5” diameter ceramic – Clarkson Calcium Phosphates, Williamsport, PA) in batch cultures at 37°C and 5% CO₂ (Koo et al., 2003; Chatfield et al., 2005). Biofilms of S. mutans were formed in ultra-filtered (Amicon 10 kDa molecular weight cut-off membrane; Millipore Co., MA, USA) tryptone-yeast extract broth with addition of 30 mM sucrose containing [¹⁴C]-glucose-sucrose (0.74 kBq mL⁻¹) (Koo et al., 2003). The biofilms were grown undisturbed for 24 h to allow initial biofilms formation. At this point (24 h-old), the biofilms were treated twice daily (10 a.m. and 4 p.m.) until the fifth day of the experimental period (126 h-old biofilm) with one of the test agents (CE, PAC, A, FLAV, PAC+A, PAC+FLAV, A+FLAV, PAC+A+FLAV) or vehicle control. The biofilms were exposed to the treatments for 1 min, double-dip rinsed in sterile saline solution and transferred to fresh culture medium as detailed elsewhere (Koo et al., 2003). Each biofilm was exposed to the respective treatment a total of eight times. The treated biofilms were analyzed for biomass (biofilm wet weight) and bacterial viability (colony forming units – CFU mg⁻¹ of biofilm dry weight). The polysaccharide composition (extracellular water-soluble and insoluble glucans, and intracellular idiophilic polysaccharides) was determined by colorimetric assays and scintillation counting as detailed by Koo et al. (2003). The acid production by the biofilms that were treated with the test agents (or control) was determined by glycolytic pH drop after addition of glucose solution (final concentration of 1%, w/v) by means of a glass electrode (Futura Micro Combination pH electrode, 5 mm diameter, Beckman Coulter, Inc, CA) (Belli et al., 1995).

Statistical analyses

The data were analyzed using ANOVA, and the F-test was used to test any difference among the groups. When significant differences were detected, pairwise comparisons were made between all the groups using Tukey’s method to adjust for multiple comparisons. Triplicates from at least two separate experiments were conducted in each of the assays. Statistical software JMP version 3.1 (SAS Institute, Cary, NC) was used to perform the analyses. The level of significance was set at 5%.

Results and discussion

The basic chemical structures of three of the most abundant flavonoid classes found in cranberry are shown in Fig. 1. The PAC extract consisted of monomers and polymers with the degree of polymerization ranging from dimers to octamers. MALDI-TOF mass spectra analysis confirmed A-type oligomers of epicatechin from trimers to octamers which is similar to those from cranberry reported by Foo et al. (2000), except with the addition of octamers. The flavonols were mostly quercetin glycosides, including the most abundant flavonol, quercetin-3-β-galactoside consistent with a previous study by Vvedenskaya et al. (2004). Cranberry offers to be a source of some unique PAC and FLAV, e.g. epicatechin-(4β → 8, 2β → O → 7)-epicatechin-(4β → 8)-epicatehin and quercetin-3-α-arabinopyranoside. The anthocyanin fraction which gives the characteristic red color of cranberry is composed largely of galactoside and arabinoside conjugates of cyanidin and peonidin (Hong & Wrolstad, 1990). We focused on each of the components at concentrations normally found in cranberries to identify the most effective compound(s).

The data presented in Fig. 2 indicate that FLAV (125 µg mL⁻¹) and PAC (500 µg mL⁻¹), alone or in combination, significantly inhibited the activities of surface adsorbed GTF B and C (30-60% inhibition). Combination of fractions PAC+FLAV and PAC+A+FLAV approached the effectiveness of CE solution, suggesting a synergistic action between the fractions. The inhibition of GTF B and C has many implications for biofilms development because large proportion of the insoluble glucans synthesized by these surface-adsorbed enzymes is retained on the pellicle promoting accumulation of Streptococcus mutans and other cariogenic bacteria on the tooth surface, and contributing to the formation of the matrix of the biofilms (Schilling & Bowen, 1992). These enzymes are critical in the expression of virulence by S. mutans in the pathogenesis of dental caries (Yamashita et al., 1993). We have shown that flavonols aglycones, such as quercetin, myricetin and kaempferol, are effective GTF inhibitors (Koo et al., 2002); the inhibitory
effects could be associated with the presence of an unsaturated double bond between C-2 and C-3 (Fig. 1), which may provide a site for nucleophilic addition by side chains of aminoacids in GTFs. It is noteworthy that anthocyanins, which lack a double bond between C-2 and C-3 (Fig. 1), exhibited only modest inhibitory activities (Fig. 2). PAC are known to bind proteins forming protein–polyphenol complexes (Haslam, 1996; Bravo, 1998), which could inhibit the activity of GTFs. The protein binding capacity of PACs depends on their degree of polymerization and access to the proteins (Bravo, 1998). Further research with individual compounds is needed to elucidate the mechanistic details of GTF inhibition by these groups of flavonoids.

The activity of *S. mutans* membrane associated F-ATPase was also inhibited by PAC, alone or in combinations (>85% of inhibition, Fig. 3). Flavonols also significantly inhibited the activity of F-ATPase (*P* < 0.05), although the inhibitory effect was modest compared to that of PAC. F-ATPase comprises two major complexes, the F1 water-soluble catalytic site and the proton-conducting hydrophobic F0 complex (Sturr & Marquis, 1992). Flavonoids have been shown to inhibit various forms of ATPases (Havsteen, 1983). Quercetin is a known non-competitive inhibitor of proton-translocating F-ATPases by binding to F1 catalytic complex (Zheng & Ramirez, 2000); whether its glycosides display similar mechanisms of enzyme inhibition remain to be elucidated. In contrast, little is known on the effects of PAC on the ATPases; this is the first study showing that PAC effectively inhibit the activity of F-ATPase of bacterial membranes.

Proton translocating F-ATPases (*H*⁺-ATPase) play major roles in protecting *S. mutans* against environmental stress caused by acidification of the biofilms. By taking up or releasing *H*⁺ as they synthesize or hydrolyze ATP, F-ATPases help maintain Δ*H* across the membrane, which is critical for the optimum function of glycolysis. Enolase and other enzymes of the glycolytic pathway and the sugar transport system are sensitive to cytoplasmic acidification (Belli et al.,

![Fig. 1. Structure of main classes of flavonoids found in cranberry. FLAV, flavonols; A, anthocyanins; PAC, proanthocyanidins.](https://academic.oup.com/femsle/article-abstract/257/1/50/490573)
1995). By inhibiting the activity of these enzymes, CE solution and its bioactive components (PAC and to a lesser extent FLAV) can affect the activity of acid sensitive glycolytic enzymes. Acid sensitization can be readily seen in glycolytic pH-drop experiments in which cells are given lytic enzymes. Acid sensitization can be readily seen in glycolytic pH-drop experiments in which cells are given lytic enzymes. Acid sensitization can be readily seen in glycolytic pH-drop experiments in which cells are given lytic enzymes.

Streptococcus mutans cells are able to rapidly degrade glucose and lower the pH value of the suspension to some minimum value at which they can no longer maintain a cytoplasmatic pH compatible with glycolytic enzymes. As shown in Fig. 4, the presence of CE solution and, especially, PAC (alone or in combinations) sensitized cells of S. mutans to acidification so that the final pH value was about 4.7–4.9, compared with about 3.7 for cells not exposed to the test agents (vehicle control). Whether these extracts can actually prevent enamel demineralization awaits further evaluation since the final pH values (4.7–4.9) are still slightly below the critical pH for enamel dissolution (pH 5.0–5.5).

Viability of the biofilms as assessed by determination of CFU mg⁻¹ of biofilm dry weight was not impacted by cranberry extract and its components (data not shown). Nevertheless, short-term topical application of PAC and FLAV (one-minute exposure, twice daily) significantly disrupted the accumulation and polysaccharide composition of S. mutans biofilms compared with the control (Table 1, P < 0.05), reducing both the biomass (dry weight) and total amount of insoluble glucans. In contrast, the influence of some minimum value at which they can no longer maintain a cytoplasmatic pH compatible with glycolytic enzymes. As shown in Fig. 4, the presence of CE solution and, especially, PAC (alone or in combinations) sensitized cells of S. mutans to acidification so that the final pH value was about 4.7–4.9, compared with about 3.7 for cells not exposed to the test agents (vehicle control). Whether these extracts can actually prevent enamel demineralization awaits further evaluation since the final pH values (4.7–4.9) are still slightly below the critical pH for enamel dissolution (pH 5.0–5.5).

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### Fig. 2. Influence of cranberry components on the activities of glucosyltransferase B adsorbed onto a saliva-coated hydroxyapatite surface. The concentrations of each of the extracts were 125 µg mL⁻¹ (FLAV, flavonols), 500 µg mL⁻¹ (PAC, proanthocyanidins), and 200 µg mL⁻¹ (A, anthocyanins). The percentage of inhibition was calculated considering the vehicle control as 100% glucosyltransferase (GTF) activity. A similar inhibitory profile was obtained for GTF C. Values (SD, N=9) connected by lines are not significantly different from each other (P > 0.05, ANOVA, comparison for all pairs using Tukey test).

### Fig. 3. Influence of cranberry components on F-ATPase of permeabilized cells of Streptococcus mutans UA159. The concentrations of each of the extracts were 125 µg mL⁻¹ (FLAV, flavonols), 500 µg mL⁻¹ (PAC, proanthocyanidins), and 200 µg mL⁻¹ (A, anthocyanins). The percentage of inhibition was calculated considering the vehicle control as 100% F-ATPase activity. Values (SD, N=9) connected by lines are not significantly different from each other (P > 0.05, ANOVA, comparison for all pairs using Tukey test).

### Fig. 4. Influence of cranberry components on glycolytic pH drop of Streptococcus mutans UA159 in suspensions. The concentrations of each of the extracts were 125 µg mL⁻¹ (FLAV, flavonols), 500 µg mL⁻¹ (PAC, proanthocyanidins), and 200 µg mL⁻¹ (A, anthocyanins). Values (N=9, SD not shown) from A, FLAV, PAC, A+PAC, FLAV+PAC, A+FLAV+PAC and crude extract solution are not significantly different from each other at each time point (P > 0.05, ANOVA, comparison for all pairs using Tukey test). Whether these extracts can actually prevent enamel demineralization awaits further evaluation since the final pH values (4.7–4.9) are still slightly below the critical pH for enamel dissolution (pH 5.0–5.5).
the flavonoids on the production of soluble glucans and intracellular iodophilic polysaccharides was negligible ($P > 0.05$, data not shown). As with other assays, the $A$ extract had no significant effect against biofilms. Although our mono-species biofilm model does not mimic the complex microbial community found in dental plaque, it is more advantageous when examining specific actions of test agents on $S. mutans$ physiology, especially on the glucan-mediated processes involved in the biofilm development. Clearly, PAC and FLAV, alone or in combinations, as well as CE solution reduced the formation and accumulation of $S. mutans$ biofilms by mostly diminishing the amounts of insoluble glucans in the biofilms matrix. This observation is consistent with the effective inhibition of GTF B and C observed in this study.

Furthermore, PAC+FLAV, PAC+A+FLAV and CE solution treatments also reduced the acidogenicity of the biofilms (Table 1), although this effect was less than that observed in glycolytic pH-drop assays using suspension cells of $S. mutans$ (Fig. 4). Biofilms are known to be more resistant to antimicrobial agents than cells in suspension because of higher biomass densities and decreased metabolic activities in biofilms, which affect the effectiveness of the therapeutic agents (Lewis, 2001).

Overall, the data show that the fractions in cranberries that are biologically active against the virulence traits of $S. mutans$ involved in acidogenicity and biofilm development are mainly PAC and to a lesser extent FLAV; the $A$ extract, in contrast, had no significant effects against $S. mutans$. Combinations of PAC+FLAV and PAC+A+FLAV displayed the highest biological activity in vitro and potency comparable to crude cranberry extract (CE) solution. The putative pathways by which flavonols and proanthocyanidins affect the virulence of $S. mutans$ may involve several routes. We propose at least three: (1) inhibition of insoluble glucans synthesis by surface-adsorbed GTF B and C; (2) inhibition of the proton-translocating F-ATPase activity; and (3) disrupting acid production. Having shown clearly the potential of cranberry flavonols and proanthocyanidins to interfere with virulence traits of $S. mutans$, the identification of the individual active compound(s) is worthy of exploration.

**Acknowledgements**

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**References**


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**Table 1.** Dry-weight, acidogenicity, and total amount of insoluble glucans in the biofilms after treatments

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>Dry-weight (mg)</th>
<th>Insoluble glucans (mg)</th>
<th>pH (30 min after glucose pulse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>9.1 ± 0.6a</td>
<td>2.09 ± 0.31a</td>
<td>4.91 ± 0.04ab</td>
</tr>
<tr>
<td>Anthocyanins (A)</td>
<td>8.7 ± 1.1ab</td>
<td>1.90 ± 0.40ab</td>
<td>4.84 ± 0.03a</td>
</tr>
<tr>
<td>Flavonols (FLAV)</td>
<td>7.3 ± 1.4c</td>
<td>1.48 ± 0.38bc</td>
<td>4.95 ± 0.04ab</td>
</tr>
<tr>
<td>Proanthocyanidins (PAC)</td>
<td>7.0 ± 1.4c</td>
<td>1.35 ± 0.32c</td>
<td>5.02 ± 0.11b</td>
</tr>
<tr>
<td>A+FLAV</td>
<td>7.7 ± 0.6bc</td>
<td>1.61 ± 0.29bc</td>
<td>5.00 ± 0.04b</td>
</tr>
<tr>
<td>A+PAC</td>
<td>7.3 ± 0.5c</td>
<td>1.35 ± 0.21c</td>
<td>4.98 ± 0.05b</td>
</tr>
<tr>
<td>FLAV+PAC</td>
<td>6.8 ± 0.9c</td>
<td>1.20 ± 0.28c</td>
<td>5.39 ± 0.02c</td>
</tr>
<tr>
<td>A+FLAV+PAC</td>
<td>7.1 ± 0.8c</td>
<td>1.34 ± 0.27c</td>
<td>5.19 ± 0.09f</td>
</tr>
<tr>
<td>Crude extract</td>
<td>6.8 ± 0.7c</td>
<td>1.22 ± 0.32c</td>
<td>5.41 ± 0.05c</td>
</tr>
</tbody>
</table>

The concentrations of each of the extracts were 125 μg mL⁻¹ (FLAV), 500 μg mL⁻¹ (PAC), and 200 μg mL⁻¹ (A).

Values in the same column followed by the same superscripts are not significantly different from each other ($P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

*Twice daily with one minute exposure for each treatment.

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