

## Research Article

# Inhibition of *Streptococcus gordonii* Metabolic Activity in Biofilm by Cranberry Juice High-Molecular-Weight Component

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Previous studies demonstrated that a cranberry high-molecular-mass, nondialyzable material (NDM) can inhibit adhesion of numerous species of bacteria and prevents bacterial coaggregation of bacterial pairs. Bacterial coaggregation leads to plaque formation leading to biofilm development on surfaces of oral cavity. In the present study, we evaluated the effect of low concentrations of NDM on *Streptococcus gordonii* metabolic activity and biofilm formation on restorative dental surfaces. We found that the NDM selectively inhibited metabolic activity of *S. gordonii*, without affecting bacterial viability. Inhibiting the metabolic activity of bacteria in biofilm may benefit the health of the oral cavity.

## 1. Introduction

For a successful bacterial colonization of the oral cavity, adherence mechanisms are essential, otherwise the bacteria get washed away and swallowed by the salivary flow. Oral bacteria have evolved several mechanisms to withstand the salivary flow and succeed in adhesion to and subsequently form biofilm on surfaces of the oral cavity. *Streptococcus gordonii* has been considered to play an important role in cariogenesis because it readily colonizes the clean tooth surfaces and is capable of forming biofilm. Among the oral bacteria, *S. gordonii* appears to have highest affinity to hard surfaces of the oral cavity [1]. *S. gordonii* biofilm forms an important component of human dental plaque by virtue of its ability to adhere to tooth surfaces [1]. Formation of dental plaque precedes cariogenesis; thus, interfering with *S. gordonii* adhesion and biofilm formation of hard tissue is likely to improve the oral health.

Dietary agents that interfere with adhesion of and biofilm formation by bacteria has been the focus of intensive research because such natural agents are likely to be nontoxic to the host [2]. Furthermore, the identified active components

can be used as supplement to oral health hygiene product negating the necessity to adhere to a particular diet. Perhaps most important advantage of searching dietary agents is that approval of clinical trials would be easier to obtain, as toxicity is not an issue. In this respect, cranberry juice and isolated fractions/constituents which inhibit adhesion of bacteria to various surfaces have been studied the most [3].

Phenolic compounds of cranberry were shown to prevent adherence of uropathogen to animal cells [4, 5]. The cranberry components were also shown to reduce the risk of cardiovascular disease [6], periodontal disease [7], and inhibit host inflammatory response [8]. Earlier studies demonstrated a high-molecular-weight mass, nondialyzable material (NDM) prepared from cranberries to contain polyphenolic compounds that inhibited the secretion of proteolytic enzymes by periodontopathogens [9], adhesion of a number of bacterial species [10, 11], and were also shown to interfere with coaggregation of oral bacterial species and biofilm formation by *Streptococcus mutans* [3, 12]. The polyphenol fraction of cranberry was reported to decrease the hydrophobicity of streptococcal species [12, 13].

In the present study, we sought to determine the ability of the high-molecular-weight component from cranberry (NDM) to interfere with biofilm formation by *S. gordonii* in general and in particular on dental composites and titanium discs. We hypothesize that the cranberry NDM will have a beneficiary role by interfering with streptococcal biofilm formation on dental materials. Prevention or reduction of oral bacterial load on the surfaces of the oral cavity will have a beneficial role in improving the oral health.

## 2. Materials and Methods

**2.1. Preparation of NDM.** NDM was obtained as described previously [3] from concentrated cranberry juice made from the American cranberry, *Vaccinium macrocarpon*, and provided by Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA, USA. Briefly, NDM was obtained after lyophilization of the material retained in a dialysis bag (12,000 molecular weight cutoff) following extensive dialysis. The retentate of the bag designated NDM, is soluble in water up to 4 mg/mL, devoid of proteins, carbohydrates, and fatty acids, and was found to exhibit tannin-like properties suggests that it is rich in phenolic compounds (e.g., proanthocyanidins) [8]. Further analysis performed by Ocean Spray Inc. revealed that this fraction is devoid of sugars, acids, and nitrogen and contains 0.35% anthocyanins (0.055% cyanidin-3-galactoside, 0.003% cyanidin-3-glucoside, 0.069% cyanidin-3-arabinoside, 0.116% peonidin-3-galactoside, 0.016% peonidin-3-glucoside, and 0.086% peonidin-3-arabinoside) and 65.1% proanthocyanidins [9, 12].

**2.2. Bacterial Strains and Culture Conditions.** *Streptococcus gordonii* Challis (ATCC, Rockville, MD) was grown in trypticase soy broth (TSB; Difco Labs) for 48 hours at 37°C. Cells were washed in PBS and resuspended to contain  $5 \times 10^7$  cells/mL.

**2.3. XTT Metabolic Assay.** Following the treatment of bacteria with NDM, *S. gordonii* cells were incubated with 50  $\mu$ L of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) for 4 hours at 37°C. In this assay, the tetrazolium salt XTT is cleaved to an orange-colored formazan product by mitochondrial dehydrogenase in viable cells [14]. At the end of incubation period, the absorbance of the resulting supernatant was measured at 490 nm using an ELISA reader (Bio Rad Laboratories). Prior to the measurement of number of cells in the biofilm, a standard curve was prepared with known numbers of bacteria.

**2.4. Quantification of Streptococcal Metabolic Activity by XTT Assay and Biofilm Mass by Crystal Violet Staining.** Following the treatment of bacteria with NDM, *S. gordonii* cells were incubated with 50  $\mu$ L of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) for 4 hours at 37°C. In this assay, the tetrazolium salt XTT is cleaved to an orange-colored formazan product by mitochondrial dehydrogenase in viable cells [14]. At the

end of incubation period, the absorbance of the resulting supernatant was measured at 490 nm using an ELISA reader (Bio Rad Laboratories). Prior to the measurement of number of cells in the biofilm, a standard curve was prepared with known numbers of bacteria.

We also used the crystal violet staining method to assess the effect of NDM on the bacterial biofilm formation in a 96-well microtiter plate [15]. Adherent bacteria in the wells were fixed with methanol for 15 min, extensively washed with distilled water, and then stained with 0.4% crystal violet (100  $\mu$ L) for 15 min. Wells were rinsed with distilled water and dried at 37°C for 2 h. After adding 100  $\mu$ L of 95% (v/v) ethanol to each well, the plate was shaken to release the stain. The absorbance at 550 nm was recorded using a microplate reader. All assays were run in triplicate, and the means  $\pm$  SD of three independent experiments were calculated.

**2.5. Effect of Cranberry NDM on the Metabolic Activity of *S. gordonii* Growing in Culture Media Measured by XTT Assay.** Freshly cultured bacterial suspension containing  $5 \times 10^7$  cells/mL was prepared, from which 0.1 mL suspension was placed in each of a 96-well microtiter plate. An equal volume of serially diluted NDM (5.0 to 400  $\mu$ g/mL) in phosphate-buffered saline (PBS; 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M KH<sub>2</sub>PO<sub>4</sub>, 0.15 M NaCl, pH 7.4) was added to each well containing the bacteria and incubated for 24 hours at 37°C. After the incubation, unattached bacteria were washed off by rinsing the plate with PBS. The microtiter plate was replenished with fresh TSB media (0.2 mL) supplemented with 0.2% sucrose and incubated for an additional 24 hours. At the end of incubation, nonadherent cells were removed and the metabolic activity of the bacteria in the biofilm was assessed by XTT assay and by staining with crystal violet, as described above. For control purposes, 2.0 mL of standard bacterial suspension was immersed in a beaker of boiling water for 5 minutes and then treated with XTT reagent as the experimental cells.

**2.6. Effect of Cranberry NDM Treatment of *S. gordonii* on Metabolic Activity of Biofilm Formed on Dental Composite, Amalgam, Hydroxyl Apatite, and Titanium Discs.** ESPE composite material was molded into a 6 mm disc and cured for 10 seconds as recommended by the manufacturer. In a similar manner, amalgam discs (6 mm) were also prepared. Hydroxyl apatite discs were purchased from Berkley Advanced Biomaterials, Inc., Berkley, CA. Polished 6 mm titanium discs were supplied by Dr. Bumgardner, University of Memphis, TN. All discs were sterilized by nitrous oxide. An aliquot (0.5 mL) of standard bacterial suspension was incubated with cranberry NDM (5 to 200  $\mu$ g/mL) for 60 min at 37°C. Bacteria were then added to the discs placed in a 24-well culture dish and further incubated for 24 hours at 37°C. After gentle washing to remove the nonadherent bacteria, discs were incubated with 0.5 mL of TSB supplemented with 0.2% sucrose for an additional 48 hours to facilitate biofilm formation. At the end of incubation, discs were rinsed once and then incubated with XTT reagent to assay for their metabolic activity [15].

**2.7. Live/Dead BacLight Assay.** Bacteria were stained using the Live/Dead BacLight Kit (Molecular Probes-Invitrogen, Carlsbad, CA). This stain distinguishes live cells from dead bacteria based on membrane integrity and two nucleic acid stains. The green fluorochrome (SYTO 9) can penetrate intact membranes, while the larger red fluorochrome (propidium iodide) penetrates only compromised membranes of dead bacteria. The dye was prepared according to the manufacturer's specifications. Cells treated with NDM (25  $\mu\text{g}/\text{mL}$  for 60 minutes) or not (control) were stained with the dye for 15 minutes in dark. Cells were mounted on a slide and evaluated by confocal microscope.

**2.8. Flow Cytometry Analysis.** Bacterial cells ( $5 \times 10^6$  cells) were pelleted, and the pellet was resuspended in 1.0 mL of NDM (25  $\mu\text{g}/\text{mL}$ ) and incubated for 60 min. The cells were stained with Live/Dead kit (Molecular Probes-Invitrogen, Carlsbad, CA) fluorescent dyes, calcein AM, and ethidium homodimer diluted according to the manufacturer's recommendation for 15 minutes in dark at room temperature. Similar numbers of bacteria were incubated with 70% isopropanol for 45 minutes to generate dead cells, which were also stained similarly (dead cell control). The effect of NDM on bacterial cells was analyzed by FACScan flow cytometry (Becton Dickinson) using  $520 \pm 20$  nm excitation for measuring calcein green fluorescence emission and ethidium homodimer red fluorescence emission using  $615 \pm 30$  nm. The data was processed with Flowmax software (Partec), and electronic gating with the software was used to separate positive signals from noise. Between 7,500 and 50,000 events were acquired using linear amplification for forward and side scatter and logarithmic amplification for fluorescence. Samples were measured in triplicate, and selected samples were controlled with epifluorescence microscopy to confirm the bacterial nature of stain.

**2.9. Confocal Microscopy.** Bacterial samples were stained with the Live/Dead BacLight bacterial viability kit (Invitrogen L-13152), a rapid epifluorescence staining method as specified by the manufacturer. The bacteria were incubated for 15 minutes in dark and then examined for the difference in live and dead cells between NDM-treated and control *S. gordonii* by confocal microscope. Images were analyzed by using COMOS software (Bio Rad), and green and red images were merged and formatted on Confocal Assistant software (Bio Rad).

**2.10. Statistical Analyses.** Data are expressed as the means  $\pm$  standard deviations of three independent experiments with triplicate samples in each experiment. Analyses of variance were performed to compare the means of the different conditions. Differences were considered significant at a  $P$  value of  $<0.05$ .

### 3. Results

**3.1. Effect of Cranberry NDM on the Metabolic Activity of *S. gordonii* Grown in Culture Media.** To investigate the effect of NDM on metabolic activity of *S. gordonii* in biofilm,

TABLE 1: Effect of cranberry NDM on the metabolic activity of *S. gordonii* growing in culture media measured by XTT assay.

NDM Concentration ( $\mu\text{g}/\text{mL}$ )	Metabolic activity
0	$2.4 \pm 0.3$
2.5	$1.73 \pm 0.2$
5.0	$1.38 \pm 0.11$
7.5	$1.19 \pm 0.24$
10	$0.86 \pm 0.12$
20	$0.65 \pm 0.11$
25	$0.37 \pm 0.1$
50	$0.23 \pm 0.02$
100	0
200	0
Heat-killed bacteria (control)	0

Bacterial biofilm prepared as described previously was treated with varying concentrations of NDM (2.5  $\mu\text{g}/\text{mL}$  to 100  $\mu\text{g}/\text{mL}$ ) for 60 minutes and 4 hours. The metabolic activity was determined by XTT assay and the biofilm mass determined by the crystal violet staining.

bacteria were treated with serially diluted NDM and then measured their metabolic activity by XTT metabolic assay. Cranberry NDM inhibited *S. gordonii* metabolic activity in a dose-dependent manner (Table 1). A 50% reduction in bacterial metabolic activity was seen when treated with 7.5  $\mu\text{g}/\text{mL}$  of NDM, while 50  $\mu\text{g}/\text{mL}$  of NDM inhibited by 96%. Total inhibition in metabolic activity was seen with 100  $\mu\text{g}/\text{mL}$  of NDM. Heat-killed bacteria did not show any metabolic activity (Table 1). Colony forming units of the NDM treatment of bacteria revealed that viability was not affected at any of the concentrations tested suggesting that the inhibition of metabolic activity was not related to NDM toxicity to bacteria (data not shown).

**3.2. Effect of Cranberry NDM Pretreatment of *S. gordonii* on the Bacterial Metabolic Function and Biofilm Formation.** The results (Figure 1) show that the time of treatment and concentration of NDM affected the *S. gordonii* metabolic activity. Pretreatment of bacteria with 2.5  $\mu\text{g}/\text{mL}$  of NDM for 15 minutes reduced bacterial metabolic activity as estimated by XTT assay by 20% of the bacteria when compared to the untreated bacteria (control). Bacteria treated with 25  $\mu\text{g}/\text{mL}$  NDM showed metabolic reduction of 66% (Figure 1). Higher concentrations of NDM (100  $\mu\text{g}/\text{mL}$ ) completely abolished the metabolic activity (data not shown).

Contrary to the reduction in metabolic activity of *S. gordonii* by NDM treatment, there was no reduction in biofilm mass when stained with crystal violet (Figure 2). Biofilm mass was reduced by only 8% of the control when bacteria were treated with 100  $\mu\text{g}/\text{mL}$ . There was no significant difference in biofilm mass between the groups treated with different concentrations of NDM. The data suggests that the cranberry NDM at relatively low concentrations does not appear to inhibit biofilm formation as estimated by the total mass but the metabolic activity of the formed biofilm was affected by NDM. In Figures 1 and 2, One mL aliquots of standard bacterial suspension in triplicate was incubated

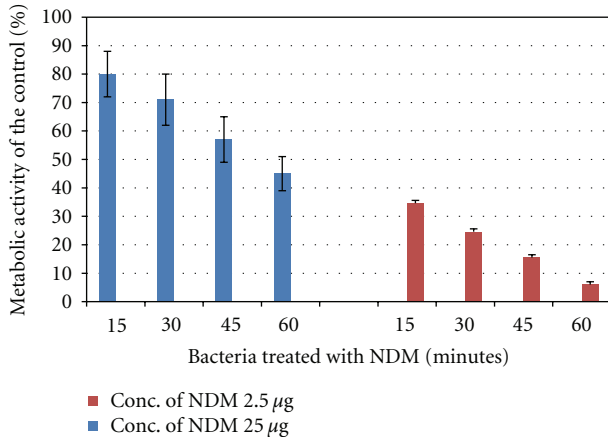


FIGURE 1: Effect of NDM treatment of bacteria on their metabolic activity determined by XTT assay.

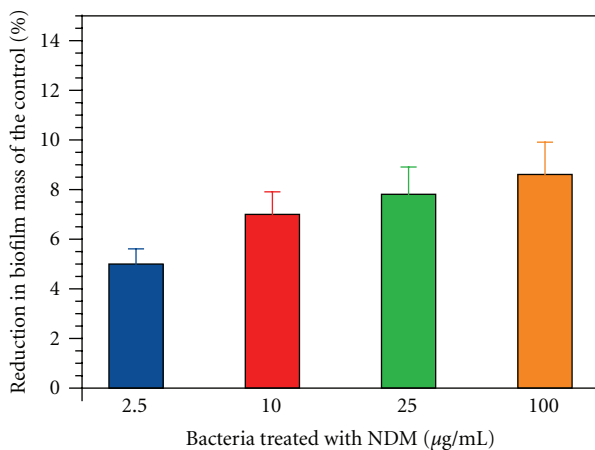


FIGURE 2: Effect of NDM treatment of bacteria on the biofilm mass as determined by crystal violet staining.

with varying concentrations of NDM (5.0 to 200 µg/mL) for 15, 30, 45, and 60 minutes. After the incubation, bacteria were centrifuged and the pellet was washed with PBS once and suspended in 0.2 mL of TSB culture media supplemented with 0.2% glucose for an additional 48 hours and measured their metabolic activity in the biofilm by XTT. Another parallel set of bacterial cells treated in a similar way was stained with crystal violet, and the optical density was measured at 550 nm. For control purposes, 1.0 mL of standard bacterial suspension was immersed in a beaker of boiling water for 5 minutes and then treated as above with XTT and crystal violet.

**3.3. Effect of NDM on Preformed Bacterial Biofilm Formation.** Next, we investigated the effect of cranberry NDM on preformed *S. gordonii* biofilm and assayed metabolic activity by XTT and biofilm mass by crystal violet method. The results (Table 2) demonstrate that the NDM failed to detach the bacteria from the preformed biofilm mass, even at a concentration of 3 mg/mL. On the other hand, it is of interest to note that the bacterial metabolic activity was reduced

TABLE 2: Effect of cranberry NDM on preformed *S. gordonii* biofilm.

NDM concentration (µg/mL)	Metabolic activity	Biofilm mass
0 (control)	2.21 ± 0.3	2.36 ± 0.41
2.5	2.14 ± 0.18	2.33 ± 0.33
5.0	2.24 ± 0.22	2.39 ± 0.27
7.5	2.06 ± 0.4	2.35 ± 0.44
10	1.96 ± 0.33	2.41 ± 0.31
20	1.84 ± 0.59	2.34 ± 0.41
25	1.97 ± 1.1	2.36 ± 0.29
50	1.86 ± 0.69	2.39 ± 0.3
100	1.08 ± 0.44	2.35 ± 0.28

Bacterial biofilm prepared as described previously was treated with varying concentrations of NDM (2.5 µg/mL to 100 µg/mL) for 60 minutes and 4 hours. The metabolic activity was determined by XTT assay and the biofilm mass determined by the crystal violet staining.

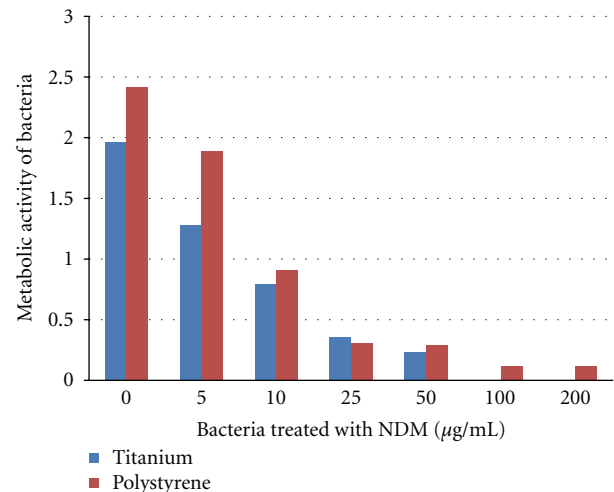


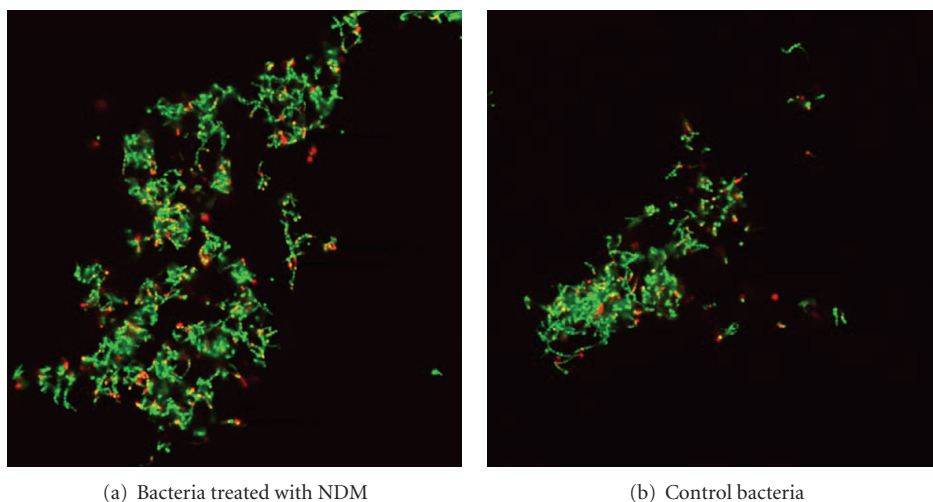
FIGURE 3: Effect of cranberry NDM treatment of *S. gordonii* on metabolic activity of biofilm formed on titanium and polystyrene surfaces. Bacteria treated with cranberry NDM for 4 hours were added to the titanium discs and in parallel to polystyrene 24-well culture dish. The metabolic activity of adherent bacteria was determined by XTT assay. Total biomass of biofilm bacteria was not significantly affected by none of the indicated NDM concentrations as determined by crystal violet assay (data not shown).

by NDM treatment (Table 2). NDM at a concentration of 100 µg/mL for 60 minutes reduced the metabolic activity by 50%, but the same concentration had no effect on biofilm mass. Increasing the length of treatment of preformed biofilm up to 8 hours also showed similar results (data not shown).

**3.4. Metabolic Activity and Total Mass of *S. gordonii* Biofilm Formed on Cranberry NDM-Coated Titanium and Polystyrene Surfaces.** The results (Figure 3) demonstrate that the NDM treatment of *S. gordonii* showed a reduction in metabolic activity of bacteria adherent to both titanium and polystyrene surfaces. Greater than 50% reduction in metabolic activity was seen with 10 µg/mL concentration of NDM. Higher concentration of NDM (>25 µg/mL) reduced the metabolic

TABLE 3: Inhibition of *S. gordonii* metabolic activity measured by XTT assay in biofilm formed on dental restorative surfaces by NDM.

NDM Conc ( $\mu\text{g/mL}$ )	Metabolic activity of biofilm cells on		
	Amalgam discs	Composite discs	HA discs
Zero	$2.66 \pm 0.44$	$2.51 \pm 0.41$	$2.37 \pm 0.22$
10	$1.84 \pm 0.36$	$1.97 \pm 0.31$	$2.01 \pm 0.26$
25	$1.33 \pm 0.12$	$1.52 \pm 0.24$	$1.79 \pm 0.19$
50	$0.94 \pm 0.08$	$1.14 \pm 0.18$	$1.21 \pm 0.15$
75	$0.42 \pm 0.05$	$0.56 \pm 0.11$	$0.74 \pm 0.13$
100	0.0	0.0	0.0

FIGURE 4: Confocal microscopic analysis of *S. gordonii* biofilm stained with Live/Dead *BacLight*.

activity of the adherent bacteria by greater than 98%. In contrast, NDM at the same concentrations reduced total bacterial mass by only 5–8 percent, when stained with crystal violet (data not shown), consistent with the data shown in Figure 2.

**3.5. Inhibition of *S. gordonii* Metabolic Activity in Biofilm Formed on Dental Restorative Surfaces by NDM.** We tested the NDM effect on metabolic activity of biofilm created on amalgam, composite, and hydroxyl apatite discs by XTT assay as described previously [14].

The results (Table 3) of the study showed that NDM treatment inhibited *S. gordonii* biofilm metabolic activity on the two dental restorative materials as well as on polystyrene surface. 25  $\mu\text{g/mL}$  of NDM inhibited approximately 50% of bacterial metabolic activity on all three discs. No measurable inhibition of metabolic activity was seen when bacteria were treated with NDM concentrations greater than 100  $\mu\text{g/mL}$ . In contrast, the total bacterial mass of the biofilm bacteria was reduced by only 5–8% as determined by crystal violet staining (data not shown) consistent with the data shown earlier.

**3.6. Analysis of NDM-Treated *S. gordonii* Biofilm by Live/Dead *BacLight* Staining, Confocal Microscopy, and Flow Cytometry.** The data obtained in this study so far suggested that the cranberry NDM inhibited the metabolic function of *S. gordonii* without being bactericidal. In order to confirm

this observation, we stained the biofilm of *S. gordonii* created on polystyrene with Live/Dead *BacLight* according to the recommended protocol by the manufacturer (Invitrogen). Stained bacteria were first viewed by a fluorescent microscope and then by confocal microscope. Figure 4 shows that NDM treatment did not appear to cause cell death, and both NDM treated (Figure 4(a)) and untreated bacteria (Figure 4(b)) appear to contain similar proportion of live and dead bacteria. This observation confirms that cranberry NDM is not bactericidal for *S. gordonii*.

Further analysis of the live and dead cells was performed by flow cytometry. The results of flow cytometry (Figures 5(a) and 5(b)) indicate that NDM treatment did not change in the ratio of live to dead bacteria consistent with our microscopic observation that the NDM has no cytotoxic effect on *S. gordonii* cells.

## 4. Discussion

Early colonization on the tooth surface and subsequent biofilm formation by *S. gordonii* and their ability to coaggregate with several oral microorganisms result in the formation of dental plaque. The plaque and biofilm formation leads to caries and subsequently leads to periodontal disease if left untreated. Constituents of cranberry were demonstrated to decrease the hydrophobicity of streptococcal species [12, 16]. Cranberry high-molecular-weight component was shown to inhibit secretion of glucosyl and fructosyltransferases by oral

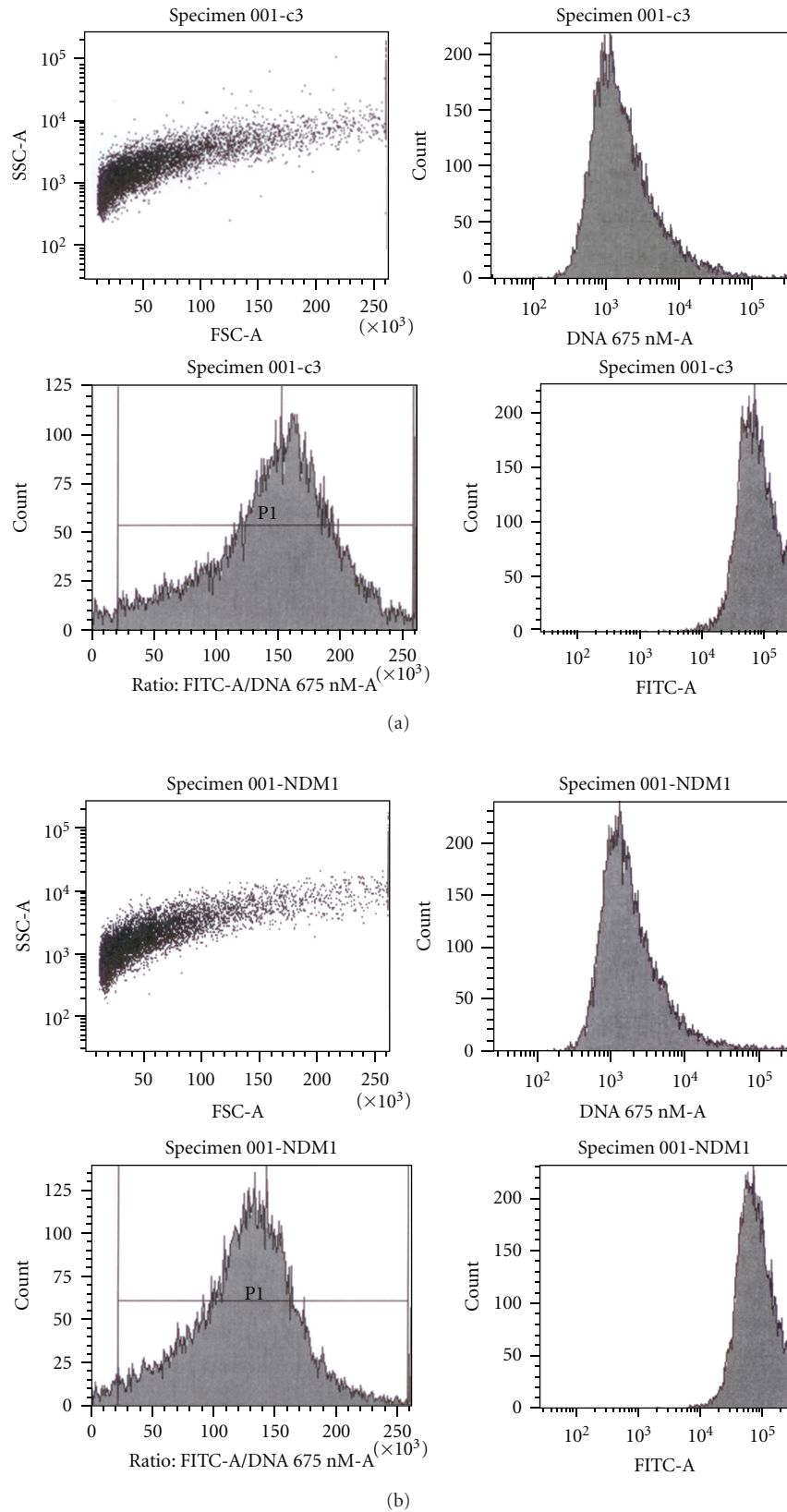


FIGURE 5: (a) *S. gordonii* (control) stained with Live/Dead BacLight and analyzed by flow cytometry. (b) *S. gordonii* (NDM treated) stained with Live/Dead BacLight and analyzed by flow cytometry.

streptococci [12] and coaggregation of bacteria [12]. The high-molecular-weight component of cranberry was shown to be highly soluble in water, lacking proteins, carbohydrates, and fatty acids [17, 18].

Previous studies have shown that relatively high concentrations of NDM (e.g., 0.5–2 mg/mL) were required to cause 80% or more reduction of biofilm formation by *S. mutans* on saliva-coated HA [16] and by *P. gingivalis* on polystyrene surfaces [7]. In the present study, we show that lower concentrations of NDM (0.05–0.1 mg/mL) selectively inhibited the metabolic activity of oral bacteria, *S. gordonii*. This conclusion is based on (i) the effect of NDM on metabolic activity of preformed biofilm that was more profound than on the total biofilm mass as measured by crystal violet stain and (ii) the confocal microscopy stain with Live/Dead stain showing no effect on the ratio of viable/dead *S. sanguis*. The effect of NDM on metabolic activity may indirectly interfere with the ability of the bacteria to adhere and form biofilm onto various dental surfaces as shown in the present study and in other studies [7, 12, 16]. Previous studies have shown that NDM affects various physicochemical properties of uropathogenic bacteria [4, 9]. The present study shows an effect on metabolic activity, and further studies are needed to see how these two effects are connected. Perhaps most important is to study how these effects of relatively low concentrations of NDM on metabolic activity of *S. sanguis* biofilm influences cariogenicity of the bacteria such as acid formation. Either way, NDM seems to affect *S. sanguis* adhesion and biofilm formation mainly by inhibiting metabolic activity of the cariogenic bacteria. It is expected that supplementing oral health product such as mouth wash with NDM will affect not only *S. mutans* total counts as shown previously [18] but also *S. sanguis* cariogenic activity.

Our study revealed the beneficial role of cranberry NDM in reducing the *S. gordonii* metabolic activity in the biofilm created on various dental surfaces such as titanium implant material, amalgam, and composite materials. The potential use of cranberry NDM in oral rinse merits further investigation, since it appears to benefit the health of the oral cavity, by reducing the metabolic activity of *S. gordonii*.

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