Potential immunomodulatory effects of non-dialyzable materials of cranberry extract in poultry production

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ABSTRACT Studies on effects of cranberry products in animals, especially in chickens, are very scarce or even lacking. This study investigated the immunomodulatory effect of high molecular weight non-dialyzable materials (NDMs) of cranberry extract. The in vitro antioxidant and anti-inflammatory activities were investigated. The ability of NDMs (0, 1, 2, or 4 mg/mL) to enhance phagocytic activities was also evaluated using chicken heterophils (CHEs) against Staphylococcus aureus. Furthermore, a broiler model was used to determine the effect of NDMs on the humoral immune response. Seven-d-old chicks were vaccinated with the infectious bursal disease virus (IBDV) vaccine S-706, and treated orally with 0, 2, 4, or 8 mg/mL/bird NDMs for five consecutive days. Serum immunoglobulin level (Ig), and antibody concentration against IBDV, infectious bronchitis virus (IBV), Newcastle disease virus (NDV), and avian reovirus (ARV) were measured weekly by enzyme-linked immunosorbent assay (ELISA). NDMs showed >five-fold higher antioxidant activity (oxygen radical absorbance capacity = 222.7 mg trolox/g) than the commercial raw cranberry juice from which it derived (oxygen radical absorbance capacity = 39.6 mg trolox/g). Likewise, NDMs demonstrated anti-inflammatory activities comparable to Naproxen but better than those of Ibuprofen. The susceptibility of S. aureus to phagocytosis by CHEs increased significantly (P < 0.05) at 4 mg/mL NDMs in the medium. While no intracellular bacteria were counted in CHEs after phagocytosis in the presence of 2 and 4 mg/mL NDMs, 1 mg/mL NDMs demonstrated a significant (P < 0.05) intracellular killing activity in CHEs against S. aureus compared to the untreated CHEs. Results from the in vivo studies indicated that birds receiving 2 and 4 mg/mL/bird NDMs had a higher serum IgM level (P < 0.05), and their antibody titers against IBDV tended to increase with NDMs administration (P = 0.06) on d 35. These results suggest that NDMs enhances bacterial susceptibility to immuno-defense mechanisms, and may be useful as immunomodulators against infections.

Key words: cranberry, non-dialyzable material, chicken heterophil, phagocytosis, antibody

INTRODUCTION

Cranberry (Vaccinium macrocarpon Ait.) is an increasingly significant commercial crop in Canada, a major producer of this important fruit, second only to the United States (FAO, 2010). Cranberry has recently received considerable attention in medical research because of its demonstrated potential effects in promoting human health (Neto, 2007; Wu et al., 2008; Pappas and Schaich, 2009; Rodriguez-Pérez et al., 2016). In addition to health benefit, cranberries have for centuries been used as a meat preservative (Pappas and Schaich, 2009). In vitro chemical assays rated cranberries as having the highest antioxidant values of over 21 fruits, and the overall phenolic content appears to correlate with the level of antioxidant activity (Vinson et al., 2001; Sun et al., 2002). The phenolic classes identified in cranberry include phenolic acids, anthocyanins, flavonols, and flavan-3-ols, which consist of both the monomers and polymers of procyanidins and proanthocyanidins (Vinson et al., 2001; Sun et al., 2002). Few studies have examined the biological effect of cranberry using animal models despite being proven as a powerful source of antioxidants with beneficial effects on health and immunity.

In majority of the birds, heterophils are major cell types involved in initial response to various pathogens and irritants (Kogut et al., 1998; Swaggerty et al., 2005). Heterophils play an important role in pathogen recognition followed by cytokine initiation and chemokine production to relay information to other immune cell types (Swaggerty et al., 2004; Swaggerty et al., 2006). Phagocytosis by immune cells is...
also an important part of the host defense mechanisms against pathogenic bacteria (Leijh et al., 1986). Unfortunately, many obligate and facultative intracellular bacteria such as *Staphylococcus aureus* have developed sophisticated systems to avoid phagocytosis and intracellular killing by phagocytic cells. In poultry, *S. aureus* - an opportunistic pathogen, induces infections having a major impact on the fertility and productivity of a breeder flock. Moreover, treating infections caused by *S. aureus* becomes increasingly challenging due to its capacity to develop enhanced antibiotic resistance. In *S. aureus*, the cranberry extracts induced a transcriptional signature similar to that of peptidoglycan-acting antibiotics, while downregulating the *spa* genes encoding IgG binding protein and *capA-O* involved in the capsular polysaccharide biosynthesis (Diarra et al., 2013).

The chickens raised under intensive production are genetically very homogenous thereby increasing the concern for enabling highly productive meat-type chickens to build sufficient immune responses against bacteria during the rearing period (Koenen et al., 2002). Prophylactic measures such as vaccination and chemophrophylaxis have been used against infectious diseases. However, health and immune responses also require an adequate supply of nutritional components to the birds. Nutritional methods including addition of probiotics or natural additives have been previously investigated to modulate chicken immunity (Koenen et al., 2004; Bai et al., 2013; Palamidi et al., 2016).

While the broiler producers are under pressure to decrease or even eliminate the use of antibiotics as feed additives, researchers are facing a tremendous task to develop alternative approaches that can improve chicken health, production, and food safety by replacing or at least reducing the use of antibiotics as growth promoters (Buchanan et al., 2008). The present study was designed to determine the antioxidant and anti-inflammatory effects of non-dialyzable materials (NDMs) of cranberry extract, and its effect on phagocytosis and intracellular killing activity by phagocytic cells. Furthermore, the effect of NDMs on humoral immune response of the broiler chickens, which is important in broiler production, was also evaluated.

### MATERIALS AND METHODS

#### Non-Dialyzable Materials of Cranberry

NDMs of a commercial cranberry juice (Decas Botanical Synergies, Wareham, MA), were prepared by extensive dialysis using dialysis bags according to Shmueli et al. (2004). The dialysis process removed the molecules smaller than 12,000-molecular-weight from NDMs to concentrate high molecular-weight phenolic compounds. Phenolic contents were determined according to Harrison et al. (2013), using a Dionex, Ultimate 3000 high-performance liquid chromatography (HPLC) system (Bannockburn, IL) which consisted of a pump, auto sampler, column compartment, and photodiode array detector controlled with Chromelone 6.80 software. The separations were obtained using a Zorbax SB-C18 HPLC column (250 mm × 3.0 mm, 5 μm; Agilent, Palo Alto, CA) and a guard column of the same material (12.5 mm × 4.6 mm, 5 μm) at 35°C column temperature. The auto sampler was set at 15°C and the injection volume was 5 μL for standards and 20 μL for NDMs. The mobile phases consisted of 50 mM phosphoric acid (solvent A) and 100% methanol (solvent B) with a flow rate of 0.4 mL/min and a gradient of 5% B from 0 to 5 min, 5 to 55% B from 5 to 51 min; 51 to 100% B from 51 to 61 min, maintained at 100% B until 68 min then decreased 100 to 5% B from 68 to 73 min, and maintained at 5% B until 83 min. Samples and standards were prepared and diluted to 2.5 and 5 mg/mL in 50% methanol then filtered through glass fiber hydrophilic polypropylene (GHP) 13 mm acrodisc filters of 2 μM (Pall Life Sciences, Mississauga, ON, Canada).

Standards were purchased from multiple suppliers as follows: Epicatechin, chlorogenic acid, chrys, cinamic acid, cyanin chloride, ferulic acid, gentisic acid, em-coumaric acid, o-coumaric acid, p-coumaric acid, proteocatechuic acid, rutin, vanillin, and vanillic acid were purchased from Sigma Aldrich (St. Louis, MO). Kaempferol, keracayn chloride, and quercetin-3-β-D-glucoside were from Fluka (Buchs, Switzerland). Calstablein chloride, delphinidin chloride, ideain chloride, isoqueritrin, kaempferol-3-O-rutinoside, kuromanin chloride, pelargonidin chloride, and peonidin chloride were sourced from Extrasynthese (Genay Cedex, France). Ombuin, quercetagatin, quercetin-3,7-dimethyl ether, quercitin-3,7,3′4′-tetramethyl ether, quercitin-3-methyl ether, quercetin-4-methyl ether, quercitin-7-methyl ether, and quercitrin were obtained from Apin Chemicals Ltd. (Abingdon, UK). Methanol (HPLC grade) was obtained from Caledon and phosphoric acid was supplied by EMD (85% HPLC grade) and diluted with milli-Q water.

#### Antioxidant Activity

The antioxidant activity was determined by the PhotoChem instrument (Analytik Jena, USA Inc., Delaware, OH) using the standard “ACW” kit protocol provided by the manufacturer. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%, Sigma, St. Louis, MO) was used for a calibration standard rather than ascorbic acid (Prior et al., 2003; Oomah et al., 2010).

#### Anti-inflammatory Activity

The anti-inflammatory activity was assessed by measuring cyclooxygenase (COX) enzymes inhibition using a colorimetric COX (ovine) inhibitor screening kit (Cayman Chemical Co., Ann Arbor, MI) as previously described (Seeram et al., 2001; Oomah et al., 2010).
Briefly, NDMs solution was incubated with 0.1 M Tris-HCl buffer (pH 8), heme, and ovine COX-1 or COX-2, for 5 min at 25°C. Then \( N,N,N,N \)-tetramethyl-\( p \)-phenylenediamine (TMPD; Sigma) was added and the reactions were started with arachidonic acid (1.1 mM). Absorbance of the cleaved TMPD substrate was monitored at 590 nm with a spectrophotometer (SpectraMax Plus 384, Molecular Devices Corp., Sunnyvale, CA) after 5 min incubation. The inhibitor concentration versus percentage inhibition was plotted, and the 50% inhibitory concentration (IC\(_{50}\)) was determined by taking the half-maximal point along the isotherm and intersecting the concentration on the X-axis.

**Isolation of Heterophils**

Blood samples were collected by cardiac puncture from 100 four-d-old broiler chicks, and chicken heterophils (CHEs) were isolated from the pooled samples as described elsewhere (Genovese et al., 2006) with some modification. Briefly, blood samples were mixed with 1% methylcellulose (1:1 v/v) at 1:5:1 ratio and centrifuged at 50 × g for 15 min (lowest setting on centrifuge). The serum and buffy coat layers were retained and suspended in Hank’s balanced salt solution (HBSS CMF 1:1). This suspension was layered over a discontinuous Ficoll-Hypaque gradient (specific gravity 1.077 over specific gravity 1.119) 2:1:1. After centrifugation at 700 × g for 30 min the interface layer was removed and washed twice with RPMI 1640 and re-suspended in RPMI 1640. Viable cell was counted using a haemacytometer and 0.08% trypan blue. Cell viability was >95%. The cell concentration was adjusted to 2 × 10\(^6\) cells/mL.

**In Vitro Phagocytosis and Killing Assays**

The minimal inhibitory concentration (MIC) of the NDMs against three \( S. \) aureus strains SHY97-4320, ABB03-2426 and ABB03-2487 was predetermined to be 2 mg/mL by broth dilution method. Since the MIC against all three strains was similar, we chose \( S. \) aureus strain SHY97-4320 for further experiments. This strain was previously reported to be resistant to penicillin (Diarra et al., 2003). The effect of NDMs at 0, 1/2 MIC (1 mg/mL), MIC (2 mg/mL), and 2 × MIC (4 mg/mL) on the phagocytosis of \( S. \) aureus SHY97-4320 by heterophils from broiler chicks was evaluated for 2 h (120 min) according to Diarra et al. (2003). Overnight bacterial culture was re-suspended in RPMI 1640 medium (Sigma) at a final concentration of 2 × 10\(^7\) cfu/mL (confirmed by triplicate determination of viable bacterial counts). A volume of bacterial suspension (2 × 10\(^7\) cfu) was added to the heterophils (10\(^6\) cells/well) in RPMI 1640 containing 10% of heat inactivated chicken serum, and incubated at 37°C to allow phagocytosis in the presence or absence of different NDMs concentrations. Aliquots removed from each mixture every 30 min were quantified for viable bacteria present in the culture supernatants (Diarra et al., 2003).

Killing assay was performed as described elsewhere (Cuffini et al., 1993; Diarra et al., 2003) using \( S. \) aureus SHY97-4320 for the reason mentioned above. Briefly, mixture of \( S. \) aureus (2 × 10\(^7\) cfu) and 10\(^6\) heterophils in RPMI 1640 containing 10% of heat-inactivated chicken serum were incubated in the presence of different NDMs concentrations at 37°C for 30 min to allow phagocytosis. After 30 min, an aliquot of bacteria containing CHEs was withdrawn to determine viable bacteria (T0). The plates containing CHEs and \( S. \) aureus SHY97-4320 were incubated for 6 h, after which the number of surviving intracellular bacteria were determined by viable count (Tx). Killing was expressed as the survival index (SI) calculated as follows: SI = (T0 + Tx)/T0. When Tx = 0 then SI = 1, and bacterial killing was 100% effective. In contrast, when Tx ≥ T0, SI ≥ 2 indicating no killing and/or possible re-growth of the bacteria (Diarra et al., 2003). The phagocytosis and killing assays were performed in a triplicate experiment.

**In Vivo Broiler Chickens: Housing, Study Design and Treatment**

A total of 1,200 Ross 308 d-old male broiler chicks were obtained from a local commercial hatchery and were randomly assigned to 24 pens (50 birds per pen) at an experimental farm in British Columbia, Canada. Before placement, all chicks were visually examined for health and inferior chicks were excluded from the trial. The temperature and lighting program was similar to commercial practice. The starter and grower diets were formulated with wheat, barley, and corn as principal cereals, and meat and fish meal as protein concentrates (Leusink et al., 2010) to meet the National Research Council nutrient requirements for broiler chickens (NRC, 1994). All experimental procedures were approved by the Animal Care Committee of the Pacific Agri-Food Research Center and followed principles described by the Canadian Council on Animal Care (http://www.ccac.ca/en/standards/guidelines).

All birds were initially (on d 0) vaccinated against Marek’s disease and infectious bronchitis virus (IBV) as a routine practice at the hatchery. To evaluate the systemic antibody response, all birds were also vaccinated against infectious bursal disease virus (IBDV) vaccine S-706 (Canadian Poultry Consultants Ltd. Abbotsford, BC, Canada) by gavages (as recommended by the manufacturer) on d 7 of age. The birds were then randomly divided into four treatment groups with six replications (pens): a control untreated group, and 3 groups orally administrated once per d from d 7 to 11 (birds weighting approximately 148 g on d 7) with 2, 4 or 8 mg/mL/bird NDMs. Each bird in control group received 1 mL of drinking water. The tested concentrations were chosen to have approximately 0, 13.5, 27,
RESULTS

Composition of the Non-Dialyzable Materials (NDMs)

The pH values of 100 mg/mL of the used commercial cranberry juice and its NDMs solution were 4.1 and 4.5, respectively (not significantly different). While the commercial cranberry products contained phenolic compounds within reported range in the literature, the NDMs contained 8.7- to 13.6-fold higher levels of phenolic acids, tartaric esters, flavonols, and anthocyanins than the initial material (Table 1).

Nineteen phenolic compounds were identified in NDMs by high-performance liquid chromatography-mass spectrometry based on retention times and spectral matches to authentic standards. Quercetin was the main phenolic (976.6 µg/g of powder), followed by vanillic acid, protocatechuic acid, myricetin, cyanidin-3-O-arabinoside, peonidin-3-O-galactoside, cyanidin-3-O-arabinoside, cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, quercetin-3-methyl ether (ombuin), quercetin-3-D-galactoside, quercetin-4-methyl ether, malvin chloride, peonidin-3-O-glucoside, quercetin-3-B-D-glucoside, cyanidin-3-O-rutinoside, quercetin-3-O-rutinoside (rutin), kaempferol, and quercetin-3-O-rhamnoside (quercitrin) (Table 2). The order of anthocyanin abundance identified in NDMs from highest to lowest was cyanidin-3-O-arabinoside, peonidin-3-O-galactoside, peonidin-3-O-arabinoside, cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, malvin chloride, peonidin-3-O-glucoside, and cyanidin-3-O-rutinoside. Quercetin and myricetin were the most abundant identified flavonols, whereas vanillic and protocatechuic acids were the main phenolic acids. At least 24 unknown phenolic compounds were also observed in NDMs which predominantly consisted of phenolic acids and flavonols.

Statistical Analyses

Statistical analyses were conducted according to a randomized complete block design using the GLM procedure of SAS (2000) with treatment groups as sources of variations. Repeat measurement analyses were performed on the log_{10}-transformed data of bacterial numbers. Least significance difference was used to separate treatment means whenever the P-value was significant. The P-value of 0.05 was used to declare significance.

Antioxidant and Anti-inflammatory Activities

Antioxidant activity correlated well with the concentrations of phenolic compounds in the commercial cranberry juice and its NDMs. The commercial product had less than one-fifth the total antioxidant activity (oxygen radical absorbance capacity, ORAC = 39.6 vs. 222.7 mg trolox equivalent/g) than its NDMs (Table 1). Both products had higher antioxidant

and 54 mg/kg of body weight. No additional anticoccidials or antibiotics were administered to the birds throughout the trial. The birds were kept in the experimental farm until 35 d in the rearing conditions described above. General health, body weight, feed intake, feed conversion efficiency, and intestinal integrity of the broiler chickens were determined.

Blood Collection and Antibody Analyses

Blood samples (1 mL/bird) were collected from two birds per pen (12 birds/treatment) on d 7 (prior to vaccination and administration of NDMs), and thereafter 14, 21, 28, and 35 d of age. The blood samples were allowed to clot at room temperature and centrifuged. The sera were then transferred to sterile Eppendorf tubes and stored at −20°C prior to determination of total serum antibodies. Serum immunoglobulin levels of the broiler chickens were determined. General health, body weight, feed intake, feed conversion efficiency, and intestinal integrity of the broiler chickens were determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenolic acids</th>
<th>Tartaric esters</th>
<th>Flavonols</th>
<th>Anthocyanins</th>
<th>Antioxidant activity</th>
<th>pH</th>
<th>°Brix 100 mg/mL</th>
<th>°Brix 10 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial product</td>
<td>41.3</td>
<td>3.3</td>
<td>2.8</td>
<td>2.2</td>
<td>39.6 (158)</td>
<td>4.1</td>
<td>9.6</td>
<td>ND</td>
</tr>
<tr>
<td>NDMs</td>
<td>562.8</td>
<td>28.8</td>
<td>28.0</td>
<td>28.6</td>
<td>222.7 (891)</td>
<td>4.5</td>
<td>ND</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Results are expressed as µg equivalents of catechin, caffeic acid, quercetin, cyanidin-3-O-glucoside, or trolox per g of dried extract, respectively, for phenolic acids, tartaric esters, flavonols, anthocyanins, and antioxidant activity (ORAC). Values in brackets are trolox µM equivalents per g of powder. Samples were diluted 100 mg/mL for pH analysis, and 100 mg/mL and/or 10 mg/mL for °Brix.

ND = Not determined, ORAC = oxygen radical absorbance capacity.
Table 2. Concentrations of known phenolic compounds in non-dialyzable materials (NDMs) of cranberry extract.

<table>
<thead>
<tr>
<th>Identified compounds</th>
<th>Detection (nm)</th>
<th>Peak match</th>
<th>RT (min)</th>
<th>Peak area (mAu×min)</th>
<th>Concentration¹ (μg/g)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanidin-3-O-galactoside</td>
<td>520</td>
<td>938</td>
<td>29.7</td>
<td>0.824</td>
<td>122.3</td>
<td>15.5</td>
</tr>
<tr>
<td>cyanidin-3-O-glucoside</td>
<td>520</td>
<td>596</td>
<td>31.1</td>
<td>1.461</td>
<td>143.5</td>
<td>ND</td>
</tr>
<tr>
<td>cyanidin-3-O-arabinoside</td>
<td>520</td>
<td>889</td>
<td>32.3</td>
<td>1.870</td>
<td>278.0</td>
<td>16.1</td>
</tr>
<tr>
<td>cyanidin-3-O-rutinoside (keracyanin chloride)</td>
<td>520</td>
<td>744</td>
<td>32.5</td>
<td>0.268</td>
<td>26.4</td>
<td>1.5</td>
</tr>
<tr>
<td>peonidin-3-O-galactoside</td>
<td>520</td>
<td>327</td>
<td>34.8</td>
<td>0.512</td>
<td>62.9</td>
<td>47.8</td>
</tr>
<tr>
<td>peonidin-3-O-glucoside</td>
<td>520</td>
<td>658</td>
<td>35.9</td>
<td>1.151</td>
<td>182.7</td>
<td>33.4</td>
</tr>
<tr>
<td>malvin chloride (catechin like compound co-eluting)</td>
<td>520</td>
<td>615</td>
<td>29.0</td>
<td>0.589</td>
<td>65.4</td>
<td>75.6</td>
</tr>
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<td>65.4</td>
<td>75.6</td>
</tr>
</tbody>
</table>

¹Concentration calculated as mg equivalents of catechin, quercetin, or cyanidin-3-O-glucoside based on peak areas at 280, 360, or 520 nm respectively.

²Cyanidin-3-O-glucoside was only detected in one of five injections.

mAu = milli ampere units, ND = Not determined, RSD = Relative standard deviation, RT = Retention time.

Table 3. COX comparison of a commercial cranberry product and its non-dialyzable materials (NDMs).

<table>
<thead>
<tr>
<th>Sample</th>
<th>COX-I IC₅₀ (mg/mL)</th>
<th>COX-I Slope</th>
<th>COX-I Intercept</th>
<th>COX-II IC₅₀ (mg/mL)</th>
<th>COX-II Slope</th>
<th>COX-II Intercept</th>
<th>Inhibition range observed (%)</th>
<th>Ideal concentration range for IC₅₀ curves (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial product</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>&gt;10</td>
<td>ND</td>
<td>ND</td>
<td>0 to 50</td>
<td>0 to 30</td>
</tr>
<tr>
<td>NDMs</td>
<td>1.1</td>
<td>38.96</td>
<td>46.5</td>
<td>1.1</td>
<td>36.41</td>
<td>47.41</td>
<td>0 to 125</td>
<td>0 to 115</td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 to 5</td>
<td></td>
</tr>
<tr>
<td>Naproxen</td>
<td>0.01</td>
<td>5.36</td>
<td>77.87</td>
<td>1</td>
<td>8.63</td>
<td>49.68</td>
<td>50 to 95</td>
<td>30 to 80</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.3</td>
<td>10.90</td>
<td>63.43</td>
<td>3</td>
<td>19.51</td>
<td>27.18</td>
<td>20 to 95</td>
<td>0 to 90</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>6</td>
<td>25.74</td>
<td>3.33</td>
<td>11.5</td>
<td>20.89</td>
<td>16.09</td>
<td>10 to 95</td>
<td>0 to 75</td>
</tr>
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</table>

Values are expressed in mg/mL of 95% ethanol. Ten μL of solution (mg/mL) was placed in each well for analyses. Slopes and intercepts for log regression of y = m×ln(x) + b.

ND = not determined.

activities than grains corn, emmer, millet, wheat, rice, oats, rye, wheat germ, wheat bran, buckwheat, and most bean varieties (data not shown). In this study, the dialysis removed unwanted simple sugars and acids from the original product to generate more purified and potent phenol extracts with high antioxidant capacities. The raw commercial cranberry juice was a weak COX inhibitor with an IC₅₀ of 10 mg/mL for COX-I and an IC₃₀ of 10 mg/mL for COX-II. NDMs however, showed COX-I and COX-II IC₅₀ inhibitory activities of 1.1 mg/mL each which was in the range of Naproxen’s activity but lower than those of Ibuprofen (Table 3).

Thirty minutes after incubation of S. aureus and CHEs, no viable bacteria were found in the cell lysate of culture treated with 2 or 4 mg/mL NDMs, whereas bacterial population of 3.3 and 2.5×10⁵ cfu/mL were recovered from lysates of the control and culture treated with 1 mg/mL, respectively (Figure 1B). Data indicated that ¹/₂ MIC (1 mg/mL) NDMs significantly reduced (P < 0.05) the number of surviving intracellular S. aureus 6 h after phagocytosis compared to untreated CHEs. Our results suggest that NDMs could enhance S. aureus susceptibility to phagocytosis by CHEs was not significantly affected (P > 0.05) in the untreated control culture or cultures containing 1 (¹/₂ MIC) and 2 (MIC) mg/mL NDMs, but increased significantly (P < 0.05) in the presence of NDMs at 4 mg/mL (2 × MIC) in the culture medium.

Phagocytosis and Killing

None of the NDMs concentrations affected S. aureus SHY97-4320 growth after 2 h incubation without CHEs (data not shown). Susceptibility of S. aureus to phagocytosis by CHEs was not significantly affected (P > 0.05) in the untreated control culture or cultures containing 1 (¹/₂ MIC) and 2 (MIC) mg/mL NDMs, but increased significantly (P < 0.05) in the presence of NDMs at 4 mg/mL (2 × MIC) in the culture medium. After 2 h co-culture of S. aureus and CHEs, the bacterial counts were 6.3, 7.1, 6.3, and 10 to 95 cfu/mL in the presence of 0, 1, 2, and 4 mg/mL NDMs, respectively (Figure 1A). NDMs at 1 mg/mL had no effect (P > 0.10) on the number of staphylococci phagocytosed by CHEs after 60, 90, and 120 min.
immuno-defence mechanisms, and may be useful immunomodulators against infections.

Broiler Performance and Immunity Profile in a Chicken Model

Broiler Performance. The effects of 2, 4, and 8 mg/mL NDMs per bird for five d on general health and broiler performance were estimated. Body and intestinal weights, feed intake and efficiency of broiler chickens were generally higher in the treatment groups compared to control, however, these differences were not statistically significant ($P > 0.05$; data not shown).

Total Immunoglobulin. We evaluated the effects of various high molecular weight NDMs concentrations orally administrated on d 7 for five d on the humoral response of broiler chickens every week from d 7 until the end of trial on d 35. The antibodies titers were characterized by a high intra-sample variability. Regardless of the treatments, a remarkable inconsistent increase was observed in the total IgA and IgY antibodies concentrations in some treatment groups compared to control, throughout the trial period; however, treatment effect was insignificant ($P > 0.05$) on the levels of these two antibodies titers (data not shown). The two immunoglobulins increased systematically as the birds aged, except for IgY on d 35. Likewise, IgM concentration increased from d 7 to 21 and decreased thereafter.

On d 35, treatment effect was significant ($P = 0.04$) on IgM level (Figure 2). The sera from birds treated with 2 mg/mL/bird (223.9 μg/mL) and 4 mg/mL/bird (213.6 μg/mL) NDMs showed the highest IgM concentration on d 35, compared to the control untreated birds (177.9 μg/mL) and birds treated with 8 mg/mL/bird (158.1 μg/mL).

Antivirus Antibodies. Despite vaccination with IBDV vaccine on d 7, the antibodies against this virus gradually decreased from d 14 to 35, with no significant response of birds to this vaccine. This may be due to the quality of our vaccine preparation and/or the neutralization by maternal antibody titers. However, on d 35 birds receiving 8 mg/mL/bird NDMs tended to have the highest anti-IBDV antibody concentration ($P = 0.06$) compared to birds from the control and other treatments (Figure 3A). As compared to the control, birds receiving 2 and 8 mg/mL/bird NDMs showed the highest anti-IBV concentration on d 14 and 21, whereas those receiving 4 mg/mL/bird NDMs exhibited the highest antibody titers against this virus on d 28 and 35 ($P > 0.05$; Figure 3B). The serum antibody levels against NDV and ARV decreased steadily with the bird’s age, except for anti-ARV on d 35 (Figure 3C,D). On d 14, the antibody titers against these two viruses were higher in the control birds followed by the birds treated with 8 mg/mL/bird NDMs. The highest anti-ARV concentrations observed in birds receiving 2 and 8 mg/mL/bird NDMs compared to control birds and the birds treated with 4 mg/mL/bird NDMs on d 28 and 35, but these differences were not significant ($P > 0.05$).

DISCUSSION

Cranberry is reported to have antibacterial, antiviral, antimutagenic, anticarcinogenic, antitumorigenic, antiangiogenic, antioxidant, and anti-inflammatory activities associated with its phytochemical contents (Wu et al., 2004; Johnson-White et al., 2006; Côté et al., 2010; Blumberg et al., 2013; Diarra et al., 2013;
Rodriguez-Pérez et al., 2016). In the present study, phenolic acids, tartaric acids, flavonols, and anthocyanin contents of NDMs were higher than those previously reported from cranberry juice and pomace (Harrison et al., 2013), suggesting our success in enriching active compounds by dialysis. Numerous researchers described cranberries to contain a relatively high content of total phenolics, and have been shown to possess a high antioxidant capacity (Wang and Stretch, 2001; Sun et al., 2002; Zheng and Wang, 2003; Ehala et al., 2005). All anthocyanins, except cyanidin-3-O-rutinoside and malvin chloride, detected in this study were also identified by Labrecque et al. (2006) in cranberry NDMs. In our NDMs, quercetin and myricetin were the most abundant flavonols in accordance with previous reports (White et al., 2010; Blumberg et al., 2013). Thus the composition of NDMs used in this study demonstrates that it could offer promising new approaches in promoting health.

The NDMs used in the present study showed >9-fold higher antioxidant activity than the value reported by Wu and colleagues (2004) probably due to its very high anthocyanin, flavonol, and phenolic acid contents (Wang et al., 1997; Wang and Stretch, 2001; Pappas and Schaich, 2009). Berry antioxidant activities may be positively correlated with activities of antioxidant enzymes which include superoxide dismutase, per-oxidase, catalase, and glutathione peroxidase (Wang, 2011). These enzymes neutralize free radicals created by oxidative stress, and thus protect cells from the damage caused by reactive oxygen species (Heinonen et al., 1998). However, one should keep in mind that total antioxidant capacity, as measured by ORAC or any other in vitro methods, may not reflect the in vivo antioxidant effects. Thus the in vivo antioxidant activities of NDMs in our study remained to be established.

The cyclooxygenase enzymes (COX-I and COX-II) are involved in the conversion of arachidonic acid to prostaglandins, which evoke the physiological response of inflammation (Meade et al., 1993). In the present study, NDMs exhibited a stronger inhibitory effect (IC₅₀ = 1.1 mg/mL) on both COX-I and COX-II compared to its commercial cranberry product and Ibuprofen (Table 3). Seeram et al. (2001) reported that antioxidant and COX inhibitory activities increased with a decreasing number of sugar residues attached to the cyanidin moiety (anthocyanin 1 to 2). This study used cranberry NDMs where most of the low molecular-weight simple sugars were removed by dialysis to generate potent phenol extracts with high antioxidant capacities, which may make NDMs even more effective on COX activities (Bodet et al., 2006). Given that antioxidant and anti-inflammatory activities of cranberry are associated with polyphenols capable of inhibiting COX...
enzymes, our results raise the possibility of using NDMs to protect against chronic inflammatory diseases. However, additional research is required to identify the specific bioactive component/s of our cranberry NDMs in order to optimise its health benefit.

When an infection occurs in birds, heterophils (CHEs) are among the first line of defense involved in a series of intra- and extra-cellular antimicrobial mechanisms. The CHEs enable phagocytosis, bacterial killing and limited oxidative burst (lack of myeloperoxidase) and degranulation. Like its equivalent neutrophils in livestock, the CHEs have been reported to form extracellular traps against invading microorganisms (Chuanmitriti et al., 2009). The effectiveness of dialyzed cranberry juice extracts (MW 6–8000) against different pathogenic bacteria including S. aureus has earlier been revealed by Johnson-White and coauthors (2006). Yet the limited scope of current knowledge in heterophil responses during exposure to immunomodulators prompted us to perform an in vitro study of CHEs defense mechanisms against S. aureus using NDMs as an immunomodulatory agent. Our results showed that 4 mg/mL NDMs significantly ($P < 0.05$) enhanced S. aureus SHY97-4320 phagocytosis by CHEs, while 1 mg/mL significantly ($P < 0.05$) killed intracellular phagocyted S. aureus (Figure 1A,B). This is probably due to interference of NDMs constituents with S. aureus resistance such as reduced expression of IgG binding protein and capsular polysaccharide biosynthesis (Diarra et al., 2013). Our results further demonstrate that NDMs potentiates CHEs function coinciding with data reported on the decreased early mortality in broiler by cranberry products (Leusink et al., 2010). To our knowledge, this is the first documented report describing NDMs effects on phagocytosis and killing of S. aureus by CHEs.

Consumers generally seek reduction or the elimination of non-therapeutic antibiotic use in animal production. Consequently, there is an urgent need to develop more natural approaches to improve birds’ immunity against pathogenic microorganisms in poultry. In a previous study, we suggested that cranberry derivatives could be developed to improve poultry chicken health and on-farm safety while reducing the use of antibiotics as growth promoters (Leusink et al., 2010). This study investigated 2, 4, and 8 mg/mL/bird NDMs on the humoral immunity of broilers from d 14 to 35. Broiler chickens are reported to generate a good IgM, but a poor IgY response (Neu et al., 1984; Koenen et al., 2002), which is inconsistent with the present study as we observed very high IgY level compared to other two isotypes of immunoglobulins upon NDMs administration. The antibodies titers were characterized by high intra-sample variability. Regardless of the treatments, IgA and IgY antibodies titer increased significantly ($P < 0.05$) with no significant treatment effect on the level of these two antibodies titers. The serum from birds treated with 2 or 4 mg/mL/bird NDMs showed the highest concentration of IgM on day 35 compared to control birds and those receiving 8 mg/mL/bird NDMs, suggesting that NDMs could modulate this serum immunoglobulin production in chickens. Nevertheless, the reason for detecting a relatively low level of IgM in this work is not known as IgM response could be associated with various factors such as type and age of bird, flock size, housing type, and temperature of the house, ventilation and light management etc. (Wit et al., 2010). It would be interesting to evaluate immunoglobulin concentrations beyond 35 d, however, broilers in British Columbia are usually slaughtered at around 35 d of age. One of the explanations for the lack of substantial treatment effects may be due to the short duration (five d) of application. Longer treatment duration might be effective in improving humoral response of birds, however, more studies in both broilers (organic chickens raised beyond 35 d) and layers (up to one year) are warranted to investigate the full prospect in vivo effects of NDMs on the immunity of poultry.

Despite considerable efforts to control infectious diseases, the IBDV, IBV, NDV, and ARV viruses continue to be important health issues for broilers (Hoerr, 2010). The antibody titers against IBDV in response to vaccination have been used to measure the humoral responses (Eterradossi and Saif, 2013). In this study, the effect of NDMs on antibody concentration against IBDV tended to be significant on d 35 ($P = 0.06$). Our results noticeably showed NDMs ability to stimulate humoral immunity and demonstrated the likelihood of using this cranberry constituent as a vaccine adjuvant in poultry production. NDMs administration displayed some treatment effects on the anti-IBV titers compared to the control birds throughout the trial, particularly on d 35. However, these effects were not statistically significant ($P > 0.05$) probably due to high variabilities. The titer values against NDV and ARV were higher in 14 d old control chickens than those aged 21 d and older, suggesting the transfer of maternal antibody from parents that fades from the circulation after three weeks. Overall, these results of antibody titer increase against the tested viruses in the treatment groups over the control offer the possibility of using NDMs as an immune-enhancing agent. Our data show for the first time that NDMs of cranberry can modulate humoral response of birds against pathogenic viruses.

The results showed that the high phenolic contents and antioxidant activity of cranberry NDMs may potentially contribute to the control of infections. However, the mechanisms by which NDMs significantly enhance phagocytosis and killing of the pathogenic bacteria such as S. aureus need to be determined. Our data also demonstrated that the short-term NDMs administration in early life enhanced serum immunoglobulins levels which could improve humoral responses against pathogenic viruses in broiler chickens. Cranberry products including NDMs would be useful in poultry production due to their potential promotion of bird’s health. Overall, the present data show for the first time that cranberry constituents can modulate
humoral responses of birds, and that further investigations on cranberry bioactives as a stimulator of immunity in broilers are warranted.

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