

## Supplementation of diets with bovine colostrum influences immune function in dogs

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(Submitted 6 June 2012 – Final revision received 2 May 2013 – Accepted 2 May 2013 – First published online 18 June 2013)

### Abstract

While the need for colostrum in neonates is well established, the systemic effect of feeding bovine colostrum (BC) to adult humans is gaining increasing attention. However, no systematic studies evaluating the immunomodulatory effect of BC in dogs have been reported. The aim of the present study was to evaluate the immunomodulatory effect of dietary supplementation of BC in dogs. The study was conducted in two phases: pre-test (8 weeks) and test (40 weeks), with twenty-four dogs (mean age 2.5 years) randomised into two groups. In the 'pre-test' phase, both groups were fed a nutritionally complete diet. At the end of the 'pre-test' phase, all dogs received a canine distemper virus (CDV) vaccine, and dogs in the 'test group' were switched to a diet supplemented with 0.1% spray-dried BC. Response to the CDV vaccine was evaluated by measuring vaccine-specific plasma IgG levels. Gut-associated lymphoid tissue response was assessed by measuring faecal IgA levels. Gut microbiota were evaluated by the temporal temperature gel electrophoresis methodology. Dogs fed the BC-supplemented diet demonstrated a significantly higher vaccine response and higher levels of faecal IgA when compared with the control group. Supplementing diets with BC also resulted in significantly increased gut microbiota diversity and stability in the test group. In conclusion, diets supplemented with BC significantly influence immune response in dogs.

**Key words:** Bovine colostrum; Nutritional immunology; Immunity; Gut health; Exercise

Colostrum (early milk produced during the first few days after parturition) not only meets the unique nutritional needs of neonates, but also transfers passive immunity and promotes the growth and development of the gastrointestinal tract<sup>(1,2)</sup>. While the need for colostrum in neonates is well established, the systemic effect of feeding BC orally to adult humans is gaining increasing attention<sup>(3)</sup>. Bovine colostrum (BC) contains several bioactive components<sup>(4)</sup>, including growth factors such as insulin-like growth factor-1, insulin-like growth factor-2, transforming growth factor  $\beta$  and epidermal growth factor, antimicrobial compounds such as lactoferrin, and immunomodulatory compounds such as Ig, transferrin and cytokines. The presence of these closely homologous bioactive ingredients in BC has led to its use in the treatment and prevention of diseases in humans and animals<sup>(2,5)</sup>. In several studies, BC has been shown to be effective in treating gastrointestinal disorders (for a review, see Playford *et al.*<sup>(6)</sup>) as well as helping athletes in endurance and speed training<sup>(7,8)</sup>. In human trials, BC containing specific antibodies has also been shown to be effective against enteropathogenic and enterotoxigenic *Escherichia coli*<sup>(9,10)</sup>, *Cryptosporidium*<sup>(11)</sup>,

*Helicobacter pylori*<sup>(12)</sup>, rotavirus<sup>(13–15)</sup> and *Shigella flexneri*<sup>(16)</sup>. However, no studies evaluating the immunomodulatory benefits of BC in dogs have been reported. The aim of the present study was to evaluate the immunomodulatory effect of BC in dogs.

### Materials and methods

#### Animals and diets

A total of twenty-four adult dogs (Husky crosses, 2–7 years, mean 2.5 years) were randomised into two groups balanced for sex, age and faecal IgA (which was used as a marker to evaluate immune status) and were fed one of two rations during the test phase of the trial. Dogs consumed fresh water *ad libitum* and were housed and fed individually. Dogs were exercised 3 d a week during the 40-week study, using a standard exercise protocol as part of their normal routine. Each exercise session involved sprint-racing as part of a team, in which dogs in harness pulled an unladen sledge for distances starting at 3 miles and gradually increasing to 14 miles per session. The trial protocol was conducted in

**Abbreviations:** BC, bovine colostrum; CDV, canine distemper virus; CRP, C-reactive protein; GALT, gut-associated lymphoid tissue; TTGE, temporal temperature gel electrophoresis.

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strict accordance with the guidelines established by the Nestlé Purina Pet Care (NPPC) Advisory Committee.

The trial was conducted in two phases: 'pre-test' (8 weeks) and 'test' (40 weeks). During the 'pre-test' phase, both groups were fed a commercial, nutritionally complete and balanced extruded dry dog food 'control diet' (Nestlé Purina product: approximately 29% protein, 36% carbohydrate, 19% fat and 1.4% fibre; metabolisable energy 16 292.4 kJ/kg (3894 kcal/kg)). At the end of the 'pre-test' phase, all dogs received a canine distemper virus (CDV) booster vaccine (Intervet Pro Progard-5 Vaccine, Canine Distemper–Adenovirus Type 2–Parainfluenza–Parvovirus Vaccine) as part of normal veterinary care. Dogs in the control group continued to be fed the 'control diet', while dogs in the test group were fed the 'control diet' supplemented with 0.1% commercially obtained spray-dried BC (Sterling Technology, Inc.). Dogs were fed their respective diets until the end of the study. Food intake was measured daily. At the conclusion of the trial, dogs were switched to a maintenance diet.

Every 4 weeks, 5 ml of jugular blood samples were collected (using BD Vacutainer™ with sodium citrate as the anticoagulant; Becton & Dickenson). To obtain plasma, blood samples were centrifuged at 10 000 rpm for 10 min at 6°C, and plasma was stored at –80°C until assayed for immune markers. Faecal samples were processed every 4 weeks and immediately stored in a –80°C freezer. Faecal scores were recorded daily during the trial using a seven-point scale with a score of 1 representing firm, hard faeces and a score of 7 representing liquid diarrhoea. On this scale, scores of 2 or 3 are ideal (Table S1, available online). Body weights of the dogs were recorded weekly.

#### *Measurement of antibodies in plasma*

Response to the CDV vaccine was evaluated by measuring CDV vaccine-specific IgG levels using a calibrated ELISA. Briefly, a ninety-six-well plate was coated overnight at 4°C with CDV antigen (VMRD, Inc.) in borate buffer (pH 7). Free binding sites were blocked with PBS containing 5% fetal calf serum and 0.1% Tween (ELISA buffer) for 2 h at 37°C. Plasma samples were placed in the wells and incubated for 2 h at 37°C, followed by several washes with PBS–0.1% Tween. Horseradish peroxidase-conjugated rabbit anti-canine IgG (Bethyl Laboratories, Inc.) diluted in ELISA buffer was applied to the plate, and the plate was incubated for 1 h at 37°C and then washed with PBS–0.1% Tween. Finally, colour development was done with 50 µl of the 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate system (KPL, Inc.) according to the manufacturer's instructions. The reaction was stopped with 50 µl of 1 M-H<sub>2</sub>SO<sub>4</sub>. Colour development was read at 450 nm, and results are expressed as µg/ml using a canine IgG standard.

#### *Measurement of antibodies in the faecal samples*

The effect of the test diet on the gut-associated lymphoid tissue (GALT) was assessed by evaluating secretory IgA levels in the faecal samples. Using 1.5 ml of the extraction

buffer (50 mM-EDTA and 100 mg/l soybean trypsin inhibitor in PBS/1% bovine serum albumin from Sigma), 0.5 g of faeces were vortexed. Phenylmethanesulphonyl fluoride (50 µl, 350 mg/l from Sigma) was added to each tube, and the samples were centrifuged at 10 000 g for 20 min. The supernatants were collected and frozen at –80°C until assayed for IgA by ELISA as follows: a ninety-six-well plate was coated overnight at 4°C with a 1:100 dilution of mouse anti-canine IgA (Serotec) in 50 µl of borate buffer (6.2 g H<sub>3</sub>BO<sub>3</sub>/l, 9.54 g Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O/l and 4.4 g NaCl/l, pH 7) and then washed with PBS–Tween-20. Free binding sites were blocked with 100 µl of PBS containing 5% fetal calf serum and 0.1% Tween-20 (ELISA buffer) for 1 h at 37°C. Duplicate faecal extracts were incubated with ELISA buffer (final volume 50 µl) for 2 h at 37°C and then washed with PBS–Tween-20. The plate was incubated with a 1:10 000 dilution of polyclonal goat anti-canine IgA conjugated with horseradish peroxidase (Serotec) in ELISA buffer (final volume 50 µl) for 1 h at 37°C, washed with PBS–Tween-20 and developed with 50 µl of the TMB peroxidase substrate system (KPL, Inc.) according to the manufacturer's instructions. The reaction was stopped with 50 µl of 1 M-phosphoric acid. Colour development was read at 450 nm, and results are expressed as µg/ml using a canine IgA standard.

#### *Measurement of C-reactive protein*

C-reactive protein (CRP) was measured as a general marker of inflammation to confirm that immune enhancement was not a result of or did not lead to a generalised inflammatory condition. Plasma CRP levels were measured in all dogs towards the end of the trial using a canine CRP kit (BD Canine CRP ELISA Kit; BD Bioscience) according to the manufacturer's directions.

#### *Measurement of the effect of exercise on gut microbiota*

During week 38, following a 2 d rest period, all dogs participated in a standard exercise protocol. In this event, dogs sprint-raced 10 miles in harness as part of a team pulling an unladen sledge. Each team contained roughly equal numbers of test and control dogs. The time to perform the task averaged 33 min and 30 s; there was less than a 30 s difference between the slowest team and the fastest team. The effect of BC on gut microbiota and its stability in response to exercise were evaluated by profiling changes in the gut microbiota. Rectal swabs were collected from the dogs 24 h before ('pre' sample) and 24 h after exercise protocol for temporal temperature gel electrophoresis (TTGE) (see below) assays. TTGE assays evaluate microbial profiles that consist of amplification products of the bacterial 16S rRNA gene, which are separated on the basis of sequence differences and yield a pattern of bands (gut microbiota profile). Each band, in theory, consists of one distinct 16S rRNA gene sequence and represents a major bacterial species of the gut microbiota (or very closely related species). 'Pre' samples collected before exercise were used to characterise baseline species diversity and 'evenness' (see below)<sup>(17,18)</sup>. The effect of exercise on

gut microbiota was evaluated by assessing the per cent similarity of 'pre'- and 'post'-exercise TTGE profiles. Similarity scores (see below) of the test groups were compared with those of the control group.

### Temporal temperature gel electrophoresis

**DNA extraction and purification for temporal temperature gel electrophoresis.** Genomic DNA from the rectal swabs was obtained using a modified extraction method described by Tsai & Olson<sup>(19)</sup>. Rectal swabs were collected, and 0.5 g of faecal sample were suspended in 1.5 ml of PBS (0.85% NaCl and 120 mM-NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0), and after three freeze-thaw cycles, 1.5 µl of Proteinase K (Fisher) were added to each sample (final concentration 50 µg/ml) and incubated in a 37°C shaking water bath for 30 min with constant agitation. DNA was precipitated by adding 400 µl of ice-cold isopropanol (Sigma) containing 96 µl of 10.5 M-ammonium acetate (1.125–1.26 M final concentration) to 400 µl of the supernatant.

**Temporal temperature gel electrophoresis.** TTGE was performed using a Bio-Rad D-Code system™. PCR fragments were separated on a 10% polyacrylamide denaturing gel (7 M-urea). Running buffer was 1.25 × TAE buffer (50 mM-Tris acetate, pH 7.4, 25 mM-sodium acetate and 1.25 mM-Na<sub>2</sub>EDTA). Separation was accomplished using a temperature gradient ramping from 59 to 69°C at a rate of 0.6°C/h. Electrophoresis was performed at 50 V and lasted for 16 h 20 min. Bacterial standard ladders were constructed by individually PCR amplifying DNA extracted from predominant intestinal strains and combining the PCR products. Primers used for the construction of the ladders were labelled with 6-carboxyfluorescein. The ladders were loaded together with the sample in each lane and were used to map the gel contours and correct for differences in the length of migration within and among the gels. Gel images were captured using a Hitachi Fluorometer and digitised using the FMBIOI (version 1.1) software (Hitachi). Digitised images were analysed using the GelCompar II (version 3.0) gel analysis software (Applied Maths). Band classes were established, and band densities (on height and band surface) within each class were tabulated.

**Species diversity.** Molecular profiles of baseline samples (collected 24 h before exercise) were used to estimate the species diversity of gut microbiota. The Shannon–Wiener index of species diversity was used for examining overall community characteristics<sup>(20,21)</sup>. The Shannon–Wiener index starts at '0' representing a single species and keeps increasing with a higher number representing a greater diversity.

### Statistical analysis

Repeated-measures ANOVA was used to test for overall differences between the groups on all measures. Dunnett's test was used to adjust for multiple comparisons with the control group. Data are presented as means with their standard errors. For all tests, the level of significant difference was set at  $P < 0.05$ .

## Results

### General physiological status

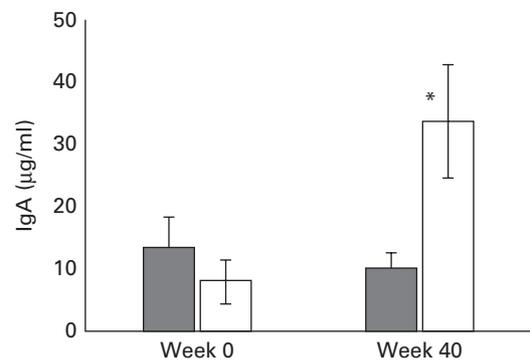
At the start of the trial, average body weights of dogs in the test group and the control group were 20.3 (SEM 0.7) and 20.7 (SEM 0.9) kg, respectively. Food intake (data not shown) and body weights did not differ between the two groups during the trial. No significant differences in faecal scores were observed between the control group and the test group (data not shown). Diets did not have an impact on blood cell counts and blood chemistry (data not shown). Levels of CRP, a marker of inflammation, measured towards the end of the trial were well within the normal range (0.8–16.4 µg/ml)<sup>(22)</sup> in both the groups (4.42 (SEM 1.09) µg/ml in the test group; 3.69 (SEM 1.81) µg/ml in the control group,  $P=0.74$ ).

### Immune response in the gut

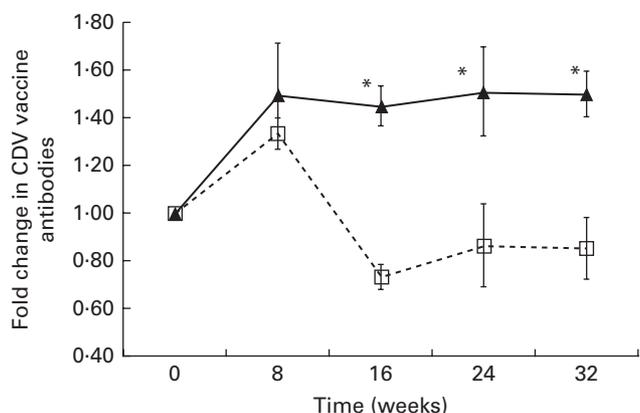
IgA was extracted from the faecal samples and assayed using a modified ELISA, and its concentration is expressed as µg/ml. As can be seen in Fig. 1, dogs fed the BC-supplemented diet had higher faecal IgA levels when compared with those fed the control diet ( $P < 0.05$ ).

### Response to canine distemper virus vaccine

All dogs were administered a CDV booster vaccine at week '0' before the 'test' phase of the trial. Blood samples were analysed every 8 weeks for CDV vaccine-specific IgG levels and data were normalised by dividing each value by the baseline value obtained at the start of the 'test' phase to eliminate dog-to-dog variation. As can be seen in Fig. 2, CDV antibody concentration gradually rose following the administration of the booster vaccine and reached a peak in 8 weeks in both the groups (indicating a response to the vaccination). In the control group, it declined to baseline values 16 weeks after the administration of the booster vaccine. However, in dogs fed the diet supplemented with BC, CDV antibody levels did not decline, but remained high until the end of the trial.



**Fig. 1.** Total IgA levels in the faecal samples collected at weeks 0 and 40 from dogs fed diets with or without bovine colostrum. Values are means, with their standard errors represented by vertical bars ( $n$  12). \* Mean values were significantly different from that of the control group ( $P < 0.05$ ). ■, Control; □, colostrum.



**Fig. 2.** Fold change over baseline in specific anti-canine distemper virus (CDV) IgG levels in plasma samples collected at weeks 0, 8, 16, 24 and 32 from dogs fed diets with or without bovine colostrum. Values are means, with their standard errors represented by vertical bars ( $n$  12). \*Mean values were significantly different from those of the control group ( $P < 0.05$ ). -□-, Control; -▲-, colostrum.

Dogs fed the diet supplemented with colostrum exhibited a significantly higher vaccine response when compared with the control group ( $P < 0.05$ ).

### Species diversity

Molecular profiles of baseline samples (collected 24 h before exercise) were used to estimate the species diversity of gut microbiota. Dogs fed the BC-supplemented diet (3.01 (SEM 0.11),  $P < 0.05$ ) showed higher Shannon–Wiener index values (i.e. a greater species diversity in their gut microbiota) when compared with those fed the control diet (2.64 (SEM 0.12)).

### Effect of bovine colostrum on gut microbiota

When the ‘pre-exercise microbiota pattern was compared with the ‘post-exercise microbiota pattern, dogs fed the diet supplemented with BC had increased gut microbiota stability (82 (SEM 13.1)% similarity *v.* 46 (SEM 5.2)% similarity in the control group,  $P < 0.05$ ).

### Discussion

The present study shows for the first time that feeding a complete and balanced diet supplemented with BC enhances immune response as well as increases gut microbiota diversity and stability in dogs. No detectable side effects were observed in the dogs, as the BC-supplemented diet did not have an impact on food intake, body weight and blood composition including CRP, a marker of inflammation. A previous publication by Giffard *et al.*<sup>(23)</sup> has reported gut health benefits of BC, showing that puppies fed diets supplemented with BC demonstrated improved faecal scores when placed in a new environment that favoured stress-related diarrhoea. However, no immune benefits or other gut health benefits were documented in their study.

To study the effect of colostrum supplementation on the GALT, we measured IgA levels in the faecal samples. IgA, a key protein produced by the GALT, has an important protective role in the gut, helping to prevent microbial adherence and colonisation, blocking viral adhesion and neutralising toxins<sup>(24–26)</sup>, and is hence used to show enhanced GALT activity. While at the outset both the groups exhibited similar levels of faecal IgA, at 40 weeks, dogs fed the diet supplemented with BC had significantly higher faecal IgA levels ( $P < 0.05$ ; Fig. 1). These data show that BC supplementation enhanced GALT function, resulting in higher IgA production, suggesting that these dogs would experience a higher level of protection from gut pathogens.

Dogs included in the study were routinely vaccinated with CDV vaccine as part of their normal veterinary care. We, therefore, decided to measure the effect of feeding diets supplemented with BC on the systemic immune status, using CDV vaccine response as a marker. All dogs were administered a CDV booster vaccination immediately before the ‘test phase’ of the trial, and their response to the CDV vaccine was measured every 2 months during the trial and normalised to their baseline levels to eliminate dog-to-dog variability. There was a statistically significant increase ( $P < 0.05$ ; Fig. 2) in specific anti-CDV IgG levels in dogs fed the diet supplemented with BC. These results suggest that the diet supplemented with BC increased priming of B-cell response to CDV vaccination. This enhanced response to CDV vaccine may enhance the effectiveness of the vaccine in preventing CDV infections. It has been shown previously that CDV-specific antibodies are very effective in neutralising extracellular CDV and preventing the intercellular spread of the virus *in vitro*<sup>(27)</sup>. Vaccine responses demonstrate clinically relevant alterations in an immune response to a challenge under well-controlled conditions and therefore are often used as a surrogate for responses to an infectious challenge. In human studies, for example, individuals who respond poorly to vaccines have greater susceptibility to infectious agents when compared with those with better vaccine responses<sup>(28,29)</sup>. These data suggest that dogs fed a diet supplemented with BC are likely to respond better to vaccines that are administered as part of routine veterinary care and will, therefore, be more effective in protecting themselves from naturally occurring infectious agents.

In addition to the immune measures, plasma samples were assayed for CRP. CRP is an acute-phase protein that is produced by the liver in response to inflammation<sup>(30)</sup>. CRP is normally measured during routine clinical examination to rule out any ongoing inflammation in the subject, in both human and veterinary medicine<sup>(31)</sup>. Normal levels of plasma CRP in dogs are between 0.8 and 16.4  $\mu\text{g/ml}$ <sup>(22,32)</sup>. Dogs suffering with inflammatory conditions tend to have much higher levels. For example, in a published study, it has been observed that dogs with pancreatitis had CRP levels of 56.4  $\mu\text{g/ml}$ <sup>(33)</sup>. Clearly, all dogs in the study had CRP levels well within the normal range, showing that BC did not negatively influence the immune system.

In addition to the immune measures, we also evaluated the effect of BC on gut microbiota. While enhanced mucosal and

systemic immunity is likely to promote healthy gut microbiota, natural antibodies and other bioactives in the colostrum have been shown to help balance beneficial and potentially harmful intestinal bacteria<sup>(24)</sup>. We characterised the gut microbiota using the Shannon–Wiener index. The Shannon–Wiener index is a widely used species diversity index for examining overall community characteristics<sup>(20,21)</sup>. Species diversity is an expression of the number and variety of species found in a given microbial community. A community has a high species diversity if many equally or nearly equally abundant species are present. Species diversity is used as a measure of community stability in which a low or changing species diversity may indicate a stressed or unstable environment<sup>(34)</sup>. Establishment of mucosal and/or luminal colonisation is the first step in the pathogenesis of many gastrointestinal bacterial pathogens. The pathogen must be able to establish itself in the face of competition from the complex microbial community that is already in place. A greater level of species diversity reduces the opportunity for potential pathogens to colonise the gut<sup>(35)</sup>. The Shannon–Wiener index starts at ‘0’ representing a single species and keeps increasing with a higher number representing a greater diversity. Dogs fed the BC-supplemented diet showed higher Shannon–Wiener index values when compared with those fed the control diet, showing that the BC-supplemented diet encouraged greater gut microbiota species diversity. This suggests that dogs fed diets supplemented with BC would potentially be able to better resist infections or colonisation by gut pathogens.

Stress, both physical and mental, has a significant negative impact on the immune system, irrespective of age<sup>(36)</sup>. The health of the immune system can be evaluated by how subjects respond to exercise<sup>(17,18)</sup> and can be helpful in assessing how they would respond to stress. The immune system plays an important role in the maintenance of a healthy gut microbiota balance<sup>(37)</sup>. Exercise can temporarily lower immune status, and this is often reflected in changes in the gut microbiota. A subject with a healthy immune system is able to prevent this drift in the gut microbiota, and this can be used as an indirect measure of the health of the immune system. At 38 weeks, all dogs participated in an exercise protocol involving a 2 d rest period followed by a 10-mile sledge run. The effect of BC on gut microbiota diversity and stability during exercise was evaluated by TTGE profile changes in the gut microbiota. The effect of exercise on gut microbiota was measured by assessing the per cent similarity of the ‘pre’- and ‘post’-exercise TTGE profiles. Similarity scores of the test groups were then compared with those of the control group. Dogs fed the diet supplemented with BC exhibited much greater similarity between the ‘pre’- and ‘post’-exercise microbial patterns when compared with those fed the control diet ( $P < 0.05$ ). Dogs fed the BC-supplemented diet handled the ‘exercise challenge’ significantly better than the non-supplemented dogs as reflected in their ability to prevent changes in microbiota after exercise. Clearly, supplementing diets with BC enabled this group of dogs to handle ‘exercise challenge’ better when compared with the control group.

In conclusion, the results reported in the present study show that diets supplemented with BC influence immune

response in dogs at both the mucosal and systemic levels and enhance gut microbiota diversity and stability. These findings could be relevant for improving protective immune and gut responses to various stress factors including infections.

### Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S000711451300175X>

### Acknowledgements

The present study was entirely funded by Nestlé Purina Research. The authors acknowledge Amy Dunlap and Yvonne Leavitt in supporting the feeding study, Julie Spears for helping analysing the microbiota data and Wendell Kerr for the statistical analysis. E. S. and A. R. designed the study, interpreted the results and prepared the manuscript. P. Z. helped with the initial study design. R. P., J. L. and P. S. carried out the assays described in the study. E. S. and A. R. had primary responsibility for the final content. All authors read and approved the final manuscript. None of the authors has any conflicts of interest associated with the present study.

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