

Repeated oral administration of lipopolysaccharide from *Escherichia coli* 0111:B4 modulated humoral immune responses in periparturient dairy cows

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Abstract

The objective of this study was to evaluate the effects of repeated oral exposure to LPS on humoral immune responses of periparturient dairy cows. Sixteen Holstein cows were assigned to two treatment groups 2 wk before the expected day of parturition. Cows were administered orally, twice weekly at wk –2, –1 and +1 around parturition, with the following treatments: 3 ml saline; or 3 ml of saline containing LPS from *Escherichia coli* 0111:B4. The amount of LPS administered during wk –2, –1, and +1 was 0.01, 0.05, or 0.1 µg/kg body weight, respectively. Multiple blood samples were collected by jugular vein and various immune and clinical variables were measured. Results indicated that, on one hand, concentrations of plasma IgG anti-LPS Abs decreased ($P < 0.01$) and those of IgM anti-LPS Abs increased ($P < 0.01$) in cows treated with oral LPS. On the other hand, there were no overall differences ($P > 0.05$) in the concentrations of serum amyloid A, LPS-binding protein, haptoglobin, cortisol, IgA anti-LPS Abs in the plasma, feed intake, body temperature and rumen contractions rate between the control and treatment groups. To our knowledge, this study is the first to show that repeated oral administration with LPS from *E. coli* 0111:B4 has the potential to stimulate humoral immune responses in periparturient dairy cows.

Keywords

Dairy cow, lipopolysaccharide, oral immunization, immunoglobulin, acute phase response

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Introduction

During the periparturient period, dairy cows experience a state of immunosuppression.¹ The mechanism(s) behind immunosuppression in periparturient dairy cows are not well understood yet; however, an altered immune responsiveness of early postpartum dairy cows is associated with a high susceptibility to infectious and metabolic diseases.^{2,3} Several lines of evidence indicate that immune responsiveness decreases gradually in the prepartum period and reaches its lowest point immediately before calving.^{3,4}

Research has also demonstrated that one of the most important immunogenic substances that mucosal sites in dairy cows are exposed to during the postpartum period is LPS, a cell-wall component of Gram-negative bacteria with highly pro-inflammatory properties, commonly known as endotoxin.⁵ Exposure of mucosal layers to endotoxin in postpartal cows occurs mainly

as a result of the dramatic accumulation of cell-free LPS in the rumen when cows are switched from a roughage-rich to a grain-rich diet,^{6,7} at the onset of lactation. Eventually, the accumulation of large amounts of endotoxin in the rumen causes endotoxemia⁷ associated with multiple metabolic, endocrine, mineral and immune responses.^{5,8,9}

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The interest in mucosal immune stimulation and, most importantly, in using the mucosally-induced tolerance as a form of immunomodulation to prevent against certain pathogens has increased recently. For example, pretreatment with LPS by the oral route was able to protect rats against sepsis through regulation of pro-inflammatory response and IgM anti-LPS Ab production.¹⁰ Moreover, intramammary mucosal pretreatment of cows with LPS was shown to protect against experimental *Escherichia coli* mastitis.¹¹ A recent study conducted by Zebeli et al.¹² indicated that parenteral administration of repeated doses of LPS in periparturient dairy cows stimulates a humoral immune response against LPS.

However, several investigators have shown that a single intravenous administration of large LPS doses in dairy cows is associated with enhanced plasma concentrations of different cytokines, such as TNF- α , IL-1, IFN- γ , and acute phase proteins (APP), such as serum amyloid A (SAA), LPS-binding protein (LBP), C-reactive protein and haptoglobin.^{13,14}

Indeed, most research conducted in dairy cows with regards to LPS has involved investigation of the host responses to its intravenous or intramammary administration. To our best knowledge, there are no reports dealing with the cow's response to oral administration of LPS. Because dairy cows are exposed to large amounts of cell-free LPS, in particular in gastrointestinal mucosal tissues,^{6,7} especially during the postpartum period, it would be of interest to evaluate whether exposure of cows to LPS before this critical period may modulate immune responsiveness of the cows. Therefore, we hypothesized that repeated oral exposure of the periparturient dairy cows to increasing doses of LPS before and immediately after parturition might modulate their humoral immune responses during the periparturient period. The objective of this investigation was to study the effects of repeated oral administration (twice-weekly) with increasing doses of LPS from *E. coli* 0111:B4, 2 wk before, and 1 wk after, calving, on selected APP, plasma anti-LPS Abs and clinical variables, such as daily feed intake, body temperature, rumen contraction rate and respiration rate in periparturient Holstein dairy cows.

Materials and methods

Animals and treatments

Sixteen clinically healthy, pregnant Holstein dairy cows at Dairy Research and Technology Centre, University of Alberta, were included in this study. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock, and animals were cared for in accordance with the guidelines of the Canadian Council on

Animal Care.¹⁵ At 2 wk before the expected day of calving (i.e. -2 wk), cows blocked by parity, body conditioning score (BCS), and the anticipated day of calving were randomly allocated to one of the two treatment groups (eight cows in each) according to a randomized block design. The eight cows assigned to the control group (CTR) were orally administered 3 ml saline (0.9% w/v NaCl) solution using disposable plastic syringes. The other eight cows pertaining to the LPS group (TRT) were orally administered with the same amount of saline containing three different doses of LPS (from *E. coli* strain 0111:B4 supplied by Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). Three increasing doses of LPS were administered to the treatment cows during the third week around parturition. Doses of LPS used were based on previous research conducted with dairy cows and on clinical and pathological responses to those doses.^{13,16,17} The initial crystalline *E. coli* LPS containing 10 mg of purified LPS was dissolved in 10 ml of distilled water, as suggested by the manufacturer, and stored in the refrigerator at 4°C. The doses and the schedule of LPS administration were as follows: (1) 0.01 μ g of LPS/kg body weight (BW) twice on d -14 and -10; (2) 0.05 μ g of LPS/kg BW twice on d -7 and -3; and (3) 0.1 μ g of LPS/kg BW twice on d +3 and +7 postpartum. The administration of saline to the CTR cows occurred according to the same schedule as the LPS cows. The experiment lasted 6 wk (i.e. 2 wk before and 4 wk after parturition) and cows were housed in tie stalls with free access to water throughout the experiment. Shortly before parturition cows were transferred to the parturition stalls and returned to their stalls on the next day of parturition. Animals were fed once daily at 08:00 h and milked twice at 05:00 h and 15:30 h in their stalls. All cows were fed the same close up diet starting 3 wk before the day of parturition. After parturition, cows were gradually switched during the first 7 d to a fresh-lactation diet. Diets were the same as in study by Zebeli et al.¹² and all diets were formulated to meet, or exceed, the nutrient requirements of dry and early lactating cows as per National Research Council (NRC) guidelines.¹⁸ Daily ration was offered as total mixed rations for ad libitum intake to allow approximately 5% feed refusals throughout the experiment.

Sample collection

Blood samples were collected on the day of LPS administration, always at the same time (07:00 h) and 15 min prior to treatment of the experimental cows. Cows were moved to the maternity barn and were restrained to take blood samples from an intravenous catheter introduced an hour before sampling into the jugular vein. Ten millilitres of blood was collected through a plastic syringe and poured gently into a tube containing Na-EDTA (Preanalytical Systems Beliver Industrial

Estate, Plymouth, UK). Blood samples were immediately put in ice, centrifuged within 20 min (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany), and plasma was separated and stored at -20°C until analyses. Immediately after blood withdrawal, indwelling catheters were filled with 2–3 ml of 0.85% sterile saline containing 50 IU heparin (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) to prevent clot formation. Blood was withdrawn twice per week beginning at wk -2 and -1 before the expected day of parturition and at $+1$ wk after parturition, as well as once per week beginning wk $+2$, $+3$ and $+4$ after parturition. No stress responses from cows were observed during blood withdrawal.

Clinical measurements

Rectal temperature, and respiratory and rumen contraction rates were measured 15 min before the treatment, as well as 30 min and every hour, up to 6 h, after administration of the treatment. Feed intake was recorded daily during the entire administration period (i.e. from -2 wk to 1 wk postpartum). Cows were supervised daily by herd veterinary technician for signs of clinical disease and distress. All disease and medication history was recorded for each cow throughout the entire experimental period.

Sample analyses

Concentrations of SAA in the plasma were determined by commercially available ELISA kit (Tridelta Development Ltd, Greystones C, Wicklow, Ireland), with monoclonal Abs specific for SAA, as described previously.¹² In brief, samples were initially diluted 1:500 and if some of the samples had optical density values below the range of the standard curve they were re-analyzed in lower dilutions. All samples were tested in duplicate and the optical densities values were read on microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA) at 450 nm. The detection limit of the assay was 18.8 ng/ml.

A commercially-available ELISA kit was used to quantify plasma LBP (Cell Sciences, Inc., Norwood, MA, USA). The Ab used to coat the wells cross-reacts with bovine LBP. Plasma samples were initially diluted 1:500 and samples with optical density values lower than the range of the standard curve were tested with a lower dilution. Samples were tested in duplicate and the optical densities were measured at 450 nm on a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA, USA).

Concentrations of haptoglobin in the plasma were measured with an ELISA kit provided by Tridelta Development Ltd. (Greystones C., The detection limit of the assay was 0.25 ng/ml, as defined by the linear

range of the standard curves. All samples were tested in duplicate and the optical densities were measured at 630 nm on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation).

Concentrations of anti-LPS core immunoglobulin (Ig)A, IgG and IgM Abs in the plasma were measured using commercially-available ELISA kits, as described previously.¹² Abs directed against the core structure of endotoxin (i.e. EndoCab) are cross-reactive against most types of LPS.^{19,20} The EndoCab ELISA is solid-phase ELISA based on the sandwich principle with a working time of 2.5 h. The color developed is proportional to the amount of anti-endotoxin core Abs present in the sample. The absorbance was measured at 450 nm with a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation). The minimum detection levels of IgA, IgG and IgM EndoCab Abs were at 0.156 MU/ml, 0.0125 MU/ml and 0.055 MU/ml.

Plasma cortisol was measured by commercially-available EIA kit (Diagnostic Systems Laboratories Inc., Webster, TX, USA). The procedure involved the basic principle of enzyme immunoassay where there is competition between an unlabelled antigen and an enzyme-labeled antigen for a fixed number of Ab-binding sites. The amount of enzyme-labeled antigen is inversely proportional to the concentration of the unlabelled analyte present in the solution. Unbound materials were removed by decanting and washing the wells. All samples were in duplicate. The optical densities were measured at 450 nm in a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation) and the concentrations were calculated by using a four-parameter curve fit.

Statistical analyses

Data were analyzed using the MIXED procedure of SAS,²¹ as described by the following model

$$Y_{ijkl} = \mu + t_i + w_j + tw_{ij} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the observations for the dependent variables, μ represent the population mean, t_i is the fixed effect of treatment, w_j is the fixed effect of time, tw_{ij} is the interaction between treatment and time, and ε_{ijkl} is the residual error assumed to be normally distributed. Measurements on the same animal at different times were considered as repeated measures. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the BIC (Bayesian information criteria). Data are shown as least-squares means (LSM) and standard error of the mean (SEM). Multiple comparisons of LSM were conducted by probability difference (PDIF) option of SAS. The significance

limit was declared at $P < 0.05$, and a tendency was considered up to $0.05 \leq P < 0.10$.

Results

Plasma immunoglobulins

Data obtained by ANOVA indicated that immunoglobulin M anti-LPS Abs in the plasma were lower in

the CTR cows versus cows treated orally with LPS ($P < 0.01$; Figure 1A). No time effect or treatment by time interaction was obtained regarding plasma IgM (Figure 1A).

Results showed differences between plasma IgG anti-LPS Abs between the two groups, with the CTR group having greater concentrations starting immediately after calving up to 4 wk after parturition ($P < 0.01$; Figure 1B). There was also a time effect for plasma IgG with values increasing continuously from the lower concentrations before parturition to the greater concentration at 4 wk after calving ($P = 0.04$). No treatment by time interaction was observed between the two treated groups ($P = 0.84$).

Data indicated no effects of treatment or time for IgA anti-LPS Abs in the plasma ($P = 0.25$ and $P = 0.24$; Figure 1C). In fact, plasma concentrations of IgA anti-LPS Abs ranged in both groups from the lower level of 0.04 MU/ml to the upper concentration of 0.07 MU/ml during the whole experimental period. Also, no treatment by time interaction was evidenced for plasma IgA ($P = 0.86$).

Acute phase proteins

Statistical processing of the data showed no differences in the concentrations of LBP in the plasma between the TRT and the CTR groups (Figure 2A). Results also demonstrated an effect of time on plasma LBP ($P = 0.01$). Concentrations of LBP increased in both groups immediately after parturition. However, the increase in the concentration of LBP from the week before to the week after parturition was greater ($P < 0.05$) for CTR group (19.1–65.9 $\mu\text{g/ml}$) than TRT group (20.7–39.2 $\mu\text{g/ml}$). No overall treatment by time interaction was obtained for plasma LBP ($P = 0.35$).

Concentrations of SAA in the plasma were not different between the two treatment groups ($P = 0.18$; Figure 2B). There was an increase in SAA in both groups immediately after parturition ($P < 0.01$). Thus, plasma SAA increased almost threefold from the week before to the week after parturition in both groups (i.e. CTR group from 12.7–37.8 $\mu\text{g/ml}$ and TRT group from 17.0–51.3 $\mu\text{g/ml}$, respectively). Moreover, the multiple comparison of LSM indicated greater SAA concentrations in the plasma of TRT cows at wk 3 postpartum ($P < 0.05$). Thereafter, concentrations of SAA decreased gradually until wk 4 after parturition. Also, ANOVA indicated no overall treatment by time interaction for plasma SAA ($P = 0.13$).

No differences between the two treatment groups were obtained regarding concentrations of haptoglobin in the plasma (Figure 2C). However, time had an effect on plasma haptoglobin ($P = 0.01$). Haptoglobin concentrations increased from 141–891 $\mu\text{g/ml}$ and from 218–788 $\mu\text{g/ml}$ between the week before and the week after parturition in the TRT and CTR groups,

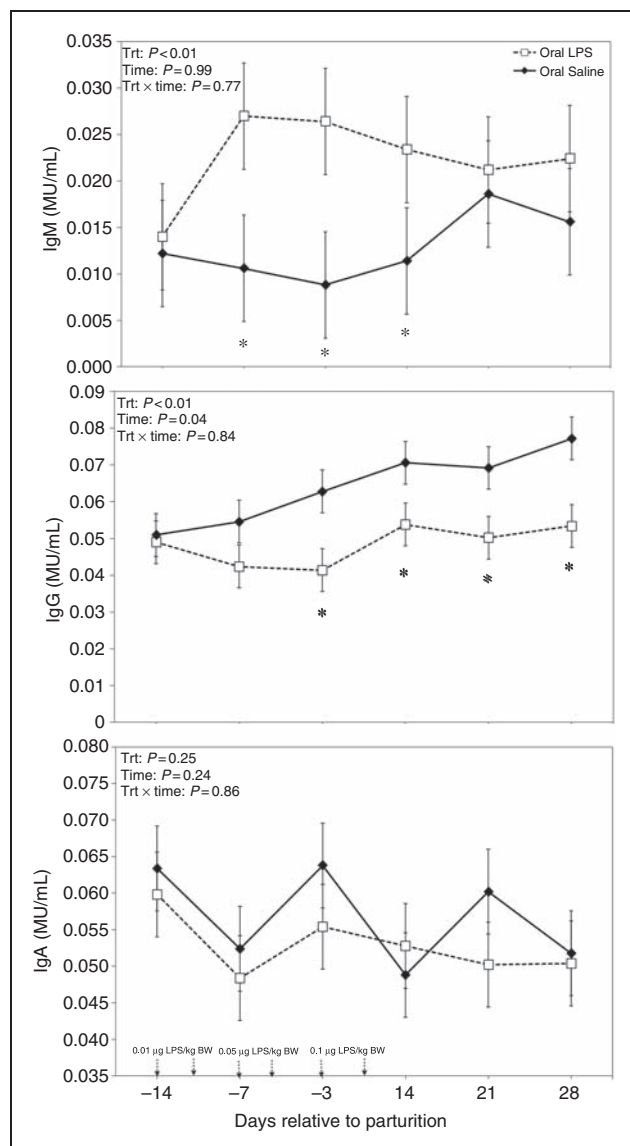


Figure 1. Effect of oral administration of increasing doses of LPS twice per week –2, –1 and 1 wk postpartum on plasma anti-LPS Ab concentrations of (A) immunoglobulin M (IgM), (B) immunoglobulin G (IgG) and (C) immunoglobulin A (IgA) in the peripartal period. Arrows indicate the schedule of LPS administration with doses. Data are shown as least-squares means and respective standard errors. $n = 8$. trt, effect of treatment. *Indicates differences between treatments at certain times, $P < 0.05$.

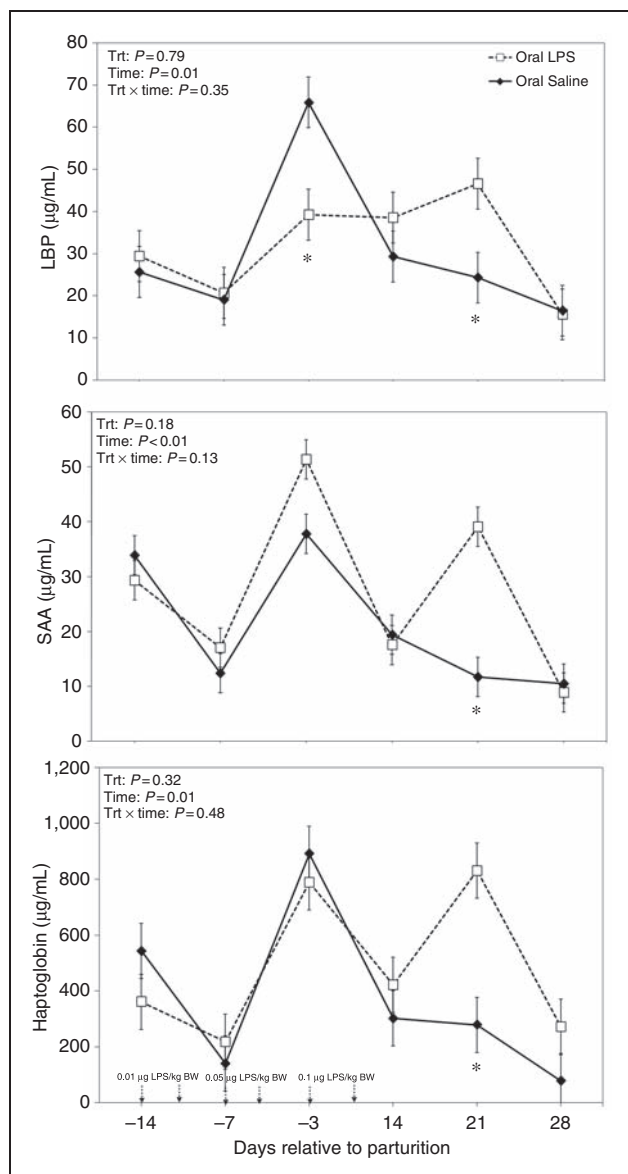


Figure 2. Effect of oral administration of increasing doses of LPS twice per week -2 , -1 and 1 wk postpartum on plasma acute phase protein concentrations of (A) LBP, (B) SAA, and (C) haptoglobin in the peripartal period. Arrows indicate the schedule of LPS administration with doses. Data are shown as least-squares means and respective standard errors. $n = 8$. trt, effect of treatment. *Indicates differences between treatments at certain times, $P < 0.05$.

respectively. Similarly to plasma SAA and LBP, the concentration of haptoglobin increased at wk 3 postpartum in the TRT cows ($P < 0.05$) and declined thereafter, reaching the lowest concentrations at 4 wk after calving. No overall interaction between treatment and time was evidenced ($P = 0.48$)

Plasma cortisol and clinical observations

There were no differences between plasma concentrations of cortisol between the two groups (Figure 3).

A time effect was obtained with regards to plasma cortisol with greater concentrations 2 wk before calving and declining almost threefold the week before calving and remaining at this levels for the remaining of the experiment ($P < 0.01$). No treatment interactions were obtained for plasma cortisol (Figure 3).

Data of feed intake measured during the time of LPS administration (i.e. -2 wk to 1 wk postpartum) indicated no differences among cows in the TRT and CTR groups (Figure 4). Time relative to parturition alone, or in combination with the treatment, also did not affect feed intake of cows in this experiment.

Data for body temperature shortly before and after each oral treatment with LPS are shown in Figure 5. Results showed that during the administration of the first and second dose of oral LPS no differences were observed in body temperatures between the two groups (Figure 5A, B). However, during the first hours after the first challenge, a slight increase in body temperature was found, which decreased again 3 h post-challenge ($P = 0.05$; Figure 5A). Time did not affect the response of body temperature during the first (Figure 5A) and second (Figure 5B) oral LPS dose-challenge. After the administration of the third LPS dose, the overall body temperature tended to increase ($P = 0.07$; Figure 5C).

Rumen contractions were not affected by any of the three oral challenges with LPS during this trial (Figure 6). Respiration rate increased after each LPS administration ($P < 0.01$; Figure 7). Time relative to LPS administration alone did not affect respiration rate in this trial (Figure 7). There was a treatment by time interaction for the respiration rate after administration of the first and second dose of LPS (Figure 7A, B).

Discussion

Dairy cows undergo a state of immunosuppression during the periparturient period, which makes them highly susceptible to various infectious and metabolic diseases. There is a sudden exposure of the cow's gastrointestinal mucosal tissues to LPS, immediately after parturition, as a result of the rapid dietary change.^{6,7} For example, the amount of cell-free LPS in the rumen fluid of mid-lactation dairy cows fed 30% and 45% barley grain (on a dry matter basis) in the diet is increased 6- and 14-fold, respectively.⁶ There is no information about the amount and toxicity of endotoxin in the rumen fluid around parturition; however, based on the composition of diets fed to dairy cows before and during the first week postpartum, endotoxin challenge from the rumen during this time may be low.⁵ However, starting after the second week, when the amount of grain in the diet is increased, the amount of endotoxin released in the rumen fluid, as a result of lysis of Gram-negative bacteria, increases dramatically.^{6,22} Although rumen endotoxins are neutralized by multiple mechanisms including deacylating

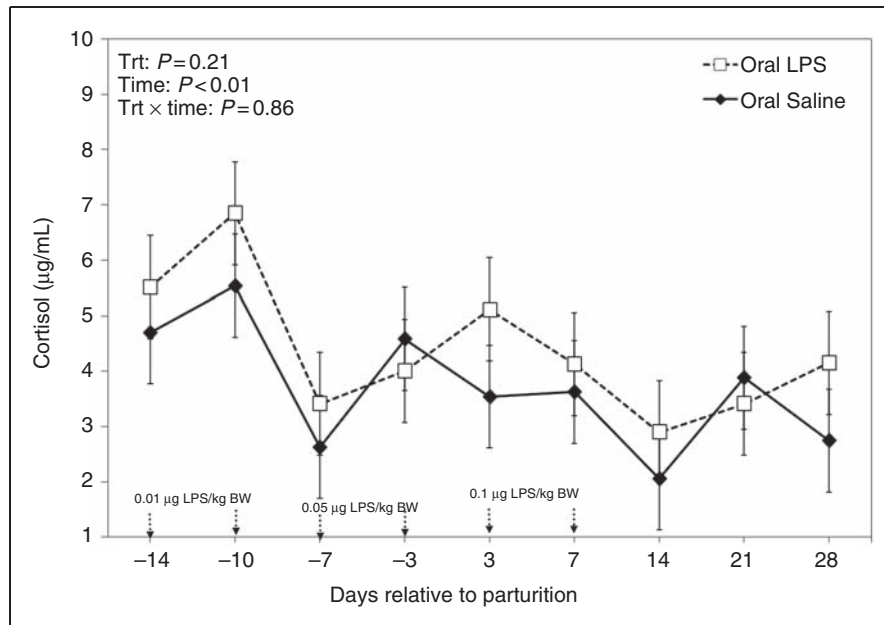


Figure 3. Effect of oral administration of increasing doses of LPS twice per week -2 , -1 and 1 wk postpartum on plasma cortisol in the peripartal period. Arrows indicate the schedule of LPS administration with doses. Data are shown as least-squares means and respective standard errors. $n=8$. trt, effect of treatment.

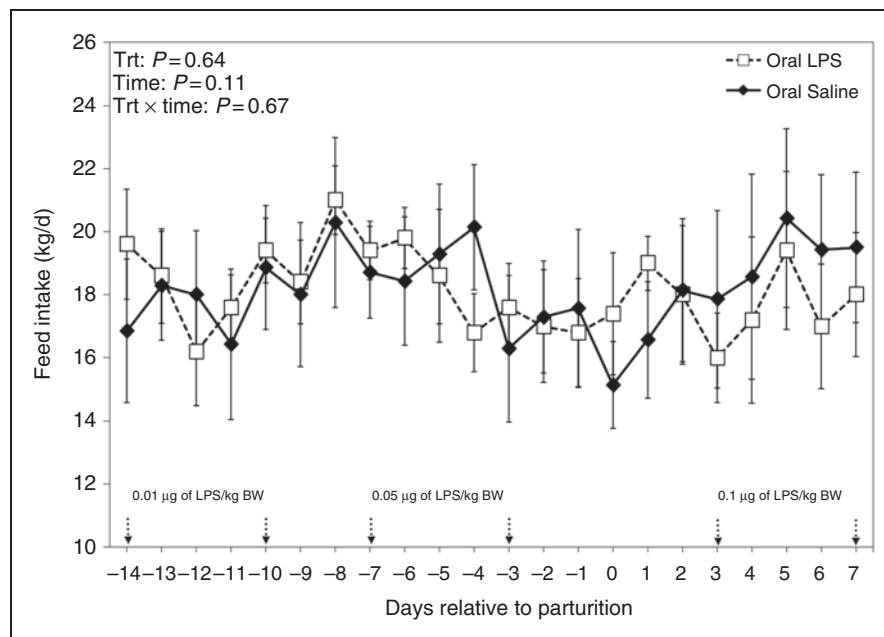


Figure 4. Effect of oral administration of increasing doses of LPS twice per week -2 , -1 and 1 wk postpartum on daily feed intake in the peripartal period. Arrows indicate the schedule of LPS administration with doses. Data are shown as least-squares means and respective standard errors. $n=8$. trt, effect of treatment.

and dephosphorylating enzymes and, as a consequence, lose their toxicity, part of the endotoxins might translocate into the host systemic circulation.^{22,23} For this reason, administration of LPS was done in the oral cavity, before this potential rumen endotoxin assault on the host, to prepare the cows' immune responses.

Therefore, this study was undertaken to evaluate whether repeated oral administration of increasing doses of LPS from *E. coli* 0111:B4 in dairy cows, before the exposure to large amounts of gastrointestinal LPS, would be able to modulate humoral immune responses in periparturient dairy cows. In agreement with our hypothesis, repeated oral exposure to LPS

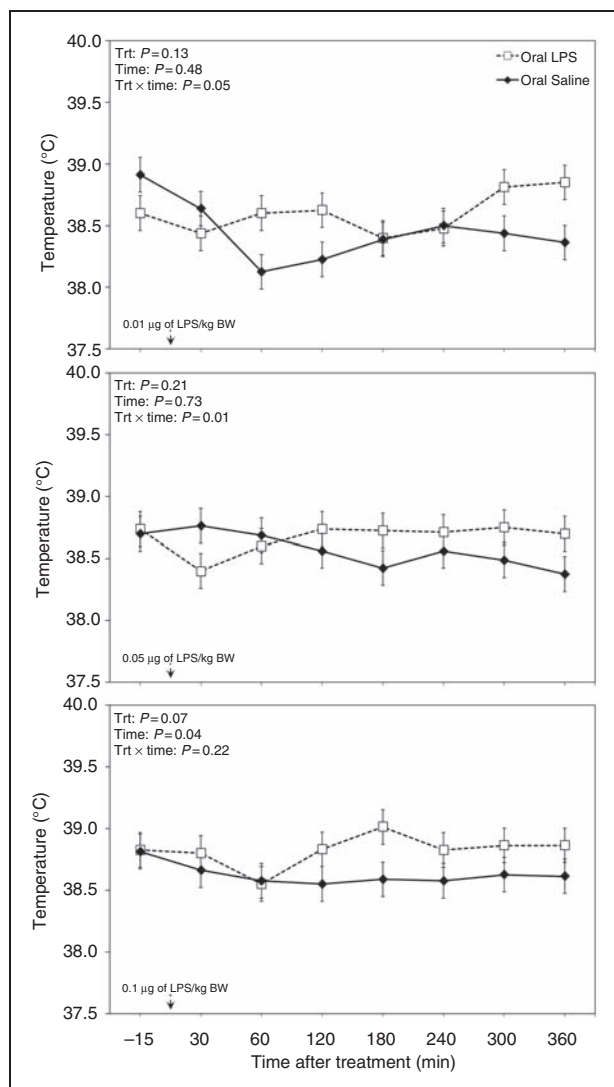


Figure 5. Effect of oral administration of increasing doses of LPS twice per week -2 , -1 and 1 wk postpartum on the fluctuations of rectal temperature measured on the days (A) -14 (i.e. after administration of $0.01 \mu\text{g}$ LPS/kg body weight), (B) -7 (i.e. after administration of $0.05 \mu\text{g}$ LPS/kg body weight) and (C) 3 postpartum (i.e. after administration of $0.1 \mu\text{g}$ LPS/kg body weight). Data are shown as least-squares means and respective standard errors. $n=8$. trt, effect of treatment.

modulated humoral immune responses in periparturient dairy cows. More specifically, oral treatment with LPS decreased concentrations of IgG anti-LPS antibodies in the plasma and increased those of anti-LPS IgM Abs. The significance of these findings is not clear at present and requires further research. However, previous research indicated that high plasma anti-LPS IgG Abs were associated with a low herd pregnancy rate and preceded occurrence of mastitis, reproduction diseases and digestion disorders in dairy cows.²⁴ Lower plasma anti-LPS IgG Abs in treated cows in our experiment suggests that repeated oral administration of LPS induced mucosal immunity against LPS. These findings may relate to enhanced mucosal immunity against LPS

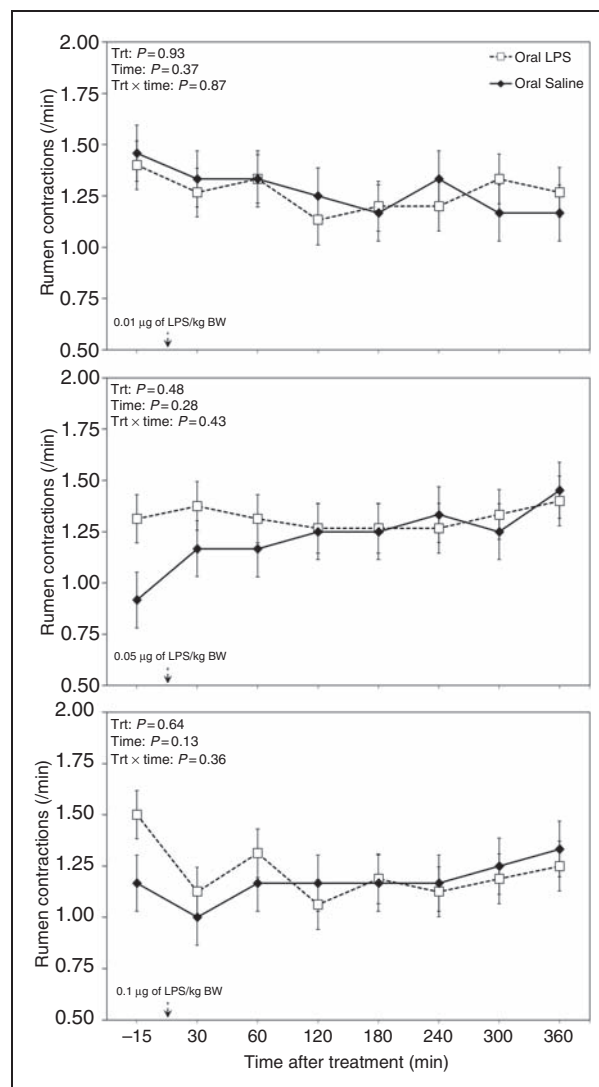


Figure 6. Effect of oral administration of increasing doses of LPS twice per week -2 , -1 and 1 wk postpartum on the fluctuations of rumen contractions measured on the days (A) -14 (i.e. after administration of $0.01 \mu\text{g}$ LPS/kg body weight), (B) -7 (i.e. after administration of $0.05 \mu\text{g}$ LPS/kg body weight) and (C) 3 postpartum (i.e. after administration of $0.1 \mu\text{g}$ LPS/kg body weight). Data are shown as least-squares means and respective standard errors. $n=8$. trt, effect of treatment.

and to the phenomenon of oral tolerance. Indeed, oral immunization with an antigen results in the development of suppressor T cells, which inhibit IgG responses. Richman et al.²⁵ showed that orally-fed antigen (e.g. ovalbumin) induced IgG-specific suppressor T cells that lowered systemic IgG concentrations. The opposite was true for the CTR cows in our experiment, which had greater concentrations of IgG anti-LPS Abs in their plasma. This suggests that CTR cows developed a secondary humoral immune response characterized by development of memory B cells to LPS. It is speculated that this might be related to translocation of endotoxin from gastrointestinal tract into the blood circulation of CTR cows.

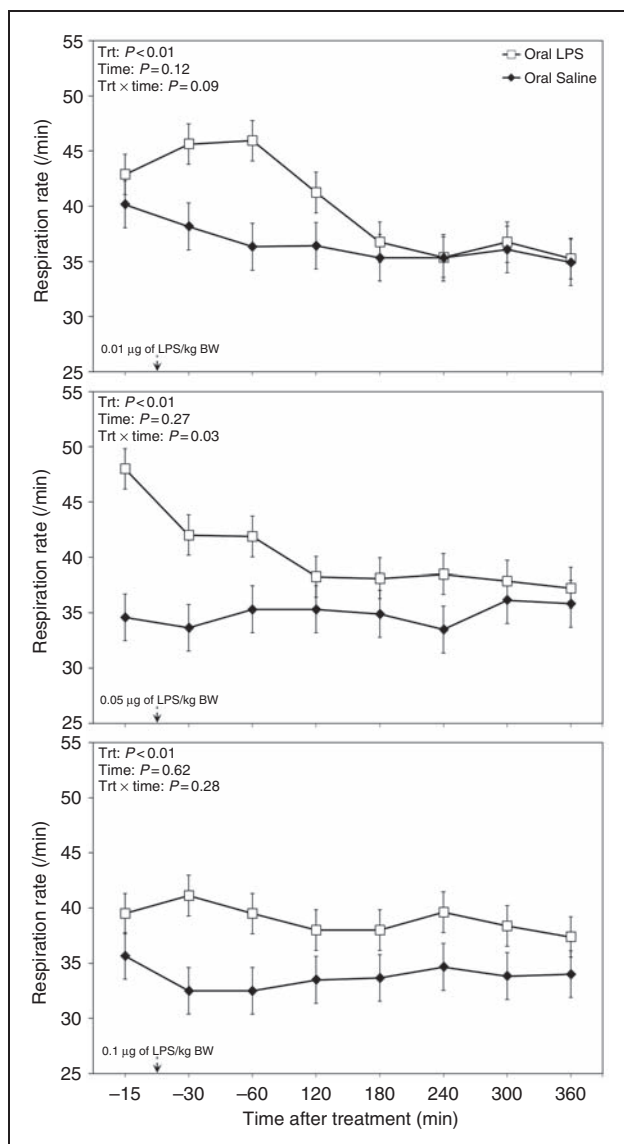


Figure 7. Effect of oral administration of increasing doses of LPS twice per week -2 , -1 and 1 wk postpartum on fluctuations of respiration rate measured on the days (A) -14 (i.e. after administration of $0.01 \mu\text{g LPS/kg body weight}$), (B) -7 (i.e. after administration of $0.05 \mu\text{g LPS/kg body weight}$) and (C) 3 postpartum (i.e. after administration of $0.1 \mu\text{g LPS/kg body weight}$). Data are shown as least-squares means and respective standard errors. $n = 8$. trt, effect of treatment.

Another interesting finding of this research was that plasma IgM anti-LPS Abs were greater in the LPS-treated cows. Greater concentrations of IgM anti-LPS Abs suggest that repeated oral administration of LPS stimulated the primary humoral immune response in the TRT group. These data are in agreement with previous research indicating that pretreatment of mice with oral LPS increased plasma anti-LPS IgM and protected them from sepsis induced by cecal ligation and puncture.¹⁰ In addition, human research indicates that low anti-LPS IgM Abs are associated with high morbidity in postoperative patients.²⁶

The protective role of IgM in sepsis models has been reported to be critical for clearance of circulating LPS.²⁷ IgM is important not only as a primary response to LPS, but also for mucosal immunity. This type of immunoglobulin has been shown to play a significant role in mucosal immunity, together with secretory IgA.²⁸ Our results suggest that oral treatment with LPS might be used to enhance humoral immunity and lower morbidity related to endotoxin in periparturient dairy cows. However, further research is warranted to elucidate the mechanism(s) by which oral LPS increased IgM anti-LPS Abs in the plasma of periparturient dairy cows.

Results showed that plasma IgA anti-LPS Abs were numerically lower in treated cows; however, the difference did not reach significance. This was expected as oral immunization usually stimulates production of secretory IgA in the mucosal membranes but not plasma IgA. Similar results were reported in mice treated orally with LPS to prevent sepsis induced by cecal ligation and puncture.¹⁰ Generally, IgA is found in all external secretions, such as bile and intestinal fluids, tears, saliva, milk or other mucosal membranes.²⁹ Oral administration of antigen has been shown to induce preferentially antigen-specific IgA responses in mucosa-associated tissues.

Repeated oral treatment with LPS did not have an overall effect on concentrations of plasma SAA, LBP and haptoglobin. However, multiple comparisons of LSM indicated greater concentrations of plasma LBP in the CTR cows during the first week postpartum (i.e. 3 d after calving). LBP is a known APP that is over-expressed during endotoxemia and whose function is to bind plasma endotoxin and carry it to the immune cells. Overall, all three APPs increased immediately after parturition when compared with prepartal values. Also, a second rise of APP was observed during the 3 -wk postpartum period in the cows in the TRT group. Plasma APPs are part of a general, nonspecific immune response and are produced by the liver under the stimulation of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α .^{30,31} Two of the proteins, SAA and LBP, are released into the systemic circulation or into mucosal layers to bind and neutralize endotoxin, whereas haptoglobin dampens the severity of cytokine release by macrophages and protects against endotoxin effects.^{32,33} The reason for the peak plasma SAA, LBP and haptoglobin in all cows on the first week after calving, as well as during the third week postpartum, is not well understood; however, it is speculated that it may be related to the pro-inflammatory cytokines coming from the Gram-negative contaminated tissues immediately after calving.³⁴ Indeed, clinical observations of cows in the present study indicated that one cow in the CTR group failed to expulse the placenta after calving, developing puerperal metritis. Another two cows of the same group were diagnosed with mastitis

and subclinical ketosis. Also, one animal in TRT group developed lameness and mastitis postpartum. Metritis and mastitis have often been associated with the release of endotoxin in the plasma postpartum.^{35,36} Owing to the limited number of observations (i.e. $n=8$) and also the subjective evaluation of the severity of the disease, the clinical data of this study should be considered with caution—although clinical observations indicated that cows of the TRT group had a better clinical health status, it needs to be verified in further studies. Also, the fact that cows in the CTR and TRT group developed diseases postpartum indicates that various sources of endotoxin may have been involved in the APP responses postpartum in this study.

Endotoxin translocated into the portal or systemic circulation is removed from the blood by Kupffer cells in the liver, which are activated to release pro-inflammatory cytokines. Cytokines then induce production of APPs in the hepatocytes.^{31,34} In fact, our data are in agreement with previous reports of enhanced APP in dairy cows after calving³⁷ and confirm previous findings that parturition is associated with the mounting of an acute phase response.³⁸

Different investigators have demonstrated that activation of the immune system by intravenous administration of endotoxin in ruminants is associated with stimulation of the hypothalamo-pituitary-adrenal axis and the release of cortisol in systemic circulation.^{39, 40} Based on the latter findings, we included measurement of plasma cortisol in our study to evaluate whether oral exposure to LPS would induce the release of cortisol into the blood stream. Data from our study showed that concentration of cortisol in the plasma was numerically greater in cows treated with oral LPS; however, the difference did not reach significance. Thus, the results suggest that repeated oral administration of LPS is safe and does not induce the activity of the hypothalamo-pituitary-adrenal axis.

The data for cortisol were supported by findings of feed intake and, most importantly, from clinical variables taken at multiple time points shortly before and up to 6 h after LPS treatment. Although oral LPS administration tended to increase the body temperature, this effect was not comparable with previous studies in cattle, where intravenous administration of LPS induced hyperthermia.¹⁴ Even with the highest dose of LPS, in this study the body temperature was below 39.5°C, which is often considered as the threshold of hyperthermia in cattle.⁴¹ Respiration rate increased following oral administration of LPS, although our results of 30–45 respirations/min were still within the healthy ranges, they were slightly greater than rates commonly observed in cows at rest (18–28 respirations/min).⁴¹ In a previous study, intravenous administration of increasing doses of LPS resulted in highly increased respiration rate during a 6-h period post-challenge with a critical maximum rate of 65–75 respirations/min.¹⁷

In conclusion, repeated oral administration of LPS from *E. coli* 0111:B4 stimulated a humoral immune response characterized by lower IgG anti-LPS and greater IgM anti-LPS Abs. No effects on plasma anti-LPS IgA Abs were observed. Results also showed that all plasma APP measured SAA, LBP and haptoglobin, as well as cortisol, feed intake, rumen contractions and body temperature were not affected by oral administration of LPS at the doses used in this experiment. The increase of LBP early postpartum in CTR cows and of other APPs during the first 3 wk postpartum in the TRT group appears to be related to host responses to various Gram-negative contaminated tissues postpartum rather than oral LPS challenge. Further research with larger cohorts of animals is warranted to investigate the significance of the modulation of humoral immune responses as a result of repeated oral administration of LPS on immune responsiveness and health status of dairy cows during the transition period.

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