

## **Beta-Hydroxybutyrate Alters the mRNA Cytokine Profile from Mouse Macrophages Challenged with *Streptococcus uberis***

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### **Summary**

The objective of this study was to determine if  $\beta$ -hydroxybutyrate (BHB) altered inflammatory responses in macrophages challenged with a common mastitis pathogen, *Streptococcus uberis*. Mouse macrophages (RAW 264.7 line) were cultured either in the presence or absence of BHB for 24 h, and then challenged or not with *S. uberis*. Relative transcript abundance of cell membrane receptors (TLR2 and GPR109a), cytokines (IL-1 $\beta$ , IL-10, TNF $\alpha$ , and TGF $\beta$ ), and chemokines (CXCL2 and CCL5) were determined using quantitative real-time polymerase chain reaction (qPCR) and normalized against the geometric mean of HPRT and B2M. *Streptococcus uberis* activated the macrophages, noted by greater transcript abundance of analyzed genes. Intriguingly, *S. uberis* increased GPR109a mRNA abundance, a receptor that is activated by BHB. Consequently, BHB dose-dependently increased transcript abundance of the pro-inflammatory cytokine (IL-1 $\beta$ ) and the anti-inflammatory cytokine (IL-10) but had no effect on TNF $\alpha$  or TGF $\beta$ . Moreover, BHB increased mRNA abundance of the chemokines, CXCL2 and CCL5. These data suggest a dysregulated immune response toward *S. uberis* due to BHB treatment, similar to what is seen in transition dairy cows. Future studies should be conducted in vivo to test the effect of BHB on immune function during an intramammary challenge.

### **Introduction**

Mastitis is the most common and costly disease in the dairy industry, impairing animal welfare and decreasing milk production. The incidence of clinical mastitis is dramatically greater during the first few weeks after calving than in the rest of the lactation. At the beginning of lactation, a depression of feed intake occurs simultaneously with an increase in energy demand, resulting in metabolic stress and negative energy balance. Consequently, dairy cattle mobilize fat reserves, liberating non-esterified fatty acids (NEFA). These fatty acids are transported to the liver for energy production. However, not all NEFA are completely oxidized, resulting in the production of  $\beta$ -hydroxybutyrate (BHB), a major ketone body. This ketone body has long been associated with disease in early lactation, but it is important to recognize that association does not discriminate between causative disease mediators and adaptive responses that help resolve the disease. The association between ketosis and mastitis is likely due to the decreased function of host innate immune cells exposed to BHB. When neutrophils were cultured

with BHB at various concentrations (0.1 to 8.0 mM), there was a stepwise reduction in extracellular killing of bacteria. Additionally, leukocytes from ketotic cows had a reduced ability to migrate toward an inflammatory response relative to those isolated from non-ketotic cows.

*Streptococcus uberis* is a common environmental mastitis pathogen that is responsible for a large proportion of mastitis during the first month of lactation, when negative energy balance is exacerbated. Therefore, it seems likely that BHB may be impairing immune responses toward this pathogen in early lactation dairy cattle. Hence, the objective of this experiment was to examine the effect of BHB on inflammatory mediators from macrophages during a *Streptococcus uberis* challenge. We hypothesized that BHB would attenuate inflammatory responses in a dose-dependent manner.

## Experimental Procedures

### *Bacterial Strain and Conditions*

*Streptococcus uberis* (kindly provided by Dr. Petersson-Wolfe, Department of Dairy Science, Virginia Tech) was originally isolated from a dairy cow with mastitis and stored in 10% skim milk at -80°C. Bacteria were streaked on an esculin blood agar plate and incubated for 24 hours. Five colonies were then cultured in Todd-Hewitt broth and incubated for 7 hours at 37°C on an orbital shaker. Bacterial suspension was pelleted with centrifugation, washed with sterile phosphate-buffered saline, and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 1% L-glutamine and 10% heat-inactivated fetal bovine serum. Serial dilutions were used to achieve the desired concentration of colony-forming units for challenge, and challenge inoculum concentration was verified using drop plating onto esculin blood agar.

### *Cell Culture Conditions and Treatments*

Mouse macrophages (RAW 264.7 line) were cultured in DMEM supplemented with 1% L-glutamine, 10% heat-inactivated fetal bovine serum, and 0.2% penicillin-streptomycin. Twenty-four-well plates (n = 8 wells per treatment group) were seeded with  $1 \times 10^5$  cells and incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. Cells were then either treated with  $\beta$ -hydroxybutyrate (Sigma Aldrich) at various concentrations (0 mM, 0.6 mM, 1.2 mM, or 1.8 mM) or not for 24 hours to mimic ketosis. To maintain a neutral pH in culture media,  $\beta$ -hydroxybutyrate was added as sodium salt, and a treatment group with 1.8 mM added NaCl was included as an osmotic control. After the 24-hour incubation step, the medium was removed and fresh medium without antibiotics containing BHB at various concentrations (or not) and with or without  $5 \times 10^5$  CFU/mL of *S. uberis* were added for 6 hours. Cells were then lysed and stored at -80°C.

### *RNA Isolation and qPCR*

Total RNA was isolated from cell lysates using the RNeasy kit (Qiagen) and was quantified using spectroscopy (NanoDrop Technologies Inc., Wilmington, DE). One microgram of total RNA was used as template for the reverse transcriptase reaction using random primers. Quantitative real-time PCR was performed in duplicate with 200 nM gene-specific forward and reverse primers with real-time SYBR green fluorescent detection (7500 Fast Real-Time PCR System, Applied Biosystems). Primers were designed from mouse GenBank sequences and were designed to amplify an intron-spanning

region of the gene. Relative mRNA abundance was quantified by the  $2^{-\Delta C_t}$  method with the geometric mean of HPRT and B2M used to normalize values.

### *Statistical Analyses*

Statistical analyses were conducted in PROC GLIMMIX SAS v. 9.4 (SAS Inst., Cary, NC). Orthogonal contrasts were performed to test the effect of *S. uberis*, overall effect of BHB within *S. uberis* challenged treatment groups, as well as linear and quadratic contrasts to test BHB dose responses. To meet the assumption of normality (PROC UNIVARIATE), all response variables required natural logarithmic transformation; least square means and standard errors were back-transformed. An outlier was defined if the observation had a studentized residual greater than 3 in absolute value, and therefore was removed from the analysis. Significance was declared at  $P \leq 0.05$ .

## Results and Discussion

### *Streptococcus uberis Effects*

As expected, *Streptococcus uberis* induced the immune activation in macrophages. This was evident through increased mRNA abundance of a pathogen recognition receptor, toll-like receptor 2 (TLR2,  $P = 0.03$ ), and thus downstream increases of both pro- and anti-inflammatory cytokine mRNA abundance. In particular, *S. uberis* increased mRNA abundance (all  $P < 0.01$ ) of pro-inflammatory cytokines interleukin (IL)-1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), as well as anti-inflammatory cytokines IL-10 and transforming growth factor  $\beta$  (TGF $\beta$ ). Moreover, *S. uberis* increased mRNA abundance of two chemokines (CXCL2 and CCL5), which are proteins used to attract immune cells into inflamed tissue. Lastly, *S. uberis* increased mRNA abundance of GPR109a ( $P < 0.01$ ), a receptor for BHB that has known anti-inflammatory effects when activated. This is intriguing, as these data could imply that *S. uberis* promotes immunological tolerance, thus impairing the immune system's ability to kill this pathogen. Regardless, an increase in GPR109a should result in macrophages that are more responsive to BHB ligation.

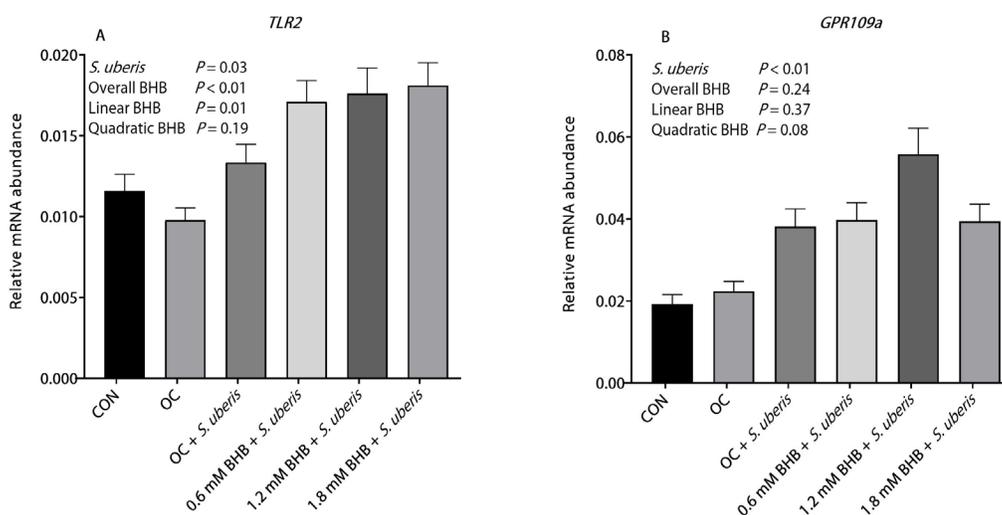
### *Beta-Hydroxybutyrate Effects*

Beta-Hydroxybutyrate increased mRNA abundance of both pro- and anti-inflammatory cytokines, although these data should be interpreted with caution. First, BHB dose-dependently increased mRNA abundance of the potent anti-inflammatory cytokine IL-10 (overall BHB effect,  $P = 0.01$ ; linear BHB effect,  $P < 0.01$ ) when compared to *S. uberis* challenged cells. Yet, BHB also dose-dependently increased mRNA abundance of the pro-inflammatory cytokine IL-1 $\beta$  (overall BHB effect,  $P < 0.01$ ; linear BHB effect,  $P < 0.01$ ). Interleukin-1 $\beta$  data should be interpreted cautiously; this cytokine is post-translationally regulated more so than other cytokines, so a future study must confirm if the active, secreted protein form of this cytokine was also increased by BHB. An increase in both IL-1 $\beta$  and IL-10 is likely due to increased abundance of the receptor that controls their expression, TLR2 (overall BHB effect,  $P < 0.01$ ; linear BHB effect,  $P = 0.01$ ). Lastly, BHB increased mRNA abundance of chemokines CXCL2 (overall BHB effect  $P = 0.01$ ; linear BHB effect,  $P = 0.04$ ) and CCL5 (overall BHB effect,  $P = 0.03$ ; linear BHB effect,  $P = 0.07$ ). Again, these data could imply a more robust immune response, as a greater abundance of chemokines should result in more immune cells migrating into inflamed tissue in vivo. However, in a similar

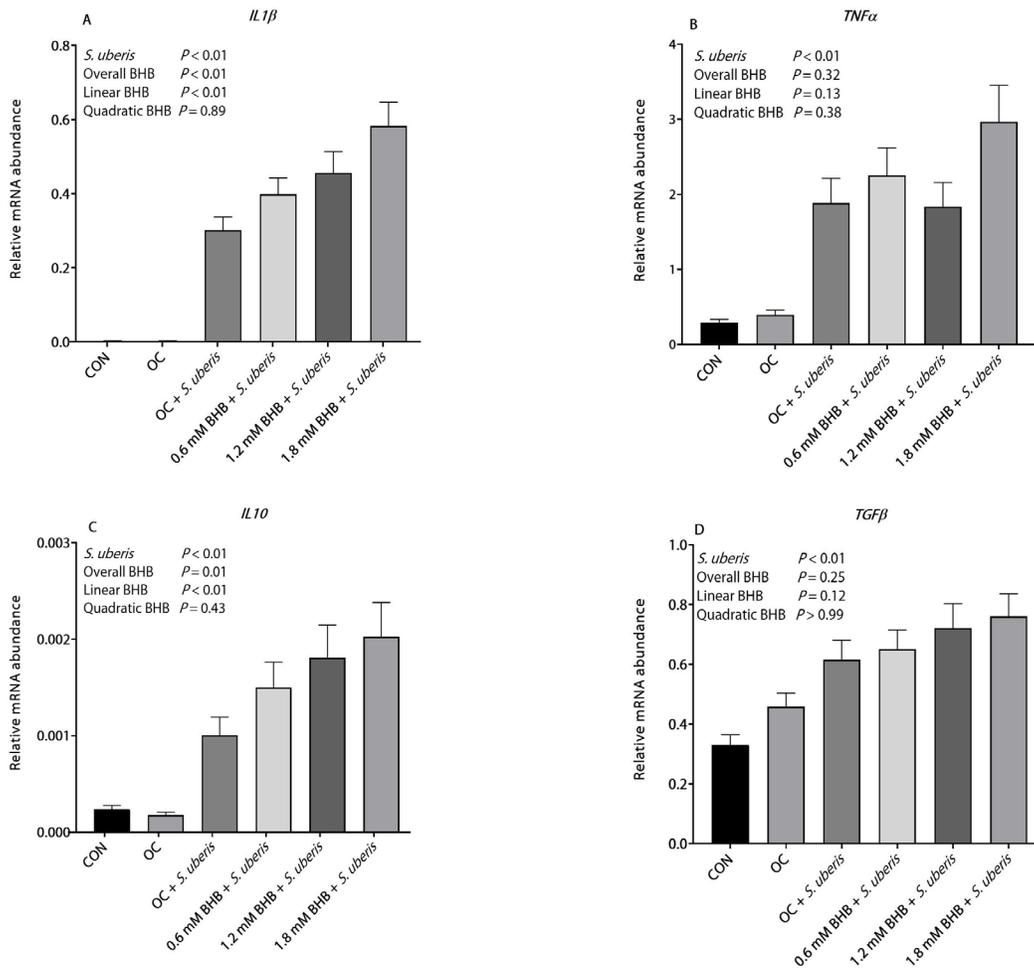
study, BHB also increased chemokine mRNA abundance, yet this did not result in an increase in immune cells migrating into tissue. Thus, an increase in chemokine mRNA abundance may simply be a compensatory response in attempt to overcome a reduction in their efficacy. Future studies should examine protein abundance of these cytokines to ensure the transcriptional effects from BHB treatment indeed alter cytokine and chemokine protein profiles.

## Conclusions

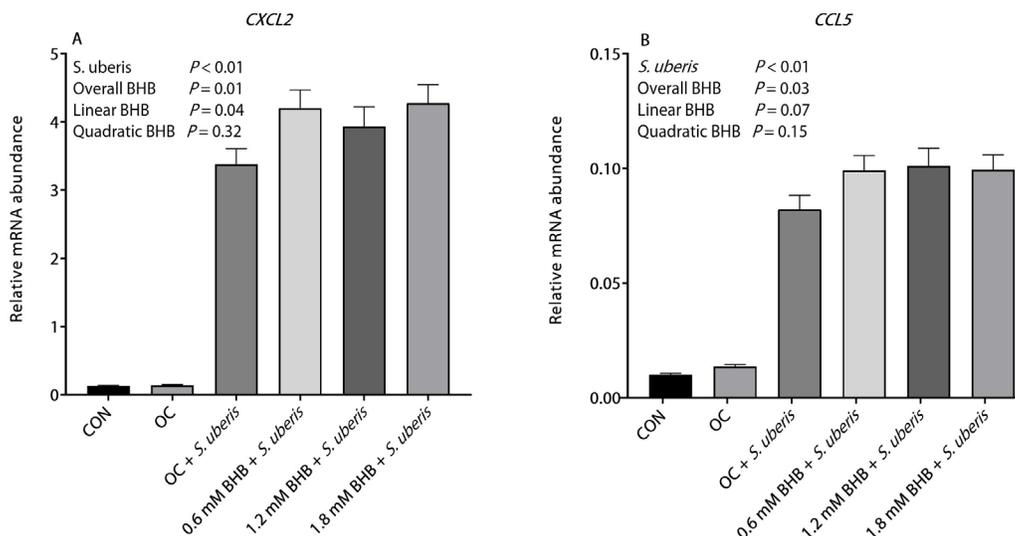
*Streptococcus uberis* is responsible for a large proportion of mastitis during the first month of lactation. In the present study, *S. uberis* increased GPR109a mRNA abundance, a receptor that is ligated by BHB. This resulted in a dose-dependent increase in mRNA abundance of both pro- and anti-inflammatory cytokines, including IL-1 $\beta$  and IL-10. An increase in the abundance of these cytokines could be indicative of the immune dysfunction that is typically seen in transition dairy cows. Future studies should be conducted in vivo to test the effect of BHB on immune function during an intramammary challenge.



**Figure 1.** Effect of *S. uberis* and BHB on receptor (TLR2, A; GPR109a, B) transcript abundance in RAW 264.7 mouse macrophages. *Streptococcus uberis* challenge increased mRNA of the receptor for identification of Gram-positive pathogens, TLR2 ( $P = 0.03$ ), as well as mRNA abundance of the receptor for BHB, GPR109a ( $P < 0.01$ ). Beta-Hydroxybutyrate treatment linearly increased TLR2 mRNA abundance ( $P = 0.01$ ) when compared to cells treated with just *S. uberis*. Treatment groups include: control (CON), OC (osmotic control), OC + *S. uberis*, 0.6 mM BHB + *S. uberis*, 1.2 mM BHB + *S. uberis*, and 1.8 mM BHB + *S. uberis*.



**Figure 2. Effect of *S. uberis* and BHB on pro-inflammatory (IL-1 $\beta$ , A; TNF $\alpha$ , B) and anti-inflammatory (IL-10, C; TGF $\beta$ , D) cytokine transcript abundance in RAW 264.7 mouse macrophages. *Streptococcus uberis* challenge activated the macrophages, increasing both pro- and anti-inflammatory cytokine mRNA abundance (all  $P < 0.01$ ). Beta-Hydroxybutyrate treatment linearly increased IL-1 $\beta$  ( $P < 0.01$ ) and IL-10 ( $P < 0.01$ ) mRNA abundance when compared to cells treated with just *S. uberis*, however, no effect of BHB was found on either TNF $\alpha$  or TGF $\beta$ . Treatment groups include: control (CON), OC (osmotic control), OC + *S. uberis*, 0.6 mM BHB + *S. uberis*, 1.2 mM BHB + *S. uberis*, and 1.8 mM BHB + *S. uberis*.**



**Figure 3. Effect of *S. uberis* and BHB on chemokine (CXCL2, A; CCL5, B) transcript abundance in RAW 264.7 mouse macrophages. *Streptococcus uberis* challenge activated the macrophages, increasing both CXCL2 and CCL5 mRNA abundance (both  $P < 0.01$ ). Beta-Hydroxybutyrate treatment linearly increased CXCL2 ( $P = 0.04$ ) as well as increased CCL5 ( $P = 0.03$ ) mRNA abundance when compared to cells treated with just *S. uberis*. Treatment groups include: control (CON), OC (osmotic control), OC + *S. uberis*, 0.6 mM BHB + *S. uberis*, 1.2 mM BHB + *S. uberis*, and 1.8 mM BHB + *S. uberis*.**