Interpretive Summary

Variation of total immunoglobulin G and β-lactoglobulin concentrations in colostrum and milk from Canadian Holsteins classified as High, Average or Low immune responders. *By Fleming et al.* Two host defense molecules were measured in colostrum and milk from cows classified as High, Average or Low immune responders according to the adaptive immune system. High antibody-mediated immune response cows had higher concentrations of IgG and β-LG in colostrum compared to Average and Low immune responders. Breeding for dairy cattle with optimal antibody-mediated immune responses may improve colostrum quality. Enhancing the quality of colostrum may decrease the risk of failure of passive transfer in calves and provide a more efficient source of ingredients for use as supplements or for incorporation into common human foods.

SHORT COMMUNICATION: IMMUNE CLASSIFICATION AND COLOSTRAL QUALITY

Variation of total immunoglobulin G and β-lactoglobulin concentrations in colostrum and milk from Canadian Holsteins classified as high, average or low immune responders

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ABSTRACT

The objective of this study was to evaluate variation in total immunoglobulin G (**IgG**) by radial immunodiffusion and β-lactoglobulin (**β-LG**) by enzyme-linked immunosorbent assay (ELISA) in colostrum and milk from Canadian Holsteins (n=108) classified as having High (**H**), Average (**A**) or Low (**L**) antibody-mediated (**AMIR**) or cell-mediated immune responses (**CMIR**) based on estimated breeding values. It was hypothesized that cows classified as H for AMIR and CMIR produce colostrum and milk with higher concentrations of immunologically active components. Colostrum and milk samples were collected on days 0 and 5 relative to calving, respectively. Data for IgG and β-LG in colostrum and milk were analyzed independently using mixed linear models; Tukey's test was used to compare least square means. Cows classified as H for AMIR had higher IgG and β-LG concentrations in colostrum compared to A and L immune responders. There were no differences in IgG and β-LG concentrations in colostrum among cows ranked on CMIR and in milk among cows ranked on AMIR. There were no differences in milk IgG concentrations among cows ranked on CMIR. Concentrations of β-LG were positively correlated with IgG concentrations in colostrum. These results suggest that breeding cows for H AMIR status may enhance passive transfer of IgG contributing to improved health in their calves, and that β-LG may play a role in the bovine immune system. Colostrum from H AMIR cows may also serve as a more economical feedstock source for natural health product manufacturing for human or animal use.

Key words: immunoglobulin, β-lactoglobulin, immune response, colostrum and milk quality

Optimal dairy cattle health is important for high quality milk production. Disease results in economic losses due to decreased milk quality, colostrum quality, and milk production, and increased labour costs, treatment costs and culling (Zwald et al., 2006). Disease also raises concerns regarding food safety, food quality and animal welfare. One potential solution to improve the health of dairy cattle is identifying and selectively breeding for High Immune Response (**HIR**) cows (Wagter et al., 2000; Mallard et al., 2011; Thompson-Crispi et al., 2012a).

The HIR test identifies cattle with balanced and robust levels of cell-mediated (**CMIR**) and antibody-mediated (**AMIR**) immune responses compared to other cows within and across herds (Wagter et al., 2000; Hernandez et al., 2005; Mallard et al., 2011; Thompson-Crispi et al., 2013). HIR cows have lower occurrence of mastitis, metritis, ketosis and retained placenta (DeLapaz, 2008; Thompson-Crispi et al., 2012a, 2013) compared to Low (**L**) and Average (**A**) responders. AMIR and CMIR are heritable (Thompson-Crispi et al., 2012b), suggesting that immune response traits are amenable to genetic selection.

Bovine colostrum and milk are rich sources of bioactive components important in animal and human health (Severin and Wenshui, 2005), respectively. Total immunoglobulin (Ig) G (consisting of IgG1, IgG2 and IgG3 subisotypes) and β-lactoglobulin (β-LG) were components of particular interest in this study. In bovine colostrum, immunoglobulins comprise greater than 95% of total whey protein, with IgG1 accounting for approximately 80% of the immunoglobulins (Butler, 1983). Adequate colostral quality is generally defined as IgG concentrations greater than 5,000 mg/dL and is associated with enhanced calf health (Weaver et al., 2000). Bovine IgG has anti-bacterial activities against mastitis-causing pathogens (e.g. *Escherichia coli*, *Staphylococcus aureus*) (Butler, 1983).

In bovine milk, whey protein is approximately 50% β-LG (Hambling et al., 1992). The primary function of β-LG remains unclear (Kontopidis et al., 2004), but it may function in transporting hydrophobic molecules (fatty acids, vitamin A and vitamin D) (Pérez and Calvo, 1995), in absorption and metabolism of fatty acids (Kushibiki et al., 2001) and in passive immunity (Ouwehand et al., 1997). β-LG has been shown to exert anti-bacterial activities against the mastitis-causing pathogens, *Staphylococcus aureus* and *Streptococcus uberis* (Chaneton et al., 2011). Proteolytic fragments of β-LG generated by trypsin hydrolysis also are bactericidal to Gram-positive organisms (Pellegrini et al., 2001). Furthermore, β-LG has anti-viral (Neurath et al., 1997), anti-cancer (Nakajima et al., 2006) and anti-oxidant (Elias et al., 2005) activities.

Because HIR cows (particularly cows classified as H for AMIR) have decreased incidence rates and severity of clinical mastitis (Thompson-Crispi et al., 2013), and because bovine IgG and β-LG exert anti-bacterial activities against mastitis-causing pathogens, it was hypothesized that HIR cows have increased concentrations of IgG and β-LG in colostrum and milk compared to cows with other immune response phenotypes. The objective of this study was thus to evaluate IgG and β-LG concentrations in colostral whey and early lactation milk from H, A and L immune response cows using radial immunodiffusion (**RID**) and enzyme-linked immunosorbent assay (**ELISA**), respectively.

Immune Response Phenotyping

Holstein cattle from the University of Guelph research herd (Elora, Ontario, ≈300 cows) and a commercial herd (Drayton, Ontario, ≈350 cows) were classified for AMIR and CMIR using a standardized 15 day protocol (Hine et al. 2011). Briefly, cows were immunized intramuscularly with type-one and type-two test antigens. Blood samples were taken on day 0 and day 14 to

evaluate primary IgG AMIR responses to the type-two test antigen by ELISA (using mouse monoclonal antibody anti-bovine IgG, clone BG-18, Sigma-Aldrich, Oakville, ON). A delayed-type hypersensitivity test to the type-one antigen was initiated at day 14 (Thompson-Crispi et al. 2012b) and evaluated 24 hours later to evaluate CMIR responses.

Statistical Analysis - Research Herd

Immune response phenotypes in the research herd were evaluated (once per animal) over a 4 year period, with data available on 390 animals. Full pedigree files including 16,763 related animals were provided by the Canadian Dairy Network (CDN, Guelph, Ontario). CMIR and AMIR were analyzed using univariate linear models as follows:

$$y_{ijklm} = \mu + \alpha \text{ x } d_i + \beta \text{ x } a_j + p_k + g_l + c_m + e_{ijklm}$$

where y_{ijklm} = AMIR or CMIR, μ = overall mean, d_i = day 0 data for AMIR or control site for CMIR, a_j = age in months at immune response test, α and β are regression co-efficients, p_k = pregnancy status (not pregnant, 1-100, or 101-200 days in calf), g_l = immune response test group (year of testing), c_m = random effect of animal and e_{ijklm} = residual error. Heritabilities of 0.29 for AMIR and 0.19 for CMIR were estimated previously (Thompson-Crispi et al. 2012b).

Statistical Analysis - Commercial Herd

The commercial herd was phenotyped using different parameters than the research herd. Animals were categorized into 7 groups as follows: bred heifers, cows being bred, calves, dry cows, high grain, low grain and transition cows. Data were available for 265 animals. Full pedigree files including 15,449 related animals were provided by CDN. CMIR and AMIR were analyzed using univariate linear models as follows:

$$y_{ijkl} = \mu + \alpha \text{ x } d_i + gl_j + gp_k + c_l + e_{ijkl}$$

where y_{ijkl} = AMIR or CMIR, μ = overall mean, d_i = day 0 data for AMIR or control site for CMIR, α is a regression co-efficient, gl_j = group lactation effect (lactation 0, 1, 2, 3 and ≥4), gp_k = group pregnancy status effect (not pregnant, 1-100, or 101-200, >200 days in calf), c_l = random effect of animal and e_{ijkl} = residual error. Heritabilities of 0.29 for AMIR and 0.19 for CMIR were used as estimated previously (Thompson-Crispi et al. 2012b).

Estimation of Breeding Values, Immune Response Classification

Breeding values were estimated for the research and commercial herd separately and the genetic analysis was performed with ASReml software (Gilmour et al., 1995). Cattle were ranked as H or L if EBVs were greater than one SD above or more than one SD below the mean response for each trait, respectively. Average AMIR and CMIR cows had EBVs within one SD of the mean.

Colostrum and milk samples from a total of 88 cows from the research herd were tested, including 21, 45 and 22, H, A and L responders (respectively) for AMIR and 15, 58, and 15, H, A, and L responders (respectively) for CMIR. For the commercial herd, a total of 20 cows were tested including 4, 10, and 6, H, A, and L responders (respectively) for AMIR and 4, 11, and 5, H, A and L responders (respectively) for CMIR. The day 5 milk average somatic cell counts for the research and commercial herds were 348,000 cells/mL and 1,067,000 cells/mL respectively, with an average somatic cell count of 488,000 cells/mL.

Colostrum and Milk Samples

Composite samples were collected on day 0 (day of calving, colostrum) and day 5 (milk). Samples were defatted by centrifugation (11,000xg for 30 min) and the whey fraction stored at -80 °C.

Total IgG Quantification

RID (Mancini et al., 1965) was used to determine total IgG concentrations using commercially prepared plates (Triple J Farms, Bellingham, WA). IgG standards (196, 1,402 and 2,748 mg/dL) for colostrum and (10, 50, 100 mg/dL) for milk, provided by the manufacturer were used to generate standard curves. Precipitate ring size (zone diameter) was measured 17 hours post-loading of each plate. Samples were diluted in PBS and run at initial dilutions of 1/4 and undiluted for colostrum and milk samples, respectively. If the diameter of the zone of precipitation was greater than that of the most concentrated standard, the sample was diluted further and rerun.

β-Lactoglobulin Quantification

β-LG was assayed using an ELISA kit (Bethyl Laboratories, Montgomery, TX). MaxiSorp™ 96-well plates (Fisher Scientific, Waltham, MA) were coated with affinity purified rabbit anti-bovine β-LG antibody diluted 1/100 in coating buffer (0.05 M carbonate-bicarbonate, pH=9.6) (Fisher), 100 µL/well and incubated at room temperature (RT) for one hour. Plates were washed using wash buffer (WB, 50mM Tris, 0.14M NaCl and 0.05% Tween 20, pH=8.0; Sigma). Plates were blocked with 200 µL/well WB and incubated at RT for 30 minutes. Diluted standard or sample was added at 100 µL/well in duplicate and incubated at RT for 1 hour. Standards were diluted using a two-fold dilution series to generate a standard curve. Colostrum

and milk samples were diluted using a four-fold dilution series in WB. Plates were washed and 100 µL/well of horseradish peroxidase-conjugated rabbit anti-bovine β-LG antibody diluted 1/100,000 in WB was added. Plates were incubated at RT for 1 hour before washing. Enzyme substrate, tetramethylbenzidine (Bethyl) was added at 100 µL/well for 8 minutes. Stop solution (0.18M H_2SO_4, Fisher) was subsequently added (100 µL/well). Optical density was measured at 450 nm using a BioTek (Winooski, VT) plate reader.

Total IgG and β-Lactoglobulin Statistical Analysis

Data for IgG and β-LG in colostrum and milk were analyzed independently using linear mixed models (PROC MIXED, SAS Version 9.1.3, SAS Institute, Cary, NC). The statistical model was:

$$y_{ijkl} = \mu + p_i + w_j + b_k + i_l + e_{ijkl}$$

where y_{ijkl} = IgG or β-LG; μ = the overall mean; p_i = parity (1, 2, 3 and ≥4); w_j = colostrum or milk weight (kg) of complete composite milking; b_k = herd (commercial or research); i_l = immune response category (H, A or L for AMIR and CMIR); e_{ijkl} = the residual error. Covariates with *P*-values > 0.10 were removed from the model. Interactions were tested and removed if non-significant preserving hierarchy. Normality was tested using the Shapiro-Wilk test and data that were not normally distributed were log transformed. Least square means were estimated and Tukey's test was used to compare contrasts between immune response groups. A Pearson correlation coefficient was used to determine associations between IgG and β-LG in colostrum and milk.

Results and Discussion

Concentrations of IgG in colostrum ranged from 399 to 20,864 mg/dL, (mean 7,048 mg/dL, SD 3,556). IgG concentrations in milk ranged from 19 to 2,096 mg/dL (mean 90 mg/dL, SD 216). β-LG concentrations in colostrum ranged from 2.5 to 12 mg/mL (mean 6.6 mg/mL, SD 2.0). β-LG concentrations in milk ranged from 0.2 to 4.2 mg/mL (mean 1.3 mg/mL, SD 0.89).

Colostrum samples for 78 cows out of 108 (72%) contained more than the suggested minimum of 5,000 mg/dL of IgG (Weaver et al., 2000). Twenty-one out of 25 (84%) of H AMIR, 38 out of 55 (69%) of A AMIR, and 19 out of 28 (68%) of L AMIR cows had over 5,000 mg/dL IgG in their colostrum. Fourteen out of 19 (74%) H CMIR, 49 out of 69 (71%) A CMIR and 15 out of 20 (75%) L CMIR had over 5,000 mg/dL of IgG in their colostrum.

Parity was found to have a significant ($P<0.01$) effect on IgG concentrations in colostrum, with parity 2 cows having the lowest concentrations of IgG, compared to parity 1, 3 and ≥4 cows (Figure 1A). Parity was also found to have a significant ($P<0.01$) effect on β-LG in milk, with parity 1 cows having lower β-LG concentrations in milk compared to parity 2 and 3 cows (Figure 1B).

Herd had a significant ($P<0.0001$) effect on IgG and β-LG concentrations in colostrum and milk, with cows from the research herd having significantly greater IgG and β-LG concentrations in colostrum and significantly lower IgG and β-LG concentrations in milk compared to cows from the commercial herd.

The trait AMIR, was found to have a significant ($P<0.01$) effect on colostral IgG and β-LG concentrations, with H AMIR cows having significantly more IgG and β-LG in colostrum compared to A and L immune responders. Cows H for AMIR had 2,472 mg/dL and 1,599 mg/dL higher concentrations of IgG in colostrum on average than cows with A and L AMIR,

203 respectively (Figure 2). This result is not surprising because H AMIR cows were classified as
204 having higher serum IgG antibody responses to a type-2 antigen. IgG1 in cattle is considered to
205 be characteristic of type-2 responses and IgG1 constitutes the major immunoglobulin component
206 in bovine colostrum (Butler, 1983).
207 H AMIR cows also had 1.8 mg/mL and 1.6 mg/mL higher concentrations of β-LG in
208 colostrum compared to A and L AMIR cows, respectively (Figure 3). However, no significant
209 differences in colostral IgG and β-LG concentrations were found among cows classified based
210 on CMIR. There were no significant differences in milk IgG concentrations among cows
211 classified according to AMIR or CMIR.
212 IgG concentrations in colostrum and milk were negatively correlated (r = -0.23; $P<0.05$).
213 Similarly, milk weight was negatively correlated (r = -0.20; $P<0.05$) with IgG concentration in
214 milk. β-LG concentrations in milk had a tendency ($P=0.10$) to be negatively correlated (r = -
215 0.18) with milk weight. IgG and β-LG concentrations in colostrum were positively correlated (r
216 = 0.48; $P<0.0001$). Colostrum and milk yield were positively correlated (r = 0.33, $P<0.001$).
217 Milk weight was (significantly) negatively correlated with IgG concentration and had a
218 tendency toward a negative correlation with β-LG in milk. These results suggest that lower
219 concentrations of IgG and β-LG are present in larger milk volumes, similar to studies conducted
220 by Guidry et al. (1980) for IgG and Ng-Kwai-Hang et al. (1987) for β-LG.
221 At the genetic level, single nucleotide polymorphisms (Martin et al., 2002) or epigenetic
222 modifications (Rijnkels et al., 2010) may drive varied expression of Ig genes as shown with other
223 bioactive milk components. Decreased IgG concentrations in colostrum may increase the
224 likelihood of mammary gland infection during late pregnancy, but increased IgG concentrations
225 in day five milk may reduce the risk post-calving. IgG antibodies in milk (following vaccination

with a J5 mutant *E. coli* bacterin), have been associated with protection against mastitis after experimental intramammary infusion of *E. coli* (Wilson et al., 2007).

Herd had a significant effect on IgG and β-LG concentrations in colostrum and milk such that the research herd had greater IgG and β-LG in colostrum and less IgG and β-LG in milk, compared to the commercial herd. Factors such as housing type and diet may specifically influence whey protein quantities. Housing type (i.e. free-stall or tie-stall) has been associated with the incidence of mastitis pathogens (Olde Riekerink et al., 2008) and thus exposure to different pathogen loads in the environment may stimulate the immune response to various degrees. Mean somatic cell counts (SCC) were higher in the commercial herd, but SCC were not found to be significant covariates in the statistical models. Feed supplementation with selenium (Ceballos-Marquez et al., 2010) and chromium (Burton et al., 1993) has been associated with udder health and immune function, respectively, suggesting that diet may influence IgG and β-LG concentrations in colostrum and milk. Previous immunizations and disease incidence may partly explain the observed variation of IgG and β-LG concentrations in colostrum and milk concentrations between herds.

Parity had a significant effect on IgG concentrations in colostrum. Higher IgG concentrations were present in colostrum of later parity cows compared to second parity cows, similar to studies conducted by Gulliksen et al. (2008). Because H AMIR is associated with longevity (Thompson-Crispi, 2012), breeding for H AMIR status may lead to additional gains in IgG in colostrum associated with longer herd life.

Parity also had a significant effect on β-LG concentrations in milk, with parity 2 and 3 cows having significantly more β-LG in milk than parity 1 cows, consistent with findings of Ng-Kwai-Hang et al. (1987). Parity did not have a significant effect on colostral β-LG

concentrations. A stable proportion of β-LG may suggest an important function for the protein in colostrum. The primary function of β-LG is unclear. In this study, cows with H AMIR produced colostrum containing higher concentrations of IgG and β-LG . Based on H AMIR cows having more colostral IgG and β-LG, and because IgG primarily functions as an immune response molecule, it could be hypothesized that β-LG also contributes to immune system function.

In this study, colostrum from H AMIR cattle had higher concentrations of IgG on average compared to A or L AMIR cows. Enhanced colostrum quality can be defined as having greater concentrations of IgG than 5,000 mg/dL and has been associated with enhanced calf health. Failure of passive transfer (**FPT**) (calf serum IgG concentration <1,000 mg/dL at 24 to 48 h of age, Weaver et al., 2000) occurs when calves receive inadequate quantities of colostral IgG and is associated with increased susceptibility to alimentary and respiratory tract infections, as well as death within the first weeks of life. Eighty-four percent of H AMIR cows produced colostrum containing greater than 5,000 mg/dL of IgG per dL compared to 69% of A AMIR and 68% of L AMIR cows. These results suggest that feeding calves colostrum from H AMIR cows may reduce the incidence of FPT in calves. In situations where H AMIR cows produce more colostrum than needed for their own calf, excess colostrum could be used to benefit additional calves.

The health benefits of whey are under investigation in humans, with whey supplements manufactured from byproducts from cheese-making (Marshall, 2004). Colostrum from H AMIR cows may provide a more efficient source of ingredients for future manufacturing of natural health products for human consumption. Future research is required to determine if β-LG plays a role in preventing septicemia, diarrhea and respiratory infections in calves after birth and if this molecule could be used to compensate for lack of protective immunoglobulins in colostrum.

Quantifying total IgG and β-LG in colostrum and milk from cows with different adaptive immune response phenotypes was the main focus of this study. H AMIR cows had significantly greater concentrations of IgG and β-LG in colostrum compared to A and L AMIR cows. H-AMIR cows were also the most likely to have the recommended minimum concentration of 5,000 mg/dL in colostrum. Concentrations of IgG were positively and significantly correlated with β-LG concentrations in colostrum. Ultimately, breeding for cattle with H antibody-mediated immune responses (AMIR) may lead to the production of better quality colostrum to improve passive protection in young calves.

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Figure 1.

A: Total immunoglobulin isotype G (IgG) (mg/dL) concentrations in colostrum based on parities of 1 (n=43), 2 (n=28), 3 (n=27) and ≥ 4 (n=10), n=108. **B:** β-lactoglobulin (mg/mL) concentrations in milk based on parity 1 (n=42), 2 (n=27), 3 (n=25) and ≥ 4 (n=9), n=103. Columns represent least squares means with bars indicating standard errors of the means. Columns with different letters differ significantly ($P < 0.05$).

Figure 2. Total immunoglobulin isotype G (IgG) (mg/dL) concentrations in colostrum (first milk post-calving) from High (n=25), Average (n=55) and Low (n=28) cows ranked for antibody-mediated immune response (AMIR) and from High (n=19), Average (n=69) and Low (n=20) cows ranked for cell-mediated immune response (CMIR). Columns represent least squares means with bars indicating standard errors of the means. Within an immune response trait, columns with different letters differ significantly ($P < 0.05$).

Figure 3. β-lactoglobulin (mg/mL) concentrations in colostrum (first milk post-calving) for High (n=25), Average (n=55) and Low (n=28) cows ranked for antibody-mediated immune response (AMIR) and for High (n=19), Average (n=69) and Low (n=20) cows ranked for the cell-mediated immune response (CMIR). Columns represent least squares means with bars indicating standard errors of the means. Within an immune response trait, columns with different letters differ significantly ($P < 0.05$).