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Immunological effects of altering the concentrate inclusion level in a grass silage based diet for early lactation Holstein Friesian cows

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Abstract:	Concentrate inclusion levels in dairy cow diets are often adjusted so that the milk yield responses remain economic. While changes in concentrate level on performance is well known, their impact on other biological parameters, including immune function, is less well understood. The objective of this study was to evaluate the effect of concentrate inclusion level in a grass silage based mixed ration on immune function. Following calving 63 (45 multiparous and 18 primiparous) Holstein Friesian dairy cows were allocated to one of three iso-nitrogenous diets for the first 70 days of lactation. Diets comprised of a mixture of concentrates and grass silage, with concentrates comprising either a low (30%, LC), medium (50%, MC) or high (70%, HC) proportion of the diet on a DM basis. Daily DM intakes, milk yields and body weight were recorded, along with weekly body condition score, milk composition and vaginal mucus scores. Blood biochemistry was measured using a chemistry analyzer, neutrophil phagocytic and oxidative burst assessed using commercial kits and flow cytometry, and interferon gamma production evaluated by ELISA after whole blood stimulation. Over the study period cows on HC had a higher total DM intake, milk yield, fat yield, protein yield, fat + protein yield, protein content, mean body weight and mean daily energy balance, and a lower body weight loss than cows on MC, whose respective values were higher than cows on LC. Cows on HC and MC had a lower serum non-esterified fatty acid concentration than cows on LC (0.37, 0.37 and 0.50 mmol/L, respectively, P = 0.005, SED = 0.032), while cows on HC had a lower serum beta-hydroxybutyrate concentration than cows on MC and LC (0.42, 0.55 and 0.55 mmol/l, respectively, P = 0.002, SED = 0.03). Concentrate inclusion level had no effect on vaginal mucus scores. At week 3 postpartum, cows on HC tended to have a higher percentage of

	oxidative burst positive neutrophils than cows on LC (43.2 and 35.3 %, respectively, P = 0.078, SED = 3.11), although at all other times concentrate inclusion level in the total mixed ration had no effect on neutrophil phagocytic or oxidative burst characteristics, or on interferon gamma production by pokeweed mitogen stimulated whole blood culture. This study demonstrates that for high yielding Holstein Friesian cows managed on a grass silage-based diet, concentrate inclusion levels in early lactation affects performance but has no effect on neutrophil or lymphocyte immune parameters.

Immunological effects of altering the concentrate inclusion level in a grass silage based diet for early lactation Holstein Friesian cows

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Short title: Early lactation concentrate level and immunity

Abstract

Concentrate inclusion levels in dairy cow diets are often adjusted so that the milk yield responses remain economic. While changes in concentrate level on performance is well known, their impact on other biological parameters, including immune function, is less well understood. The objective of this study was to evaluate the effect of concentrate inclusion level in a grass silage based mixed ration on immune function. Following calving 63 (45 multiparous and 18 primiparous) Holstein Friesian dairy cows were allocated to one of three iso-nitrogenous diets for the first 70 days of lactation. Diets comprised of a mixture of concentrates and grass silage, with concentrates comprising either a low (30%, LC), medium (50%, MC) or high (70%, HC) proportion of the diet on a DM basis. Daily DM intakes, milk yields and body weight were recorded, along with weekly body condition score, milk composition and vaginal mucus scores. Blood biochemistry was measured using a chemistry analyzer, neutrophil phagocytic and oxidative burst assessed using commercial kits and flow cytometry, and interferon gamma production evaluated by ELISA after whole blood stimulation. Over the study period cows on HC had a higher total DM intake, milk yield, fat yield, protein yield, fat + protein yield, protein content, mean body weight and mean daily energy balance, and a lower body weight loss than cows on MC, whose respective values were higher than cows on LC. Cows on HC and MC had a lower serum non-esterified fatty acid concentration than cows on LC (0.37, 0.37 and 0.50 mmol/L, respectively, P = 0.005, SED = 0.032), while cows on HC had a lower serum beta-hydroxybutyrate concentration than cows on MC and LC (0.42, 0.55 and 0.55 mmol/l, respectively, P = 0.002, SED = 0.03). Concentrate inclusion level had no effect on vaginal mucus scores. At week 3 postpartum, cows on HC tended to have a higher percentage of oxidative burst positive neutrophils than cows on LC (43.2 and 35.3 %,

respectively, P = 0.078, SED = 3.11), although at all other times concentrate inclusion level in the total mixed ration had no effect on neutrophil phagocytic or oxidative burst characteristics, or on interferon gamma production by pokeweed mitogen stimulated whole blood culture. This study demonstrates that for high yielding Holstein Friesian cows managed on a grass silage-based diet, concentrate inclusion levels in early lactation affects performance but has no effect on neutrophil or lymphocyte immune parameters.

Key words: dairy cows, concentrate level, immune function, neutrophils, interferon gamma.

Implications

This study demonstrates that for high yielding Holstein Friesian cows managed on a grass silage-based diet, concentrate inclusion levels in early lactation can be substantially increased or decreased, with corresponding effects on performance, energy balance and body tissue mobilization. However, concentrate level had no effect on the immune parameters examined in this study, including the ability of neutrophils to phagocytose bacteria and respond with oxidative burst, and interferon gamma production of lymphocytes following whole blood stimulation.

Introduction

The intakes of dairy cows during early lactation are often unable to keep pace with the rapid increase in energy requirements associated with milk production, and as a

consequence, cows enter negative energy balance (EB) (Ingvartsen, 2006). Negative nutrient balance (including negative EB) is associated with an increase in both production and inflammatory disease, leading to significant economic loss and animal welfare problems (Mulligan and Doherty, 2008). In addition, changes in blood metabolite profiles due to the catabolic state of body tissue mobilization have been shown to impair immune function. For example, *in vitro* studies have demonstrated that low glucose concentrations reduce the energy available for many neutrophil functions (Newsholme *et al*., 1986; Roche *et al*., 2013), higher non-esterified fatty acid (NEFA) concentrations decrease neutrophil viability (Scalia *et al*., 2006), and higher beta-hydroxybutyrate (BHB) concentrations impair neutrophil phagocytic and bactericidal capacity (Suriyasathaporn *et al*., 2000). However, few *in vivo* studies have directly examined the relationship between EB and immune function in early lactation. In early lactation cows it might be expected that higher concentrate inclusion levels would improve nutrient balance (including EB), improve immune function and decrease the risk of health problems.

Achieving high energy intakes in early lactation are expected to reduce negative EB at this time, with Ferris *et al*. (2003) advocating the use of diets with a high intake potential and/or with a high energy density. While this can be achieved by increasing the proportion of concentrates in the diet, concentrates are generally more expensive than pasture and conserved forage. Consequently, concentrate feed levels on commercial farms will be influenced by economic factors. For example, concentrate feed levels may be increased under a high milk price and/or low concentrate cost scenario or reduced under a low milk price and/or high concentrate cost scenario. Indeed, concentrate feed levels adopted in many countries have changed

considerably in recent years, reflecting volatility in global dairy markets and fluctuations in the costs of feed ingredients. While many studies have examined the impact of postpartum concentrate feed levels on DM intake (DMI), milk production and tissue changes (for example, Ferris *et al*., 1999; Andersen *et al*., 2003; Sterk *et al*., 2011), few have examined the impact of postpartum concentrate feed level on immune function *in vivo*.

Therefore, the objectives of the current study were to investigate the effect of concentrate inclusion level in a grass silage-based diet on the performance, metabolic and immune function of early lactation Holstein Friesian cows. We hypothesized that increasing the proportion of concentrates in early lactation would improve EB and result in improvements in measures of immune function.

Material and methods

Animals and Housing

This study was conducted at the Agri-Food and Biosciences Institute, Northern Ireland (from September 2014 to January 2015), and involved 45 multiparous (mean parity, 3.5; SD, 1.27) and 18 primiparous Holstein Friesian dairy cows. Cows had a mean Predicted Transmitting Ability $(PTA)_{2015}$ for milk yield of 132 (SD, 128.1) kg, milk fat plus protein yield of 20.6 (SD, 9.20) kg and a mean Profitable Lifetime Index $(PLI)_{2015}$ of £260 (SD, 85.7). These cows were within the top 1% of UK genetics in terms of PLI.

Throughout the experiment all cows were housed together in a free stall cubicle house with concrete flooring, which was scraped every 3 hours by an automated system. The

cubicle to cow ratio was ≥ 1:1 at all times. Cubicles were fitted with rubber mats and were bedded three times each week with sawdust.

Experimental Design, Diets and Feeding

All cows were managed identically during the prepartum period. Within 24 hours of calving, cows were transferred from a maternity pen to the free stall cubicle house described earlier. Cows were randomly assigned to one of three treatments at calving, namely low concentrate (LC), medium concentrate (MC) and high concentrate (HC), with primiparous and multiparous cows being assigned separately. However, throughout the allocation process a check was made to ensure that the three treatment groups remained 'balanced' for parity, PTA for fat plus protein (kg), pre-calving body weight (BW) and body condition score (BCS), and in the case of multiparous cows, for previous lactation 305-day milk yield.

The treatment diets were offered as a partial mixed ration comprising concentrates and grass silage in differing ratios (30:70, 50:50 and 70:30) on a DM basis for LC, MC and HC, respectively. The concentrates offered with each treatment (Table 1) were formulated using the FeedByte® rationing model version 3.78 (available at http://www.sruc.ac.uk/info/120110/dairy/354/dairy_services-key_features) so as to achieve a common total diet crude protein concentration of 150 g/kg DM with each of LC, MC and HC. Concentrate formulations were based on the quality of grass silage offered and estimated intakes of silage and concentrates by cows on each treatment. The grass silage offered was produced from a primary growth herbage that was harvested from predominantly perennial ryegrass-based swards and ensiled following

148 a 24 to 48 hours period of field wilting. Rations were prepared daily using a complete
149 diet mixer wagon (Redrock Varicut, Redrock, County Armagh, Northern Ireland), and
150 transferred directly to feed-boxes mounted on weigh cells. Access to treatment rations
151 were controlled by a Calan Broadbent feeding system (American Calan Inc.,
152 Northwood, NH, USA) linked to an electronic identification system, thus enabling
153 individual cow intakes to be recorded daily. Uneaten ration was removed daily at
154 approximately 08.00, while the fresh ration was offered between 09.00 to 10.00. To
155 ensure *ad libitum* consumption, the diets for each treatment were offered at 107 % of
156 the previous day intake. To maintain efficient cow flow onto the milking parlour, all
157 cows were offered an additional 0.5 kg concentrate at each milking via an in-parlor
158 feeding system (Table 1). The study was conducted over the first 70 days post-calving.
159
160 *Feed analysis*
161 Samples of grass silage were taken daily (n = 110), dried at 85^{o}C for 18 hours to
162 determine oven DM content and milled through a sieve with 0.8 mm apertures.
163 Subsamples of the dried milled silages were collected twice weekly and composited
164 every 14 day (n = 8), with the composited sample analyzed for neutral detergent fibre
165 (NDF), acid detergent fibre (ADF) and ash. In addition, a sample of the silage was
166 taken every 7 day (n = 16), split into two portions, one for analysis of pH and
167 concentrations of gross energy, crude protein (CP), (nitrogen(N) × 6.25), ammonia-N,
168 and fermentation acids (lactic and acetic acid), and one for estimation of metabolizable
169 energy (ME) concentration. Volatility coefficients were used to convert the oven DM
170 contents of the grass silages offered to a volatile-corrected DM basis. A sample of
171 each of the concentrates offered was taken weekly, dried at 100^{o}C for 24 hours before
172 milling through a 0.8 mm sieve, composited every 14 day (n = 8) and analyzed for

NDF, ADF, ash, gross energy and CP (N × 6.25) concentrations. An additional concentrate sample was taken at the same frequency, dried at 60^{o}C for 48 hours, and milled (0.5 mm sieve) before analysis for starch concentration. All lab analyses were conducted as described by Little *et al*. (2017).

Measurements of cow performance

Ration intakes for each individual cow were recorded daily using the intake recording system described earlier. Cows were milked twice daily, between 05.30 and 07.00, and 15.30 and 17.00, through a 50-point rotary milking parlor. Individual cow milk yields were automatically recorded at each milking and a mean daily milk yield was calculated for each cow on a weekly basis.

On the same day each week throughout the study, milk samples were obtained from two consecutive milkings (am and pm separately), a preservative tablet added (Broad Spectrum Microtabs II, D and F Control Systems, Massachusetts, USA), and samples stored at 4 ^{o}C until analyzed. Samples were analyzed for fat and protein content by Fourier transform infrared spectroscopy. The instrument used was a Standard Lactoscope FT-MIR automatic (Delta Instruments, Drachten, The Netherlands), with fat and protein contents predicted using the models provided by the manufacturer. A weighted milk composition was subsequently calculated for each sampling occasion. In addition, on one occasion each month, samples from two consecutive milkings, bulked in proportion to yield, were collected and somatic cell count (SCC) measured using flow cytometry (SomaScope, Delta Instruments, Drachten, The Netherlands). Cow BW were recorded twice daily and cow BCS were recorded weekly as described by Little *et al*. (2017). Mean daily ME requirements were calculated as described by

198 Little *et al*. (2017), while energy corrected milk yield (ECMY) were calculated using
199 (ME_{milk} / 3.1). Vaginal mucus was assessed and scored by a single operator at weeks
200 2 (11 to 17 days), 3 (18 to 24 days), and 4 (25 to 31 days) postpartum, as described
201 in detail by Little *et al*. (2017). Briefly, vaginal mucus was accessed for color, proportion
202 and volume of pus, and a character score assigned as follows: (0) clear or translucent
203 mucus; (1) mucus containing flecks of white or off-white pus; (2) <50 mL exudate
204 containing ≤50% white or off-white mucopurulent material; and (3) >50 mL exudate
205 containing purulent material, usually white or yellow, but occasionally sanguineous.
206 The vaginal mucus was also assessed for odor, and given a score 0 for normal odor
207 or a score of 1 if a fetid odor was detected.
208
209 *Blood measurements*
210 *Blood Biochemistry.* Blood samples were collected at weeks 1 (4 to 10 days), 2 (11 to
211 17 days), 3 (18 to 24 days), 4 (25 to 31 days), 5 (32 to 38 days), 6 (39 to 45 days) and
212 10 (67 to 73 days) postpartum, for the measurement of albumin, BHB, glucose,
213 globulin, NEFA, total protein, and urea concentrations. These were stored and
214 analyzed on a Randox Imola chemistry analyzer system (Randox, County Antrim,
215 United Kingdom), as described in detail by Little *et al*. (2017).
216
217 *Neutrophil Phagocytic and Oxidative Burst Measurements.* An additional blood sample
218 was collected in a lithium heparin tube (BD, Oxford, UK) at weeks 1 (6 to 8 days), 2
219 (13 to 15 days) and 3 (20 to 22 days) postpartum for the measurement of the *in vitro*
220 phagocytic capacity and oxidative burst activity of neutrophils. The test and control
221 samples were processed in duplicate within 3 hours of collection, using Phagotest and
222 Phagoburst kits, respectively (Orpegen Pharma GmbH, Heidelberg, Germany), with

modifications to manufacturer's instructions as described in detail by Little *et al*. (2017). These were then analyzed in duplicate by flow cytometric analysis. A live gate identifying the neutrophil population was set using the forward and side scatter properties of these cells, and the corresponding green fluorescence histogram (FL1) was analyzed. The control sample was used to set a threshold for fluorescence so that only 1 to 3 % of the acquired events were positive. The number of events above this threshold was counted as the percentage of neutrophils actively carrying out phagocytosis or oxidative burst. The mean fluorescence intensity (MFI) correlates to the number of bacteria phagocytosed by each cell, or the mean oxidative burst activity by a single cell. The phagocytic or oxidative burst index is the percentage of active neutrophils multiplied by the fluorescence intensity.

Interferon Gamma Production. An additional blood sample was collected in a lithium heparin tube at weeks 1 (6 to 8 days), 2 (13 to 15 days), 3 (20 to 22 days), 4 (27 to 29 days), 5 (34 to 36 days) and 6 (41 to 43 days) postpartum for the ELISA measurement of Interferon gamma (IFN-γ) production from stimulated lymphocytes, as described in detail by Little *et al*. (2017). Briefly, whole blood samples were incubated for 24 hours at 37^{o}C and 5% carbon dioxide with phosphate buffered saline as a negative control to look at the inherent IFN-γ level, and also with pokeweed mitogen used as a positive stimulant of peripheral blood mononuclear cells to produce IFN-γ.

Statistical Analysis

Two multiparous cows were removed from the experiment (one due to a chronic displaced abomasum and one due to injury), and their data excluded from the

248 statistical analysis, leaving 14 multiparous cows and 6 primiparous cows on LC and
249 MC, and 15 multiparous cows and 6 primiparous cows on HC. Data were analyzed
250 using GenStat Version 16.2 (VSN International, Oxford, UK). Data describing BW
251 change to nadir, days to nadir BW, BW change to day 70, BCS and the flow cytometric
252 analysis of neutrophil phagocytosis and oxidative burst were analyzed using analysis
253 of variance (ANOVA) with treatment and parity (primiparous or multiparous) as factors.
254 Where significant in the model, appropriate pre-experimental variables were included
255 as covariates when analyzing corresponding dependent variables. Where $P < 0.05$ for
256 the fixed effect of treatment, differences between treatments were tested using
257 Fisher's protected adjusted multiple comparisons. Data describing DMI, milk yield, milk
258 composition, somatic cell score ($\log^e$ transformed somatic cell count to make the data
259 normally distributed), mean BW, mean daily EB, serum and plasma biochemistry and
260 IFN-γ production were analyzed using repeated measures Residual Maximum
261 Likelihood (REML) analysis. The mixed model used included the following terms as
262 fixed effects: treatment + week + parity + (treatment × week) + (parity × week) +
263 (treatment × parity). Cow within week were included as random effects, to which an
264 antidependence order 1 covariance structure was applied. Parity was categorized as
265 primiparous or multiparous. Where significant in the model, appropriate pre-
266 experimental variables were included as covariates in the mixed model. For
267 multiparous cows, milk yield was analyzed with the addition of previous lactation 305-
268 day milk yield in the model. Similarly, for multiparous cows, milk fat composition, milk
269 crude protein composition, fat yield, crude protein yield, fat + protein yield, and mean
270 BW were analyzed with the addition of previous lactation fat composition, crude protein
271 composition, fat yield, crude protein yield, fat + protein yield, and pre-experimental
272 BW, respectively, in the model. The absence of data for primiparous animals for these

variables meant that ‘actual’ previous lactation data could not be used for multiparous cows. Rather, for each variable, a covariate value for each multiparous cow within each treatment was calculated as the difference between the value for each individual cow during the previous lactation, and the mean value for all cows on that treatment during the previous lactation, with a value of ‘zero’ used for primiparous cows. Residual plots were used to check the normality assumption and homogeneity of variance for model validity. Where $P < 0.05$ for the fixed effect of treatment in the F-test, differences between treatments were tested using Fisher’s unprotected least significant difference test. The relationships between mean weekly immune parameters (IFN-γ, neutrophil phagocytic index and neutrophil oxidative burst index) and mean weekly EB, ECMY, serum NEFA and BHB, and plasma glucose concentrations were evaluated using simple linear regression analysis and differences were considered statistically significant when $P < 0.05$. Data describing vaginal mucus scores at each week were analyzed using generalized linear model regression analysis with the logit link function. The model included treatment as a term and significance was identified using chi squared testing. Vaginal mucus score data were translated into one integer; 0 = 0, 0; 1 = 1, 0; 2 = 2, 0; 3 = 3, 0; 4 = 2, 1; 5 = 3, 1, and for analysis were grouped into 2 categories, ≤ 1 and > 1.

Results

The grass silage offered was of good quality and was well fermented (Table 2). The concentrates offered differed in crude protein content so that the mixed rations would be isonitrogenous. This was achieved, with rations offered with LC, MC and HC having CP contents of 152, 152 and 154 g/kg DM, while the calculated ME content was 12.0, 12.4 and 12.8 MJ/kg DM, respectively. Rations offered with LC, MC and HC were

298 calculated (using FeedByte® version 3.78) to supply 1 556, 1 997 and 2 420 g effective
299 rumen degradable protein (ERDP)/cow per day, and 559, 733 and 888 g digestible
300 undegradable protein (DUP)/cow per day, compared to requirements of 1 235, 1 700
301 and 2 175 g ERDP/cow per day, and 790, 788 and 875 g DUP/cow per day. Similarly,
302 rations with LC, MC and HC were calculated to supply 1 346, 1 817 and 2 275 g
303 MP/cow per day, compared to requirements of 1 577, 1 872 and 2 262 g MP/cow per
304 day. Thus only HC was calculated to fully meet the DUP and MP requirements of the
305 cows.
306
307 Cows on LC and MC had a higher silage DMI ($P < 0.01$) than those on HC, while
308 multiparous cows had a higher silage DMI ($P < 0.01$) than primiparous cows (Table 3).
309 Cows on LC had a lower milk fat yield than those on MC and HC ($P < 0.01$), while
310 multiparous cows had a higher milk fat yield ($P < 0.01$) than primiparous cows. Milk fat
311 + protein yield increased from LC through to HC ($P < 0.01$), while multiparous cows
312 had a higher milk fat + protein yield ($P < 0.01$) than primiparous cows. Milk crude
313 protein content increased ($P < 0.01$) from LC through to HC, while milk crude protein
314 content ($P = 0.95$) was unaffected by parity. Milk somatic cell score was unaffected by
315 treatment ($P = 0.46$) and parity ($P = 0.39$). Cows on HC had a higher mean BW ($P =$
316 0.01) than those on LC, while multiparous cows had a higher BW ($P = 0.02$) than
317 primiparous cows. Cows on HC had a lower BW change to nadir ($P = 0.02$) than those
318 on LC, while BW change to nadir ($P = 0.14$), was unaffected by parity. Days to nadir
319 BW ($P < 0.01$) decreased from LC through to HC, while days to nadir BW ($P = 0.45$)
320 was unaffected by parity. Bodyweight change to day 70 ($P < 0.01$) decreased from LC
321 through to HC, while multiparous cows tended to lose less BW to day 70 ($P = 0.08$)
322 than primiparous cows. Treatment did not affect BCS at day 70 ($P = 0.17$) nor BCS

change to day 70 (P = 0.11), while parity did not affect BCS at day 70 (P = 0.10) or BCS change to day 70 (P = 0.70). Mean daily negative EB decreased from LC through to HC (P < 0.01), while multiparous cows had a negative mean daily energy balance compared to a positive mean daily energy balance in primiparous cows (P < 0.01). There was no difference in serum albumin (P = 0.11) and globulin concentrations (P = 0.12) between treatments. Cows on HC had a lower serum BHB concentration (P < 0.01), higher plasma glucose concentration (P = 0.02), and higher serum total protein concentration (P < 0.01) than those on MC and LC, while cows on LC had a higher serum NEFA concentration (P < 0.01) than those on MC and HC. Multiparous cows had a higher serum albumin (P = 0.01), BHB (P = 0.02), NEFA (P < 0.01), and total protein concentration (P < 0.01), tended to have a higher serum globulin concentration (P = 0.06), and had a lower plasma glucose concentration (P = 0.03). There were no significant (P < 0.05) concentrate level × parity interactions for any of the parameters presented in Table 3.

There was a significant treatment × parity interaction for concentrate DMI (P < 0.01), total DMI (P < 0.01), milk yield (P = 0.02), milk crude protein yield (P < 0.01), milk fat composition (P < 0.01) and blood serum urea concentration (P < 0.01) (Table 4). For each of concentrate DMI, total DMI and crude protein yield, values increased with increasing concentrate inclusion level, while the magnitude of the increase was greater with multiparous than primiparous cows. Concentrate level had no effect on milk yield of primiparous cows (P > 0.05) while milk yield of multiparous cows increased with increasing concentrate level (P = 0.02). With primiparous cows, LC had a lower milk fat composition than MC and HC, while with multiparous cows, milk fat composition was unaffected by concentrate level (P > 0.05). Blood serum urea concentrations

348 decreased with increasing concentrate levels, with the magnitude of this decrease
349 greatest with primiparous cows ($P < 0.01$).
350
351 All the parameters presented in Table 3 and Table 4 changed with time ($P < 0.05$),
352 with the changes in total DMI, concentrate DMI, BW and milk yield presented in Figure
353 1. There was a significant treatment × time interaction ($P < 0.05$) for total DMI,
354 concentrate DMI, milk yield, crude protein yield, fat + protein yield, mean BW, BHB,
355 globulin and total protein. At weeks 1 and 2, cows on LC had a lower total DMI than
356 cows on MC and HC, whilst at weeks 3 to 10 total DMI increased from LC through to
357 HC ($P < 0.05$; Figure 1a). At weeks 1 to 10, concentrate DMI increased from LC
358 through to HC ($P < 0.05$; Figure 1b). At weeks 5 and 6, cows on LC had a lower BW
359 than cows on HC, at week 7 cows on LC had a lower BW than cows on MC and HC,
360 whilst at weeks 8 to 10 BW increased from LC through to HC ($P < 0.05$; Figure 1c). At
361 weeks 3 to 5 cows on LC had a lower milk yield than cows on HC, whilst at weeks 6
362 to 10 milk yield increased from LC through to HC ($P < 0.05$; Figure 1d). At weeks 2 to
363 5 cows on LC had a lower crude protein yield than cows on HC, at week 6 and 7 cows
364 on LC had a lower crude protein yield than cows on MC and HC, whilst at weeks 8 to
365 10 crude protein yield increased from LC through to HC ($P < 0.05$). At weeks 2 to 4
366 cows on LC had a lower fat + protein yield than cows on HC, whilst at weeks 5 to 10
367 fat + protein yield increased from LC through to HC ($P < 0.05$). The changes in serum
368 NEFA, BHB, urea and plasma glucose concentrations over time are presented in
369 Figure 2. At week 2, serum BHB increased from HC through LC, at week 3 cows on
370 MC had a higher BHB than cows on LC which had a higher BHB than cows on HC. At
371 weeks 4, 5 and 6 cows on MC and LC had a higher BHB than cows on HC, while at
372 week 10 cows on LC had a higher BHB than cows on HC ($P < 0.05$); Figure 2b). At

weeks 4 and 5 cows on HC had a higher serum globulin than cows on LC, at week 6 serum globulin increased from LC through HC, while at week 10 cows on HC had a higher globulin than cows on MC and LC ($P < 0.05$). At week-5 cows on HC had a higher total protein than cows on LC, while at weeks 6 and 10 cows on HC had a higher total protein than cows on MC and LC ($P < 0.05$).

Concentrate inclusion level had no statistical difference on the probability of obtaining different vaginal mucus scores at weeks 2, 3 or 4 of lactation ($P = 0.87$, 0.53 and 0.19, respectively).

There were no significant ($P > 0.05$) concentrate level × time interactions for any of the neutrophil function parameters examined, and as such only the main effects of treatment and parity over the three measurement periods are presented in Table 5. Concentrate inclusion level had no effect ($P > 0.05$) on the percentage of phagocytic neutrophils, the phagocytic MFI or the phagocytic index of neutrophils, the percentage of oxidative burst neutrophils, the oxidative burst MFI or the oxidative burst index of neutrophils (Table 5). While the percentage of phagocytic neutrophils was unaffected by parity ($P = 0.11$), primiparous cows had a higher MFI of phagocytic neutrophils ($P = 0.01$), phagocytic index ($P = 0.01$), percentage of oxidative burst neutrophils ($P < 0.01$), oxidative burst index ($P < 0.01$), and tended to have a higher MFI of oxidative burst neutrophils ($P = 0.10$) compared with multiparous cows (Table 5). While the oxidative burst measures did not change with time, the percentage of phagocytic neutrophils (45.7, 44.3 and 41.7; $P = 0.01$), MFI of phagocytic neutrophils (94.8, 73.3

397 and 63.0, $P < 0.01$) and phagocytic index (44.1, 33.5 and 27.9; $P < 0.01$) all decreased
398 with time from calving (values, for weeks 1, 2 and 3 post calving, respectively).
399
400 While treatments had no statistically significant effect ($P > 0.05$) on the IFN-γ
401 production by PWM stimulated whole blood culture, IFN-γ production changed over
402 time ($P < 0.01$), reaching a peak at week 3 post calving and then decreased during
403 week 4, 5 and 6. However, there was no treatment × time interaction ($P > 0.05$; Figure
404 3a). While multiparous cows had a higher ($P < 0.01$) IFN-γ production than primiparous
405 cows, IFN-γ production increased to a peak at week 3 and then decreased ($P < 0.01$),
406 while there was no treatment × time interaction ($P > 0.05$; Figure 3b).
407
408 Linear regression analysis identified no significant ($P < 0.05$) relationships or strong
409 model fit ($R^2 > 0.25$) between EB and IFN-γ production, ECMY and IFN-γ production,
410 NEFA and IFN-γ production, or BHB and IFN-γ production. There was a positive
411 relationship between plasma glucose and neutrophil phagocytic index ($R^2 = 0.27$, $P <$
412 0.01) at week 1 of lactation. No significant relationships were identified between
413 neutrophil function and any of the parameters examined (P>0.05).
414
415 **Discussion**
416 As the milk price to concentrate cost ratio changes in response to volatility in global
417 dairy markets and fluctuations in the costs of feed ingredients, concentrate inclusion
418 levels in dairy cow diets are often adjusted to ensure that an economic milk production
419 response is achieved. While the impact of changes in concentrate levels on cow
420 performance is well known, their impact on other biological parameters such as
421 immune function have received much less focus *in vivo*. Thus, the objectives of the

current study were to investigate the effect of concentrate inclusion level in a grass silage based diet on immune function of early lactation Holstein Friesian cows.

Dry Matter Intake, Milk Production and Bodyweight Changes

With multiparous cows, total DMI increased as concentrate inclusion level in the diet increased, in agreement with others (Ferris *et al*., 1999; Sterk *et al*., 2011; McCarthy *et al*., 2015a). This increase in DMI was expected as a decrease in the forage component of the ration generally decreases the restriction on rumen fill and allows total DMI to increase (Allen, 2000). However, with primiparous cows, total DMI increased between LC and MC but not with HC, with this consistent with the fact that milk yield of primiparous cows did not increase with increasing concentrate level, while crude protein yield increased to a lesser extent with primiparous than multiparous cows. With multiparous cows, as concentrate inclusion level in the diet increased, there was associated with an increase in milk yield, and milk crude protein yield. The increase in milk crude protein content as concentrate inclusion level increased was likely due to increased microbial protein synthesis (Jenkins and McGuire, 2006). Although a reduction in milk fat content has been reported with higher concentrate (higher starch, lower NDF) rations (Ferris *et al*., 1999; Sterk *et al*., 2011), the opposite was observed with primiparous cows in the current study, while milk fat of multiparous cows was unaffected by concentrate level. However, the effect of concentrate level on milk fat content can be inconsistent, with Machado *et al*. (2014) and Rabelo *et al*. (2003) both observing milk fat content to be unaffected by concentrate level. The increase in serum total protein with cows on HC was driven by the numerical increase in serum globulin however, these values remain within normal reference ranges (Radostits *et al*., 2007).

As the concentrate inclusion level in the diet increased, the extent of negative EB decreased, while cows on HC had a shorter duration of negative EB compared to cows on MC and LC. This improved EB as concentrate inclusion level increased was manifested in lower body tissue mobilization (lower BW loss, lower serum NEFA and BHB concentrations and a tendency for a lower BCS loss), which agrees with the findings of others (Andersen *et al*., 2003; McCarthy *et al*., 2015b). While postpartum negative EB is a normal physiological occurrence in dairy cows (Grummer *et al*., 2004; Ingvartsen, 2006), a shorter duration and lesser depth of negative EB may minimize the detrimental impacts on immune function, and may result in improved cow health and welfare (Ingvartsen and Moyes, 2013; Sordillo, 2016). In addition to a negative EB, DUP requirements with LC and MC were not fully met (559 and 773 g DUP compared to requirements of 790 and 788 g for LC and MC, respectively) and may have contributed to tissue catabolism with these treatments to help meet the protein requirements of the cow.

Immune Function

It was hypothesized that increasing the proportion of concentrates in the diet in early lactation would reduce negative EB and as such, improve immune function. The functional capacity of blood neutrophils were evaluated as effective neutrophil function is required to resolve bacterial infections that occur in early lactation, such as mastitis and metritis (Paape *et al*., 2002; Sheldon *et al*., 2009). Neutrophil function, EB and uterine disease have been linked, with Galvão *et al*. (2010) demonstrating that cows which developed uterine disease had a greater postpartum negative EB and lower neutrophil intracellular glycogen (needed for neutrophil phagocytosis and microbial

killing) than healthy cows. However, despite significant differences in EB across the 3 treatments in the current study, concentrate inclusion level did not influence circulating neutrophil physiology as measured by phagocytic or oxidative burst capacity. This is perhaps surprising, especially as cows on the LC diet had lower plasma glucose, and higher serum BHB and NEFA concentrations, compared with those on the HC diet, with these changes in metabolic profiles normally associated with reduced neutrophil function (Suriyasathaporn *et al*., 2000; Scalia *et al*., 2006). However, the differences in EB, plasma glucose, serum BHB and serum NEFA concentrations between treatments may not have been sufficiently large to impact on neutrophil function. A similar finding was observed in a recent study (McCarthy *et al*., 2015a; McCarthy *et al*., 2015b; Yasui *et al*., 2016) in which cows offered a high starch ration had improved early lactation EB and higher plasma glucose concentrations, lower serum NEFA and BHB concentrations (compared with cows offered a low starch ration), but yet cell physiology, as measured by neutrophil phagocytosis and oxidative burst, did not differ between treatments. In addition, neutrophil function has been shown to decline around calving, with nadir function a few days before (Kimura et al., 1999) or after (Gilbert et al., 1993) parturition, remaining low for approximately 15 days postpartum, before increasing until at least 6 weeks postpartum (Gilbert et al., 1993). However, a depression in neutrophil function at the time of calving does not always occur (Llamas Moya et al., 2008; Little et al., 2016), with the current study showing neutrophil phagocytic function to decline during the first three weeks postpartum. This occurs despite serum BHB and NEFA concentrations decreasing and serum glucose concentrations increasing with time postpartum, with these changes in the metabolic profile normally associated with improved measures of neutrophil function (Suriyasathaporn *et al*., 2000; Scalia *et al*., 2006).

Lymphocyte physiology, as measured by the functional ability to produce the cytokine IFN-γ was also examined using PWM in a whole blood culture. Interferon gamma is a cytokine that is synthesized by activated T-lymphocytes and functions to enhance immune surveillance and activate the cellular immune response during infection (Schroder *et al*., 2004), while reduced IFN-γ production is associated with increased susceptibility to infectious diseases (Nonnecke *et al*., 2003). The current study provides no evidence that an increase in negative EB in early lactation had a detrimental effect on lymphocyte function. With regards IFN-γ production, there is conflicting evidence that lymphocyte function is influenced by metabolites associated with negative EB. For example, an *in vitro* study by Ster *et al*. (2012) demonstrated that incubating isolated peripheral blood mononuclear cells in an environment comprising an increasing NEFA concentration, resulted in decreasing IFN-γ production. Similarly, Loiselle *et al*. (2009) demonstrated increased IFN-γ production at day 5 and day 14 postpartum when cows had a lower serum NEFA concentrations. However, in agreement with the outcomes of the current study which involved a direct measure of cell physiology, Carbonneau *et al*. (2012) observed no increase in IFN-γ production in cows with an improved EB (as demonstrated by lower serum NEFA concentrations) in early lactation. This may be due to the relatively small difference (0.13 mmol/L) in mean serum NEFA concentration between LC and HC in the current study. In addition, no relationships were identified between serum NEFA and IFN- γ production in the current study, perhaps a reflection of the BCS of the cows on the study. For example, Lacetera *et al*. (2005) observed no relationship between serum NEFA concentration and IFN-γ production in thin (BCS ≤ 2.5) cows, while a negative relationship between serum NEFA and IFN-γ production was observed in medium (2.6

522 < BCS < 3.5) and over conditioned (BCS ≥ 3.5) cows. As the mean BCS of cows in
523 the current study was 2.5, the absence of a relationship between serum NEFA
524 concentration and IFN-γ production is largely in agreement with the findings of
525 (Lacetera *et al*., 2005). Immune responses also play an important role in resolving
526 unavoidable bacterial contamination of the uterus that occurs after calving (Sheldon
527 *et al*., 2009). Vaginal mucus scores, which were used to assess the extent of uterine
528 bacterial infection, provided no evidence that concentrate proportion in the diet in early
529 lactation affected the risk of uterine infection. These observations are consistent with
530 the study by Yasui *et al*. (2016), in which rations that supplied different starch levels
531 and altered EB in early lactation had no effect on the incidence of cytological
532 endometritis.
533
534 Given the link between increasing NEFA concentrations in the cell environment, and
535 decreasing IFN-γ production (Ster *et al*. 2012), it might have been expected that IFN-
536 γ production would continue to rise throughout the 6 week measurement period.
537 However, IFN-γ production in the current study increased from calving until week 3
538 postpartum, before declining, in common with the findings of Little *et al*. (2016). In
539 addition, Heiser *et al*. (2015) reported a reduction in IFN-γ mRNA expression around
540 calving, which overshot to increased levels 2 weeks postpartum, before falling.
541
542 Although it is well known that a deficiency of protein impairs immune function and
543 increases susceptibility to infectious diseases in humans (Li *et al*.,2007), much less is
544 known about the relationship between protein supply and immune function in animals.
545 Increasing dietary protein supply increased intestinal mucosal mast cells and
546 eosinophils, and significantly reduced the worm burden in rats (Jones *et al*., 2011),

547 while increasing the dietary protein supply in sheep increased local leukocytes and
548 IgE antibodies against gastrointestinal nematode parasites (Houdijk *et al*., 2005).
549 While diets offered to cows on LC and MC were deficient in metabolisable protein
550 (1346 and 1817 g MP/cow per day compared to requirements of 1577 and 1872 g for
551 LC and MC, respectively), the potential impact of this on the immune function of cows
552 on these treatments is unknown. Recognising the absence of information on this issue,
553 both Dann *et al*. (2013) and Roche *et al*. (2013) have advocated further research on
554 the effects of dietary protein level, protein type, and essential amino acids, on cow
555 health and immunity during the transition period and in early lactation.
556
557 Multiparous cows had a lower phagocytic MFI, phagocytic index, percentage of
558 oxidative burst neutrophils and oxidative index, compared with primiparous cows. This
559 lower neutrophil function in multiparous cows is consistent with the limited information
560 published previously. For example, neutrophils from multiparous cows had lower
561 superoxide anion production (Gilbert *et al*., 1993) and lower mean oxidative burst
562 activity (Llamas Moya *et al*., 2008), compared with those from primiparous cows.
563 However, regarding the acquired immune function, multiparous cows had a higher
564 production of the lymphocyte cytokine IFN-γ after stimulation, compared with
565 primiparous cows, in agreement with others (Lessard *et al*., 2004; O'Driscoll *et al*.,
566 2012). An increased IFN-γ production is associated with a heightened immune
567 surveillance and function during infection (Schroder *et al*., 2004) and increased
568 resistance to infectious diseases (Nonnecke *et al*., 2003). Thus, these findings suggest
569 that during early lactation, multiparous cows have a greater decrease in cellular
570 immunity but a lesser decrease in humoral immunity, compared with primiparous
571 cows. This may have negative implications for disease in multiparous cows, as the

572 main infectious challenge in early lactation, such as those responsible for mastitis
573 (Paape *et al*., 2002) and metritis (Sheldon *et al*., 2009) are bacterial in origin.
574
575 In summary, this study demonstrates that increasing concentrate inclusion level in a
576 grass silage-based mixed ration resulted in an increase in total DMI, milk yield, milk
577 protein composition and yields of fat and protein. With the higher concentrate inclusion
578 level, EB also increased, with an associated reduction in tissue mobilization as
579 evidenced by less BW and BCW loss and lower serum NEFA and BHB concentrations.
580 However, in contrast to the hypothesis, a lower concentrate inclusion level in early
581 lactation had no impact on immune function as measured by neutrophil phagocytosis
582 and oxidative burst, and lymphocyte IFN-γ production.
583
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594
595 **Declaration of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics committee

All procedures described in this paper were approved by the animal research ethics committee at the Agri-Food and Biosciences Institute, Hillsborough and were conducted under an experimental license granted by the Department of Health, Social Services & Personal Safety for Northern Ireland, in compliance with the United Kingdom (UK) Animals (Scientific Procedures) Act 1986.

Software and data repository resources

None of the data or models were deposited in an official repository.

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Table 1 The ingredient compositions (g/kg fresh basis) of the concentrates offered with the low (LC), medium (MC), and high (HC) concentrate treatments, and the concentrate offered via the in-parlor concentrate feeder to Holstein Friesian dairy cows.

	Treatment concentrates			Concentrate offered in-parlor
	Low	Medium	High	
Maize grain	300	250	172	300
Wheat grain	100	83	114	100
Soya bean meal	136	83	58	50
Soya hulls	100	125	200	200
Barley	100	83	100	-
Rapeseed meal	50	62	77	-
Sugar beet pulp	100	125	114	100
Maize gluten feed	50	125	114	175
Mineral/vitamin mix	25	17	14	25
Maxfat CS [1]	13	25	17	25
Cane molasses	13	13	14	25
Acidbuf[2]	13	9	6	-

LC = low concentrate, MC = medium concentrate, HC = high concentrate

[1] Maxfat CS, Rumen protected fat, Trouw Nutrition, Cheshire, UK

[2] Acidbuf, Rumen acid buffer, Celtic sea minerals, Cork, Ireland

Table 2 The chemical compositions (and standard deviation in parenthesis) of the grass silage and concentrates offered to Holstein Friesian dairy cows during the study

	Grass silage	Treatment concentrates			Concentrate offered in-parlor
		LC	MC	HC	
Oven DM (g/kg)	206 (30.8)	891 (5.5)	895 (8.0)	894 (8.7)	898 (11.3)
VCODM[1] (g/kg)	227 (12.5)	-	-	-	-
pH	3.85 (0.234)	-	-	-	-
Ammonia nitrogen (g/kg total nitrogen)	95 (9.2)	-	-	-	-
Composition of DM (g/kg)					
Crude protein	137 (9.6)	182 (5.6)	168 (6.6)	162 (2.7)	166 (5.1)
Lactic acid	127.8 (47.70)	-	-	-	-
Acetic acid	25.9 (8.33)	-	-	-	-
Acid detergent fibre	316 (7.2)	105 (11.7)	152 (19.9)	167 (12.4)	161 (26.9)
Neutral detergent fibre	559 (5.8)	211 (22.2)	285 (33.5)	306 (16.4)	320 (43.5)
Ash	91 (4.2)	92 (5.3)	84 (5.4)	80 (3.8)	75 (6.9)
Gross energy (MJ/kg DM)	19.1 (1.15)	17.7 (0.14)	18.0 (0.12)	17.9 (0.08)	18.3 (0.23)
Metabolisable energy (MJ/kg DM)	11.4 (0.32)[2]	13.1[3]	13.4[3]	13.4[3]	13.4[3]

LC = low concentrate, MC = medium concentrate, HC = high concentrate

[1]VCODM, volatile corrected oven dry matter

[2] Predicted using Near Infrared Reflectance Spectroscopy

[3] Calculated from standard values

Table 3: Effects of concentrate inclusion level in the mixed ration (low, LC; medium, MC; high, HC) on dry matter intake, milk production, milk crude protein, somatic cell score, body weight, body condition score, energy balance and on serum and plasma biochemistry during the first 70 days of lactation of Holstein Friesian dairy cows.

	Concentrate level				Parity			*P*-value	
	LC	MC	HC	SED[1]	Primiparous	Multiparous	SED[1]	Concentrate level	Parity
Silage dry matter intake (kg/cow per day)	9.8[a]	9.1[a]	6.4[b]	0.36	7.2	9.5	0.30	<0.01	<0.01
Yield (kg/cow per day)									
Fat	1.05[a]	1.22[b]	1.31[b]	0.056	0.88	1.51	0.05	<0.01	<0.01
Fat + Protein	2.80[a]	2.10[b]	2.37[c]	0.091	1.53	2.64	0.07	<0.01	<0.01
Milk crude protein (g/kg)	29.4[a]	31.4[b]	33.7[c]	0.51	31.6	31.4	0.41	<0.01	0.95
Somatic Cell Score (1000/ml $\log_e$)	10.8	10.7	11.1	0.29	11.0	10.8	0.23	0.46	0.39
Bodyweight									
Mean (kg)	599[a]	609[ab]	620[b]	6.8	600	618	8.0	0.01	0.02
Change to nadir (kg)	-65[a]	-49[ab]	-39[b]	6.1	-64	-46	10.4	0.02	0.14
Days to nadir body weight	52[a]	39[b]	29[c]	3.2	39	42	3.5	<0.01	0.45
Body weight change to day 70 (kg)	-53[a]	-30[b]	-9[c]	7.0	-47	-24	11.9	<0.01	0.08
Body Condition Score									
At day 70	2.37	2.42	2.46	0.049	2.52	2.68	0.065	0.17	0.10
Change to day 70	-0.33	-0.28	-0.12	0.076	-0.21	-0.26	0.089	0.11	0.70
Mean daily energy balance (MJ/cow per day)	-26.0[a]	-8.1[b]	7.5[c]	7.63	8.7	-26.4	6.23	<0.01	<0.01
Blood Biochemistry									
Albumin (g/L)	32.6	33.6	33.2	0.45	32.4	33.8	0.36	0.11	0.01

Beta-hydroxybutyrate (mmol/L)	0.55[a]	0.55[a]	0.42[b]	0.03	0.45	0.56	0.03	<0.01	0.02
Globulin (g/L)	34.5	35.3	38.5	1.17	35.1	37.1	0.93	0.12	0.06
Glucose (mmol/L)	3.27[a]	3.32[a]	3.44[b]	0.043	3.41	3.28	0.034	0.02	0.03
Non-esterifed fatty acid (mmol/L)	0.50[a]	0.37[b]	0.37[b]	0.032	0.32	0.51	0.026	<0.01	<0.01
Total protein (g/L)	67.1[a]	68.9[a]	71.6[b]	0.95	67.5	70.8	0.76	<0.01	<0.01

LC = low concentrate, MC = medium concentrate, HC = high concentrate

[1] SED, standard error of the difference

[a,b,c] Means with different superscripts, within a row, differ ($P < 0.05$)

Table 4: Effects of concentrate inclusion level in the mixed ration (low, LC; medium, MC; high, HC) on concentrate and total dry matter intake, milk and crude protein yield, milk fat content and serum urea concentration during the first 70 days of lactation of Holstein Friesian dairy cows

	Primiparous			Multiparous				*P*-value
	LC	MC	HC	LC	MC	HC	SED[1]	Concentrate level × Parity
Dry matter intake (kg/cow per day)								
Concentrate	4.5[a]	8.0[b]	12.1[d]	5.4[a]	10.4[c]	17.4[e]	0.48	<0.01
Total	13.2[a]	15.8[b]	17.3[b]	16.2[b]	20.8[c]	25.2[d]	0.89	<0.01
Yield (kg/cow per day)								
Milk	19.8[a]	20.6[a]	21.9[a]	31.3[b]	35.8[c]	41.5[d]	1.82	0.02
Crude protein	0.57[a]	0.66[ab]	0.74[b]	0.93[c]	1.10[d]	1.38[e]	0.05	<0.01
Milk fat (g/kg)	38.7[a]	44.7[c]	43.4[bc]	43.1[bc]	42.7[bc]	40.8[ab]	1.63	<0.01
Blood serum urea (mmol/L)	4.20[e]	3.14[c]	2.18[a]	3.46[d]	3.07[c]	2.58[b]	0.133	<0.01

LC = low concentrate, MC = medium concentrate, HC = high concentrate

[1] SED, standard error of the difference

[a,b,c,d,e] Means with different superscripts, within a row, differ ($P < 0.05$)

Table 5: Effects of concentrate inclusion level in the mixed ration (low, LC; medium, MC; high, HC) on the mean phagocytic and oxidative burst measures of neutrophils during the first 3 weeks postpartum of Holstein Friesian dairy cows.

	Concentrate level				Parity			*P*-value		
	LC	MC	HC	SED[1]	Primiparous	Multiparous	SED[1]	Concentrate level	Parity	Concentrate level × Parity
Phagocytic measures										
% phagocytic neutrophils	43.9	43.3	44.6	1.52	44.9	42.9	1.24	0.62	0.11	0.93
MFI[2]	71.5	79.5	80.1	7.30	84.4	69.7	5.96	0.80	0.01	0.12
Phagocytic index[3]	32.1	36.4	37.0	3.99	39.2	31.1	3.26	0.72	0.01	0.17
Oxidative burst measures										
% oxidative burst neutrophils	39.6	42.9	46.3	3.08	47.8	38.1	2.51	0.34	<0.01	0.17
MFI[2]	70.0	72.0	73.9	4.57	75.1	68.8	3.73	0.62	0.10	0.21
Oxidative burst index[4]	30.0	32.0	36.8	3.85	37.6	27.6	38.5	0.40	<0.01	0.14

LC = low concentrate, MC = medium concentrate, HC = high concentrate

[1] SED, standard error of the difference

[2] MFI = mean fluorescence intensity (x1000)

[3] Phagocytic index = [(% phagocytic neutrophils) × (MFI)] / 100

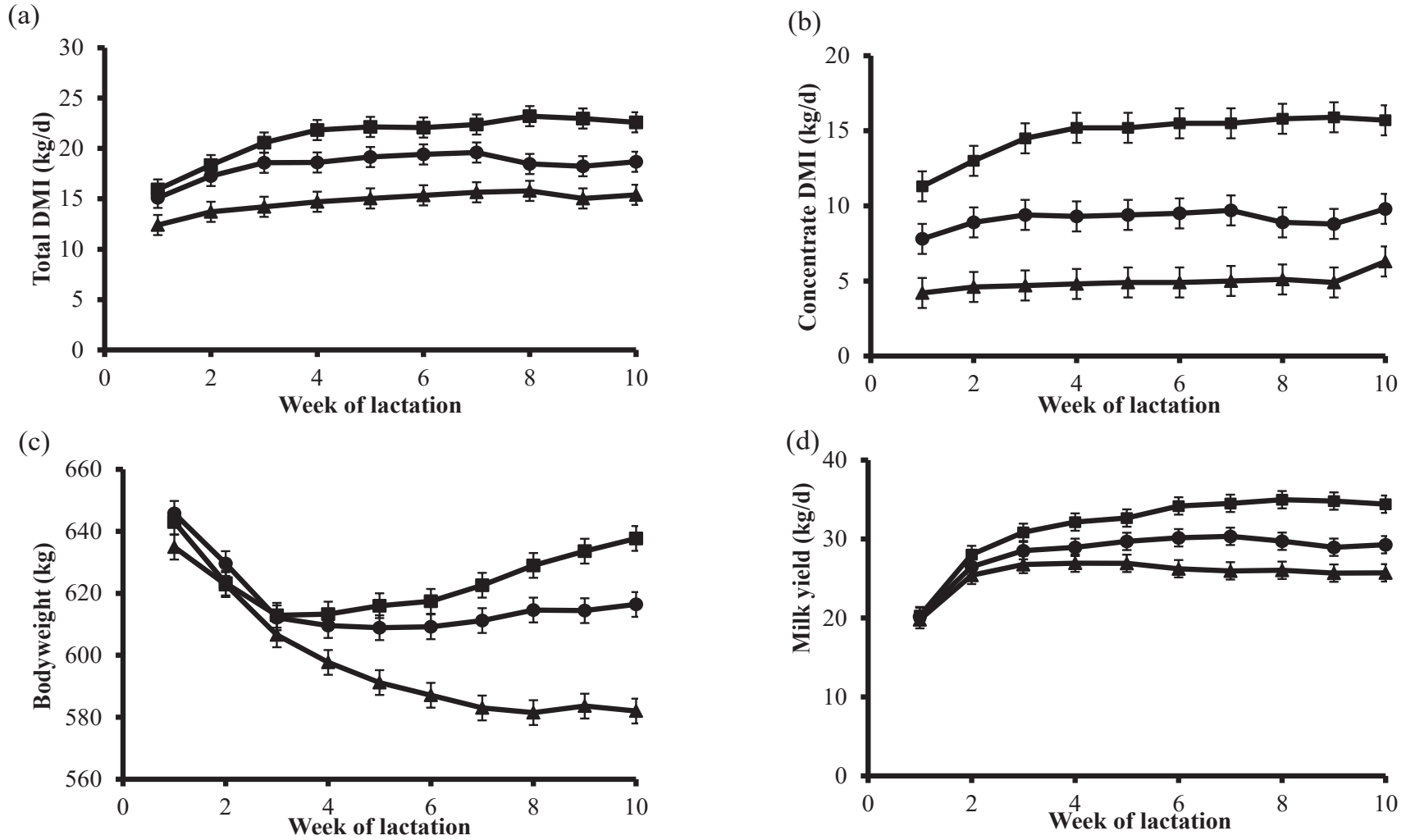
[4] Oxidative burst index = [(% oxidative burst neutrophils) × (MFI)] / 100

Figure 1: Effects of concentrate inclusion level in the mixed ration (low, LC, ; medium, MC ; and high, HC,) on (a) total dry matter intake (DMI) (b) concentrate dry matter intake (DMI), (c) body weight and (d) mean daily milk yield of Holstein Friesian dairy cows. Data presented is the mean data each week, with error bars representing the SEM

Figure 2: Effects of concentrate inclusion level in the mixed ration (low, LC, ; medium, MC ; and high, HC,) on (a) serum non-esterified fatty acid (NEFA) concentration, (b) serum beta-hydroxybutyrate (BHB) concentration, (c) plasma glucose concentration and (d) serum urea concentration of Holstein Friesian dairy cows. Data presented is the mean data each week, with error bars representing the SEM.

Figure 3: Effects of (a) concentrate inclusion level in the mixed ration (low, LC, ; medium, MC ; and high, HC, ; treatment, SED = 2.06, $P = 0.31$; time, SED = 1.21, $P < 0.01$; treatment × time, SED = 2.58, $P = 0.18$), and (b) parity (multiparous, primiparous; parity, SED = 1.68, $P < 0.01$; time, SED = 1.21, $P < 0.01$; parity × time, SED = 2.00, $P = 0.44$) on the mean interferon gamma (IFN-γ) production of whole blood due to pokeweed mitogen stimulation of Holstein Friesian dairy cows. Data presented is the mean data each week, with error bars representing the SEM.

Figure 1 hi-resolution



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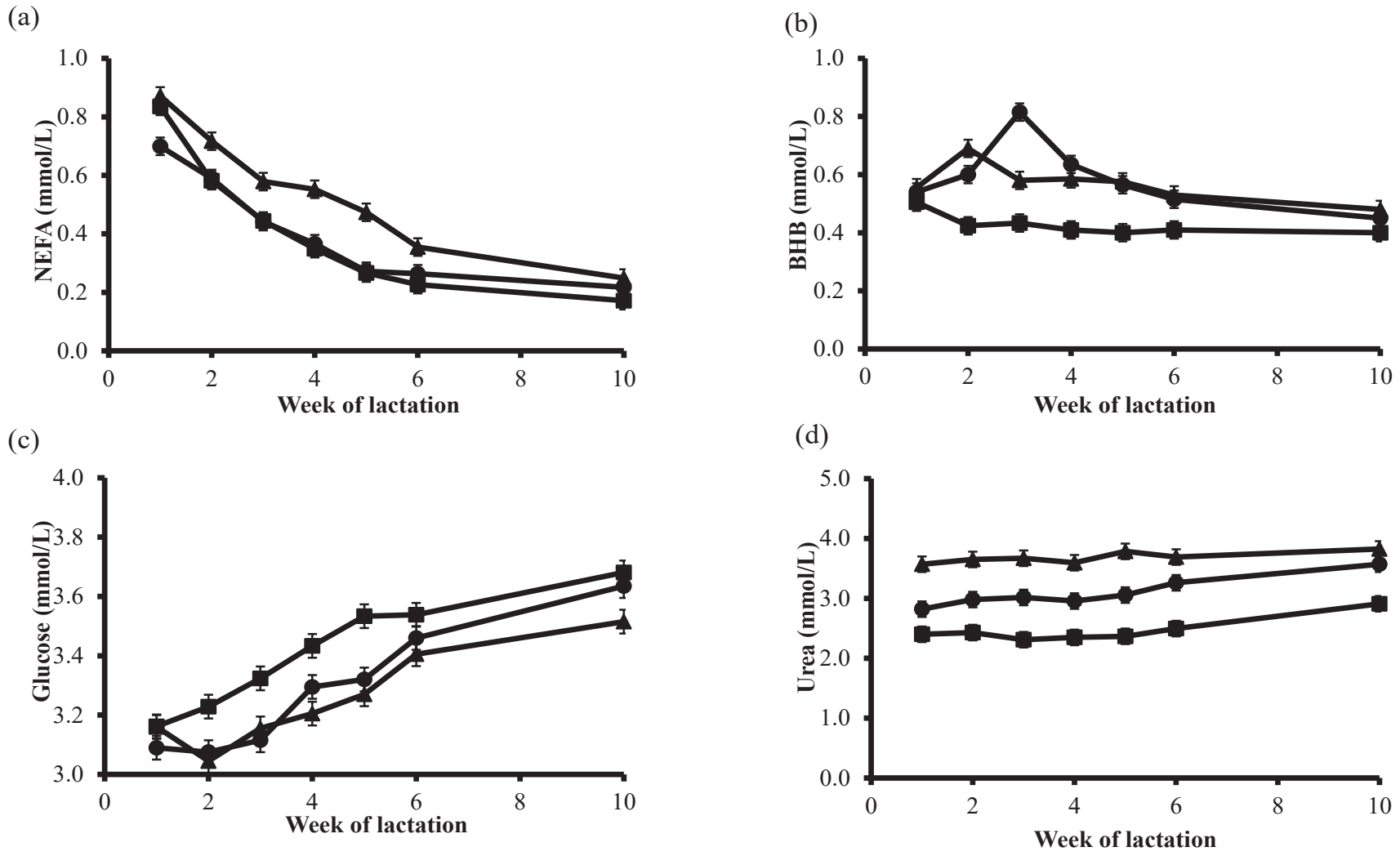

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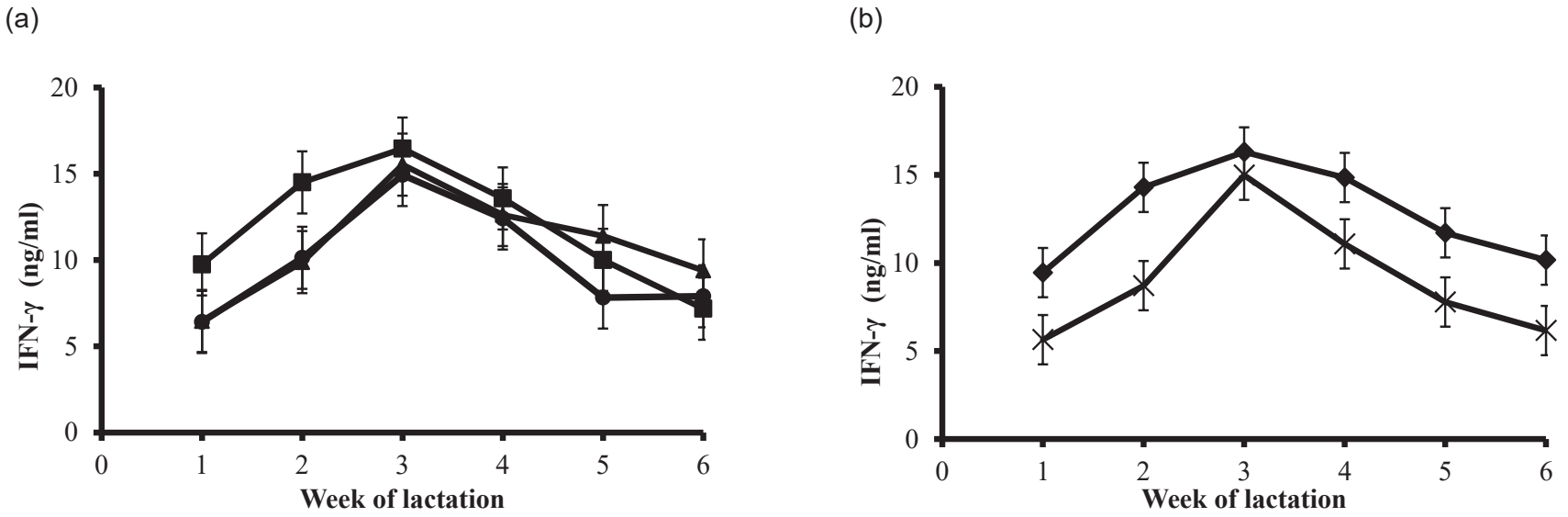

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- Berry DP, Wall E and Pryce JE 2014. Genetics and genomics of reproductive performance in dairy and beef cattle. Animal 8 (suppl. 1), 115–121.
- Knowles TG, Kestin SC, Haslam SM, Brown SN, Green LE, Butterworth A, Pope SJ, Dirk Pfeiffer D and Nicol CJ 2008. Leg disorders in broiler chickens: prevalence, risk factors and prevention. PLoS ONE 3, e1545.
- Pérez-Enciso M, Rincón JC and Legarra A 2015. Sequence- vs. chip-assisted genomic selection: accurate biological information is advised. Genetics Selection Evolution 47, 43. doi:10.1186/s12711-015-0117-5.
- When the article is online but not yet printed, the right format is:
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- Littell RC, Milliken GA, Stroup WW and Wolfinger RD 1996. SAS system for mixed models. Statistical Analysis Systems Institute Inc., Cary, NC, USA.
- Martin P and Bateson P 2007. Measuring behaviour. Cambridge University Press, Cambridge, UK.
- National Research Council (NRC) 2012. Nutrient requirements of swine, 11th revised edition. National Academy Press, Washington, DC, USA.

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- Nozière P and Hoch T 2006. Modelling fluxes of volatile fatty acids from rumen to portal blood. In Nutrient digestion and utilization in farm animals (ed. E Kebreab, J Dijkstra, A Bannink, WJJ Gerrits and J France), pp. 40–47. CABI Publishing, Wallingford, UK.

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- Martuzzi F, Summer A, Malacarne M and Mariani P 2001. Main protein fractions and fatty acids composition of mare milk: some nutritional remarks with reference to woman and cow milk. Paper presented at the 52nd Annual Meeting of the European Association for Animal Production, 26-29 August 2001, Budapest, Hungary.

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