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RESEARCH PAPER

Growth, weaning performance and blood indicators of humoral immunity in Holstein calves fed supplemental flavonoids

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Summary

The primary objective was to test the hypothesis that flavonoids mediate immune response and affect calf performance. Twenty Holstein calves [7 ± 2 days age; 41.4 ± 0.7 kg body weight (BW)] were randomly assigned to four treatments of (i) no; (ii) low (7.3×10^{-5} g/kg BW); (iii) medium (7.3×10^{-4} g/kg BW); and (iv) high (3.6×10^{-3} g/kg BW) doses of flavonoids intake in a completely randomized design. Calves received the treatments as a tablet until weaning or a daily intake of 680 g starter. After weaning, calves received no supplemental flavonoids and monitored until 120 days of age. The flavonoids were extracted from propolis. Treatments did not affect body length, wither height and the severity of scours. At week 5 of age, BW was higher when calves fed the high compared to the low dose of flavonoids. At week 6, calves fed the high dose of flavonoids had higher BW than those fed no or low doses of flavonoids. The serum immunoglobulin G (IgG) concentrations remained lower at the first 3 weeks of the experiment when calves received the low but not the high doses of flavonoids. At week 4, both medium and low doses of flavonoids moderated serum IgG. At week 8, the medium and high but not the low doses of flavonoids lowered serum IgG. At week 6, calves fed high and medium flavonoids doses had lower blood immunoglobulin M (IgM) than control calves. Results suggest that flavonoids affect the humoral immune response and can improve growth in young calves. This response depended on calf age. Future studies are needed to further evaluate the premise that dietary forages or the main source of flavonoids are helpful for a less stressful weaning in the modern calf raising.

Introduction

Flavonoids are products of the phenylpropanoid biosynthetic pathway with putative therapeutic effects (Coronado et al., 1995; Havsteen, 2002). Higher plants synthesize flavonoids during growth and development, which accumulate in the green cells (Havsteen, 2002). Flavonoids are involved in plant sensitivity to photons, energy transfer, endocrine regulation of growth, control of respiration and morphogenesis (Middleton and Kandaswami, 1992; Harborne and Baxter, 1999). Also, flavonoids have recently received much medical research interest (Harborne and Williams, 2000; Middleton et al., 2000). This is because flavonoids may possess anti-inflammatory, estrogenic, enzyme-inhibitory, antimicrobial, immunostimulatory, antiallergic, antioxidant and antitumour activities (Havsteen, 1983, 2002;

Harborne and Williams, 2000). As such, young growing ruminants may have evolutionarily depended on flavonoids. The main source of flavonoids in calf diets are forages, particularly fresh and non-ensiled types. Due to the stimulatory effect of highly fermentable carbohydrates on rumen papilla growth (Nocek et al., 1980; Heinrichs and Lesmeister, 2005), both the industrial and research interests to dietary use of forages for young calves have been diminishing. Also, forages have been highlighted mainly as a source of physical fibre and a stimulator of chewing and salivation. The evolutionary fact that young ruminants have been raised on fresh and green forages seems to have largely been overlooked. Thus, it remains a major question if flavonoids are important in microbial establishment and epithelial expansion of the reticulorumen. It is also unknown if flavonoids play a role in a smooth transition of the neonatal calf into a functioning fermentor. Our primary objective was to determine the effects of flavonoids on circulating indicators of humoral immunity and weaning performance of Holstein calves.

Materials and methods

Experimental design and calf management

Twenty Holstein calves [eight male and 12 female; 7 ± 2 days old; 41.4 ± 0.7 kg body weight (BW); mean ± SE] were divided into four groups based on dam parity, sex and birth weight. Five calves were assigned to each of four treatments in a completely randomized design with repeated measures. Treatments were tablets containing (i) 0 (CO); (ii) low or 7.3×10^{-5} g/kg BW (LF); (iii) medium or 7.3×10^{-4} g/kg BW (MF); and (iv) high or 3.6×10^{-3} g/kg BW (HF) flavonoids. Calves received no antibiotics at any time during the study. A commercial milk replacer was offered daily at 10% of BW until weaning. The milk replacer contained 220 g protein, 180 g fat, 9 g ash, 1 g crude fibre, 7 g calcium, 7 g phosphorus, 40 000 IU vit A, 5000 IU vit D3, 80 mg vit E, 100 mg Fe, 90 mg Zn and 10 mg I per kg. One kilogram of the milk replacer was mixed with 10 l of water and fed to individual calves. Weaning occurred when calves consumed a daily amount of 0.68 kg starter for two consecutive days (NRC, 2001; Franklin et al., 2003). Calves had unlimited access to fresh water and a starter concentrate. After weaning, calves were offered alfalfa hay in addition to the starter concentrate. The starter concentrate contained 11% barley grain, 32% soybean meal, 51.5% corn grain, 4.5% wheat bran and 1% vitamins and minerals supplement, on a dry matter basis. Calves were monitored until 4 months of age and housed outdoors in individual calf hutches at the Dairy Facilities of the Lavark Research Station (Isfahan University of Technology, Isfahan, Iran).

Flavonoids extraction and preparation

The flavonoids used were extracted from propolis (Yaghoubi et al., 2007b). Propolis samples were collected from an experimental apiary in Lavark (Isfahan, central Iran). Propolis was at 4 °C until processing. The propolis was cut into small pieces, ground, and washed with 80% ethanol (1:10 w/v) in a shaker (Ogawa Seiki Co., Ltd., Model PM 17, Tokyo, Japan) (300 rpm) at room temperature for 48 h. The ethanol extract solution was subsequently filtered through a filter paper (whatman # 41). The extracted solution was incubated at 35–40 °C for 2 weeks to be free of ethanol. The resultant brownish resin was mixed with 40% barley meal and used to make flavonoid tablets. Total flavonoids were determined using a colorimetric method (Popova et al., 2005). Briefly, 0.25 ml of the propolis extract was diluted with 1.25 ml of distilled water. Next, 75 μl of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, the mixture was added with 150 μl of a 10% $AlCl_3.6H_2O$ solution. Following 5 min, 0.5 ml of 1 M NaOH was added to the mixture, and the solution was made up to 2.5 ml with distilled water. The solution was mixed and the absorbance was read at 510 nm immediately afterwards. Total flavonoids were estimated against standard pyrocathecol concentrations.

Feed, fecal and blood sampling and analysis

Amount of starter offered and refused was recorded daily for individual calves. Body weight was recorded at birth and weekly until weaning. The BW was measured weekly until 6 weeks of age as well as at 30-day post-weaning and 120-day of age. Wither height and body length (withers to pins) were also recorded at birth and weaning (Larson et al., 1977). Fecal physical form was scored at morning and evening liquid-feed deliveries. The fecal scores of 1, 2, 3 and 4, respectively, represented the physical forms of firm, slightly loose, loose and watery (Larson et al., 1977). Jugular blood was sampled weekly through 8 weeks of the experiment. Blood was centrifuged at 3000 *g* for 15 min. The serum harvested was frozen at −20 °C until analysis for immunoglobulin G (IgG) and immunoglobulin M (IgM) by ELISA (Kruger et al., 2003; Pinchuk et al., 2003). Serum

samples were diluted at $1:10^6$ (vol/vol) for determining IgG and at $1:10^5$ (vol/vol) for IgM. The quantitative ELISA protocols were according to BETHYL procedures (Bethyl, Montgomery, TX, USA) using specific Starter Accessory Kits for IgG (Catalog No. E118) and IgM (Catalog No. 101). The kit performance was optimized for standard dilutions from 7.8–500 ng/ml of bovine IgG and 15.625–1000 ng/ml of bovine IgM.

Statistical analysis

The general linear models procedure of SAS Institute (2003) was used to analyse non-repeated measures. For the repeated measures of blood immunoglobulins and BW, the Mixed Model Procedure of SAS Institute (2003) was used. The mixed model consisted of the fixed effects of treatments, week (as a repeated effect), and their interaction, plus the random effect of calf. The method for estimating least square mean values was Restricted Maximum Likelihood and the method for calculating denominator degrees of freedom was Between–Within (SAS Institute, 2003). The covariance structure with the least Akaike's Information Criterion (Wang and Goonewardene, 2004) was adopted for repeated measures. For BW data, the initial weights were modelled as a covariate to further control the experimental error. The PDIFF option of SAS Institute (2003) was used to separate least square mean values. Significant differences were set at $p \leq 0.05$ and trends at $p < 0.10$.

Results

The HF fed calves achieved a daily starter intake of 680 g earlier than LF fed calves ($p = 0.05$) and numerically earlier than control and MF groups. The HF group tended to have a greater weaning BW ($p = 0.06$) than the LF group (Table 1). Weaning age and BW did not significantly differ between the control group with any of the LF, MF, or HF groups. The calf BW increased with age ($p < 0.05$). Calves on HF had higher BW than did calves on LF at week 5 of age ($p = 0.04$). At week 6 of age, the HF group had higher ($p = 0.03$) BW than control ($p = 0.03$) and LF ($p = 0.06$) groups but not the MF group ($p > 0.15$, Fig. 1). Weaning withers height and body length were unaffected ($p > 0.10$) by treatments. The serum concentrations of IgG exhibited a time dependent response to treatments (Fig. 2). The LF group had lower concentrations of circulating IgG at week 3 ($p = 0.03$) and tended to have lower serum IgG at week 1 ($p = 0.10$), week 2 ($p = 0.08$) and week 4 ($p = 0.10$) than did the control group (Fig. 2). At week 8, circulating IgG were elevated in control group ($p < 0.01$) and tended to decrease ($p = 0.09$) in the LF group compared to the MF group (Fig. 2). Also at week 8, the serum IgG concentrations in HF fed calves was lower ($p = 0.03$) than in control calves but did not differ with that in LF and MF groups (Fig. 2). Serum IgM showed a response to treatments only at week 6 (Fig. 3). Calves receiving medium and high doses of

Table 1 Weaning and overall performance of control and flavonoids-fed calves during the 4-month study*

Variable	Level of flavonoids consumption†				SEM	Overall p-value
	CO	LF	MF	HF		
Weaning age, day‡	54.7	63.9	50.0	41.7	9.8	0.31
Weaning body weight, kg§	53.7	59.4	57.2	52.7	3.1	0.30
Weaning wither height, cm	81.3	83.0	83.3	81.1	2.6	0.62
Weaning body length, cm	47.7	47.3	45.5	45.7	2.5	0.40
Initial body length, cm	41.0	41.0	40.6	43.2	1.6	0.65
Initial wither height, cm	79.4	79.0	80.2	79.3	1.3	0.92
Weight gain/feed intake, pre-weaning	1.51	1.69	1.67	1.87	0.3	0.56
Weight gain/feed intake, 30-day post-weaning	0.78	0.77	0.78	0.71	0.1	0.69
Dry starter intake, kg	11.5	9.0	12.5	11.7	2.8	0.52
Days with fecal score >2¶	54.2	43.7	58.3	48.4	10.1	0.40
Body weight at 30-day post-weaning, kg	81.7	87.8	79.7	78.4	8.8	0.65
Body weight at 120 days of age, kg	101.6	109.8	111.3	111.3	10.9	0.67

*Weaning occurred when calves consumed 0.68 kg of starter/day (NRC, 2001).

†CO, no flavonoids; LF, low or 7.3×10^{-5} g/kg BW; MF, medium or 7.3×10^{-4} g/kg BW; HF, high or 3.6×10^{-3} g/kg BW.

‡LF vs. HF, $p = 0.05$.

§LF vs. HF, $p = 0.06$.

¶Scored based on physical appearance of feces: 1 = firm, 2 = slightly loose, 3 = loose, 4 = watery.

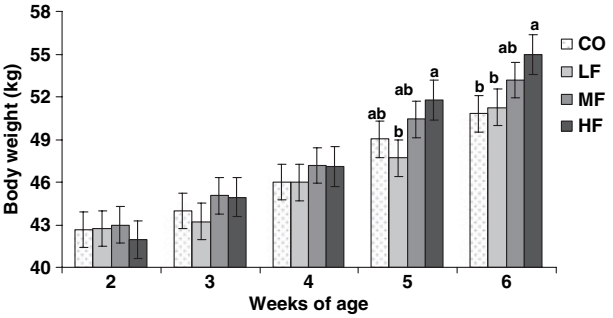

Fig. 1 Body weight of calves fed either no-flavonoids (CO), low-flavonoids (LF), medium- flavonoids (MF), or high-flavonoids (HF). LF, low or 7.3×10^{-5} g/kg BW; MF, medium or 7.3×10^{-4} g/kg BW; HF, high or 3.6×10^{-3} g/kg BW. Data were presented as least squares mean values ± SE. [ab]Within each week, bars with different superscripts differ as following: week 5: LF vs. HF, p = 0.04. Week 6: CO vs. HF, p = 0.03; LF vs. HF, p = 0.06.

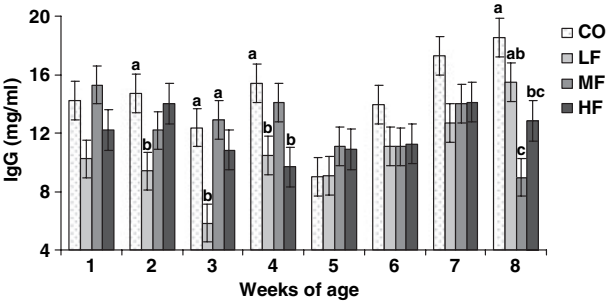

Fig. 2 Serum IgG concentrations in calves fed either no-flavonoids (CO), low-flavonoids (LF), medium-flavonoids (MF), or high-flavonoids (HF). LF, low or 7.3×10^{-5} g/kg BW, MF, medium or 7.3×10^{-4} g/kg BW; HF, high or 3.6×10^{-3} g/kg BW. Data were presented as least squares mean values ± SE. Weaning ages: CO = 54.7, LF = 63.9, MF = 50.0, HF = 41.7 days. [abc]Within each week, bars with different superscripts differ statistically as following: week 2: LF vs. HF, p = 0.08. Week 3: CO vs. LF, p = 0.03; MF vs. LF, p = 0.02. Week 4: CO vs. LF, p = 0.10; CO vs. HF, p = 0.07. Week 8: CO vs. MF, p < 0.01; CO vs. HF, p = 0.09; LF vs. MF, p = 0.03.

flavonoids had lower (p < 0.01) blood IgM at week 6 than control calves (Fig. 3).

Discussion

The BW data suggested that flavonoids at higher doses can be beneficial around weaning. The greater BW in the HF group than in control and LF groups at week 6 was in line with earlier weaning, suggesting a less stressful transition to dry feed. Early and less-stressful weaning is an influential management strategy to improve calf health and calf-raising economics (NRC, 2001). Early weaning without a major stress to the calf depends on an early establishment

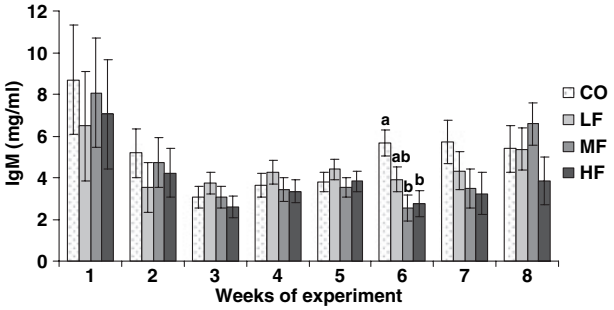

Fig. 3 Serum IgM concentrations in calves fed either no-flavonoids (CO), low-flavonoids (LF), medium-flavonoids (MF), or high-flavonoids (HF). LF, low or 7.3×10^{-5} g/kg BW; MF, medium or 7.3×10^{-4} g/kg BW; HF, high or 3.6×10^{-3} g/kg BW. Data were presented as least squares mean values ± SE. Weaning ages: CO = 54.7, LF = 63.9, MF = 50.0, HF = 41.7 days. [abc]Within week 6, bars with different superscripts differ at p < 0.01.

of mixed microbial population in the reticulorumen. Early settlement of rumen fermentation leads to an early and capacious release of volatile fatty acids (VFA) (Davis and Drackley, 1998). The VFA, particularly butyric and propionic acids, are essential for the timely rumen epithelial and papillae growth. A well-developed rumen epithelia will facilitate and increase VFA delivery to the liver. As a result, the hepatic metabolism of VFA and related substances will expand earlier (Baldwin et al., 2004). Accordingly, the increased BW around weaning by the high doses of flavonoids suggests a role for flavonoids in alleviating the stress of transition from the neonate non-ruminant into a functioning fermentor. Increased BW around weaning in HF calves was associated with reduced weaning age. This indicates that high doses of flavonoids enabled early weaning while promoting tissue growth. It would, thus, be logical to suggest that high rather than low doses of flavonoids alleviated the stress of calf transition from liquid to dry feed. Therefore, flavonoids may contribute to the development of a desirable microbial population in the neonate reticulorumen. This suggestion needs to be substantiated in further details using larger sample sizes. The BW data lends support to the notion that the evolutionary reliance of young ruminants on herbages is, at least partly, linked to flavonoids and their impacts on reticulorumen development.

The comparable body length and wither height among treatments indicated that the carcass frame configuration was unaltered by flavonoids. Flavonoids have similar structure as estrogenic hormones (Sonnenbichler and Pohl, 1980). As such, flavonoids have been postulated to possess anabolic effects as

do estrogenic compounds (Sharma et al., 1971; Sokolova et al., 1978; Havsteen, 2002). With respect to tissue accretion, the results of the current study support this postulation. Unchanged wither height along with increased BW might raise the possibility that HF-fed calves accumulated more visceral tissue mass than control calves, in addition to non-splanchnic tissue gain. Although, the possibility of increased visceral mass may have not been major in the present study because feed intake did not differ among treatments. Nonetheless, this possibility warrants full attention in future studies.

Fecal score was not affected by flavonoids. Flavonoids possess antimicrobial activities (Cushnie and lamb, 2005). The authors have recently shown (Yaghoubi et al., 2007a,b) that propolis flavonoids can act against protozoa and gram positive bacteria. Adding 8 ml of the rumen fluid of forage-fed cows to milk replacer has been shown (Muscato et al., 2002) to improve calf growth and lower the frequency of scours. Given that the autoclaved rumen fluid was as effective as the fresh rumen fluid in improving calf performance, factors such as non-pathogenic bacteria or microbial polysaccharides may have contributed to improved calf health (Muscato et al., 2002). Moreover, the findings of Muscato et al. (2002) indicate that some unknown compounds with pro-immunity role may exist in the rumen contents of forage-fed animals. As such, an industrial impetus for the current study was that flavonoids are the unknown compounds which can help to improve gut health and attenuate diarrhoea in young calves. This would be due to enhancing immunity and maintaining a desirable balance in microbial populations of the neonate gut. Probably, the post-rumen gut health and its microbial stability were not highly stressed under the environmental conditions of the current study. A certain level of stress such as disease models would be needed for occurring a gut health response to any treatments such as flavonoids.

Flavonoids can activate the humoral immunity (Goodwin and Webb, 1980). No research has documented the influence of flavonoids on humoral immunity of the young calf. The neonatal calf does not possess a well-responsive immune system. As a result, the young calf may not effectively manage combating the external stressors during the early stages of growth (Franklin et al., 2003). We hypothesized that flavonoids at a given dose stimulate the humoral immunity, thereby enabling the young calf to transit through the weaning phase less stressfully. During the first few days of birth, serum IgG would typically reflect the efficacy of acquiring passive immunity by the adequate and timely consumption of colostrum (Quigley et al., 1995). Later on, as milk replacer and dry feed intakes increase, the young calf develops its own systematic humoral immune function. Hence, the serum IgG levels long after transitioning the calf from colostrum to milk, milk replacer, and dry feed may correlate positively with the presence and degree of environmental stresses. Importantly, the IgG is the major component of humoral immunity composing >70% of body's immunoglobulin pool. Therefore, the circulating levels of IgG after the first week of birth may be used to assess the immune response to the occurrence of detrimental environmental antigens. Such antigens could seriously threaten the calf health before and during weaning (Davis and Drackley, 1998). The significance levels of the treatment differences in the present study were prominent. Although flavonoids did not show a consistent weekly picture on blood IgG concentrations, these data suggest that during the first month of age, low does of flavonoids can affect the immune response. At week 8, the medium rather than the low and high doses of flavonoids were effective in moderating serum IgG. It is suggested that as dry feed intake increases and rumen develops further, a higher dose of flavonoids may benefit microbial populations in the rumen. In addition, it appears that flavonoids should not exceed a certain level, which otherwise may depress feed intake.

Flavonoids can interfere with cellular integrity and activity of some gram negative and gram positive bacteria as well as protozoa (Mirzoeva et al., 1997; Yaghoubi et al., 2007a,b). The immune response observed in the current study may, in part, be linked to such microbiological impacts of flavonoids along the transition gut. The circulating IgG acts as an antibody to identify and trace harmful foreign particles such as viruses and bacteria. By limiting the activity of certain types of bacteria, flavonoids may contribute to maintaining the integrity of the gut epithelium, and thereby, to moderating the circulating levels of IgG. This was more noticeable during the initial phase of transition from liquid to solid feed (1–4 weeks) when the young calf is more sensitive to viral, bacterial and other stressors (Davis and Drackley, 1998).

The findings provide no basis for a non-gut mechanism for the effects of flavonoids on humoral immunity, although this possibility cannot be ruled out. The results led to four major conclusions. First, flavonoids affected the immune response in young calves. Secondly, the low but not the medium and

high doses of flavonoids modulated the immune response during the first 4 weeks of calf age. Thirdly, later on around weaning (i.e. 8 weeks of age), the medium and high rather than the low doses of flavonoids affected the indicators of humoral immunity. Finally, the altered humoral immune response around weeks 4 and 5 of age were followed by and associated with an improved BW at week 5 and 6 of age in calves receiving high doses of flavonoids compared to those receiving no or low doses of flavonoids. Future larger studies are essential to further specify this immune reaction and to assess the growth and dry feed intake responses to flavonoids. Such information is needed before one can recommend that young calves should be fed forages or a main source of flavonoids along with highly fermentable concentrate. Additional information will allow insight into whether flavonoids play an evolutionary role in the adaptation of calves to solid feed, and thus, may be overlooked in the modern nutrition of calves fed limited fresh forages.

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