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Skeletal health, redox balance and gastrointestinal functionality in dairy cows: connecting bugs and bones

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Abstract

This research reflection examines the physiological links between redox balance, skeletal health and gastrointestinal functionality in dairy cows. With the increase in demand of animal products caused by the growth in human population, the dairy industry needs to develop and implement innovative strategies which are profitable, sustainable and cow friendly. Redox balance, skeletal heath and gastrointestinal functionality are three key physiological systems that are often seen as independent entities. In this research reflection we intend to stress that the antioxidant system, bone health and the microbiome are intimately intertwined. Antioxidants are crucial for the maintenance of redox homeostasis and optimal immune function. Optimal gastrointestinal functionality is important to maintain animal performance, health and welfare. In particular, the intestinal microbiome is increasingly seen as a driver of health and disease. Vitamin D metabolism is pivotal not only for optimal skeletal health, but in light of all the extra-skeletal effect of vitamin D, it is the foundation for optimal productive life. It makes sense to ask the question 'how are redox balance and the microbiome involved in the modulation of bone health and immune function?' In other words, are bugs and bones connected in dairy cows! The existing data available in the literature suggests that this might be the case. The characterization of the interactions between redox balance, skeletal health and the microbiome, will allow the development of a multisystem biological approach to refine nutritional interventions to improve dairy cattle health, welfare and productive longevity.

With the current forecast for the world's population to reach close to 11 billion by 2100, it comes with no surprise that the demand for animal food products including dairy will increase. The implications for the dairy industry are numerous and far-reaching as it will have to develop and adopt strategies to increase productivity while maintaining cow health and welfare and safeguarding the environment. Notwithstanding the recent advances in dairy health and production, new developments in the dairy industry must encompass strategies that increase lifetime performance in order to meet the ever-growing demand for dairy products from the rapidly growing world population (McGrath *et al*., 2019). In the future, dairy farms will be bigger and highly modernized and for dairy farmers to feed the world's growing population within planetary boundaries, they will have to adopt technologies and husbandry practices that will provide improved cow lifetime performances, profitable dairy farms and sustainable agricultural practices (Britt *et al*., 2018).

Dairy cows of the future will be more robust with improved health and longevity, driven principally by improvements in genomic selection schemes. Welfare of dairy cattle will continue to receive increased attention, and dairy farm facilities will be modified to improve welfare of animals (Britt *et al*., 2018). Considering that nutritional interventions are arguably the easiest strategy that can be implemented at farm level (McGrath *et al*., 2019), the dairy industry has the responsibility to develop and implement alternative nutritional strategies which are good for its people (profitable), planet (sustainable) and for the cows (health and welfare). The maintenance of redox balance, skeletal heath and gastrointestinal functionality have been identified as key pillars around which veterinarians and nutritionist should develop interventions that would result in more resilient and healthy dairy cattle (Celi *et al*., 2017).

In this research reflection we intend to highlight the physiological links between the antioxidant system, vitamin D metabolism and the rumen microbiome. Antioxidants play a key role in the maintenance of redox homeostasis which is crucial for immune system, while bone metabolism and health are the foundation for optimal productive life. The role of the microbiome as driver of health and diseases has received significant attention by the scientific community, however there are very few studies investigating its role in the modulation of bone health and immune function. Our intent is to draw the reader's attention to the fact that biological systems, namely, redox balance, skeletal health and the microbiome, are connected. The characterization of the interactions between these systems will allow the development of targeted nutritional interventions that would enable the improvement of dairy cattle health, welfare and productive longevity.

Redox balance

When oxidant activity exceeds the neutralizing capacity of antioxidants it can result in oxidative stress which in ruminants has been associated with several diseases, including conditions that are relevant for animal production and welfare (Celi, 2011). An imbalance between pro-oxidants and antioxidants lead to oxidative stress, therefore the maintenance of redox balance is crucial for optimal ruminant health (Chauhan *et al*., 2014). The main pro-oxidants (reactive oxygen species, reactive nitrogen species) and antioxidants (endogenous and dietary) involved in the maintenance of redox balance in ruminants have been reviewed extensively (Miller *et al*., 1993; Lykkesfeldt and Svendsen, 2007; Celi, 2011; Chauhan *et al*., 2014; Sordillo and Mavangira, 2014).

Redox balance plays a key role in the modulation of metabolism, immunity, reproduction, health and welfare. In dairy cattle, redox balance can be disrupted at any time, however, the time around calving (transition period) and the first few weeks of life (neonatal period) are characterized by a higher risk of oxidative stress and incidence of diseases (Ranade *et al*., 2014; Abuelo *et al*., 2019). During these periods the requirement for nutrients and micronutrients, including antioxidants, is considerably increased (Pedernera *et al*., 2010), therefore, supplementing diets with micronutrients and antioxidants can prove to be useful in maintaining redox balance (Abuelo *et al*., 2015; Surai *et al*., 2019).

A relationship between dairy calf growth rates and antioxidant status has been reported (McGrath, 2016) and it has been proposed that dietary antioxidants can maintain redox balance in young ruminants, reducing the risk of disease and mortality while maintaining productive performance. In light of the observed accelerated growth of dairy calves and its association with increased lifetime productivity of dairy cows (Soberon and Van Amburgh, 2013), the use of dietary antioxidants seems to be a valid practical option to increase lifetime performances of dairy cattle (McGrath, 2016).

Redox balance is also implicated in the modulation of reproductive physiology in light of its involvement in the regulation of events such as oocyte maturation, steroidogenesis, regulation of follicular fluid environment, folliculogenesis, corpus luteal function, luteolysis and embryonic mortality (Talukder *et al*., 2017). The fact that antioxidant supplementation can improve reproductive outcomes in dairy cattle does not come as a surprise. While it is clear that maintaining redox balance in dairy cattle is crucial to maximize fertility (Talukder *et al*., 2017) and health (Abuelo *et al*., 2019), a better understanding of the factors involved in the control of redox balance will allow the verification of their success and effectiveness (Chauhan *et al*., 2016). More importantly, as antioxidants are routinely supplemented in dairy diets (Lean *et al*., 2013), the question is how much of which formulation(s) of antioxidants should be used to maintain redox balance. There is practical evidence in the literature suggesting that the supplementation of ruminant diets with supra-nutritional levels of antioxidants such as vitamin E and selenium can not only sustain redox balance but also maintain crucial physiological functions like thermoregulation, feed intake, respiratory physiology, rectal temperature, acid-base balance, inflammation and gastrointestinal functionality (Chauhan *et al*., 2014; Celi and Gabai, 2015).

Skeletal health and redox balance

Skeletal health is another of the many physiological function regulated by redox balance as suggested by epidemiological evidence linking dietary antioxidant intake and bone health (Rao and Rao, 2013). Redox balance seems to play a role in bone remodeling (Domazetovic *et al*., 2017). A disruption in redox balance results in decreased bone formation as the result of reduced differentiation and survival of osteoblasts, and activation of osteoclasts resulting in bone reabsorption (Rao and Rao, 2013). Moreover, antioxidant deficiency has been associated with reduction in bone formation ultimately resulting in osteoporosis (Domazetovic *et al*., 2017). Antioxidant enzymes such as glutathione peroxidase and catalase are considered markers of antioxidants defence mechanisms against bone resorption and osteoporosis (Rao and Rao, 2013). Recently, many dietary antioxidants have been reported to restore redox balance and skeletal health (Rao and Rao, 2013). For example, antioxidants such as vitamin C contribute to the maintenance of skeletal health by suppressing osteoclast activity and promoting osteoblast differentiation. Although vitamin C can be synthesized in tissues by ruminants, it is important to emphasize that vitamin C is an important cofactor for collagen formation and synthesis of hydroxyproline and hydroxylysine, indicating that vitamin C interacts with other nutritional factors such as vitamin E, vitamin D and calcium in the control of skeletal health (Sahni *et al*., 2015). Finally, dietary carotenoids have also been reported to play an important role for improving bone health (Sahni *et al*., 2015).

Extraskeletal effects of vitamin D

The mechanisms of disrupted skeletal health in dairy cows impairing their ability to maintain effective bone and mineral homeostasis have been recently reviewed (McGrath *et al*., 2015). The role of vitamin D and its metabolites in maintaining skeletal health and mineral homeostasis are very well established. However, the actions of vitamin D are not confined to the skeleton as vitamin D receptors (VDR) and vitamin D hydroxylases CYP24A1 and CYP27B1 are nearly ubiquitous, advocating for a variety of extraskeletal actions of the vitamin D endocrine system (Bikle, 2016; Bouillon *et al*., 2019). The potential for the bone to now be considered an endocrine organ firmly puts skeletal health of the modern day dairy cow at the forefront of science (McNeill and Anderson, 2012; Lean *et al*., 2014).

In both swine and poultry, the use of vitamin D_3 and 25-OH-D_3 (25-hydroxycholecalciferol) has been shown to improve bone strength, calcification, immunity and muscle content, often resulting in less morbidity and mortality as well as greater productivity (Chou *et al*., 2009; Sugiyama *et al*., 2013). The next frontier of bone health research in dairy cows should be the characterization of the extraskeletal effects, such as the effects on metabolism, reproduction, muscle biology, immunity, and gastrointestinal functionality.

There is substantial evidence from published and new data that supports the hypothesis that in dairy cattle the skeleton has a pivotal role during the homeorhetic adaptation to lactation and that this relationship may be influenced by nutrition. Indeed, recent studies have demonstrated a link between 25-OH-D_3 and energy metabolism in dairy cows (Lean *et al*., 2014; Rodney *et al*., 2018). In dairy cows, a dramatic increase in energy and nutrient requirements can be observed during the transition period, exposing them to negative energy and nutrient balance. The metabolic

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adaptations to negative energy balance require interactions of metabolic fuels and its failure may occur in various tissues resulting in several metabolic diseases (Drackley, 1999). Despite the transition period being the most studied period of the productive life of a dairy cow, the long-term physiological implications on responses like mammary gland function and fertility require a thorough assessment. Essential physiological function such as calcium homeostasis, lipid metabolism, insulin secretion, tissue sensitivity to insulin and the integration of these processes with other functions like redox balance are not fully elucidated yet. It has been observed that nutritional interventions administered before calving can increase milk yield, improve fertility and reduce the risk of metabolic diseases (Lean *et al.*, 2014). It is also worth noting that correlations between energy and bone metabolism have been observed in dairy cows providing evidence to support a homeorhetic role for calcium metabolism in dairy cattle (Lean *et al.*, 2014; Martinez *et al.*, 2018*b*; Rodney *et al.*, 2019). However, it is worth mentioning that vitamin D supplementation around the time of calving has not always succeeded in preventing hypocalcemia (Weiss *et al.*, 2015). Considering the complex mechanisms involved in the control of calcium homeostasis in dairy cattle (DeGaris and Lean, 2008), further studies are required to evaluate the effectiveness of vitamin D supplementation to reduce hypocalcemia during early lactation. Although it has been reported that vitamin D has a high margin of safety when used at recommended levels (Celi *et al.*, 2018), excessive intakes of vitamin D can result in intoxication in ruminants and future studies need to evaluate the safety of vitamin D supplementation for long periods of time.

Considering that VDRs are expressed in numerous tissues of the reproductive tract (Dokoh *et al.*, 1983; Stumpf *et al.*, 1987), reproductive physiology should also be considered in regard to skeletal health. It has been reported that vitamin D has a positive effect on reproductive physiology in cattle (Ward *et al.*, 1971; Panda *et al.*, 2001; Kemmis *et al.*, 2006). Indeed, it has been observed that dairy cattle reproductive performance improved after vitamin D supplementation (Ward *et al.*, 1971), and that calcitriol blood concentration is elevated during pregnancy (O'Brien *et al.*, 2014). A recent study in transition dairy cows has reported that dietary 25-OH-D_3 tended to improve pregnancy rate and reduced the days to pregnancy during the 305-d lactation (Martinez *et al.*, 2018*a*). The authors argue that metabolites of vitamin D such as 25-OH-D_3 might have directly stimulated the VDRs in reproductive tissues (Lou *et al.*, 2010), which might have resulted in positive effects on fertility. Another possibility is that the positive effect on reproductive function might have been indirect as cows fed 25-OH-D_3 presented improved neutrophil function and reduced incidence of inflammatory diseases such as metritis, which are known to decrease fertility (Ribeiro *et al.*, 2016). Therefore, the observed improved pregnancy rate might have been the consequence of the better health status induced by dietary 25-OH-D_3 (Martinez *et al.*, 2018*a*).

Considering that VDR and 1-α-hydroxylase are expressed in muscle fibers and myoblasts, it is not surprising that vitamin D plays a direct regulatory role in muscle physiology. Indeed, vitamin D is involved in the regulation of myogenesis, cell proliferation, differentiation, regulation of protein synthesis and mitochondrial metabolism (Montenegro *et al.*, 2019). Lack of VDR disrupts muscle growth and development, as observed in VDR knockout mice which have smaller muscle fibers and aberrant myogenic regulatory factor expression (Dzik and Kaczor, 2019). Moreover, as the inflammatory processes reduce muscle protein accretion, decreasing the impact of the inflammatory response is a key strategy to sustain production performances. Considering that dairy beef cross cattle production is becoming a financially attractive opportunity for the dairy industry as it allows producers to produce calves that will yield better carcasses than purebred dairy breeds and thus attract higher prices, dietary supplementation of dairy cattle's diet with vitamin D, in light of its the well-known anti-inflammatory effects, represents a great opportunity for the dairy industry to increase profitability and sustainability. Indeed, dairy beef production generates around 33% of the greenhouse gases equivalents per unit weight of meat compared to traditional beef cattle production and thus 25-OH-D_3 supplementation can increase meat yield and lower the carbon footprint of the dairy industry at the same time (Britt *et al.*, 2018).

It is well known that both the innate and acquired immune responses are modulated by ligand dependent VDR functions. Indeed, VDR and vitamin D metabolic enzymes can be found in all cells of the innate and adaptive arms of the immune system (Bouillon *et al.*, 2019). In cattle, calcitriol augments the production of nitric oxide and β-defensin antimicrobial peptides (Merriman *et al.*, 2015), molecules that are toxic to bacteria, indicating a potential for targeted enhancement of defence against bacterial infections *via* the vitamin D pathway (Nelson *et al.*, 2012). Macrophages are the main sources of the calcitriol that controls vitamin D-mediated immune responses. In bovine macrophages CYP27B1 is stimulated *via* toll-like receptor recognition of pathogen associated molecular patterns such as lipopolysaccharide, peptidoglycan, and mycobacterial lipopeptides. In macrophages, CYP27B1 facilitates the conversion of calcidiol to calcitriol, activating vitamin D-mediated immune responses (Nelson *et al.*, 2010). Interestingly, CYP27B1 is expressed in the udder during mastitis in dairy cattle (Nelson *et al.*, 2010). Although vitamin D treatments does not seem to prevent or cure mastitis, it reduced its negative impact on the cow (Lippolis *et al.*, 2011). It has been reported that dietary 25-OH-D_3 decreased the incidence of retained placenta and metritis and the percentage of cows with multiple diseases during the first 30 d in milk, which are likely related to the improved measures of immune function evaluated in neutrophils (Martinez *et al.*, 2018*a*). While it is clear that vitamin D can modulate the immune system in dairy cattle, we still need clarity of understanding of the immunomodulatory effects of dietary 25-OH-D_3 as this will allow the development of nutritional strategies to increase the resilience of dairy cattle to bacterial infection. These strategies have the potential to reduce the use of antibiotics in the dairy industry and thus might contribute to the decrease in antimicrobial resistance.

VDR and vitamin D metabolic enzymes have been localized in virtually all cells of both the innate and adaptive immune system. Moreover, there is now consensus that cells of the immune system produce 1,25(OH)2D_3 locally and that expression of CYP27B1 in the immune system is regulated independently of that in the endocrine system that controls calcium homeostasis (Nelson *et al.*, 2012). For example, there is evidence that in activated immune cells VDR controls several immune responses (Hewison, 2011). It has been observed that in cattle, 1,25(OH) 2D_3 increases the production of antimicrobial peptides such as nitric oxide and β-defensin, (Nelson *et al.*, 2012). Considering that vitamin D metabolism might be quite different in each system (skeletal, immune, digestive, etc.), it is reasonable to argue that the vitamin D requirements for each of these systems may also differ (Nelson *et al.*, 2016). Nevertheless, the optimal 25(OH) D_3 concentration for the dairy cattle immune system has not been established thus far. In vitro studies investigating the effect of different 25(OH)D_3 concentrations on macrophage host defence responses suggest that 100 ng/ml should be an effective level (Nelson *et al.*, 2010). However, despite the association between vitamin D deficiency and risk of respiratory infections, calves with a serum 25(OH)D_3 concentration of about 175 ng/ml and experimentally challenged with respiratory syncytial virus (RSV), did not perform better than calves with serum 25(OH) D_3 concentration of about 30 ng/ml in regard to severity the infection (Sacco *et al.*, 2012). As reported above, however, there is evidence for dietary 25(OH)D_3 to reduce the incidence of some peripartum diseases in dairy cattle (Martinez *et al.*, 2018*a*); this apparent discrepancy needs to be addressed with further work investigating the relationship between serum 25(OH)D_3 and infectious disease outcome in dairy cattle.

Vitamin D deficiency has been linked to disrupted gastrointestinal functionality (Christakos, 2012; De Santis *et al.*, 2015), and although vitamin deficiencies do not seem to occur in modern dairy production systems, it should be noted that vitamin requirements of the modern dairy cow may be higher than those currently recommended by the NRC. The current understanding of gastrointestinal functionality encompasses not only the gastrointestinal (GI) microbiota including pathogens causing diseases, mortality and morbidity in dairy cattle, but also other key components such as diet, effective structure of the gastrointestinal barrier, host interaction with the GI microbiota, effective digestion and absorption of feed and effective maturation and development of innate and acquired immune functions (Celi *et al.*, 2017). This novel definition of gut health focuses on the functionality of the whole GI system and on the complex interactions between all its components mentioned above. With this definition in mind, it is not surprising that skeletal health is also influenced by the GI microbiota and vice versa. Indeed, it has been reported that vitamin D influences the composition of the microbiota (Sun, 2018; Waterhouse *et al.*, 2019), and that the gut microbiota regulates endocrine vitamin D metabolism (Bora *et al.*, 2018). Vitamin D regulates the expression of tight junction proteins in the intestinal epithelial cells, thereby maintaining optimal intestinal barrier function (De Santis *et al.*, 2015). In the intestinal mucosa, vitamin D, together with vitamin A, supports innate lymphoid cells that produce IL-22, suppressed IFN-γ and IL-17 by T cells, and induces regulatory T cells (Cantorna *et al.*, 2019). Vitamin D seems to shape the gut microbiota by modulating the intestinal epithelium and mucosal immune system and thus maintaining intestinal homeostasis (Cantorna *et al.*, 2019). On the other end, the intestinal microbiota may regulate bone metabolism by influencing the relative activities of osteoclasts and osteoblasts through effects on the immune system and host metabolic pathways, as well as through the production of metabolites (Charles *et al.*, 2015). It has been proposed that during eubiosis (balance between the gut microbiome and their host), there is a balance between the anti-osteoclastogenic and pro-osteoclastogenic pathways, however, during dysbiosis (disturbance in the balance between the gut microbiome and the host), the gut microbiome may promote osteoclast-mediated bone loss as a result of the increase in inflammatory cytokines which recruit more osteoclast precursors, promote osteoclast activity and reduce anti-osteoclast T cells (Charles *et al.*, 2015). Several cytokines are involved in skeletal health and proinflammatory cytokines are believed to have osteoclastogenic effects (Jonsson *et al.*, 2013). For example, tumor necrosis factor α (TNF-α) and IL-1 have been shown to modulate the expression of receptor activator of nuclear factor κ-B (RANK), its ligand (RANKL), and osteoprotegerin (Jonsson *et al.*, 2013). Osteoclastogenesis is the differentiation of osteoclast into multinucleated cells which leads to bone degradation and calcium mobilization. Osteoclastogenesis is initiated by the binding of RANKL to its receptor on osteoclasts. Osteoprotegerin on the other hand, inhibits osteoclastogenesis by acting as a decoy receptor for RANKL (Hatate *et al.*, 2020). Changes in cytokine production in tissues and blood seems also to modulate calcium homeostasis during the parturient period, with potential implications for the incidence of peripartum hypocalcemia (Gray *et al.*, 2007). While specific studies in this area are lacking in dairy cattle, the literature clearly highlights the biological links between redox balance, skeletal health and gastrointestinal functionality.

In conclusion, future dairy production and its sustainability depends on the development a larger understanding and practical application of concepts related to gastrointestinal functionality that imply complete holistic management of the production system. Nutritional strategies can reduce the incidence of health and welfare issues, but they need to be strategically integrated with management practices and breeding programs. The success of these strategies, however, is often hindered by the complexity of the interactions between cows and their environment. In dairy cattle, effective gastrointestinal functionality is vital in determining health, welfare and productive performance. Optimization of gastrointestinal functionality is crucial to increase nutrient digestibility and thus maximizing value from feed, to sustain host physiological functions such as innate and adaptive immunity and thus increasing resilience to environmental challenges, and finally, to maintain eubiotic conditions. The characterization of the interactions between the antioxidant system, vitamin D metabolism and the rumen microbiome will allow the development of strategic nutritional interventions aimed at improving skeletal health, redox balance and gastrointestinal functionality that would enable achieving optimal lifetime performance.

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