Short communication: Influence of intramuscular injection of vitamin B₁₂ in early-lactation dairy cows on Mozzarella cheese quality and vitamin B₁₂ stability

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ABSTRACT

The current study explored the effect of intramuscular injection of vitamin B₁₂ (VB₁₂) in early-lactation dairy cows on subsequent low-moisture part-skim Mozzarella cheese quality and VB₁₂ levels during cheese processing and storage. Twenty-four peripartum dairy cows were blocked based on parity and milk yield and randomly assigned into 2 treatments: basal diet (CON) and basal diet with an intramuscular injection of 10 mg of VB₁₂ per cow per week (VB₁₂). Raw milk was collected to determine VB₁₂ content and then used to make low-moisture part-skim Mozzarella cheese 8 wk after injection. The VB₁₂ content of raw milk and cheese was determined using ultra-performance liquid chromatography coupled with tandem mass spectrometry. We found that VB₁₂ content was significantly increased in milk (15.43 vs. 3.30 ng/mL) and fresh cheese (3.72 ng/g vs. undetectable) from the VB₁₂ group compared with the CON group. However, approximately 70% of VB₁₂ was lost in the whey during cheese making, and no VB₁₂ was detectable in either cheese treatment after 8 wk of storage. Furthermore, no significant differences were observed in fat and protein contents in the cheese between the 2 groups. For cheese color, the *b** value increased and the *a** value decreased slightly in fresh VB₁₂ cheese. Functional properties of stretchability, flowability, and meltability of VB₁₂ cheese were initially comparable to that of CON cheese, but higher flowability and meltability was observed in VB₁₂ cheese after 8 wk of storage. In summary, intramuscular injection of VB₁₂ in early-lactation dairy cows increases the content of VB₁₂ in milk and fresh cheese with no adverse effect on cheese quality, but substantial VB₁₂ is lost during cheesemaking and declines rapidly during storage.

Key words: dairy cow, vitamin B₁₂, intramuscular injection, cheese quality, retention

Short Communication

Cyanocobalamin, a water-soluble vitamin known as vitamin B₁₂ (VB₁₂), plays a key role in cellular metabolism in the body, including DNA synthesis and energy supply, and also has certain anti-inflammatory effects (Hosseinzadeh et al., 2012). It is recognized that VB₁₂ is able to regenerate red blood cells, prevent anemia, and is essential for the metabolism of carbohydrates, fats, and proteins. A VB₁₂ deficiency is touted as a crucial reason for several human neurological and hematological disorders (Green et al., 2017). Long-term deficiency of VB₁₂ has adverse effects on pregnancy, vascular function, bone, and eye health (Green et al., 2017).

The recommended dietary intake of VB₁₂ for adults is 2.4 µg/d, and 2.6 to 2.8 µg/d of VB₁₂ is required for pregnancy or lactation (Institute of Medicine, Food and Nutrition Board, 1998). Foods of animal origin, including liver, meat, eggs, and dairy foods (especially foods of ruminant origin), are excellent sources of VB₁₂ (Nohr et al., 2011), usually providing 2.4 µg/d of VB₁₂ dietary intake (Green et al., 2017). Dietary intake of VB₁₂ is usually higher than the reference intake in subjects consuming a Western diet (Dullemeijer et al., 2013); however, VB₁₂ deficiency is prevalent in developing countries due to the low intake of animal products (Refsum et al., 2001; Siekmann et al., 2003; Hao et al., 2007). Additionally, vegans, who consume no animal-origin foods, and elders (over 50 yr of age) are often subjected to VB₁₂ deficiency due to the lack of VB₁₂ intake. Milk and dairy products are popular foods worldwide, and even though milk and dairy products have lower VB₁₂ than meat, the VB₁₂ in dairy products appears to be more bioavailable (Gille and Schmid, 2015), significantly increasing plasma VB₁₂ concentration (Vogiatzoglou et al., 2009). Milk and dairy products are thus considered as ideal natural-delivery vehicles for large amounts of bioactive compounds (Yeum and Rus-

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sell, 2002). Increasing VB₁₂ concentration in milk and dairy products is a potentially efficient way to improve VB₁₂ intake and plasma bioavailability.

As with most water-soluble vitamins, VB₁₂ loss is closely associated with the amount of water used during cooking. Lešková et al. (2006) reported that thermal processing and pH level generally influence the stability of VB₁₂. Our previous study found that intramuscular injections of VB₁₂ in dairy cows significantly improved milk yield, milk-protein yield, and VB₁₂ content in raw milk (Wang et al., 2018). Gagnon et al. (2015) also found that weekly VB₁₂ injections stimulated cell differentiation and ovulation. However, it is still unclear whether intramuscular injection of VB₁₂ can influence cheese quality and VB₁₂ stability due to the stretching and heating process during Mozzarella cheese making and subsequent cheese storage. The current study aimed to explore the effect of VB₁₂ injection in early-lactation dairy cows on low-moisture part-skim Mozzarella cheese quality and VB₁₂ cheese retention during the cheese-making process, as well as stability during the subsequent cheese storage period.

Experimental milk samples in this study were obtained from the commercial dairy farm Hangjiang (Hangzhou, China), based on our previous animal trial (Wang et al., 2018). In brief, 24 peripartum dairy cows were blocked based on parity and milk yield, then randomly assigned into 2 treatments: basal TMR diet (CON) and basal diet with an intramuscular injection of 10 mg of VB₁₂ per cow per week (VB₁₂) during the prepartum period and 8 wk after calving. The TMR ingredients and chemical profiles are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2020-18568>).

Milk samples from the 2 groups were collected after 8 wk of feeding and intramuscular injections during the early-lactation stage, and stored at -20°C until analysis. Raw milk was thus collected 3 times and then Mozzarella cheese was manufactured. Milk composition was measured by infrared spectroscopy using a MilkoScan FT 120 (Foss Electric, Hillerød, Denmark), shown in Supplemental Table S2 (<https://doi.org/10.3168/jds.2020-18568>). Thirty liters of fresh raw milk was collected and standardized through partial skimming, subsequently pasteurized for 30 min at 63°C, and then cooled to 35°C. The Mozzarella cheese-making process was conducted according to the modified method of previous studies (Sheehan and Guinee, 2004; Liu et al., 2018). The resulting cheese was cut into several pieces, vacuum packaged, and refrigerated at 4°C. Raw milk samples from each group for cheese making were collected every 2 d at 0700 h during the ninth week. Compositions and properties of all cheese samples were analyzed in triplicate.

Cheeses made from the 2 groups were analyzed according to common methods, including the Gerber method (NSAI, 1955) for fat determination, oven-drying method (IDF, 1958) for moisture determination, and macro-Kjeldahl method (IDF, 1993) for protein determination. Moisture content in nonfat substances and fat content in DM were calculated based on compositional results. A hand-held Minolta CR-20 colorimeter (Minolta Laboratories, Osaka, Japan) was used to measure fresh cheese color. The lightness (L^* value, varies from 0% dark to 100% light), red-green (a^* value, varies from -60% green to +60% red), and yellow-blue chromaticity (b^* value, varies from -60% blue to +60% yellow) were determined based on the CIELAB color space (Pinho et al., 2004) with 5 readings per sample. Fresh cheese pH was measured using a calibrated Unicam glass Ag-AgCl combination pH probe meter (Mettler-Toledo, Zurich, Switzerland). Cheese functionality properties were measured after heating the cheese, with flowability analyzed by a modified Schreiber method (Guinee et al., 2000). Meltability was determined using the Arnott test (Park et al., 1984). Stretchability was measured using a fork test according to Wadhvani et al. (2011). All tests were conducted in triplicate at 1, 4, and 8 wk of aging time.

The VB₁₂ content of raw milk and cheese was analyzed using ultra-performance liquid chromatography coupled with tandem MS according to Zironi et al. (2013). Ten milliliters of raw milk was centrifuged at $4,000 \times g$ for 10 min to separate and eliminate the fat. Five grams of cheese was mixed with 5 mL of 200 mM sodium acetate buffer and 150 μ L of 1% potassium cyanide solution, and then the sample was heated to 90°C for 1 h in the presence of cyanide. All forms of VB₁₂ were converted to cyanocobalamin. After cooling, samples were purified by a single solid-phase extraction step (Zironi et al., 2013), after which 10 mL of samples were analyzed in positive electrospray ionization mode.

The cheese quality properties and VB₁₂ contents were analyzed using Proc GLM program of SAS 9.4 (SAS Institute, Inc., Cary, NC). The model for chemical compositions, quality properties, and VB₁₂ contents was as follows: $Y_{ij} = \mu + T_i + e_i$, where Y_{ij} is the observation of dependent variables i ; μ is the population mean of variable; T_i is the effect of VB₁₂ injection on cheese quality and VB₁₂ content; e_i is the random error. Significance was proclaimed at $P < 0.05$, and trends were proclaimed at $P < 0.10$.

The VB₁₂ concentration of milk and cheese are shown in Table 1. A significantly higher VB₁₂ concentration was observed in VB₁₂ milk compared with CON milk (15.43 vs. 3.30 ng/mL). A previous Canadian survey showed that the concentration of VB₁₂ in milk from

commercial dairy herds was 2.3 to 3.9 µg/L (Duplessis et al., 2016). As reported in the national food composition databases of Denmark and Switzerland, the concentration of VB₁₂ in bovine milk is 0.8 to 4.9 µg/L (Gille and Schmid, 2015). The VB₁₂ concentration of CON milk was thus consistent with normal milk test values, but VB₁₂ injection significantly increased VB₁₂ content of milk, which is consistent with Akins et al. (2013). Duplessis and Girard (2019) also found that intramuscular injections of VB₁₂ increased VB₁₂ content of colostrum. Therefore, it can be concluded that intramuscular injection of VB₁₂ is an efficient way to produce VB₁₂ rich milk.

Most VB₁₂ is water soluble to whey and thus separated from the curd during the cheese-making process (Gille and Schmid, 2015). As shown in Table 1, approximately 70% of VB₁₂ was lost in whey during cheese making, leading to an undetectable VB₁₂ concentration in CON fresh cheese and 3.72 ng/g in VB₁₂ fresh cheese. Arkbåge et al. (2003) also found that 82, 50, and 38% of VB₁₂ in milk was removed with whey during cottage, hard, and blue cheese-making processes, respectively. We found that VB₁₂ reduction continued after cheese making, with undetectable levels of VB₁₂ left in VB₁₂ cheese after 8 wk of storage, indicating that VB₁₂ was unstable and decomposed during cheese storage. Arkbåge et al. (2003) found that VB₁₂ concentration of fermented milk decreased significantly during storage due to *Lactobacillus bulgaricus* and *Streptococcus thermophilus* starter cultures, which suggested that bacterial strains in starter culture consumed or damaged VB₁₂. Thus, the starter culture used during cheese making may have exacerbated the decrease of VB₁₂ during cheese storage.

Effects of intramuscular injection of VB₁₂ in early-lactation dairy cows on chemical compositions of cheese are shown in Table 2. No significant differences were observed in protein, fat, or moisture content between the 2 cheeses ($P > 0.05$), which may be attributed to the similar raw-milk fat and protein content between CON and VB₁₂ milk, as well as the standardized ratio of protein to fat in milk before cheese making. As shown in our previous study, the VB₁₂ injection increased milk yield with no influence on milk protein and fat content (Wang et al., 2018). Duplessis et al. (2017) also detected no influence of VB₁₂ on milk protein and fat content. The chemical profiles of milk and cheese indicated that VB₁₂ injection did not affect milk and cheese macronutrients and moistures. The pH values of CON and VB₁₂ low-moisture part-skim Mozzarella cheese are shown in Table 2. The pH value of CON cheese was significantly higher than that of VB₁₂ cheese (5.30 vs. 5.25, $P < 0.01$) after 1 wk of storage. The pH of low-

Table 1. Vitamin B₁₂ (VB₁₂) concentration and distribution in raw milk and low-moisture part-skim Mozzarella cheese with and without (CON) VB₁₂ intramuscular injection

Item	Treatment	
	CON (n = 3)	VB ₁₂ (n = 3)
Milk (ng/mL)	3.30 ^b	15.43 ^a
Fresh cheese (ng/g)	Undetectable	3.72
Cheese (2 mo) (ng/g)	Undetectable	Undetectable
Whey (ng/mL)	2.57 ^b	11.54 ^a
Stretch water (ng/mL)	ND ¹	ND
Brine (ng/mL)	ND	ND

^{a,b}Means within a row with different superscripted letters are significantly different ($P < 0.05$).

¹ND = not detected.

moisture part-skim Mozzarella cheese was influenced by lactic acid content (Guinee et al., 2002).

The color properties of fresh low-moisture part-skim Mozzarella cheese are shown in Table 3. The VB₁₂ injection increased b^* value (16.00 vs. 15.06, $P < 0.01$) and decreased a^* value (−3.38 vs. −3.78, $P = 0.02$) of VB₁₂ cheese compared with CON cheese, suggesting a much more intense greenness and yellowness in VB₁₂ cheese. However, VB₁₂ injection did not influence L^* value of cheese ($P = 0.82$), indicating lightness was not affected by VB₁₂ injection. The color of milk and cheese can be influenced by diet (Calderón et al., 2007), thus the increased yellowness of VB₁₂ cheese may be attributed to the increased content of VB₁₂, which has a yellow or orange color. As shown in the Table 1, the VB₁₂ content in CON cheese was significantly lower (undetectable vs. 3.72 ng/g). Furthermore, although fat content of CON cheese did not statistically differ from that of VB₁₂ cheese, the 1.5% greater fat content in VB₁₂ cheese may have also contributed to the enhancement of cheese color (Paulson et al., 1998). Color is one of the main sensory properties that determine consumer acceptance. Wadhvani and McMahon (2012)

Table 2. Composition of fresh low-moisture part-skim Mozzarella cheese from dairy cows receiving or not receiving (CON) vitamin B₁₂ (VB₁₂) injection

Item	Treatment		SEM	P-value
	CON (n = 3)	VB ₁₂ (n = 3)		
Protein (%)	22.80	21.65	0.71	0.37
Fat (%)	17.21	18.73	0.42	0.16
Moisture (%)	51.37	50.10	0.39	0.08
FDM ¹ (%)	35.12	35.87	0.32	0.57
MNFS ² (%)	61.77	62.11	0.58	0.70
pH	5.30	5.25	0.02	<0.01

¹FDM = fat in DM.

²MNFS = moisture in nonfat substances.

found that consumers prefer low-fat cheese made with an intermediate level of annatto color that imparts a pleasing yellowish-orange color. Thus, the increased yellowness of cheese made from VB₁₂ rich milk may be more attractive to consumers.

Higher stretchability and meltability of low-moisture part-skim Mozzarella cheese is highly appreciated in pizza making (Jana, 2001). The meltability, stretchability, and flowability changes of the 2 cheeses during 8 wk of storage are displayed in Figure 1. No significant difference was observed between the 2 cheeses at wk 1 ($P > 0.05$), indicating that VB₁₂ injection did not affect the functionality of fresh cheese. After 8 wk of storage, greater flowability (CON vs. VB₁₂: 342 vs. 402%) and meltability (CON vs. VB₁₂: 66 vs. 85%) were observed in VB₁₂ cheese ($P < 0.05$). However, the stretchability of VB₁₂ cheese increased initially and decreased after 8 wk of storage, which agrees with Rowney et al. (1999). Proteolysis of intact casein by microorganisms and enzymes during 8 wk of storage can contribute to functionality changes (Sheehan and Guinee, 2004). Cortez et al. (2008) detected that cheese meltability is closely associated with moisture content in nonfat substances and pH; lower pH improves cheese meltability. Thus, the lower pH and higher fat may have contributed to the greater meltability in VB₁₂ cheese.

Dairy products supply a highly bioavailable source of VB₁₂, and adequate intake of enhanced milk could be an improved therapeutic strategy to reduce VB₁₂ deficiency. A dramatic positive relationship was detected between dietary VB₁₂ intake and plasma VB₁₂ concentration in a large population-based study (Vogiatzoglou et al., 2009). Naik et al. (2013) found that milk consumption increased VB₁₂ status, increased circulating VB₁₂, and potentially reduced the incidence of cardiovascular disease. Thus, higher VB₁₂ concentration in milk and fresh cheese via bovine intramuscular injection could be beneficial to supply VB₁₂, especially in pregnant woman or elders in developing countries, at a relatively low expense. Because large amounts of VB₁₂ were lost to the whey and decomposed during cheese

Table 3. Color properties of fresh low-moisture part-skim Mozzarella cheese made from milk with or without (CON) vitamin B₁₂ (VB₁₂) intramuscular injection

Item ¹	Treatment		SEM	P-value
	CON (n = 3)	VB ₁₂ (n = 3)		
L*	88.98	89.06	0.24	0.82
a*	-3.38	-3.78	0.06	<0.01
b*	15.06	16.00	0.24	0.02

¹L* value varies from 0% dark to 100% light; a* value varies from -60% green to +60% red; b* value varies from -60% blue to +60% yellow.

Figure 1. Stretchability, flowability, and meltability changes of resulting low-moisture part-skim Mozzarella cheeses made from cow milk with (gray) or without VB₁₂ intramuscular injection (black). Error bars represent SD; bars with different letters (a-c) are significantly different ($P < 0.05$).

storage, the current study suggests that the VB₁₂ injection is better suited for drinking-milk fortification, rather than aged cheese production.

In conclusion, intramuscular injection of VB₁₂ increased VB₁₂ content in raw milk; however, approximately 70% of VB₁₂ was lost in whey during cheese making with no additional VB₁₂ remaining in cheese after 8 wk of storage. The VB₁₂ injection had no significant effect on cheese fat and protein content at 8 wk of age, but cheese color (*a** and *b** value), meltability, and flowability were slightly affected during storage. Therefore, intramuscular injection of VB₁₂ in early-lactation dairy cows increases VB₁₂ content in milk and has no adverse effect on cheese quality, but VB₁₂ is unstable during cheese-making and storage.

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