Short communication: Effects of dietary *N*-carbamoylglutamate supplementation on the milk amino acid profile and mozzarella cheese quality in mid-lactating dairy cows

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ABSTRACT

N-Carbamoylglutamate (NCG) is an enhancer of Arg, which is a functional AA and could prevent cardiovascular disease and improve immunity. The present study was conducted to investigate the effects of supplementing NCG in diets of lactating cattle on the NCG concentration and AA composition of raw milk and on mozzarella cheese quality. Thirty multiparous cows with a mean body weight of 669 kg (standard deviation = 71) and days in milk of 176 (standard deviation = 55) were blocked based on parity and milk production and randomly assigned to 1 of 2 treatments: basal diet (CON) and basal diet supplemented with 40 g of NCG per day per cow (NCG). After 8 wk of treatment, raw milk samples were collected from the 2 groups for AA analysis and mozzarella cheese-making. Furthermore, the NCG concentration and distribution in milk and mozzarella cheese were detected. The AA concentration in milk was greater and the NCG concentration in raw milk was approximately 6 times greater in the NCG group than in the CON group. No NCG was detected in cheese from the CON group, and very little NCG (<1.0 µg/kg) of cheese was detected in the NCG group. Most of the dietary NCG was transferred into whey, stretch water, and brine during cheese production. No significant difference was found between the 2 groups on cheese texture and color except that hardness was lower in the NCG group. Overall, the results indicated that dietary supplementation of NCG could improve the NCG and AA concentrations in raw milk without affecting the quality of cheeses such as mozzarella.

Key words: dairy cow, *N*-carbamoylglutamate, amino acid profile, mozzarella cheese

Short Communication

Cardiovascular disease is a global epidemic, representing a significant socioeconomic burden with a high rate of morbidity and mortality in all countries. According to a report of the World Health Organization, 17.5 million people died of cardiovascular diseases in 2012, twice the number of people who died of cancer (Nichols et al., 2014). Go et al. (2013) predicted that this figure will reach 25 million by 2030. The AA Arg is a conditionally essential AA for most mammals and considered as a functional AA (Wu et al., 2009). Numerous studies reported that Arg can relax blood vessels and prevent cardiovascular disease (Rosenthal et al., 2016) and increase immune function (Ren et al., 2012; Tan et al., 2015). However, the cost of supplemented exogenous Arg is high and there are concerns about safe dosage (Wu et al., 2009; Rosenthal et al., 2016).

Alternatively, *N*-carbamoylglutamate (NCG) is a structural analog of *N*-acetylglutamate, which is an allosteric activator of the essential auxiliary factor ammoniacal phosphate synthetase in the endogenous synthesis of Arg (Gessler et al., 2010). Many studies have reported that supplementation with NCG can increase plasma Arg concentration in animals (Chacher et al., 2014; Cao et al., 2016; Xiao et al., 2016). In addition, NCG is widely used in humans, and in addition to being a nutritional supplement that can significantly increase muscle production (Frank et al., 2007), it can also be used as a medicine for the treatment of certain rare diseases such as reducing elevated serum ammonia levels in patients with propionic and methylmalonic aciduria during acute metabolic decompensation (Filippi et al., 2010; Kasapkara et al., 2011). Thus, NCG could be an enhancer of Arg and serve as a healthy product.

Milk and cheese are popular foods around the world and important sources of nutrition for humans. Milk has long been regarded as a natural delivery vehicle for many bioactive compounds (Yeum and Russell., 2002). Furthermore, mozzarella cheese is one of the most popular cheeses, used widely in foods such as pizza and as

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a snack popular among children (Liu et al., 2018). With the improvement of living standards, consumer interest in the nutritional, functional, and health properties of foods has increased (Jones and Jew, 2007; Annunziata and Vecchio, 2011). Thus, the quality and health benefits of milk and cheese have been the focus of many studies (Settanni and Moschetti, 2010; Liu et al., 2015).

Milk AA composition is an important factor associated with milk quality and cheese flavor (Engels, 2002). Our previous studies showed that supplementing 40 g of NCG per day to a dairy cow could increase the concentration of plasma AA and improve milk protein concentration (Gu et al., 2018). However, it remains unclear how much NCG is transferred from the diet to the milk and whether dietary NCG supplementation affects milk AA composition and dairy product quality. Therefore, the aim of the present study was to investigate the NCG concentration and distribution in milk and mozzarella cheese under dietary NCG supplementation and the effects of dietary NCG on milk AA composition and mozzarella cheese quality.

The experiment design was same as described in our previous study (Gu et al., 2018). In brief, 60 multiparous Holstein cows in mid lactation were selected and assigned to 15 blocks based on parity and milk yield and then randomly allocated into 4 groups with basal diets supplemented with different doses of NCG (0, 10, 20, or 40 g/d per cow). Milk samples of the current study were collected from the control group (CON) and 40 g/d per cow group (NCG) after 8 wk of that study. The ingredients and chemical compositions of the diets are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-17385>). The milk components were analyzed via infrared spectroscopy using a Milko Scan FT 120 (Foss Electric, Denmark). Milk samples were collected and stored at -20°C for AA profile analysis and NCG concentration determination. The AA profiles in the milk were determined with an Automatic AA Analyzer (Hitachi High-Technologies Corp., Tokyo, Japan) as previously described (Wang et al., 2016).

According to Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-17385>), after 8 wk of feeding, the milk yield of the NCG group (22.8 kg/d) was similar to the CON group (22.4 kg/d); however, higher protein and fat content (3.73 and 4.45%, respectively) was found in the NCG group raw milk than the CON group (3.57 and 4.20%, respectively). Fresh milk for making cheese was collected from dairy cows of each group at 0600 h. The mixed raw milk (30 L) was standardized to a ratio of protein to fat of 1:1.2 by partial skimming, and the milk was pasteurized at 63°C for 30 min and then cooled to 35°C . Mozzarella cheese was made as described by Sheehan and Guinee (2004). After the

cheese was made, samples of cheese, whey, hot water, and brine corresponding to the 2 treatment groups were collected and frozen until NCG concentration analysis. Mozzarella cheese was cut, vacuum packaged (0.5 kg), and then stored in a refrigerator (-4°C). Cheese samples were analyzed after 1 wk of storage.

The fat, moisture, and protein concentrations of the cheese were determined with the Gerber method, oven-drying method, and macro-Kjeldahl method, respectively (Liu et al., 2018). The textural properties were measured using a texture analyzer (TA-XT Plus, Stable Micro System, Surrey, UK) fitted with a P/N 0.5 probe (10-mm diameter) moving at a speed of 1 mm/s for a distance of 8 mm using a cycle or normal program. The hardness and adhesiveness of cheese were determined as described previously (Ayad et al., 2004). The analyses were conducted in triplicate. Color analyses were performed using a handheld colorimeter (Minolta CR-20, Minolta Laboratories, Osaka, Japan). The L^* , a^* , and b^* color measurements were determined according to the Commission Internationale d'Eclairage LAB color space. On average, 5 readings per sample were recorded.

The concentration of NCG concentration in the milk, cheese, whey, stretch water, and brine were analyzed using HPLC electrospray ionization tandem MS. One milliliter of milk or other liquid sample was weighed accurately and placed into a tube, to which 4 mL of anhydrous methanol was added. The mixture was then centrifuged at $4,000 \times g$ and 4°C for 20 min. The supernatant was then collected and passed through a $0.22\text{-}\mu\text{m}$ aqueous filter and an organic filter. Next, 0.5 mL of the supernatant was collected and stored in a gas phase vial for subsequent NCG detection. For the cheese samples, 1 g of cheese was dissolved in 1 mL of water and then subjected to the same procedures as described for the liquid samples. Five milligrams of NCG standard (purity 98%, purchased from Sigma, St. Louis, MO) was added to a 50-mL brown volumetric flask, dissolved to volume with pure water to produce 100 mg/L standard stock solution, and stored at 4°C .

The instrument used to detect NCG was the Agilent 6460 LC-Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, CA) with an Electrospray Ion Source and Mass Hunter Acq B0400 Data Processing System, the 1290 Chromatography System with Binary Gradient Pump, an online degasser, an autosampler, and a column oven (Agilent Technologies). The liquid chromatography conditions were as follows: column: Unimicro C18 ($250\text{ mm} \times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$); mobile phase: methanol and 0.1% (vol/vol) aqueous formic acid (5:95, vol/vol); column temperature: 20°C ; flow rate: 0.6 mL/min; and injection volume: 10 μL . The MS conditions were as follows: ionization

source: electrospray ion source; scanning mode: positive ion scanning mode (positive); detection mode: multiple reaction detection; capillary voltage: 4.0 kV; ion source temperature: 100°C; drying gas temperature: 300°C; dry gas flow rate: 11 L /min; atomizing gas pressure: 1.03×10^5 Pa; fragmentor voltage: 70; collision energy: 24.

The AA data were analyzed using SAS software (version 9.0, SAS Institute Inc., Cary, NC) with the covariance type AR (1) for repeated measures and employing a randomized complete block design. Cheese data from at least 3 replicates were evaluated for each index. Independent *t*-tests were applied to analyze the cheese data. All differences were considered statistically significant at $P < 0.05$.

The results of NCG concentration in the CON and NCG groups are shown in Table 1. After 2 mo of dietary NCG supplementation (40 g/d per cow) or the CON diet, the NCG concentration in the NCG group was 6 times greater than that in the CON group (6.6 vs. 1.1 µg/L). The NCG is a synthetic analog of *N*-acetylglutamate and is known as an enhancer of Arg (Chacher et al., 2014), which has 2 negatively charged carboxyl groups and no positive charge. Chacher et al. (2012) reported that degradation of NCG in the rumen was approximately 18%. A previous study showed that the NCG is absorbed via excitatory AA 3 transporters into the small intestine and liver and used for Arg synthesis (Burckhardt and Burckhardt, 2017). Based on the milk yield reported in our previous study (Gu et al., 2018), approximately 0.37% of dietary NCG is transferred to the milk. The remaining NCG might be transferred to the blood, feces, urine, or other disposal routes. Wu et al. (2015) demonstrated that NCG is a nontoxic substance with no genotoxicity; therefore, the milk with NCG would be a safe product for humans. Previous studies showed that supplementation with NCG could improve liver function and intestinal health and alleviate oxidative stress in animals (Cao et al., 2016; Xiao et al., 2016; Zhang et al., 2019); additionally, it is used to treat hyperammonemia in rare inherited disorders, such as carbamoyl phosphate synthetase I and *N*-acetylglutamate synthase deficiency, ornithine transcarbamylase deficiency, and methylmalonic acidemia (Tuchman et al., 2008; Yap et al., 2019). The underlying mechanism may be that NCG promotes endogenous synthesis of Arg (Wu et al., 2004, 2010), and this was also reflected in our previous study (Gu et al., 2018). Arginine is a functional AA with many biological functions in reproduction, immunity, neonatal growth, and wound healing in monogastric animals and humans (Wu et al., 2009). The more important biological function is that Arg is the precursor of nitric oxide (NO), which can regulate angiogenesis, dilate blood vessels,

Table 1. *N*-Carbamoylglutamate concentrations and distribution among the different components of milk and cheese, expressed as mean $\pm$ SD¹

Item	CON	NCG
Milk (µg/L)	1.1 $\pm$ 0.07 ^a	6.6 $\pm$ 1.11 ^b
Whey (µg/L)	0.8 $\pm$ 0.05 ^a	4.0 $\pm$ 1.53 ^b
Cheese (µg/kg)	ND	<1.0
Stretch water (µg/L)	ND	ND
Brine (µg/L)	ND	ND

^{a,b}Means within a row with different superscript letters differ ($P < 0.05$).

¹CON = basal diet; NCG = basal diet supplemented with 40 g of *N*-carbamoylglutamate per day per cow. ND = not detected.

and accelerate blood flow (Jobgen et al., 2006; Morris, 2009). Numerous studies reported that Arg and NO can relax blood vessels and prevent cardiovascular disease (Bondonno et al., 2016; Rosenthal et al., 2016; Farah et al., 2018). The above information indicates that milk with NCG could be a safe and healthy product and would be conducive to human health.

The NCG concentration and distribution among different components during cheese production are shown in Table 1. Following cheese production, most of the NCG (~60–73%) was in the whey (0.8 µg/L in the CON group and 4.0 µg/L in the NCG group). The remaining NCG (~27–40%) was in the cheese, stretch water, and brine. A low NCG concentration (<1 µg/kg) was found in the cheese of cows from the NCG group. Burckhardt and Burckhardt (2017) reported that NCG is a water-soluble substance, which can explain why most of the NCG (~60–73%) in the milk was transferred to the whey during the cheese-making process and not stored in the cheese. In addition, the stretching and brining processes might have destroyed or extracted NCG from the curd and cheese.

Compared with the CON cows, the cows that received NCG supplementation had a greater milk AA concentration (Table 2). The Arg concentration in the CON and NCG groups was 1.82 and 2.19 µmol/L, respectively. The concentrations of most AA were increased in the NCG group, including EAA and NEAA. This could be attributed to the higher milk protein concentration in the NCG group than in CON (3.73 vs. 3.57%, respectively; Table 2). These results are consistent with our previous study in which plasma AA and NO concentrations were increased with NCG addition (Gu et al., 2018). Moreover, NO is a metabolite of Arg and could accelerate mammary blood flow (Morris, 2009). Therefore, the AA supply for the mammary gland may be increased with NCG addition. An increased AA supply can be expected to increase the amount of AA substrate used for protein synthesis (Rius et al., 2010),

Table 2. Milk AA concentrations from cows receiving or not receiving dietary *N*-carbamoylglutamate¹

AA	CON	NCG	SEM	<i>P</i> -value
Milk protein (%)	3.57	3.73	0.06	0.05
EAA (μmol/L)				
Arg	1.82	2.19	0.10	0.04
Thr	3.45	4.10	0.20	0.05
Val	5.05	6.10	0.30	0.04
Met	1.63	1.98	0.10	0.04
Ile	3.67	4.45	0.23	0.05
Leu	7.12	8.69	0.43	0.04
Phe	2.70	3.26	0.16	0.04
Lys	5.37	6.40	0.32	0.06
His	1.66	2.04	0.10	0.02
Subtotal	32.5	39.2	1.92	0.04
NEAA (μmol/L)				
Cys	0.24	0.30	0.02	0.08
Glu	14.9	17.9	0.86	0.04
Tyr	2.42	2.85	0.14	0.07
Gly	2.55	3.11	0.14	0.03
Ala	3.57	4.22	0.20	0.05
Ser	5.00	5.97	0.28	0.04
Pro	5.79	6.98	0.33	0.04
Asp	5.58	6.70	0.31	0.04
Subtotal	40.0	48.1	2.27	0.04
Aromatic AA (μmol/L)	5.12	6.11	0.30	0.05
BCAA (μmol/L)	15.9	19.2	0.96	0.04
TAA (μmol/L)	72.6	87.2	4.19	0.04

¹CON = basal diet; NCG = basal diet supplemented with 40 g of *N*-carbamoylglutamate per day per cow. BCAA = branched-chain amino acid; TAA = total amino acids.

which might explain the increased milk AA concentration in the NCG group. The free AA composition and concentration in cheese are strongly and positively correlated with the AA concentration and composition of the source raw milk (Eren-Vapur and Ozcan, 2012). In addition, AA may influence flavor either directly or indirectly by serving as precursors for volatile aroma compounds, such as aldehydes, acids, alcohols, esters, and sulfur compounds (Engels and Visser, 1996; Ayad et al., 2001; Ardo et al., 2002).

The composition, texture, and color of the cheese derived from the CON and NCG groups are shown in Table 3. The results were similar between the 2 groups. High fat concentration was found in the cheese from the NCG group, potentially due to the high fat concentration in the milk as described in our previous study (Gu et al., 2018). In the present study, lower hardness was found in the NCG cheese than in the CON cheese, possibly due to the higher fat concentration in the NCG cheese. Other texture parameters and cheese color were not affected by NCG treatment.

In conclusion, dietary NCG supplementation could increase milk NCG and AA concentration in raw milk. During the production of mozzarella cheese, most of the NCG was lost in the whey. None of the cheese quality parameters were affected by dietary NCG except

Table 3. The composition (mean ± SD) and color of cheese made from milk of cows receiving or not receiving dietary *N*-carbamoylglutamate¹

Item	CON	NCG
Protein (%)	24.9 ± 0.25	24.8 ± 0.41
Fat (%)	22.8 ± 0.68 ^b	24.3 ± 0.57 ^a
Moisture (%)	44.7 ± 1.27	43.9 ± 2.21
Hardness (g)	332 ± 13 ^a	263 ± 21 ^b
Cohesiveness (ratio)	0.60 ± 0.05	0.52 ± 0.48
Color ²		
L* (%)	87.8 ± 0.45	91.4 ± 0.41
a* (%)	−1.18 ± 0.06	−0.80 ± 0.05
b* (%)	14.0 ± 0.34	14.8 ± 0.29

^{a,b}Means within a column with different superscript letters differ (*P* < 0.05).

¹CON = basal diet; NCG = basal diet supplemented with 40 g of *N*-carbamoylglutamate per day per cow.

²Commission Internationale d'Éclairage L*a*b* color space.

for cheese hardness. These results suggest that dietary NCG may have the potential to produce milk or milk products that are rich in NCG and have high AA and protein concentrations. However, cheese quality was not affected, as the NCG was removed with the whey during cheese production.

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