PAPER

Lecithin: a by-product of biodiesel production and a source of choline for dairy cows

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Abstract

The aim of the present study was to compare soy lecithins (L), a by-product of the biodiesel production process, with choline chloride microencapsulated with hydrogenated vegetable oils (C), to verify their effects on dry matter intake, milk yield, milk quality traits, milk choline and haematological profile of dairy cows. A total of 12 mid-lactating Holstein Friesian cows were assigned to one of two experimental groups and fed according to cross-over design (2 diets x 2 periods). Diets were isoenergetic, isofibrous and isonitrogenous and had the same content of choline. Dry matter intake, milk yield and the 3.5% fat-corrected milk (FCM) were not affected by the diet. The milk choline content and the milk quality did not differ between treatments, with the exception of milk fat ($P<0.05$) and urea ($P<0.01$), which resulted lower for the L-group. Milk fat, protein and choline yields remained unaffected. With regard to the haematological profile, all of the parameters fell within the physiological range of lactating dairy cows, and the only difference was represented by the lower urea level of the L fed cows ($P<0.01$), that could be explained by a better N metabolic efficiency. Results indicated that soy lecithins can be used as an available source of choline in mid-lactating dairy cows.

Introduction

Rumen-protected choline (RPC) supplementation to both transition and early lactating dairy cows, usually provided as choline chloride coated with hydrogenated oils (Pinotti *et al*., 2002), has been demonstrated to improve milk production (Erdman and Sharma, 1991; Pinotti *et al*., 2003; Ardalan *et al*., 2010) and the metabolism of lipids and methyl groups (Piepenbrink and Overton, 2003; Baldi and Pinotti, 2006). Moreover, supplemental choline may improve the transport of lipids in the blood, thus reducing the risk of fatty liver disease and ketosis in periparturient dairy cows, as choline is an essential component of the phospholipids that are at the core of the structure of lipoproteins (Pinotti *et al*., 2002). Some authors have shown that unprotected choline chloride fed to ruminants is extensively degraded in the rumen (Sharma and Erdman, 1989); by comparing choline chloride to RPC, Bonomi *et al*. (1996) found that 10 g of unprotected choline chloride has the same effects as 2 g of RPC in the improvement of milk yield and milk fat and protein in dairy cows, confirming the high extent of unprotected choline degradation in the rumen. In the light of these findings it was concluded that unprotected choline naturally present in the diet makes an insignificant contribution to the choline pool in the body. Atkins *et al*. (1988) have indicated that choline chloride was more degradable than naturally occurring choline in feed, and Jenkins *et al*. (1989) have suggested that intact phospholipids, where most of the choline is present as phosphatidylcholine (PtdCho) (Pinotti *et al*., 2002), might reach the hind-gut. Nevertheless, to our knowledge, no studies have investigated the role of lecithins as potential sources of RPC.

It has been reported that crude lecithins may contain approximately 20% phosphatidylcholine (Grummer *et al*., 1987), phosphatidylethanolamine and phosphatidylinositol and 30% to 40% oil (Zinn *et al*., 1989) and could possibly represent a source of rumen-protected choline. In fact, lecithins could be obtained as a by-product of the water degumming process in biodiesel production (Van Gerpen, 2004). Therefore, the aim of this study was to compare the effects of supplements based on soy lecithins and RPC on the dry matter intake (DMI), milk yield, milk quality traits, milk choline and haematological profile. Milk choline was taken into account because it is reported to be a suitable indicator of post-ruminal choline supply and bioavailability in dairy cows (Deuchler *et al*., 1998; Pinotti *et al*., 2003).

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Key words: Mid-lactating dairy cows, Rumen-protected choline, Lecithin, Phosphatidylcholine, Milk quality.

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Materials and methods

Animals, experimental design and dietary treatment

The Padova University Animal Care and Use Committee approval was obtained before beginning the study.

During a trial lasting 42 days, twelve Holstein Friesian cows were assigned to one of two experimental groups that were balanced for milk yield (36.5±6.2 kg/d), days in milk (DIM, 123±65 d), parity (1.7±0.9), body weight (BW, 668±30 kg) and body condition score (BCS, 3.0±0.3). Two weeks before the beginning of the trial, the cows were fed the experimental total mixed ration (TMR), but without the supplements; values of feed intake, 3.5% fat-corrected milk (FCM), milk choline and haematological profile (*data not reported*) were measured, in order to know their basal values, which were 20.5 kg/d, 35.3 kg/d and 96 mg/kg, respectively. The cows were housed in a free stall, in two different pens, fed *ad libitum* at 9.00 and milked twice a day with an automated milking plant. The BW and BCS were measured at days 0, 21 and 42 of the trial.

The animals were assigned to two experi-

mental groups and exposed to one of two dietary treatments in a 2×2 cross-over design (2 diets x 2 periods), with a period of 21 days (14 days of an adjustment phase followed by 7 days of data collection). The treatments consisted of the following supplements added to the TMR: a control supplement (C) based on 25% choline chloride microencapsulated with hydrogenated vegetable oils and an experimental supplement (L) based on soy lecithins derived from a biodiesel production process. The supplements were added in the mixing wagon and incorporated into a maize silage-based TMR prior to be given to the animals. The dietary treatments were formulated to be isoenergetic, isonitrogenous and isofibrous and to have the same content of choline; the diet ingredients and composition are presented in Tables 1 and 2. Orts proximate composition did not show any differences from the diets (*data not presented for the sake of brevity*). Based on the choline content of supplements each cow was fed 100 g of C (25% of choline) or 700 g of L (3.5% of choline), for a total amount of 25.0 and 24.5 g/d of choline for the C- and L-diets respectively, according to the range suggested in the literature (Erdman and Sharma, 1991; Pinotti *et al*., 2005; Elek *et al*., 2008). The cows wore identification collars, and feed intake was individually and continuously recorded using an automated feeding control system (Biocontrol A/S, Rakkestad, Norway). The TMR, orts and supplements were sampled weekly during the experiment and analysed for chemical composition according to AOAC (2000) and van Soest *et al*. (1991) using a Fibre Analyser (ANKOM/2000, ANKOM Technology, New York, NY, USA). The choline content of the lecithins was determined using the Phosphatidylcholine Assay Kit (Cayman Chemical Company, Ann Arbour, MI, USA). Briefly, a 10-mL sample of lecithins was diluted 1:100 with de-ionised water, and 100 µL of buffer was added to 20 µL of the diluted sample for choline analysis. Phosphatidylcholine-specific phospholipase D was first used to hydrolyse the PtdCho to choline and phosphatidic acid. The newly formed choline was then used to generate hydrogen peroxide in a reaction catalysed by choline oxidase. Catalysed by peroxidase, the last step was the reaction of hydrogen peroxide with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline and 4-aminoantypyrine to generate a blue dye with a recommended absorption at 585-600 nm. After an incubation at 37°C for 60 min, the absorbance was read at 590 nm using the Packard Spectra Count microplate reader (Packard Instrument Co, Downers Grove, IL, USA) (modified from Takayama *et al*., 1977).

After lipid extraction by chromatography using a dichloromethane/methanol solution (2:1 v/v), the fatty acids (FA) composition of the supplements was then determined. Aliquots of the extracts were trans-esterified according to the procedure reported by Christie (1982), and the fatty acid methyl esters (FAME) were detected as described below. An aliquot of 100 mL was homogenised in a 100 mL solution of anhydrous sodium sulphate (0.47 M) and centrifuged (4000 g, 10 min, 4°C), and 100 mg of surfaced fat was mixed with 4 mL methanol and 4 mL n-heptane and centrifuged again (4000 g, 5 min, 4°C). An aliquot of the upper phase (2 mL) containing the ether extract was trans-esterified with sodium methoxide, and the FAME were quantified by gas chromatography (Shimadzu GC17A, equipped with an FID detector, using an Omegawax 250 column of 30 m x 0.25 µm x 0.25 µm). Helium was used as the carrier gas at a constant flow of 0.8 mL/min, and injector

Table 1. Formulation and composition of C-diet (choline chloride microencapsulated with hydrogenated vegetable oils) and L-diet (soy lecithin).

	Dietary treatment	
	C-diet	L-diet
Ingredients		
Cereal mix°, % DM	22.1	22.0
Maize silage, % DM	26.3	26.4
Soybean based blend#, % DM	16.3	16.2
Alfalfa hay 2nd crop, % DM	22.9	22.8
Sugar beet dry pulps, % DM	8.1	8.1
Crushed linseed, % DM	1.3	1.3
Straw, % DM	2.1	2.1
Choline chloride§, % DM	0.4	-
Hydrogenated fat, % DM	0.5	-
Soy Lecithin^, % DM	-	1.1
Diet composition		
Crude protein, % DM	15.5	15.7
NDF, % DM	28.5	29.3
Ether extract, % DM	4.5	4.4
Crude ash, % DM	7.2	7.6
Non-fibre carbohydrates, % DM	44.3	43.0
Net energy for lactation, MJ/kg DM	7.4	7.4

°70% maize meal and 30% barley meal; #40.5% soybean meal; 34.5% extruded de-hulled soybean expeller; 9.6% vit-mineral mix; 10.8% extruded soybean seeds; 3.1% extruded flax seeds; 1.5% maize meal; §daily dose of supplemented choline: 25.0 g/cow; ^daily dose of supplemented choline, 24.5 g/cow. NDF, neutral detergent fibre.

Table 2. Proximate composition, choline contents and fatty acids profiles of the supplements.

	Dietary treatment	
	C-diet	L-diet
Proximate composition		
Dry matter, %	90.1	34.9
Crude protein, % DM	17.9	3.8
Ether extract, % DM	51.0	60.7
Choline, % DM	27.7	10.9
FA, g/100 g of total detected FA		
C16:0	58.0	17.0
C18:0	39.6	4.2
C18:1 n-9	0.19	12.0
C18:2 n-6	0.02	57.9
C18:3 n-3	0.02	6.6
Saturated FA	99.7	21.6
Unsaturated FA	0.3	78.4

C, choline chloride microencapsulated with hydrogenated vegetable oils; L, soy lecithin; FA, fatty acids.

and detector temperatures were 260°C. Peaks were identified based on commercially bought FAME mixtures (37-Component FAME Mix, Supelco Inc., Bellefonte, PA, USA). The obtained data were expressed as g/100 g of total detected FAME.

Milk analysis

During the trial, the milk production was recorded at each milking during the last week of each experimental period. The milk samples from each animal, pooled from two consecutive milkings, were collected on days 15 and 21 of each experimental period and divided into two subsamples; the first fraction was treated with preservative (sodium azide) and stored at 4°C until analysis. The samples were analysed for fat, protein and lactose by a Milk-o-Scan 4000 infrared analyser (Fossomatic, Foss Electric, Hillerød, Denmark). The milk urea content was determined using differential pH-metry (EUROCHEM CL 10 plus, Microlab EFA). The second fraction was freeze-dried and stored frozen at -80°C for milk choline analysis. Milk choline was determined by the enzymatic method of Woollard and Indyk (2000). Briefly, 4 g of freeze-dried sample was digested by 30 mL of 1.0 M hydrochloric acid at 70°C for 3 h to release the majority of bound choline. After cooling at room temperature, pH was adjusted with 3 N NaOH to 3.5 to 4.0. The hydrolysate was diluted to 50 mL with deionised water, centrifuged (4000 g, 5 min, 10°C) and filtered. The residual choline from phospholipids was cleaved with phospholipase D (P0065-25KU, from *Streptomyces chromofuscus*, ≥50,000 unit/mL, Sigma-Aldrich, St. Louis, MO, USA). Free choline reacted with choline oxidase (C-5896, from Alcaligenes species, 10 unit/mg, unit definition, Sigma-Aldrich) liberating hydrogen peroxide. In the presence of peroxidase (Type I, P-8125, from horseradish, 80 unit/mg, Sigma-Aldrich), phenol is oxidized, forming a chromophore with 4-aminoantipyrine (A-4382; Sigma-Aldrich). Absorbance of this compound was measured at 505 nm. Choline level was determined as choline hydroxide by the mean of a standard solution prepared by dissolving 523 mg of choline bitartarate (C-2654; Sigma-Aldrich) in 100 mL of water, which was equal to 2500 mg/mL choline hydroxide solution. The five-point standard curve (50, 100, 150, 200 and 250 mg/mL choline hydroxide equivalent) was prepared by further diluting the standard solution in water. This method measures the content of the total choline in milk: free choline, choline bound as acetylcholine, phosphatidylcholine, lysophosphatidylcholine, sphingomyelin and glycerophosphocholine (Elek *et al*., 2008).

Blood analysis

After the morning milking, blood samples from the jugular vein were taken twice during each experimental week and were collected in lithium-heparin tubes (Vacuette, Greiner Bio-One, Kremsmuenster, Austria) and centrifuged (1500 g, 15 min, 4°C) for plasma separation. The plasma was analysed for the following haematological parameters: total protein, albumin, globulin, total bilirubin, aspartate aminotransferase (AST), γ-glutamyl transferase (γGT), creatine kinase (CK), urea, glucose, triglycerides, non-esterified fatty acids (NEFA), cholesterol, β-hydroxybutyrate (β-HB), calcium (Ca), phosphorus (P), magnesium (Mg) and the NEFA/cholesterol ratio. The haematological parameters were measured with reagents supplied by Roche Diagnostics and Randox Laboratories Ltd. (Indianapolis, IN, USA) for the Roche Hitachi 912 Plus automatic analyser.

Statistical analysis

After verifying the normality and variance homogeneity (PROC UNIVARIATE and Shapiro-Wilk test), a mixed model procedure (PROC MIXED) was performed to evaluate the data on the BCS, BW, feed intake, milk yield, milk fat, protein, lactose, choline and haematological profile. The linear random model

Table 3. Effects of the C-diet (choline chloride microencapsulated with hydrogenated vegetable oils) and L-diet (soy lecithin) on the dry matter intake, milk yield, 3.5% fat-corrected milk, milk composition, milk choline, milk fat, protein and choline yield.

	Dietary treatment		SEM	P
	C-diet	L-diet		
Dry matter intake, kg/d	21.0	20.7	0.6	ns
Choline intake, g/d	24.5	24.1	0.6	ns
Milk yield, kg/d	35.0	36.0	1.4	ns
3.5% FCM, kg/d	35.6	35.2	1.3	ns
Milk fat, %	3.59	3.39	0.13	*
Milk protein, %	3.21	3.23	0.07	ns
Milk urea, mg/dL	23.4	20.6	1.0	**
Milk choline content, mg/kg	116	108	5	ns
Milk fat yield, g/d	1255	1213	56	ns
Milk protein yield, g/d	1132	1160	51	ns
Milk choline yield, g/d	4.08	3.85	0.27	ns

FCM, fat-corrected milk; *P<0.05; **P<0.01; ns, not significant.

Table 4. Haematological profile of cows fed the C-diet (choline chloride microencapsulated with hydrogenated vegetable oils) and L-diet (soy lecithin).

	Dietary treatment		SEM	P
	C-diet	L-diet		
Total protein, g/L	77.2	77.5	1.2	ns
Albumin, g/L	35.1	35.0	0.4	ns
Globulin, g/L	42.0	42.5	1.1	ns
Total bilirubin, μmol/L	1.56	1.62	0.12	ns
AST, U/L	85.9	86.2	4.2	ns
γGT, U/L	23.2	23.2	1.4	ns
CK, U/L	188	179	15	ns
Urea, mmol/L	4.72	4.24	0.13	**
Glucose, mmol/L	3.50	3.54	0.06	ns
Triglycerides, mmol/L	0.12	0.13	0.01	ns
NEFA, mmol/L	0.16	0.17	0.01	ns
Total cholesterol, mmol/L	5.15	5.12	0.20	ns
β-hydroxybutyrate, mmol/L	0.59	0.59	0.04	ns
Calcium, mmol/L	2.47	2.50	0.03	ns
Phosphorus, mmol/L	1.61	1.53	0.04	ns
Magnesium, mmol/L	0.96	0.96	0.01	ns
NEFA/Cholesterol	0.03	0.03	0.01	ns

AST, aspartate aminotransferase; γGT, γ-glutamyl transferase; CK, creatine kinase, NEFA, non-esterified fatty acids; **P<0.01; ns, not significant.

included the fixed effects of dietary treatment and period along with their interaction, the random effect of cow and the random residual. All of the statistical analyses were conducted using SAS (2008).

Results and discussion

The DMI, the daily intake of choline, the milk yield and the 3.5% FCM were not affected by the dietary treatment (Table 3), and the mean BW (673 *vs* 682 kg; P>0.05) and BCS (3.0 *vs* 3.0; P>0.05) did not differ between the diets and did not change significantly throughout the experiment. As reported in Table 3, the lecithin supplement did not change the milk choline content nor the milk quality, with the exception of the milk fat and urea, which were both significantly lower in the L-diet group. With regard to the daily output of milk nutrients and choline no differences were observed between dietary treatments. Among the blood parameters the dietary treatment significantly modified only the urea concentration which resulted lower in L, as reported in Table 4.

The lack of difference in the milk yield, the 3.5% FCM and the milk choline content between the treatments suggests that lecithins and RPC are equivalent sources of rumen protected choline for mid-lactating dairy cows. Other authors (Deuchler *et al*., 1998; Pinotti *et al*., 2003) in fact reported that milk choline content is an indicator of post ruminal choline availability; the similar milk choline concentration and milk choline daily output between the treatments of this study suggest a similar post-ruminal choline bioavailability. The composition of milk, and the haematological profile however, indicated some differences between the metabolism of RPC and lecithins. The lower urea concentration in both the milk and plasma of the L-diet-fed cows could reflect a more effective use of the degradable proteins by the micro-organisms present in the rumen (Frand *et al*., 2003; Piepenbrink and Overton, 2003; Moharrery, 2004), while the lower milk fat content resulting from the L-diet could be due to a diversified metabolic route between lecithins and RPC. Choline plays a major role in lipid transport because PtdCho is an essential component of the very low-density lipoproteins (VLDL) produced in the liver and cannot be substituted by other phospholipids. As the long-chain FA in milk are obtained from the blood triacylglycerols of VLDL, which arise from absorbed fat and, endogenously via the mobilisation of adipose fat stores, a different metabolic use of the PtdCho present in lecithins, could have reduced the VLDL formation and led to a lower milk fat production in high-producing dairy cows. On the other hand, the higher milk fat content from the C-diet fed cows is in line with the increase of the milk fat percentage reported by Pinotti *et al*. (2002), mainly in mid-lactating dairy cows following the supplementation with RPC.

With the exception of the blood urea, no statistical differences in the blood parameters were found between the diets, and all of the values fell within the physiological range of lactating dairy cows (Cozzi *et al*., 2010). The similar values of blood glucose between C- and L-groups seemed to confirm the hypothesis that both supplements do not differ in the function of sparing methyl groups. Pinotti *et al*. (2002, 2004) in fact have found that during lactation the extra demand for methyl groups can be met by choline supplementation, which reduces the need for *de novo* methyl group synthesis via the tetrahydrofolate system, that is reported to consume gluconeogenic precursors (Baldi and Pinotti, 2006). Finally the value of NEFA to cholesterol ratio, which can be considered a measure of the fat retained in or metabolised by the liver, was more than five times lower than what reported by Pinotti *et al*. (2003) for both diets, suggesting an efficient liver function and proper lipid metabolism (Piepenbrink and Overton, 2003). This finding should be interpreted in light of the stage of mid-lactation in the present study, compared with periparturient cows in the research of Pinotti *et al*. (2003).

Conclusions

Based on these results, we conclude that soy lecithins obtained as a by-product of the biodiesel production process can be used as an available source of choline in mid-lactating dairy cows. Lecithins resulted in the same milk yield and 3.5% FCM production than choline chloride microencapsulated with hydrogenated vegetable oils, even though led to a slightly lower milk fat concentration.

Further investigation is required to determine how different forms of choline could influence choline bioavailability for the different metabolic functions. Moreover, additional extensive feeding experiments are required on animals in early lactation to verify whether lecithins could prevent or alleviate hepatic lipid accumulation.

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