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The effect of dietary rapeseed on milk protein fractions in dairy cows

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Abstract

Significant interest exists on the inclusion of oilseeds in dairy cows' diets to improve the polyunsaturated fatty acid (PUFA) profile of their milk due to PUFA's effects on human health. However, this strategy can affect milk protein fractions, compounds that play an important role in the dairy industry, and also affect consumers' health. The present work thus aimed to investigate the effect of rapeseed in dairy cows' diets on milk protein fractions such as caseins [αs_1-casein (αs_1-CN), αs_2-casein (αs_2-CN), β-casein (β-CN), k-casein (k-CN)], whey proteins [β-lactoglobulin (β-Lg), α-lactalbumin (α-La)], and minor proteins [lactoferrin (LF), bovine serum albumin (BSA), immunoglobulin G (IgG)]. The animals were randomly distributed in two groups, each fed with a control diet and a rapeseed-based diet, respectively. The protein fractions were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Results indicated that the inclusion of rapeseed in dairy cows' diets led to a significant decrease in total milk protein (from 3.73 ± 0.03 to 3.58 ± 0.03%, $p < 0.05$) and total casein content (from 2.96 ± 0.03 to 2.80 ± 0.03%, $p < 0.05$). Additionally, a significant decrease was also observed in the content of k-CN (from 32275 to 25883 A.U., $p = 0.015$), an important protein involved during milk coagulation; and a decrease tendency for β-CN also occurred (from 44746 to 41441 A.U., $p = 0.087$), the high content of which is associated with some human diseases. Dietary rapeseed also decreased the β-Lg content (from 61256 to 52694 A.U., $p = 0.016$), which is positively correlated with cheese quality. Other minor protein fractions such as BSA and IgG were also decreased by the rapeseed diet.

Introduction

Most strategies applied in ruminants' feeding involving oily seeds are directed toward a better fat quality in the farm animals' products (*i.e.,* milk and meat). Rapeseed is one of the feedstuffs used for implementing such strategies; having a beneficial composition of unsaturated fatty acids, including high amounts of oleic, linoleic, and linolenic fatty acids (Harstad and Steinshamn, 2010). Due to this composition, the inclusion of rapeseed in ruminants' diets can positively influence the fatty acid profile of their milk. While numerous studies focus on dietary rapeseed as a source of lipids and its effect on the resulting milk fat quality, much less attention has been directed toward its potential effect on milk protein fractions. In fact, rapeseed is also a proteinaceous feedstuff with a crude protein content of approximately 20% (dry matter basis), and has been previously reported to alter milk protein (Martineau *et al.,* 2013; Butler *et al.,* 2019).

However, the studies on the effect of dietary rapeseed on milk protein fractions is scarce. These fractions may be important, both from a technological point of view, due to their role in dairy product production, and also from the consumer-health point of view, as some of them have bioactive properties. Therefore, the purpose of the present work was to investigate whether the feeding strategy of using rapeseed in dairy cows' diets to increase the content of polyunsaturated fatty acids (PUFA) in their milk has secondary effects on milk protein fractions.

Materials and methods

Experimental design and sampling

The experiment was conducted on a dairy cow farm belonging to the National Research-Development Institute for Animal Biology and Nutrition which was suitable for the feeding trials. A total of 30 dairy cows in mid-lactation (156 DIM) and having a moderate production level (23 kg/day) were randomly distributed in two groups within an experimental design of diet (control *versus* rapeseed-based), breed

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(Holstein Friesian (HF) *versus* Montbeliarde (Mntb)), and repetition (day one *versus* day two) as factors. The experiment lasted for eight weeks. The animals were housed in a free-stalls shelter, fed *ad libitum*, provided with free access to water, and milked twice a day.

The two groups were fed two isonitrogenous and isoenergetic diets. The control diet (C) consisted of corn silage, alfalfa hay, and a compound feed composed of corn grain, wheat grain, wheat bran, sunflower meal, soybean meal, macro-minerals, and a tailored mineral-vitamin premix. The experimental diet (E) was identical, except for the structure of the compound feed: half of the soybean meal and 40% of the corn grains were replaced with rapeseed. Thus, the two diets had similar nutritive supply, while the nature of the energy supply differed; mainly starch in diet C and both starch and fat in diet E.

The milk samples for the analysis of the milk proteins were collected on the same day as the milk samples for primary chemical analyses. Milk was sampled from all the test dairy cows used in the experiment, and transported on ice. The milk samples for the determination of the crude protein and total casein were kept overnight at 4°C; the milk samples for the assessment of protein fractions were defatted by centrifugation at 2640 rpm at 4°C for 20 min, and stored at -20°C until further analysis.

Analysis of milk proteins

Milk crude protein and total casein were determined using a CombiScope FTIR 200 device (Delta Instruments, Drachten, Holland) according to the standard ISO 9622:2013.

Milk protein fractions were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Mini-Protean 3 BioRad system, BioRad, Hercules CA, USA) using 15% polyacrylamide gel. For each sample, 25 µL were diluted 1:1 with 2× Laemmli sample buffer (containing β-mercaptoethanol), and heated at 95°C for 10 min. Samples were then migrated for 2 h at 150 V. After migration, the gels were stained with Coomassie Brilliant Blue G-250 for 90 min at room temperature. Precision Plus Protein Standards Kaleidoscope from BioRad containing proteins at various known molecular weights was used for milk protein identification. The gels were scanned, and the GelQuant software was used for quantitative band analyses, indirectly determining the content of the protein fractions in the milk (Anema, 2009). The relative amounts of milk proteins were expressed as arbitrary units (A.U.).

Statistical analysis

The effect of the diet was tested using ANOVA GLM procedure of the Minitab software, followed by the Tukey's *post-hoc* test (version 16, Minitab® Statistical Software), with diet, breed, and repetition as fixed effects. Statistical significance was set at $p < 0.05$. For p-values between 0.05 and 0.10, the differences were considered as tendencies.

Results and discussions

The present work aimed to determine the differences between milk collected from cows fed with a diet based on traditional ingredients, typical of ruminants' nutrition in Eastern Europe, and those fed with an experimental diet containing rapeseed, as a strategy to enhance milk fat quality. The effects of the experimental diet on milk yield, primary constituents (fat, lactose, *etc.*), and fatty acid profile are reported in a twin article (currently under review). The present article refers to the effects of the experimental diet on milk protein fractions.

Effect on the primary parameters of milk quality

In the present work, the main difference between the control and experimental diets was the energy source of the compound feeds; in the group given the experimental diet, the corn grain and soybean meal were partially replaced with rapeseed. Thus, the starch-to-fat ratio was 94:6 in the control diet, and 77:23 in the experimental diet. The diets were isoenergetic and isonitrogenous. The main effect of this replacement was reported in the twin article; the experimental diet was associated with an improvement on the milk PUFA profile. This result is in line with previously reported studies, although those studies were performed in different feeding conditions (Ryhänen *et al.,* 2005; Stergiadis *et al.,* 2014). However, the side effects of such feeding strategies, such as their influence on the other milk components, are worthy of attention. Some of these effects, such as the content of total protein and fat, can affect milk price and transformation yield (Lerch *et al.,* 2012).

Along with the positive effect on milk fatty acid profile, the experimental diet resulted in a decrease in the protein content (from 3.73 ± 0.03 to 3.58 ± 0.03%, $p < 0.05$) and total casein content (from 2.96 ± 0.03 to 2.80 ± 0.03%, $p < 0.05$) of the milk, as presented in Figure 1. However, since the milk yield also increased, the quantity of protein produced daily by the cows did not change significantly.

Typically, both parameters, milk protein

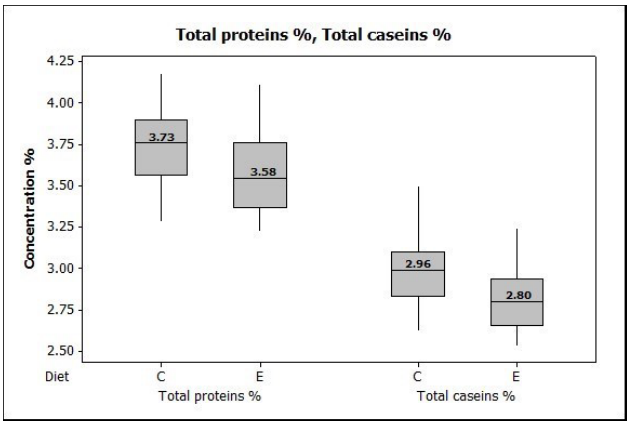

Figure 1. Percentages of total proteins and total caseins in control (C) and experimental (E) groups.

content (%) and output (g/day), are assessed in experiments that study the effect of diet on milk composition. However, in the present work, further analysis of the effect of rapeseed inclusion on milk protein fractions was done by focusing on the milk protein content, which is the relevant parameter in terms of both consumer health and manufacturing considerations.

Effect on the distribution of milk protein fractions

SDS-PAGE electrophoresis is an inexpensive and rapid means of separating individual protein fractions from a mixture of proteins. In the present work, this method was used to quantify the following protein fractions: αs_1-casein (αs_1-CN), αs_2-casein (αs_2-CN), β-casein (β-CN), k-casein (k-CN), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), lactoferrin (LF), bovine serum albumin (BSA), and immunoglobulins (Ig). Cumulatively, these protein fractions accounts for 97% of the total milk proteins (Dupont *et al.,* 2013).

The electrophoretic pattern of the milk samples indicated the presence of the four main caseins namely αs_1-CN, αs_2-CN, β-CN, k-CN, with bands that appeared approximately between 20 and 25 kDa (Farrell *et al.,* 2004); γ-casein fraction could not be identified; also the bands of the two main whey proteins, β-Lg and α- La appeared at approximately 14 and 18 kDa (Farrell *et al.,* 2004; Anema, 2009). In addition, three other milk proteins, LF, BSA, and IgG were determined; their corresponding bands appeared between 50 and 100 kDa (Franco *et al.,* 2010; Costa *et al.,* 2014; Morgan *et al.,* 2016). Figure 2 presents a representative selection of the electrophoretic pattern of the milk protein fractions. The effect of the experimental diet on the milk protein fractions, assessed through densitometry analysis of the milk samples, is presented in Table 1. The content of k-CN was

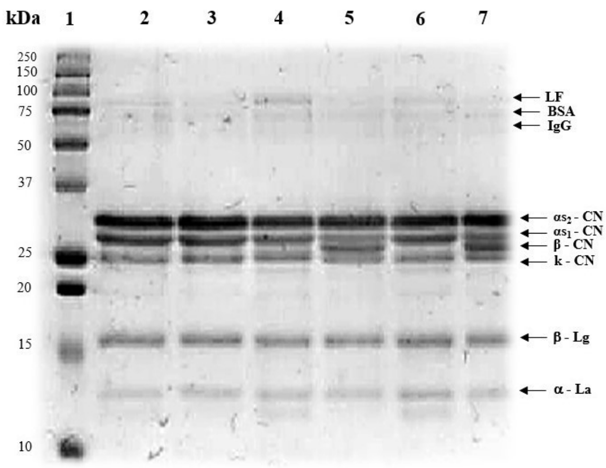

Figure 2. Electrophoretic pattern of milk proteins in milk samples.

significantly lower in the experimental group (p = 0.015). This decrease may affect cheese production, as k-CN has an important role in curd firming and milk coagulation processes, helping to form and stabilise globular structures (casein micelles) at the surface. Coagulation begins when k-CN is hydrolysed; various studies have demonstrated that an optimal proportion of k-CN can shorten coagulation time and increase curd firming. Additionally, a decrease tendency for β-CN was observed (p = 0.087), a decrease which may not affect milk coagulation (Amalfitano *et al.,* 2019) but may be a risk factor for human health conditions such as heart disease, type 1 diabetes, and atherosclerosis (Kamiński *et al.,* 2007). In contrast, the content of α s-caseins (αs_2-CN and αs_1-CN), the most abundant casein fractions, did not significantly change (Veloso *et al.,* 2002). Aside from having properties similar to k-CN in relation to coagulation time and curd firming, αs_1-CN may encourage the retention of a larger quantity of nutrient in the curd, while the coagulation time and curd firming processes earlier described can be delayed by αs_2-CN (Cipolat-Gotet *et al.,* 2018; Amalfitano *et al.,* 2019).

As shown in Table 1, the rapeseed diet also affected the content of β-Lg, which significantly decreased (p = 0.016), but did not alter the content of α-La (p = 0.527). Previous studies have reported that these whey proteins have a minor role in coagulation when compared with casein fractions; however, they may influence cheese-making as they are strongly negatively correlated with protein recoveries in curd (Cipolat-Gotet *et al.,* 2018; Amalfitano *et al.,* 2019).

Another consideration is the fact that casein and whey protein are the major compounds involved in the development of cow-milk allergy, a major health problem for some consumers (Boettcher and

Table 1. Densitometry analysis of the Holstein and Montbeliarde cow milk proteins during the treatment periods.

Parameter	C (A.U.)	E (A.U.)	HF (A.U.)	Mntb (A.U.)	SEM diet	SEM breed	*p* diet	*p* breed
$\alpha_{S2\text{-CN}}$	105410[a]	105653[a]	106558[a]	104505[a]	2616	2611.5	0.948	0.580
$\alpha_{S1\text{-CN}}$	48420[a]	51736[a]	55838[a]	44318[b]	1810.5	1805	0.198	0.000
β-CN	44746[a]	41441[a]	44551[a]	41636[a]	1351	1346.5	0.087	0.130
k-CN	32275[a]	25883[b]	28396[a]	29762[a]	1816	1814	0.015	0.596
β-Lg	61256[a]	52694[b]	56531[a]	57419[a]	2459.5	2450	0.016	0.799
α-La	34875[a]	32948[a]	35820[a]	32003[a]	2140	2128	0.527	0.210
LF	23483[a]	23352[a]	23893[a]	22942[a]	1101	1098	0.933	0.543
BSA	19114[a]	16934[b]	18858[a]	17190[a]	751	736.8	0.039	0.113
IgG	21554[a]	17997[b]	19995[a]	19556[a]	1008.1	1007	0.014	0.759

Means within a row with different lowercase superscripts are significantly different ($p < 0.05$). C: control diet group; E: rapeseed diet group; HF: Holstein Friesian breed; Mntb: Montbeliarde breed, αs_1-CN: α s_1-casein; αs_2-CN: αs_2-casein; β-CN: β-casein; k-CN: k-casein; β-Lg: β-lactoglobulin; α-La: α-lactalbumin; LF: lactoferrin; BSA: bovine serum albumin; and IgG: immunoglobulin G.

Crowe, 2013; Mousan and Kamat, 2016). In this context, it is important and noteworthy that the experimental diet did not induce an increase of these fractions.

The effect of the rapeseed diet on minor whey protein fractions such as BSA, IgG, and LF was also assessed, as these components may have implications on consumer health. The content of BSA significantly decreased in the experimental group, from 19114 to 16934 A.U. ($p = 0.039$; Table 1). This has practical importance, as various studies have reported that BSA is a potential triggering factor for the onset of autoimmune disease, leading to human insulin-dependent diabetes (Persaud and Barranco-Mendoza, 2004; Morgan *et al.,* 2016). It is also an allergenic component of milk that can induce symptoms such as urticaria, cough, lipoedema, and rhinitis (Restani *et al.,* 2004).

The content of IgG was 16.5% lower in the rapeseed diet as compared to the control diet ($p = 0.014$; Table 2). This may have negative practical implications, as IgG contributes to an effective immune system in consumers (Hurley and Theil, 2011; Geer and Barbano, 2014).

In contrast, the rapeseed diet did not significantly modify the LF content (Table 1); this is important due to the involvement of this protein fraction in the defence system. Moreover, LF has antibacterial effects against a wide range of bacteria; it can hydrolyse and generate peptides with antibacterial activities such as lactoferricin and lactoferrampin (Dupont *et al.,* 2006; Franco *et al.,* 2010). Other studies have reported other important biological properties of LF, such as anti-inflammatory

Table 2. Casein fraction profile (% of total casein).

Parameter	C	E	HF	Mntb	SEM diet	SEM breed	*p* diet	*p* breed
Total casein (A.U.)	225561[a]	226917[a]	229872[a]	222606[a]	4510	4509.5	0.832	0.257
$\alpha_{S2\text{-CN}}$ (%)	45.66[a]	47.09[a]	45.19[b]	47.56[a]	0.718	0.717	0.165	0.022
$\alpha_{S1\text{-CN}}$ (%)	20.47[b]	22.38[a]	23.61[a]	19.45[b]	0.634	0.633	0.360	0.000
β-CN (%)	18.73[a]	18.30[a]	18.77[a]	18.26[a]	0.501	0.501	0.540	0.471
k-CN (%)	14.03[a]	11.63[b]	12.30[a]	13.37[a]	0.751	0.750	0.027	0.317

Means within a row with different lowercase superscripts are significantly different ($p < 0.05$). C: control diet; E: rapeseed diet; HF: Holstein Friesian breed; Mntb: Montbeliarde breed; αs_1-CN: α s_1-casein; αs_2-CN: αs_2-casein; β-CN: β-casein; and k-CN: k-casein and antitumor (Duarte *et al.,* 2011; Niaz *et al.,* 2019). Moreover, some medical approaches are based on the isolation of this milk protein fraction and its use as a dietary supplement or as a treatment for patients with saliva deficiency (Franco *et al.,* 2010). As shown in Table 1, there were no significant differences in the milk from the two different breeds, except for the αs_1-CN level, which was higher in the product of the HF breed (55838 A.U.) than in that of the Mntb breed (44318 A.U.; p = 0.000).

Over time, a few studies focusing on the effects of the rapeseed inclusion in ruminants' diets on their milk content reported contradictory results. Some studies reported that short-term supplementation with rapeseed did not influence the total casein and whey protein contents (Šustala *et al.,* 2003). In contrast, Emanuelson *et al.* (1993) observed after long-term feeding of rapeseed, the concentration of total milk protein was significantly higher (Emanuelson *et al.,* 1993). Other studies even reported a decrease in milk casein content by increasing canola oil supplementation (Jenkins and Jenny, 1992).

The effect of rapeseed can be attributed to several properties; the content of plants' secondary metabolites, specific profile of fatty acids, and overall increase of dietary fats. Supplementation with conjugated linoleic acid had no effect on the contents of total casein and whey protein (Perfield *et al.,* 2002). On the other hand, duodenal infusion with a high amount of linolenic acid (400 g/day) changed the composition of milk protein fractions, increasing β-CN, αs_1-CN, and albumin (Yang *et al.,* 2013). After infusion of either *trans-* or *cis-*fatty acids, the total contents of casein and whey protein decreased. The casein fractions were not affected by the treatment, with the exception of γ_2-casein, which increased in correlation with β-casein proteolysis. Other milk protein fractions such as serum albumin, lactoferrin, immunoglobulins, and whey proteins were not influenced by the experimental diet (Romo *et al.,* 2000).

The effect of dietary fatty acids on milk protein was observed not only for rapeseed but also for other oilseeds. Studies have shown that whole cottonseeds resulted in a decrease in the content of total casein (DePeters *et al.,* 1985), and the same trend has been reported after supplementing dairy cows' diets with various amounts of flaxseed (Santillo *et al.,* 2016). Given the fact that caseins are the most abundant protein fractions in milk, a decrease in total milk protein after supplementation of dairy cows' diets with a fat source is mainly attributable to a decrease in casein content. This may be explained by the dilution effect, which appears after increasing milk yields by using fat as an energy source (Schroeder *et al.,* 2004). Conversely, infusion with a high amount of fatty acids can increase proteolysis in milk, resulting in a higher content of proteins with lower molecular weight, such as casein fractions (Yang *et al.,* 2013).

Effects on casein fraction profile

The influence of diet and breed on casein fractions' relative proportions was also assessed, as these relative proportions may be relevant for producing favourable characteristics for processing the milk into dairy products (Wedholm *et al.,* 2006). The analysis of the casein profile (relative proportion of each fraction: total caseins) revealed that αs_2-CN composed the highest proportion in both groups, followed by αs_1- N, β-CN, and k-CN. The results indicated that the relative proportion of k-CN was significantly decreased by the rapeseed diet (p = 0.027); however, the effect observed in the case of β-CN was not significant (p = 0.540; Table 2). The α-caseins' relative proportions were also not significantly changed by the rapeseed diet.

A breed effect was observed with the αs_2-CN content; the relative proportion was higher (p = 0.022) in the milk of the Mntb breed (47.56 ± 0.762%) in comparison to the HF breed (45.19 ± 0.672%). In contrast, the percentage of αs_1-CN (p = 0.000) was higher in the milk produced by the HF

Table 3. Whey fraction profile (% of total whey).

Parameter	C	E	HF	Mntb	SEM diet	SEM breed	*p* diet	*p* breed
Total whey protein (A.U.)	95431[a]	88230[a]	95548[a]	88113[a]	2884	2863	0.080	0.071
β-Lg (%)	66.51[a]	64.98[a]	63.63[a]	67.87[a]	1.621	1.613	0.507	0.068
α- La (%)	33.49[a]	35.02[a]	36.37[a]	32.13[a]	1.621	1.613	0.507	0.068

Means within a row with different lowercase superscripts are significantly different (p < 0.05). C: control diet; E: rapeseed diet; HF: Holstein Friesian breed; Mntb: Montbeliarde breed; β-Lg: β-lactoglobulin; and α-La: α-lactalbumin.

breed (23.41 ± 0.596%) as compared to that of the Mntb breed (19.45 ± 0.670%). For the other two caseins, β-CN and k-CN, no differences between the two breeds were detected. Previous studies states that breed can influence the profile of milk protein fractions (Gustavsson *et al.,* 2014). However, there is a paucity of information specific to the breeds used in the present work; in the case of Mntb and HF, the data are controversial at best (Malossini *et al.,* 1996; Sanchez *et al.,* 2016). More data are therefore necessary to clearly assess the influence of breed.

Effect on whey protein fraction distribution

The two main whey proteins were analysed and expressed as a percentage of total whey protein, and the results revealed that β-Lg was proportionally the highest, at the expense of α-La (Table 3), which is consistent with the study (Bonfatti *et al.,* 2008). Although β-Lg, as a proportion of total milk protein, was significantly influenced by the experimental diet, its relative proportion in total whey proteins was not affected (p = 0.507). The diet also had no significant effect on the relative proportion of α-La, consistent with the result obtained for α-La content in the milk proteins. The HF breed tended to produce milk with a lower relative proportion of β-Lg (67.87 *versus* 63.63%) and a higher relative proportion of α-La (32.13 *versus* 36.37%).

Conclusion

The inclusion of rapeseed in dairy cows' diets as a strategy to enhance their milk's PUFA profile also influenced milk protein fractions. The rapeseed diet induced a significant decrease in k-CN content, with negative effects in cheese-making. Conversely, the decrease of the β-Lg content may be beneficial, as it is negatively correlated with protein recovery in curd. The inclusion of rapeseed also influenced some of the protein fractions known for their potential to influence consumers' health. The BSA content decreased, a beneficial result as milk BSA is known to be associated with diabetes incidence. On the other hand, IgG also decreased, which may decrease the milk's potential benefit to the defence system of consumers. The diet had no significant effects on the other protein fractions (α-CN, β-CN, α-La, and LF). The effect of breed on the milk protein fractions was statistically insignificant, with the exception of αs-CN. When proportions of casein fractions (relative to total casein) and whey fractions (relative to total whey) were considered, statistical significance was detected in both cases of α-CN proteins. These results are not yet conclusive as the data available on the comparison between HF and Mntb are too scarce.

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