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Impact of herbage proportion, animal breed, lactation stage and season on the fatty acid and protein composition of milk

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ABSTRACT

Impact of herbage proportion, breed, average days in lactation (ADiL), and season on bulk milk composition of 12 dairy farms were investigated over a year using a mixed effect model approach. A higher proportion of herbage led to higher contents of omega 3 (n-3) fatty acids (FAs), conjugated linoleic acids (CLA), vaccenic acid, and branched chain FAs (+0.08, +0.08, +0.19, +0.05 g 100 g^{-1} fat, respectively). Breed influenced n-3 FAs and CLA (+0.31, +0.17 g 100 g^{-1} fat, respectively), κ-casein, lipoprotein lipase, β-lactoglobulin, total whey protein, fatty acid synthase, and glycoprotein-2, resulting in +0.96, –0.019, –0.83, –1.12, +0.009, and +0.01 g 100 g^{-1} measured proteins, respectively, in Brown Swiss milk. ADiL influenced lactoferrin and crude protein; season affected all FAs and proteins examined. The identification of these quantitative relationships allow predictions that provide new insights for the production, processing and commercialisation of grassland-based dairy products.

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1. Introduction

In grassland dominated regions fresh or conserved herbage provides an important feed resource for ruminants (Lüscher et al., 2019). Swiss dairy farms traditionally have a high proportion of herbage in the diet of cows – 85% for dairy farms and 62% for mixed farms with milk production and arable farming (Schmid & Lanz, 2013). Utilisation of herbage for dairy production has a number of important advantages, as this promotes the utilisation of locally available resources (Winkler, Cutullic, & Aeby, 2012), contributes to animal welfare (Peyraud, Delaby, Delagarde, & Pavie, 2014), reduces feed-food competition (Ertl, Klocker, Hörtenhuber, Knaus, & Zollitsch, 2015) and can improve the nutritional quality of the products (Bisig et al., 2008).

Concomitant with increasing consumer demands for more sustainable and animal friendly food, in several countries, including Switzerland, commercial milk produced with a higher-than-average proportion of herbage is therefore marketed successfully. However, so far, studies on the impact of herbage on milk composition of different cow breeds, particularly comprehensive analysis that includes the influence of the breed, lactation stage or season, as well as the quantification of minor proteins, have been lacking. Over the last five decades, various studies have been published on the influence of cows' diet on fatty acids (FAs) in milk fat. Increased proportions of herbage in the diet reduce the amount of saturated FAs (SFAs) in milk fat (Bisig et al., 2008; Collomb et al., 2008a; Couvreur, Hurtaud, Lopez, Delaby, & Peyraud, 2006; Ferlay et al., 2008; Leiber, Kreuzer, Wettstein, & Scheeder, 2005). In the past, SFAs of dairy origin were generally associated with cardiovascular disease (CVD) in humans. However, more recent scientific evidence indicates that the consumption of regular fat dairy products does not increase the risk of CVD and that it is inversely correlated with weight gain and the risk of obesity (Astrup et al., 2016; Drouin-Chartier et al., 2016). The levels of polyunsaturated FAs (PUFAs) in milk fat increase with an increased proportion of herbage (Bisig et al., 2008; Collomb et al., 2008a; Couvreur et al., 2006; Leiber et al., 2005; O'Callaghan et al., 2016). This is favourable as the two FAs linoleic acid (LA, C18:2 n-6) and α-linoleic acid (ALA, C18:3 n-3), that are essential from a nutritional point of view, as they cannot be synthesised by humans, are part of this group, along with other favourable FAs such as conjugated linoleic acids (CLAs; Fuke & Nornberg, 2017) and long-chain PUFAs (Sanders, 2009). Moreover, the content of branched chain FAs (BCFAs) also increases with increasing proportions of herbage (Collomb et al., 2008a, Couvreur et al., 2006, Hurtaud, Agabriel, Dutreuil, & Rouille, 2010). This is especially interesting, as it has been reported that BCFAs introduce apoptosis of human breast cancer cells, inhibit tumour growth in a mouse model and in cultured cells, and may be important for the development and maintenance of the human microbiota (Ran-Ressler, Bae, Lawrence, Wang, & Brenna, 2014). Furthermore, they also increase pancreatic β-cell function (Bainbridge, Cersosimo, Wright, & Kraft, 2016).

In contrast to the numerous studies on the influence of feed on milk fat composition, surveys examining the correlation between the composition of cows' diet and the content of individual milk proteins are sparse. One possible reason for this might be either the fact that a straightforward method for absolute quantification of the most abundant milk proteins was missing or that laboratory tests are time-consuming and, therefore, past studies focused only on a few major milk proteins (Brodziak, Barłowska, Król, & Litwińczuk, 2012; Gellrich, Meyer, & Wiedemann, 2014; Heck, van Valenberg, Dijkstra, & van Hooijdonk, 2009; Król, Litwińczuk, Brodziak, & Sawicka-Zugaj, 2010; Schwendel et al., 2017). Another reason for the scarce studies on detailed milk protein composition might be that the protein content in milk depends on numerous aspects such as breed, feed, milking technique, lactation, lactation stage, and season (Kukovics & Németh, 2013), to name the most important. Therefore, the interpretation of results requires an extensive data framework (Schwendel et al., 2015; Stergiadis et al., 2015). Moreover, the content of individual milk proteins appears less influenced by the feed (Schwendel et al., 2012; Walker, Dunshea, & Doyle, 2004). Nevertheless, Brodziak et al. (2012) and Król et al. (2010) found higher absolute contents of the bioactive whey proteins lactoferrin, α-lactalbumin, and β-lactoglobulin in the milk of cows that received higher proportions of grass in their diet than in the milk of cows kept in a total mixed ration (TMR) system. Furthermore, Brodziak et al. (2012) found a higher content of bovine serum albumin (BSA) and lysozyme in the milk of those grass-fed cows.

In the present study, we investigated the influence of herbage proportion, cattle breed, lactation stage, and season on the composition of the bulk milk produced on 12 farms during one year by determining over 70 FAs and 19 key milk proteins, resulting in 119 FAs, FA groups, proteins, and protein groups. For protein analysis, a newly developed multiple reaction monitoring (MRM) method was used, thereby enabling the simultaneous quantification of not only the main caseins and whey proteins but also 9 minor proteins associated with the milk fat globule membrane (Bär et al., 2019). Further, the influence of the statistically significant contributing factors on the 119 measured parameters was quantified with mixed effect models. As a result, it is possible for the first time to make quantitative statements on the relationship between herbage content, breed, lactation stage and season on the detailed milk composition.

2. Methods

2.1. *Selection of farms, sampling, and feed ration surveys*

Twelve dairy farms, located in the central lowland of Switzerland, were selected for contrasting proportions of fresh and conserved herbage in feed rations (Table 1) and different cattle breeds (Table 2). The farms were not subject to seasonal calving and the calving time was not synchronised (Table 2). Feed rations were determined on the basis of a combined approach of an on-farm survey counterchecked with an energy/protein balance, taking into account the energy and protein requirements of the herd depending on the production level, feed intake, energy, and protein requirements of the herds were calculated for each month and farm, based on Jans, Kessler, Münger, Schori, and Schlegel (2016), with additional data available from breeding associations and the national animal traffic database (i.e., lactation stage, parity, and conformation traits). The calculated ration composition was checked for plausibility with the purchased and on-farm produced feed during a farm visit. The available energy and protein for the on-farm produced feed was estimated, using standard values according to the Swiss Feed Database (Agroscope, 2018).

Most of the on-farm produced meadow fodder came from natural meadows consisting of grasses, legumes and herbs. The artificial meadows were mainly composed of perennial ryegrass, annual ryegrass, meadow fescue, timothy, buttercup, red clover and white clover. In the case of purchased feed, the manufacturer's declaration was used. All farms were members of a breeding association, thereby ensuring that the milk quantity and content of each cow was measured monthly over a year. Herbage was defined as fresh herbage (pasture and fresh herbage indoor feeding), hay and silage. On an annual average, the diet of lactating dairy cows on the 12 farms contained 67% herbage, with a considerable variation between the individual farms (Table 1). The annual average corresponds well to values found by Ineichen, Sutter, and Reidy (2016) and Schmid and Lanz (2013) for lowland farms. Average milk yield was 25.27 kg day^{-1} cow^{-1}. For classification according to breed, at least 95% of the herd had to belong to this breed. Consequently, 9 farms were assigned to the breed group RED and 3 farms to the breed group BS. The composition of the individual herds is specified in Table 2.

2.2. Collection of samples

For milk analysis, 2000 mL bulk milk samples were collected once a month and on the same day after morning milking and thorough mixing from the milk tank of the individual farms. Samples were transported refrigerated to the laboratory for FA and protein analysis within 48 h. Out of 144 samples, 5 samples had to be excluded from the evaluation, resulting in 139 samples.

2.3. Fatty acid analysis

Milk samples were prepared according to Collomb et al. (2008a). High-resolution gas chromatography combined with flame ionisation detection and 54 reference FAs (Collomb & Bühler, 2000) were used to determine the FA composition. Fourteen CLA isomers were determined by silver-ion high-performance liquid chromatography (HPLC) combined with a photodiode array detector, three reference CLA isomers and synthesised CLA isomers (Collomb, Sieber, & Bütikofer, 2004). The most abundant six isomers were evaluated. The total fat content was determined in accordance with Roese-Gottlieb, ISO 1211:2010 /IDF 1 (ISO, 2010).

2.4. Protein analysis

Milk samples were processed and analysed using MRM in accordance with Bär et al. (2019). The samples were analysed at different time intervals after the sampling was completed. When comparing the results, we identified a temporal bias in our data, leading to an overall decrease of the sum of all analysed proteins, which might be due to storage time before measurement. Therefore, to eliminate this temporal bias, we restricted our analysis of the 19 measured proteins to relative values only, i.e., for each farm the monthly values obtained for the 19 proteins were summed up and set to 100%, and the values for the individual proteins were given in g per 100 g measured proteins (g 100

g^{-1}). The crude protein content of milk samples was calculated by multiplying the total nitrogen content, determined in accordance with Kjeldahl, ISO 8968-3:2007/IDF 20-3 (ISO, 2007), by a conversion factor of 6.38.

2.5. Statistical methods

2.5.1. Basic model

A linear mixed effects model was fitted to several variables, as described below. As fixed effects, herbage, breed, average days in lactation (ADiL), and the month (as a categorical variable) were used, a random intercept was used to model farm effects. To account for residual temporal autocorrelation (within each farm, residuals closer in time may be more highly correlated), a first-order autoregressive AR(1) correlation structure was used. The need for temporal autocorrelation was assessed by calculating a confidence interval for the autocorrelation parameter. The significance of the fixed effects and the categorical variables was assessed with marginal F-tests. Model diagnostics revealed problems with normality for certain models, which were mostly caused by outliers. Cook's D was used to identify influential data points for each model and the results with and without these points were compared. All statistical analyses were performed using R 3.5.3 (R Core Team, 2019) and RStudio 1.2.1335 (RStudio Team, 2018) using the packages nlme 3.1-137 (Pinheiro et al. 2018) and car 3.0-3 (Fox & Weisberg, 2019).

2.5.2. Variable selection approach

As a first step, the model described above was applied to 7 FA and protein variables which had been selected a priori by research interest: n-3s, CLA, BCFAs, total casein, κ-casein, lactoferrin, total whey protein. In the second step, the model was applied to all the 119 FA and protein variables to screen for further interesting results. Results were pre-selected according to their *p*-values (for herbage, breed, ADiL variables) that were adjusted for multiple testing by employing the Bonferroni-Holm adjustment. The variables with an adjusted *p*-value below 0.01 were further analysed. Consequently, the following additional variables were analysed (in total 31): lauric acid C12; methyl-dodecanoic acid C13 iso; myristic acid C14; myristoleic acid C14:1 cis; methyl-tetradecanoic acid C15 iso; palmitic acid C16:0; trans-hexadecenoic acid C16:1 trans; methyl-hexadecanoic acid C17 iso;

stearic acid C18; VA C18:1 trans-11; ratio C18:1 trans-10/trans-11, C18:1 cis-9; octadecenoic acid C18:1 cis-12; rumenic acid C18:2 cis-9,trans-11; octadecadienoic acid C18:2 cis-9,trans-12 and cis,cis methylene interrupted diene (MID) and trans-8,cis-13; C18:2 trans-7,cis-9; C18:2 trans-11,cis-13; C18:2 trans-11,cis-13 and cis-9,cis-11; α-linolenic acid C18:3 cis-9,cis-12,cis-15; trans-total with CLA trans; total C18:2 trans,trans non methylene interrupted diene (NMID); eicosatrienoic acid C20:3 (n-3); eicosapentaenoic acid C20:5 EPA (n-3); SFA; PUFA; α_{S1}-casein; β-casein; lipoprotein lipase; crude protein; β-lactoglobulin; FAS and GP2.

3. Results and discussion

3.1. *Fat content and fatty acids*

3.1.1. *Fat content*

The monthly fat content of the milk from the farm with the lowest proportion of herbage (32.5%) was found to be consistently higher in comparison with the milk of the farm with the highest annual proportion of herbage (99.5%) — with the exception of October, in which they contained equal amounts (Supplementary material Table S24). However, comparing the average fat content of the milk of the 12 farms, this is attributable to an individual farm effect, which could be explained, for example, by the different genetics of the animals. During the warm season, the total fat content decreased in the milk of all farms. In the bulk milk from the farms with the highest proportion of herbage, the fat content had a higher variability and was lowest in June (30.1 g kg^{-1} milk) and highest in April (39.9 g kg^{-1} milk). Milk of other farms with comparable diets showed a similar behaviour in relation to the variations. Herd management with a high proportion of herbage in the diet is more complex, as nutrient content varied depending on weather conditions and the development stage of grass and herbs. Especially young grass with little fibre can lead to milk fat depression, which could explain these variations in total fat content (Rivero & Anrique, 2015).

3.1.2. *Fatty acids*

3.1.2.1. Seasonal variation of fatty acids

The content of several FAs in milk fat varied significantly over a year (Table 3). With a high proportion of herbage (99.5%), the n-3 FAs content steadily increased during the summer season, with fresh grass and herbs being part of the feed from May to October (1.16–2.12 g 100 g^{-1} fat; Fig. 1A), confirming the results observed by Collomb et al. (2008b). A similar seasonal increase was also observed for CLA (Fig. 1 B; Supplementary material Table S24). The sum of BCFAs in the milk produced with a high proportion of herbage increased earlier in the year and reached maxima of 2.37 and 2.08 g 100 g^{-1} fat in April and October, respectively, and a minimum of 1.55 g 100 g^{-1} fat in February (Supplementary material Table S24). With a low proportion of herbage (32.5%), the contents of n-3 FAs varied less at a lower level — in a range from 0.59 g to 1.03 g 100 g^{-1} fat. A similar behaviour was observed for CLA (Fig. 1B; Supplementary material Table S24). For BCFAs, also with a low proportion of herbage, the content increased from 1.45 to 1.98 g 100 g^{-1} fat early in the year, from February to April (Supplementary material Table S24).

3.1.2.2. Influence of herbage proportion on fatty acids

A significant influence of the proportion of herbage in the cow`s diet was found for 20 FAs. With an increase of the herbage proportion from 32.5% to 99.5% the content of n-3 FAs, CLA, and BCFAs in the milk increased significantly ($p < 0.001$; Fig. 2 A and B; Table 3). For a 10%-points higher proportion of herbage in the cows' diet the model yielded an increase of n-3 FAs, CLA, and BCFAs by 0.082, 0.084, and 0.053 g 100 g^{-1} fat, respectively (Table 4). The contents of n-3 FAs and CLA ranged from 0.46–2.12 g 100 g^{-1} and 0.28–2.09 g 100 g^{-1} fat (Table 3 and Supplementary material Table S1), reaching the highest content with proportions of herbage up to 99.5%, similar to Collomb et al. (2008b), Dhiman, Anand, Satter, and Pariza, (1999), and Hofstetter, Frey, Gazzarin, Wyss, and Kunz (2014). Similar to the results of Collomb et al., (2008b), CLA isomer C18:2 trans-11,cis-13 was the second most abundant isomer (Table 3 and Supplementary material Table S1), but different to their results it did not increase in content with a higher herbage proportion (Table 4). The content of BCFAs ranged from 1.22 to 2.37 g 100 g^{-1} fat (Table 3 and Supplementary material Table S1), which is in the range found by Collomb et al. (2008b) and Gomez-Cortes, Juarez, and de la Fuente (2018). Milk fat contains BCFAs that mainly originate from membranes of ruminal bacteria (Vlaeminck, Fievez, Cabrita, Fonseca & Dewhurst, 2006) and has been reported to play an essential role in the human gut, especially in the intestine of a new-born baby, where they support the colonisation of specific microorganisms in the gut microbiome (Ran-Ressler, Devapatla, Lawrence, &

Brenna, 2008). In addition, BCFAs may have other beneficial functions related to CVD, cancer, obesity, and inflammation (Gomez-Cortes et al., 2018).

Conversely, Lauric acid (C12:0) and ∑SFAs decreased with increasing proportion of herbage (Table 4). Moreover, there was a decrease in a minor monounsaturated FA (C18:1 cis-12), as well as for C18:2 cis-9,trans-12 and cis,cis MID and trans-8,cis-13 (Table 4). The increasing individual FAs were VA (C18:1 trans-11), rumenic acid (C18:2 cis-9,trans-11), ALA C18:3 cis-9,cis-12,cis-15 (n-3), the long chain n-3 FA EPA (C20:5), a few minor trans-FAs, and FAs belonging to BCFAs and CLAs. Since the isomerisation and hydrogenation of the α-linolenic acid contained in herbage to VA in the rumen occurs very quickly, VA accumulates in the blood and milk. Therefore, VA increased the most, 10%-points more herbage led to 0.189 g 100 g^{-1} fat more VA (Table 4). Various positive bioactivities in humans, such as anti-inflammatory and anti-carcinogenic effects, have been described for VA (Gomez-Cortes et al., 2018). A large increase was also found in the total PUFA (Table 4).

3.1.2.3. Influence of breed and average days in lactation on the composition of milk fat

Significant effects of the two breed groups RED and BS on the content of milk FAs were found. The contents of n-3 FAs and total CLA were 0.306 and 0.174 g 100 g^{-1} fat higher respectively in milk from BS (Fig. 3A,B; Table 4), while the total BCFAs were not significantly different. In contrast to our results, Bainbridge et al. (2016) found no significant difference in total n-3 FAs and total CLA content in milk of Holstein, Jersey, and crossbreeds of the two, but a higher content of BCFAs in the milk of Jersey cows. The difference in those findings could be explained by the different breeds studied.

In the presented study, milk of the two breed groups showed also a significant difference in the content of the CLA isomer C18:2 trans-11,cis-13 and a minor monounsaturated FA, C14:1 cis, which were 0.052 g 100 g^{-1} fat and 0.100 g 100 g^{-1} fat higher in milk of BS (Table 4). With CLA 18:2 trans-11,cis13, also the sum of this isomer and C18:2 cis-9,trans-11 was higher in BS milk. C14:1 cis-9 in milk is to 90% a product of Δ9-desaturase activity (Shingfield, Bonnet, & Scollan, 2013). In contrast to our results Soyeurt, Dehareng, Mayeres, Bertozzi, and Gengler (2006) found lower Δ9-desaturase activity for BS and Jersey cows compared with Holstein. However, as in this study, they also observed a slight increase of C14:1 over the lactation period. Per ADiL, the content of this FA in our study increased by 0.001 g 100 g^{-1} fat, the content of the CLA isomer C18:2 trans-7,cis-9 increased by 0.00031 g 100 g^{-1} fat and that of stearic acid C18:0 decreased by 0.007 g 100 g^{-1} fat (Table 4). Contrary to our findings, Stoop, Bovenhuis, Heck, and van Arendonk (2009) observed a minimum in mid lactation for unsaturated FAs and a decrease from days 80 to 150 for C18:0 remaining stable afterwards, whereas Samková et al. (2018) found a minimum for C18:0 in mid lactation. The differently described course of the C18:0 content in milk over lactation could be explained by the fact that the results of the two studies are based on different breeds (Friesian Holstein and Czech Fleckvieh, respectively).

3.2. Protein

3.2.1. Crude protein content

The crude protein content in the bulk milk from 12 farms with different ratios of herbage varied between 29.10 and 38.15 g kg^{-1} milk and followed a seasonal pattern (Supplementary material Table S24; Fig. 1C), decreasing from winter to summer, with the lowest concentration from June to September and the highest concentration from October until March. The urea concentrations were lower in winter and higher in summer (Supplementary material Table S24). These results confirm the findings of Křížová et al. (2014), who observed a decrease in the content of fat, crude protein, true protein, casein, and whey protein in the milk of all investigated Czech Fleckvieh and Holstein herds in summer and a simultaneous increase of non-protein-nitrogen (NPN). The breed influenced the fat, crude protein, true protein, casein and NPN content, in favour for the milk of Czech Fleckvieh. Concerning the type of feeding, the fat content was higher and the concentrations of lactose, urea and all protein fractions were lower in the milk of the grazing herds compared with the non-grazing herds. Similar results were found by Ozrenk and Selcuk Inci (2008). However, in this study, no significant effect of the proportion of herbage in the fodder on the crude protein content was found (Fig. 2C; Table 5). Moreover, no influence of the breed was found in this regard (Fig. 3C; Table 5). In contrast, the ADiL significantly increased the average crude protein content by 0.014 g kg^{-1} per day after calving (Fig. 4C; Table 5). In good agreement with our findings, Auldist, Napper, and Kolver (2000) observed that the seasonal variation of total protein content showed no significant difference in the milk from pasture-fed cows and those fed with TMR.

3.2.2. Casein

Caseins are the major proteins in milk and function as important protein sources. They are relevant for calcium transport and absorption (Holt, 2011) and serve as a source for a variety of bioactive peptides that may influence human health in many different ways (Mohanty, Mohapatra, Misra, & Sahu, 2016). Caseins determine the yield in cheese manufacturing, as only the casein is coagulated by renneting. Hence, it is not surprising that the total casein content in the bulk milk from the 12 farms followed a similar pattern as the crude protein content (Supplementary material Table S24). However, the total casein content showed a stronger decrease from April until November and December, respectively. Bernabucci et al. (2015) stated that the reduction of total casein concentration is primarily responsible for the reduction in milk protein in summer, which is in agreement with our findings. Apart from the season, no significant influence for the variables examined (herbage, breed, ADiL) on the total casein content was found. Although the effect of lactation stage for the full data set was significant, the elimination of three outliers resulted in a non-significant effect. Therefore, no clear-cut conclusion could be drawn with regard to the influence of the lactation stage.

Examining the seasonal trend of the values, Gellrich et al. (2014) observed a comparable pattern in the milk from Holstein-Friesian cows. Similar to the current study, the concentration of α- and κ-casein was found in tendency to be lower in summer. However, in contrast to our results, the content of β-casein in the milk was found to be higher during summer.

The results presented here are also in good agreement with those of Bernabucci et al. (2015), who were able to link the change in the coagulating properties of milk during the warm season to the lower content of α-casein and β-casein. At the same time, they observed an increase in the content of unidentified proteins. The observation that the rennet coagulation time and curd firmness are dependent on the relative concentration in total casein of α_{S1}-casein, β-casein, respectively, of κ-casein was previously described by Joudu, Henno, Kaart, Püssa, and Kärt (2008). Bonfatti et al. (2011) found that the concentration of κ-casein is decisive for rennet coagulation time. However, κ-casein is not only important for stabilising the casein micelle and, therefore, for rennet coagulation during cheese manufacturing, but also serves as an ion-carrier and precursor for bioactive peptides (Holt, 2011; Korhonen, 2009; Mohanty et al., 2016; Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Like the content of total casein, the concentration of κ-casein varied highly between the herds and over the year and was the lowest in summer (Fig. 1D; Supplementary material Table S24). No significant correlation was found between the herbage proportion and the κ-casein content (Fig. 2D; Table 5 and Supplementary material Table S11), while a significant influence of the breed was observed (Fig. 3 D; Table 5 and Supplementary material Table S11) resulting in a 0.96 g 100 g^{-1} measured proteins higher content in the milk of BS than in the milk of RED. In BS populations the allelic frequency of κ-casein B is higher than in Holstein related breeds (Boettcher et al., 2004). As a consequence, the production of κ-casein is higher in BS (Bonfatti et al., 2011). A significant influence of the breed was also found for lipoprotein lipase, a protein which is loosely attached to the casein micelles in fresh milk (Deeth, 2006, Table 5). In contrast to κ-casein, the content of lipoprotein lipase was found to be –0.019 g 100 g^{-1} measured proteins lower in the milk from BS than the milk of RED (Table 5 and Supplementary material Table S23).

3.2.3. Whey proteins

The concentration of α-lactalbumin in milk varied over the season (Supplementary material Table S24), which confirmed the results of Heck et al. (2009). The statistical evaluation of the data revealed that not only the season had a significant impact on the sum of all investigated whey proteins, but also the breed and the days in lactation (Table 5 and Supplementary material Table S15). The effect of the days in lactation was negative with one additional day in lactation leading to a decrease of 0.007 g 100 g^{-1} measured proteins of total whey protein in the milk. Furthermore, BS milk on average had 1.12 g 100 g^{-1} measured proteins less total whey protein.

The content of β-lactoglobulin in the bulk milk varied considerably between the farms and over the year, and was lowest in February and September (Fig. 1E; Supplementary material Table S24). As illustrated in Fig. 3E, a significant effect was found between breed and the β-lactoglobulin concentration in the milk (–0.832 g 100 g^{-1} measured proteins in the milk of BS, Fig. 3E; Table 5 and Supplementary material Table S17).

Similarly, the content of lactoferrin, a protein that is known for its antioxidant, bacteriostatic, anti-viral, and immunomodulatory properties as well as for the function of iron transfer and absorption (Korhonen, 2009; Korhonen & Marnila, 2011; Lönnerdal & Iyer, 1995; Tomita et al., 2009), varied over the course of the year (Fig. 1F; Supplementary material Table S24), with a higher content in the winter months and a lower concentration in the summer in the milk of all farms. The highest variation over the year was observed in the milk of the cows fed with high herbage content in the ration, while the lowest variation was found in the milk from the farms that fed low amounts of herbage (Fig. 2F). Lactoferrin concentration in milk has been shown to positively correlate with the somatic cell count (SCC) (Kuczynska et al., 2012; Litwińczuk, Król, Brodziak, & Barłowska, 2011; Turner, Williamson, Thomson, Roche, & Kolver, 2003). Since an increased SCC in the milk of one cow has a greater influence on the bulk milk on smaller farms, the management resulting in a consistent milk quality may be more challenging for smaller farms producing milk under high herbage feeding conditions. However, in our study no correlation was found, neither between the lactoferrin content and SCC in the milk (Supplementary material Table S24), nor between the herbage proportion or the breed and the content of lactoferrin in bulk milk (Figs. 2F and 3F; Table 5 and Supplementary material Table S13). Yet, the lactation stage and the season had a significant effect on the lactoferrin content (Figs. 4F and 1F; Table 5 and Supplementary material Table S13). A correlation of the concentration of lactoferrin with the season was also stated by Król et al. (2010). Contrasting the results shown, they found that the milk from local Polish breeds had a higher content of lactoferrin (average 129.2 mg L^{-1}) in summer than in winter.

3.2.4. Proteins associated with the milk fat globule membrane

In the case of the minor proteins associated with the milk fat globule membrane, a correlation with breed was found for two proteins. One of these proteins is FAS, which is involved in the biosynthesis of long-chain FAs (Roy et al., 2006). In the investigated bulk milk, the content of FAS varied over the year (Supplementary material Table S24). The mixed model showed a significant month and breed effect (0.009 g 100 g^{-1} measured proteins higher FAS content for BS milk than for RED cows, Table 5 and Supplementary material Table S19), no significant effect of the lactation stage or the herbage proportion on the FAS content could be found. In recent years, it was found that the variability of the gene coding for FAS (*FASN*) is high, not only among different breeds but also within the same breed (Ciecierska et al., 2013), which affects the milk fat composition, the total protein and fat content of the milk, as well as the total milk yield. Similarly, the content of the GP2 — a protein that binds to pathogens and might be protective against infections (Murgiano et al., 2009) — was not found to be affected by the proportion of herbage or the ADiL (Table 5 and Supplementary material Table S21). A significant breed effect on the average GP2 content was found (Table 5 and

Supplementary material Table S21). The milk of BS had a 0.011 g 100 g^{-1} measured proteins higher average amount of GP2 than RED milk. The finding that the concentration of GP2 is breed-correlated is supported by Murgiano et al. (2009), who found that GP2 is overexpressed in milk from Holstein cattle compared with milk from Chianina cows.

4. Conclusions

The impact of higher proportions of herbage in the cows' diet on the detailed milk composition and the interaction with breed, season and ADiL was so far only incompletely investigated. Owing to the mixed model approach this study contributes to an increased knowledge on this matter. The results for FAs not only showed that a higher proportion of herbage had an important positive influence on FA composition, such as an increase in n-3 FAs, CLA, VA, and BCFAs, confirming previous studies, but also provided information on the influencing parameters for six individual CLA isomers and ten BCFAs. Unexpectedly, the proportion of herbage had no significant influence on the content of the analysed 19 milk proteins.

However, the breed (RED compared with BS) influenced κ-casein levels (BS > 0.96 g 100 g^{-1} measured proteins), which is favourable for clotting properties during cheese-making. This effect is even enhanced in animals with genotype BB for κ-casein, which is more frequent in BS than in Holstein, thereby making the milk of BS cows highly suitable for cheese production. Furthermore, β-lactoglobulin (–0.83 g 100 g^{-1} measured proteins) and total whey protein (–1.12 g 100 g^{-1} measured proteins) were lower in the milk from BS cows; nevertheless, this breed still had a higher crude protein content compared with RED and a more favourable FA content with 30% more n-3s and 22% more CLA. The ADiL led to a decrease of 13% in stearic acid C18:0 between 86 and 245 ADiL and an increase of crude protein (+7%). The season had an influence on all the FAs; FA groups, all proteins, protein groups as well as the total fat content and the crude protein.

Above all, the mixed model approach used in this study provided for the first time quantitative statements on the relationship of different influencing parameters, such as herbage proportion, cattle breed, lactation stage, and season and the bulk milk composition from 12 Swiss farms, described by 119 examined parameters, which was tracked over the course of one year. Furthermore, these quantitative relationships allows quantitative predictions on the content of nutritionally relevant milk components, such as beneficial FAs, β-lactoglobulin, and total whey protein. Hence, an increase of the herbage proportion from for example 40% to 90% leads predictably to an increase in n-3 FAs content from approximately 0.95 to 1.36 g 100 g^{-1} fat (within the same breed and month). Therefore, the applied mixed effect model has the potential to provide a highly valuable input for the dairy industry in the future.

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Figure legends

Fig. 1. Content of (A) omega 3 fatty acids (n-3 FA, g 100 g^{-1} fat), (B) conjugated linoleic acids (CLA, g 100 g^{-1} fat), (C) crude protein (g kg^{-1} milk), (D) κ-casein (g 100 g^{-1} measured proteins) (E), β-lactoglobulin (g 100 g^{-1} measured proteins) and (F) and lactoferrin (g 100 g^{-1} measured proteins) in the bulk milk of the 12 farms analysed monthly over a year (n = 139).

Fig. 2. Content of (A) omega 3 fatty acids (n-3 FA, g 100 g^{-1} fat), (B) conjugated linoleic acids (CLA, g 100 g^{-1} fat), (C) crude protein (g kg^{-1} milk), (D) κ-casein (g 100 g^{-1} measured proteins), (E) β-lactoglobulin (g 100 g^{-1} measured proteins), and (F) lactoferrin (g 100 g^{-1} measured proteins) in the bulk milk of the 12 farms analysed monthly over a year (n = 139) as a function of the proportion of herbage.

Fig. 3. Relationship between the content of (A) omega 3 fatty acids (n-3 FA, g 100 g^{-1} fat), (B) conjugated linoleic acids (CLA, g 100 g^{-1} fat), (C) crude protein (g kg^{-1} milk), (D) κ-casein (g 100 g^{-1} measured proteins), (E) β-lactoglobulin (g 100 g^{-1} measured proteins), and (F) lactoferrin (g 100 g^{-1} measured proteins) and the breed [Red Holstein, Swiss Fleckvieh, Simmental cattle (RED) or Brown Swiss (BS)] in the bulk milk of the 12 farms (n = 139).

Fig. 4. Relationship between the content of (A) omega 3 fatty acids (n-3 FA, g 100 g^{-1} fat), (B) conjugated linoleic acids (CLA, g 100 g^{-1} fat), (C) crude protein (g kg^{-1} milk), (D) κ-casein (g 100 g^{-1} measured proteins), (E) β-lactoglobulin (g 100 g^{-1} measured proteins), and (F) lactoferrin (g 100 g^{-1} measured proteins) and the averaged days in lactation (ADiL) in the bulk milk of the 12 farms (n = 139).

Table 1

Annual average of monthly ration shares per farm in 2014 and the mean value, standard deviation, minimum and maximum over all 12 farms (n = 139).

Ration (%)	Farm												Mean	SD	Min.	Max.
	1	2	3	4	5	6	7	8	9	10	11	12				
Total herbage	0.35	0.45	0.43	0.42	0.71	0.52	0.94	0.99	0.78	0.85	0.85	0.78	0.68	0.22	0.32	1.00
Monthly min.	0.32	0.43	0.40	0.38	0.66	0.50	0.88	0.96	0.71	0.74	0.62	0.65				
Monthly max.	0.39	0.48	0.53	0.47	0.75	0.55	0.96	1.00	0.90	0.98	0.95	0.93				
Herbage, fresh	0.09	0.02	0.07	0.08	0.21	0.12	0.57	0.69	0.39	0.48	0.48	0.42	0.31	0.36	0.00	0.99
Herbage, conserved (hay/silage)	0.26	0.43	0.35	0.34	0.50	0.40	0.37	0.30	0.40	0.37	0.38	0.36	0.37	0.28	0.00	1.00
Whole-crop maize	0.37	0.33	0.35	0.37	0.16	0.33	0.00	0.00	0.09	0.05	0.06	0.06	0.18	0.16	0.00	0.42
Other feedstuff	0.04	0.01	0.10	0.04	0.03	0.03	0.00	0.00	0.00	0.04	0.00	0.01	0.03	0.04	0.00	0.13
Concentrates	0.24	0.21	0.13	0.16	0.10	0.11	0.06	0.01	0.13	0.05	0.08	0.15	0.12	0.07	0.00	0.28

Table 2

Annual average values per farm of monthly milk production in 2014, and the mean value, standard deviation, minimum and maximum over all 12 farms (n = 139).[a]

Parameter	Farm												Mean	SD
	1	2	3	4	5	6	7	8	9	10	11	12		
Cows in milk (farm $^{-1}$)	53	56	31	43	24	49	14	22	21	14	22	21	30	15
Brown Swiss (farm $^{-1}$)							14	22	21					
RED* (farm $^{-1}$)	53	56	31	43	24	49				14	22	21		
Calving	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.	n. syn.		
Energy corrected milk (kg day^{-1} cow $^{-1}$)	30.09	32.83	29.26	30.30	19.45	29.25	21.95	17.44	21.76	24.70	22.70	24.38	25.27	5.02
Fat (g kg^{-1} milk)	41.66	42.97	37.73	39.27	31.78	36.08	40.86	36.02	41.90	44.36	41.63	38.82	39.56	4.52
Protein (g kg $^{-1}$ milk)	34.79	33.86	33.26	33.41	30.83	34.02	35.04	33.63	34.56	35.69	33.70	35.66	34.07	1.69

[a] RED, Red Holstein, Swiss Fleckvieh or Simmental; n. syn., not synchronised.

Table 3

Annual average values, standard deviation, minimum, and maximum of selected fatty acids and groups in bulk milk samples analysed monthly on 12 farms (n = 139). [a]

Fatty acid or group of FA (g 100 g^{-1} fat)	Mean	SD	Min.	Max.
C16:0 Palmitic acid	26.52	2.53	20.15	34.56
C18:0 Stearic acid	8.54	1.15	5.11	11.91
C18:1 t11 Vaccenic acid	1.68	0.81	0.65	3.74
C18:1 c9 Oleic acid	16.28	1.65	11.89	20.28
C18:2 c9,t11 (CLA) Rumenic acid	0.66	0.35	0.20	1.85
C18 :2 t11,c13 (CLA)	0.04	0.03	0.01	0.15
C18 :2 t7,c9 (CLA)	0.03	0.01	0.02	0.05
C18 :2 t9,t11 (CLA)	0.03	0.01	0.01	0.06
C18 :2 t11,c13 and c9,c11 (CLAs)	0.05	0.03	0.01	0.16
C18:2 c9,t11 and t8,c10 and t7c9 (CLAs)	0.70	0.36	0.22	1.91
∑SFA	60.54	2.53	52.51	66.38
∑MUFA	23.59	2.07	18.16	28.73
∑PUFA	3.82	0.73	2.35	6.15
∑n-3	1.02	0.35	0.46	2.12
∑n-6	2.07	0.36	1.47	2.97
∑CLA	0.78	0.38	0.28	2.09
∑Branched-chain fatty acids	1.80	0.24	1.22	2.37

[a] Abbreviations are: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. ∑CLA: (C18:2 c9t11+t8c10+t7c9), (C18:2 t11c13+c9c11), C18:2 t9t11; ∑Branched chain fatty acids: C13 iso+aiso, C14 iso, C15 iso+aiso, C16 iso, C17 iso+aiso, C18 iso+aiso. For definitions of further groups see Supplementary material Table S1. Single values are listed in Supplementary material Tables S1 (per farm) and S16 (per farm and month).

Table 4

Influence of the factors herb, breed, average days in lactation and month on the content of selected fatty acids (FAs) and groups of FAs in the bulk milk of 12 farms (n = 139).[a]

Fatty acids ($g\ 100\ g^{-1}$ fat)	Intercept		Herbage		Breed (Brown Swiss)		Average days in lactation		Month
	Est.	*p*	Est.	*p*	Est.	*p*	Est.	*p*	*p range*
C12:0 Lauric acid	3.389	**<0.001**	-0.537	**0.026**	-0.087	0.542	0.000	0.599	**<0.001–**0.936
C13:0 iso (BCFA)	0.011	**0.007**	0.021	**<0.000**	-0.004	0.125	0.000	0.836	**<0.001**–0.949
C14:0 Myristic acid	10.45	**<0.001**	-0.685	0.157	0.164	0.557	0.001	0.560	**<0.001–**0.922
C14:1 cis	0.817	**<0.001**	-0.078	0.205	0.100	**0.011**	0.001	**<0.001**	**<0.001–**0.317
C15:0 iso (BCFA)	0.119	**<0.001**	0.120	**<0.001**	-0.011	0.374	0.000	0.054	**0.001**–0.913
C16:0 Palmitic acid	27.979	**<0.001**	-0.555	0.764	-0.660	0.541	0.005	0.408	**<0.001**–0.677
C16:1 trans	0.032	0.240	0.156	**<0.001**	0.007	0.642	0.000	0.546	**0.006**–0.799
C17:0 iso (BCFA)	0.115	**<0.001**	0.110	**<0.001**	0.004	0.712	0.000	0.952	**<0.001**–0.160
C18:0 Stearic acid	8.817	**<0.001**	-0.154	0.839	-0.590	0.194	-0.007	**0.006**	**<0.001**–0.745
C18:1 t11 Vaccenic acid	-0.041	0.898	1.887	**<0.001**	0.194	0.316	-0.001	0.620	**<0.001**–0.974
C18:1 cis-9 (oleic acid)	15.622	**<0.001**	0.469	0.720	0.139	0.855	-0.006	0.092	**<0.001**–0.629
C18:1 cis-12	0.377	**<0.001**	-0.170	**<0.001**	-0.011	0.575	0.000	0.995	**<0.001**–0.257
C18:2 c9,t11 (CLA) Rumenic acid	-0.088	0.490	0.794	**<0.001**	0.138	0.074	0.000	0.761	**<0.001**–0.746
C18:2 c9,t12 and c,c MID and t8,c13	0.306	**<0.001**	-0.130	**<0.001**	0.010	0.372	0.000	0.467	**<0.001**–0.474
C18:2 t7,c9 (CLA)	0.037	**<0.001**	-0.004	0.473	0.000	0.983	3.1×10^{-5}	**0.036**	**<0.001**–0.255
C 18:2 t11,c13 (CLA)	0.005	0.764	0.014	0.524	0.052	**0.004**	0.000	0.625	**0.004**–0.980
C18:2 t11,c13 and c9,c11 (CLAs)	0.006	0.707	0.029	0.164	0.045	**0.004**	0.000	0.512	**0.003**–0.648
∑ CLA	-0.003	0.982	0.835	**<0.001**	0.174	**0.048**	0.000	0.776	**<0.001**–0.523
C18:3 c9,c12,c15 α-Linolenic acid (n-3)	0.182	**0.011**	0.491	**<0.001**	0.184	**0.002**	0.000	0.620	**0.008–0.815**
∑ C 18:2 t with CLA t	0.551	**0.016**	1.031	**<0.001**	0.319	**0.029**	0.000	0.864	**<0.001**–0.506
∑ trans with CLA t	2.313	**<0.001**	2.611	**<0.001**	0.558	0.124	-0.001	0.794	**<0.001**–0.987
∑ C18:2 t,t NMID	0.015	0.459	0.105	**<0.001**	0.002	0.856	0.000	0.740	**<0.001**–0.983
C20:3 (n-3)	0.003	0.575	0.022	**<0.001**	0.003	0.187	0.000	0.098	**<0.001**–0.889
C20:5 EPA (n-3)	0.028	**<0.001**	0.040	**<0.001**	0.008	0.135	0.000	0.817	**<0.001**–0.331
∑ n-3	0.378	**0.002**	0.822	**<0.001**	0.306	**0.002**	0.000	0.458	**<0.001**–0.925
∑ SFA	63.934	**<0.001**	-3.012	**0.031**	-0.781	0.308	-0.002	0.717	**<0.001**–0.927
∑ PUFA	2.784	**<0.001**	1.117	**0.004**	0.458	0.057	-0.001	0.299	**<0.001**–0.964
∑ Branched chain FA	1.150	**<0.001**	0.525	**<0.001**	-0.026	0.688	0.001	0.120	**<0.001**–0.124

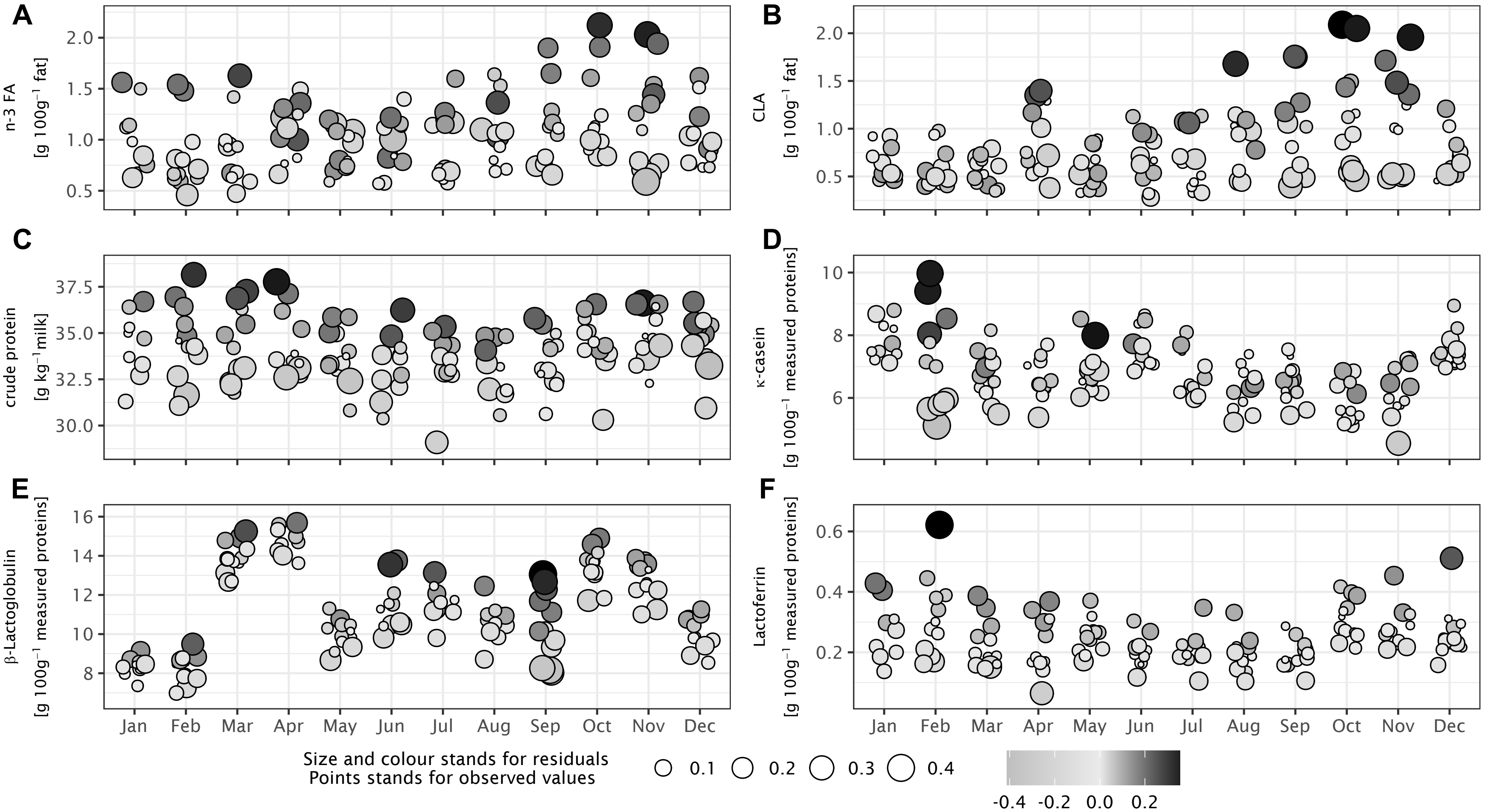
[a] Abbreviations are: BCFA, branched chain fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid c, cis; t, trans; c,c MID, cis methylene interrupted diene; t,t NMID, trans trans non methylene interrupted diene. For definition of groups see Table 3 and Supplementary material Table S1. Estimates at intercept are for 0% herbage, Red Holstein, Swiss Fleckvieh, Simmental, one average day in lactation and January 2014. Estimate for herbage quantifies the estimated increase in the content of the FAs or group of FAs in $g\ 100\ g^{-1}$ fat compared with the intercept value if the proportion of herbage is 100%. Estimate for breed indicates how much the content of the FAs or group of FAs in $g\ 100\ g^{-1}$ fat is higher in milk of Brown Swiss cows compared with Red Holstein, Swiss Fleckvieh or Simmental. Estimate for average days in lactation indicates the effect of one additional average day in lactation on the content of the FAs and groups of FAs compared with the intercept value. *p*-Values for the month effect vary from month to month and are indicated as a range. *p*-values in bold indicate a significant effect ($p < 0.05$).

Table 5

Influence of the factors herb, breed, average days in lactation and month on the content of selected proteins and groups of proteins in the bulk milk of 12 farms (n = 139). [a]

Protein	Intercept		Herbage		Breed (Brown Swiss)		Average days in lactation		Month
	Est.	p	Est.	p	Est.	p	Est.	p	p-range
Crude protein	32.62	**<0.001**	-0.867	0.553	0.608	0.599	0.014	**<0.001**	**0.003–**0.994
κ-casein	6.972	**<0.001**	0.596	0.273	0.955	**0.012**	0.001	0.394	**<0.001–**0.211
Lipoprotein lipase	0.048	**<0.001**	-0.002	0.729	-0.019	**<0.001**	-0.000	0.781	**<0.001–**0.679
Total whey proteins	10.541	**<0.001**	1.033	0.132	-1.122	**0.011**	-0.007	0.003	**<0.001–**0.002
β-Lactoglobulin	8.710	**<0.001**	0.834	0.223	-0.832	**0.045**	-0.004	0.050	**0.001**–0.860
Lactoferrin	0.105	**0.047**	0.093	0.146	-0.039	0.294	0.001	**0.002**	**0.012**–0.863
Fatty acid synthase	0.028	**<0.001**	-0.002	0.704	0.009	**0.002**	-0.000	0.922	**0.012**–0.246
Glycoprotein-2	0.021	**<0.001**	-0.003	0.699	0.011	**0.018**	-0.000	0.314	**<0.001**–0.172

[a] Estimates at intercept are for 0% herbage, Red Holstein, Swiss Fleckvieh or Simmental, one average day in lactation and January 2014. Estimates for herbage indicate the increase of the content of the protein or protein group compared with the intercept if the proportion of herbage is 100%. Estimates for breed indicate how much the content of the protein or group of proteins is higher or lower in the milk from Brown Swiss cows compared with that from the Red Holstein, Swiss Fleckvieh or Simmental group. Estimates for average days in lactation indicates the effect of an additional average day in lactation on the content of the proteins compared with the intercept value. Values for crude protein are g kg^{-1} milk; values for individual proteins are in g 100 g^{-1} measured proteins. p-Values for the monthly influence vary from month to month. A significant effect is indicated by a p-value in bold ($p < 0.05$).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n-3 FA
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0.5
1.0
1.5
2.0
B
CLA
[g 100g^{-1} fat]
0.5
1.0
1.5
2.0
C
crude protein
[g kg^{-1}milk]
30.0
32.5
35.0
37.5
D
κ-casein
[g 100g^{-1} measured proteins]
6
8
10
E
β-Lactoglobulin
[g 100g^{-1} measured proteins]
8
10
12
14
16
F
Lactoferrin
[g 100g^{-1} measured proteins]
0.2
0.4
0.6
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Size and colour stands for residuals
Points stands for observed values
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0.2
0.3
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-0.4
-0.2
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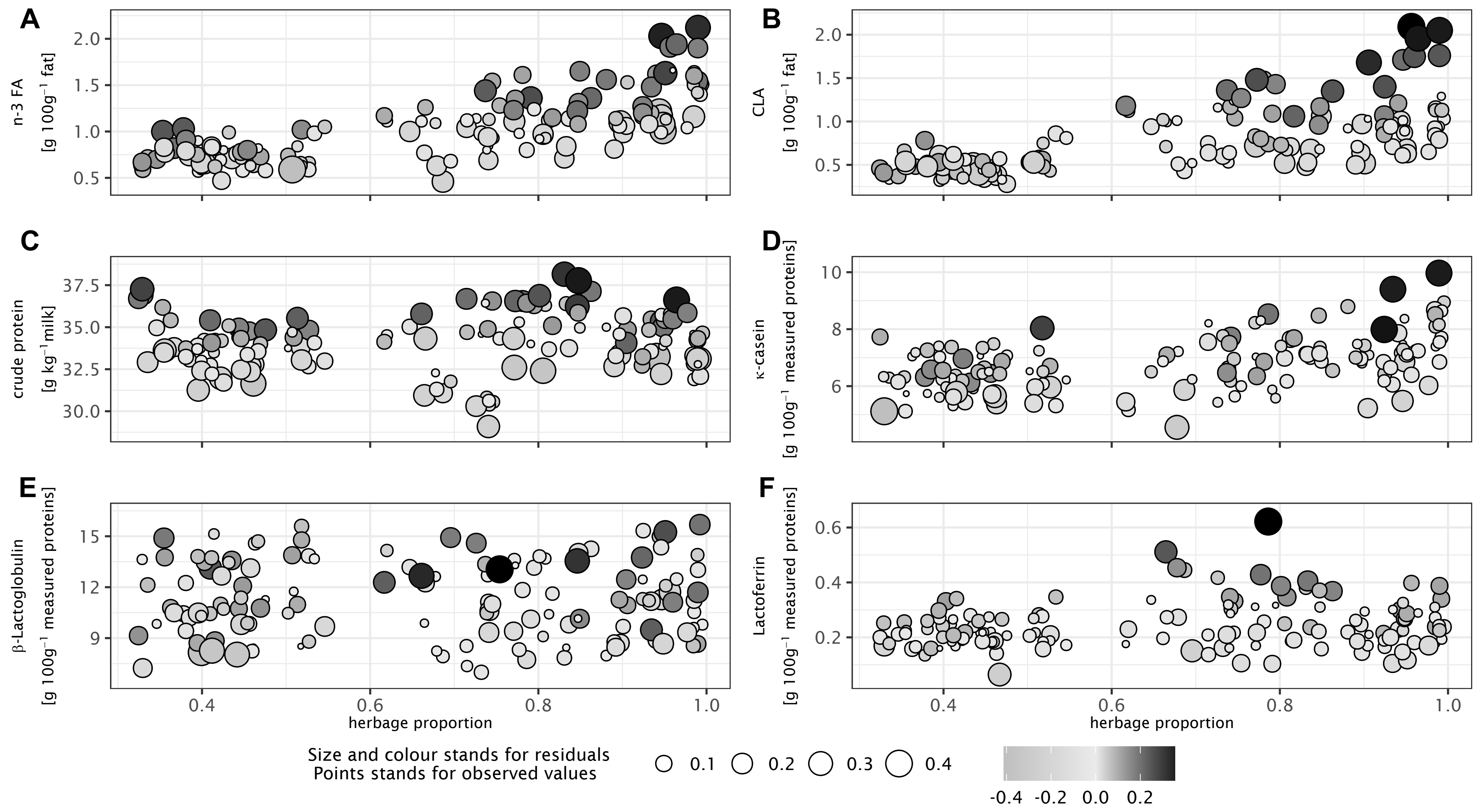
A
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[g 100g^{-1} fat]
2.0
1.5
1.0
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B
CLA
[g 100g^{-1} fat]
2.0
1.5
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0.5
C
crude protein
[g kg^{-1}milk]
37.5
35.0
32.5
30.0
D
κ-casein
[g 100g^{-1} measured proteins]
10
8
6
E
β-Lactoglobulin
[g 100g^{-1} measured proteins]
15
12
9
F
Lactoferrin
[g 100g^{-1} measured proteins]
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0.4
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herbage proportion
Size and colour stands for residuals
Points stands for observed values
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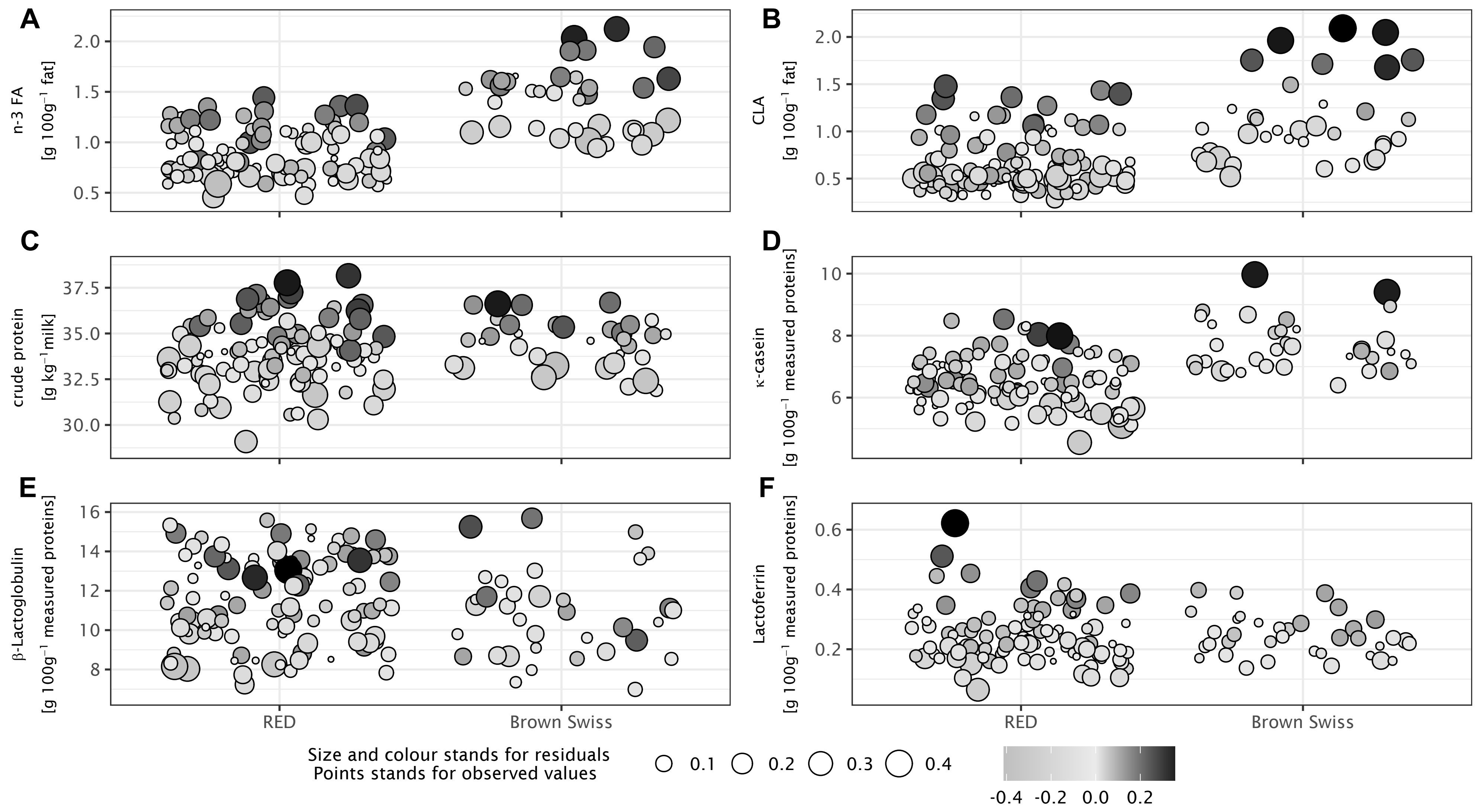
A
n-3 FA
[g 100g^{-1} fat]
2.0
1.5
1.0
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B
CLA
[g 100g^{-1} fat]
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1.5
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0.5
C
crude protein
[g kg^{-1}milk]
37.5
35.0
32.5
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D
κ-casein
[g 100g^{-1} measured proteins]
10
8
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E
β-Lactoglobulin
[g 100g^{-1} measured proteins]
16
14
12
10
8
F
Lactoferrin
[g 100g^{-1} measured proteins]
0.6
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RED
Brown Swiss
RED
Brown Swiss
Size and colour stands for residuals
Points stands for observed values
0.1
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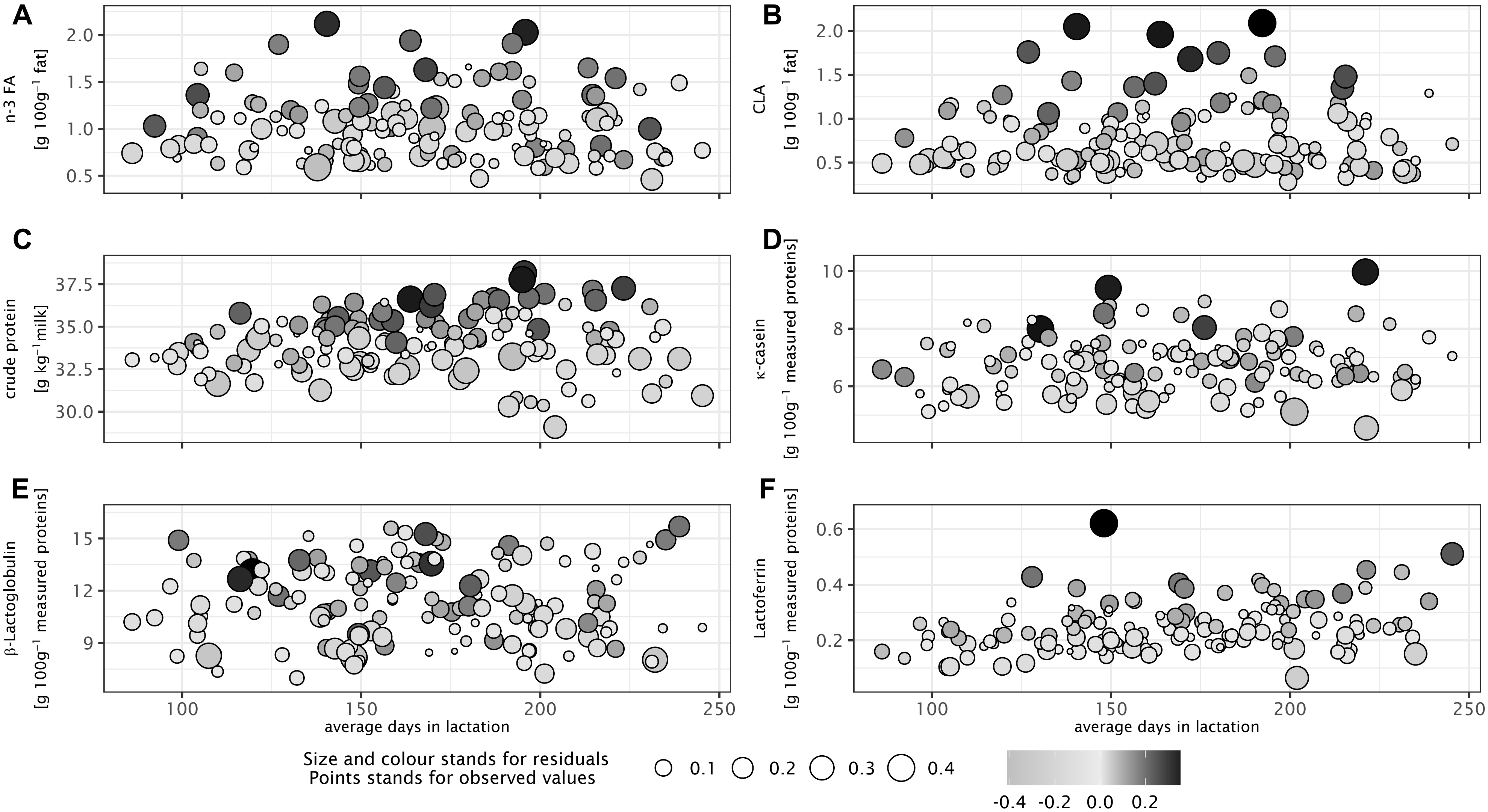
A
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[g 100g^{-1} fat]
B
CLA
[g 100g^{-1} fat]
C
crude protein
[g kg^{-1}milk]
D
κ-casein
[g 100g^{-1} measured proteins]
E
β-Lactoglobulin
[g 100g^{-1} measured proteins]
F
Lactoferrin
[g 100g^{-1} measured proteins]
average days in lactation
100
150
200
250
0.5
1.0
1.5
2.0
30.0
32.5
35.0
37.5
6
8
10
9
12
15
0.2
0.4
0.6
Size and colour stands for residuals
Points stands for observed values
0.1
0.2
0.3
0.4
-0.4
-0.2
0.0
0.2