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# Dairy Science & Technology

## Mineral, vitamin A and fat composition of bulk milk related to European production conditions throughout the year --Manuscript Draft--

<b>Manuscript Number:</b>	DSTE-D-16-00007R2	
<b>Full Title:</b>	Mineral, vitamin A and fat composition of bulk milk related to European production conditions throughout the year	
<b>Short Title:</b>	Annual changes of bulk milk micronutrients	
<b>Article Type:</b>	Original Article	
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<b>Abstract:</b>	<p>In many Western countries, milk and dairy products provide both mineral and lipid fractions relevant to our health. The milk contents of these compounds are highly variable and the identification of husbandry conditions leading to milks favourable for both mineral and lipid fractions is of interest. The aims of this study were to describe throughout the year the changes in bulk milk compounds and to relate them to husbandry practices. Ten compounds with nutritional requirements were selected: a minimum amount is required for Ca, Mg, P, Zn, vitamin A, n-3 fatty acids (FA), oleic acid and n-6 FA, and a maximum amount for trans FA and C12-14-16 FA. Milks and their associated husbandry practices were collected 5 times during one year from 74 groups of farms located in France, Norway, Slovakia and Slovenia. The Ca, Mg, P and Zn contents varied in different ways throughout the year and independently of each</p>	

	<p>other. We showed the absence of milks with the highest contents in these four minerals simultaneously but we identified groups of farms where milk had high content in Mg or in Zn. Positive links have been confirmed between grass-based-diets and milk n-3 FA content, maize silage-based-diets and n-6 FA content, and diets rich in concentrate and oleic acid content. These results showed that it was possible to identify husbandry conditions leading to milk favourable throughout the year for one or several compounds but not for all compounds considered in this study.</p>
<p><b>Response to Reviewers:</b></p>	<p>Reviewers' comments: *****</p> <p>Reviewer #1: General comments: Most of the comments from my original review have been addressed but not all and I feel the text is still not very good, making it challenging to read. In addition the discussion is fairly naive and descriptive rather than explaining the potential impact of the differences identified. I think this should be improved before publication. AU: We followed the reviewers queries related to the discussion. We proposed an improved version including more focused aspects on the explanation of the links between feeding and milk characteristics (See in particular lines 328-340 for minerals, lines 353-355 for vitamin A, lines 418-421 for n-3 FA, and lines 437-442 for trans FA) and on the potential nutritional impact of the differences identified (See in particular lines 466-479).</p> <p>Specific point - as before these suggestions and questions are not be exhaustive and apologies if many appear as editing rather than reviewing, but the text is flawed if not implemented:</p> <p>-line 61: do you mean "the multiple compounds of interest in milk" ? AU: yes, modified in the text</p> <p>-line 71 &amp; 74 explain n-3 FA and n-6 FA AU: done</p> <p>-line 77 clarify if 17 g is the combined intake or applies to each FA AU: Yes, the text was modified accordingly</p> <p>-line 77 - why are trans fats 'in contrast' - in the context being mentioned, relating to health? AU: the text was clarified</p> <p>-line 80 again make it clear - is this total trans fats? AU: the text was clarified according to the definitions of the FAO.</p> <p>Also, the negative aspects of trans fats is mentioned here but then not picked up again in the discussion AU: This aspect was picked up again in the section 4.4 "Nutritional interest of bulk milk according to GF classes". The negative aspect of trans FA in milk was discussed (see lines 472-475).</p> <p>-line 80-84 - this needs a source (either a citation or the values) for the assumed dairy fat composition used in these calculations AU: The calculations were done according to the values published by Coppa et al., 2013. This reference was chosen because it depicts the fat composition of a large number of tanker milks (1248) collected throughout Europe.</p> <p>-lines 115-117 this does not make sense AU: The sentence was reformulated and clarified. -line 123 - DMI needs explanation AU: Dry Matter Intake (DMI) was added in the text.</p> <p>-lines 134-6 - clarify the difference between these 2 scenario - which results in proportional contribution to the bulk milk and which gives the same impact regardless of milk supply from individual farms? AU: done</p>

Comment citer ce document :

-line 138 - divided into how many sub-samples?

AU: 3 subsamples spread into 3 laboratories analyzing minerals, vitamins and fatty acids respectively. These details were added in the text.

-line 142 omit the 1st or 2nd 'milk'

AU: done

-line 150-151 are unnecessary

AU: deleted

-lines 202-206 The 4 simple sentences could be combined to improve readability.

AU: this paragraph was simplified and reformulated (see lines 198-205)

-line 213 - were farms described by 'changes' to diet or by the diet fed at each of the sample times?

AU: reformulated

-line 241 The text following this sub-heading does not cover seasonal changes since it discussed PCA output before results in figure 2 which are seasonal changes.

AU: in order to avoid any misinterpretations, the sub-heading was removed and sub-heading 3.1 was changed as "3.1 Bulk milk composition and changes throughout the year" (line 236).

I think it much better to start by discussing the more 'simple' seasonal variation shown in fig 2

AU: The variation of milk composition shown in fig 2 cannot be described before the results of the PCA and HCA because the latter were used to build the 4 GF classes presented in Fig 2.

-line 242 and 244- state the variation 'explained' by each PC1 and 2 (%TV) - as you have for PC3 and 4

AU: done

-line 243: is there a bit missing - if the 1st part of this sentence states PC1 'opposed' n-3.. then you need to say it supported or was associated with n-6 - or something to this effect?

AU: The sentence was reformulated (lines 242-245)

-line 249 useful to comment on the numbers of groups allocated to each of these classes - I thought these were equal until looking at table 3

AU: the number of GF in each class was added in the text (lines 249-251).

-lines 287-293: I think more quantification out to be reported by using text such as "Class 3 was mainly represented by Norwegian farms with 86% Norwegian Red cattle"  
AU: figures were added in the text (lines 286-293).

-lines 316-326 the discussion on Ca, P and Mg ought to be related to DIM as well as seasonality - with comments on other published work if this related to block calving studies. Also, as mentioned before it is relevant to mention the potential impact of pasture composition as a high content of clover will change the dynamics compared to grass only swards - noting the proportion of clover will change substantially throughout the season, being highest in autumn. This has not been picked up from previous comments (and could also relate to n-3 PUFA concentrations)

AU: The link between mineral contents in milk and DIM (lines 312-316), season (lines 322-325) and diet (lines 325-340) was discussed in section 4.1. In particular, we enriched the discussion and proposed hypotheses related to a potential impact of pasture composition. In particular we picked up the suggestions of the reviewer. We have nevertheless to underline (we did into the text) that our design doesn't allow to validate or not these hypotheses.

The link between the n-3 content in milk and the diet was discussed in section 4.3 (lines 418-421).

-line 347-350 as stated before you cannot discuss vit A supply to cows without mentioning carotenoid intake - also much higher from silage than hay - so these

Comment citer ce document :

sentences ought to be reversed

AU: The different sentences from this paragraph have been reversed as suggested. We mentioned the carotenoid intake earlier in the paragraph (lines 353-355).

-lines 361-364 - is this repetition of results rather than discussion?

AU: We agree: this sentence was removed.

-lines 398-426 any discussion on n-3 and n-6 (and C18:1t11 and CLA) in milk fat ought to mention rumen retention time and the potential impact on hydrogenation hence differentiating between highly digestible lush grazing vs more mature conserved forage

AU: the discussion on fatty acids in general was improved. The supplements suggested by the reviewer were added, in particular for the interpretation of the seasonal pattern of trans FA (lines 437-442). Other supplements were also included, in order to explain better our results or to propose hypotheses (See in particular lines 328-340 for minerals, lines 353-355 for vitamin A, lines 418-421 for n-3 FA).

Figure 1: add the meaning of '(1-4)' in the legend

AU: done

Figure 2 I still think it would be useful to show the p-values for sampling time and interactions between GF and Date (some of which must be significant looking at the charts)

AU: the P values for the effects of sampling time, GF class and interaction sampling time\*GF class were previously reported in the text but as suggested by the reviewer, they were added into the charts.

Figure 3 needs a key for abbreviations and is still ~FG not GF

AU: taken into account

Editor's comment:

Thank you for your revision. The manuscript has been much improved but it still needs polishing before acceptance.

Please also check if classes 2 and 3 of GF were differentiated both on PC2 and PC4 as stated in the text (Line 251-252), as it is unusual to have the same variables differentiated by 2 different axes...

AU: Indeed, you are right: it was a mistake. PC4 differentiated Classes 2 and 4. Change was made in the text (line 252).

## Major changes made in the manuscript

We followed all the reviewers' queries in order to improve the readability of the text and the interest of the discussion.

### **Introduction and Materials and methods sections**

All the details that are required were added.

### **Results section**

The 3.1 and 3.2 former sections were pooled.

### **Discussion**

We proposed an improved version including more focused aspects on the explanation of the links between feeding and milk characteristics.

The potential nutritional impact of the differences identified between GF classes was investigated, in particular for classes 1 and 3.

1 **Mineral, vitamin A and fat composition of bulk milk related to European production**  
2 **conditions throughout the year**

3 **Short title: Annual changes of bulk milk micronutrients**

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5 **Jaroslav Golecký • Bruno Martin • Anne Ferlay • Isabelle Constant • Carole Delavaud •**

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36 **Abstract.** In many Western countries, milk and dairy products provide both mineral and lipid  
37 fractions relevant to our health. The milk contents of these compounds are highly variable and  
38 the identification of husbandry conditions leading to milks favourable for both mineral and lipid  
39 fractions is of interest. The aims of this study were to describe throughout the year the changes  
40 in bulk milk compounds and to relate them to husbandry practices. Ten compounds with  
41 nutritional requirements were selected: a minimum amount is required for Ca, Mg, P, Zn,  
42 vitamin A, n-3 fatty acids (FA), oleic acid and n-6 FA, and a maximum amount for *trans* FA  
43 and C12-14-16 FA. Milks and their associated husbandry practices were collected 5 times  
44 during one year from 74 groups of farms located in France, Norway, Slovakia and Slovenia.  
45 The Ca, Mg, P and Zn contents varied in different ways throughout the year and independently  
46 of each other. We showed the absence of milks with the highest contents in these four minerals  
47 simultaneously but we identified groups of farms where milk had high content in Mg or in Zn.  
48 Positive links have been confirmed between grass-based-diets and milk n-3 FA content, maize  
49 silage-based-diets and n-6 FA content, and diets rich in concentrate and oleic acid content.  
50 These results showed that it was possible to identify husbandry conditions leading to milk  
51 favourable throughout the year for one or several compounds but not for all compounds  
52 considered in this study.

53 **Keywords,** micronutrients, milk composition, nutritional quality, farming practices



## 54 **1 Introduction**

55 Improving the nutritional quality of milk is of concern to current producers, the dairy industry  
56 and consumers. Indeed, the dairy industry and retailers include increasing nutritional data in  
57 their public communication (Menard et al. 2012), and this trend is driven by the increasing  
58 number of well-known links between dairy products and human health (Visioli and Strata  
59 2014). In many Western countries, the contribution of milk and dairy products in a balanced  
60 diet is recognized for the supply of both macro-constituents (proteins, fat and minerals) and  
61 micro-constituents (vitamins and trace elements). Considering the multiple **compounds of**  
62 **interest in milk**, the present study will explore, throughout the year, the changes in a set of  
63 nutritional relevant compounds: some minerals, fatty acids (FA) and vitamin A in relation with  
64 the associated husbandry conditions.

65 For minerals, the sole consumption of liquid milk in the European Union (170 mL / day / capita,  
66 IDF 2013) provides approximately 20-25 % of Ca and P and 10 % of Mg and Zn of the  
67 minimum daily amounts that are required for human metabolism (1000 mg of Ca, 750 mg of P,  
68 260 mg of Mg and 7 mg of Zn) (WHO and FAO 2004). The contribution of dairy products to  
69 our fat consumption is also well known. Some FA are considered desirable and a daily  
70 minimum intake is required, while others are usually consumed in excess and, therefore a  
71 limited daily intake is recommended. The n-3 FA (**mainly  $\alpha$ -linolenic acid in milk and other**  
72 **minor long chain n-3 FA**) limit metabolic syndrome and type 2 diabetes (Palmquist 2009), and  
73 thus a minimum daily consumption is required (2 g for C18:3n-3, the major n-3 FA) (FAO  
74 2010). For oleic acid, the daily intake may range from 32 to 43 g (FAO 2010). The n-6 FA  
75 (**mainly linoleic acid in milk**) may activate adipose tissue development, and their daily intake  
76 may range from 4.3 to 8.5 g (FAO 2010). Among saturated FA (SFA), C12:0, C14:0 and C16:0  
77 when consumed in excess may increase the risk of cardiovascular diseases (Shingfield et al.  
78 2008). **The daily intake of the sum of these three FA** should not exceed 17 g (FAO 2010). **The**

79 intake of *trans* FA from ruminant products don't seem to increase the risk of cardiovascular  
80 diseases in contrast to *trans* FA produced industrially (Gayet-Boyer et al. 2014). Nevertheless,  
81 for the FAO (2010), the daily *trans* FA intake (typically isomers of C18:1 *trans* derived from  
82 partially hydrogenated vegetable oils) should not exceed 1 % of the energy intake i.e. 2.4 g.  
83 Based on an average daily consumption of 18 g per capita of dairy fat in the European Union  
84 (estimated from IDF, 2013) and on the fat composition reported by Coppa et al. (2013), dairy  
85 fat provides approximately 6 % and 3 % of the recommendations for n-3 and n-6 FA, 9 % for  
86 oleic acid and up to 47 % and 22 % for the sum of C12:0, C14:0, C16:0 and *trans* FA,  
87 respectively. Dairy fat also provides lipophilic vitamins, in particular one-third of the vitamin  
88 A daily recommendation, which is 600 µg Eq retinol (WHO and FAO 2004).

89 The milk concentrations of all these compounds are highly variable throughout the year and  
90 vary according to the husbandry conditions. For dairy industries, there is an interest to  
91 characterize this variability and its variation factors. They are partially described for minerals  
92 and are well known for FA and vitamin A. The milk mineral content varies mainly according  
93 to the lactation stage and the nutritional and health status of the dairy cows (Gaucheron 2005),  
94 whereas the vitamin A content is mainly affected by the nature of the forage, breed or calving  
95 period (Nozière et al. 2006). The concentrations of the various FA change according to the  
96 cow's diet and to a lesser extent to animal characteristics, such as breed and lactation stage  
97 (Chilliard et al. 2007). Among the mentioned factors, the cow's diet and lactation stage have a  
98 short-term effect on milk composition (< 1 month). Therefore, changes in the farmer's practices  
99 throughout the year result in seasonal changes in bulk milk composition.

100 The variations in milk mineral contents or fat composition over one year have been described,  
101 in field studies, in herd milks (Rodriguez Rodriguez et al. 2001) or in dairy plant milks in  
102 Denmark (Poulsen et al. 2015), the Netherlands (Heck et al. 2009; van Valenberg et al. 2013),  
103 the US (O'Donnell-Megaró et al. 2011) and the UK (Butler et al. 2011). The relationships

104 between milk minerals and production systems have rarely been studied in on-farm conditions  
105 (Hurtaud et al. 2014). In contrast, many authors have reported changes throughout the year in  
106 milk fat composition (Butler et al. 2008; Ferlay et al. 2008; Collomb et al. 2008; Larsen et al.  
107 2010; Borreani et al. 2013) or in fat-soluble vitamins (Agabriel et al. 2007; Ellis et al. 2007)  
108 according to the production systems. To the best of our knowledge, no study has investigated  
109 concomitantly minerals, FA and vitamin A in bulk milk and the associated husbandry  
110 conditions. Thus, the identification of husbandry conditions leading to the production of milks  
111 favourable for both mineral and lipid fractions throughout the year is the challenge of this study.  
112 Our strategy to achieve this aim is (1) to classify farms on the basis of the changes in mineral,  
113 vitamin A contents and fat composition in milk throughout the year and for each class, (2) to  
114 characterize the milk changes across the year and (3) to explain these changes according to the  
115 husbandry conditions.

## 116 **2 Materials and methods**

### 117 2.1 Study design

118 **In our study, the bulk milk composition and the associated production conditions are studied at**  
119 **the scale of a group of farms (GF).** In this manner, 74 GF were selected in France, Norway,  
120 Slovakia and Slovenia (Table 1) due to their large diversity of cow husbandries. Farms of each  
121 GF were geographically close and had similar milk production conditions. The different GF  
122 were located with a latitude range of 45.5 to 69.5°N and an altitude ranging from 40 to 1,400  
123 m.a.s.l. The dairy cow diets were very diverse, ranging from diets based on grass (grass silage,  
124 hay or pasture according to the season) to diets including a large part of maize silage throughout  
125 the year. The proportion of concentrates ranged from nearly zero to more than 50 % of the **dry**  
126 **matter intake** (DMI). The annual milk yield ranged from approximately 3,000 to more than  
127 9,000 kg·cow<sup>-1</sup>·year<sup>-1</sup>. The Holstein breed was the most common, with 22 GF having herds of  
128 more than 50 % Holstein cows. The Norwegian Red was the only breed that was present in

129 Norway, while the Slovak Spotted and Holstein breeds were found in Slovakia (Table 1). Six  
130 other breeds (Brown, Montbeliarde, Simmental, Normande, Tarentaise and Abondance) were  
131 only present in France or in Slovenia.

132 Repeated measures - milk samplings and surveys - were performed on the 74 GF 5 times  
133 throughout 2008, over different periods depending on the country: twice during the wintering  
134 season, from January to March (winter1) and from February to May (winter2) and 3 times  
135 during the grazing season from May to June (spring), from June to July (summer) and mostly  
136 in September (autumn) (Table 1). Milk samples were collected from two or four successive  
137 milkings: in France and Slovenia, herd milk samples were equally pooled, (which gives the  
138 same impact regardless of milk supply from individual farms) and in Norway and Slovakia,  
139 milk samples were directly collected in the collecting tanker truck (which results in proportional  
140 contribution to the bulk milk). Each of the 360 samples that were collected (10 missing samples)  
141 was then divided in 3 subsamples devoted to the analysis of minerals, vitamins and fatty acids.  
142 They were frozen without a preserving agent at  $-20^{\circ}\text{C}$  and stored until further analysis.  
143 Surveys were carried out to record the milk production conditions, particularly the cow's diets,  
144 to be further linked to the milk characteristics. The national milk recording organizations  
145 provided milk yield according to the International Committee for Animal Recording standards.

## 146 2.2 Choice of milk compounds and analyses

147 The selected milk compounds were chosen among those used by the World Health  
148 Organization's published human nutritional requirements (WHO and FAO 2004; FAO 2010):  
149 Ca, Mg, P, Zn, vitamin A, the sum of n-3 FA (C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3),  
150 oleic acid, the sum of n-6 FA (C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, and  
151 C22:4n-6), the sum of C12:0, C14:0 and C16:0 (denoted C12-14-16), and finally, the sum of  
152 the *trans* FA (t9-C14:1; t11-C16:1; t4-, t5-, t6/7/8-, t9-, t10-, t11- and t12-C18:1; t9t12-C18:2;  
153 c9t13-C18:2; c9t14-C18:2; c9t12-C18:2; t9c12-C18:2; t11c15-C18:2; t11t13-Conjugated

154 Linoleic Acid (CLA), tt-CLA and c9t11-CLA). Minerals, vitamins and fatty acids were  
155 analysed by three laboratories respectively at the end of the collection period.

### 156 2.2.1 Minerals

157 Frozen milk samples were thawed overnight and homogenised by shaking and sonication at  
158 room temperature for 1 h. Closed microwave-assisted digestion (ETHOS 1600 microwave lab  
159 station, Milestone, Bergamo, Italy) was performed on 1,000 g of a separate sample using 7 mL  
160 of 65 % nitric acid (HNO<sub>3</sub>, SUPRAPUR, Merck, Darmstadt, Germany) plus 1 mL of 30 %  
161 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, SUPRAPUR, Merck, Darmstadt, Germany) in PTFE vessels.  
162 Digested samples were diluted to 50 mL with MILLI-Q grade water. The concentrations of Ca,  
163 Mg and Zn in the digested milk samples were determined by flame atomic absorption  
164 spectroscopy (FAAS, AAnalyst 800, Perkin Elmer, Germany) as described in ISO 8070:(2007)  
165 and ISO 11813:(1998). The concentrations of P were determined by measuring the absorbance  
166 of a molybdenum-phosphate complex at 430.0 nm on a UV/VIS spectrometer (Cary 100,  
167 Varian, Mulgrave, Australia). For each mineral, the instrument was calibrated with 5 calibration  
168 solutions that were prepared by the dilution of commercial standard solutions (1,000 mg·L<sup>-1</sup> Ca,  
169 1,000 mg·L<sup>-1</sup> Mg, 1,000 mg·L<sup>-1</sup> Zn and 10,000 mg·L<sup>-1</sup> P, all CERTIPUR, Merck, Darmstadt,  
170 Germany). To control for contamination, a blank test was performed in each series. To confirm  
171 the accuracy of the analysis, a certified reference material, skim milk powder (CRM 063R  
172 Commission of the European Communities, Community Bureau of Reference, Brussels,  
173 Belgium), was also analysed in each series of digestions.

### 174 2.2.2 Vitamin A

175 Two volumes of milk were hydrolysed with three volumes of 12.5 M KOH/ethanol (2:1 v/v)  
176 for 25 min at 80 °C. BHT was added as an antioxidant. After cooling, retinol was extracted with  
177 hexane/toluene (1:1 v/v). The extract was then subjected to chromatographic analysis on an HP  
178 1100 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) equipped with an HP

179 1100 fluorescence detector, em: 325 nm ex: 480 nm. Vitamin A compounds were separated on  
180 a 4.6 mm × 100 mm normal phase silica column using 2 % 2-propanol in hexane as the mobile  
181 phase. A three-point calibration curve was used for quantification.

### 182 2.2.3 Fatty acids

183 Milk samples were lyophilised (Thermovac<sup>TM</sup>-20, Froilabo, Ozoir-la-Ferriere, France). The  
184 FA in lyophilised milk samples were methylated directly according to Ferlay et al. (2008). The  
185 FA methyl esters (FAME) were injected by auto-sampler into a Trace-GC 2000 series gas  
186 chromatograph that was equipped with a flame ionization detector (Thermo Finnigan, Les Ulis,  
187 France). The FAMEs were separated on a 100 m × 0.25 mm i.d. fused silica capillary column  
188 (CP-Sil 88, Chrompack, Middelburg, Netherlands). The injector temperature was maintained  
189 at 255 °C, and the detector temperature was maintained at 260 °C. The initial oven temperature  
190 was held at 70 °C for 1 min, increased to 100 °C at 5 °C min<sup>-1</sup> (held for 2 min) and then to  
191 175 °C at 10 °C min<sup>-1</sup> (held for 42 min) and 5 °C min<sup>-1</sup> to a final temperature of 225 °C (held  
192 for 15 min). The carrier gas was hydrogen. The pressure was maintained constant during the  
193 analysis. Peaks were routinely identified by retention time comparisons with commercial  
194 authentic standards containing a mixture of FAMEs (NCP #463, Nu Check Prep, Elysian, USA;  
195 Supelco #37, Supelco, Bellefonte, PA, and O5632, Sigma).

## 196 2.3 Data Analysis

### 197 2.3.1 Typology of GF according to the milk composition throughout the year

198 **An innovative approach combining** principal component analysis (PCA) and hierarchical  
199 cluster analysis (HCA) was performed to classify the **74** GF on the basis of the changes in the  
200 milk composition throughout the year (SPAD 7.4.56. Software for Data Mining, Data Analysis  
201 and Text Mining, Coheris SPAD, Suresnes, France). Using a standard PCA, the original set of  
202 50 variables, consisting of 10 milk compounds measured 5 times, was transformed into a small  
203 number of uncorrelated variables known as principal components (PC) that retain as much of

204 the information in the original variables as possible. HCA was applied to investigate nearness  
205 or similarities between GF, **by maximizing** intra-group homogeneity and inter-group diversity.  
206 The significance of the differences between classes for each of the 10 selected milk compounds  
207 was assessed using linear mixed model analysis (PROC MIXED, SAS, 2006-2010) in which  
208 the GF class was considered as a fixed factor, the sampling period as a repeated factor, and the  
209 GF as a random factor. The interaction between GF class and sampling period was included in  
210 the model.

### 211 2.3.2 Characterization of cow husbandry conditions

212 Cow husbandries were described by the location (country and altitude), lactating cows'  
213 characteristics (breed and daily milk yield), **and by the diet fed at each sampling period**. The  
214 diets were described using the database of the Norwegian Agricultural Authority or, in other  
215 countries, using the percentages of the different feeds as calculated according to the farmers'  
216 declarations. In France and Slovenia, an average diet was calculated during each period for each  
217 GF. When an ingredient was not quantified (pasture grass in particular), its quantity was  
218 estimated as the difference between the energy requirements of the herd and the energy that  
219 was provided by the known amounts of feed that were offered in the diet, assuming that the  
220 energy balance of the animals was zero. The maintenance requirements were estimated  
221 according to the average weight of each breed and milk production requirements according to  
222 the milk yield. The animal requirements and energy value of feeds were assigned according to  
223 the national feeding systems of each country. Among the variables describing the cow  
224 husbandry, the altitude (of the farmstead) and cow breed (proportions of the cows of the nine  
225 breeds) were considered constant over the year (i.e., 11 variables). One-way ANOVA was  
226 performed on these latter variables to determine the significance of the differences between the  
227 4 GF classes. In contrast, the cow's diet was described 5 times a year by the proportion of hay,  
228 grass silage, maize silage and concentrate during the winter season and by the proportion of the



229 same feeds plus pasture for the grazing season. The significance of the differences between the  
230 4 GF classes for these diet variables and for the milk yield (average production / day / cow) was  
231 assessed using linear mixed model analysis (PROC MIXED, SAS, 2006-2010) in which the GF  
232 class was considered as a fixed factor, the sampling period as a repeated factor and the GF as a  
233 random factor. The interaction between GF class and sampling period was included in the  
234 model.

### 235 **3 Results**

#### 236 **3.1 Bulk milk composition and changes throughout the year**

237 The average composition and variability of the 360 analysed milks are described in Table 2.  
238 The coefficient of variation (CV) was low (< 10 %) for Mg and P and slightly higher for Ca,  
239 Zn and vitamin A (CV ranged from 13 to 18 %). Concerning FA, the variability of C12-14-16  
240 and oleic acid was considerably lower (CV < 10 %) than that of the other FA that were  
241 considered (CV between 20 and 36 %).

242 The results of PCA are reported in Fig. 1. The n-3 FA at the 5 sampling periods were associated  
243 to *trans* FA during the grazing season on the negative part of PC1 (20.5% of the total variability-  
244 TV) and were opposed to n-6 FA at the 5 sampling periods and Zn in the autumn on the positive  
245 part of PC1. PC2 (14.2% TV) differentiated *trans* FA during the winter and oleic acid at the 5  
246 sampling periods, from C12-14-16 at the 5 sampling periods. PC3 (9.2 % TV) mainly opposed  
247 Mg to n-6 FA at the 5 sampling periods, and PC4 (8.2 % TV) distinguished mainly Ca at the 5  
248 sampling periods (data not shown). HCA, performed on 74 GF coordinates on the 4 first PC of  
249 the PCA (57.50 % TV), split the GF into 4 classes. Classes 1 and 4, with 13 and 24 GF  
250 respectively, were projected on each side of PC1 and classes 2 and 3, with 23 and 14 GF  
251 respectively, on each side of PC2 (Fig. 1). PC3 differentiated the classes 3 and 4 and PC4  
252 differentiated the classes 2 and 4 (data not shown).



253 Significant differences in the contents ( $P < 0.001$ ) between the 4 GF classes were observed for  
254 all 10 of the milk compounds (Fig. 2). The sampling period had a significant effect for all of  
255 the compounds, except for n-6 FA ( $P < 0.05$ ), and the interaction between the class and the  
256 period had significant effects ( $P < 0.01$ ) for all compounds, except for n-6 FA and oleic acid.  
257 Milks produced from class 1 had the highest content of n-3 FA throughout the year and the  
258 highest *trans* FA content in the grazing season. They had low oleic acid and n-6 FA contents  
259 throughout the year. The milks from class 1 presented the highest contrasting composition  
260 throughout the year, with the strongest decrease in C12-14-16 content and the highest increase  
261 in *trans* FA content when cows turned out to grazing. They had lower P and vitamin A contents  
262 than those of other classes and were richer in Ca, particularly in the spring. Milks from class 2  
263 were relatively rich in C12-14-16 and had an intermediate content in n-3 FA. They were  
264 characterized by low mineral contents, particularly for Ca and Mg. The contents of the different  
265 compounds in milks from class 3 remained relatively constant throughout the year. The C12-  
266 14-16 contents were low, while oleic acid, Mg and vitamin A contents were globally high.  
267 Milks from class 4 contained the highest amount, throughout the year, in n-6 FA and Zn (except  
268 in winter<sup>1</sup>) as well as in C12-14-16 during the grazing season. Taking into account the overall  
269 mineral contents, the milks from class 4 presented the most balanced mineral contents.

### 270 3.2 Cow husbandry conditions associated with GF classes

271 The diet changes throughout the year according to the GF classes are described in Figure 3. The  
272 diets were significantly different between the classes ( $P < 0.005$ , not shown), and as expected,  
273 the diets changed throughout the year ( $P < 0.005$ ) with an interaction between the period and  
274 class for all feeds ( $P < 0.005$ ). In class 1, the cow's diets were based on conserved grass in the  
275 winter (52 % hay, 20 % grass silage) and fresh grass in the grazing season (77 %), associated  
276 with concentrate (20 % in the winter and 12 % during the grazing season, respectively). In class  
277 3, the cow's diets were also principally based on grass, grass silage during the winter (51 %)

278 and fresh grass during the grazing season (55 %), and concentrate was distributed throughout  
279 the year (40 %). In classes 2 and 4, maize silage represented a significant proportion of forages  
280 in the diet: 20 % in the winter and 12 % in the grazing season for class 2 and 33 % in the winter  
281 and 25 % in the grazing season for class 4. The maize silage was mainly associated during the  
282 winter with grass silage (35 and 25 % for classes 2 and 4, respectively) and in the grazing season  
283 with fresh grass (45 and 23 % in classes 2 and 4, respectively). The concentrate was distributed  
284 throughout the year, 22 and 28 % on average for classes 2 and 4, respectively. The other  
285 production conditions are listed in Table 3. In class 1, GF were mainly located in France (70 %),  
286 where 8 cow breeds were used, and the milk yield was significantly the lowest (17.1 kg per cow  
287 and per day). The GF from class 2 were widespread in Slovenia (48 %), France (26 %) and  
288 Norway (26 %). The major breeds were Holstein, Norwegian Red, Simmental and Brown, with  
289 an intermediate milk yield (20.2 kg per cow and per day). Class 3 was mainly represented by  
290 Norwegian farms with 86 % of Norwegian Red cattle. Slovakian GF constituted the main part  
291 (75 %) of class 4. Holstein was the major breed, followed by the Slovak Spotted (57 and 29 %  
292 of the total cows, respectively). The milk yield was the highest in both classes 3 and 4 (24.3  
293 and 25 kg per cow and per day, respectively).

294 A slight variation of milk yield was recorded in the spring (turn out to grazing): there was an  
295 increase in classes 1 and 4 and a decrease in class 2. The milk yield remained stable throughout  
296 the year for class 3 (data not shown).

## 297 **4 Discussion**

### 298 4.1 Milk mineral composition

299 The average mineral contents found in our study were within the range of those reported in herd  
300 milk by Rodriguez Rodriguez et al. (2001) and Lucas et al. (2006) and in dairy plant milk by  
301 Poulsen et al. (2015), except for Mg, which was 10 to 20 % higher in this study. The variability  
302 of the mineral contents of our bulk milk (pooled in transport tankers) was often close to that

303 observed with herd milks in the literature. This variability highlights the possibility of  
304 distinguishing milks according to their mineral composition.

305 In our study, the 4 mineral contents in the different classes fluctuated according to different  
306 patterns throughout the year and did not show a general trend (Fig. 2). In contrast, Poulsen et  
307 al. (2015) reported that the contents of Ca, Mg, and P were strongly associated and all showed  
308 similar seasonal variations, potentially due to their common metabolic pathway. These authors  
309 reported for Ca, Mg and P a positive correlation with milk protein content and noted the lowest  
310 values in the summer (July and August) and the highest values in winter (November to  
311 February). Similarly, Hurtaud et al. (2014) showed a common trend for the changes in Ca  
312 content in herd milk with the lowest values found in May and the highest in January. These  
313 seasonal patterns could be linked to block calving practices but the data are not reported in these  
314 studies. In our case, the average lactation stage ranges from 158 to 202 days according to the  
315 sampling period and to the GF class (data not shown). This difference seems too limited to  
316 affect mineral content in milk (Gaucheron 2005). It is nevertheless noticeable that similarly to  
317 Poulsen (2015) milk Ca and protein contents were positively correlated within the GF  
318 ( $P < 0.001$ , data not shown): it certainly reflects the functional link between Ca and the casein  
319 micelles. Hurtaud et al. (2014) suggested that the seasonal Ca content changes may be due to  
320 day length and hot temperature. Boudon et al. (2016), in an experimental study, demonstrated  
321 indeed that the Ca content significantly decreased during long and sunny days compared to  
322 short days. In our study, the average Ca content was also significantly lower in long vs. short  
323 days (1391 and 1470 mg·kg<sup>-1</sup>, respectively) but we did not evidence that according to the GF  
324 latitude (highly variable in our design) the higher the difference between long and short days  
325 was, the greater the seasonal difference of milk Ca content was (data not shown). Considering  
326 that all the minerals in animal body and products come exclusively from the diet, the mineral  
327 content in milk could be also affected by the nutritional status of dairy cow (Gaucheron 2005).

328 Nevertheless, little is known on the possible effect of minerals intake on milk composition.  
329 Maize is an extremely poor source of minerals compared to grass and the mineral composition  
330 of the herbage differs greatly according to the species and their phenological stage. For  
331 example, the Ca content in plant is higher in legumes than in grasses (Pirhofer-Walzl et al.  
332 2011). In our study, the richness in minerals of the milks from class 4 could be linked with a  
333 large proportion of clover and alfalfa in the forage and concentrate distributed in GF from  
334 Slovakia. It can also be noted that on pasture, a high content of clover may change the dynamics  
335 of milk minerals compared to grass only swards because the proportion of clover change  
336 substantially throughout the season, being highest in autumn. Moreover, the high Mg contents  
337 of milks from class 3 could be associated with the abundance in herbs in half of the GF, as herbs  
338 are particularly rich in this mineral. The low variability of the mineral contents in milks from  
339 class 3 could also be related to the constant and significant amount of commercial concentrates,  
340 which generally are enriched with minerals. In our study, it is nevertheless difficult to elucidate  
341 the effect of diet on milk minerals because of probable confounded effects with mineral  
342 supplementation, fertilization practices, pedo-climatic context and cow breed. These different  
343 effects cannot be segregated with our dataset. A breed effect on the milk mineral content has  
344 also been reported (Poulsen et al. 2015). In our study, in the sole situation where two breeds  
345 were reared in the same country (Slovakia) with similar diets, we did not found any difference  
346 between Holstein and Slovak Spotted breeds on the mineral contents in milk.

#### 347 4.2 Milk vitamin A composition

348 The vitamin A contents that were measured in our study were approximately 40 % lower than  
349 the average value reported by Lucas et al. (2006) and Agabriel et al. (2007) in bulk milk, and  
350 within the range of those reported by Martin et al. (2002) in an experimental study. The  
351 observed annual change in vitamin A content is consistent with the literature: it increased  
352 between the winter and grazing periods, simultaneously with the increasing proportions of fresh

353 herbage in the diet (Fig. 2 and 3). This high milk content in vitamin A is explained by the higher  
354  $\beta$ -carotene content in fresh grass than in all conserved forages (Nozière et al. 2006). Indeed, the  
355  $\beta$ -carotene acts as the main precursor of vitamin A for ruminants (Nozière et al. 2006). During  
356 the winter season, we observed a large diversity in the vitamin A content in milk. The highest  
357 contents were associated with diets including a major proportion of both grass silage and  
358 concentrate (Fig. 2 and 3, class 3). The grass silage contains 3 times more carotenoids than hay  
359 which increase vitamin A in milk. In addition, commercial concentrate is very often enriched  
360 with vitamin A, which is directly transferred into the milk (Nozière et al. 2006). On the opposite,  
361 the lowest vitamin A contents were associated with diets including a large part of hay, which  
362 contain very few carotenoids and a low proportion of concentrate (Fig. 2 and 3, class 1).

#### 363 4.3 Milk fat composition

364 The effect of diet on FA concentrations in milk has been clearly shown under experimental  
365 conditions (Chilliard et al. 2007; Shingfield et al. 2013) and validated on commercial farms  
366 (Ferlay et al. 2008; Slots et al. 2009; Borreani et al. 2013). Coppa et al. (2013) have even  
367 predicted bulk milk FA composition using data describing farming practices via on-farm  
368 surveys. Our results, at the level of a large-scale design, are completely consistent with these  
369 publications.

370 In our study, the average content of C12-14-16 was consistent with those of Ferlay et al. (2008)  
371 and Heck et al. (2009), higher than those observed by Collomb et al. (2008) and O'Donnell-  
372 Megaro et al. (2011) and lower than that shown by Butler et al. (2011). The lower content of  
373 C12-14-16 reported by Collomb et al. (2008) (34.9 and 41.1 g·100 g<sup>-1</sup> fat in the summer and in  
374 the winter, respectively) could be explained by the fact that cows were fed diets high in grass  
375 (74% of fresh grass in the summer and 69 % of hay in the winter) from the Swiss mountains.  
376 Feeding the cows with these diets, which are rich in polyunsaturated fatty acids (PUFA),  
377 explains the decrease in these SFA, via an inhibition of *de novo* mammary synthesis of all

378 C12:0, C14:0 and half of C16:0 by *trans* FA resulting from the rumen biohydrogenation of  
379 PUFA ingested (Chilliard et al. 2007). This mechanism could explain the low C12-14-16  
380 content observed in our study during summer in class 1, where the proportion of grazed grass  
381 is very high (Fig. 2 and 3). Moreover, O'Donnell-Megaró et al. (2011) associated the low  
382 content of C12-14-16 they found in dairy plant milk (40.5 g·100 g<sup>-1</sup> fat) to the low forage diet  
383 and the routinely use of lipid supplement and by-product in the US. The unsaturated FA from  
384 the latter feedstuffs as well as the reduction in forage intake depress the *de novo* mammary  
385 lipogenesis, thereby reducing the SFA content in milk (Chilliard et al. 2007). Similar  
386 hypotheses could explain the low values of C12-14-16 found in our study for the class 3 mainly  
387 made of Norwegian farms where cow diets included a high and constant proportion of  
388 commercial concentrate (40%) comprising fat added from different rape products (70-75 % of  
389 the fat added). In the UK, Butler et al. (2011) proposed that the high C12-14-16 level (50.3  
390 g·100 g<sup>-1</sup> fat) in some of their dairy plant milks could be associated with the wide use of  
391 commercial feed products, including palm oil, a practice known to increase the C16:0  
392 concentration in milk (Larsen et al., 2010).

393 The average values of oleic acid that were observed in our study were higher than the average  
394 values that were observed by Collomb et al. (2008) and Heck et al. (2009) (+ 1.9 and + 1.4  
395 g·100 g<sup>-1</sup> fat, respectively). Our findings were consistent with those of Ferlay et al. (2008), and  
396 they were lower than those observed by Butler et al. (2011) in conventional milks collected in  
397 retail outlets and particularly by O'Donnell-Megaró et al. (2011) (-2.9 and -4.6 g·100 g<sup>-1</sup> fat,  
398 respectively), who linked this high milk oleic acid content with dietary lipid supplements.  
399 Moreover, Glasser et al. (2008) have shown that milk oleic content increased significantly with  
400 lipid supplements rich in long chain unsaturated FA (with atoms of carbon  $\geq 18$ ). The high milk  
401 concentration of oleic acid shown by O'Donnell-Megaró et al. (2011) could be explained by  
402 the fact that this FA is an intermediate of ruminal biohydrogenation of unsaturated FA from

403 dietary lipid supplements. Milk oleic acid results also from the mammary  $\Delta$ -9 desaturation of  
404 stearic acid which is the end-product of the ruminal biohydrogenation of dietary lipids  
405 (Chilliard et al. 2007). In our study, the incorporation of lipids from rape (rich in oleic acid) in  
406 Norwegian commercial concentrates is probably the main reason why milk oleic acid content  
407 is constantly the highest across the year (class 3).

408 The n-3 FA average contents in milk from our study were comparable to the annual average  
409 values that were observed by Butler et al. (2011) and van Valenberg et al. (2013). In contrast,  
410 Ferlay et al. (2008) and Collomb et al. (2008) reported a higher n-3 FA content (+0.19 and  
411 +0.45 g·100 g<sup>-1</sup> fat, respectively) with grass-based diets. Similarly, the highest n-3 FA contents  
412 in milk observed all over the year for class 1 was associated with the highest proportion of grass  
413 in the diet (hay and grass silage during winter and pasture during summer) and low amount of  
414 concentrate. Grass-based diets are rich in C18:3 n-3 and therefore are known to increase the n-  
415 3 FA milk content up to 5 times when fresh grass is compared to maize-silage diet (Ferlay et  
416 al. 2006). In addition, Collomb et al. (2008) also suggested that the mountain location could  
417 reinforce the effect of grass due to a combined effect of altitude via cow hypoxia and of  
418 botanical diversity of the permanent grassland. The latter may provide plant secondary  
419 compounds (terpenoids and phenolic compounds) able to limit rumen biohydrogenation and  
420 therefore increase milk C18:3 n-3 content. Indeed, in this study, the GF located at the highest  
421 altitude are all included in class 1.

422 The milk in our study had n-6 FA contents that were comparable to those observed by Ferlay  
423 et al. (2008) and van Valenberg et al. (2013) and lower than those that were found by Butler et  
424 al. (2011) (-0.3 g·100 g<sup>-1</sup> fat). These latter milks were produced in conventional farms where  
425 cows could have been fed high-maize silage diets. In our study, the highest n-6 FA contents  
426 were associated with diets including the highest part of maize silage (Fig. 2 and 3, class 4). This  
427 fodder is rich in C18:2 n-6 (nearly 1.5 % DM) and therefore increases milk C18:2 n-6 content



428 (Chilliard et al. 2007). Moreover, Slots et al. (2009) have highlighted that the n-6 FA milk  
429 contents were positively linked to commercial concentrate mix. In contrast to our results, they  
430 showed no correlation between the maize silage intake (10 to 30 % of DMI) and the n-6 FA  
431 content in the milk.

432 The average *trans* FA content in our milks was equivalent to that reported by Ferlay et al.  
433 (2008). No other comparison could be made, because the method of calculation for the *trans*  
434 FA sum was different according to the authors and all of the data were not available.  
435 Considering the changes in *trans* FA content throughout the year, our results agree with the  
436 literature, i.e., a strong elevation in milk *trans* FA content **observed in class 1** when cows grazed  
437 pasture (Butler et al. 2008; Ferlay et al. 2008; Collomb et al. 2008; Larsen et al. 2010). **The**  
438 **richness in C18:3 n-3 of pasture could explain the ruminal production of *trans* FA from ruminal**  
439 **biohydrogenation of C18:3 n-3, and then the high milk content of *trans* FA. The higher levels**  
440 **of *trans* FA during grazing than winter season for class 1 could also be reinforced by the**  
441 **phenological stage of the grass (more digestible lush grazed grass versus mature conserved**  
442 **forage) that influences the ruminal biohydrogenation pathway of PUFA (Chilliard et al. 2007).**  
443 In addition, we observed that *trans* FA content did not increase with pasture diets including a  
444 large part of concentrate (Fig 2 and 3, class 3) and therefore a least part of grazed grass in the  
445 diet.

446 Moreover, we have highlighted 3 out of 4 classes of GF mainly gathering farms located in the  
447 same country. This result could indicate that specific production conditions exist within the  
448 country and affect the milk characteristics, as shown by Gaspardo et al. (2010), with  
449 neighbouring Italian and Slovenian farms having similar practices.

#### 450 4.4 Nutritional interest of bulk milk according to GF classes

451 For minerals, in order to compare the nutritional interest of our milks according to the RNI, we  
452 only considered the average daily liquid milk consumption in the European Union, i.e., 170 mL



453 in 2012 (IDF 2013). Indeed, it was difficult to estimate the total mineral intake from all dairy  
454 products then anyway, when milk is transformed, a variable fraction of minerals moves from  
455 milk to whey according to the cheese-making process (range from 1 to 10 for Ca, for example)  
456 (Feinberg et al. 1987). As the mineral content of most dairy products is driven by the process,  
457 the variability of the mineral content of milk affects human mineral intake mainly through the  
458 consumption of liquid and fermented milk like yoghurt. The total fat dairy intake can be  
459 estimated more easily because most of the processes don't modify the fat composition (Lucas  
460 et al., 2006). In Europe, it approximates 18 g per day and per capita (IDF 2013).

461 According to the GF classes, the average supply of the RNI (WHO and FAO 2004; FAO 2010)  
462 ranged from 23.6 % (class 2) to 25.8 % (classes 1 and 4) for Ca, from 18.4 % (class 4) to 28.7  
463 % (class 1) for *trans* FA, from 4.4 % (class 4) to 8.8 % (class 1) for n-3 FA and from 44.6 %  
464 (class 3) to 48.6 % (class 4) for C12-14-16. On the opposite, the average supplies of the RNI  
465 for P, Mg, Zn, vitamin A, n-6 FA, and oleic were almost similar for the different classes  
466 (maximum difference about 1%). Therefore, the proposed GF classification seems meaningful  
467 from a nutritional point of view only for Ca, n-3 FA, C12-14-16 and *trans* FA. Considering  
468 these four compounds we underline the absence of milks with the highest nutritional interest in  
469 order to reduce simultaneously the *trans* FA and C12-14-16 intake and to increase the n-3 FA  
470 and Ca intake. For example, the choice of milks produced by farms from class 1 seems relevant  
471 to increase the intake of Ca and n-3 FA throughout the year and to reduce the C12-14-16 intake  
472 during the pasture season but meanwhile it dramatically increases the *trans* FA intake. We have  
473 nevertheless to consider that in class 1 *trans* FA mainly consist in vaccenic acid (data not shown)  
474 which is converted to rumenic acid by desaturation in human metabolism. Rumenic acid is only  
475 found in ruminant fat and has been linked to many potential health benefits. The choice of milks  
476 from class 3 could be a lever to reduce the C12-14-16 intake throughout the year but it is  
477 irrelevant for Ca and n-3 FA supplies. Finally the potential nutritional impact of the milk

478 composition differences found here needs to be evaluated in the context of the overall diet. For  
479 heavy consumers of dairy product the effects previously highlighted could be reinforced.

480

## 481 **5 Conclusion**

482 Based on a wide-ranging framework, this study investigated changes throughout the year in the  
483 contents of bulk milk compounds with nutritional requirements. The approach focused on a set  
484 of minerals, fat-soluble vitamins and FA with the associated husbandry conditions. We  
485 observed that the Ca, Mg, P and Zn contents evolved in different ways throughout the year, and  
486 they evolved independently of each other. We showed the absence of milks with the highest  
487 contents in these four minerals simultaneously, except in the autumn. Moreover, we identified  
488 GF where milk had high content in Mg or in Zn throughout the year. We confirmed that the FA  
489 profile and the vitamin A contents remained strongly associated with the diet composition. We  
490 also highlighted that no GF produce milks with a significant contribution to the 5 FA  
491 requirements, independent of the sampling dates. Our results suggested that groups of farms  
492 could be selected to collect milks with some specific characteristics throughout the year or  
493 during some periods. Throughout the year, grass-based diets remained the best method to  
494 produce milk with high n-3 FA content. Maize silage-based diets resulted in milk with high n-  
495 6 FA content while those rich in commercial concentrate including rape induced an increase in  
496 oleic acid content in milk. During the outdoor period, grazing remained clearly the most  
497 effective means with a low C12-14-16 FA content in milk, accompanied by a concomitant  
498 increase in *trans* FA. These FA did not increase with diets rich in concentrate. During the indoor  
499 period, diet with both grass silage and commercial concentrate led to high milk vitamin A  
500 content. Thus, in practice, it is possible for dairy industry to take advantage of the location  
501 collection area and the diversity of the farming systems, to segment the dairy products using

502 individual specific milk compounds. Nevertheless, it was not possible to identify milks relevant  
503 to our health for both mineral and lipid fractions throughout the year.

504

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#### 515 **Conflict of interest**

516 Chantal Chassaing, Cécile Sibra, Jože Verbič, Odd Magne Harstad, Jaroslav Golecký,  
517 Bruno Martin, Anne Ferlay, Isabelle Constant, Carole Delavaud, Catherine Hurtaud, Vida  
518 Žnidaršič Pongrac, and Claire Agabriel declare no conflict of interest.

519 **Compliance with Ethics Guideline** This article does not contain any studies with human or  
520 animal subjects performed by any of the authors.

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642 GF is identified by the class **number (1 to 4)** to which it belongs.

643 Most correlated variables with the factor plan: P (phosphorus); Zn (zinc); n3FA (sum of  
644 C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3); n6FA (sum of C18:2n-6, C18:3n-6, C20:2n-6,  
645 C20:3n-6, C20:4n-6, C22:4n-6); oleic (oleic acid); C12-14-16 (sum of C12:0, C14:0, C16:0);  
646 transFA (sum of t9-C14:1, t11-C16:1, t4-, t5-, t6/7/8-, t9-, t10-, t11- and t12-C18:1, t9t12-  
647 C18:2, c9t13-C18:2, c9t14-C18:2, c9t12-C18:2, t9c12-C18:2, t11c15-C18:2, t11t13-  
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649 (spring); Su (summer); Au (autumn).

650 **Fig. 2** Bulk milk composition throughout the year according to the group of farms classes and  
651 the sampling period

652 Sampling period: **W1** January - March, **W2** February – May, **Sp** May – June, **Su** June – July,  
653 **Au** September

654 Results are represented as adjusted mean and standards errors of means (error bars). The overlap  
655 of two errors bars indicates that values are not significantly different ( $P>0.05$ ). **The significance**  
656 **of the effects of the GF class on milk composition, the sampling period and of the interaction**  
657 **between the period and the class are noted for each compound.**

658 **Fig. 3** Composition of cow's diet at milk sampling times according to the **GF** classes

659 **DMI: Dry matter intake. Sampling period: W1 January - March, W2 February – May, Sp**  
660 **May – June, Su June – July, Au September**

661



1 **Mineral, vitamin A and fat composition of bulk milk related to European production**  
2 **conditions throughout the year**

3 **Short title: Annual changes of bulk milk micronutrients**

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36 **Abstract.** In many Western countries, milk and dairy products provide both mineral and lipid  
37 fractions relevant to our health. The milk contents of these compounds are highly variable and  
38 the identification of husbandry conditions leading to milks favourable for both mineral and lipid  
39 fractions is of interest. The aims of this study were to describe throughout the year the changes  
40 in bulk milk compounds and to relate them to husbandry practices. Ten compounds with  
41 nutritional requirements were selected: a minimum amount is required for Ca, Mg, P, Zn,  
42 vitamin A, n-3 fatty acids (FA), oleic acid and n-6 FA, and a maximum amount for *trans* FA  
43 and C12-14-16 FA. Milks and their associated husbandry practices were collected 5 times  
44 during one year from 74 groups of farms located in France, Norway, Slovakia and Slovenia.  
45 The Ca, Mg, P and Zn contents varied in different ways throughout the year and independently  
46 of each other. We showed the absence of milks with the highest contents in these four minerals  
47 simultaneously but we identified groups of farms where milk had high content in Mg or in Zn.  
48 Positive links have been confirmed between grass-based-diets and milk n-3 FA content, maize  
49 silage-based-diets and n-6 FA content, and diets rich in concentrate and oleic acid content.  
50 These results showed that it was possible to identify husbandry conditions leading to milk  
51 favourable throughout the year for one or several compounds but not for all compounds  
52 considered in this study.

53 **Keywords,** micronutrients, milk composition, nutritional quality, farming practices

## 54 **1 Introduction**

55 Improving the nutritional quality of milk is of concern to current producers, the dairy industry  
56 and consumers. Indeed, the dairy industry and retailers include increasing nutritional data in  
57 their public communication (Menard et al. 2012), and this trend is driven by the increasing  
58 number of well-known links between dairy products and human health (Visioli and Strata  
59 2014). In many Western countries, the contribution of milk and dairy products in a balanced  
60 diet is recognized for the supply of both macro-constituents (proteins, fat and minerals) and  
61 micro-constituents (vitamins and trace elements). Considering the multiple compounds of  
62 interest in milk, the present study will explore, throughout the year, the changes in a set of  
63 nutritional relevant compounds: some minerals, fatty acids (FA) and vitamin A in relation with  
64 the associated husbandry conditions.

65 For minerals, the sole consumption of liquid milk in the European Union (170 mL / day / capita,  
66 IDF 2013) provides approximately 20-25 % of Ca and P and 10 % of Mg and Zn of the  
67 minimum daily amounts that are required for human metabolism (1000 mg of Ca, 750 mg of P,  
68 260 mg of Mg and 7 mg of Zn) (WHO and FAO 2004). The contribution of dairy products to  
69 our fat consumption is also well known. Some FA are considered desirable and a daily  
70 minimum intake is required, while others are usually consumed in excess and, therefore a  
71 limited daily intake is recommended. The n-3 FA (mainly  $\alpha$ -linolenic acid in milk and other  
72 minor long chain n-3 FA) limit metabolic syndrome and type 2 diabetes (Palmquist 2009), and  
73 thus a minimum daily consumption is required (2 g for C18:3n-3, the major n-3 FA) (FAO  
74 2010). For oleic acid, the daily intake may range from 32 to 43 g (FAO 2010). The n-6 FA  
75 (mainly linoleic acid in milk) may activate adipose tissue development, and their daily intake  
76 may range from 4.3 to 8.5 g (FAO 2010). Among saturated FA (SFA), C12:0, C14:0 and C16:0  
77 when consumed in excess may increase the risk of cardiovascular diseases (Shingfield et al.  
78 2008). The daily intake of the sum of these three FA should not exceed 17 g (FAO 2010). The

79 intake of *trans* FA from ruminant products don't seem to increase the risk of cardiovascular  
80 diseases in contrast to *trans* FA produced industrially (Gayet-Boyer et al. 2014). Nevertheless,  
81 for the FAO (2010), the daily *trans* FA intake (typically isomers of C18:1 *trans* derived from  
82 partially hydrogenated vegetable oils) should not exceed 1 % of the energy intake i.e. 2.4 g.  
83 Based on an average daily consumption of 18 g per capita of dairy fat in the European Union  
84 (estimated from IDF, 2013) and on the fat composition reported by Coppa et al. (2013), dairy  
85 fat provides approximately 6 % and 3 % of the recommendations for n-3 and n-6 FA, 9 % for  
86 oleic acid and up to 47 % and 22 % for the sum of C12:0, C14:0, C16:0 and *trans* FA,  
87 respectively. Dairy fat also provides lipophilic vitamins, in particular one-third of the vitamin  
88 A daily recommendation, which is 600 µg Eq retinol (WHO and FAO 2004).

89 The milk concentrations of all these compounds are highly variable throughout the year and  
90 vary according to the husbandry conditions. For dairy industries, there is an interest to  
91 characterize this variability and its variation factors. They are partially described for minerals  
92 and are well known for FA and vitamin A. The milk mineral content varies mainly according  
93 to the lactation stage and the nutritional and health status of the dairy cows (Gaucheron 2005),  
94 whereas the vitamin A content is mainly affected by the nature of the forage, breed or calving  
95 period (Nozière et al. 2006). The concentrations of the various FA change according to the  
96 cow's diet and to a lesser extent to animal characteristics, such as breed and lactation stage  
97 (Chilliard et al. 2007). Among the mentioned factors, the cow's diet and lactation stage have a  
98 short-term effect on milk composition (< 1 month). Therefore, changes in the farmer's practices  
99 throughout the year result in seasonal changes in bulk milk composition.

100 The variations in milk mineral contents or fat composition over one year have been described,  
101 in field studies, in herd milks (Rodriguez Rodriguez et al. 2001) or in dairy plant milks in  
102 Denmark (Poulsen et al. 2015), the Netherlands (Heck et al. 2009; van Valenberg et al. 2013),  
103 the US (O'Donnell-Megaró et al. 2011) and the UK (Butler et al. 2011). The relationships

104 between milk minerals and production systems have rarely been studied in on-farm conditions  
105 (Hurtaud et al. 2014). In contrast, many authors have reported changes throughout the year in  
106 milk fat composition (Butler et al. 2008; Ferlay et al. 2008; Collomb et al. 2008; Larsen et al.  
107 2010; Borreani et al. 2013) or in fat-soluble vitamins (Agabriel et al. 2007; Ellis et al. 2007)  
108 according to the production systems. To the best of our knowledge, no study has investigated  
109 concomitantly minerals, FA and vitamin A in bulk milk and the associated husbandry  
110 conditions. Thus, the identification of husbandry conditions leading to the production of milks  
111 favourable for both mineral and lipid fractions throughout the year is the challenge of this study.  
112 Our strategy to achieve this aim is (1) to classify farms on the basis of the changes in mineral,  
113 vitamin A contents and fat composition in milk throughout the year and for each class, (2) to  
114 characterize the milk changes across the year and (3) to explain these changes according to the  
115 husbandry conditions.

## 116 **2 Materials and methods**

### 117 2.1 Study design

118 In our study, the bulk milk composition and the associated production conditions are studied at  
119 the scale of a group of farms (GF). In this manner, 74 GF were selected in France, Norway,  
120 Slovakia and Slovenia (Table 1) due to their large diversity of cow husbandries. Farms of each  
121 GF were geographically close and had similar milk production conditions. The different GF  
122 were located with a latitude range of 45.5 to 69.5°N and an altitude ranging from 40 to 1,400  
123 m.a.s.l. The dairy cow diets were very diverse, ranging from diets based on grass (grass silage,  
124 hay or pasture according to the season) to diets including a large part of maize silage throughout  
125 the year. The proportion of concentrates ranged from nearly zero to more than 50 % of the dry  
126 matter intake (DMI). The annual milk yield ranged from approximately 3,000 to more than  
127 9,000 kg·cow<sup>-1</sup>·year<sup>-1</sup>. The Holstein breed was the most common, with 22 GF having herds of  
128 more than 50 % Holstein cows. The Norwegian Red was the only breed that was present in

129 Norway, while the Slovak Spotted and Holstein breeds were found in Slovakia (Table 1). Six  
130 other breeds (Brown, Montbeliarde, Simmental, Normande, Tarentaise and Abondance) were  
131 only present in France or in Slovenia.

132 Repeated measures - milk samplings and surveys - were performed on the 74 GF 5 times  
133 throughout 2008, over different periods depending on the country: twice during the wintering  
134 season, from January to March (winter1) and from February to May (winter2) and 3 times  
135 during the grazing season from May to June (spring), from June to July (summer) and mostly  
136 in September (autumn) (Table 1). Milk samples were collected from two or four successive  
137 milkings: in France and Slovenia, herd milk samples were equally pooled, (which gives the  
138 same impact regardless of milk supply from individual farms) and in Norway and Slovakia,  
139 milk samples were directly collected in the collecting tanker truck (which results in proportional  
140 contribution to the bulk milk). Each of the 360 samples that were collected (10 missing samples)  
141 was then divided in 3 subsamples devoted to the analysis of minerals, vitamins and fatty acids.  
142 They were frozen without a preserving agent at  $-20^{\circ}\text{C}$  and stored until further analysis.  
143 Surveys were carried out to record the milk production conditions, particularly the cow's diets,  
144 to be further linked to the milk characteristics. The national milk recording organizations  
145 provided milk yield according to the International Committee for Animal Recording standards.

## 146 2.2 Choice of milk compounds and analyses

147 The selected milk compounds were chosen among those used by the World Health  
148 Organization's published human nutritional requirements (WHO and FAO 2004; FAO 2010):  
149 Ca, Mg, P, Zn, vitamin A, the sum of n-3 FA (C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3),  
150 oleic acid, the sum of n-6 FA (C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, and  
151 C22:4n-6), the sum of C12:0, C14:0 and C16:0 (denoted C12-14-16), and finally, the sum of  
152 the *trans* FA (t9-C14:1; t11-C16:1; t4-, t5-, t6/7/8-, t9-, t10-, t11- and t12-C18:1; t9t12-C18:2;  
153 c9t13-C18:2; c9t14-C18:2; c9t12-C18:2; t9c12-C18:2; t11c15-C18:2; t11t13-Conjugated

154 Linoleic Acid (CLA), tt-CLA and c9t11-CLA). Minerals, vitamins and fatty acids were  
155 analysed by three laboratories respectively at the end of the collection period.

### 156 2.2.1 Minerals

157 Frozen milk samples were thawed overnight and homogenised by shaking and sonication at  
158 room temperature for 1 h. Closed microwave-assisted digestion (ETHOS 1600 microwave lab  
159 station, Milestone, Bergamo, Italy) was performed on 1,000 g of a separate sample using 7 mL  
160 of 65 % nitric acid (HNO<sub>3</sub>, SUPRAPUR, Merck, Darmstadt, Germany) plus 1 mL of 30 %  
161 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, SUPRAPUR, Merck, Darmstadt, Germany) in PTFE vessels.  
162 Digested samples were diluted to 50 mL with MILLI-Q grade water. The concentrations of Ca,  
163 Mg and Zn in the digested milk samples were determined by flame atomic absorption  
164 spectroscopy (FAAS, AAnalyst 800, Perkin Elmer, Germany) as described in ISO 8070:(2007)  
165 and ISO 11813:(1998). The concentrations of P were determined by measuring the absorbance  
166 of a molybdenum-phosphate complex at 430.0 nm on a UV/VIS spectrometer (Cary 100,  
167 Varian, Mulgrave, Australia). For each mineral, the instrument was calibrated with 5 calibration  
168 solutions that were prepared by the dilution of commercial standard solutions (1,000 mg·L<sup>-1</sup> Ca,  
169 1,000 mg·L<sup>-1</sup> Mg, 1,000 mg·L<sup>-1</sup> Zn and 10,000 mg·L<sup>-1</sup> P, all CERTIPUR, Merck, Darmstadt,  
170 Germany). To control for contamination, a blank test was performed in each series. To confirm  
171 the accuracy of the analysis, a certified reference material, skim milk powder (CRM 063R  
172 Commission of the European Communities, Community Bureau of Reference, Brussels,  
173 Belgium), was also analysed in each series of digestions.

### 174 2.2.2 Vitamin A

175 Two volumes of milk were hydrolysed with three volumes of 12.5 M KOH/ethanol (2:1 v/v)  
176 for 25 min at 80 °C. BHT was added as an antioxidant. After cooling, retinol was extracted with  
177 hexane/toluene (1:1 v/v). The extract was then subjected to chromatographic analysis on an HP  
178 1100 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) equipped with an HP

179 1100 fluorescence detector, em: 325 nm ex: 480 nm. Vitamin A compounds were separated on  
180 a 4.6 mm × 100 mm normal phase silica column using 2 % 2-propanol in hexane as the mobile  
181 phase. A three-point calibration curve was used for quantification.

### 182 2.2.3 Fatty acids

183 Milk samples were lyophilised (Thermovac<sup>TM</sup>-20, Froilabo, Ozoir-la-Ferriere, France). The  
184 FA in lyophilised milk samples were methylated directly according to Ferlay et al. (2008). The  
185 FA methyl esters (FAME) were injected by auto-sampler into a Trace-GC 2000 series gas  
186 chromatograph that was equipped with a flame ionization detector (Thermo Finnigan, Les Ulis,  
187 France). The FAMEs were separated on a 100 m × 0.25 mm i.d. fused silica capillary column  
188 (CP-Sil 88, Chrompack, Middelburg, Netherlands). The injector temperature was maintained  
189 at 255 °C, and the detector temperature was maintained at 260 °C. The initial oven temperature  
190 was held at 70 °C for 1 min, increased to 100 °C at 5 °C min<sup>-1</sup> (held for 2 min) and then to  
191 175 °C at 10 °C min<sup>-1</sup> (held for 42 min) and 5 °C min<sup>-1</sup> to a final temperature of 225 °C (held  
192 for 15 min). The carrier gas was hydrogen. The pressure was maintained constant during the  
193 analysis. Peaks were routinely identified by retention time comparisons with commercial  
194 authentic standards containing a mixture of FAMEs (NCP #463, Nu Check Prep, Elysian, USA;  
195 Supelco #37, Supelco, Bellefonte, PA, and O5632, Sigma).

## 196 2.3 Data Analysis

### 197 2.3.1 Typology of GF according to the milk composition throughout the year

198 An innovative approach combining principal component analysis (PCA) and hierarchical  
199 cluster analysis (HCA) was performed to classify the 74 GF on the basis of the changes in the  
200 milk composition throughout the year (SPAD 7.4.56. Software for Data Mining, Data Analysis  
201 and Text Mining, Coheris SPAD, Suresnes, France). Using a standard PCA, the original set of  
202 50 variables, consisting of 10 milk compounds measured 5 times, was transformed into a small  
203 number of uncorrelated variables known as principal components (PC) that retain as much of

204 the information in the original variables as possible. HCA was applied to investigate nearness  
205 or similarities between GF, by maximizing intra-group homogeneity and inter-group diversity.  
206 The significance of the differences between classes for each of the 10 selected milk compounds  
207 was assessed using linear mixed model analysis (PROC MIXED, SAS, 2006-2010) in which  
208 the GF class was considered as a fixed factor, the sampling period as a repeated factor, and the  
209 GF as a random factor. The interaction between GF class and sampling period was included in  
210 the model.

### 211 2.3.2 Characterization of cow husbandry conditions

212 Cow husbandries were described by the location (country and altitude), lactating cows'  
213 characteristics (breed and daily milk yield), and by the diet fed at each sampling period. The  
214 diets were described using the database of the Norwegian Agricultural Authority or, in other  
215 countries, using the percentages of the different feeds as calculated according to the farmers'  
216 declarations. In France and Slovenia, an average diet was calculated during each period for each  
217 GF. When an ingredient was not quantified (pasture grass in particular), its quantity was  
218 estimated as the difference between the energy requirements of the herd and the energy that  
219 was provided by the known amounts of feed that were offered in the diet, assuming that the  
220 energy balance of the animals was zero. The maintenance requirements were estimated  
221 according to the average weight of each breed and milk production requirements according to  
222 the milk yield. The animal requirements and energy value of feeds were assigned according to  
223 the national feeding systems of each country. Among the variables describing the cow  
224 husbandry, the altitude (of the farmstead) and cow breed (proportions of the cows of the nine  
225 breeds) were considered constant over the year (i.e., 11 variables). One-way ANOVA was  
226 performed on these latter variables to determine the significance of the differences between the  
227 4 GF classes. In contrast, the cow's diet was described 5 times a year by the proportion of hay,  
228 grass silage, maize silage and concentrate during the winter season and by the proportion of the



229 same feeds plus pasture for the grazing season. The significance of the differences between the  
230 4 GF classes for these diet variables and for the milk yield (average production / day / cow) was  
231 assessed using linear mixed model analysis (PROC MIXED, SAS, 2006-2010) in which the GF  
232 class was considered as a fixed factor, the sampling period as a repeated factor and the GF as a  
233 random factor. The interaction between GF class and sampling period was included in the  
234 model.

### 235 **3 Results**

#### 236 **3.1 Bulk milk composition and changes throughout the year**

237 The average composition and variability of the 360 analysed milks are described in Table 2.  
238 The coefficient of variation (CV) was low (< 10 %) for Mg and P and slightly higher for Ca,  
239 Zn and vitamin A (CV ranged from 13 to 18 %). Concerning FA, the variability of C12-14-16  
240 and oleic acid was considerably lower (CV < 10 %) than that of the other FA that were  
241 considered (CV between 20 and 36 %).

242 The results of PCA are reported in Fig. 1. The n-3 FA at the 5 sampling periods were associated  
243 to *trans* FA during the grazing season on the negative part of PC1 (20.5% of the total variability-  
244 TV) and were opposed to n-6 FA at the 5 sampling periods and Zn in the autumn on the positive  
245 part of PC1. PC2 (14.2% TV) differentiated *trans* FA during the winter and oleic acid at the 5  
246 sampling periods, from C12-14-16 at the 5 sampling periods. PC3 (9.2 % TV) mainly opposed  
247 Mg to n-6 FA at the 5 sampling periods, and PC4 (8.2 % TV) distinguished mainly Ca at the 5  
248 sampling periods (data not shown). HCA, performed on 74 GF coordinates on the 4 first PC of  
249 the PCA (57.50 % TV), split the GF into 4 classes. Classes 1 and 4, with 13 and 24 GF  
250 respectively, were projected on each side of PC1 and classes 2 and 3, with 23 and 14 GF  
251 respectively, on each side of PC2 (Fig. 1). PC3 differentiated the classes 3 and 4 and PC4  
252 differentiated the classes 2 and 4 (data not shown).

253 Significant differences in the contents ( $P < 0.001$ ) between the 4 GF classes were observed for  
254 all 10 of the milk compounds (Fig. 2). The sampling period had a significant effect for all of  
255 the compounds, except for n-6 FA ( $P < 0.05$ ), and the interaction between the class and the  
256 period had significant effects ( $P < 0.01$ ) for all compounds, except for n-6 FA and oleic acid.  
257 Milks produced from class 1 had the highest content of n-3 FA throughout the year and the  
258 highest *trans* FA content in the grazing season. They had low oleic acid and n-6 FA contents  
259 throughout the year. The milks from class 1 presented the highest contrasting composition  
260 throughout the year, with the strongest decrease in C12-14-16 content and the highest increase  
261 in *trans* FA content when cows turned out to grazing. They had lower P and vitamin A contents  
262 than those of other classes and were richer in Ca, particularly in the spring. Milks from class 2  
263 were relatively rich in C12-14-16 and had an intermediate content in n-3 FA. They were  
264 characterized by low mineral contents, particularly for Ca and Mg. The contents of the different  
265 compounds in milks from class 3 remained relatively constant throughout the year. The C12-  
266 14-16 contents were low, while oleic acid, Mg and vitamin A contents were globally high.  
267 Milks from class 4 contained the highest amount, throughout the year, in n-6 FA and Zn (except  
268 in winter<sup>1</sup>) as well as in C12-14-16 during the grazing season. Taking into account the overall  
269 mineral contents, the milks from class 4 presented the most balanced mineral contents.

### 270 3.2 Cow husbandry conditions associated with GF classes

271 The diet changes throughout the year according to the GF classes are described in Figure 3. The  
272 diets were significantly different between the classes ( $P < 0.005$ , not shown), and as expected,  
273 the diets changed throughout the year ( $P < 0.005$ ) with an interaction between the period and  
274 class for all feeds ( $P < 0.005$ ). In class 1, the cow's diets were based on conserved grass in the  
275 winter (52 % hay, 20 % grass silage) and fresh grass in the grazing season (77 %), associated  
276 with concentrate (20 % in the winter and 12 % during the grazing season, respectively). In class  
277 3, the cow's diets were also principally based on grass, grass silage during the winter (51 %)

278 and fresh grass during the grazing season (55 %), and concentrate was distributed throughout  
279 the year (40 %). In classes 2 and 4, maize silage represented a significant proportion of forages  
280 in the diet: 20 % in the winter and 12 % in the grazing season for class 2 and 33 % in the winter  
281 and 25 % in the grazing season for class 4. The maize silage was mainly associated during the  
282 winter with grass silage (35 and 25 % for classes 2 and 4, respectively) and in the grazing season  
283 with fresh grass (45 and 23 % in classes 2 and 4, respectively). The concentrate was distributed  
284 throughout the year, 22 and 28 % on average for classes 2 and 4, respectively. The other  
285 production conditions are listed in Table 3. In class 1, GF were mainly located in France (70 %),  
286 where 8 cow breeds were used, and the milk yield was significantly the lowest (17.1 kg per cow  
287 and per day). The GF from class 2 were widespread in Slovenia (48 %), France (26 %) and  
288 Norway (26 %). The major breeds were Holstein, Norwegian Red, Simmental and Brown, with  
289 an intermediate milk yield (20.2 kg per cow and per day). Class 3 was mainly represented by  
290 Norwegian farms with 86 % of Norwegian Red cattle. Slovakian GF constituted the main part  
291 (75 %) of class 4. Holstein was the major breed, followed by the Slovak Spotted (57 and 29 %  
292 of the total cows, respectively). The milk yield was the highest in both classes 3 and 4 (24.3  
293 and 25 kg per cow and per day, respectively).

294 A slight variation of milk yield was recorded in the spring (turn out to grazing): there was an  
295 increase in classes 1 and 4 and a decrease in class 2. The milk yield remained stable throughout  
296 the year for class 3 (data not shown).

## 297 **4 Discussion**

### 298 4.1 Milk mineral composition

299 The average mineral contents found in our study were within the range of those reported in herd  
300 milk by Rodriguez Rodriguez et al. (2001) and Lucas et al. (2006) and in dairy plant milk by  
301 Poulsen et al. (2015), except for Mg, which was 10 to 20 % higher in this study. The variability  
302 of the mineral contents of our bulk milk (pooled in transport tankers) was often close to that

303 observed with herd milks in the literature. This variability highlights the possibility of  
304 distinguishing milks according to their mineral composition.

305 In our study, the 4 mineral contents in the different classes fluctuated according to different  
306 patterns throughout the year and did not show a general trend (Fig. 2). In contrast, Poulsen et  
307 al. (2015) reported that the contents of Ca, Mg, and P were strongly associated and all showed  
308 similar seasonal variations, potentially due to their common metabolic pathway. These authors  
309 reported for Ca, Mg and P a positive correlation with milk protein content and noted the lowest  
310 values in the summer (July and August) and the highest values in winter (November to  
311 February). Similarly, Hurtaud et al. (2014) showed a common trend for the changes in Ca  
312 content in herd milk with the lowest values found in May and the highest in January. These  
313 seasonal patterns could be linked to block calving practices but the data are not reported in these  
314 studies. In our case, the average lactation stage ranges from 158 to 202 days according to the  
315 sampling period and to the GF class (data not shown). This difference seems too limited to  
316 affect mineral content in milk (Gaucheron 2005). It is nevertheless noticeable that similarly to  
317 Poulsen (2015) milk Ca and protein contents were positively correlated within the GF  
318 ( $P < 0.001$ , data not shown): it certainly reflects the functional link between Ca and the casein  
319 micelles. Hurtaud et al. (2014) suggested that the seasonal Ca content changes may be due to  
320 day length and hot temperature. Boudon et al. (2016), in an experimental study, demonstrated  
321 indeed that the Ca content significantly decreased during long and sunny days compared to  
322 short days. In our study, the average Ca content was also significantly lower in long vs. short  
323 days (1391 and 1470 mg·kg<sup>-1</sup>, respectively) but we did not evidence that according to the GF  
324 latitude (highly variable in our design) the higher the difference between long and short days  
325 was, the greater the seasonal difference of milk Ca content was (data not shown). Considering  
326 that all the minerals in animal body and products come exclusively from the diet, the mineral  
327 content in milk could be also affected by the nutritional status of dairy cow (Gaucheron 2005).

328 Nevertheless, little is known on the possible effect of minerals intake on milk composition.  
329 Maize is an extremely poor source of minerals compared to grass and the mineral composition  
330 of the herbage differs greatly according to the species and their phenological stage. For  
331 example, the Ca content in plant is higher in legumes than in grasses (Pirhofer-Walzl et al.  
332 2011). In our study, the richness in minerals of the milks from class 4 could be linked with a  
333 large proportion of clover and alfalfa in the forage and concentrate distributed in GF from  
334 Slovakia. It can also be noted that on pasture, a high content of clover may change the dynamics  
335 of milk minerals compared to grass only swards because the proportion of clover change  
336 substantially throughout the season, being highest in autumn. Moreover, the high Mg contents  
337 of milks from class 3 could be associated with the abundance in herbs in half of the GF, as herbs  
338 are particularly rich in this mineral. The low variability of the mineral contents in milks from  
339 class 3 could also be related to the constant and significant amount of commercial concentrates,  
340 which generally are enriched with minerals. In our study, it is nevertheless difficult to elucidate  
341 the effect of diet on milk minerals because of probable confounded effects with mineral  
342 supplementation, fertilization practices, pedo-climatic context and cow breed. These different  
343 effects cannot be segregated with our dataset. A breed effect on the milk mineral content has  
344 also been reported (Poulsen et al. 2015). In our study, in the sole situation where two breeds  
345 were reared in the same country (Slovakia) with similar diets, we did not found any difference  
346 between Holstein and Slovak Spotted breeds on the mineral contents in milk.

#### 347 4.2 Milk vitamin A composition

348 The vitamin A contents that were measured in our study were approximately 40 % lower than  
349 the average value reported by Lucas et al. (2006) and Agabriel et al. (2007) in bulk milk, and  
350 within the range of those reported by Martin et al. (2002) in an experimental study. The  
351 observed annual change in vitamin A content is consistent with the literature: it increased  
352 between the winter and grazing periods, simultaneously with the increasing proportions of fresh

353 herbage in the diet (Fig. 2 and 3). This high milk content in vitamin A is explained by the higher  
354  $\beta$ -carotene content in fresh grass than in all conserved forages (Nozière et al. 2006). Indeed, the  
355  $\beta$ -carotene acts as the main precursor of vitamin A for ruminants (Nozière et al. 2006). During  
356 the winter season, we observed a large diversity in the vitamin A content in milk. The highest  
357 contents were associated with diets including a major proportion of both grass silage and  
358 concentrate (Fig. 2 and 3, class 3). The grass silage contains 3 times more carotenoids than hay  
359 which increase vitamin A in milk. In addition, commercial concentrate is very often enriched  
360 with vitamin A, which is directly transferred into the milk (Nozière et al. 2006). On the opposite,  
361 the lowest vitamin A contents were associated with diets including a large part of hay, which  
362 contain very few carotenoids and a low proportion of concentrate (Fig. 2 and 3, class 1).

#### 363 4.3 Milk fat composition

364 The effect of diet on FA concentrations in milk has been clearly shown under experimental  
365 conditions (Chilliard et al. 2007; Shingfield et al. 2013) and validated on commercial farms  
366 (Ferlay et al. 2008; Slots et al. 2009; Borreani et al. 2013). Coppa et al. (2013) have even  
367 predicted bulk milk FA composition using data describing farming practices via on-farm  
368 surveys. Our results, at the level of a large-scale design, are completely consistent with these  
369 publications.

370 In our study, the average content of C12-14-16 was consistent with those of Ferlay et al. (2008)  
371 and Heck et al. (2009), higher than those observed by Collomb et al. (2008) and O'Donnell-  
372 Megaro et al. (2011) and lower than that shown by Butler et al. (2011). The lower content of  
373 C12-14-16 reported by Collomb et al. (2008) (34.9 and 41.1 g $\cdot$ 100 g<sup>-1</sup> fat in the summer and in  
374 the winter, respectively) could be explained by the fact that cows were fed diets high in grass  
375 (74% of fresh grass in the summer and 69 % of hay in the winter) from the Swiss mountains.  
376 Feeding the cows with these diets, which are rich in polyunsaturated fatty acids (PUFA),  
377 explains the decrease in these SFA, via an inhibition of *de novo* mammary synthesis of all

378 C12:0, C14:0 and half of C16:0 by *trans* FA resulting from the rumen biohydrogenation of  
379 PUFA ingested (Chilliard et al. 2007). This mechanism could explain the low C12-14-16  
380 content observed in our study during summer in class 1, where the proportion of grazed grass  
381 is very high (Fig. 2 and 3). Moreover, O'Donnell-Megaró et al. (2011) associated the low  
382 content of C12-14-16 they found in dairy plant milk (40.5 g·100 g<sup>-1</sup> fat) to the low forage diet  
383 and the routinely use of lipid supplement and by-product in the US. The unsaturated FA from  
384 the latter feedstuffs as well as the reduction in forage intake depress the *de novo* mammary  
385 lipogenesis, thereby reducing the SFA content in milk (Chilliard et al. 2007). Similar  
386 hypotheses could explain the low values of C12-14-16 found in our study for the class 3 mainly  
387 made of Norwegian farms where cow diets included a high and constant proportion of  
388 commercial concentrate (40%) comprising fat added from different rape products (70-75 % of  
389 the fat added). In the UK, Butler et al. (2011) proposed that the high C12-14-16 level (50.3  
390 g·100 g<sup>-1</sup> fat) in some of their dairy plant milks could be associated with the wide use of  
391 commercial feed products, including palm oil, a practice known to increase the C16:0  
392 concentration in milk (Larsen et al., 2010).

393 The average values of oleic acid that were observed in our study were higher than the average  
394 values that were observed by Collomb et al. (2008) and Heck et al. (2009) (+ 1.9 and + 1.4  
395 g·100 g<sup>-1</sup> fat, respectively). Our findings were consistent with those of Ferlay et al. (2008), and  
396 they were lower than those observed by Butler et al. (2011) in conventional milks collected in  
397 retail outlets and particularly by O'Donnell-Megaró et al. (2011) (-2.9 and -4.6 g·100 g<sup>-1</sup> fat,  
398 respectively), who linked this high milk oleic acid content with dietary lipid supplements.  
399 Moreover, Glasser et al. (2008) have shown that milk oleic content increased significantly with  
400 lipid supplements rich in long chain unsaturated FA (with atoms of carbon  $\geq 18$ ). The high milk  
401 concentration of oleic acid shown by O'Donnell-Megaró et al. (2011) could be explained by  
402 the fact that this FA is an intermediate of ruminal biohydrogenation of unsaturated FA from



403 dietary lipid supplements. Milk oleic acid results also from the mammary  $\Delta$ -9 desaturation of  
404 stearic acid which is the end-product of the ruminal biohydrogenation of dietary lipids  
405 (Chilliard et al. 2007). In our study, the incorporation of lipids from rape (rich in oleic acid) in  
406 Norwegian commercial concentrates is probably the main reason why milk oleic acid content  
407 is constantly the highest across the year (class 3).

408 The n-3 FA average contents in milk from our study were comparable to the annual average  
409 values that were observed by Butler et al. (2011) and van Valenberg et al. (2013). In contrast,  
410 Ferlay et al. (2008) and Collomb et al. (2008) reported a higher n-3 FA content (+0.19 and  
411 +0.45 g·100 g<sup>-1</sup> fat, respectively) with grass-based diets. Similarly, the highest n-3 FA contents  
412 in milk observed all over the year for class 1 was associated with the highest proportion of grass  
413 in the diet (hay and grass silage during winter and pasture during summer) and low amount of  
414 concentrate. Grass-based diets are rich in C18:3 n-3 and therefore are known to increase the n-  
415 3 FA milk content up to 5 times when fresh grass is compared to maize-silage diet (Ferlay et  
416 al. 2006). In addition, Collomb et al. (2008) also suggested that the mountain location could  
417 reinforce the effect of grass due to a combined effect of altitude via cow hypoxia and of  
418 botanical diversity of the permanent grassland. The latter may provide plant secondary  
419 compounds (terpenoids and phenolic compounds) able to limit rumen biohydrogenation and  
420 therefore increase milk C18:3 n-3 content. Indeed, in this study, the GF located at the highest  
421 altitude are all included in class 1.

422 The milk in our study had n-6 FA contents that were comparable to those observed by Ferlay  
423 et al. (2008) and van Valenberg et al. (2013) and lower than those that were found by Butler et  
424 al. (2011) (-0.3 g·100 g<sup>-1</sup> fat). These latter milks were produced in conventional farms where  
425 cows could have been fed high-maize silage diets. In our study, the highest n-6 FA contents  
426 were associated with diets including the highest part of maize silage (Fig. 2 and 3, class 4). This  
427 fodder is rich in C18:2 n-6 (nearly 1.5 % DM) and therefore increases milk C18:2 n-6 content

428 (Chilliard et al. 2007). Moreover, Slots et al. (2009) have highlighted that the n-6 FA milk  
429 contents were positively linked to commercial concentrate mix. In contrast to our results, they  
430 showed no correlation between the maize silage intake (10 to 30 % of DMI) and the n-6 FA  
431 content in the milk.

432 The average *trans* FA content in our milks was equivalent to that reported by Ferlay et al.  
433 (2008). No other comparison could be made, because the method of calculation for the *trans*  
434 FA sum was different according to the authors and all of the data were not available.  
435 Considering the changes in *trans* FA content throughout the year, our results agree with the  
436 literature, i.e., a strong elevation in milk *trans* FA content observed in class 1 when cows grazed  
437 pasture (Butler et al. 2008; Ferlay et al. 2008; Collomb et al. 2008; Larsen et al. 2010). The  
438 richness in C18:3 n-3 of pasture could explain the ruminal production of *trans* FA from ruminal  
439 biohydrogenation of C18:3 n-3, and then the high milk content of *trans* FA. The higher levels  
440 of *trans* FA during grazing than winter season for class 1 could also be reinforced by the  
441 phenological stage of the grass (more digestible lush grazed grass versus mature conserved  
442 forage) that influences the ruminal biohydrogenation pathway of PUFA (Chilliard et al. 2007).  
443 In addition, we observed that *trans* FA content did not increase with pasture diets including a  
444 large part of concentrate (Fig 2 and 3, class 3) and therefore a least part of grazed grass in the  
445 diet.

446 Moreover, we have highlighted 3 out of 4 classes of GF mainly gathering farms located in the  
447 same country. This result could indicate that specific production conditions exist within the  
448 country and affect the milk characteristics, as shown by Gaspardo et al. (2010), with  
449 neighbouring Italian and Slovenian farms having similar practices.

#### 450 4.4 Nutritional interest of bulk milk according to GF classes

451 For minerals, in order to compare the nutritional interest of our milks according to the RNI, we  
452 only considered the average daily liquid milk consumption in the European Union, i.e., 170 mL

453 in 2012 (IDF 2013). Indeed, it was difficult to estimate the total mineral intake from all dairy  
454 products then anyway, when milk is transformed, a variable fraction of minerals moves from  
455 milk to whey according to the cheese-making process (range from 1 to 10 for Ca, for example)  
456 (Feinberg et al. 1987). As the mineral content of most dairy products is driven by the process,  
457 the variability of the mineral content of milk affects human mineral intake mainly through the  
458 consumption of liquid and fermented milk like yoghurt. The total fat dairy intake can be  
459 estimated more easily because most of the processes don't modify the fat composition (Lucas  
460 et al., 2006). In Europe, it approximates 18 g per day and per capita (IDF 2013).

461 According to the GF classes, the average supply of the RNI (WHO and FAO 2004; FAO 2010)  
462 ranged from 23.6 % (class 2) to 25.8 % (classes 1 and 4) for Ca, from 18.4 % (class 4) to 28.7  
463 % (class 1) for *trans* FA, from 4.4 % (class 4) to 8.8 % (class 1) for n-3 FA and from 44.6 %  
464 (class 3) to 48.6 % (class 4) for C12-14-16. On the opposite, the average supplies of the RNI  
465 for P, Mg, Zn, vitamin A, n-6 FA, and oleic were almost similar for the different classes  
466 (maximum difference about 1%). Therefore, the proposed GF classification seems meaningful  
467 from a nutritional point of view only for Ca, n-3 FA, C12-14-16 and *trans* FA. Considering  
468 these four compounds we underline the absence of milks with the highest nutritional interest in  
469 order to reduce simultaneously the *trans* FA and C12-14-16 intake and to increase the n-3 FA  
470 and Ca intake. For example, the choice of milks produced by farms from class 1 seems relevant  
471 to increase the intake of Ca and n-3 FA throughout the year and to reduce the C12-14-16 intake  
472 during the pasture season but meanwhile it dramatically increases the *trans* FA intake. We have  
473 nevertheless to consider that in class 1 *trans* FA mainly consist in vaccenic acid (data not shown)  
474 which is converted to rumenic acid by desaturation in human metabolism. Rumenic acid is only  
475 found in ruminant fat and has been linked to many potential health benefits. The choice of milks  
476 from class 3 could be a lever to reduce the C12-14-16 intake throughout the year but it is  
477 irrelevant for Ca and n-3 FA supplies. Finally the potential nutritional impact of the milk

478 composition differences found here needs to be evaluated in the context of the overall diet. For  
479 heavy consumers of dairy product the effects previously highlighted could be reinforced.

480

## 481 **5 Conclusion**

482 Based on a wide-ranging framework, this study investigated changes throughout the year in the  
483 contents of bulk milk compounds with nutritional requirements. The approach focused on a set  
484 of minerals, fat-soluble vitamins and FA with the associated husbandry conditions. We  
485 observed that the Ca, Mg, P and Zn contents evolved in different ways throughout the year, and  
486 they evolved independently of each other. We showed the absence of milks with the highest  
487 contents in these four minerals simultaneously, except in the autumn. Moreover, we identified  
488 GF where milk had high content in Mg or in Zn throughout the year. We confirmed that the FA  
489 profile and the vitamin A contents remained strongly associated with the diet composition. We  
490 also highlighted that no GF produce milks with a significant contribution to the 5 FA  
491 requirements, independent of the sampling dates. Our results suggested that groups of farms  
492 could be selected to collect milks with some specific characteristics throughout the year or  
493 during some periods. Throughout the year, grass-based diets remained the best method to  
494 produce milk with high n-3 FA content. Maize silage-based diets resulted in milk with high n-  
495 6 FA content while those rich in commercial concentrate including rape induced an increase in  
496 oleic acid content in milk. During the outdoor period, grazing remained clearly the most  
497 effective means with a low C12-14-16 FA content in milk, accompanied by a concomitant  
498 increase in *trans* FA. These FA did not increase with diets rich in concentrate. During the indoor  
499 period, diet with both grass silage and commercial concentrate led to high milk vitamin A  
500 content. Thus, in practice, it is possible for dairy industry to take advantage of the location  
501 collection area and the diversity of the farming systems, to segment the dairy products using

502 individual specific milk compounds. Nevertheless, it was not possible to identify milks relevant  
503 to our health for both mineral and lipid fractions throughout the year.

504

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#### 515 **Conflict of interest**

516 Chantal Chassaing, Cécile Sibra, Jože Verbič, Odd Magne Harstad, Jaroslav Golecký,  
517 Bruno Martin, Anne Ferlay, Isabelle Constant, Carole Delavaud, Catherine Hurtaud, Vida  
518 Žnidaršič Pongrac, and Claire Agabriel declare no conflict of interest.

519 **Compliance with Ethics Guideline** This article does not contain any studies with human or  
520 animal subjects performed by any of the authors.

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643 Most correlated variables with the factor plan: P (phosphorus); Zn (zinc); n3FA (sum of  
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650 **Fig. 2** Bulk milk composition throughout the year according to the group of farms classes and  
651 the sampling period

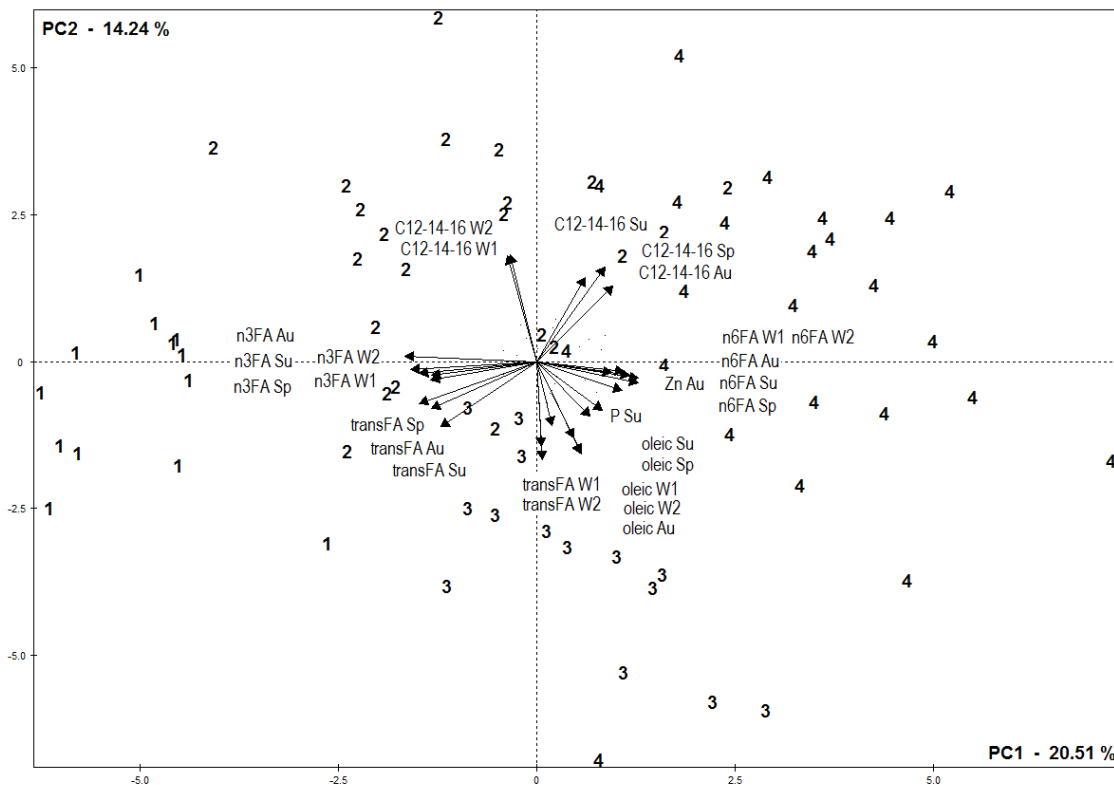
652 Sampling period: **W1** January - March, **W2** February – May, **Sp** May – June, **Su** June – July,  
653 **Au** September

654 Results are represented as adjusted mean and standards errors of means (error bars). The overlap  
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658 **Fig. 3** Composition of cow's diet at milk sampling times according to the GF classes

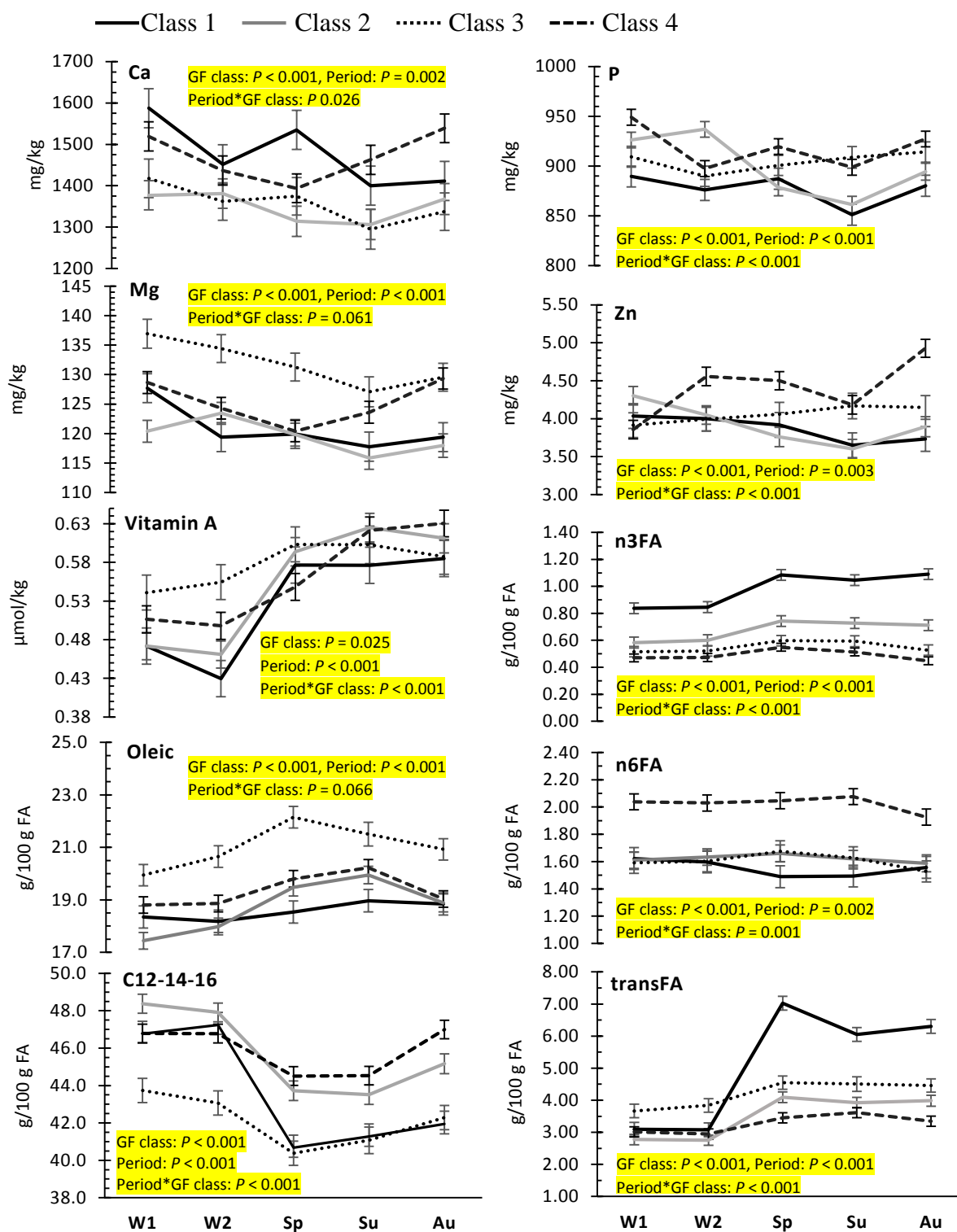
659 DMI: Dry matter intake. Sampling period: W1 January - March, W2 February – May, Sp  
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661

**Fig. 1** Projection on the PC1-PC2 plane of the 74 groups of farms (GF) and the most correlated variables with the factor plan



GF are identified by the class **number (1 to 4)** to which they belong. Most correlated variables with the factor plan: P (phosphorus); Zn (zinc); n3FA (sum of C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3); n6FA (sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, C22:4n-6); oleic (oleic acid); C12-14-16 (sum of C12:0, C14:0, C16:0); transFA (sum of t9-C14:1, t11-C16:1, t4-, t5-, t6/7/8-, t9-, t10-, t11- and t12-C18:1, t9t12-C18:2, c9t13-C18:2, c9t14-C18:2, c9t12-C18:2, t9c12-C18:2, t11c15-C18:2, t11t13-Conjugated Linoleic Acid (CLA), tt-CLA and c9t11-CLA) - W1 (winter1); W2 (winter2); Sp (spring); Su (summer); Au (autumn).

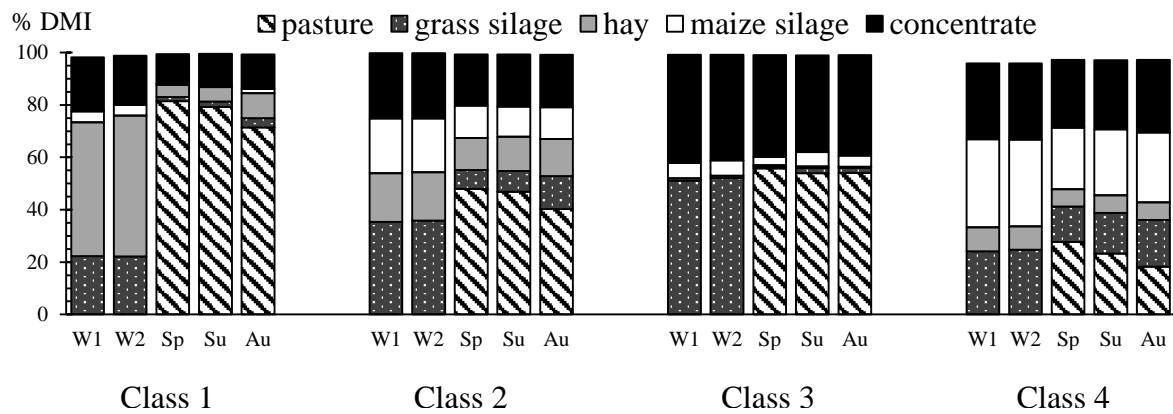
**Fig. 2** Bulk milk composition throughout the year according to the classes of group of farms and the sampling period.



Sampling period: **W1** January - March, **W2** February – May, **Sp** May – June, **Su** June – July, **Au** September

Results are represented as adjusted mean and standards errors of means (error bars). The overlap of two errors bars indicates that values are not significantly different ( $P > 0.05$ ). The significance of the effects of the GF class on milk composition, the sampling period and of the interaction between the period and the class are noted for each compound.

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**Fig. 3** Composition of cow's diet at milk sampling times according to the GF classes

DMI: Dry matter intake. Sampling period: W1 January - March, W2 February - May, Sp May - June, Su June - July, Au September

**Table 1** Description of the study design

Country	France <sup>a</sup>	Norway	Slovakia	Slovenia <sup>a</sup>	Total
Number of GF	20	19	20	15	74
Number of farms	100	436	20 <sup>b</sup>	144	700
Number of lactating cows	5463	9224	3881	2854	21422
Breed of cows (number of GF)	Holstein (5) Tarentaise and Abondance (2) Holstein and Normande or Montbeliarde or Brown (13)	Norwegian Red (19)	Holstein (10) Slovak Spotted (10)	Holstein (3) Simmental (5) Brown (5) Brown and Simmental (2)	
Periods of milk sampling and survey with farmers in 2008 (Month-Date)					
Winter1	01-02 to 01-28	03-17 to 04-06	03-01	01-28 to 02-06	
Winter2	02-04 to 03-25	04-06 to 06-08	04-19	03-31 to 04-08	
Spring	04-28 to 06-25	05-26 to 06-20	06-09	05-16 to 06-05	
Summer	07-07 to 08-01	06-14 to 06-27	08-16	06-06 to 06-26	
Autumn	08-27 to 10-03	08-18 to 09-21	10-05	09-02 to 09-05	

GF: group of farms

<sup>a</sup> GF constituted for this study

<sup>b</sup> Each farm consisted of one GF, considering the very high number of cows per farm in this country

**Table 2** Bulk milk composition (n = 360 samples)

	Mean	Coefficient of variation (%)	Minimum	Maximum
Minerals, mg·kg <sup>-1</sup>				
Calcium	1414	13.0	1022	2205
Magnesium	124	8.2	97	151
Phosphorus	903	5.0	758	1033
Zinc	4.1	16.3	2.5	6.5
Vitamin A, μmol·kg <sup>-1</sup>	0.59	18.1	0.35	1.31
Fatty acids, g·100g <sup>-1</sup> of fat				
n-3 FA <sup>a</sup>	0.64	36.1	0.32	1.60
Oleic acid	19	9.6	15	25
n-6 FA <sup>b</sup>	1.74	20.1	0.94	3.49
C12-14-16 <sup>c</sup>	45	7.5	36	54
<i>trans</i> FA <sup>d</sup>	3.84	34.1	1.82	8.68

<sup>a</sup> Sum of C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3

<sup>b</sup> Sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, C22:4n-6

<sup>c</sup> Sum of C12:0, C14:0, C16:0

<sup>d</sup> Sum of t9-C14:1, t11-C16:1, t4-, t5-, t6/7/8-, t9-, t10-, t11- and t12-C18:1, t9t12-C18:2, c9t13-C18:2, c9t14-C18:2, c9t12-C18:2, t9c12-C18:2, t11c15-C18:2, t11t13-Conjugated Linoleic Acid (CLA), tt-CLA and c9t11-CLA



**Table 3** Characterization of cow husbandry conditions on the 4 GF classes

	Class 1	Class 2	Class 3	Class 4	<i>P</i> *
Number of GF	13	23	14	24	
Country, number of GF					
France	9	6	1	4	
Norway	0	6	12	1	
Slovakia	1	0	1	18	
Slovenia	3	11	0	1	
Altitude of farm, m a.s.l.	519 <sup>a</sup>	443 <sup>a</sup>	97 <sup>b</sup>	324 <sup>a</sup>	***
Major breeds of cows, % of total cows					
Hostein	13 <sup>b</sup>	29 <sup>b</sup>	7 <sup>b</sup>	57 <sup>a</sup>	***
Norwegian Red	0 <sup>c</sup>	26 <sup>b</sup>	86 <sup>a</sup>	4 <sup>c</sup>	***
Slovak Spotted	8 <sup>b</sup>	0 <sup>b</sup>	7 <sup>b</sup>	29 <sup>a</sup>	*
Brown	22 <sup>a</sup>	14 <sup>ab</sup>	0 <sup>bc</sup>	0 <sup>c</sup>	*
Montbéliarde	19 <sup>a</sup>	9 <sup>ab</sup>	0 <sup>b</sup>	2 <sup>b</sup>	*
Simmental	2 <sup>b</sup>	17 <sup>a</sup>	0 <sup>b</sup>	3 <sup>b</sup>	+
Normande	18 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	***
Tarentaise	10 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	*
Abondance	6 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	*
Milk yield, kg·cow <sup>-1</sup> ·d <sup>-1</sup>	17.1 <sup>c</sup>	20.2 <sup>b</sup>	24.3 <sup>a</sup>	25.0 <sup>a</sup>	***

GF: group of farms

\* Statistical significance: means in the same row, followed by a different letter are statistically different ( $P < 0.05$ ) +  $P < 0.01$  \*  $P < 0.05$  \*\*\*  $P < 0.001$