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Association between Ruminal pH and Rumen Fatty Acids Concentrations of Holstein Cows during the First Half of Lactation

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Abstract: Ruminal pH in dairy cows follows a diurnal fluctuation; low values are indicative of subacute ruminal acidosis and are associated with alterations of rumen fatty acids concentrations. The objective of the present study was to prospectively study the associations between ruminal pH and the rumen fluid concentrations of short, medium, and long chain fatty acids, under field conditions during the first half of lactation in 53 Holstein cows of a dairy farm. Ruminal fluid was obtained by rumenocentesis, which was performed at 30, 90, and 150 days in milk (DIM). Ruminal pH was measured immediately after collection with a portable pH meter, whereas gas chromatography was used for the determination of ruminal fatty acid concentrations. Mixed linear regression models were used for data analysis. The prevalence of cows with low ruminal pH (<5.5) was 45.3%, 54.7%, and 66.0% at 30, 90, and 150 DIM, respectively. The concentrations of acetic, propionic, butyric, valeric, isovaleric, caproic and linoleic acids were negatively associated with ruminal pH values, whereas the acetic to propionic ratio was positively associated with rumen pH. Under field conditions and naturally occurring low ruminal pH cases, ruminal concentrations of most fatty acids are negatively related with ruminal pH values

Keywords: ruminal fluid pH; dairy cows; ruminal fatty acids

1. Introduction

Ruminal pH in dairy cows follows a diurnal fluctuation. In high yielding dairy cows fed increased quantities of concentrates, ruminal pH ranges from 6.6 to 5.3 (or even 5.0 during the intensive rumen fermentation phases). Ruminal pH can remain below average levels for a considerable time during a 24 h period [1]. Low ruminal pH in cows is associated with the ingestion of rich in readily fermentable carbohydrates diets and the accumulation of excessive amounts of short chain fatty acids (SCFA), due to their slow absorption by the rumen wall [2].

The lower than normal ruminal pH is indicative of subacute ruminal acidosis (SARA), an important disease of dairy cattle that has significant physiological and economic effects [2–4]. The pH threshold to define SARA is still a matter of debate, with researchers using different pH cut-off values of 5.5 [2], 5.6 [5] or 5.8 [6]. The consequences of SARA in dairy cows may include reduced milk production and milk fat and protein content, due to alterations in rumen fermentation patterns [7,8]. These changes favour the production of...
certain long chain fatty acids (LCFA), which are absorbed into the blood stream, and inhibit udder milk fat synthesis [9].

Studies regarding the association between suboptimal ruminal pH and rumen fluid fatty acids concentrations [9,10] have produced controversial results. In some studies, it is reported that propionate increases [9,10] and acetate declines [9,10] with the decrease in ruminal fluid pH, whereas in others that they remain unaffected [10,11]. Similarly, butyric and valeric acid concentrations are reported to increase [10,12], to decrease [9,13] or to remain unaffected [11] when ruminal pH drops. In addition, most of them deal with experimentally induced ruminal pH reduction (SARA-induction), in early lactation cows. Relevant field studies, performed in commercial dairy herds, to assess the association between ruminal pH and ruminal fluid fatty acids concentrations, in different stages of lactation are lacking from the accessible literature.

Therefore, the objective of this study was to assess, under field conditions, the association between ruminal pH and ruminal fatty acids concentrations, by repeatedly evaluating the same cows, fully covering the first half of lactation.

2. Materials and Methods

2.1. Animals and Study Design

Fifty-three randomly selected lactating Holstein cows (34 primiparous and 19 multiparous; parity 2 and 3, median parity 1.49, SD: 0.69) from a commercial dairy farm in Greece were included in the study. The farm was selected based on high SARA prevalence (farm #11, [14]). For 15 consecutive months, the farm was visited for sample collection and clinical assessment of the animals three times every week.

The study was conducted between mid-September and late May. Each cow entered the study at 30 days in milk (DIM) and the study period lasted until 150 DIM. At 30, 90, and 150 DIM the body condition score (BCS) was recorded by the same author (GCK) using a five-degree scale with increments of 0.25 [15] and a rumen fluid sample was obtained for pH measurement and for the determination of short and medium chain fatty acids concentrations. Based on pH measurements and using the cut-off value of 5.5 the pH status of cows at each rumenocentesis was categorized as: (i) low (pH values \( \leq 5.5 \)) or (ii) normal (pH values > 5.5). All animals were routinely clinically examined at the aforementioned DIM and whenever the farmer observed clinical illness or drop in milk production. Milk yield was recorded daily. The mean milk yield of cows for the whole study period (30–150 DIM) was 25.44 kg (SD: 5.13).

2.2. Housing and Nutrition

Springing heifers and dry cows were housed in a bedded pack shed as a single group; primiparous and multiparous lactating cows were also housed as a single group in a two-row free-stall barn. Heifers and dry cows were offered, ad libitum, a low energy total mixed ration (TMR) consisting of corn silage (37.5% of dry matter-DM), wheat straw (37.5% of DM), wheat bran (11.25% of DM), soybean meal (11.25% of DM) and a mineral/vitamin supplement (2.5% of DM). Lactating cows were offered, ad libitum, a TMR adjusted during the study according to herd milk production level and consisting of corn silage (45% of DM), alfalfa hay (11% of DM), wheat straw (4–9% of DM), corn grain (12–15% of DM), wheat bran (6% of DM), soybean meal (12–14% of DM), Ca-soap fat supplement (1.5% of DM) and a mineral/vitamin supplement (3.5% of DM). Both TMRs were formulated to meet net energy and metabolizable protein recommendations of the National Research Council [16]). Sodium bicarbonate was included in the lactation ration (0.75% of DM) but no other buffer was used. Bunk space for dry cows was 70 cm and for lactating cows 60 cm. The stocking density for the lactating group throughout the study was ranged from 90% to 125%.
2.3. Rumen Fluid Sampling and Analyses

Rumen fluid was aspirated via rumenocentesis as described previously [17], without sedation. After local anaesthesia by injecting 4 mL of 2% Xylocaine (AstraZeneka, Athens, Greece), at the puncture site (2 mL subcutaneously and 2 mL intramuscularly), a 16 G and 13 cm long stainless-steel needle (H. Hauptner & Richard Herberholz GmbH & Co. KG, Solingen, Germany) was inserted to aspirate 2 to 3 mL of rumen fluid, within 20 sec, into a 5 mL disposable syringe. Rumenocenteses were consistently performed 5–8 h after the morning feeding, as suggested [17], between 12:00 and 14:00.

In total, 159 rumenocenteses were performed. All cows were monitored for the presence of complications such as, hematoma, abscess formation at the puncture site or peritonitis for the following 14 days. Minor complications were detected in 3 cases; a large abscess (approximately 10 cm) in one cow and a smaller one (<3 cm) in two cows, all after the 1st rumenocentesis (i.e., at 30 DIM). They all resolved spontaneously within two weeks, and the daily milk yield of these cows was not affected during this period.

Ruminal fluid pH was measured immediately after collection on-site with a portable pH meter (Horiba, B-213, Kyoto, Japan) in room conditions. The remaining rumen fluid was transferred to the laboratory and stored at \(-20^\circ\)C, until it was further assayed for fatty acid concentration.

The following short and medium chain fatty acids concentrations were determined with gas chromatography [18,19], using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany): acetic (2:0), propionic (3:0), butyric (4:0), isobutyric (iso4:0), valeric (pentanoic, 5:0), isovaleric (iso5:0), caproic (hexanoic, 6:0), and heptanoic (7:0). Long chain fatty acids, palmitic (hexadecanoic, 16:0), palmitoleic (cis-9-hexadecenoic, c9-16:1), stearic (octadecanoic, 18:0), trans-octadecenoic (t-18:1), oleic (cis-9-octadecenoic, c9-18:1), cis-vaccenic (cis-11-octadecenoic, c11-18:1), and linoleic (cis,cis-9,12-octadecadienoic, 18:2) concentrations were also determined with gas chromatography [20,21], in a 5890 Series II Hewlett-Packard gas chromatographer (Hewlett-Packard GmbH, Waldbronn, Germany).

2.4. Statistical Analysis

Data were analysed using SPSS 21. The differences regarding the prevalence of cows with low ruminal pH status among the three sampling occasions were assessed using the chi-square test.

Afterwards, a series of mixed linear regression models were built to assess the statistical effects of (i) ruminal pH status (low and normal) and (ii) ruminal pH values (as continuous variable) on the concentration of the studied fatty acids.

The model used to quantify these effects for the gth sampling occasion of the hth cow is described below (Model 1):

\[
Y_{gh} = \mu + RpH_{gh} + P_h + G_h + \beta_2 \times S + \gamma_h + \delta_h + e_{gh} \quad (Model \ 1)
\]

where:

- \(Y_{gh}\) = Ruminal fluid concentration for each one of the 15 studied fatty acids, \(\mu\) = intercept, \(RpH_{gh}\) = fixed effect of ruminal pH status [2 levels; 0 = low pH (\(\leq 5.5\)) and 1 = normal pH (>5.5)], \(P_h\) = fixed effect of parity number (2 levels; 1st and \(\geq 2\)nd parity), \(G_h\) = fixed effect of days in milk (3 levels; 30, 90, and 150 DIM), \(\beta_2\) = fixed effect of the regression coefficient of BCS (S) \(\gamma_h\) = repeated variation in the hth cow, \(\delta_h\) = random variation in the hth cow, and \(e_{gh}\) = residual error.

In the second model, the fixed effect of ruminal pH status (low and normal) was replaced by the fixed effect of the regression coefficient of the ruminal pH value. Otherwise, it was built using the same explanatory variables and setting up, to estimate the effects of ruminal pH on the concentration of the studied ruminal fatty acids.

Among first order autoregressive (ARH1), compound symmetry (CS) and unstructured (UN), the covariance structure with the lowest Akaike’s information criteria (AIC) was included in case a significant improvement of the model was observed (\(p < 0.05\)). The
assumptions of normal distribution, homoscedasticity, and linearity for the models were checked by visually assessing the plots of standardized residuals against standardized predicted values and histograms, as well as the probability-probability and quantile-quantile plots of standardized residuals.

The significance level was set at \( a = 0.05 \) for all the comparisons.

3. Results

Eighty-eight cases of low (\( \leq 5.5 \)) ruminal pH status were recorded throughout the study; 67 in primiparous and 21 in multiparous cows. The distribution of cases at the three sampling points in relation to parity is presented in supplementary Table S1. The prevalence of low ruminal pH cases increased from DIM 30 through DIM 150 (45.28%, 54.72%, and 66.04% for DIM 30, DIM 90, and DIM 150, respectively) and it was significantly higher at DIM 150 compared to DIM 30 (\( p \leq 0.05 \)). Twelve out of the 53 cows tested had low and seven had normal ruminal pH status in all sampling occasions. Box plots of ruminant pH, BCS, and concentrations or ruminal fatty acids (short, medium and long chain) during the three sampling occasions (30, 90 and 150 DIM) are presented in Supplementary Figures S1–S3.

The statistical effects of ruminal pH and of the rest of the parameters forced into the two models on rumen content’s fatty acids concentration are presented in Supplementary Tables S2 and S3. While several associations regarding pH and DIM were detected, parity and BCS were not found to be associated with fatty acid concentrations, with the exception of cis-vaccenic acid (c11-18:1) which was positively associated with BCS.

Linear regression models (Table 1) revealed that low pH status cows had significantly higher concentrations of acetic (\( p < 0.001 \)), propionic (\( p < 0.001 \)), butyric (\( p < 0.001 \)), valeric (\( p < 0.001 \)), palmitic (\( p < 0.01 \)), oleic (\( p < 0.01 \)), and linoleic (\( p < 0.001 \)) acids; moreover, they also had a lower acetic to propionic acid ratio (\( p < 0.001 \)). On the other hand, concentrations of isobutyric, isovaleric, caproic, heptanoic, palmitoleic, stearic, trans-octadecenoic, and cis-vaccenic acids were not significantly affected by pH status.

### Table 1. Association between ruminal pH status [low (\( \leq 5.5 \)) and normal values (>5.5)] and ruminal fatty acids concentration.

| Parameter | \( B \) | SE | \( p \)-Value | 95% CI |
|-----------|--------|----|----------------|-------|
| Acetic acid—2:0 (mmol/L) | 13.60 | 2.614 | 0.000 | 8.43 18.76 |
| Propionic acid—3:0 (mmol/L) | 13.12 | 1.841 | 0.000 | 9.48 16.76 |
| Acetic:propionic acid ratio | -0.52 | 0.097 | 0.000 | -0.71 -0.33 |
| Butyric acid—4:0 (mmol/L) | 8.84 | 1.468 | 0.000 | 5.94 11.74 |
| Isobutyric acid—iso 4:0 (mmol/L) | 0.01 | 0.037 | 0.857 | -0.07 0.08 |
| Valeric acid—5:0 (mmol/L) | 0.61 | 0.174 | 0.001 | 0.27 0.96 |
| Isovaleric acid—iso5:0 (mmol/L) | 0.18 | 0.099 | 0.073 | -0.02 0.37 |
| Caproic acid—6:0 (mmol/L) | 0.11 | 0.084 | 0.195 | -0.06 0.28 |
| Heptanoic acid—7:0 (mmol/L) | -0.01 | 0.015 | 0.524 | -0.04 0.02 |
| Palmitic acid—16:0 (mmol/L) | 0.15 | 0.051 | 0.004 | 0.05 0.25 |
| Palmitoleic acid—c9—16:1 (mmol/L) | 0.00 | 0.002 | 0.470 | -0.01 0.00 |
| Stearic acid—18:0 (mmol/L) | 0.15 | 0.120 | 0.203 | -0.08 0.39 |
| Trans-octadecenoic acid—t—18:1 (mmol/L) | 0.04 | 0.034 | 0.203 | -0.02 0.11 |
| Oleic acid—c9—18:1 (mmol/L) | 0.08 | 0.027 | 0.006 | 0.02 0.13 |
| Cis-vaccenic acid—c11—18:1 (mmol/L) | 0.01 | 0.018 | 0.562 | -0.03 0.05 |
| Linoleic acid—18:2 (mmol/L) | 0.06 | 0.015 | 0.000 | 0.03 0.09 |

B: regression coefficients of ruminal pH status, SE: standard error, CI: confidence interval.

When the pH value of the ruminal fluid was forced into the model as a continuous variable (Table 2), a one-unit increase in ruminal pH was associated with a significant decrease on the concentration of acetic (by 27.6 mmol/L, \( p < 0.001 \)), propionic (by 24.0 mmol/L, \( p < 0.001 \)), butyric (by 16.0 mmol/L, \( p < 0.001 \)), palmitic (by 1.1 mmol/L, \( p < 0.001 \)), isovaleric (by 0.4 mmol/L, \( p < 0.01 \)), caproic (by 0.4 mmol/L, \( p < 0.001 \)), and
linoleic (by ca. 0.1 mmol/L, $p < 0.001$) acids; moreover, it was associated with increased acetic to propionic ratio by 0.98 ($p < 0.001$). No significant effects ($p > 0.05$) of ruminal pH on isobutyric, heptanoic, palmitic, palmitoleic, stearic, trans-octadecenoic, oleic, and cis-vaccenic acids were observed.

### Table 2. Association between ruminal pH values (as continuous variable) and ruminal fatty acids concentration.

| Parameter                     | B     | SE    | $p$-Value | 95% CI Lower | 95% CI Upper |
|-------------------------------|-------|-------|-----------|--------------|--------------|
| Acetic acid—2:0 (mmol/L)      | −27.60| 2.92  | 0.000     | −33.38       | −21.82       |
| Propionic acid—3:0 (mmol/L)   | −23.98| 2.07  | 0.000     | −28.06       | −19.89       |
| Acetic:propionic ratio        | 0.98  | 0.12  | 0.000     | 0.75         | 1.21         |
| Butyric acid—4:0 (mmol/L)     | −16.00| 1.755 | 0.000     | −19.46       | −12.53       |
| Isobutyric acid—iso 4:0 (mmol/L)| −0.02 | 0.048 | 0.003     | −0.11        | 0.07         |
| Isovaleric acid—iso5:0 (mmol/L)| −0.40 | 0.130 | 0.003     | −0.66        | −0.14        |
| Valeric acid—5:0 (mmol/L)     | −1.12 | 0.217 | 0.000     | −1.55        | −0.69        |
| Caproic acid—6:0 (mmol/L)     | −0.40 | 0.106 | 0.000     | −0.61        | −0.19        |
| Heptanoic acid—7:0 (mmol/L)   | 0.02  | 0.021 | 0.327     | −0.02        | 0.06         |
| Palmitic acid—16:0 (mmol/L)   | −0.10 | 0.070 | 0.179     | −0.23        | 0.04         |
| Palmitoleic acid—c9—16:1 (mmol/L)| 0.001| 0.003| 0.810     | −0.005       | 0.006        |
| Searic acid—18:0 (mmol/L)     | 0.07  | 0.159 | 0.668     | −0.25        | 0.38         |
| Trans-octadecenoic acid—t—18:1 (mmol/L)| −0.004| 0.044| 0.926     | −0.09        | 0.08         |
| Oleic acid—c9—18:1 (mmol/L)   | −0.05 | 0.036 | 0.201     | −0.12        | 0.03         |
| Cis-vaccenic acid—c11—18:1 (mmol/L)| −0.02 | 0.023| 0.474     | −0.06        | 0.03         |
| Linoleic acid—18:2 (mmol/L)   | −0.09 | 0.019 | 0.000     | −0.13        | −0.06        |

B: regression coefficients of ruminal pH (as continuous variable), SE: standard error, CI: confidence interval.

### 4. Discussion

The objective of this study was to investigate the associations between ruminal pH and the concentrations of ruminal fatty acids. Although there are relevant reports in the available literature [9,10], the novelty of this study is that low ruminal pH status was not experimentally induced and that pH and ruminal fatty-acid concentrations were repeatedly determined for each animal; the study was conducted at a commercial dairy farm with naturally occurring cases of low ruminal pH and each cow was sampled three times, at 60 days intervals, fully covering the first half of lactation.

The pH cut-off value of 5.5 considered in our study for the classification of cows into the low and normal pH status groups was selected on the basis of being the most commonly used threshold for the detection of SARA [22].

The prevalence of low ruminal pH status detected in this study was high. This was rather expected, since the only criterion for the selection of this farm for the study was the high prevalence of SARA [14]. In contrast to our findings, in other studies involving more farms, the prevalence of SARA recorded in early and mid-lactation cows was lower; for example, 19.0% vs. 26.0% [17], 11.0% vs. 18.0% [23], and 29.3% vs. 26.4% [24], in early and mid-lactation cows, respectively. In any case, differences regarding SARA prevalence during the various stages of lactation are likely to be associated with different management practices followed among herds, in different countries and regions over time.

The prevalence of low ruminal pH status in mid-lactation (DIM 150) was significantly higher compared to early lactation (DIM 30). In early lactation, the over-accumulation of SCFA that decreases ruminal pH results from their low absorption rate, which is related with the short length of rumen papillae, due to inappropriate transition management [2]; in mid-lactation, the accumulation of high amounts of SCFA results from the high intake of high energy rations with low buffering capacity (low in effective fibre) [8]. Moreover, continuous bouts of acidosis make it more difficult for cows to restore normal ruminal pH [25]; this might explain the higher prevalence of low ruminal pH status cases as lactation progressed.
Ruminal acetic acid and propionate concentrations were negatively associated with ruminal pH values. Regarding the acetic acid, our results are in contrast with previous findings derived either from in vitro [13] or dietary SARA-induced experiments [11], where production of acetic acid declined with the decrease in ruminal fluid pH. However, in another study, mean concentrations of acetic acid in ruminal fluid was numerically higher (no statistical comparison was provided) in SARA-positive Italian herds compared to those considered in critical or in normal condition [11], which is in agreement with our findings. In particular, values measured in all herds of the latter study were higher than those proposed by the previous authors [11] and about only 6% lower than those recorded in our study, during the same stage of lactation (DIM 5–60 vs. DIM 30, respectively). Concentrations of propionic acid were elevated in some [11] but not all studies [10] when ruminal pH was low.

In agreement with our findings, the acetate to propionate ratio has been found to be decreased in cows with low ruminal pH [9,10,13,26], attributed to a lower acetate production. In contrast, the decreased ratio detected here resulted from a much higher increase in propionic compared to acetic acid production.

Butyric and valeric acid ruminal concentrations were negatively associated with ruminal pH in the present study. However, the results from former reports are contradictory; some researchers observed that their concentration increased [10,12] and others decreased [9,13] or remained unaffected [11] when ruminal pH dropped.

Direct comparisons of the results obtained here for short and medium chain ruminal fatty acids with those of other studies are not feasible due to major differences in the study design and the methodology followed; SARA-inducing protocols (e.g., Latin square experiments lasting only for some weeks) do not represent actual on-farm conditions, where SARA risks are permanently challenging and affected herds and cows are long-term adapted to this condition.

The low ruminal pH status was significantly associated with the concentrations of palmitic, oleic, and linoleic acids at the present study; specifically, low pH status cows had higher concentrations of these fatty acids in ruminal fluid compared to cows with normal ruminal pH. When ruminal pH was considered as a covariate, a statistically significant association with ruminal concentration of linoleic acid was also observed. The exact mechanism by which low ruminal pH is related with these fatty acids is poorly elucidated and further investigation is needed. A more extended biohydrogenation of unsaturated c:16 acids under rumen acidotic conditions cannot be substantiated here, as concentrations of palmitoleic acid were very similar between low and normal pH status cows. Increased oleic acid concentration in low pH status cows may be linked with those of linoleic acid. Ruminal fluid concentration of linoleic acid is considered diet-dependent [27] but in this study, all cows were fed the same ration.

5. Conclusions

Under field conditions and naturally occurring low ruminal pH cases, concentrations of acetic, propionic, butyric, valeric, isovaleric, caproic and linoleic acids were found to be negatively associated with ruminal pH values. At the same time, the acetic to propionic ratio was positively associated with rumen pH.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants2040026/s1, Figure S1: Box-plots of ruminal pH and body condition score during the three sampling occasions (30, 90 and 150 days-in-milk); Figure S2: Box-plots of short and medium chain fatty acids content of ruminal fluid during the three sampling occasions (30, 90 and 150 days-in-milk); Figure S3: Box-plots of long chain fatty acids content of ruminal fluid during the three sampling occasions (30, 90 and 150 days-in-milk); Table S1: Prevalence (number and %) of low ruminal pH status (pH ≤ 5.5) cases at the three sampling points [30, 90 and 150 days in milk (DIM)] in relation to parity; Table S2: Associations between ruminal pH status and the rest of the parameters forced into model 1 with rumen content’s fatty acids concentration in the studied cow population; Table S3: Association between ruminal pH as a continuous variable and
the rest of the parameters forced into model 1 with rumen content’s fatty acids concentration in the studied cow population.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to further processing for other studies.

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