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Associations among post-partum rumen fill and motility, subclinical ketosis and fertility in Holstein dairy cows

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HIGHLIGHTS

- Low rumen fill and motility early post-partum are negatively associated with reproduction.
- Low rumen fill and motility early post-partum are positively associated with hyperketonemia.
- Assessment once, at DIM 7 or 8, is a time-efficient method of fresh cow monitoring.
- Twice more and mostly different cows are identified compared to a hyperketonemia test.

ABSTRACT

This prospective observational study aimed to investigate the association of rumen fill and motility in post-partum Holstein cows with their future reproductive performance and subclinical ketosis (SCK). The study population consisted of two independent data sets: the first (DS1) included 237 cows from 6 herds and the second one (DS2) 709 cows from 9 herds. Rumen Fill Score (RFS) was transformed into a 3 level-trait, representing very low, low and adequate dry matter intake, respectively. A binary Rumen Contraction Score (RCS) was defined as: 0: <2 contractions/2 min, impaired rumen motility and 1: ≥2 contractions/2 min, normal rumen motility. A combined binary trait based on RFS and RCS (RFCS) was also established, representing unsatisfactory and satisfactory rumen function. Three SCK traits were defined, based on 3 different thresholds, SCK_I: BHB≥1,000mmol/L, SCK_II: BHB≥1,100mmol/L and SCK_III: BHB≥1,200mmol/L. Scores were assessed and blood samples collected on day 7 (DS1) or day 8 (DS2), postpartum. Kaplan-Meier survival analysis, multivariable Cox proportional hazards models and Generalized Linear Mixed Models were performed to evaluate the association of rumen and SCK traits with reproduction. Herd, parity, calving season and several postparturient diseases were also included as potential explanatory variables. Mean days from calving to pregnancy after the 1st artificial insemination (AI) and from calving to pregnancy (all AIs) were shorter for levels of rumen traits representing adequate DMI and normal rumen motility; in most cases these differences were statistically significant in both datasets. Cows with adequate DMI and normal rumen motility (only in DS2) had greater hazard (hazard ratio [HR] = 1.84 and 1.61, for RFS and RFCS, respectively) and odds (odds ratio [OR] = 2.49 and 1.98, for RFS and RFCS, respectively) for pregnancy at 1st AI. Assessment of the association of examined rumen traits with hazard and odds for pregnancy at all AIs yielded statistically significant results in both datasets. For RFS, RCS and RFCS, HRs ranged from 1.57 to 3.31 and ORs from 1.95 to 4.83. No statistically significant associations with hazard and odds for pregnancy at 1st or all AIs were detected, for any of the 3 SCK traits, in either dataset.

Overall, the combined RFCS trait constantly identified more than twice the number of cows with future reproductive problems than a positive SCK blood test.

KEYWORDS

Dairy cow reproduction, rumen fill and motility, subclinical ketosis

1. INTRODUCTION

A long-standing industry recommendation to dairy farmers has been to check fresh dairy cows , every day or every other day, during the post-partum transition period, usually the first 10-14 days after calving **[1,2]**. This *"fresh cow monitoring"* practice is proposed to include both visual/clinical- (general brightness and alertness, rumen fill and appetite, udder fill, manure consistency, hydration status, respiration pattern and effort, nasal, ocular and uterine discharge, lung and rumen auscultation, body temperature and lameness) and record-oriented (first test-day milk production, mature-equivalent 305-days projected milk (ME305), milk components and ratios, somatic cell counts) observations, coupled with the ubiquitous blood or milk/urine ketosis-test **[3-5]**.

Early detection of metritis can be achieved when applying this practice **[1]** despite that diagnostic criteria are not uniformly followed across farms or reported in research studies. This unconformity has been linked to antimicrobial abuse, raising severe concerns **[6,7]**. In any case, adoption of these complete monitoring programs is rather limited **[3,4]**, mainly because other benefits remain subtle; few of the suggested observations have been clearly associated with impaired production while, at the same time, this task is laborious and requires qualified personnel which is difficult to find and recruit.

Benefits on reproductive performance can provide a strong motivation for dairy farmers to adopt fresh-cow monitoring practices; however, subclinical ketosis (SCK) seems to have monopolized the research interest. During the first two weeks post-partum SCK was found to be associated with impaired reproduction in two studies **[8,9]** and overall, screening for SCK was strongly suggested as a measure for early detection of metabolic disorders **[10]**. However, other studies **[11-13]** did not show an association of SCK, mostly at the herd level, with reproduction, increasing the need for more studies to advance our knowledge regarding the negative association of SCK with reproductive performance **[14]**. Recent publications have provided such evidence, for cows sampled at days-in-milk (DIM) 1 to 7 **[17]**. A recent meta-analysis, despite high data heterogeneity, reported a hazard ratio (HR) for time to pregnancy of 0.77 for SCK-positive cows **[18]**.

During the post-parturient transition period, SCK results from decreased dry matter intake (DMI) due to excess body condition at calving and inflammation/immune system activation **[19-21]**. Depending on the threshold used for SCK (ranging usually between 1,000 and 1,400 μmol/L **[14]**) and the ability to efficiently metabolize non-esterified fatty-acids (NEFA) **[22-23]**, cows experiencing a severe negative energy balance (NEB) may not show up as subclinically ketotic within or at the end of the first week post-partum [**14**]. However, timely diagnosis and treatment of this condition is essential in limiting future economic losses **[24]**. Apparently, targeted evaluation of cow appetite can bridge this gap and for this reason both group (feed-bunk scoring) and individual (rumen fill score and motility) observations should be incorporated in fresh-cow monitoring schemes. To the authors' best knowledge, the latter individual rumen traits have never been associated with reproductive outcomes, so far; nor have they ever been directly linked with SCK.

Therefore, the main objective of the present study was to investigate the association of rumen fill and motility during the immediate post-partum period with future reproductive performance. A second objective was the assessment of the concomitant association of SCK with these rumen traits, as well as with the reproductive outcomes examined.

2. MATERIALS AND METHODS

2.1 Study Population and general management

This was a prospective observational study that included a total of 946 cows from 15 confined dairy herds located in Northern Greece. Two independent data sets were considered: the first (DS1) included 237 cows (1st parity: n=14, 2nd parity: n=93, 3rd+ parity: 130) from 6 herds **[25]** and the second one (DS2) 709 cows (1st parity: n=347, 2nd parity: n=173, 3rd+ parity=189) from 9 herds **[26]**. Extensive description of the herds, rations fed and management practices can be found in relevant publications **[25,26]**. Briefly, herd size ranged from 90 to 300 milking cows and milk production averaged between 9,000 and 12,000 kg per cow per lactation in both data-sets. Cows were milked twice daily and housed in free-stall or bedded pack pens. Total mixed rations for dry and fresh cows were formulated to meet or exceed net energy and metabolizable protein requirements, according to NRC-2001 **[27]** recommendations.

2.2 Observations, sample collection and analysis

In all 15 dairy farms, cows were properly restrained in headlock-equipped feed-bunks to ensure their welfare and all tasks were performed and recorded by the first authors of the aforementioned publications **[25,26]**, both qualified veterinarians, one for each dataset. Farms were visited daily between 08:00 – 10:00 a.m. for clinical examination of cows, data and sample collection.

Rumen fill was assessed after the morning milking as cows were restrained at the feed-bunk, at day 7 (d7) or at day 8 (d8) relative to calving, for DS1 and DS2, respectively. Rumen fill was scored in a five-point scale **[28]**, based on left paralumbar fossa depth (**1**: a more than one hand deep dip is visible behind the last rib in the left flank, which has a deep rectangular hollow shape, indicative of very low DMI; **2**: a one hand deep dip is visible behind the last rib in the left flank, which has a triangular hollow shape, indicative of low DMI; **3**: a slight dip is visible in the left flank, behind the last rib, the skin under the lumbar vertebrae runs vertically down for one hand's width and then curves outwards, the skin fold from the hook bone is hardly visible; **4**: the rumen fossa behind the last rib is not visible and the skin under the lumbar vertebrae curves outwards; and **5**: skin is flat, or slightly bulging, on the left flank, behind the last rib. The skin under the lumbar vertebrae curves outwards, so that hook bones are not visible). The categorical trait Rumen Fill Score (RFS) included 3 levels, namely **RFS1, RFS2** and **RFS3**. The first two levels included cows with a rumen fill score of 1 and 2, representing very low and low DMI, respectively. The third level (RFS3) included cows with rumen fill scores 3, 4 (n=8 cows, 3 for DS1 and 5 for DS2) and 5 (none had such score in either dataset), all representing adequate DMI. In DS1, number of cows with RFS1, RFS2 and RFS3 were 11, 98 and 128, respectively. In DS2, number of cows with RFS1, RFS2 and RFS3 were 138, 405 and 166, respectively.

Rumen motility was assessed by auscultation, once, immediately after the assessment of rumen fill; the stethoscope was placed at the center of the left paralumbar fossa for 2 minutes and the number of contractions was recorded. The binary trait Rumen Contraction Score (RCS) included: **RCS1** (0-1 contraction/2 min, impaired rumen motility) and **RCS2** (≥2 contractions/2 min, normal rumen motility). In DS1, number of cows with RCS1 and RCS2 were 39 and 198, respectively, while in DS2, number of cows with RCS1 and RCS2 were 86 and 623, respectively.

Moreover, a combined "Rumen Fill & Contraction Score" binary trait (RFCS) was established, defined as follows: a) **RFCS1**: all cows with RCS1 (irrespective of RFS) and cows with RCS2/RFS1; these cows represented unsatisfactory rumen status and b) **RFCS2**: Cows with RFS2/RCS2 or RFS3/RCS2, representing cows with acceptable rumen status. In DS1, number of cows with RFCS1 and RFCS2 were 48 and 189, respectively, while in DS2, number of cows with RFCS1 and RFCS2 were 192 and 517, respectively.

A blood sample was collected from each cow at d7 (DS1) or d8 (DS2), by coccygeal venipuncture into 10-ml vacuum glass tubes without anticoagulant (BD Vacutainer, Plymouth, United Kingdom). Details regarding sample transportation to the lab, serum harvest and analysis for β-hydroxybutyrate (BHB), are provided in **[25]** and **[26]** **(**a spectrophotometric kinetic method and using the Siemens ADVIA 1800 Chemistry System, respectively)**;** precision test results (intra- and inter-assay coefficients) are provided in **[25]** and **[26]**, as well. Prevalence of SCK (calculated as the number of cows with BHB above the threshold used divided by the total number of cows) was calculated using 3 different thresholds, **SCK_I**: BHB≥1,000 mmol/L (23/237, 9.7% for DS1 and 99/709, 14.0% for DS2), **SCK_II**: BHB≥1,100 mmol/L (21/237, 8.9% for DS1 and 82/709, 11.6% for DS2), **SCK_III**: BHB≥1,200 mmol/L (19/237, 8.0% for DS1 and 73/709, 10.3% for DS2). Thus, these represented 3 different SCK binary (2-level) traits. All clinical cases of retained fetal membranes (RFM), metritis (MET), mastitis (MAST), ketosis (KET) and displaced abomasum (DA) were diagnosed by the two respective veterinarians, using standardized disease definitions (described in **[25]** for DS1 and **[26]** for DS2) and recorded as binary traits. Moreover, a combined binary clinical disease trait was established, defined as “at least one of the aforementioned clinical disorders”, between days 1 and 8 after calving (CD_1-8). Clinical cases were promptly treated according to the established farm protocols.

2.3 Reproductive management and data

All herds implemented a voluntary waiting period (VWP) of 50 days after calving. After the VWP, estrus detection was performed by visual observation for 20-30 min twice daily; standing to be mounted was used to categorize a cow as being in heat. Then, AI was applied by farm personnel following the am/pm rule. No hormonal interventions were used on any enrolled farm for the first AI before 100 DIM (DS1) or 120 DIM (DS2); cows not pregnant or inseminated by 100 DIM (DS1) or 120 DIM (DS2) entered an Ovsynch synchronization protocol. Pregnancy was diagnosed by the farms’ veterinarians at 35-50 days post-AI by ultrasonography or rectal palpation, and confirmed by 90 post-AI. A cow was considered pregnant only after the second positive diagnosis. Reproductive data were retrieved from farm records at the end of the study period and included: a) time interval from calving to detected pregnancy (date of successful 1st AI) until the 100th or the 120th DIM, for DS1 and DS2, respectively, b) detected pregnancy at 1st AI performed until the 100th or the 120th DIM (PREG_1stAI_100, PREG_1stAI_120), for DS1 and DS2, respectively, c) time interval from calving to detected pregnancy until the 150th or the 200th DIM, for DS1 and DS2, respectively and d) detected pregnancy (all AIs) by the 150th or 200th DIM (PREG_150, PREG_200), for DS1 and DS2, respectively. Calendar calving season was recorded as follows: winter (December, January and February), spring (March, April and May), summer (June, July and August) and autumn (September, October and November).

2.4 Statistical analysis

Statistical analyses were performed separately for DS1 and DS2. Kaplan-Meier survival analysis was performed to estimate the mean and median days to PREG_1stAI_100, PREG_1stAI_120 and the mean and median days to PREG_150 and PREG_200; pairwise comparisons among the levels of the 3 rumen (RFS, RCS and RFCS) and the 3 SCK (SCK_I, SCK_II and SCK_III) traits were performed using the log rank statistic. Censored cases for PREG_1stAI_100 and PREG_1st AI_ 120 were n_1=133 and n_2=337, respectively. Censored cases for PREG_150 and PREG_200 were n_1=104 and n_2=402, respectively.

Multivariable Cox proportional hazards (Cox-PH) models were performed to evaluate the association of each of the 3 rumen and the 3 SCK traits with the hazard for PREG_1stAI_100 (DS1) and PREG_1stAI_120 (DS2) and PREG_150 (DS1) and PREG_200 (DS2). Potential explanatory variables that were initially entered to the models were the fixed effect of herd (6 levels for DS1 and 9 levels for DS2), RFS (3 levels), RCS (2 levels), RFCS (2 levels), parity (3 levels: 1st, 2nd and 3rd+), calendar season at calving (4 levels), and postparturient diseases (binary variables). Separate models were fitted for each rumen and SCK traits. Potential 2-way interactions were studied during the preliminary Cox-PH model-building; however, no significant associations were acquired. Finaly, the six Cox-PH models (each for rumen and SCK trait) were built using a backward stepwise elimination procedure (Wald) in order to remove all variables with $P > 0.05$. The proportional hazards assumption was tested by visually assessing the estimated log $[-logS(t)]$ survival curves over different levels of tested categorical variables, with the assumption that plotted curves would be parallel **[29]**. The assumption was met for all variables. Moreover, scaled Schoenfeld residuals were plotted against ln(time) **[30]**. These plots did not trend up or down, but rather hovered around the "zero" line, indicating no trend over time. Independent censoring assumption was evaluated by performing complete positive correlation; that is, refitting the models after re-entering all the censored observations as if they conceived at the time of censoring, instead of being censored. There was no evident violation of this assumption. The functional form of each variable was evaluated using Martingale residuals; these residuals were computed from a null model, with no variables included. Plots of Martingale residuals against parity, an ordinal variable, were approximately straight, indicating that the relationships were linear **[30]**. The overall fit of the models was evaluated using Cox-Snell residuals. Plots of cumulative hazard estimations against Cox-Snell residuals of explanatory variables, which were allowed to vary with ln(time), were approximately straight lines with an intercept of 0 and a slope of 1, suggesting that models with no time varying covariates were more appropriate. Covariate-adjusted Cox hazard curves were generated showing the proportion of open cows by 150 (DS1) and 200 (DS2) DIM by level of rumen and SCK traits.

The association of the 3 rumen and the 3 SCK traits with the odds of PREG_1stAI_100 (DS1) and PREG_1stAI_120 (DS2) and PREG-150 (DS1) and PREG_200 (DS2) was evaluated with the binary logit function of multivariable Generalized Linear Mixed Model (GLMM-BLR) of SPSS. Cow was set as the experimental unit. Models were built using the same variables as with the Cox-PH; RFS1, RCS1 and RFCS1 and the non-SCK categories were set as the reference categories. A backward stepwise elimination procedure (Wald) was used to remove all variables with P > 0.05. Potential 2-way interactions were studied during the preliminary model-building; however, no significant associations were acquired.

Finally, differences in prevalence of SCK (all 3 traits) among the rumen variables were evaluated with the chi-square test. All statistical analyses were conducted using SPSS 25.0 (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Statistical significance was set at $P < 0.05$.

RESULTS

For the 3 rumen traits, a) mean and median days from calving to pregnancy after the 1st AI at natural estrus and b) mean and median days from calving to pregnancy (all AIs) for the two datasets, estimated by Kaplan-Meier survival analysis are shown in Tables 1 and 2, respectively. Overall, time intervals were shorter for levels representing adequate DMI and rumen motility (RFS3, RCS2 and RFCS2) compared to the other levels; in most cases these differences were statistically significant in both datasets.

The association of RFS and RFCS with hazard and odds for pregnancy at 1st AI at a natural estrus, yielded statistically significant results only for DS2. Compared to cows with RFS1, those with RFS3 had a greater hazard (HR =1.84, 95% CI: 1.05 – 3.23; $P=0.033$), and those with RFS2 and RFS3 had greater odds (OR=2.02, 95% CI: 1.08 – 3.77, $P=0.027$ and OR=2.49, 95% CI: 1.26 – 4.92, $P=0.009$, respectively) of PREG_1stAI_120. Similarly, compared to cows with RFCS1, those with RFCS2 had a greater hazard (HR=1.61, 95% CI: 1.03 – 2.54; $P=0.039$), and greater odds (OR=1.98, 95% CI: 1.15 – 3.42; $P=0.014$) of PREG_1stAI_120.

The association of RFS, RCS and RFCS with hazard and odds for PREG_150 (DS1) and PREG_200 (DS2) and the proportion of open cows by 150 (DS1) and 200 (DS2) DIM are shown in Table 3.

a) Regarding RFS: In DS1, cows with RFS3 had a greater hazard for PREG_150 (HR=3.31, $P=0.043$) compared to cows with RFS1. In DS2, cows with RFS2 and RFS3 had a greater hazard for PREG_200 (HR=1.58, *P=0.005,* and HR=1.73, *P=0.005,* respectively), compared with RFS1 cows. The covariate-adjusted proportion of open cows was 80.7%, 64.0% and 50.4% for DS1 (150 DIM) and 72.7%, 60.4% and 57.6% for DS2 (200 DIM), for cows with RFS1, RFS2 and RFS3, respectively. In DS1, cows with RFS3 had greater odds for PREG_150 (OR=4.83, *P=0.029*) compared to cows with RFS1. In DS2, cows with RFS2 and RFS3 had greater odds for PREG_200 (OR=1.95, *P= 0.004,* and OR=2.22, *P= 0.003,* respectively), compared with RFS1 cows.

b) Regarding RCS: Cows with RCS2 had a greater hazard for PREG_150 (HR=1.57, *P=0.041,* DS1) and for PREG_200 (HR=1.73, *P=0.017,* DS2) compared to RCS1 cows. The covariate-adjusted proportion of open cows was 71.1% and 54.2% for DS1 (150 DIM) and 75.0% and 60.0% for DS2 (200 DIM), for cows with RCS1 and RCS2, respectively. Moreover, cows with RCS2 had greater odds for PREG_150 (HR=2.02, *P=0.038,* DS1) and for PREG_200 (HR=2.21, *P=0.007,* DS2) compared to RCS1 cows.

c) Regarding RFCS: Cows with RFCS2 had a greater hazard for PREG_150 (HR=1.70, *P=0.033,* DS1) and for PREG_200 (HR=1.70, *P=0.001,* DS2) compared to RFCS1 cows. The covariate-adjusted proportion of open cows was 71.1% and 54.2% for DS1 (150 DIM) and 72.8% and 58.3% for DS2 (200 DIM), for cows with RFCS1 and RFCS2, respectively. Moreover, cows with RFCS2 had greater odds for PREG_150 (HR=2.13, *P=0.031,* DS1) and for PREG_200 (HR=2.20, *P<0.001,* DS2) compared to cows RFCS1 cows .

Covariate-adjusted survival curves of the 3 rumen traits for PREG_150 (DS1) and PREG_200 (DS2) are shown in Figure 1. Cows with RFS2 differed from RFS1 and RFS3 cows in DS1; they were very similar to RFS3 cows in DS2, though. Levels of binary RCS and RFCS traits were distinctively different from each other.

For the 3 SCK traits, a) mean and median days from calving to pregnancy after the 1st AI and b) mean and median days from calving to pregnancy for the two datasets, estimated by Kaplan-Meier survival analysis are shown in Suppl. Tables 1 and 2, respectively. Mean days from calving to pregnancy were numerically greater in SCK-positive cows compared to SCK-negative ones, across all thresholds in both datasets; however, differences were not statistically significant. Moreover, no statistically significant associations with hazard and odds for PREG_150 (DS1) and PREG_200 (DS2) were detected, for any of the 3 SCK traits, in either dataset.

Associations of metritis, parity and calving season with hazard for pregnancy and those of metritis and calving season with odds for pregnancy are presented in Suppl. Tables 3 and 4.

a) No associations were found for metritis in DS1; however, in DS2, cows with metritis had a lower hazard (HR=0.64, 0.66 and 0.68, for RFS, RCS and RFCS, respectively) and lower odds (OR=0.43, 0.56 and 0.44) for PREG_200.

b) Parity was retained in the model for the Cox-PH analysis (only for DS1) but not for the GLMM analysis. Cows of parities 2 and 3+ had lower hazard for PREG_150 compared to parity 1 cows (RCS and RFCS traits).

c) Cows calving during summer and autumn had generally greater hazard and odds for PREG_150 and PREG_200.

Prevalence of SCK in both datasets, for all 3 thresholds used and per rumen trait and level are shown in Table 4. The number of "positive" cows identified per rumen trait in all three SCK traits are shown in Table 4, as well. Differences in SCK prevalence among levels of rumen traits were statistically significant (*P=0.05*). Prevalence of SCK was highest in RFS1, RCS1 and RFCS1 but interestingly, also present in cows with adequate rumen fill and motility ranging from 1.8% to 6.3% in RFS3, from 8.0% to 14.0% in RCS2 and 4.8%-11.0% in RFCS2. Moreover, the combined RFCS trait would constantly identify more than twice the number of cows with future reproductive problems than the BHB blood test; e.g., 48 vs. 19 cows in DS1 and 192 vs. 73 cows in DS2, for the SCK_III trait.

DISCUSSION

The aim of this prospective observational study was to investigate the association of post-partum rumen fill and motility, as well as that of SCK, with future reproductive performance of Holstein cows. Additionally, the association of rumen fill and motility with SCK prevalence was also explored. Nutritional, reproductive and welfare management in all farms of both data-sets were above average and quite consistent; farms were visited daily and compliance was assured. To the authors' best knowledge, the associations detected, have never been published before.

Although rumen fill itself is affected by many factors (ration type and speed of gastric content transit among others), Rumen Fill Score was found in previous research to be well correlated with DMI and presents a substantial intra- and inter-observer repeatability **[31]**; this has a considerable clinical importance from a practical point of view, providing an easy means of assessing cow appetite. On the other hand, counting rumen contractions by auscultation is an objective clinical observation assessing rumen motility. Of course, since references associating rumen traits with reproduction are lacking in the literature, the present study should be replicated and results confirmed by other researchers (also exploring causes of infertility), before wider adoption of the practices outlined here can be recommended. The fact, though, that very similar outcomes resulted from two independent datasets that: a) included multiple farms and hundreds of cows each, b) cows in each data-set were evaluated by two different observers, one for each data-set, following the same protocol and c) were analyzed separately, strengthens our confidence regarding the validity of our findings. Cows with low scores (RFS1, RCS1 and RFCS1) had clearly impaired short- and long-term reproductive performance. Therefore, we can reasonably support that visual observation (RFS) and clinical examination (rumen auscultation for RCS), either alone or in combination (RFCS) in the early post-partum period can serve as an alert of suboptimal ruminal function and can identify cows at risk for future reproductive failure and in need of some kind of support to overcome this post-partum challenge.

Interestingly, and contrary to rumen traits, SCK at d7 or d8 was not associated with impaired reproductive performance in the current study. This is not unusual; the relatively low prevalence of SCK during the 1st week after calving in our datasets is probably the explanation. Moreover, even in studies where no association of hyperketonemia with reproductive performance was found, the latter was associated with elevated NEFA concentrations instead **[11-13,32]**, that is, with NEB. Rumen traits, as defined in our study (low DMI and motility) imply an association with NEB but not necessarily with BHB, which may or may not be elevated (yet) at testing-day **[14]**. It would be interesting to re-check cows with low RFS and normal serum BHB values at RFS testing day, a few days later in order to explore whether BHB concentrations do increase. On the other hand, higher prevalence of SCK in rumen trait levels denoting low DMI and motility (RFS1, RCS1 and RFCS1) compared to levels denoting adequate intake and rumen function (RFS3, RCS2 and RFCS2) appears logical. There are no other similar published studies to compare our results with.

Maximization of nutrient intake is one of the main goals in transition management **[33]**. Although this is widely accepted, adoption rate of individual cow appetite monitoring practices by farmers has been rather moderate to low. In Germany, only 35% of the respondents (n=429) used rumen fill scoring **[3]**. In a California study (n=45), rumen fill scoring, alone or in combination with feed-bunk scoring was applied in 35% of the dairies, as well **[4]**. In the same study, auscultation was used to diagnose displaced abomasum (DA) and pneumonia cases in 20% and 9% of the dairies, respectively; however, rumen contractions were counted by auscultation in 2% of the dairies, only **[4]**. Moreover, in Canada, cow appetite (method not reported) was systematically evaluated only in 27% of the farms (n=78) **[34]**. Interestingly, adoption of SCK testing, either using blood or milk/urine samples, was even lower in these studies (3% in **[3]** and 7% in **[4]**), despite the extensive research regarding the effects of hyperketonemia on health and reproduction.

All authors of the aforementioned studies stress the restricted time and human resources to perform these tasks and the rudimentary application of monitoring programs, in most cases. A recent study provided valuable information regarding the duration of various examinations and the time cows remained blocked in headlocks **[35]**. Clearly, a strong motivation is needed for farm managers and personnel alike, to implement fresh cow health monitoring, whatever checks these may include. Motivation would stem from definitive associations with economically important outcomes and the availability of effective preventive and corrective measures.

Research so far, has focused mainly on SCK in fresh cows as a factor associated with future health and reproductive problems. Abomasal displacement (DA) has attracted considerable attention and although not all studies had a definitive outcome **[11,36,37]**, this issue has been thoroughly reviewed **[14]** and it is now widely accepted that SCK and DA are strongly associated. However, with a mean incidence of 2.2% **[38]** and an alarm rate at ≥6% **[2]**, DA may not provide a motive strong enough for rigid fresh cow monitoring. Considerable improvements in milk production would certainly be a stronger motive but research results are ambiguous and after accounting for various diseases, it appears that the benefit is actually less than 1kg/cow/day **[14]**. Early diagnosis of MET (checking for abnormal vaginal discharge and fever) is probably the main reason dairy farmers implement a fresh cow monitoring program **[1,4,6]**; based on their experience, MET is negatively associated with future reproductive performance **[39,40]**. The latter is undoubtedly the stronger motivation due to the economic losses it incurs **[41-43]**.

Daily checking of general cow appearance and disposition, presence of abnormal discharges or elevated body temperature is desirable; however, specific diagnostic tasks should be focused on fixed time-points in order to be logistically effective and thus, easily adopted. Obviously, blood or milk/urine samples for SCK checking are not expected to be collected daily and monitoring schemes proposed call for one or two checks between 3 and 14 DIM **[44-46]**. In this respect and considering the results of the present study that clearly associate rumen traits with future reproductive performance, checking once for rumen fill and motility at DIM 7 or 8 can be more efficient as twice the number of cows needing help can be detected without using any invasive techniques. Availability of effective corrective measures once the problem has been identified would also stimulate farmers' interest in fresh-cow monitoring schemes. Again, research has focused on SCK treatment and data have been systematically reviewed **[47]**; administration of propylene glycol (PG) is effective **[44,45]** but the number of available well-designed studies is small. Moreover, in our case, the point of interest is low DMI and rumen motility, not SCK itself. The Hepatic Oxidation Theory **[23,48,49]** links decreased DMI with elevated blood NEFA concentrations. By experience, strategies suggested in **[23]** aiming to avoid decreased DMI are quite useful but should be considered as a way to prevent the problem, not to resolve it. Whether feed intake increases (and how fast) after the administration of PG or of any other product reviewed in **[47]** has not been investigated; resolution of SCK certainly implies this but direct (actual intakes) or indirect evidence (RFS) is lacking. It is reasonable to assume that cows with low rumen fill and motility scores would benefit from measures proposed for subclinically ketotic cows; and since more cows are identified, herd-level results would subsequently improve. However, there are still several subclinically ketotic cows assigned favorable (RFS3, RCS2 and RFCS2) scores (see Table 4). These cows would not be identified if we omit the BHB test. There is a debate amongst farmers and veterinarians, whether cows with a "positive" BHB test but perfectly fit otherwise (high milk production and DMI, no signs of disease) should be treated for SCK. The negative association of hyperketonemia with health and production is well established **[50,51]**, linked to impaired immune function and oxidative stress **[52-55].** Moreover, the period of interest extends beyond the immediate post-partum period and both short- (pregnancy at 1st AI) and long-term reproductive outcomes are of interest. Cows with low rumen scores at d7 or d8 but not classified as subclinically ketotic at that point (depending on threshold used), may well have one or more positive BHB tests during the 3rd, 4th or 5th week of lactation, even later on. We recently showed that SCK extends well beyond the post-partum transition period and repeated cases in the same cow are common (39.4% and 27.3%, when incidence is high [46.7%] and low [19.2%], respectively) **[56]**. Impaired immune function, extended beyond the typical post-partum transition period (3 weeks) due to SCK, can impact clinical and subclinical endometritis incidence in dairy cows; these have detrimental effects on overall reproductive performance **[57-59]**. Therefore, we believe that rumen fill and motility scores and BHB tests should complement each other; this way, repetitive BHB tests will not be needed.

One other issue was how to treat the intermediate RFS2 level when building the combined RFCS trait. We allocated it to the unsatisfactory status when combined with RCS1 but not when combined with RCS2. In **[28]**, it is stated that RFS2 is common in early (1st week) postparturient cows. Certainly, common does not mean normal, rather, we considered this as “expected”; and common it was as 41% and 57% of cows were classified as RFS2 in DS1 and DS2, respectively. Our choice was based on the notion that normal rumen motility is a promising sign that DMI will improve. This point needs refining in future studies considering that reproductive outcomes were different in DS1 between RFS2 and RFS3 (but not in DS2, see Figure 1). Investigation of the overall association of rumen fill and motility scores with rumination time could provide useful insights for this specific issue, as well. The last decade, rumination time has been associated with several disease outcomes **[60-65]** and with SCK in particular in the early post-partum period **[66-68].** It has not been associated with reproductive performance, yet. If associations between rumen traits and rumination time exist, smaller farms or those at developing countries that may not have access to modern technology could benefit from the simplicity of monitoring the rumen traits used in this study.

Metritis was found to be negatively associated with future reproductive performance in this, as in numerous other studies **[39,40,69,70]**. Other post-partum diseases or the combined CD_1-8 were not, but our health monitoring period was short (8 days). Diseases like MAST, KET, DA and SCK during the 2nd or the 3rd week post-partum might also have a negative association with reproduction as was the case in other studies

[39,71,72] but such records were not available. Finally, to our knowledge, no attempt has been made so far to find associations of rumen fill and motility with post-partum transition diseases.

The use of two separate datasets originating from studies with different objectives and observers provided data from a large number of cows of multiple farms but we were not able to investigate the potential effect of the observer assessing the rumen traits. This was the main limitation of this study. Nevertheless, these heterogeneous datasets yielded similar associations with the tested reproductive traits, suggesting generalizability of our results. On the other hand, close and consistent monitoring of the enrolled cows during the study period by the same veterinarian within each original cohort study was a strength of our study. A study including multiple observers, allowing for an estimation of the inter-observer agreement, with more frequent measurements during an extended period post-partum, designed to investigate the association of early post-calving rumen traits with targeted fertility traits would provide answers in more in-depth issues.

CONCLUSIONS

Rumen fill and motility scores at d7 or d8 post-partum appear to be associated with future reproductive performance in Holstein dairy cows. Combined with a concurrent blood BHB test, the majority of cows needing assistance to overcome transition challenges can probably be identified while allowing farm personnel to focus on a specific time-point, enhancing labor and at the same time, production efficiency, through improved cow health and welfare.

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DECLARATION OF INTEREST

The authors declare no financial or commercial conflicts of interest.

ETHICS STATEMENT

The research was conducted in compliance with institutional guidelines and approved by the Research Committee of the Aristotle University of Thessaloniki, Greece (Approval protocol numbers 62/15-12-2015 and 5/14-12-2010). An informed consent was obtained from all farmers involved in the study.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

G. E. Valergakis: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **N. Siachos**: Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing - review & editing. **A. Kougioumtzis**: Data curation, Software, Validation, Writing - review & editing. **G. Banos:** Data curation, Formal analysis, Writing - review & editing. **N. Panousis**: Formal analysis, Investigation, Writing - review & editing. **V. Tsiamadis**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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