animal

Combinations of non-invasive indicators to detect dairy cows submitted to high-starch-diet challenge

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*High-starch diets (**HSDs**) fed to high-producing ruminants are often responsible for rumen dysfunction and could impair animal health and production. Feeding HSDs are often characterized by transient rumen pH depression, accurate monitoring of which requires costly or invasive methods. Numerous clinical signs can be followed to monitor such diet changes but no specific indicator is able to make a statement at animal level on-farm. The aim of this pilot study was to assess a combination of non-invasive indicators in dairy cows able to monitor a HSD in experimental conditions. A longitudinal study was conducted in 11 primiparous dairy cows fed with two different diets during three successive periods: a 4-week control period (P1) with a low-starch diet (**LSD**; 13% starch), a 4-week period with an HSD (P2, 35% starch) and a 3-week recovery period (P3) again with the LSD. Animal behaviour was monitored throughout the experiment, and faeces, urine, saliva, milk and blood were sampled simultaneously in each animal at least once a week for analysis. A total of 136 variables were screened by successive statistical approaches including: partial least squares-discriminant analysis, multivariate analysis and mixed-effect models. Finally, 16 indicators were selected as the most representative of a HSD challenge. A generalized linear mixed model analysis was applied to highlight parsimonious combinations of indicators able to identify animals under our experimental conditions. Eighteen models were established and the combination of milk urea nitrogen, blood bicarbonate and feed intake was the best to detect the different periods of the challenge with both 100% of specificity and sensitivity. Other indicators such as the number of drinking acts, fat:protein ratio in milk, urine, and faecal pH, were the most frequently used in the proposed models. Finally, the established models highlight the necessity for animals to have more than 1 week of recovery diet to return to their initial control state after a HSD challenge. This pilot study demonstrates the interest of using combinations of non-invasive indicators to monitor feed changes from a LSD to a HSD to dairy cows in order to improve prevention of rumen dysfunction on-farm. However, the adjustment and robustness of the proposed combinations of indicators need to be challenged using a greater number of animals as well as different acidogenic conditions before being applied on-farm.*

Keywords: multi-parametric analysis, starch-rich ration, proxies, dairy cattle, proof of concept

Implications

High-starch diets can cause digestive disorders that negatively impact dairy cow welfare and result in large economic loss to farmers. The decrease in rumen pH is mainly responsible for the subsequent digestive and metabolic disorders, but no specific clinical sign reflects animal's health status. This pilot study proposes combinations of non-rumen indicators in several models able to detect animals affected by nutritional high-starch diet challenge in experimental conditions. Hence, we built the proof of concept that simultaneous analyses of non-rumen indicators could be used for on-farm detection of risky diets fed to dairy cows.

Introduction

Due to intensification of animal production, precision nutrition is of major concerns since it allows the farmer to preserve animal welfare and maximize benefits of the farm. In this context,

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feeding high-starch diets (**HSDs**) is a solution to meet high-producing dairy cows requirements. According to animal susceptibility, a HSD may impair rumen function and animal health leading to large economic losses to farmers.

The HSDs fed to dairy cows are responsible for digestive disorders initiated by the drop in rumen pH, which is commonly associated to sub-acute ruminal acidosis (**SARA**) syndrome (Plaizier *et al.*, 2008). A multiplicity of non-specific clinical signs can emerge from SARA with negative effects on production and health in dairy cows, which include milk yield drop, milk fat depression, laminitis or liver abscesses (Martin *et al.*, 2006) as well as locomotion disorders (Garrett *et al.*, 1997), erratic feed intake (Li *et al.*, 2011) or animal behaviour disorders (Soriani *et al.*, 2012). Furthermore, increased concentrations of stress and inflammation biomarkers (Gozho *et al.*, 2007; Trevisi *et al.*, 2014) have been reported under SARA conditions in different biological matrices that include blood and faeces (Plaizier *et al.*, 2017), urine (Vagnoni and Oetzel, 1998) and saliva (Tapio *et al.*, 2016). Milk fatty acid composition is also related to changes in rumen pH and affected by SARA in dairy cows (Colman *et al.*, 2010; Fievez *et al.*, 2012). Nevertheless, no study has examined the potential interest to mathematically combine indicators to monitor the impact of HSDs more accurately.

Due to the lack of specific clinical signs to identify cows under SARA conditions, we performed a pilot study using a nutritional model where dairy cows were challenged with a HSD known to induce ruminal dysfunction at risk of SARA (Villot *et al.*, 2018). Our aim was to propose an original approach by developing combination of non-invasive indicators based on multi-parametric models to detect dairy cows at risk of digestive disorders.

We analysed non-invasive variables obtained from 11 dairy cows fed a HSD for 4 weeks. First, a large number of variables known to be modified under SARA were screened. Then, the most relevant indicators were classified according to multivariate analyses before being used to highlight potential combinations able to discriminate animals affected by a HSD challenge in experimental conditions. Finally, the abilities of the established combinations of indicators were compared.

Materials and methods

Animals, diets and experimental procedures

Eleven primiparous Holstein dairy cows, including six rumen-fistulated animals, were in individual stalls on concrete floors with rubber mats and had *ad libitum* access to water. At the start of the experiment, dairy cows had an average body weight of 658±37 kg (mean±SD), were 135±7 days in milk and had a milk yield of 27.5±2.3 kg/day. Two different total mixed diets (**TMDs**) were distributed over 12 weeks of the longitudinal experiment as described precisely by Villot *et al.* (2018). Briefly, cows received a low-starch diet (**LSD**) as a control containing 13% starch (on a DM basis) for a 4-week period (**P1**). The amount of starch was then increased gradually every 2 days, from 13% to 35% during a 6-day transition period.

An HSD containing 35% starch was distributed for a 4-week period in order to maintain a long-term HSD challenge (**P2**). Finally, cows received the initial diet (LSD), without transition for a 3-week recovery period (**P3**). All diets were formulated to cover at least 105% of energy ($NE_L = 1.59$ Mcal/kg DM for LSD and 1.73 Mcal/kg DM for HSD) and protein (metabolizable protein = 95 g of protein digestible in the intestine (**PDI**)/kg DM for LSD and 101 g of PDI/kg DM for HSD) requirements for lactating dairy cows based on a milk production of 27 kg/day (INRA, 2007). Portions of 60% and 40% of the daily amount of the TMD were offered twice daily at 0900 h and 1630 h, respectively. The desired quantity of TMD was obtained by daily adjustment of the amounts offered, depending on the refusals of the previous day. Feed intake was measured and recorded twice daily throughout the experiment by individually weighing the feed offered and subtracting the refusal weight of the TMD. Throughout the experiment, cows were milked twice daily at 0700 h and 1600 h.

Sampling and analyses

Eight weeks of measurements were spread throughout the experiment on weeks 3 and 4 (P1), weeks 6, 7, 8 and 9 (P2) and weeks 10 and 12 (P3). A large number of variables were screened in different matrices during this experiment (Supplementary Table S1), but only the detailed analysis of the final indicators selected is described in this section.

Milk. Samples were collected over day 2, and day 3 (four consecutive milkings) of each measurement week. Individual samples (10 ml) of each milk were stored at 4°C with potassium dichromate (Merck Chimie SAS, Fontenay-sous-Bois, France) before analysis of urea, fat and protein content by mid-infrared analysis (AOAC International, 1997) using a three-channel spectrophotometer (MilkoScan 4 000 (Foss Electric A/S, Hillerod, Denmark); Laiterie Interprofessionnelle Auvergne Limousin, Aurillac, France). A second sample (day 3) was freeze-dried and then composited based on AM and PM milk yields before fatty acid (**FA**) analysis as described in Ferlay *et al.* (2010). Briefly, the FAs were transmethylated to FA methyl esters (**FAME**) and then FAME peaks were routinely identified by a comparison of the retention times with authentic FAME standards (GLC 463, Nu-Chek Prep Inc., Elysian, MN, USA; reference mixture 47 885, Supelco Inc., Bellefonte, PA, USA) and a mixture of C18:1, C18:2 and conjugated linoleic acid (**CLA**) isomers (Loor *et al.*, 2005). Isomers of CLA were identified using authentic CLA methyl ester standards (O5632; Sigma–Aldrich Chemie S.a.r.l.) and the elution order of the isomers reported in the literature (Shingfield *et al.*, 2008). A reference standard butter (CRM 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to estimate the correction factors for the short-chain FAs (4:0 to 10:0).

Blood. Samples were individually collected before the morning feed distribution on day 2 of each week of measurement. Blood was collected from the jugular vein into a lithium heparinized 10-ml tube (Elvetec Services, Meyzieu, France).

Each capped tube was placed on ice and analysis of bicarbonate concentration and pH was performed within 30 min of collection with a blood gas analyser (ABL5, Radiometer, Copenhagen, Denmark). Another 10-ml blood sample was collected at the same time using EDTA-collecting tubes (Elvetec Services, Meyzieu, France) and plasma was separated immediately after sampling by centrifugation at $3500 \times g$ for 15 min at 4°C. Plasma samples were frozen at −20°C until urea, cholesterol, β-hydroxybutyrate (**BHB**) and glucose determinations on an Arena 20XT (Thermo Scientific, Vaanta, Finland) automated analyser. Spectrophotometric enzymatic kits were specific: 981 820 kit for urea, 981 379 kit for glucose and 984 325 kit for BHB, from Thermo Scientific. Cholesterol was analysed at 37°C by means of a clinical autoanalyser (ILAB 650, Wefen, Instrumentation Laboratory, Lexington, MA, USA), using IL Test kit (0018255740, Wefen, Instrumentation Laboratory, Lexington, MA, USA).

Faeces and urine. Urine and faecal samplings (500 ml each) were synchronized with blood collection. Faecal samples were collected via grab sampling from the rectum. Faecal pH was measured immediately after the collection with a digital pH-meter (VWR pH100) with a precision of 0.1 unit, calibrated with standard solutions (pH = 4, 7 and 10). Faecal sieving was performed on a 425-ml faecal sample to establish the total and relative proportions of different particle sizes. First, faecal density was calculated by weighing each sample. Then, the particle size distribution of faeces was determined by commercial wet sieving (Lallemand SAS, France) using sieve apertures of 5 and 2 mm. Faecal samples were washed for 5 min using the same water nozzle and pressure. After sieving, faecal residuals from each sieve were back-weighed and the proportions of different sized particles were calculated based on the total material retained.

Urine was collected during spontaneous urination before the morning feed distribution on day 2 of each week of measurement. A sample of urine was collected in a 300-ml container in which pH was immediately recorded (pH parameter as described previously).

Behaviour. Rumination time (**RTime**) was measured continuously using the HR-Tag (SCR Engineers Ltd, Netanya, Israel) previously validated on dairy cattle (Schirmann *et al.*, 2009). This system is held on a nylon collar positioned on the left side of the cow's neck. The logger contained a microphone and microprocessor that recorded the distinctive sounds of rumination in 2-h blocks. Daily RTime was calculated individually for the 11 cows by adding the 2-h blocks values over 24 h. The time budget of all dairy cows was estimated from 24-h video recordings. The behaviour of each cow was scanned every 5 min by a skilled technician (used to describe video recording without errors) to describe the pattern of daily activities during day 7 of each week of measurement. Times spent in behaviour categories (eating, drinking, standing and lying) were isolated and calculated as previously described by Mialon *et al.* (2008). Drinking acts were calculated daily based on a drop in rumen temperature of at least

−0.2°C (Gasteiner *et al.*, 2015) in a local minimum within a 30-min interval. This calculation was internally validated using the 24-h video recordings of the cows during the experiment. Rumen temperature was measured by a commercial reticular sensor (eCow, Exeter, UK). One sensor per cow was placed in the reticulum through the oesophagus. Each sensor was set up to record temperature over 15 min (96 data points/day) with an accuracy of ±0.1. Data were downloaded every 15 days using the eCow handset (smartphone+antenna) with the eCow Android application.

Statistical analysis
A total of 136 variables were obtained from 5 different matrices (faeces, blood, milk, urine and saliva) and behaviour, and then were screened throughout the experiment to establish the models. Milk data were averaged per day based on AM and PM milk yields. Repeated data over a day or several days were averaged over their respective week of measurement. All data were analysed using R 3.2.3 software, 2016. When necessary, data were log-transformed before analysis. Four steps of data analysis (Table 1) were performed to identify plausible and robust combination of indicators able to discriminate HSD from LSD:

(1) The variables modified statistically during the experiment were determined using a linear mixed model (see Supplementary Material S1). The model was implemented with the lme4 and lmerTest packages (lmer function) as follows: $Y_{ijk} = \mu + A_i + B_j + \varepsilon_{ijk}$, where $Y_{ijk} =$ dependent variables ($k = 136$), $\mu =$ general mean, $A_i =$ fixed effect of the week considered as the within-subject factor (mean of weeks 3 and 4 (P1), weeks 6, 7, 8 and 9 (P2) and weeks 10 and 12 (P3)), $B_j =$ random animal effect ($j \in [1, n]$) and $\varepsilon_{ijk} =$ residual error term (DDFM = Kenward–Roger) taking into account the repeated measures. When a variable had missing values, the df were calculated by the Kenward–Roger's approximation. The results of the linear mixed models and the least square means with the SE of the models are reported in Supplementary Table S1. Since no statistical significance was observed between weeks 3 and 4 of the LSD period (P1) for all the variables, the average value from these 2 weeks of P1 was calculated as the control value for each variable of the cow. The Dunnett's *post hoc* test comparing LSD *v.* each other weeks of measurement was then applied in order to observe statistically significant changes in each variable during the experiment. A first and broad selection was performed by retaining variables for which the P value of the mixed model was lower than 0.1 between LSD and each other individual week during HSD challenge.

(2) A second selection of the most discriminant variables was made by combining two multivariate approaches: (i) a principal component analysis (**PCA**, see Supplementary Material S1) to establish the correlations between variables and to illustrate the ability of these variables to discriminate LSD and HSD and (ii) a partial least squares-discriminant analysis (**PLS-DA**, see Supplementary Material S1) to rank the importance of the variables in this discrimination which includes a leave-one-out cross-validation procedure (ropls function of Bioconductor package in R). The PCA and PLS-DA were performed on data of LSD during P1 and HSD periods for the 11 cows. We implemented an individual normalization cow by cow in order to take into account the repeated measures without being

Table 1 *Four steps in statistical analyses of data to elaborate combination of indicators to detect dairy cows submitted to HSD challenge*

Steps	Statistical analysis	Data set used	Variables analysed	Objective
1	(a) Linear mixed model (b) Dunnett's *post hoc* test	P1, weeks 6, 7, 8, 9, 10 and 12 ($w=77$)	50 in blood 46 in milk 23 in saliva 9 for behaviour 7 in faeces 1 in urine	(a) Identification of variables modified during the experiment ($P<0.1$) (b) Comparison of P1 mean *v.* weeks 6, 7 ,8, 9, 10 and12
2	Multivariate analysis: PCA and PLS-DA	P1 and P2 ($w=22$)	25 in blood 35 in milk 13 in saliva 5 for behaviour 6 in faeces 1 in urine	Selection of best discriminating variables (LSD control (P1) *v.* HSD (P2)) with the lowest multi-collinearity
3	Model development : Generalized linear mixed model	P1 and P2 ($w=22$)	4 in blood 6 in milk 3 for behaviour 2 in faeces 1 in urine	Looking for the best predictive binary models with 2 or 3 indicators
4	Validation of each model: confusion matrix	*Data set #1* ($w=66$): weeks 3, 4, 6, 7, 8 and 9 *Data set #2* ($w=88$): Data set #1 + weeks 10 and 12 *Data set #3* ($w=77$): Data set #1 + week 12	18 models with 2 or 3 indicators	Detection abilities of HSD and LSD of the established models with individual week data

PCA = principal component analysis; PLS-DA = partial least squares-discriminant analysis; $w=$ number of observation used to perform the analysis; P1, P3 = LSD (low-starch diet); P2 = HSD (high-starch diet).

impacted by the high inter-animal variability. Variable importance in projection (**VIP**) scores of the PLS-DA estimates the importance of each variable (among each other) in the projection to the latent structure. Non-correlated variables with the highest VIP scores can be considered as the most important to discriminate LSD (P1) and HSD and have been named as indicators.

(3) Statistical models were built in order to calculate the probability that a cow was affected by HSD challenge or not from the combination of two or three indicators. Since a linear regression model has no limit boundaries, the use of a logistic function (sigmoid curve) was required as link function between the probability to be affected by HSD challenge and the explanatory indicators. The classical logit function was chosen, and the models can be written as follows:

$$\ln\left(\frac{P(\text{HSD}|X_i)}{1 - P(\text{HSD}|X_i)}\right) = a_1 X_1 + a_2 X_2 + \ldots + a_p X_p + b$$

equivalent to

$$P(\text{HSD}|X_i) = \frac{\exp\left(a_1 X_1 + a_2 X_2 + \ldots + a_p X_p + b\right)}{1 + \exp\left(a_1 X_1 + a_2 X_2 + \ldots + a_p X_p + b\right)}$$

with $b=$ constant; $a_{1, 2,., p}=$ coefficients; $X_{1, 2, \ldots, p}=$ explanatory indicators.

To take into account the repeated measurements, a generalized linear mixed model (**GLMM**) was performed with lme4 and lmerTest packages (glmer function). The models were calculated with period values of indicators for LSD (P1) and HSD for each cow. A model selection was performed according to their Bayesian Information Criterion and to maximize their likelihood function. Model by model, the P value of each selected indicator was lower than 0.05.

(4) Individual probability that a cow was under HSD challenge or was not calculated for each model. Whereas the models were developed based on average data of each period (number of observation used, e.g. $w=$ (11 cows×4 weeks) = 44), the ability of each model to detect HSD accurately was tested on individual week data (data set #1):

- data set #1 ($w=66$): weeks 3 and 4 (P1) for LSD and weeks 6, 7, 8, and 9 (P2) for HSD.
  Then, the ability of each model to identify accurately when animals had totally recovered from an HSD challenge was tested by comparing two different weeks' data sets including recovery data as LSD period: #2 and #3:
- data set #2 ($w=88$): weeks 3 and 4 (P1), and weeks 10 and 12 (P3) for LSD and weeks 6, 7,8 and 9 (P2) for HSD.
- data set #3 ($w=77$): weeks 3 and 4 (P1), and week 12 (P3) for LSD and weeks 6, 7, 8 and 9 (P2) for HSD.

The first week of recovery P3 was not taken into account in data set #3, whereas it was included in data set #2.
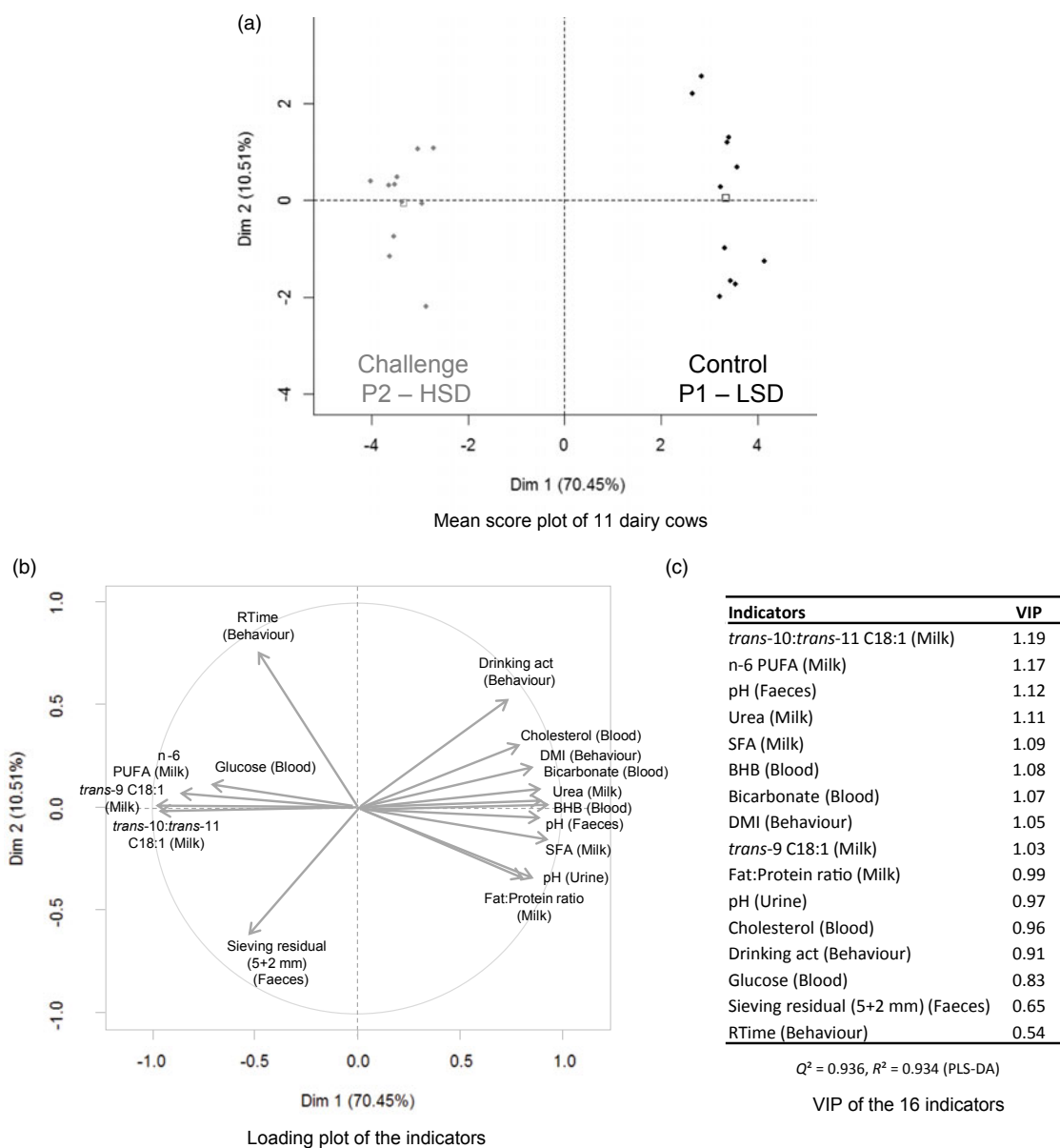
(a)

Mean score plot of 11 dairy cows

(b)

Loading plot of the indicators

(c)

Indicators	VIP
trans-10:*trans*-11 C18:1 (Milk)	1.19
n-6 PUFA (Milk)	1.17
pH (Faeces)	1.12
Urea (Milk)	1.11
SFA (Milk)	1.09
BHB (Blood)	1.08
Bicarbonate (Blood)	1.07
DMI (Behaviour)	1.05
trans-9 C18:1 (Milk)	1.03
Fat:Protein ratio (Milk)	0.99
pH (Urine)	0.97
Cholesterol (Blood)	0.96
Drinking act (Behaviour)	0.91
Glucose (Blood)	0.83
Sieving residual (5+2 mm) (Faeces)	0.65
RTime (Behaviour)	0.54

$Q^2 = 0.936$, $R^2 = 0.934$ (PLS-DA)

VIP of the 16 indicators

Figure 1 Principal component analysis (PCA) of indicators discriminating cows from the HSD challenge (P2) to the LSD control (P1), and the VIP scores of each indicator calculated with the PLS-DA. Results of PCA presented as (a) score plot of 11 dairy cows and (b) loading plot of the different indicators (mean of week 3 and week 4 with 2 observations/cow for LSD control (P1), and mean of 4 observations/cow for HSD (P2)) and (c) the VIP scores of each indicator calculated with the PLS-DA. This PCA was designed to illustrate mixed model results. HSD = high-starch diet; LSD = low-starch diet; VIP = variable importance in projection; PLS-DA, partial least square discriminant analysis; RTime = rumination time; BHB = β-hydroxybutyrate; SFA = saturated fatty acid; PUFA = poly-unsaturated fatty acids; DMI = DM intake; R^2 = coefficient indicating the predictive accuracy of the PLS-DA; Q^2 = coefficient indicating the quality of the leave-one-out cross-validation of the PLS-DA.

A value (or score) was a true positive (**TP**) when the probability calculated by the model was strictly above 0.50 during HSD period and a true negative (**TN**) when the probability calculated by the model was below 0.50 during LSD period. In contrast, a false-negative (**FN**) value was noted when the model calculated a probability strictly below 0.50 during HSD challenge and a false-positive (**FP**) value was attributed when the model calculated a probability strictly above 0.50 during LSD period. The true positive rate (**TPR** = sensitivity) and true negative rate (**TNR** = specificity) were then calculated as follows: TPR = TP/(TP+FN), TNR = TN/(FP+TN).

Description of statistical techniques can be found in Supplementary Material S1.

Results

Screening of non-invasive variables
A total of 85 among 136 variables were substantively modified, from a statistical point of view, between week measures throughout the experiment ($P<0.1$) (Supplementary Table S1). A large majority of changed variables were measured in milk (41% of total modified indicators), blood (30%) and saliva (15%), whereas variables

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related to faeces (7% of total modified indicators), behaviour (5%) and urine (<5%) were lower.

The selection of 85 variables was then refined using a multivariate analysis to identify which ones were the most appropriate to discriminate animals fed HSD from animals fed LSD (P1). Only one variable per group of variables presenting multi-collinearity was kept (e.g. for milk composition, fat:protein ratio was more discriminant in comparison to individual fat or protein concentrations). Hence, PLS-DA discriminated fewer variables and a classification was performed with their individual VIP. The results of the successive PLS-DA and PCA (with 135, 85 and 16 variables) are presented in Supplementary Figures S1, S2 and Figure 1, respectively, and were able to discriminate the animals undergoing HSD challenge from animals fed LSD control with high-quality metrics ($R^2 = 0.936$ and $Q^2 = 0.934$ for the PLS-DA with 16 variables). As resume in Figure 1, the PCA dimensions 1 and 2 accounted for 70.5% and 10.5% of the total dispersion, respectively. On dimension 1, animals fed HSD are all clearly plotted on the left part, whereas the cows are plotted on the right part when fed LSD in P1 (Figure 1a). Selected indicators were mostly well represented on the dimension 1 of the loading plot (Figure 1b) except for faecal sieving residual and RTime, which contributed less than the others on this dimension, and which has the lowest VIP scores (Figure 1c). Indicators were able to characterize both LSD (P1) and HSD periods, meaning that the variability in the data is first explained by the two different periods. The loading plot (Figure 1b) showed that lower values in several indicators, such as blood bicarbonate concentration, fat:protein milk ratio, milk urea and faecal pH, were closely related to HSD, while other indicators, such as percentage of n-6 poly-unsaturated fatty acid (**PUFA**) and milk *trans*-10: *trans*-11 C18:1 ratio, had higher values.

Table 2 summarizes the 16 indicators and details about these variables are reported on the trajectory graphs (Supplementary Figure S3). Most of the indicators were modified for several weeks when cows were fed HSD and recovered rapidly as soon as they were in the recovery: week 10 (LSD, P3). Faecal pH, blood bicarbonate, BHB and cholesterol concentrations, milk fat:protein ratio and urea concentration were lower during HSD challenge in comparison to the LSD control (P1) and LSD recovery (P3) ($P<0.001$). The milk n-6 PUFA and *trans*-9 C18:1 concentrations and the *trans*-10: *trans*-11 C18:1 ratio were increased, whereas saturated FA (**SFA**) decreased ($P<0.001$) in cows fed HSD in comparison to cows fed LSD control. The initial values of those indicators were only reached again in the last week of the recovery period, week 13 (P3). Urea concentrations measured in milk decreased ($P<0.001$) in cows fed HSD in comparison to LSD, whereas blood urea concentration only decreased in week 8 during HSD challenge.

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Finally, in these particular experimental conditions, 18 different models able to detect animals affected by HSD challenge

among all possible equations built from the 16 explanatory indicators were selected (Table 3): 13 models with 2 indicators and 5 models with 3 indicators.

The models were classified according to their sensitivity and specificity in detecting animals submitted to HSD challenge. One model (no. 1) including DM intake (**DMI**), blood bicarbonate and milk urea concentrations perfectly identify (TPR = 100%) when cows were fed HSD. In this study, 15 of the developed models allowed to classify correctly animals fed the HSD challenge with a sensitivity higher than 85% and a specificity higher than 90%.

With the exceptions of model no. 7 and no. 11, model specificity is increased by including only the data of the last week of the recovery period (data set #3) in contrast to including both the first and last weeks of the recovery period (data set #2).

Discussion

Combinations of non-invasive indicators to detect animals submitted to high-starch-diet challenge

We propose here an original approach to detect, with a combination of non-invasive indicators, animals at risk of SARA presenting ruminal dysfunction consecutively to an HSD challenge. Since cows with ruminal dysfunctions present different clinical signs resulting to a poor diagnosis of SARA disease, we used a nutritional model known to induce ruminal dysfunction at risk of SARA. This approach supplied a pool of models balancing goodness of fit with simplicity. Moreover for variables selection, we did not use the actual statistical agreement that makes consensus in science for the *P-value* threshold of 0.05 but we rather choose 0.1. Since a *P value*>0.05 does not necessary means a failure of rejecting the null-hypothesis but only inconclusive results, the choice to use 0.1 threshold was made to keep more variables, which in combinations with others can better explain differences between periods than a single one. Models with a biological justification and that could be used on-farm were finally chosen. Eighteen combinations were established using the data of the LSD and HSD periods of the 16 indicators previously highlighted in different matrices. The 16 indicators used to build the proposed combinations are already well known in the literature as potential biomarkers of SARA. The decrease in pH in different matrices (faeces, urine and blood) during SARA has been reported in several studies and could biologically been explained by the increase in acid load in blood that cannot be compensated by the bicarbonate buffer system resulting in the increase in acid secretion by the kidneys (Danscher *et al.*, 2015). Nevertheless, conflicting results about pH measures in faeces or urine investigated during HSD challenges lead to a dissensus about pH as biomarker of SARA on-farm (Morgante *et al.*, 2009; Gianesella *et al.*, 2010). In addition, a decrease in blood bicarbonates can be indicative to some extent of physiological compensation for increased absorption of ruminal Volatile Fatty Acids (VFA) during SARA (Burrin and Britton, 1986). Urea-nitrogen was decreased in milk matrix during HSD challenge. A high level

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Table 2 *Dairy cow indicators affected by experimental HSD challenge*

Periods	LSD[1] control (P1)	HSD[2] (P2)				LSD[1] recovery (P3)		P value[3]	RSD	Variance	
Indicators weeks	Mean of 3, 4	6	7	8	9	10	12	Week	Model	Cow	residual
Blood											
Bicarbonate, mmol/l	33.3±2.6	26.6±2.8**	ND	25.6±4.7**	29.2±2.8*	30.6±4.6	31.2±2.7	<0.001	3.6	3.719	13.21
BHB, mmol/l	0.429±0.085	0.239±0.037**	0.231±0.055**	0.306±0.082**	0.332±0.077**	0.398±0.083	0.42±0.052	<0.001	0.066	0.004	0.004
Cholesterol, mmol/l	5.08±0.67	3.93±0.39**	3.95±0.33**	4.31±0.40**	4.14±0.55**	4.67±0.7	4.81±0.6	<0.001	0.51	0.685	0.264
Glucose, mmol/l	4.17±0.27	4.19±0.25	4.48±0.23**	4.61±0.22**	4.5±0.19**	4.35±0.24	4.26±0.19	<0.001	0.2	0.001	0.001
Milk											
Fat:protein ratio	1.21±0.16	1.02±0.12**	0.92±0.16**	0.94±0.10**	0.99±0.10**	1.1±0.14	1.16±0.07	<0.001	0.12	0.027	0.015
Urea, mmol/l	4.43±0.42	3.52±0.33**	3.22±0.36**	3.15±0.37**	3.39±0.19**	4.89±0.46	4.71±0.3	<0.001	0.35	0.088	0.12
FA, g/100g of total FA											
SFA	69.5±2.3	61.6±1.9**	64±3.4**	66.9±1.9*	65±3.8**	63.9±2.1**	67.9±2.4	<0.001	2.5	11.97	6.42
n-6 PUFA	2±0.14	2.69±0.17**	2.65±0.30**	2.78±0.21**	2.75±0.32**	2.53±0.23**	2.15±0.12	<0.001	0.21	0.055	0.046
trans-10:*trans*-11 C18:1	0.3±0.1	1.57±1.10**	2.66±1.70**	2.25±1.05**	2.28±1.10**	0.71±0.48**	0.66±0.96	<0.001	1.05	0.097	0.035
trans-9 C18:1	0.21±0.04	0.33±0.04**	0.31±0.07**	0.3±0.06**	0.35±0.10**	0.27±0.05**	0.22±0.04	<0.001	0.06	0.006	0.007
Behaviour											
Drinking act, no./day	8±0.7	6.8±0.6**	6.6±0.6**	7.5±0.6	7.1±0.6**	8.2±0.6	7.8±0.6	<0.001	0.5	1.14	0.275
RTime, min/day	485±47	500±25	542±31**	519±47	507±53	521±31	506±52	0.016	41	4768	1698
DMI, kg/day	19±1.2	16.9±1.2**	16±1.0**	17.2±0.5**	16.1±1.1**	16.1±0.9**	19.2±0.9	<0.001	0.9	0.768	0.775
Faeces											
pH	6.71±0.27	6.38±0.39**	6.13±0.30**	6.17±0.17**	5.89±0.18**	6.41±0.18	6.46±0.2	<0.001	0.24	0.021	0.057
Sieving residual (5+2 mm), %	13.7±3	18.8±6.1*	17.5±3.4	15.1±4	15.2±2.7	9.3±2.6	11.9±2.6	<0.001	3.7	1.424	13.91
Urine											
pH	8.19±0.09	7.72±0.18**	7.93±0.11**	8.11±0.17	8.02±0.22*	8.02±0.13*	7.99±0.05	<0.001	0.15	0.001	0.023

HSD = high-starch diet; LSD = low-starch diet; RSD = relative standard deviation of the model; ND = not determined; BHB = β-hydroxybutyrate; FA = fatty acids; SFA = short chain fatty acids; PUFA = poly-unsaturated fatty acids; RTime = rumination time; DMI = DM intake.

Eight-week measurements were performed: 2 weeks in period 1 (P1): weeks 3 and 4 which were averaged; 4 weeks in period 2 (P2): weeks 6, 7, 8 and 9; and 2 weeks in period 3 (P3): weeks 10 and 12.

Dunnett's *post hoc* test was performed to compare LSD control *v.* each other individual weeks of the experiment. Values within a row differ significantly from LSD control at *P<0.1 and **P<0.05 from a statistical point of view. Values are means (n = 11 cows) ± SD.

[1] Cows were fed with LSD: 32% concentrate+68% forage, containing 13% starch.

[2] Cows were fed with an HSD: 54% concentrate+46% forage, containing 35% starch.

[3] A linear mixed model was used to compare weeks of measurements during the experiment.

Table 3 *Generalization of multiple linear regression model of indicators and their ability to classify dairy cows in LSD and HSD periods*

| | | | | | | | Classification's abilities of the models | | | |
| | | | | | | | Data set #1 | | Data set #2 | Data set #3 |
No.	Matrix	Indicator 1	Matrix	Indicator 2	Matrix	Indicator 3	TPR = Se	TNR = Sp	TNR = Sp	TNR = Sp
1	Behaviour	DMI, kg/day	Blood	Bicarbonate, mmol/l	Milk	Urea, mmol/l	100.0	100.0	87.1	100.0
2	Blood	Cholesterol, mmol/l	Milk	n-6 PUFA, g/100 g FA			95.5	90.9	70.5	90.9
3	Behaviour	DMI, kg/day	Milk	n-6 PUFA, g/100 g FA			95.5	90.9	70.5	87.9
4	Blood	Bicarbonate, mmol/l	Milk	Urea, mmol/l			93.9	100.0	87.1	90.0
5	Blood	Bicarbonate, mmol/l	Milk	Fat:protein ratio			93.9	90.0	75.0	81.0
6	Behaviour	RTime, min/day	Behaviour	DMI, kg/day	Milk	n-6 PUFA, g/100 g FA	93.2	100.0	75.0	97.0
7	Blood	BHB, mmol/l	Milk	Urea, mmol/l			93.2	95.2	97.7	96.9
8	Behaviour	DMI, kg/day	Milk	Urea, mmol/l			93.2	95.2	90.7	96.9
9	Blood	BHB, mmol/l	Blood	Glucose, g/l	Urine	pH	93.2	90.9	70.5	84.8
10	Milk	Urea, mmol/l	Urine	pH			93.0	100.0	94.7	100.0
11	Behaviour	Drinking act, no./day	Faeces	pH	Milk	Urea, mmol/l	91.4	100.0	97.1	96.2
12	Behaviour	Drinking act, no./day	Faeces	pH	Milk	SFA, g/100 g FA	88.6	94.4	76.5	88.5
13	Faeces	pH	Blood	BHB, mmol/l			88.4	90.9	81.8	87.9
14	Faeces	pH	Milk	Urea, mmol/l			86.0	100.0	93.0	96.9
15	Faeces	pH	Milk	*trans*-10:*trans*-11 C18:1, g/100 g FA			86.0	95.5	79.5	84.8
16	Faeces	pH	Milk	Fat:protein ratio			86.0	90.9	81.8	84.8
17	Faeces	pH	Behaviour	DMI, kg/day			83.7	90.9	75.0	93.9
18	Faeces	Sieving residual (5+2 mm), %	Urine	pH			79.1	94.4	61.5	64.3

LSD = low-starch diet; HSD = high-starch diet; no. = number; TPR = true positive rate; Se = sensitivity; TNR = true negative rate; Sp = specificity; DMI = DM intake; PUFA = poly-unsaturated fatty acids; FA = fatty acids; RTime = rumination time; SFA = short-chain fatty acids; BHB = β-hydroxybutyrate.
Data set #1: weeks 3, 4, 6, 7, 8 and 9.
Data set #2: weeks 3, 4, 6, 7, 8, 9, 10 and 12.
Data set #3: weeks 3, 4, 6, 7, 8, 9 and 12.
True positive (TP) value (or score) was when the model identifies a cow fed HSD with a probability strictly above 50% during HSD challenge.
True negative (TN) value was when the model identifies a cow fed HSD with a probability strictly below 50% during P1.
False negative (FN) value was noted when the model identifies a cow fed HSD with a probability strictly below 50% during P2.
False positive (FP) value was attributed when the model identifies a cow fed HSD with a probability strictly above 50% during P1.
True positive rate (TPR = Se), and true negative rate (TNR = Sp) were then calculated as follows: TPR = TP/(TP+FN); TNR = TN/(FP+TN).

of fermentable carbohydrates in the diet favours microbial protein synthesis, which reduces rumen urea concentration. Therefore, the formation of hepatic urea is reduced and less urea is excreted in blood and milk (Enemark *et al.*, 2002). These results have been confirmed by Gao and Oba (2015), who were able to separate (or distinguish) cows with low or high risk of SARA according to milk urea-nitrogen (MUN) and milk fat content. The decrease in MUN can also be linked to a lower ingestion (Bertoni *et al.*, 2008) and therefore a lower protein intake during HSD challenge. In our study, milk urea concentration was correlated with saliva and blood concentrations, as reported previously (Broderick and Clayton, 1997). Milk fat:protein ratio is the most widespread indicator to detect SARA and was significantly impacted during the HSD challenge of our study, nevertheless it is insufficient on its own to be a specific marker of rumen dysfunction such as SARA, since it can

be affected by other metabolic disorders (Vlcek *et al.*, 2016). Even if n-6 PUFA, SFA, *trans*-10:*trans*-11 C18:1 and *trans*-9 C18:1 can be related to milk fat depression itself, those biomarkers are known to be affected during SARA and were selected to be part of the proposed combinations. Many authors report that those specific FA are good predictors of rumen function and therefore could be informative about rumen disorders induced in SARA challenge (Vlaeminck *et al.*, 2006; Colman *et al.*, 2010). The daily DMI and drinking acts of the animals were decreased during HSD compared to LSD. Reduced and erratic feed intakes have been seen as important signs of SARA (Kleen *et al.*, 2003). The decrease in drinking acts and water consumption during SARA could be an indicator of sickness for animals, as suggested by authors. Therefore, for the future, we could improve the monitoring of drinking behaviour by estimating daily water consumption based on rumen temperature

measured with bolus, as it has already been initiated by Gasteiner *et al.* (2009). Even though the literature had few references for indicators of SARA in saliva, this matrix was promising and easy to sample. Saliva is closely related to blood and can thus reflect blood variations in metabolites, hormones or inflammatory markers (Lamy and Mau, 2012) and proteome and microbiota compositions in saliva samples have been used as non-invasive approaches to study animal diseases (Gutierrez *et al.*, 2013). Out of 23 indicators analysed in saliva during our trial, only urea concentration and pH discriminated HSD and LSD. Nevertheless, those indicators did not return to their control values even at the end of the recovery period. In our study, buccal samples (effectively saliva mixed with feed particles) were collected before the morning feed distribution on day 2 of each week of measurement. Saliva samples were obtained by allowing cows to chew on two sterile gauze pads for 1 min. The pads were then centrifuged (2 min × 2500×**g**, at 21°C) to extract saliva. The changes in saliva composition in response to ingestion, rumination (Kittelmann *et al.*, 2015) or to the animal physiological status might lead to important variations leading to inconsistent measurement in this matrix. It is mandatory to find a good standardization of saliva collection in order to compare indicators between animals and studies.

In a recent review (Plaizier *et al.*, 2018), authors suggest to combine clinical examinations of cows including milk, blood urine and faeces indicators as well as diet characteristics and herd management to accurately detect SARA. In this pilot study, we developed an original approach, where quantitative combinations including non-invasive indicators seem promising in terms of sensitivity to detect dairy cows affected by an HSD challenge presenting a high risk of SARA. However, the proposed combinations were calculated based on a single experimental HSD challenge involving 11 primiparous animals, where only one specific acidogenic diet was used to increase the risk of SARA. Primiparous dairy cows were used in this HSD challenge since they are known to be more susceptible to acidogenic diets than multiparous cows due to multiple factors (feeding pattern behaviours, rumen pH, etc.) (DeVries *et al.*, 2011; Humer *et al.*, 2015). Therefore, those combinations need to be challenged with different nutritional conditions, with multiparous animals presenting less susceptibility to rumen dysfunction, and higher number of animals. The variability provided by other risky diets may impair the ability of the combinations to classify correctly the animals presenting a rumen dysfunction. Diet characteristics and herd management factors could be further implemented in the models to improve detection of animals undergoing rumen dysfunction.

On-farm detection of animals submitted to high-starch-diet challenge
Out of the 16 indicators selected, several can be measured on-farm following easy sample collection. For instance, milk FA composition was measured by gas chromatography in our study. However, recent studies have yielded good results in prediction using mid-infrared spectroscopy (Coppa *et al.*,

2010). Calamari *et al.* (2016) have also proposed using spectrophotometry to determine some common plasma indicators. Even though further improvements in mid-infrared spectroscopy are still required to obtain high standard predictive equations, it is nevertheless a promising technique for on-farm measurement of indicators. In comparison with other standard methods, it can be faster and cheaper. The pH measurements in faeces and urine are heterogeneously affected during SARA challenge (Luan *et al.*, 2016), but such measures can improve detection of animals affected by HSD challenge on-farm when combined with other indicators.

With a sensitivity higher than 85% for most of our established models, we propose to evaluate their usefulness for on-farm detection of animals affected by HSD challenge. Model no. 1, which includes daily DMI, blood bicarbonate and milk urea concentration, showed 100% sensitivity and 100% specificity and is thus promising. Feed consumption is not yet measured individually and routinely on-farm. With the implementation of sensors and precision livestock technologies, farmers are already able to measure rumination and ingestion time, we can expect that feed intake will be available for individual cows at an affordable price in the very near future. This model also requires blood and milk samples. Milk urea nitrogen can be measured during routine checks on milk performance, but blood sampling requires veterinary involvement.

The model validation of this pilot study is only based on the P value of the variables used to build the models. The justification of model assumptions is rather weak due mainly to the small numbers of individuals, which were necessary to screen a large number of variables in a repeated measurements experiment. We therefore strongly recommend to deploy more internal validation (e.g. residuals analysis and heterogeneity) for the future. In consequence, the Se and Sp values of the models presented in this pilot study are high. However, we used different grading scales of the training data set: (i) period means to develop the models and (ii) weekly means coupled to a leave-one-out cross-validation to test their ability. The next step of this proof of concept study would be to evaluate the robustness of our results with an external data set to test the models. We suggest to use different and larger number of diets inducing rumen dysfunction as well as herd characteristics (e.g. feeding strategy and stocking rate) to assess the proposed combinations. The models also require to be validated in respect to the lactation period since the occurrence and severity of SARA could vary with the days in milk (Penner *et al.*, 2007). The models also need to be specific of rumen dysfunction and therefore they should be challenged with other diseases to evaluate their specificity.

Lag time to recover after high-starch-diet challenge
The models were selected according to the best combination of both sensitivity and specificity in detection of cows fed HSD challenge, with data at the week scale: data set #1 (average data by period was used to build the models). Then, the abilities of the established models to properly classify animals

during the recovery period were calculated with week data using two different data sets (data sets #2 and #3). Sensitivity was the same for the three tested data sets since only one period of HSD challenge was performed during our study but specificities of the models were mainly higher when only week data from the LSD period (data set #1) were used. These results are consistent with the fact that models were established with mean values over the periods of the same data set. Specificity was then higher with week data of the recovery period without the first week (week 10 (P3), data set #3), compared to data set #2 containing this previous week 10 (P3) and week 12 (P3) of the recovery period. This result is in accordance with the data showing that animals recovered their initial rumen function 3 weeks after they were fed HSD (Villot *et al.*, 2018). Krause and Oetzel (2005) also demonstrated that cows need several days to recover from a SARA challenge. Our combination of indicators reflects the physiological status of the animals that have not fully recovered from the HSD challenge and discriminate animals experiencing rumen dysfunction and not only the diet changes. In particular, a combination involving DMI, blood bicarbonate and milk urea concentrations seems able to detect both when cows were affected by HSD challenge and when they totally recovered from this challenge. Nevertheless, all the combinations of our study have been developed using a single SARA induction diet; they must be evaluated with different conditions with more animals and tested with other diseases to confirm their specificity.

Conclusion

A large screening of variables ($k = 136$) measured simultaneously on the same dairy cows gave us the opportunity to propose combinations of two or three non-invasive indicators to detect animals submitted to HSD challenge in our experimental conditions. Eighteen combinations were proposed with specificity and sensitivity above 90% and 75%, respectively, to detect animals fed a HSD. This study highlights that it is pertinent to combine non-invasive indicators to enhance the ability of future models to detect animals submitted to nutritional changes at risk of digestive disorders such as SARA in dairy cows.

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Declaration of interest

The present article does not involve any conflict of interest.

Ethics statement

The trial was conducted at the dairy cow research facilities of the French National Institute for Agricultural Research in France (INRA, Herbipôle, UE 1414, Theix) and was approved by a French ethics committee (approval #8 803- 2 017 020 115 479 420).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731119001629

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