THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Institut National Polytechnique de Toulouse (INP Toulouse)

Discipline ou spécialité :

Pathologie, Toxicologie, Génétique et Nutrition

Présentée et soutenue par :

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le mardi 27 février 2018

Titre :

Effect of live yeast on the fermentation and microbiological physico-chemical parameters of the rumen, depending on the nature of the diet: modeling and validation in ruminant

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Ecole doctorale :

Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

Unité de recherche :

Génétique, Physiologie et Systèmes d'Elevage (GenPhySE)

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Acknowledgments

Cette thèse a été réalisée dans le cadre d'un contrat de Recherche Industrie CIFRE, financée par l'Association National de la Recherche Technique (ANRT) et Phileo Lesaffre Animal Care. Le manuscrit vient clôturer 3 ans d'expérience scientifique et humaine, je remercie toutes les personnes qui ont contribué à l'aboutissement de ce projet.

Je présente tout d'abord mes remerciements aux membres du jury: M. Jan DIJKSTRA (Wageningen University), M. Francis ENJALBERT (ENVT), M. Olivier HUSSON (CIRAD), Mme Christine JULIEN (Phileo Lesaffre Animal Care), M. Pierre NOZIERE (INRA de Theix) pour avoir accepté de participer à ce jury. Je remercie tout particulièrement Christine pour tout le temps qu'elle m'a accordé en répondant à mes questions.

Je tiens à remercier mes directeurs de thèse, Corine Bayourthe et Qingxiang Meng pour leurs encadrements et leur appui scientifique. Merci à Corine de m'avoir accordé la confiance et permis d'effectuer cette thèse dans les meilleures conditions. Merci à Professeur Meng pour m'avoir accueilli et m'avoir aidé depuis plusieurs années.

Je suis très reconnaissant à Phileo Lesaffre Animal Care qui a assuré le financement de cette thèse, en particulier sa directrice Frederique Clusel et son directeur R&D Eric Auclair. Je remercie mon responsable Jean Philippe Marden qui s'est attaché à m'offrir les meilleures conditions matérielles pour réaliser mes études et notamment mon essai à « The Farm ». Je remercie Brigitte Geste et Anaïs Fernandez pour leur aide tout au long de l'expérimentation à « The Farm ». Merci à Johanna Haroux et à Cendrine Luzuy pour leurs aides lors de mes déplacements à Lille. Je remercie également les collègues Phileo en Chine : Xiaosong Liu, Bo Zhang, Wei Ji et Lei Zhou pour leurs aides lors de mes déplacements à Shanghai.

Je remercie ensuite les membres de mon comité de thèse qui m'ont formulé de précieux conseils : Daniel Sauvant et Djamel Ali-Haimoud.

Je souhaite remercier tous les collègues de l'UMR GenPhySE pour leur investissement dans ce travail. Merci à Xavier Fernandez, Martine Bouissou-Matet Yerle et Christèle Rober-Granie pour leur accueil au sein de l'UMR GenPhySE. Merci à Sylvie Combes, Laurent Cauquil, Géraldine Pascal, Béatrice Gabinaud, Muriel Segura, Olivier Zemb, Caroline Achard, Annabelle Meynadier-Troegeler, Asma Zened, Laurence Lamothe, Thierry Gidenne, Michèle Theau-Clement, Corinne Pautot, Carole Bannelier, Michel Bouillier-Oudot, Herve Rémignon,

Hélène Manse, Nathalie Marty-Gasset, Stéphane Seidlinger, Cécile Bonnefont, Yves Farizon, Marie-Luce Chemit pour m'avoir accompagné tout au long de cette thèse. Je remercie également les gestionnaires de l'unité : Viviane, Manuela, Florence, Nancy, Valérie G, Valérie L, Evelyne et surtout Chrystelle pour avoir assuré tous mes ordres de missions. Merci à Philippe Schmidely de AgroParisTech pour m'avoir appris à faire des méta-analyses avec SAS. Merci aussi à Cécile Canlet de l'UMR 1331 ToxAlim pour nous avoir aidés pour les analyses métabolomiques.

Je remercie également Defa Li pour son accueil au sein de la China Agricultural University. Merci également à Hailing Luo, Yanling Li, Zhengming Zhou, Hao Wu, Rui Li, Fei Wang, Yuanlong Yang, Qianglin Liu, Deyong Li, Liweng He, Ping Liu, Xin Wu，Xinzhuang Zhang, Liping Zhao, Yunlong Huo, Yuming Jiang, Guoyin Wang, Wanbao Chen, Yan Li, Lei Shi pour m'avoir aidé à Pékin. Merci également à Shujie Liu, Shatuo Chai, et Lizhuang Hao pour leur aide à différentes occasions.

J'ai une pensée toute particulière pour Professeur Liping Ren qui m'a aidé depuis de nombreuses années et qui nous a quitté pendant mon séjour à la China Agricultural University.

Je tiens à remercier les stagiaires qui ont travaillé sur ce projet de thèse : Océane Cartoscelli et Gerges Hanna, grâce à vous ce travail a pu avancer.

Je remercie tous mes jeunes compagnons du couloir : Héloïse, Christelle, Clémentine, Céline, Davi, Ivan, Malo, Charlotte, Marie, Manon, Marie-Léa, Chloé, Juliette, Tehya, Tao. Avec vous j'ai partagé de très bons moments. J'ai beaucoup apprécié la petite balade du midi avec vous accompagnée de pleine de discussions intéressantes qui m'ont amenées à des réflexions originales sur la vie professionnelle et privée.

Avant de rejoindre Toulouse, j'ai parcouru un long chemin. Je tiens spécialement à remercier toutes les personnes qui m'ont donné ma chance en me laissant l'opportunité de faire une thèse :

Sophie Prache pour m'avoir initié à faire de la recherche, et surtout m'a appris la fraternité ; Jean-François Hocquette pour m'avoir aidé à plusieurs reprises pour mettre mes compétences en valeurs ; Dominique Pomiès, Isabelle Veissier, Jacques Agabriel, Bruno Martin qui m'ont beaucoup appris sur les ruminants ; Catherine Jondreville, Elisabeth Baéza et Angelique Travel pour m'avoir accueilli à Nancy. Je remercie également Benoit Malpaux, ancien chef de département PHASE; Dominique Gigan et

Jean-Luis Porry du Ministère Français de l'Agriculture ; Jean-Baptiste Coulon, président du centre INRA de Auvergne-Rhône-Alpes; Erwin Dreyer, président du centre INRA de Nancy-Lorraine ; Anne Mrowicki, sous-directrice de l'Accueil International de l'Université de Lorraine ; Sandrine et Marie-Lise, gestionnaires RH du centre INRA de Nancy-Lorraine ; Maryse Teyssèdre et Annie Royet, gestionnaires de l'UMRH ; Gong Aiqi du Ministère de l'Agriculture de Chine.

Je tiens à remercier Luc Delaby pour m'avoir transmis la passion de travailler avec les ruminants.

Merci à William Martin-Rosset pour m'avoir appris les moindres détails de l'ingestion et la digestion des aliments chez différentes espèces d'herbivores.

Merci à Djamel Ali-Haimoud et Marie Odile Bisch, grâce à vous, j'ai pu avoir des expériences d'enseignement à l'école d'ingénieurs de Purpan pendant la thèse.

Merci à Maryline Kouba pour m'avoir reçu en 2008, où commence ma vie en France.

Merci à Marie-Jeanne et Robert Rahn pour m'avoir accueilli au Caradeuc et à Pont-Aven et surtout m'avoir aidé à trouver mon premier stage en ferme laitière.

Merci à l'experte anonyme qui a validé mon dossier CIFRE pour que je puisse apprendre la nouvelle le jour de mon anniversaire.

Un grand merci à Céline Chevance, grâce à toi j'ai pu rester en France. Merci à Patrick et Olivier de la chèvrerie de Corps-Nuds qui m'ont appris que les chèvres savent pâturer.

En fin, merci à ma famille qui m'a soutenu de loin. Merci grand-mère.

List of publications and communications

Publications

Article 1: Huang Y., Marden J.P., Julien C., Bayourthe C. Redox potential: an intrinsic parameter of the rumen environment. Journal of Animal Physiology and Animal Nutrition (In press).

Article 2: Huang Y., Marden J.P., Benchaar C., Julien C., Auclair E., Bayourthe C. 2017. Quantitative analysis of the relationship between ruminal redox potential and pH in dairy cattle: influence of dietary characteristics. Agricultural Sciences, 8, 616-630.

Article 3: Huang Y., Marden J.P., Julien C., Auclair E., Benchaar C., Bayourthe C. Relationship between ruminal redox potential and fermentation parameters from in vivo experiments in dairy cattle: influence of dietary characteristics. In prep. Journal of Dairy Science.

Article 4: Huang Y., Marden J.P., Julien C., Auclair E., Bayourthe C. Response of redox potential and fermentation parameters to live yeast supplementation in dairy cow: quantitative analysis. In prep. Livestock Science.

Article 5: Huang Y., Marden J.P., Julien C., Auclair E., Bayourthe C. Effect of live yeast supplementation in early-lactating cows fed diets differing in content of soluble sugars. In prep. Animal.

Article 6: Huang Y., Castoscelli O., Canlet C. Cauquil L. Marden J.P., Julien C., Auclair E., Bayourthe C. Effects of live yeast supplementation and dietary soluble sugars content on rumen microbial composition and metabolomic profile in early-lactating cows. In prep. Environmental Microbiology.

Communications

Huang Y., Julien C., Marden J.P., Bayourthe C. 2016. Relationship between ruminal redox potential and pH in dairy cattle. 20th Congress of the European Society of Veterinary and Comparative Nutrition. 123. (Poster).

Huang Y., Julien C., Marden J.P., Bayourthe C. 2016. Analyse quantitative de l'effet des levures vivantes sur le potentiel redox ruminal chez la vache laitière. Rencontres autour des Recherches sur les Ruminants 23, 41. (Poster).

Huang Y., Marden J.P., Julien C., Auclair E., Bayourthe C. 2017. Improvement of ruminal fermentation by live yeast in dairy cattle : a quantitative analysis. Journée scientifique de l'école doctorale SEVAB, 27 mars, Toulouse, France. (Oral communication).

Huang Y., Marden J.P., Julien C., Auclair E., Bayourthe C. 2017. Improvement of ruminal fermentation by live yeast in dairy cows. ADSA Annual Meeting, June 25-28, Pittsburgh, Pennsylvania, USA. (Oral communication).

Huang Y., Marden J.P., Julien C., Auclair E., Hanna G. Bayourthe C. 2017. Changes in ruminal redox potential and pH of lactating cows during a dietary transition. ADSA Annual Meeting, June 25-28, Pittsburgh, Pennsylvania, USA. (Oral communication).

Contents

List of Tables

List of Figures

Abbreviations

ACE: acetate;

ADF: Acid Detergent Fiber

ADL: Acid Detergent Lignin

ANOVA: Analysis Of Variance

ATP: Adenosime Tri-Phosphate

BUT: Butyrate;

BW: Body Weight

CV: Coefficient of Variation

DACD: Dietary Anion Cation Difference

DIM: Days In Milk

DM: Dry Matter;

DMI: Dry Matter Intake

DNA: Deoxyribonucleic Acid

EB: Electrolytic Balance

E_h: Redox Potential

HAC: Hierarchical Ascendant Classification

HS: High Soluble Sugars

LS: Low Soluble Sugars

LY: Live Yeasts

NAD: Pyridine Nucleotides

NDF: Neutral Detergent Fiber

NDFF: Neutral Detergent Fibre from Forages

NH_3: Ammonia

NMR: Nuclear Magnetic Resonance

OM: Organic Matter

OPLS-DA: Orthogonal Projection of Latent Structures–Discriminant Analysis

OUT: Operational Taxonomic Unit

PCA: Principal Component Analysis

PCO: Proportion of Concentrate

PCR: Polymerase Chain Reaction

PRO: Propionate

RFC: Readily Fermentable Carbohydrates

rH: Clark's exponent

RPB: Rumen Protein Balance

SARA: Sub-acute Ruminal Acidosis

SEM: Standard Error Mean

TMR: Total Mixed Ration

tVFA: total Volatile Fatty Acids

VIP: Variable Importance in Projection

VFA: Volatile Fatty Acids

GENERAL INTRODUCTION

Agriculture, in particular ruminant livestock farming, presents today strong societal issues rhyming with environment and animal welfare that sometimes put a veil on the economic issues of production for society. The farmers, by setting goals, direct their strategic and technical choices for the management of their production playing on the shifting equilibrium between productivism, quality and economic profitability. At the scale of the herd, in the case of the search for an optimized production of ruminants, the farmers will focus on the control of the feeding of animals, sensitive lever of evolution and control of the production giving rise to a great deal of work in the field of research and development.

In fact, in ruminant, production and quality have their origin in the quantity and quality of products derived from ruminal fermentations. Feeding a ruminant feed is first of its ruminal microbiota which lives in symbiosis with the animal: microbial digestion in the rumen could be improved. The optimization of this system through improved ruminal fibrolysis, microbial synthesis, lower amylolysis and proteolysis to promote the arrival of glucose and protein in the intestine. The choice of the quantity and quality of feedstuffs can design a diet adapted to the requirements of animals as well as the production target set by the farmer. Recommendations for feeding ruminants are all responses to the optimization of the production system at the animal and herd scale.

Feeding high-producing dairy cows with high-readily fermentable carbohydrates (RFC, such as starch and sugar) diets is common practice to meet the energy requirements for milk production. However, this feeding practice can contribute to create fermentation disorder, such as ruminal acidosis characterized by more or less extended periods of pH depression (Plaizier et al., 2008). Sub-acute ruminal acidosis (SARA) is one of the major concerns of current dairy farms because it is poorly detected in herds and has many consequences, such as feed intake depression, reduced fiber digestibility, milk fat depression, diarrhea and laminitis (Plaizier et al., 2008).

To improve ruminal fermentation, various additives (including probiotics) can be incorporated into their diet. Their use is particularly aimed at modifying the balance of microbial populations in the rumen in order to redirect the fermentative facies towards the formation of final products of digestion that are more beneficial for the animal's metabolism. As an alternative to growth promoting antibiotics, probiotics remain at the forefront of the animal feed industry. Among the probiotics, the positive effect of the yeast *Saccharomyces cerevisiae* (Figure 1) on performance of beef and dairy cattle has been widely demonstrated (Bach et al., 2007; Thrune

et al., 2007; Marden et al., 2008; Desnoyers et al., 2009). Their main positive effects include an increase in rumen pH and a reduction in lactic acid, especially in cases of higher proportion of concentrate in the diet and to higher intake levels (Desnoyers et al., 2009). Until now, pH has been one of the most commonly used descriptors to define acidotic conditions. However pH is a measure that merely reflects one aspect of the rumen environment.

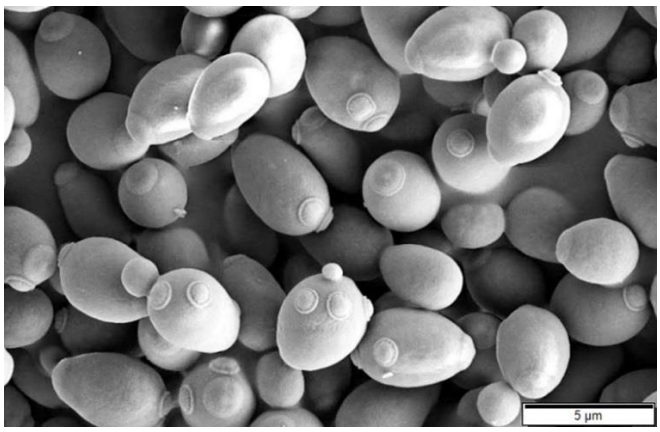

Figure 1. Electron microscopy image of yeast *Saccharomyces cerevisiae* (Murtey and Ramasamy 2016).

Recently, measurement of ruminal redox potential (E_h, in mV) has been considered as an interesting tool to indicate ruminal fermentation disorder, such as SARA (Marden et al., 2005; Marden et al., 2008; Julien et al., 2010). The role of E_h has been reported in many biological media such as dairy products (Brasca et al., 2007), wine (Tomlinson and Kilmartin, 1997) and rumen fluid (Marounek et al., 1982; Marden et al., 2005; Julien et al., 2010). In fact, E_h is a basic physicochemical measurement characterizing the reducing status of a milieu. Each bacteria has its favorable range of E_h (Husson, 2013; Friedman et al., 2017), and the negative E_h seems to be favorable to strict anaerobic bacteria such as fibrolytic and lactate utilizing bacteria (Pinloche et al., 2013; Friedman et al., 2017). The positive effect of live yeasts (LY) on ruminal Eh has been reported (Mathieu et al., 1996; Marden 2007). The decrease of ruminal Eh (enforcement of reducing power) following LY might favorited fibrolytic and lactate utilizing bacteria and contributed to stabilization of rumen function. Marden (2007) then proposed to use ruminal E_h as a key tool for understanding the mode of action of this additive **(Figure 2).**

Figure 2. Simplified mode of action of live yeasts proposed by Marden (2007).

However, effects of LY on the digestion, the metabolism and the performances remain variable according to the experimental conditions: strain and dose of LY, physiological stage of the animal, and also dietary characteristics (Julien 2010). **Our knowledge on mode of actions of LY is still limited, the challenge of this work was to improve our understanding on the mode of actions of LY, and to define the optimal condition of LY utilization in livestock production.** On the one hand, from a purely cognitive point of view, there is a need to specify the relationships between the two physicochemical parameters (pH and E_h), the fermention parameters, and the dietary chractaristics, in order to have an integrated comprehention of rumen function. On the other hand, producers and users of LY are seeking practice to optimize its condition of utilization and to predict its effect.

Ruminal E_h is rarely discussed in dairy cows due to the difficulty of measurement (Marden et al., 2005). The accurate ruminal E_h measurement requires strict anaerobic conditions which are not always satisfied (Marden et al., 2005). For several years, our research team has conducted numerous experiments with simultaneous measurements of ruminal E_h of dairy cows fed various diets under anaerobic conditions, many of these experiments also investigated the effect of LY in dairy cattle. Analysis of these measurements could provide better understanding of i) factors controlling ruminal E_h, ii) relationship between ruminal E_h and other ruminal parameters such as pH and volatile fatty acids (VFA) profile. In addition, associate the response of ruminal E_h and other parameters following LY supplementation might provide new knowledge about mode of actions of LY. Therefore, a great part of this work consisted to quantitative analysis of existing results from 22 experiments with cannulated dairy cattle and try to:

- investigate the relationship between ruminal E_h and other main ruminal parameters including pH and VFA profile;
- quantify the influence of dietary characteristics on ruminal E_h, in order to predict the ruminal E_h and evaluate the risk level of given diet;
- investigate the effect of LY on ruminal E_h and other parameters in order to improve our understanding on the mode of actions of LY.

The manuscript is organized in three parts:

- **part I** focused on ruminal E_h as an important parameter of the ruminal biotope, which include i) a literature review of published articles on ruminal E_h (Article 1) and ii) quantitative analysis of internal dataset on the relationship between ruminal redox potential and other ruminal parameters such as pH and VFA profile, as well as the influence of dietary characteristics (Article 2 and 3).

- By using quantitative analysis of internal dataset**, Part II** studied the effect of live yeast on ruminal redox potential in dairy cattle (Article 4).

- **part III** consisted to verify some of the findings from quantitative analysis by an *in vivo* experiment. Since the quantitative analysis revealed strong influence of soluble sugars on ruminal E_h, and greater response of ruminal E_h following LY supplementation was related to higher intake of soluble sugars, the experiment aimed to validate these findings by investigating the effect of LY supplementation on ruminal E_h in early-lactating cows fed high amount of soluble sugars (Article 5). In addition to ruminal physicochemical and fermentation parameters, rumen microbial composition and metabolomic profile were analyzed to explain the mode of actions of LY (Article 6).

Part I. Redox potential: an important parameter of the ruminal biotope

Chapter 1. Redox potential: an intrinsic parameter of the rumen environment (Article 1)

REVIEW **Article 1**

Redox potential: an intrinsic parameter of the rumen environment

Y. Huang[1,2], J. P. Marden[3], C. Julien[3] and C. Bayourthe[1,2*]

Journal of Animal Physiology and Animal Nutrition (In press)

Summary

The ruminal ecosystem is a fermentative milieu which is interesting to discuss in terms of its redox status as a reflection of an intense bacterial activity. The objective of this systematic literature review is to quantitatively analyze data collected on redox potential (E_h) in the rumen and to explore relationship between ruminal E_h, nature of the diet fed to ruminants, and ruminal fermentation parameters. Data obtained from 15 studies comprising 24 different diets was used in the analysis. Studies included in the data file were selected based on the criteria of: (i) the nature of the reference electrode was specified, (ii) the ruminal E_h was recorded simultaneously to pH, (iii) animal body weight (BW) and dry matter intake (DMI) were reported and (iv) the composition of the diet was precisely described. Results showed a high variability in ruminal E*h* values. However, all recorded values are markedly negative reflecting the absence of oxygen and the strong reducing power of the rumen environment. The main factors contributing to the variation were the method of E_h measurement and the nature of the diet fed to ruminants. Redox potential was positively correlated with DMI and proportion of concentrate in the diet, and tended to be positively correlated with soluble carbohydrates content. In contrast, it was negatively correlated with neutral detergent fibre from forages. The hierarchical ascendant classification discriminated two groups of diets leading to significant change in ruminal E_h level. Ruminal E_h was negatively correlated with pH, total volatile fatty acids produced and proportion of acetate, and positively correlated with proportion of propionate. This review revealed E_h to be dependent on the diet composition and DMI, at least within the range of diets included in the database.

Keywords diet composition, ruminal redox status

Introduction

The rumen is an open ecosystem in which lives a highly diversified and predominantly strictly anaerobic microflora. The physico-chemical conditions of the biotope (high moisture content, temperature of 39°C, negative redox potential) are very favorable to the development and the fermentative activity of this flora. Like in other biological media, redox potential (E_h) is an important parameter because oxidation-reduction reactions are essential for the maintenance of all living microorganisms (Falkowski et al., 2008; Husson, 2013). Each microorganism type is adapted to specific E_h conditions and is characterized by its ability to develop within a range of E_h (Husson, 2013): in general, aerobes require a range of E_h between +500 to +300 mV, facultative anaerobes between +300 to -100 mV, and anaerobes between +100 to less than -250 mV (Ray, 2004). Redox potential may occur at different levels in microorganisms and so, potentially modify their growth capacity and production of metabolites. Van Dijk and Veeger (1981) and Kalachniuk et al. (1994) demonstrated that the metabolic activity of some key ruminal bacteria (*Selenomonas ruminantium*, *Streptococcus bovis*, *Megasphera elsdneii*) was affected by redox conditions, as previously reported by some authors in other biological media during fermentation processes (Vivas and Glories, 1995; Tomlinson and Kilmartin, 1997; Picek et al., 2000; Hirano, 2008; Escalante-Minakata et al., 2009).

Ruminal oxidation-reduction conditions are regularly disturbed by the entrance of oxygen *via* feeding cycle and blood. The consumption of oxygen by facultative anaerobic bacteria allows to maintain the state of anaerobiosis of the ruminal medium as indicated by the very low values of oxygen partial pressure i.e. 10^{-66} - 10^{-60} atm (Marden et al., 2005) and, inside of the rumen, reducing conditions are essential for anaerobic bacteria such as fibrolytic populations. These bacteria are unable to use O_2 as the final electron acceptor and thus derive their energy from fermentation reactions in which the electron acceptors are various organic compounds (acetate, butyrate, formiate, propionate, oxalate, fumarate, CO_2). So, ruminal reducing conditions directly originated from microbial activity. This was demonstrated by Mathieu et al. (1996) who reported E_h value of -322 mV in faunated animals and -282 mV in defaunated animals, and by Julien et al. (2010b) who reported positive E_h values (+ 270 mV) in sterilized ruminal fluid and negative E_h values (from −220 to −110 mV) measured *in vivo*. Moreover, the hypothesis of a relation between the ruminal E_h and the activity of some bacterial populations,

particularly the strict anaerobic bacteria such as cellulolytic and lactate-utilizing bacteria (Marden et al., 2008) was confirmed by Pinloche et al. (2013).

Since the E_h has an important effect on the enzymatic processes which are essential for bacteria metabolism, it seems important to investigate the reductive characteristics of rumen environment. However, very little information is available concerning the E_h of rumen contents and how this changes with type of diet, known to also impact the profile of microbiota. Thus, the aim of this literature review is to provide a comprehensive overview on the current knowledge regarding the ruminal E_h and to identify the relationships between the E_h, the nature of the diet and the activity of the rumen microbiota.

Literature review

The redox conditions in the digestive tract of animals determine whether aerobic oxidation or anaerobic fermentation of nutrients should prevail and, consequently, may have a major impact on the digestion, metabolism, and assimilation of ingested nutrients. Few authors have assessed the redox conditions in different parts of the digestive tract of animals. The first researchers were Veivers et al. (1982) and Brune (1998) in herbivorous insects (termites). They observed that the dilated hindgut is a strictly anoxic habitat (E_h values from -230 to -270 mV) while E_h in the midgut content is markedly positive with values greater than +100 mV. Similarly, in the gastrointestinal tract of goat and sheep, Marounek et al. (1987) found that the range of E_h was rather very wide ranging from +100 mV (in the abomasum) to -220 mV (in the rumen and the colon). In piglet, Stewart (1997) showed a drastic fall of E_h towards negative values from the beginning of the small intestine (+150 mV) to the large intestine (-250 mV), associated with a progressive increase in the number of bacteria. The concentration of oxygen decreases from the median segments of the small intestine and the proportion of anaerobic bacteria species begins to increase at the expense of aerobic species (Pidello, 2014). Thus, whether in monogastrics or ruminants, in the digestive compartment where fermentation processes occur (rumen, caecum and colon), E_h values are lower than those recorded in other parts of digestive tract. However, E_h values in these biological fermenters differ between animals: from -210 to -290 mV in the caecum of horse (Da Veiga et al., 2005; Philippeau et al., 2009), -210 mV in the caecum of rabbit (Kimsé et al., 2009), -185 mV in the colon of swine (Lizardo et al., 2012), -322 mV in the first compartment stomach of alpaca (Liu et al., 2009), and from -115 to -300 mV in the rumen of ruminants (Table 1).

Table 1. Range of values of the ruminal redox potential measured in various ruminants

Animal	Measurement method	Diet composition	E_h (mV)	Reference
Heifer	RFS	Crushed oat/Straw	-385 to -430	Broberg et al. (1957a,b)†
Sheep	RFS	Meadow hay	-195	Barry et al. (1977)
Sheep	RFS	Timothy grass hay/Pelleted barley/Soybean meal	-302 to -340	Mathieu et al. (1996)†
Sheep	RFS	Timothy hay/Alfalfa hay/Concentrate	-306	Mwenya et al. (2004)‡
Sheep	RFS	Italian ryegrass/Alfalfa hay cubes/Concentrate	-319	Sar et al. (2005)†
Goat	RFS	Meadow hay/Ground barley	-157	Marounek et al. (1982)
Goat	RFS	Meadow hay	-176	Marounek et al. (1982)
Goat	RFS	Meadow hay/Ground barley/Urea	-160	Marounek et al. (1987)
Goat	RFS	Corn silage/Alfalfa hay/Concentrate	-327 to -352	Andrade et al. (2002)†
Dairy cow	RFS	Alfalfa hay/Wheat straw/Concentrate	-248	Mwenya et al. (2005)
Alpaca	RFS	Sorghum sudan/Concentrate	-349	Liu et al. (2009)‡
Alpaca	RFS	Alfalfa hay/Concentrate	-314	Liu et al. (2009)‡
Alpaca	RFS	Fresh alfalfa/Concentrate	-303	Liu et al. (2009)‡
Dairy cow	*Ex vivo*	Corn silage/Concentrate	-134	Marden and Bayourthe (2005)
Dairy cow	*Ex vivo*	Corn silage/Hay/Concentrate	-177	Marden and Bayourthe (2005)
Dairy cow	*Ex vivo*	Corn silage/Orchard grass - fescue hay/Concentrate	-195	Marden et al. (2005)
Dairy cow	*Ex vivo*	Corn silage/Dehydrated alfalfa/Ground corn/Concentrate	-115	Marden et al. (2008)
Dairy cow	*Ex vivo*	Grass hay/Ground barley/Ground wheat/Soybean meal	-206 to -213	Julien et al. (2010a)§
Dairy cow	*Ex vivo*	Alfalfa hay/Ground corn/Wheat straw	-222	Michelland et al. (2011)
Dairy cow	*Ex vivo*	Corn silage/Ground corn/Soybean meal	-168	Michelland et al. (2011)
Dairy cow	*Ex vivo*	Corn silage/Ground corn/Ground wheat/Soybean meal	-177	Marden et al. (2013)
Dairy cow	*Ex vivo*	Grass hay/Soybean meal	-173	Julien et al. (2014)
Dairy cow	*Ex vivo*	Corn silage/Ground corn/Soybean meal	-168	Julien et al. (2014)
Dairy cow	*Ex vivo*	Corn silage/Ground wheat/Soybean meal	-179	Julien et al. (2014)
Dairy cow	*Ex vivo*	Corn silage/Wheat/Tanned soybean meal	-166	Julien et al. (2015)
Dairy cow	*Ex vivo*	Corn silage/Wheat/Soybean meal	-147	Julien et al. (2015)
Dairy cow	*In vivo*	Freshly cut alfalfa	-226	Waghorn (1991)
Dairy cow	*In vivo*	Corn silage/Alfalfa hay/Concentrate	-273	Richter et al. (2010)
Dairy cow	*In vivo*	Corn silage/Alfalfa hay/Concentrate	-272	Krizova et al. (2010)
Dairy cow	*In vivo*	Corn straw/Concentrate	-384	Qin et al. (2017)†
Dairy cow	*In vivo*	Corn silage/Alfalfa hay/Concentrate	-362	Qin et al. (2017)†
Goat	*In vivo*	Grass hay/Sugar beet silage/Concentrate	-142 to -154	Giger-Reverdin et al. (2014)¶

RFS, rumen fluid samples: measurements performed by potentiometry on collected hand rumen fluid samples; *ex vivo:* measurements performed with probes on continuously pumped rumen fluid (Marden *et al*., 2005); *in vivo*: measurements performed continuously with probes inside the rumen and wireless device; E*h*, redox potential.
†E*h* expressed as a potential difference (E_0) between a platinum electrode and a reference electrode, i.e., calomel or silver : silver chloride.
‡No precision about the reference electrode used.
§Four experimental hay-based diets consisting of 0, 25, 42 and 56% of ground wheat and barley concentrate mixture were tested in this trial.
¶Two experimental diets consisting of 30 and 60% of concentrate were tested in this trial.

Compared to pH, ruminal E_h has rarely been measured. It is mainly due to difficulties of measurement since the E_h is sensible to air contamination. Table 1 shows E_h values obtained in the rumen of various ruminants according to the method of measurement. Three techniques of E_h potentiometric measurements were reported in the literature. The first one (rumen fluid samples) consists of a manual suction-strainer device that pumps out ruminal fluid from a cannulated animal to measure E_h on collected hand-samples in contact with atmospheric air, after a 25-min stabilization period as recommended by Andrade et al. (2002). The second method (*ex vivo*) was developed by Marden et al. (2005) and allows continuous measurements of E_h on ruminal fluid pumped out of the rumen by a peristaltic pump into a thermostatic vessel maintained at 39°C. The third method allows *in vivo* measurements performed continuously with probes inside the rumen and wireless device (Richter et al., 2010; Qin et al., 2017). This latter technique allows simultaneous measurement of E_h, pH and temperature in the rumen, also allowing the monitoring of daily variations under strictly anaerobic conditions.

As shown in Table 1, whatever the method used, all recorded values are markedly negative reflecting the absence of oxygen and a strong reducing power of the rumen environment: the average E_h value is -238.3 mV (± 85.5; CV = 35.9%; n = 39). Particular attention should be paid to the high variability in E_h values. The main factor explaining this variability is mainly the mode of expression of E_h. By definition, the E_h is the potential difference measured between a platinum electrode and a standard hydrogen electrode. In practice, this standard electrode is never used and all recorded values must be corrected according to the equation: $E_h = E_0 + C$, where E_0 is the potential difference measured between a platinum electrode and a reference electrode (calomel or silver-silver chloride) and C is the potential of the reference electrode used relative to the standard hydrogen electrode i.e. +199 mV at 39°C (Nordstrom, 1977). Values reported in the studies of (1957a,b), Mathieu et al. (1996), Andrade et al. (2002), Sar et al. (2005), and Qin et al. (2017) are relative to a potential difference (E_0) (measured between a platinum electrode and a reference electrode, i.e., calomel or silver-silver chloride). After correcting values (E_0 + 199 mV) recorded by these authors, the new average E_h value is -178.1 mV (± 42.1; CV = 23.6%; n = 35). However, the correction of values did not reduce the variability that remains relatively high (CV = 23.6%). This variability could be partly explained by the difference in measurement techniques used which are not made under the same anaerobic conditions.

Table 2. Description of ruminal redox potential (mV) data (n = 35) depending on the measurement method used

Measurement method	N	Mean	SEM	CV (%)	Min	Max	Q1	Median	Q3
Rumen fluid samples	12	-166.5	12.5	26.0	-248	-103	-188.1	-158.5	-137.7
Ex vivo	16	-176.2	8.1	18.4	-222	-115	-207.0	-176.5	-161.2
In vivo	7	-202.1	20.8	27.2	-273	-142	-249.0	-185.0	-158.5

Rumen fluid samples, measurements performed by potentiometry on collected hand rumen fluid samples; *Ex vivo,* measurements performed with probes on continuously pumped rumen fluid (Marden *et al*., 2005); *In vivo*, measurements performed continuously with probes inside the rumen and wireless device; N, number of E_h values per measurement method; SEM, standard error of the mean; CV, coefficient of variation.

Table 2 shows the description of E_h data (n = 35) for each of the three different techniques of measurement reported in the literature. A one-way ANOVA (with E_h value and measurement method as dependent and independent variable respectively), performed to compare the mean E_h values, showed no significant difference ($P = 0.207$) between all the techniques. However, mean E_h value measured *in vivo* (-202.1 mV) was numerically lower than that obtained by *ex vivo* method (-176.2 mV) or by collected hand rumen fluid samples (-166.5 mV), probably because measurements are made under more strict anaerobic conditions. Compared to the *ex vivo* method, the average E_h value measured on collected hand rumen fluid samples was slightly higher by about 9.7 mV. A trial (Marden et al., 2005) carried out simultaneously on the same animal to compare these two methods showed a difference seven times greater (about 70 mV) than that recorded (9.7 mV) in this review. Moreover, in addition to influence of measurement technique, other factors such as dietary characteristics (Julien 2010) might also affect ruminal E_h values.

Analyses of relationships between E_h, dietary factors and fermentation parameters

There were only 24 potential references (Table 1) available for studying the relationships between the E_h, the nature of the diet and the activity of the rumen microbiota. Studies included in the data file were selected based on the criteria of: (i) the nature of the reference electrode was specified, (ii) the ruminal E_h was recorded simultaneously to pH, (iii) animal body weight (BW) and dry matter intake (DMI) were reported and (iv) the composition of the diet was precisely described. Thus, 7 studies were excluded, such as those of Broberg (1957a,b), Mathieu et al. (1996), Andrade et al. (2002), Sar et al. (2005), and Qin et al. (2017), because it was not specified if E_h values were corrected in relation to the standard hydrogen electrode or not. The study of Liu et al. (2009) was excluded because no precision was given about the reference electrode used. The study of Mwenya et al. (2004) and that of Michelland et al. (2011) were also excluded because animal BW and/or DMI were not specified. When several diets were tested within a study, they were considered as separate treatments in the statistical analysis. This left a database of 15 studies and 24 different diets.

Of the 15 studies, 8 did not specify the chemical composition of the diet (Marounek et al., 1982; Marounek et al., 1987; Waghorn, 1991; Marden et Bayourthe, 2005; Marden et al., 2005; Krizova et al., 2010; Marden et al 2013; Julien et al., 2014) and the others specified only OM,

CP, NDF and starch contents. In addition, data relative to the quantification of VFAs were not available in 7 trials (Barry et al., 1977; Marounek et al., 1982; Marounek et al., 1987; Waghorn, 1991; Marden et al., 2005; Krizova et al., 2010; Richter et al., 2010). Because of incomplete data, the Systool program and tables from Institut National de la Recherche Agronomique (2010) were used to estimate the chemical composition of the 24 diets, the daily ruminal production of tVFA and the proportion of each VFA. The Clark's exponent (rH) which is a true index of the reducing status of a given milieu was also calculated by integrating both pH and E_h values in the Nernst's equation: $rH = E_h/30 + 2\ pH$ (Marounek et al., 1987). Descriptive statistics for data set used in the analysis are presented in tables 3 and 4.

Table 3. Descriptive statistics for data set (n = 24) used to analyze relation between ruminal E_h and diet composition

Item	Mean	SD	Minimum	Maximum
DMI (% BW)	2.31	1.17	1.14	4.44
Concentrate (% DM)	29,7	20.4	0	73.0
Digestible organic matter (g/kg DM)	641.4	50.7	531.5	735.9
Fermentable organic matter (g/kg DM)	496.0	59.7	376.2	605.4
Soluble carbohydrates (g/kg DM)	35.8	31.4	0	111.6
Starch (g/kg DM)	211.7	146.6	0	473.3
Rapidly fermentable carbohydrates (g/kg DM)	247.5	170.5	0	528.6
Rumen degradable starch (g/kg DM)	158.7	106.7	0	361.9
Neutral detergent fibre (g/kg DM)[3]	379.8	134.4	167.9	619.5
Digestible neutral detergent fibre (g/kg DM)	258.9	85.4	151.9	414.3
Crude protein (g/kg DM)	138.7	28.7	106.0	206.0
DCAD (mEq/kg DM)[4]	40.1	14.7	26.1	74.1

DMI, dry matter intake; BW, body weight; NDF, neutral-detergent fibre from forages; DACD, dietary anion cation difference: DACD (mEq / Kg DM) = $(Na^+ + K^+) - (Cl^- - S^-)$; SD, standard deviation.

Table 4. Descriptive statistics for data set (n = 24) to analyze relation between ruminal E_h and pH, rH, and VFA

Item	Mean	SD	Minimum	Maximum
E_h (mV)	-193.7	42.1	-272.7	-115.0
pH	6.36	0.31	5.81	6.80
rH	6.27	0.93	4.49	8.04
Total volatile fatty acids (moles/kg DM)	4.17	0.55	3.10	4.97
Acetate (% total VFA)	64.9	3.6	58.8	70.7
Propionate (% total VFA)	19.6	4.3	7.4	26.6
Butyrate (% total VFA)	12.1	1.7	10.9	19.0

E*h*, redox potential; rH, Clark's exponent = (E*h* / 30) + (2 × pH); DM, dry matter; VFA, volatile fatty acids; SD, standard deviation.

Relationships between the E_h and, dietary factors and some ruminal fermentation parameters were evaluated from principal component analysis (PCA) loading plots, based on the correlation matrix, consisting respectively of 12 variables (DMI, percentage of concentrate, digestible OM, fermentable OM, soluble carbohydrates, starch, rapidly fermentable carbohydrates, rumen degradable starch, NDF, digestible NDF, CP and dietary anion cation difference content) and 6 variables (pH, Clark's exponent, total volatile fatty acids produced, individual proportion of acetate, propionate and butyrate), using XLSTAT software (XLSTAT 2014.4.10 for Windows, Addinsoft, New York, NY). Dietary factors that presented significant correlation with E_h were used to identify groups of diets by using Hierarchical Ascendant Classification (HAC). Due to the limited number of available data, the HAC was partitioned in two main groups which mainly differed by their E_h value. To determine how the dietary factors discriminated the groups of diets in the HAC, a one-way ANOVA including group effect was performed for each factor. Tukey's test was then applied to separate least square means that differed significantly. Statistical significance was established for $p < 0.05$ and tendencies discussed for $0.05 < p \leq 0.10$.

Results and Discussion

Impact of the diet on ruminal redox status

The first objective of this review was to assess the relationships between the E_h and some dietary factors. The loading scores for the variables from the PCA are presented in **Figure 3.**

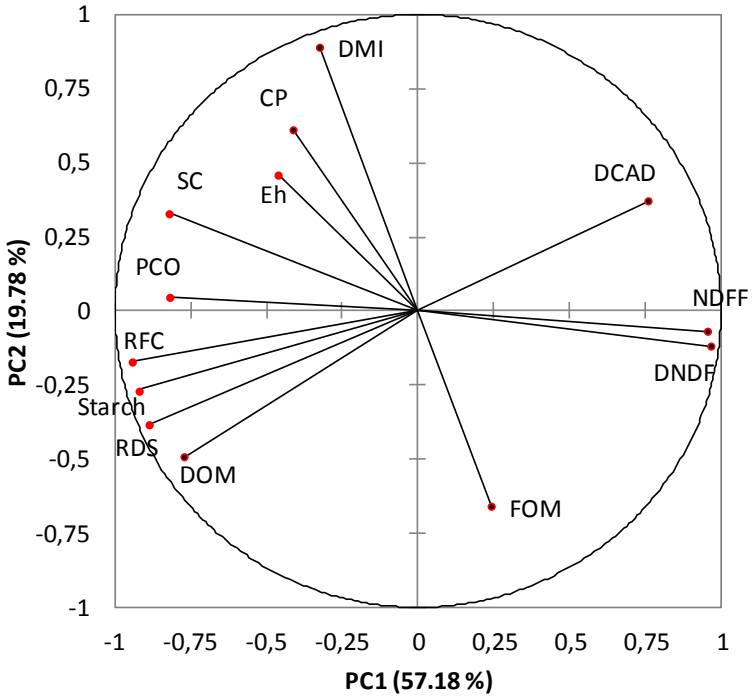

Figure 3. Loading plot describing the relationships between E_h and diets composition derived from a principal component analysis.

CP, crude protein; DCAD, dietary cation anion difference; DMI, dry matter intake; DNDF, digestible neutral detergent fibre; DOM, digestible organic matter; E_h, redox potential; FOM, fermentable organic matter; NDFF, neutral detergent fibre from forages; PCO, percentage of concentrate; RDS, rumen degradable starch; RFC, rapidly fermentable carbohydrates; SC, soluble carbohydrates

The cross-validation technique showed that two first components were responsible for 77% of the total variance in the pooled data. There were three positive correlations between E_h and some dietary factors. The highest r value was observed between E_h and DMI ($r = 0.651$; $p = 0.001$) followed by the proportion of concentrate in the diet (PCO; $r = 0.497$; $p = 0.015$) and soluble carbohydrates content (SC; $r = 0.391$; $p = 0.059$). On the contrary, E_h was negatively correlated with neutral detergent fibre from forages (NDFF; $r = -0.441$; $p = 0.031$) and tended to be negatively correlated with digestible neutral detergent fibre (DNDF; $r = -0.368$; $p = 0.077$). The HAC classified the diets in two groups (Table 5) depending on the E_h level. Diets in group 1 led to stronger reducing conditions than diets in group 2 ($p = 0.013$): -203 (sd = 42.5) *vs* -163 mV (sd = 29.1). Compared to diets in group 2, diets in group 1 were characterized by a NDFF content 1.9 times higher ($p < 0.0001$) and three times lower proportion of concentrate ($p < 0.0001$). This group consists mainly of hay-based diets composed (DM basis) of 100% hay

(meadow or alfalfa) or hay associated with barley, wheat or corn-silage. However, in these diets, the proportion of cereals or corn-silage did not exceed 20%. The stronger reducing conditions induced by these diets could be related to their high NDFF content. This is in accordance with the results of Giger-Reverdin et al. (2006) who recorded a decrease in ruminal E_h when chewing activity of goats increased due to a greater amount of forage in the diet i.e. a greater amount of NDFF . On the contrary, increasing the proportion of concentrates or adding starch and soluble carbohydrates to diets led to less reducing conditions. This was the case for diets in group 2 which had a higher ($p < 0.0001$) soluble carbohydrates content compared to diets in group 1. All these diets were corn silage-based diets associated with crushed or ground corn. In group 2, we also found a diet consisting of a mixture of chopped grass hay (27%), ensiled sugar beet pulp (13%) and concentrate (60%). These preliminary results showed that the level of E_h in the rumen seemed to be directly related to the dietary characteristics (starch vs. cellulose).

Table 5. Hierarchical ascendant classification in two groups according E_h value (n = 24).

	Group 1 n = 11	Group 2 n = 13	Pooled SD	p-value
E*h* (mV)	-203.0	-162.9	35.8	0.013
DMI (% BW)	1.8	3.0	1.0	0.01
PCO (% DM)	0.16	0.47	0.13	<0.0001
SC (g/kg DM)	17.8	61.0	23.2	<0.0001
NDFF (g/kg DM)	471.8	251.0	77.2	<0.0001
DNDF (g/kg DM)	314.4	181.4	54.2	<0.0001

E_h, redox potential; DMI, dry matter intake; PCO, percentage of concentrate; SC, soluble carbohydrates; NDFF, neutral detergent fibre from forages; DNDF, digestible neutral detergent fibre; SD, standard deviation; n, number of diets.

These trends are in agreement with the results of other studies. Monteils et al. (2009) showed that ruminal E_h in dairy heifers fed a fibre diet (70% of NDF on a DM basis) was lower than that recorded in animals fed a diet containing a high proportion of starch (46% on a DM basis): -210 vs. -171 mV, even though the difference disappears with time and with adaptation of animals to their diet **(Figure 4).** Similarly, a sudden increase of readily fermentable carbohydrates (RFC) content in the diet fed to dry dairy cows led to an abrupt change of the ruminal redox status (Michelland et al., 2011): from -222 mV for a high fibre diet to -168 mV for a high RFC diet. This change was accompanied by a concomitant decrease in the diversity of rumen bacterial communities at the time of disruption. Friedman et al. (2017) also observed a significantly higher E_h in the rumen fluid originating from cows fed a high-grain (65%) diet

than in the rumen fluid originating from cows fed a non-grain diet. This change could be induced by the diet and/or metabolic activities of microbiota members, which in turn modify the community.

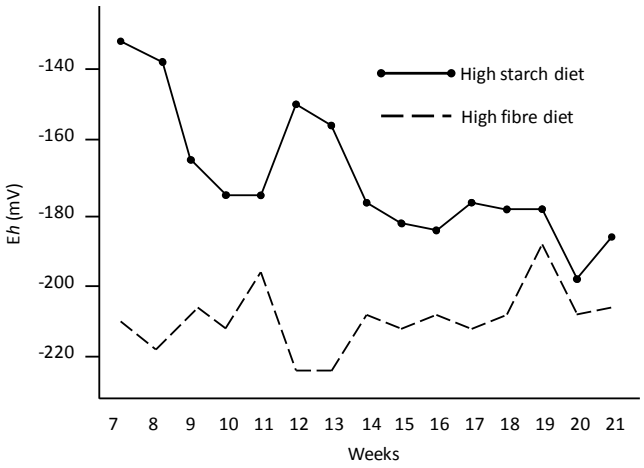

Figure 4. Changes in ruminal redox potential (E_h) in heifers fed high starch-diet or fed high fibre diet (from Monteils et al., 2009).

Ruminal redox conditions and bacterial activity

Another output of this study was to identify the relationships between the E_h and bacteria activity, evaluated by the measurement of the pH and VFA produced in the rumen content. This question is relevant because, in the rumen, numerous chemical reactions are involved in metabolic pathways which ensure both a transfer and utilization of energy. Microbial cell metabolism is thermodynamically driven by different mixed oxidation-reduction systems **(Figure 5)** (Ungerfeld and Kohn, 2006). Therefore, a relationship was expected between ruminal E_h and metabolic activity of the microorganisms during fermentation, as already observed by Baldwin and Emery (1960) and Kalachniuk et al. (1994). The loading scores for the variables from the PCA are presented in **Figure 6**. The cross-validation technique established that two first components are responsible for about 81% of the total variance in the pooled data. Redox potential was negatively correlated with pH ($r^2 = 0.747$; $p = 0.03$; **Figure 7)**. Some studies demonstrated a linear relationship between these two parameters in the digestive compartments where fermentation processes occur: the caecum of rabbit (Kimsé et al., 2009), the caecum of pig (Lizardo et al., 2012) and the rumen of small ruminants (Baldwin and Emery, 1960; Marounek et al., 1987; Giger-Reverdin et al., 2006). In the present study, we obtained a non-linear correlation and it appears that below a pH of 6, the E_h value no longer

varies **(Figure 7).** This concerns only diets with a NDFF content less than 30% containing corn silage as sole forage and for a DMI between 3 and 4.5% BW. In any case, relationship between E_h and pH reflects that many biochemical reactions in digestive ecosystems depend on redox couples with exchanges of protons.

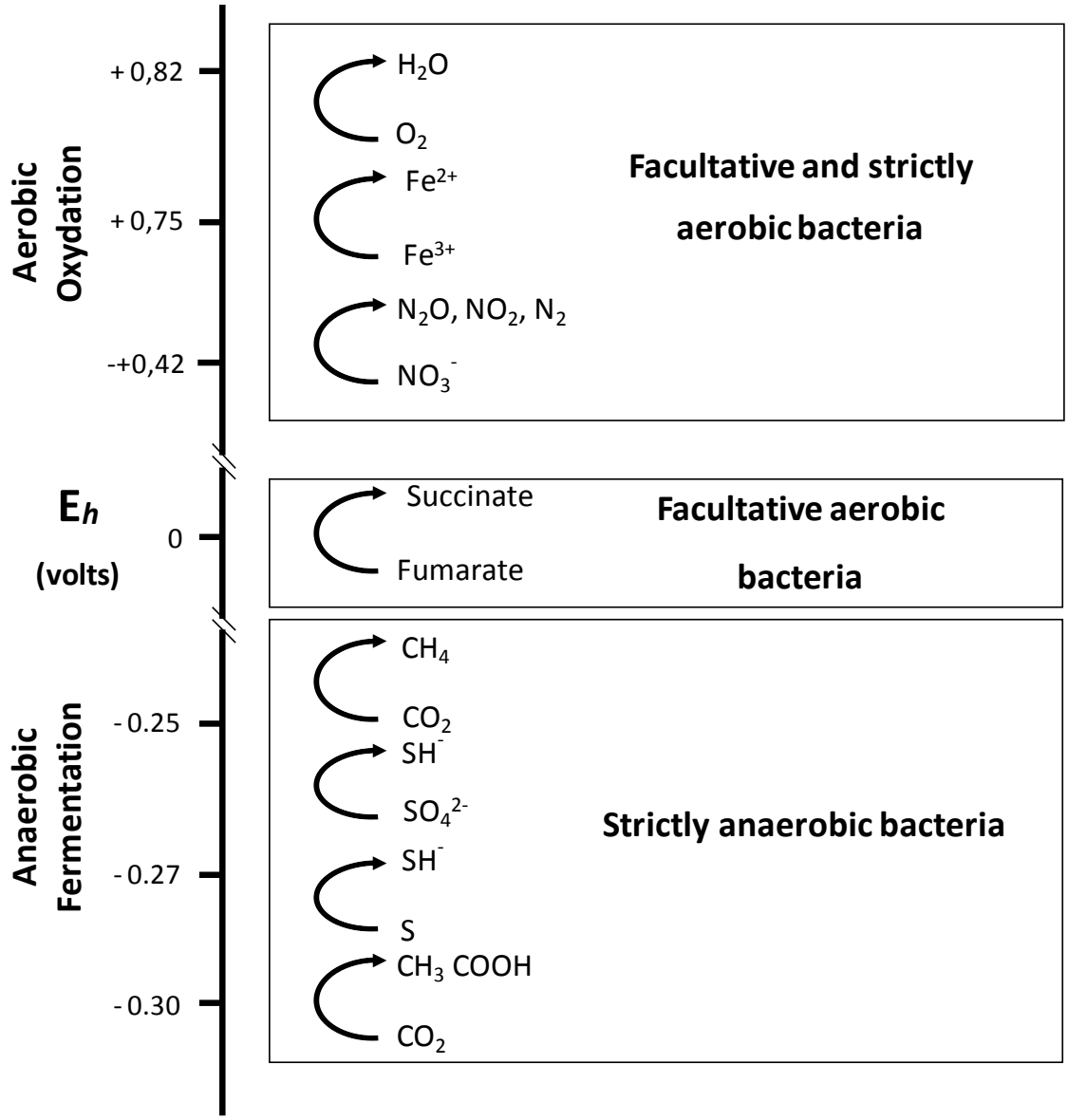

Figure 5. Redox potential and energetic pathways: tower showing electron cascade (from Ungerfeld and Kohn, 2006).

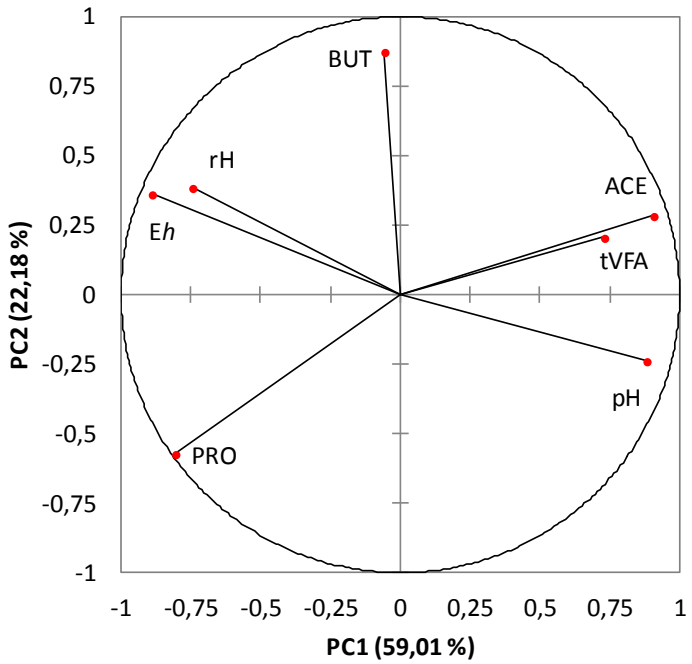

Figure 6. Loading plot describing the relationships between redox potential (E_h) and fermentative parameters derived from a principal component analysis.

ACE, acetate; BUT, butyrate; E*h*, redox potential; PRO, propionate; rH, Clark's exponent; tVFA, total volatile fatty acids.

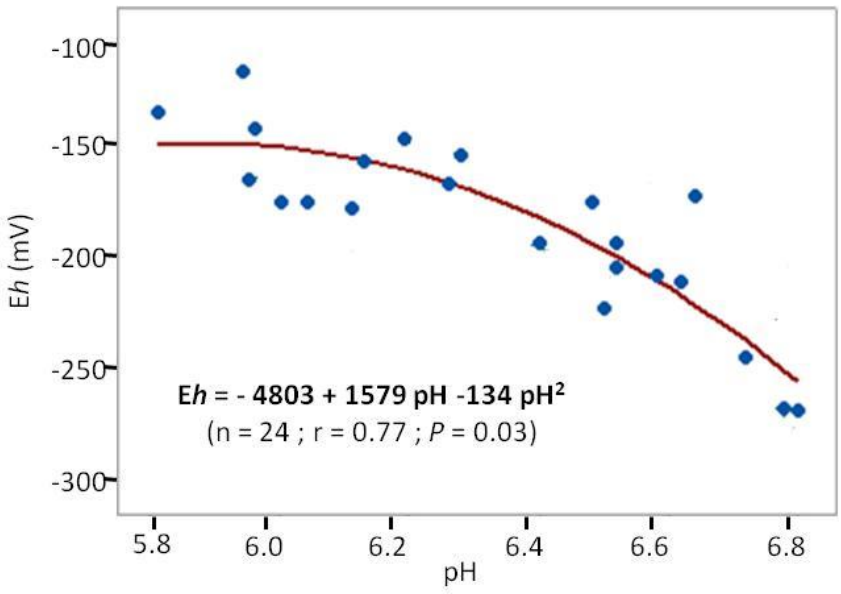

Figure 7. Relationship between ruminal redox potential (E_h) and pH.

Redox potential was negatively correlated with tVFA produced ($r^2 = 0.21$; $p = 0.048$; **Figure 8a**) and proportion of acetate ($r^2 = 0.42$; $p = 0.003$; **Figure 8b**), and positively correlated with

proportion of propionate ($r^2 = 0.26$; $p = 0.027$; **Figure 8c**) as previously observed by Lizardo et al. (2012) in the caecum of pig. Barry et al. (1977a) hypothesized that hydrogen produced by the metabolism of the microorganisms could be responsible for the preservation of the reducing conditions of the ruminal milieu. Indeed, the production of acetate drives the production of H_2 while that of propionate is consumer of H_2. The orientation of ruminal fermentations induced by the nature of the diet, producing more or less H_2, could be an explanation in the correlation established between E_h and tVFA but also between E_h and, acetate or propionate.

Some experiments have illustrated the fact that E_h is an indicator of bacterial activity. Studies conducted in calves from birth to weaning by Rey et al. (2012) and Julien et al. (2015) have shown that the E_h in the rumen takes positive values in the first day of life then declines over few days to reach highly negative values, characteristic of a strong reducing environment. The values range from +224 mV at birth to -141 mV at weaning for Rey et al. (2012) and from +253 mV to -159 mV for Julien et al. (2015). The positive values at birth are consistent with the E_h value (+270 mV) reported by Julien et al. (2010b) in sterilized rumen fluid devoided of living organisms. Observed variations of E_h in calves reflect disruption in the milieu and successive implantation of different microorganisms. If E_h appeared to be as influential as pH on bacterial activities, it was identified as a mechanism by which diet could impact ruminal microbiome composition (Ungerfeld and Kohn, 2006). Correlations between the E_h and the richness of bacterial community have been demonstrated by Julien et al. (2010c), indicating a close relationship between the metabolic activity of the microorganisms and the E_h in the rumen. The oxidation-reducing status of the rumen, which affects the digestive microbiota of herbivores and its activity, appeared to be a potential indicator of ruminal functioning.

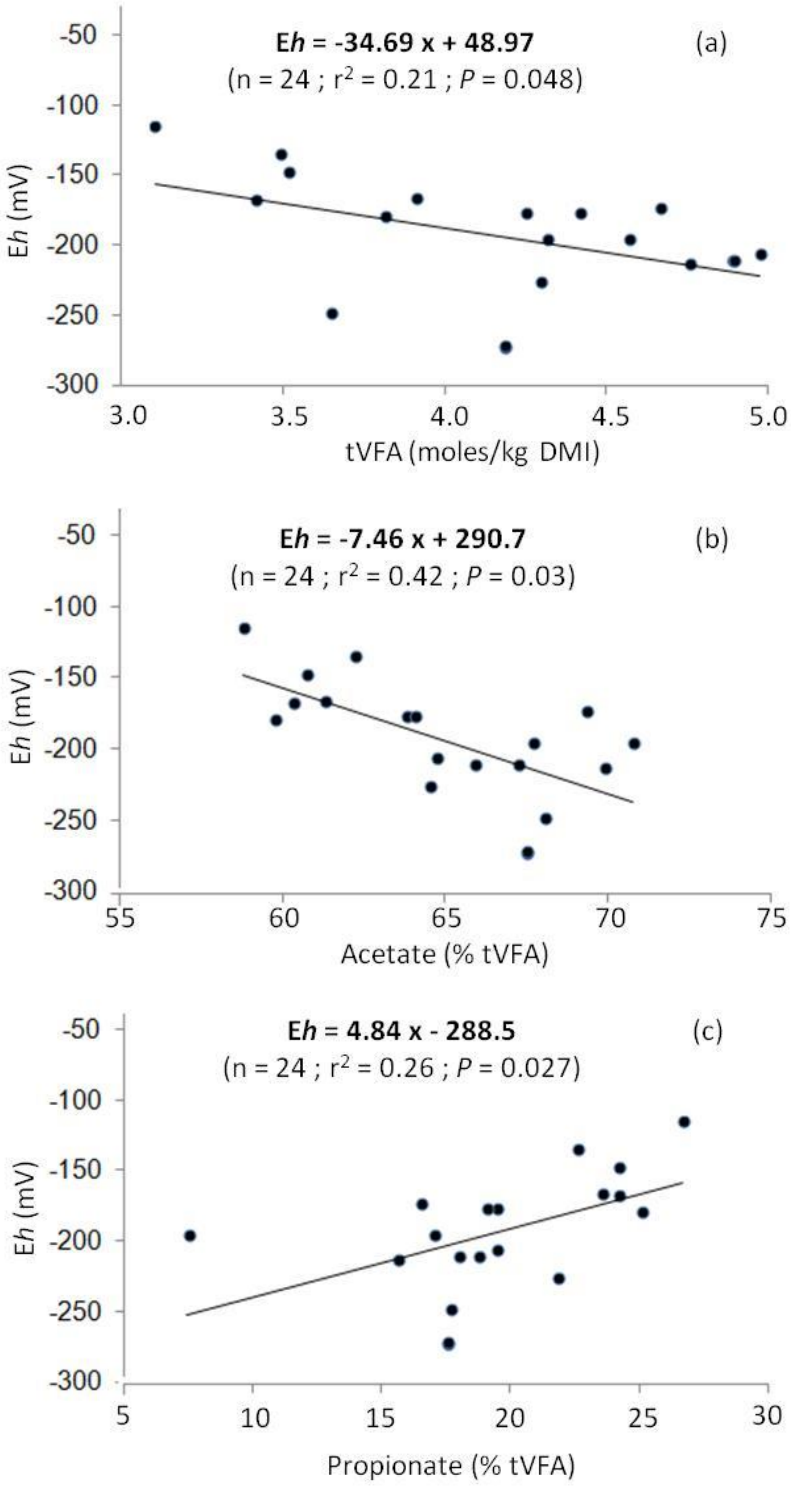

Figure 8. Relationship between E_h and tVFA produced (moles/d) (a), proportions (% tVFA) of acetate (b) and propionate (c).

Conclusions

The ruminal ecosystem is a complex environment which is interesting to discuss in terms of its redox status as a reflection of an intense bacterial activity. This review revealed E_h to be dependent on the diet composition and DMI, at least within the range of diets studied here. Only the measurements carried out under strict anaerobic conditions, such as those with *in vivo* method, make it possible to have an objective and accurate value of E_h. Published data concerning the *in vivo* measurement of ruminal E_h are scarce and much more *in vivo* trials involving such physico-chemical measurement with various types of diets would validate this preliminary analysis.

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Chapter 2. Study of the relationship between ruminal redox potential, pH, and fermentation parameters and diet composition: a meta-analysis approach

A. Presentation of the database

Introduction

The ruminal E_h is rarely discussed in dairy cows mainly due to the difficulty of its measurement: it takes long time to become stable and requires strict anaerobic conditions which are not always satisfied (Marden et al., 2005). Also, some authors (Andrade et al., 2002; Giger-Reverdin et al., 2014) who used a reference electrode of calomel or silver chloride did not correct the raw E_h data (+ 199 mV at 39 ºC). Indeed, E_h is the potential difference between a platinum electrode and a standard hydrogen electrode. Therefore, considerable difference in ruminal E_h values has been reported (Huang et al., in press). There are three methods of E_h potentiometric measurements reported in the literature. The first one consisted of a manual suction-strainer device that pumped out ruminal fluid from a cannulated animal to measure E_h on collected hand-samples in contact with atmospheric air, after a stabilization period of 25 to 30 min as recommended by Andrade *et al*. (2002) and adapted by Giger-Reverdin *et al*. (2014). The two others are *ex vivo* measurements performed on continuously pumped rumen fluid without air contact (Marden et al., 2005) and *in vivo* measurements performed continuously by wireless probes inside the rumen as described by Penner *et al*. (2006). For several years, our research team has conducted numerous experiments with simultaneous measurements of ruminal E_h and pH of dairy cows fed various diets **under anaerobic conditions by *ex vivo* and *in vivo* methods.** Analysis of these aggregated measurements could provide a better understanding of factors controlling ruminal E_h, and demonstrate a quantifiable relationship between ruminal E_h, pH and fermentation parameters.

Selection of studies and data collection

A database was constructed by the results from 22 experiments **(Table 6).** We included in the database only experiments conducted by our research group and two others conducted in Agriculture and Agri-Food Canada (Research and Development Centre, Sherbrook, QC) to ensure a consistency of measurement methods among studies. It includes either published or unpublished studies. Both lactating (12 experiments) and non-lactating cows (10 experiments) were used. Qualitative factors such as physiological status of animals (lactating vs. non-lactating) and site of the experiment (France vs. Canada) were collected. Each experimental period covered an adaptation period (2 to 3 weeks) to the different dietary treatment and a measurement period (3 days).

Table 6. Summarize of 22 experiments in the database.

N_{exp}[1]	Physiological status	Experimental design	Method[2] for measuring E_h	Main ingredients of diets	Reference
1	Non-lactating	Latin square	1	Corn silage/wheat/corn/soybean meal	Unpublished
2	Lactating	Latin square	1	Corn silage/alfalfa hay/composed concentrate	Marden 2007
3	Lactating	Randomized block	1	Corn silage/wheat/composed concentrate	Unpublished
4	Non-lactating	Latin square	1	Corn silage/wheat grain/corn/soybean meal	Unpublished
5	Non-lactating	Randomized block	1	Corn silage/alfalfa hay/corn/soybean meal	Monteils et al., 2011
6	Non-lactating	Latin square	1	Grass hay/barley/wheat/soybean meal	Julien et al., 2010
7	Non-lactating	Randomized block	1	Alfalfa hay/corn silage/wheat straw/corn/soybean meal	Michelland et al., 2011
8	Lactating	Latin square	1	Corn silage/wheat/soybean/meal/tanned soybean meal	Julien et al., 2015
9	Lactating	Latin square	1	Corn silage/wheat/corn/soybean meal	Julien 2010
10	Lactating	Latin square	1	Corn silage/wheat/corn/soybean meal	Julien 2010
11	Lactating	Latin square	2	Alfalfa silage/corn silage/grass hay/corn/soybean meal	Benchaar *et al*., unpublished
12	Non-lactating	Randomized block	1	Corn silage/wheat/corn/soybean meal	Unpublished
13	Lactating	Latin square	2	Corn silage/alfalfa hay/soybean meal/composed concentrate	Unpublished
14	Non-lactating	Latin square	2	Grass hay/soybean meal	Unpublished
15	Lactating	Latin square	1	Grass hay/wheat/corn/soybean meal/composed concentrate	Unpublished
16	Non-lactating	Latin square	1	Corn silage/wheat/corn/soybean meal	Unpublished
17	Non-lactating	Latin square	1	Corn silage/wheat/corn/soybean meal	Unpublished
18	Lactating	Latin square	1	Corn silage/alfalfa hay/composed concentrate	[Marden 2007
19	Non-lactating	Randomized block	1	Corn silage/wheat/corn/soybean meal	Unpublished
20	Lactating	Latin square	2	Barley silage/corn silage/barley/corn/soybean meal	Benchaar *et al*., unpublished
21	Lactating	Latin square	1	Corn silage/alfalfa hay/composed concentrate	Marden et al., 2008
22	Lactating	Latin square	1	Corn silage/wheat/composed concentrate	Unpublished

[1]N_{exp} = number of experiments; [2]Method 1 = measurements performed with probes on continuously pumped rumen fluid [6]; Method 2 = measurements performed continuously with probes inside the rumen and wireless device [15].

The diets were formulated to meet energy and protein requirements, with two equal distributions at 0900 and 1700h. The composition of the diets **(Table 7)** varied widely (e.g. the proportion of concentrate ranged from 0 to 63%). Some of the dietary characteristics such as neutral detergent fiber from forages (NDFf), ruminally degradable starch, rumen protein balance (RPB) were estimated by the online software "systool.fr" (Chapoutot et al., 2013) using the equations published in Sauvant and Nozière (2016). The influence of dietary ionic balance on acid-base balance of animal has been reported (Ross et al., 1994; Meschy 2010; Apper-Brossard et al., 2010), it can be expressed (in mEq/kg of DM) as the dietary cation anion difference (DCAD = Na+K-Cl-S) or electrolytic balance (EB = Na + K - Cl). We also calculated these values according to the INRA tables (2007) for all the diets used in the data base.

Table 7. Descriptive variables of the diets composition (n = 57) for data set used in the meta-analysis.

Item	Mean	SD	Minimum	Maximum
Intake, kg DM/cow per d	16.6	7.3	7.7	27.3
Proportion of concentrate, % DM	37.7	13.6	0.0	62.6
OM, g/kg DM	946.2	16.2	891.8	968.1
RPB, g/kg DM	4.0	17.8	-27.0	79.4
NDF, g/kg DM	368.5	73.8	263.3	566.3
NDFf, g/kg DM	303.0	92.3	178.5	566.3
Starch, g/kg DM	293.6	126.6	0.0	503.2
Degradable starch, g/kg DM	217.9	102.5	0.0	440.4
CP, g/kg DM	149.0	23.9	101.1	222.3
Soluble sugars, g/kg DM	50.6	28.4	0.0	105.4
DCAD, mEq/kg DM	173.3	99.3	59.1	438.0
EB, mEq/kg DM	276.9	119.5	133.8	638.0

DM = dry matter; OM = organic matter; RPB = rumen protein balance; NDF = neutral detergent fibre; NDFf = NDF from forages; CP = crude protein; DCAD = dietary cation anion difference (Na+K-Cl-S, in mEq/kg of DM); EB = electrolytic balance (Na + K - Cl , in mEq/kg of DM); SD = standard deviation.

All E_h and pH values were measured under strict anaerobic conditions, by *ex vivo* (**Method 1**), or *in vivo* method (**Method 2**). In Method 1, rumen fluid was pumped continuously through a rubber tube into a 50-mL-double-walled thermocontrolled vessel outside the rumen, the Eh and pH were measured by electrodes dipped in the collected rumen fluid without air contamination **(Figure 9)**. In Method 2, a wireless real-time data logger (Dascor, Escondido, CA, USA) was submersed into the ventral rumen sac via the ruminal cannula after calibration, and the Eh and pH were measured by external sensors of the data logger and stored in the memory chip **(Figure 10)**. For

both methods, the accuracy Eh electrode was checked by measuring the standard solution at 220 mV (Fishier Scientific) before and after each measurement.

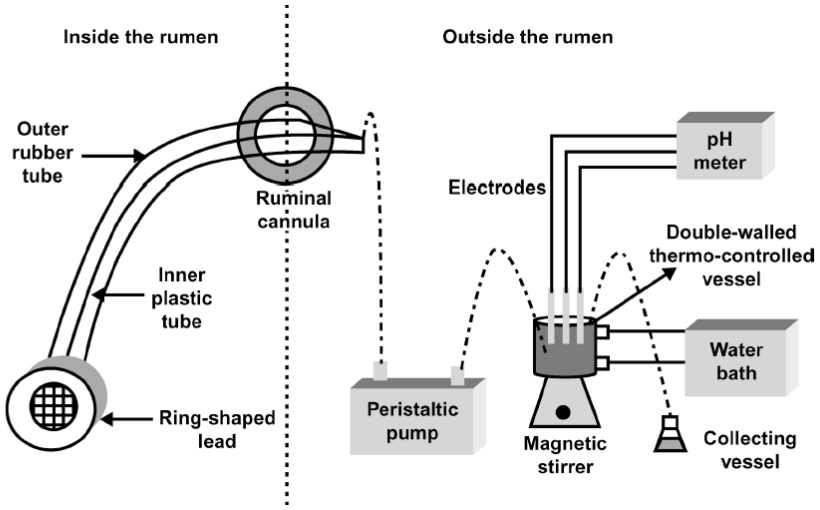

Figure 9. The *ex vivo* measurement (Method 1) of E_h and pH performed on continuously pumped rumen fluid without air contact (Marden et al., 2005).

Figure 10. Wireless data logger (Dascor, Escondido, CA, USA) used for *in vivo* mesurements of ruminal Eh and pH (Method 2).

A total of 775 kinetics of ruminal E_h and pH measurements were gathered together. Each kinetic includes 9 measurements of ruminal pH and E_h taken at 1 h intervals from the morning diet distribution to 8 hours after. The average E_h and pH of these 9 measurements have been calculated for each kinetic. The measurement of ruminal E_h and pH on each animal under each dietary treatment was repeated in three consecutive days during the measurement period. Considering both methods used an Eh platinum electrode, all records of the potential difference were corrected relative to the standard hydrogen electrode (+199 mV at 39°C, Nordstrom 1977). Moreover, as Huang et al. (2016) **(Annex I)** observed an effect of the method on the E_h value, due to the difference of sensors and location of measurements, the E_h values measured by Method 2 were corrected (+35.4 mV) to avoid the influence of method effect.

The complet database included 22 experiments with cannulated dairy cattle including 57 dietary treatments (Table 6). Whole set or sub-database were used for different studies depending on the availability of the variable:

- All 22 experiments were used for quantitative analysis of the relationship between ruminal E_h and pH as well as the influence of dietary characteristics on ruminal Eh (Article 2).
- Data from 9 experiments were used for quantitative analysis of relationship between ruminal redox potential and fermentation parameters (Article 3).
- Data from 16 experiments were used to study the response of E_h and fermentation parameters to live yeast supplementation in dairy cow (Article 4).

B. Quantitative analysis of the relationship between ruminal redox potential and pH in dairy cattle: influence of dietary characteristics (Article 2)

Article 2

Agricultural Sciences, 2017, 8, 616-630
http://www.scirp.org/journal/as
ISSN Online: 2156-8561
ISSN Print: 2156-8553

Quantitative Analysis of the Relationship between Ruminal Redox Potential and pH in Dairy Cattle: Influence of Dietary Characteristics

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Abstract

The ruminal redox potential (E_h) can reflect the microbiological activity and dynamics of fermentation in the rumen. It might be an important indicator of rumen fermentation in combination with pH. However, the ruminal E_h has been rarely studied in dairy cows due to the difficulty of its measurement, and the relationship between ruminal E_h and pH is not clear. The objective of this study was to investigate the relationship between ruminal E_h and pH of dairy cows by meta-analysis of systematic measurements from different experiments. A database was constructed from 22 experiments on cannulated dairy cattle including 57 dietary treatments. The ruminal pH and E_h were measured without air contact between 0 and 8 h post-feeding. The results demonstrated a quadratic correlation between ruminal E_h and pH with a reliable within-animal variation (E_h = -1697 +540.7 pH – 47.7 pH^2, $n_{observation}$ = 70, n_{animal} = 26, $P < 0.001$, RMSE = 16, AIC = 597). The dietary characteristics (NDF, NDFf, OM, starch, degradable starch, soluble sugars contents, and the dietary ionic balance) influencing the ruminal pH also affected the ruminal E_h, but not always to the same extent. Some of them still influenced the relationship between ruminal E_h and pH. While the mechanism of the interaction between ruminal E_h and pH remains to be elucidated, it would be interesting to associate E_h to microbial profile, ruminal VFA concentration and milk production performance in future studies.

Key words

Redox potential, pH, Rumen, Diet, Dairy cow

1. Introduction

Oxidation-reduction conditions are classically assessed by measuring the redox potential (E_h), also called oxidation-reduction potential (usually named ORP) expressed in millivolts (mV). It measures the ability of a solution to accept or donate electrons and corresponds to the potential difference between a platinum electrode and a standard hydrogen electrode [1]. Oxidation-reduction and acid-base reactions are essential for the maintenance of all living organisms. The chemistry of living organisms relies even more on oxidation-reduction reactions than it does on acid-base reactions, which are more focused on proton transfers [1] [2].

The role of E_h has been reported in many biological media such as dairy products [3], wine [4] and rumen fluid [5] - [7]. The ruminal E_h can reflect the microbiological activity and dynamics of fermentation in the rumen [8]. As a matter of fact, ruminal E_h is a mixed potential because of the strong fermentative activity involving numerous oxido-reduction couples. It reflects a weighted average of the potentials contributed by each of the redox couples as mentioned by De Laune and Reddy [9] for soil. The ruminal milieu is anaerobic with an E_h markedly negative, reflecting a strong reducing power in absence of oxygen [6]. It has been reported that dry matter intake can cause an increase of E_h, and the higher E_h also seems to be associated with higher concentrate proportions in the diet and lower ruminal pH [7], which may indicate digestive disorder. Indeed, a low E_h seems to be more favorable to the strict anaerobic bacteria such as fibrolytic and lactate utilizing bacteria [10]. Therefore, the ruminal E_h might be an important indicator of rumen function along with other ruminal variables. Until now, no threshold of ruminal E_h value has been proposed to evaluate rumen function. Since the ruminal pH is considered as the most direct indicator of the rumen digestive disorder and has been extensively studied [11] [12], comparing with ruminal pH could be helpful to interpret ruminal E_h value.

However, compared to other ruminal parameters, the E_h is rarely discussed in dairy cows, and the relationship between ruminal E_h and pH is not clear. Indeed, the ruminal E_h measurement method is not standardized. Three methods of E_h potentiometric measurements have been reported in the literature. The first one consisted of a manual suction-strainer device that pumped out ruminal fluid from a cannulated animal to measure E_h on collected hand-samples in contact with atmospheric air, after a stabilization period of 25 to 30 min as recommended by Andrade *et al*. [13] and adapted by

Giger-Reverdin *et al*. [14]. The two others are *ex vivo* measurements performed on continuously pumped rumen fluid without air contact [6] and *in vivo* measurements performed continuously by wireless probes inside the rumen as described by Penner *et al*. [15] and adapted by Qin *et al*. [16]. Considerable difference in ruminal E_h values has been reported. The major difference is due to the different reference electrodes used. By definition, E_h is the potential difference between a platinum electrode and a standard hydrogen electrode. Some authors [13] [17] who used a reference electrode of calomel or silver chloride did not correct the raw E_h data (+ 199 mV at 39 ºC). Also, the accurate ruminal E_h measurement requires strict anaerobic conditions which are not always satisfied [6].

For several years, our research team has conducted numerous experiments with simultaneous measurements of ruminal E_h and pH of dairy cows fed various diets under anaerobic conditions by *ex vivo* and *in vivo* methods. Analysis of these aggregated measurements could provide a better understanding of factors controlling ruminal E_h and pH, and might demonstrate a quantifiable relationship between ruminal E_h and pH. The objective of this study was to investigate the relationship between ruminal E_h and pH of dairy cows by meta-analysis of systematic measurements from different experiments.

2. Materials and methods

2.1. Selection of studies

A database was constructed from 22 experiments with cannulated dairy cattle including 57 dietary treatments (Table 6). As explained above, due to the heterogeneity of the ruminal Eh values reported in the literature, associated with time of measurement, anaerobic conditions and electrode used [5] [7] [8] [13] [14] [18] [37], we included in the database only experiments conducted by our research group and two others conducted in Agriculture and Agri-Food Canada (Research and Development Centre, Sherbrook, QC) to ensure a consistency of measurement methods among studies. It includes either published [7] [18]-[21] and unpublished studies [22] [23]. Both lactating (12 experiments) and non-lactating cows (10 experiments) were used. Qualitative factors such as physiological status of animals (lactating vs. non-lactating) and site of the experiment (France vs. Canada) were collected.

All animal housing and handling procedures were in accordance with the guidelines for animal research of the French Ministry of Agriculture [24]. Cannulation techniques provided for humane treatment of cows, adhering to locally approved procedures, and were similar to those described by Streeter et al. [25]. All animals were housed in individual tie stalls throughout the experiment with free access to water. Each experimental period covered an adaptation period (2 to 3 weeks) to the different dietary treatment and a measurement period (3 days).

The diets were formulated to meet energy and protein requirements, with two equal distributions at 0900 and 1700h. The composition of the diets (**Table 7**) varied widely (e.g. the proportion of concentrate ranged from 0 to 63%). Some of the dietary characteristics such as neutral detergent fiber from forages (**NDFf**), ruminally degradable starch, rumen protein balance (**RPB**) were estimated by the online software "systool.fr" [26] using the equations published in Sauvant and Nozière [27]. The influence of dietary ionic balance on acid-base balance of animal has been reported [28]-[30], it can be expressed (in mEq/kg of DM) as the dietary cation anion difference (**DCAD** = Na+K-Cl-S) or electrolytic balance (**EB** = Na + K - Cl). We also calculated these values according to the INRA tables [31] for all the diets used in the data base.

2.2. Measurement of ruminal E_h and pH

A total of 775 kinetics of ruminal E_h and pH measurements were gathered together. Each kinetic includes 9 measurements of ruminal pH and E_h taken at 1 h intervals from the morning diet distribution to 8 hours after. The average E_h and pH of these 9 measurements have been calculated for each kinetic. The measurement of ruminal E_h and pH on each animal under each dietary treatment was repeated in three consecutive days during the measurement period.

All E_h and pH values were measured under strict anaerobic conditions, by *ex vivo* (method 1) [6], or *in vivo* method (method 2) [15]. In Method 1, rumen fluid was pumped continuously through a rubber tube into a 50-mL-double-walled thermocontrolled vessel outside the rumen, the E_h and pH were measured by electrodes dipped in the collected rumen fluid without air contamination. In Method 2, a wireless real-time data logger (Dascor, Escondido, CA, USA) was submersed into the ventral rumen sac via the ruminal cannula after calibration, and the E_h and pH were measured by external sensors of the data logger and stored in the memory chip. For both methods, the accuracy

E_h electrode was checked by measuring the standard solution at 220 mV (Fishier Scientific) before and after each measurement.

Considering both methods used an E_h platinum electrode, all records of the potential difference were corrected relative to the standard hydrogen electrode (+199 mV at 39°C) [32]. Moreover, as Huang *et al*. [33] observed an effect of the method on the E_h value, due to the difference of sensors and location of measurements, the E_h values measured by method 2 were corrected (+35.4 mV) to avoid the influence of method effect.

2.3. Statistical Analysis

Interpretation of the database was based on a statistical meta-analysis [34] [35]. At each step of the meta-analysis process, graphical observations were made to check the coherence of relationships and to identify obviously abnormal values. All analyses were performed using the statistical software R version 2.15.1 (R Development Core Team, 2012).

2.3.1. Influence of dietary characteristics on E_h and pH

The average E_h and pH of each dietary treatment were calculated for this analysis. The experiment effect was considered to be random. The within-experiment correlation was calculated using a mixed model. The general form of the mixed model was:

$$Y_{ij} = B_0 + B_1X_{ij} + s_i + b_i X_{ij} + e_{ij},$$

where i = number of studies, j = number of observations, $B_0 + B_1X_{ij}$ is the fixed effect part of the model and $s_i + b_i X_{ij} + e_{ij}$ is the random effect part of the model. The goodness of fit of the model was evaluated using the Akaike Information Criterion (**AIC**) [36]. Because a reliable within-experiment response requires a minimal variation of descriptive variables, only the experiments tested a sufficient range of dietary characteristics (OM > 25 g/kg, starch > 70 g/kg, soluble sugar > 20 g/kg, CP > 18 g/kg, NDF >80 g/kg, DCAD > 50 mEq/kg, EB > 100 mEq/kg) were selected for within-experiment analysis.

For each relationship, the number of treatments (n_{treat}) and of experiments (n_{exp}) used in the analysis are reported. Treatments with high normalized residuals (< - 3 or > + 3) were identified and discarded from the model as statistical outliers if they had a high leverage effect based on *Hi*

calculation (*Hi* > 3× k/n, where k is number of independent variables in the model and n is the number of observations) and Cook distance (Cook > 1) [34]. A one-way ANOVA was used to test whether ruminal E_h or pH varied according to the qualitative factors such as physiological status and site of the experiment.

2.3.2. Relationship between ruminal E_h and pH

Since the individualized ruminal E_h and pH measurements are available, the average E_h and pH of each animal in each dietary treatment (3 repetitions) were calculated to take into account the variability within one animal under different dietary treatments. Only the animals (70 observations from 26 animals) presenting a sufficient range of ruminal pH (≥ 0.2) were selected to this analysis. The within-animal correlation was calculated using a mixed model. The animal effect was considered to be random. The model was:

$$Y_{ij} = B_0 + B_1X_{ij} + s_i + b_i X_{ij} + e_{ij},$$

where i = number of animals, j = number of observations, $B_0 + B_1X_{ij}$ is the fixed effect part of the model and $s_i + b_i X_{ij} + e_{ij}$ is the random effect part of the model.

The influence of co-variables (OM, NDF, NDFf, total starch, degradable starch, CP, soluble sugars, DCAD, EB, and RPB contents in diets) on the relationship between ruminal E_h and pH was tested. The first step consisted in highlighting the co-variables influencing the residuals (i.e. the difference between observed E_h and predicted E_h by the equation). The influence of all co-variables on residuals (observed minus predicted E_h) was tested using the Stepwise procedure. In the second step of the analysis, the significant co-variables were included in the model.

3. Results

A summary of E_h and pH value in the database is given in **Table 8**. Both E_h (ranged from -233.4 to -99.6 mV) and pH (ranged from 5.48 to 6.76) covered a wide range.

Table 8. Summary of the redox potential and pH value in the database.

	n	Mean	SD[1]	Minimum	Maximum
E_h (mV)	775	- 179.8	25.9	- 233.4	- 99.6
pH	775	6.15	0.30	5.48	6.76

[1]SD = standard deviation.

3.1. Influence of dietary characteristics on ruminal E_h and pH

Table 9 reports the relationship between ruminal E_h and dietary characteristics. Ruminal E_h was positively correlated to OM ($P = 0.022$), total starch ($P = 0.012$), degradable starch ($P = 0.041$), and soluble sugars ($P < 0.001$) contents, and negatively correlated to total NDF ($P = 0.024$), NDFf ($P = 0.049$), DCAD ($P < 0.001$), and EB ($P < 0.001$). The ruminal E_h was not related to CP ($P = 0.713$), and RPB ($P = 0.209$). No experiment tested the effect of intake and only two experiments tested a sufficient range of proportion of concentrate (≥ 30%), which did not permit the analysis of within-experiment relationship between ruminal E_h and these two parameters.

The quadratic adjustment was significant between ruminal E_h and DCAD ($E_h = -122 - 0.462$ DCAD + 0.000596 DCAD^2, $P = 0.010$, RMSE = 9, AIC = 187) and between ruminal E_h and EB ($E_h = -107 - 0.368$ EB + 0.000313 EB^2, $P = 0.003$, RMSE = 8, AIC = 183). The ruminal E_h was significantly affected by physiological status (–188.5 ± 24.0 and – 169.1 ± 20.8 mV for non-lactating and lactating cows respectively, $P = 0.002$), but not affected by the site of experiment ($P = 0.353$).

Table 9. Relationship between ruminal redox potential and dietary characteristics.

Item	n_{exp}	n_{treat}	Intercept	Slope	*P*-value	RMSE	AIC
OM, g/kg DM	6	18	-718	0.559	0.022	13	151
RPB, g/kg DM	7	20	NS	NS	NS	NS	NS
NDF, g/kg DM	5	15	-143	-0.126	0.024	14	129
NDFf, g/kg DM	5	15	-165	-0.086	0.049	15	131
Starch, g/kg DM	6	18	-215	0.088	0.012	13	153
Degradable starch, g/kg DM	6	18	-210	0.089	0.041	14	155
CP, g/kg DM	6	18	NS	NS	NS	NS	NS
Soluble sugars, g/kg DM	6	18	-215	0.696	< 0.001	10	137
DCAD, mEq/kg DM	8	22	-154	-0.145	< 0.001	11	179
EB, mEq/kg DM	8	22	-141	-0.141	< 0.001	12	174

OM = organic matter; DM = dry matter; RPB = rumen protein balance; NDF = neutral detergent fibre; NDFf = NDF from forages; CP = crude protein; DCAD = dietary cation anion difference (Na+K-Cl-S, in mEq/kg of DM); EB = electrolytic balance (Na + K - Cl , in mEq/kg of DM); n_{exp} = number of experiments; n_{treat} = number of treatments; RMSE = residual mean standard error; AIC = akaike information criterion.

Table 10 reports the relationship between ruminal pH and dietary characteristics. Ruminal pH was positively correlated to NDF ($P = 0.008$), NDFf ($P = 0.012$), DCAD ($P = 0.004$), and EB ($P = 0.001$), and was negatively correlated to OM ($P = 0.018$), starch ($P = 0.004$), degradable starch ($P = 0.018$), and soluble sugars ($P < 0.001$) contents. It was not related to CP ($P = 0.195$) and RPB ($P = 0.518$).

No quadratic adjustment was significant for relationship between ruminal pH and dietary characteristics (data not shown). The ruminal pH was significantly affected by physiological status (6.32 ± 0.25 and 5.99 ± 0.17 for non-lactating and lactating cows respectively, $P < 0.001$), but not affected by the measurement method of E_h ($P = 0.942$), and the site of the experiment ($P = 0.950$).

Table 10. Relationship between ruminal pH and dietary characteristics.

Item	n_{exp}	n_{treat}	Intercept	Slope	*P*-value	RMSE	AIC
OM, g/kg DM	6	18	10.93	-0.0049	0.018	0.11	2.1
RPB, g/kg DM	7	20	NS	NS	NS	NS	NS
NDF, g/kg DM	5	15	5.98	0.0011	0.008	0.10	3.5
NDFf, g/kg DM	5	15	6.14	0.0008	0.012	0.10	4.9
Starch, g/kg DM	6	18	6.57	-0.0008	0.004	0.10	2.9
Degradable starch, g/kg DM	6	18	6.52	-0.0008	0.018	0.11	5.4
CP, g/kg DM	6	18	NS	NS	NS	NS	NS
Soluble sugars, g/kg DM	6	18	6.54	-0.0055	< 0.001	0.06	-14.3
DCAD, mEq/kg DM	8	22	6.05	0.0011	0.004	0.09	2.1
EB, mEq/kg DM	8	22	5.97	0.0010	0.001	0.11	5.5

OM = organic matter; DM = dry matter; RPB = rumen protein balance; NDF = neutral detergent fibre; NDFf = NDF from forages; CP = crude protein; DCAD = dietary cation anion difference (Na+K-Cl-S, in mEq/kg of DM); EB = electrolytic balance (Na + K - Cl , in mEq/kg of DM); n_{exp} = number of experiments; n_{treat} = number of treatments; RMSE = residual mean standard error; AIC = akaike information criterion.

3.2 Relationship between ruminal E_h and pH

The relationship between ruminal E_h and pH is presented in **Figure 11**. The ruminal E_h and pH were negatively correlated. The linear relationship (equation 1) and quadratic adjustment (equation 2) were both significant ($P < 0.001$):

$$E_h = 104 - 46.3\ pH\ (n_{obs} = 70,\ n_{anim} = 26,\ RMSE = 17,\ AIC = 609) \quad \text{Eq 1}$$

$$E_h = -1697 + 540.7\ pH - 47.7\ pH^2\ (n_{obs} = 70,\ n_{anim} = 26,\ RMSE = 16,\ AIC = 597) \quad \text{Eq 2}$$

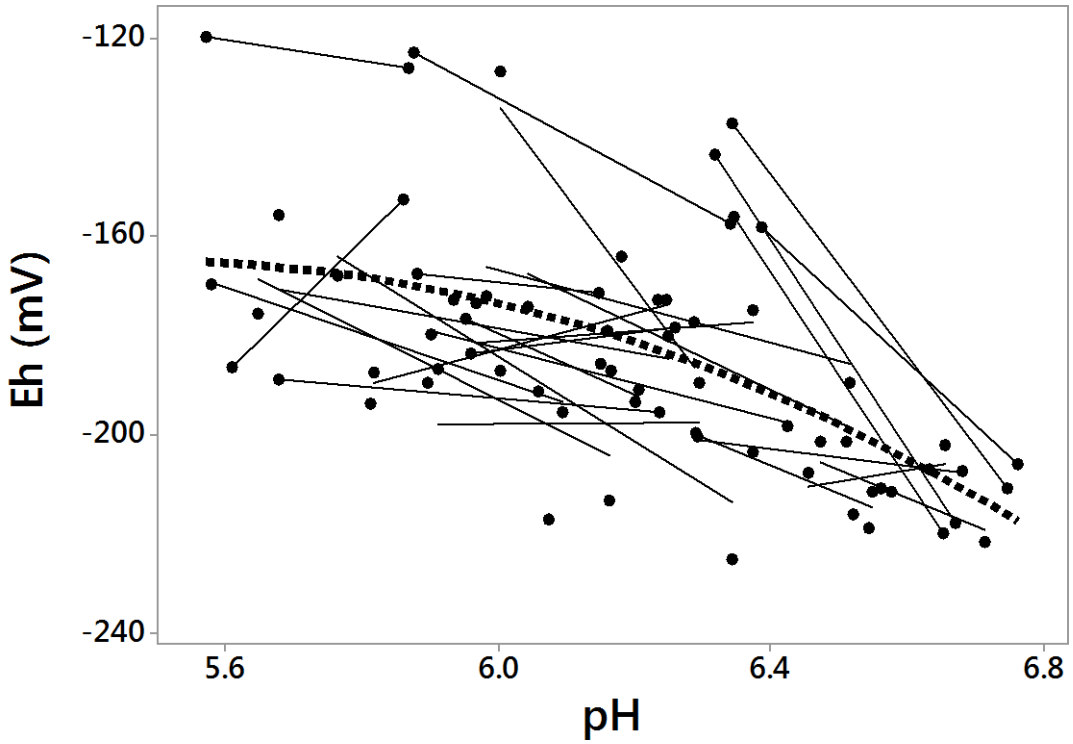

Figure 11. Relationship between ruminal redox potential (E_h) and pH.

Each symbol represents the data from one animal in one experiment. The solid lines represent the linear regression of the data from each animal. The dotted line represents the average within-animal quadratic adjustment of all observations ($E_h = -1697 + 540.7\ pH - 47.7\ pH^2$, $n_{observations} = 70$, $n_{animals} = 26$, $P < 0.001$, RMSE = 16, AIC = 597, R^2=0.77).

3.3 Variables influencing the relationship between ruminal E_h and pH

The intake ($P < 0.001$), soluble sugars contents ($P = 0.008$), DCAD ($P = 0.003$) were selected by the Stepwise analysis and significantly influenced the residuals of equation 2. Once included in equation 2, only the DMI was significant ($P = 0.03$) and slightly improved the equation:

$$E_h = -2097 + 690.2\ pH - 60.7\ pH^2 - 1.27\ DMI \qquad \text{Eq 3}$$

($n_{obs} = 70$, $n_{anim} = 26$, RMSE = 16, AIC = 591)

4. Discussion

Meta-analyses use scientific methods based on statistics to summarize and quantify knowledge acquired through previously conducted studies [34]. Until now, there is a limited number of studies reporting ruminal E_h measurements. Unlike a classical empirical modeling of biological responses based on exhaustive data collection from published experimental results, our study used the aggregation of measurements from our experiments in order to ensure the homogeneity of E_h values and avoided the considerable influence of measurement method explained previously. Use of such analysis leads to a better understanding of factors controlling the variables.

The database of present study covered a wide range of ruminal E_h and pH values. The range of ruminal E_h value in dairy cattle in our database (-233.4 to -99.6 mV) is comparable with that in sheep (-260 to -150 mV) [8] [37], in goat (-190 to -145 mV) [5] and in dairy cow (-241 to -185 mV) [38]. Some authors reported much lower ruminal E_h values: from -302 to -340 mV in sheep [17] and from -327 to -352 mV in goat [13]. It is due to the different reference electrodes used as explained above. The significant effect of physiological status on ruminal E_h and pH was expected and could be explained by dietary difference between lactating and non-lactating cows.

4.1 Dietary characteristics influencing ruminal E_h

The influence of dietary concentrate proportion on ruminal E_h observed in previous studies [5] [8] [14] was not confirmed by our analysis due to the limited number of experiments (n = 2) presenting a sufficient range of dietary concentrate proportion. However, the variables associated with slowly or rapidly degradable materials contents (NDF, NDFf, OM, starch, degradable starch and especially soluble sugars, which resulted low RMSE and AIC) showed consistent correlation with ruminal E_h.

Few studies investigated the influence of these dietary characteristics on ruminal E_h. However, the effect of slowly or rapidly degradable diet on ruminal E_h has been reported. Andrade *et al*. [13] observed a higher ruminal E_h for the goats fed rapidly degradable diet (-327 mV) compared to that of goats fed slowly degradable diet (-352 mV). These E_h values were lower than ours due to the different reference electrodes used, but the difference of ruminal E_h caused by two type of diet was significant ($P < 0.001$). Our results are in agreement with these observations.

To our knowledge, the effect of dietary ionic balance (DCAD and EB) on ruminal E_h has never been reported. According to our results, the DCAD and EB showed consistent correlation with

ruminal E_h. The quadratic adjustment of the within-experiment relationship resulted slightly higher AIC (187 and 183 for DCAD and EB respectively) but lower RSME (9 and 8 for DCAD and EB respectively). The mechanism of this effect remains unclear. But it is known that E_h can affect mineral availability. As demonstrated in soil, E_h is a factor that strongly influence the mobility of many elements such as N, P, S, K and Na. Conversely, E_h is influenced by the various elements [1]. Considering that the effect of dietary ionic balance was not investigated as a determining factor by the experiments in the database, it deserves to be confirmed by a classic experiment with *in vivo* measurements.

4.2 Dietary characteristics influencing ruminal pH

The influence of OM, NDF, NDFf, starch, degradable starch and soluble sugars contents on ruminal pH is well documented. Among these variables, the relationship between NDF and starch content and ruminal pH are frequently studied. The relationship ($y = 5.53 + 0.022\ x$) between pH and diet NDF content (% DM) reported by Pitt *et al*. [39] is close to the relationship obtained in our study. By analyzing results from 23 studies of lactating dairy cows fed pasture, Kolver and de Veth [40] reported a within study equation between ruminal pH and NDF content (% DM) with a numerically lower slope than ours ($y = 5.84 + 0.0075\ x$, $P = 0.014$, $n = 100$), when taking into account the difference of unit of NDF (g/kg DM in our analysis). Regarding the influence of degradable starch in the rumen (% of intake dry matter) on ruminal pH (dairy and beef cattle), Sauvant and Peyraud [11] reported a similar relationship ($y = 6.4 – 0.01x$) compared to ours.

The DCAD and EB are close (the only difference is that the EB does not consider sulfur ions) and highly correlated [41]. Both influence ruminal pH. Their influence on acid-base balance of animal has been described [42]. Indeed, Na and K are absorbed from the gastrointestinal tract in exchange for the secretion of a proton, whereas Cl and S are often absorbed in exchange for the secretion of a bicarbonate ion [30] [43]. Increasing DCAD in the diet allows the cows to overcome the saturation of the renal mechanisms for saving HCO_3 and contributes to increase blood bicarbonate concentration which could be recycled into the rumen to limit the decrease of ruminal pH. Several studies reported that a shift from negative or null DCAD to highly positive values increases DMI and milk yield [42] [44]. A meta-analysis [29] grouping 27 experiments reported positive relationship between EB and blood pH, EB and bicarbonate content in blood, EB and pH of urine. Our results showed clear positive relationship between DCAD or EB and ruminal pH,

which is in agreement with the hypothesis of the acid-base balance mechanism in ruminant. The equation between ruminal pH and DCAD obtained by our analysis is consistent with that of Iwaniuk and Erdman [45], obtained by a meta-analysis of 63 published journal articles ($y = 6.31 + 0.0003$ x, $P = 0.034$, $r^2 = 0.19$, n = 83). Considering these results, DCAD and EB deserve to be more often measured and taken into account in future studies.

4.3 Relationship between ruminal E_h and pH

The results of present study confirmed the negative relationship between ruminal E_h and pH reported by previous studies in goats [5] [13] [46]. The slope of the linear relationship in our study is similar to that of Giger-Reverdin et al. [46]. The lower average ruminal E_h value (-354 ± 22 mV) reported by these authors could be explained by the different measurement methods used as explained previously. By gathering together a large data base of wide range ruminal E_h and pH values, we further demonstrated a quadratic correlation (Eq 2) between ruminal E_h and pH with a reliable within-animal variation of the variable. Considering that in biological media, such as rumen, many oxidation-reduction reactions involve protons, it is not surprising that ruminal E_h and pH are related [1] [13] as is shown by the Nernst's equation [47].

It is noteworthy that the diet characteristics (NDF, NDFf, OM, starch, degradable starch, soluble sugars contents, and the dietary ionic balance) influencing the ruminal pH also affected ruminal E_h, but not always in same extent. Indeed, the complex reactions which determine E_h are not necessarily the same reactions which determine pH: for example, when rapidly-oxidizable organic matter is added, the E_h could be changed without changing pH [48]. Also, Friedman et al. [49] highlighted the E_h as a key factor in the structuring of anaerobic microbial communities through their experimental system separating E_h from pH effect.

In our database, we can observe some high pH values (e.g. pH > 6, without SARA according to the ruminal pH thresholds proposed in the literature) associated with high E_h which is unfavorable to activities of fibrolytic and lactate utilizing bacteria, and also some low E_h values associated with low pH (**Figure 11**). Therefore, in some circumstances, the E_h could better reflect the fermentation dynamics than pH and *vice versa.*

The measurement of ruminal pH alone might not be sufficient for diagnosing digestive disorder in some cases. The simultaneous measurement of ruminal E_h and pH could be useful to provide complementary information about the rumen fermentation. Nevertheless, no threshold has been

proposed to evaluate the rumen digestive disorder. In order to initiate the use of ruminal E_h, we could propose a preliminary threshold of ruminal E_h > - 166 mV (correspond to pH < 6 according to Eq 2) indicating digestive disorder.

5. Conclusions

By gathering together a large database of uniformly measured ruminal E_h and pH under anaerobic conditions, the present study demonstrated a quadratic correlation between ruminal E_h and pH. The analysis highlights the influence of dietary characteristics on ruminal E_h. Within experiments, a good prediction of ruminal E_h could be made using soluble sugars content and the dietary ionic balance. The dietary characteristics (NDF, NDFf, OM, starch, degradable starch, soluble sugars contents, and the dietary ionic balance) influencing the ruminal pH also affected the ruminal E_h, but not always in same extent. Some of them still influence the relationship between ruminal E_h and pH. The mechanism of the interaction between ruminal E_h and pH remains to be elucidated, it would be interesting to associate microbial profile and ruminal VFA concentration and milk production performance in future studies.

Acknowledgements

We would like to thank Prof. Philippe Schmidely for his help in the statistical analysis of the results.

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C. Relationship between ruminal redox potential and fermentation parameters from *in vivo* experiments in dairy cattle: influence of dietary characteristics (Article 3)

In preparation for the Journal of Dairy Science Article 3

Short Communication: *Relationship between ruminal redox potential and fermentation parameters from in vivo experiments in dairy cattle: influence of dietary characteristics*

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Abstract

The present study explored the relationship between ruminal redox potential (E_h) and fermentation parameters e.g. VFA and ammonia (NH_3-N), and the influence of diet composition and DMI. Data from 9 *in vivo* trials conducted in ruminally cannulated Holstein dairy cows were compiled for meta-analysis. The data set was constructed using individual cow observations (n = 162). Total ruminal VFA concentration ($P = 0.004$), propionate ($P = 0.002$) and butyrate proportions ($P = 0.009$) were positively correlated to E_h, whereas acetate proportion ($P = 0.016$), acetate to propionate (A:P) ratio ($P = 0.036$) and NH_3-N concentration ($P = 0.031$) were negatively correlated to E_h. Inclusion of dietary characteristics in the models did not always result in improved predictions. A reduction ($P < 0.001$) in error for acetate, butyrate, A:P, and NH_3-N was observed once soluble sugars content (g/kg DM) was included in the models. The relationship between ruminal E_h and main fermentation parameters was for the first time quantified.

Key words: redox potentiel, fermentation parameters, rumen, dairy cow

Introduction

Both oxidation-reduction and acid-base reactions are essential for the maintenance of all living organisms. However, the chemistry of living organisms relies more on oxidation-reduction reactions than it does on acid-base reactions, which are more focused on proton transfers (Falkowski et al., 2008; Husson 2013). Oxidation-reduction conditions are usually assessed by measuring redox potential (**E_h**, in mV), also called oxidation-reduction potential. It measures the ability of a medium to accept or donate electrons and corresponds to the potential difference (mV) between a platinum electrode and a standard hydrogen electrode (Husson 2013).

The role of E_h has been reported in many biological media such as dairy products (Brasca et al., 2007), wine (Tomlinson and Kilmartin 1997) and rumen fluid (Marounek et al., 1982; Marden et al., 2005; Julien et al., 2010). The ruminal milieu is anaerobic with an E_h markedly negative i.e. from -115 to -300 mV (Huang et al., In press), reflecting a strong reducing power in absence of oxygen (Marden et al., 2005). It has been reported that dry matter intake can cause an increase of E_h, and the higher E_h seems also associated with higher concentrate proportions in the diet (Julien et al. 2010). In a recent meta-analysis, Huang et al. (2017a) further demonstrated a quadratic correlation between ruminal E_h and pH in dairy cows and highlighted the influence of some dietary characteristics on ruminal E_h which reflects the microbiological activity and dynamics of fermentation in the rumen (Broberg 1958).

Volatile fatty acids produced in the rumen by microbial fermentation are main energy source for ruminants. The VFA profile, especially the acetate:propionate (**A:P**) ratio is an important indicator of rumen function (Sauvant et al., 2011). It depends on the type of fermented substrate, the microbial population and ruminal environment affecting microbial metabolism. Dijkstra (1994) was the first to stress the need to maintain a low E_h in the rumen through reduction and oxidation of pyridine nucleotides (**NAD**) as the driving force for rumen VFA production. It is therefore necessary to understand the influence of E_h on VFA profile. The objective of this study was to investigate the relationship between ruminal E_h and fermentation parameters from *in vivo* experiments in dairy cows and the influence of diet characteristics.

Materials and methods

A data set was constructed using individual cow observations (n = 162) from 9 trials (Table 11) (including two trials conducted in collaboration with Agriculture and Agri-Food Canada, Research and Development Centre, Sherbrook, QC).

Table 11. Description of each trial involved in the meta-analysis.

Trial	Physiological status	Experimental design	E_h Method[1]	Main ingredients of diets
Julien et al. 2010	Non-lactating	Latin square	1	Grass hay/barley/wheat/soybean meal
Michelland et al. 2011	Non-lactating	Randomized block	1	Alfalfa hay/corn silage/wheat straw/corn/soybean meal
Julien et al. 2015	Lactating	Latin square	1	Corn silage/wheat/soybean meal/tanned soybean meal silage
Julien 2010	Lactating	Latin square	1	Corn silage/wheat/corn/soybean meal
Julien 2010	Lactating	Latin square	1	Corn silage/wheat/corn/soybean meal
Benchaar et al. unpublished	Lactating	Latin square	2	Alfalfa silage/corn silage/grass hay/corn/soybean meal
Unpublished	Lactating	Latin square	2	Corn silage/alfalfa hay/soybean meal/composed concentrate
Unpublished	Non-lactating	Latin square	2	Grass hay/soybean meal
Benchaar et al. unpublished	Lactating	Latin square	2	Barley silage/corn silage/barley/corn/soybean meal

[1]Method 1 was performed with probes on continuously pumped rumen fluid (Marden et al. 2005); Method 2 was performed continuously with probes inside the rumen and wireless device (Penner et al., 2006); E_h = redox potential.

The trials were performed through different years with different dietary treatments. Only the experiments testing at least two diets were included in the database to take into account the within-experiment variation. It included either published (Julien et al., 2010; Michelland et al., 2011; Julien et al., 2015) and unpublished studies (Julien, 2010). All trials used ruminally cannulated Holstein dairy cows. Cannulation technique provided for treatment of cows, adhering to locally approved procedures, and were similar to those described by Streeter et al. (1990). Both lactating (6 trials) and non-lactating cows (3 trials) were used. All animals were housed in individual tie stalls throughout the experiment with free access to water. Each

experimental period covered an adaptation period (2 to 3 weeks) to the different dietary treatments followed by a 3 d measurement and sampling period.

The diets were formulated to meet energy and protein requirements, with two equal distributions at 0900 and 1700h. The composition of the diets (Table 12) varied considerably, especially for soluble sugars (CV = 63%), dietary cation anion difference (DCAD, CV = 50%) and starch (CV = 49%) contents. The proportion of concentrate and the DMI ranged from 0 to 56% and from 8 to 26 kg/cow/d, respectively.

Table 12. Summary of dietary characteristics of data set.

Item	Mean	SD	Minimum	Maximum
Intake (kg DM/cow/d)	18.7	7.0	8.0	26.2
Proportion of concentrate (% DM)	34.2	12.4	0.0	55.9
OM (g/kg DM)	939.4	18.5	891.8	960.3
NDF (g/kg DM)	391.7	69.6	316.4	566.3
Starch (g/kg DM)	237.9	116.1	0.0	393.1
CP (g/kg DM)	155.7	22.6	119.1	222.3
Soluble sugars (g/kg DM)	42.9	27.3	0.0	72.2
DCAD[1] (mEq/kg DM)	207.9	104.1	76.8	438.0
EB[2] (mEq/kg DM)	311.3	128.8	133.8	638.0

[1]DCAD = dietary cation anion difference (calculated as $[Na^+ + K^+] - [Cl^- - S^-]$).

[2]EB = electrolytic balance (calculated as $[Na^+ + K^+] - Cl^-$).

The details of E_h and pH measurements were reported in Huang et al. (2017a). Briefly, E_h and pH were measured under strict anaerobic conditions, by *ex vivo* (method 1; Marden et al. 2005) or *in vivo* method (method 2; Huang et al., 2017b). In method 1, rumen fluid was pumped continuously through a rubber tube into a 50-mL-double-walled thermocontrolled vessel outside the rumen, the E_h and pH were measured by electrodes dipped in the collected rumen fluid without air contamination. In method 2, a wireless real-time data logger (Dascor, Escondido, CA, USA) was submersed into the ventral rumen sac via the ruminal cannula after calibration, and the E_h and pH were measured by external sensors of the data logger and stored in the memory chip. For both methods, the accuracy E_h electrode was checked by measuring the standard solution at 220 mV (Fisher Scientific) before and after each measurement. For each

cow, ruminal E_h was recorded hourly from morning feeding to 8 h after. Thereafter, the daily E_h and pH were averaged.

Ruminal fluid (20 ml) was collected from cows before and at 1, 2, 4, 6, and 8 h after the morning feeding. Each sample was preserved by the addition of 1 ml of H_2SO_4 (50%) and were frozen at - 20°C for subsequent determination of VFA and NH_3-N concentrations. Analysis of VFA was performed using a gas chromatographic method as described in Marden et al. (2008). Ammonia concentration was analyzed by colorimetry with Nessler's reagent using the method adapted by Hach et al. (1985, 1987) on the separated liquid phase of ruminal samples centrifuged at 4000 × g for 20 min.

The method used in meta-analysis of our data was that developed by St-Pierre (2001) and Sauvant et al. (2008). At each step of meta-analysis process, graphical observations were made to check the coherence of relationships and to identify obviously abnormal values. The average E_h, total VFA, molar proportion of individual VFA, A:P ratio, and NH_3-N concentration measured on each animal for each dietary treatment were calculated to take into account the intra-animal variability. The ruminal E_h was analyzed as an explanatory variable. The correlations between E_h and ruminal fermentation parameters were calculated using mixed model (St-Pierre 2001). The animal effect was considered to be random. The best fit chosen was the one with the lowest root mean square error (**RMSE**). The goodness of fit of the models was evaluated using the Akaike information criterion (**AIC**) (Wang and Goonewardene, 2004). The influence of co-variables (DMI, percentage of concentrate, OM, NDF, total starch, CP, soluble sugars, DCAD and EB contents in the diets) on the relationship between ruminal E_h and fermentation parameters was tested. The first step consisted in highlighting the co-variables influencing the residuals (i.e. the difference between observed and predicted values) by using the Stepwise procedure. In the second step of the analysis, the significant co-variables were included in the model. All analyses were performed using the statistical software R version 2.15.1 (R Development Core Team, 2012).

Results and discussion

Data gathered from the trials included ruminal E_h, total VFA, molar proportion of individual VFA, and NH_3-N concentration (Table 13).

Table 13. Statistical descriptions of variables measured in the data set.

	Mean	SD	Minimum	Maximum
E_h (mV)[1]	- 182.5	26.4	- 233.4	- 114.7
Total VFA (m*M*)	89.4	17.8	52.2	136.0
Acetate (%)	63.2	6.2	48.8	74.3
Propionate (%)	20.8	5.1	12.5	35.2
Butyrate (%)	11.7	2.3	7.1	20.5
Acetate: propionate	3.29	1.03	1.48	5.51
NH_3-N (mg/L)	127.1	33.0	59.6	233.3

[1]E_h = redox potential.

The ruminal E_h ranged from -233.4 to -114.7 mV, total VFA from 52.2 to 136.0 m*M*, acetate proportion from 48.8 to 74.3 %, propionate proportion from 12.5 to 35.2%, butyrate proportion from 7.1 to 20.5%, A:P ratio from 1.48 to 5.51, and NH_3-N concentration from 59.6 to 233.3 mg/L. Relationships between E_h and, total VFA, molar proportion of individual VFA, A:P ratio, and NH_3-N concentration are presented in **Figure 12**.

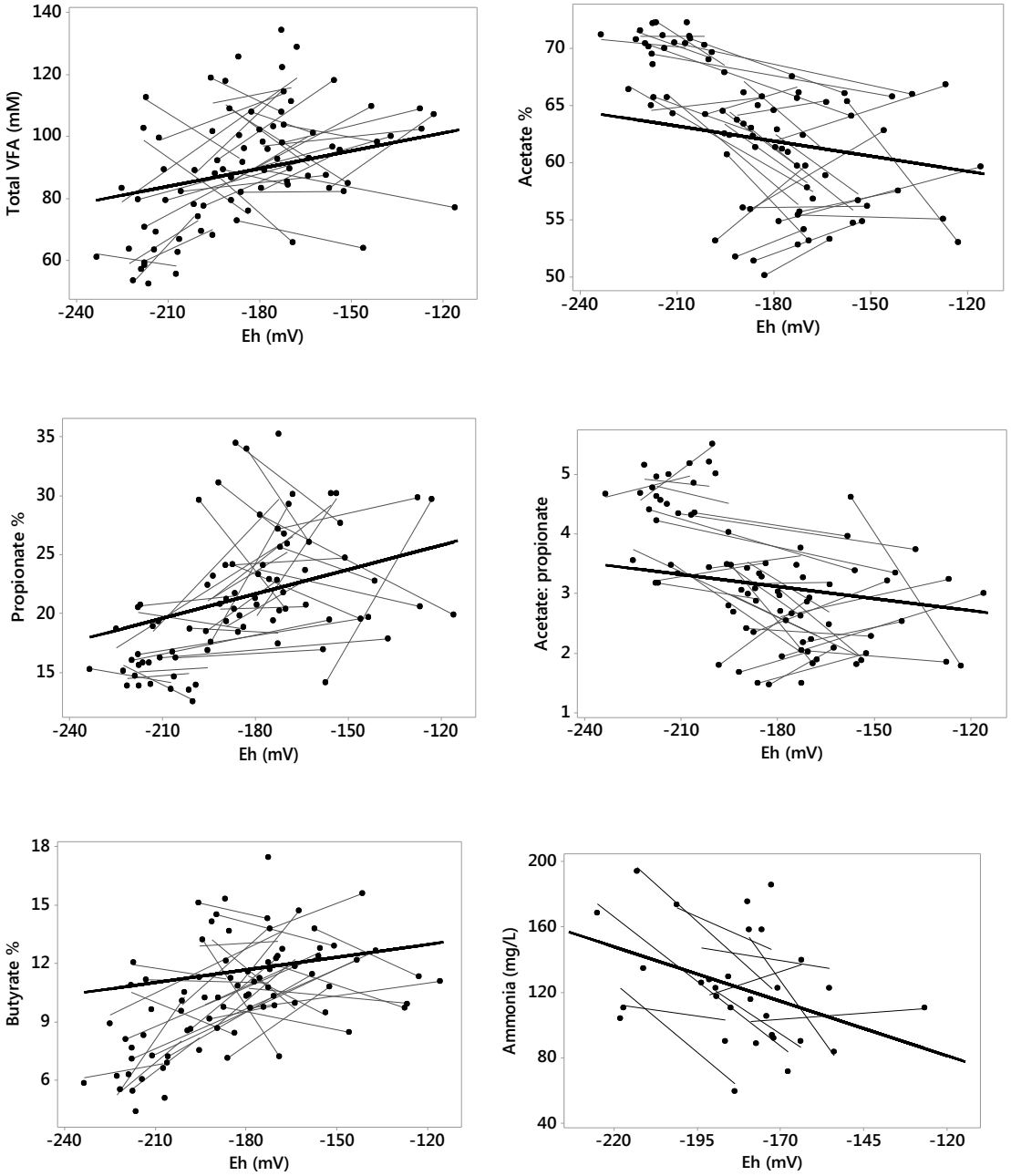

Figure 12. Relationship between E_h and, total VFA (mM), acetate (%), propionate (%), butyrate (%), acetate:propionate ratio, and ammonia (NH_3-N, mg/L).

Each point represents the data from one animal in one experiment. The fine lines represent the linear regression of the data from each animal. The thick lines represent the average within-animal adjustment of all observations.

Total ruminal VFA concentration (Equation 1), propionate (Equation 3) and butyrate (Equation 4) proportions were positively correlated to E_h, whereas acetate proportion (Equation 2), A/P ratio (Equation 5) and NH_3-N concentration (Equation 6) were negatively correlated to E_h.

Total VFA (m*M*) = 124.0 + 0.191 E_h (*P* = 0.004; n = 81, RSME = 12.1; AIC = 657.7) [1]

Acetate (%) = 54.0 – 0.044 E_h (*P* = 0.016; n = 81, RSME = 3.2; AIC = 459.4) [2]

Propionate (%) = 34.1 + 0.069 E_h (*P* = 0.002; n = 81, RSME = 4.0; AIC = 490.6) [3]

Butyrate (%) = 15.6 + 0.022 E_h (*P* = 0.009; n = 81, RSME = 1.7; AIC = 333.2) [4]

A:P = 1.90 - 0.0068 E_h (*P* = 0.036; n = 81, RSME = 0.58; AIC = 183.1) [5]

NH_3-N (mg/L) = 1.5 - 0.665 E_h (*P* = 0.031; n = 30, RSME = 29.4; AIC = 297.0) [6]

In the present study, some of the diet characteristics still influenced the relationship between ruminal E_h and fermentation parameters. None of diet characteristics influenced the residual variation of total VFA. Residual of propionate proportion was influenced by soluble sugar and NDF contents ($P < 0.001$), acetate proportion by soluble sugar and DCAD contents ($P < 0.001$), butyrate proportion by NDF ($P < 0.001$), CP ($P < 0.05$) and EB ($P < 0.01$) contents, and A:P ratio and NH_3-N concentration by DMI and soluble sugar content ($P < 0.001$). However their inclusion in the models did not always result in improved predictions. Only a minor reduction ($P < 0.001$) in error for acetate, butyrate, A:P, and NH_3-N was observed:

Acetate (%) = 64.6 - 0.0091 E_h - 0.09323 Soluble sugar
(n = 81; RSME = 2.7; AIC = 447; $P < 0.001$)

A:P = 4.64 – 0.0040 E_h – 0.082 DMI – 0.0102 Soluble sugar
(n = 81; RSME = 0.54; AIC = 181; $P < 0.001$)

Butyrate (%) = 18.5 + 0.0026 E_h – 0.0166 NDF
(n = 81; RSME = 1.5; AIC = 323; $P < 0.001$)

NH_3-N = -272.3 - 0.175 E_h + 19.2 DMI- 2.501 Soluble sugar
(n = 30; RSME = 17.8; AIC = 273; $P < 0.001$)

Equation 1 shows that the higher E_h, the higher VFA concentration is ($P = 0.004$). This result was expected given the known negative relationships between VFA concentration and pH (Sauvant et al., 2006) on the one hand and, pH and E_h (Huang et al., 2017a) on the other hand. The fact that the VFA concentration varies with the reducing level of the medium is consistent with the results of bioenergetic studies according to which the ruminal fermentation is mainly under thermodynamic control (Ungerfeld and Kohn 2006).

Redox potential is closely associated with the VFA profile (equations 2, 3, and 4) derived from dietary carbohydrate fermentations in the rumen. In our study, the negative correlation between E_h and acetate proportion and, the positive correlation between E_h and propionate proportion are in agreement with *in vitro* results (Wang et al., 2012; Nerdahl and Weimer 2015). Consequently, the A:P ratio which is related to the energetic status of microorganisms of the ruminal ecosystem, is highly correlated with E_h in intra-experiment (equation 5). The inverse relationship between A:P ratio and the amount of concentrate or starch in the diet is well accepted. This relationship is often explained by the tendency of fibrolytic bacteria to produce acetate and amylolytic bacteria to produce propionate (Blaxter 1962; Enjalbert et al., 1999). However, the metabolic characteristics of fibrolytic and amylolytic bacteria is not entirely convincing. If many amylolytic ruminal bacteria produce significant amounts of propionate, some fibrolytic ruminal bacteria produce large amounts of succinate, which can be converted to propionate (Hungate 1966).

The partition among the fermentative pathways in the rumen can be regulated by energy status (Nozière et al., 2010). When soluble energy sources are limiting in the rumen, it is likely that fermentation pathways that yield maximum ATP per unit of substrate will dominate, which favors acetate production (Russell and Wallace 1988). Since a low ruminal E_h may also result from poor availability of soluble energy sources (Huang et al., 2017a), the decrease of A:P ratio with increasing E_h was expected.

Since Dijkstra (1994) recognized the importance of maintaining a low E_h in the rumen through reduction and oxidation NAD for rumen VFA production, other studies also considered the effect of E_h on partition of fermentative pathways (Offner and Sauvant 2006). Indeed, most of reactions producing VFA in the rumen involve electron transfers (Table 14, Offner and Sauvant 2006; Wang et al., 2012). The conversion of carbohydrates to acetate by rumen microorganisms yields reducing equivalents, which may enhance the reducing power and

decrease ruminal E_h (Wang et al., 2012). On the other hand, the formation of propionate consumes reducing equivalents which may lead to increase of ruminal E_h.

This hypothesis is supported by the fact that ruminal methane production and A/P ratio are highly correlated (Russell 1998; Sauvant et al., 2011). Since methane is an electron sink products (CO_2 +8 H^+ + 8 e^- → CH_4 + 2 H_2O) that drives to a loss of reducing power (Pidello 2014), its production might be inhibited with a higher E_h (lower reducing power) and promoted with a lower E_h (higher reducing power). Thus, the negative relationship between ruminal E_h and A/P ratio observed in the present study was expected. Indeed, the competition between propionate production and methanogenesis for reducing equivalents is widely recognized (Van Nevel and Demeyer 1977; Russell 1998; Ungerfeld 2013).

Table 14. Typical reactions producing VFAs in the rumen (adapted from Offner and Sauvant 2006; Wang et al., 2012)

Glucose ↔ $2Pyruvate^- + 6H^+ + 4e^-$
$Pyruvate^- + 2H^+ + 2e^-$ ↔ $Lactate^-$
$Pyruvate^- + H_2O$ ↔ $Acetate^- + CO_2 + 2H^+ + 2e^-$
$Pyruvate^- + 4H^+ + 4e^-$ ↔ $Propionate^- + H_2O$
$Lactate^- + 2H^+ + 2e^-$ ↔ $Propionate^- + H_2O$
$2Pyruvate^- + H^+$ ↔ $Butyrate^- + 2CO_2$

Ruminal fermentation is generally considered to be unstable when A:P < 3.0 (Sauvant and Peyraud 2010; Sauvant et al., 2011) and this value corresponds approximately to a pH of 6.0. According to equation 5, an A:P ratio lower than 3 leads to an E_h > - 162 mV. This value is very close to that predicted (E_h > - 166 mV) by Huang et al. (2017a) for a pH below 6. Although the mechanism of the interaction between different ruminal parameters is not entirely clear, we can still confirmed that an E_h value around -165 mV could be considered as a threshold value for assessing rumen function. Thus, we can assume that ruminal fibrolytic activity would be highly compromised when the rumen E_h exceeds -165 mV.

Compared to acetate and propionate proportion, butyrate proportion is less discussed. Despite this, we can still hypothesize the influence of E_h on butyrate proportion in ruminal content. Since production of butyrate allows electron from lactate oxidation to be used (Nagaraja and Lechtenberg 2007), it could drive to an increase of ruminal E_h.

Ammonia is the main source of N for microbial protein synthesis and bacteria can grow with NH_3-N as sole N source. Ruminal NH_3-N concentration is inversely related to carbohydrates availability (Russell et al., 1983; Heldt et al., 1999). If energy is limiting in the rumen, microorganisms degrade feed protein to NH_3-N but NH_3-N uptake by ruminal microorganisms is inhibited (Nocek and Russell 1988). An improved microbial activity might be responsible for a greater incorporation of NH_3-N into microbial protein (Erasmus et al., 1992; Lascano and Heinrichs 2009). Since E_h increases with carbohydrates availability in the rumen (Huang et al., 2017a), the negative relationship between E_h and NH_3-N concentration was expected. Nonetheless, considering that bacterial growth is also a possible electron sink (Freguia et al. 2007), the bacterial growth could decrease both NH_3-N concentration and reducing power in the rumen, therefore the negative relationship between E_h and NH_3-N concentration could be explained.

By gathering together a dataset of ruminal E_h, VFA profile and NH_3-N measurements in dairy cattle, the relationship between ruminal E_h and other fermentation parameters was for the first time quantified. The electron transfers of reactions producing VFA in the rumen might cause the change of ruminal E_h, which confirms the interest of ruminal E_h measurements. To understand the mechanism of such effect, further investigation is required.

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Part II. Response of redox potential and fermentation parameters to live yeast supplementation in dairy cow: a quantitative analysis (Article 4)

In preparation for Livestock Science

Article 4

Response of redox potential and fermentation parameters to live yeast supplementation in dairy cow: a quantitative analysis

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Abstract

Subacute ruminal acidosis (SARA) is one of the major concerns of current ruminant production. Supplementation of live yeast (LY) in the diet is an interesting practice to limit the negative effects of SARA. The measurement of ruminal redox potential (E_h, mV) was raised to be a key tool for understanding the mode of action of LY. The present study aimed to investigate the influence of dietary characteristics on response of ruminal E_h following LY supplementation, and to analyse the relationship between response of ruminal E_h and response of total volatile fatty acids (VFA), molar proportions of individual VFA, and ammonia. Experimental results from 16 trials (including 49 treatments) on cannulated dairy cattle were gathered together for meta-analysis. A total of 575 pH and E_h kinetics describing the post-prandial evolution of ruminal pH and E_h were analyzed. Continuous ruminal pH and E_h data are summarized for each 9-h period for each kinetic and each cow by calculating: mean pH, amount of time (h/d) below pH 6, area (time × pH) below pH 6, and mean E_h, amount of time (h/d) above E_h -160, area (time × E_h) above E_h -160 mV. They were calculated to compare if any of these criteria can better reflect the effect of LY. Results suggest that high response of ruminal E_h following LY supplementation would be related to high daily intake of DM and of soluble sugars. The relationship between response of ruminal E_h and that of control group shows that the regulation of ruminal E_h by LY would be particularly effective when risk of digestive disorder is high. The relationship between response of rumen fermentation parameters and that of E_h further suggests the implication of electron transfer in production of volatile fatty acids (VFA), and the improvement of fermentation by LY might be explained by a better transfer and use of electrons. In this study, a novel approach to analyzing ruminal E_h is introduced.

Keywords: Live yeast, Redox potential, Dairy cow

1. Introduction

Feeding high-energy diet to high-producing dairy cows is a common practice in dairy farm which can lead to digestive disorder, such as acidosis. Subacute ruminal acidosis (SARA) is one of the major concerns of current ruminant production because it is poorly detected in herds and has many consequences, such as feed intake depression, reduced fiber digestibility, milk fat depression, diarrhea and laminitis (**Plaizier et al., 2008**). Ruminal acidosis is characterized by abnormal and intermittent drops in rumen pH. To define the occurrence of SARA, daily mean pH thresholds of 6.0 have been proposed (**Sauvant et al., 1999)** and the definitions

sometimes include the time spent under these thresholds which, from an experimental point of view, implies a continuous monitoring of the evolution kinetics of rumen acidity.

Supplementation of live yeast (LY) in the diet is an interesting practice to limit the negative effects of SARA. The effect of LY on SARA has been widely investigated (**Desnoyers et al., 2009**) and the studied parameters were mainly ruminal pH, volatile fatty acids (VFA), ammonia (NH_3-N) concentration and milk production performance.

Recently, the measurement of ruminal redox potential (E_h, in mV) has been considered as an interesting tool to reflect microbiological activity (**Marden et al., 2005; Julien et al., 2010; Huang et al., In press**) and to indicate digestive disorder in the rumen (**Huang et al., 2017**). It is negatively related to the ruminal pH and can provide additional information about fermentation in the rumen. In fact, E_h is a basic physicochemical measurement characterizing the reducing status of a milieu. Each bacteria has its favorable range of E_h (**Husson, 2013; Friedman et al., 2017**), and very negative E_h seem to be favorable to strict anaerobic bacteria such as fibrolytic and lactate utilizing bacteria (**Pinloche et al., 2013; Friedman et al., 2017**). The effect of LY on ruminal E_h has been reported (**Marden et al., 2008**) and was raised to be a key tool for understanding the mode of action of LY.

However, due to heterogeneity of ruminal E_h values reported in the literature, the effect of LY on E_h has been rarely discussed, and no quantitative analysis of the effect of LY on ruminal E_h has been reported. Indeed, the ruminal E_h measurement method is not standardized. The studies associated with different measurement methods reported considerable difference in ruminal E_h values (**Huang et al., in press**). The major difference is due to the different reference electrodes used. Also, an accurate ruminal E_h measurement requires strict anaerobic conditions which are not always satisfied (**Marden et al., 2005**).

During several years, numerous experiments conducted by our research team have investigated the effect of LY on ruminal E_h and pH (measured under strict anaerobic conditions) in dairy cows fed various diets. Since the interaction between diet characteristics and effect of LY on ruminal E_h has already been observed (**Julien 2010**), the first objective of the present study was to quantify the influence of diet characteristics on response of ruminal E_h to LY supplementation. Furthermore, the relationship between response of ruminal E_h and other rumen fermentation parameters was analyzed to provide a better understanding of mode of action of LY.

2. Materials and methods

2.1. Selection of studies

A database was constructed from 16 experiments (including 49 dietary treatments) on cannulated Holstein dairy cattle. As explained above, due to the heterogeneity of the ruminal E_h values reported in the literature (associated with time of measurement, anaerobic conditions and electrode used), we include in the database only the experiments conducted by our research group to ensure a consistency of measurement methods among the experiments. It includes either published (**Marden et al., 2008; Julien et al., 2015**) and unpublished studies (**Marden 2007; Julien 2010**). Both lactating (10 experiments) and non-lactating cows (6 experiments) were used. Cannulation techniques provided for humane treatment of cows, adhering to locally approved procedures, and were similar to those described by **Streeter et al. (1990)**. All animals were housed in individual tie stalls throughout the experiment with free access to water. Each experimental period consisted of an adaptation period (21 days) to the different dietary treatment following by a 3-d measurement period. The diets were formulated to meet energy and protein requirements, with two equal distributions at 0900 and 1700h. The LY used in these studies was *Saccharomyces cerevisiae* (Actisaf® Sc 47) provided by Phileo Animal Care (Marcq-en-Baroeul, France) at 10^{10} cfu/g DM. For LY supplemented cows, the recommended dose of 5g/cow/d was top-dressed on the total mixed ration (TMR) during the morning feeding. The composition of the diets was widely varied (**Table 15**).

Table 15. Description of diet characteristics in the data set.

Item[1]	Mean	SD	Minimum	Maximum
Intake, kg DM/cow /d	16.7	7.1	7.7	27.3
Proportion of concentrate, % DM	37.4	15.6	0.0	62.6
OM, g/kg DM	947.8	14.5	918.0	968.1
CP, g/kg DM	149.8	21.5	101.1	206.0
NDF, g/kg DM	366.4	80.5	267.8	566.3
NDFf, g/kg DM	304.1	103.0	178.5	566.3
Starch, g/kg DM	291.2	136.5	0.0	503.2
Degradable starch, g/kg DM	217.0	108.3	0.0	440.4
Soluble sugars, g/kg DM	55.9	28.9	0.0	105.4
CRDM, g/kg DM	257.0	128.3	0.0	438.7
DCAD, mEq/kg DM	170.6	90.9	59.1	438.0
EB, mEq/kg DM	279.8	118.3	133.8	638.0
Daily intake, g/cow				
Starch	4904	2961	0	9244
Degradable starch	3523	1985	0	6723
Soluble sugars	1056	764	0	2876
CRDM	4575	2970	0	9454

[1]DM = dry matter, OM = organic matter, CP = crude protein, NDF = neutral detergent fibre, NDFf = neutral detergent fibre from forages, CRDM = dietary content of rapidly degradable carbohydrates from the concentrate, DCAD = dietary cation-anion difference ($[Na^+ + K^+)] - [Cl^- - S^-]$); EB = electrolytic balance ($[Na^+ + K^+] - Cl^-$), SD = standard deviation.

The feed value of the diet was calculated according to the **INRA Tables (2007)**. Some of the diet characteristics such as neutral detergent fiber from forage (NDFf) and rumen degradable starch were calculated by the online software "systool.fr" (**Chapoutot et al., 2013**) using the equations published in **Sauvant and Nozière (2016)**. The dietary content of rapidly degradable carbohydrates from the concentrate (CRDM in g/kg DM) was estimated from the DM disappearance of concentrate after 4 h of *in sacco* incubation.

2.2. Measurement of ruminal E_h and pH

Detailed E_h and pH measurement was described by **Huang et al. (2017)**. All values were measured under strict anaerobic conditions. A total of 575 kinetics of ruminal E_h and pH measurements were gathered together. Each kinetic includes simultaneous measurements of ruminal pH and E_h at every hour from the morning feeding to 8 hours after. The measurement of ruminal E_h and pH on each animal under each diet treatment was repeated during 3 days. Continuous ruminal pH and E_h data are summarized for each 9-h period measurements for each

kinetic and each cow by calculating pH and E_h indicators: mean pH, amount of time (h/d) below pH 6, area (time × pH) below pH 6, mean E_h, amount of time (h/d) above E_h -160 mV, area (time × E_h) above E_h -160 mV. These E_h thresholds were selected because previous study (**Huang et al., 2017**) proposed using E_h around -165 mV as an indicator of rumen digestive disorder.

2.3. Statistical Analysis

The method used in meta-analysis of our data was that developed by **St-Pierre (2001)** and **Sauvant et al. (2008**). At each step of the meta-analysis process, graphical observations were made to check the coherence of relationships, and to identify obviously abnormal values. All analyses were performed using the statistical software R version 3.4.0 (R Core Team, 2017).

Firstly, the LY effect on ruminal E_h and pH indicators, total VFA concentration, molar proportion of individual VFA, A/P (Acetate/Propionate) ratio, and NH_3-N concentration was tested separately for dry cows and lactating cows. Secondly, the response to LY supplementation (difference between LY and control) of each parameter was calculated for each treatment (including dry cow data set and lactating cow data set) to investigate the interactions between factors. Previous study has highlighted that the effect of LY on ruminal E_h may be limited when the risk of SARA is low (**Julien 2010**). Therefore, the relationship between response of ruminal E_h (mean E_h) and that of control group was analyzed to investigate if the extent of response depends on the initial status of digestive disorder. Thereafter, the influence of diet characteristics on E_h response was analyzed. Finally, the response of mean E_h was used to explain the response of VFA and NH_3-N. The study effect was considered to be random. The statistical model used was:

$$Y_{ij} = B_0 + B_1X_{ij} + s_i + b_i X_{ij} + e_{ij},$$

where i = number of studies, j = number of treatments, $B_0 + B_1X_{ij}$ is the fixed effect part of the model and $s_i + b_i X_{ij} + e_{ij}$ is the random effect part of the model.

3. Results

3.1 Qualitative analysis: LY vs. control

In dry cows (**Table 16**), the effect of LY was significant on mean pH ($P = 0.006$), time ($P = 0.007$) and area ($P = 0.017$) of pH < 6.0, and on ammonia concentration ($P < 0.001$). Effect of

LY on area of E_h > -160 mV showed a tendency (P = 0.072). In lactating cows (**Table 17**), the effect of LY was significant on E_h and pH indicators, total VFA concentration, molar proportion of acetate and propionate (P < 0.001), but not on molar proportion of butyrate (P = 0.185).

Table 16. Effect of live yeast supplementation on rumen parameters in dry cows.

Item[1]	Control	LY	RMSE	*P*-values
E_h indicators				
Mean E_h, mV	- 197.3	- 195.9	11.7	0.762
Time E_h > -160 mV, h/d	0.80	0.90	1.43	0.915
Area E_h > -160 mV, mV × h/d	9.0	21.9	46.9	0.072
pH indicators				
Mean pH	6.34	6.40	0.17	0.006
Time pH < 6.0, h/d	1.64	0.90	1.70	0.007
Area pH < 6.0, pH × h/d	0.54	0.24	0.74	0.017
Total VFA, m*M*	74.4	74.7	4.65	0.924
Acetate, %	69.7	70.3	0.97	0.255
Propionate, %	15.2	15.3	0.92	0.683
Butyrate, %	11.1	10.5	1.41	0.465
A/P	4.60	4.62	0.25	0.267
NH_3-N, mg/L	153.2	141.9	10.9	< 0.001

[1]E_h = redox potential, VFA = volatile fatty acid, A/P = Acetate/Propionate ratio, LY = live yeast, RMSE = root mean square error.

Table 17. Effect of live yeast supplementation on rumen parameters in lactating cows.

Item[1]	Control	LY	RMSE	*P*-values
E_h indicators				
Mean E_h, mV	- 173.5	- 186.2	29.9	< 0.001
Time E_h > -160, h/d	3.40	2.19	2.581	< 0.001
Area E_h > -160, mV × h/d	101.6	47.2	105.4	< 0.001
pH indicators				
Mean pH	5.94	6.11	0.230	< 0.001
Time pH < 6.0, h/d	4.74	3.36	2.40	< 0.001
Area pH < 6.0, pH × h/d	1.79	0.92	1.20	< 0.001
VFA, m*M*	91.7	99.2	4.38	< 0.001
Acetate, %	59.6	59.4	2.17	0.002
Propionate, %	22.3	23.1	2.86	< 0.001
Butyrate, %	11.2	10.5	1.32	0.185
A/P	2.79	2.69	0.376	< 0.001
NH_3-N, mg/L	128.5	114.5	19.38	< 0.001

[1]E_h = redox potential, VFA = volatile fatty acid, A/P = Acetate/Propionate ratio, LY = live yeast, RMSE = root mean square error.

3.2. Response of rumen parameters to LY supplementation

The summary of response of rumen parameters to LY supplementation is given in **Table 18**. Mean E_h response averaged -8.45 mV and ranged from -54.5 to 17.1 mV; mean pH response averaged 0.13 and ranged from -0.12 to 0.43; total VFA response averaged 2.52 m*M* ranged from -9.40 to 18.3 m*M*, acetate proportion response averaged - 0.5 % and ranged from -7.2 to 2.6%; propionate proportion response averaged 0.9% and ranged from -3.7 to 9.0%; butyrate proportion response averaged -0.3% and ranged from -2.2 to 3.6%; A/P ratio response averaged -0.12 and ranged from -1.12 to 0.58; NH_3-N concentration response averaged -12.5 mg/L and ranged from -78.0 to 24.7 mg/L.

Table 18. Summary of response of rumen parameters to live yeast supplementation in dry and lactating cows.

Item[1]	Mean	SD	Minimum	Maximum
Mean E_h response, mV	- 8.45	17.5	-54.5	17.1
Mean pH response	0.13	0.12	-0.12	0.43
Total VFA response, m*M*	2.52	8.43	-9.40	18.3
Acetate response, %	- 0.5	2.6	-7.2	2.6
Propionate response, %	0.9	3.1	-3.7	9.0
Butyrate response, %	- 0.3	1.2	-2.2	3.6
Acetate/propionate response	- 0.12	0.45	-1.12	0.58
NH_3-N response, mg/L	- 12.5	27.1	-78.0	24.7

[1]E_h = redox potential, VFA = volatile fatty acid.

3.3. Relationship between response of ruminal E_h and that of control group

The relationship between response of mean ruminal E_h, time and area of E_h > -160 mV, and that of control group is given in **Table 19**. All these responses were significantly correlated with those of control group. According to the equation, the mean E_h response became negative only when that of control group was higher than -189.5 mV; the decrease of mean E_h could reach 20 mV when the mean E_h of control group was equal to -149.3 mV.

Table 19. Relationship between response of ruminal E_h and that of control group.

Equations[1]	n_{exp}	n_{treat}	RMSE	*P*-values
Mean E_h Response = - 94.19 - 0.497 mean E_h control	16	27	7.67	0.003
Time E_h > -160 response = 1.248 - 0.723 time E_h > -160 control	16	27	0.76	0.0003
Area E_h > -160 response = 34.12- 0.8409 area E_h > -160 control	16	27	21.8	0.0001

[1]E_h = redox potential, n_{exp} = number of experiments, n_{treat} = number of treatments, RMSE = root mean square error.

3.4. Influence of dietary characteristic on ruminal E_h response

The influence of concentrate proportion of diet (*P* = 0.850), OM (*P* = 0.269), NDF (*P* = 0.891), NDFf (*P* = 0.735), starch (*P* = 0.574), rumen degradable starch (*P* = 0.735), soluble sugars (*P* = 0.183), CRDM (*P* = 0.596), EB (*P* = 0.806) and DCAD (*P* = 0.691) contents of diet on E_h response was not significant. The CP content (g/kg DM) of the diet significantly influenced the E_h response (Y = 55.1 – 0.438 X, *P* = 0.015, RSD = 10.9) which becomes negative when the CP content exceed 127 g/kg DM. The DMI (kg DM/cow/d) showed a strong tendency to influence the E_h response (Y = 12.9 - 1.39 X, *P* = 0.054, RSD = 9.3) and the E_h response

becomes negative only when the DMI exceed 9.3 kg/cow/d. The daily intake (g/d) of starch (P = 0.363), rumen degradable starch (P = 0.814), and CRDM (P = 0.579) did not influence the E_h response. The daily intake (g/d) of soluble sugars significantly influenced the E_h response (Y = 5.67- 0.014 X, P = 0.034, RSD = 9.5). According to the equation, the E_h response becomes negative as soon as the daily intake of soluble sugars exceed 405 g, and the decrease of E_h following LY supplementation could achieve 30 mV once the daily intake of soluble sugars reaches 2548 g.

3.5. Relationship between response of rumen fermentation parameters and that of E_h

All ruminal VFA responses were significantly correlated to mean E_h response (Table 20). The decrease of mean E_h following LY treatment was associated to an increase of total VFA concentration ($P < 0.01$) and molar proportion of propionate ($P < 0.001$). It was associated to a decrease of molar proportion of acetate ($P < 0.001$), molar proportion of butyrate ($P < 0.05$) and A/P ratio ($P < 0.001$). The ammonia concentration response (P = 0.323) was not influenced by the E_h response.

Table 20. Relationship between responses of rumen fermentation parameters and mean Eh response.

Equations[1]	n_{exp}	n_{treat}	P-values	RMSE
total VFA $_{response}$ = -0.897- 0.330 mean $E_{h\ response}$	11	20	0.002	4.65
Acetate $_{response,\ \%}$ = 0.846 + 0. 109 mean $E_{h\ response}$	11	20	0.0007	1.39
Propionate $_{response}$ % = -0.911- 0.148 mean $E_{h\ response}$	11	20	0.0001	1.62
Butyrate $_{response,\ \%}$ = 0.176 + 0.035 mean $E_{h\ response}$	11	20	0.035	0.90
A/P $_{response}$ = 0.132 + 0.020 $E_{h\ response}$	11	20	0.0003	0.268

[1]VFA = volatile fatty acid, E_h = redox potential, A/P = Acetate/Propionate ratio, n_{exp} = number of experiments, n_{treat} = number of treatments, RMSE = root mean square error.

4. Discussion

4.1. Qualitative analysis: LY vs. Control

The bibliography relating to the study of the effect of LY *Saccharomyces cerevisiae* as a feed additive in ruminants is wide and varied, and the numerous research works (**Martin and Nisbet 1992; Newbold et al., 1996; Fonty and Chaucheyras-Durand 2006; Moallem et al.,**

2009; Bitencourt et al., 2011; Ferraretto et al., 2012; Dehghan-Banadaky et al., 2013; Bayat et al., 2015; Tristant and Moran 2015; Chaucheyras-Durand et al., 2016; Ambriz-Vilchis et al., 2017) conducted in this area reveal variable and non-systematic responses. In dairy ruminants in particular, it seems that the response of animals varies according to their physiological stage, the dose used, and the diet fed. The effect of LY on ruminal pH is central to the action of this additive in improving ruminant productivity (**Wallace and Newbold 1992**). Indeed, the main effect of LY is to limit the post-prandial drop in ruminal pH below the threshold value of 6, thus preventing the installation of ruminal acidosis in dairy cows fed on acidogenic diets (**Marden et al., 2008; Desnoyers et al., 2009**).

In our study, addition of LY in diets for lactating cows significantly increased the mean pH (+0.17 unit-pH) and total VFA concentration (+8.2%) and decreased the mean E_h (from -173.5 to -186.2 mV). Similar responses have been reported by others. The decrease of ruminal E_h in lactating cows observed by **Marden et al. (2008**) is confirmed in the present study. The increase in ruminal pH and VFA concentration observed in lactating cows is in agreement with **Robinson (2002)** who demonstrated an increase in pH (+0.1 unit-pH) and rumen VFA (+5.4%). **Lescoat et al. (2000)** did not observe any effect of LY on ruminal pH, but observed a similar increase in VFA concentration (+5.2%). A recent meta-analysis (**Desnoyers et al., 2009**) based on a large data base (157 experiments) also reported an increase in pH (+ 0.03 unit-pH) and an increase in VFA concentration (+5.2%) related to LY supplementation and dosage. Others reported no effect of LY on ruminal fermentation parameters: **Bitencourt et al.** (**2011**) and **Bayat et al.** (**2015**) with 1g/cow/d at 10^{10} cfu/g, and **Ferraretto et al.** (**2012**) and **Dehghan-Banadaky et al.** (**2013**) with 4g/cow/d at 15×10^{9} cfu/g. Ruminal sampling method (collected by rumenocentesis or stomach tube at a single time vs in rumen-cannulated animals or in animals equipped with indwelling probes taking into account rumen pH variations throughout the day) but also physiological stage, yeast strain and composition of diets could explain the difference in response to LY supplementation. Our results also demonstrated the limited effect of LY in dry cows due to low risk of SARA: low intake (on average 8.3 kg DM) and high proportion of NDF in the diet (on average 403g/kg DM). This corroborates the results of **Julien et al.** (**2011**) who reported that LY have no major effect on the fermentation parameters in cows with a low level of intake, fed with a diet leading to strongly reducing ruminal conditions (E_h close to -200 mV) and a pH close to 6.50. Consequently, these different results raise the question of a threshold of efficacy of LY: when the ruminal E_h is initially low, LY are ineffective to strengthen reducing conditions of ruminal environment.

The decrease in NH_3-N concentration following LY supplementation was significant both in dry and lactating cows. The recorded decrease can be explained by a less intense degradation of proteins and / or dietary peptides in the rumen. It does not exclude a greater incorporation of NH_3-N into microbial protein (**Erasmus et al., 1992; Lascano and Heinrichs, 2009**). Indeed, NH_3-N is the main source of N for microbial protein synthesis and bacteria can grow with NH_3-N as sole N source. Ruminal NH_3-N concentration is inversely related to carbohydrate availability (**Heldt et al., 1999**). If energy is limiting in the rumen, microorganisms degrade feed protein into NH_3-N, and NH_3-N uptake by ruminal microorganisms is inhibited (**Nocek and Russell, 1988**). According to **Bach et al. (2005)**, cellulolytic bacteria primarily utilize NH_3-N while amylolytic preferentially utilize amino acids.

The calculation of threshold-related variables (time and area of E_h and pH, lower or higher than thresholds) seems to be useful to highlight the effect of LY. A meta-analysis (**Dragomir et al., 2008**) based on 48 studies (including 219 pH curves) resulted significant correlations between threshold-related variables and mean pH. These authors further suggested that threshold-related variables might carry supplementary information to explain the variation in ruminal pH induced by within-study factors. The present study proposed for the first time the calculation of threshold-related variables of ruminal E_h and demonstrated their usefulness.

4.2. Relationship between response of ruminal E_h and that of control group

The close relationship between response of ruminal E_h and that of control group suggests that the regulation of ruminal E_h by LY would be particularly effective when risk of digestive disorder is high. Such tendency is in accordance with that has already been observed on ruminal pH. **Meschy et al. (2004)** conducted a meta-analysis on 40 studies in dairy cows and demonstrated greater response of ruminal pH to buffers addition when that of control group was low ($pH_{response} = 1.43 - 0.21\ pH_{control}$). **Lettat et al. (2012)** are in line with this hypothesis, their quantitative analysis of bacterial probiotics effect also associated greater response of ruminal pH to the high risk (low pH) of control group. Morevoer, in the present study, the relationship between response of area of $E_h > -160$ mV and that of control group was characterized by a lower *P*-value (< 0.001) and a higher slope (- 0.841), which permitted to better highlight the effect of LY. These results confirmed again the usefulness to calculate the area of $E_h > -160$ mV.

4.3. Influence of dietary characteristics on ruminal E_h response

The influence of DMI on ruminal E_h response confirmed again the limited effect of LY in dry cows might be due to low risks of SARA (**Julien 2010**). Previous study reported a strong influence of rapidly degradable matter (OM, starch, soluble sugars) content of the diet on ruminal E_h (**Huang et al., 2017**). In the present study, these diet characteristics poorly influenced the ruminal E_h response. A possible explanation may be the interaction between diet composition and the daily intake of the diet. By taking into account the daily intake of soluble sugars, we successfully demonstrated its influence on E_h response following LY supplementation. Indeed, soluble sugars ferment faster than starch, high concentration of sugars presents a potential risk for lactic acidosis (**Nagaraja and Titgemeyer 2007; Lean et al., 2014**), while LY are able to outcompete lactate-producing bacteria for the utilization of sugars, and at the same time stimulates lactate fermentation by *Megasphaera elsdenii* (**Chaucheyras et al., 1996**). This is confirmed by **Lascano et al. (2015)** who observed a greater drop of ruminal lactate concentration in LY-supplemented dairy heifers fed high-sugar diet compare to dairy heifers fed high-starch diet. Therefore, greater E_h response at high amount of soluble sugars in our study might be related to these stimulatory activities of LY.

4.4. Relationship between response of rumen fermentation parameters and that of E_h

The increase in total VFA concentration could be associated to a decrease of ruminal E_h due to LY supplementation. Indeed, **Dijkstra (1994)** firstly recognized the need to maintain a low E_h in the rumen through reduction and oxidation of pyridine nucleotides (NAD) as the driving force for rumen VFA production. The conversion of carbohydrate to acetate by rumen microorganisms yields reducing equivalents (**Wang et al., 2012**), whereas the formation of propionate consumes reducing equivalents. Therefore, the decrease of ruminal E_h (increase of reducing power) following LY supplementation might promoted the production of propionate and inhibited that of acetate. In addition, the increase of propionate proportion could also be resulted from transformation of lactate into propionate promoted by a decrease of E_h (enhance of reducing power) created by LY (**Pinloche et al., 2013**). Indeed, the lactate utilizing bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium* are strictly anaerobic, they can ferment lactic acid to propionic acid via the acrylate pathway (**Nisbet and Martin, 1991; Nagaraja and Titgemeyer 2007; Wang et al., 2012**). The associated decrease of E_h and increase of propionate proportion is then explained. The lactate concentration was not available

in present study but the enhanced conversion of lactate to propionate by LY has already been observed in previous study (**Marden et al., 2008**).

5. Conclusions

The present study suggests that high response of ruminal E_h would be related to high daily intake of DM and of soluble sugars. The relationship between response of ruminal E_h and that of control group suggests that the regulation of ruminal E_h by LY would be particularly effective when risk of digestive disorder is high. These results could be used to define the optimal conditions for LY utilization in dairy cattle. The relationship between response of rumen fermentation parameters and that of E_h further confirmed the implication of electron transfer in production of VFA, and the improvement of fermentation by LY might be explained by a better transfer and use of electrons.

Conflict of interest statement

Authors declare no conflict of interest.

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Part III. Soluble sugar content of the diet and live yeast supplementation in dairy cattle

Chapiter 1 Effect of live yeast supplementation in early-lactating cows fed diets differing in content of soluble sugars (Article 5)

In preparation for Animal

Article 5

Effect of live yeast supplementation in early-lactating cows fed diets differing in content of soluble sugars

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Live yeasts (LY) have been extensively used in dairy cows for normalizing rumen fermentations when animals are fed on acidogenic diets. The positive effect of LY on ruminal redox potential (E_h) has been reported and was raised to be a key tool for understanding their mode of action. A previous quantitative analysis showed a strong effect of LY on E_h for high daily intake of soluble sugars. The aim of this study was to validate the effect of LY supplementation on ruminal E_h in early-lactating cows fed high amount of soluble sugars, and try to understand the mode of action of LY in such conditions. Eight multiparous lactating Holstein cows fitted with ruminal cannulas were used to investigate the effect of LY supplementation in early-lactating cows fed high (HS) or low (LS) soluble sugars diet. Live yeast supplementation decreased ruminal E_h (-241.6 vs. -265.0 mV, P = 0.005) and increased pH (6.02 vs. 6.15, P < 0.001). The response of ruminal E_h was greater (P = 0.048) when cows were fed HS diet than LS diet (-34.3 vs. -12.6 mV). These results confirmed greater efficiency of LY supplementation on E_h when cows were fed high amount of soluble sugars as predicted by previous meta-analysis. Moreover, LY supplementation tended (P = 0.097) to decrease milk urea content (199 vs 217 mg/kg) and increased (P < 0.001) urine pH (6.98 vs. 7.40) 4 h post-feeding. Live yeast supplementation also decreased the amplitude of ruminal temperature fluctuation (3.4 vs 2.6 °C, P < 0.001) and increased the minimum temperature (36.6 vs. 37.3°C, P < 0.001).

Keywords: live yeast, redox potential, soluble sugar, rumen, dairy cow

Introduction

Ruminal acidosis is one of the major concerns of current dairy farms because it is poorly detected in herds and has many consequences, such as feed intake depression, reduced fiber digestibility, milk fat depression, diarrhea and laminitis (Plaizier et al., 2008). It is caused by feeding high-readily fermentable carbohydrates (RFC) diets such as starch and sugar to meet energy requirements of high-producing dairy cows. However, this feeding practice can contribute to create fermentation disorders, such as ruminal acidosis characterized by more or less extended periods of pH depression (Plaizier et al., 2008).

Sub-acute ruminal acidosis (SARA) is the most important nutritional disease in dairy cattle (Enemark 2009) and improvement of rumen pH can be achieved through the use of probiotics. In this area, live yeasts (LY) have been extensively studied and used in dairy cows for normalizing rumen fermentation (Bach et al., 2007; Thrune et al., 2007; Marden et al., 2008; Desnoyers et al., 2009). Their main positive effects include an increase in rumen pH and a reduction in lactic acid, especially in cases of higher proportion of concentrate in the diet and to higher intake levels (Desnoyers et al., 2009). Until now, pH has been one of the most commonly used descriptors to define acidotic conditions. However pH is a measure that merely reflects one aspect of the rumen environment.

Recently, measurement of ruminal redox potential (E_h, in mV) has been considered as an interesting tool for identifying ruminal fermentation disorders, such as SARA (Marden et al., 2005; Marden et al., 2008; Julien et al., 2010). It is negatively related to ruminal pH and can provide additional information about fermentation in the rumen (Huang et al., 2017a). In fact, E_h is a basic physicochemical measurement characterizing the reducing status of a milieu. Each bacteria has its favorable range of E_h (Husson, 2013; Friedman et al., 2017), and the negative E_h seems to be favorable to strict anaerobic bacteria such as fibrolytic and lactate utilizing bacteria (Pinloche et al., 2013; Friedman et al., 2017). The positive effect of LY on ruminal E_h has been reported (Marden et al., 2008), and was raised to be a key tool for understanding the mode of action of this additive. However, effect of LY remains variable according to the experimental conditions: strain and dose of LY, physiological stage of the animal, and also diet characteristics. Previous quantitative analysis also observed stronger effect of LY on E_h with high amount of daily intake of

soluble sugars (Huang et al., Artcicle 4). In the present study, we aimed to validate this hypothesis by studying the effect of LY supplementation on ruminal E_h in early-lactating cows fed low and high amount of soluble sugars, and try to understand the mode of action of LY in such conditions.

Materials and methods

The experiment was conducted at the research station of Phileo Lesaffre Animal Care (The Farm, Chemin de Vallesvilles, Seysses, F-31600) from October 2016 to February 2017. Animal procedures were conducted in strict accordance with the European Union recommendations on the protection of animals used for scientific purposes (Directive 2010/63/EU). The protocol was approved by the french Ethical Committee for Animal Experimentation, Animal Sciences and Health N°115.

Cows, experimental design, and treatments

Eight multiparous early-lactating Holstein cows fitted with ruminal cannulas were used in a 4 by 4 crossover design. They were assigned into two groups (LY supplemented group vs. control group). Each group was matched for days in milk (DIM), milk production, and BW during a 7-d pretrial adjustment to stalls. At the start of the experiment, cows averaged 72 ± 33 DIM with an average BW of 619 ± 91 kg and milk yield of 31 ± 8 kg/d. They were fed a high soluble sugars diet (HS) or a low soluble sugars diet (LS), supplemented or not with LY. During the first experimental period, the control group fed the HS diet with no supplemental LY added, whereas the LY group were fed the LY-supplemented HS diet. Treatments were switched for the second period. The same design was reproduced with the LS diet during the third and fourth periods.Each experimental period consisted of 28 days in duration with a 21 d adaptation period to the treatment followed by 7 days for sampling and measurements. The difference between high soluble sugars (8.6%, HS) and low soluble sugars (2.1%, LS) content of the diets was achieved by adding cane molasses in the HS diet. For LY (Actisaf® Sc 47, Phileo Animal Care, Marcq-en-Baroeul, France) supplemented cows, the recommended dose of 5 g (10^{10} cfu/g DM) per cow and per day with 100g of maize as an extender was top-dressed on the total mixed ration (TMR) during the morning feeding. Each diet was provided *ad libitum* (5% orts, on an as-fed basis) and cows were housed

in individual tie-stalls with free access to water throughout the experiment. The ingredients and chemical composition of the two experimental diets are shown in Table 21.

Table 21. Ingredient and chemical composition of experimental diets

	Diet[1]	
Item	HS	LS
Ingredients (% DM)		
Soybean meal	14	15
Alfalfa hay	5	5
Corn silage	48	56
Cane molasses	9	-
Ground wheat	7	7
Corn grain, rolled	16	17
Mineral-vitamin mix[2]	1	1
Chemical composition (% of DM)[3]		
OM	94.3	95.1
CP	15.2	15.7
NDF	28.7	30.2
ADF	14.6	14.5
Starch	31.1	37.1
Soluble sugars	8.6	2.1

[1]HS = high soluble sugars; LS = low soluble sugars.

[2]Contained (per kg, DM basis): 70 g of P, 230 g of Ca, 50 g of Mg, 4.5 g of Zn, 4 g of Mn, 25 mg of Co, 25 mg of Se, 1.5 g of Cu, 320,000 IU of vitamin A, 100,000 IU of vitamin D3, 900 IU of vitamin E.

[3]Calculation according to chemical composition of each ingredient collected in each experimental period.

Measurement and sample collection

Diets were offered in equal amounts twice daily (0900 and 1800 h). For each cow, amounts of diet offered and refused were weighed every morning before the morning feeding, to determine daily intake. Samples of TMR and of individual orts were collected daily during each 7-d sampling and measurement period, dried at 60°C for 48 h in a forced-air oven to determine DM content, ground through a 1-mm screen using a hammer mill and stored until further analysis in 100-ml plastic bottles. Individual water consumption was also recorded daily throughout the experiment. Cows were milked twice daily at 0700 and 1700 h in their stalls and individual milk production was recorded electronically at each milking throughout the experiment. On d 24 and d 25, milk samples

were collected from individual cow at each milking, treated by preservative and stored at 4°C until analyzed for milk fat, true protein, lactose, and urea by infrared analysis (CIALSO, Auch, F-32000). Milk composition was corrected for differences in milk yield between a.m. and p.m. milking.

For each cow, ruminal pH and E_h were recorded on 3 successive days, from d 23 to d 26. At d 22 (5 h after the morning feeding) a wireless real-time data logger (Dascor, Escondido, CA, USA) was submersed into the rumen via the ruminal cannula after calibration, and the E_h and pH were measured by external sensors of the data logger and stored in the memory chip (Huang et al., 2017b). At d 26 (1 h before the morning meal), the probe was removed and after extraction of the data, cleaned successively with distilled water and an enzymatic solution. Redox potential measures the ability of a solution to accept or donate electrons and corresponds to the potential difference (mV) between a platinum electrode and a standard hydrogen electrode. Since an Ag-AgCl reference electrode was used, all records of the potential difference were corrected (+199 mV at 39°C, Nordstrom, 1977).

Sample analysis

Samples of TMR and orts were dried at 60°C for 48 h in a forced-air oven to determine DM content, ground through a 1-mm screen using a hammer mill and stored until further analysis in 100-ml plastic bottles. Urine samples were collected from individual cow on d 22 and d 25, at 9 a.m. and 1 p.m. Urine pH was measured immediately by mobile pH meter. Samples of ruminal fluid (20 ml) were collected from individual cow on d 24 and d 25, at 0 h (just before morning feeding) and, at 1, 2 and 4 h after morning feeding. Each sample was preserved by addition of 1 ml of H_2SO_4 (50%) and were frozen at - 20°C for subsequent determination of VFA and ammonia (NH_3-N) concentrations. Analysis of VFA was performed using a gas chromatographic method of Playne (1985), modified by Marden et al. (2008). Ammonia concentration was analyzed by colorimetry with Nessler's reagent using the method adapted by Hach et al. (1985; 1987) on the separated liquid phase of ruminal samples centrifuged at 4,000 × g for 20 min. Lactate concentration (D-lactate and L-lactate) was determined using a commercial kit (cat. no. 11 112 821 035, Boehringer Mannheim/R-Biopharm, St. Didier au Mont d'Or, France).

Statistical analysis

All analyses were performed using the statistical software R version 3.4.0 (R Core Team, 2017). The statistical model included treatment and period as fixed effects, and cow as random effects. Rumen parameters (E_h, pH, VFA, NH_3-N, temperature) were analyzed by ANOVA for repeated measures using the same model, that included additional fixed effects of day, time and all interactions. Differences between treatment effects were assessed by pairwise comparisons (Tukey's test). Treatment effects were declared significant at $P < 0.05$, and a trend was assumed for probabilities < 0.1 and > 0.05.

Results

DM and water intake

The results about DM and water intake are presented in Table 22. The effect of LY supplementation was not significant on DM ($P = 0.501$) and water intake ($P = 0.160$). However, LY-supplementation numerically decreased water intake (79.0 vs. 76.6 L/d). Relative to the HS diet, LS diet increased DM (19.7 vs. 23.6 kg/d, $P < 0.001$) and water intake (75.2 vs. 80.3 L/d, $P = 0.003$).

Table 22. Effect of live yeast supplementation and soluble sugars level on DMI and water intake

	Treatment[1]					*P*-value[2]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
DMI (kg/d)	19.6	19.8	24.1	23.1	0.4	0.501	< 0.001	0.259
Water intake (L/d)	75.5	74.9	82.5	78.2	1.2	0.160	0.003	0.282

[1]HS = high soluble sugars; LS = low soluble sugars; LY = live yeast.
[2]LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction.

Ruminal Eh, pH and temperature

Circadian E_h changes (Figure 13) showed systematically lower E_h values with the LY-supplemented HS diet than with the HS control diet. The difference was smaller between the LY-supplemented LS diet than with the LS control diet.

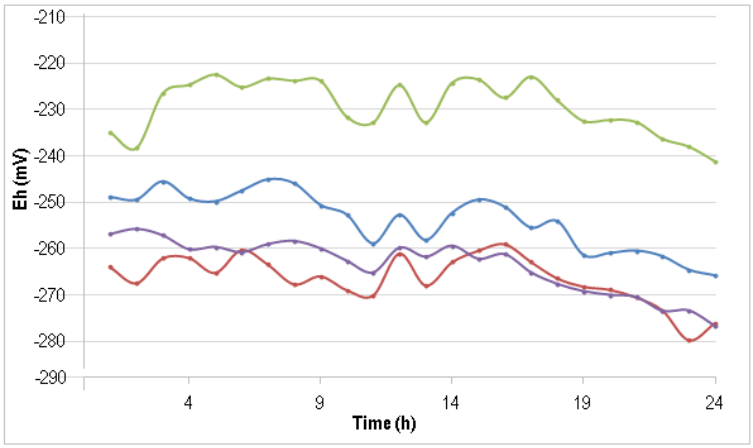

Figure 13. Circadian redox potential (Eh) changes of cows fed LS diet, LY-supplemented LS diet HS diet and LY-supplemented HS diet.

(LS diet –●– , LY-supplemented LS diet –●– , HS diet –●– , LY-supplemented HS diet –●–)

At the same time, circadian pH changes (Figure 14) showed systematically lower pH values with the HS diet than with the LS diet, and systematically higher pH values with the LY-supplemented HS diet than with the HS control diet.

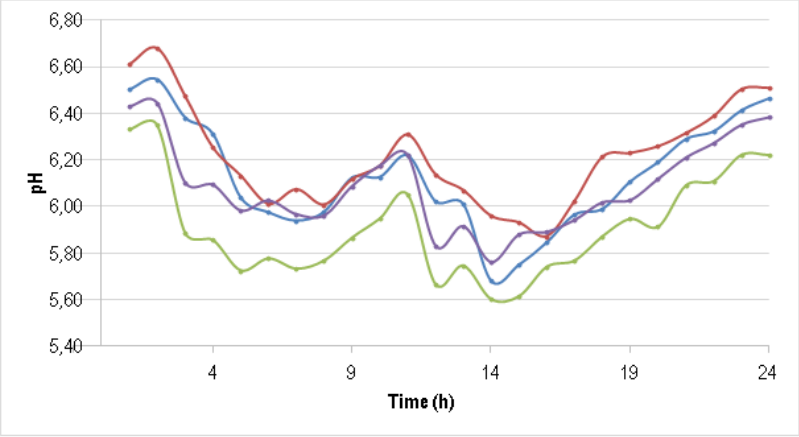

Figure 14. Circadian pH changes of cows fed LS diet , LY-supplemented LS diet, HS diet , LY-supplemented HS diet.

(LS diet –●– , LY-supplemented LS diet –●– , HS diet –●– , LY-supplemented HS diet –●–)

The results about the effect of LY supplementation and soluble sugars level on ruminal E_h, pH and temperature were presented in Table 23.

Table 23. Effect of live yeast supplementation and soluble sugars level on ruminal redox potential, pH and temperature.

	Treatment[1]					*P*-value[2]		
Item[3]	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
E_h (mV) 24h	-229.3	-263.6	-253.9	-266.4	5.0	0.005	0.097	0.186
pH 24h	5.91	6.09	6.13	6.22	0.02	< 0.001	< 0.001	0.114
T (°C) 24h	39.1	39.1	39.1	39.1	0.03	0.605	0.667	0.782
T min (°C)	36.9	37.3	36.3	37.3	0.13	< 0.001	0.171	0.126
T max (°C)	39.9	39.8	40.1	39.9	0.03	0.076	0.003	0.296
T amp (°C)	3.0	2.5	3.8	2.6	0.14	< 0.001	0.049	0.102

[1]HS = high soluble sugars; LS = low soluble sugars; LY = live yeast.

[2]LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction.

[3]E_h = redox potential; T = temperature; T min = minimum temperature; T max = maximum temperature; T amp = amplitude of temperature fluctuation.

Live yeast supplementation decreased mean E_h (-241.6 vs. -265.0 mV, P = 0.005), and amplitude of ruminal temperature fluctuation (3.4 vs 2.6 °C, P < 0.001), and increased mean pH (6.02 vs. 6.15, P < 0.001) and minimum temperature (36.6 vs. 37.3°C, P < 0.001) (Table 3). Compared with LS diet, HS diet decreased mean pH (6.18 vs 6.00, P < 0.001), maximum temperature (40.0 vs 39.8 °C, P = 0.003) and amplitude of temperature fluctuation (3.2 vs 2.7, P < 0.05), and tended to increase mean E_h (-260.2 vs -246.5mV, P = 0.097). The response of ruminal E_h was greater when cows were fed HS diet than LS diet (-34.3 vs. -12.6 mV, P = 0.048). The response of ruminal pH was also greater when cows were fed HS diet than LS diet (0.18 vs. 0.09, P = 0.046).

The results about the effect of LY supplementation and soluble sugars level on ruminal E_h,urine pH are presented in Table 24.

Table 24. Effect of live yeast supplementation and soluble sugars level on urine pH.

	Treatment[1]					*P*-value[2]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Before feeding	7.43	7.70	7.95	7.92	0.06	0.285	0.001	0.175
4 h after feeding	6.56	7.18	7.40	7.63	0.08	< 0.001	< 0.001	0.101

[1]HS = high soluble sugars; LS = low soluble sugars; LY = live yeast.

[2]LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction.

Live yeast supplementation did not influence urine pH before morning feeding (at 9 a.m.) but increased significantly the urine pH (6.98 vs. 7.40, $P < 0.001$) 4 h after. Cows fed HS diet showed lower urine pH both before morning feeding (7.57 vs. 7.94, $P < 0.001$) and 4 h after (6.86 vs. 7.51, $P < 0.001$).

Ruminal fermentation end products

Live yeast supplementation did not influence most of the fermentation end-products excepted A:P ratio which was increased (2.52 vs. 2.61, $P = 0.015$) (Table 25). High sugars content in the diet increased total VFA concentration (119.5 vs 140.7 m*M*, $P < 0.001$), acetate (70.1 vs. 83.8 m*M*, $P < 0.001$), propionate (29.9 vs. 32.9 m*M*, $P = 0.003$), butyrate (13.6 vs. 18.4 m*M*, $P = 0.001$), valerate (2.27 vs. 2.48 m*M*, $P = 0.018$), A:P ratio (2.47 vs., 2.67 m*M*, $P = 0.001$), D-lactate (0.87 vs. 2.62 m*M* $P < 0.001$), L-lactate (0.88 vs. 1.76, $P = 0.002$) and total lactate (1.75 vs. 4.38m*M*, $P < 0.001$) concentration in the rumen, and decreased the isobutyrate (1.17 vs 1.02 m*M*, $P < 0.001$), isovalerate (2.45 vs 2.09 m*M*, $P < 0.001$) and NH_3-N concentration (144.6 vs 128.9 mg/L, $P < 0.001$).

Table 25. Effect of live yeast supplementation and soluble sugars level on ruminal fermentation characteristics.

	Treatment[1]					*P*-value[2]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Total VFA (m*M*)	142.2	139.0	116.7	122.3	1.82	0.794	< 0.001	0.115
Acetate (m*M*)	84.46	83.12	68.03	72.18	1.09	0.420	< 0.001	0.125
Propionate (m*M*)	33.21	32.59	29.79	29.97	0.62	0.596	0.003	0.481
Butyrate (m*M*)	18.97[a]	17.75[a]	13.10[b]	14.07[b]	0.35	0.964	0.001	0.039
Valerate (m*M*)	2.50	2.45	2.31	2.24	0.06	0.229	0.018	0.720
Isobutyrate (m*M*)	1.01	1.04	1.13	1.22	0.02	0.166	< 0.001	0.112
Isovalerate (m*M*)	2.10[a]	2.09[a]	2.32[ab]	2.59[b]	0.04	0.183	< 0.001	0.019
A:P ratio	2.65	2.69	2.38	2.56	0.04	0.015	0.001	0.332
D-lactate (m*M*)	2.72	2.50	0.83	0.90	0.25	0.937	< 0.001	0.762
L-lactate (m*M*)	1.81	1.71	0.99	0.77	0.16	0.659	0.002	0.662
Total lactate (m*M*)	4.53	4.21	1.82	1.67	0.40	0.827	< 0.001	0.989
NH_3-N (mg/L)	131.7	125.9	144.0	145.2	2.8	0.431	< 0.001	0.270

[1]HS = high soluble sugars; LS = low soluble sugars; LY = live yeast.

[2]LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction.

[a,b]Mean values within the same row with different superscripts differ ($P < 0.05$).

Milk production and milk composition

Milk production and milk composition was not significantly affected by LY supplementation, although LY-supplemented cows produced numerically more milk (28.0 vs 27.6 kg), FCM (30.0 vs 28.8 kg), fat (44.6 vs 42.8 g/kg), protein (38.3 vs 35.6 g/kg) and lactose (48.3 vs. 47.5 g/kg) content, and more fat (1.25 vs 1.19 kg/d), protein (1.02 vs 0.98 kg/d) and lactose (1.35 vs 1.32 kg/d) yield (Table 26). Live yeast supplementation tended to decrease milk urea content (199 vs 217 mg/kg, *P* = 0.097) and milk urea yield (5.63 vs 5.93 g/d, *P* = 0.089).

Table 26. Effect of live yeast supplementation and soluble sugars level on milk production and milk composition

	Treatment[1]					*P*-value[2]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Production (kg/d)								
Milk yield	25.1	26.9	30.1	29.1	0.72	0.881	<0.001	0.050
FCM[3]	27.3	29.5	30.3	30.5	0.89	0.637	0.061	0.395
Component								
Fat (g/kg)	45.3	46.7	40.3	42.6	0.86	0.455	0.001	0.350
Protein (g/kg)	35.6	36.2	35.6	36.5	0.32	0.184	0.386	0.363
Lactose (g/kg)	45.8[a]	47.4[ab]	49.2[b]	49.2[b]	0.30	0.121	<0.001	0.044
Urea (mg/kg)	170.4	140.8	263.4	256.9	10.9	0.097	<0.001	0.130
Yield								
Fat (kg/d)	1.15	1.25	1.22	1.26	0.04	0.564	0.398	0.716
Protein (kg/d)	0.89	0.97	1.08	1.07	0.03	0.549	<0.001	0.110
Lactose (kg/d)	1.16	1.28	1.48	1.43	0.04	0.773	<0.001	0.054
Urea (g/d)	3.95	3.83	7.91	7.43	0.32	0.089	<0.001	0.505

[1]HS = high soluble sugars; LS = low soluble sugars; LY = live yeast.

[2]LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction.

[3]FCM = 0.4 × milk yield (kg/d) + 15 × fat yield (kg/d).

[a,b]Mean values within the same row with different superscripts differ (*P* < 0.05).

Compared with LS diet, HS diet significantly decreased milk yield (26.0 vs 29.6 kg/d, *P* < 0.001), lactose (46.5 vs. 49.2 g/kg, *P* < 0.001) and urea (155.6 vs. 260.1 mg/kg, *P* < 0.001) content, protein (0.93 vs. 1.07 kg/d, *P* < 0.001) and lactose (1.22 vs. 1.46 kg/d, *P* < 0.001) yield, but

increased fat content (46.0 vs. 41.4 g/kg). HS diet also tended to decrease FCM yield (28.4 vs 30.4 kg/d, $P = 0.061$).

Discussion

Relative to the thresholds established by Sauvant and Peyraud (2010) for a corn silage based diet (no more than 25% of concentrates and 25% of rapidly fermentable carbohydrates (RFC), such as starch and soluble sugars, and no less than 35% of NDF and 25% of NDF from forages (NDFf)), the LS and HS diets (Table 1) can be considered as acidogenic. However, the HS diet was characterized by a higher proportion of concentrates (47%) and a lower NDFf content (19.9%) than the LS diet (39% and 25%, respectively). The RFC:NDFf ratio is also considered as another indicator of the acidogenicity of a diet. This ratio was 1.40 and 1.84 for LS and HS diets respectively, well above the threshold value of 1 established by Sauvant and Peyraud (2010). Thus, the HS diet seems more acidogenic than the LS diet, which is confirmed by the results on fermentative parameters.

Indeed, compared to the LS diet, the HS diet induces a lower pH (5.91 vs 6.13), and a higher total VFA and lactate concentrations (respectively + 22% and + 167%). The lower ruminal pH in cows fed on the HS diet compared with cows fed on the LS diet is explained by the faster and more extensive fermentation of sugarcane molasses (Oliveira et al., 2003). This is in accordance with studies conducted in Friesian male cattle (Khalili and Huhtanen 1991), in sheep (Haji-Hajikolaei et al., 2006), and in calves (Oltramari et al., 2016). Heldt et al. (1999) found no difference in average ruminal pH of beef steers fed supplemental sugars (glucose, fructose, or sucrose) or starch, but observed an extremely rapid drop in pH with all three sugars (lowest pH 3 h after supplementation) compared with starch (lowest pH 9 h after supplementation). In our study, the acidic pH of 5.91 is a consequence of the rapid and intense use of rapidly fermentable sugars, immediately available to lactate producing bacteria. In the acid environment induced by the HS diet, the balance between lactate producing bacteria and lactate users is disturbed, for the benefit of lactate producers less sensitive to acidic pH. In such conditions, lactate accumulates, leading to a drop in pH below 6. The comparison between pH and E_h induced by the LS diet and those induced by the HS diet showed an inverse relationship between these two physico-chemical parameters. This result is in accordance with the negative relationship between ruminal pH and

E_h established by Huang et al. (in press) from literature data and from an internal database (Huang et al. 2017a). Moreover, the relationship established by these authors between soluble sugars content of the diet and E_h is well confirmed in the present study. In such conditions, a positive effect of LY should be expected.

From quantitative analysis of the literature carried out by Desnoyers et al. (2009), it appears that LY supplementation in dairy cow had a positive effect on milk yield and DMI, due to an improve rumen function. In the present study, no effect of LY supplementation on DMI and on any of the milk parameters measured was observed which is in accordance with other recent studies (Al Ibrahim et al., 2010; Ferraretto et al., 2012; Tristant and Moran 2015; Ambriz-Vilchis et al., 2017; Uyeno et al, 2017). It is noteworthy that in the current study, LY supplementation tended to decrease urea content in milk, as observed by Tristant and Moran (2015), which could reflect a better use of nitrogen by the rumen microbiota. This hypothesis would be in line with the results of Julien et al. (2015) who showed a better efficiency of nitrogen utilization in supplemented LY dairy cows.

The E_h values recorded in the present study were lower than those recorded by Marden et al. (2005; 2008) and Julien et al., (2010), thereby confirming the method effect (*ex vivo* vs *in vivo* measurements) reported by Huang et al. (2016). In previous studies, rumen fluid was pumped continuously through a rubber tube into a 50-mL-double-walled thermocontrolled vessel outside the rumen, the E_h was measured by electrodes dipped in the collected rumen fluid *(ex vivo* measurements). Although the air contamination was avoid, the redox conditions in a thermocontrolled vessel could not exactly reflect that in the rumen (*in vivo* measurements used in our experiment). Nevertheless, the decrease of ruminal E_h following LY supplementation is confirmed here. Although the diet effect on E_h only showed a tendency, the response of E_h (difference between LY-supplemented group and control group) was significantly greater in HS diet (-34.3 mV) than LS diet (-12.5 mV), which is in accordance with the prediction of Huang et al (Article 4). According to these authors, LY supplementation would induce a decrease in E_h as soon as intake of soluble sugars reaches 405g per day. For a soluble sugars intake of 2548 per day, the decrease in E_h could reach 30 mV. In the present study, the difference in the daily intake of soluble sugars (500 vs 1700g) would explain the difference in response of E_h. The 34 mV difference we recorded between E_h induced by the HS diet and that induced by the LY-supplemented HS diet validates the equation proposed by Huang et al. (Article 4) and is consistent

with the results of other studies. Thus, Mathieu et al. (1996) recorded a deviation of 21 mV in sheep receiving LY at a dose of 1.0×10^9 cfu/d and Marden et al. (2008) recorded a difference of 34 mV in dairy cows receiving LY at a dose of 5.0×10^{10} cfu/d.

The increase of ruminal pH following LY supplementation is also confirmed in our study. Giger-Reverdin and Duvaux-Ponter (2016) observed that the increase of milk urea content was correlated to the decrease of ruminal pH, and proposed to use milk urea as a non-invasive indicator for SARA. Thus, the tendency of a lower milk urea content following LY supplementation observed in our study may reflect a lesser risk of developing ruminal acidosis in cows fed on the HS diet. A supplementary evidence is the positive effect of LY supplementation on urine pH 4h post feeding. In fact, the urinary excretion of proton is the only way for the animal to evacuate nonvolatile acids and is a major contributor to the acid-base balance of the animals (Shapiro et al., 1992; Patience and Chaplin, 1997). In our study, the positive effect of LY on ruminal pH could be partly explained by an increase in urinary H^+ excretion and accelerated bicarbonate reabsorption (Fürll 1994). This would need to be verified.

The response of pH (difference between LY-supplemented group and control group) to LY supplementation is greater with HS diet than with LS diet. This result is partially supported by the meta-analysis of Julien (2010) which demonstrated higher response of pH when that of control group was already low (higher risk of SARA). Indeed, soluble sugars are used by microbiota faster than starch, diet with high sugars content presents a higher potential risk for ruminal acidosis (Nagaraja and Titgemeyer 2007; Lean et al., 2014). This may be the reason for both increase of E_h and decrease of pH. Thereby, LY are able to outcompete lactate-producing bacteria for the utilization of sugars (Chaucheyras et al., 1996), and at the same time stimulates lactate fermentation by *Megasphaera elsdenii.* The ability of LY to strengthen the reducing power (decrease of E_h) of the ruminal milieu could explain the improvement in activity of strictly anaerobic bacteria, such as lactate utilizing bacteria, as demonstrated by Marden et al. (2008) and Pinloche et al. (2013). The stabilization of pH following LY supplementation could be the result of a decrease in ruminal lactate concentration (Marden et al., 2008). Thus the associated decrease of E_h and increase of pH could be explained. In our study, the effect of LY on lactate concentration was not significant, probably because of inter-individual variability. However, the total lactate concentration in the rumen is numerically lower and is significantly decreased one hour post-feeding (data not shown). Because LY can use soluble sugars, their activity could be promoted

by the HS diet. This could explain the decrease in lactate concentration recorded in this study. All these first results would attest of the positive effect of LY in dairy cow fed on an acidogenic diet.

Most of the fermentation end-products were not influenced by LY supplementation, except for A:P ratio. Thus, the current study did not confirm the decrease in A:P ratio induced by LY supplementation observed in the study of Huang et al. (Article 4). Many other factors may affect the ruminal VFA concentration, such as absorption, as well as the amount of ruminal liquid into which the mass of VFA is diluted (Dijkstra et al., 1993; Hall et al., 2015). These factors may increase the variability of VFA concentration in the rumen making the effect of treatment non-detectable. Moreover, the effect of LY supplementation on VFA profile could be interfered by dietary characteristics. In the study of Chademana and Offer (1990), LY supplementation tended to decrease the A:P ratio in sheep fed high concentrated diet, and to increase the A:P ratio in sheep fed medium concentrate diet.

Continuously recorded ruminal temperature is rarely reported due to the difficulty of its measurement. Recently, the development of technologies such as submersible rumen data loggers permitted systematical measurement of ruminal temperature and its utility is discovered by researchers. Castro-Costa et al. (2015) observed an increase of rumen temperature after feeding (+1.4°C) and Pourazad et al. (2016) associated higher ruminal temperature to SARA in dairy cattle. AlZahal et al. (2008; 2009), showed that time spent above 39°C is negatively associated with ruminal pH and positively associated with time spent under pH 6.0. In the present study, supplementation with LY tended to decrease the maximum ruminal temperature, probably reflected its ability to limit ruminal acidosis as shown by increased ruminal pH.

The increased minimum temperature with LY supplementation in our study could reflect changes of intake behavior. Indeed, the decrease of ruminal temperature is mainly due to water drinking (Castro-Costa et al., 2015; Petersen et al., 2016) which is closely related to feed intake behavior. This is supported by Devries and Chevaux (2014) who observed that with LY supplementation, cows tended to have more meals (9.0 vs. 7.8 meals/d) which tended to be smaller in size (3.4 vs. 3.8 kg/meal). These authors hypothesized that increased meal frequency with LY supplementation may be translated into more frequent drinking bouts per day. While compare to abrupt drinking of high amount of water, frequent drinking bouts could prevent great drop of ruminal temperature following drinking. Since gas production and NDF disappearance would be reduced when incubation temperature was below 39°C (Petersen et al., 2016), the

increase of minimum ruminal temperature in our study provided evidence that LY supplementation had some effect on the rumen environment. The eating and drinking behaviors of animal were not recorded in the present study and it would be of interest to verify in future studies if the effect of LY supplementation on ruminal temperature resulted from healthier intake behavior. Following advanced device development, the measurement of ruminal temperature will become easier in next future. These results showed great application potential of ruminal temperature measurement in field condition to monitor rumen function.

Conclusion

The present study demonstrated greater response of ruminal E_h following LY supplementation in HS diet compare to LS diet, therefore confirmed greater effect of LY supplementation when cows were fed high amount of soluble sugars as predicted by previous meta-analysis. Further studies on ruminal microbiota composition and metabolomic profile could be useful to clarify the mode of action of LY in such conditions. In addition, our results showed a great application potential of ruminal temperature measurement in field condition to monitoring the rumen function.

Acknowledgements

The present research (PhD) of Yayu Huang was financially supported by Phileo. The authors particularly thank the personnel of The Phileo experimental unit, Brigitte Geste and Anaïs Fernandez, for their skilled technical assistance in cow welfare, feeding and sampling.

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Chapter 2. Effects of live yeast supplementation and dietary soluble sugars content on rumen microbial composition and metabolomic profile in early-lactating cows (Article 6)

In preparation for Environmental Microbiology

Article 6

Effects of live yeast supplementation and dietary soluble sugars content on rumen microbial composition and metabolome profile in early-lactating cows

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Summary

Pyrosequencing strategy and ^{1}H Nuclear Magnetic Resonance technique were applied to investigate the effects of live yeast (LY) supplementation and dietary soluble sugars content on rumen microbial composition and metabolome profile of early-lactating cows. Eight multiparous lactating Holstein cows fitted with ruminal cannulas were used to investigate the effect of LY supplementation in early-lactating cows fed high (HS) or low (LS) soluble sugars diet. Live yeast supplementation tended to impact the richness of the liquid-associated bacterial fraction. Live yeast supplementation in HS diet increased the relative abundance of *Ruminococcus* and decreased that of *Ruminobacter*. PLS-DA analysis on metabolome data showed a better separation between the HS and HS+LY groups compare to LS and LS+LY groups. For the HS diet, LY supplementation significantly decreased relative proportions of acetate and butyrate, and increased relative proportions of propionate and glucose. The higher relative proportion of glucose following LY supplementation suggested a slow-down of starch degradation by LY.

Introduction

Ruminants rely on their microbiota to ferment plant cell wall material in order to meet their energy requirement. Thus ruminants live in an obligate symbiotic relationship with a complex population of microbes (Russell, 2002). As a result of dietary changes the bacterial population in the rumen is constantly challenged, and the relative abundance of each species can vary dramatically according to the nature of the daily diet of the host animal (Belanche et al., 2010; Fernando et al., 2010; Pitta et al., 2010). In intensive systems of dairy production, the rumen has to cope with rapid and large shifts in diet from primarily forage-based to progressively more readily fermentable carbohydrate (RFC) feedstuffs (Taniguchi et al., 2010). Such diets lead to fermentation patterns at the edge of the rumen physiological equilibrium and even a slight perturbation can lead to metabolic disorders (Owens et al., 1998; Nagaraja and Titgemeyer, 2007).

Previous studies have shown the ability of live yeast (LY) to improve rumen fermentation pattern in lactating cows fed a high RFC diet (Bach et al., 2007; Thrune et al., 2007; Marden et al., 2008; Desnoyers et al., 2009). In such conditions, improvement in growth and activity of cellulolytic and lactate-consuming bacterial populations have been reported (Chaucheyras-Durand et al., 1997; Pinloche et al., 2013; Friedman et al., 2017). A previous quantitative analysis showed improvement in rumen fermentation with LY supplementation in lactating cows fed high amount of daily intake of soluble sugars (Huang *et al*., Article 4). The mechanism of this improvement remains unclear since rumen microbial composition was not available. Recently, metabolome analysis has opened new perspectives in the field of nutrition research, allowing scientists to explore the complex metabolic pathways in response to diets (Ametaj et al., 2010; Lee et al.,2012; Mao et al., 2016). We hypothesized that the rumen microbial composition and metabolome analysis would provide a more comprehensive view on how rumen metabolism changes after LY supplementation. So, the objective of this companion paper was to investigate the effects of LY supplementation and dietary soluble sugars content on rumen microbial composition and metabolome profile in early-lactating cows.

Experimental procedures

The experiment was conducted at the research station of Phileo Lesaffre Animal Care (The Farm, Chemin de Vallesvilles, Seysses, F-31600) from October 2016 to February 2017. Animal procedures were conducted in strict accordance with the European Union recommendations on the protection of animals used for scientific purposes (Directive 2010/63/EU). The protocol was approved by the french Ethical Committee for Animal Experimentation, Animal Sciences and Health N°115.

Animals and experimental design

All details about animal feeding and experimental design are described previously by Huang et al. (Article 5). Briefly, eight multiparous early-lactating Holstein cows fitted with ruminal cannulas were used in a 4 by 4 crossover design. They were housed in individual tie-stalls with free access to water throughout the experiment. They were assigned into two groups (LY supplemented group vs. control group). Each group was matched for DIM, milk production, and BW during a 7-d pretrial adjustment to stalls. At the start of the experiment, cows averaged 72 ± 33 DIM with an average BW of 619 ± 91 kg and milk yield of 31 ± 8 kg/d. They were fed a high soluble sugars diet (HS) or a low soluble sugars diet (LS), supplemented or not with LY. During the first experimental period, the control group fed the HS diet with no supplemental LY added, whereas the LY group were fed the LY-supplemented HS diet. Treatments were switched for the second period. The same design was reproduced with the LS diet during the third and fourth periods. Each experimental period consisted of 28 days in duration with a 21 d adaptation period to the treatment followed by 7 days for sampling and measurements. The difference between high soluble sugars (8.6%, HS) and low soluble sugars (2.1%, LS) content of the diets was achieved by adding cane molasses in the HS diet. Diets were offered in equal amounts twice daily (0900 and 1800 h) and each diet was provided *ad libitum* (5% orts, on an as-fed basis). The ingredients and chemical composition of the two experimental diets are described in Table 27.

Table 27. Ingredient and chemical composition of experimental diets.

	Diet[a]	
Item	HS	LS
Ingredients (% DM)		
Soybean meal	14	15
Alfalfa hay	5	5
Corn silage	48	56
Cane molasses	9	-
Ground wheat	7	7
Corn grain, rolled	16	17
Mineral-vitamin mix[b]	1	1
Chemical composition (% of DM)[c]		
OM	94.3	95.1
CP	15.2	15.7
NDF	28.7	30.2
ADF	14.6	14.5
Starch	31.1	37.1
Soluble sugars	8.6	2.1

a. HS = high soluble sugars; LS = low soluble sugars.
b. Contained (per kg, DM basis): 70 g of P, 230 g of Ca, 50 g of Mg, 4.5 g of Zn, 4 g of Mn, 25 mg of Co, 25 mg of Se, 1.5 g of Cu, 320,000 IU of vitamin A, 100,000 IU of vitamin D3, 900 IU of vitamin E.
c. Calculation according to chemical composition of each ingredient collected in each experimental period.

For LY (Actisaf® Sc 47, Phileo Animal Care, Marcq-en-Baroeul, France) supplemented cows, the recommended dose of 5 g (10^{10} cfu/g DM) per cow and per day with 100g of maize as an extender was top-dressed on the total mixed ration (TMR) during the morning meal.

Rumen sampling

Ruminal content (250 ml) was collected from individual cow on d 24 at 13 h (4 hours after the morning meal) then separated into two fractions. A liquid fraction was obtained by forced filtration at 54 N through a 250 µm sieve for 60 s. A solid fraction was composed of the remaining fibres on the filter. For microbial analysis, samples of liquid and of solid fractions were weighed (80 to 90 µg and 50 to 60 µg, respectively) and promptly stored at -20 °C until further treatment. For metabolome analysis, samples (5 ml) from liquid fraction were immediately immersed in liquid nitrogen and immediately stored at - 80°C.

PCR amplification of bacterial 16S ribosomal genes for Illumina MiSeq pyrosequencing

Total genomic DNA from a 200 mg sample was extracted and purified with the ZYMO (ZR-96soil microbe DNA kit, Epigenetics Compagny, USA) according to the manufacturer's instructions. The V3-V4 regions of 16S rRNA genes of samples were amplified from purified genomic DNA with the primers F343 (5' -CTT TCC CTA CAC GAC GCT CTT CCG ATC TTA CGG RAG GCA GCA G - 3') (Liu et al., 2007) and reverse R784 (5' - GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA CCA GGG TAT CTA ATC CT - 3') (Andersson et al., 2008).

The PCR was carried out with an annealing temperature of 65°C for 30 amplification cycles to minimize PCR biases. As MiSeq enables paired 250-bp reads, the ends of each read are overlapped and can be stitched together to generate extremely high-quality, full-length reads of the entire V3 and V4 region in a single run. Single multiplexing was performed using 6 bp index, which were added to R784 during a second PCR with 12 cycles using forward primer (AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC) and reverse primer (CAA GCA GAA GAC GGC ATA CGA GAT GTG ACT GGA GTT CAG ACG TGT). The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge (Illumina, San Diego, CA, USA) at the Genomic and Transcriptomic Platform (INRA, Toulouse, France) according to the manufacturers' instructions. Each pair-end sequence was assigned to its sample with the help of the previously integrated index. The raw sequences were cleaned, clustered in OTU (operational taxonomic unit) and affiliated to taxa using FROGS pipeline (Escudie et al., 2015). Briefly, sequences were filtered removing sequences that did not match the proximal PCR primer sequences (with 2 mismatches allowed), with a minimum and maximum sequencing length (less than 397 nucleotides and higher than 432), with at least one ambiguous base. Chimeric DNA sequences were detected using UCHIME and removed (Edgar et al., 2011). Representative sequences were clustered into operational taxonomic unit (OTU) using SWARM (Mahé et al., 2014). OTU taxonomic assignment was performed using the SILVA SSU Ref NR 115 database (Pruesse et al., 2007) using the BLAT algorithm (Kent 2002).

Sample preparation for Proton Nuclear Magnetic Resonance (^{1}H-NMR)

The samples (200 µl) of liquid fraction were diluted into 500 µl of phosphate buffer (0.2M; pH 7.0) prepared in deuterated water (D_2O) with sodium trimethylsilylpropionate (TMSP, 10 mg of TMSP into 100 ml of D_2O) and centrifuged at 4600 × g at 4°C. A volume of 600 µl was sampled and transferred into NMR tubes. All ^{1}H-NMR spectra were obtained using a Bruker Avance III HD NMR spectrometer operating at 600.13 MHz for the 1H resonance frequency and an inverse detection 5 mm 1H-13C-15N-31P cryoprobe. They were acquired at 300 K using the Carr-Purcell-Meiboom-Gill spin-echo pulse sequence with presaturation and a total spin-echo delay (2 n τ) of 240 ms to attenuate broad signals from proteins and lipoproteins. A total of 128 transients were collected into 32 K data points using a spectral width of 20 ppm, a relaxation delay of 2 s, and an acquisition time of 1.36 s. Prior to Fourier transformation, an exponential line broadening function of 0.3 Hz was applied to the free induction decay. All spectra were manually phased and baseline corrected using Topspin (V3.2, Bruker, Biospin, Munich, Germany). They were referenced to TMSP at 0 ppm. They were then data reduced using AMIX software (version 3.9, Bruker Biospin) to integrate 0.01 ppm wide regions corresponding to the δ 10 to 0.5 ppm regions. The region containing the residual water (5.1-4.5 ppm) was removed. Each integrated region was normalized to the total intensity of spectrum to generate quantitative variables. A total of 791 buckets were included in the data matrices.

Statistical analysis

For microbial data, all analyses were performed using the statistical software R version 3.4.0 (R Core Team, 2017). The statistical model included treatment and period as fixed effects and square and cow within square as random effects.

For metabolomic data, statistical analyses were performed using the web interface Workflow4Metabolomics (http://workflow4metabolomics.org/) to identify the buckets that discriminated i) LY supplementation and ii) soluble sugars content in the diets. First, a multilevel analysis was performed to split the variability into two parts thanks to paired data: between-cow variability and within-cow variability. Multivariate statistical analyses were performed on within-cow variability. Then, a principal component

analysis (PCA) was performed to observe data and eliminate outlier samples. Then the orthogonal projection of latent structures–discriminant analysis (OPLS-DA) supervised method was performed. The OPLS-DA is similar to PCA but uses discriminant variables that correlate to class membership. It permits better discrimination of 2 groups. The axes represent the latent variables. Discriminant buckets were determined using variable importance in projection (VIP), an appropriate quantitative statistical parameter ranking the buckets according to their ability to discriminate different groups. The buckets were referenced by their chemical shift, expressed in ppm. The performance of the OPLS-DA model was evaluated by R2Y and Q2 parameters, which informed about the explained variance and the predictive ability of the model, respectively. All OPLS-DA models were constructed using a 7-fold cross-validation method to determine the number of latent variables to include in the OPLS-DA model and further assessed with a 200-permutation test to calculate the robustness and validity of the OPLS-DA results. For each bucket, the significance was checked by kruskal-wallis test. The *p* - values were corrected for multiple test with the false discovery rate (Hochberg and Benjamini, 1990) using the R software (version 2.14.1). Discriminant buckets were identified by matching 1D and 2D NMR spectra of reference compounds with the NMR spectra of ruminal samples recorded in the same conditions.

Results

Rumen microbiota

The effects of LY supplementation and diet on richness (estimated by the Chao1 value) and diversity (estimated by the Shannon index) of ruminal bacterial communities in the liquid and solid fractions are shown in Figure 15.

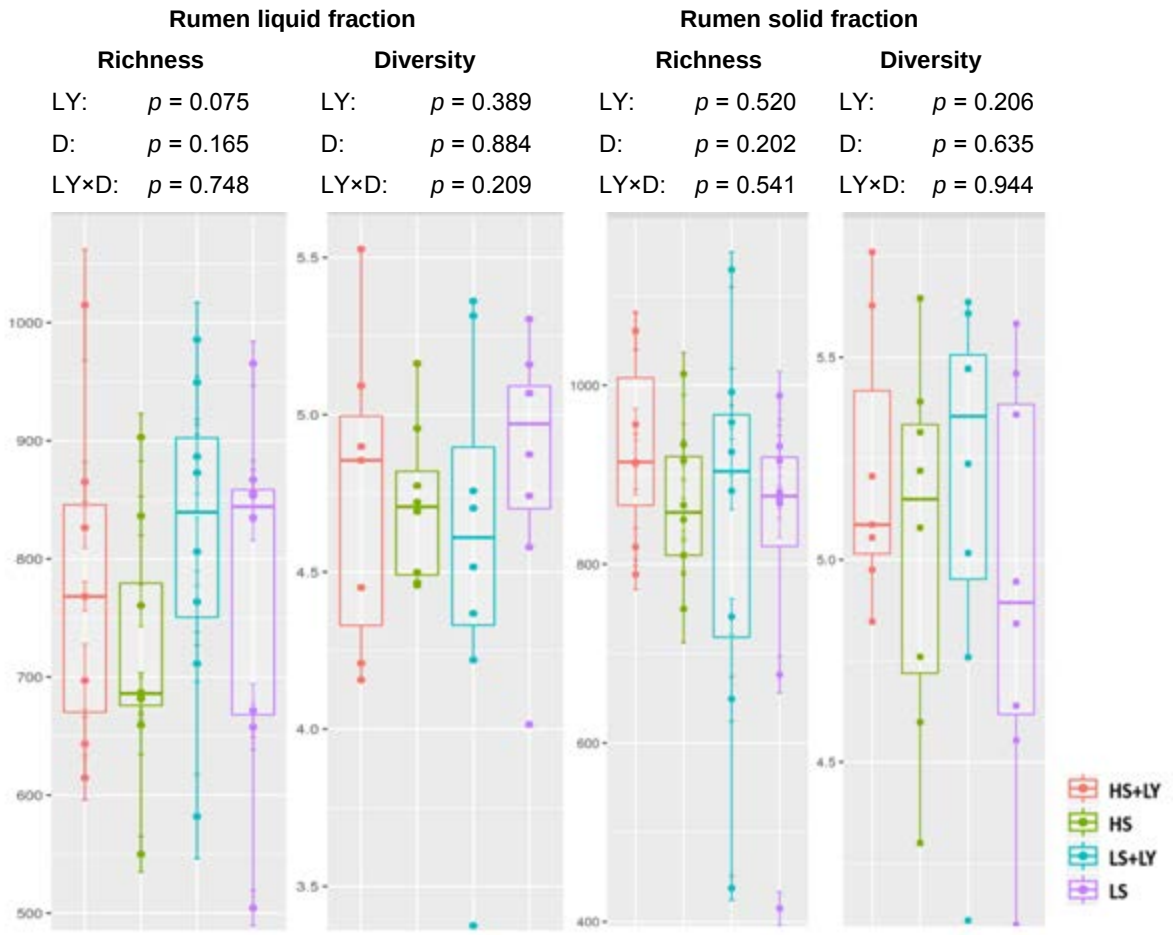

Figure 15. Effect of live yeast (LY) supplementation and soluble sugars content of the diet (D) on richness (estimated by the Chao1 value) and diversity (estimated by the Shannon index) of ruminal bacterial communities of liquid and solid fractions.

The four treatments consisted in a high soluble sugars diet (HS) or a low soluble sugars diet (LS), supplemented or not with LY.

The richness and diversity of ruminal bacterial communities were affected neither by the soluble sugars content of the diet nor by LY supplementation. Live yeast supplementation tended (p = 0.075) to impact the richness of the liquid-associated bacterial fraction.

Three phyla represented 94 and 91% of the bacterial population, for liquid and solid fraction respectively (Tables 28 and 29). *Firmicutes* was the most abundant phyla followed by *Bacteroidetes* and *Proteabacteria*, while 6 other minor phyla were also detected.

Table 28. Effect of live yeast supplementation and dietary soluble sugars content on relative abundance (%) of bacterial phyla present in the liquid fraction.

	Treatment[a]					*p*-value[b]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Actinobacteria	2.09	1.54	3.57	4.04	0.5	0.70	0.21	0.62
Bacteroidetes	31.9	32.0	30.5	28.8	0.9	0.46	0.56	0.59
Fibrobacteres	1.49	1.31	1.30	1.36	0.1	0.88	0.62	0.68
Firmicutes	50.9	46.8	45.0	42.5	1.8	0.47	0.09	0.75
Fusobacteria	0.00	0.00	0.03	0.00	0.01	0.14	0.16	0.32
Proteobacteria	11.6	15.0	17.5	21.7	2.0	0.21	0.08	0.88
Spirochaetae	0.89	1.43	0.80	0.71	0.1	0.74	0.75	0.10
SR1(Absconditabacteria)	0.03	0.06	0.08	0.14	0.01	0.08	0.13	0.54
Tenericutes	0.14	0.13	0.18	0.11	0.03	0.37	0.64	0.59
Unidentified	1.04	0.91	1.05	0.69	0.09	0.11	0.95	0.49

a. HS = high soluble sugars diet ; LS = low soluble sugars diet; LY = live yeast.
b. LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction effect.

In liquid fraction and compared to LS diet, HS diet tended (p = 0.09) to increase the relative abundance of the *Firmicutes* phylum by 5.1 points, and tended (p = 0.08) to decrease *Proteobacteri*a phylum by 6.3 points. Live yeast supplementation tended (p = 0.08) to increase the relative abundance of phylum SR1 (*Absconditabacteria*). Live yeast supplementation tended to increase the relative abundance of phylum *Spirochaetae* with HS diet (interaction effect, p = 0.1).

Table 29. Effect of live yeast supplementation and dietary soluble sugars content on relative abundance (%) of bacterial phyla present in the solid fraction.

	Treatment[a]					*p*-value[b]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Actinobacteria	1.64	1.13	2.14	2.79	0.2	0.24	0.37	0.14
Bacteroidetes	28.9	25.2	24.1	27.3	0.9	0.19	0.05	0.05
Fibrobacteres	2.96	4.18	2.56	3.72	0.4	0.20	0.66	0.97
Firmicutes	51.7	52.8	47.1	49.9	1.2	0.34	0.12	0.69
Proteobacteria	11.0	11.8	20.2	12.5	1.4	0.01	<0.01	0.04
Spirochaetae	2.90	4.16	3.00	2.95	0.3	0.95	0.91	0.33
SR1 (Absconditabacteria)	0.01	0.03	0.04	0.03	0.01	0.21	0.01	0.04
Tenericutes	0.14	0.16	0.20	0.16	0.03	0.72	0.52	0.69
Unidentified	0.71	0.51	0.60	0.64	0.04	0.60	0.19	0.03

a. HS = high soluble sugars diet ; LS = low soluble sugars diet; LY = live yeast.
b. LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction effect.

In solid fraction and compared to LS diet, HS diet decreased *Proteobacteria* and SR1 (*Absconditabacteria*) phyla by 4.95 ($p < 0.01$) and 0.015 points ($p = 0.01$) respectively, and tended ($p = 0.05$) to increase the relative abundance of *Bacteroidetes* phylum by 1.35 points. Following LY supplementation, *Proteobacteria* phylum increased for cows fed HS diet, and decreased for cows fed LS diet ($p < 0.05$).

Relative abundances (%) of bacterial genera present in the liquid and solid fraction were presented in tables 30 and 31 respectively.

Table 30. Effect of live yeast supplementation and dietary soluble sugars content on relative abundance (%) of bacterial genera present in the liquid fraction.

	Treatment[a]					*p*-value[b]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Ruminantium group	0.50	0.50	0.61	0.59	0.05	0.74	0.18	0.85
Bifidobacterium	1.16	0.75	2.99	3.61	0.56	0.59	0.12	0.62
Butyrivibrio 2	0.15	0.14	0.18	0.15	0.02	0.35	0.27	0.68
Fibrobacter	1.49	1.31	1.30	1.36	0.14	0.88	0.62	0.67
Lachnospira	0.03	0.05	0.01	0.03	0.01	0.16	0.19	0.83
Lactobacillus	0.04	0.02	0.04	0.05	0.01	0.59	0.84	0.44
Prevotella	19.74	19.34	17.53	17.80	1.02	0.91	0.38	0.94
Ruminococcus	5.49	10.38	9.33	6.50	0.99	0.20	0.08	0.02
Selenomonas 1	0.24	0.13	0.20	0.16	0.02	0.41	0.55	0.42
Streptococcus	0.01	0.00	0.02	0.01	0.00	0.73	0.62	0.47
Succinivibrio	0.10	0.22	0.18	0.30	0.06	0.33	0.57	0.82

a. HS = high soluble sugars diet ; LS = low soluble sugars diet; LY = live yeast.

b. LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction effect.

Table 31. Effect of live yeast supplementation and dietary soluble sugars content on relative abundance of bacterial genera present in the solid fraction.

	Treatment[a]					*p*-value[b]		
	HS	HS+LY	LS	LS+LY	SEM	LY	D	LY × D
Ruminantium group	1.17	1.28	0.99	1.14	0.09	0.44	0.35	0.89
Bifidobacterium	0.49	0.35	1.53	2.24	0.29	0.24	0.08	0.36
Fibrobacter	2.96	4.22	2.56	3.72	0.41	0.20	0.66	0.96
Lactobacillus	0.01	0.01	0.04	0.03	0.00	0.17	0.01	0.68
Prevotella	19.92	15.92	15.58	18.21	1.05	0.33	0.11	0.08
Ruminobacter	0.54	0.13	0.23	0.96	0.18	0.10	0.49	0.07
Ruminococcus	6.84	8.83	7.91	7.61	0.68	0.86	0.54	0.36
Selenomonas 1	0.13	0.09	0.28	0.19	0.03	0.14	0.02	0.55
Streptococcus	0.01	0.00	0.01	0.01	0.00	0.50	0.40	0.90
Succinimonas	0.01	0.09	0.03	0.04	0.01	0.79	0.64	0.20
Succinivibrio	0.06	0.16	0.28	0.22	0.05	0.54	0.04	0.20
Butyrivibrio 2	0.94	0.82	1.07	1.12	0.14	0.87	0.69	0.70
Lachnospira	0.02	0.04	0.02	0.01	0.01	0.41	0.99	0.13

a. HS = high soluble sugars diet; LS = low soluble sugars diet; LY = live yeast.
b. LY = live yeast effect; D = diet effect; LY × D = live yeast by diet interaction effect.

Whatever the treatment, only 4 genera were present in liquid and solid fraction at more than 1% of the bacterial population: Prevotella (18.6% ± 1.1), Ruminoccocus (7.93% ± 2.3), Bifidobacterium (2.13% ± 1.3), and Fibrobacter (1.37% ± 0.09) for liquid fraction; Prevotella (17.4% ± 2.0), Ruminococcus (7.8% ± 0.8), Fibrobacter (3.37% ± 0.75), and Ruminantium (1.15% ± 0.12) for solid fraction.

Relative to LS diet, HS diet tended (p = 0.08) to increase Ruminococcus genera in liquid fraction. In solid fraction, it decreased the relative abundances of *Lactobacillus* (0.025 points), *Selenomonas* 1 (0.13 points), *Succinivibrio* (0.14 points), and tented to decrease relative abundance of *Bifidobacterium* (*p* = 0.08). Live yeast supplementation tended (*p* = 0.10) to decrease the relative abundance of *Ruminobacter* for cows fed HS diet, and to increase relative abundance for cows fed LS diet.

Rumen metabolome

For the four experimental treatments, the analysis generated a PLS-DA model with five latent components, characterized by a faithful representation of the data (R2Y = 77.6%) and, more important, by a good cumulative predictive capacity (Q2 = 0.45) (Figure 16).

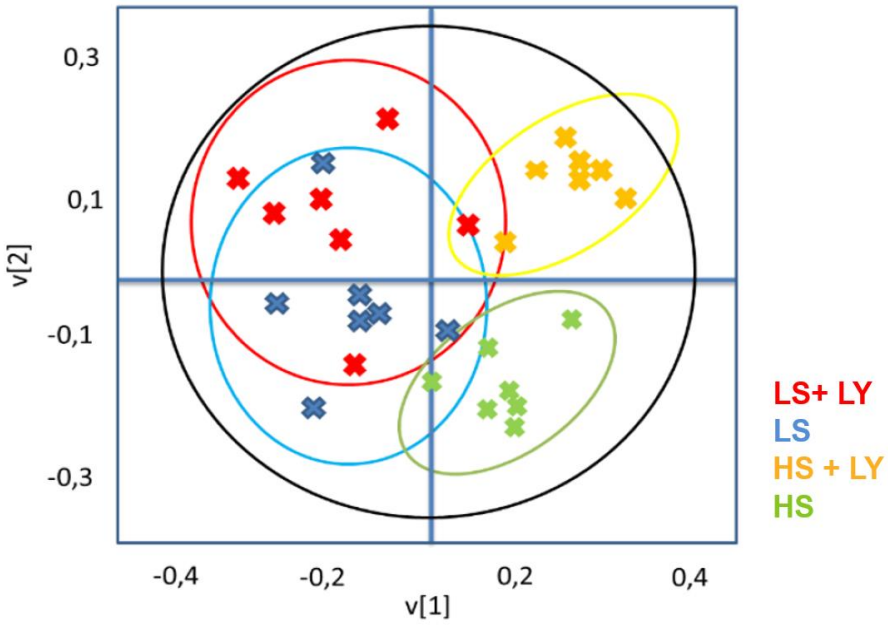

Figure 16. Two-dimensional PLS-DA score plot of integrated ^{1}H-NMR spectra to explore dissimilarities among treatments consisting in a high soluble sugars diet (HS) or a low soluble sugars diet (LS), supplemented or not with live yeast (LY).

(Predictive ability $Q^2 = 0.45$; explained variability $R^2 = 77.6\%$; v corresponds to the latent variable).

The score plot of the PLS-DA showed a clear separation between the HS and the HS+LY treatments. In order to improve the discrimination between treatments, a pairwise comparison was performed.

The discriminant metabolites between LS diet and HS diet are presented in Table 32. Compare to LS diet, HS diet significantly decreased the relative proportions of acetate, branched VFAs (iso butyrate and iso valerate), 3-phenylpropionate, glucose and maltose, ethanol and putrescine, and significantly increased the relative proportions of butyrate and 3-hydroxybutyrate, methionine, phenylalanine and ethanolamine.

Table 32. Discriminant rumen metabolites from cows fed a low or a high soluble sugars diet.

Metabolite	Chemical shift (ppm)	Variation	VIP[a] value	*p*-value
Acetate	1.92	↘	1.42	<0.05
Butyrate	0.88; 1.56	↗	5.44; 5.98	<0.01;<0.01
Isobutyrate	1.06	↘	4.98	<0.05
Isovalerate	2.06; 0.92	↘	1.62; 1.59	<0.05;<0.01
3-hydroxybutyrate	2.31; 2.42; 4.16	↗	0.98;0.82; 0.80	<0.01;<0.01;<0.05
3-phenylpropionate	7.30; 7.37	↘	1.01; 1.20	<0.01; <0.05
Glucose	5.24; 3.77; 3.72; 3.53; 3.48; 3.40; 3.25	↘	2.36; 2.17; 2.31; 2.21; 2.17; 1.92; 2.08	<0.01; <0.01; <0.05; <0.05; <0.05; <0.01; <0.05
Maltose	3.85; 3.59; 3.28	↘	2.01; 1.50; 1.21	<0.05; <0.05;<0.05
Ethanol	3.66; 1.18	↘	1.90; 2.60	<0.05; <0.05
Putrescine	3.04	↘	1.08	<0.05
Ethanolamine	3.81; 3.13	↗	1.14; 0.93	<0.01; <0.01
Methionine	2.14; 3.86	↗	3.91; 0.99	<0.01
Phenylalanine	3.12	↗	0.81	<0.01

a. Variable importance in projection.

The discriminant metabolites between the high and low soluble sugars diets and the same diets supplemented with LY are presented in Table 33.

Table 33. Discriminant rumen metabolites from cows fed a high or a low soluble sugars diet, supplemented or not with live yeast.

Metabolite	Chemical shift (ppm)	Variation	VIP[a] value	*p*-value
HS diet vs LY-supplemented HS diet				
Acetate	1.92	↘	5.05	<0.01
Butyrate	0.90 ; 1.55; 2.15	↘	3.57; 2.28;1.87	<0.01; <0.01; <0.01
Propionate	1.05 ; 2.19	↗	4.37; 2.67	<0.05; <0.05
Glucose	3.72; 3.77; 3.84	↗	0.94; 0.85; 0.88	<0.01; <0.01; <0.05
LS diet vs LY-supplemented LS diet				
Butyrate	2.15	↘	1.398	<0.05
Propionate	1.05; 2.19	↘	4.127; 2.85	<0.05; <0.05
Valerate	1.29	↗	1.028	<0.05
Lactate	1.33	↗	1.626	<0.01
Glucose	3.48; 3.72; 3.84	↗	0.82; 0.85; 0.90	<0.05; <0.05; <0.05

a. Variable importance in projection.

For the HS diet, LY supplementation significantly decreased relative proportions of acetate and butyrate, and increased relative proportions of propionate and glucose. For the LS diet, LY supplementation significantly decreased relative proportions of butyrate and propionate, and increased relative proportions of valerate, lactate and glucose.

Discussion

High-RFC diets were used to improve performance of high producing dairy cows alter microbial communities in the rumen and the symbiosis between the host and these communities by causing the production of excessive amounts of organic acids (volatile organic acid and lactate (Plaizier et al., 2009; Zebeli and Metzler-Zebeli 2012; Marchesini et al., 2013). This results in major changes in the rumen environment, such as a pH depression, higher redox potential (E_h), and reduction in the populations of many beneficial bacteria (Marden et al., 2008; Fernando et al., 2010; Khapifour et al., 2010; Hook et al., 2011). Our study was conducted to investigate the effects of LY supplementation on the rumen microbial composition and metabolome profile in early-lactating cows fed diets differing by their soluble sugars content.

Effects on rumen microbiota

In the present study, the HS diet induced a decrease in relative abundance of *Succinivibrio* genus (belonging to *Proteobacteria* phylum). Although the bacteria constituting this bacterial genus are not fibrolytic, they can still interact with fibrolytic bacteria (Koike *et al*., 2003). This is in agreement with decrease of relative proportion of acetate which is the main end product of fiber degradation (Enjalbert et al., 1999). The ruminal butyrate content is significantly higher with HS diet, which is consistent with other results showing that butyric acid production was increased with diets rich in soluble sugars, such as beets (sucrose) or whey (lactose) (Jouany et al., 1995). Our results about changes in butyrate and acetate concentration with the HS diet are in agreement with other studies conducted in sheep (Syrjälä 1972; Chamberlain et al., 1985) and cattle (Huhtanen, 1988; Khalili and Huhtanen, 1991) which reported that sucrose and molasses supplements increase the molar proportion of butyrate at the expense of acetate.

As Huang et al. (Article 5) in the same experimental trial observed greater effect of LY on ruminal redox potential (E_h) in cows fed HS diet than LS diet, changes of microbiota following LY supplementation were expected to be different between these two diets. With LS diet, LY promote the development of bacteria of the *Bacteroidetes* phylum at the expense of *Spirochaetae*, SR1 (*Absconditabacteria*) and *Proteobacteria* phyla whose relative abundances are reduced. When the diet was enriched in soluble sugars, LY inversely modified the microbiota profile. Regarding the bacterial genera in solid fraction, the LY-supplemented HS diet seems to increase the occurence of two well-known cellulolytic flora such as *Ruminococcus* and *Fibrobacter* and, conversely, decreased the occurence of *Ruminobacter* which is a well-known amylolytic bacteria with a very high growth rate mainly favored by high grain diets (Cotta, 1988; McAllister et al., 1990). Our results are in agreement with Chaucheyras-Durand and Fonty (2001) who suggested that LY could stimulate the development of cellulolytic bacteria. Later, Pinloche et al. (2013) found an increase in relative abundance of major fibrolytic species and a decrease in proteolytic species, following LY supplementation. More recently, Jiang et al. (2016) also found that supplementation with LY in a diet composed of silage and cereals led to an increase in the relative abundance of cellulolytic bacteria. It is generally admitted that most of fibre-degrading microorganisms are sensitive to oxygen. A significant reduction in oxygen fugacity of the ruminal milieu was observed when LY was supplemented in a high concentrate diet (Marden and Bayourthe, 2005). Consequently, LY decreases the ruminal E_h by scavenging O_2 and then conferred a better reducing environment more favorable for growth and activities of these anaerobic bacteria.

Fibrobacter is a plant cell wall degrading bacterial group and was shown to be the most prolific endoglucanase producer in the firmly attached population (Michalet-Doreau et al., 2001). The fact that this bacterial group was more abundant in the solid fraction with LY-supplemented HS diet might suggest that LY stimulate its attachment to solid material. When considering only *Ruminococcus* and *Fibrobacter*, 41% were found in the solid fraction with LY-supplemented HS diet but only 29% with HS diet. Recent data (Kong et al., 2012) suggest that these bacteria account for about 50% of the total active cellulolytic bacteria. If we also consider the fact that LY tended to increase the bacterial richness in the solid fraction then one might conclude that LY

stimulate either growth or attachment of bacteria on the solid particles. Furthermore, it was observed in a previous study that the attachment of *Ruminococcus* and *Fibrobacter* were positively correlated with a decrease in E_h (Roger et al., 1990). Huang et al. (Article 5) have actually observed a decrease in E_h with the LY-supplemented HS diet compared to HS diet. Considering that effects of LY on *Ruminococcus* and *Ruminobacter* could also be observed in cows fed high starch diet, the effect of LY recorded in our study may not specific to the soluble sugars content but rather to the high content of RFC, including starch and soluble sugars.

However, the reason that LY favored the relative abundance of *Ruminobacter* and decreased that of *Ruminococcus* in the rumen of cows fed LS diet is unclear. It is important to mention that the beneficial effects of LY have been attributed to only a few of the diverse microorganisms in the rumen but many unidentified microorganisms could also be affected by LY supplementation and involved in the mechanism. In addition, the methods used in our study may also introduce some variabilities. Indeed, Jiang et al. (2016) reported weak relationship between qPCR and MiSeq sequencing for *Megasphaera elsdenii*, *Ruminococcus flavefaciens* and *S. ruminantium.*

Effects on rumen metabolome

The increase in soluble sugars content of the diet led to a change in the ruminal metabolomic profile. A significant decrease in the relative proportions of 3-phenylpropionate, glucose, maltose, ethanol and putrescine, as well as a significant increase in the relative proportions of 3-hydroxybutyrate, methionine, phenylalanine and ethanolamine were observed in our study. It is difficult to explain these variations because, at present, there is very little information to reliably connect the metabolites present in the rumen content to the composition of microbial community. However, variations in the ruminal metabolome profile have been observed in cows fed with a high proportion of starch concentrates (= 45%). Saleem et al. (2012) observed an increase in the concentrations of several toxic, inflammatory and unnatural compounds, including putrescine, methylamines, ethanol, urea, ethanolamine and short chain fatty acids. Changes in the relative proportions of several amino acids (phenylalanine, ornithine, lysine, leucine, arginine, valine and phenylacetylglycine) and a decrease in 3-phenylpropionate concentration have also been recorded (Ametaj et al., 2010;

Saleem et al., 2012). The difference of metabolomic profile introduced by HS diet in our study corroborate some of these observations: higher relative proportions of methionine, phenylalanine, 3-hydroxybutyrate and ethanolamine, a metabolite derived from phosphatidylethanolamine, which is the major phospholipid of enterocyte membranes (Kawai et al., 1974); lower relative proportion of 3-phenylpropionate, a metabolite that plays a role in the growth of rumen bacteria and also a protective role against oxidative stress (Turlin et al., 2005). Our results revealed higher risk introduced by HS diet. Again, since these changes can be observed in cows fed high starch diet, the effect observed in our study may not specific to the soluble sugars content but rather due to the risk introduced by high content of all RFC.

Regarding the effect of LY, PLS-DA analysis on metabolomic data showed a better separation between the HS and HS+LY diets compare to LS and LS+LY diets. This might be resulted from higher response of ruminal E_h to LY supplementation in HS diet compare to LS diet observed by Huang et al. (Article 5). However, few discriminant buckets were identified. The decrease of relative proportion of acetate and butyrate and the increase of relative proportion of propionate of samples from cows fed on LY-supplemented HS diet is in agreement with previous quantitative analysis (Huang et al., Article 4). This confirmed our hypothesis that the decrease of ruminal E_h (increase of reducing power) following LY supplementation might promoted the production of propionate and inhibited that of acetate, which could be interpreted to a better use of energy. In addition, conversion of lactate produced in the rumen to propionate by lactate utilizer could also contributed to the increase of relative proportion of propionate (Marden et al., 2008). It is interesting to observe the increase of relative proportion of glucose following LY supplementation whatever the treatment (Table 7). Indeed, starch is first degraded to maltose by amylase, then catalyzed by maltase or maltose phosphorylase and finally degraded to glucose in the rumen (Zhang et al., 2017). Recent metabolomic analysis (Saleem et al., 2012; Zhang et al., 2017) has already related high glucose concentration in rumen fluid from cows fed high grain diet. Knowing that in our study the samples were taken 4 hours after morning feeding, the higher relative proportion of glucose could result from a slow-down of starch degradation due to LY supplementation. In fact, the effect of LY to slow-down starch degradation could be due to an increase in the activity of protozoa: LY supplementation is often associated with increased protozoa counts (Plata et al., 1994; Mathieu et al.,

1996) and protozoa are known to store starch after feeding (Abou Akadda and Howard, 1960), therefore delaying starch digestion by bacteria.

Conclusion

Live yeast supplementation changed microbiota composition in the rumen by increasing the occurrence of two well-known cellulolytic flora such as *Ruminococcus* and *Fibrobacter* and led to a slow down degradation of starch. These modifications were partially explained by the attenuation of the post-prandial drop in pH and the more reducing environment caused by this additive. Live yeast increased the microbiota diversity in term of richness, suggesting a benefit in using LY in dairy cow fed a high soluble sugars diet.

Acknowledgments

The present research (PhD) of Yayu Huang was financially supported by Phileo. The authors particularly thank the personnel of The Phileo experimental unit, Brigitte Geste and Anaïs Fernandez, for their skilled technical assistance in cow welfare, feeding and sampling. This work was carried out within the French Infrastructure for Metabolomics and Fluxomics (MetaboHUB-ANR-11-INBS-0010).

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GENERAL DISCUSSION

As an alternative to growth promoting antibiotics, probiotics remain at the forefront of the animal feed industry. Live yeast *Saccharomyces cerevisiae* has been wildly used in commercial farms of beef and dairy cattle and the researches on effects of the LY are numerous. While the effects of LY on the digestion, the metabolism and the performances are very variable depending on the conditions. There is a need for the scientists to understand the mode of actions of LY in different conditions and for the farmers to define the optimal condition of LY utilization in livestock production. The current work fits into these contexts and follows on from the PhD work done previously by Marden (2007) and Julien (2010). We discuss here the main results from this thesis work.

1. Ruminal E_h and rumen function

1.1. Ruminal E_h could be a potential indicator of digestive disorder

Subacute ruminal acidosis (SARA) is a common digestive disorder in cattle fed high concentrate diets and affects 20–40% of animals in high producing dairy herds (Kleen, 2004). Fermentation of feedstuffs in rumen produces volatile fatty acids (VFA) and lactic acid. These acids can accumulate and reduce ruminal pH if the absorption and rumen buffering cannot keep pace with their accumulation (Plaizier et al., 2009). Ruminal pH is the most frequently used indicator of SARA. Low rumen pH for prolonged periods can negatively affect feed intake, microbial metabolism, and nutrient degradation. Low ruminal pH is also related to inflammation, laminitis, diarrhea and milk fat depression (Stone, 2004; Krause and Oetzel, 2006; Enemark, 2008). **However, the extent of problems that occur and the precise mechanisms by which low ruminal pH increases these disorders have not been fully characterized.**

In fact, ruminal pH is determined by the concentration of protons in the rumen fluid, which depends on the equilibrium between supply from fermented feeds and the buffering capacity of saliva, the absorption through the rumen wall and the passage to the lower digestive tract (Allen, 1997). It is commonly accepted that the reduction in pH drives the change in the VFA profile towards more propionate and less acetate and, when pH drops below 5.5, then lactic acid accumulates (Dirksen, 1969). However, when the fermentation profile changes due to feeding high concentrate and low forage diets, it is not clear if the observed reduction in the acetate to propionate ratio and other fermentation indicators are due to the reduction of pH or to a change in the microbial fermentation pathways of feeds (Calsamiglia et al., 2012). In vivo,

the reduction of pH occurs when feeding high concentrate diets and, therefore, the causal effect is confounded.

Actually, digestive disorder can occur without low ruminal pH. After evaluating 172 cows suspected at risk of SARA (based on lameness prevalence, low milk production, low fat and low fat-to protein ratio in milk) from 24 herds in Belgium, Lessire et al. (2017) observed no pH value lower than 5.5, and only 10 cows could be considered at risk for SARA (pH < 5.8). Palmonari et al. (2010) also reported that milk fat depression could occur at intermediate ruminal pH. In our database for meta-analysis (Huang et al., Articles 3 and 4), about 10 % of the observations (77 of 775 kinetics) showed high pH values (e.g. pH > 6, without SARA according to the ruminal pH thresholds proposed in the literature) but high E_h (e.g. E_h > -160 mV) which is considered to be unfavorable to activities of fibrolytic and lactate utilizing bacteria). These observations were associated with low acetate: propionate ratio (averaged 2.88) and high ammoniac concentration (108.1 mg/L), which revealed digestive disorder, or at least suboptimal rumen function.

On the other hand, some decrease of ruminal pH does not necessarily cause any particular damages as evaluated by inflammatory response and milk production performance. According to Li et al. (2012), alfalfa-pellet SARA challenge can cause the decrease of ruminal pH to the same level than grain-based SARA challenge, but resulted in a much lower increase in rumen lipopolysaccharide endotoxin (LPS), and only grain-based challenge increased LPS concentration in cecal digesta, and in wet feces. Later, Coombe et al. (2015) in their study reported that some cows had low ruminal pH did not initiated inflammatory response evaluated by haptoglobin concentration. Humer et al. (2015) found that the metabolic activity of the liver were not necessarily associated with low ruminal pH that was defined as acidotic from commonly accepted thresholds. Also, Gao and Oba (2016) observed a decrease of ruminal pH without affect milk yield and milk fat content.

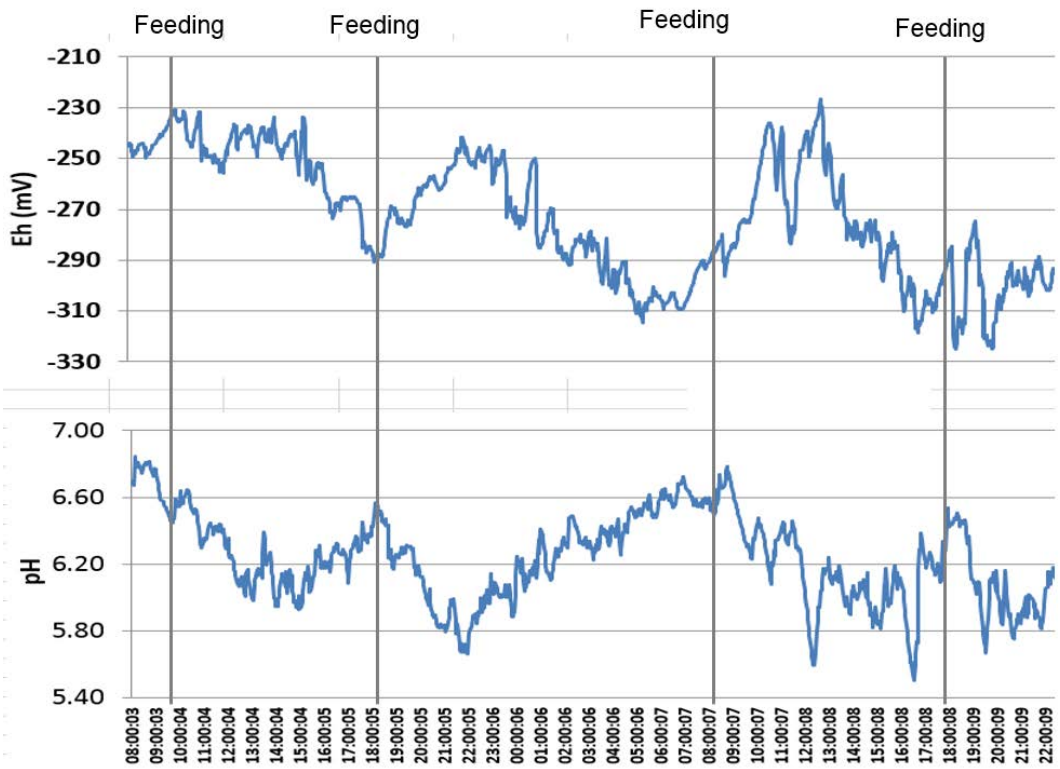

Figure 17. Normal kinetic of ruminal E_h and pH (during 36 hours) in dairy cow.

In addition, our simultaneous measurement of pH and E_h revealed that in most of the cases, the decrease of pH occurred at almost the same time of increase of E_h (**Figure 17**, unpublished data); and in other cases, especially in some abnormal situation, the decrease of pH did not follow the increase of E_h, as can be show in **Figure 18** which recorded a digestive disorder during the period of adaptation. We can observe that after 23:00, ruminal E_h stayed in a high level during 18 hours, ruminal pH fluctuated during around 8 hours and quickly dropped below 5.6 after 9:00 the second day. From the evening feeding (distributed 10.8 kg of DM) of the second day, the intake reduced dramatically (7.8 kg refused, corresponded to 3.0 kg of DMI at the third day morning). The abnormal evolution was firstly revealed by a long period of high ruminal E_h, the drop of pH occurred only 10 hours after. **It is likely that ruminal E_h could better reflect the real time fermentation condition in the rumen than pH which is the result of acid production, buffering and absorption.** In addition, from this example we can also conclude that continuous record of both pH and E_h could be very helpful in detection of digestive disorder compare to traditional measurement, because even one extreme value (a very high E_h or a very low pH) could be uninterpretable.

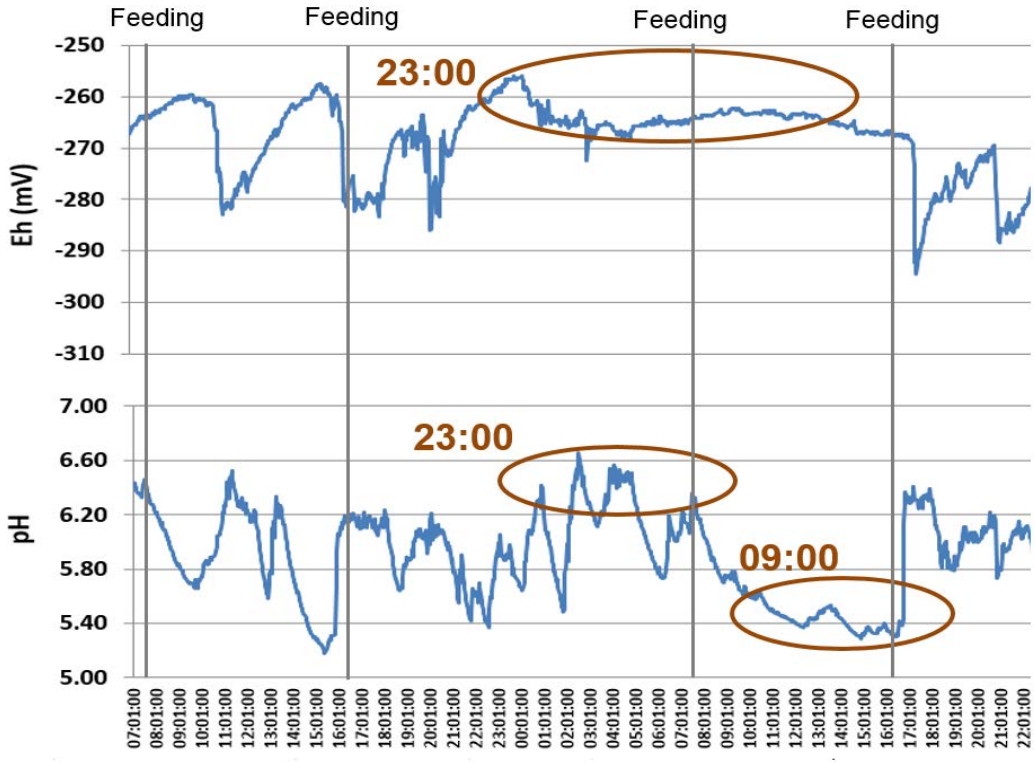

Figure 18. Example of digestive disorder recorded by kinetic of ruminal E_h and pH in dairy cow.

These observations suggest that the measurement of ruminal pH alone might not be a reliable method of diagnosing digestive disorder in some cases. The opinion that a reduced ruminal pH is the sole reason for digestive and metabolic disorders in dairy cattle is likely an oversimplification of the complexity of the ruminal ecosystem and its effect on the host animal. Therefore, in some circumstances, the E_h could better reflect the fermentation dynamics than pH. On the contrary, the pH may better reflect the rumen function than E_h in other circumstances. The simultaneous measurement of ruminal E_h and pH at experimental level could be useful to provide complementary information about the rumen fermentation.

1.2. Ruminal E_h was related to classical parameters and could reflect the transfer and use of electrons in the rumen

As has been explained (Huang et al., in press; Huang et al., 2017), ruminal E_h is rarely reported in dairy cows mainly due to the difficult of measurement: the accurate ruminal E_h measurement requires strict anaerobic conditions which are not always satisfied (Marden et al., 2005). Our studies clearly showed that ruminal E_h is related to all main ruminal parameters.

Firstly, ruminal E_h is negatively related to pH. The analysis based on 15 studies from the literature (Huang et al., Japan) obtained a negative quadratic relationship between E_h and pH (E_h = - 4803 + 1579 pH - 134 pH^2, n = 24, P = 0.03). Further, the meta-analysis (Huang et al., 2017) of internal database (systematic measurements from 22 experiments conducted by our research team or in collaboration during last ten years) resulted in a similar relationship (E_h = –1697 +540.7 pH – 47.7 pH^2, n_{obs} = 70, n_{anim} = 26, $P < 0.001$).

Secondly, ruminal E_h is related to VFA profile. Based on measured ruminal VFA concentration from internal database (9 trials), we (Article 3) found that the increase of ruminal E_h was associated to the decrease of acetate proportion (Acetate % = 54.0 – 0.044 E_h, P = 0.016) and the increase of propionate proportion (Propionate % = 34.1 + 0.069 E_h, P = 0.002), and therefore related to the decrease of Acetate to Propionate ratio (A:P = 1.90 - 0.0068 E_h, P = 0.036). In addition, these results are consistent with that has been measured in cecum of pigs (Acetate % = 43.7 – 0.084 E_h, Propionate % = 47.6 + 0.13 E_h).

It was expected that ruminal E_h and these VFA parameters were related because they were all related to ruminal pH. Indeed, the proportion of acetate generally decreases during subacute acidosis to the benefit of an increase in the proportion of butyrate alone (Eadie et al., 1970, Michalet-Doreau and Morand 1996, Doreau et al., 2001), propionate and butyrate (Mackie et al 1978, Burrin and Britton 1986, Coe et al 1999) or propionate alone (Fulton et al 1979, Kennelly et al 1999, Hristov et al. 2001, Tajima et al 2001). According to Sauvant et al. (2006), A: P ratio = 3 corresponds approximately pH= 6.0, and A: P ratio < 3 could be consider as indicator of subacute acidosis. However, the mechanism of these changes is not clear, variations of ruminal pH cannot clearly explain that of VFA profile.

Our study (Article 3) hypothesized that E_h variations may be related to the transfer of electrons in the reactions producing VFAs in the rumen **(Table 14)**. For example, the conversion of carbohydrates to acetate by rumen microorganisms yields reducing equivalents, which may enhance the reducing power and decrease ruminal E_h (Wang et al., 2012). On the other hand, the formation of propionate consumes reducing equivalents which may lead to increase of ruminal E_h. This hypothesis is supported by the fact that ruminal methane production and acetate to propionate ratio are highly correlated (Russell 1998, Sauvant et al., 2011). Since methane is an electron sink products (CO_2 +8 H^+ + 8 e^- → CH_4 + 2 H_2O) that drives to a loss

of reducing power (Pidello 2014), its production might be inhibited with a higher E_h (lower reducing power) and promoted with a lower E_h (higher reducing power). Thus, the negative relationship between ruminal E_h and A:P ratio observed in the present study was expected. Indeed, the competition between propionate production and methanogenesis for reducing equivalents is widely recognized (Van Nevel and Demeyer 1977; Russell 1998; Ungerfield 2013).

Furthermore, by calculating the response of each ruminal parameters (difference between yeast treatment and control group) following live yeast supplementation, we (Article 4) found that the ruminal VFA responses were also significantly correlated to E_h response: the decrease of E_h following LY treatment was associated with the decrease of molar proportion of acetate and acetate to propionate ratio, it was associated with the increase of molar proportion of propionate **(Figure 19)**. **Therefore, the hypothesis that ruminal E_h was related to the transfer of electrons in the reactions producing VFAs become more reliable because the variations of these parameters were also related.**

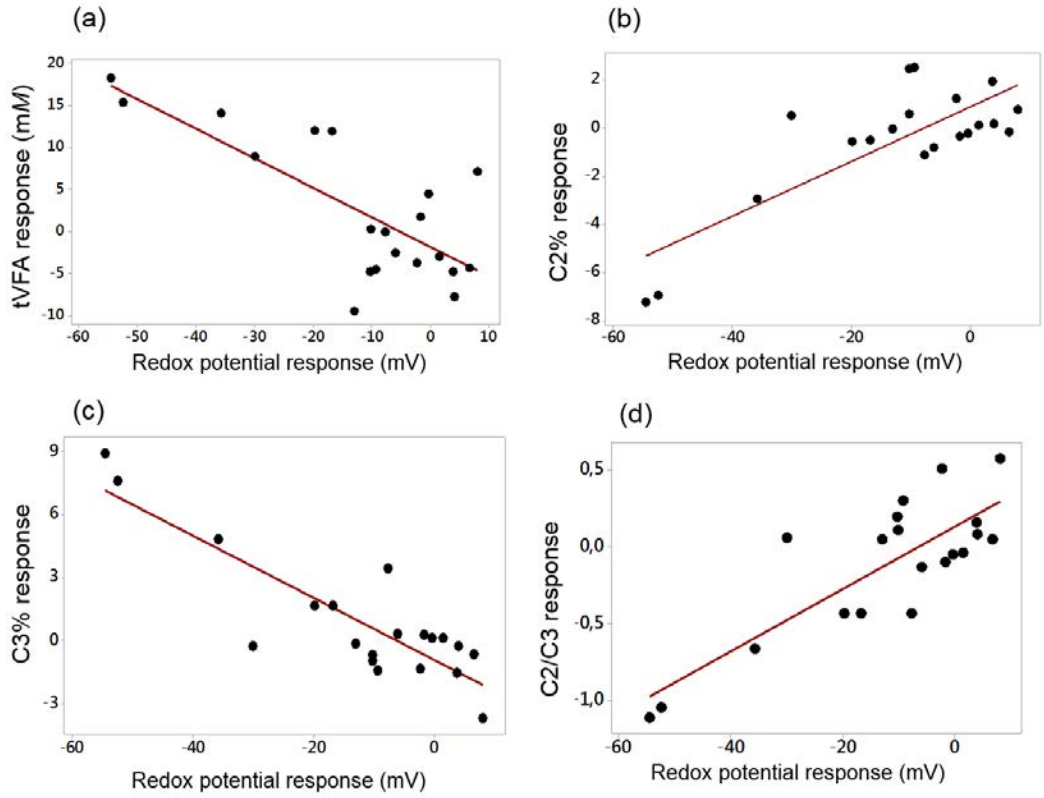

Figure 19. Relationship between ruminal VFA profile responses and that of E_h: (a) total VFA responses and that of E_h, (b) acetate proportion response and that of E_h, (c) propionate proportion response and that of E_h, (d) acetate to propionate ratio response and that of E_h (results from Article 4). Each point represents mean observation of one treatment, the read line represents the obtained equation.

1.3. Ruminal E_h was influenced by dietary characteristics

Our works demonstrated the influence of dietary characteristics on ruminal E_h. Based on 15 studies from the literature, Huang et al., (Japan) already found that ruminal E_h was positively correlated with DMI and proportion of concentrate in the diet, and was negatively correlated with NDF from forages. By using internal database, Huang et al. (2017) confirmed these findings, and further emphasized the influence of soluble sugars content in the diet on ruminal E_h. Latter, the in vivo trial on cannulated early lactating cows further validated the influence of soluble sugars. Although the experimental design of Huang et al. (Article 5) was planned to favor the effect of LY compared to the effect of diet, which allowed confounding effect between diet, period, and physiological stage, a pre-trial (Huang et al., 2017b) clearly demonstrated the increase of ruminal E_h during a dietary transition from a low to a higher level of soluble sugars. Thus, the dietary risk level of SARA could be estimated by the predictive equations resulted in our studies. **Although the dataset did not permit the integration of all dietary characteristics in one equation, the separated calculation of each factor can still indicate the risk level.**

2. Effect of LY on ruminal E_h in dairy cattle

2.1 Mode of actions of LY explained by E_h measurement

In agreement with previous studies, our quantitative analysis (Article 4) confirmed the effect of LY on most of ruminal parameters in lactating cows: the decrease in ruminal E_h and the increase in ruminal pH and rumen VFA concentration. LY supplementation also decreased the molar proportion of acetate and increased that of propionate, and decreased the A:P ratio. One of the main challenge of this work was to clarify the mode of actions of LY.

By specifying the mode of action of a chemical buffer and that of LY, Marden (2007) proposed the first mode of actions of LY taking into account the decrease of E_h following LY supplementation: LY would reinforce the reducing power in the rumen favoring the activity of strict anaerobic bacteria such as lactate utilizer and the conversion of lactate produced in the rumen to propionate would resulted in a stabilization of the ruminal pH despite the higher total VFA content.

In our experiment trial in early lactating cows (Article 5) we did not confirm the significant effect of LY on lactate concentration, but observed a numerical decrease of total lactate concentration following LY treatment in cows fed HS diet. In agreement with Julien (2010), we also observed a quick increase of lactate concentration right after the feeding (the peak occurred only one hour after feeding). Thus, the 4 sampling time (just before and 1, 2 and 4 hours after feeding) set out in our trial might be not well adapted to analysis this metabolite, the pic of increase could have occurred between the first (just before feeding) and second (1 hour after feeding) sampling. Also, accumulation of acids in the rumen may accelerate their absorption (Sauvant et al., 1999), this may reduce the difference between control and LY treatment, making LY effect more difficult to be observed. Nevertheless, it is clear that there was no accumulation of lactic acid as show by (Marden 2007), the experimental conditions and the digestive disorder level were different.

By calculating the response of each ruminal parameters following live yeast supplementation, Article 4 related for the first time the decrease of E_h following LY treatment to the decrease of molar proportion of acetate and acetate to propionate ratio, and to increase of molar proportion of propionate. As mentioned previously, the conversion of carbohydrate to acetate by rumen microorganisms yields reducing equivalents, whereas the formation of propionate consumes reducing equivalents (Wang et al., 2012). Therefore, we hypothesized that the decrease of ruminal E_h (increase of reducing power) introduced by LY might promoted the production of propionate and inhibited that of acetate, which could be interpreted to a better use of energy. **These findings strongly suggested that the effect of LY on VFA profile was achieved via the increase of reducing power, possibly reflected improved electron transfer and use in the rumen. Thus, these explications could complete the mode of action proposed by Marden (2007).**

Nevertheless, our experimental trial (Article 5) did not confirm the decrease of A:P ratio following LY supplementation resulted from previous quantitative analysis of Article 4 **(Figure 20).** Many other factors may affect the ruminal VFA concentration, such as absorption, as well as the amount of ruminal liquid into which the mass of VFA is diluted (Dijkstra et al., 1993; Hall et al., 2015). These factors may increase the variability of VFA concentration in the rumen making the effect of treatment non-detectable. Moreover, the effect of LY supplementation on VFA profile could be interfered by dietary characteristics. For example, in the study of Chademana and Offer (1990), LY supplementation tended to decrease the A:P ratio in sheep

fed high concentrated diet, and to increase the A:P ratio in sheep fed medium concentrate diet. Moreover, propionate is not the only end product of lactate utilizing bacteria, by the presence of glucose there was an increase in the production of butyrate, caporate, and valerate with a concurrent reduction in propionate (Marounek et al., 1989).

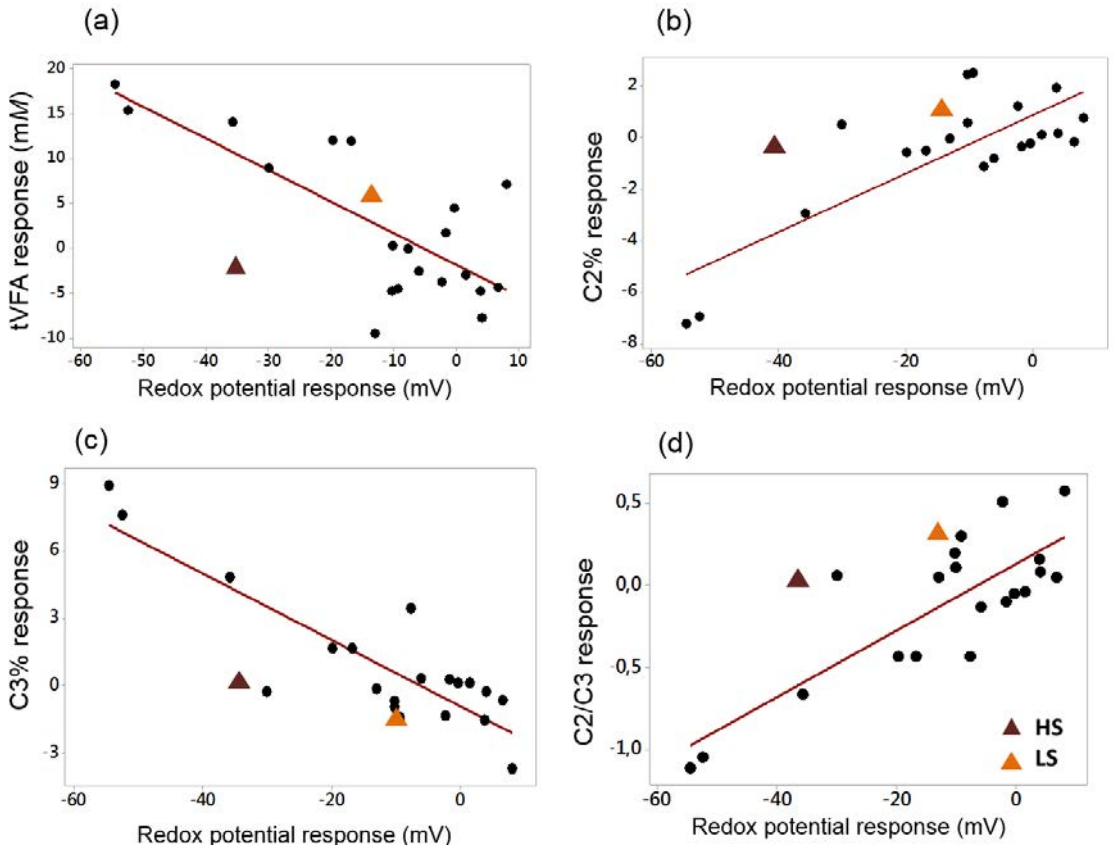

Figure 20. Positioning of results from experimental trial (Article 5) in the database of Article 4: relationship between (a) total VFA responses and E_h response, (b) acetate proportion response and E_h response, (c) propionate proportion response and E_h response, (d) Acetate to propionate ration. Each point represents mean observation of one treatment, the read line represents the obtained equation. HS: high soluble sugars diet; LS: low soluble sugars diet.

The analysis on microbiota of cows in the experimental trial (Article 6) by MiSeq illumina pyrosequencing confirmed some of previous findings observed in diets composed by high rapidly degradable carbohydrate, such as the increase of relative abundance of *Ruminococcus* and the decrease of *Ruminobacter*. The PLS-DA analysis on metabolomics data from Proton nuclear magnetic resonance (H-NMR) clearly discriminated rumen fluid samples of LY-supplemented from control cows fed HS diet while for samples from cows fed LS diet,

samples of LY-supplemented cows were not well distinguished that of control cows. This finding is in accordance with greater E_h response of LY supplementation in cows fed HS compare to LS diet. The changes of metabolites may resulted from enforcement of reducing power introduced by LY supplementation. This analysis took into account the integrated ^{1}H-NMR spectra, since few discriminant metabolites has been identified, some unidentified metabolites may also influence by LY supplementation. Further studies should be focused on these unidentified metabolites.

2.2 Prediction of LY effect on ruminal E_h

The work of Julien (2010) showed that *in vitro* on sterile ruminal contents, LY exerted an intrinsic reducing power, whereas *in vivo*, the effect of LY on reducing power of the rumen was variable and not systematic. In our work, Article 4 clearly quantified the relationship between the response of ruminal E_h and that of control group, which confirmed the proposition of Julien (2010). According to obtained equation, enforcement of reducing power by LY supplementation could be expected only when that of control group is higher than -189.5 mV. This result suggests that the regulation of ruminal E_h by LY would be particularly effective when risk of digestive disorder is high. Indeed, Julien (2010) based on trials from her PhD work has already observed that LY seems able to exercise a reducing power when the rumen E_h is higher than -174 mV, very close to the threshold resulted from our work.

Since dietary characteristics strongly influenced ruminal E_h, it becomes logical to question the possibility to predict the effect of LY depending on dietary characteristics**.** According to Article 4, the daily intake of soluble sugars (in g/day) significantly influenced the E_h response ($Y = 5.67 - 0.014\ X$, $P = 0.034$). The effect of LY on ruminal E_h was enhanced by the high amount of soluble sugars intake. According to the equation, the E_h response become negative as soon as the daily intake of soluble sugars exceed 405 g, and the decrease of E_h following LY supplementation could achieve 30 mV once the daily intake of soluble sugars reaches 2548 g. We then decided to verify the influence of soluble sugars in our experimental trial (Article 5 and 6), and **successfully confirmed a greater response of ruminal E_h (Figure 21) when cows were fed HS diet compared to LS diet** (-34.3 vs. -12.6 mV, P =0.048), already predicted by equation obtained from Article 4. Indeed, sugar ferment faster than starch, high sugar concentration presents a higher potential risk for acidosis (Nagaraja and Titgemeyer 2007; Lean et al., 2014). This may be the reason for both increase of E_h and decrease of pH. Thereby,

LY are able to outcompete lactate-producing bacteria for the utilization of sugars (Chaucheyras et al., 1996).

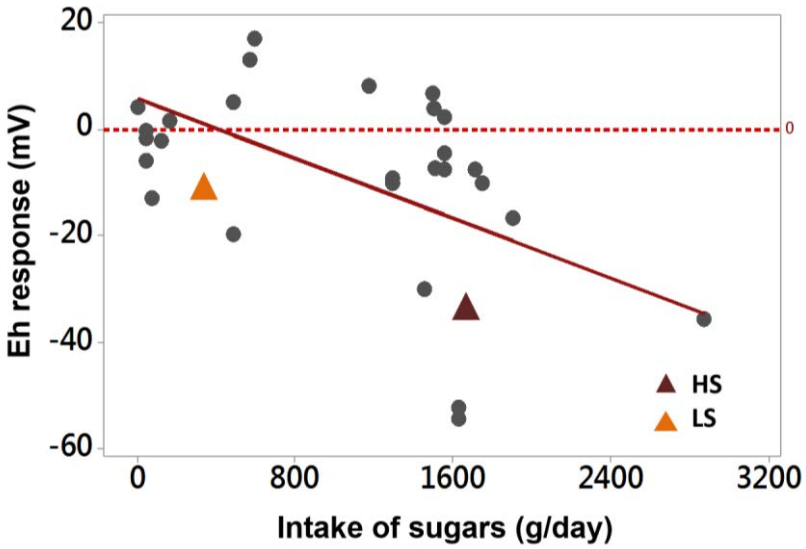

Figure 21. Positioning of E_h responses following LY supplementation from experimental trial (Article 5) in the database of Article 4. HS: high soluble sugars diet; LS: low soluble sugars diet.

3. Contributions for application purposes from this work

3.1 Prediction tool of ruminal E_h

Although there are still some inconvenient and difficulties to measure ruminal E_h on commercial farms, the risk level of a given diet could be evaluated by predictive equations according to its characteristics (Huang et al., 2017). The effect of LY on E_h should also be indirectly predicted by integrating the equations from Huang et al. (2017) and Article 4. The effect of LY supplementation in diets rich in soluble sugars can be evaluated directly by equation proposed by Article 4. In addition, there are already some simple tools such as methylene blue reduction test to use on commercial farms to evaluate proximately ruminal E_h (Lessire et al., 2017). Finally, as discussed previously, evaluation of rumen function should not relays on only one ruminal parameter, several parameters should be considered together.

3.2 Alternative indicators of acidosis

Urine pH

The effect of LY on urine pH in Article 5 provided a supplementary evident which confirmed its influence on acid-base balance of cows. In fact, the urinary excretion of proton is the only way for the animal to evacuate nonvolatile acids and is a major contributor to the acid-base balance of the animals (Shapiro et al., 1992; Patience and Chaplin, 1997). Since the sampling method is simple and noninvasive, urine pH may be used as an alternative indicator of acidosis under farm condition.

Ruminal Temperature

In Article 5, LY-supplementation decreased the maximum ruminal temperature. It has been reported (AlZahal et al., 2008, 2009), that duration of time above 39°C is negatively associated with ruminal pH and positively associated with duration of time with ruminal pH <6.0. Our observation probably reflected the effect of LY on SARA (e.g. increase of ruminal pH). LY-supplementation also increased the minimum ruminal temperature (Article 5), it is possible that this translated into more frequent drinking bouts per day. Since gas production and NDF disappearance would be reduced when incubation temperature was below 39 °C (Petersen et al., 2016), the increase of minimum ruminal temperature in our study provided evidence that the yeast supplementation was having some effect on the rumen environment. Ruminal temperature is relatively stable and easier to record compare to ruminal redox potential and pH. Although the effect of LY on eating and drinking behavior of animal remains to be elucidated in future studies, great application potential of ruminal temperature measurement was put forward to monitoring the rumen function on commercial farms.

4. Limits and perspectives for future studies

4.1. Meta-analysis of existing data from experiments

Meta-analysis permit to summarize and quantify knowledge acquired through previously conducted studies. For factors that had only a secondary or minor role in prior experiments, meta-analysis of a great number of results can provide a better understanding of them and generate some hypothesis for future studies. Our work confirmed the great interests of meta-analysis of existing data from previously conducted experiments. Especially for studies

focus on rarely investigated parameters, such as ruminal E_h. Although in some cases, we may have limited number of data to establish reliable prediction models, quantitative analysis of all exiting results on candidate factors at least provided better understanding of main factor influencing ruminal E_h. However, cautions should be taken during each step of analysis, to verify the relevancy of selected data, and of results obtained. The chose of analysis and interpretation of results should be based on existing knowledges on correspondent subject.

4.2. Measurement of ruminal E_h

The sensor of E_h measurement usually takes longtime to become stable, which is one of the main difficulties for its measurement in biological conditions. Andrade et al. (2002) then proposed a 25-min stabilization period for *in vitro* measurement of rumen fluid samples. This method was not considered in our studies since air contamination could highly influence the measured values (Marden et al., 2005). Although the *ex vivo* proposed by Merden et al. (2005) excluded the influence of air contamination, the measured values were still different compare to in vivo measurement by submersible data logger (Huang et al., 2016). According to *ex vivo* method, rumen fluid was pumped continuously through a rubber tube into a 50-mL-double-walled thermocontrolled vessel outside the rumen, the E_h was measured by electrodes dipped in the collected rumen fluid. Submersible data logger resulted lower E_h values, which suggests that the redox condition in the thermocontrolled vessel used by *ex vivo* method could not exactly reflect that in the rumen. In our meta-analysis, we corrected E_h values from *in vivo* method according to the analysis of Huang et al. (2016) in order to facility the discussion by taking previous published results (which used *ex vivo* method) as reference. Since the values measured by submersible data logger are more relevant, the difference between two methods should be taken into account in use of equations and threshold E_h values proposed by our studies based on meta-analyses. Nonetheless, the effects of LY on ruminal E_h resulted from all studies are relative to correspondent control groups, thus the responses of ruminal E_h by LY supplementation are comparable as demonstrated by **Figure 3**. Finally it is still not certain that the E_h values measured by our submersible data loggers are real E_h values because the calibration can only be done with positive standard buffer solution (e.g. standard buffer solution of +220 mV was used in our studies), and no negative standard buffer solution is available today to verify the devices. Since development of materials and methods is very quick, improved measurement method in near future could be helpful for researches on ruminal E_h.

4.3. Experimental design

The 4 by 4 crossover design in our experimental trial (Article 5 and 6) was not an optimal one. It was planned to favor the investigation of LY effect: cows received HS diet during the two first periods and then changed to LS diet during the two last periods; in each period, cows were randomly divided into two groups (LY supplemented group vs. control group) to compare the effect of LY. Therefore, the diet effect was confounded to the effect of lactating stage of cows. For example, it is difficult to conclude if the higher DM (19.7 vs. 23.6 kg/d, $P < 0.001$) and water intake (75.2 vs. 80.3 l/d, $P = 0.003$) of LS groups compare to HS groups should be attributed to diet effect or effect of physiological stage of cows.

In fact, before the trial, two cows were in suboptimal status and risked to be excluded from the trial. In Latin square design, exclusion of one animal could greatly imbalance the experimental groups, while in crossover design the cows can be divided only into two groups in which the imbalance could be corrected easier, and the results could still be useful. Therefore, in future studies, it would be better to use a Latin square design for similar experimental trials when experimental conditions are optimal.

CONCLUSION

The aim of this work was to provide better understanding of the mode of actions of LY, and to define the optimal condition of LY utilization in dairy cattle. In order to meet this aim, we investigated the interaction between LY, ruminal parameters and dietary characteristics.

By using quantitative analysis of existing data from previously conducted experiments, we clarified the relationship between ruminal redox and other main ruminal parameters such as pH and VFA profile. Ruminal E_h was related to all classical ruminal parameters and can provide additional information about rumen function. **The results suggested that E_h variations might be related to the transfer of electrons in the reactions producing VFAs in the rumen.** Latter, by calculating the response of each ruminal parameters (difference between yeast treatment and control group) following live yeast supplementation, we found that the ruminal VFA responses were also significantly correlated to E_h response. These results confirmed that ruminal E_h was related to the transfer of electrons in the reactions producing VFAs and further suggested that the effect of LY on VFA profile was achieved via the increase of reducing power, possibly reflected improved electron transfer and use in the rumen. **Therefore, the increase of reducing power might not only favorite the activities of fibrolytic and lactate utilizing bacteria but also improved electron transfer and use during the fermentation in the rumen. These findings could complete the mode of actions of LY proposed by Marden (2007).**

We successfully quantified the influence of dietary characteristics on ruminal E_h. Although the dataset did not permit the integration of all dietary characteristics in one equation, the separated calculation of each factor can still indicate the risk level. The analysis also demonstrated that the regulation of ruminal E_h by LY would be particularly effective when risk of digestive disorder is high. By associating of these equations, the effect of LY in a given diet could be indirectly estimated. Since the analysis related the E_h response following LY to daily soluble sugars intake, and the *in vivo* experiment validated this relationship, the effect of LY supplementation in diets rich in soluble sugars can be evaluated directly by equation proposed. **Therefore, the findings of this work provided some tools to define the optimal condition of LY utilization.**

The *in vivo* experiment in early-lactating cows not only confirmed greater effect of LY on ruminal Eh in diet rich in soluble sugars, but also demonstrated that i) LY supplementation tended to impact the richness of the liquid-associated bacterial fraction, and ii) some unidentified metabolites were also changed following LY supplementation, probably associated to the decrease of ruminal E_h.

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ANNEX

In Proceedings of the 20th congress of the ESVCN. 15-17 September 2016, Berlin, Germany.

Relationship between ruminal redox potential and pH in dairy cattle

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Introduction: The ruminal redox potential (E_h) and pH provide the basis for understanding of microbiological activity[1,2]. A high ruminal E_h might be associated to a low pH and vice versa[1], but the relationship between ruminal E_h and pH is not precisely quantified. Since the E_h is very sensitive to oxygen contamination, its measurement must be carried out under anaerobic conditions[3]. However, few studies have measured the ruminal E_h in this way. This study investigated the relationship between ruminal E_h and pH by meta-analysis of a large database.

Animals, material and methods: A total of 642 kinetics of ruminal E_h and pH measurements from 20 experiments carried out with both lactating (449 kinetics) and dry cows (193 kinetics) were gathered together. The composition of the diets was very varied (e.g. the proportion of concentrate ranged from 0 to 63%). Two measurement methods (both under anaerobic conditions) were used: the *ex vivo* method[3] and the *in vivo* method using a submersible data logger (Dascor, Escondido, CA). For all the experiences, each kinetic includes 10 measurements of ruminal E_h and pH from one hour before the diet distribution to 8 hours after. The average E_h (ranged from -290.7 to -82.5 mV) and pH (ranged from 5.09 to 6.92) values were calculated for each kinetic. The relationship between average E_h and pH values was analyzed using a mixed model, the effect of the measurement method was investigated as qualitative factor.

Results and discussion: Ruminal E_h was negatively related to pH (E_h = 111.0 - 50.2 pH, $P < 0.001$, RSD = 20.5, R^2 = 54.0%, figure 1). The method effect was significant ($P < 0.001$): the E_h value measured by *in vivo* method was 35.4 mV lower than by *ex vivo* method (interaction between measurement method and pH: $P = 0.219$). This could be explained by the difference of sensors and location of measurements.

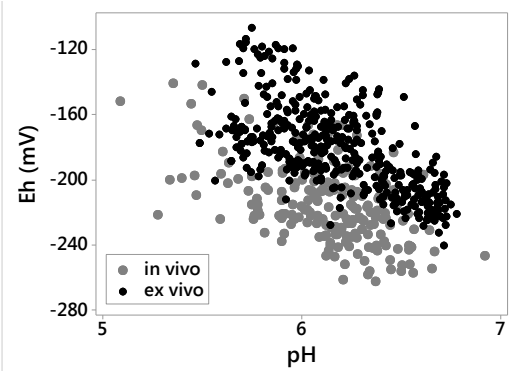

Figure 1: Relationship between ruminal redox potential (E*h*) and pH by two measurement methods (*in vivo* and *ex vivo*)

Conclusion: The ruminal E_h was negatively related to pH, both ruminal E_h and pH could reflect the fermentation activity in the rumen. The influence of the measurement method should be considered in the future studies.

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Table 1 (abstract 432). Ruminal Eh and pH in lactating dairy cows fed increasing proportions of readily fermentable carbohydrates

	Treatment					
	Basal diet	Diet 1	Diet 2	Diet 3	SEM	*P*-value
Daytime ruminal Eh, mV	−317.7[a]	−292.7[ab]	−280.8[b]	−261.0[b]	42.9	<0.001
24 h Ruminal Eh, mV	−318.3[a]	−291.6[ab]	−282.7[b]	−261.9[b]	42.3	<0.001
Daytime ruminal pH	6.25[a]	6.16[b]	6.13[b]	5.95[b]	0.17	<0.001
24 h Ruminal pH	6.25[a]	6.14[b]	6.09[bc]	5.93[c]	0.17	<0.001

nitrogen ($P \leq 0.01$). Total volatile fatty acids did not change with transition from AH to CS, but decreased with transition from CS to AH. Nonetheless, after transition back to original forage in the third period, rumen fermentation variable returned to initial levels with no difference from those on d 0. Our finding suggested that abrupt forage substitution with large nutrients difference could influence rumen function during the immediate transition to some extents, but it can eventually recover within 2 wk without detrimental effects. The first 6 d after forage transition when the rumen fermentation was critically disrupted are the key times that need further concern.

Key Words: forage transition, rumen fermentation variables, sheep

432 Changes in ruminal redox potential and pH of lactating cows during a dietary transition. Y. Huang*[1], J. P. Marden[2], C. Julien[2], E. Auclair[2], G. Hanna[1], and C. Bayourthe[1], [1]*GenPhySE, Université de Toulouse, INRA, INPT, INP-ENVT, Castanet-Tolosan, France,* [2]*Phileo Lesaffre Animal Care, Marcq-en-Baroeul, France.*

The objectives of the present study were (i) to investigate the changes in ruminal E_h and pH of lactating cows during a dietary transition from a low to a higher level of readily fermentable carbohydrates (RFC), and (ii) to compare the daytime and 24-h measurement of these 2 parameters. The experiment lasted 37 d. Eight early (averaged 47 DIM) lactating Holstein cows fitted with ruminal cannulas were fed a basal diet (67.7% maize silage, 10.8% alfalfa hay, and 21.5% concentrate, DM basis) with low level of RFC (% DM) (1.4% of soluble sugars, 18.2% of starch) for 21 d. Thereafter, they were fed 3 successive diets (containing 3.5%, 5.6% and 8.6% soluble sugars; 16.4%, 17.7%, 19.4% starch, respectively) at d 22, d 27 and d 32 to manage a progressive transition. Diets were offered *ad libitum* in equal amounts twice daily. The DMI and milk production were recorded individually. Ruminal E_h and pH were continuously measured for 3 d at the end of each dietary treatment, by using a ruminal submersible data logger (Dascor, Escondido, CA). The E_h and pH data were summarized as mean E_h and pH over daytime (from 1 h before morning feeding to 8 h after) and over 24 h. Dry matter intake ($P = 0.361$) and milk yield ($P = 0.868$) did not change during the dietary transition: in average 18.2 kg DM/d and 32.6 kg/d respectively. Increasing proportions of dietary RFC increased significantly E_h (+ 56 mV) and decreased pH (- 0.32). Compare with mean daytime pH, mean pH over 24 h allows a better distinction between treatments (Table 1). In conclusion, a long-term continuous 24-h measurement shows an effect of increased proportions of RFC in the diet on the diurnal pattern of ruminal E_h and pH.

Key Words: redox potential, rumen, dietary transition

433 Impact of dietary starch concentration formulated with two types of corn silage on the performance of dairy cows. J. I. Sanchez-Duarte*[1] and K. F. Kalscheur[2], [1]*South Dakota State University, Brookings, SD,* [2]*US Dairy Forage Research Center, USDA, ARS, Madison, WI.*

This study explored the effect of feeding different starch concentrations and conventional or brown midrib corn silage on the performance of lactating dairy cows. Forty-eight Holstein cows were assigned to 1 of 4 diets using a randomized complete block design with a 2-wk covariate period followed by 8-wk experimental period. Experimental diets were arranged as a 2 × 2 factorial with 2 types of corn silages [conventional (CS) and brown midrib (BMR) corn silage] and 2 dietary starch concentrations (19 and 25% of DM). Diets were formulated to contain 60.7% forage and 39.3% concentrate on DM basis. Dried corn grain was replaced with soyhulls and beet pulp to decrease dietary starch concentration. Cow was the experimental unit. Silage × starch interactions were detected ($P \leq 0.05$) for milk yield, energy-corrected milk (ECM), and feed efficiency (ECM/DMI). Milk yield was similar between cows fed BMR-25% starch and CS-19% starch, but was greater than for cows fed other diets. ECM was greatest for cows fed BMR-25% starch compared with the other 3 diets. Feed efficiency was greatest for cows fed CS-19% starch and BMR-25% starch and least for cows fed BMR-19% starch. Milk protein percentage was affected by starch concentration, resulting in greater protein concentration for cows fed 25% starch compared with cows fed the 19% starch diet. There was no effect of diet on DMI, milk fat percentage, milk fat and protein yield, and MUN. Overall, the milk and ECM of lactating dairy cows was superior when feeding BMR-25% starch, but cows fed BMR-19% starch responded similarly to cows fed CS diets at either 19 or 25% starch.

Table 1 (abstract 433).

	CS		BMR			
Item	19%	25%	19%	25%	SEM	$P > F$[1]
DMI, kg/d	25.9	26.8	27.0	26.8	0.54	NS
Milk, kg/d	44.1[ab]	43.4[b]	43.4[b]	45.7[a]	0.69	I
ECM, kg/d	45.1[b]	44.9[b]	44.2[b]	46.8[a]	0.71	I
ECM/DMI	1.76[a]	1.69[ab]	1.66[b]	1.76[a]	0.04	I
Fat, %	3.83	3.89	3.87	3.88	0.09	NS
Fat, kg/d	1.66	1.68	1.63	1.73	0.05	NS
Protein, %	2.91	3.00	2.90	2.98	0.04	S
Protein, kg/d	1.27	1.27	1.23	1.32	0.02	NS
MUN, mg/dL	11.1	10.5	11.4	11.0	0.35	NS

[ab]Means with different superscripts differ ($P \leq 0.05$).

[1]S = starch effect; I = silage by starch effect; NS = not significant.

Key Words: BMR corn silage, milk yield, starch concentration

434 Effects of replacing corn with different levels of starch degradability with beet pulp as a source of soluble fiber on fermentation in continuous culture. L. E. Koch*, B. M. Koch, R.

105 Improvement of ruminal fermentation by live yeast in dairy cows. Y. Huang*[1], J. P. Marden[2], C. Julien[2], E. Auclair[2], and C. Bayourthe[1], [1]*GenPhySE, Université de Toulouse, INRA, INPT, INP-ENVT, Castanet-Tolosan, France,* [2]*Phileo Lesaffre Animal Care, Marcq-en-Baroeul, France.*

Supplementation of live yeast (LY) in the diet is an interesting practice to limit the negative effects of SARA. Measurement of ruminal redox potential (E_h) has been shown to be a tool to understand the mode of action of LY in rumen. The objective of this study was to quantify the effect of LY (5g/d of *Saccharomyces cerevisiae*, 10^{10} cfu/g DM, CNCM I-4407, Phileo Animal Care, France) on ruminal E_h of lactating and dry cows via quantitative analysis of data from 16 experiments (including 27 LY treatments) conducted by our research team. A total of 575 kinetics (each established from morning feeding to 8 h after) of ruminal E_h and pH were gathered together. Yeast effect on ruminal E_h, pH, VFA and ammonia concentration was tested qualitatively (control vs. LY). The relationship between response of ruminal E_h (difference between yeast treatment and control group) and that of control group was analyzed by a liner model. Thereafter, the relationships between response of E_h and response of VFA and NH_3 concentration were also analyzed by liner model. In lactating cows, addition of LY significantly decreased ruminal E_h (from – 173.5 to – 186.2 mV, $P < 0.001$) and increased pH (from 5.94 to 6.11, $P < 0.001$) and total VFA content (from 92.3 to 99.2 m*M*, $P < 0.001$). In dry and lactating cows, analysis of relationship between E_h response and E_h of control groups showed that the regulation of ruminal E_h by LY would be particularly efficient when risk of digestive disorder is high i.e., E_h control > –195.7 (E_h response = – 72.4 – 0.37 E_h control, n = 27, $P < 0.01$, $R^2 = 0.33$, RSD = 14.6). Moreover, E_h response is associated with the increase of VFA content response (E_h response = – 8.2 – 1.63 VFA response, n = 20, $P < 0.001$, $R^2 = 0.57$, RSD = 12.2) and the decrease of ammonia content response (E_h response = – 6.5 + 0.41 NH_3 response, n = 18, $P < 0.05$, $R^2 = 0.34$, RSD = 15.9), which suggest an improvement of ruminal fermentation by LY.

Key Words: rumen, live yeast, ruminal redox

106 Evaluation of supplementing brewer's yeast to lactating dairy cows. T. C. Aubrey*[1], J. L. Anderson[1], and A. R. Boyer[2], [1]*Dairy and Food Science Department, South Dakota State University, Brookings, SD,* [2]*Kent Nutrition Group, Muscatine, IA.*

The objective of the study was to evaluate supplementing concentrated brewer's yeast in the ration of dairy cows on lactation performance. We hypothesized that diets containing a concentrated brewer's yeast supplement would benefit feed efficiency and increase milk and component yields. Thirty-six Holstein cows (24 multiparous and 12 primiparous; DIM = 71.17 ± 16.42) were used in an 8-wk randomized complete block design experiment. Cows were blocked by milk yield, DIM, and parity. Treatments included (1) control with no yeast (CON), (2) a concentrated brewer's yeast product (Y1), and (3) a commercial yeast product (Y2). Cows were fed a common TMR, except for yeast supplements (14.2 g/h/d), once daily at 0800h using the Calan Broadbent feeder system to determine daily individual DMI. Cows were housed in a free stall barn and milked 2×/d and all milk weights were recorded. One day each week milk samples were collected for compositional analysis. Body condition scores (BCS) and body weights were obtained each week. Blood for plasma urea nitrogen (PUN) analysis was taken during wk 7 and 8. Data were analyzed using MIXED procedures with repeated measures and means were compared using Tukey's test. Dry matter intake was similar (24.2, 24.6 and 24.1 kg/d for CON, Y1, and Y2, respectively; SEM = 0.82; $P = 0.88$); but there was a week by treatment interaction ($P < 0.01$) with cows fed Y1 having greater DMI during wk 2, 3, 4 of the study. Milk production (34.6, 34.6, 33.2 kg/d; SEM = 0.82; $P = 0.28$), milk fat (1.32, 1.29, 1.29 kg/d; SEM = 0.068; $P = 0.41$), and protein (0.97, 0.96, 0.94 kg/d; SEM = 0.033; $P = 0.84$) yields and other components were similar ($P > 0.05$) among treatments. Feed efficiencies, calculated as energy corrected milk/DMI, were similar among treatments (1.51, 1.36, 1.51; SEM = 0.063; $P = 0.15$), but there was a treatment by week interaction ($P < 0.01$). A treatment effect for PUN was detected (16.86, 14.10, 16.15; SEM = 0.444; $P < 0.01$). No statistical significance was determined for BCS and body weights ($P > 0.05$). Yeast products maintained performance, rather than improving production as hypothesized.

Key Words: yeast supplement, lactation performance, dairy cow

107 Effects of *Saccharomyces cerevisiae* fermentation products and subacute ruminal acidosis (SARA) on apparent digestibility of dry matter, NDF, and phosphorus in lactating dairy cows. V. P. Senaratne*[1], H. Khalouei[1], K. Fehr[1], J. Guo[1], I. Yoon[2], E. Khafipour[1], and J. C. Plaizier[1], [1]*Department of Animal Science, University of Manitoba, Winnipeg, Canada,* [2]*Diamond V, Cedar Rapids, IA.*

The effects of *Saccharomyces cerevisiae* fermentation products (SCFP) on the apparent digestibilities of dry matter (DM), neutral detergent fiber (NDF) and phosphorus (P) in lactating cows during control feeding and during grain-based subacute ruminal acidosis (SARA) challenges were investigated. Thirty-two Holstein lactating dairy cows were assigned to 4 treatments, i.e., control, and 3 different SCFP supplementations. Cows in the 3 SCFP treatment groups received 14 g/d Diamond V Original XPC (XPC), 19 g/d NutriTek (NTL), or 38 g/d NutriTek (NTH) mixed with 126, 121, and 102 g/d ground corn, respectively, while the cows in the Control group received 140 g/d ground corn only. Supplements were top dressed once daily immediately after feed delivery from 4 wk pre-calving to 11 wk post-calving. At wk 5 and 8 after calving one-week grain-based SARA challenges were conducted by switching from a lower to a higher concentrate diet (50% to 70% concentrate, DM basis). Diet samples were collected weekly and fecal samples of individual cows were collected twice weekly. Samples were pooled for wk 1–4 after calving (preSARA), wk 5 after calving (first SARA challenge), and wk 8 after calving (second SARA challenge). Samples were analyzed for DM, acid insoluble ash (AIA), NDF and P (% DM basis). Apparent total-tract digestibilities for DM, NDF and P were calculated using AIA as an internal marker. The apparent total-tract digestibility of DM and P were not affected by the SARA challenges and SCFP, and averaged 68.9 and 52.6%, respectively, across treatments and weeks. The SARA challenges reduced the apparent total-tract digestibility of NDF from 61.1 to 49.0% ($P < 0.01$), but the NTH supplementation increased NDF digestibility from 52.7 to 61.8% ($P < 0.02$). Our results show that SCFP can increase fiber digestion, which is particularly important during high grain feeding.

Key Words: dairy cow, SARA, *Saccharomyces cerevisiae* fermentation product

108 Effects of *Saccharomyces cerevisiae* fermentation products on endotoxins and acute phase proteins in lactating dairy cows. J. Guo[1], H. Khalouei[1], K. Fehr[1], V. Senaratne[1], Z. Zhang[1], H.

Abstract

Ruminal acidosis is one of the major concerns of current dairy farms. Live yeasts (LY) have been extensively studied and used in dairy cows for stabilization of rumen fermentation. Recently, measurement of ruminal redox potential (E_h, in mV) has been considered as an interesting tool to indicate ruminal fermentation disorder. The positive effect of LY on ruminal E_h has been reported, but it remains variable according to the experimental conditions. The aims of this work was to provide better understanding of mode of actions of LY, and to define the optimal condition of LY utilization in dairy cows. The first part of this work consisted to quantitative analysis of existing results from 22 experiments with cannulated dairy cattle. The second part of this work consisted to verify some of the results from quantitative analysis by an *in vivo* experiment in lactating cows. By using quantitative analysis of existing data from previously conducted experiments, we clarified the relationship between ruminal redox and other main ruminal parameters such as pH and VFA profile, and suggested that E_h variations might be related to the transfer of electrons in the reactions producing VFAs in the rumen. Moreover, response of ruminal E_h following live yeast supplementation was also related to that of ruminal VFA profile, which suggested that the effect of LY on VFA profile was achieved via the increase of reducing power, possibly reflected improved electron transfer and use in the rumen. The analysis further demonstrated that the regulation of ruminal E_h by LY would be particularly effective when risk of digestive disorder is high. Since the influence of dietary characteristics on ruminal E_h was quantified, the effect of LY in a given diet could be indirectly estimated. In addition, quantitative analysis also associated the response of ruminal E_h following LY supplementation to the intake of soluble sugars. The *in vivo* experiment in early-lactating cows confirmed greater effect of LY on ruminal E_h in diet rich in soluble sugars, and further demonstrated that i) LY supplementation tended to impact the richness of ruminal bacteria, and ii) some unidentified metabolites were also influenced by LY supplementation, probably associated to the decrease of ruminal E_h.

Résumé

L'acidose ruminale est l'une des préoccupations majeures des exploitations laitières actuelles. Les levures vivantes (LV) ont été largement étudiées et utilisées chez les vaches laitières pour stabiliser la fermentation ruminale. Récemment, la mesure du potentiel redox ruminal (E_h, en mV) a été considérée comme un outil intéressant pour indiquer le trouble de la fermentation ruminale. L'effet positif de LV sur E_h ruminal a été rapporté, mais il reste variable selon les conditions expérimentales. Les objectifs de ce travail étaient de fournir une meilleure compréhension du mode d'action de LV et de définir la condition optimale de l'utilisation de LV chez les vaches laitières. La première partie de ce travail a consisté en une analyse quantitative des résultats de 22 expériences avec des vaches laitières canulées. La deuxième partie de ce travail a consisté à vérifier certains des résultats de l'analyse quantitative par une expérience chez des vaches en lactation. En utilisant l'analyse quantitative de données existantes provenant d'expériences antérieures, nous avons clarifié la relation entre le E_h ruminal et d'autres paramètres ruminaux principaux tels que le pH et le profil VFA, et suggéré que les variations de E_h pourraient être liées au transfert d'électrons dans les réactions dans le rumen. En outre, la réponse du E_h après la supplémentation en LV était également liée à celle du profil AGV ruminal, suggérant que l'effet de LV sur le profil VFA était atteint par l'augmentation du pouvoir réducteur, reflétant un meilleur transfert d'électrons dans le rumen. L'analyse a en outre démontré que la régulation du E_h ruminal par LV serait particulièrement efficace lorsque le risque de troubles digestifs est élevé. Puisque l'influence des caractéristiques de la ration sur le E_h ruminal a été quantifiée, l'effet de LV dans un régime donné pourrait être estimé indirectement. En outre, l'analyse quantitative a également révélé que la réponse de E_h suite à la supplémentation en LV était associée à la quantité de sucres solubles ingérée. L'expérience *in vivo* chez des vaches en début de lactation a confirmé un effet plus important de LV sur E_h ruminal avec une ration riche en sucres solubles, et a démontré que la supplémentation en LV avait un impact sur la richesse des bactéries, et que les métabolites ont également été influencés par la supplémentation en LV, probablement associée à la diminution du E_h ruminal.