Chapter 7

Use of Yeast Probiotics in Ruminants: Effects and Mechanisms of Action on Rumen pH, Fibre Degradation, and Microbiota According to the Diet

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1. Introduction

The valorization of fibrous feed sources by ruminants is possible thanks to their unique digestive system involving an intensive preliminary ruminal fermentation step prior to a more classical enzymatic phase. The reticulo-rumen hosts a highly specialized anaerobic microbial community responsible for fibre breakdown, which is influenced by biochemical and microbial characteristics of the rumen environment. In particular, the role of the different microbial species involved in pH regulation and the influence of feed management are presented in section 2. Indeed, intensive farming pratices may disturb the microbial balance due to an excessive high fermentable carbohydrate supply required to sustain high animal performance, and it can turn into metabolic disorders that are likely to impact animal health as reviewed in section 3. This is one area where yeasts probiotics can help the ruminant and the feed nutritionist optimizing the cows nutrition owing to an increasingly well understood proper mode of action. Section 4 reports the positive effects these feed additives, under the form of active dry yeast, have on rumen fermentation, feeding behaviour and feed efficiency, as well as tips to properly assess these effects.

Once the optimal rumen conditions are set up (section 6), fibre will be efficiently digested. It becomes then interesting to dive into the world of the fibrolytic microbiota in section 5 to truly percieve the unicity of the fibre rumen degradation process, bearing in mind that the nature of fibre will impact its digestibility and subsequent animal production response. In addition to its role on rumen pH stabilization that directly affects the fibrolytic microflora, yeast probiotics represent a valuable tool to optimize cow nutrition as detailed in section 7.

However, section 8 will emphasize the yeast strain effect and the need of a viable feed additive to be able to offer a comprehensive solution to ruminants' diet formulation. Finally, besides the clearly established benefits on rumen management and fibre degradation, live yeast as probiotics are also currently being assessed in other promising fields of applications (section 9).

2. Rumen pH: A key parameter linked to rumen function

Due to intense microbial activity, fermentation of feedstuffs in the reticulo-rumen produces a wide range of organic acids. Some of these acids can accumulate and reduce ruminal pH if rumen buffering systems are unable to counteract their impact. Low rumen pH for prolonged periods can negatively affect feed intake, microbial metabolism, and nutrient degradation, and leads to acidosis, inflammation, laminitis, diarrhea and milk fat depression. High yielding dairy cows and fattening beef cattle fed diets rich in readily fermentable starch or sugars at high feed intake levels are particularly susceptible to acidosis, and goats, sheep and other ruminants are also prone to the disease. It is now recognized that subacute ruminal acidosis (SARA) affects from 10% to 40% of dairy cattle in a herd, resulting in large financial losses and major concern for animal welfare reasons. Therefore, rumen pH regulation is a key determinant in the maintenance of an optimal rumen function.

2.1. How to measure rumen pH accurately

Common field techniques for pH measurement have been relied on collection of samples by rumenocentesis or oral stomach tubing [1,2]. Rumenocentesis has proven to be a more reliable technique for the determination of ruminal pH than oral stomach tubing because saliva contamination is often associated with the stomach tubing technique [3,4]. If rumenocentesis may be done with minimal disturbance [5], frequent sampling raises ethical issues and is not without risk for the animal health. Enemark et al. [2] conducted a study to evaluate the potential of biochemical markers in blood, feces, and urine to predict ruminal pH. They concluded that no peripheral markers could properly predict ruminal pH. A permanent surgical modification, such as rumen cannulation, and the use of an external data logger connected to a pH probe immerged into the rumen [3,6] have been successful in well controlled research studies to monitor rumen pH kinetics, which allow to better characterize microbial fermentations and predict acidosis situations. Recently, telemetric boluses able to measure and record rumen pH in cattle continuously have been developed by different companies. When interrogated by wireless, the bolus transmits the recorded data to an operator standing beside the cow with a receiving station. These rumen pH boluses methods offer a simple, accurate and long lasting measurement of pH in intact cattle [7]. They have been successfully applied in controlled animal studies and offer the opportunity to link pH kinetics to measurements in field situations, but clarifications are still needed about the location of the probes (reticulum, rumen) and thereby the representativeness of the measure, their calibration, long-term measure accuracy, and life time. Moreover, the cost of these

systems are still high and the current proposed boluses are not yet applicable to non cannulated small ruminants.

2.1.1. Microbial mechanisms which lead to pH modulation and acidosis

Rumen microbial populations hydrolyze and ferment dietary compounds into volatile fatty acids (VFAs), whose amounts drive pH evolution. Moreover, lactic acid is a common product of carbohydrate fermentation, produced by bacterial species such as *Streptococcus bovis*, *Selenomonas ruminantium*, *Mitsuokella multiacidus, Lachnospira multipara* or *Lactobacillus sp*. *S. bovis* is considered as a major contributor in lactate production from high fermentable diets. Indeed, it is able of very rapid growth, is acid-resistant and produces extracellular and intracellular amylases which hydrolyze raw starch and soluble starch, respectively [8]. Moreover, it has been shown that *S. bovis* produces mainly L-lactate under moderately acidic pH but shifts its metabolism towards D-Lactate production when the pH decreases [9], this latter isoform being more toxic as it is less efficiently re-utilized by the microbiota and the animal tissues. *Megasphaera elsdenii* is considered as the predominant lactate-utilizing bacterial species in the rumen and can be found in large numbers in the rumen of cereal grain-fed cattle [10]. *Selenomonas ruminantium* subsp *lactylitica* is another important lactate-utilizing species. Contrary to *S. ruminantium*, *M. elsdenii* is not submitted to catabolite repression by soluble sugars [11] and ferments lactate to propionate via the acrylate pathway [10]. It exhibits also a lactate racemase activity which is involved in the conversion of D- into L-lactate, which is more easily metabolized. Nevertheless, with high amounts of readily fermentable carbohydrates, or during adaptation from forage to concentrate diets, acid overload of the rumen is possible and may lead to a strong decline in rumen pH, which may trigger acidosis in cattle [1]. Indeed, as rumen pH falls, lactate producers may outnumber lactate utilizers, leading to an accumulation of this metabolite in the rumen. Due to the low pK_a (3.7) of lactic acid compared to the pK_a of the major VFAs (4.8-4.9 for acetate, propionate and butyrate), even low amounts of lactic acid may play a major role on the onset of acidosis. If rumen pH continues to fall, *Lactobacilli* may replace *S. bovis*, initiating a spiraling effect with excessive D-lactate accumulation [9].

Thanks to their capacity to engulf and slowly ferment starch granules into VFAs (particularly butyrate), rumen protozoa can compete with lactate-producing amylolytic bacteria and lactic acid can be actively taken up by entodiniomorphid ciliates [12]. Overall these processes have a beneficial effect on pH stabilization and may participate to limit the severity of acidosis.

2.1.2. Effect of the diet on rumen microbiota, microbial fermentations and pH evolution

The effect of a diet shift (from high forage to high concentrate) on the composition of the rumen microbiota has been extensively studied, in particular since the last 10 years because of the development of culture-independent techniques quantifying microbial abundance and assessing population dynamics. Tajima et al. [13] have shown that a diet shift from high forage to high grain in steers induced profound changes in bacterial abundances, an increase

in *S. bovis* and *Prevotella ruminicola* 16S *rrs* gene copy numbers and a decline in fibrolytic *Fibrobacter succinogenes* population densities being measured. Using quantitative PCR, Mosoni et al. [14] measured significant decrease in *F. succinogenes, Ruminococcus albus* and *R. flavefaciens* 16S *rrs* gene copy numbers/g of rumen contents in sheep fed 50% concentrate 50% hay, compared with a 100% hay diet. In lambs, the effect of hay *vs* concentrate diet fed at weaning was studied on abundance of different species of the rumen microbiota [15]. Whereas abundance of total bacteria, measured by qPCR, was significantly higher with concentrate diet than with hay diet, the relative abundance of the fibrolytic species *F. succinogenes* and that of methanogens were significantly lowered in the presence of concentrate. *R. flavefaciens* abundance was 2.5-fold lower with the concentrate diet. The rumen microbiome of dairy cows in which subacute ruminal acidosis (SARA) had been induced with either grain or alfalfa pellets has also been analysed [16]. T-RFLP analysis indicated that the most predominant shift during SARA was a decline in Gram-negative *Bacteroidetes* organisms. However, the proportion of *Bacteroidetes* was greater in alfalfa pellet-induced SARA than in mild or severe grain-induced SARA. This shift was also evident from real-time PCR data for *P. albensis, P. brevis,* and *P. ruminicola*, belonging to the phylum *Bacteroidetes*. The real-time PCR analysis also indicated that in severe grain-induced SARA, *S. bovis* and *Escherichia coli* were dominant, *M. elsdenii* dominated in mild grain-induced SARA, and *P. albensis* was abundant in alfalfa pellet-induced SARA. Comparing 16S rRNA gene libraries of hay *vs* high grain-fed beef cattle, Fernando et al. [17] reported significantly higher numbers of bacteria of the phylum *Fibrobacteres* in libraries of hay-fed cattle whereas the libraries of grain-fed animals contained a significantly higher numbers of bacteria of the phylum *Bacteroidetes*. Real-time PCR analysis revealed increases in *M. elsdenii, S. bovis, S. ruminantium*, and *P. bryantii* populations during adaptation to the high-grain diet, whereas the fibre-degrading *Butyrivibrio fibrisolvens* and *F. succinogenes* populations gradually decreased as the animals were adapted to the high-concentrate diet. All together, these studies indicate a negative effect of low pH on cellulolytic bacteria. Indeed, they cannot grow with a low intracellular pH, and an increase in pH gradient leads to an entry of undissociated VFAs in the cells and an accumulation of dissociated anions in the intracellular compartment induces severe toxicity for the bacteria [18].

An increase in the percentage of rapidly degradable starch in the diet generally favors the development of protozoa as soon as the rumen pH is not below 5.5 [19]. The genus *Entodinium* can then represent up to 95% of the total ciliate community. When rumen pH is below 5.5, ciliate protozoa populations are decreased and defaunation can even be observed transiently [20].

A low rumen pH has also a strong impact on rumen fungi. Indeed, the production of zoospores by *Caecomyces* have been sharply decreased *in vitro* at pH 5.5. Zoospore numbers were below 10^3/ml or even not detected in animals fed diets inducing low rumen pH [21]. Moreover, the presence of large amounts of soluble sugars, as with high concentrate diets, may induce saturation of the spore adhesion sites and reduce fungal colonization [22].

Changes in the structure of the rumen microbiota are generally accompanied with modifications of fibrolytic activities. Indeed, compared with a forage diet, cereal grain

supplementation induces a decrease in specific and total polysaccharidase activities of the solid-associated microorganisms, whereas the response of glycosidase activities is more variable [19]. A relationship between the decrease in polysaccharidase activities (xylanase, avicelase) of these microorganisms and the decrease in ruminal fibre degradation rate has been found by several authors [23-25]. Low pH seems to be more detrimental to growth and survival of cellulolytic microorganisms than to microbial cellulases whose activities are generally optimal at moderately acidic pH (between 5.5 and 6.0) [18]. However, Martin et al. [23] have quantified cellulase and hemicellulase activities and 16S rRNA of cellulolytic bacteria in rumen contents of cows fed a 40% barley diet, and found that cereal supplementation modified the activity but not the abundance of cellulolytic bacterial community.

Sauvant et al. [26] summarized studies conducted on 14 feedstuffs and showed that a strong relationship exists between rumen pH values induced *in vitro* by each feedstuff's fermentation and its percentage of Dry Matter (DM) degradation (Figure 1), indicating that the nature of the feedstuff impacts on its acidogenic potential. Indeed, rapidly degradable starch (as in barley or wheat) will more strongly impact rumen pH than slowly degradable starch (as in corn or sorghum).

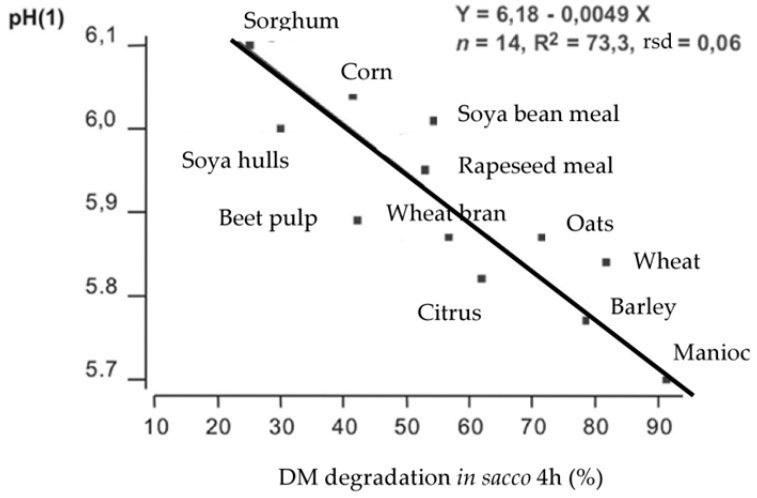

Figure 1. Relationships between acidogenic potential of feedstuffs and their degradation *in sacco*. From [26].

For example, when comparing wheat and corn supplementation in beef steers, mean pH was less and time below pH 6.2 was greater for the wheat based diet than for the corn based diet, which was linked to a higher lactate and VFA concentration [27]. The effect of 3 dietary challenges differing by the nature and degradation rate of their carbohydrates (wheat, corn or beet pulp) was investigated on rumen pH kinetics and fermentation profile in sheep [28]. Mean ruminal pH was significantly less for wheat than for corn and beet pulp at 4.85, 5.61,

and 6.09, respectively. This was correlated with a change in the fermentation profile: ruminal lactic acidosis was induced by wheat, whereas butyric and propionic SARA were respectively provoked by corn and beet pulp after the 3 day challenge.

The particle length of forages can greatly affect rumen pH. Indeed, physically effective Neutral Detergent Fibre (peNDF) represents the physical characteristics of fibre by accounting for particle length and NDF content, which promote chewing and the flow of salivary buffers to the rumen [29]. Yang and Beauchemin [30] compared rumen pH response when short (7.9 mm) or long (19 mm) cut alfalfa silage was included in either high or low concentrate diets. They showed that increasing peNDF intake reduced ruminal acidosis; mean ruminal pH and the duration that pH remained below 5.8 were highly correlated to intake of long particles.

3. Impact of a lowered rumen pH on rumen efficiency and animal productivity

3.1. Consequences of a low rumen pH: acidosis, inflammation, rumen wall integrity and impact on animal health

Acute acidosis occurs after the consumption of an excessive quantity of readily fermentable carbohydrates that rapidly alters ruminal function and can have irreversible metabolic consequences. Ruminal perturbations include an increased concentration of lactate (up to 100mM) and a decrease in VFA concentration after 8 to 24h, this latter being the result of poor microbial activity and/or of quicker absorption of the VFA from the rumen to the blood in response to pH fall [31]. Rumen pH values can then drop under 5.0 and trigger metabolic acidosis with an accumulation of D-lactate in the bloodstream. SARA is probably more difficult to characterize because biological parameters in the rumen fluctuate within physiological limits and are difficult to maintain [31]. This unstable state may reflect the oscillatory behavior of the ruminal microbial population in response to diet-based fermentative jolts. According to Kleen and Canizzo [32], the exact definition of SARA remains debatable, but it is certain that SARA is present in a large number of dairy herds. SARA is characterized by a drop of ruminal pH to non-physiological levels; pH values of 5.5 and 5.8 and the duration per day below these threshold values are used to define individuals or groups experiencing SARA or being at risk for SARA. SARA is frequent in high producing cattle and has wide-reaching economic consequences, as it has been estimated to cost $1.12 /d per cow in USA [33]. In Europe, field studies data indicate that SARA prevalence would range between 10 and 30% in dairy herds [32]. In these studies, the pH thresholds of 5.5 and 5.8 were generally used, rumenocentesis being the reference method for collecting rumen fluid.

The microbial dysbiosis occurring in the rumen during acidosis may trigger the release of potential harmful molecules which may impact the animal health. Indeed, due to an increase of the death and lysis of Gram-negative bacteria under low pH, free lipopolysaccharide (LPS) concentration is increased in the rumen fluid and translocation of

this endotoxin can occur across the rumen mucosa [34]. Endotoxin release can trigger an inflammatory response, with an increase in acute phase protein concentrations in peripheral blood [34-37]. Endotoxin is suggested to be involved in metabolic disorders such as laminitis, abomasal displacement, fatty liver or sudden death syndrome [38].

Moreover, the low pH of rumen digesta may have a negative impact on rumen wall integrity. Repeated aggressions by fermentation acids may cause papillar atrophy, diffuse areas of acute or chronic lesions, scars resulting from severe local rumenitis, perforations and mucormycosis which are at the origin of pain, discomfort, as well as erratic feed intake and alteration of rumen function [39].

Low ruminal pH is often associated with increased occurrence of bloat, which is characterized by an accumulation of gas in the rumen and reticulum. Indeed, frothy bloat is caused by entrapment of gas produced from fermentation of readily digestible feeds (high digestible legumes or cereals). Bloat can impair both digestive and respiratory function, and can occur both in cattle raised on pasture or in confinement [40]. Abscessed livers are generally considered to be associated with both acute and subacute ruminal acidosis. Ulcerative lesions, hairs, and other foreign objects that become embedded in the ruminal epithelium can provide routes of entry into the portal blood for microbes that cause liver abscesses [41]. *Fusobacterium necrophorum* (and/or *F. funduliforme*), a commensal rumen Gram-negative species, has been identified as a causative agent of liver abscess; as it is able to use lactate as its major substrate, and its population increases in the rumen of cattle fed high-grain diets [42]. Diarrhea has been very frequently associated with ruminal acidosis and microbial dysbiosis [1]. Changes in fecal consistency, color, brightness, and odour are generally observed; presence of undigested whole grains and large size particles is also a sign of rumen dysfunction [43].This phenomenon may be linked to excessive hindgut fermentation because too much readily fermentable carbohydrates reach the post-ruminal compartments [36] but also the increase in osmolarity of the digesta would lead to soften the fecal mass [43].

Under low rumen pH conditions, erratic feed intake is generally observed but a decrease in intake, mostly on acidogenic feed, has also been reported [44]. In fattening bulls fed high concentrate diets, it has been observed that animals change their feeding behavior to counteract acidosis by spreading their meals over the day [45]. A 10-30% increase in water intake was observed in sheep submitted to acidotic challenges [46]. Water intake could represent a means to dilute acidity but also to reduce rumen fluid viscosity. An increase in salt licking has been also measured in the same study and in goats fed with high concentrate diets [47]. Licking would favor salivary bicarbonate production. Animals under acidosis would also be able to modify their dietary choice to optimize their digestive comfort. Acidosis and low rumen pH conditions may also have consequences on social behavior. For example, sheep undergoing successive acidotic challenges were more active and more aggressive towards each other, spent more time standing, adopted alarm postures more often, and reacted more slowly to hot stimulus during the acidosis bouts [46]. These discomfort signs would not be only linked to rumen pH evolution but to the set up of an inflammatory status in the rumen triggered by changes in microbiota balance.

3.2. Effect of rumen pH on milk yield and quality

From a dietary standpoint, rumen pH is a function of the dry matter intake (DMI) where it becomes below 6 when DMI exceeds 3.8% body weight, i.e. high producing animals with elevated nutritional requirements are more at risk [26]. The quality of the ingested feed directly matters too where pH turns out below 6 when the rumen digested starch accounts for more than 40% of the diet DM [26].

Cows fed high-concentrate diet (nadir 75:25 concentrate:forage ratio) will have a lower ruminal pH, acetate, and butyrate concentrations, whereas propionate concentration will go up. When the rumen acidity is alleviated with a buffer, total VFA production increases, and so does milk production and milk fat content, especially for high concentrate fed cows. Milk fatty acid profile gives also a good insight of what happened in the rumen and more trans 10-11 C18:1 is well correlated to a depressed milk fat due to its inhibitory effect on de novo fatty acids synthesis in the mammary gland [48]. In addition, the stage of lactation may modulate the animal sensitivity to high-concentrate diet with a better resistance to less optimal rumen fermentation conditions for late lactation cows [49]. However, not only the forage:concentrate ratio matters on rumen pH but the nature or technological process of the grains [50] and the frequency of distribution of the concentrate [51] also do.

High fibre diets will not sustain an elevated production of propionate that will negatively impact the milk lactose synthesis and overall milk yield. The cow will thus mobilize her body fat reserves (ketone bodies metabolized in the liver from butyrate) to compensate for this lack of energy.

4. Benefits of using yeast probiotics to control pH stability

4.1. Targets

pH evolution is the result of impaired microbial balance and animal compensation mechanisms. Strategies aiming to induce beneficial effects on the balance of the rumen microbiota and thereby stabilize rumen pH can represent interesting means to reduce the risk of acidosis. This may be achieved by targeting microbial populations involved in massive release of fermentation acids, and/or those implicated in lactic acid removal.

4.2. How best measuring a probiotic effect on animal performance?

Two types of experimental design are basically available to the scientist: contemporaneous or crossover. Parallel designs (i) can be completely randomized design with only one explanatory variable or (ii) randomized complete block design in presence of 2 factors where the experimenter divides animals into subgroups called blocks (eg. sex, origin, size…) such that the variability within blocks is less than the variability between blocks. In crossover design, each experimental unit receives two or more treatments through time, and as the comparison of treatments is made within subjects, each subject acts as its own control which increases statistical power to detect a direct treatment effect [52] and makes it more

efficient than the randomized complete block design. However, there are limitations important to bear in mind amongst with a carryover effect is likely to occur between periods, the latter being able to vary between treatments.

The particular nature of probiotics as live microorganisms impacting the rumen flora balance and fermentations make their comparative assessment critical when using experimental design encompassing a carry-over effect. The inclusion of a washout period between successive treatments is a good way of minimizing the remanent treatment effect over time, but there is good evidence suggesting that the 15-28 days usually applied are not long enough.

Indeed, in a complete rumen content transfer study between two cows, Weimer et al. [53] showed that it could last up to 65d for the bacterial community composition to reach back its original profile. A measurement of methanogens population dynamics over time [54] indicated that 4 weeks were not enough to adapt from the dietary shift of grazing to concentrate. These recent microbial studies support questioning about the relevance of crossover type of designs in assessing probiotics effect on rumen parameters [55]. However, it would not be fair omitting to report studies where such a design allowed displaying significant probiotic effects, but the inconsistence or absence of response with a latin-square design may also be due to the tested probiotic strains themselves or to the too short adaptation period.

4.3. Experimental proofs

Stabilization of ruminal pH in the presence of yeast probiotics has been reported by several authors [56-59]. In a meta-analysis, Sauvant et al. [26] concluded that yeast supplementation increased ($P<0.05$) rumen pH *in vitro*, but did not find any significant *in vivo* effect neither on pH, nor on VFAs or lactate. However, the authors admitted that the studies selected for the meta-analysis had used different strains of *S. cerevisiae*, or yeast culture which is defined to be mainly composed by dead cells and fermentation products. More than an increase in mean rumen pH, reductions in duration within a day under a certain pH threshold, as well as in area under the pH curve have been measured in the presence of live yeast probiotics [56, 59]. A recent study conducted in a commercial dairy herd [60] compared sodium bicarbonate and live yeast supplementation in 2 pens of 60 cows on milk production and feed efficiency and rumen pH was monitored every 5 min during 5 weeks in 4 cows equipped with a pH probe. Sodium bicarbonate is very often used as an efficient buffer to overcome pH fall in dairy cows. Mean pH remained consistently higher for the live yeast supplemented cows when compared to the control group cows (6.22 vs 6.03). In addition, live yeast supplemented cows spent less time below a pH threshold of 5.6.

4.4. Modes of action on rumen microbiota and lactate accumulation

Effects of live yeasts have been studied on lactate-metabolizing bacteria. *In vitro*, one strain of *S. cerevisiae* was able to outcompete *S. bovis* for the utilization of sugars; due to a

higher affinity of the yeast cells for sugars, the reduction in quantity of fermentable substrate available for the bacterial growth consequently limited the amount of lactate produced [61]. Dead cells had no effect on lactate production. Moreover, stimulation of growth and metabolism of lactate-utilizing bacteria, such as *M. elsdenii* or *S. ruminantium*, was observed *in vitro* in the presence of different live yeasts [61-64] through a supply of different growth factors such as amino acids, peptides, vitamins, and organic acids, essential for the lactate-fermenting bacteria. The impact of yeast probiotics on ruminal lactate concentration has been confirmed in *in vivo* studies. In sheep receiving a live yeast product during their adaptation to a high-concentrate diet, ruminal lactate concentration was significantly lower compared to control animals. Consequently, rumen pH was maintained at values compatible with an efficient rumen function, as shown by higher fibrolytic activities in the rumen of the supplemented animals [24, 65]. In dairy cows, reductions in ruminal lactate concentrations have also been observed with yeast probiotics [66-67].

According to the composition of the diet, the fermentation pattern can be shifted to butyric orientated acidosis [28]. Brossard et al. [6,12] reported the pH stabilising effect of one strain of *S. cerevisiae* in sheep fed a high-wheat diet under a butyric latent acidosis. Authors suggested that this strain could act by stimulating ciliate Entodiniomorphid protozoa, which are known to engulf starch granules very rapidly and thus compete effectively with amylolytic bacteria for their substrate [68]. In addition, starch is fermented by protozoa at a slower rate than by amylolytic bacteria and the main end-products of fermentation are VFAs rather than lactate, which may explain why these ciliates had a stabilizing effect in the rumen by delaying fermentation.

When ruminants encounter successive acidotic bouts, it is not well known whether live yeast supplementation could alter rumen microbiota and fermentations. Indeed, the severity of acidosis may change with repeated challenges, partly because of modifications in feeding behavior [69], and because of possible shifts in rumen microbial communities leading to selection of the most acid resistant species. Studies in sheep submitted to acidotic challenges showed that cellulolytic bacterial culturable population was greatly decreased after a first acidotic challenge but that after 3 challenges, the level of population came back to normal [70]. However, it is probable that this population, enumerated in a filter paper-based medium, had encountered profound changes in its structure and/or diversity. In this study, with repeated challenges, a positive evolution of rumen pH parameters were observed in live yeast supplemented animals which was accompanied with decreased numbers of lactate producing bacteria and a beneficial effect on bacterial diversity which was maintained at a higher level [71].

Provided an adequate balance between soluble nitrogen and carbohydrate supply, it is likely that live yeast probiotics can enhance microbial growth; indeed, more digested carbohydrates would be incorporated into microbial mass thanks to an optimized fermentation coupling and not “wasted” under the form of VFAs, thereby the risk of acidosis would be reduced [72].

4.5. Beneficial consequences of yeast probiotics on rumen fermentations, feeding behavior, feed efficiency, and animal production

Bach et al. [56] reported that the supplementation of live yeast increased average rumen pH and average maximum pH by 0.5 units, and average minimum pH by 0.3 units in loose-housed lactating cows (Figure 2). In this study, a significant change was observed in the eating behavior of the animals. Cows supplemented with live yeast had a shorter inter-meal interval (3.32h) than unsupplemented cows (4.32h). This change in feeding behavior could help in rumen pH recovery, or the beneficial effect of live yeast on pH stabilization could induce a change in eating behavior.

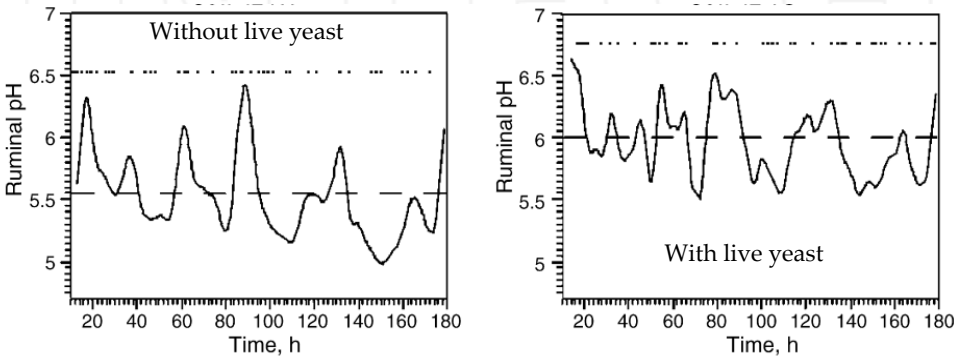

Figure 2. Ruminal pH pattern (solid line) during the 8 days of sampling as affected by live yeast supplementation.The dashed line depicts average ruminal pH. The dots indicate the beginning of a meal. From [56], example shown with one cow.

A meta-analysis conducted on all types of yeast (including live yeast and yeast culture) and all types of dairy ruminants (cows, goats, ewes) [58] concluded that the addition of yeast improved milk yield by 1.2 g/kg body weight. In their multi-analysis reporting data collected from 14 dairy cow trials fed the same live yeast strain, De Ondarza et al. [73] found that live yeast improved ($P < 0.0001$) milk yield by 1.15 kg/day. The effect was slightly greater for cows in early lactation (<100 Days In Milk, DIM) than for cows >100 DIM, suggesting that animal performance is improved when the acidosis risk is high, notably at critical periods of the lactation cycle.

The effect of yeast probiotics on DM intake shows either no effect [73] or a significant increase in DMI [58]. Live yeast supplementation seems to have an effect on intake pattern rather than on intake *per se* [56]. As a result, feed efficiency is generally improved in the presence of live yeast [73,74]. Milk composition is generally not or only slightly affected by yeast supplementation. Milk fat and protein percentages have been found to be slightly but significantly lower in the presence of live yeast [73], but due to the increase in milk yield, yields of milk fat and true protein were higher than in control cows.

5. Fibre digestion in the rumen: a key process in ruminant nutrition

By symbiosis with specific micro-organisms, ruminants possess a unique ability to use plant cell wall components as energy and nutrient sources and thereby convert plant biomass into milk, meat, wool and hides. A large proportion of energy intake of ruminant comes in the form of structural complex polysaccharides (cellulose, hemicelluloses, pectins), which are mainly present in the plant cell walls. Indeed, the rumen harbors an abundant and diversified community of bacteria, fungi and protozoa able to thoroughly hydrolyze plant cell wall polysaccharides. Effective degradation is the result of microbial adhesion to plant tissue and production of active enzymatic machinery well adapted to plant cell wall breakdown.

5.1. Relation between fibre digestion and intake and productivity

Digestion of fibre is the result of the competition between rates of passage and degradation and the ruminal passage rate (%/h) depends on fibre particles size and digestibility [75]. Reducing particle size will increase DMI but the effect on total digested fibre is also related to the quality of the roughage and its nature: legumes NDF is quicker digested than perennial grass NDF despite a higher lignification, but less resistance to breakdown [76]. Particle size also affects the reticulo-omasal passage kinetics along with the intrinsic fragility of the fibre, its density and shape. The importance of particle size on forage rumen degradation has been recently highlighted [77] as the adjustment parameter to increase the available surface area for attachment of ruminal fibrolytic bacteria and protozoa without negatively affecting cellulolytic activity and other fermentation processes in the rumen.

Fibre occupies space and limit intake by filling the rumen as they are hollow and therefore fill a bigger volume than their mass indicates. In addition, a fraction of the dietary fibre will remain undigested or slowly degraded and will accelerate the rumen filling [78] reducing thus the entrance of other important ingredients to meet the animal nutritional requirements. Knowing that feed intake is the main predictive variable of milk yield [79], the increase of dietary forage will lead to a milk yield reduction besides isonitrogenous rations [80]. Rinne et al. [81] also concluded to a linear decrease of milk yield when the corn silage NDF content increased due to later harvest.

5.2. How to measure fibre digestion

Different methods can be used to measure fibre digestion in the rumen. This compartment is mostly targeted because in general the proportion of fibre which is digested in the hindgut is small. However, the contribution of the large intestine to plant cell wall digestion may increase with the proportion of cereal in the diet [82].

Degradation of dry matter, and NDF fraction of raw materials or more complex mixture of ingredients can be assessed with various *in vitro* techniques requiring mixed rumen contents [83,84], *in situ* (nylon bags) kinetics [82,85] or rumen evacuation [86] in rumen cannulated animals, or in non cannulated ruminants (total fecal collection). The measurement of

particle sizes in the fecal material using the Penn State forage and total mixed ration particle separator can be of interest to estimate fibre digestibility [60].

Fibre degrading functional groups can be enumerated on complex culture media in which a source of polysaccharide is added as sole energy source. Measurement of fibrolytic activities can be performed on pure cultures as well as on rumen contents samples. After extraction of ruminal microbial enzymes, activities are measured against various polysaccharides and the concentration of reducing sugars released after enzyme action is determined [19]. PCR-based techniques using specific primer sets are powerful to quantify absolute or relative abundance of targeted fibrolytic species within a complex sample [14,87,88], or to specifically detect and quantify *in vivo* the expression of cellulase or hemicellulase genes from selected microorganisms [89].

5.3. Microbial communities involved in fibre degradation in the rumen

In the rumen, degradation and fermentation of plant cell wall polysaccharides is achieved by bacteria, protozoa and fungi. The different fibrolytic species, or even strains, are specialized to a various extent in the degradation of specific substrates. The overall effective degradation is the result of these different capacities, related to substrate composition and to interactions existing between these communities and also between the fibrolytic and the non-fibrolytic microorganisms within the ecosystem.

In the Bacteria domain, the cellulolytic function is covered by a very limited number of cultivated species. These species are established a few days after birth in the newborn ruminant, although no solid feed penetrates into the rumen [90]. Indeed, from one week of age, the size of the cellulolytic bacterial community is close to that found in adult animals. Cellulolytic bacteria are unable to properly colonize the rumen in absence of a complex and diversified bacterial fermentative community [91,92]. In young lambs kept without contact with their dams or other adults, cellulolytic bacteria were not detected in the rumen during three months after birth, which suggests the essential role of newborn-dam contacts in the transmission of rumen microbiota and rumen maturation [92].

The concentration of fibrolytic bacteria is generally close to 10^9 culturable cells/g of rumen content. Quantitative PCR studies have shown that the main cellulolytic species *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* represent 1-5% of the total bacteria [14, 93] but recent data suggest that these bacteria account for about 50% of the total active cellulolytic bacteria [94]. *F. succinogenes* is very active on crystalline cellulose and hemicelluloses (xylans). However, it is only able to use products of cellulose hydrolysis [94]. *R. albus* and *R. flavefaciens* are active on cellulose, xylans and pectins. Other species are considered as secondary fibrolytic species such as *Butyrivibrio fibrisolvens* and *P. ruminicola*, because they are not able to breakdown the cellulose polymer. However, they possess high carboxymethylcellulose-, xylan- and pectin-degrading activities and probably play an important role in overall fibre digestion [95,96].

The enzymatic equipment of the three main cellulolytic species has been well studied since the last 20 years. In the database CAZy (Carbohydrate Active enZymes, http://www.cazy.org ;

[97]) are referred protein sequences involved in carbohydrate binding and hydrolysis. The recent whole genome sequencing programs confirm that a huge number of genes is involved in fibre breakdown in each bacterial cell, demonstrating great functional redondancy, which is essential for the good functionning of the ecosystem. Genome sequences of strains belonging *to F. succinogenes, R. flavefaciens, R. albus, P. ruminicola,* and *P. bryantii* are now available. From these genome sequences, 183 putative CAZymes have been found for *F. succinogenes*, and more than 140 for *R. flavefaciens* and *R. albus* [98].

Efficacy of fibrolytic bacteria to degrade plant cell wall components are explained by their adhesion capacities and the production of a well adapted enzymatic equipment. Bacteria use different strategies to colonize plant material: for example, *Ruminococci* exhibit several structures on their cell surface, such as type IV pili and components of glycocalyx. Moreover, they produce an elaborate cellulosomal enzyme complex that is anchored to the bacterial cell wall [99,100]. In *F. succinogenes*, attachment to the substrate is mediated by fibro-slime proteins and type IV pilin structures attached to the outer membrane; 13 cellulose binding proteins anchored on the outer membrane seem to be important in effective adhesion to crystalline cellulose [101].

Ciliate protozoa also participate to fibre degradation. Characterization of their ability to directly process plant material have been addressed by diverse strategies, such as direct, biochemical detection of specific fibrolytic enzymes in extracts derived from individual protozoan species [102], or by molecular cloning studies to directly identify protozoal genes encoding enzymes capable of degrading cellulose or hemicellulose [103]. Among protozoa, only Entodiniomorphs (*Polyplastron*, *Eudiplodinium*, *Epidinium)* are considered as cellulolytic. Their abundance is between 10^4 and 10^6 cells/g of rumen content. Ciliates are able to engulf whole plant particles, and digest plant polymers in digestive vacuoles. They synthesize a well adapted enzymatic equipment composed of cellulases and hemicellulases [104,105]. Up to now, about a dozen of fibrolytic genes have been identified in the various protozoa species. An activity-based metagenomic study of a bovine ruminal protozoan-enriched cDNA expression library identified four novel genes possibly involved in cellulose and xylan degradation [106]. Several studies have reported that defaunation, i.e. removal of protozoa, can have a negative effect on fibre degradation in the rumen [107,108]. Mosoni et al. [88] showed that long term defaunation had rather a beneficial effect on the abundance of fibrolytic bacterial species *R. flavefaciens* and *R. albus*, quantified by qPCR, but not on that of *F. succinogenes*, which is the most efficient in low digestible plant cell wall degradation, which could explain at least in part, the observed negative effect on fibre digestion.

Anaerobic fungi are also involved in digestion of plant material. They represent a very homogenous phylogenetic group (phylum *Neocallimasticota*) and a very specialized functional group as all species are fibrolytic [109]. The fungal biomass is estimated to represent between 5 and 10% of the total microbial mass. During their life cycle, flagelatted zoospores alternate with filamentous sporangia which are tightly attached to plant tissues, thanks to their cellulosome-like complexes [110]. Rumen fungi produce a very efficient set of cellulases and hemicellulases, whose specific activities are higher than that of bacteria [111]. They also possess esterase activities which contribute to the cleavage of ester bridges which

link phenolic compounds of lignin to structural carbohydrates [112,113]. Moreover, thanks to the development of a rhizoidal network they are able to weaken and even disrupt plant tissue which enhances accessibility to digestible structures [114]. Studies carried out with gnotoxenic lambs harbouring or not fungi confirmed their important role in fibre breakdown in the rumen [115].

6. Limiting factors in fibre digestion

6.1. Animal characteristics

A cow chews during eating and rumination to reduce feed (forage) particle sizes and allow the best fermentation process possible via a better distribution of feedstuff and bacteria in the rumen as well through rumen pH maintenance (high buffer capacity of the saliva). Indeed, this first step of the digestive process stimulates saliva production (274 ml/ min chewing and 6g sodium bicarbonate/ liter of saliva) and rumen motility. With an average daily time spent eating, ruminating and resting of 1/3, a production of up to 150 l of saliva per day is achieved. However, about half of the saliva will be produced during rumination, whereas eating will account for 20% and resting 30% [116].

The chewing responses to forage fragility and digestibility have been described [117]: at equal particle size, a low NDF Digestibility (NDFD) rate and less fragile forage increase by about 30 min/day the chewing time when compared to a high NDFD and fragile hay, whereas fragility appears less related to chewing when forage NDFD is similar. These results suggest that increased dietary physically effective NDF may affect chewing activity either through prolonging chewing time or increasing chewing rate. In addition, longer particle size will promote salivation and thus a shorter time with rumen pH<5.8 [118].

From a species standpoint, chewing activity is highly related to the intake capacity and body weight. Animals with a greater intake capacity seem to chew feed more efficiently (i.e. goat, sheep), while heavier animals (cows) can cope with relatively more fibre, because rumination capacity is in line with body size [119].

6.2. Composition of the diet and structure of fibre

Many biotic and abiotic factors may limit the efficacy of fibre degradation in the rumen which may be driven by changes in fibre colonization efficacy. For example, the chemical composition of the plant material modulates the rate and extent of fibre digestion [120]. Digestibility of forage fibre (cell walls) has long been known to be negatively associated with lignin concentration. This relationship between lignin and fibre digestibility is very strong for a same forage compared according to different maturity stages, but it is less clear when comparing different forages harvested at a similar maturity stage, so with similar lignin concentrations [121]. To explain the observed variation in fibre digestibility of forages with similar lignin concentrations, composition of lignin and chemical cross-linking of lignin to cell wall polysaccharides have been suggested as involved additional factors. For example, cross-linking of lignin and arabinoxylans may limit cell wall digestibility by

placing lignin in very close proximity to the polysaccharides and preventing physical access by hydrolytic microbial enzymes [120]. The slow entrance of microbial cells into some plant cell tissues such as sclerenchyma and also their slow diffusion capacities down the lumina represent also an important limitation factor for totally efficient fibre digestion [122].

Several studies have shown that the feed particle size may influence the degradation rate of fibre fractions as well as the bacterial colonization of the feed particles. Witzig et al. [123] investigated the effect of the forage source and particle size on the composition of the ruminal *Firmicutes* community assessed by qPCR and Fluorescent In Situ Hybridization *in vitro*. They found that *Ruminococcus albus* was more abundant on short particle size of forage, whereas the xylanolytic *Roseburia* sp. was favored by coarse particle grass silage based diets, and that abundance of *Clostridium* cluster XIV was higher with increasing grass silage proportion in the diet.

6.3. Characteristics of the rumen environment

As described earlier in this chapter, it has been demonstrated that a diet rich in readily fermentable carbohydrates can adversely alter the structure and/or activities of fibre-degrading community, because of a decline in ruminal pH and acidosis occurrence. As a consequence, ruminal digestion of NDF is decreased [124] (Figure 3).

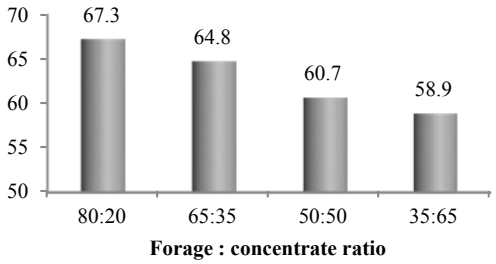

Figure 3. Effect of forage:concentrate ratio on apparent rumen NDF digestibility (%) in cows. From [124].

It is generally admitted that most of fibre-degrading microorganisms are sensitive to oxygen because most of them lack detoxification enzymes necessary for removal of reactive oxygen species. The presence of dissolved oxygen in the rumen ecosystem has been demonstrated [125,126] and oxygen regularly enters the rumen due to feed and water uptake and mastication, which can be illustrated by a greater post-feeding redox potential as measured in dairy cows by Marden et al. [57,127]. Newbold et al. [128] measured the concentration of cellulolytic bacteria in Rusitec in which either normal or low O_2 concentrations had been maintained. Oxygen concentration significantly influenced cellulolytic bacteria, whose numbers were increased by almost 15-fold when low O_2 concentrations were applied in the fermenters. Adhesion of cellulolytic bacteria to cellulose has been shown to be inhibited in the presence of oxygen *in vitro* [129].

6.4. Physiology of fibrolytic microorganisms and microbial interactions

Among biotic factors, the existence of a complex set of interactions between fibrolytic microbes and the other actors of feed digestion does impact fibre degradation. For example, synergistic cross feeding interactions have been described between cellulolytic and non cellulolytic species which lead to a global improvement in degradation [130]. A relevant example is the interaction between proteolytic bacteria and cellulolytic bacteria, the former releasing ammonia, used as preferential nitrogen source for the latter, and the latter releasing soluble sugars from cellulolysis, which will be metabolized by proteolytic bacteria. Moreover, hydrogen transfer between fibre degrading organisms and hydrogen consuming methanogens is necessary for an optimal functioning of fibre degradation mechanisms. Indeed, methanogens help to reduce the hydrogen partial pressure and thereby avoid the inhibition of ferredoxine oxidoreductase which has an essential role on NADH re-oxidation [130]. The result of this interaction is a gain in energy for both partners and an increase in fibre digestion. On the opposite, competition mechanisms have been described between cellulolytic bacterial species for adhesion on cellulose [131,132]. Secretion of inhibitory peptides by *Ruminococcus* strains have been shown *in vitro* to impact growth of rumen fungi [133]. Finally, the physiology of the microorganisms plays also an essential role on overall fibre digestion. Indeed, there are great differences between species regarding their preference and affinity for substrates, their energy requirements, or their capacity to resist to environmental stresses.

7. Benefits of using yeast probiotics to promote fibre digestion

7.1. Targets

To optimize fibre digestion, there is a need to minimize the indigestible fibre fraction, maximize rate of fibre digestion, and maintain a ruminal environment that promotes the population of fibre-digesting bacteria. The indigestible fibre in forages (iNDF) is related to lignin concentration, but also contains structural carbohydrates (cellulose and hemicellulose) which are 'trapped' with lignin. Whereas lignin, of which biochemical degradation process involves oxidative pathways, is considered not digested in the animal gastro-intestinal tract, the release of the carbohydrates bound to lignin would be interesting in terms of increasing feed value of the forage.

To achieve these goals with probiotics, several strategies may be developed depending on the dietary conditions of the animals. Indeed, indirect or direct effects can be sought. Indirect benefits could be mediated through pH stabilization effects (see section 4), or modification of the environment of the microbiota which will definitely sustain or promote fibre-degrading microbiota and their action on plant cell walls. Direct effect of probiotics on fibrolytic microorganisms can also be wished to exist, as nutritional requirements for peptides, amino acids, ammonia, organic acids or branched chain fatty acids have been described for bacteria and fungi and the supply of these components might be achieved through the use of probiotics.

7.2. Experimental proofs

Using different methods, it has been reported that live yeast supplementation improves rumen fibre digestion *in vivo* [85,134-137], although this has not always been observed [138].

7.3. Modes of action on rumen microbiota

In vitro, the potential of probiotic yeasts to enhance growth and activity of fibre-degrading rumen microorganisms has been established. Fungal zoospore germination and cellulose degradation were increased in the presence of a strain of *S. cerevisiae* [139]; the authors suggested that yeasts could enhance fungal colonization of plant cell walls, which was confirmed recently [136]. The effectiveness of some yeast strains to stimulate growth or/and activities of fibrolytic bacteria has also been demonstrated. *In vitro*, a *S. cerevisiae* strain stimulated growth of *Fibrobacter succinogenes* S85 and reduced the lag time for growth of *Ruminococcus albus* 7, *Ruminococcus flavefaciens* FD1, and *Butyrivibrio fibrisolvens* D1 [140]. Callaway and Martin [141] showed that the same yeast could accelerate the rate, but not the extent, of cellulose filter paper degradation by *F. succinogenes* S85 and *R. flavefaciens* FD1. *In vivo*, in gnotoxenic lambs harbouring three species of bacteria (*F. succinogenes, R. albus,* and *R. flavefaciens*) as sole cellulolytic organisms, cellulolytic bacteria became established earlier and remained at a high and stable level even after a stressful period (lambs were fitted with a rumen cannula) in the lambs receiving a probiotic yeast daily [137]. Ciliate protozoa, which are not able to establish unless bacterial communities have previously colonized the rumen [142], appeared more rapidly in the rumen of conventional lambs in the presence of live yeasts [143].This supports the hypothesis that live yeast supplementation accelerates maturation of the rumen microbial ecosystem. Fibre degradation processes would thereby be set up more efficiently in the early age of the animal, as shown by the increase in polysaccharidase and glycoside-hydrolase activities in the presence of yeast in the rumen of gnotoxenic lambs [137].

There are some evidence that live yeast additives indirectly promote fibre degradation or fibrolytic microbial activities by stabilizing rumen pH in case of ruminal acidosis (see section 4). Greater polysaccharide-degrading activities of the solid-associated bacterial fraction in rumen-cannulated adult sheep fed a high-concentrate diet were measured in the presence of yeasts [144]. The proportions of 16S rRNA of *F. succinogenes, R. albus,* and *R. flavefaciens* have been shown to increase in the rumen of sheep receiving another yeast product [145]. A 2 to 4-fold increase in the number of 16S rRNA gene copies of *R. albus* and *R. flavefaciens* was also measured with real-time PCR in rumen contents of sheep receiving a high-concentrate diet and a live yeast probiotic [14].

Guedes et al. [85] reported that a live yeast strain increased NDF degradation of different corn silage samples incubated *in sacco*. In their study, cows were fed with grass silage-corn silage based diet and the rumen pH was not indicative of SARA situation. However, it is noteworthy that a yeast effect was observed on pH and lactate concentration but the authors suggested that the yeast efficacy was not only attributable to a pH stabilization effect. Using

the same technique, Chaucheyras Durand et al. [136, unpublished] have studied the effect of the same yeast strain on fibre degradation of different substrates and followed the kinetics of colonization by fibre-degrading bacteria and fungi using qPCR in rumen cannulated cows. In this study, the diet offered to the cows was composed of grass silage and hay and was not at risk regarding SARA. Results showed that the supplementation of 10^{10} cfu/day/cow of the yeast additive promoted colonization of fibrous substrates by cellulolytic bacteria (*F.succinogenes, R.flavefaciens, B.fibrisolvens*) and fungi but that the degree of stimulation was depending on the nature of the substrate, and on the microbial species targeted. It was noticed that feedstuffs with highest levels of lignin and thereby with less easily accessible digestible carbohydrates were better degraded in the presence of yeast, suggesting a particularly marked impact on the microbial breakdown of lignin-polysaccharide linkeages. The same strain of *S. cerevisiae* significantly improved NDF degradation of 40 corn silages samples incubated *in sacco* in rumen cannulated cows, with differences in the degree of improvement according to the degradability of the corn silage [85]. Indeed, the yeast probiotic increased NDF degradation of the low digestible corn silages more strongly than that of the high digestible corn silages (Figure 4). These results suggest that live yeast could help to reduce indigestible NDF by promoting the action of bacteria and fungi involved in the hydrolysis of lignin-polyholoside bonds (Figure 5).

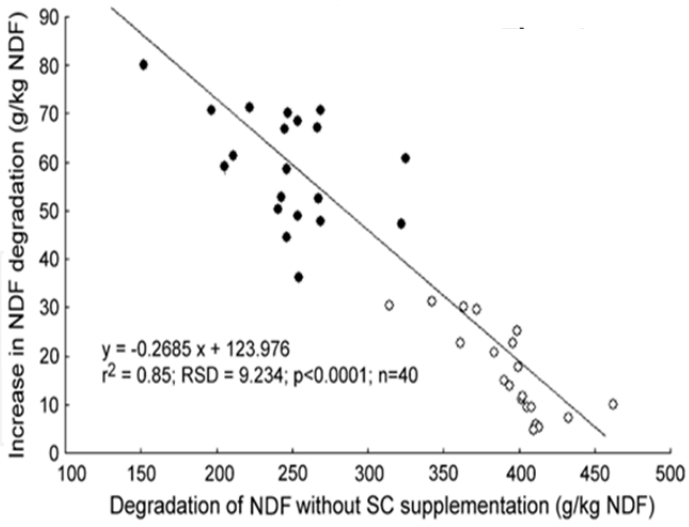

Figure 4. Figure 4. Effects of supplementation with a yeast probiotic (*Saccharomyces cerevisiae* CNCM I-1077) on fibre (NDF) degradation of maize silages after 36h of incubation in the rumen of cows: open circles, high fibre degradation group , full circles, low fibre degradation group. From [85].

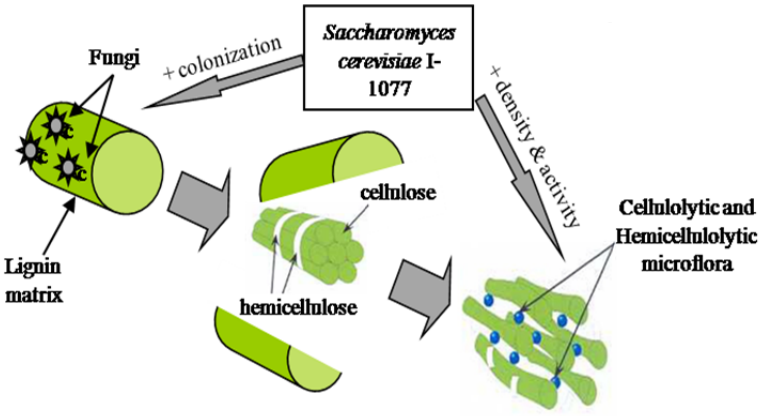

Figure 5. A proposed scheme for mode of action of *Saccharomyces cerevisiae* CNCM I-1077 on fibre degrading communities.

In the study of Chaucheyras-Durand et al. [136, unpublished], a positive effect of live yeast was demonstrated for the first time on *Butyrivibrio fibrisolvens* abundance on fibrous substrates. The hemicellulose fraction of forages consumed by ruminants consists mainly in xylan substituted with acetyl, arabinosyl, and glucuronyl residues. Xylan is also cross-linked via ferulic and p-coumaric acids which are esterified to the arabinose side chains. It is supposed that the ester linkages between these phenolic acids and polysaccharides provide a steric hindrance to the degradation of fibre by rumen microbiota. Consequently, the promotion of *B. fibrisolvens*, that possesses ferulic and p-coumaric acid esterases which hydrolyze these ester linkages [146] appears particularly interesting.

One of the main factors implicated in the beneficial effect of live yeasts on fibre-degrading bacteria is probably the capacity of yeast cells to scavenge oxygen. Indeed, although the rumen environment is known to be strictly anaerobic, dissolved oxygen can be detectable *in situ*; as high as 16 liters of oxygen can enter an ovine rumen daily during feed and water intake, rumination or salivation [147]. Most of ruminal microorganisms are considered to be highly sensitive to oxygen, but this is particularly true for fibre-degrading organisms. Respiratory-deficient mutants of *S. cerevisiae* were unable to stimulate bacterial numbers in rumen-simulating fermenters, whereas the wild-type parent strains, able to consume oxygen, did effectively stimulate bacterial activities [128]. Other studies have reported that redox potential of rumen fluid was lowered in the presence of live yeasts in lambs [143], in sheep [148] and in cows [57] suggesting that live yeast cells could create more favorable environmental conditions for growth and activities of the cellulolytic microbiota. Due to the fact that live yeasts could release vitamins or other growth factors to closely associated bacterial cells [149], yeast impact could also be mediated through the interplay between different bacterial species (i.e. non cellulolytic species) and would not only be explained by a direct effect on oxygen consumption.

7.4. Consequences on rumen fermentations, feed efficiency, and animal production

The beneficial effects on fibre digestion can be partly at the origin of the increase in dry matter intake often observed with yeast supplementation [149], but more generally a better fibre digestion is recognized to benefit the animal rumen health and its function by improvement of feed efficiency. The study carried out by Bitencourt et al. [150] did support this assumption with cows fed a corn silage, soybean meal, citrus pulp and steam-flaked corn based TMR. The diet NDF digestibility was improved by 11.3% in presence of 10^{10} cfu/day of the live yeast and the milk production tended to be improved by 0.9 kg/d. Cows were not in SARA situation (6.43<pH<6.5). In De Ondarza et al. multi-analysis [73], live yeast effect was particularly strong in low yielding cows. In addition, feed efficiency of the supplemented animals was improved which illustrates a better use of the diet. When targeting the cows fed diet above 30% NDF, feed efficiency was higher than the overall mean and the live yeast treated animals gained an extra 40g of milk per kg DMI. The shorter intervals between meals of live yeast fed cows reported in [56] strongly suggests the fact that the TMR digestibility was improved as the meal size and length were not affected by the treatment. As mentioned earlier, improvement of rumen pH for the cows receiving the live yeast at the same dose than the previously cited studies would also support a higher activity of the cellulolytic flora and thus explain the higher meal frequency.

8. Importance of yeast viability and strain selection

A better understanding of the modes of action of live yeast probiotics is important to further select of new yeast strains acting on specific key target microorganisms and areas of ruminal fermentation. Therefore, strain selection process is obviously critical in terms of safety; chosen organisms should be on the GRAS (Generally Recognized As Safe) list, or sufficient evidence would have to be provided to guarantee their innocuity for the animal, consumer and environment. Moreover, strain selection is important as different probiotics clearly exhibit markedly different effects on digestive microbiota of the same targeted organism. Dose response effects have also been reported for a same strain within the same experiment [63,85], suggesting that an optimal concentration of live cells has to be defined precisely according to the product application.

Efficacy of probiotics is strongly related to cell viability and metabolic activity [151], therefore, stability within the rumen is also an important consideration. Although yeast strains cannot properly colonize the rumen for a long period of time, certain strains can remain metabolically active in rumen fluid for more than 24 h [152] and live cells may be recovered from the faeces of treated animals up to several days after their initial incorporation in the diet. One objective when selecting a new probiotic strain will then be to assess its capacity to persist for a long time at a significant concentration in the targeted digestive compartment. Production, storage, and delivery protocols for yeast products should be designed to maintain yeast cell viability. High temperature storage, or in the presence of components such as minerals acting as oxidizing agents, may compromise

viability [153]. The most common and officially recognised method for quantification of viable yeast probiotics is the colony forming unit (CFU) plate counting technique. Although it is perfectly adapted to take into account cells which have the capacity to multiply in optimal environmental conditions, it has long been recognized that microbial cells may exist in a latent state, in which they will not form colonies on nutrient media but may have other measurable activity [154]. For example, throughout alcoholic fermentation, *Saccharomyces cerevisiae* cells have to cope with stress conditions that could affect their viability and thereby enter into a Viable But Not Culturable (VBNC) state [155,156]. Further methodological developments would be necessary in order to take into account this status, which would improve our understanding on adaptive responses of probiotic yeasts to digestive conditions.

9. Conclusions and future work

Yeast probiotics benefit from a natural and well-accepted image by the consumer, as they are not involved in health disorders and do not have any detrimental impact on environment. Moreover, yeasts have been used for a long time in human nutrition. More and more well controlled research studies indicate that they can be useful to positively balance the rumen microbiota, stabilize rumen pH, and promote microbial degradation of plant cell walls. Thanks to their action, improvement in animal production and health can be obtained and in that sense one can expect a promising future for these additives in ruminant nutrition. As particularly shown for fibre degradation, the nature of dietary ingredients has a great influence in the rumen response to yeast probiotics. More research is needed to enlarge the efficacy data base using various diets and raw materials, which in term would lead to elaboration of predictive tools applicable on farms.

In the context of a high feed cost, fermentation aids such as live yeast represent a valuable nutritional tool which allows increasing the forage portion of the diet and consequently limiting the costly sources of energy. In addition, current intensive farming practices require high levels of fermentable carbohydrates which put the animal at risk of developing metabolic disorders. In that sense, yeast probiotics become even more relevant when the digestive microbiota is challenged, for example during a feed transition (weaning, grazing, step up feeding programs) or during periods of stress (hot temperature, transportation). In these particular conditions, higher yeast doses appear to better support rumen challenges. As differences have been reported in terms of response of the ruminal microbiota to different yeast additives (strain and capacity to retain metabolic activity), it is important to focus on the way the yeast strain is selected. Future research will also need to address the behavior of the yeast cells in the digestive environment. Indeed, identification of specific metabolic and physiologic characteristics exhibited by the yeast strains would allow a better understanding of their interactions within the animal gut and will help to further select more targeted additives for improved benefits in ruminant nutrition.

During plant cell wall breakdown and fermentation, most of cellulolytic bacteria, with the exception of *Fibrobacter succinogenes*, produce a lot of hydrogen, which is used to reduce

carbon dioxide by *Archaea* methanogens to produce methane. This hydrogen transfer is important for a good functioning of the rumen ecosystem, but at the same time methane formation represents a loss of energy (10-12% of the metabolizable energy of the host animal) and this gas being a potent greenhouse gas, it should be decreased [157]. Studies with gnotobiotically-reared lambs have shown that animals inoculated with *F. succinogenes* were less prone to produce methane than lambs inoculated with *Ruminococci* and fungi, without significant modifications of rumen fibre degradability and volatile fatty acid concentrations [158]. The use of microbial solutions to promote *F. succinogenes* would then appear interesting to be able to mitigate methane emissions by cattle.

It is noteworthy that the increase in feed efficiency reported in presence of yeast probiotics has already an indirect effect on polluting outputs as it will decrease the amount of output/kg of milk/meat produced, but targeting microorganisms directly involved in these fermentative processes may be of interest.

Biohydrogenation mechanisms would also be a good target as they appear to be involved in milk fat depression which is very commonly observed in high-yielding cows, at risk for SARA. Under certain conditions, rumen microbial biohydrogenation results in the formation of fatty acids that are potent inhibitors of milk fat synthesis, i.e. trans10,cis12-CLA, and of possibly related intermediates from linolenic acid and other polyunsaturated fatty acids [48]. It has been shown that *Butyrivibrio sp.*is able to produce mainly trans-11,vaccenic acid via cis9, trans11-CLA instead of trans10,cis12-CLA from linolenic acid. By increasing the *Butyrivibrio* sp. population so that they utilize more linolenic acid at the expense of the organisms which form the detrimental isomer trans10,cis12 CLA, the potential exists to avoid a decrease in milk fat content. Stabilising ruminal pH through the addition of live yeasts should be beneficial for improved growth of these organisms which are sensitive to low pH. Moreover, promising data have been recently obtained that show a stimulation of *B. fibrisolvens* colonization on plant cell walls.

Yeast probiotics which have a good survival beyond the rumen may have interesting effects on intestinal homeostasis, and could thereby positively influence immune system and animal health. Indeed, certain strains of *Saccharomyces* may reduce pathogen load or their effects through competitive exclusion, cell binding or degradation of the toxins produced by intestinal pathogens. The beneficial effect that live yeast can have on pH regulation could also limit the release of inflammatory molecules, such as lipopolysaccharide or biogenic amines, and counteract the set up of acid-resistance mechanisms which may increase the virulence of certain pathogens. It has been reported that acidification of the rumen environment may increase mycotoxin absorption at low pH and decrease microbial detoxication mechanisms [159], so a better control of rumen pH by probiotic yeast may also aid in decreasing mycotoxin animal exposure.

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