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Research article

Effects of *Saccharomyces cerevisiae*, medium and forage type and their interactions on *in vitro* ruminal fermentation

A. Russouw [a], E. Chevaux [b], F. Chaucheyras-Durand [b,c], G. Esposito [a,d], E. Raffrenato [a,d,*]

[a] Department of Animal Sciences, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa
[b] Lallemand SAS, 19 rue des briquetiers, 31702, Blagnac, France
[c] Université Clermont Auvergne UMR MEDIS, INRA, 63122, Saint-Genès Champanelle, France
[d] RUM&N Sas, 42123, Reggio Emilia, Italy

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ABSTRACT

The objective of this study was to investigate the effects of a live yeast, *Saccharomyces cerevisiae* CNCM I-1077, at four doses (0, 1×10^5, 1×10^6 and 1×10^7 cfu/mL) according to the reducing medium used [Goering-Van Soest (GV), McDougall (MD) or Kansas State (KS)] on *in vitro* ruminal neutral detergent fibre digestibility (NDFd), rate of digestion of NDF (kd), organic matter digestibility (OMd), dry matter digestibility (DMd), pH as well as volatile fatty acids (VFA) concentration, using two forages (oat hay and wheat straw) with differing chemical composition. The maximum *in vitro* NDFd, DMd, OMd as well as kd were obtained with dose 1×10^6 cfu/mL, although differences between doses were not always significant. The pH estimates were the lowest with the 1×10^7 cfu/mL dose, but the differences were not all significant; however, 1×10^7 cfu/mL corresponded to significantly lower pH estimates compared to the control and 1×10^5 (6.51 vs. 6.60 and 6.59, respectively). The decrease in pH was accompanied by an increase in VFA concentrations as the yeast dose increased. The KS medium resulted in the lowest digestibility estimates, pH estimates as well as kd, regardless of yeast dose. The 1×10^6 cfu/mL was the better performing yeast dose *in vitro* resulting in higher digestibility estimates which indicates the yeasts ability to stimulate the microorganisms within the rumen by beneficially modifying rumen environment, thus promoting microbiota activity. The MD and GV media provide better environments for fermentation than the KS medium, resulting in higher *in vitro* NDFd, DMd, OMd, pH estimates as well as rate of NDF digestion. The MD and GV are also the media that resulted in more consistent results when analysing the effects of the live yeast. Our data suggest that the *in vitro* conditions have to be carefully chosen to be able to demonstrate rumen fermentation shifts with the use of live microbial additives.

1. Introduction

In recent years, more emphasis is being placed on substitutes to antibiotics in the dairy industry with the main focus being on direct fed microbials (DFM) as they are recognized as being a safer alternative to both animal and consumer (Thrune et al., 2009). Yeast additives or direct-fed microbials (DFM) have been reported to increase animal performance and health (Chaucheyras-Durand et al., 2008), however, the responses have been somewhat inconsistent (Carro et al., 1992a; Doreau and Jouany, 1998) or dismissive (Raeth-Knight et al., 2007). Many studies attribute the variable effects to the differences in experimental diets, feeding systems, different doses and strains of yeast being used (Chaucheyras-Durand et al., 2008; Patra, 2012; Wang et al., 2016).

Chaucheyras-Durand et al. (2008) previously stated that not all yeast strains should be classified as the same, and neither should different species of yeast, as seen in the study by Wang et al. (2016). Three different species of yeast were compared *in vitro* with regards to the fermentation of cereal straws. When looking at the *in vitro* fermentation of cereal grains, digestibility values [dry matter digestibility (DMd) and neutral detergent fibre digestibility (NDFd)] are regarded as the most important parameters (Wang et al., 2016). Wang et al. (2016) found noticeable differences in both *in vitro* DMd and NDFd between three different yeast species incubated at different doses. *Candida tropicalis* improved both DMd and NDFd for maize stover and rice straw, while *Candida utilis* decreased both DMd and NDFd compared to the control. *Saccharomyces cerevisiae* showed non-significant differences when

* Corresponding author.
E-mail address: emiliano@sun.ac.za (E. Raffrenato).

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compared to the control, but the differences were dependant on the dose (Wang et al., 2016). It should be noted that the highest dose of yeast did not always result in the highest digestibility values as seen in *in vivo* studies as well (Nocek et al., 2002). Furthermore, when the objective is to quantify the effect of a DFM (yeast) using an *in vitro* fermentation, the stimulated rumen environment itself could interact with the yeast, the specific dose and the sample(s) used.

Currently, *Saccharomyces cerevisiae* is the most common yeast species being used as a supplement for high producing dairy cows (Chaucheyras-Durand et al., 2008; Opsi et al., 2012; Thrune et al., 2009). Many *in vitro* studies, to test the effects of *S. cerevisiae*, make use of the GV medium (Bossen et al., 2008; Elghandour et al., 2014; Goering and Van Soest, 1970) or the MD incubation medium (Carro et al., 1992b; McDougall, 1948; Kung et al., 1997), with some studies also making use of the KS medium (Holden, 1999) but, according to our knowledge, no studies have been conducted to compare the three media and the presence of rumen modifiers. Leo Penu et al. (2012) compared the GV and KS incubation media on gas production *in vitro* and found significant differences between the two. The GV medium allowed for better gas production at 24, 48 and 72 h and for better buffering capacity than the KS medium (Leo Penu et al., 2012). This study suggests different fermentative behaviour of the rumen microbial populations, that could impact their response to rumen modifiers such as live yeasts. The hypothesis is therefore that the choice of a reducing medium for an in vitro fermentation will affect the outcome when testing a rumen modifier at different inclusions, such as a yeast, and result in different conclusion about the specific additive, according to the specific medium. The sample, and its characteristics, used could also possibly interact with the medium, besides the additive.

The objective of this study was thus to investigate the effects of a live yeast, *Saccharomyces cerevisiae* CNCM I-1077, at four doses, 0, 1×10^5 (10^5), 1×10^6 (10^6) and 1×10^7 (10^7) cfu/mL, according to the reducing medium used (GV, MD or KS) on *in vitro* NDFd, kd, OMd, DMd, pH and volatile fatty acids (VFA) concentration using two forages (oat hay and wheat straw) with differing chemical compositions.

2. Materials and methods

2.1. Forages and chemical analysis

Two forages, chosen to have a wide range of cell wall quality and proportion, oat hay (OH) and wheat straw (WS), were dried at 60 °C for 48 h and then ground through a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA). Samples were analysed for moisture and ash (AOAC, 2002; method 934.01 and 942.05), neutral detergent fibre (aNDFom), acid detergent fibre (ADFom), acid detergent lignin (ADL), ether extract (EE), crude protein (CP) and starch. Neutral detergent fibre was analysed as described by Mertens (2002) using Gooch crucibles with porosity 2, and with the addition of heat-resistant alpha-amylase and sodium sulphite. Acid detergent fibre (ADFom) and ADL were analysed as described by Raffrenato and Van Amburgh (2011). Both aNDFom and ADFom were ash-corrected. All fibre fractions were analysed with Gooch crucibles fitted with glass fibre filter with porosity 1.5 μm (934-AH™ by Whatman®, Whatman Limited, GE Healthcare, Maidstone, UK; Raffrenato and Van Amburgh, 2011). Ether extract was determined using Tecator Soxtec System HT 1043 Extraction Unit (AOAC, 2002; Method 920.39). Crude protein was measured with a Leco N analyser ("FP-528" Leco Africa (Pty), Ltd, Kempton Park, South Africa), while starch was determined as described by Hall (2008). The chemical composition of the two forages used in this study can be seen in Table 1. The oat hay used in the study had unexpectedly low CP, probably due to a combination of factors: low N fertilization, a late cutting in the season and a high hay harvesting, that determined a dilution from the high starch content (Coblentz et al., 2017).

2.2. In vitro fermentation

The experiment was performed as a $4 \times 3\times2$ factorial arrangement in a completely randomized design. Factors for this experiment consisted of three media, three doses of yeast and two forages. Combinations of feed sample, medium and yeast dose were incubated in quadruplicates for 0, 12, 24 and 48 h, following the procedure described by Goering and Van Soest (1970) with adjustments to the medium used, for all determinations except NDF rate of digestion (NDF-kd). Samples were also incubated for 120 and 240 h to estimate indigestible NDF and calculate the rate of NDF digestion (kd) according to Raffrenato et al. (2019). Feed samples were weighed (0.5 ± 0.05 g) into 125-mL Erlenmeyer flasks and 40 mL of medium was added to each flask. Three different media were used for this trial under strictly anaerobic conditions, namely KS, MD and GV. All three media were prepared as described by Marten and Barnes (1980), McDougall (1948) and Goering and Van Soest (1970), respectively.

An active dry yeast, *S. cerevisiae* CNCM I-1077, was prepared using peptone water (FDA and US Food and Drug Administration, 2001) and was injected at 0 h into each flask to make up a final dose of 10^5, 10^6 or 10^7 cfu/mL yeast within the flasks. Flasks with no yeast were present to serve as controls for each time point and blank flasks were also used to rectify for any particles present in the rumen fluid. Both blanks and control flasks were injected with the same amount of peptone water. Flasks were incubated with 10 mL rumen fluid as described by Goering and Van Soest (1970) to obtain a final 1:4 ratio between rumen fluid and medium. Rumen fluid was collected before the morning feeding from two lactating Holstein cows receiving a total mixed ration (TMR; Table 2) and being housed at the Stellenbosch University research farm, Western Cape, South Africa. The trial was approved by the Stellenbosch University Research Ethics Committee (Animal Care and Use; Approval SU-ACUD15-00060). Rumen fluid was transported, in filled-up thermos flasks, to the laboratory where it was mixed and filtered through four layers of cheese cloth into a pre-warmed flask kept at 39 °C. Once all fluid had been filtered, the air space above the fluid in the flask was purged with CO_2, before it was injected into the flasks.

Duplicates of the fermentations' residuals were analysed for aNDFom (Mertens, 2002) and pH was measured at 0, 12, 24 and 48 h. Volatile fatty acids were also determined via gas-liquid chromatography according to Siegfried et al. (1984). The VFA analysed included acetate (A), propionate (P), butyrate (B), isobutyrate (IB), valerate (V), isovalerate (IV) and total VFA (TVFA), although more emphasis was placed on A, P, B, TVFA and A:P ratio. Extra flasks with each treatment combination were added and removed at 0 h to measure initial pH and VFA as initial reference points. After ruminal *in vitro* fermentation, the other flasks were

Table 1. Chemical composition of the forages used in the study on a DM basis (%).

Forages	Item[1]							
	aNDFom	ADFom	ADL	EE	CP	Starch	Moisture	Ash
Oat hay	60.6	34.0	5.27	3.06	3.12	16.14	5.50	6.62
Wheat straw	83.1	54.1	6.71	0.82	7.85	0.12	10.89	4.13

[1] aNDFom: Ash-corrected, amylase-treated Neutral Detergent Fibre; ADFom: Ash-corrected Acid Detergent Fibre; ADL: Acid Detergent Lignin; EE: Ether Extract; CP: Crude Protein.

Table 2. Total mixed ration fed to the donor cows.

Ingredient	% DM[1]
Ground maize	38.30
Lucerne hay	28.31
Maize gluten	7.25
Wheat straw	6.60
Sugarcane molasses	5.62
Soybean meal	3.07
Barley malt	3.03
Potato by-product meal	2.17
Dry molasses	1.84
Feather meal with blood	1.54
Limestone	0.85
Blood meal	0.65
Salt	0.44
Urea	0.15
Monocalcium phosphate	0.13

[1] Expressed on Dry Matter basis.

subjected to acid-pepsin digestion and analysed for dry and organic matter digestibility values (DMd and OMd) as described by Tilley and Terry (1963). All fermentations and digestions were run three times with each forage run separately.

2.3. Statistical analyses

In vitro NDF digestibility values, the estimated rates, DMd, OMd, pH and VFA were analysed as response variables by the GLIMMIX procedure of SAS using a factorial arrangement of forage, medium, yeast and all interactions. Run was added as random factor with time treated as repeated measure. The highest order interaction (dose × medium × forage × time) was removed from the model because non-significant and because its presence did not result in an improvement of the Bayesian Information Criterion (Kass and Raftery, 1995; Schwarz, 1978). The control parameters for NDF were the digestibility and rates of NDF digestion, when forages were fermented alone. Differences between means and the control were declared significant at $P \leq 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P \leq 0.10$ were considered tendencies. Treatments are reported as least squares means and because of the high number of interactions, only the most significant ones will be presented and discussed.

3. Results

3.1. Neutral detergent fibre digestibility

A summary of the results of all response variables analysed is shown in Table 3. Of the main effects, medium, yeast level and time were significant, while forage tended to be significant.

Specifically, for yeast, when pooling all other factors, NDF digestibility was the highest for the 10^6-dose, which corresponded to about 16% increase in NDFd compared to the control. Differences between the other levels were not always significant. Yeast also significantly interacted with medium and time (Figure 1, Table 4; $P < 0.05$), resulting in different responses when considering these variables, but not with forage ($P = 0.18$). The highest NDFd was obtained when using 10^6 for both the MD and VS media (0.367 and 0.362 g/g NDF). However, when using the KS medium, yeast did not have any effect. When looking at the interaction yeast × time, 10^6 was consistently effective in increasing digestibility after 12 h (Table 4).

Moreover, both forage and medium interacted significantly ($P = 0.0015$), resulting in the KS medium having lower estimates, for both forages, compared to MD and GV (not shown).

Medium and forage interacted significantly with time ($P < 0.0001$). When looking at the combinations resulting from the interaction medium × time, the KS medium presented the lowest NDFd up to 48 h (Figure 2), with 120 and 240 h showing similar values (not shown). Of the 3-way interactions, the interaction between medium, forage and yeast resulted significant showing how the yeast had different effects across media and forage (not shown).

3.2. Rate of NDF digestion

Medium had a significant effect ($P < 0.0001$) on the rate of NDF digestion, while forage and yeast did not ($P = 0.53$ and 0.44). For yeast, the highest numerical rate (0.0340 h^{-1}) was observed for the 10^6-dose, when pooling all other factors, consistent with the results from NDFd, but

Table 3. P-Values of the fixed effects and their respective interactions, for all response variables tested.

Response variable[1]	NDFd	NDF-kd	DMd	OMd	TVFA	A	P	B	IB	V	IV	pH
Effect												
Medium	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Forage	0.0693	0.5358	<0.0001	<0.0001	0.0014	0.1113	0.0008	<0.0001	0.0016	0.0061	0.0092	<0.0001
Yeast	0.0001	0.4382	0.0079	0.0011	0.0023	0.0043	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Time	<0.0001	n.a.[2]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0506	0.0013	0.0175	<0.0001
Medium×Forage	0.0015	0.0159	<0.0001	<0.0001	0.0014	0.0005	0.0424	0.0028	<0.0001	0.0019	0.0014	0.1020
Medium×Yeast	0.0005	0.0239	0.5938	0.6041	<0.0001	0.0006	0.0367	0.1074	<0.0001	<0.0001	<0.0001	<0.0001
Medium×Time	<0.0001	n.a. [2]	<0.0001	<0.0001	0.0008	0.0091	0.0457	0.0399	<0.0001	<0.0001	<0.0001	<0.0001
Forage×Yeast	0.1817	0.5385	0.0647	0.0910	0.0492	0.6122	0.9542	0.0241	0.1494	0.5201	0.2267	0.3194
Forage×Time	<0.0001	n.a. [2]	<0.0001	<0.0001	0.2416	<0.0001	0.0008	0.0381	0.1340	0.5787	0.7452	<0.0001
Yeast×Time	0.0459	n.a. [2]	<0.0001	<0.0001	0.9345	0.1116	0.0265	0.0013	0.1209	0.2581	0.4317	0.0018
Medium×Forage×Yeast	0.0033	n.a.[3]	0.8490	0.6406	<0.0001	<0.0001	0.0016	0.9855	0.2759	0.1661	0.3096	<0.0001
Medium×Forage×Time	<0.0001	n.a. [2]	<0.0001	<0.0001	0.5333	0.9109	0.7530	0.1826	0.0231	0.3749	0.6733	<0.0001
Medium×Yeast×Time	0.5687	n.a. [2]	0.8714	0.7480	0.0144	0.3511	0.4867	<0.0001	0.0957	0.0475	0.0486	0.3794
Forage×Yeast×Time	0.5600	n.a. [2]	0.0743	0.0718	0.8511	0.0034	0.0199	<0.0001	0.0651	0.5324	0.2708	0.9885

[1] Response variables were: Neutral detergent fibre digestibility (NDFd), rate of digestion for NDF (NDF-kd), dry and organic matter digestibility (DMd and OMd), total volatile fatty acids concentration (TVFA), acetic acid concentration (A), propionic acid concentration (P), butyric acid concentration (B), iso-butyric acid concentration (IB), valeric acid concentration (V), iso-valeric acid concentration (IV) and rumen pH (pH).

[2] Time was not included in the model when NDF-kd was the response variable.

[3] Interaction excluded from the model when NDF-kd was the response variable because non-significant.

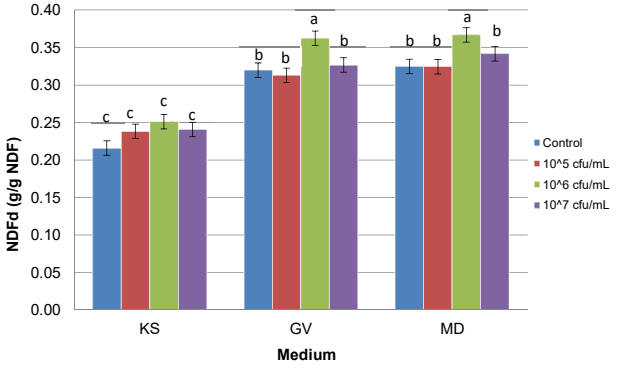

Figure 1. The effect of medium and yeast on NDFd (Neutral Detergent Fibre digestibility), when pooling time points (KS: Kansas State medium; GV: Goering and Van Soest medium; MD: McDougall medium). Interaction medium × yeast for NDFd was significant with *P*-value = 0.0005. Different lowercase letters indicate significant differences ($P < 0.05$).

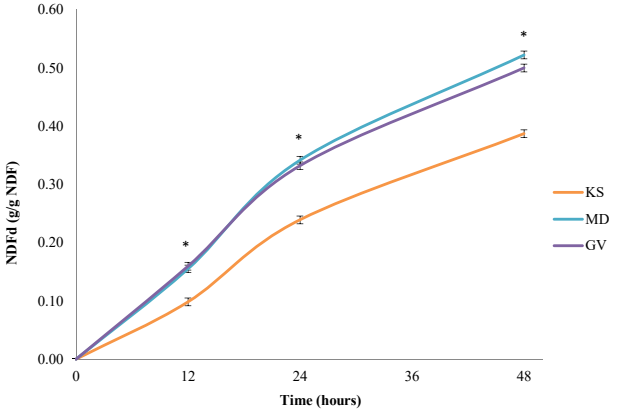

Figure 2. The effect of medium on Neutral Detergent Fibre digestibility (NDFd; g/g NDF), over time (KS: Kansas State medium; GV: Goering and Van Soest medium; MD: McDougall medium). The interaction medium × time for NDFd was significant with *P*-value < 0.0001. * NDFd at 12h and 24h for GV and MD was higher than KS; NDFd at 48h for MD was higher than GV, and NDFd for GV was higher than KS ($P < 0.05$).

the result was not significant. Yeast also interacted with medium ($P = 0.02$), but not with forage ($P = 0.53$).

The KS medium consistently resulted in the lowest estimates ($P < 0.0001$) of NDF rates of digestion, when pooling all factors, when compared to GV and MD, reflecting the results from the NDFd estimates. The significant interaction between medium and yeast ($P = 0.0239$) resulted in non-consistent differences between the doses across medium used, with the KS showing no difference across doses and the other two media showing some level of differences (Figure 3). Yeast did not interact with forage, resulting in consistent differences between the forages across doses.

The interaction medium × forage was significant ($P = 0.01$) resulting in inconsistent differences between the forages across media (Table 5).

3.3. Organic matter and dry matter digestibility

Yeast had a significant effect on both OMd and DMd ($P = 0.0011$ and 0.0007, respectively). The largest OMd and DMd corresponded to the 10^6 yeast dose (0.446 and 0.423, respectively). The yeast dose significantly interacted with time for both OMd and DMd ($P < 0.0001$), while only showing a tendency to interact with forage for OMd ($P = 0.0910$). Dose 10^6 also in this case resulted in the highest estimates for OMd and DMd, but only for the 24 and 48 h time points ($P < 0.05$). Oat hay had significantly higher ($P < 0.0001$) DMd and OMd estimates compared to WS, with dose 10^6 resulting in the highest digestibility for WS forage and only in a numerical difference for OH.

When looking at the interaction of medium and time, at 12 h none of the media were different ($P > 0.05$) from each other, while at 24 and 48 h all the media resulted in different OMd and DMd ($P < 0.01$). The MD medium resulted in the highest ($P < 0.01$) DMd and OMd estimates at 24 h (0.43 g/g DM and 0.45 g/g OM) and 48 h (0.56 g/g DM and 0.58 g/g OM) followed by GV and KS.

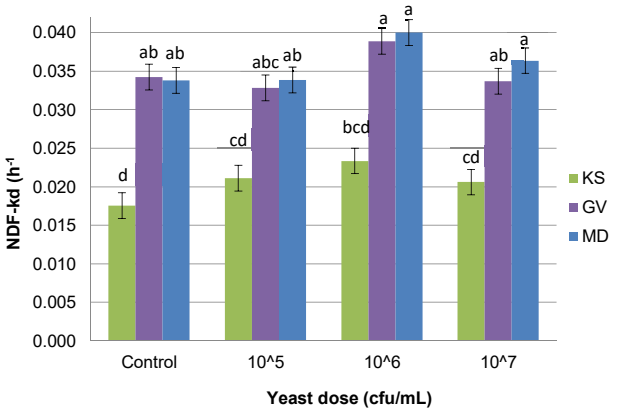

Figure 3. The effects of the interaction between medium and yeast dose on NDF-kd (rate of digestion for Neutral Detergent Fibre), when pooling forages (KS: Kansas State medium; GV: Goering and Van Soest medium; MD: McDougall medium). The interaction medium×yeast for NDF-kd was significant with P-value = 0.0239. Different lowercase letters indicate significant differences ($P < 0.05$).

3.4. Volatile fatty acids

For the total VFA and most of the individual VFA analysed, all the fixed effects were significant. The highest dose of yeast corresponded to the highest total concentration of VFA (i.e. 84.98 mM) and this result was confirmed for all the others VFA quantified, except for P. Yeast interacted significantly with medium, time and forage for A, P, B, V, IV and TVFA.

The GV medium resulted in the highest concentrations for all VFA followed by MD and KS. The concentrations of VFA did increase over

Table 4. The effect of yeast dose on Neutral Detergent Fibre digestibility (g/g NDF) over time, when pooling media.

Time (hours)	Yeast dose (cfu/mL)					
	Control	1×10^5	1×10^6	1×10^7	SEM[1]	*P* value
12	0.135^{a}	0.126^{ab}	0.154^{a}	0.135	0.009	<0.0001
24	0.288^{b}	0.295^{ab}	0.334^{a}	0.300^{ab}	0.009	<0.0001
48	0.438^{b}	0.454^{ba}	0.512^{a}	0.475^{ab}	0.009	<0.0001

[a-b]Means within a row with different superscripts differ ($P < 0.05$).
[1] Standard error of the means.

Table 5. The effect of medium and forage on rate of digestion for Neutral Detergent Fibre (h^{-1}; $P = 0.0239$).

Forage	Medium[1]				
	KS	GV	MD	SEM[2]	*P* value
Oat hay	0.0189[b]	0.0343[a]	0.0337[a]	0.0016	<0.001
Wheat straw	0.0223[b]	0.0354[a]	0.0382[a]	0.0014	<0.001

[a-b]Means with different superscripts within rows differ ($P < 0.05$).
[1] KS: Kansas State medium; GV: Goering and Van Soest medium; MD: McDougall medium.
[2] Standard error of the means.

time, with dose 10^7 resulting in the highest concentrations across all times, therefore resulting in no significant interaction between time and yeast, for most VFA. The difference in concentration between forages was significant for all except for A, while the interaction between forage and medium being significant for all VFA and TVFA. Oat hay resulted in higher concentrations compared to WS, although not always significantly.

Although the interaction between yeast and time was non-significant for most VFA, the interaction between yeast and time for the A:P ratio was significant ($P = 0.0009$; Table 6). The A:P ratio when no yeast was applied remained significantly lower compared to the other doses. The highest A:P ratios were however observed for 10^6 which followed the trend seen for NDFd.

3.5. pH

All fixed effects included in the model were significant. The highest yeast dose corresponded to the lowest pH value ($P < 0.005$) with no differences among the other yeast levels. Yeast interacted significantly with medium ($P < 0.0001$) and time ($P = 0.0018$) but not with forage ($P = 0.3194$). The KS medium resulted in the overall lowest ($P < 0.0001$) pH estimates followed by MD and GV. The interaction between medium and time was also significant ($P < 0.0001$), with differences in pH across time points and media not consistent (Table 7). The pH decreased for all doses as time increased ($P < 0.05$; statistical differences not shown). At 0 h there were no significant differences ($P = 0.12$) between the pH estimates for either the control or yeast treatments (Table 8).

Wheat straw resulted in a pH higher than oat hay (6.63 vs. 6.51; $P < 0.0001$) specifically at 12 and 24 h ($P < 0.01$; Figure 4). There were no significant differences between the pH estimates at 48 h ($P > 0.05$). The interaction between medium, forage and dose and the interaction between medium, forage and time were also both highly significant ($P < 0.0001$).

4. Discussion

4.1. Neutral detergent fibre digestibility

The intermediate yeast dose (10^6) corresponded to the highest NDFd. This was similar to what previously presented by Nocek et al. (2002) and Wang et al. (2016). This result, however, differs from the corresponding *in vivo* dose often suggested (1×10^5 cfu/ml) by yeast producing companies based upon a daily yeast supply of 1×10^{10} cfu in an average rumen volume of 100 L. In our study, live yeast supplementation occurred once at time 0 and in a closed batch system as opposed to a dynamic rumen environment. In vivo studies with young ruminants showed that live yeast clearance from the rumen starts within 30 h post administration (Durand-Chaucheyras et al., 1998), thus 48 h time point was already starting to become challenging from a true expected live yeast effect. The 10^5 dose did not actually differ from the control and

Table 6. The effect of yeast dose on volatile fatty acids over time.

Time (hours)	Yeast dose (cfu/mL)				
	0	1×10^5	1×10^6	1×10^7	SEM[1]
Acetate (mM)					
12	31.40[b]	31.94[b]	33.38[b]	37.19[a]	2.3065
24	40.25[b]	40.96[b]	44.19[a]	48.30[a]	2.2912
48	50.79[b]	49.56[b]	50.89[b]	55.99[a]	2.2912
Propionate (mM)					
12	15.87[a]	14.97[abc]	12.87[c]	15.61[ab]	0.9844
24	21.38[a]	18.56[bc]	17.28[b]	20.68[ac]	0.9811
48	25.07[a]	21.30[b]	20.57[b]	24.60[a]	0.9811
Acetate:Propionate ratio					
12	2.21[d]	2.15[d]	2.58[a]	2.41[abc]	0.0676
24	2.15[d]	2.22[cd]	2.55[a]	2.30[abcd]	0.0673
48	1.93[d]	2.34[ab]	2.47[ab]	2.21[c]	0.0673
Butyrate (mM)					
12	6.59[a]	5.89[a]	7.41[b]	8.55[c]	0.4250
24	8.29[ab]	7.35[a]	8.57[b]	9.82[c]	0.4226
48	9.06[a]	7.72[b]	8.74[a]	10.51[c]	0.4226
Total VFA (mM)					
12	54.87[a]	56.00[a]	57.17[a]	65.85[b]	3.4874
24	71.92[ab]	72.02[a]	75.31[a]	85.35[b]	3.4682
48	84.54[a]	84.24[bc]	86.09[ac]	98.75[a]	3.4682

[a-d]Means within a row with different superscripts differ ($P < 0.05$).
[1] Standard error of the means.

Table 7. The effect of medium and yeast on pH.

Medium[1]	Yeast dose (cfu/mL)				
	Control	1×10^5	1×10^6	1×10^7	SEM[2]
KS	6.09[b]	6.17[a]	6.18[a]	6.04[b]	0.0181
MD	6.79[ab]	6.76[bc]	6.74[c]	6.67[d]	0.0192
GV	6.90[a]	6.87[ab]	6.85[bc]	6.82[cd]	0.0161

[a-d]Means within a row with different superscripts differ ($P < 0.05$).
[1] KS: Kansas State Medium; GV: Goering and Van Soest Medium; MD: McDougall Medium.
[2] Standard error of the means.

Table 8. The effect of yeast dose on pH over time.

Time (hours)	Yeast dose (cfu/mL)				
	Control	1×10^5	1×10^6	1×10^7	SEM[1]
0	6.88	6.86	6.84	6.85	0.0226
12	6.63[a]	6.62[a]	6.57[a]	6.51[b]	0.0213
24	6.50[a]	6.50[a]	6.48[b]	6.43[b]	0.0163
48	6.42[a]	6.45[a]	6.44[a]	6.34[b]	0.0153

[ab]Means within a row with different superscripts differ ($P < 0.05$).
[1] Standard error of the means.

from the 10^7 dose. Our results also show that the effect was consistent only after 12 h of in *vitro* fermentation (Table 4) and when using two (MD and GV) of the three media tested.

It is interesting to note how the difference in digestibility apparently increased between the highest and lowest dose with the progress in time (Figure 2), with a net separation between the the 10^6 dose and the other levels. The increase in the digestibility estimates are assumed to be due to some of the proposed modes of action of the yeast supplement which include the ability to provide vitamins, nutrients and dicarboxylic acids (Newbold et al., 1998) as well as through the removal of O_2 which enters the rumen via feed or water, etc., from the rumen fluid which may hinder the growth of strict anaerobic bacteria (Chaucheyras-Durand et al., 2016). Interestingly, more live yeast does not always translate into greater effect as illustrated by Brassard et al. (2006) where 10 times the recommended dose did not provide a stronger rumen response to a challenging diet. Indeed, we can hypothesize that beyond a certain threshold of live yeast fed to the rumen, some competition for nutrients may occur between rumen microbiota and the added yeast, mitigating then the expected proportional effect. Here, the 10^7 dose was likely responding according to this hypothesis.

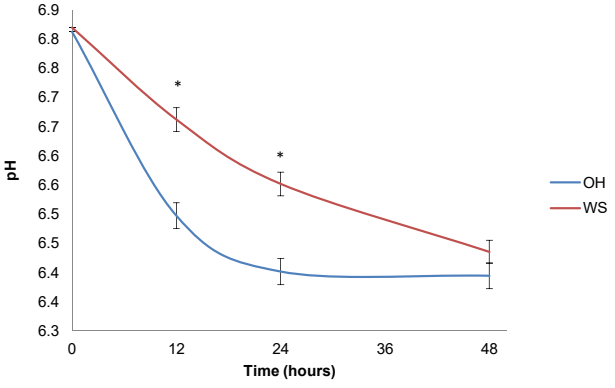

Figure 4. The effect of forage (OH: Oat hay; WS: Wheat straw) on pH over time. The interaction forage × time for pH was significant with *P*-value < 0.0001. *pH at 0h was similar between forages (P = 0.32); pH at 12 and 24h was higher for WS (P < 0.0001), and similar at 48h (P = 0.22).

The use of the yeast tended to be more effective with WS, resulting in increased proportional effect between 10^6 and the control for WS (+19%) rather than for OH (+12%). Independent of the yeast, WS interestingly tended to have higher NDFd when compared to OH. This was not expected as OH had lower NDF and lignin contents (60.72 %DM and 5.22 %DM) compared to WS (83.41 and 6.78 %DM for aNDFom and ADL, respectively). This trend was also seen when comparing both forages with different media and yeast doses as well as over time. This clearly demonstrates that the amount of cell wall present in a forage is not necessarily a measurement of forage quality, especially when comparing different species, but *in vitro* NDF digestibility estimates are necessary to characterize a forage. Lignin is known to affect cell wall digestibility (Van Soest, 1994). In fact, even if OH is characterized by a lower lignin value than WS on DM basis, the trend is the opposite when lignin is expressed on NDF basis (8.6% vs. 8.1% for OH and WS, respectively). The unexpected tendency of the wheat straw to be a better forage, in terms of NDF digestibility, when compared to oat hay could have also been supported by other chemical analyses for antimicrobial components that may have been present in the oat hay. Unfortunately, these analyses were not performed during the study. The CP content of the oat hay (3.12%) was also very low, compared to the wheat straw (12.85%), and that could have contributed to less N available during the fermentation *in vitro*. It is well known how readily available non-protein N (e.g. NH_3) during rumen fermentation is captured by fibrolytic bacteria and stimulates their activity (Van Soest, 1994). The presence of larger amount of starch in the oat hay might have also played a role in lowering fibrolytic activity. However, this last hypothesis is indeed supported by a lower pH when OH was fermented, when compared to WS, but the level of pH should have not affected fibrolytic activity.

Our initial hypothesis, that a specific reducing medium could affect the outcomes when testing a rumen modifier, and interact with the sample, was confirmed. In fact, the lower digestibility values for the KS medium up to 48 h could be seen as a result of the micro-environment provided for by the medium, with the KS not providing a suitable environment for fibrolytic bacteria, such as having a lower pH, compared to GV and MD. The KS medium did also not give a suitable environment for the yeast to contribute to the increased NDFd, like it happened for the GV and MD media (Figure 1). Furthermore, the interaction of medium with forage and yeast confirmed how WS and the media MD and GV responded better to the yeast. The interaction of forage with medium and time

showed again the net separation between the KS and the other two media especially at 24 h and the higher NDFd of WS, especially at 48 h (data not shown).

4.2. Rate of NDF digestion

Results for rate of NDF digestion only partially confirmed the NDF digestibility results. The tendency of the difference between the forages was not confirmed. The higher NDF digestibility for WS was compensated by the lower potentially digestible NDF and rates were similar to oat hay's digestion rates. The significant yeast effect for NDFd was also not confirmed, even if the trend was still observed. Rates of digestion are very sensitive to the anchor points used in the non-linear estimation (Raffrenato et al., 2019) and therefore the large standard errors might have nullified the significance in NDFd differences among the doses. However, the interaction of the medium with both yeast and forage confirmed the KS medium being the only one that cancelled out the differences between forages or among the doses of yeast. The yeast effect was in fact significantly higher for the 10^6-dose for both the MD and GV media, while the KS medium did not show any difference. Thus, the rates' results confirmed how the KS medium created a lower quality microenvironment for cell wall fermentation, independent of the yeast presence. The interaction with yeast showed how yeast efficacy was lost with KS. When using different media, comparing either NDF digestibility or rate of digestion values should therefore be avoided.

4.3. Organic matter and dry matter digestibility

The organic and dry matter digestibility results confirmed that 10^6 was the most effective dose when pooling all time points and media used. This was not seen in the study by O'connor et al. (2002) who found that yeast had little effect on the extent of *in vitro* DMd for different yeast cultures as well as two varying doses. The interaction between yeast and time confirmed the efficacy of the yeast only after 12 h of *in vitro* fermentation with the 10^6 dose being the most effective for both 24 and 48 h OMd and DMd, but not for 12 h.

The second stage of the *in vitro* digestion resulted in the OH having higher OM and DM digestibility values. This was unlike the results seen for NDFd, where WS had higher digestibility values than OH, which was unexpected and reflected the apparently better quality NDF present in the WS compared to OH. A possible hypothesis for the higher DMd and OMd estimates for OH could be due to the acid-pepsin digestion, where a large part of the digested matter is non-fibre and therefore being higher in OH than WS.

While the MD medium proved to provide a better environment for the microorganisms compared to GV and KS, during the initial rumen *in vitro* stage, it is assumed that during the second stage of the Tilley and Terry (1963) procedure, all the digestible matter is digested by the acid-pepsin treatment and therefore the differences between the media mainly originate from the first stage when fibre is degraded as well. The medium that resulted in extra fibre degradation during the ruminal fermentation can also free more digestible matter trapped by the cell wall. In other words, the more fibre was degraded in the first stage of the Tilley and Terry procedure, the more OMd and DMd in the second stage.

4.4. Volatile fatty acids

The volatile fatty acids not always confirmed the results from the NDFd. While the dose of 10^6 was among the highest numerical values, the 10^7 resulted in the significantly highest VFA production within the *in vitro* fermentation, demonstrating how yeast promotes fermentative bacterial activity. Apparently, there was no correspondence between the NDFd results and the VFA quantified. This may be due to the lower disappearance of the cell wall when the 10^7-dose was applied, while the higher activity of the bacteria was supported by the presence of the larger concentration of yeast. The highest ratio (i.e. 2.53; $P < 0.0001$) between acetic and propionic acid corresponded to the 10^6-dose, confirming however the results from NDFd.

Even though significant, the difference between the GV and the MD medium was opposite to the results for NDFd. Therefore, the GV medium would favour microbial activity, not necessarily related to cell wall disappearance. However, the differences for both NDFd and VFA for the two media were relatively small and probably not biologically important. It is relevant to note that for the NDF procedure, after *in vitro* fermentation, the samples were not centrifuged (as originally suggested in the procedure; Van Soest, 1994) and one of the media might interact with the cell wall recovery. On the other hand, it is also possible that the medium itself may interact with the VFA analysis, biasing the results or degrading the VFA at different rates for the two media. According to our knowledge, there is no published work looking at the effect of medium on NDF or VFA analysis and degradation.

As expected, oat hay fermentation resulted in the highest VFA production. While the TVFA produced by the OH corresponds to the larger portion of non-fibre organic matter of the OH, the results of the two forages for acetic acid followed a different trend when compared to the NDFd results and reflected the higher potentially digestible NDF pool. Thus, the higher NDFd for wheat straw was counteracted by the larger digestible fibre for OH. The highest dose (10^7) resulted in highest TVFA concentrations for both forages, indicating the increase in rumen microbial fermentation. An alternative hypothesis is that the yeast itself would contribute to some organic acid production (i.e. acetate) according to (Chaucheyras-Durand et al., 2016).

The higher A:P value for the 10^6-dose indicate increased degradation of fibre resulting in increased amounts of A compared to P. Regardless, A:P for all doses remained higher compared to the control across time, although not always significant. These findings agree with findings by O'connor et al. (2002) but not with Wang et al. (2016) who found a significant decrease in the A:P ratio compared to the control with an increase in yeast dose, after a threshold of yeast dose of 0.25×10^7 cfu/mL, which is also the closest to our 10^6-dose. In our case a negative effect was indeed reached after this threshold for A:P ratio, isobutyrate and pH as well, suggesting that the 10^6 cfu optimum dose identified in this study may correspond to a critical yeast concentration beyond which benefits are no longer linearly increasing. The A:P ratio gives the indication as to which direction fermentation is favouring, either towards acetic acid which is fermented from fibre or towards propionic acid which is starch derived.

4.5. pH

The significant difference between the 10^7 dose and the rest of the yeast levels were too small to be considered biologically relevant, with the largest pH difference, within each medium, being between 0.08 and 0.14. The findings were however similar to those found by Wang et al. (2016). In that case, the pH for maize stover and rice straw also decreased when the dose of *S. cerevisiae* increased, although not always significantly. In our study the lowest pH consistently corresponded to the highest dose, with the other yeast levels not being consistent in pH values. However, the trend was consistent when analysed within the MD or the GV medium. In fact, for the MD and GV media, the highest pH was observed for the control and decreased as the yeast dose increased (Table 6) and this might be related to a more active fermentation in the presence of yeast, and a contemporary mitigation of rumen pH due to stimulation of lactate utilizers and presence of a more competitive environment for lactate producers (Chaucheyras-Durand et al., 2008). The KS medium resulted in the shortest buffering capacity among the media utilized. The pH dropped below 6.0 for the KS medium which could explain why greater digestibility differences were observed at 24 and 48 h compared to MD and GV, whose pH values remained above 6.0 for all time points. Previously, it had been reported that fibre digestibility is hindered when pH drops below 6 (Rode, 2000) as was also seen by Shriver et al. (1986) who observed a decrease in *in vitro* NDFd as pH

decreased from 6.2 to 5.8. It is interesting to notice how the pH consistently decreased with time for the KS medium resulting in the time points being always different from each other in pH (i.e.: 6.49 to 5.87, from 0 to 48 h, respectively), while differences were not always significant for the other media. This result suggests a stronger buffering capacity for the GV and MD media.

The lower pH levels observed for OH could be attributed to the higher amount of starch and increased concentrations for A and P compared to WS. Higher concentrations of A and P were observed for OH compared to WS (62.95 vs. 49.97 mM and 26.80 vs. 20.82 mM, respectively). There was no significant difference seen for butyrate between the two forages.

5. Conclusions

The 10^6 cfu/mL was the best performing live yeast dose *in vitro* resulting in higher digestibility estimates. Organic and dry matter digestibility were also positively affected with increased values for the 10^6-dose as well. The highest dose (10^7) instead resulted in significantly higher VFA concentrations demonstrating the yeast's ability to facilitate fibre digestion *in vitro* and by supplying the microorganisms with a better micro-environment. Somewhat dismissive effects by the yeast were seen on the pH due to the control having higher pH estimates compared to the all three yeast treatments. The significantly higher concentrations of VFA as yeast dose increased, corresponds with the decrease in pH. It should, however, be emphasized that even small differences in pH of 0.1 could result in significant differences between treatments. Taking that into account, yeast was capable of stimulating fermentation while maintaining a relatively stable pH. However, all pH estimates remained within the normal ranges for fermentation to occur, therefore it cannot be determined as to whether the yeast had the ability to stabilize rumen pH. The repeatability of the *in vitro* fermentations was high enough to detect small differences even if they were not biologically important.

The MD and GV media provide a better environment for fermentation than the KS medium, resulting in higher NDFd, DMd, OMd, pH estimates as well as rate of digestion and VFA. This study gives important insights regarding different media used *in vitro* and how they can result in variable digestibility values as well as their interaction with *Saccharomyces cerevisiae*. Thus, when testing or quantifying various parameters, in vitro fermentations should be standardized, with possibly having the goal of measuring the intrinsic characteristics of the feed tested. It is important that care should be taken when effects of feed additives on *in vitro* fermentations parameters are evaluated, especially when comparing studies with different forages and media as interactions between variables may result in different outcomes.

Declarations

Author contribution statement

A. Russouw: Performed the experiments; Wrote the paper.

E. Chevaux, F. Chaucheyras-Durand: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

G. Esposito: Contributed reagents, materials, analysis tools or data.

E. Raffrenato: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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