Influence of Living and Autoclaved Yeasts of *Saccharomyces boulardii* on In Vitro Ruminal Microbial Metabolism

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ABSTRACT

Experimental data on the effects of *Saccharomyces boulardii* on rumen microbial metabolism are scarce. The aim of this study was to examine whether *S. boulardii* had an effect on parameters of rumen microbial metabolism at different dosages and whether the yeast would be suitable as a probiotic agent for ruminants. To test whether the potential positive effects of *S. boulardii* could be attributed to the yeast's viability or to its content of nutrients, living and autoclaved yeasts were tested simultaneously. For this purpose, incubation trials were carried out using the long-term rumen simulation technique. Living and autoclaved yeasts were added to fermentation vessels at a concentration of 0.5 or 1.5 g/d. The addition of living and autoclaved yeasts stimulated microbial metabolism, with no major differences between the treatments. It was concluded that ruminal microbes digested the supplied yeast of *S. boulardii* as an additional substrate and that *S. boulardii*, at least in ruminants, is utilized as a prebiotic rather than as a probiotic agent.

(Key words: *Saccharomyces boulardii*, yeast, ruminal microbial metabolism)

Abbreviation key: SCFA = short-chain fatty acids.

INTRODUCTION

Dietary supplements of yeast cultures, based on freeze-dried *Saccharomyces cerevisiae*, have been reported to improve health and productivity of ruminants. In comparison with antimicrobial agents, yeast cultures offer a natural alternative to manipulate animal performance. They have been shown to improve feed intake (Phillips and von Tungeln, 1985; Harris and Lobo, 1988; Williams et al., 1991), milk production (Harris and Webb, 1990; Piva et al., 1993; Kung et al., 1997; Dann

et al., 2000), and weight gain (Phillips and von Tungeln, 1985; Fallon and Harte, 1987; Hughes, 1988; Drennan, 1990). However, yeast cultures have not been found to alter ruminal metabolism or improve animal performance in all cases (Adams et al., 1981; Harrison et al., 1987; Arambel and Kent, 1990; Chademana and Offer, 1990; Williams et al., 1991; Cabrera et al., 2000; Garcia et al., 2000). Some of the reasons for these inconsistencies may be strain-dependent (Newman and Spring, 1993; Newbold et al., 1995), or due to specific differences between commercial additives (Arcos-García et al., 2000; Miller-Webster et al., 2002), or mediated by differences in dietary compositions (Fiems et al., 1993; Wallace, 1994; Adams et al., 1995). More than 1000 strains of *S. cerevisiae* are listed in the American Type Culture Collection catalogue (ATCC, 1990) and it is still not known how widespread the so-called probiotic activity is among these strains of yeast. It has been observed that different strains of *Saccharomyces cerevisiae* had distinct effects on rumen bacteria under in vitro conditions and in in vivo experiments with sheep (Newbold et al., 1995). *Saccharomyces boulardii* is a nonpathogenic yeast that was originally used in humans to prevent or treat diarrhea of various origins (Czerucka and Rappal, 2002). In addition, it has been shown in recent in vitro experiments in Ussing chambers with mucosal tissues from the mid-jejunum of pigs that prophylactic treatment of the animals with *S. boulardii* can significantly diminish an artificially induced secretory response of the jejunal mucosa due to application of theophylline (Schroeder et al., 2004). Theophylline increases intracellular cyclic nucleotide levels, which can stimulate mucosal Cl⁻ secretion. Lowering this effect, as seen with *S. boulardii*, at least supports the concept that probiotics may exert beneficial effects in the gastrointestinal tract.

In contrast, the effects of *S. boulardii* on ruminal microbial metabolism have not yet been investigated. It was the aim of this study to investigate potential positive effects of this strain on short-chain fatty acid (SCFA) production rates, protein synthesis, and digestibility of organic matter. We specifically addressed the

Received July 28, 2004.

Accepted January 31, 2005.

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Table 1. Chemical compositions of the diets (%).

Ingredient	Hay	Concentrate
DM	93.94	90.48
CP	7.27	15.96
Crude lipids	0.88	2.32
Crude fiber	27.59	10.49
N-free extract	46.01	43.46
Total ash	6.13	8.83

question of whether the effects of *S. boulardii* required viable probiotic yeasts or would also be observed with the autoclaved (prebiotic) form.

MATERIALS AND METHODS

Incubation Technique

The study was carried out using the rumen simulation technique (RUSITEC; Czerkawski and Breckenridge, 1977). The experiment consisted of 9 plexiglass 1-L vessels (fermentors) maintained at constant temperature (39°C). The nominal volume in each vessel was 750 mL. The vessels were inoculated on d 1 with liquid (gauze-filtered) and solid rumen contents taken from 2 rumen cannulated adult donor sheep that were maintained on a diet of hay and concentrates. Animals had free access to hay, water, and a vitamin-enriched salt lick. The same dietary components were used for in vitro fermentation trials. The chemical composition is presented in Table 1. Each vessel was loaded with 2 nylon bags (pore size, 150 μ m). At the start of the trial, one bag was filled with 80 g of solid rumen contents (fresh weight) and the other with the daily diet, a mixture of 5 g of hay and 4 g of pelleted concentrate. The nylon bag with solid rumen contents was replaced after 24 h of incubation with a bag containing the diet. The feed bag was changed after 48 h so that 2 bags were always present. This gave a retention time of 48 h for feed. When the bag was being changed, the vessels were flushed with nitrogen to maintain anaerobic conditions. The liquid flow through the vessels was maintained by continuous infusion of a buffer solution of pH 7.4 with 293 mosm/L at a rate of 750 mL/d. The buffer composition is presented in Table 2. Starting with the collection

Table 2. Chemical composition of the buffer solution (mmol/L).

Ingredient	
NaCl	28.00
KCl	7.69
CaCl ₂ ·2 H ₂ O	0.22
MgCl ₂ ·6 H ₂ O	0.63
NH ₄ Cl	5.00
¹⁵ NH ₄ Cl	0.07
Na ₂ HPO ₄ ·12 H ₂ O	10.00
NaH ₂ PO ₄ ·H ₂ O	10.00
NaHCO ₃	97.90

period, a solution of ¹⁵NH₄Cl (98% excess ¹⁵N, Promochem, Wesel, Germany) was added to the buffer solution as a marker for calculating net microbial protein synthesis.

Experimental Procedure

To examine the role of living and autoclaved *S. boulardii*, 4 experiments were performed; each experiment lasted for 16 d. The first 8-d period represented an adaptation period (to achieve steady state conditions) and was followed by an 8-d collection period. At the start of the collection period, living or autoclaved yeasts of *Saccharomyces cerevisiae* Hansen CBS 5926 (Hexal AG, Holzkirchen, Germany) were added to the respective fermentation vessels. The product was delivered in lyophilized form containing at least 2×10^{10} cfu/g. During the collection period of experiment 1, the 9 vessels were divided into 3 groups. One group served as controls; 0.5 and 1.5 g/d of living yeast was added to the second and third group, respectively. This experimental setup was repeated in experiment 3. Experiments 2 and 4 were performed under the same conditions as the others, except that autoclaved yeast was used. The following considerations were the basis for the chosen yeast dosages. With respect to the literature, inclusion rates in the diet are typically in the range of 4 to 100 g/d (Newbold, 1995). As described, the fermentor content was 750 mL to which 0.5 or 1.5 g of *S. boulardii* was added per d. Extrapolating these values to the in vivo condition of sheep rumen liquid content (10 L), this would result in 6.7 or 20 g of *S. boulardii* given to the animal, respectively.

Inactivation of *S. boulardii* was achieved by autoclave standard operating procedures (213 kPa at 121°C for 20 min). To test if the inactivation was complete, 10 mg of autoclaved yeasts were incubated at 30°C in yeast extract/peptone/dextrose liquid medium (Serva, Heidelberg, Germany) in a shaking incubator (GFL shaking incubator 3031, Burgwedel, Germany) at 200 rpm for 16 h. Then, 100 μ L of inoculate the liquid medium was plated onto yeast extract/peptone/dextrose agar, and incubated for 3 d at 30°C. No living yeasts were detected.

Analytical Procedures and Samplings

The pH values and redox potentials were measured daily in each vessel at the time of feeding using a pH electrode (Typ 408 and Pt 4805-S7/120, Mettler Toledo, Steinbach, Germany) connected to a Knick pH meter (digital pH meter 646, Knick, Berlin, Germany). Liquid effluent was collected daily and samples were taken for analyses of SCFA, NH₃-N, and ¹⁵N enrichments. The

overflow flasks were placed into ice to stop microbial activity and preserve fermentation products. An aliquot of effluent was centrifuged at $40,000 \times g$ for 20 min at 4°C. The resulting supernatant was acidified with 0.1 mL of 98% formic acid and then centrifuged at $4000 \times g$ for 10 min at 4°C. The supernatant was analyzed for SCFA by gas chromatography (model 5890 II, Hewlett Packard, Böblingen, Germany) equipped with a 1.8 m $\times$ 2 mm glass column packed with Chromosorb WAW (mesh 80/100) with 20% neopentyl glycol succinate and 2% ortho phosphoric acid. Helium was used as a carrier gas with a flow rate of 25 mL/min. Injection port, detector, and oven temperatures were 220, 250, and 130°C, respectively. Daily production rates of SCFA were estimated by multiplying the respective concentration by the volume of effluent collected. Ammonia N was measured using the steam distillation method of Kjeldahl (Stuck et al., 1995). Bacterial fractions were isolated from respective effluents using differential centrifugation according to the method of Brandt and Rohr (1981). Enrichment of ^{15}N was analyzed by ^{15}N emission spectrometry (NOI-7, Fischer Analysen Instrumente, Leip-

zig, Germany) in $\text{NH}_3\text{-N}$ fractions of the infusion buffer, the effluents, and bacterial fractions at d 6 to 8. At these times, the steady state conditions were reached for ^{15}N enrichment in the microbial cell mass. The daily production of microbial N was calculated by the equation of Nolan and Leng (1983). This value was multiplied by 6.25 to calculate microbial protein synthesis.

Statistical Analyses

Because the number of fermentation vessels was limited, the experiments were divided into 4 consecutive trials in a mixed sequence (living/autoclaved/living/autoclaved yeasts). To rule out potential time-dependent differences between trials, we used identical liquid and solid rumen contents sampled and mixed from the same 2 donor sheep in each experiment. All other technical applications remained unchanged. Therefore, it seemed appropriate to treat the data as randomized across the experimental period. The results from d 1, 5, and 8, and the results of the microbial protein synthesis from

Table 3. Results of 2-way ANOVA of the factors dose and status at different days of *Saccharomyces boulardii* application.

Parameters of microbial metabolism	Day of application	Dose of additive ¹	Status of yeast ²	Interaction dose $\times$ status
pH	1	**	NS	NS
	5	NS	NS	NS
	8	NS	NS	NS
Redox potential	1	NS	NS	NS
	5	NS	NS	NS
	8	NS	NS	NS
Total short-chain fatty acids	1	NS	NS	NS
	5	***	NS	NS
	8	***	NS	NS
Acetate	1	NS	NS	NS
	5	***	NS	NS
	8	*	NS	NS
Propionate	1	NS	NS	NS
	5	*	NS	NS
	8	NS	NS	NS
Butyrate	1	*	NS	NS
	5	***	*	NS
	8	***	*	NS
Isovalerate	1	***	*	NS
	5	***	***	***
	8	***	***	***
Valerate	1	NS	NS	NS
	5	***	NS	NS
	8	***	NS	NS
$\text{NH}_3\text{-N}$	1	***	NS	NS
	5	***	**	NS
	8	***	*	NS
Microbial protein	6	***	NS	NS
	7	***	***	NS
	8	***	**	NS

¹Dose = addition of 0, 0.5, or 1.5 g/d of *Saccharomyces boulardii*.

²Status of yeast = living or autoclaved.

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; NS $P > 0.05$.

Figure 1. pH values as affected by increasing dose (0, 1.0, and 1.5 g/d) of living or autoclaved *Saccharomyces boulardii* at d 1, 5, and 8 after beginning of application (mean ± SEM, n = 6; different lowercase superscripts on a given day represent significant effects of dose with at least $P \leq 0.05$).

d 6, 7, and 8 were statistically evaluated by 2-way ANOVA using the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). Effects included in the model were dose of yeast (0, 0.5, and 1.5 g) and status (living or autoclaved). In case of a significant result for the effects of dose of *S. boulardii* and status of *S. boulardii*, individual mean values were checked in pairs using a 2-tailed t-test for unpaired observations.

RESULTS AND DISCUSSION

pH Values and Redox Potentials

Throughout the experiment, pH values ranged between 6.82 and 6.92 and were thus within the physiological range of rumen fluid pH. Irrespective of the type of additive at d 1, pH values showed a significant dose-dependent decrease after yeast application (Table 3, Figure 1). This might be explained by adaptation of rumen microbes to the new conditions. However, it must also be considered that the overall pH differences were rather low and this most probably rules out a significant biological importance of this effect. It is generally accepted that redox potentials can serve as a measure of the anaerobic conditions in the rumen (Broberg, 1957; Mishra et al., 1970). If a range between -250 and -350 mV is assumed for physiological conditions in the rumen, all data obtained from the present study are well within this range (Figure 2). There was no statistically significant effect of the additives. This observation is consistent with the almost unaffected pH values.

Figure 2. Redox potentials as affected by increasing dose (0, 1.0, and 1.5 g/d) of living or autoclaved *Saccharomyces boulardii* at d 1, 5, and 8 after beginning of application (mean ± SEM, n = 6).

SCFA

Irrespective of *S. boulardii* status, total SCFA production was significantly increased in a dose-dependent manner at d 5 and 8 (Table 3, Figure 3a). This was mainly due to respective changes in acetate production rates (Figure 3b). In contrast, propionate production showed no significant differences in response to either living or autoclaved *S. boulardii* (Figure 3c). On the other hand, the addition of both yeasts increased the production rates of butyrate, isovalerate, and valerate at d 5 and 8 (Table 3, Figure 3, parts d, e, and f). At d 5 and a dosage of 1.5 g, the stimulatory effect on butyrate production was higher in the presence of autoclaved yeasts than with living yeasts. A potential explanation for the stimulatory effects of yeasts or yeast products on SCFA production could be related to the composition of the cell wall or other cell contents of *S. boulardii*. The cell wall comprises 3 components, namely glucans, mannoproteins, and chitin, and represents 20% of the dry weight of the cell. It consists of a layered structure, with an internal layer made up of β -1,3 and β -1,6 glucans, as well as small amounts of chitin and mannoproteins, and an outer layer of mannoproteins (Fleet, 1991; Klis, 1994; Moukadiri et al., 1997). These structures are appropriate substrates for microbial fermentation in the rumen irrespective of the yeast status.

The production of isovalerate formed the most remarkable difference between living and autoclaved yeasts because the increased production by living yeasts was significantly higher than by autoclaved yeasts. In the presence of living yeasts, the molar proportion of isovalerate increased from approximately 1 to 5%. This increase is probably based on the production

Figure 3. Daily total short-chain fatty acids (a), acetate (b), propionate (c), butyrate (d), isovalerate (e), and valerate (f) production as affected by increasing dose (0, 1.0, and 1.5 g/d) of living or autoclaved *Saccharomyces boulardii* at d 1, 5 and 8 after beginning of application. (mean $\pm$ SEM, $n = 6$; different lowercase superscripts on a given day represent significant effects of dose with at least $P \leq 0.05$, different uppercase superscripts at a given concentration represent significant effects of *S. boulardii* status with at least $P \leq 0.05$).

Figure 4. Daily NH₃-N production as affected by increasing dose (0, 1.0, and 1.5 g/d) of living or autoclaved *Saccharomyces boulardii* at d 1, 5, and 8 after beginning of application (mean $\pm$ SEM, n = 6; different lowercase superscripts on a given day represent significant effects of dose with at least $P \leq 0.05$).

of ethanol. Ethanol may be converted by ruminal microorganisms into acetate and into isovalerate, which was shown by studies with 2-¹⁴C ethanol and by feeding experiments with ethanol (Orskov and Hemken, 1967; Pradhan and Hemken, 1970).

NH₃-N and Microbial Protein Synthesis

Regardless of additive type, NH₃-N concentrations were characterized by dose-dependent differences during the experimental period (Table 3, Figure 4). Whereas the average NH₃-N concentrations ranged around 7 mmol/L in all control vessels, the application of 0.5 g of *S. boulardii* per d increased the NH₃-N concentrations to 8.5 mmol/L. They were further increased to about 12 mmol/L at a dosage of 1.5 g/d of *S. boulardii*. The chemical composition of the yeast has to be discussed as a major factor for these differences. Respective analyses have shown that CP contents of *S. boulardii* ranged around 39% (G. Breves, unpublished data, 2003). Thus, substantial increases in NH₃-N concentrations will occur in response to microbial degradation of yeast cells, which does not depend on *S. boulardii* status.

Similar changes as for NH₃-N concentrations were recorded for microbial protein synthesis as measured at d 6, 7, and 8 after reaching steady state enrichment of ¹⁵N in microbial cell mass. At each sampling day, the application of 0.5 or 1.5 g/d of *S. boulardii* (except autoclaved yeast on d 6) resulted in significantly higher values for microbial protein synthesis compared with control vessels irrespective of type of *S. boulardii* (Fig-

Figure 5. Daily microbial protein production as affected by increasing dose (0, 1.0, and 1.5 g/d) of living or autoclaved *Saccharomyces boulardii* at d 6, 7, and 8 after beginning of application (mean $\pm$ SEM, n = 6; different lowercase superscripts on a given day represent significant effects of dose with at least $P \leq 0.05$; different uppercase superscripts at a given concentration represent significant effects of *S. boulardii* status with at least $P \leq 0.05$).

ure 5). In a few cases at d 7 and 8 and at dosages of 0 to 0.5 g/d of *S. boulardii*, living yeasts lead to significantly higher values for microbial protein synthesis than did the application of autoclaved yeasts. This observation cannot be explained, but it seems unlikely that this effect was *S. boulardii*-dependent because it also occurred in the absence of additives. In summary, the latter data are not in accordance with assumptions that the stimulatory effects of yeasts on rumen microbial growth depend on living yeasts (Dawson et al., 1990; Kumar et al., 1994; Newbold et al., 1998). The reasons for this discrepancy are not yet fully understood. It cannot be excluded that stimulatory factors for microbial growth are released when yeast cells are microbially degraded.

CONCLUSIONS

The aim of the present study was to measure the effects of living and autoclaved *S. boulardii* on major biochemical parameters of rumen microbial metabolism. Through the application of yeasts, rumen microbial metabolism was stimulated. For most parameters, no advantages of living yeasts could be detected and from this it may be concluded that yeasts act through prebiotic rather than probiotic effects.

ACKNOWLEDGMENTS

The authors are grateful to G. Becker, M. Burmester, U. Dringenberg, and K. Hansen for their skilled technical assistance.

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