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Concentrate levels and *Saccharomyces cerevisiae* affect rumen fluid-associated bacteria numbers in dairy heifers ☆

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ABSTRACT

Total viable rumen bacteria counts through the use of colony-unit forming assays lack accuracy because they only include culturable bacteria capable of initiating cell division. Thus, bacterial counts can be underestimated. The use of fluorescent characteristics of cell membranes allows flow cytometry to enumerate and distinguish dead from live bacteria cells. The objective of this experiment was to investigate the viable and total ruminal bacteria counts when 3 levels of forage:concentrate in diets were fed at restricted levels with the addition of *Saccharomyces cerevisiae* (YC). Three cannulated post-pubertal Holstein heifers (age 18 ± 1.0 months) were fed corn silage (CS)-based diets in a 3-period (35 d) Latin square design. Heifers were fed the diets for 21 d with no yeast addition, followed by 14 d where yeast culture (YC) was added (1 g/kg as-fed basis); (Yea-Sacc^{1026}, Alltech, Inc., Nicholasville, KY). A low concentrate (LC) TMR (80% CS, 20% concentrate; 12.4% CP, 35% NDF), a medium concentrate (MC) TMR (60% CS, 40% concentrate; 12.3% CP. 28% NDF), and a high concentrate (HC) TMR (40% CS, 60% concentrate; 12.6% CP, 25% NDF), were fed once per day. Rumen fluid was sampled −2, 0, 2, 4, 6, 8, 10, 12 h after feeding. Samples were immediately stained with fluorescent dyes using the *Bac*Light kit (Molecular Probes Inc., Eugene, OR) and analyzed with a Coulter XL-MCL single laser flow cytometer. Mean rumen viable bacteria counts linearly increased among treatments (4.96, 4.78, $6.73 \times 10^{11} \pm 0.53 \times 10^{11}$ cells/ml; $P = 0.02$) for LC, MC and HC respectively, and YC addition increased number of viable bacteria cells ($P < 0.01$). Total and viable bacteria counts decreased for the first 2 h after feeding then increased 4 h post-feeding. Dietary concentrate level and YC can alter rumen bacteria counts as measured by this method.

1. Introduction

Bacteria cell viability is often referred to its ability to reproduce and to form a colony. Total viable bacteria counts through the use of colony forming unit assays often lack accuracy because they only include culturable bacteria capable to initiate cell division (Davey et al., 2004). In addition, substrate affinity may vary greatly within organisms (Russell and Hespell, 1981) and intermediate states of some bacteria are undetectable and may not be culturable (Berney et al., 2007). Conventional methods of microscopically counting rumen bacteria are laborious and time-consuming. Thus, viable bacterial counts are often not done or may be underestimated. There are several methods that can assess the viability of single cells without culturing cells. For example, Zhang and Fang (2004) showed that cell metabolic activity, an indicator of its viability, can be measured by specific staining procedures or by the integrity of its cell membrane, thus this method can measure live bacteria cells through counting. Methylene blue, a dye that reacts with the oxidoreductases of the viable cells is also often used for this purpose. New methodologies have been developed that enable the recognition of different bacteria types and different degree of activity (Vaque et al., 2001). The use of direct fluorescent labels of bacteria cells seizes this property

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and can provide actual numbers of viable, dead and total bacteria in fluid samples. Fluorescent characteristics of cell membranes allow flow cytometry to enumerate and distinguished dead from live bacteria cells. The kit, LIVE/DEAD *Bac*Light (Molecular Probes Inc., Eugene, OR), was developed recently to detect Gram-negative and Gram-positive bacteria viability and has been used widely to enumerate bacteria in other fields (Boulos et al., 1999; Gasol et al., 1999; Janssen et al., 2002). This kit utilizes two nucleic acid stains: 1) SYTO-9 which penetrates and stains all the cells regardless of their viability and emits green fluorescence; and 2) propidium iodide (PI) that is excluded by intact membranes. Therefore, PI penetrates cells when membrane integrity is compromised and it stains membranes with an intense red fluorescence. This enables viable cells to be stained green and non viable cells red. Membrane integrity is highly correlated to active (high DNA) and inactive (low DNA) bacteria, which at the same time has been interpreted to correspond to live and dead bacteria (Gasol et al., 1999; Vaque et al., 2001).

Changes in bacteria numbers and shifts in the microbial population influenced by modification of diets from roughage to grain have been reported in ruminants (Tajima et al., 2001). Fermentation end products such as volatile fatty acids and microbial protein are the result of the degradation of feed ingredients by the microbial population in the rumen. Thus, nutrients available for absorption can be affected by population sizes of the microbial species in the rumen and have an effect in animal performance (Weimer, 2001) Even though rumen fluid-associated bacteria (FAB) are estimated to be around 30% of the total population (Miron et al., 2001). Accurate determination of FAB numbers is necessary and can further be applied to particle associated bacteria (PAB) numbers. On the other hand, the use of *Saccharomyces cerevisiae* as an additive has resulted on changes of microbial population and numbers in the rumen (Mutsvangwa et al., 1992; Wallace, 1996). Therefore, the aim of this experiment was to monitor rumen bacteria numbers and bacteria viability in growing dairy heifers as affected by different ratios of forage to concentrate (F:C) with or without the addition of yeast culture (YC).

2. Materials and methods

Animal care procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Three Holstein dairy heifers (18.0 ± 1.2 months of age and 449.6 ± 19.7 kg BW), each previously fitted with a 10.6 cm rumen cannula (Bar Diamond, Parma, ID) under anesthesia, were randomly assigned to 1 of 3 treatment sequences in a 3×3 Latin square design and housed in a mechanically ventilated and environmentally controlled tie stall barn.

Heifers were weighed weekly, with measurements at 0800 and 1800 h, 2 h prior and 8 h after feeding, except the week immediately prior to intensive sampling. The amount of experimental diet was adjusted weekly, based on the previous BW, to a level formulated to allow 800 g/d ADG. Total mixed rations (TMR) contained corn silage as the sole forage source, ground corn, soybean meal and heat-treated soybean meal (Table 1). Diets provided 2.6, 2.7 and 2.8 Mcal/kg DM metabolizable energy (ME) for low (LC), medium (MC), and high (HC) concentrate respectively, with a formulated level of 13% CP to target an average daily gain of 800 g/d. Animals were fed once daily at 1000 h and no refusals were observed during the trial. Rations were mixed daily in a rotary mixer (Calan Super Data Ranger; American Calan, Northwood, NH) for approximately 5 min. Animal management and feed sampling followed the procedure used by Lascano and Heinrichs (2009). In brief, feedstuffs and TMR samples were collected daily and composited for each period; dried in a forced air oven (55 °C) immediately after collection for 72 h, and stored for further analysis. Changes in BW determined the quantity of TMR received for the following 7 d; however, DM intake was not changed immediately prior to sampling, as that could have increased variation in the results. Heifers had ad libitum access to water and were released 1 h post-feeding for approximately 1 h/d to a paved exercise lot, except on intensive sampling days. Each of the 3 periods of the experiment consisted of 35 d. Heifers were fed treatment diets for 21 d with no YC addition (14 d of adaptation and 7 d of sampling); (Yea-Sacc^{1026}, Alltech, Inc., Nicholasville, KY), followed by 14 d when YC was added to the diet [7 d adaptation to YC (Nocek et al., 2002) and 7 d sampling period].

Rumen samples were taken from 5 locations in the rumen (dorsal, ventral, anterior, caudal, and central) on days 18 and

Table 1
Ingredient and nutrient composition of low concentrate (LC), medium concentrate (MC), and high concentrate (HC) rations fed to heifers.

Composition	Treatment			SE
	LC	MC	HC	
Ingredients (% DM)				
Corn silage [a]	80.00	60.00	40.00	.
Ground corn	5.67	29.60	47.70	.
Soybean meal (SBM)	9.47	9.94	9.00	.
Heat treated SBM [b]	1.60	0.80	0.00	.
Sodium bicarbonate	0.35	0.35	0.35	.
High mineral mix [c]	0.00	1.23	2.95	.
Low mineral mix [d]	2.45	1.48	0.00	.
Nutrients [e]				
DM %	41.93	50.61	56.5	1.03
CP (% DM)	12.93	12.38	13.17	0.10
Soluble (% CP)	43.65	43.65	33.84	0.76
ADF %	20.45	17.93	12.97	0.19
NDF %	34.18	30.03	23.23	0.38
NFC %	48.05	53.30	58.48	0.14
TDN %	72.13	75.13	78.63	0.11
ME, Mcal/kg DM [f]	2.63	2.72	2.84	0.00
Ca %	0.38	0.37	0.36	0.02
P %	0.30	0.28	0.30	0.01
Mg %	0.20	0.22	0.23	0.01
K %	1.33	1.12	1.02	0.03

[a] Corn silage contained: 33.7% DM, 38.4% NDF, 23.9% ADF, 9.1% CP, and 36.32% starch on DM basis.
[b] Turbo meal.
[c] High mineral mix contained: 7.8% vitamin E, 2.6% vitamin ADE, 28.6% distillers corn with soluble vitamin D, 14.6% plain salt, 36.5% limestone, 2.6% magnesium oxide, 5.7% trace mineral premix, and 1.6% selenium premix on a DM basis.
[d] Low mineral mix contained: 7.4% vitamin E, 2.5% vitamin ADE, 28.6% distillers corn with soluble vitamin D, 13.9% plain salt, 34.8% limestone, 6.0% magnesium oxide, 5.5% trace mineral premix, and 1.5% selenium premix on a DM basis.
[e] $n = 6$ composite samples representing 42 samples per treatment taken daily throughout the collection periods.
[f] Estimated: metabolizable energy (ME) = TDN × 0.04409 × 0.82.