In situ identification and quantification of protein-hydrolyzing ruminal bacteria associated with the digestion of barley and corn grain

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In situ identification and quantification of protein-hydrolyzing ruminal
bacteria associated with the digestion of barley and corn grain

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Running title: *in situ* identification of ruminal protein hydrolysers

Abstract

In this study, BODIPY FL DQTM casein staining combined with fluorescence *in situ* hybridization (FISH) was used to detect and identify protein-hydrolyzing bacteria within biofilms that produced active cell-surface-associated serine- and metallo-proteases during the ruminal digestion of barley and corn grain in cows fed barley-based diets at two different levels. A doublet coccoid bacterial morphotype associated with barley and corn grain particles fluoresced after BODIPY FL DQTM casein staining. They accounted for 3-10% of the total bacteria attached to surface of cereal grain particles, possibly indicative of an important role in the hydrolysis of the protein matrix within the endosperm. However, the identity of these predominant proteolytic bacteria could not be determined using FISH. Quantitative FISH revealed that known proteolytic species, *Prevotella ruminicola*, *Ruminobacter amylophilus* and *Butyrivibrio fibrisolvens* were attached to particles of various cultivars of barley grain and corn, confirming their role in the proteolysis of cereal grains. Differences in chemical composition among different barley cultivars did not affect the composition of proteolytic bacterial populations. However, the concentrate level in the basal diet did have an impact on the relative abundance of proteolytic bacteria and thus possibly their overall contribution to the proteolysis of cereal grains.

Key words: rumen, proteases, barley, corn, FISH, bacteria.

Ruminal proteolysis determines the amount of NH_3 , amino acids, peptides, and branched-chain volatile fatty acids available for growth and proliferation of ruminal microbiota and is an important determinant of protein nutrition in the ruminant host (Wallace and Brammall 1985). The breakdown of dietary protein is mainly carried out by bacteria (40-60%). The composition of the proteolytic microbial populations is influenced by the diet of the animal and the physical form of the feed (Attwood et al. 1996). Proteolytic activity of the ruminal digesta is almost entirely associated with microbial cells, as cell-free rumen fluid has little activity toward soluble proteins (Broderick 1998). Cysteine proteases are predominant in the rumen (40–60%) with additional activities arising from serine- and metallo-proteases (McAllister et al. 1993; Ramirez Piñeres et al. 1997). Knowledge of the composition of proteolytic microbial populations and their activity could provide insight into new strategies for improving the efficiency of protein utilization in the rumen (Broderick et al. 1991).

In this study we used BODIPY (boron-dipyrromethene) FL DQTM casein staining to label protein-hydrolyzing bacteria with active cell-surface associated (CSA) serine- and metallo-proteases attached to barley and corn grain in the rumen of cows fed barley-based diets. Fluorescence *in situ* hybridization (FISH) (Amann 1995) with oligonucleotide probes specifically designed for known proteolytic bacterial species were then used to identify protein hydrolysing organisms (PHOs) that were or were not stained by BODIPY FL DQTM. Effects of barley-based diets with two different levels of concentrate on the relative abundance of the PHOs attached to four varieties of barley grain and one variety of dent corn were investigated. Three

rumen-cannulated beef heifers (BW: 308kg $\pm$ 32 SD) were used in this crossover study. In the first month, heifers were fed a low-concentrate barley silage-based diet (17% barley grain and 3% of dry cow mineral plus 80% barley silage) and in the second month, a high-concentrate barley grain-based diet (60% barley grain plus 37% barley silage and 3% of dry cow mineral; Xia et al. 2015). To investigate the attachment of proteolytic bacteria, ruminal incubation experiments were carried out using Dacron bags (5 $\times$ 10 cm internal dimension, 51 $\pm$ 2 μ m pore size; Ankom, Fairport NY, USA) (Batajoo and Shaver, 1998). Four different barley grain varieties (Fibra, Hilose, McGwire and Xena) and dent corn were selected specifically as substrates for ruminal incubation on the basis that they differed substantially in chemical composition (Xia et al. 2015). All barley and corn samples were ground through a 6 mm screen and after mixing, triplicate sub-samples (5 g) of each sample were placed in Dacron bags and incubated in the rumen of each heifer for 4, 12, 24 and 48 h. Bags were collected at each time point and transferred to the lab within 20 min for analyses. Experiments were conducted at Lethbridge Research Centre, Agriculture and Agri-Food, Canada and the study was approved by the Lethbridge Research Centre animal care committee.

To collect bacteria attached to the barley or corn kernels for protease staining and FISH probing, bags were gently rinsed with 1 $\times$ PBS buffer and drained for 3–5 min on a sieve. Contents from individual bags were then transferred to sterilised containers. The contents of triplicate incubated samples were combined and homogenized manually with a glass rod. A portion (8 g) of each sample was suspended in 35 mL of

phosphate buffer (Kong et al. 2010). The mixture was subjected to mechanical pummelling for 6 min in a Colworth Stomacher 400 (A. J. Seward & Co. Ltd, London) before filtration through sterilized eight-layer cheesecloth. A portion of the filtered fluid was fixed with 50% ice-cold ethanol or 4% paraformaldehyde (PFA) for FISH probing. Other aliquots (0.4 mL) were used for BODIPY FL DQTM casein staining.

BODIPY FL DQTM casein staining was performed as previously described for biodigester sludge by Xia et al. (2011) with slight modifications. Briefly, 400 μ L of filtrated bag fluids were transferred into 1.5-mL Eppendorf tubes and centrifuged (4500 g for 10 min). The pellets were re-suspended in 400 μ L 1 $\times$ MES (2-N-morpholino ethane sulfonic acid) buffer (pH 6.2, 10mM) and 200 μ L of a working solution of BODIPY FL DQTM casein was added. The mixture was placed in a 10-mL serum bottle wrapped in aluminum foil and incubated at 25 °C for 30 min on a rotating disk (220 r.p.m) before microscopic examination. Samples were spread evenly on three-well (5 μ L in each well) gelatin-coated Teflon printed slides (Electron Microscopy Sciences, 1560 industry road, Hatfield, PA 19440, USA) and dried in a dark room before mounting with antifade reagent CITI fluor and examined microscopically. Images of bacterial samples stained with BODIPYFL casein, FISH and DAPI were examined and captured using an epifluorescence microscope (Leica DM6000B, Wetzlar, Germany) equipped with a Leica DFC500 camera. Statistical analyses were carried out using student T test with significance declared at $P < 0.05$.

Regardless of the diet used, bacteria displaying a doublet or single coccoid morphotype were observed in washings from all barley and corn samples as identified

by BODIPY FL DQTM casein staining (Fig. 1A). BODIPY FL DQTM casein staining combined with DAPI (4',6-diamidino-2-phenylindole; Xia et al. 2011) showed that these PHOs were more abundant (5-10% of total microbial cells) in the low concentrate than in the high concentrate diet (3-6% of total microbial cells), suggesting that they played a more important role in proteolysis of the low concentrate diet. We used BODIPY FL DQTM casein staining combined with FISH (Xia et al. 2007) to identify PHOs with CSA serine- and metallo-protease activity. The PHOs failed to hybridize with FISH probes Bru597 (5'-CGGCTACATTTTCACGTCACGC-3', optimal formamide concentration 40%) and Bam120 (5'-CAGGCAGCTCCCCAGGTA-3', optimal formamide concentration 10%) designed for *P. ruminicola* and *R. amylophilus*, respectively. Furthermore, they also did not hybridize with any of the probes selected from Probebase (Loy et al. 2007) that are known to target ruminal proteolytic bacteria. These include the probe mixture of Bfi826 and But1243 designed for *B. fibrisolvens*-related species, probe Strc493 designed for *Streptococcus bovis*, probe Snm1418 designed for *Selmononas ruminantium*, probe Erec482 designed for *Clostridium coccooides* and *Eubacterium*-related bacteria, probe Lach571 designed for *Lachnospira multiparus*, probe Bru597 designed for *P. ruminicola* and probe Bam 120 designed for *R. amylophilus*. A probe mixture of Eub338, Eub338II and Eub338III (Eubmix) designed for most bacteria (Loy et al. 2007) did hybridize with PHOs, but FISH probes at various family, class or phylum level were unable to further classify PHOs. Therefore, the phylogeny of these protein-hydrolyzing bacteria with active CSA

serine and/or metallo protease activity remains unknown. *In vitro* studies have shown that *P. ruminicola* possesses mainly CSA cysteine type proteases (Wallace, 1996) and *B. fibrisolvens*, *R. amylophilus*, *S. ruminantium* and *S. bovis* possess mainly extracellular serine-protease activity (Wallace, 1996). BODIPY FL DQTM casein staining performed in this study did not positively stain these bacteria, a result that aligns with these previous *in vitro* studies.

After 48 h of incubation, more ($P < 0.05$) corn and Hilose protein (of the total protein in bag contents) was hydrolyzed with the low than with the high concentrate diet (76.5% vs 54.2%; 91.4% vs 82.3%); while similar percentages ($P > 0.05$) of protein in Fibar, McGwire and Xena was hydrolyzed in both diets (86.3% vs 81.2%, 93.7% vs 89.6%, and 87.0% vs 85.4%, respectively).

We used quantitative FISH (Kong et al., 2010) to determine the percentage of the known proteolytic bacterial species attached to barley grain and corn particles within the rumen of these heifers. In heifers fed either the low or the high concentrate diet, *P. ruminicola*- (Fig. 1B), *R. amylophilus*- (Fig. 1C) and *B. fibrisolvens*- (Fig. 1D) like proteolytic bacteria were commonly associated ($>1\%$ of total bacterial present) with the grains, while *S. bovis*-, *L. multiparus*- and *S. ruminantium*-like bacteria as well as *C. coccoides* and *Eubacterium*-related proteolytic bacteria were rarely observed. This *in situ* result generally aligns with culture-based methods, which have defined *P. ruminicola*-, *R. amylophilus*- and *B. fibrisolvens*-like proteolytic bacteria as being the predominant culturable proteolytic bacteria within the rumen (Cotta and Hespell 1986).

The percentages of *P. ruminicola*-, *R. amylophilus*- and *B. fibrisolvens*-like proteolytic bacteria attached to barley and corn particles differed among samples, but trends were similar across all grain types. During 48 h of incubation, the percentages of *P. ruminicola*-like bacteria with the low concentrate diet peaked at 4 or 12 h with maximum levels of between 8.2 and 11.9% and thereafter fluctuated between 1.6-2.9%. With the high concentrate diet, percentages of this bacterial population reached a maximum of between 2.6 and 5.6% after 4 h and then decreased to 1.6-3.0% after 48 h of incubation. The percentage profiles of *R. amylophilus*-like bacteria with the high concentrate diet were similar to those of *P. ruminicola*-like bacteria with levels peaking after 4 h of incubation at between 1.7 and 3.4% and thereafter ranging between 1.0 and 2.9%. In contrast, with the low concentrate diet the percentages of *R. amylophilus*-like bacteria gradually increased and peaked at 24 h at 4.6 to 5.6% of the total bacterial numbers before decreasing to between 1.8 and 3.3% at 48 h. The percentage profiles of *B. fibrisolvens*-like bacteria differed from those of *P. ruminicola*- and *R. amylophilus*-like bacteria. With the high concentrate diet, levels of *B. fibrisolvens*-like bacteria on barley and corn grain were higher ($P < 0.05$) than those of *P. ruminicola*- and *R. amylophilus*-like bacteria accounting for 11.2% and 18.7% of total bacteria after 48 h. With the low concentrate diet the percentage profiles of *B. fibrisolvens*-like bacteria attached to barley and corn grain increased between 4 to 12 h, plateaued after 24 h before finally increasing to 5.4 to 9.6% after 48 h. These results suggest that the physiochemical differences among barley grain varieties and between barley grain and corn did not substantially affect the

composition or abundance of these proteolytic bacteria in the present study.

Diet had a large impact on the relative abundance of *R. amylophilus*-, *P. ruminicola*- and *B. fibrisolvens*-like bacteria. During 48 h of incubation the percentages of *R. amylophilus*- and *P. ruminicola*-like bacteria with the low concentrate diet were higher ($P < 0.05$) than with the high concentrate diet, suggesting that they may have played a greater role in proteolysis with this diet. In contrast, the percentages of *B. fibrisolvens*-like bacteria associated with the high concentrate diet were higher ($P < 0.05$) than with the low concentrate diet, suggesting that they played a greater role in the proteolysis of barley and corn grain. Moreover, with the low concentrate diet the percentages of these three proteolytic populations peaked at different time points suggesting that their roles in the proteolysis of barley and corn grain might differ, but the mechanisms remain unclear. Although the abundances of *R. amylophilus*-, *P. ruminicola*- and *B. fibrisolvens*-like bacteria were generally in line with the protein digestion profiles of different barley grains, their involvement in degradation of carbohydrates at the same time cannot be fully ruled out as several of these bacteria can also hydrolyze starch, maltose and use glucose directly as an energy source (Marounek and Bartos 1987).

To our knowledge this is the first attempt at visualizing PHOs with active proteases in the rumen. BODIPY FL DQTM casein staining combined with FISH allowed high resolution in situ detection of PHOs with CSA serine- and metallo-protease activity in the rumen of cows fed barley-based diets. Two control measures were adopted in this study to ensure reliable detection and enumeration of

PHOs with CSA protease activity. Firstly, diets with two different concentrate ratios were used to encompass the possible effect of diet on composition of PHOs (Wallace and Brammall, 1985). Secondly, corn was used as an alternative substrate to barley grain to rule out possible selection of PHOs specific to barley as diet could affect the spectrum of ruminal proteases and the composition of proteolytic bacteria (Reilly et al. 2002). Under both incubation conditions, the ratios of PHOs detected at different time points over 48 h of incubation ranged between 5-10% and 3-6% of total bacteria observed in the low and high concentrate diet, respectively. This suggests that a group of unknown PHOs with CSA serine- and metallo-protease activity were also important participants in the proteolysis of barley and corn within the rumen.

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REFERENCES

- Amann, R.I. 1995. In situ hybridization of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, part 3.3.6. Molecular Microbial Ecological Manual (Akkermans A.D.L., van Elsas J.D., and de Bruijn, F.J. eds), pp. 1–15. Kluwer Academic Publications, London.
- Attwood, G.T., Reilly, K., and Patel, B.K.C. 1996. *Clostridium proteoclasticum* sp. nov., a novel proteolytic bacterium from the bovine rumen. *Internat. J. Syst. Bacteriol.* **46**(3): 753-758.
- Batajoo, K.K., and Shaver, R.D. 1998. In situ dry matter, crude protein, and starch

degradabilities of selected grains and by-product feeds. *Anim. Feed. Sci. Technol.* **71(1-2)**: 165-176.

Broderick, G. A., Wallace, R. J., Orskov, E. R. 1991. Control of rate and extent of protein degradation. In: T. Tsuda, Y. Sasaki and R. Kawashima (Ed.) *Physiological aspects of digestion and metabolism in Ruminants*. pp. 541-592. Academic Press, London.

Broderick, G. A. 1998. Can cell-free enzymes replace rumen microorganisms to model energy and protein supply? Occasional Publication No. 22. *In vitro Techniques for Measuring Nutrient Supply to Ruminants*. (Deaville, E.R., Owens, E., Adesogan, A.T., Rymer, C., Huntington, J.A., and Lawrence, T.L.J. eds), pp. 99-114. British Society of Animal Sciences, Edinburgh.

Cotta, M.A., and Hespell, R.B. 1986. Proteolytic activity of the ruminal bacterium *Butyrivibrio fibrisolvens*. *Appl. Environ. Microbiol.* **52(1)**: 51-58.

Kong, Y.H., He, M., McAllister, T., Seviour, R., and Forster, R. 2010. Quantitative fluorescence in situ hybridization of microbial communities in the rumens of cattle fed different diets. *Appl. Environ. Microbiol.* **76(20)**: 6933-6938.

Loy, A., Maixner, F., Wagner, M., and Horn, M. 2007. probeBase - An online resource for rRNA-targeted oligonucleotide probes: New features 2007. *Nucleic Acids Res.* **35(SUPPL. 1)**: D800-D804.

Marounek, M., and Bartos, S. 1987. Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. *J. Appl. Bacteriol.* **63(3)**: 233-238.

McAllister, T.A., Phillippe, R.C., Rode, L.M., and Cheng, K.J. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. *J. Anim. Sci.* **71(1)**: 205-212.

Ramirez Piñeres, M.A., Ellis, W.C., Wu, G., and Ricke, S.C. 1997. Effects of protease inhibitors on degradation of H₃[¹⁴C]-casein by ruminal microorganisms. *Anim. Feed. Sci. Technol.* **64(2-4)**: 113-126.

Reilly, K., Carruthers, V.R., and Attwood, G.T. 2002. Design and use of 16S ribosomal DNA-directed primers in competitive PCRs to enumerate proteolytic bacteria in the rumen. *Microbial Ecol.* **43(2)**: 259-270.

Wallace, R.J., and Brammall, M.L. 1985. The role of different species of bacteria in the hydrolysis of protein in the rumen. *J. Gen. Microbiol.* **131(4)**: 821-832.

Wallace, R.J. 1996. The proteolytic systems of ruminal microorganisms. *Anim. Res.*

45(SUPPL.): 301-308.

Xia, Y., Kong, Y.H., and Nielsen, P.H. 2007. In situ detection of protein-hydrolysing microorganisms in activated sludge. *FEMS Microbiol Ecol.* **60(1)**: 156-165.

Xia, Y., Massé, D.I., McAllister, T.A., Beaulieu, C., Talbot, G., Kong, Y.H., and Seviour, R. 2011. In situ identification of keratin-hydrolyzing organisms in swine manure inoculated anaerobic digesters. *FEMS Microbiol Ecol.* **78(3)**: 451-462.

Xia, Y., Kong, Y.H., Seviour, R.J., Yang, H.E., Forster, R.J., Vasanthan, T., and McAllister, T. 2015 In situ identification and quantification of starch-hydrolyzing bacteria attached to barley-grain in the rumen of cows fed barley-based diets. *FEMS Microbiology Ecology* **91(8)**: fiv077. doi: 10.1093/femsec/fiv077.

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Figure Legend

Figure 1. Four sets of fluorescence images taken with ruminal samples rinsed from Hilose particles in the rumen of a heifer fed a 17% barley based diet. (A) a color combined image from a DAPI stained image (blue) and an image after BODIPY DQTM casein staining (green) showing bacterial cells with a coccoid morphotype in doublets or as single images are positively stained with BODIPY DQTM casein. The two images were intentionally separated along the X axis to clearly show the same cells after staining. Cells within the squares are still present after staining, while those in circles positively stained with BODIPY DQTM casein, but were washed from the slides after DAPI staining. Images (B), (C) and (D) are color combined images of a FISH image (red) with Cy3 labeled probes and a DAPI image (blue) showing the yellow-colored (combination of red and green) cells hybridizing with probe Bru597 targeting *Prevotella ruminicola* (image B), probe Bam120 targeting *Ruminobacter amylophilus* (image C) and a probe mixture of Bfi826 and But1243 targeting *Butyrivibrio fibrosolvens*-like bacteria (image D). The bar in image A represents 10 μm and was used for all images.

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