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Original article

Degradation of scrapie associated prion protein (PrP^{Sc}) by the gastrointestinal microbiota of cattle

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Abstract – A food-borne origin of the transmission of bovine spongiform encephalopathy (BSE) to cattle is commonly assumed. However, the fate of infectious prion protein during polygastric digestion remains unclear. It is unknown at present, whether infectious prion proteins, considered to be very stable, are degraded or inactivated by microbial processes in the gastrointestinal tract of cattle. In this study, rumen and colon contents from healthy cattle, taken immediately after slaughter, were used to assess the ability of these microbial consortia to degrade PrP^{Sc}. Therefore, the consortia were incubated with brain homogenates of scrapie (strain 263K) infected hamsters under physiological anaerobic conditions at 37 °C. Within 20 h, PrP^{Sc} was digested both with ruminal and colonic microbiota up to immunochemically undetectable levels. Especially polymyxin resistant (mainly gram-positive) bacteria expressed PrP^{Sc} degrading activity. These data demonstrate the ability of bovine gastrointestinal microbiota to degrade PrP^{Sc} during digestion.

transmissible spongiform encephalopathy / prion / degradation / microbiota / gastrointestinal tract

1. INTRODUCTION

Transmissible spongiform encephalopathies (TSE) or "prion diseases" form a group of fatal neurodegenerative disorders, which include, among others, scrapie in sheep, bovine spongiform encephalopathy (BSE), and Creutzfeldt-Jakob disease (CJD) of humans [14]. According to the "protein-only" hypothesis, TSE is caused by an infectious agent that is partially, if not entirely composed of a pathological form (PrP^{Sc}) of a host-encoded protein, the PrP protein [14]. No differences in primary structure have been found between PrP^{C} and PrP^{Sc} [18]. PrP^{Sc}, which is predominantly β-sheet structured, is considered to be remarkably resistant to inactivation [20]. Digestion with proteinase K

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leads to an N-terminally truncated form of 27–30 kDa, designated PrP^{Sc} [15]. PrP^{Sc} is resistant towards other proteolytic enzymes like trypsin, V8 protease [11] and pepsin [8]. Recently, data were published indicating that extra cellular proteases of *Streptomyces*, *Bacillus* and anaerobic thermophilic prokaryotes are able to decrease the PrP^{Sc} level in infected brain homogenates of mice and hamsters [7, 8, 11, 12]. Thus, TSE agents are sensitive towards proteases, although such proteases rarely appear to occur.

Feedstuffs consumed by ruminants are initially exposed to microbial fermentation in the rumen prior to gastric and intestinal digestion [9]. The influence of a complex microbiota composed of a large number of different predominantly anaerobic bacteria (10^{10} to 10^{11}/g), protozoa (10^4 to 10^5/g) [9] and fungi located in the gastrointestinal tract on the characteristic resistance of PrP^{Sc} has not been described yet. Transmission of prions by food-borne infection has been linked to BSE and kuru in humans [6, 22]. Usually, dietary proteins are degraded almost completely by passing the stomach, small intestine and large intestine during digestion. The polygastric digestion of ruminants in particular represents an efficient system to degrade food proteins by microbial fermentation processes in the rumen and colon [9, 19]. Due to the polypotent metabolic activity of the complex microflora in the gastrointestinal tract this should also apply to the protein structure of prions. The purpose of this study was to investigate in vitro the ability of PrP^{Sc} degradation by the complex ruminal and colonic microbiota of cattle.

2. MATERIALS AND METHODS

2.1. Experimental material

Degradation assays were done using uninfected and scrapie-infected (strain 263K) hamster brain homogenates. Syrian hamsters were infected intra-cerebrally with 20 μL of 10% scrapie-infected hamster brain homogenates in phosphate buffered saline (PBS). After 60 to 70 days post-infection, the animals developed clinical symptoms [13]. The excised brains were stored at –70 °C until use. A 20% homogenate of the hamster brain tissue was prepared in homogenisation buffer (0.32 M sucrose, 0.5% sodium desoxycholate, 0.5% Nonidet P 40, pH 7) or in sterile mineral salt buffer solution of McDougall (10.45 mM KCl, 8.04 mM NaCl, 0.49 mM $MgSO_4$, 0.36 mM $CaCl_2$, 110.7 mM $NaHCO_3$ and 27.38 mM Na_2HPO_4, pH 8.3) using a glass douncer. All homogenates were stored in aliquots at –70 °C until use. All experiments were carried out in agreement with the national guidelines in a class 3** laboratory of the institute.

Rumen content and the ligatured section of the *colon ascendens* from healthy fattened beef bulls with an average age of twenty months were taken under sterile conditions immediately after slaughtering in the abattoir near the research centre. The microbiota of rumen/intestine contents were inactivated by autoclaving at 121 °C for 15 min to obtain controls. A 10% homogenate of either the active and inactive microbiota of rumen/intestine contents was prepared with sterile mineral salt buffer solution of McDougall in the absence or presence of soluble carbohydrates (6.7 g/L maltose; 3.3 g/L xylose; 3.3 g/L soluble starch; 2.1 g/L $NaHCO_3$; 3.3 g/L citrus pectin), respectively [4].

2.2. Degradation assay

The rumen/intestine homogenates were filtrated in order to remove crude suspended particles, which could impair the detection of PrP^{Sc}. Samples were prepared in the ratio of 10 to 1, concerning intestinal homogenate to brain homogenate,

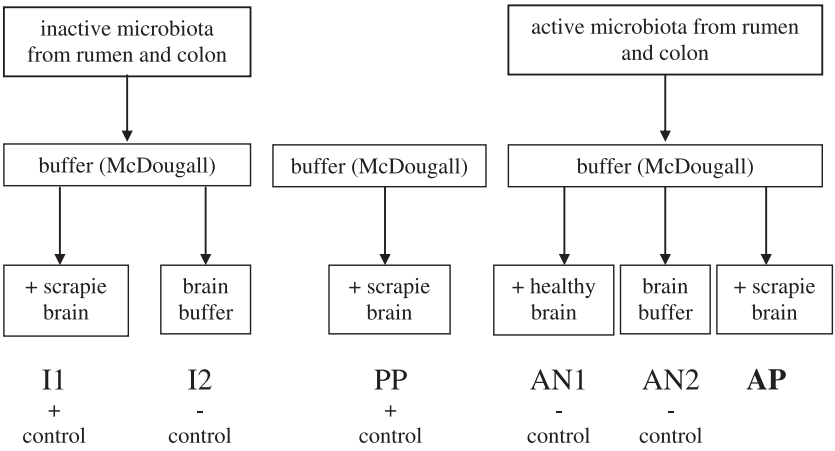

Figure 1. Experimental flow scheme for in vitro degradation studies of the prion protein (PrP^{Sc}) by complex microbiota of the bovine rumen and colon. Samples were prepared in the ratio of 10 to 1 (intestine content to brain material) including negative and positive controls.

including negative and positive controls according to the scheme in Figure 1. Immediately after sample preparation, the references at 0 h incubation time were taken and stored at –70 °C until further treatment. Incubation of the samples was carried out at 37 °C for 20 h under anaerobic conditions. According to the scheme in Figure 1, both inactivated rumen/colon samples and mineral salt buffer solution of McDougall samples with the addition of scrapie brain homogenate represented the positive controls. Negative controls were prepared by adding homogenisation buffer or healthy brain homogenate. In order to differentiate the PrP^{Sc} degrading microbiota, antimicrobial substances were added to selected samples. For that purpose 100 mM Polymyxin-B-Sulfate (Serva, Heidelberg, Germany) and Vancomycin-Kanamycin-Supplement (Oxoid, Wesel, Germany) were used as additives.

2.3. Proteinase K treatment

Aliquots of 100 μL from each sample were digested with 10 μL Proteinase K (Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 100 μg/mL for 1 h at 37 °C. The reactions were stopped by addition of 20 μL 100 mM PMSF (Sigma-Aldrich), a protease inhibitor and incubation for 15 min at room temperature [15].

2.4. Gel electrophoresis, Western blotting and immunodetection of PrP^{Sc}

Proteins were subjected to electrophoresis using precasted 12.5% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) from BioRad (Criterion) together with molecular weight marker Precision Plus Protein standards, dual colour (Bio-Rad Laboratories, Munich, Germany), and they were transferred by western blotting to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) [5, 15]. Prion protein was detected by immunostaining with specific monoclonal anti-prion antibody 3F4 (Sigma-Aldrich) and 6H4 (Prionics, Schlieren, Switzerland) at 0.2 μg IgG/mL, and secondary antibody conjugated to horseradish peroxidase (Dianova, Hamburg, Germany) [15]. The

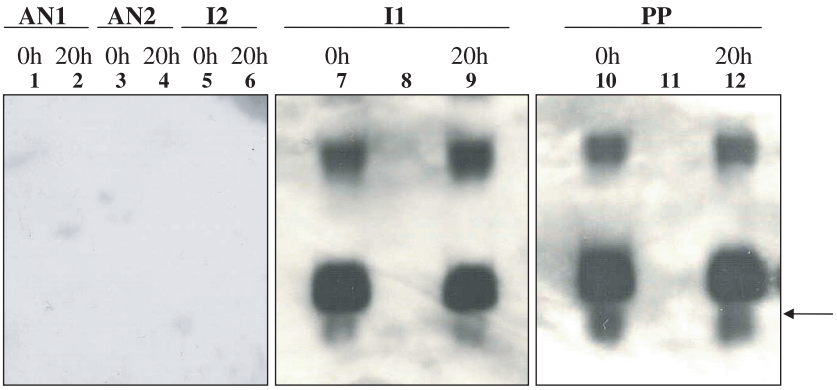

Figure 2. Control experiments of in vitro prion protein (PrP^{Sc}) degradation studies by complex ruminal and colonic microbiota of cattle. Complex intestinal microbiota of cattle were incubated with healthy hamster brain homogenate (PrP^{C}) (lanes 1 and 2) and brain homogenisation buffer (lanes 3 and 4) under anaerobic conditions for 0 and 20 h. Inactivated complex intestinal microbiota of cattle were incubated with brain homogenisation buffer (lanes 5 and 6) and scrapie (strain 263K) infected brain homogenate (lanes 7 and 9) under anaerobic conditions for 0 and 20 h. The McDougall mineral salt buffer solution was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 h (lanes 10 and 12). Arrows indicate the position of molecular-weight marker (25 kDa). Lanes 8 and 11 are empty.

reaction was visualised using a highly sensitive chemoluminescence-based detection technique (ECL, Amersham Bioscience, Freiberg, Germany) following the supplier's instructions. The signals were recorded on photographic film.

3. RESULTS

In vitro degradation assays of infectious prion protein (PrP^{Sc}) by complex microbiota of the bovine rumen and colon were established according to the experimental flow scheme in Figure 1. Due to the performed control experiments, artificial effects, like failure of PrP^{Sc} detection by sticking to the complex matrix of intestine contents, could be excluded. In all of the in vitro degradation experiments (n = 21), each composed of the set of samples shown in Figure 1, the inactive microbiota (sample I1) and the McDougall buffer solution only (sample PP) did not affect the PrP^{Sc} signal in western blot (Fig. 2, lanes 7, 9, 10 and 12). As expected, the negative controls with healthy brain homogenate (sample AN1) and without brain (samples AN2 and I2) showed no immunodetectable signals at all (Fig. 2, lanes 1–6). Neither inactivated microbial consortia nor cell-free supernatant obtained after centrifugation ($6000 \times g$ for 10 min) of intestine contents (data not shown) affected the PrP^{Sc} signal.

The present study demonstrates the ability of microorganisms of the gastrointestinal tract of cattle to significantly degrade PrP^{Sc}. In 18 out of 21 incubation experiments with rumen contents and 19 out of 21 incubation experiments with colon contents, a substantial degradation of PrP^{Sc} was determined as shown in Table I. Following incubation for 20 h under physiological anaerobic conditions, active ruminal and colonic microbiota reduced the PrP^{Sc} signal in western blot up to immunochemically undetectable levels (Fig. 3, lanes 2 and 4).

Table I. Determination of PrP^{Sc} degradation activity after an in vitro incubation assay under anaerobic conditions.

Numbers of in vitro incubation experiments under anaerobic conditions	PrP^{Sc} degradation				
	Complete[a]	Almost complete[b]	Substantial[c]	Weak[d]	No[e]
Rumen (n = 21)	9	4	5	2	1
Colon (n = 21)	11	4	4	1	1

[a] No PrP^{Sc} signal; [b] very weak PrP^{Sc} signal; [c] weak PrP^{Sc} signal; [d] significant PrP^{Sc} signal; [e] PrP^{Sc} signal remained stable on immunoblot after incubation.

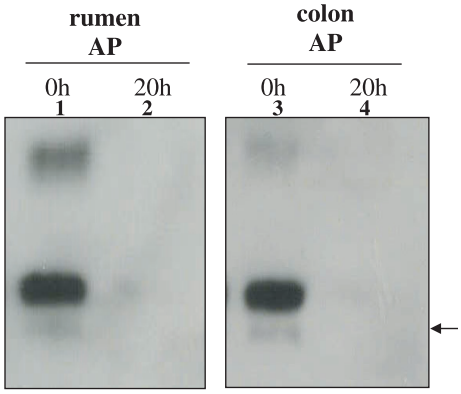

Figure 3. In vitro prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen and *Colon ascendens*. Complex ruminal microbiota of cattle were incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 h (lanes 1 and 2). Complex microbiota from *Colon ascendens* of cattle were incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 h (lanes 3 and 4). The arrows indicate the position of the molecular-weight marker (25 kDa).

In order to differentiate the PrP^{Sc} degrading microbiota, incubation experiments with selective mixed cultures were performed. The PrP^{Sc} signal was reduced to immunochemically undetectable levels by polymyxin-resistant (mainly gram-positive) microbiota of bovine rumen and colon (Fig. 4, lanes 2 and 6). In contrast, the PrP^{Sc} signal remained stable after incubation for 20 h with predominant gram-negative microbiota of the rumen and colon (Fig. 4, lanes 4 and 8).

To examine the influence of detergents on scrapie associated prion protein degrading capacities of bovine intestinal microorganisms and to create more physiological conditions, scrapie infected hamster brains were homogenised in sterile McDougall mineral salt buffer solution in the absence of detergents and incubated with buffered gastrointestinal contents. In the presence of soluble carbohydrates, PrP^{Sc} was almost fully digested after 20 h by bovine gastrointestinal microbiota (Fig. 5A, lanes 1 and 2; Fig. 5B lanes 1 and 2), whereas the PrP^{Sc} signal remained stable without available carbohydrates (Fig. 5A, lanes 3 and 4; Fig. 5B, lanes 3 and 4).

4. DISCUSSION

The infectious isoform of the prion protein is considered to be very stable towards conventional decontamination procedures [18]. Its partial resistance to proteinase K digestion is used for immunochemical detection [20]. However, despite a high stability, PrP^{Sc} degradation has been demonstrated. McKinley et al. [11] reported that proteinase K digestion for more than 2 h leads to hydrolysis. In recent publications, thermostable alkaline serine proteases of *Thermococcus*, *Thermosipho, Thermoanaerobacter* and *Streptomyces* were able to degrade PrP^{Sc} under special conditions [7, 12, 21]. Denaturation

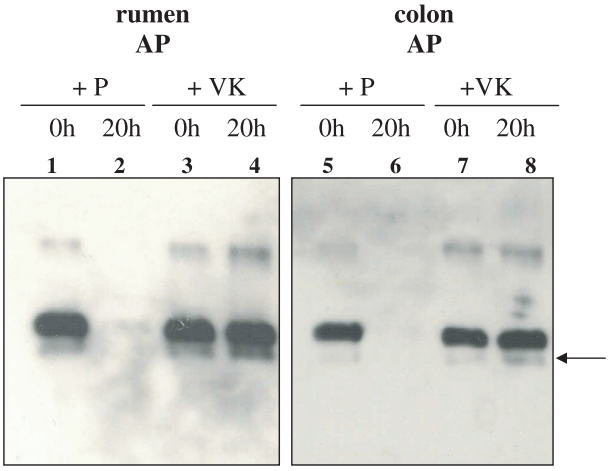

Figure 4. In vitro prion protein (PrP^{Sc}) degradation studies by selected complex microbiota of bovine rumen and *Colon ascendens*. Complex ruminal microbiota of cattle were incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 h in the presence of 100 mM polymyxin (lanes 1 and 2) or vancomycin-kanamycin supplement (lanes 3 and 4). Complex microbiota from *Colon ascendens* of cattle were incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 h in the presence of 100 mM polymyxin (lanes 5 and 6) or vancomycin-kanamycin supplement (lanes 7 and 8). The arrows indicate the position of the molecular-weight marker (25 kDa).

leads to a conformation of PrP^{Sc} susceptible to proteolysis. Heat pre-treatment and the presence of a detergent result in degradation of PrP^{Sc} by PWD-1 keratinase from *Bacillus licheniformis*, proteinase K, alkalase, and subtilisin Carlsburg [8]. In contrast to the bacterial proteases, digestion of heated samples with pepsin or trypsin was incomplete [8]. Therefore, it has to be assumed that PrP^{Sc} in contaminated foodstuff will survive treatment with endogenous proteases during digestion. Our degradation studies were carried out in the presence of 0.05% detergent. These conditions may facilitate PrP^{Sc} solubilisation, so that PrP^{Sc} is more capable for degradation. But moreover, the results of the degradation assay obtained in the absence of any detergent showed that this fact could be excluded. Furthermore, an effective microbial degradation of PrP^{Sc} is rather dependent on the presence of soluble carbohydrates than on detergents. According to Broderick et al. [4] using a defined medium in addition to soluble carbohydrates as a standard technique to estimate the rates of in vitro ruminal protein degradation, microorganisms utilise the supplements for growth. Enhanced microbial numbers result in higher proteolytic activity and in a greater protein hydrolysis.

Feedstuffs consumed by ruminants are all initially exposed to the fermentative activity in the rumen prior to gastric and intestinal digestion. A large but variable proportion (60 to 90%) of the dietary protein is degraded by the rumen microorganisms [9]. Ruminal microbial consortia are composed of a large variety of predominantly anaerobic bacteria (10^{10} to 10^{11}/g) and protozoa (10^4 to 10^5/g) [9]. Due to the polypotent metabolic activity of this complex microflora a degradation of PrP^{Sc} may occur. Not yet digested material finally reaches the large intestine. The colon is colonised by more than 400 different

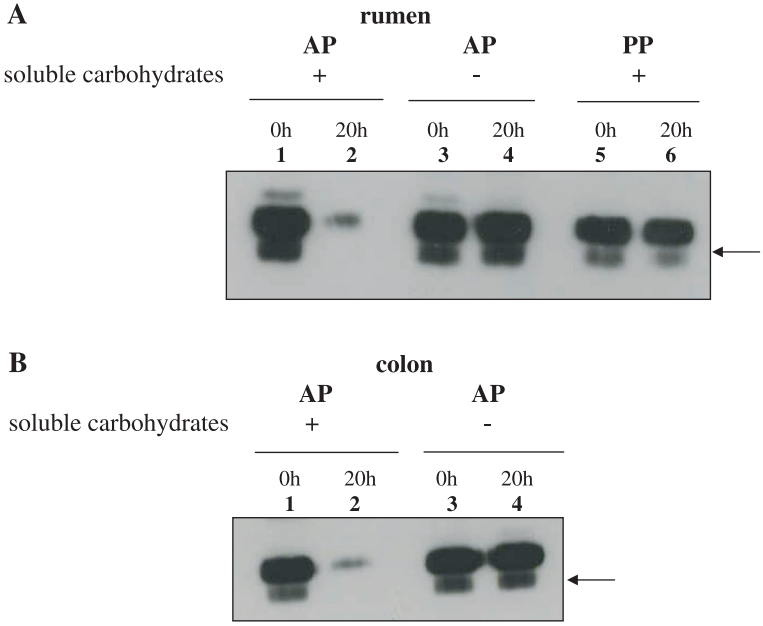

Figure 5. In vitro prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen and *Colon ascendens* in different buffers without the presence of detergents. (A) Complex ruminal microbiota of cattle were incubated with scrapie (strain 263K) infected brain homogenated in McDougall mineral salt buffer solution under anaerobic conditions in the presence and absence of soluble carbohydrates for 0 and 20 h (lanes 1, 2, 3 and 4). McDougall mineral salt buffer solution in addition to soluble carbohydrates were incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 h (lanes 5 and 6). (B) Complex microbiota from *Colon ascendens* of cattle were incubated with scrapie (strain 263K) infected brain homogenated in a McDougall mineral salt buffer solution under anaerobic conditions in the presence and absence of soluble carbohydrates for 0 and 20 h (lanes 1, 2, 3 and 4). The arrows indicate the position of the molecular-weight marker (25 kDa).

bacterial species, fungi and protozoa [9]. Under predominant anaerobic conditions, an additional degradation of food components may happen. Based on these results, this section of the gastrointestinal tract is also capable of PrP^{Sc} degradation. Within the average food passage of two days through the whole digestion tract of cattle [16], degradation of PrP^{Sc} by an intact ruminal and colonic microbiota is assumed. However, manipulation of ruminal fermentation by feed additives (e.g. antibiotics) may reduce PrP^{Sc} degradation capacities. For example, the feed supplement monesin inhibits gram-positive bacteria, which, according to our result, are responsible for ruminal and colonic PrP^{Sc} degradation.

The ability of intestinal microorganisms to degrade PrP^{Sc} might justify an alternate route of prion neuroinvasion prior to entering the lower gastrointestinal tract, which is composed of the stomach and intestine. Bartz et al. [2] suggested that prion infection via tongue-associated cranial nerves may be an alternate route of prion neuroinvasion following oral exposure. Their findings indicate that a low dose of prions, which is more likely to exemplify a natural infection, is unable to

cause disease when orally ingested, but may cause disease when inoculated into the tongue [2]. Therefore, abrasions of the tongue may predispose a host to oral prion infection of the tongue-associated cranial nerves [2]. Also, an increased risk of ruminants becoming infected with prions indicates this way of neuroinvasion. During rumination, foodstuff is transported back into the oral cavity, which could result in an increased risk of infection via abrasions of the tongue. After oral ingestion of high doses of scrapie agent the disease-specific isoforms of the prion protein, PrP^{Sc}, are found in the enteric nervous system of the submucosal and myenteric and the gut-associated lymphoid tissue [10]. Presumably, PrP^{Sc} degradation capacities of the intestinal microbiota are limited to low doses of prions, which probably more likely represent natural infections.

The present study shows the ability of complex ruminal and colonic microbiota of cattle to decrease scrapie associated prion protein in vitro up to immunochemically undetectable levels. It is highly possible that prion protein derived from BSE infected cattle would also be digested by bovine gastrointestinal microorganisms based on the similarity of the three-dimensional structures. Further studies are now underway for its verification. Comprehensive examinations in which PrP^{Sc} was visualised by western blotting after immunolabelling with the monoclonal antibody 3F4 have previously shown a close quantitive correlation between PrP^{Sc} amounts and infectivity in the brains of hamsters infected with a 263K scrapie agent [3]. Additionally, studies have demonstrated an inactivation of 263K scrapie agent concomitant with the disappearance of PK-resistant PrP in hamster brain homogenates [1]. Therefore, an inactivation of infectious prion protein by microbial PrP^{Sc} degrading processes during digestion is assumed. As a future task, this correlation will be validated by in vivo hamster bioassays, as Solassol et al. [17] found that reduced PrP^{Sc} levels in immunoblots do not correspond with in vivo data. However, the in vitro prion protein degradation procedure does not yet allow a final conclusion about the real in vivo conditions. Our in vitro model only exemplifies the facultative anaerobes of ruminal and colonic microbiota, while the obligate anaerobes cannot be included due to the experimental design. Altogether, degradation of PrP^{Sc} by complex bovine gastrointestinal microflora appears, at least in vitro.

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