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Degradation of Prion Protein by the Gastrointestinal Microbiota of Cattle

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List of abbreviations

aa	amino acid
BSE	bovine spongiform encephalopathy
°C	degree Celsius
cfu	colony forming units
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CWD	chronic wasting disease
DNA	deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
E. coli	*Escherichia coli*
e.g.	(lat. exempli gratia); for example
ENS	enteric nervous system
FFI	fatal familial insomnia
g	gram
GALT	gut associated lymphoid tissue
GI	gastrointestinal
GSS	Gerstmann-Sträusler-Scheinker syndrome
h	hours
ID50	infectious dose
kDa	kilo dalton
kg	kilogram
l	litre
LRS	lymphoreticular system
mA	milli ampere
mab	monoclonal antibody
MBM	meat and bone meal
M-cells	membranous epithelial cells
mg	milligram
min	minute
ml	millilitre
mM	millimolar
µl	microlitre

µg	microgram
no.	number
p.i.	post inoculation
pH	pondus hydrogenii
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PMSF	phenylmethylsulfonylfluoride
PrP	prion protein
PrP^{BSE}	BSE associated prion protein
PrP^{C}	cellular prion potein
PrP^{res}	proteinase K resistant prion protein
PrP^{Sc}	disease / scrapie associated prion protein
PVDF	polyvinylidenfluoride
rpm	rotation per minute
SAF	scrapie associated fibril
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sp.	species
STI	Standard I agar
TME	transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathies
UK	United Kingdom
UV	ultraviolet
V	volt
vCJD	variant Creutzfeldt-Jakob disease

Summary

The influence of complex microflora residing in the gastrointestinal tract of cattle on prion protein plays a crucial role with respect to early TSE pathogenesis and the potential infectivity of faeces resulting in environmental contamination. However, it is unknown whether infectious prion proteins (PrP^{Sc}), considered to be very stable, are inactivated by microbial processes in the gastrointestinal tract of animals. Feedstuffs consumed by ruminants are initially exposed to microbial fermentation in the rumen prior to gastric and intestinal digestion. Particularly, the polygastric digestion of ruminants represents an efficient system to degrade food proteins by microbial fermentation processes in rumen and colon.

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 inactivate PrP^{Sc}. For that purpose, the consortia were incubated with brain homogenates of scrapie (strain 263K) infected hamsters and BSE infected cattle, respectively.

Biochemical analyses indicate the ability of complex ruminal and colonic microbiota of cattle to decrease scrapie associated prion protein up to immunochemically undetectable levels in Western blot under physiological conditions. In contrast, incubation with BSE associated prion protein did not result in degradation. This implicates a greater stability of BSE associated prion protein towards microbial degradation processes in the gastrointestinal tract.

In vivo hamster bioassays were performed with degraded samples of scrapie brain homogenates in order to prove the concomitance of the loss of anti-prion antibody 3F4 immunoreactivity and the inactivation of PrP^{Sc}. The results demonstrated significant prion infectivity after degradation of infected hamster brain through the gastrointestinal microflora of cattle.

Thus, infectivity is still present, even in the absence of Western blot signals. This might be caused by PrP^{Sc} at levels below the threshold of immunochemical detection, or by a sub-fraction of infectious prion protein not detectable by immunochemical methods. Finally, the possibility of present infectious molecules or structures other than PrP^{Sc} must be considered. Conclusively, these data highlight the deficiency of using Western blot or immunoassay formats in TSE inactivation assessment studies, and raise the possibility that the environment might be contaminated through cattle shedding infected faeces.

Zusammenfassung

Ziel der Arbeit war es, die Stabilität von Prion-Proteinen (PrP^{Sc}) im Gastrointestinaltrakt von Rindern zu untersuchen, um Aussagen zur Verbreitung und Ausscheidung von TSE-Erregern treffen zu können. Bislang ist nicht bekannt, ob infektiöse Prion-Proteine während der Verdauung durch mikrobielle Prozesse abgebaut und inaktiviert werden. In der Regel werden Proteine aus Futtermitteln im polygastrischen Verdauungssystem der Wiederkäuer nahezu vollständig verdaut. Während 70-90 % der Proteine im Pansen vorwiegend durch Bakterien abgebaut werden, erfolgt ein weiterer Protein-Abbau durch proteolytische Bakterien der Mikroflora im Colon. Um zu überprüfen, ob dies auch auf die Struktur des Prion-Proteins zutrifft, wurde die komplexe Mikroflora des bovinen Gastrointestinaltraktes auf die Fähigkeit des PrP^{Sc}-Abbaus getestet.

Hierfür wurden Inkubationsversuche mit den komplexen Pansen- bzw. Coloninhalten von Mastbullen und Scrapie-infizierten Hamsterhirnhomogenaten (Stamm 263K) bzw. BSE-infizierten Rinderhirnhomogenaten durchgeführt.

Dabei konnte Scrapie-assoziiertes PrP^{Sc} nach einer Inkubation von bis zu 40 Stunden sowohl mit Pansen- als auch mit Coloninhalt im Western Blot immunochemisch nicht mehr nachgewiesen werden, während BSE-assoziiertes PrP^{Sc} unter identischen Bedingungen nicht abgebaut werden konnte. Um Aussagen über eine Inaktivierung von PrP^{Sc} durch die bovine Gastrointestinalflora treffen zu können, wurden Tierversuche durchgeführt und die Infektiosität in den Ansätzen nach der Degradation von PrP^{Sc} bestimmt. Obwohl PrP^{Sc} im Western Blot immunochemisch nicht detektierbar war, konnte im Bioassay dennoch signifikante Prioninfektiosität nachgewiesen werden. Folglich korrelieren die Resultate der biochemischen Analyse nicht mit denen des Bioassays.

Dies wäre durch eine fehlende Sensitivität der immunochemischen Nachweismethode erklärbar, mit der Folge, dass gewisse Mengen an PrP^{Sc} unterhalb der Nachweisgrenze des Western Blots bzw. eine nicht detektierbare Subfraktion von PrP^{Sc} die Infektiosität verursachen. Darüber hinaus könnten andere infektiöse Moleküle und Strukturen unabhängig von PrP^{Sc} vorhanden sein. Letztendlich kann eine Kontamination der Umwelt durch das Ausscheiden von infektiösen Faeces nicht ausgeschlossen werden. Des Weiteren zeigen diese Daten den Bedarf an alternativen Nachweismethoden bezüglich der Diagnose von TSE-Erkrankungen.

1. Introduction

The present work was part of the project “Occurrence and Stability of the BSE Agent in Foodstuff (primarily in Milk and Milk Products) and in the Environment” (Grant No. 1205 TG 81 LMU 19a) within the Bavarian Research Cooperation FORPRION. After the occurrence of the first cases of BSE in Bavaria the Bavarian Government decided to fight prion diseases. At the beginning of the year 2001 a research initiative was started, called the "Bavarian Research Cooperation Prions (FORPRION)". This research consortium was granted by the Ministry of Science, Research and Art and the Ministry of Health, Food and Consumer Protection. Through basic and applied research the consortium aims to make progress in the diagnosis and therapy of human and animal prion diseases, as well as in the field of preventive consumer protection.

1.1 Transmissible spongiform encephalopathies (TSE)

1.1.1 Definition

Transmissible spongiform encephalopathies (TSE) or prion diseases are a group of fatal neurodegenerative disorders of humans and animals, which always lead to death. Incubation periods prior to clinical symptoms range from months to years. So far, there is no prophylaxis or therapy for any form of TSE. These diseases are experimentally and naturally transmissible to individuals of the same or other species. They are usually characterized by spongiform degeneration of the brain accompanied by the deposition of amyloid plaques consisting of abnormal protease resistant prion protein (PrP) (Soto, 2006 in Prions: The new biology of proteins).

1.1.2 Human TSE diseases

Familial forms of TSE are associated with the presence of an autosomal dominant genetic mutation of the human the prion protein gene (*Prnp*) (Hsiao et al., 1989). These diseases include familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI).

Sporadic CJD accounts for the majority of TSE disease cases in humans at present with an incidence of approximately 0.6-1.2 x 10^{-6} per year (Ladogana et al., 2005). There is no

association with a mutant PrP allele or evidence for exposure to TSE agent (Harries-Jones et al., 1988). The transmitted and iatrogenic forms of humans TSE are represented by kuru, iatrogenic CJD and variant CJD (vCJD). Kuru occurred among the aborigines in Papua New Guinea throughout the 1950s and 1960s. Ritual acts of mortuary cannibalism seemed to be responsible for epidemic transmission (Gajdusek, 1977). Iatrogenic CJD has been induced by transplantation of infected tissues, administration of pituitary hormones derived from deceased individuals suffering from unrecognized TSEs, or by neurosurgery using instruments incompletely sterilized following use from TSE patients (Brown et al., 1992).

In 1996, vCJD was described, which is now believed to be a zoonotic disease of bovine spongiform encephalopathy (BSE) agent (Will et al., 1996). The unusual young age range of these patients and their distinctive pathology implies a new clinical form of TSE disease. Biochemical and histopathological evidence suggests that vCJD represents transmission of BSE prions to humans (Bruce et al., 1997; Hill et al., 1997). Since there is no association of occupational exposure of vCJD patients to cattle on farms or in abattoirs, spread may have occurred through consumption of BSE-contaminated meat products. As of 2006, 164 cases of vCJD have been reported, mostly from UK (EUROCJD data, 31.10.2006: http://www.eurocjd.ed.ac.uk/vcjdworldeuro.htm). The incidence of vCJD in humans is low and appears to be stabilizing or even falling since 2003 (Figure 1).

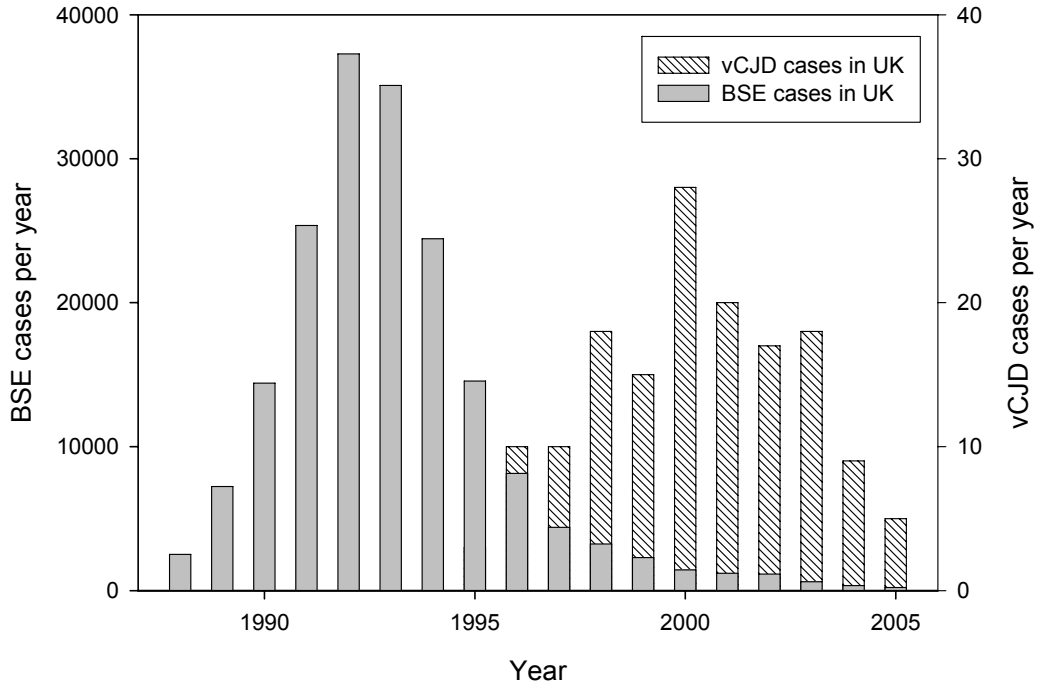


Figure 1: Comparison of the incidence of BSE in cattle and vCJD in humans in the UK.

A large proportion of the British population may have been exposed to BSE infection. Furthermore, animal experiments indicate that the infectious dose (ID50) for oral cross-species transmission of BSE is as low as 500 mg of brain tissue (Foster et al., 1996). Considering that only approximately 160 humans have contracted vCJD, it is likely that vCJD susceptibility is controlled by endogenous and/or exogenous factors other than the amount of ingested infectious agent (Bons et al., 1999).

1.1.3 Animal TSE diseases

1.1.3.1 Bovine spongiform encephalopathy (BSE)

In 1986, bovine spongiform encephalopathy (BSE) was diagnosed and described in UK for the first time (Wilesmith et al., 1988). Thus far, more than 190 000 cases of BSE have been reported world wide. About 97 % of the BSE cases have been confirmed in UK, while 404 cases have been approved in Germany (OIE, 18.12.2006). The OIE report summarizes all cases of BSE positive tested animals regardless of clinical signs (http://www.oie.int/eng/info/en_esb.htm).

BSE is a massive common-source epidemic caused by contaminated meat and bone meal (MBM) fed primarily to dairy cows (Nathanson et al., 1997). The MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. It is assumed that a change in rendering process in the late 1970s allowed prions to survive rendering and to be passed into cattle (Morgan, 1988). However, it remains unclear whether BSE originated by adaptation from a scrapie strain of sheep or from an unrecognised bovine TSE case. The disease has a long incubation period of 4-5 years and it is fatal for cattle within weeks to months of its onset.

Measures for preventing human exposure have been identified and enforced. They include the ban on meat and bone meal in animal feed in 1994, testing of slaughtered animals since 2001, systemic removal of "high-risk material" from carcasses since 2000 and destruction of suspected and confirmed bovine cases as well as the control of animals potentially exposed at the same time. The peak of the BSE epidemic in cattle occurred in 1992-1993, and the incidence has declined dramatically since regulations have been established preventing feeding of ruminant MBM (Figure 1).

1.1.3.2 Scrapie

Scrapie, a fatal, degenerative disease affecting the central nervous system of sheep and goats, has been concerned since the 19th century (Parry, 1962). Signs of scrapie vary among individual animals and develop very slowly. Due to damage to nerve cells, affected animals usually show behavioural changes, tremor, rubbing, and motoric incoordination that progress to death. A crucial breakthrough was achieved in the 1930s by the experimental transmission of scrapie to goats. Subsequently, the scrapie agent has been transmitted to hamsters, mice, rats, mink, guinea pigs and some species of monkeys by inoculation (Barlow and Rennie, 1976; Crozet et al., 2001; Gibbs, Jr. and Gajdusek, 1972; Hanson et al., 1971). The scrapie agent is thought to be spread most commonly from the ewe to her offspring and to other lambs through contact with the placenta and placental fluids (Detwiler and Baylis, 2003).
Scrapie prions appear to persist for years in the environment. When scrapie infected brain was mixed with soil and buried in the garden, 2-3 log units of infectivity of an initial of 4.8 log units remained after three years (Brown and Gajdusek, 1991).

1.1.3.3 Chronic wasting disease (CWD)

Chronic wasting disease (CWD), a transmissible spongiform encephalopathy (TSE) affecting cervids in North America and Canada, is recognized since the late 1970s (Williams and Young, 1980). CWD has been diagnosed in mule deer, white-tailed deer, and Rocky Mountain elk in captive herds and in the wild. Symptoms of CWD include excessive salivation, teeth grinding, lowering of the head, and dropping ears.
CWD is thought to be transmitted horizontally from infected to susceptible animals. Residual infectivity in contaminated environments also appears to be important in sustaining epidemics (Miller and Williams, 2004). Recently, data were published indicating the presence of infectious prions in saliva and blood of deer with CWD (Mathiason et al., 2006). This may explain a facile transmission of the disease via body fluids.

1.1.3.4 Other animal TSE diseases

Transmissible mink encephalopathy (TME) is a rare foodborne disease of ranch-raised mink produced by an as yet unidentified contaminated feed ingredient (Marsh and Bessen, 1993). TME has an average incubation period of more than 7 months before the onset of clinical signs. TME-infected animals may exhibit severe incoordination, difficulty in walking, and pronounced jerkiness of hind limbs. Feline spongiforme encephalopathy (FSE) affects domestic cats and felidae in zoos. Cases of sporadic spongiform encephalopathies were also

diagnosed in hoofed zoo species, like kudus and elands. Epidemiological research and strain typing indicated a link to BSE in cattle. The most widely accepted hypothesis is that affected animals were exposed to BSE infectivity through contaminated feed.

1.2 Nature of the infectious agent

1.2.1 Viral hypothesis

Due to the discovery of the prion protein (PrP) an increase in knowledge has been made concerning many aspects of TSE diseases. However, thus far there are only rare informations about the structure and composition of the infectious agent. Early ultrafiltration studies suggested that the infectious particle was very small and might be a virus (Tateishi and Kitamoto, 1986). Based on resistance to inactivation by heat and acid it might also be difficult to rule out the presence of a virus in TSE infectivity. It has been concluded that there is a common agent ("slow virus"), and like conventional viruses, it has many strains confirmed by the existence of different TSE strains (Bruce and Dickinson, 1987; Carp et al., 1989; Kuczius and Groschup, 1999).

The resistance towards a wide range of physical and chemical treatments is the remarkable feature of TSE agent (Rohwer, 1984). It has been suggested that TSE agent is devoid of nucleic acid due to the resistance of scrapie agent to nuclease digestion, and therefore it has been concluded that the TSE agent may be a protein due to the sensitivity to protein destruction procedures (Prusiner, 1982). However, the stability of common viruses is also dependent on their protein coat, and thus treatment with proteases reduces infectivity (Bolton et al., 1982). In the case of TSE agent the viral nucleic acids might be covered with host encoded protein (PrP). UV and ionizing radiation inactivation studies have not concluded the absence of a nucleic acid suggesting that the DNA must be small (Rohwer, 1984). Furthermore, small nucleic acid molecules have been found in purified infectious scrapie samples (Kellings et al., 1994).

1.2.2 Prion hypothesis

The prion theory postulates that proteinaceous infectious particles, which are largely, if not entirely composed of abnormally folded host-encoded prion proteins, are the causative agents of TSE diseases (Prusiner, 1982). Prions are defined as "small infectious particles which resist inactivation by procedures which modify nucleic acids" (Prusiner, 1982). The best proof for this concept would be the *in vitro* generation of infectious prions from synthetic sources, which could not be demonstrated up to date, although substantial efforts have been made (see 1.3.3).

1.3 Prion protein (PrP)

1.3.1 Function of prion protein (PrP)

Physiological cellular prion protein (PrP^C), encoded by the *Prnp* gene, is found in the membrane of normal, healthy mammalian cells, particularly in neuronal tissues. Accumulating data suggest that PrP^C is also found in a broad spectrum of non-neuronal tissue. PrP^C is selectively localized in bovine podocytes and in mammary gland epithelial cells (Amselgruber et al., 2006; Didier et al., 2006). The function of the cellular prion protein is still unclear. Several strains of mice that lack prion proteins have been generated to elucidate normal prion function. The first *Prnp* knockout mice were generated in 1992 and showed no significant phenotype compared to wild-type mice (Bueler et al., 1992). Other $PrP^{0/0}$ mice showed some alterations in their circadian rhythms and sleep behaviour (Tobler et al., 1997).

The only confirmed function of PrP^C is its necessity for the infection with prions. Mice homozygous deleted in the *Prnp* gene were not infectable whereas heterozygous mice were only partially protected against scrapie infection indicated by a prolonged incubation period (Bueler et al., 1993).

Some reports also indicate a role of PrP^C in synaptic processes (Collinge et al., 1994). Since prions are present in high concentrations on neurons, they may facilitate signal transduction across synapses between nerve cells. Prions may also act as antioxidants by facilitating the transport of copper into the cell where it can be incorporated into the antioxidant enzyme superoxid dismutase and reduce oxidative damage (Jackson et al., 2001; Klamt et al., 2001; Kramer et al., 2001).

1.3.2 Structure of prion protein (PrP)

Prions are 33 to 35 kDa sized glycoproteins comprised of about 250 amino acids with a glycosylphosphatidylinositol (GPI) anchor for the connection to the cell membrane and with two sites for attachment of oligosaccharides. The structure of prion protein varies somewhat among different species of animals, but certain parts of the molecule are more highly conserved than others (Wopfner et al., 1999). Within the amino (N) terminus a highly conserved region is located consisting of five repeats of an octamer (octarepeats) capable to bind copper *in vivo* (Brown et al., 1997). While the amino terminal part of the prion protein is vastly flexible, the carboxyl (C) terminal part shows different structural features containing a globular domain comprised of α-helices and β-sheets (Zahn et al., 2000; Zahn et al., 2003) (Figure 2).

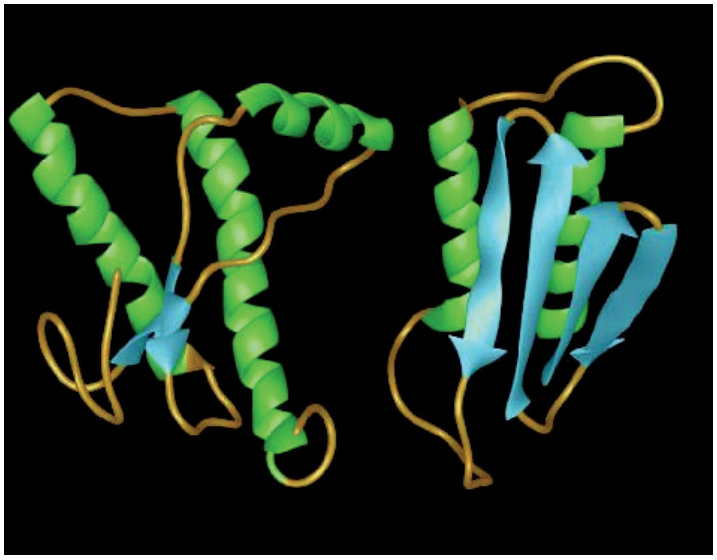


Figure 2: Molecular structure models of the C-terminal region of PrP^{C} (left) and PrP^{Sc} (right). (from Fred Cohen laboratory, UCSF: www.cmpharm.ucsf.edu/cohen). The α-helices (curled structures in green) and β-sheets (arrows in blue) are indicated.

1.3.3 Conversion of prion protein (PrP)

The prion protein (PrP) exists in two different conformational forms, the physiological cellular isoform (PrP^{C}) and the pathogenic isoform (PrP^{Sc}). PrP^{C} and PrP^{Sc} contain exactly the same amino acids (primary structure), but they differ in the bending and folding of the molecules (secondary structure). PrP^{C} has more α-helical structures, while PrP^{Sc} is comprised of more β-sheets (Figure 2).

According to the "protein-only" hypothesis the conversion of PrP^{C} to PrP^{Sc} is the central event in prion diseases (Prusiner, 1991). The introduction of PrP^{Sc} causes PrP^{C} to change to the abnormal shape and aggregate to form amyloid fibrils (Clarke et al., 2001; Prusiner, 2001). The conversion reaction has been successfully performed *in vitro*, but generation of infectivity failed (Caughey et al., 1995; Caughey, 2000). The infectivity of *in vitro* converted PrP^{Sc} could for the first time be demonstrated with the "protein misfolding cyclic amplification" (PMCA) technique (Castilla et al., 2005; Saborio et al., 2001). However, with the PMCA method PrP^{Sc} is propagated in a highly complex mixture of homogenized brain tissue. Therefore, the precise physical nature of the infectious agent as well as the involvement of other cellular factors remains enigmatic.

The change in the secondary structure from α-helices to β-sheets has dramatic effects on the properties of the prion protein. PrP^{C} is soluble and completely sensitive towards proteinase K (PK) treatment (Prusiner et al., 1981). In contrast, PK digestion of PrP^{Sc} results in an N-terminal truncated fragment of the prion protein with a molecular size of 27-30 kDa also termed as PrP^{res}. The detection of PrP^{res} as a surrogate marker for prion infection remains the gold standard for biochemical diagnosis of prion diseases and forms the basis for all of the currently marketed BSE tests. The extreme stability of PrP^{Sc} leads to aggregation into scrapie associated fibrils (SAFs) resulting in cell death (Prusiner et al., 1983; Prusiner, 1991). PrP^{Sc} is also notoriously resistant to heat, UV irradiation and other conventional decontamination procedures (Bellinger-Kawahara et al., 1987; Brown et al., 1990).

1.3.4 Pathogenesis

Prion pathogenesis is characterised by distinct phases: infection and peripheral replication, neuroinvasion, and neurodegeneration. The oral route of infection is widely assumed to be important in the natural pathogenesis of scrapie (Hoinville, 1996), BSE (Wilesmith et al., 1988), and vCJD (Will, 2003), but the processes involved from the movement of the infectious agent to the central nervous system (CNS) are incompletely understood.

For the infection with prions the presence of PrP^{C} is obligatory (Bueler et al., 1993). PrP^{C} expression is required for transporting the infectious agent from the periphery to the CNS and within the CNS (Blattler et al., 1997; Brandner et al., 1996). The lymphoreticular system (LRS) has been implicated as the route of transmission from the gut to the brain. It is suggested that hematopoietic cells transport prions from the entry site to the LRS, which accumulates and replicates prions (Blattler et al., 1997). Membranous epithelial cells (M-cells) and dendritic cells of Peyer's patches are important in the movement of infectious prions

across the gastrointestinal (GI) epithelium (Press et al., 2004). From there, PrP^{Sc} propagation requires B-lymphocytes, dendritic cells and follicular dendritic cells of lymphatic organs like the spleen (Klein et al., 1997; Klein et al., 1998; Prinz et al., 2003b; Prinz et al., 2003a) Once infection replicates in the lymphoid nodules of the gut associated lymphoid tissue (GALT), dissemination to other LRS tissues and the transport to the CNS via the peripheral nervous system can occur (Glatzel et al., 2004).

Moreover, the early accumulation of the disease-causing agent in the plexuses of the enteric nervous system (ENS) supports the contention that the autonomic nervous system is important in disease transmission (van Keulen et al., 1999). As ENS fibres are connected with parasympathetic terminals of the vagus nerve, the ENS is regarded as the site of initial neuroinvasion for scrapie agent.

1.4 The gastrointestinal system

1.4.1 Dietary habits

The dietary habits of domestic animals range from flesh-eating (carnivore) to plant-eating (herbivore). Carnivores, like dogs and cats, obtain most of their food by eating other animals, and their digestion relies largely on enzymes rather than microorganisms. Omnivores, like pigs, fed on both plants and animals, and their digestion is also mainly enzymatic. Herbivores consume plants, which makes cellulose digestion essential. Cellulose, a carbohydrate polymer which is extremely insoluble and remarkably resistant, is the most important structural material of plants. Plant material is lower in energy content, and so herbivores must consume a large quantity to satisfy their energy requirements. Cellulose-containing feed is mostly bulky, and the processes involved in its digestion are relatively slow and take time. Much space is required, and so the part of the digestive tract used for cellulose digestion is large including several compartments.

The domesticated herbivores separate into two groups: (1) non-ruminants are animals with simple stomachs, like horses, in which microbial fermentation takes place in the distal part of the digestive tract (hindgut fermentation); (2) ruminants, like cattle, sheep and goats, possess a specialised region of the digestive tract (rumen) in which extensive fermentation of the plant material occurs prior to digestion with alimentary enzymes (foregut fermentation) (Stevens and Hume, 1998).

1.4.2 Digestion

1.4.2.1 Definition

Digestion is the physical and chemical breakdown of feeds by passing through the gastrointestinal tract. The structures of the gastrointestinal tract include the mouth, the oesophagus, the stomach and the intestines. Digestion breaks down and releases the nutrients in feeds so they can be absorbed by the blood stream and transported to cells to support metabolism (Jeroch et al., 1999).

1.4.2.2 Digestion of non-ruminants

Non-ruminants are animals with a monogastric digestion system containing a simple, one-compartment stomach. For example, the human digestion system is monogastric. After food reaches the stomach a chemical break down occurs by digestive enzymes. The milieu there is extremely acidic with a pH of about 2 caused by the secretion of hydrochloric acid. The monogastric stomach acts primarily as a storage structure, whereas most digestion occurs in the small intestine composed of duodenum, jejunum and ileum. Any remaining material then enters the large intestine or colon, where it is prepared for excretion (Jeroch et al., 1999; Engelhardt and Breeves, 2000).

1.4.2.3 Digestion of ruminants

The ruminants are typical of rechewing food and have a complex polygastric digestion system which is highly adapted to cellulose digestion by symbiotic microorganisms. Digestion of cellulose by microbial enzymes arises in the forestomach which consists of several compartments (Figure 3).

The reticulum and the rumen are the first two stomachs of ruminants. The content of the reticulum is mixed with that of the rumen almost continuously. Both stomachs, often referred to as the reticulo-rumen, share a dense population of microorganisms, like bacteria, protozoa and fungi. The rumen is a large vessel that contains as much as 100 to 120 kg of digesting material mixed with saliva. Fiber particles remain there from 20 to 48 hours. However, particles that digest faster tend to stay in the rumen for a shorter period of time. About 60-75% of ingesta are fermented by microbes before exposition to gastric juices. The third stomach or omasum is round and has a capacity of about 10 litres. The omasum is a small organ with great absorption capacity. It allows the recycling of water and minerals which return to the rumen through saliva. The fourth stomach is the abomasum which corresponds to

the stomach of non-ruminants. It secretes a strong acid and many digestive enzymes. In non-ruminants, ingested feeds are first digested in the abomasum. However, the material entering the abomasums of ruminants is made up primarily of unfermented feed, some end products of microbial fermentation and microbes which grew in the rumen.

After rumen fermentation the products can pass through the small and the large intestines for further digestion and absorption. This special digestion system, together with the habit of regurgitating and rechewing partly digested food, allows ruminants to extract nutrients from high-fibre, poor-quality plant material (Jeroch et al., 1999; Engelhardt and Breeves, 2000).

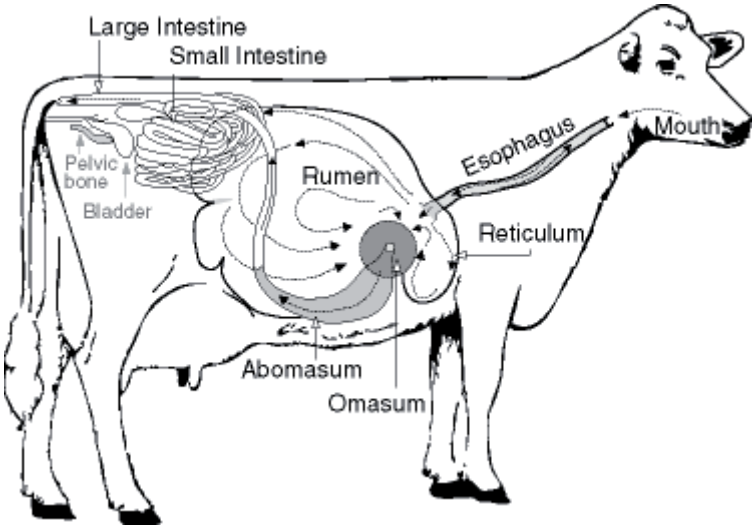


Figure 3: The digestive system of cattle.

(from http://babcock.cals.wisc.edu/downloads/de/01.en.pdf).

1.4.3 Microbiology of gastrointestinal tract

The gastrointestinal tract of mammals is colonised by a huge variety of microbes, which occupy different sites. Each habitat is dynamic, and the conditions are continuously modified by diet, by the host, and by the metabolic activity of the microbial inhabitants. However, homeostatic control operates to ensure a certain stability of the gastrointestinal environments (Engelhardt and Breeves, 2000).

1.4.3.1 Rumen

The rumen microbial ecosystem is a complex consortium of different microorganisms living in a symbiotic relationship with the host. The well stabilized milieu is buffered in a narrow range of pH from 6.0-7.0 to avoid disturbances by incoming microbial contaminants through feed and water. The efficiency of ruminants to utilize a wide variety of feeds is due to the highly specified rumen microbial system consisting of bacteria (10^{10}–10^{11} cells/ml, representing by more than 50 genera), protozoa (10^{4}–10^{6} /ml, from 25 genera), fungi (10^{3}–10^{5} spores/ml, from 5 genera) and bacteriophages (10^{8}–10^{9} /ml) (Stevens and Hume, 1998). The anaerobiosis inside the rumen is a major constraint. The anaerobic conditions are maintained by gases, e. g. carbon dioxide, methane and hydrogen. Some of the oxygen entrapped in the feed is utilized by facultative anaerobes. For example, the most numerous facultative anaerobe, *Streptococcus bovis*, is usually present in numbers of 10^{5}–10^{7} cfu/ml and coliforms are present in numbers of 10^{3}–10^{5} cfu/ml. Interactions among the bacteria are quite diverse, but they collectively ferment carbohydrates, utilize protein and other nitrogenous compounds for synthesis of microbial protein, synthesize B vitamins, hydrolyze lipids, and hydrogenate fatty acids (Table 1).

Table 1: Grouping of rumen bacterial species according to the type of fermented substrates.

Major Cellulolytic Species	**Major Proteolytic Species**	**Major Acid-utilizing Species**
Bacteroides succinogenes *Ruminococcus flavefaciens* *Ruminococcus albus* *Butyrivibrio fibrisolvens*	*Bacteroides amylophilus* *Bacteroides ruminicola* *Butyrivibrio fibrisolvens* *Streptococcus bovis*	*Megasphaera elsdenii* *Selenomonas ruminantium*
Major Lipid-utilizing Species *Anaerovibrio lipolytica* *Butyrivibrio fibrisolvens* *Treponema bryantii* *Eubacterium* sp. *Fusocillus* sp. *Micrococcus* sp.	**Major Ureolytic Species** *Succinivibrio dextrinosolvens* *Selenomonas* sp. *Bacteroides ruminicola* *Ruminococcus bromii* *Butyrivibrio* sp. *Treponema* sp.	**Major Pectinolytic Species** *Butyrivibrio fibrisolvens* *Bacteroides ruminicola* *Lachnospira multiparus* *Succinivibrio dextrinosolvens* *Treponema bryantii* *Streptococcus bovis*
Major Hemicellulolytic Species *Butyrivibrio fibrisolvens* *Bacteroides ruminicola* *Ruminococcus* sp.	**Major Amylolytic Species** *Bacteroides amylophilus* *Streptococcus bovis* *Succinimonas amylolytica* *Bacteroides ruminicola*	**Major Sugar-utilizing Species** *Treponema bryantii* *Lactobacillus vitulinus* *Lactobacillus ruminus*
Major Methane-producing Species *Methanobrevibacter ruminantium* *Methanobacterium formicicum* *Methanomicrobium mobile*	**Major Ammonia-producing Species** *Bacteroides ruminicola* *Megasphera elsdenii* *Selenomonas ruminantium*	

from Church, D. C. (ed.). The Ruminant Animal: Digestive Physiology and Nutrition. Englewood Cliffs, N.J.: Prentice Hall, 1988.

1.4.3.2 Small intestine

The numbers of bacteria in the mammalian midgut are generally much lower than in rumen. For example, human small intestine contains 10^4–10^6 predominantly anaerobic microorganisms per gram of ingesta (Savage, 1986). Most of the bacteria are believed to be transients, and the impact of microbes on digestion in the small intestine is minimal.

1.4.3.3 Large intestine

Microbial fermentation forms a major part of the digestive process in the large intestine. Bacterial cell counts in the hindgut of mammals run up from 10^7–10^{12} /g. The bacterial species inhabiting the large intestine are generally similar to those found in rumen (Wolin, 1981). However, coliforms, particularly *E. coli*, may be present in higher amounts. The *colon ascendens* is responsible for fermenting carbohydrates, while the *colon descendens* breaks down proteins and amino acids. Most bacteria come from the genera *Bacteroides*, *Clostridium*, *Fusobacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium*. Facultative anaerobes such as *Escherichia* and *Lactobacillus* are also present to a less extent.

1.5 Aim of the work

The influence of a complex microbiota composed of a variety of different protease-secreting bacteria, protozoa and fungi located in the gastrointestinal tract on the characteristic resistance of infectious prion proteins has not been described. Hitherto it is unknown, whether infectious prion proteins (PrP^{Sc}), considered to be very stable, are degraded or inactivated by microbial processes in the gastrointestinal tract of cattle. In this context early pathogenesis and the potential infectivity of faeces resulting in contamination of the environment is of general interest.

An oral route of infection is commonly assumed to be important in the natural pathogenesis of bovine spongiform encephalopathy (BSE) of cattle following the ingestion of infected tissues via contaminated feed (Wilesmith et al., 1988). Usually, the proteins of feedstuff are degraded almost completely by passing 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 rumen and colon (Mackie and White, 1990). A large but variable proportion (60 to 90 %) of the dietary protein is degraded by the rumen microorganisms prior to gastric and intestinal digestion. Due to the polypotent metabolic activity of the complex microbiota in the gastrointestinal tract this also should apply to the protein structure of prions. The purpose of this study was to investigate *in vitro* the ability of PrP^{Sc} degradation and inactivation by the complex ruminal and colonic microbiota of cattle.

2. Materials and Methods

2.1 Preparation of brain homogenates

Excised brains from healthy and scrapie (strain 263K) infected Syrian hamsters (Charles River) were provided by the Friedrich-Loeffler-Institute in Riems. A 20 % homogenate of the hamster brain tissue was prepared in brain homogenisation buffer or in sterile mineral salt buffer solution of McDougall by using a glass douncer:

Brain homogenization buffer solution	sucrose ultrapure	107 g
	sodiumdesoxycholate	5 g
	Nonidet P 40	5 ml
	aqua dest.	ad 1000 ml
Mineral salt buffer solution (McDougall)	sodium hydrogen carbonate	9.8 g
	sodium phosphate	9.3 g
	potassium chloride	0.57 g
	sodium chloride	0.47 g
	magnesium sulphate	0.12 g
	calcium chloride	0.04 g
	aqua dest.	ad 1000 ml
	pH 8.3	

Additionally, brain homogenates from BSE-positive and negative cattle were provided by the Friedrich-Loeffler-Institut in Riems in different dilutions (50 %, 25 %, 10 %, 5 %, and 1 %). All homogenates were stored in aliquots at –70 °C until use.

2.2 Preparation of intestinal homogenates

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 nearby the research centre (Figure 4).

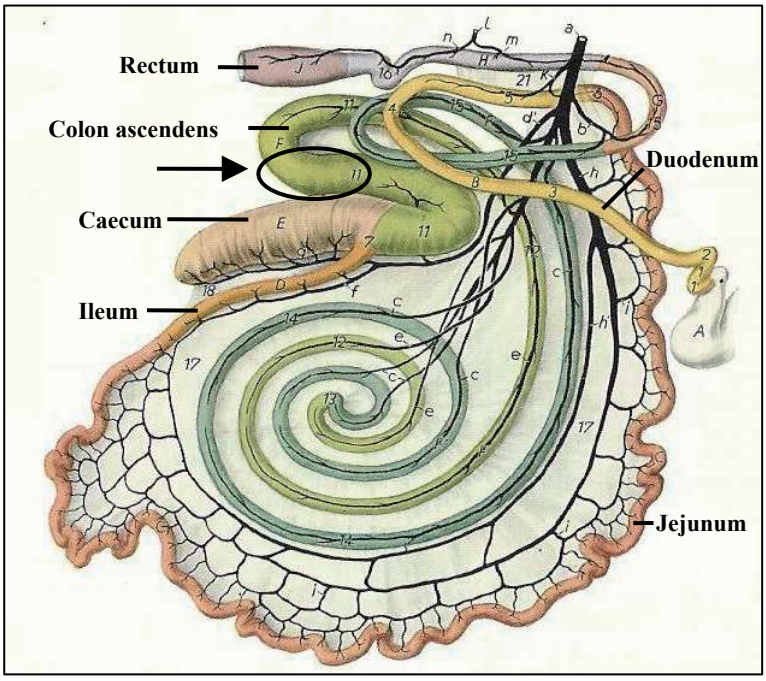


Figure 4: Intestinal tract of cattle.

(from Nickel, Schummer, Seiferle. Lehrbuch der Anatomie der Haustiere Band II. Berlin: Paul Parey Verlag, 1960). Arrow indicates the sampling section.

The microbiota of rumen/intestine contents was 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, respectively (Broderick et al., 2004; Hobson et al., 1968, Kollarczik et al., 1994).

Mineral salt buffer solution (McDougall) with soluble carbohydrates	sodium hydrogen carbonate	11.90 g
	sodium phosphate	9.30 g
	potassium chloride	0.57 g
	sodium chloride	0.47 g
	magnesium sulphate	0.12 g
	calcium chloride	0.04 g
	maltose	6.7 g
	xylose	3.3 g
	soluble starch	3.3 g
	citrus pectin	3.3 g
	aqua dest.	ad 1000 ml
	pH 8.3	

2.3 *In vitro* 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, including negative and positive controls according to the scheme in Figure 5. Immediately after sample preparation the references at 0 hours incubation time were taken and stored at –70 °C until further treatment. Incubation of the samples was carried out at 37 °C for several hours under anaerobic and aerobic conditions. According to the scheme in Figure 5 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-Sulfat (Serva, Heidelberg) and Vancomycin-Kanamycin-Supplement (Oxoid, Wesel) were used as additives. Polymyxin-B-Sulfat, a polypeptide antibiotic, is exclusively effective against gramnegative bacteria. Vancomycin-Kanamycin-Supplement is used as additive in SCHAEDLER-Agar (Oxoid) for the detection of gramnegative germs and is effective against grampositive bacteria.

Additionally, a degradation assay was performed with *Streptococcus bovis* (DSM-No. 20480) isolated from faeces of cattle. *Streptococcus bovis* is the most numerous facultative anaerobe in bovine gastrointestinal tract, and responsible for proteolysis (Griswold et al., 1999b). Therefore, an overnight culture of *Streptococcus bovis* grown in Standard I (STI) bouillon (Merck, Darmstadt) was incubated in a ratio of 10 to 1 with PrP^{Sc} in brain homogenate as mentioned above.

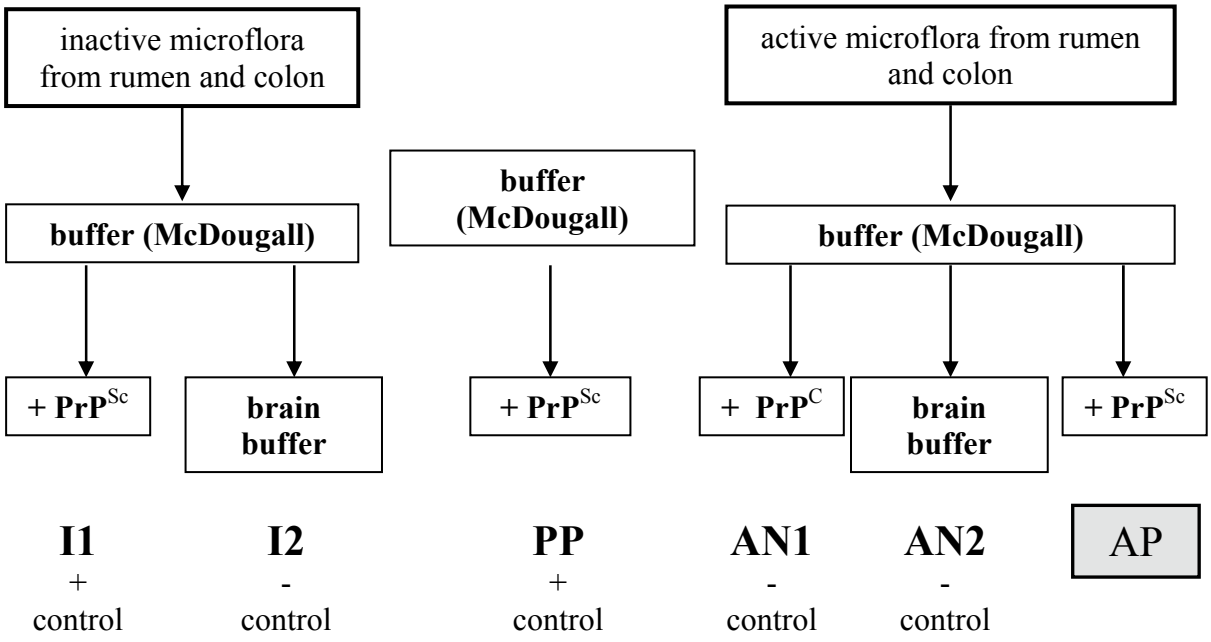


Figure 5: Experimental flow scheme for *in vitro* degradation studies of prion protein (PrP^{Sc}) by complex microbiota of bovine rumen and colon.

Samples were prepared in the ratio of 10 to 1 (intestine content to brain material) including negative and positive controls.

2.3.1 Proteinase K treatment

Aliquots of 100 µl from each sample were digested with 10 µl Proteinase K (Sigma-Aldrich, Taufkirchen) at a final concentration of 100 µg/ml for 1 hour at 37 °C. Reactions were stopped by adding 20 µl 100 mM PMSF (Sigma-Aldrich, Taufkirchen), a protease inhibitor and incubation for 15 min at room temperature (Schaller et al., 1999).

2.3.2 Gel electrophoresis

Proteins were subjected to electrophoresis in the PerfectBlue Doppelgelsystem Twin ExW S (Peqlab, Erlangen) using 12 % sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE). All samples were boiled for 5 min in Laemmli buffer (BioRad Laboratories, Munich) in a ratio of 1 to 3 concerning sample volume to buffer volume. A volume of 25 µl sample and 15 µl molecular weight marker Precision Plus Protein standard dual color (BioRad Laboratories) per slot were subjected to electrophoresis for 15 min at 200V (collecting gel) and 45 min at 100V (separating gel).

2.3.3 Western blot

Proteins were transferred by semi dry method to a nitrocellulose (Schleicher & Schuell, Dassel) or PVDF membrane (Schleicher & Schuell), respectively (Burnette, 1981). Therefore filter paper (Hybond Blotting Papier RPN 6102M, Amersham Bioscience, Freiberg) and membrane were soaked in blotting buffer after moistening the PVDF membrane with methanol for 15 sec.

Blotting buffer (10 x)	Tris	60.6 g
	glycine	29.3 g
	methanol	200 ml
	SDS ultrapure	0.39 g
	aqua dest.	ad 1000 ml

Subsequently, a gel sandwich was built on the cathode of the chamber (PerfectBlue Semi-Dry Elektroblotter, Peqlab). The transfer was performed at 140 mA for 75 min.

2.3.4 Immunochemical detection

Prion protein (PrP) was detected by immunostaining with specific monoclonal anti-prion antibodies (mabs) (Table 2).

Table 2: Specific monoclonal anti-prion antibodies for immunodetection.

anti-PrP antibody	binding site	supplier
mab 3F3	aa 109-112	Sigma-Aldrich, Taufkirchen
mab 6H4	aa 144-152	Prionics, Schlieren
mab 14D11	aa 180-200	Roboscreen, Leipzig

After blocking with 5 % skim milk in PBST for 60 min at room temperature the membrane was incubated under slight shaking with primary antibody 3F4 (Sigma-Aldrich), 6H4 (Prionics) or 14D11 (Roboscreen) for 90 min at 0.2 µg IgG/ml PBST, washed thrice with

PBST for 10 min and subsequently incubated with the secondary antibody conjugated to horseradish peroxidase (Dianova, Hamburg) in a dilution of 1:5000 in PBST for 60 min.

PBST solution	sodium chloride	8 g
	potassium chloride	2 g
	potassium hydrogen phosphate	2 g
	sodium hydrogen carbonate	1.15 g
	calcium chloride	0.13 g
	magnesium chloride	0.1 g
	Tween-20	1 ml
	aqua bidest.	ad 1000 ml

The reaction was visualized using a highly sensitive chemiluminescence-based detection technique (ECL) (Amersham Bioscience). Therefore the membrane was incubated for 60 sec with 7 ml of the both ECL detection solutions, respectively. The luminescence detection was performed in a dark chamber using an exposition time of 1 min to 15 min. The lightened film was developed for 1 min and rinsed for 3 min in water. After fixing, the film was rinsed in water again and dried.

2.3.5 Removal of antibodies (Stripping)

The membrane was washed under slight shaking with stripping buffer for 60 min, subsequently blocked again with 5 % skim milk in PBST for 60 min at room temperature and reprobed with a second specific monoclonal anti-prion antibody as mentioned above.

stripping buffer solution	glycine	15 g
	SDS ultrapure	1 g
	Tween 20	10 ml
	aqua bidest.	ad 1000 ml
	pH 2.2	

2.4 Animal bioassay

2.4.1 Methods for sample preparation

To reduce bacterial cell count without impairing PrP signal on Western blot, several attempts were performed. The intestinal contents were prepared as mentioned above, mixed with scrapie brain homogenate in a ratio of 10 to 1 and treated differently by

- autoclaving:
1 ml per sample was autoclaved for 10 min at 121 °C, 115 °C and 110 °C, respectively.

- sterilfiltration:
1 ml of the sample was filtrated through 0.22 µm filter (Millipore, Schwalbach).

- tyndallisation:
Tyndallisation is a method to sterilize food without using high temperature compared with autoclaving. Therefore 1 ml per sample was heated trice at 65 °C, 80 °C and 90 °C, respectively, and cooled down to room temperature in intervals of 24 hours.

- mechanical cell lysis with the Hybaid RiboLyserTM Cell Disrupter (Thermo Labsystems, Waltham, USA):
Bacterial cell membranes were destroyed mechanically with expedited particles of ceramic, glass and silicate. Therefore ribolyser tubes lysing matrix E (Qbiogene, Grünberg) with 300 µl of the sample were placed in a ribolyser for 45 sec (speed 5.5). The particles were removed by centrifugation for 2 min at 8000 rpm, and the supernatant was collected in fresh tubes.

After the different treatments bacterial cell count and PrP^{Sc} signal was determined. Therefore a dilution line was prepared with saline 1 to 10. 100 µl per dilution were plated on STI agar plates (Merck, Darmstadt) and incubated over night at 37 °C. The determination of PrP^{Sc} signal was performed via immunoblotting as mentioned above.

2.4.2 Sample preparation for *in vivo* hamster bioassays

After performing *in vitro* PrP^{Sc} degradation assays, selected samples were prepared using the ribolyser method (see above). Supernatant was incubated at 75 °C for 5 min to inactivate proteases and frozen. For bioassays 1 ml of each sample was sent to the Friedrich-Loeffler-Institute in Riems.

2.4.3 *In vivo* hamster bioassay

Each sample (30 µl) of pretreated scrapie and normal hamster brain homogenate was inoculated intra-cerebrally into 8 Syrian hamsters (Charles River, Wilmington, USA), respectively. Animals were checked every second day during the first two months post inoculation. Thereafter, hamsters were assessed daily for the onset of clinical scrapie signs and clinical states were recorded as scores ranging from 0 (healthy) to 4 (severely sick). Hamsters suffering for three consecutive days from severe clinical symptoms (stage 4) were euthanized and the brains were taken and tested by Western blotting using mab 3F4 for the presence of PrP^{Sc} (Schaller et al., 1999). Mean incubation periods and standard errors of the mean were determined for each group and scrapie agent infectivity titres calculated on the basis of these data (Prusiner et al., 1980). The animal bioassays were carried out in collaboration with the Friedrich-Loeffler-Institute in Riems.

3. Results

3.1 *In vitro* degradation assay of scrapie associated prion protein (263K)

In vitro degradation assays of infectious scrapie associated prion protein (PrP^{Sc}) by complex microbiota of bovine rumen and colon were established according to the experimental flow scheme in Figure 5.

3.1.1 Control experiments of degradation assay

Several control experiments were performed to exclude artificial effects, like failure of PrP^{Sc} detection by sticking to the complex matrix of intestine contents (Figure 6). In all of the *in vitro* degradation experiments (n=21) the inactive microbiota (sample I1) and the McDougall buffer solution only (sample PP) did not affect the PrP^{Sc} signal in Western blot (Figure 6, 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 (Figure 6, lanes 1-6).

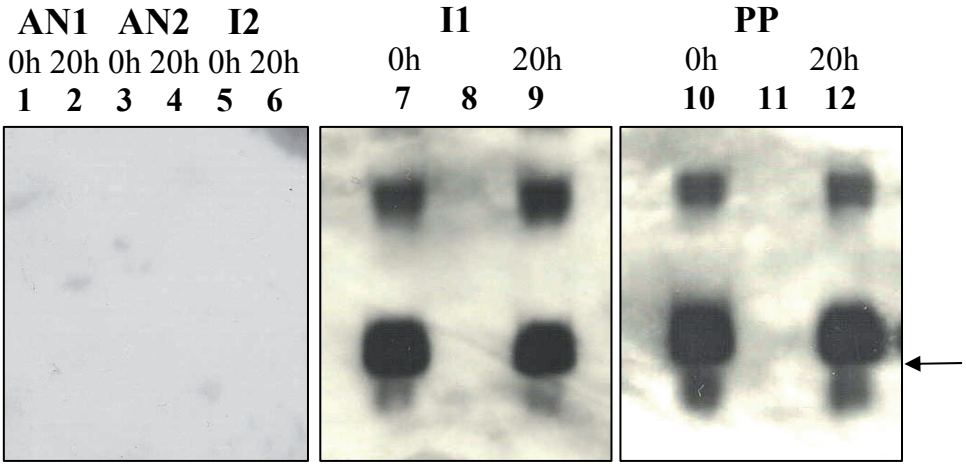


Figure 6: Control experiments of *in vitro* prion protein (PrP^{Sc}) degradation studies by complex ruminal and colonic microbiota of cattle.

Complex intestinal microbiota of cattle was 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 hours. Inactivated complex intestinal microbiota of cattle was 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 hours. Mineral salt buffer solution of McDougall was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours (lanes 10 and 12). Arrow indicates the position of molecular-weight marker (25 kDa). Lanes 8 and 11 are empty.

3.1.2 Degradation assay with microbial active samples

Following incubation for 20 hours under physiological anaerobic condition, active ruminal and colonic microbiota reduced the PrP^{Sc} signal in Western blot up to immunochemically undetectable levels (Figure 7, lanes 2 and 4).

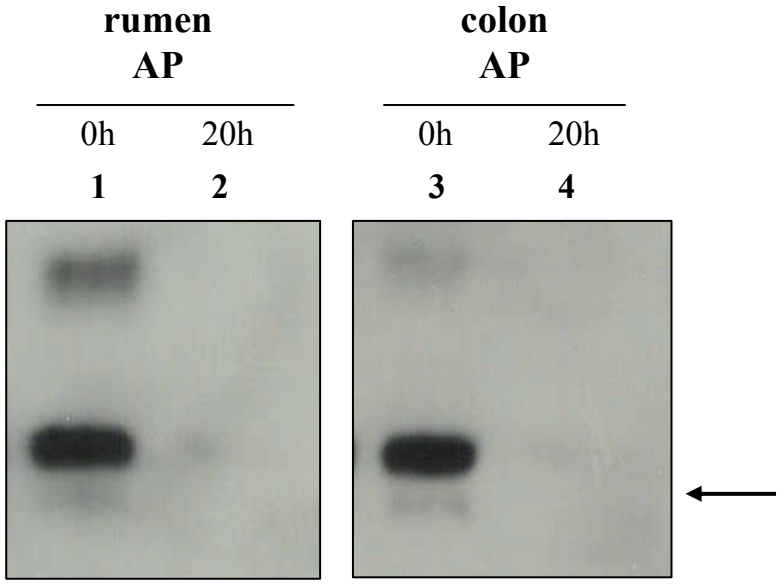


Figure 7: *In vitro* prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen and *colon ascendens*.

Complex ruminal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours (lanes 1 and 2). Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours (lanes 3 and 4). Arrows indicate the position of molecular-weight marker (25 kDa).

3.1.3 Repeatability of degradation assay

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 content and 19 out of 21 incubation experiments with colon content a substantial degradation of PrP^{Sc} was determined as summarized in Table 3.

Table 3: Determination of PrP^{Sc} degradation capacity after *in vitro* incubation assay under anaerobic condition.

Numbers of *in vitro* incubation experiments under anaerobic condition	**PrP^{Sc} degradation**				
	complete[1]	almost complete[2]	substantial[3]	weak[4]	no[5]
rumen (n = 21)	9	4	5	2	1
colon (n = 21)	11	4	4	1	1

[1] no PrP^{Sc} signal; [2] very weak PrP^{Sc} signal; [3] weak PrP^{Sc} signal; [4] significant PrP^{Sc} signal; [5] PrP^{Sc} signal remained stable on immunoblot after incubation.

3.1.4 Characterisation of PrP^{Sc} degrading microbiota

To differentiate the PrP^{Sc} degrading microbiota incubation experiments with selective mixed cultures were performed. PrP^{Sc} signal was reduced to immunochemically undetectable levels by polymyxin-resistent (mainly grampositive) microbiota of bovine rumen and colon (Figure 8, lanes 2 and 6). In contrast, PrP^{Sc} signal remained stable after incubation for 20 hours with predominant gramnegative microbiota of rumen and colon (Figure 8, lanes 4 and 8).

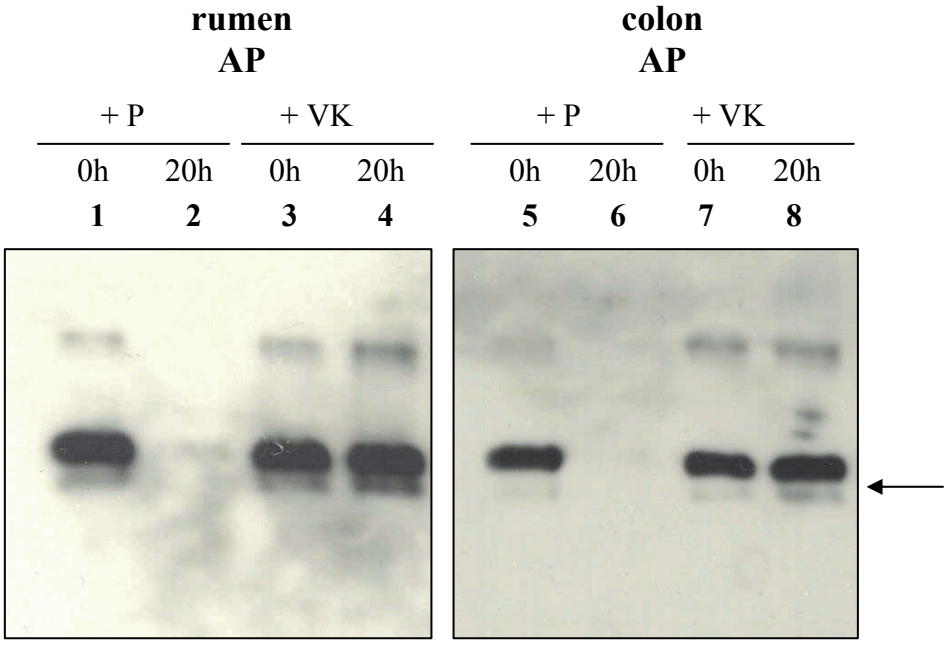


Figure 8: *In vitro* prion protein (PrP^{Sc}) degradation studies by selected complex microbiota of bovine rumen and *colon ascendens*.

Complex ruminal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours in the presence of 100mM polymyxin (lanes 1 and 2) or vancomycin-kanamycin supplement (lanes 3 and 4). Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours in the presence of 100mM polymyxin (lanes 5 and 6) or vancomycin-kanamycin supplement (lanes 7 and 8). Arrow indicates the position of molecular-weight marker (25 kDa).

3.1.5 Degradation assay under physiological condition

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 homogenized in sterile mineral salt buffer solution of McDougall 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 hours by bovine gastrointestinal microbiota (Figure 9A, lanes 1 and 2; Figure 9B lanes 1 and 2), whereas PrP^{Sc} signal remained stable without available carbohydrates (Figure 9A, lanes 3 and 4; Figure 9B, lanes 3 and 4).

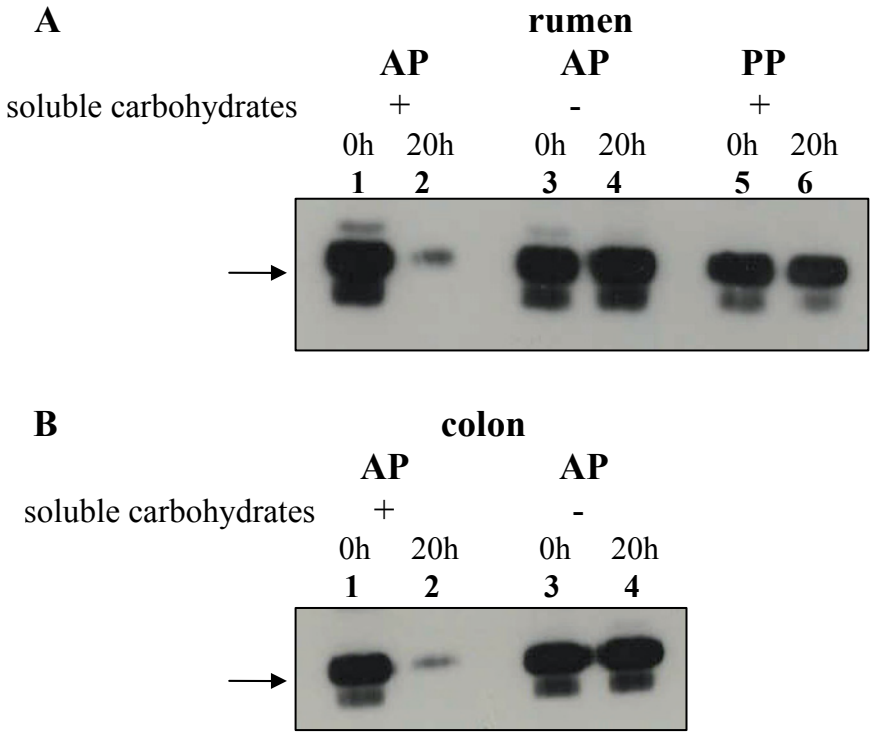


Figure 9: *In vitro* prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen and *colon ascendens* in different buffers without the presence of detergents.

A) Complex ruminal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic conditions in the presence and absence of soluble carbohydrates for 0 and 20 hours (lanes 1, 2, 3 and 4). Mineral salt buffer solution of McDougall in addition of soluble carbohydrates was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours (lanes 5 and 6).

B) Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenated in mineral salt buffer solution of McDougall under anaerobic conditions in the presence and absence of soluble carbohydrates for 0 and 20 hours (lanes 1, 2, 3 and 4). Arrows indicate the position of molecular-weight marker (25 kDa).

3.1.6 Degradation assay with *Streptococcus bovis*

Additionally, a degradation assay was performed with *Streptococcus bovis* (DSM-No. 20480) isolated from faeces of cattle. *Streptococcus bovis* is the most numerous facultative anaerobe in bovine gastrointestinal tract, and is responsible for proteolysis (Griswold et al., 1999). After incubation of *Streptococcus bovis* with scrapie brain homogenate under anaerobic conditions PrP^{Sc} was almost fully digested, whereas incubation in the presence of oxygen did not alter PrP^{Sc} signal (Figure 10, lanes 3 and 7).

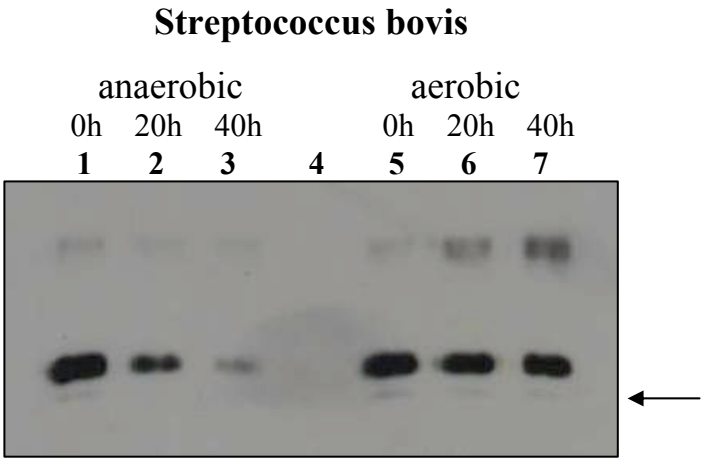


Figure 10: Prion protein (PrP^{Sc}) degradation assay with *Streptococcus bovis*.

An overnight culture of *Streptococcus bovis* was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic (lanes 1, 2 and 3) and aerobic (lanes 5, 6 and 7) conditions for 0, 20 and 40 hours. Arrow indicates the position of molecular-weight marker (25 kDa). Lane 4 is empty.

3.1.7 Detection of PrP^{Sc} with different antibodies

To examine the complete degradation of PrP^{Sc} the immunodetection was accomplished with several monoclonal antibodies (mabs). While the mab 14D11 binds at the C-terminal region of prion protein (aa 180-200), the commonly used mab 3F4 binds at the N-terminal region (aa 109-112). PrP^{Sc} pattern after immunodetection by the mabs with different binding sites is identical (Figure 11). However, the intensity of PrP^{Sc} signals using mab 14D11 is reduced due to the lower specifity of the antibody, which is raised against recombinant human PrP with a cross reactivity to bovine and ovine PrP. Following incubation with complex ruminal microbiota under anaerobic condition PrP^{Sc} is undetectable with both antibodies (Figure 11, lane 2).

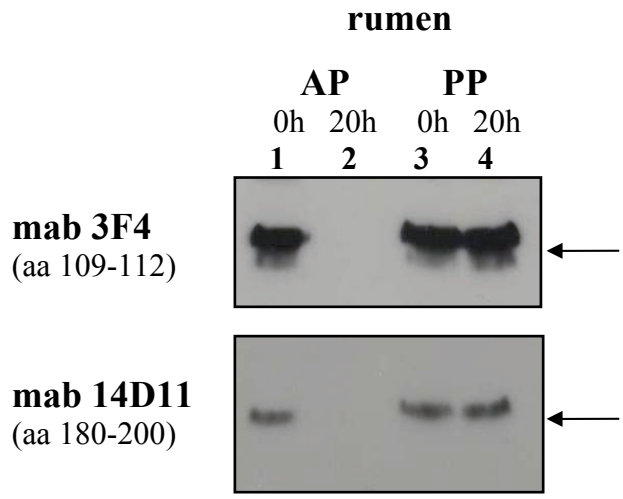


Figure 11: Immunodetection of prion protein (PrP^{Sc}) with different antibodies following degradation by complex microbiota of bovine rumen.

Complex ruminal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenated in mineral salt buffer solution of McDougall under anaerobic conditions in the presence of soluble carbohydrates for 0 and 20 hours (lanes 1 and 2). Mineral salt buffer solution of McDougall in addition of soluble carbohydrates was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 20 hours (lanes 3 and 4). For the immunodetection two different antibodies were used. After the detection of PrPSc with mab 3F4, the membrane was washed with stripping buffer and reprobed with mab 14D11. Arrows indicate the position of molecular-weight marker (25 kDa).

3.2 Animal bioassay with scrapie associated prion protein (263K)

3.2.1 Biochemical analysis of the samples used for animal bioassay

Prior to performing animal bioassays the selected samples were analysed biochemically for PrP^{Sc} degradation. While inactive microbiota (sample I1) did not affect the PrP^{Sc} signal in Western blot (Figure 12, lanes 1 and 2), following incubation for 40 hours under physiological anaerobic conditions, active ruminal (sample ruAPan) and colonic (sample coAPan) microbiota reduced the PrP^{Sc} signal significantly up to immunochemically undetectable levels (Figure 12, lanes 5 and 8), as shown previously. Additionally, colonic microbiota (sample coAPa) eliminated all 3F4-immunoreactive material after incubation for 40 hours in the presence of oxygen (Figure 12, lane 11). As expected, the negative control with normal brain homogenate (sample AN1) showed no immunodetectable signals at all (Figure 12, lanes 12 and 13).

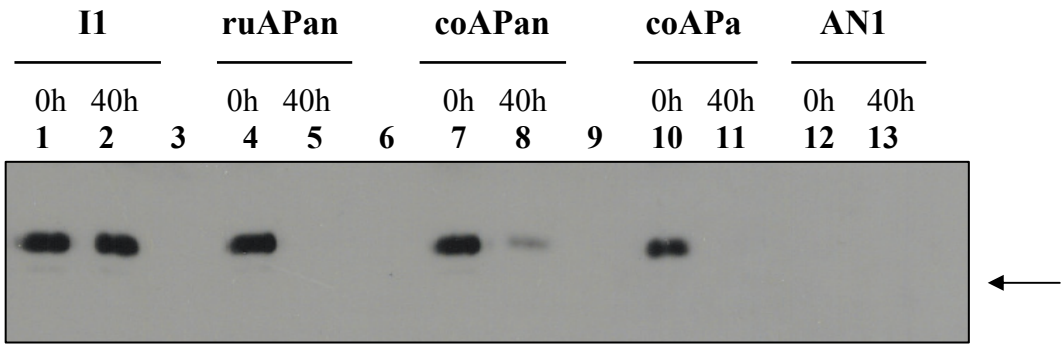


Figure 12: Biochemical analysis of the samples including controls used for animal bioassay.

Inactivated complex intestinal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate for 0 and 40 hours (lanes 1 and 2). Complex ruminal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 40 hours (lanes 4 and 5). Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 40 hours (lanes 7 and 8). Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenate under aerobic conditions for 0 and 40 hours (lanes 10 and 11). Complex intestinal microbiota of cattle was incubated with healthy hamster brain homogenate (PrP^{C}) (lanes 12 and 13) for 0 and 40 hours. Arrow indicates the position of molecular-weight marker (25 kDa). Lanes 3, 6 and 9 are empty.

3.2.2 Sample preparation for animal bioassay

To reduce bacterial cell count before inoculation without impairing PrP signal on Western blot, several methods were explored (Table 4).

Table 4: Comparison of different methods for the potential of sample preparation prior to animal bioassay.

Method	Condition	Bacterial cell count (cfu/ml)	PrP^{Sc} signal
Autoclaving	121 °C	-	-
	115 °C	-	-
	100 °C	-	-
Sterilfiltration	0.22 µm	-	(+)
Tyndallisation	65 °C	4.3×10^6	++
	80 °C	2.0×10^5	++
	90 °C	2.0×10^2	+
Ribolyser	speed 5.5 45 sec	3.3×10^2	+++

- no PrP^{Sc} signal; (+) very weak PrP^{Sc} signal; + weak PrP^{Sc} signal; ++ significant PrP^{Sc} signal; +++ PrP^{Sc} signal remained stable on immunoblot after treatment.

Sterilisation processes like autoclaving and sterilfiltration removed the microbial load of the samples completely, but they also eliminated the PrP^{Sc} signal in Western blot. Tyndallisation processes slightly affect PrP^{Sc} signal at lower temperatures, although the bacterial cell count with values around 10^5-10^6 cfu/ml is too high for inoculation. Tyndallisation at 90 °C leads to a significant reduction of the bacterial cell count to values around 10^2 cfu/ml suitable for hamster inoculation, but as well the PrP^{Sc} signal in Western blot declines. The ribolyser method reduced the bacteria cell count to 10^2 cfu/ml without impairing PrP^{Sc} signal on Western blot (Table 4).

Using the Ribolyser method the risk of bacterial brain lesions could be minimized before inoculation without affecting PrP^{Sc} signal in Western Blot (Figure 13).

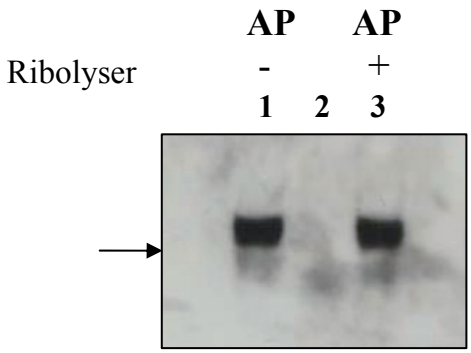


Figure 13: Detection of PrP^{Sc} signal after mechanic cell lysis (Ribolyser method).

Samples were transferred into Ribolyser Tubes Lysing Matrix E containing beads (Q-Biogene) and placed in a Hybaid RiboLyserTM Cell Disrupter (Thermo Labsystems) for 45 sec at speed 5.5. Disrupted cells were separated by short spin centrifugation (15 sec, 8000 rpm). Lanes 3 and 11 are empty. Arrow indicates the position of molecular-weight marker (25 kDa).

3.2.3 *In vivo* hamster bioassay

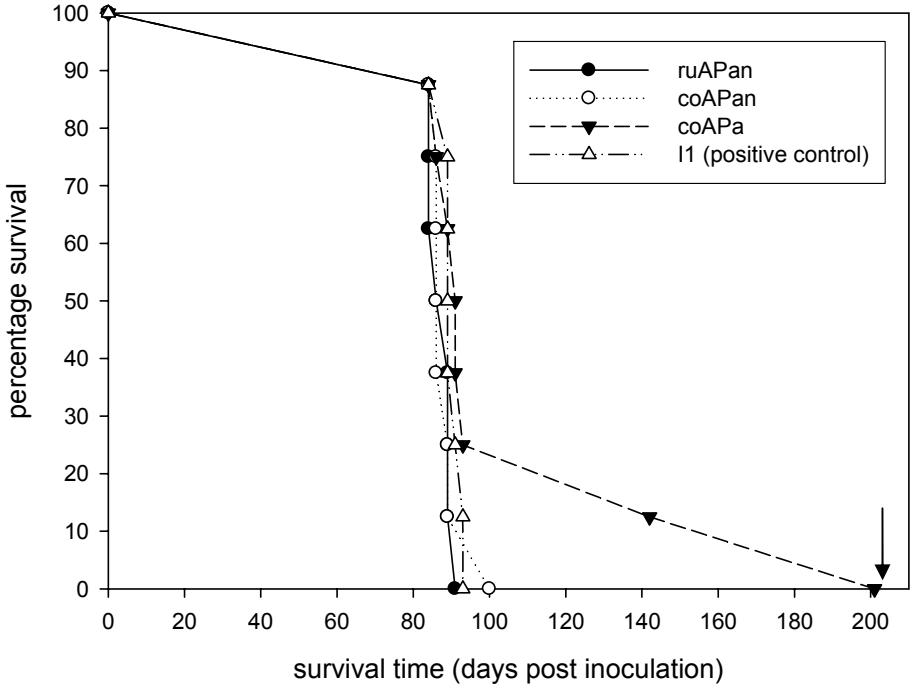
In order to assess the concomitance of PK-resistant prion protein disappearance in hamster brain homogenates and the inactivation of 263K scrapie agent, *in vivo* hamster bioassays with the samples were performed (Figure 12). The inoculation of the animals was carried out in the Institute of Novel and Emerging Infectious Diseases in Riems. The results of the animal bioassay are shown in Table 5. Negative effects caused by the residual bacterial count onto the animals could be excluded. All animals inoculated with the sample of the negative control survived for more than 201 days post inoculation (Table 5; sample AN1).

Table 5: Mean survival time of hamsters following intra-cerebral inoculation of normal and scrapie hamster brain homogenate after incubation with gastrointestinal microbiota of cattle and *Streptococcus bovis* under different conditions.

Sample number	Pre-treat-ment	Incubation conditions 40 hours	Scrapie hamster brain homo-genate	Normal hamster brain homo-genate	Number of affect-ed versus inoculated hamsters	Mean survival time (days)	Standard error of the mean (days)
AN1 (negative control)	active ruminal microbiota	anaerobic	-	+	0/8	> 201.0	-
I1 (positive control)	inactive ruminal microbiota	anaerobic	+	-	8/8	89.6	0.72
ruAPan	active ruminal microbiota	anaerobic	+	-	8/8	87.0	0.88
coAPan	active colonic microbiota	anaerobic	+	-	8/8	88.3	1.17
coAPa	active colonic microbiota	aerobic	+	-	**7/8**	96.6	4.91
Str.bovis	*Strepto-coccus bovis*	anaerobic	+	-	8/8	92.3	1.26

However, significant residual prion infectivity was retained after degradation of infected hamster brain through the gastrointestinal microbiota of cattle and *Streptococcus bovis*, respectively. Infectivity levels in all treated or nontreated samples were determined by hamster bioassays. Measurements of incubation period length are in direct reciprocal correlation to the titer of the agent and the dilution of the inoculated sample, and can be calculated on the basis of a standard curve (Prusiner et al., 1980). Treatment of scrapie

associated prion protein under anaerobic conditions with both ruminal and colonic microbiota of cattle showed no extension of mean survival time in comparison to the positive control (Figure 14 and Table 5; samples ruAPan and coAPan). A slight increase of the mean survival time from 89.6 ± 0.72 days to 96.6 ± 4.91 was noted for the group of hamsters inoculated with samples of the colonic degradation experiment (Table 5; sample coAPa). However, this prolongation in the incubation time was due to one hamster which lacked any clinical signs and PrP^{Sc} formation most likely for artfactual reasons (Figure 14). Excluding this animal from the calculation diminished the one log infectivity titer reduction (corresponding in the 7 day prolongation in the medium incubation time) we conclude that the treatment had no effect on the scrapie infectivity level at all.



note: arrow indicates the animal without clinical signs and PrP^{Sc} deposits

Figure 14: Bioassay survival curves of scrapie (strain 263K) brain homogenates after pre-treatment with gastrointestinal microbiota of cattle.

3.3 *In vitro* degradation assay of BSE associated prion protein

In vitro degradation assays (n=6) of infectious BSE associated prion protein (PrP^{Sc}) with the complex microbiota of bovine rumen and colon were established under different conditions in accordance to the experimental flow scheme in Figure 5. Following incubation with both ruminal and colonic microbiota of cattle for up to 40 hours did not result in PrP^{Sc} degradation in any instance (Figure 15 and Figure 16).

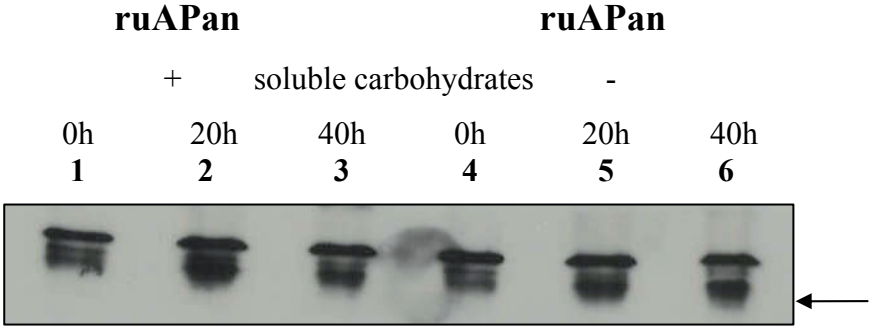


Figure 15: *In vitro* BSE associated prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen in different buffers.

Complex ruminal microbiota of cattle was incubated with BSE infected brain homogenate (10 %) in mineral salt buffer solution of McDougall under anaerobic conditions in the presence (lanes 1, 2 and 3) and absence (lanes 4, 5 and 6) of soluble carbohydrates for 0, 20 and 40 hours. Arrow indicates the position of molecular-weight marker (25 kDa).

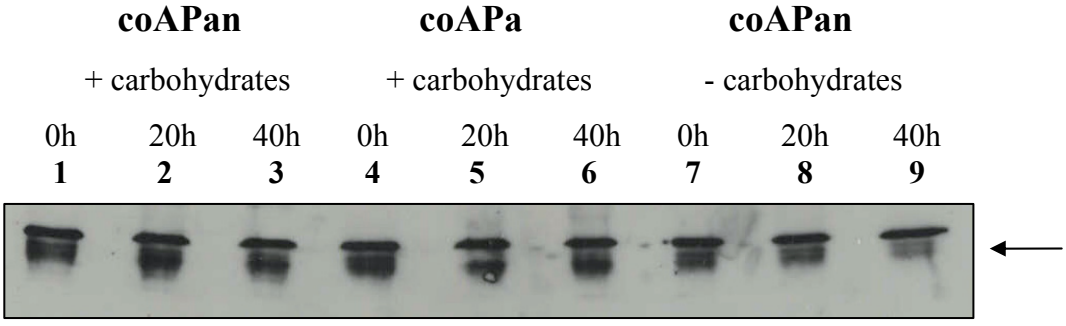


Figure 16: *In vitro* BSE associated prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine *colon ascendens* in different buffers.

Complex colonic microbiota of cattle was incubated with BSE infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic (lanes 1, 2 and 3) and aerobic (lanes 4, 5 and 6) conditions in the presence soluble carbohydrates for 0, 20 and 40 hours. Complex colonic microbiota of cattle was incubated with BSE infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic conditions in the absence of soluble carbohydrates for 0, 20 and 40 hours (lanes 7, 8 and 9). Arrow indicates the position of molecular-weight marker (25 kDa).

Additionally, a degradation assay of infectious BSE associated prion protein (PrP^{Sc}) and the complex microbiota of bovine rumen and colon was performed with prolonged incubation times for up to six days. The long-term incubation of BSE associated prion protein (PrP^{Sc}) together with both ruminal and colonic microbiota of cattle obviously diminished the PrP^{Sc} signal in western blot, whereas the PrP^{Sc} signal of the positive control remained stable (Figure 17).

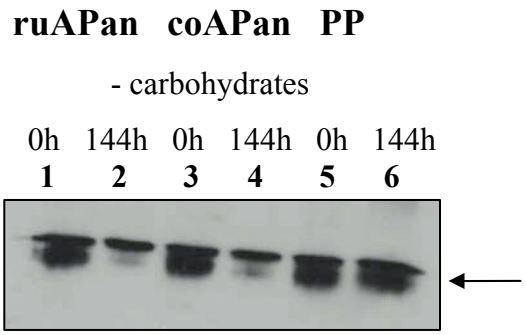


Figure 17: *In vitro* BSE associated prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen and *colon ascendens* with long-term incubation.

Complex ruminal and colonic microbiota of cattle were incubated with BSE infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic conditions in the absence soluble carbohydrates for 0 and 144 hours (lanes 1, 2, 3 and 4). Mineral salt buffer solution of McDougall (positive control) was incubated with BSE infected brain homogenate in under anaerobic conditions in the absence of soluble carbohydrates for 0 and 144 hours (lanes 5 and 6). Arrow indicates the position of molecular-weight marker (25 kDa).

4. Discussion

4.1 Biochemical evidence of scrapie associated prion protein degradation by the gastrointestinal microbiota of cattle

The detection of proteinase K resistant prion protein remains the gold standard for biochemical diagnosis of prion diseases, and is basis for all of the currently marketed BSE tests. Digestion with 50 µg/ml of PK at 37 °C for two hours does not degrade the carboxy terminal domain of PrP^{Sc} (McKinley et al., 1983), nor decrease the infectious titre of prion preparation. PrP^{Sc} can be digested by more vigorous enzymatic treatment with prolonged incubation time and higher concentrations of PK, which results in a decrease of prion infectivity titers. The correlation between TSE infectivity and the level of PK-resistant PrP^{Sc} served as the main biochemical evidence supporting the role of prion protein as a component of TSE agents (Diringer et al., 1983). This remarkable discovery identified PrP^{Sc} as the first reliable marker of prion infection.

Prions are characterized by an extraordinary resistance to most physical and chemical treatments classically used for inactivation of conventional pathogens. Many studies have investigated the contribution of various processes to either the reduction of infectivity or to inactivation of TSE agents (Brown et al., 2003; Grobben et al., 2005; Peretz et al., 2006; Race and Raymond, 2004; Scott, 2006; Taylor and Woodgate, 2003).
Biological decontamination is usually based on an enzymatic breakdown of PrP^{Sc}. This was shown by several groups using a Western blot method based on a biochemical detection of PrP^{Sc}. However, no information about the correlation with the levels of infectivity in bioassays is given in these studies.
For example, extra cellular proteases of *Streptomyces* sp. (Hui et al., 2004) and *Nocardiopsis* sp. TOA-1 (Mitsuiki et al., 2006) are able to decrease PrP^{Sc} levels in scrapie infected brain homogenates under special conditions (60 °C and pH 11).
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 (Langeveld et al., 2003). In contrast to the bacterial proteases, digestion of heated samples with pepsin or trypsin was incomplete.

Also, the use of anaerobic thermophilic prokaryotes for biodegradation of PrP^{Sc} was investigated (Tsiroulnikov et al., 2004). To improve the efficiencies of composting and decontamination of slaughterhouse and rendering effluents, collections of anaerobic thermophilic bacteria from deep-sea and terrestrial sources, as well as representatives from the genus *Streptomyces* were screened for their ability to use keratin, which forms similar structures to prion protein amyloids, and recombinant ovine prion protein. They proposed the decontamination of animal meal containing prion protein using proteolytic enzymes secreted by *Thermoanaerobacter*, *Thermosipho*, and *Thermococcus* spp. and mesophilic soil bacteria *Streptomyces* spp.

Recently, some foodborne bacteria, most of them originally isolated from cheese surfaces, were identified able to degrade PrP^{Sc} by secreted proteases under more or less native conditions (30 °C and pH 8) (Muller-Hellwig et al., 2006). About 200 protease secreting isolates belonging to the *Actinomycetales* and *Bacillales* were screened for PrP^{Sc} degrading capacity. Only 6 strains expressed PrP^{Sc} degrading activities represented by *Arthrobacter nicotinae*, *Bacillus licheniformis*, *Brachybacterium conglomeratum*, *Brachybacterium tyrofermentans*, *Staphylococcus sciuri* and *Serratia* spp.

These findings are in accordance with our results of the biochemical evidence of scrapie associated prion protein degradation. On account of easier handling and availability of scrapie material, initial studies were performed with scrapie (strain 263K) infected hamster brain homogenates. Moreover, the Syrian hamster model infected with hamster-adapted 263K strain of scrapie agent is well-validated and highly sensitive. Apart from a short incubation period (60-80 days) and a high titre of infectivity in the brain (up to 10^{11} ID_{50}/g), the 263K strain is also appreciated because of its high resistance to decontamination methods (Ernst and Race, 1993; Golker et al., 1996).

Within 20 hours, PrP^{Sc} was digested both with ruminal and colonic microbiota up to immunochemically undetectable levels. Using various antibodies with different binding sites within the prion protein it could be suggested that PrP^{Sc} is fully digested. Conclusively, the eventuality of a partially degraded prion protein, not detectable due to a lack of antibody binding site, could be excluded. Since our initial 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. This could be excluded concerning the results of degradation assays obtained under more physiological conditions in the absence of any

detergent. 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. (2004) using a defined medium in addition of soluble carbohydrates as standard technique to estimate the rates of *in vitro* ruminal protein degradation, microorganisms utilize the supplements for growth. Enhanced microbial numbers result in higher proteolytic activity and in a greater protein hydrolysis.

These data implicate the ability of bovine gastrointestinal microbiota to degrade PrP^{Sc} during digestion. Feedstuffs consumed by ruminants are all initially exposed to the fermentative activity of the complex consortium of symbiotic microorganisms in the rumen. A large but variable proportion (60 to 90 %) of the dietary protein is degraded by the rumen microorganisms prior to gastric and intestinal digestion (Mackie and White, 1990). Ruminal microbial consortia are composed of large variety of predominantly anaerobic bacteria (10^{10}–10^{11} cells/ml, representing more than 50 genera), protozoa (10^4–10^6 /ml, from 25 genera), fungi (10^3–10^5 spores/ml, from 5 genera) and bacteriophages (10^8–10^9 /ml) (Stevens and Hume, 1998). Due to our results a degradation of PrP^{Sc} in this section of the bovine gastrointestinal tract may occur. Unfermented material finally reaches the large intestine colonized by more than 400 different bacterial species, fungi and protozoa (Mackie and White, 1990). Based on the biochemical results, this segment of the gastrointestinal tract is also capable of PrP^{Sc} degradation. Within the average food passage of 2 days through the whole digestion tract of cattle (Jeroch et al., 1999; Engelhardt and Breeves, 2000), 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 are responsible for ruminal and colonic PrP^{Sc} degradation according to our studies with selective gastrointestinal cultures.

The most numerous facultative anaerobe in rumen, *Streptococcus bovis*, is usually present in numbers of 10^5–10^7 /ml. It is one of the bacteria commonly linked with high levels of protein in the diet (Attwood and Reilly, 1995; Hazlewood et al., 1983). Since *Streptococcus bovis* is classified as a proteolytic rumen bacterium (Russell et al., 1981), a prion protein degradation assay with an isolate of this strain was performed. The results indicate PrP^{Sc} degrading ability only in the absence of oxygen. In contrast, no PrP^{Sc} degradation arises in the presence of oxygen.

Recently, a study was published investigating the early alimentary pathogenesis of scrapie in sheep (Jeffrey et al., 2006). In accordance with our results, only trace amounts of PrP^{Sc} were detected by western blotting after successive incubation of scrapie brain homogenates with alimentary tract fluids from ovine rumen, abomasum, bile and intestine *in vitro,* suggesting that the majority of scrapie prion protein of the inoculum is readily digested.

Although infectivity is generally related to the presence of the disease associated prion protein (PrP^{Sc}) (Caughey et al., 1997; McKinley et al., 1983), biochemical degradation of PrP^{Sc} is not equivalent to inactivation of PrP^{Sc} at all. A verification of the biochemical data is obligatory by performing animal bioassays.

4.2 Significant infectivity of scrapie associated prion protein in animal bioassay following in vitro digestion with bovine gastrointestinal microbiota

The biochemical analysis by immunoblotting has given the evidence of PrP^{Sc} degradation by the bovine intestinal microbiota. Comprehensive examinations in which PrP^{Sc} was visualized by Western blotting after immunolabeling with the monoclonal antibody 3F4 have previously shown a close quantitative correlation between PrP^{Sc} amounts and infectivity in the brains of hamsters infected with 263K scrapie agent (Beekes et al., 1996). Additionally, studies have demonstrated an inactivation of 263K scrapie agent concomitant with the disappearance of PK-resistant PrP in hamster brain homogenates (Baier et al., 2004).

To investigate the inactivation of infectious prion proteins by microbial processes in the gastrointestinal tract infection studies were performed. Efforts were made to determine prion infectivity of scrapie brain homogenates in hamster bioassays following digestive pretreatment with gastrointestinal microbiota of cattle. Consequently, significant residual prion infectivity was retained after degradation of infected hamster brain through the gastrointestinal microbiota of cattle. None of the pretreated samples showed a significant extension of mean survival time compared to the positive control. Moreover, the present study shows that the loss of anti-prion antibody 3F4 immunoreactivity is obviously not correlated with a biological inactivation of PrP^{Sc}, as significant prion infectivity was present even in the absence of a Western blot signal.

Similar results were also obtained in a proteolytic inactivation study of the bovine spongiform encephalopathy agent (McLeod et al., 2004). Several proteases were screened for a loss of anti-prion antibody 6H4 immunoreactivity under different conditions, and protease treated infectious brain homogenate was assessed in mouse bioassay. Despite a number of proteases eliminating all 6H4 immunoreactive material, only one enzyme (properase) showed a significant extension in incubation period in bioassays. Thus, the removal of all immunoreactive PrP^{Sc} correlates poorly with the levels of infectivity measured by bioassay. This is also confirmed by a study investigating the influences of chemical and physical factors on the protease resistance and the infectivity of scrapie strain 263K associated prion protein as chemical and physical processes disrupt the PK resistance but did not diminish scrapie infectivity under the same conditions (Yao et al., 2005).

In summary, the data presented here together with previous reported results (McLeod et al., 2004; Somerville et al., 2002, Yao et al., 2005) suggest that it is impropriate to use PrP^{Sc} as surrogate marker for TSE infectivity in inactivation experiments. Conclusively, the use of Western blots or immunoassay formats may not provide an indication of the levels of prion inactivation. Therefore, the relationship between the loss of signals based on immunodetection *in vitro* and the loss of infectivity *in vivo* of PrP^{Sc} has to be analysed comparatively for each inactivation experiment.

The results may reflect the relative insensitivity of the biochemical detection method with sufficient material being present to cause disease in the absence of visible immunoreactive bands. Recently, the ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification (PMCA) was described (Saa et al., 2006). It was shown that PMCA enables the specific and reproducible amplification of exceptionally minute quantities of PrP^{Sc}, and is similar to the effectiveness of PCR amplification of DNA (Sykes et al., 1992). A comparison of the sensitivity of some of the current methodologies used for PrP^{Sc} detection demonstrates that the PMCA technology is several billion times more sensitive than the standard western blot or ELISA-based assays and at least 4000-times more sensitive than animal bioassay (Saa et al., 2006). Therefore, PMCA offers a new opportunity for the development of an ultra-highly sensitive diagnosis of TSE.

A probable alternative explanation for the discrepancies of the results of biochemical analysis and infection assays is that the residual infectivity is found in a sub fraction of the prion

protein that is not detectable by immunoblotting due to different conformation. Using various antibodies with different binding sites within the prion protein the selective degradation of only the antibody binding domain could be excluded.
However, the presence of molecules in pretreated brain homogenates apart from PrP^{Sc} which may also contribute to infectivity must be considered, as the particular structure of the infectious agent is not known and is still a matter of some controversy.

The prion hypothesis proposes that the agent is comprised of a host glycoprotein (PrP), which alters its conformation into an infectious form (Prusiner, 1982). In contrast, the virino hypothesis suggests that the agent comprises an unidentified informational molecule, probably a nucleic acid that is protected by the host protein PrP (Farquhar et al., 1998), and the virus hypothesis favours a structure sharing the essential elements of conventional viruses (Chesebro, 1998).
To characterise the thermodynamic diversity between TSE agent strains the effect of heating was compared on infectivity and biochemical properties of PrP^{Sc} (Somerville et al., 2002). The residual PrP^{Sc}, which resists digestion with proteinase K after heating, does not follow the decrease of infectivity as monitored by bioassay, that means proteinase K resistant PrP^{Sc} could be detected by immunoblotting, whereas >99 % of infectivity was destroyed after heating to a temperature of 100 °C. This result showed that the heat-sensitive component of the infectious agent is not associated with the structure of PrP^{Sc} responsible for proteinase K resistance. In other respects, the association between PrP^{Sc} and TSE infectivity can also be altered concerning the presented results.

All these observations concerning the independency of PrP^{Sc} detection and TSE infectivity would apply for the virino hypothesis, which proposes that PrP and an informational molecule, most likely to be a nucleic acid encoding strain specifities and infectivity, comprise the infective TSE agent (Manuelidis et al., 1987). Moreover, small nucleic acid molecules have been found in purified infectious scrapie samples (Kellings et al., 1994).
Unique virus-like tubulofilamentous particles, termed nemavirus, have been consistently observed in spongiform encephalopathic brains by electron microscopy in thin sections (Narang, 1993). In contrast to common viruses they have a three-layer structure consisting of an outer protein coat, which can be digested by proteolytic enzymes, a middle layer of ssDNA removable by DNase, and inner protease resistant PrP/SAF.

4.3 Stability of the BSE associated prion protein towards the gastrointestinal microbiota of cattle

An oral route of infection is commonly assumed to be important in the natural pathogenesis of BSE in cattle following the ingestion of infected tissues via contaminated feed (Wilesmith et al., 1988). The BSE strain is the most virulent TSE strain and is orally transmissible to a new host such as cats, mink, cows, and humans.

To highlight the mechanisms of PrP^{Sc} transmission and pathogenesis several studies were performed. Recently, a study was published investigating the early alimentary pathogenesis of scrapie in sheep (Jeffrey et al., 2006). Only trace amounts of PrP^{Sc} were detected by western blotting after successive incubation of scrapie brain homogenates with alimentary tract fluids from ovine rumen, abomasum, bile and intestine *in vitro,* suggesting that the majority of scrapie prion protein of the inoculum is readily digested. To understand the mechanism of PrP^{Sc} uptake from contaminated food by the intestinal epithelial cells, the *in vitro* transport of human PrP^{Sc} from sporadic CJD brain tissue (sCJD-PrP^{Sc}) across a layer of human intestinal epithelial cells was investigated (Mishra et al., 2004). It was shown that digestive enzyme treatment of sporadic CJD brain homogenate simulating the human digestion process generates a C-terminal fragment similar to the proteinase K-resistant PrP^{Sc} core of 27-30 kDa.

Nonetheless, the stability of the BSE associated prion protein towards the digestion processes in the gastrointestinal tract has not been described yet. Here we demonstrate that following incubation with both ruminal and colonic microbiota of cattle for up to 40 hours did not result in BSE associated prion protein (PrP^{Sc}) degradation in any instance. These results are contrary to the data of scrapie associated prion protein (PrP^{Sc}) degradation assays, although it was shown that BSE agents are less resistant to PK digestion than scrapie (263K) strains (Kuczius and Groschup, 1999). This implicates that the BSE associated prion protein (PrP^{Sc}) resists gastrointestinal digestion processes in cattle and raises the possibility that infected faeces might contaminate the environment.

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 can not be included due to the experimental design. Also the disproportional ratio of infected brain

homogenate to gastrointestinal contents must be kept in mind. In case of transferring our *in vitro* conditions to the *in vivo* ingestion of infected brain material in ruminants, one single cow has to consume a 25 kg portion of infected brain material in contaminated feed. This seems to be rather unlikely as the daily feed amount is about 20 kg of maize silage.

Probably, PrP^{Sc} degradation capacities of the intestinal microbiota are limited to low doses of prions, which represent more likely natural infections. Findings indicate that a low dose of prions is unable to cause disease when orally ingested, but may cause disease when inoculated into the tongue (Bartz et al., 2003). Prion infection via tongue-associated cranial nerves may be an alternate route of prion neuroinvasion following oral exposure. Therefore, abrasions of the tongue may predispose a host to oral prion infection of the tongue-associated cranial nerves. Moreover, an increased risk of ruminants becoming infected with prions suggests 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.
Recently, data were published indicating the presence of infectious prions in saliva and blood of deer with CWD (Mathiason et al., 2006). This study aimed to investigate the transmission of CWD via body fluids. Therefore, cohorts of CWD-naive deer were exposed to saliva, blood, or urine and faeces from CWD-positive deer. Serial tonsil biopsy of each recipient deer revealed that infectious CWD prions were present in saliva and blood from CWD-positive deer. By contrast, CWD prions were not detectable in tissues from deer inoculated orally with urine and faeces from CWD infected deer. This fact may favour the theory that PrP^{Sc} degradation capacities of the intestinal microbiota are limited to low doses of prions.

The results of the long-term incubations studies indicate that BSE associated prion protein is susceptible to degradation by the bovine gastrointestial microbiota. Extended incubation time might further reduce PrP^{Sc} signal to undetectable levels. Though, the inactivation of BSE associated prion protein by the gastrointestinal microbiota of cattle has to be further validated independently in animal bioassays.

To gain more insight into the mechanisms of PrP^{Sc} transmission, a comprehensive pathogenesis study of BSE in cattle is presently performed at the Friedrich-Loeffler-Institute in Riems. Following the way of infection from the very beginning until the onset of clinical signs, samples are taken at regular intervals. Due to the fact that the spread of the infectious agent from the alimentary tract to the brain still remains unclear, this topic is of special

interest. Furthermore, the results of the pathogenesis study are supposed to provide a better understanding of possible food borne exposition risks for consumers concerning BSE infectivity in beef and environment. However, a further task should address the presence of infectious prions in faeces of infected animals using sensitive transgenic mice models. The sample preparation method for animal bioassay described here may offer a suitable tool for this challenge.

5. Conclusion

In summary, the results implicate that TSE infectivity survives gastrointestinal digestion processes in cattle and raise the possibility that infected faeces might contaminate the environment.

Moreover, the impropriety to use PrP^{Sc} as a surrogate marker for TSE infectivity was shown in the present study. The results may also reflect the relative insensitivity of the biochemical detection method and raise a number of important issues concerning TSE diagnosis for food safety reasons. Diagnosis of TSE`s is based on identification of characteristic lesions or on detection of PrP^{Sc} in tissues by the use of the partial PK resistance property. The detection of PrP^{Sc} as a surrogate marker for prion infection remains the gold standard for biochemical diagnosis of prion diseases. Most of the officially approved rapid or screening tests are based on Western blot and immunoassay formats. For food safety reasons the development of new tests with higher sensitivity and of more reliable diagnostic applications for live animals is essential.

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Original papers

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Poster

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Education

06/1996	Abitur Frankenwald-Gymnasium Kronach
10/1996-01/2003	Diploma studies of Biology University Bayreuth
03/2003-12/2006	Dissertation thesis at the Institute for Microbiology and Toxicology, Federal Research Centre for Nutrition and Food, Kulmbach

Experience

08/1999-09/1999	Practical training in the microbiological laboratory (8 weeks) Kulmbacher Brauerei AG
01/2002-09/2002	Diploma thesis: Characterisation of *hesA* from *Anabaena* spp. Department for Microbiology, University Bayreuth
Since 03/2003	Research scientist at the Institute for Microbiology and Toxicology, Federal Research Centre for Nutrition and Food, Kulmbach

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