# 2 Materials and methods

## 2.1 Experimental animals

A total of 24 of German dairy sheep of different sex were used in this study. Animals were 9-10 month old at time of the experiment, and their weights ranged between 33.5-50 kg. The animals were allotted to 8 groups of three animals. The sheep were kept individually in pens, so as to control the feed intake. The sheep were weighed weekly and at the end of each trial (before slaughter).

## 2.2 Feedings

Prior to the experiment the sheep had been fed only with hay ad libitum for at least 8 weeks, in order to adapt them to a low-energy feeding. After that, Sheep were either solely fed on hay (1-1.5 kg hay/day) (control-group) or received in addition 780 g concentrate (experimental-groups). The animals were assigned to 8 groups according to the type and duration of feeding as follow:

1. Hay fed animals (control-group): The animals in this group were fed only hay
2. Sheep fed hay ad libitum and concentrate-diet for 2 days.
3. Sheep fed hay ad libitum and concentrate-diet for 4 days.
4. Sheep fed hay ad libitum and concentrate-diet for 1 week.
5. Sheep fed hay ad libitum and concentrate-diet for 2 weeks.
6. Sheep fed hay ad libitum and concentrate-diet for 4 weeks.
7. Sheep fed hay ad libitum and concentrate-diet for 6 weeks.
8. Sheep fed hay ad libitum and concentrate-diet for 12 weeks.

Introduction of concentrate diet was preceded by an adaptation period of 4 days in which the concentrate feeding was increased gradually. The concentrate diet was supplied at equal portions at 07.00 am and 02.30 pm. All animals had free access to tap water and salt block.

## 2.3 Compositions of diet

The nutrients content of both concentrate and hay rations were shown in tables 1 and 2, respectively.

**Tab. 1** Composition of the concentrate

| Nutrients | % | Nutrients | % |
|---|---|---|---|
| Dry matter | 89.1 | Organic ADF | 13.28 |
| Crude ash | 6.42 | Organic NDF | 25.46 |
| Crude protein | 18.03 | ADL | 3.99 |
| Crude fiber | 9.65 | Vitamin A | 7200 Iu |
| Calcium | 0.65 | Vitamin D3 | 1800 Iu |
| Phosphorus | 0.59 | Selenium | 0.5 mg |
| Magnesium | 0.27 | Copper | 10 mg |
| Potassium | 1.35 | DCAB | +299 meq/kg DM |
| Sodium | 0.42 | Net energy lactation (NEL) | 6.7 MJ/kg |
| Chloride | 0.46 | Metabolic energy (ME) | 10.41 MJ/kg |
| Sulfur | 0.21 | | |

**Tab. 2** Composition of hay

| Nutrients | % | Nutrients | % |
|---|---|---|---|
| Dry matter | 93.5 | ADL | 4 |
| Crude ash | 4.9 | Non fibers carbohydrates (NFC) | 22.5 |
| Crude protein | 8.8 | Metabolizable energy (ME) | 9.3 MJ/Kg |
| Crude fiber | 29.3 | Net energy lactation (NEL) | 5.5 MJ/Kg |
| Potassium | 1.44 | Usable crude protein (nXP) | 120.3 g/Kg |
| Sodium | 0.032 | Degradable crude protein (UDP) | 17.6 g/Kg |
| Organic ADF | 34 | Ruminal nitrogen balance (RNB) | -5.2 g/Kg |
| Organic NDF | 56.5 | | |

## 2.4 Sampling and processing of the epithelial tissues

Following the feeding trail and at the end of each of experimental period, the sheep were slaughtered according to the good manufacturing practice standards of meat production, i.e, stunning by a captive-bolt pistol and killing by exsanguination in the faculty abattoir. Two to three minutes after the death of the animal, the gastro-intestinal tract was removed from the abdominal cavity and spread in a clean table. The rumen was taken out and opened by a horizontal incision along the left side of the ventral sac of the rumen. After the removal of the ingesta, the samples were obtained from identical site of the most cranial part of the ventral ruminal sac (left wall of the recessus ruminis ventralis sac; adjacent to the left longitudinal groove) (Gäbel et al, 1987), as demonstrated in figure 1.

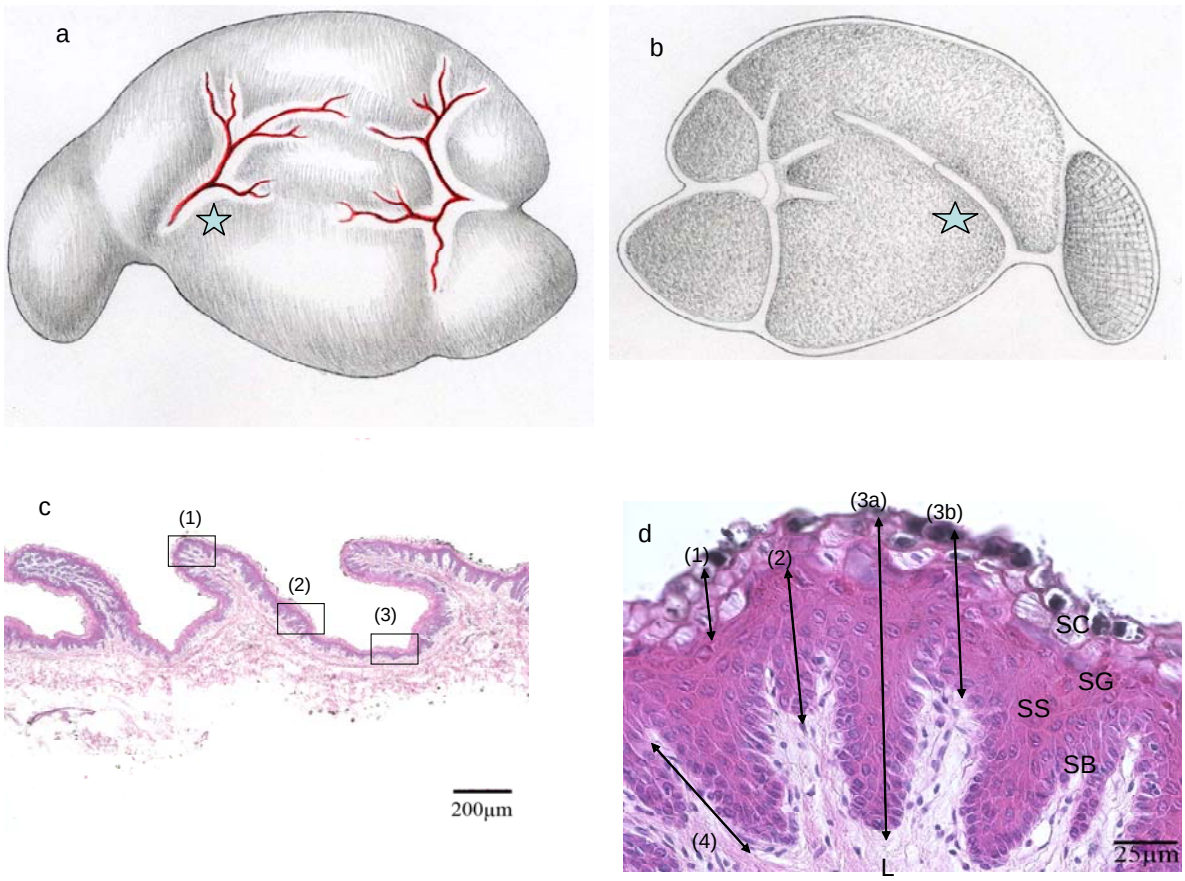


**Fig. 1: Site of both sampling:** a. from outside of the left wall of the rumen b. from inside of the left wall of the rumen; c. from different locations of the ruminal papilla included tip of papilla (1), base of the papilla (2) and interpapillary region (3) d. measurements at the tip of papilla included thickness of the stratum corneum (dead cells) (1), thickness of the stratum granulosum and germinativum (stratum basale + stratum spinosum) (living cells) (2), total thickness of the epithelium at two levels (3a and 3b) and length of papillary body (4).

After that, the samples were first carefully cleaned by immersion in a warmed (37 º C) and carbon gassed (95% $O_2$ + 5% $CO_2$) transportation buffer solution (composition see table 3), and several times washed, until the liquid remained clear. The epithelium mucosa were then stripped from the muscle layers using artrial forceps scissors and scalpel. The ruminal mucosa, then, was sliced into sections and immersed in different solutions: 4 % paraformaldehyde, liquid nitrogen and Karnovsky's fixative solution for both light and electron microscopy.

**Tab. 3** Composition of the transportation buffer

| Compound | Concentration (mmol / L) |
|---|---|
| Sodium | 145.2 |
| Potassium | 5 |
| Calcium | 1 |
| Magnesium | 1 |
| Bicarbonate | 25 |
| Chloride | 120 |
| Dihydrogenphosphate | 0.4 |
| Hydrogenphosphate | 2.4 |
| Glucose | 5 |
| pH value | 7.4 |
| Osmolarity | 300 mosmol/L |

### 2.4.1 Gross anatomy

Rumen epithelium sections (3 x 3 cm) from the left lateral wall of the recessus ruminis ventralis sac were brought to the laboratory in 4% formalin and examined under stereomicroscope to characterize the gross morphology of the ruminal papillae, their color, clumping of the papillae and the fodder precipitations on the papillae. Both color and consistency of the ruminal mucosa were determined. Macroscopic observations with attention paid to grooves covering papillary surface were also done, using a binocular microscope.

### 2.4.2 Histology

For light microscopy, rumen epithelial tissues were fixed in 4% formalin solution for 24 hours (Romeis, 1989). After rinsing with water, samples were stored in 0.1 moll-1 phosphate buffer (pH 7.2). They were then dehydrated in graded series of ethanol alcohol (30%, 50%, 70%, and 90%), cleared with xyline, saturated with and embedded in paraffin. Tissue blocks were then cut at 5-7µm thickness and mounted onto glass slides. The sections were further cleared in xylene and rehydrated in descending grades of alcohol, washed in water and stained. After staining with the following stains, the sections were dehydrated in ascending grades of alcohol, cleared with xyline and finally, cover slipped with mounting media (Canada balsam).

1. Haematoxylin and eosin (H / E) (Romeis, 1989): for general histology.
2. Modified trichrome stain with gallocyanin, chromotrop 2R and anilinblue (GRA) (Romeis, 1989): for the differentiation of connective tissue and smooth muscle fibres.
3. Periodic acid–Schiff-Reaction (PAS) to McManus (Romeis, 1989): for detection of glycogen, glycoprotein and glycolipids. Control sections for glycogen were treated with a 0.1% diastase, used in solution buffered to pH 6 at 37 º C for 30 minutes.

### 2.4.3 Morphometry

#### 2.4.3.1 Quantitative morphological analysis

To study the effect of type of diet and the duration of the concentrate feeding on the development of the ruminal papillae, five papillae (selected randomly) were measured from each animal, so a total of 120 papillae were measured (15 papillae per group of treatment). The morphometric procedure was carried out with a stereomicroscope and standard measuring slide at 10x magnification and the following morphometric parameters were measured:

1. Length of papillae (distance between the base and the tip of the papillae).
2. Width of papillae (at the middle of the papillae).
3. Density of papillae (number of papillae / $cm^2$ mucosa).
4. Total surface of papillae per $cm^2$ mucosa: was determined as length x width x 2, multiplied by the number of papillae / $cm^2$ mucosa (density of papillae).

### 2.4.3.2 Qualitative histological analysis

For histology, the morphometric procedure was carried out with a computerized image analysis program Lucia 32-G Corona, version 4.11 (Co. Laboratory Imaging) on Haematoxylin and Eosin stained tissue sections. For image processing, Photoshop program, version 7.0 (Co. Adobe, San Jose/USA) was used. 3 papillae were randomly selected from each animal (9 papillae / group of treatment). In each papilla, 3 regions of mucosa (papillary tip, papillary base and interpapillary mucosa) were included. On each location 10 measurements were done randomly for each parameter at 125x magnification (giving a total of 90 measurements / group of treatment), and the following parameters were determined:

A. Thickness of the strata germinativum and granulosum at the tip of the papilla, base of the papilla and inter-papillar region.

B. Thicknesses of the stratum corneum at the tip of the papilla, base of the papilla and inter-papillar region.

C. Thickness of the rumen epithelium at the tip of the papilla, base of the papilla and inter-papillar region.

D. Length of the papillary bodies located at the tip of the papillae.

Variable studies included the relation between stratum (germinativum + granulosum) and stratum corneum was done.

### 2.4.3.3 Statistical analysis

In this study, by each experimental animal several epithelial tissues were always examined. The morphometrical data were prepared with the Excel program version XP (Co. Microsoft, Redmond / USA) and were finally statistically analyzed using SPSS program, version12.0 (Co. SPSS Software GmbH, München, Germany). Type and duration of feeding were considered as main effects. The statistical analyses were founded on the mean values per animal. The results are shown as the mean average ± standard error. Values were analyzed by analysis of variances procedure (ANOVA, statistical software STATGRAPHICS version 12, Statistical Graphics Cooperation, and USA). We used a mixed model with animal as random factor and group is a fixed factor. In the case were ANOVA was significant, Post-Hoc-tests (Scheffe) were carried out in order to study the significant differences among the distinct groups. Results were regarded to be significantly different at $P < 0.05$. An example of the analytical method used for the statistical analysis of the data throughout this study is attached to the appendix (table 1).

## 2.4.4 Immunohistochemistry

### 2.4.4.1 Tissue collection and preparation

Immunohistochemical staining was performed on cryostat sections (Gap junction-Connexin 43), and on deparaffinized sections (NHE3 and αSMA) of the rumen epithelium. Full thickness mucosa or rumen papillae isolated directly with scissors from the left wall of the most cranial part of the ventral sac (recessus ruminis) of the rumen, snap frozen with liquid nitrogen, and stored at -76 ºC. Cryostat sections were cut 5 µm thick and collected on microscope slides precoated with Silane solution (3-Aminopropyl) (Co. Sigma, Code No. A3648). The sections were fixed in acetone for 9 sec. and dried for at least 1h at room temperature, then refrozen and stored at -20 ºC. Prior to staining the sections were dried 1h at room temperature, and fixed in cold aceton for 8 min. Fresh rumen- mucosal tissues were formaldehyde-fixed, processed by standard paraffin-embedding methods, and sectioned 5-7 µm thick and collected on microscope slides precoated with Silane solution. Tissues were deparaffinized, hydrated, rinsed twice with distilled water before staining (table 4).

### 2.4.4.2 Antibodies

#### 2.4.4.2.1 α-Actin smooth muscles (α SMA)

**Primary antibody:**

Mouse monoclonal anti-human smooth muscle Actin, (Fa. Serotec, Oxford, UK) Catalog Nr. MCA 1905.

**Secondary antibody:**

ShpXMs (Fab`2) Ig Biotin conjugate, Cat. No. AQ300B, (Fa Chemicon, California, USA)

#### 2.4.4.2.2 $Na^+/ H^+$ exchanger-3 (NHE3)

**Primary antibody:**

Rabbit $Na^+/H^+$ exchanger-3 (NHE3) polyclonal antibody (Fa.Chemicon, USA and Canada) Catalog Nr.AB 3085.

**Secondary antibody:**

Peroxidase labelled polymer conjugated to goat anti-rabbit immunoglobulins in Tris-HCl buffer containing carrier protein and an anti-microbial agent, Code N0. K0492 (Fa DakoCytomation, Carpinteria, USA)

#### 2.4.4.2.3 Connexin 43 (Cx43)

**Primary antibody:**

Mouse anti- Connexin 43 (Cx43) monoclonal antibody (Fa.Chemicon, USA and Canada) Catalog Nr.MAB3067.

**Secondary antibody:**

Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer containing carrier protein and an anti-microbial agent, Code N0. K4007 (Fa DakoCytomation, Carpinteria, USA)

### 2.4.4.3 Procedure of immunohistochemistry

For immunolabeling of the gap junction-Connexin 43 (Cx43), Sodium / Hydrogen-Exchanger type1 and 3 (NHE1, NHE3), both deparaffinized and cryostat sections were stained according to the protocol Dako Envision+System, Peroxidase (DAB) (Fa DakoCytomation, Carpinteria, USA) (Tab. 4). Cx43 and NHE3 were detected in cryostat and deparaffinized sections, respectively. However, NHE1 was not detected with this method, using both frozen and deparaffinized sections and different antibody-dilutions.

The specificity of the staining reaction was determined in control experiments. These comprised either substitution of the primary antibody by PBS, normal mouse serum 1:200 (Cx43 and αSMA) and normal rabbit serum 1:200 (NHE3), or omission of the primary antibody; in all cases, sections were negative. All immunohistochemistry was repeated for material obtained from three animals and examined directly under a light microscope.

**Tab. 4** Protocols of using different antibodies

| Steps | α SMA | NHE3 | Cx43 |
|---|---|---|---|
| 1.Deparaffinization with zylin and rehydration in descending grades of alcohol | X | X | _ |
| 2. Cold acetone at 4 ºC | _ | _ | 8 min |
| 3.Rinse with distilled water | _ | 2 x5 min | 2 x5 min |
| 4. Rinse in washing PBS buffer | 5 min | 2 x5 min | 2 x5 min |
| 5.Methanol-hydrogen peroxide-solution | 20 min | _ | _ |
| 6. Peroxidase block | _ | 5 min (incubation bei RT). | 5 min (incubation bei RT). |
| 7.Rinse with distilled water | _ | 2 x3 min | 2 x3 min |
| 8. Rinse in washing PBS buffer | 10 min | 10 min | 10 min |
| 9.Protein block | 30 min | _ | _ |
| 10. Primary antibody | 1:200 in PBS buffer (overnight at 4 ºC) | 1:100 in PBS buffer (incubation for 30 min) | 1:200 in PBS buffer (incubation for 30 min) |
| 11.Control serum | 1:200 | 1:200 | 1:200 |
| 12. Rinse in washing PBS buffer | 10 min | 2 x3 min<br>10 min | 2 x3 min<br>10 min |
| 13.Secondary antibody | 1:200 for 30 min bei RT | 35 min bei RT | 35 min bei RT |
| 14. Rinse in washing PBS buffer | 10 min | 2 x3 min<br>10 min | 2 x3 min<br>10 min |
| 15.Detection system (Streptavidin-Biotin-Peroxidase) | 30 min. RT | _ | _ |
| 16.Rinse in washing PBS buffer | 10 min | _ | _ |
| 17.DAB + Chromogen- substrate solution | _ | 5-10 min | 5-10 min |
| 18.POD-nachweis | 5-20 min | _ | _ |

| | | | |
|---|---|---|---|
| 19.Washing with running tap water | 10 min | _ | _ |
| 20. Rinsing with ice cold-distilled water | _ | 2 times (5 min each) | 2 times (5 min each) |
| 21.Stainning with Mayer's Haematoxylin | 10 sec. | 5 min | 5 min |
| 22.Rinse and washing with distill and running tap-water, respectivlly | 5 min | 5 min | 5 min |
| 23.Dehydration Through 70% ethanol for 5 min, 100% ethanol for 3x5 min | X | X | X |
| 24.Xyline | 3x5 min | 3x5 min | 3x5 min |
| 25.Coverslips with mounting media (Canada balsam) | X | X | X |

### 2.4.4.4 Details of some steps present in table 4

**Steps 4., 8., 12.,14., 16.** PBS-Puffer to Dulbecco Cat. No.47302 (Fa. Serva, Heidelberg, Deutschland)

**Step 5.** Methanol-hydro-peroxides-solution (100 ml Methanol + 3 ml $H_2O_2$)

**Step 6.** Peroxidase block: 0.03% hydrogen peroxide containing sodium azide (to block endogenous peroxidase activity).

**Step 9.** Protein block

**Step 10.** Primary antibody (see above), incubation with antibodies was done in a humid chambers

**Step 11.** Control serum: normal mouse or rabbit serum, negative control Ig GI Cat, No. X0931 (Fa DakoCytomation, Carpinteria, USA)

**Step 13.** Secondary antibody (see above)

**Step 15.** Detection system Strept Avidin-Biotin-Peroxidase Complex (SABC) / HRP K0377 (Fa DakoCytomation, Carpinteria, USA) at room temperature

**Step 17.** DAB+ substrate-Chromogen solution: 1ml (Buffered substrate solution, pH 7.5, containing hydrogen peroxide and a preservative) + one drop (3,3-Diaminobenzidin Chromogen solution)

**Step 18.** POD-detection with b3.3`-Diaminobenzidine Tablets D-5905 (Fa. Sigma-Aldrich, Taufkirchen, Deutschland) at room temperature in darkness

### 2.4.5 Electron microscopy

#### 2.4.5.1 Transmission electron microscopy (TEM)

Fresh ruminal tissue samples were cut into smaller pieces (2-3 $mm^2$) and immediately immersed in modified Karnovsky's fixative solution (Romeis, 1989) for 12 h in a refrigerator (4°C). Samples were post fixed in 1% Osmium tetroxide buffered with cacodylate (pH 7.2), and then washed several times in cacodylate Buffer (0.1 M) and dehydrated in a graded series of ethanol alcohol. After dehydration, samples were washed with propylenoxid and immersed in a mixture of propylenoxid and Agar (1:1) overnight. At the next day, samples were embedded in pure Agar (Agar 100 Resin, Co. Agar Scientific Ltd, UK) and put in oven (45-50°C) for polymerization. Semithin sections of 0.5µm thick were sectioned and stained with Richardson solution (1%Methyleneblue/AzurII solution) (Romeis, 1989). Ultrathin sections (50-80 nm thick) were taken from selected areas using Ultramicrotome Ultracut E (Fa. Reichert-Jung, Heidelberg), and mounted on copper grids. To increase structural contrast for electron microscopy, sections were counterstained with uranyl acetate and lead citrate. Tissue observation and photographic documentation were made with transmission electron microscope EM 10 (Fa. Zeiss, Oberkochen, Germany).

#### 2.4.5.2 Scanning electron microscopy (SEM)

For observations of surface structures, small pieces of tissue (0.5 x 0.5 cm), were fixed in modified Karnovsky's fixative solution (7.5% Glutaraldehyde, 3% Paraformaldehyde) (Romeis, 1989), and postfixed with 1% Osmiumtetroxide (OsO4) buffered with cacodylate (pH 7.2). After washing several times in cacodylate Buffer (0.1 M), specimens were dehydrated through a graded series of ethanol alcohol, and dried using hexamethyldisilazane solution (HMD) (Co. Roth, Karlsruhe, Germany) overnight. The specimens were mounted onto aluminum stubs with Leit-C glue (Co. Plano, Marburg, Germany) and sputter-coated with gold (2min, 30-40 nm). SEM examination was made with a scanning electron microscope (DSM 950, Co. Zeiss, Oberkochen, Germany) at an accelerating voltage of 10 kV. Samples were examined and at magnification of 30x to 7500x. Photos of the scanning electron microscope were taken in digital form directly to the personal computer.