Characterization of mucosa-associated bacterial communities in abomasal ulcers by pyrosequencing

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Abstract: Abomasal ulcers are important pathological alterations of the gastrointestinal tract in cattle and are exceptionally hard to diagnose in vivo. The microbiome of the abomasum in cattle with or without ulcers has hardly been studied to date, and if so, the studies used culture-dependent methods. In the present study, the bacterial communities associated with abomasal ulcers of slaughter cows, bulls, and calves in Austria were described using 16S rRNA gene pyrosequencing. Sequences were clustered into 10,459 operational taxonomic units (OTUs), affiliating to 28 phyla with Proteobacteria, Firmicutes, Bacteroidetes and Tenericutes dominating (96.4 % of all reads). The most abundant genera belonged to Helicobacter, Acetobacter, Lactobacillus, and novel Mycoplasma-like phylotypes. Significant differences between the microbial communities of healthy and ulcerated calves compared to cows and bulls could be observed. However, only few statistically significant differences in the abundances of certain OTUs between healthy and ulcerated abomasal mucosa were found. Additionally, near full-length 16S rRNA gene sequences of the most abundant phylotypes were obtained by cloning and Sanger sequencing (n=88). In conclusion, our results allow the first deep insights into the composition of abomasal mucosal bacterial communities in cattle and describe a hitherto unknown high diversity and species richness of abomasal bacteria in cattle. Our results suggest that bacteria may have only limited involvement in the etiology of abomasal ulcers. However, future research will be needed to verify the contribution of bacteria to abomasal ulcer formation as presence or absence of bacteria does not necessarily correlate with etiology of disease.

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Characterization of mucosa-associated bacterial communities in abomasal ulcers by pyrosequencing

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Abstract

Abomasal ulcers are important pathological alterations of the gastrointestinal tract in cattle and are exceptionally hard to diagnose in vivo. The microbiome of the abomasum in cattle with or without ulcers has hardly been studied to date, and if so, the studies used culture-dependent methods. In the present study, the bacterial communities associated with abomasal ulcers of slaughter cows, bulls, and calves in Austria were described using 16S rRNA gene pyrosequencing. Sequences were clustered into 10,459 operational taxonomic units (OTUs), affilating to 28 phyla with Proteobacteria, Firmicutes, Bacteroidetes and Tenericutes dominating (96.4% of all reads). The most abundant genera belonged to Helicobacter, Acetobacter, Lactobacillus, and novel Mycoplasma-like phylotypes. Significant differences between the microbial communities of healthy and ulcerated calves compared to cows and bulls could be observed. However, only few statistically significant differences in the abundances of certain OTUs between healthy and ulcerated abomasal mucosa were found. Additionally, near full-length 16S rRNA gene sequences of the most abundant phylotypes were obtained by cloning and Sanger sequencing (n=88). In conclusion, our results allow the first deep insights into the composition of abomasal mucosal bacterial communities in cattle and describe a hitherto unknown high diversity and species richness of abomasal bacteria in cattle. Our results suggest that bacteria may have only limited involvement in the etiology of abomasal ulcers. However, future research will be needed to verify the contribution of bacteria to abomasal ulcer formation as presence or absence of bacteria does not necessarily correlate with etiology of disease.

Keywords: Cattle, Abomasum, Abomasal ulcers, Bacterial microbiome, 16S rRNA amplicon pyrosequencing
1. Introduction

Abomasal ulcers are an important cause of indigestion in cattle of all breeds and ages and production systems (Smith, 2009). Until now, the exact etiological agents of abomasal ulcer formation are not completely understood. Different factors like stress, proliferation of bacteria within the gut, abrasion of the abomasal mucosa due to roughage, and mineral deficiencies have been associated with abomasal ulceration (Eddy, 2004; Jelinski et al., 1996; Mills et al., 1990).

Physiologically, the mucosa of the abomasum is protected by a mucous layer, bicarbonate buffer, and high rate of blood circulation in the submucosal tissue. However, if the balance between ulcerogenic factors and protective mechanisms is disturbed, the acidic contents of the abomasum damage the lining and cause ulceration (Smith, 2009).

In cattle, ulcers occur in several forms and have been classified according to Fox (1980) and Whitlock (1980) as lesions of four types: i) a superficial erosion of the mucous membrane, ii) a deeper lesion eroding larger blood vessels leading to substantial haemorrhage and iii) perforating the abomasa wall leading to local or iv) generalized peritonitis, respectively. Braun et al. (1991) introduced a more detailed subtyping of type one ulcers ranging from a discoloration of the epithelium (type 1a) to distinct craters (type 1d) without reaching the submucosal layer, which are therefore not classified as type two lesions. All four types can cause severe illness or are potentially fatal. However, the consequences of the disease are highly dependent on the severity of the lesions. Generally, the diagnosis of ulcers is difficult because affected cattle show typically non-specific clinical signs. An accurate clinical diagnosis is only possible with bleeding ulcers (Eddy, 2004).

The prevalence of abomasal ulcers varies significantly due to differences in the examined population, case definitions and different means of diagnosis. Clinically apparent abomasal ulcers were reported with a prevalence of 0.2 % in young beef calves on herd level
(Katchuik, 1992) and were found in 2.2 % of adult dairy cattle (Smith et al., 1983). Jensen et al. (1976) showed that 1.6 % of necropsied yearling feedlot cattle had abomasal ulcers with fatal perforations or haemorrhages. Studies in slaughtered animals are more sensitive in detecting low-grade ulcers and demonstrate that between 67 % and 87 % of veal calves are affected (Welchman and Baust, 1987; Wiepkema et al., 1987). Overall, abomasal ulceration can cause considerable economic losses for the cattle industry and represents a significant challenge for animal welfare.

Previous studies suggested several microbiological agents to be associated with the formation of gastric ulcers in cattle. Gitter and Austwick (1957) found fungal hyphae in abomasal ulcers while Roeder et al. (1987) and Mills et al. (1990) reported Clostridium perfringens to be the major bacterial agent in abomasal ulcers. Isolation of Campylobacter supports an association with abomasal ulceration. However, the relationship between Campylobacter and neonatal abomasal ulceration is undetermined (Jelinski et al., 1995). In humans, Helicobacter pylori is known as an important etiological agent of peptic ulcers (Kuipers et al., 1995). However, H. pylori were not isolated in any sample of ulcerated or healthy abomasum of cattle (Jelinski et al., 1995; Valgaeren et al., 2013).

Knowing the composition of the bacterial abomasal microbiota may provide additional insights into the ecology of species related to cattle disorders. However, the task of describing the microbial composition involved in the etiology of abomasal ulcers by traditional microbiological methods is seriously hampered by the fact that the majority of the microorganisms present in the environment are not cultivable under standard laboratory conditions (Handelsman, 2004). Sequencing technologies such as Roche/454-pyrosequencing or particularly Illumina MiSeq technology are fundamentally changing the way in which microbial communities can be studied. To our knowledge, no study exists to date where the microbiome of abomasal ulcers was captured entirely and compared to tissue samples from
healthy animals. It was the objective of this study to utilize 16S rRNA gene-targeted pyrosequencing technology to characterize the bacterial communities associated with abomasal ulcers of slaughter cows, bulls and calves in Austria to verify the following hypotheses: Cattle with abomasal ulcers have a distinct microbiome of the abomasal mucosa which is different from healthy cattle and might contain infective agents involved in the pathogenesis of abomasal ulcers; the microbiome of adult cattle differs from that in calves.
2. Materials and Methods

2.1. Animals and sampling

The samples for the study were obtained at two different abattoirs in Austria on five different days over the course of four months. A total of 215 fattening bulls, cows and calves were examined immediately after slaughter. Of these 215 animals, 42 were randomly selected for microbiome analyses.

The animals were stunned and hung by the hind legs, exsanguinated, and skinned. Then the abdomen was opened; the gastrointestinal tract (GIT) was removed entirely from the carcass and moved to a separate room, where the parts of the GIT were separated for further processing or disposal. The abomasa were opened along the greater curvature including the pylorus and evaluated for abnormal contents such as gravel or sand. The pH of the abomasal content was measured, and subsequently the abomasum was cleaned under running water until the mucosa was completely cleaned from digesta. Additionally, the mucosa was individually rigorously rinsed with sterile ice-cold phosphate buffered saline times to remove remains of free-floating bacteria. Abomasal mucosa samples for microbiological analysis were taken from the corpus and the pyloric region of bulls, cows and calves (Table 1, Fig. S1).

2.2. Ulcer documentation

The abomasal mucosa was examined for ulcers, which were typed according to a modified version of the system by Fox (1980) and Whitlock (1980), and subtyped according to Braun et al. (1991) as shown in Table 1. The size, location and number of ulcers were recorded, and each ulcer was photographed.
2.3. Abomasal mucosa sampling for microbiological analysis

Initially, ulcers were excised with a margin of healthy mucosa immediately after slaughter at the abattoir. The samples were rinsed and stored immediately in sterile Dulbecco’s phosphate buffered saline without Ca$^{2+}$ and Mg$^{2+}$ (PAA Laboratories GmbH, Pasching, Austria) in 50 ml tubes, and cooled within 10 minutes after slaughter as well as during transport to the laboratory. There they were stored at -80°C until further analysis. For DNA isolation, ulcers were precisely dissected from the samples taken at the abattoir to exclude surrounding tissue. Samples were taken from three different groups: fattening bulls (B), cows (C), and calves (V). Within each group, the ulcerated abomasal mucosa of affected animals (I), unaffected mucosa of these same animals with ulcers (II), and abomasal mucosa of healthy animals (III) were sampled, resulting in nine groups in total. In the groups containing healthy animals (VIII, BIII, and CIII), samples from seven animals per group were selected randomly. In the groups containing animals with ulcers (I and II), samples from group II (ulcerated region) were paired with samples from group I (healthy region of abomasum) within the same animal. Here, the selection was blocked to yield three to four samples each from the region of the pylorus and fundus to account for potential differences in the etiology of abomasal ulcers in the two regions. In total, 63 samples from 42 animals were analysed.

2.4. DNA extraction

All samples were individually processed. Isolation of total genomic DNA was performed from 250 mg tissue by using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA concentration was determined by using a Qubit® 150 fluorometer (Invitrogen, Carlsbad, CA, USA).
2.5 PCR amplification of the V1-V2 region of bacterial 16S rRNA genes and pyrosequencing

The V1-V2 hypervariable regions of 16S rRNA genes were individually amplified from each sample using a composite pair of primers containing a unique 10-base barcode and the Lib-L kit, primer A-primer B, Roche 454 Life Science, Branford, Connecticut, USA. The forward primer used was 5’-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG NNN NNN NAG AGT TTG ATC CTG GCT CAG-3’: the underlined sequence is the GS Titanium A Adaptor, followed by four nucleotides to help diminish any effect the composite primer and the italicized sequence represents the universal bacterial primer 27F (Weisburg et al., 1991). The reverse primer used was 5’ – CCT ATC CCC TGT GTG CCT TGG CAG TCC TGC TGC CTY CCG TA- 3’, without barcode sequence: the underlined sequence represents the GS FLX Titanium B Adaptor, and the italicized sequence is the broad-range bacterial primer R357 (Dorsch and Stackebrandt, 1992). The sequence NNN NNN NNN N is identical in the forward and reverse primer of each pair and designates the unique 10-base barcode used to tag each amplicon.

Each PCR was performed in triplicate in a final volume of 25 μl, containing 1 × Fast Start Buffer, 2.5 U High Fidelity Enzyme, 200 μM each of dATP, dTTP, dGTP, dCTP, 0.4 μM barcoded primers (Eurofins MWG, Ebersberg, Germany), 2.5 mM MgCl₂ and PCR grade water (Roche Diagnostics, Mannheim, Germany). 100 ng total genomic DNA was added to the PCR. Amplification was performed in a standard thermocycler after initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 45 sec, at 55°C for 45 sec, at 72°C for 60 sec, and a final extension at 72°C for 7 min. Amplicons were purified with a WAVE® System (Transgenomic Inc., Omaha, NE, USA) and eluted by a linear gradient of acetonitrile in 0.1 M triethylammoniumacetate. Subsequently, amplicon DNA was purified on NucleoFast® 96 PCR plates (Macherey-Nagel, Düren, Germany). The DNA concentrations were determined using a PicoGreen® dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA). The barcode-labeled amplicons were pooled and analyzed using a 2100 Bioanalyzer
(Agilent Technologies, Waldbronn, Germany) with the Agilent DNA 7500 kit. Emulsion PCR of pooled samples was performed with the GS FLX Titanium Sequencing Kit (Roche Life Science, Branford, Connecticut, USA) according to the manufacturer’s instructions. Blank controls, in which no DNA was added to the reaction, were performed simultaneously. Library preparation and pyrosequencing was performed at the Medical University of Graz, Austria (Center for Medical Research, Core Facility Molecular Biology).

2.6. Pyrosequencing data analysis

All reads (n=268,479) from the 63 samples were analyzed using the mothur software package version 1.30.2 (Schloss et al., 2011). The following parameters were used: minimum quality score=30 with a window size=50, minimum length=162, allowed number of differences in the primer sequence=2, maximal homopolymer length=8, maximum number of differences to barcode sequence=1, no ambiguous bases allowed. All high-quality reads were aligned to the SILVA SSURef database (v102) (Quast et al., 2013). To reduce sequencing error and remove chimeric sequences, pre.cluster and chimera.uchime were used, respectively.

A total of 156,536 (58.3 %) sequences remained after quality control. Sequences were clustered into OTUs at 97 % similarity. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) and the SILVA SSU reference database (v102) (Quast et al., 2013). For calculation of diversity indices data were normalized based on a random selection of 900 sequences per sample using mothur. Yue & Clayton Theta similarity coefficients were calculated with mothur and visualized in a heatmap using JColorGrid (Joachimiak et al., 2006). Rarefaction and rank-abundance curves for each group were calculated with mothur and visualized using SigmaPlot 11.0. The similarity between bacterial communities was analyzed using Principal Component Analysis (PCA) based on a quantitative matrix of abundances of all OTUs with JMP 9.0.0.
The mothur implementation of Metastats (White et al., 2009) was used to determine which OTUs showed a statistically significant different abundance between the groups using 1000 permutations to compute \( q \)-values (false discovery rate (FDR) corrected \( p \)-value) of 0.01 as a maximum. \( P \)-value threshold of significance as 0.05 was set additionally. To control FDR within the tests, only \( q \)-values and \( p \)-values below 0.05 were selected.

2.7. 16S RNA gene clone libraries

In order to get better phylogenetic resolution, cloning and Sanger sequencing of near full-length 16S rRNA genes was performed. PCR and cloning was performed with the same DNA extractions used for pyrosequencing. For cloning, six DNA extractions (5 ng/µl each) were pooled and used as template for PCR. Near full-length 16S rRNA gene sequences were obtained by PCR using the primers 27F (5’-AGA GTT TGA TYM TGG CTC- 3’, *E.coli* 16S rRNA positions 8-27) and 1492R (5’-GGY TAC CTT GTT ACG ACT T- 3’, *E. coli* positions 1492 to 1510). Each PCR reaction was performed in a final volume of 50 µl, containing 20 mM Tris-HCl, 50 mM KCl, 3.5 mM MgCl\(_2\), 0.2 pmol/µl of each 16S rRNA gene primer, 200 µM dNTP mix, 1.5 U of Platinum® Taq DNA polymerase (Invitrogen, Vienna, Austria), DEPC-treated water (Thermo Scientific, Vienna, Austria) and 5 µl template. Amplification was performed in 30 cycles after initial denaturation at 94°C for 5 min, 94°C for 40 sec, 52°C for 40 sec, 72°C for 1 min and a final elongation at 72°C for 7 min.

PCR products were ligated into the pSC-A-amp/kan PCR cloning vector using the StrataClone PCR Cloning Kit (Agilent Technologies, Vienna, Austria) according to manufacturer’s instructions. PCR of cloned products with vector-specific M13 primers was performed. M13 PCR amplicons were screened for different patterns with restriction fragment length polymorphism (RFLP) using the FastDigest restriction enzyme AluI (Thermo Scientific, Vienna, Austria) and stained on 2% TBE agarose gels with ethidium bromide. A total of 94 different RFLP patterns were obtained and 96 clones were chosen and sequenced.
(LGC Genomics, Berlin, Germany). The sequence data of near full-length 16S rRNA gene sequences were analyzed for taxonomic affiliation by RDP Classifier (Wang et al., 2007) and for the closest match to sequences in GenBank by BLASTn. To obtain better phylogenetic resolution of the pyrosequencing OTUs, the 16S rRNA clone sequences were matched with the pyrosequencing reads. For this, three representative sequences of each pyrosequencing OTU were searched as a query against a database containing the near full-length 16S rRNA clone sequences by BLASTn. All pairs with more than 99% similarity were considered matching.

2.8. Generation of phylogenetic trees

The phylogenetic relationships of selected 16S rRNA clone sequences representing potential novel lineages within the Actinobacteria and Tenericutes were calculated with ARB (Ludwig et al., 2004) using the SILVA 111 SSU Ref NR database (Quast et al., 2013) applying 50% conservation filters for the respective phyla and the maximum parsimony and RaxML treeing algorithm using the GTRMIX rate distribution model and advanced bootstrap analysis with 1000 × bootstrapping included in ARB.

2.9. Accession numbers

The 16S rRNA gene clone sequences were submitted to the European Nucleotide Archive with the accession numbers LN612638-LN612725. Roche/454 pyrosequencing data were submitted to the European Nucleotide Archive and can be found here: http://www.ebi.ac.uk/ena/data/view/PRJEB7320.
3. Results

Detailed information on the samples used in this study is shown in Table 1.

3.1. Pyrosequencing

The microbial communities of 63 abomasum samples (see Table 1) were studied using pyrosequencing. A total of 268,479 sequencing reads with an average read length of 202 bp were obtained. After quality control, 156,536 reads (58.3 %) remained. The number of reads per sample varied from 947 to 6410, with an average of 2,484 reads per sample. The sequences were assigned to 10,459 OTUs (of which 1658 were singleton and 5462 doubleton OTUs). The 10 OTUs detected with the highest relative abundance across all 63 samples were assigned to Helicobacter (OTU 1), Acetobacter (OTU 2), Mycoplasma-like (OTU 3, OTU 5, OTU 6, OTU 8, and OTU 10), Lactobacillus (OTU 4), Bifidobacterium-like (OTU 7), and Clostridium sensu stricto (OTU 9) (Table S1, Fig. 1). Although the majority of OTUs showed highest similarity to GIT bacteria, it should however be noted that the possibility of introducing environmental bacteria during sample processing cannot be entirely excluded.

28 phyla were identified with Proteobacteria, Firmicutes, Bacteroidetes, and Tenericutes being most abundant; 96.4 % of all reads affiliated to these four phyla. Statistically significant differences in the microbial communities on phylum level of adult animals and calves were found. Proteobacteria, and Tenericutes were dominant in bulls and cows, while Firmicutes and Bacteroidetes were dominant in calves (Fig. 2).

3.2. Species richness and alpha-diversity analysis

Species richness and diversity estimations are shown in Table S2 and Table S3. When comparing ulcerated mucosa, unaffected tissue of the animals with ulcerated mucosa and mucosa from healthy animals no statistically significant differences of the diversity estimators were found. Only significant differences for the different animal groups (cows, bulls, calves)
and also when comparing adult (cows and bulls) and calves (p<0.004) were found. Rarefaction curves showed that the coverage of the present diversity was high for most samples (Fig. S2).

3.3. Beta-diversity analysis

The Yue & Clayton theta similarities (Fig. S3) revealed significantly similar community structures in bulls and cows compared to calves (p<0.001) (Table S4). The comparison between samples from animals with and without ulcers did not show any significant differences. Principle Components Analysis (PCA) of community composition based on UniFrac distances showed similar bacterial communities within groups. Communities of samples with and without ulcers showed no differentiation (Fig. 3).

3.4. Comparison of bacterial communities between groups

Considerable differences between the bacterial communities between adults and calves are obvious (Fig. 1, 2, 3 and S3). The most abundant OTUs in bulls and cows (>10 %) affiliated to Helicobacter canadensis (OTU 1), Acetobacter tropicalis (OTU 2), and Mycoplasma-like (OTU 3), accounting for 35.1 %, 12.0 % and 11.0 %, respectively. Lactobacillus taiwanensis (OTU 4) dominated in calf samples (22.6 %). Other OTUs with a higher relative abundance (>2 %) in the abomasum of calves were Lactobacillus (OTU 11, OTU 13) and Clostridium uliginosum (OTU 15) (Fig. 1).

Metastats was used to determine which OTUs showed a statistically significant different abundance between the groups (White et al., 2009). Only 16 out of all OTUs were found to be significantly differentially abundant between different animals groups and subgroups (Table S5).
3.5. Comparison of clone sequences with pyrosequencing reads

After quality control, 88 near full-length sequences were obtained from the cloning and Sanger sequencing approach. Nineteen pyrosequencing OTUs had a matching clone sequence (>99% similarity). While some clones showed high similarity (≥99%) to type strains (e.g. the clones affiliating to OTU 4 and OTU 9), a number of clone sequences – among others - those affiliating to OTUs 1, 3, 5, 6, 7, 8, 10 showed low similarities (≤90%) to type strains and to their closest hit in GenBank (Table S1).

Due to their low similarity (≤ 86%) to type strains and their relatively high abundance, the clone sequences affiliating to OTUs 3, 5, 6, 8, 10, 16, 17, 27, 31 (Tenericutes, clones 24a, 44a and 78a) and OTUs 7 and 12 (Actinobacteria, clones 14b, 48a and 33a) were subjected to phylogenetic analysis. Phylogenetic trees revealed that the clone sequences affiliated to the Bifidobacteriaceae and to the Mycoplasmataceae, respectively (Figures S4-S5). The Tenericutes clone sequences showed highest similarity to sequences retrieved from fecal samples from porpoise (JN792314.1, 91%). The Actinobacteria clone sequences had highest similarity (99%) to sequences from bighorn sheep feces (EU466336.1). Based on recently published similarity thresholds for genus definition (94.5%) (Yarza et al., 2014) and our phylogenetic analyses, these clone sequences obtained in our study thus represent novel candidate genera within the Bifidobacteriaceae and Mycoplasmataceae.
4. Discussion

To our knowledge, this is the first application of 16S rRNA gene based pyrosequencing technology to characterize the abomasal mucosal microbial communities in cattle with and without abomasal ulcers. Previous culture-based studies have focused on culturable bacteria from the abomasum associated with ulcers or were restricted to clinical case reports (Braun et al., 1997; Braun et al., 1991; Valgaeren et al., 2013).

The only study analyzing abomasal microbiomes by 16S rRNA gene targeted pyrosequencing analyzed the microbiota of abomasal content - not mucosa – in response to infection by the nematode *Ostertagia ostertagi* (Li, 2011). Therefore, this and the present study can only be compared to a very limited extent. In line with this, Li et al. (2011) found a very different community structure from our study, with *Bacteroidetes* (60.5 %), *Firmicutes* (21.1 %), *Proteobacteria* (7.2 %), *Spirochaetes* (2.9 %) and *Fibrobacteres* (1.5 %) being most abundant phyla. These differences are likely attributable to fundamental differences between the studies such as abomasal content vs. abomasal mucosa.

In general, it should be noted that due to the relatively short read lengths, it may not be possible to classify some pyrosequencing OTUs reliably on species level. We found *Helicobacter* (OTU 1) to be the most abundant OTU in abomasum mucosa samples. Similar to humans, *Helicobacter* species were suggested to be a gastric pathogen in cattle. In this study – among type strains - OTU 1 was classified as *Helicobacter canadensis* (94 % similarity), an emerging human pathogen associated with diarrhea and bacteremia (Fox et al., 2000). *H. canadensis* has also been isolated from a number of other animals (Goto et al., 2004; Inglis et al., 2006; Nebbia et al., 2007; Waldenstrom et al., 2003). Interestingly, OTU 1 showed 99 % similarity to ‘*Candidatus Helicobacter bovis*’, which has been found in the abomasum of cattle in previous studies (De Groote et al., 1999; Haesebrouck et al., 2009). Unfortunately, no information about a possible nexus of *H. bovis* with bovine abomasal
ulceration is available (Jelinski et al., 1995; Valgaeren et al., 2013). In the present study, no significant difference in the presence of *Helicobacter* in abomasum from healthy and ulcerated mucosa has been detected. Thus, a role of *Helicobacter* in the pathogenesis of ulceration in cattle seems to be unlikely. However, it should be noted that presence or absence or changes in the relative abundances of certain bacteria may not necessarily imply or rule out a role in disease. Although a limited number of animals have been examined in this study, it seems reasonable to conclude that *Helicobacter* may belong to the normal flora of the bovine abomasum. In agreement with this, a recent study did not detect *Helicobacter* in equine glandular stomach lesions (Husted et al., 2010).

Our results showed a 10.1 % relative abundance of *Acetobacter* (OTU 2, highest similarity to *Acetobacter tropicalis*) in all samples with clearly higher abundance in cows and bulls. *Acetobacter xylinum* (X75619, similarity 87 %) was described as part of the microbial community of the dairy cow rumen (Weimer et al., 2000). Interestingly, acetic acid bacteria are also often found in the GIT of various insects (Crotti et al., 2010; Kounatidis et al., 2009; Wong et al., 2013).

*Mycoplasma*-like OTUs (e.g. OTU 3, 5, 6, 8, 10, with a total relative abundance of 13.6 %) were among the most abundant and diverse phylotypes in the abomasum samples. Taxonomic assignment revealed highest similarity to *Mycoplasma alvi* (OTUs 3, 10; 85 %) and *Mycoplasma muris* (OTUs 5, 6, 8; 86 %). Members of the Tenericutes, including *Mycoplasma, Anaeroplasma*, and *Asteroleplasma*, can be pathogens and have also been detected from bovine and ovine rumen samples (Mao et al., 2013; Weisburg et al., 1989). *Mycoplasma alvi* was isolated from bovine gastrointestinal (including abomasum) and urogenital tracts; however, a nexus with ulceration has not been reported (Gourlay et al., 1977; Pettersson et al., 1996).
Lactic acid bacteria (LAB) belonging to the Lactobacillales, are well known as probiotic microorganisms (Herich and Levkut, 2002). We found high abundances of LAB (OTU 4-Lactobacillus taiwanensis, OTU 11-Lactobacillus reuteri, OTU 13- Lactobacillus amylovorus), primarily in calves. This is consistent with recent results, where lactobacilli were found to be abundant in the GIT of calves (Malmuthuge et al., 2014; Oikonomou et al., 2013). Milk or milk replacers are the main sources of nutrition for calves during the first weeks of life, which most likely explains the high abundance of Lactobacillus OTUs (OTU 4, OTU 11, and OTU 13).

Bifidobacteria are a part of the beneficial microbiota colonizing the GIT of calves. Interestingly, we found Bifidobacterium-like phylotypes OTU 7 (Bifidobacterium breve) and OTU 12 (Bifidobacterium adolescentis) only in adult animals, but not in calves. This is in contrast to other studies, which found bifidobacteria to be particularly abundant in calves during the milk-feeding period (Vlkova et al., 2006). However, due to the low sequence similarity (<90 %) of OTU 7 and OTU 12 to their closest related type strains, both phylotypes most likely represent novel candidate genera within the Bifidobacteriaceae. Thus, no conclusions about a possible beneficial function of these novel phylotypes can be drawn.

Considerable differences in the composition of bacterial communities between calves and the adult cattle (steers and cows) were obvious on phylum as well as on OTU level (Fig. 1, Fig. 2, Fig. 3). One hypothesis of our study was that bacteria might be involved in abomasal ulcer formation in cattle. However, our analysis revealed no clear evidence for candidate OTUs involved in ulcer formation. On community level, no differences between healthy and ulcerated animals were found as revealed by Yue-Clayton theta similarities and statistical analyses (Fig. S3 and Table S4). The pyrosequencing data showed only a few significant differences in the relative abundances of OTUs among healthy or ulcerated abomasal samples. Among all groups, only four OTUs (OTU 8, 15, 41, 49) showed...
significantly higher abundances in ulcerated abomasal mucosa in comparison to healthy mucosa. However, with the exception of OTU 8 in bulls, the differences in relative abundance were not statistically significant comparing bulls, cows, and calves. This suggests that no general bacterial candidate responsible for ulcer formation in all analyzed groups seems to be present. Nevertheless - as also stated above - it should be kept in mind that the mere presence or absence or changes in the relative abundances of certain bacteria may not necessarily imply or rule out a role of these bacteria in disease. Interestingly, the *Mycoplasma*-like OTU 3 was significantly increased in healthy mucosa samples while OTU 8 was significantly decreased. Nevertheless, e.g. OTU 8 (*Mycoplasma*-like) and 15 (*Clostridium uliginosum*) might be associated with ulcer formation in adult cattle and calves, respectively. However, more research will be needed to clarify the possible role of these phylotypes in abomasal ulcer formation.

In conclusion, we provide the first deep insights into the bacterial microbiota of healthy and ulcerated abomasal mucosa in calves, bulls and cows. We report a high diversity of bacteria attached to the abomasal mucosa in bulls, cows and calves. We could show distinct differences in the bacterial communities between calves on one hand and cows and bulls on the other hand. We did not find a relationship between microbiota communities associated with abomasal ulcers. However, since all ulcers in cattle of the study were type 1 ulcers the question remains if there could be a more distinct influence of certain bacteria on the formation of more severe lesions. Other host-dependent factors such as age, immune status, and host-independent factors (e.g. environment) may contribute to ulceration in cattle.

**Conflicts of interest**

There are no potential conflicts of interest.
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References


Figure Legends

Figure 1: Heatmap showing relative abundances (%) of the 50 most abundant OTUs in the abomasum samples based on an OTU definition threshold of 0.03 16S rRNA distance. Abbreviations: (B) bulls; (C) cows; (V) calves; (I) ulcerated mucosa; (II) unaffected part of ulcerated mucosa; (III) healthy mucosa from healthy animal. For better visualization, relative abundances higher than 30% are shown as 30% and marked by an asterisk.

Figure 2: Relative abundance of bacterial phyla in abomasum samples. Only phyla with relative abundances higher than 0.1 % are shown. Error bars represent standard errors of the mean (SEM).

Figure 3: Comparison of bacterial community composition of abomasal mucosa samples as determined by principal coordinate analysis (PCA). Percentage variation explained by each PCA is indicated on the axes. A) Comparison between calves, cows, and bulls; B) comparison between healthy and ulcerated parts of abomasum mucosa samples.
Table 1: Characteristics of abomasum samples

<table>
<thead>
<tr>
<th>Animals</th>
<th>Samples (n=63)</th>
<th>Sampling region</th>
<th>Ulcer subtype(^a) (frequency)</th>
<th>pH</th>
<th>Abnormal content</th>
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<td>BI1(^b), BI1(^c)</td>
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</table>

\(^a\) Classification of ulcers according to Braun et al. (1991): Type I, erosions and non-perforating ulcers; subtypes, Ia, erosion with minimal mucosal defects; Ib, deeper erosions combined with local hemorrhage; Ic, craters with a superficial coating of detritus, fibrin, or inflammatory products.

\(^b\) I: ulcerated mucosa

\(^c\) II: unaffected part of ulcerated mucosa

\(^d\) III: healthy mucosa from healthy animal
<table>
<thead>
<tr>
<th>OTU</th>
<th>Relative Abundance per Animal Group (%)</th>
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<tr>
<td>OTU 1</td>
<td>Bulls: 30</td>
</tr>
<tr>
<td>OTU 2</td>
<td>Bulls: 25</td>
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<tr>
<td>OTU 3</td>
<td>Bulls: 20</td>
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<td>OTU 5</td>
<td>Bulls: 10</td>
</tr>
<tr>
<td>OTU 6</td>
<td>Bulls: 5</td>
</tr>
</tbody>
</table>

Figure 1
Figure 2

The diagram illustrates the relative abundance of various bacterial phyla in different samples from cattle categorized into three groups:

- **Calves**:
  - **I** - ulcerated mucosa
  - **II** - unaffected part of ulcerated mucosa
  - **III** - healthy mucosa from healthy animal

- **Cows**:
  - **I** - ulcerated mucosa
  - **II** - unaffected part of ulcerated mucosa
  - **III** - healthy mucosa from healthy animal

- **Bulls**:
  - **I** - ulcerated mucosa
  - **II** - unaffected part of ulcerated mucosa
  - **III** - healthy mucosa from healthy animal

The relative abundances are represented by different colors and patterns for each phylum:

- **Proteobacteria** (black)
- **Firmicutes** (light gray)
- **Bacteroidetes** (dark gray)
- **Tenericutes** (white)
- **Actinobacteria** (crosshatched)
- **others**

The y-axis represents the phyla categories, and the x-axis represents the relative abundance (%) ranging from 0 to 80.