The Effect of Rumen Microbiota in The Susceptibility of Subacute Ruminant Acidosis in Dairy Cows

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Abstract

Background: Subacute ruminal acidosis (SARA) is a well-recognized metabolic disease that has negative impact on the animal performance and health. SARA in cows is mainly caused by long-term high-concentration diet (HCD) feeding, however, some cows are so well adapted to the HCD that do not develop such condition while others are more susceptible. We speculated the difference may be associated with the rumen microbiota community. Here, we analyzed the rumen bacterial and fungal microbiota from SARA-resistance and SARA-prone cows before and after feeding with HCD for six weeks.

Results: The 16S rRNA sequencing analysis showed that the rumen bacterial community in SARA-prone cows had lower bacterial diversity and higher relative abundance of *unidentified_Spirochaetaceae* and *Anaeroplasma* comparing to those of SARA-resistance cow. Moreover, the abundance of *Stenotrophomonas* were increased in SARA-positive compared to SARA-negative cows. In addition, the ITS1-IF sequencing analysis indicated that the abundance of *Fusarium_oxysporum* and *Papiliotrema_laurentii* were different in SARA-prone and SARA-resistance cows. Furthermore, feeding with HCD significantly increased the *Sarocladium_zea*, *Meyerozyma_caribbica*, and *Fusarium_oxysporum*, while decreased *Wallemia_sebi* in rumen microbiota. These results suggested that the abundance of *unidentified_Spirochaetaceae*, *Anaeroplasma*, *Fusarium_oxysporum*, and *Papiliotrema_laurentii* in rumen maybe connected to the susceptibility of SARA in dairy cows. In addition, SARA provocation was increased the pathogenic *Stenotrophomonas*, *Sarocladium_zea*, *Meyerozyma_caribbica*, and *Fusarium_oxysporum* in rumen.

Conclusions: This study suggested that manipulating rumen microbiota will serve as a novel approach for preventing the development of SARA in dairy cows in future studies.

Background

Around the beginning of the lactation period, dairy cows usually undergo dietaries change- from a high-forage diet to a high-grain diet transitions- to meet the nutritional requirement for milk production. However, long-term feeding of these readily fermentable carbohydrates diets usually leads to the occurrence of subacute ruminal acidosis (SARA), which significantly depresses the rumen pH [1-3]. SARA brought giant challenges to the dairy industry not only due to huge economic losses it brings, but also negative impact on animal welfare. Previous studies revealed that SARA was associated with the development of diarrhea, laminitis, abomasum displacement, liver abscesses, systemic inflammation, and inefficient feed utilization, as well as milk fat depression [4, 5]. Emerging evidence indicated that several factors (i.e. heat stress, rob food feed, and inadequate adaptation to highly fermentable diets) were considered to predispose dairy cows to SARA [6]. Among those factors, organic matter fermented in the rumen was considered to be one of the most important reasons for developing of SARA [5].

When dairy cows are fed with high grain diets, non-fibrous carbohydrates in the rumen will promote the fermentation of amylolytic bacteria and the production of organic acids, especially lactate [7-10]. In early

stage, a large amount of lactate can quickly be metabolized by lactate utilizers, and converted into short chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate [11]. However, once the production of lactic acid exceeds the metabolize capacity of lactate utilizers, vast lactic acid begins to accumulate in the rumen, and result in the pH in rumen fluid drops rapidly, and finally lead to SARA [12, 13]. Clinical practices show that individual cows exhibit a great variation in their susceptibility to SARA, even when fed and managed similarly [14]. What causes this variation is little known, but it is probably combined effects of physiology and behavior, including the rumen microbiota composition. In addition, an important study demonstrated that differences in rumen microbiota was an imperative factor influencing intolerance or adaption of high-grain diet. Just as previous research reported, sheep inoculated intraruminally with ruminal ingesta from a wheat-adapted sheep did not get overeating indigestion as the sheep that was of inoculated after feeding of cracked wheat [15]. These evidence suggested that rumen microbiota was critical for the development of SARA.

Thus, in this study we evaluated the diversity of bacterial and fungal community in rumen fluid from SARA-resistance and SARA-prone cows and aimed to find certain strain that adapted to high-grain feed in rumen, as well as provide a new direction for the screening for SARA resistant cows and the preventing of SARA.

Materials And Methods

Ethics

The full proposal was reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jilin University ethics committee, which approved the animal care and use permit license. All experiments comply with the manual of the care and use of laboratory animals published by the US National Institutes of Health.

Animals and protocols

Sixteen Holstein cows (2-3 years) were used for the experiment. Prior to experiment, all animals were fed a diet containing a forage-to-concentrate ratio of 60:40 and with as lib water for fifteen days. After diet adaption, all cows were fed with a high-concentration diet (HCD) comprising 30% forage and 70% mixed concentrate twice/day (5:00 and 18:00, water was available all the time) for six weeks. The rumen fluid samples were collected and divided into 4 groups, including SARARes (SARA-resistance): Rumen fluid from cows without SARA before feeding with HCD, SARAPro (SARA-prone): Rumen fluid from cow with SARA before feeding with HCD, SARANeg (SARA-negative): Rumen fluid from cows without SARA after feeding with HCD, and SARAPos (SARA-positive): Rumen fluid from cows with SARA after feeding with HCD.

Sample collection and analysis

The rumen pH was measured continuously by the radio-transmission pH-measurement system. The rumen fluid samples were collected for microbiota analysis before and after six weeks of HCD feeding.

DNA extraction, Illumina MiSeq sequencing, bioinformatics analysis

Total genome DNA samples from rumen fluid were extracted using the CTAB/SDS method and diluted to 1 ng/μL using sterile water. 16S rRNA genes or ITS1-1F of distinct regions were amplified using specific primers (16S V4:515F-806R or ITS1-1F-F-ITS1-1F-R) with barcodes. All PCR reactions were performed using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed in equal ratios and purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) as well as libraries quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the libraries were sequenced on an IlluminaHiSeq2500 platform, and 250bp paired-end reads were generated.

Sequencing depth was monitored by rarefaction curves, which had a minimum identity cutoff of 97% and a minimal alignment length cutoff of 100 bp. The alpha diversity, including chao 1, ace, shannon index, and simpson index were used to evaluate the complexity of species richness and diversity for each sample. All the indices were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Venn diagrams applied to analyze the number of core genera rumen fluid in different group samples, which were created basing on the principles of bioinformatics and evolutionary genomics. The linear discriminant analysis coupled with effect size (LEfSe) analysis was conducted to identify bacterial taxa differentially represented between rumen fluid bacterial community from deferent group samples.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6.01 (GraphPad Software, Inc., San Diego, CA). All data were expressed as the means ± SEM. Differences between date means were determined using one-way ANOVA (Dunnett's t-test) and the two-tailed t-test. $P < 0.05$ was considered to be statistically significant.

Results

pH in rumen fluid

After six weeks of HCD feeding, eight cows showed SARA-positive, which characterized as rumen fluid pH at 5.775 ± 0.10 (means ± SEM) (pH value < 5.8 was sustained for more than 3 h at different time periods in the cows fed with HCD for 6 weeks). The remained half animals showed SARA-negative, which featured as rumen fluid pH at 6.32 ± 0.09 (means ± SEM, Table 1).

Comparison of the rumen bacterial microbiota between SARA-resistant and SARA-prone cow prior to HCD feeding

Rumen fluid samples were gathered from eight Holstein dairy cows on day 0 (at the day beginning of HCD feeding) and on week six (six weeks after HCD feeding) to detect the bacterial community of rumen fluid using the V4 region of the bacterial 16S ribosomal RNA (rRNA) gene amplified PCR. Of the pyrosequencing reads that passed the quality control tests, total 2,809,712 reads, with an average of 87,830 reads per samples in rumen fluid. A species accumulation boxplot of 32 samples indicated that sampling depth had sufficient sequences to characterize the majority of bacterial communities (see supplementary fig. S1). To explore whether the susceptibility of SARA was associated the rumen microbiota in cows, we analyzed the bacterial community in rumen fluid from SARA-resistant and SARA-prone cows before feeding with HCD. Chao 1, and ace analysis showed that the bacterial richness of SARA-resistant were higher than in the SARA-prone cows (Fig. 1A-B). Shannon index and Simpson's index of the SARA-resistant and SARA-prone cows showed no significant difference in bacterial community diversity between the two groups (Fig. 1C-D).

At the phylum level the results indicated that phyla *Proteobacteria*, *Firmicutes*, *Bacteroidete*, *Tenericutes*, and *Spirochaetes* were the most abundant among rumen fluid from different treatment group cows (Fig. 1E). At the genus level, the relative abundance of *Stenotrophomonas*, *unidentified_Ruminococcaceae*, *Mycoplasma*, *unidentified_Prevotellaceae*, *unidentified_Rikenellaceae*, *Succiniclasticum*, *Succinivibrio*, and *unidentified_Bacteroidales* were the eight most prevalent genera in rumen fluid from different treatment group cows (Fig. 1F). In addition, t-test analysis was used to identify differences in bacterial genera of rumen fluid between SARA-resistant and SARA-prone group cows, and the data showed *unidentified_Spirochaetaceae* and *Anaeroplasma* were significant higher in SARA-prone group than those in SARA-resistant group cows (Fig. 1G).

Venn diagram was used to estimate whether a unique bacterial microbiota was associated with the development of SARA. As shown in Figure. 2A-B, most genera were shared between SARA-resistant and SARA-prone group cows. Core genera accounted for 66.92% of all rumen bacteria in SARA-resistant group and 75.48% in SARA-prone group cow (Fig. 2B). Since most bacterial genera were shared between SARA-resistant and SARA-prone group cows, it is likely that changes in bacterial relative abundance are more important for the susceptibility of SARA than unique differences in bacterial communities. Furthermore, the LEfSe was used to provide biomarkers at the genus-level with a linear discriminant analyses (LDA) also detected that *unidentified_Spirochaetaceae* and *Anaeroplasma* genus were enriched in SARA-prone cows (Fig. 2C-D). These results suggested that the relative abundance of *unidentified_Spirochaetaceae* and *Anaeroplasma* in rumen maybe associated with the susceptibility of SARA in cows.

The changes of rumen bacterial microbiota in SARA-resistance and SARA-prone cows before and after feeding with HCD

The α -diversity of the rumen bacterial community showed that microbiota richness had reduced in SARA-resistance before and after feeding with HCD for six weeks (Fig. 1A-B). In addition, the difference of rumen microbiota in SARA-resistance cows before and after feeding with HCD identified that the *Saccharofermentans* genus were increased in HCD feeding cows (Fig. 3A and 3D-E).

Furthermore, the richness and diversity of rumen fluid bacterial community from SARA-positive cows was significantly declined after feeding with HCD (SARA-prone vs SARA-positive cows) (Fig. 1A-D). Comparison of phyla and genus levels showed that *Proteobacteria* phyla and the genus of *Stenotrophomonas*, *Sphingomonas*, *Delftia* were significantly increased, while the relative abundance of phyla *Fimicutes*, *Bacteroidetes*, *Spirochaetes*, *Fibrobacteres*, and *Kiritimatiellaeota*, and the genus of *unidentified_Ruminococcaceae*, *unidentified_Rikenellaceae*, *unidentified_Lachnospiraceae*, *unidentified_Bacteroidales* *Succiniclasicum*, *unidentified_Spirochaetaceae*, *Saccharofermentans*, *Anaeroplasma*, *Papillibacter*, *Fibrobacter*, and *Anaerovorax* in rumen fluid were significantly decreased in SARA-positive cows before feeding with HCD when it compared to post HCD feeding (Fig 3A-C). Moreover, LEfSe analysis showed that genus *Stenotrophomonas*, which belongs to family *Xanthomonadaceae*, order *Xanthomonadales*, class *Gammaproteobacteria*, and phyla *Proteobacteria*, was identified as biomarkers in group SARA-positive cows (Fig. 3F-G).

Comparison of the rumen bacterial microbiota between SARA-resistant and SARA-prone cow after feeding with HCD

In order to compare the rumen bacterial microbiota between SARA-negative and SARA-positive cows, we analysis the rumen microbiota of SARA-resistance and SARA-prone cows after feeding with HCD. Chao 1, ace, simpson and shannon index showed that the bacterial community richness and diversity of rumen microbiota from cows of SARA-negative were higher than that in SARA-positive cows (Fig. 1A-D).

The difference of rumen bacteria in SARA-negative and SARA-positive cows were further detected at phylum and genus levels. The results showed that the relative abundance of *Proteobacteria* phyla, *Stenotrophomonas* and *Sphingomonas* genus were increased, while the relative abundance of *Bacteroidetes* and *Firmicutes* phyla and *unidentified_Lachnospiraceae* genus were reduced in rumen fluid from SARA-positive compared to the SARA-negative cows (Fig. 4A-B). Furthermore, LEfSe analysis to identity the biomarkers between SARA-negative and SARA-positive cows showed the phyla *Proteobacteria*, class *Gammaproteobacteria*, order *Xanthomonadales*, family *Xanthomonadaceae*, and genus *Stenotrophomonas* were significantly enriched in SARA-positive group cows (Fig. 4C-D).

Comparison of the rumen fungal microbiota between SARA-resistant and SARA-prone cow before feeding with HCD

For V4 amplicons, a total 2,922,553 reads for all 32 rumen samples were obtained by high-throughput sequencing (HTS), and with the average number of reads per rumen samples was 91,329. A species accumulation boxplot for a satisfactory sequencing depth to analyze the core microbiome (see supplementary Fig. S2). Comparison of α -diversity between SARA-resistance and SARA-prone cows showed that the ruminal fungi richness has no significant difference (Fig. 5A-B), while ruminal fungi diversity was obviously higher in SARA-prone cows compared to the SARA-resistance cows (Fig. 5C-D).

Further analyzed indicated that ruminal fungi population was dominated by *Ascomycota*, *Neocallimastigomycota*, *Basidiomycota*, and *Mortierellimycota* phylum among different treatment groups

(Fig. 5E). Moreover, the relative abundance of *Meyerozyma*, *Byssoschlamys*, *Fusarium*, *Candia*, *Sarocladium*, *Penicillium*, *Wallemia*, *Orpinomyces*, *Acermonium*, *Aspergillus*, *Talaromyces*, *Plectosphaerella*, *Cladosporium*, *Alternaria*, *Passalora*, *Phoma*, *Papiliotrema*, *Stephononectria*, and *Pseudeurotium* were the top 20 genus in the rumen fluid of different treatment group cows (Fig. 5F). In addition, among these fungi, the abundance of *Sarocladium* was higher, while the abundance of *Aspergillus*, and *Papiliotrema* were lower in SARA-prone cows than those in SARA-resistance cows (Fig. 5G).

Venn showed that most fungal genera were shared between SARA-resistant and SARA-prone cows (Fig. 6A-B). Of the OTUs, about 69.57% in SARA-resistance cows, and 60.72% in SARA-prone cows obtained from the core fungal microbiota (Fig. 6B). Furthermore, the LEfSe was to identify the abundance of fungi taxa with sequences in rumen fluid between SARA-resistance and SARA-prone cows. The results showed that the *Aspergillus* genus, *Caecomyces* genus, *Papiliotrema_Laurentii* species, and *Alternaria_alternata* species were enriched in SARA-resistance group cows, while the *Komagataella_pastoris*, *Sarocladium_zeae*, and *Fusarium_oxysporum* were enriched in SARA-prone group cows (Fig. 6C-D). These results suggested that *Papiliotrema_Laurentii*, *Alternaria_alternata*, *Komagataella_pastoris*, *Sarocladium_zeae*, and *Fusarium_oxysporum* in rumen maybe associated with the susceptibility of SARA in cows.

The changes of rumen fungal microbiota in SARA-resistance and SARA-prone cows before and after feeding with HCD

Comparison of rumen fungi richness and diversity among SARA-resistance, SARA-prone, SARA-positive, and SARA-negative cows demonstrated that feeding with HCD for 6 weeks significantly reduced the rumen fungi richness and diversity both in SARA-resistance and SARA-prone cows (Fig. 1A-D). The differences of rumen fungal based on t-test and LEfSe analysis showed that *Ascomycota* phylum, *Penicillium* genus, *Sarocladium* genus, *Sarocladium_zeae* species, *Meyerozyma* genus, *Meyerozyma_caribbica* species, *Fusarium* genus, *Fusarium_oxysporum* species were increased, while *Wallemia* genus, *Wallemia_sebi* species, *Plectosphaerella* genus, *Acremonium* genus, *Papillotrema* genus reduced in both SARA-resistance and SARA-prone cows (Fig. 7A-H).

Comparison of the rumen fungal microbiota between SARA-resistant and SARA-prone cow after feeding with HCD

The estimators of fungi community richness and diversity of rumen fluid in SARA-positive cows were obviously reduced compared to the SARA-negative cows after feeding with HCD for six weeks (Fig. 1A-D). Moreover, t-test and LEfSe both indicated that the levels of genus of *Fusarium* was reduced, while the relative abundance of *Pseudeurotium* was increased in SARA-negative cows compared to the SARA-positive cows. In addition, the *Cephalotrichum_nanum*, *Colletotrichum_gloeosporioides*, *Blumeria_graminis*, *Scopulariopsis_brumptii*, *Penicillium_citrinum*, *Monographella_nivalis*, and *Talaromyces_funiculosus* species were both enriched in SARA-negative cows compared to the SARA-positive cows (Fig. 8A-C).

Discussion

In recent years, milk production of dairy cows has been substantially increased worldwide. Meanwhile, feeding of concentration was increased in order to meet the nutrient requirement of lactation of dairy cows. As a result, a large amount of organic acids accumulated in the rumen and usually induced the development of SARA, which characterized as ruminal pH is lower than 5.5-5.8 more than 180 min for 24 h after high-concentration diet feeding [16, 17]. SARA often accompanied by the changes of rumen microbiota and physiology that induced systemic metabolic disorders in the cows [18], which ultimately led to significant economic losses in the dairy industry [5, 19]. Rumen microbiota, including bacteria, archaea, fungi and protozoa, in SARA cows has received extensive attention recently. With the development of high-throughput sequencing technology, the differences of rumen microbiota community between healthy and SARA cows have been revealed [20, 21]. However, whether the structure of rumen bacteria or fungal microbiota in the cows themselves is related to the susceptibility of SARA has not been reported. Indeed, it is very important to understand the mechanisms behind different susceptibilities to SARA, in particular to explore the role of rumen microbiota in the development of SARA, which would help developing the potential effective prevention strategies against SARA in dairy cows. Thus, we assessed the difference of rumen bacterial and fungal microbiota in SARA-resistance and SARA-prone cows. The results showed that increased abundance of *unidentified_Spirochaetaceae Anaeroplasma*, and *Fusarium_oxysporum* in rumen maybe associated with the increased susceptibility of SARA in dairy cows.

Rumen is considered as one of the most important organs which is responsible for affecting the occurrence and development of various of diseases in dairy cows, and the microbiota in the rumen exerts a multitude of important physiological function. Studies have showed that inoculation of rumen fluid from healthy cows accelerated recovery of rumen bacterial community homeostasis and ruminal bacteria function in sheep suffering from rumen acidosis [22]. Thus, we hypothesized that whether SARA occurrence might be due to the different structure of rumen microbiota community in cows themselves. The α -diversity analysis showed that a significant difference of bacterial richness and fungal diversity between SARA-resistance and SARA-prone cows. In addition, the abundance of rumen bacterial microbiota of *unidentified_Spirochaetaceae* and *Anaeroplasma*, and the abundance of rumen fungal microbiota of *Fusarium_oxysporum* were significantly higher, while the abundance of *Papiliotrema_laurentii* was lower in SARA-prone than those in SARA-resistance cows. Studies have been showed that *Spirochaetaceae* was associated with the fiber-degrading ability in rumen [23]. Others based on bioaugmentation with rumen-related microorganisms for lignocellulose degradation indicated the important role of *Spirochaetaceae* uncultured members for the production of volatile fatty acid in anaerobic digesters [24]. *Anaeroplasma* was first isolated from bovine rumen fluid by Robinson and Hungate [25]. It is a gram-negative bacterium, and belongs to order *Mycolasma* and possessed lytic enzymes that results in partial digestion of killed gram-negative bacteria or casein [25, 26]. Ruminal *Anaeroplasma* requires lipopolysaccharide, cholesterol and soluble starch [27], and has ability to produce ethanol and lactic acids [26]. Susanna K.P. Lau [28] suggested that *Anaeroplasma* could produce amylolytic enzymes and contribute to amylase breakdown. Jiakun Wang [29] also showed that

Anaeroplasma could ferment sugars to acetate and formate, and the relative abundance of *Anaeroplasma* in rumen fluid was positively correlated with the CH₄ production. *Fusarium_oxysporum*, an economically important filamentous fungus species of *Fusarium*, is a large species complex of among plant, animal and human pathogens that attack a diverse array of species in a host-specific manner [30, 31]. *Papiliotrema_laurentii* is a yeast that commonly inhabits soil and plant, and has ability to hydrolyze polyester coatings and coat components [32]. Studies also suggested that *Papiliotrema_laurentii* can accumulate intracellular lipids from inulin hydrolysates [33]. These results suggested that abundance of *unidentified_Spirochaetaceae*, *Anaeroplasma*, *Fusarium_oxysporum*, and *Papiliotrema_laurentii* in rumen maybe associated with susceptibility of SARA in cows.

Large amounts of evidences showed that over-feeding with HCD altered the microbiota community in cow's rumen. Similarly, bacterial community structure in rumen fluid were changed and the bacterial and fungal richness and diversity were significantly reduced in rumen fluid from cows suffering SARA [4, 19]. In addition, the relative abundance of phyla *Proteobacteria* was increased, whereas the relative abundance of phyle *Firmicutes*, *Bacteroidetes*, and *Spirochaetes* were significantly decreased. Moreover, the relative abundance of genus *Stenotrophomonas* were significantly increased in post HCD feeding cows comparing to pre-HCD feeding in SARA-positive ones. *Stenotrophomonas* was one of the most prevalent opportunistic pathogens which usually associated with the development of many infectious diseases, including bacteremia, sepsis, pneumonia, and chronic enteritis [34, 35]. Infection of diseases caused by *Stenotrophomonas* would be exceptionally difficult to treat due to its multi-antibiotic resistance ability [36, 37]. Studies showed that the SARA induced by HCD feeding was significantly increased the abundance of *Stenotrophomonas* genus in gut, which was positively associated with the concentration of total volatile fatty acid (VFA) production. It is also suggested that SARA induction diet feeding of cow increase the risk of human infection with these opportunistic pathogens[38]. In addition, studies also demonstrated that the genus of *Stenotrophomonas* act as pathogenic agents of Crohn's disease[39]. Thus, the increased abundance of them in digestive tract may be associated with damage of the gut and causing the diarrhea which often seen in SARA. Furthermore, Zhang et al.,[37] also suggested that feeding with HCD increased the abundance of *Stenotrophomonas* in milk, and it is regarded as a very important pathogen of bovine mastitis. M. Ohnishi et al., reported that *Stenotrophomonas* were closely associated with an herb outbreak of bovine mastitis to some extent, it is resistant to many antimicrobials [40]. In addition, predominance of *Stenotrophomonas* has been also indicated within the milk microbiota of bacteria culture-negative mastitis samples[41]. These may be part of the reason why mastitis is often secondary to SARA in clinical. Although the abundance of *Sarocladium_zeae*, *Meyerozyma_caribbica*, and *Fusarium_oxysporum* in rumen fungal microbiota were increased in the SARA cows compared to the no-SARA cows, there are little has been reported about the effects of these fungi on animal health. *Sarocladium_zeae* can produce bassianolide, vertilecanin A, vertilecanin A methyl ester, 2-decenedioic acid and 10-hydroxy-8-decenoic acid, which may be exert biological role in cereal[42]. *Meyerozyma_caribbica* is an indigenously isolated oleaginous yeast, and it is produced in media containing glucose a bioemulsifier that was partially characterized as a proteoglycan [43]. The role of these fungi in the SARA and other diseases that secondary to SARA need to be further studied.

Conclusions

The present study indicated that one of the potential mechanisms for the development of SARA induced by HCD may be facilitated in part by structural differences in rumen microbiota. Specifically, a high abundance of *unidentified_Spirochaetaceae* and *Anaeroplasma*, as well as *Fusarium_oxysporum* in rumen may be contributed to the increase susceptibility of SARA. Moreover, the opportunistic pathogens, *Stenotrophomonas*, and fungus *Sarocladium_zeae*, *Meyerozyma_caribbica*, and *Fusarium_oxysporum* were increased in rumen of cows suffered SARA. In-depth research will be needed to isolate and culture *unidentified_Spirochaetaceae*, *Anaeroplasma*, *Stenotrophomonas*, *Sarocladium_zeae*, *Meyerozyma_caribbica*, and *Fusarium_oxysporum* and to validate the role of these bacteria or fungus in the susceptibility and the diseases secondary to SARA in dairy cows.

Abbreviations

SARA: Subacute ruminal acidosis; HCD: High-concentration diet; SCFAs: Short chain fatty acids; LEfSe: Linear discriminant analysis coupled with effect size; LDA: Linear discriminant analyses; HTS: High-throughput sequencing; VFA: Volatile fatty acid.

Declarations

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Authors' contribution

X H contributed to article writing, literature search, results evaluation. R M, M T and J G performed the final revision of the article and expert opinions. C Z and Y C contributed to literature search and results evaluation. N Z and Y F contributed to study design.

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Ethics approval

The full proposal was reviewed by the Institutional Animal Care and Use Committee (IACUC) of Jilin University ethics committee, which approved the animal care and use permit license. All experiments comply with the manual of the care and use of laboratory animals published by the US National Institutes of Health.

Consent for publication

All the authors read and agree to the content of this paper and its publication.

Competing interests

None of the authors have a financial interest in any of the products, devices, or Materials mentioned in this manuscript. The authors declare that they have no conflicts of interest.

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Table

Table 1. The pH in the rumen fluid

Parameter	SARA-Res	SARA-Pro	SARA-Neg	SARA-Pos	<i>p</i> -Value	
					SARA-Res vs SARA-Neg	SARA-Neg vs SARA-Pos
Mean pH value (Mean±SME)	7.43±0.11	7.38±0.13	6.32±0.06	5.77±0.10	<i>p</i> < 0.01	<i>p</i> < 0.01
Time < pH 5.8, h/d	0	0	1.50±0.48	5.80±0.62	<i>p</i> < 0.01	<i>p</i> < 0.01

Figures

Figure 1

The bacterial richness, diversity, and community structure in rumen fluid. Comparison of the bacterial microbiota richness in terms of (A) chao 1 and (B) ace in rumen fluid among SARA-resistance, SARA-prone, SARA-negative, and SARA-positive cows. Comparison of the bacterial microbiota diversity in terms of (C) shannon index and (D) simpson index among SARA-resistance, SARA-prone, SARA-negative, and SARA-positive cows. (E) The relative abundance of the top 10 phyla in the rumen fluid among SARA-

resistance, SARA-prone, SARA-negative, and SARA-positive cows. (F) The relative abundance of top 25 genus in the rumen fluid among SARA-resistance, SARA-prone, SARA-negative, and SARA-positive cows. (G) The difference of bacterial community at genus level was detected by T-test analysis.

Figure 2

The multiple taxonomical level comparison of the bacterial community structure between SARA-resistance and SARA-prone cows. (A) Venn diagrams showing numbers of shared and unique genera in rumen bacterial communities among SARA-resistance, SARA-prone, SARA-negative, and SARA-positive cows. (B) Venn diagrams showing numbers of shared and unique genera in rumen bacterial communities between SARA-resistance and SARA-prone cows. (C) Linear discriminant analysis (LDA) score derived from LefSe analysis, showing the biomarker taxa (LDA score of > 2.5) in rumen bacteria between SARA-resistance and SARA-prone cows. (D) Cladogram generated from LefSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus.

Figure 3

The changes of rumen bacterial microbiota in SARA-resistance or SARA-prone cows before and after feeding with HCD. (A) The difference of bacterial community at genus level between SARA-resistance and SARA-negative cows was detected by T-test analysis. (B) The difference of bacterial community at phyle level between SARA-prone and SARA-positive cows was detected by T-test analysis. (C) The difference of bacterial community at genus level between SARA-prone and SARA-positive cows was detected by T-test

analysis. (D) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 3) in rumen between SARA-resistance and SARA-negative cows. (E) Cladogram generated from LEfSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus. (F) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 4) in rumen between SARA-prone and SARA-positive cows. (G) Cladogram generated from LEfSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus.

Figure 4

Comparison of the rumen bacterial microbiota between SARA-resistant and SARA-prone cow after feeding with HCD. (A) The difference of bacterial community at phyle level between SARA-negative and SARA-positive cows was detected by T-test analysis. (B) The difference of bacterial community at genus level between SARA-positive and SARA-negative cows was detected by T-test analysis. (C) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 4) in rumen between SARA-negative and SARA-positive cows. (D) Cladogram generated from LEfSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus.

resistance, SARA-prone, SARA-negative, and SARA-positive cows. (F) The relative abundance of top 25 genus in the rumen fluid among SARA-resistance, SARA-prone, SARA-negative, and SARA-positive cows. (G) The difference of fungal community at genus level was detected by T-test analysis.

Figure 6

The multiple taxonomical level comparison of the fungal community structure between SARA-resistance and SARA-prone cows. (A) Venn diagrams showing numbers of shared and unique genera in rumen fungal communities among SARA-resistance, SARA-prone, SARA-negative, and SARA-positive cows. (B) Venn diagrams showing numbers of shared and unique genera in rumen fungal communities between SARA-resistance and SARA-prone cows. (C) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 3.5) in rumen fungi between SARA-resistance and SARA-prone cows. (D) Cladogram generated from LEfSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus.

Figure 7

The changes of rumen bacterial microbiota in SARA-resistance or SARA-prone cows before and after feeding with HCD. (A) The difference of fungal community at phyle level between SARA-resistance and SARA-negative cows was detected by T-test analysis. (B) The difference of gungal community at phyle level between SARA-prone and SARA-positive cows was detected by T-test analysis. (C) The difference of fungal community at genus level between SARA-resistance and SARA-negative cows was detected by T-

test analysis. (D) The difference of fungal community at genus level between SARA-prone and SARA-positive cows was detected by T-test analysis. (E) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 4) in rumen fungi between SARA-resistance and SARA-negative cows. (F) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 4) in rumen fungi between SARA-prone and SARA-positive cows. (G) and (H) Cladogram generated from LEfSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus.

Figure 8

Comparison of the rumen bacterial microbiota between SARA-resistant and SARA-prone cow after feeding with HCD. (A) The difference of fungal community at genus level between SARA-positive and SARA-negative cows was detected by T-test analysis. (B) Linear discriminant analysis (LDA) score derived from LEfSe analysis, showing the biomarker taxa (LDA score of > 3) in rumen fungi between SARA-negative and SARA-positive cows. (C) Cladogram generated from LEfSe analysis indicating the relationship between taxon belong to phylum, class, order, family, and genus.

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