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Effect volatile fatty acids concentrations in the development of the caprine ruminal microflora

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Abstract

It is known that volatile fatty acids can inhibit growth of species of the family *Enterobacteriaceae* in vitro. However these volatile fatty acids affect bacterial population in the rumen of goats is unknown. Therefore, a study was conducted to investigate if changes in volatile fatty acids in rumen of goats during growth affect bacterial population. Results showed that members of *Enterobacteriaceae* and *enterococci* are present in large numbers in 3 weeks old kids and start to decrease when kids grow older. Lactobacilli are present in large number as well in 3 weeks old kids, but they remain stable during the growth of kids. Acetate, butyrate and propionate increase from undetectable levels in one week old kids to high concentration in 15 weeks old goats after which they stabilize. Significant negative correlation could be calculated between numbers of *Enterobacteriaceae* and concentration of undissociated a acetate, propionate and butyrate. It is concluded that volatile fatty acids are responsible for the reduction in numbers of *Enterobacteriaceae* in the rumen of goats during growth.

Keywords: Volatile fatty acids, Development, Caprine, Ruminal, Microflora.
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تأثير الأحماض الدهنية الطيارة في تطور النبيت الجرثومي الطبيعي للكرش في الماعز

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الخلاصة

من المعلوم إن الأحماض الدهنية الطيارة لها القدرة على تثبيط نمو الجراثيم العائلة المعوية Enterobacteriaceae خارج النسيج الحي ولكن قدرتها على التأثير على البيئة الجرثومية في كرش الماعز غير معروفة لذلك فقد تم تصميم هذه الدراسة لمعرفة تأثير التغير في تركيز الأحماض الدهنية الطيارة في كرش الماعز أثناء فترة النمو على أعداد جراثيم النبيت الجرثومي الطبيعي وأظهرت النتائج ان جراثيم Enterobcteriacea, enterococci توجد بأعداد كبيرة في الماعز بعمر 3 أسابيع ويبدأ بالتناقص مع تقدم العمر. اما العصيات اللبنية فأنها توجد بأعداد كبيرة في الماعز بعمر 3 أسابيع ويبقى ثابتا خلال فترة النمو. ان تركيز الأحماض الدهنية الطيارة acetate و butarate و propionate ارتفع من مستويات غير محسوسة في الجداء بعمر أسبوع واحد إلى تراكيز عالية بعمر 15 أسبوع ويبقى ثابتا إلى نهاية التجربة. أظهرت النتائج كذلك وجود ارتباط معنوي عكسي بين أعداد جراثيم العائلة المعوية وتركيز الأحماض الدهنية الطيارة واستنتج من هذه الدراسة بان الأحماض الدهنية الطيارة مسؤولة عن اختزال أعداد جراثيم العائلة المعوية في كرش الماعز.

Introduction

Ruminal microflora play important role in digestion of nutrients to produce volatile fatty acids and microbial proteins (1). There is also an endogenous production of acetate in the liver, adipose tissue, and other organs (2). In the rumen and blood the volatile fatty acids dissociated to acetate, propionate, and butyrate (3). Microflora consist of indigenous bacteria that permanently colonize the gastrointestinal tract. It is widely believed that part of the microflora is beneficial to health whilst other may be harmful (4).The majority of these bacteria are strict anaerobes especially those in the rumen (1). The microbial ecosystem of the gut is an a balance with all available ecological niches that it remain very stable in term of its composition. This stability limits both the available space at the gastrointestinal wall and nutrients for newly entering bacteria. Therefore the indigenous microflora tend to suppress colonization of newly entering bacteria (5). Both the variety of species as well as the microbial numbers in the gut are determined by many factors including pH and concentration of volatile fatty acids of the intestinal contents, intestinal motility, immune activity, competition for nutrients and receptor sites, production of anti microbial compounds and feed composition. Unfortunately due to its complexity,our understanding of the microflora and its interaction with these factors is still limited (6). To our knowledge the correlation between the volatile fatty acids were calculated in broiler (7) and mice (8-11), and have not been determined in ruminants yet. Therefore this study was conducted to evaluate the role of volatile fatty acids in the establishment of ruminal microflora in goats.

Materials and methods

Five kids were obtained on weeks 1, 3, 5, 8, 12, 15, 29 and 37 in this experiment, water and feed were given ad libtum. All kids received feed with growth promoting probiotics.

Bacteriological analysis

Samples for bacteriological analysis were diluted with a reduced physiological salt solution. For viable count of for a aerobic bacteria, total anaerobic, lactobacilli, Enterobacteriaceae, enterococci and *Bacteroides species*. Dilution were spread plated on appropriate selective agar plate and incubated as described by (12). *Enbacterium Spp* was counted on selective agar medium according to (13) in which Colombia blood agar was used as the base agar. Numbers of CFU are expressed as log CFU per gram. At week 24, five colonies for all selective agar plates were randomly selected and after growth in liquid medium, they were confirmed as the specific bacterial group growing on the respective selective agar medium (12).

Volatile Fatty Acids and pH Analyses

Approximately 0.4 gram of ruminal materials was resuspended in 1.6 ml of sterile water. The pH of samples was measured according to (6) and same samples were stored at -20 ºC. Concentration of acetate, propionate, butyrate and lactate were determined by high performance liquid chromatography (HPLC). Concentration of undissociated volatile fatty acids and lactate were calculated with the Henderson – Haselbach equation $pH = pK_0 = Log_{10} [A^-]/[HA]$ (where A^- is dissociated acids and HA is undissociated acids). pH values, total concentration of each volatile fatty acids and lactate and the respective pK_0 of acetic (4.75), propionic (4.87), butyric (4.81) and lactic (3.08) acids under standard condition (14).

In vitro assay

Five strains of Enterobacteriaceae and four strains of Enterococcus and one strain of Lactobacillus were randomly isolated from the selective agar plates on week 24. Acetate, propionate, butyrate and lactate were added to brain heart infusion (BHI) broth containing 0.5 gram of cystein HCl/L with the concentration of volatile fatty acids equal to concentration measured in the rumen of 3, 5, 8 and 15 weeks old goats respectively. The pH of these BHI solutions was adjusted with HCl to 5.8. These four BHI solution and control without volatile fatty acids (BHI broth, pH= 5.8) were inoculated with overnight cultures of the ten strains mention above, and incubated aerobically at 37 ºC for 12 hours, growth was monitored by measuring the change in optical density to 620 nm(OD). All strains were tested in triplicate (7).

Data analysis

Correlation between enterobacteriaceae, lactobacilli, entreococci, *Bactericides Spp*, Eubacterium and total and dissociated volatile fatty acids were calculated analyzed statistically by persons correlation with SPSS 12 software.

Results

pH, lactate and volatile fatty acids

Ruminal pH values were in the range of 5.0 – 6.2 during the growth of kids. Acetate could be detected in the ruminal content of 3 weeks old kids and concentration increased until 15 weeks old age. From this day on the acetate concentration established about 75 µmol/gr. Propionate and butyrate were detected in 12 weeks old and these concentration remain stable from week 12 onwards (propionate 15 µmol, butyrate 26 µmol/gr). Lactate was detected during the first week and increased onwards (Table 1).

Development of the bacterial flora

The development of the dominant microflora in the rumen of goats is presented in (Table 2). In early life, enterobacteriaceae, enterococci and lactobacilli were the most important groups of bacteria. After three weeks, CFU of enterobacteriaceae and enterococci started to decrease until the kids were 15 weeks old. Thereafter their numbers established. Viable count of *Bacteriodes Spp* and *Eubacterium Spp* were established after 12 weeks at stable level of 8 and 7 log CFU/gr respectively. These numbers were 3 to 4 log CFU /gr lower than total anaerobic number. This indicates that these groups were not the most dominant groups of obligate anaerobic bacteria. From week 12 onward, total numbers of anaerobically grown bacteria were approximately 2-3.5 log CFU/gr higher than those of aerobic bacteria. Microscopic examination of the bacteria from colonies from anaerobically incubated were Gram positive, Y branched bacteria.

Correlation between bacterial numbers and volatile fatty Acids

The correlation between lactate, acetate, propionate or butyrate and bacterial numbers and their significant (P< 0.05) were calculated (Table 3). There are significant negative correlations between numbers of Enterobacteriaceae and acetate, as well as the undissociated form of acetate, propionate and butyrate. Numbers of Enterococci showed only negative correlation with total and undissociated acetate. No significant correlation could be detected between numbers of lactobacilli and volatile fatty acids among the different bacterial groups.

Table (1): Concentration of volatile fatty acids in different age groups.

Week of age	pH	Lactate µmol/gr	Acetate µmol/gr	Propionate µmol/gr	Butyrate µmol/gr
1	5.3±0.4	6±0.2	ND*	ND*	ND*
3	5.7±0.3	6.5±0.1	14.5±0.1	ND*	ND*
5	6.2±0.4	7±0.3	22.6±0.4	ND*	ND*
8	5.4±0.3	7.5±0.4	33.5±0.5	ND*	ND*
12	5.0±0.2	8.2±0.2	46.5±0.6	12.2±0.3	17±0.3
15	5.3±0.4	11±0.1	72.3±0.3	15.0±0.4	26±0.2
29	5.5±0.5	13.3±0.3	75.2±0.7	15.0±0.3	26±0.1
37	6.0±0.3	41.2±0.1	75.3±0.2	15.0±0.2	26±0.3

ND*: Note detectable

Table (2): Development of the dominant microflora in the rumen of goats (Log CFU\gr).

Weeks of age	Total anaerobic bacteria	Lactobacilli	Enterobacter	Enterococci	Total aerobic bacteria	Bacteroides	Eubacteria
1	8.5±0.2	7.0±0.12	10.0±0.21	6.0±0.07	6.0±0.09	2.2±0,06	2.1±0.04
3	11.0±0.08	8.5±013	10.0±0.11	10.0±0.1	9.4±0.2	1.3±0.02	1.4±0.013
5	12.0±0.3	9.5±0. 5	8.5±0.87	8.4±0.74	8.7±0.54	3.1±0.07	3.5±0.064
8	10.0±0.2	7.3±0.8	8.5±0. 9	8.4±0.64	8.8±0.95	4.0±0.045	4.2±0.08
12	10.5±0.3	8.0±0.12	7.4±0.21	7.6±0.24	8.9±0.15	8.0±0.20	7.0±0.1
15	11.0±0.4	7.5±0.25	7.3±0.16	7.2±0.08	8.3±0.13	7.8±0.15	6.8±0.24
29	11.0±0.5	8.5±0.25	7.5±0.18	7.4±0.11	8.7±0.05	7.9±0.18	6.8±0.12
37	11.5±0.1	8.5±0.12	7.6±0.08	7.5±0.12	8.8±0.15	7.8±0.06	6.7±0.11

Table (3): Statistical data for the correlation between bacterial number and conc volatile fatty acids.

VFA conc.	Lactobacilli	Enterobacteriaceae		Enterococci	
µmol/gr	P	P	R	P	R
Total					
Lactate	NS	NS		NS	
Acetate	NS	0.0.5<	-0.593	0.05<	-0.765
Propionate	NS	NS		NS	
Butyrate	NS	NS		NS	
Undissociated					
Lactate	NS	NS		NS	
Acetate	NS	0.0.5<	-0.712	0.05<	-0.610
Propionate	NS	0.0.5<	-0.609	NS	
Butyrate	NS	0.0.5<	-0.656	NS	

In vitro studies

The correlation may suggest an effect of volatile fatty acids on the development of the microflora. These in vitro experiment shows that strains of *Enterobacteriaceae* isolated from rumen of goats are more susceptible to volatile fatty acids than enterococci or lactobacilli. For *Enterobacteriaceae* increasing concentration of volatile fatty acids caused a gradual decrease in the maximal specific growth (μ max) after 12 hours of growth (Table 4). Enterococcal strains were also affected by the volatile fatty acids. Furthermore, the relative decrease in (μ max) was less for *Enterococci* than *Enterobacteriaceae* (Table 4). Growth of Lactobacillus strain was no reduced by the addition of volatile fatty acids.

Table (4): Average M max for strains of Enterobacteriaceae enterococci and Lactobacillus growing in the presence of volatile fatty acids**.**

Medium	Enterobacteriaceae n=4		Enterococci n=4		Lactobacillus n=1	
	M max/h	% of control max	M max/h	% of control max	M max/h	% of control max
100	0.77	100	0.56	100	0.26	100
Week 3	0.59	76.6	0.44	80.0	0.32	123
Week 5	0.34	44.2	0.45	80.4	0.35	135
Week 8	0.23	29.9	0.37	66.1	0.33	127
Week 15	0.15	19.5	0.31	55.4	0.33	127

Discussion

The trend observed for the developments of the microbial groups in the rumen of goats during growth were similar to the results obtained in chickens (15,16). In this study the Gram positive, Y branched bacteria to be the dominant microflora in the rumen of 15-24 weeks old goats this observation was in agreement with those reported in chickens (17).

This study shows the presence of high concentration of acetate, propionate and butyrate in the rumen. High concentrations of volatile fatty acids are indicative that fermentation by obligate anaerobic bacteria are important (18,19). This is in agreement with the 2 to 3.5 times higher number of anaerobic bacteria than aerobic bacteria observed in this study. The concentrations of volatile fatty acids in this study are similar to result obtained in other studies (1,6). However those studies focused on volatile fatty acids alone and not on microflora. Therefore, correlations were calculated between volatile fatty acids and the log CFU of different bacterial groups per gram. A Significant negative correlation was observed between Enterobacteriaceae and acetate and undissociated from of acetate, propionate and butyrate. We made the assumption with our statistical correlation analysis that the two variables used (bacterial numbers against concentration of volatile fatty acids) are independent of any other variables. However the decrease in Enterobacteriaceae can be dependent on many others variables (e.g. competition of attachment sites competition for substrates, immunomodulation or production of antimicrobial substances). Similarly the production of acetate is dependent on other variables (e.g. bacterial metabolism). Therefore, it remains uncertain whether these significant correlation are influenced by other variables and thus if these significant correlation are causal. However, they may be an indication that the undissociated form of volatile fatty acids reduced the numbers of enterobacteriaceae in vivo. This hypothesis is supported by a proposed mechanism for fatty acids toxicity. This mechanism states that undissociated forms of these acids can diffuse freely across the bacterial membrane in to the cell. Inside the bacterial cell, the acid dissociates, thereby reducing the internal pH, which will cause internal cell damage. However, the anion itself may damage the cell as well (20, 21, and 22).

Reports concerning correlations between volatile fatty acids and Enterobacteriaceae have mainly focused on the intestines of mice (19,20,23,24). Unfortunately, in these reports, some contradictory results have been shown. In some of the studies it was observed that higher concentration of butyrate (19) or total volatile fatty acids (23) are related to reduce numbers of enterobacteriaceae, but the correlations and their significant have not been calculated. Furthermore, pH values were not shown and therefore correlations between undissociated volatile fatty acids and Enterobacteriaceae can not be deduced. In contrast, Fretter and Abrams (9) did not observed any relationship between volatile fatty acids and Enterobacteriaceae in mice. The pH values for the cecum of mice in their study ranged from 6.5 – 7.0. At these pH values the concentrations of undissociated volatile fatty acids are very low. This could be an explanation for the absence of correlation between volatile fatty acids and Enterobacteriaceae. In this study, pH values are around 5.0 – 6.2 resulting in 10 times as much of the undissociated acid at the same total volatile fatty acid concentration. This might very well result in the significant correlations we observed in the rumen of goats, in contrast to what was observed in the cecum of mice.

Further evidence that the significant correlation observed are causal came from our in vitro assay. In this assay, the situation in the rumen was mimicked by adding volatile fatty acid to BHI broth at concentrations and a pH value corresponding to the pH and concentration on a special week of age in goats. Under these conditions the growth rates of five strains of Enterobacteriaceae decreased. This strongly suggest that undissociated concentrations of volatile fatty acid are responsible for the in vivo reduction numbers of Enterobacteriaceae in the rumen of goats

Enterococcal strains are less affected by volatile fatty acid and the decrease of M max was not so pronounced as with the strains of enterobacteriaceae. This could be the reason that we found a significant negative correlations only between acetate and numbers of enterococci.It should be noted however, that volatile fatty acids are not necessarily the only mechanism behind the reduction of Enterobacteriaceae and enterococci. The Lactobacillus strain was not affected during growth in BHI broth with volatile fatty acid, which is in agreement with the lack for correlations between lactobacilli and concentration of volatile fatty acids in the rumen of kids.

One of the mechanisms by which the intestinal microflora may reduce Enterobacteriaceae is the bacteriostatic effect of volatile fatty acids in the rumen (24). The data from this study are the first to show that the volatile fatty acids are one of the mechanisms responsible for the decrease in numbers of Enterobacteriaceae in the rumen of goats during growth.

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