Efficacy of clay minerals and activated charcoal to bind endotoxins in rumen fluid

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15 january-15 april 2010

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Summary

Laminitis can be a herd health problem and is strongly associated with subacute rumen acidosis (SARA). Lipopolysaccharide concentration from Gram negative bacteria increases during periods of low ruminal pH levels and they can enter the bloodstream. LPS has a vasoactive function and is possibly responsible for laminitis to occur. It is known that clay minerals and activated charcoal are able to bind mycotoxins in vitro and they are being used as a foodadditive to prevent mycotoxicosis. Due to their structure and properties it is hypothesized that they are also capable of binding LPS in vitro. Three trials were conducted to determine if they are indeed able to bind LPS. The first two trials used a fixed amount of LPS, the third trial used an in vitro fermentation model to create an acidosis and subsequent rise in LPS. Three substances were used: activated charcoal, montmorrilonite and Amadeite. From the first two trials it appears that activated charcoal and montmorrilonite are capable to bind LPS in vitro. The third trial did not succeed in creating a rise in LPS although a acidosis was achieved.

Introduction

Lameness is a herd health problem of importance and can have a strong economical impact by causing increased veterinary cost and it has an effect on the reproductive efficiency.

There are multiple causes of lameness such as infectious diseases of the interdigital space for example foot rot or digital dermatitis. Traumatic damage to the sole can also occur when the cattle is housed in stables with hard or rough flours or when they have to walk long distances over rough roadways. Embedded stones for example can cause white line abcesses to form.

Laminitis is a condition where the bloodflow to the extremities is restricted which causes damage to the claws. Laminitis can be divided in three forms. Acute, subacute and subclinical (Greenough 2007). Acute laminitis is a consequence of a grain overload where the cow has eaten a large amount of grain and the pH of the rumen drops rapidly below 5.0. Affected animals show signs rapidly after overloading and an increase in heart rate, respiratory rate and liquid, light colored feces can be observed. Cows can become recumbent or crawl on their knees.

Subacute laminitis is a more subtle disorder were the only signs are that of a mild discomfort with cows shifting weight from leg to leg and they place their feet on the ground very carefully. Subacute laminitis is also associated with nutritional changes which can cause vasoactive substances to be released into the bloodstream that alter the bloodflow in the claw causing damage and discomfort.

Subclinical laminitis or pododermatitis aseptica diffuse is a disorder without clinical signs at the moment that structures in the claw are damaged but creates specific lesions that will show after a period of time. These lesions are caused by two different pathological processes. 1) The claw horn is weakened structurally and functionally and no longer able to endure loading and more vulnarabule to environmental agents. 2) the support system of the pedal bone and the integrity of the suspensory apparatus of the digit are weakened (Greenough 2007). Systemic vasoactive substances change the bloodpressure in the extremities and arteriovenous shunts start to form. This increases the bloodpressure in the claws and the vessels start to leak and are damaged and edema and hemorraghing occurs. The opening of the shunts also causes ischemia to occur in the dermis (Nocek et al 1997, Greenough 2007). As a result of the increased pressure and ischemia in the dermis the degeneration of the corium and the formation of horn is impaired. This can be detected in the sole 4 to 8 weeks after the initial damage is done as sole hemorraghes, white line defects, dorsal ridging of the wall or double soles (Nocek et al 1997). If the damage to the epidermis is severe the laminar layer can separate completely which will cause the pedal bone to shift. This creates even more damage to the soft tissue and necrosis can occur. The claw is tilted slightly which can be observed.

It is very difficult to distinguish between sole hemorraghes caused by subclinical laminitis or caused by traumatic damage to the sole. Housing should always be considered when sole bruising is found as a herd problem.

Subclinical laminitis has been associated with subacute rumen acidosis (SARA). This is a condition that occurs in both in dairy cattle as in beef cattle. The drop of the pH in the rumen, the formation of endotoxins (lipopolysaccharides) and damage to the rumenal wall can have a systemic effect that could possibly have an effect on the bloodflow in the extremitities and play a role in the occurence of laminitis.

For this paper I will explore the occurence of SARA and the role of LPS in the occurence of laminitis. I will also explain about clay minerals and activated charcoal to show why they could possibly be of use in preventing laminitis to occur.

Definition

There are many definitions of when to speak of SARA based on method and location of taking rumen fluid samples. Samples taken by stomach tube, rumenocentesis or through a fistula from the ventral sac of the rumen differ in pH value. Samples taken by stomach tube can be diluted by saliva and are on average 0,3 point higher than samples taken by rumenocentesis (Plaizier et al 1998). Some researchers defined SARA as an condition in which the pH falls below 5,6 (Cooper et al 1996, Gozho et al, 2006). Other researchers use the threshold of 5,8 because cellulolytic bacteria can not grow under a pH of 6,0 (Russel et al 1996). Acute rumen acidosis occurs when the pH of the rumen drops below 5,2 (Owens et al 1998). In the case of subacute rumen acidosis the drop in pH is caused by a rise in volatile fatty acid and lactic acid synthesis. In the case of acute rumen acidosis, the more severe drop in pH is caused by accumulation of lactic acid caused by a decline in lactic acid utilizing bacteria. (Owens et al 1998)
The pH of the rumen fluctuates during the day and the most accurate way to measure the pH is by using an indwelling pH probe in the ventral sac of the rumen.

Causes of ruminal pH depression

Several researchers have shown that supplementing cows with a diet high in grain can induce subacute rumen acidosis (Gozho et al 2006, 2007, Khafipour et al 2009)
The feeding of more easy degradable carohydrates and less dietary effective fiber decreases the time spent on chewing. Reduced chewing time in turn reduces the rate of saliva flow. Saliva contains sodium bicarbonate that buffers the acids produced during fermentation. (Owens et al 1998). During episodes of SARA the drop in pH is caused by accumulation of volatile fatty acids (VFA). Diets high in rapidly fermentable carbohydrates and proteins cause the fermentation rate to increase and the production rate of VFA rises accordingly. Normally the VFA are absorbed through the rumenal wall. The rumenal mucosae are made of papillae which increase the absorption surface. The papillae increase in length when a diet is fed that contains grain, the produced VFA stimulate growth of the papillae (Nocek et al 1997). Cows that make the transition from the dry period to lactation without being fed a close up diet or lactation diet before partus are at a higher risk to develop SARA because the papillae in the rumen are not fully grown yet and the absorption surface is not sufficient for the high production of VFA (Stone 2004). When the absorption rate of VFA is reduced the exchange of bicarbonate for the ionized acids also reduces. Combined with the reduced saliva rate caused by a decrease of dietary fiber the concentration of bicarbonate in the rumen fluid can be too low to effectively act as a buffer in the rumen. (Krause et al 2006)

Clinical signs of SARA

Laminitis has long been associated with SARA and other health problems such as liver abcesses, rumenitis, decreased dry matter intake (DMI) and decreased milk production can also be caused by SARA (Nocek et al 1997). Experimentally induced SARA leads to a reduced DMI (Gozho et al 2006, Keunen et al 2002) because of a decreased digestion of fiber, when the pH drops below 6.0 cellulolytic bacteria loose function and the digestion of fibers slows down (Russel et al 1996). The often recurrent drop in pH can change the rumen osmalality and when this occurs fluid is atracted to the rumen from the blood. The influx of fluid and increase of pressure can damage the rumen wall and abcesses can form. Repair of the rumen wall can lead to hyperkeratosis and this changes the permeability of the rumen wall and absorption of the VFA will decrease. (Krehbiel et al 1995)

Laminitis can be caused by the production of histamine and LPS which have a vasoactive effect. This changes the bloodflow to the extremities and ischemia can occur and damage to the tissues in the corium. (Nocek et al 1997)

Sometimes a drop in milkfat can be seen due to an inversion in the ratio of the VFA in the rumen. Normaly acetate is the most dominant followed by proprionate and butyrate. When the pH drops, butyrate and proprionate increase and the production of acetate decreases, since acetate is used for fatsynthesis the milk fat percentage will drop. (Goad et al 1998) This is however not a sign in all cases of SARA. (Garret et al 1999)

Role of LPS

Endotoxin or lipopolysaccharides (LPS) are a structural part of the bacterial cell wall of Gram negative bacteria and consists of three parts: a side chain, core polysaccharides and Lipid A which possesses the endotoxic activity. Lipid A has a negative charge (Epand et al 1999). Upon entering the bloodstream LPS is capable of activating the immune system and Gozho et al (2006, 2007) have shown that acute-phase proteins such as serum amyloid A (SAA) and haptoglobin (Hap) are elevated after inducing SARA with a rise in rumen LPS although they could not detect any LPS in the blood.

Emmanuel et al 2007 have shown that endotoxin are capable of crossing over the rumenal wall in vitro under acidotic (pH <5,0) and subacute acidotic (pH <5,6) conditions.

LPS levels in the rumen rise as the pH drops because Gram negative bacteria die and the LPS from the cell wall is released. Growing and dividing bacteria also release LPS from their outer membrane (Rietschel et al 1994). Combined with the low pH and the possible damage to the rumen epithelium it is possible for the LPS to cross the rumen wall and enter the bloodstream

A recurrent low rumen pH is accompanied by increased concentrations of endotoxins (Gozho et al, 2006, Emmanuel et al, 2008) and it has been suggested by Greenough (2007) that these endotoxins play an important role in the development of laminitis. Not al researchers were able to induce an inflammatory respons. Khafipour et al (2009a) did induce a rise in LPS in the rumen fluid without an systemic inflammatory respons in an experiment where cows were fed a diet high in alfalfa pellets. A similar experiment by Khafipour et al (2009b) using a diet high in grain in contrast did succeed in inducing a systemic respons as seen by a rise in acute phase proteins serum amyloid A, haptoglobin and LPS-binding protein.

The underlying mechanism might be that a recurrent low rumen pH damages the rumen epithelium thereby enhancing the transfer of endotoxins to the blood as shown by Emmanuel et al (2007).

Properties of clay
Clay minerals are naturally derived substances that have been used succesfully in adsorption of mycotoxin to prevent health problems in cattle, pigs and poultry due to feeding of contaminated feedstufs (Diaz et al 2002).
It is because of the large surface area due to the multilayered structure and the positive charge between the layers that many toxins can be trapped by clay minerals.
Montmorillonite is a clay aluminum silicate and has a 2:1 layer structure of tetrahedral and octahedral sheets It has the capacity to substitute the Si_{4+} for Al_{3+} in the tetrahedral sheet and substitute trivalent cations for divalent cations in the octahedral sheet. This creates a net positive charge in the sheet which can attract negatively charged ions and organic elements such as LPS (Murray 2000, G. Bugla-Płoskońska et al 2009).
Montmorillonite swells when added to water and this contributes to its large internal surface and thus a great adsorption capacity (White et al 1983)
Amedeite is a manmade product made by adding seaweed to montmorrilonite in order to increase the space between the layers so that larger particles can also be trapped.

Ditter et al (1983) showed that bentonite is a potent adsorber of endotoxin in vitro. Bentonite is mainly composed of montmorillonite.
Gardiner et al (1993) showed that terra fullonica (an aluminum silicate like montmorrilonite) significantly reduced systemic signs of endotoxeamia in rats with experimentally induced gastro-enteritis.
It has been shown that clay minerals are able to perform in the rumen. Diaz et al (2002) have shown that both clay minerals and activated charcoal have the ability to adsorp aflatoxin B1 under different pH conditions in vitro. Spotti et al (2004) have shown that clay minerals have a great adsorbing capacity for mycotoxin in rumen fluid.

Properties of activated charcoal
Activated charcoal has long been used as an oral supplement to adsorb toxins and also in haemofiltration systems to treat patients with sepsis (endotoxaemia). Charcoal is the end product of destructive distillation or organic or inorganic materials. It is then chemically treated to activate it and increase the internal surface for absorption and it can also be coated to use for example in heamofiltration systems. (Jaber 1997)
Research has shown that activated charcoal is succesful when applied in cases of gastrointestinal inflammation (Gardiner et al)
Stecko et al (2000) have shown that activated charcoal is capable of removing endotoxin in vitro from bovine plasma and in fact binds 50% of the endotoxin. Miatra et al (1981) found that activated charcoal binds 96% of endotoxin in saline but only 23% in canine serum.

Aim of the study
The aim of the current experiment is to evaluate the capacity of activated charcoal and two clay minerals: montmorillonite and Amadeite® to trap lipopolysaccharides (LPS) in vitro.

Materials and methods

Three trials were conducted in order to determine if the clay minerals and activated charcoal are capable of absorbing LPS. In the first trial only a buffer was used to see if the treatments were capable of binding the LPS. The second trial used sterilized rumen fluid as medium to see if there is any interference of components in the rumen fluid with the binding capacity of the treatments. The third and last trial used rumen fluid and ground cassava chip as a base for in vitro fermentation to see if that process, the microflora in the rumen or the diet would interfere with the LPS binding effect.

Trial 1

Two levels of pH (at 6.5 and 5.5) were studied and 5 treatments: positive control (which is LPS only), negative control (which is treatment only), activated charcoal (Sigma Aldrich), montmorillonite (Sigma Aldrich) and Amadeite® (Olmix) were employed. All adsorbents were performed in triplicate except for the positive and negative controls which were performed in duplicate. Sampling times were at 0 and 60 min for LPS analysis.

The absorbents were suspended in a phosphate buffer which was made by dissolving NaCl 80 g, KCl 2 g, Na_2HPO_4 14.2 g, $KHPO_4$ 2.4 g in 800 ml distilled water. pH was adjusted to 7.4 with HCL 1 M, solution was transferred to a graduated cylinder and adjusted to 1000 ml with distilled water. The solution was sterilised by autoclaving at 121 ºC for 20 minutes. The phosphate buffer was adjusted to pH 6.5 and 5.5 by using 1M HCL and 1M NaOH. The adsorbents were suspended in the buffer to reach final concentration of 10 mg of product/10 ml (0.1% w/v). 10 ml of the buffer was placed into 15 ml pyrogen-free centrifuge tubes. 0.01 ml (10 µl) was removed from the tubes. 10 mg of the adsorbents was then added and the suspension was thoroughly mixed with a vortex mixer.

LPS was suspended in the phosphate buffer to reach a final concentration of 20 µg/ml. 0.01 ml (10 µl) of the LPS solution was added to the adsorbent solution. The solution was then incubated for 60 minutes at 39 ^{o}C. Samples were centrifuged at 3500 rpm (Kokusan H-103n) for 15 min and then 1 ml was placed in 1.5 ml pyrogen-free tubes in duplicate and stored at -20^{o} C until LPS analysis.

Trial 2

2 levels of pH at 6.5 and 5.5 were studied and 5 treatments: positive control (which is LPS only), negative control (which is treatment only), activated charcoal, montmorillonite and Amadeite®) were employed. All adsorbents were performed in triplicate, except for the positive and negative controls wich were performed in duplicate. Sampling times were at 0 and 60 min for LPS analysis. Rumen fluid was taken from 2 rumen fistulated dry cows which were fed a diet of roughage and concentrate at a 40:60 ratio for at least 7 days before the start of the trial. The cows were fed 4 kg of rice straw and 6 kg of concentrate (1,3 kg rice bran; 2,5 kg soybean meal; 2,05 kg cassave chips; 0,15 kg Dairy premix minerals).

The rumen fluid was strained through 3 layers of gauze and then centrifuged 3 times (Hermle Z206A). Each time the supernatant was transferred to another centrifuge tube. Each time the rumen fluid was centrifuged at 3000 x g for 15 minutes. After centrifuging the rumen fluid was autoclaved at 121 ºC for 20 minutes. In two Erlenmeyer flasks capped with a stopper and a Bunsen valve, 350 ml of rumen fluid and 350 ml of sterilized mineral buffer of pH 6.8 (.408% potassium dihydrogen phosphate, .02% magnesium sulfate, .05% sodium chloride and .05% calcium chloride dihydrate) were combined.

pH was adjusted to 6.5 and 5.5 with 1 M HCL and 1 M NaOH. The flask was continuously flushed with CO_2. 20 ml of rumen buffer solution was then placed in stock bottles with 20 mg of adsorbent to reach a final concentration of 0.1% w/v. 0.02 ml was taken from the stock bottles and 0.02 ml of LPS solution at a concentration of 20 µg/ml was added. Solution was thoroughly mixed and flushed again with CO_2 before incubation for 60 min at 39 ºC. After incubation 10 ml of the sample was placed in centrifuge tubes and the tubes were centrifuged for 30 min at 3500 rpm (Kokusan H-103n). After centrifuging 1 ml was passed through a dispossible 0.22 µm sterile filter (Sartorius Stedim Biotech Minisart syringe filter) in order to eliminate any fine particles that were not suspended by centrufuging. Samples were stored in 1.5 ml pyrogen-free tubes at -20 ºC for LPS analysis.

Trial 3

Four treatments (control, activated charcoal, montmorillonite and Amadeite®) were employed. All adsorbents were performed in triplicate. Sampling times were at 0, 6 and 9 hours after incubation.

Rumen fluid, collected from 2 rumen fistulated cows fed a diet of roughage and concentrate at a 40:60 ratio for at least 7 days before the start of the trial. Fluid was kept under anaerobic conditions and at 39 ºC during transport. 900 ml of rumen fluid and 900 ml of sterilized mineral buffer of pH 6.8 according to Nagaraja 1978 (.408% potassium dihydrogen phosphate, .02% magnesium sulfate, .05% sodium chloride and .05% calcium chloride dihydrate) were combined in an Erlenmeyer flask capped with a stopper and Bunsen valve. The flask and its contents were flushed with CO_2 while on a heater and magnetic stirrer. 0.1 ml of resazurin (100 mg dissolved to 100 ml dH_20) and 0.35 g of $NaS_2.9H_20$ in 37 ml of NaOH were added to the solution, in order to see when anaerobic conditions are achieved (color change) adapted from Manke 1968. After thoroughly mixing and heating to 39 ºC, 50 ml of rumen fluid solution was extracted and added to the stock bottle containing 5 g of purified ground cassava chip and 50 mg of adsorbent to reach a final concentration of 0.1% w/v. These were thoroughly mixed and placed into the incubator at 39 ºC. After incubation pH was measured with a portable pH meter (Mettler Toledo SG2 SevenGo) and 10 ml was placed in centrifuge tube and centrifuged for 30 min at 3500 rpm (Kokusan H-103n). After centrifuging 1 ml was passed through a dispossible 0.22 µm sterile filter (Sartorius Stedim Biotech Minisart syringe filter) and placed in pyrogen-free tubes and heated at 100^{o}C for 30 min in a waterbath and stored at -20 ^{o}C until LPS analysis. An additional 9 ml was taken and placed in a bottle with 1 ml of 1 M H_2SO_4 and stored at -20 ºC for volatile fatty acid analysis.

LPS analysis

The concentration of free LPS in the rumen fluid will be determined by a chromogenic kinetic Limulus amebocyte lyase (LAL) according to Ghozo et al. (2005).

Statistical analysis and calculations

All data were analyzed with one-way analysis of variance (ANOVA) using a general linear model (GLM) and Turkey's multiple comparisons test, with differences considered significant at $P<0,01$. SPSS version 16 for windows.

Adsorption percentages were calculated as followed: EU in ml/200.000 = a. 100 x (1-a) = adsorption percentage. 200.000 EU/ml were added to the positive control samples.

Results

Trial 1

As shown in figure 1 activated charcoal and montmorrilonite show similar absorbtion percentages for the different pH levels. Only Amadeite shows a big difference between time and pH levels, for both pH levels the amount of LPS even increases after incubation.

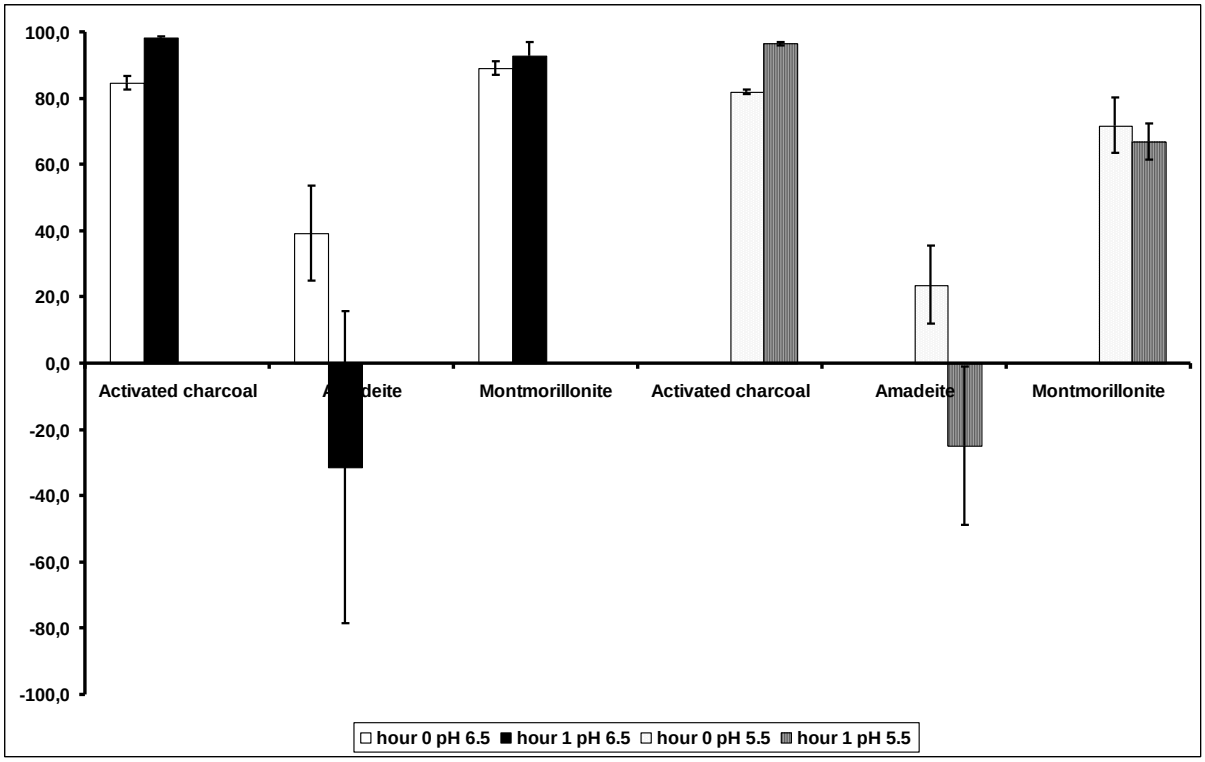

Figure 1) mean absorption percentages and standard deviations of treatments in buffer solution.

There was a significant effect of time on the absorption percentage ($P<0,01$) but no effect of pH on absorption percentage ($P>0,01$). There was a significant difference between Amadeite® and activated charcoal and between Amadeite® and montmorillonite ($P<0.01$) but not between activated charcoal and montmorillonite ($P>0,01$). There was also a signifficant effect of treatment on absorption percentage.

Trial 2

As in trial 1 montmorrilonite showed no significant difference between pH level or time of sampling.

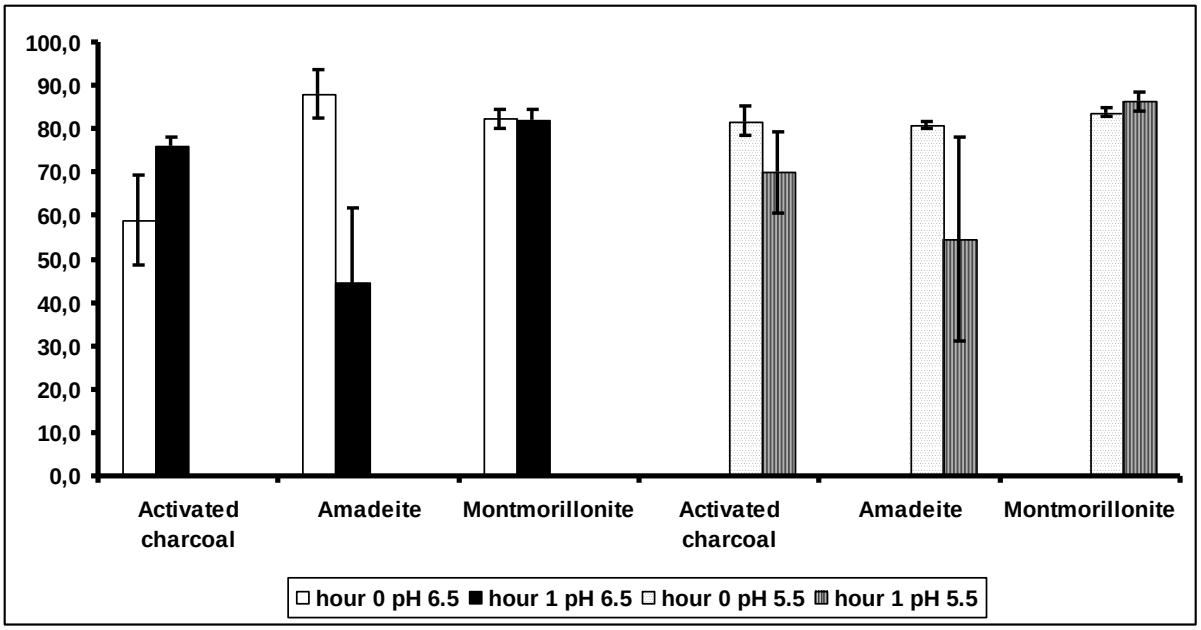

Figure 2) mean absorption percentages and standard deviations of treatments in rumen fluid.

In trial 2 there was a significant effect of time on the adsorption percentage (P<0,01) but there were no significant effects of treatment or pH on the adsorption percentage (P>0,01).

Trial 3

There was no significant difference between the pH at the start of the trial or in the drop during in vitro fermentation (P< 0,01). There was also no significant difference between the treatments for the concentration of LPS for the same time of sampling (P<0,01). There was a signifanct difference between hour 0 and 6 and between hour 6 and 9 for all the treatment groups (P>0,01).

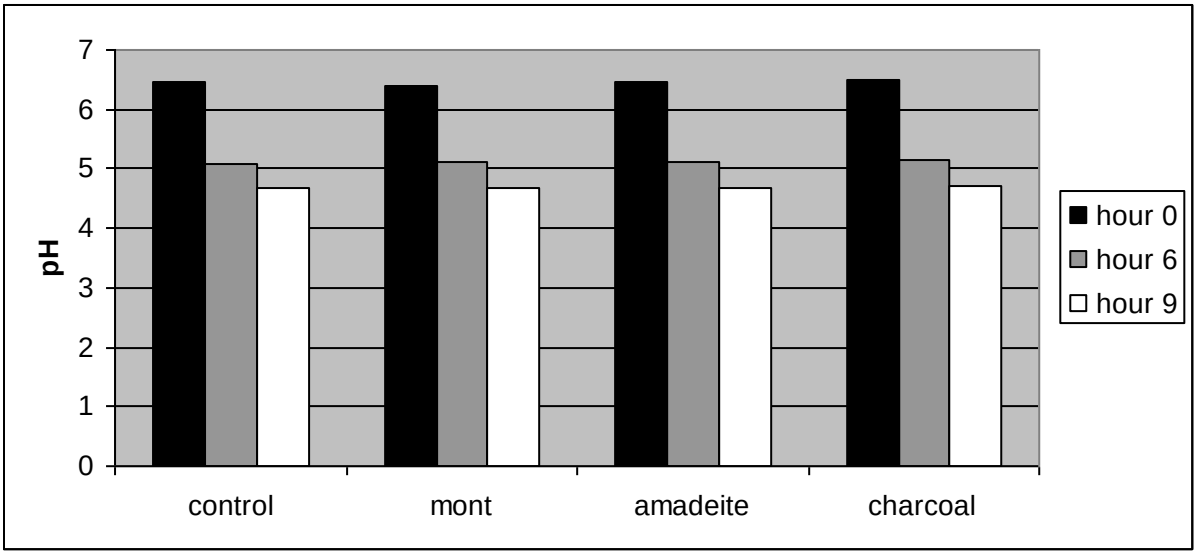

Figure 3) pH changes over time during in vitro fermentation.

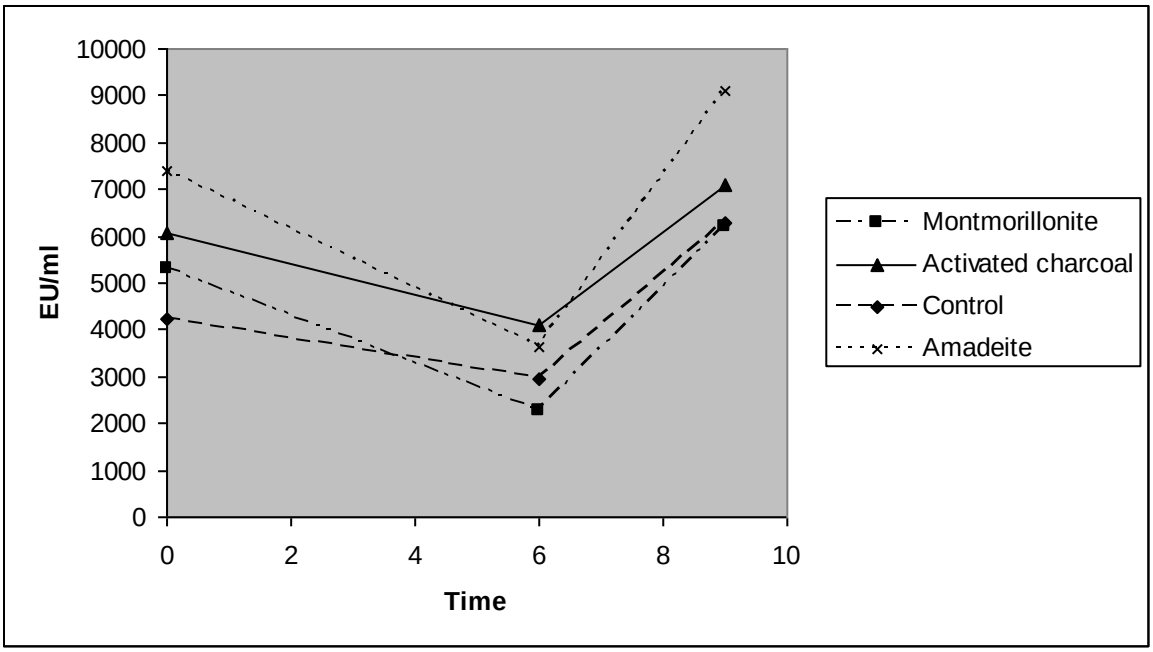

Figure 4) concentration (EU/ml) of LPS over time during in vitro fermentation.

Discussion

The significant effect of time on the absorption percentage in trial 1 can be explained by the behaviour of Amadeite over time. For both pH levels after one hour of incubtation the concentration of LPS increased significantly. One possible explanation may be that the seaweed particles of the Amadeite are interfering with the method of measuring the LPS or perhaps are capable of producing LPS.
This however does not explain why Amadeite does not show the same reaction in trial 2 were there was no increase in LPS although the adsorption percantage did decrease. Amadeite may possibly not be able to bind LPS permanently and it may be released again.

In trial 2 there was also a significant effect of time on the absorption percentage but the difference was again the largest in the Amadeite group. Both montmorrilonite and activated charcoal showed little difference in time, especially montmorrilonite showed almost no difference between sampling times at the 5.5 pH level.

In trial 3 the objective was to determine if the clay minerals and activated charcoal were capable of absorbing naturally formed LPS in an in vitro fermentation model of rumen acidosis. Although we did succeed in creating an acute acidosis with a mean pH of 4,7 in all treatment groups and the control, there was no significant rise in LPS concentration. This could possibly be explained by the fact that the trial was conducted for nine hours only and maybe this was too short a time for the ruminal bacteria to be affected by the low pH and they were not damaged enough yet and therefore no significant rise in LPS could be observed.

Conclusion

From the results of trial 1 and 2 it appears that both activated charcoal and montmorrilonite are capable of absorbing LPS in a buffer as well in rumen fluid, and that pH has no effect on the effectivity. Montmorillonite appears to have the most promise as it shows the highest absorption capacity in rumen fluid with no significant difference between the two pH levels.

Acknowledgements

I would like to thank dr. Rittichai Pilachai for letting me join him in his research and for his excellent guidance during the trials. I would also like to thank dr. Anantachai Chaiotwitiyakun, dr. Thomas Schonewille and dr. Henk Everts for their supervision and feedback on my paper and preperation for the research.

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Attachments

Table 1) absorption percentage trial 1)

Treatment trial 1	Replica	pH	Time	Abs %	Time	Abs %
Activated charcoal	R1	6,5	0	85,3	1	99,0
Activated charcoal	R2	6,5	0	88,4	1	98,7
Activated charcoal	R3	6,5	0	80,1	1	96,9
Activated charcoal	R1	5,5	0	83,6	1	97,1
Activated charcoal	R2	5,5	0	81,2	1	97,0
Activated charcoal	R3	5,5	0	81,2	1	95,2
Montmorillonite	R1	6,5	0	92,6	1	84,0
Montmorillonite	R2	6,5	0	90,3	1	99,3
Montmorillonite	R3	6,5	0	84,4	1	95,3
Montmorillonite	R1	5,5	0	78,3	1	60,4
Montmorillonite	R2	5,5	0	84,1	1	61,1
Montmorillonite	R3	5,5	0	53,0	1	79,6
Amadeite	R1	6,5	0	72,4	1	54,1
Amadeite	R2	6,5	0	23,4	1	-15,9
Amadeite	R3	6,5	0	21,8	1	-132,4
Amadeite	R1	5,5	0	50,3	1	26,9
Amadeite	R2	5,5	0	14,5	1	-34,7
Amadeite	R3	5,5	0	5,9	1	-67,2

Table 2) adsorption percentage trial 2)

Treatment trial 2	Replica	pH	Time	Abs %	Time	Abs %
Activated charcoal	R1	6,5	0	75,8	1	76,4
Activated charcoal	R2	6,5	0	65,3	1	80,0
Activated charcoal	R3	6,5	0	35,9	1	71,6
Activated charcoal	R1	5,5	0	75,6	1	50,5
Activated charcoal	R2	5,5	0	80,9	1	87,6
Activated charcoal	R3	5,5	0	88,9	1	71,7
Montmorillonite	R1	6,5	0	84,1	1	79,5
Montmorillonite	R2	6,5	0	77,2	1	79,9
Montmorillonite	R3	6,5	0	85,8	1	87,4
Montmorillonite	R1	5,5	0	83,4	1	90,0
Montmorillonite	R2	5,5	0	82,2	1	81,6
Montmorillonite	R3	5,5	0	85,8	1	87,6
Amadeite	R1	6,5	0	94,5	1	10,2
Amadeite	R2	6,5	0	75,3	1	78,2
Amadeite	R3	6,5	0	94,1	1	45,7
Amadeite	R1	5,5	0	82,8	1	83,9
Amadeite	R2	5,5	0	80,4	1	0,6
Amadeite	R3	5,5	0	79,7	1	79,3