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INFLUENCE OF MICROBIAL INTERACTIONS ON IN VITRO CELLULOLYTIC ACTIVITY OF RUMEN FUNGI.

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SUMMARY

As ruminants in Egypt are mainly fed on lignocellulosic agricultural by-products, so the present study aimed to investigate the fungal cellulolytic activity in absence of either rumen bacterial or protozoal populations after 12 and 24 hours of in vitro incubation. The rumen contents of five steers were collected immediately after slaughtering and used to generate three microbial systems, a control system (whole ruminal fluid without chemical treatment to measure activity of all microbial groups), a protozoal+fungal system (protozoal and fungal groups plus antibacterial agent) and a bacterial+fungal system (bacterial and fungal groups plus antiprotozoal agent). The fermentation patterns of cellulose due to the various treatments resulted in three distinct groups of data. Absence of either bacterial or protozoa species had a positive effect on fungal zoospores count, cellulose degradation %, total volatile fatty acids (VFAs) concentrations but negatively affected methane production without any alteration in either ammonia nitrogen concentration or CO2 production. Nevertheless, the positive effects achieved by defaunation outperformed those achieved by absence of bacterial species. In addition, defaunation was associated with increased propionates at the expense of acetates, while, absence of bacteria did not alter VFAs molar proportions. Moreover, late stages of incubation were associated with decreased bacterial and protozoal (entodinomorphs and holotrichs) counts, decreased acetic acid and methane production, increased pH value, ammonia nitrogen concentration, propionic and butyric acids molar proportions. Despite VFAs concentrations and cellulose degradation appeared numerically higher at 24 hours of incubation, the rate of production and degradation in the first 12 hours outperformed late stages of incubation. Thus it was concluded that negative effect of rumen protozoa on fungal cellulolytic activity is greater than that of rumen bacteria.

Key words: Cellulose degradation, Microbial interactions, Rumen microbes, Rumen fungi.

INTRODUCTION

Rumen fungi were originally believed to be flagellated protozoa until the pioneer discovery of Orpin (1975) that these organisms were actually flagellated zoospores of anaerobic fungi. Although counts of rumen fungi are relatively low in comparison to those of the bacteria and ciliate protozoa, they appear to be superior to the rumen bacteria in their ability to hydrolyze most of structural polysaccharides occurring in plant cell walls (Williams et al., 1994; Kopeny and Hodrova, 1995). They are able to weaken and degrade the more recalcitrant plant tissues as well as penetrating the cuticle barrier (Akin et al., 1989).

The effects of other rumen microorganisms on fungal growth and their ability to degrade structural polysaccharides are not completely understood and data regarding bacterial- fungus and protozoal-fungus interrelationships are contradictory. Although Orpin and Joblin (1997) recorded a protozoal predatory activity on rumen fungi, Williams and Withers (1993) found increased fungal counts in fauna-free animals after refaunation. However, the stabilizing role that protozoa have on the physico-chemical characteristics of the ruminal environment may have a beneficial action for fungi (William and Coleman, 1992).

On the other hand, a number of studies have revealed an increased fungal biomass and cellulolytic activity in cocultures with rumen methanogenic bacteria (Fonty and Joblin, 1991; Orpin and Joblin, 1997). However when combined in coculture with the cellulolytic ruminococci, their cellulolytic activity appeared to be inhibited (Bernalier et al., 1992).

Lee et al. (2000) concluded that interactions between rumen microbes can range from synergism to antagonism depending on the microbial groups and species involved and the type of substrate used.

The present study was undertaken to investigate to what extent cellulose degradation and growth of rumen fungi could be influenced by in vitro incubation with other types of rumen microbes.

MATERIALS AND METHODS

This experiment was conducted in Dept. of physiology, Faculty Vet. Med., Cairo University, Egypt.

1-Collection of rumen contents.

Rumen contents used to fractionate the microbial groups were collected from the rumen of five slaughtered steers. Collected rumen contents were strained through four layers of cheesecloth and brought immediately to the laboratory.

2-Separation of microbial fractions. For separation of microbial fractions from the rumen contents, we used chemical treatments previously described by Morgavi et al. (1994) and Lee et al. (2000) with some modifications. Strained rumen fluid was poured into a separating flask that had been gassed with oxygen-free CO2. The sample was then incubated under anaerobic conditions at 39°C for up to 60 min to allow small feed particles to buoy up and the microbial fractions to sediment at the bottom. Small feed particles that had floated to the surface were removed, and most of the lower liquid portion was carefully collected and divided into two parts. The first part was used to prepare the whole rumen fluid fraction that contains all types of rumen microbes, and the second part was used to prepare the different microbial systems. The following antibiotics and other chemicals were dissolved in anaerobic distilled water prepared by boiling and bubbling with CO2 gas, and used as following: antibacterial agents (streptomycin sulfate, penicillin G, potassium, and chloramphenicol [0.1 mg/ml each]) and antiprotozoal agent, sodium lauryl sulfate [0.01 mg/ml].

3-Preparation of microbial treatment systems: The following cocultural and microbial treatment systems were prepared to assess the interactions between specific microbial groups: a control system (whole ruminal fluid without chemical treatment to measure activity of all microbial groups), a protozoal+fungal system (protozoal and fungal groups plus antibacterial agents) and a bacterial+fungal system (bacterial and fungal groups plus antiprotozoal agent). Antimicrobial agents were added to the incubation tubes before inoculating them with microbial fractionates. The fluid in each system was then anaerobically mixed with phosphate buffer saline (pH 7.2) in a ratio of 1:2. After mixing, 30 ml of the diluted systems were anaerobically transferred to 60-ml aluminum tubes containing 100 mg of cellulose microfibrills, tubes were incubated at 38°C in a two separate anaerobic jars (one jar for 12 hour incubation and the other for 24 hours incubation) under CO2 and hydrogen gases released from a gas-packs purchased from oxoid. The treatment systems were conducted with duplicate tubes for each time period.

4-Sampling and analysis: Samples were taken from all tubes at 12 and 24 hours of incubation. Immediately pH was determined in the fluid. For determination of total VFAs concentrations and individual VFAs proportions 1 ml of 25% metaphosphoric acid was added to 5 ml of fermentation fluid, centrifuged (7,000 x g for 10 min) and supernatants were stored at -20°C until analyzed. For ammonia N determination, a 4-mL sample of fermented fluid was acidified with 4 mL of 0.2 N HCl and frozen. Samples were centrifuged at

5000 x g for 20 min, and the supernatant was analyzed by spectrophotometry for ammonia N (Chaney and Marbach, 1962).Total VFAs concentrations were measured by steam distillation according to Eadie et al. (1967), molar proportions of VFAs were analyzed using High Performance Liquid Chromatography (HPLC; Model Water 600; UV detector, Millipore Crop.) according to the method of Samuel et al. (1997).The total direct count of bacteria, protozoa (Holotrich and Entodiniomorph) and fungal zoospores were made using the procedure of Galyean (1989) by a haemacytometer. Differentiation of rumen fungal zoospores from small protozoa was based on characteristics of having flagellae, while large protozoa had ciliates around the cells.

5- Cellulose degradation %: The remaining contents of the tubes were filtered through a nylon filter. The retentate was washed with approximately 50 ml of distilled water and transferred to weighed aluminum dishes by being washed down with distilled water. The dishes were kept overnight in an oven set at 80°C as a predrying treatment and then dried at 135°C for 2 h. They were then weighed; cellulose degradation % was then calculated as original weight of cellulose minus dry residue weight (after incubation) divided by the original sample weight.

6-Statistical analysis: Data were analyzed by two way analysis of variance (ANOVA) test according to Snedecor and Cochran (1980). Treatment means were compared by the least significance difference (LSD) at 5% level of probability.

RESULTS

It is evident from table (1) that late stages of incubation (24 hours of incubation) were associated with a reduction in both bacterial and protozoal (both entodinomorphs and holotrichs) counts. Conversely, fungal zoospores count was increased by advancement of incubation. The overall means of different treatment systems denote that defaunation had a decreasing effect on bacterial count and similarly absence of bacterial populations negatively affected protozoal count (both entodinomorphs and holotrichs). In contrast, fungal zoospores count was increased in absence of either bacterial or protozoal species. Moreover, the decrement and increment effects induced by absence of either bacterial or protozoal species were time-dependent.

Table (1): Effect of incubation time and microbial treatment systems on microbial count

Incubation time	Control	Fungi + protozoa	Fungi + bacteria	Overall means of incubation time	L.S.D.
Bacterial count (x 10^9 /ml fermented fluid)					
12 hours of incubation	3.97 ± 0.07	0	2.52 ± 0.07	2.16 ^{b}	L.S.D. of overall mean of treatment effect = 0.1014 L.S.D. of overall mean of time effect = 0.083 L.S.D. of interaction between treatment x time effects = 0.143
24 hours of incubation	1.32 ± 0.07	0	1.2 ± 0.01	0.84 ^{b}	
Overall means of treatment effect	a 2.65	a 0	a 1.86		
Entodinomorphide ciliate count (x 10^5 /ml fermented fluid)					
12 hours of incubation	7.92 ± 0.10	3.36 ± 0.03	0	3.76 ^{b}	L.S.D. of overall mean of treatment effect = 0.094 L.S.D. of overall mean of time effect = 0.076 L.S.D. of interaction between treatment x time effects = 0.132
24 hours of incubation	2.70 ± 0.02	1.98 ± 0.01	0	1.56 ^{b}	
Overall means of treatment effect	a 5.31	a 2.67	a 0		
Holotrich ciliate count (x 10^3 /ml fermented fluid)					
12 hours of incubation	144 ± 2.28	54 ± 2.28	0	66 ^{b}	L.S.D. of overall mean of treatment effect = 3.392 L.S.D. of overall mean of time effect = 2.769 L.S.D. of interaction between treatment x time effects = 4.796
24 hours of incubation	126 ± 1.70	36 ± 1.70	0	54 ^{b}	
Overall means of treatment effect	a 135	a 45	a 0		
Fungal zoospores count (x 10^3 /ml fermented fluid)					
12 hours of incubation	132 ± 1.52	198 ± 2.35	288 ± 1.70	206.00 ^{b}	L.S.D. of overall mean of treatment effect = 3.806 L.S.D. of overall mean of time effect = 3.108 L.S.D. of interaction between treatment x time effects = 5.382
24 hours of incubation	180 ± 1.84	224 ± 1.95	378 ± 1.58	260.67 ^{b}	
Overall means of treatment effect	a 156	a 211	a 333		

Data presented as means ± SE, N =5

Values having the same letter in the same raw or column are significantly different at P <0.05

Table (2) identifies that, pH value and ammonia nitrogen concentrations were increased at late stages of incubation. It is worth noting that, despite VFAs concentrations appeared numerically higher at 24 hours of incubation, VFAs production rate at early stages of incubation outperformed late stages of incubation. Furthermore, VFAs production was increased in absence of either bacterial or protozoal species. Meanwhile, absence of bacterial species induced a time –independent increment effect on pH value. Nevertheless, absence of either bacterial or protozoal species did not alter ammonia nitrogen concentrations.

Table (2): Effect of incubation time and microbial treatment systems on pH value, total VFAs conc. and ammonia nitrogen conc.

Incubation time	Control	Fungi + protozoa	Fungi + bacteria	Overall means of incubation time	L.S.D. of overall mean of treatment effect = 0.0925 L.S.D. of overall mean of time effect = 0.076 No significant interaction between treatments x time effects.
Rumen pH value					
12 hours of incubation	6.20 ± 0.07	6.37 ± 0.05	6.31 ± 0.03	6.29 [c]	
24 hours of incubation	6.77 ± 0.02	6.82 ± 0.04	6.70 ± 0.04	6.76 [c]	
Overall means of treatment effect	a 6.49	a 6.60	b 6.51		
Rumen total VFAs conc. (µmol)					
12 hours of incubation	418 ± 10.51	570 ± 7.07	874 ± 8.92	620.67 [b]	L.S.D. of overall mean of treatment effect = 25.618 L.S.D. of overall mean of time effect = 20.917 L.S.D. of interaction between treatment x time effects = 36.229
24 hours of incubation	640 ± 16.67	722 ± 10.02	1064 ±17.49	808.67 [b]	
Overall means of treatment effect	a 529.0	a 646.0	a 969.0		
Ruminal ammonia nitrogen conc.(mg/dl)					
12 hours of incubation	13.08 ± 0.56	10.94 ± 0.57	11.80 ± 0.40	11.94 [a]	No overall mean of treatment effect L.S.D. of overall mean of time effect = 0.651 L.S.D. of interaction between treatment x time effects = 1.128
24 hours of incubation	17.75 ± 0.22	18.04 ± 0.08	18.23 ± 0.21	18.01 [a]	
Overall means of treatment effect	15.42	14.49	15.02		

Data presented as means ± SE, N =5

Values having the same letter in the same raw or column are significantly different at P <0.05

As shown in table (3), late stages of incubation revealed higher propionic and butyric acids molar proportions and lower acetic acid molar proportions than early stages of incubation. Absence of protozoal populations was associated with decreased acetates, increased propionates and non-significant difference in butyrates. Nevertheless, absence of bacterial populations did not alter acetates and propionates, however, was associated with decreased butyrates relative to control. In addition, increment and decrement effects induced by defaunation were time-dependent.

Table (3): Effect of incubation time and microbial treatment systems on VFAs molar proportions.

Incubation time	Control	Fungi + protozoa	Fungi + bacteria	Overall means of incubation time	L.S.D. of overall mean of treatment effect = 0.933 L.S.D. of overall mean of time effect = 0.762 L.S.D. of interaction between treatment x time effects = 1.320
Acetic acid (mol/100 mol)					
12 hours of incubation	64.17 ± 0.57	64.20 ± 0.54	65.44 ± 0.49	64.60 ^{b}	
24 hours of incubation	58.85 ± 0.31	57.68 ± 0.34	44.10 ± 0.41	53.54 ^{b}	
Overall means of treatment effect	a 61.51	a 60.94	a 54.77		
Propionic acid (mol/100 mol)					
12 hours of incubation	18.54 ± 1.29	20.02 ± 0.57	20.04 ± 0.74	20.20 ^{c}	L.S.D. of overall mean of treatment effect = 1.516 L.S.D. of overall mean of time effect = 1.238 L.S.D. of interaction between treatment x time effects = 2.144
24 hours of incubation	24.03 ± 0.29	24.24 ± 0.40	32.24 ± 0.68	26.84 ^{c}	
Overall means of treatment effect	b 21.29	a 22.13	ab 26.14		
Butyric acid (mol/100 mol)					
12 hours of incubation	15.65 ± 0.56	13.43 ± 0.60	13.53 ± 0.59	14.20 ^{c}	L.S.D. of overall mean of treatment effect = 1.167 L.S.D. of overall mean of time effect = 0.953 L.S.D. of interaction between treatment x time effects = 1.650
24 hours of incubation	18.15 ± 0.39	16.82 ±0.56	19.00 ± 0.66	17.99 ^{c}	
Overall means of treatment effect	a 16.90	a 15.13	16.27		

Data presented as means ± SE, N =5

Values having the same letter in the same raw or column are significantly different at P <0.05

Data presented in table (4) reveals that CO_2 production did not differ neither by advancement

Data presented in table (4) reveals that CO2 production did not differ neither by advancement of incubation nor by absence of either bacterial or protozoal species. In contrast, CH4 was decreased by advancement of incubation and in absence of either bacterial or protozoal species. However the decrement effect achieved by defaunation outperformed the decrement effect achieved in absence of bacterial species. Additionally, decreased CH4 production was time-dependent and appeared noticeable at late stages of incubation. The data also denotes that cellulose degradation percent was increased in absence of either bacterial or protozoal species. Nevertheless, increment effect of defaunation exceeds that achieved in absence of bacterial species. It is worth noting that, despite cellulose degradation percent appeared higher at 24 hours of incubation, the rate of degradation at early stages of incubation outperformed late stages of incubation.

Table (4): Effect of incubation time and microbial treatment systems on molar proportions of co_2 methane and cellulose degradation percent.

Incubation time	Control	Fungi + protozoa	Fungi + bacteria	Overall means of incubation time	No overall treatment effect No overall time effect No significant interaction between treatment x time effects.
Co_2 (mol/100 mol)					
12 hours of incubation	60.19 ± 0.98	57.75 ±1.40	58.02 ± 1.39	58.65	
24 hours of incubation	62.65 ± 1.50	60.13 ± 0.76	58.61 ± 0.60	60.46	
Overall means of treatment effect	61.42	58.94	58.32		
Methane (mol/100 mol)					
12 hours of incubation	35.28 ± 0.57	33.31 ± 0.50	34.48 ± 0.34	34.36 b	L.S.D. of overall mean of treatment effect = 0.844 L.S.D. of overall mean of time effect = 0.689 L.S.D. of interaction between treatment x time effects = 1.193
24 hours of incubation	32.50 ± 0.18	31.19 ± 0.44	23.49 ± 0.30	29.06 b	
Overall means of treatment effect	a 33.89	A 32.25	a 28.99		
Cellulose degradation percent					
12 hours of incubation	11 ± 0.35	15 ± 0.24	23 ± 0.54	16.33 b	L.S.D. of overall mean of treatment effect = 0.666 L.S.D. of overall mean of time effect = 0.544 L.S.D. of interaction between treatment x time effects = 0.941
24 hours of incubation	17 ± 0.15	19 ± 0.06	28 ± 0.35	21.33 b	
Overall means of treatment effect	a 14.00	A 17.00	a 25.50		

Values having the same letter in the same raw or column are significantly different at P <0.05

DISCUSSION

Like other tropical countries, ruminants in Egypt are fed mainly on lignocellulosic agricultural by-products which are rich in cellulose, hemicellulose and lignin. The rumen harbors various types of fungi which are active in degradation of such components. However, the antagonistic interactions among fungi and other microbial groups in the rumen limit their cellulolytic activity. The interrelationships between fungi and rumen bacteria in absence of protozoa and between fungi and rumen protozoa in absence of bacteria can be studied only in vitro.

Cellulose degradation was significantly higher during early stages of incubation (first 12 hours) than during late stages of incubation (following 12 hours). It seems that during these later stages, the efficient cellulolytic activity of fungal zoospores was responsible for the extent of cellulose degradation as late incubation was accompanied with decreased bacterial and protozoal counts and increased zoospores count. Fungal zoospores count and their cellulolytic activity appeared higher in absence of either bacterial or protozoal populations. However their count and activity were higher by defaunation than in absence of bacterial populations. This was true for both early and late stages of incubation. The antagonistic relationship between protozoa and fungi noticed in this study confirms the finding of Romulo et al. (1986) who showed two- to fourfold increase in zoospores and zoosporangia of anaerobic fungi in defaunated sheep. Moreover, Soetanto et al. (1985) and Ushida et al. (1989) found increased fungal populations in defaunated animals and observed increased digestion of the high-fiber diet fed to these animals. However it contradicted Williams and Withers (1993) who did not observe a decrease in fungal concentrations when defaunated sheep were refaunated. Also Newbold and Hillman (1990) observed only small increases in fungal zoospores in defaunated ruminants. This antagonistic effect may be mainly attributed to the predatory activity of protozoa on rumen fungi as suggested by Orpin, (1984). Another possible explanation is that fungal sporangium can be degraded by protozoal chitinolytic enzymes (Morgavi et al., 1994).

Increased fungal zoospores count and cellulolytic activity in absence of bacterial populations confirms the observed negative effects of bacteria on fungal cellulose digestion noticed by Bernalier et al. (1992, 1993) and Dehority and Tirabasso (2000). Inhibition of fungal activity is perhaps caused by an extracellular factor released by cellulolytic bacteria (Stewart et al., 1992) that interfere with attachment of rumen fungi to cellulose (Bernalier et al.1993).

Lee et al (2000) suggested that the contribution of the fungal fraction to cell wall degradation may

greatly exceed that of the bacteria. They concluded that the relative contributions of microbial fractions to the overall process of cell wall digestion are in the following order: fungal fraction > bacterial fraction > protozoal fraction.

Decreased protozoal count in absence of rumen bacteria could be due to insufficient nitrogenous compounds and other required nutrients for protozoal growth obtained by predation on rumen bacteria, a fact that was well-established by a series of studies as reported by Coleman (1975). In addition, decreased bacterial count by defaunation may be a result of little redox potential. Protozoa consume oxygen (Williams, 1986) and oxygen levels were found to increase transiently in defaunated animals (Hillman et al., 1985).

The molar proportions of volatile fatty acids produced in vitro were close to those which have been observed in ruminal fluid in vivo with similar diets showing the similarity between in vivo and in vitro fermentations and that a relatively normal rumen microbial population was maintained in this in vitro study. Increased cellulolysis in absence of bacteria did not alter VFAs proportions, however in absence of protozoa, enhanced cellulolysis was accompanied by increased propionates at the expense of acetates only at late stages of incubation. This could be attributed to the process of interspecies hydrogen transfer (Lopez et al., 1995). As Methanogenic bacteria are metabolically correlated with ciliate protozoa (Newbold et al., 1995) so defaunation reduces methanogenesis and directs hydrogen for propionates production. Santra et al. (1996) found that defaunating agents strongly inhibit methanogenesis.

Absence of either bacterial or protozoal populations did not alter pH value and ammonia nitrogen concentrations; however, both values were higher at later stages of incubation. These results when correlated with other events at late stages of incubation like, reduced bacterial and protozoal count, reduced cellulose degradation and reduced VFAs production, mostly indicates, reduced ammonia utilization for microbial protein synthesis in absence of adequate energy-yielding substrates. It is well-known that ruminal ammonia nitrogen is a good indicator for energy availability (Mabjeesh et al., 1997) and a balance between nitrogen supply and energy- yielding substrates is essential for maximization of microbial protein synthesis (Sinclair et al., 1993).

It appears that the negative effect of rumen protozoa on fungal cellulolytic activity is greater than that of rumen bacteria and attenuation of this effect would result in an overall improvement of the amount of cellulose degraded.

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تأثير التفاعلات الميكروبية على فاعلية فطريات الكرش فى هضم السليلوز خارج الكرش

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أجريت هذه الدراسة على عينات لسائل الكرش تم تجميعها من خمس عجول أصحاء بعد الذبح مباشرة بهدف دراسة تأثير ميكروبات الكرش الأخرى من بكتريا و برتوزوا على فاعلية فطريات الكرش فى هضم السليلوز خارج الكرش. و قد استخدمت هذه العينات لأنشاء ثلاثة أنظمة ميكروبية و هى: النظام الضابط (و يحتوى على جميع العشائر الميكروبية) و نظام البروتوزوا + الفطريات (و يحتوى على البرتوزوا و الفطريات بعد اضافة مضادات البكتريا) و و نظام البكتريا + الفطريات (و يحتوى على البكتريا و الفطريات بعد اضافة مضادات البروتوزوا). و قد تم تحضين هذه الأنظمة الميكروبية الثلاثة لكل عينة فى أنابيب مزدوجة تحتوى على ألياف السليلوز الدقيقة لمدة ١٢ و ٢٤ ساعة عند ٣٨ درجة مئوية فكانت النتيجة ظهور ثلاثة صور متميزة من الهضم التخمرى. فقد أثبتت الدراسة ظهور تأثير ايجابى لغياب أيا من البكتريا أو البروتوزوا على أعداد الفطريات المتحوصلة و نسبة السليلوز المهضوم و تركيز الأحماض الدهنية الطيارة و تقليل غاز الميثان دونما تأثير على تركيزات الأمونيا أو ثانى أكسيد الكربون. و قد ظهر جليا أن التأثيرات الايجابية المتحققة فى غياب البرتوزوا قد فاقت تلك المتحققة بغياب البكتريا اضافة الى أن غياب البرتوزوا صاحبه ارتفاع فى نسبة حامض البربيونيك على حساب حامض الأستيك الأمر الذى لم يحدث عند غياب البكتريا. كما أظهرت الدراسة أن المراحل المتأخرة من التحضين كانت مصحوبة بنقصان فى أعداد البكتريا و البروتوزوا و نسبة حامض الأستيك و الميثان و زيادة فى قيمة الأس الهيدروجينى و الأمونيا و حامض البربيونيك و البيوتريك. أيضا و على الرغم من ظهور زيادة رقمية فى تركيز الأحماض الدهنية الطيارة و معدلات هضم السليلوز فى المراحل المتأخرة من التحضين الا أن المراحل الأولى من التحضين قد فاقت المراحل المتأخرة فى هذا الشأن. و قد خلصت الدراسة الى أن للبروتوزوا تأثيرا سلبيا على هضم السليلوز بواسطة فطريات الكرش و أن هذا التأثير يفوق التأثير السلبى للبكتريا.