Experiment	**Microbial Diversity in the Rumen**
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Reading	Chapters in BBOM 9th: 16.15, 3.8, 14.3, 16.14, 17.1-17.3. BBOM 9th: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, Prentice Hall, 1999. ISBN: 0-13-085264-3
Objectives	• **Sampling** of microbes from a natural ecosystem • Studying microbial diversity by **microscopy** • Learning about **syntrophic interactions** • Determining **habitat conditions** • **Evaluating** experiments quantitatively • Linking microbial **physiology with biochemistry**
Background	Ruminants, e.g. cattle, sheep, camels, llamas, deer etc. are cloven-hoofed, mammals which feed on **plant materials**. They were domesticated early for meat, milk and other products and they play important roles as mediators in the global **carbon cycle**. Although they are **herbivorous**, they lack the **glycolytic hydrolases** needed to cleave the major plant polysaccharides like cellulose, pectins, hemicellulose and starch. They rely on the **glycolytic enzymes produced by prokaryotes** which they harbor in a particular digestive compartment. Digestion in ruminants is achieved by one of the most fascinating but also extremely complex **microbial ecosystems**, the **rumen**. The rumen is a large **pregastric fermentation chamber** present in the digestive tracts of all ruminants. It has a volume of up to 250 l in an adult cow and contains a **microbial community** consisting of about 10^{11} microbes per ml of rumen fluid. The microbiota comprises mostly **anaerobic bacteria** and **archaea** (about 10^8 – 10^{11} ml^{-1}, belonging to more than 200 species), **anaerobic ciliated protozoa** (10^4 to 10^6 ml^{-1}) and **anaerobic fungi** (10^2 to 10^4 zoospores ml^{-1}). They are kept by the host under more-or-less constant **anoxic conditions** at a redox potential of –350 to –400 mV, a pH of 6.5, and a temperature of 39°C. Substrates for the rumen microbes are supplied by the feed which is collected, prepared, conditioned and reconditioned by the host animal while the **microbes hydrolyze the plant polymers and ferment the hydolysis products**. Microbial digestion products are removed by **diffusion into the blood stream of the host**, gases are belched up through the esophagus. Solids, including 500 to 700 g/day of microbial biomass, are further digested in the gastric stomach (the abomasum) and in the small and large intestines. Undigested material leaves the digestive system through the rectum in fecal shapes and consistencies which are characteristic for particular ruminants, their diets and their digestive systems. Digestion in the rumen is carried out by **synergistically interacting microbes** in an **anaerobic food-web symbiosis** (figure 1). First, the plant polysaccharides are enzymatically broken down into di- and monosaccharides which are then fermented to **short chain volatile fatty acids** (VFA) like formic, acetic, propionic, butyric acid etc. CO_2, H_2 and CH_4 and, depending on the diet, small amounts of lactate and succinate are produced as well. Ammonia, branched chain fatty acids and other essential growth factors are produced by several groups of prokaryotes from plant proteins and nucleic acids. Some of the nitrogen is recycled through the **ureohepatic cycle** and added as urea to the rumen ecosystem with the saliva. While the bulk VFAs which serve as energy sources for the ruminant are taken up and distributed in the body by the blood stream, H_2 and CO_2 are converted by **methanogens** into CH_4. The gases are mostly belched into the atmosphere. Through many years of ingenious microbiological research, the prokaryotic composition of the rumen community has been able to be analyzed in great detail. Some of the prokaryotes present and their contribution to the digestion process are listed in table 1.

Table 1. Some prokaryotes of the rumen microbiota and catabolic abilities

Organisms	Catabolic Abilities												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	Starch degradation	Cellulose degradation	Pectin degradation	Hemicellulose degradation	Sugar metabolism, Glycolysis	Acetogenesis	Butyrogenesis	Lactate formation	Succinate formation	Lactate conversion to propionate	conversion to	Formate formation	from H_2, CO_2 and
Bacteroides amylophilus	X				X	X			X				
Ruminobacter amylophilus	X				X	X			X			X	
Bacteroides ruminicola	X		X		X	X				X		X	
Succinimonas amylolytica	X				X	X			X	X			
Selenomonas ruminantium	X				X	X		X			(X)		
Selenomonas ruminantium subsp. lactilytica	X				X	X			(X)	X			
Streptococcus bovis	X		X		X			X					
Ruminococcus flavefaciens		X		X	X	X			X			X	
Ruminococcus albus		X		X	X	X						X	
Fibrobacter succinogenes		X		X	X	X			X			X	
Butyrivibrio fibrisolvens		X		X	X	X	X	X				X	
Clostridium lochheadii		X			X	X	X					X	
Lachnospira multiparus			X	X	X	X		X				X	
Lactobacillus spp.					X			X					
Schwartzia succinovorans											X		
Veillonella parvula						X				X	(X)		
Megasphaera elsdenii					X	X	X			X	(X)		
Methanobrevibacter ruminantium													X
Methanomicrobium mobile													X

( ) some strains only or only by resting cells

Literature	• The chapters of BBOM 9th mentioned above • Hungate, Robert E., 1966. The Rumen and its Microbes, AP, New York. 533 pgs. A classic describing all aspects of rumen microbiology. • Dirksen G., 1969. Ist die Methylenblauprobe als Schnelltest für die klinische Pansenuntersuchung geeignet ? Deutsche Tierärztliche Wochenschrift 76/12, 305-309.
www. Links	• Images of the exterior and the interior of calf rumens (Penn. State): http://www.das.psu.edu/dcn/calfmgt/rumen/index.html • Anaerobic zoosporic fungi of the rumen: http://www.towson.edu/~wubah/rumenfungi/Anaerobic%20zoosporic%20fungi%20station.htm • Rumen physiology and rumination (Colorado State): http://arbl.cvmbs.colostate.edu/hbooks/pathphys/digestion/herbivores/rumination.html • The microbe zoo (MSU): http://commtechlab.msu.edu/sites/dlc-me/zoo/zacmain.html
Practical work	We will collect rumen fluid from a **fistulated cow** at the Animal Hospital, observe the organisms of the **microbiota** by phase contrast microscopy and make a few tests which will give a qualitative impression about the **habitat conditions** and the **activity** of the rumen microbes.
Material and Experimental Protocols	**1. Sampling rumen fluid from a fistulated cow:** sampling technique. Insert a stiff plastic tubing all the way to the **bottom of the rumen chamber**. Fill the tubing by suction and fill the collected rumen fluid into a sterile 250 ml bottle with a wide neck. Fill the bottle almost completely, leaving only a 2 ml gas space and close tightly. Keep sample at 35 to 40°C. **2. Organismic composition of rumen microbiota:** microscopy Prepare a wet mount with the rumen fluid and observe the microbiota in the **phase contrast microscope** beginning with the lowest magnification. You will be able to observe the **ciliates** best at low magnification while you need oil immersion objectives to clearly identify shapes of **bacteria** and **archaea**. Methanogens containing the blueish **autofluorescent F_{420}** are best observed in the fluorescence microscope with filter block A or D. What happens to the organisms if you aerate a small aliquote of the rumen fluid for a few seconds ? **3. Activity of rumen microbiota:** methylene reduction Prepare 3 tubes containing 9 ml of freshly collected rumen fluid each. Add 1 ml of glucose stock solution (100mM) to tubes #2 and #3 and the same volume of sterilized water to tube #1. Close the tubes airtight and keep tubes #1 and #2 in the waterbath at 39°C. Tube #3 is kept in boiling water for 5 minutes before it is transferred to the 39°C water bath. Incubate for 5 minutes. Mix 0.05 ml of a 5 mM **methylene blue** solution to each of the tubes, note the time of the addition and keep in the waterbath at 39°C. Record the time it takes for the methylene blue to loose its color. Compare with the color change in assay #3. Alternatively, you might use **resazurin** instead of the methylene blue. Why is the color change faster with resazurin ? **Methylene blue** (MW 319.86++aq.). A 5 mM (approximately) stock solution is prepared by dissolving 14.5 mg methylene blue powder in 10 ml 20 mM phosphate buffer pH 7. This stock solution is 200x concentrated. 50 µl dye stock solution are added to 10 ml culture. **Resazurin** (MW 229.18): preparation and storage of the 200x stock solution see below. **Glucose monohydrate** (MW 198): To prepare a 100mM stock solution dissolve 1.98g / 100ml distilled water and autoclave. Add 1 ml to 9 ml rumen culture.

4. Changes of habitat conditions: acid production
Prepare 3 tubes containing 9 ml of freshly collected rumen fluid each. Add 1 ml of glucose stock solution to tubes #2 and #3 and the same volume of sterilized water to tube #1. Close the tubes airtight and keep tubes #1 and #2 in the waterbath at 39°C. Tube #3 is kept in boiling water for 5 minutes before it is transferred to the 39°C water bath. After another 5 minutes, remove a 100 µl aliquote from the culture assay #1 and add to 100 µl bromothymol blue indicator prepared in a well of a white ceramic plate. Record the time it takes for the bromothymol blue to change its color from blue (pH 7.2) to green (pH 6.6) or yellow (pH < 6). Repeat with assays #2 and #3. Why is the pH decreasing during the incubation ?
Bromothymol blue: To make a 0.04% (w/v) stock solution dissolve 40 mg of bromothymol blue powder in 6 ml 0.01N NaOH and add distilled water to a final volume of 100 ml.

5. Habitat conditions: redox potential of rumen fluid
Oxygen which enters the rumen with the feed is reduced immediately by reducing compounds or through consumption by facultative aerobes. The rumen thus has a constantly low redox potential of –350 to –400 mV which can be illustrated qualitatively by adding 50 µl of resazurin solution to 10 ml of rumen fluid in a completely filled and tightly closed tube. At the concentration used, resazurin changes its color from purple to pink to colorless at a **redox potential** of approximately **– 45 mV**. As soon as the redox potential of the rumen fluid becomes low enough, the color of the indicator dye will disappear. Actively metabolizing microbes are able to maintain the low redox potential even in the test tube culture. If we allow oxygen to enter, the color changes back to pink.
Phenosafranin is a redox indicator dye with a **midpoint potential of –270 mV**.

You will obtain 5mM solutions of resazurin and phenosafranin with which you should design your redox experiments. Observe redox changes and add glucose to stimulate microbial activity if you cannot observe a color change within a few minutes. Incubation temperature is 39°C. How can you check whether or not the redox dye actually responds to reducing and oxidizing conditions ?
Resazurin (MW 229.18): 11.5 mg powder dissolved in 10 ml 20 mM phosphate buffer, pH 7 will give a 5 mM dye solution which is 200x concentrated. The solution is sterilized by filtration and kept in a brown glass bottle.
Phenosafranin (MW 322.80): 16.14 mg powder dissolved in 10 ml 20 mM phosphate buffer, pH 7 will give a 5 mM dye solution which is 200x concentrated. The solution is sterilized by filtration and kept in a brown glass bottle.

6. Volatile fatty acids: gas chromatography of the volatile metabolites
Since many of the **short chain fatty acids** are volatile when protonated they can easily be separated, detected and identified by gas chromatography coupled to **flame ionization detection** (FID). Acidify a 1 ml aliquote of each tube from the “pH-experiment” and inject 100 µl via the injection loop into the gas chromatograph. Follow separation and identify the peaks with VFA standards. Compare with a sample taken from the original rumen fluid.

7. Gas production:
Design an experiment in which you can follow the production of metabolic gases by the rumen community.

Laboratory Rules & Precautions

Use **good laboratory practice**! Do not contaminate yourself, others or the laboratory environment. All waste must be sterilized before disposal. It is necessary to work cautiously and, where necessary, aseptically.
Wash your hands before you leave the room and desinfect bench surfaces with 70 % ethanol.

Experiences gained	• Handling anaerobic microbes • Using the phase contrast microscope • Learnig how to design experiments • Detect microbial activities through product analysis and changes in habitat conditons
Timing	90 minutes
Reporting	• Make drawings of a few of the microbes observed in the microscope. Do not forget to note the magnification. • Report conclusions from the redox and the pH-indicator experiments. • What VFA are produced during glucose fermentation ?
Questions to be answered	The following exercises will allow you to quantitatively describe some of the microbiological processes which happen in the rumen. **1. Balancing fermentation** (Consult Fig. 16.41 in BBOM 9th (pg 683) before you attempt to solve this problem) Hungate observed the production of the following fermentation products in bovine rumen fluid (all values are given in µmoles per hour per gram of rumen contents): acetic acid 20.3, propionic acid 7.1, butyric acid 5.3, carbon dioxide 18.6, methane 7.8 a) Determine how much glucose must have been fermented in order to arrive at the quantities of fermentation products observed. Try to reconstruct a balanced stoichiometric equation for this mixed acid glucose fermentation. Electrons, charges and masses of all atoms involved must be balanced. b) Which organisms of the ones listed in table 1 might have been present in the rumen fluid from which the products under (a) were determined ? c) What is the fate of the fatty acids produced, what happens to the methane ? **2. Fermentation patterns** During digestion of 1044 g of hexose monomer ($C_6H_{12}O_6$, MW 180 D) *in vitro* by a rumen microbe culture the following products were detected: 356.3 g of acetic acid (MW 60), 155.9 g propionic acid (MW 74), and 134.9 g butyric acid (MW 88). The gas phase contained predominantly methane and carbon dioxide. a) Derive the balanced stoichiometry for the hexose fermentation process. b) When lactate was added to the rumen fluid one obseved an increase in the concentrations of propionate and acetate and the number of *Veillonella alcalescens* cells dramatically increased. How was lactate metabolized ? Use the metabolic summary (figure 1) to answer the question. c) One observes elevated concentrations of succinate and propionate but less methane in the rumen microbe culture when *Wolinella succinogenes* cells from a pure culture are added, and the medium is supplemented with fumarate. How can the result of this experiment be explained ? (see pg. 619 BBOM 9th for a short note on the metabolic abilities of *W. succinogenes*) d) When Monensin® (an ionophoric antibiotic acting as a growth promotor, often added to the feed of cows), was added to the complete experimental rumen microbe culture, one observed a decrease in the amount of methane formed but an increase in the concentrations of butyrate and propionate. What is the role of Monensin® as a feed additive ?

	3. Syntrophism in fermentation a) *Ruminococcus flavefaciens*, a cellulose degrader, produces in an axenic batch culture acetate, formate and succinate in molar ratios of 107:62:93 from 100 mMol/l glucose. Hydrogen and carbon dioxide were found in the gas phase, but no other organic metabolites could be detected. Reconstruct and comment the fermentation balance. (Hint: Have a quick look at question 6a) below for the enzymes involved in pyruvate conversion) b) Cont. from (3a): If *Methanobrevibacter ruminantium*, a hydrogenotrophic and formatotrophic methanogen (see BBOM tab. 14.5. and fig 14.7a), and *Ruminococcus flavefaciens* are cultured together, succinate and acetate are produced in molar ratios of 11:189. The gas phase contained CH_4 and CO_2 but no H_2. Reconstruct the fermentation balance of this mutualistic bacterial community for the degradation of 100 mMol/l glucose. c) Predict the outcome of the experiment in which Monensin® will be added to the two-membered community *in vitro*. **4. The rumen food web** a) Which enzymes are needed to hydrolyze the plant polymers, cellulose (beta-1,4-glycosidically linked glucose monomers), starch (alpha-1,4-glycosidically linked glucose monomers), and pectin (a galacturonic acid polymer) ? b) What is the role of the ciliates in the rumen ecosystem ? **5. Host microbe interactions** The production of acids during fermentation requires constant neutralization in order to maintain pH-homeostasis. Degradation would quickly be inhibited at pH values below 6. How do ruminants buffer their rumen ecosystem ? **6. Biochemistry of Pyruvate conversion** (these aspects will be studied in detail during the course "Biochemistry and Physiology of Prokaryotes" during the 4th semester) a) Pyruvate is one of the key intermediates between glycolysis and the different fermentation pathways. Different organsims employ different routes for pyruvate conversion which leads to the various fermentation patterns. Pyruvate can be converted by anaerobes … • to oxaloacetate by pyruvate carboxylase, • to acetyl~CoA by pyruvate-ferredoxin oxidoreductase, • to acetyl~CoA and formate by pyruvate-formate lyase or • to lactate by lactate dehydrogenase. Which of the organisms listed in tab.1 must contain which of these enzymes ? b) What can you find out about propionate production from lactate or succinate from the book ? (BBOM fig 13.69) c) *Ruminococcus flavefaciens* excretes succinate which it can apparently not convert further to propionate. Diagnose this inability from the point of view of the enzymes which must be lacking. d) By which enzyme do organisms which produce succinate from pyruvate carboxylate pyruvate? How is this carboxylation step achieved enzymatically by organisms which produce propionate from pyruvate via succinate ?
Outlook	Experiment 1 will be treated from a thermodynamic point of view in exercise 18.

Figure 1. Degradation of plant polymers in the rumen (the numbers refer to the catabolic abilities listed in table 1)