INTESTINAL FUNCTIONS IN ANIMALS

An Experimental Study in Horses, Pigs, Cows and Fish

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ABSTRACT

Animals and humans live in symbiosis with an active gastrointestinal ecosystem. The balance of the system is maintained by the main actors, the macroorganism, the microflora and the environment, in concert. Microflora-associated characteristics (MACs), defined as the recording of any anatomical structure, physiological, biochemical or immunological function in the host (macroorganism) that has been influenced by the intestinal microflora, are parameters reflecting the ecosystem. By utilizing six MAC-parameters the intestinal ecosystems in healthy horses, cows, pigs, salmons and cods have been studied and compared with previous results found in man, rats and mice. The following MACs were studied: conversion of bilirubin to urobilins, conversion of cholesterol to coprostanol, degradation of mucin, inactivation of tryptic activity, degradation of β-aspartylglycine and formation of short-chain fatty acids.

The found baselines for these MACs in faecal samples, as well as in different sections of the equine gastrointestinal tract and in rumen of cows, are comparative to baselines found in samples from healthy animals of other species. As the microflora in growing mammals undergoes environmental assimilation and maturation, changes of MACs were found in piglets related to ages.

The antibiotic zinc-bacitracin was found to influence upon three MAC-parameters in horses and four MACs in weaned piglets. Antibiotic treatment of salmon did also lead to different MAC-values. Four MAC-parameters in piglets at weaning were influenced by the probiotic preparation Alcare®, which contains *Bacillus licheniformis*. Different values of MACs were found in different breedings of salmons and in ecologically vs. conventionally raised piglets, which demonstrate influences of the environment on the ecosystems. Some of these parameters were found to be influenced by exogenous factors, such as physical effort of horses, as well as the diet to horses and piglets. The disease diarrhoea did also influence on MAC-values.

These results demonstrate that MAC-parameters used in these studies are applicable tools to measurements of actual complex intestinal state in horses, cows, pigs and other species.

The baselines in these species are more similar within the herbivores and within the omnivores than between these categories. When more baselines are established in more species, the concept can be of increasingly importance for studies of both general and specific factors influencing on microbial intestinal interactions with the host. As the gastrointestinal ecosystem represent a corner-stone for animal health and welfare, increased knowledge of the system creates possibilities for future strategies in animal husbandry – including fish farming.

Content

The present thesis is based on the following papers, which are referred to in the text by roman numerals:

I COLLINDER E, LINDHOLM A, MIDTVEDT T and NORIN E.
Six intestinal microflora-associated characteristics in sport horses.
Equine Veterinary Journal 2000; 32 (3), 222-227.

II COLLINDER E, BERGE G N, GRØNVOLD B, LINDHOLM A, MIDTVEDT T and NORIN E.
Influence of bacitracin on microbial functions in the gastrointestinal tract of horses.
Equine Veterinary Journal 2000; 32 (4), 345-350.

III COLLINDER E, CARDONA M E, KOZAKÓVA H, NORIN E, STERN S and MIDTVEDT T.
Biochemical intestinal parameters in pigs reared outdoors and indoors, and in germfree pigs.
Accepted for publication

IV COLLINDER E, CARDONA M E, BERGE G N, NORIN E, STERN S and MIDTVEDT T.
Influence of zinc bacitracin and *Bacillus licheniformis* on intestinal functions in weaned piglets.
Submitted

ABBREVIATIONS AND DEFINITIONS

ABG	Dept of Animal Breeding and Genetics
Carnivores	Flesh-eating animals
CONV	Conventional: An animal harbouring an uncontrolled microflora
CR	Colonization resistance: Resistance to colonization of the alimentary tract by newly ingested micro-organisms
Ecology	The branch of biology which deals with the mutual relations between organisms and their environment
Ecosystem	The fundamental unit in ecology, comprising the living organisms and nonliving elements interacting in a certain area
Foregut	Oesophagus, stomach
Forestomach	Reticulum, rumen and omasum in ruminants
GAC	Germfree Animal Characteristic: The recording of any anatomical structure, physiological, biochemical or immunological function in a macro-organism, which has not been influenced by the microflora
GF	Germfree: An organism free from all demonstrable forms of outer life including bacteria, viruses, fungi and protozoa
GI	Gastro-intestinal
GLM	Generalized linear model; statistical model
Herb	Any seed plant whose stem withers away to the ground after each season's growth
Herbivores	Animals subsisting on herbs or vegetables
Hindgut	Caecum, colon, rectum
IPs	Piglets born and raised indoors
LA	Dept of Large Animal Clinical Science
Large intestine	Caecum and colon
MAC	Microflora-Associated Characteristic. The recording of any anatomical structure, physiological, biochemical or immunological function in a macro-organism, which has been influenced by the microflora
ME	Milieu exterieur
MI	Milieu interieur

Midgut	Duodenum, jejunum, ileum
MT	Milieu total
Omnivores	Animals capable of subsisting on both animal and vegetable food
OPs	Piglets born and raised outdoors
Ruminants	1. Regurgitating and re-chewing food 2. An order of animals which have a stomach with four complete cavities (rumen, reticulum, omasum, abomasum), through which the food passes in digestion
SAS	A software system, trade mark for data analysis at SAS Institute Inc, North Carolina, USA
SCFA	Short-chain fatty acid
SUAS	Swedish University of Agricultural Sciences
TA	Tryptic activity
Terrestrial animals	Animals living on land or the ground; distinguished from aquatic, marine
ZB	Zinc bacitracin

1. INTRODUCTION

The most interesting feature of the digestive system is the fundamental intestinal ecology, the ecosystem (Fig. 1). This comprises of the living organisms, the nonliving elements and the host animal itself. Regarding the living organisms and their influence on health and prolonged life, Pasteur postulated that microbes were necessary for normal life (152) and Metchnikoff claimed that the intestinal flora was essential for prolongation of life (99, 100). This thesis deals with factors influencing ecosystems in the intestinal ecology of some animal species.

Soon after birth, the gastrointestinal (GI) tract of animals is colonized with bacteria, and complex populations are established. The number and species in the intestines are several hundreds in the terrestrial ones so far investigated (50, 67, 149). The macro-organism (host) lives in symbiosis with its intestinal microflora in an active ecosystem. This system is said to be in a balanced unbalance, namely a system based on many interactions, which in concert maintain the balance. The three major actors, the macroorganism, the intestinal microflora and the environment, are involved in this system (Fig. 1).

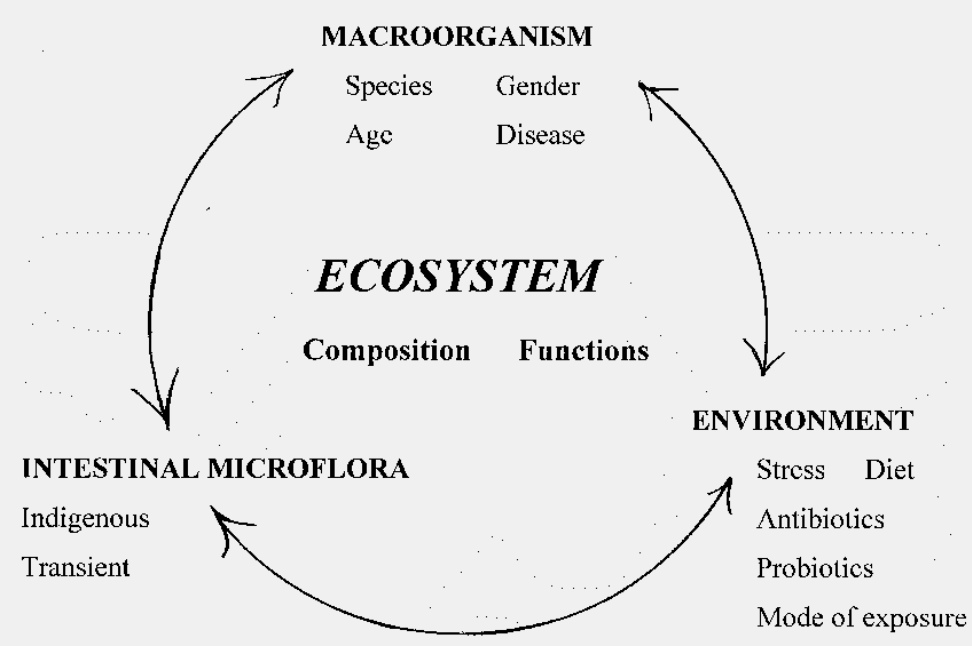


Figure 1. The gastrointestinal ecosystem in animals.

Any alteration in these three actors, such as antimicrobial treatment, various diseases, stresses, heavy changes of weather, dietary variations, including probiotics, malnutrition, starvation etc.

may affect the ecosystem (Papers I-IV). In principle, all these factors should be kept in mind when any ecosystem is studied.

The intestinal microflora is governed by a wide variety of host- and microflora-derived physico-chemical factors, such as pH value, redox-potential, nutrient availability, peristalsis and transit time. Furthermore, it is generally assumed that the GI immune system after development soon after birth coexists with the flora and maintains a balance with "nice" gut bacteria, while protecting against (and eventually eliminating) intestinal pathogens (63).

2. BACKGROUND

In an attempt to shorten down the description of intestinal physiology and microflora in the species that have been investigated, my description will mostly focus upon findings in the horse, as a model animal.

2.1. Fermentation

The equine large intestine serves to absorb electrolytes and water, both those of dietary origin and those secreted by the upper digestive tract (162), and serves as a site of microbial digestion. Both of these functions require a means of prolonging the rate of digesta passage and, although it is assumed that digesta is retained by either the presence of stationary haustral contractions or by retrograde propulsion, the actual mechanisms are poorly understood.

The GI microflora ferments both exogenous dietary and endogenous proteins and carbohydrates into several compounds, out of which short-chain fatty acids (SCFAs) are formed. They provide energy for the intestinal epithelium and other tissues. Moreover, they facilitate the host's absorption of Na and water. The flora also converts nitrogenous compounds to ammonia, amino acids, peptides and microbial proteins. Much of our understanding of the nutritional contributions of intestinal microbes comes from early studies of the ruminant forestomach and studies in germ-free (GF) animals. It is assumed that bacteria in the hindgut of mammals perform similar functions to those in rumen. Complicating factors are the different main sites of fermentation and the microenvironments in the hindgut. There are compartmentalized individual mixing pools of digesta in the horse (10). The most important population of microbes may be attached to the epithelial lining or embedded in the mucous layer, thereby difficult to investigate. Although the equine

large intestine is well developed and demonstrates a remarkable capacity for both digestion and absorption, these functions are shared to varying degrees by most mammalian species.

2.2. Gastro-intestinal environment

The luminal side of the gut is covered by a mucous layer. The mucous layer, mainly consisting of mucin, protects the mucosa, lubricates digesta to facilitate their passage, and forms a barrier which modifies the transport of solutes across the epithelium (28, 49). The mucin is highly hydrated, containing about 95 % water, and thus exists as a gel of glycoprotein net held together by hydrogen bonds and a few bridges. This layer offers little, if any, structural barrier to the movements of small ions, and therefore its ionic composition most often reflects that of the digesta. However, macromolecular particles are assumed to be excluded from the mucous layer (45), which probably prevents damaging of substances close to the mucosa.

Despite the apparently free movement of small ions through the luminal mucous layer, the H-ion concentration in the mucous layer is remarkably independent of the H-ion concentration in the bulk luminal fluid. This stable pH microclimate at the luminal epithelial surface explains why absorption of SCFAs from the colon is largely independent of the pH of the luminal fluid (66).

The environment in the hindgut of mammals is suitable for active microbial growth and metabolism of many different species. The temperature is constant, the pH value is usually between 6.5 and 7.5 throughout the intestines, and the oxygen tension is extremely low. Electro-potentials (Eh) of about – 200 mV are often recorded. Thus, most micro-organisms isolated from the hindgut are anaerobes.

Along the hindgut, the dry matter content increases as a result of the water absorption.

2.3. Physiology of the alimentary tract of horses

The small intestine of a 500 kg horse is 15 to 22 m long, and 7 to 10 cm in diameter. Much of the fat and protein, and a great deal of the soluble carbohydrates are degraded and absorbed there, together with most of the vitamins and minerals (140). Horses have a characteristic great amount of liquid secreted into the lumen, through the small intestine. In general, ingested liquid reaches caecum within 2 hours and feed particles within 6-8 hours respectively (10). The passage of both

liquids and particulate matter throughout the colon is slower and occurs over a period of some days (10, 136, 173).

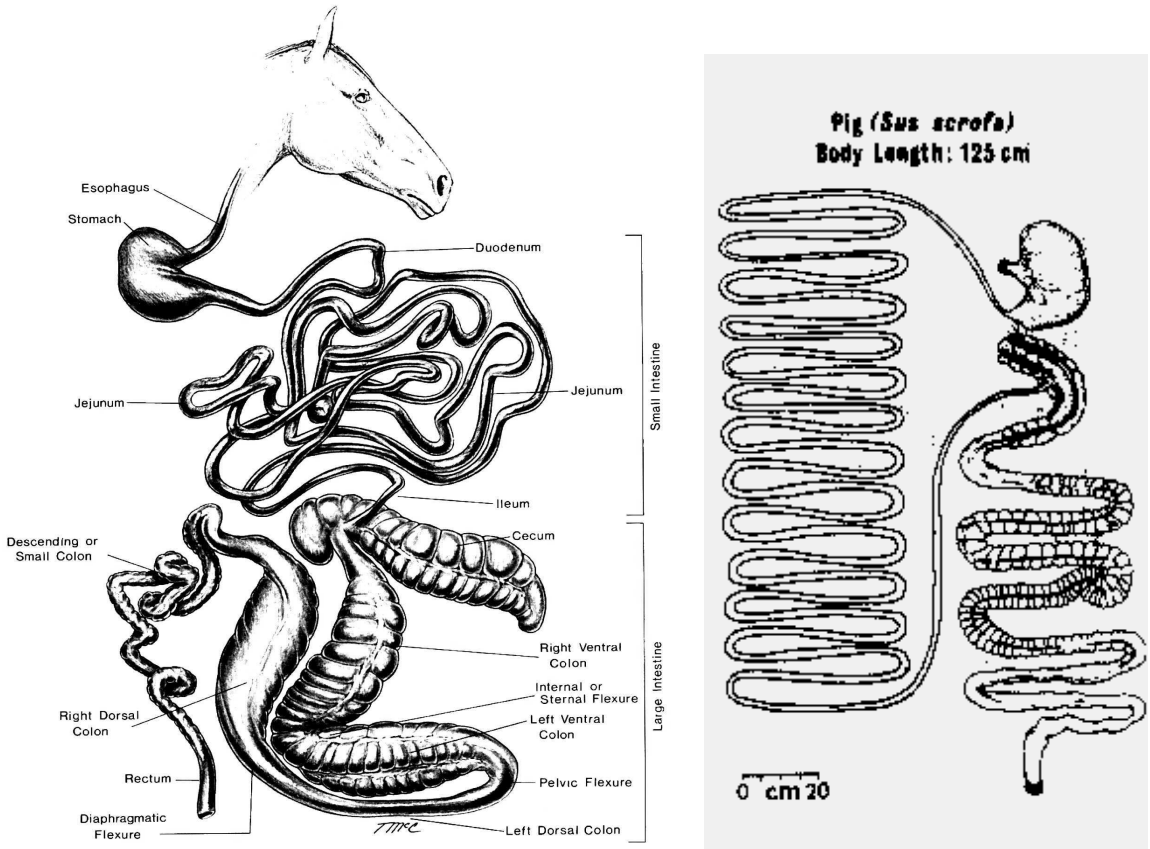


Figure 2. Gastrointestinal tracts of the horse and pig. The caecum and colon of the horse are sacculated as a result of longitudinal bands of muscle. The caecum of the horse is relatively voluminous, but its ventral and dorsal large colon are developed into even more voluminous individual compartments. A major portion of the colon of pigs is arranged in two spirals. The ascending spiral reverses itself to form the descending spiral, as indicated by the slight downward bend near the centre of the colon. The pig caecum and a considerable part of the colon are also sacculated. (Adapted from 6 and 88)

2.4. Gastrointestinal microbes in the horses

The microbes in the equine stomach and in the small intestine are mostly bacteria, but in the large intestine fungi and protozoa are present as well (74). As both fungi and protozoa have a

relatively slow multiplication rate, their survival depends on transit time of the ingesta through the large intestine, which may last for some days. Some fungi break down fibres, but their relative importance in equine digestion of cellulose has not been satisfactory investigated yet. The amount of protozoa varies from a few to one million per gram ingesta, but their ecological importance is still vague. Because of the indefinite influence of fungi and protozoa, the term 'the microbes' do still mainly include bacteria.

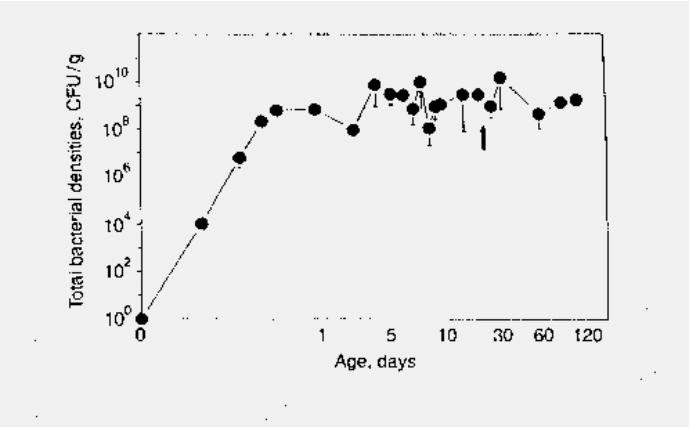
In mammals, the bacterial part consists of several hundred bacterial species, and anaerobes outnumber aerobes by a factor of 2-3 logs (156). Its composition may differ considerably among animal species, among individuals within the same species, and also during lifetime within the same individual (157). Each kind of animals and each local site of the intestine seems to have its own unique flora which has evolved with the host over millions of years, and simultaneously been adjusted to the actual habit and feed.

For a closer understanding of the intestinal flora, most efforts have been made to enumerate, isolate and identify the microbial species in the flora. Some examinations of the normal flora of horses have shown that lactobacilli are dominating in the stomach, and various proteolytic bacteria have been found in jejunum and ileum (95). Both groups are present in the large intestine. Most attention to the bacterial flora has been directed to its possible role in diseases, for instance presence of *Clostridium difficile* and its toxins.

2.5. Gastrointestinal microbes in the pigs

A great amount of anaerobic bacteria in the large intestine of adult pigs are gram-positive, mainly cocci, lactobacilli, eubacteria and clostridia (145), and the same species profile has been found in faeces (148). In piglets, as in other species, the establishment of an adult colonic microflora is a gradual process, which contains shifts in the relative abundance of various bacteria throughout the first months of their life (Fig. 3 and Fig. 4). Shifts of the flora in piglets are discussed in Paper III.

Figure 3. Total bacterial counts (aerobes + anaerobes) in faecal samples obtained from distal colon of pigs during the first 120 days of their postnatal development. Data are presented as means ± SE for log of total counts (CFU/ g faeces). The arrow indicates age of weaning (21 days). (Adapted from 169)



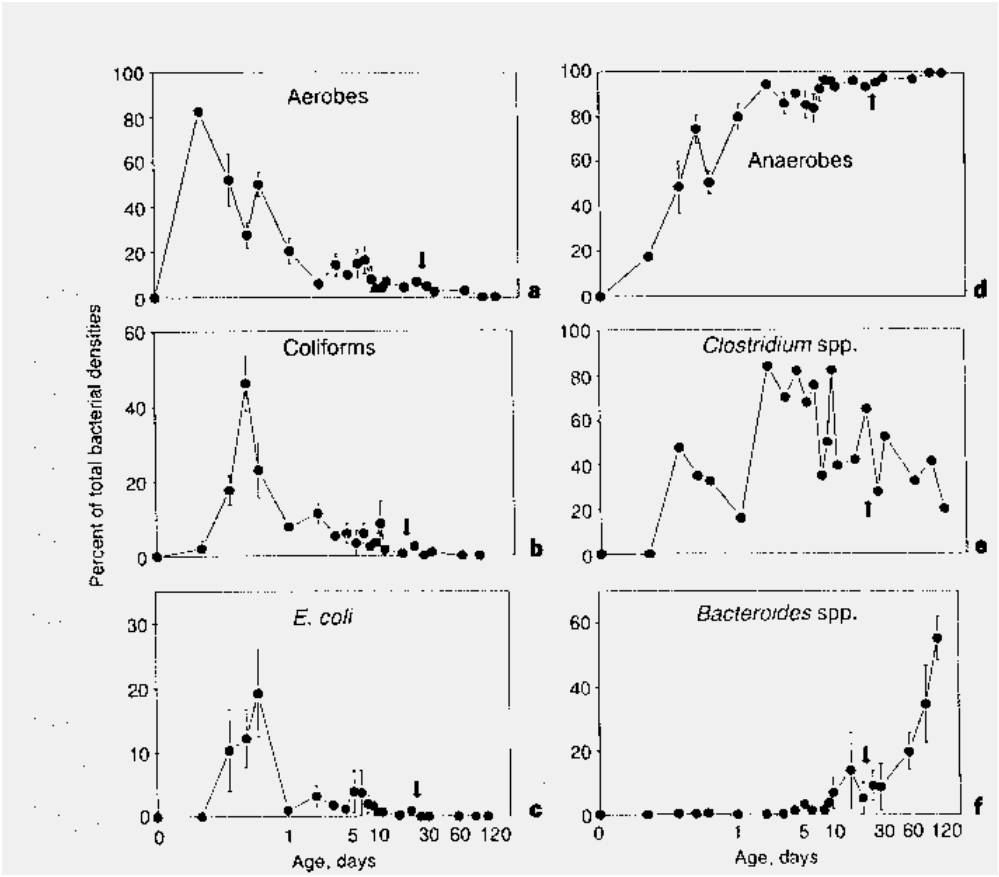


Figure 4. Changes in densities of selected bacterial groups in the distal colon of pigs during their first 120 days after birth. Arrows at 21 days indicate age of weaning. Data are presented as percentages of total bacterial counts (aerobes + anaerobes) represented by each bacterial group. Means ± SE are illustrated (Adapted from 169)

2.6. Gastrointestinal microbes in the ruminants

Rumen microbiology has been much more studied than any other GI ecosystem, and this ecological system has been used as a model for studies of different microbial interactions. The first pure culture of a cellulolytic rumen bacterium, *Ruminococcus flavefaciens*, was made by Kaars Sijpesteijn 1943 (75). Several hundred species of rumen bacteria have then been characterized by enumeration, isolation and identification (67).

The reticuloruminal contents of cattle can provide 10-15 % of the animal's body weight. They become colonized, predominantly with enterobacteria and streptococci, during the first week of life (44). These are later joined by lactobacilli, which persist, along with the streptococci, in the nursing animal. Weaning is followed by the development of a complex intestinal group of flora and fauna.

Today, major pathways involved in the microbial breakdown of feeds to compounds from which the microbes obtain energy for growth and the relevant anabolic processes are well known. Despite the enormous work that has been performed, it is still difficult to predict or model what actually happens in the rumen ecosystem. This system is very complex containing three totally different types of micro-organisms, namely bacteria, protozoa and fungi. Countless interactions occur between them and also interactions between the host and its microbes. This complex teamwork with extensive interactions makes it impossible to judge the contributions of the important groups of bacteria in the rumen without considering the fungi and protozoa. The fungi fermentation products are of a mixed acid type with the production of formate, acetate, succinate, lactate, ethanol, CO_2 and H_2. The fungi are also able to produce extracellular proteases (51, 89, 172). The rumen protozoa are mainly flagellates and ciliates. Products of the protozoal fermentation include acetate, butyrate, lactate, CO_2 and H_2 but not propionate (20). The fungi interact with both bacteria and protozoa, with variable results depending on species (2, 15, 51, 89, 172, 182). Thus, the intestinal flora and its co-functions with the host are probably influenced by fungi and protozoa, which in the rumen are known to exhibit much variation from animal to animal. Another difficulty to determine cross-talks between the ruminant and its flora is that several strains are most likely unknown and many are probably impossible to cultivate. From the thesis of M. Murphy (124), and the methods for metabolic fingerprinting and fermentative capacity (77, 81), it seems that the enzymatic profile of the flora might be a better indicator of the flora than the identified strains of bacteria. That is to say, what the flora can do.

2.7. Comparative digestion among mammals

Herbivores have evolved various strategies for exploiting plant food. Since many forage plants contain large amounts of biomass in the form of cellulose, hemicellulose and pectin, for which animal digestive systems have no required enzymes, the herbivores have anatomical and/or physiological adaptations of the digestive tract to compensate for assimilation of this material. Herbivorous fishes, reptiles, birds and mammals have an enlarged and/or elongated digestive tract, often including fermentation chambers or sacs in the foregut or in the hindgut. Two major alternatives facing the adaptation of the herbivores have been proposed (178): firstly, the utilization of the slower digested cellulosic matter via retention in a fermentative organ where symbiotic micro-organisms do the work of digestion, either in the foregut or hindgut. Secondly, in the case of high food intake, rapid passage and little utilization of the cellulosic matter, the forage source is exploited for available protein, sugar and starch. Either of these two digestive strategies can be combined with selective feeding behaviour whereby the more available and digestible plant part is utilized. The interrelationship between body size, diet and digestive strategy poses an interesting problem. The relationship between gut capacity and mass of fermentation contents is isometric with increased body size regardless of whether they are foregut or hindgut fermenting animals (137). An animal's mass-specific metabolic rate decreases as the body increases, while the ratio of gut capacity to body size remains almost constant. Hence, digestive capacity is associated with large body size while selective ability is associated with small body size. This function suggests that large animals retain slow fermenting residues longer times than smaller animals do. Retention time of digesta in horses has been shown to take from some to reach anus (173), up to several (10) days, and in pigs around 24 hours (9). The time of stained hay in cattle has been shown to take 90 and 120 hours (13, 173), and 90 % of markers administered into the abomasum (secretory stomach) were excreted in faeces within 24 hours (13). Digesta might be retained in rumen for 3-4 days.

Small animals can select more nutritious and rapidly digested material to compensate their lack of retention (Fig. 5) (42). Another strategy seen in rodents and lagomorphs (rabbits, hares, pika) is cecotrophy that can compensate for some of the problems of small size. Since the lower intestinal tract is an area of poor absorption (159), coprophagy might lead to more effective utili-

zation of nutrients, especially of the essential amino acids and vitamins synthesized in caecum by the microbes (161).

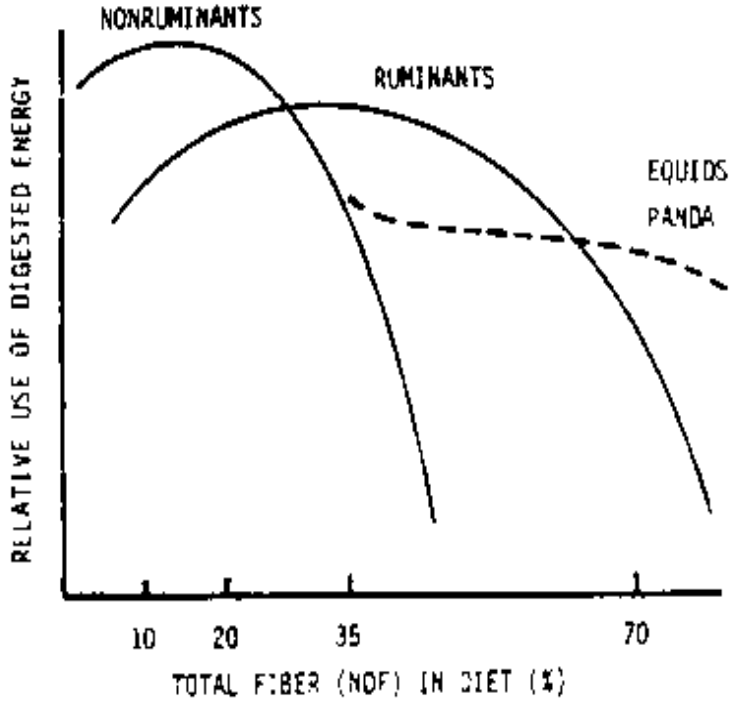


Figure 5. The optimum level of fibre for non-ruminants is less than that for ruminants because more microbial protein is lost in the faeces of non-ruminants, and the digestive capacity for fibrous carbohydrates is less than in the ruminants. A few non-ruminants tolerate high fibre diets by being able to ingest very high feed intakes to obtain their requirements from non-fibre components. This results in fast passages and low fibre digestibility. Ruminants fail on low quality of very high fibre diets because of the cost of rumination and other digestive work required to eliminate lignified fibre from their complex gut (177). (Adapted from 178)

Thus, ruminants have a markedly reduced capacity of passing poorly digestible material out, see fig. 16 (page 49).

2.8. Investigation of the normal microflora

The normal or indigenous microflora of animals and man consists of a resident part, which largely stays with the host organism, and a transient part which dynamically changes in composition. The turnover of the transient part of the microflora in a habitat of the GI tract depends both on the composition of the resident flora and on the degree of contamination (qualitatively and

quantitatively) of ingested food and beverages. Transient microbes can also be derived from other habitats or from elsewhere on the host, as skin and upper respiratory membranes. Some of the pathogens are indigenous to the GI ecosystem and can live in harmony with their hosts, and becoming pathogenic only when the ecosystem is disturbed in some way.

The GI microflora can be investigated in three different routes:

A. Enumeration, isolation and identification studies. Utilizing classic and modern molecular methods, the complexity of the GI microflora have partly been documented. However, it is well known that a quantitative and qualitative evaluation of the microflora is extremely time consuming and difficult to perform.

B. Capability. What can the microflora do? The same difficulties as in A holds true also for this way of investigating the microflora. However, the enzymatic capacity of the flora might be a better flora-indicator than A (142).

C. Performance. What has the microflora done? To investigate this, one must clarify which mechanisms and reactions are related to the host and which are related to the microflora itself. In short, the host's side of the ecosystem can be defined as Milieu Interieur (MI), the non-host side as Milieu Exterieur (ME) and MI plus ME together as Milieu Total (MT) (107). When baselines in MI are established, the normal functions of the flora as well as alterations in these functions can be worked out by applying the so-called MAC/GAC concept. A Microflora-Associated Characteristic (MAC) has been defined as the recording of any anatomical structure, physiological, biochemical or immunological function in an organism that has been influenced by the microflora (114). When active microbes that actually influence the parameter under study are absent as in GF animals, healthy newborns and sometimes following antimicrobial therapy - the particular recording is termed Germfree Animal Characteristic (GAC). Some intestinal parameters, listed in Table 3, have been studied to some extent in parallel in CONV and GF animals.

2.9. Colonisation resistance

The complex microflora confers a variety of beneficial characteristics on a host, including resistance to colonization of the GI tract by newly ingested micro-organisms. The gut microflora's protective role has been shown by successful treatment of diarrhoeal cases by administration of faecal suspensions from healthy subjects as enemas (47, 57, 153). Such protective role has also been demonstrated in large studies in poultry, where oral administration of a faecal suspension from adult birds prevented the growth of salmonellae in newly hatched chicks (132, 183).

2.9.1. Antibiotics

Since more than 40 years ago, it has been known that some antibiotics suppress that part of the flora that normally provides protection against infection of *Salmonella*, *Vibrio cholerae* and *Shigella flexneri* (21, 53, 54). Antimicrobial therapy causes alterations in the composition of the intestinal microflora. The extent of the changes depends on the antimicrobial spectrum of the drug and its pharmacokinetics and on the sensitivity profile of the microbes for that particular drug. Studies on antibiotics given to mice and rats have shown that antimicrobial compounds have different influences on the MACs (16, 17, 29, 32, 59, 108, 114, 118, 128). Disturbances in MACs have also occurred in healthy volunteers (humans) after antimicrobial treatments (normal doses) (30, 70, 71, 108, 114, 128, 129, 150, 165). Antimicrobial therapies to domestic animals are described in Paper II and Paper IV.

2.9.2. Probiotics

Probiotics can be defined as "live microbial feed/food supplements that beneficially affect the host animal by improving its microbial balance" (55). Several probiotic preparations exist commercially. They are widely used in agriculture for farming pigs and poultry, and are also popular as human food supplements. Many attempts have been made to avoid intestinal disorders seen after antibiotic treatment by giving probiotics. Much is written but little is known about the beneficial actions of probiotics (97). Important factors, such as survival of the strains and their ability for metabolic expression in the GI tract, have not been satisfactory investigated. The selection of

suitable microbes as probiotics has most often been based on experience, not science. One probiotic preparation and its influence on young pigs described in Paper IV.

Table 2. **Influences of the microflora on some major intestinal anatomic, physiological and biochemical parameters.**

Parameter	*MAC*	*GAC*	*Microbes*
Anatomical / Physiological:			
Intestinal wall	Thicker	Thinner	Unknown
Cell kinetics	Fast	Slower	Unknown
Migration motor complexes	Normal	Fewer	Unknown
Production of peptides	Normal	Altered	Unknown
Sensitivity to peptides	Normal	Reduced	Unknown
Caecum size (rodents)	Normal	Enlarged	Partly known
Osmolality	Normal	Reduced	Unknown
Colloid osmotic pressure	Normal	Increased	Unknown
Oxygen tension	Low	High (as in tissue)	Several species
Electro-potential Eh, mV	Low (< -100)	High (> -100)	Unknown
Biochemical:			
β-aspartylglycine	Absent	Present	Mixture of microbes
Bile acid metabolism	Deconjugation	No deconjugation	Many species
	Dehydrogenation	No dehydrogenation	Many species
	Dehydroxylation	No dehydroxylation	Few species
Bilirubin metabolism	Deconjugation	Little deconjugation	Many species
	Urobilin	No urobilin	One species
Cholesterol	Coprostanol	No coprostanol	Few species
Intestinal gases	Carbon dioxide	Some carbon dioxide	Many species
	Hydrogen	No hydrogen	Some species
	Methane	No methane	Few species
Mucin	Degraded	No degradation	Several species
Short-chain fatty acids	Large amounts	Far less	Many species
Tryptic activity	Little or absent	High activity	Few species

(Adapted from 110)

Table 3. Methods used and microbes involved in the studied MAC/GAC concept.

Parameters	*MACs*	*GACs*	*Microbes involved*	*Methods*	*Ref*
Conversion of bilirubin to urobilins	Urobilins present	No urobilins present	*Clostridium ramosum*	Spectrophotometry	105
Conversion of chol-esterol to coprostanol	Coprostanol present	No coprostanol present	*Eubacterium coprostanoligenes*	Gas chromatography	115
Degradation of mucin	Mucin degraded	No degradation	Several species	Electrophoresis	58
Inactivation of tryptic activity	Low or no activity	High activity	*Bacteroides distasonis*	Spectrophotometry	130
Degradation of β-aspartylglycine	Absent	Present	Mixture of microbes	Electrophoresis	179
Formation of SCFAs	High amount, several acids	Low amount, mainly acetic acid	Several species	Gas chromatography	155

3. INTESTINAL MICROFLORA-ASSOCIATED CHARACTERISTICS

The exogenous factors life habits, diets, antibiotics and probiotics most likely influence the intestinal flora. For evaluation of values reflecting cross-talks between the host and its intestinal flora, and for evaluation of such external influence on these, the following six biochemical MAC-parameters (Table 3) have been chosen:

Conversion of *bilirubin to urobilins* and of *cholesterol to coprostanol*, aimed to investigate hepatic and intestinal co-functions;

Degradation of *mucin*, in order to study an intestinal characteristic of major importance in preserving the integrity of the intestinal mucosa;

Inactivation of *tryptic activity*, reflecting complex interactions between pancreas-derived trypsinogen, dietary compounds and intestinal micro-organisms;

Degradation of *β-aspartylglycine*, reflecting the so-called colonisation resistance;

Formation of *short-chain fatty acids*, reflecting dietary intestinal co-functions.

These functions are briefly described below.

3.1. Conversion of bilirubin to urobilins

Bile pigments consists partly of bilirubin, a toxic and water insoluble end-product of the catabolism of haemoglobin, and some other heme-containing substances. Bilirubin is conjugated to the less toxic and water-soluble glucuronate in the liver, and excreted in the bile. In the intestine, bilirubin conjugates are de-conjugated and transformed to a series of urobilinogens, usually termed urobilins. A small amount of the enzymes β-glucuronidases is derived from endogenous sources (146), but most are of microbial origin. These de-conjugating enzymes are produced by a large variety of intestinal microbes (56), but the capacity to alter bilirubin to urobilins seems to be a rare property among intestinal micro-organisms. So far, only one bacterium, *Clostridium ramosum*, has been found capable of performing this transformation (60, 113).

Since bacterial strains producing β-glucuronidases are several (56, 62, 78), they are probably colonizing the intestines earlier than the few strains capable of converting de-conjugated bilirubin to urobilins. As no substantial absorption of bilirubin conjugates takes place from the human GI tract (87), it is generally believed that the bilirubin conjugates must first be de-conjugated before absorption of the liberated un-conjugated bilirubin can occur. These factors might interfere with physiological hyper-bilirubinemia (23, 111, 139), in newborn children

and piglets, before they have enough colonized microbes for each step of microbial detoxification and elimination of bilirubin.

Studies on this parameter in rats and man have shown that intake of ampicillin, bacitracin, clindamycin and erythromycin significantly suppress faecal excretion of urobilins, whereas intake of metronidazole has no effect (59, 108, 114, 128). Furthermore, intake of vancomycin suppress bilirubin metabolism strongly in man but intake of doxycycline, nalidixic acid, ofloxacin or trimethoprim/sulfamethoxazole has no significant effect on formation of urobilins (19, 108, 114, 128, 150, 165).

3.2. Conversion of cholesterol to coprostanol

Cholesterol is a component of all mammalian cellular membranes and a precursor of steroid hormones, vitamin D and bile acids. It is derived from both endogenous synthesis occurring mainly in the liver and from exogenous sources. The main elimination routes for plasma cholesterol are biliary excretion of cholesterol into the intestines. Intestinal cholesterol can be reabsorbed or undergo microbial conversion. The major microbial metabolite is the un-absorbable coprostanol, which is excreted with the faeces. The organisms responsible for the conversion are strictly anaerobic, gram-positive, nonspore-forming rods, mostly of the genus *Eubacterium*. Strains capable for this conversion have been found in rats (48), human (147) and baboon (122). Previously a strain, *Eubacterium coprostanoligenes* ATCC 5122, has been isolated (52), which has the capacity to convert cholesterol in different animal species. Oral administration of this strain to hens causes a significant increase in intestinal conversion of cholesterol to coprostanol (91), and this strain given to rabbits has a hypo-cholesterolemic effect (90). The mechanism of cholesterol reduction has been studied (144). The reaction most likely proceeds by a sequential oxidation (Fig. 6), whereby the structure changes to an un-absorbable metabolite (14). This hypothesis is further supported by the detection of coprostanone in faeces and by the reduction of 4-cholesten-3-one and coprostanone to coprostanol by faecal bacteria (48, 123).

By definition, GF animals lack the intestinal microbial excretion route for cholesterol. As early as 1959, the serum cholesterol concentrations were found higher in GF than in CONV rats fed a casein containing diet (40).

Studies in rats have shown that ampicillin, bacitracin, clindamycin, dirithromycin, erythromycin, kanamycin, penicillin and tetracycline suppress the cholesterol conversion significantly and that metronidazole has a small effect (29, 59). Studies in man have shown that an-

tibiotics with an anaerobic, gram-positive profile such as oxytetracycline, bacitracin, clindamycin, metronidazole and vancomycin significantly reduce the conversion of cholesterol to coprostanol and that antibiotics acting mainly on aerobic, gram-negative organisms have a small or no influence on this metabolism (80, 112, 117). Those antibiotic-induced alternations in microbial conversion of cholesterol to coprostanol might influence other results of cholesterol metabolism, such as serum cholesterol levels and development of atheromatous arterial disease (110).

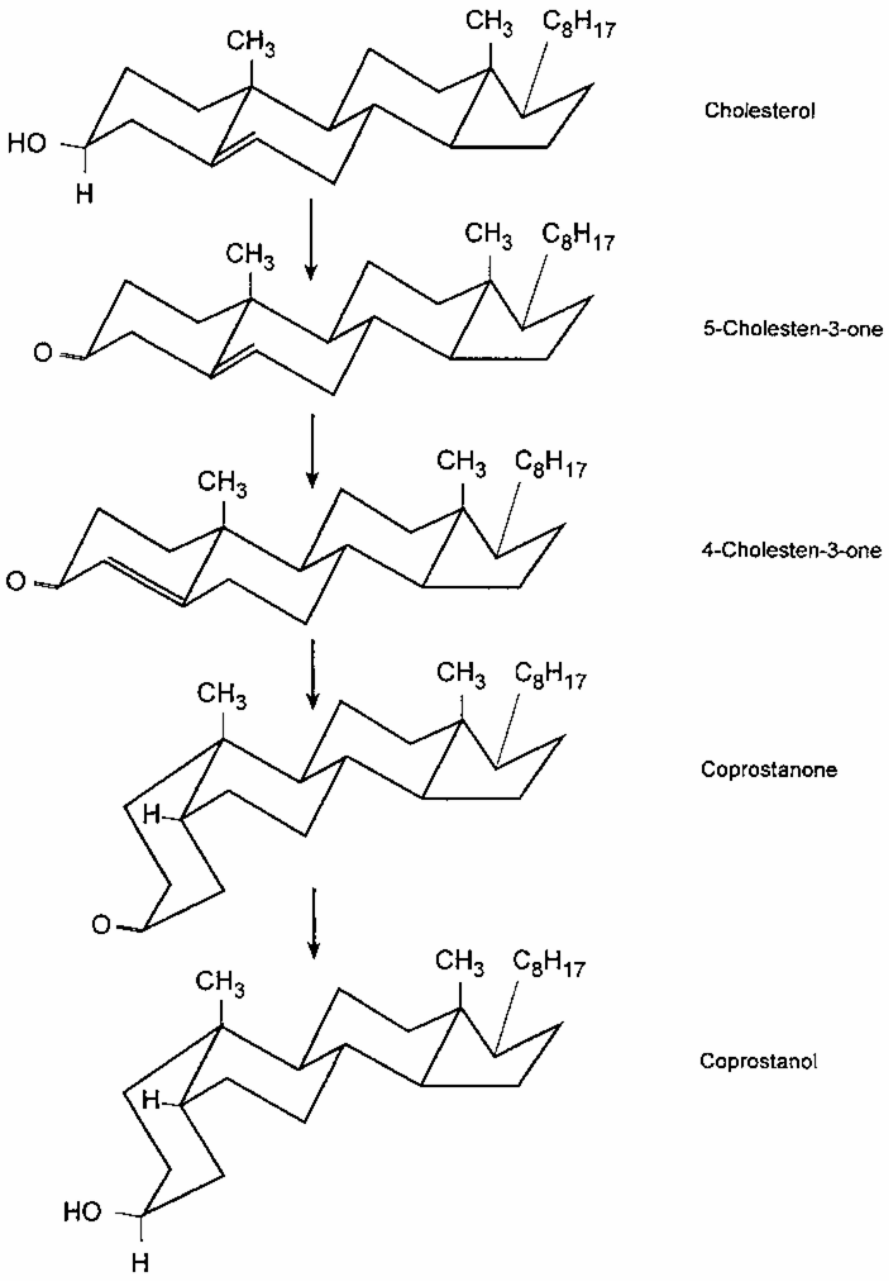


Figure 6. Proposed reaction pathway for microbial conversion of cholesterol to coprostanol. (Adapted from 144)

3.3. Degradation of mucin

Mucin in the GI tract is produced by goblet cells in the surface and crypt epithelium and in glandular mucous cells arranged in acinar structures in the submucosa. Mucin consists of a peptide core with oligosaccharide side chains *O*-glycosidically bound. Mucin has several important physiological and patho-physiological roles. It acts as lubricant, as a barrier and stabiliser for the intestinal microclimate, and as a source of energy for the microflora. There is a growing evidence that alterations of mucin may be relevant in the pathophysiology of some intestinal diseases, such as ulcerative colitis, Crohn's disease, gastric and duodenal ulceration, colon adenocarcinoma, intestinal strangulation and mucus obstruction in cystic fibrosis (28).

GF rats excrete large amounts of mucin with their faeces, whereas CONV rats and healthy adult humans do not excrete mucin (30). Some strains belonging to the *Bifidobacterium*, *Bacteroides* and *Ruminococcus* genera (65) have been isolated and related to degrading mucin. The complete degradation of mucin might require various glycosidases and peptidases, and it is possible that the degradation is a sequential action of bacterial strains (65, 76). However, one *Peptostreptococcus* strain (31) has been shown to be without glycosidases, and still degrade mucin totally with its peptidases. A markedly altered establishment of mucin degradation has been reported earlier in adult ex-GF rats (115). A pronounced difference of priming the mucin degradation has been found between breast-fed and formula-fed children, probably partly depending upon diet and environment (106). These findings and also a spontaneous reestablishment of mucin degradation after antibiotic treatment in healthy human adults (30), confirm that mucin-degrading microbes are widely distributed in the environment. This subject is discussed in paper III.

Studies in rats have shown that bacitracin, clindamycin and vancomucin suppress degradation of mucin (17, 29, 32, 59, 114, 128) and man (19, 30, 114,165).

3.4. Degradation of tryptic activity, TA

Trypsin is chosen as a model for studying endogenously derived enzymes. It is excreted as a precursor, trypsinogen, from the pancreas, and activated in the small intestine, mainly by brush border enzymes (110, 126). TA involves the net sum of processes such as the secretion of trypsinogen, the activation of trypsinogen to trypsin and the presence in the intestine of host-, microbial- and diet-derived compounds that inactivate or degrade trypsin. Faeces of GF rats contain large amounts of TA, whereas far less is found in their CONV counterparts (130).

Obviously, intestinal microorganisms are responsible for the inactivation of trypsin, and at least one strain of *Bacteroides distasonis* (143) capable of performing this inactivation has been isolated from both rats and mice. TA in CONV rats has been shown to be influenced by several antibiotics (16, 59). In the papers I-IV all tryptic activities determined are markedly low and diet, antibiotic or probiotic substance have small influence only. This is in concert with previous findings in rats and man following ingestion of antibiotics (16, 59, 114, 128, 129, 165).

3.5. Degradation of β-aspartylglycine, β-asp

Resistance

A specific group of microbes in the flora, the opportunistic pathogens, may cause disease when regulatory mechanisms are impaired (5). The resistance of the ecosystem to colonization of the alimentary tract by newly ingested micro-organisms is often called colonisation resistance (CR) (5, 174, 175, 176, 180, 181).

The presence of the dipeptide β-asp in faeces from mammals indicates a reduction in CR. The biochemical background for the presence of β-asp in the faeces is probably as follows: host-derived intestinal proteolytic enzymes break down some dietary proteins to the β-carboxyl dipeptide β-asp. The β-carboxyl dipeptide bonds are then cleaved by proteases derived from microbes (181). This is substantiated by findings in GF animals: lambs, piglets, rats and mice. In faeces from GF lambs and piglets (Welling G.W., personal communication), and adult GF rats and mice (115, 179), β-asp is always found, whereas never in samples from their CONV counterparts. Thus, the presence or absence of β-asp represents a MAC/GAC parameter, depending on (i) the presence of dietary precursors, (ii) the presence of host-derived proteolytic enzymes, and (iii) the presence/absence of microbially derived proteolytic enzymes. Previously it has been shown that the amount of β-asp gradually diminishes in faeces from ex-germfree mice, as the number of various microbes in their GI flora gradually increases (179).

This parameter has previously been found in some few humans treated with antibiotics (165, 180, 181). Also in domestic animals, treated with antibiotics, this parameter can possibly be used to mirror the level of CR.

3.6. Formation of short-chain fatty acids, SCFAs

The GI microflora ferments dietary and endogenous carbohydrates into SCFAs that provide energy for the gut epithelium and other tissues and facilitate the absorption of Na^+ and water (167). The major substrates for this fermentation, regardless of the location of the fermentation chamber, are complex carbohydrates originating from plant cells and, for the most part, consisting of cellulose, hemicellulose, pectins, starch, dextrans and soluble carbohydrates (mono- and disaccharides) (84). SCFAs are produced in the GI tract of all species, but the relative importance of this production vary from species to species. They contribute a considerable energy to herbivores, especially ruminants (70-80 %), and also a great deal of caloric requirements to omnivores, such as pigs, but less in carnivores. The formation of SCFAs is of importance for the maintenance of a balanced intestinal ecosystem (109).

The microbial origin of intestinal SCFAs has been substantiated by comparative studies in GF and CONV mice and rats (68). Faecal SCFAs represent the net sum of production, absorption and possible secretion of SCFAs throughout the GI tract. Because of the many physiological and clinical roles attributed to SCFAs, ranging from Na^+ absorption (184) to cancer pathogenesis (85, 121), there is a considerable interest in this parameter. The mere fact that intake of antibiotics (70, 71), as well as dietary changes (73, 103, 155), may cause alteration in excretion of SCFAs, underlines the importance of studying this function in greater detail. The possible consequences of such an alteration should be analysed according to an outlined scheme for domestic animals alike the outlined for humans in Table 4.

Table 4. A consequence analysis of one microflora-associated characteristic, SCFAs.

Statement: SCFAs are normally produced in high amounts by the intestinal flora; they are mainly absorbed and partly excreted in faeces			
Mechanism behind possible consequences: Biochemical: SCFAs are involved in several metabolic pathways Immunological: Uncertain consequences			
Place:		Locally:	In the intestinal lumen
			At the mucosa surface
			Within the mucosa cells
		Distant:	In the liver, pancreas, brain etc.
Form:	Direct:	Locally:	Main anions in intestinal content
			Growth promotion of some microbes
			Growth suppression of others
			Growth regulation of mucosa cells
		Distant:	Energy supply to the general metabolism
	Indirect:	Locally:	"Promoted" microbes produce suppressive bacteriocins
			Direct suppression provides niches for other microbes to grow
		Distant:	Metabolic alterations act on production of insulin, etc.
Consequence:	Physiological:		Locally the SCFAs are parts of direct/indirect regulatory mechanisms for water and electrolyte absorption; the net effect is antidiarrhoeic, involved in regulation of carbohydrate metabolism, etc.
	Pathophysiological:		Involved in hepatic coma. Probably involved in colonic cancer, ulcerative and pseudomembraneous colitis, etc.

4. Host organisms

The GI microflora has the ability to adapt itself into the most suitable in any animal. In this thesis, baselines of MACs in domestic mammals are investigated; one hindgut herbivore, one foregut herbivore and one omnivore. Additionally, three hindgut omnivores and two species of fish are included, as models for comparative studies on baselines.

4.1. Mammalian herbivores

The herbivores are the most abundant in numbers of mammals and show the widest ecological distribution. Most mammalian herbivores obtain a substantial part of their nutrients by retention and microbial fermentation of plant material in a forestomach or in the voluminous caecum, and/or in colon. Horses and cows are representative of these two types.

4.2. Mammalian omnivores

Omnivores feed on both plants and animals. In several omnivores, digesta is retained in caecum and/or in colon, thereby facilitating microbial degradation. Values of intestinal functions for pigs, rats, mice, and humans are compared in this work.

4.3. Fishes

The GI tract of fish provides a nutrient rich habitat for microbial growth (25, 151), and some data imply that the host specificity of bacteria colonizing fish is low compared with mammals (4). To reach some knowledge, four intestinal functions of farmed salmon and cods are included in the thesis.

5. AIM OF THIS STUDY

Applying the MAC/GAC concept for measurement of the intestinal ecosystem(s), baseline values for up to six parameters were established in healthy individuals; in one hindgut herbivore (horse), one foregut herbivore (cow) and one omnivore (pig). These values from three hindgut omnivores (man, rat, mouse), and some values from two species of fish, were included for comparative studies.

Additionally, some major factors known to be of importance for establishment and maintenance of intestinal ecosystems were investigated:

A. Age. (Paper III);

B. General exogenous factors: environment, physical effort, stress, diet. (Paper I, Paper III);

C. Specific exogenous factors: antibiotics, probiotics. (Paper II, Paper IV).

6. MATERIAL AND METHODS

A brief summary of the materials and methods used and some additional information are given below. For more details, see Papers I-IV.

6.1. Animals and experimental design

All studies were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

Paper I: Faecal samples from 25 horses (5 Thoroughbreds, 20 Standardbreds), weight 400-530 kg and on habitual home diets, were taken immediately at arrival to Mälaren Equine Hospital for routine health examination. These samples were investigated for presence of intestinal mucin. For the other MACs, faeces from 19 trotters (440-570 kg) were taken. They were 10 Standardbreds at a training campus and 9 Standardbreds living at Dept of Large Animal Clinical Science (LA), Swedish University of Agricultural Sciences (SUAS).

Paper II: Six clinically healthy Swedish Standardbreds (5 geldings, 1 mare), 5-8 years old, weighing 450-600 kg, were included in the study of zinc bacitracin (ZB) treatment, at LA, SUAS. Faecal samples were taken before, during and after treatment with ZB. From necropsied horses, 2 treated with ZB and 5 untreated, intestinal samples were taken from 11 sections of their GI tract. All these samples were investigated for 5 MAC-functions. Concentration of ZB was also investigated in those samples.

Paper III: Into this study, 12 litters of crossbred pigs (Swedish Landrace x Swedish Yorkshire) at Dept of Animal Breeding and Genetics (ABG), SUAS, were included. Of these, 6 litters were born and living outdoors and 6 litters were born and living indoors. In total, 58 piglets were living outdoors (OPs) and 57 piglets indoors (IPs), respectively. The sows were gilts, i.e. first time farrowing, and all litters were born in April and slaughtered the same year in the autumn. Indoors, the farrowing took place in individual pens and outdoors the gilts farrowed in individual huts in a common closure. Faecal samples were taken from Ops during their growth, from 4 days to 160 days of age, and from IPs, from 12 hours to 160 days of age. These samples were investigated for 6 MAC-functions.

Faecal samples from 10 germfree minipigs, seven days of age, from the Inst of Immunology and Gnotobiology, Czech Republic, was also brought to this study. These samples were investigated for 4 functions.

Paper IV: 47 piglets of the Swedish Yorkshire Breed, at the ABG, SUAS, were divided into four groups. The groups received separately the feed additives zinc bacitracin (Albac®), *Bacillus licheniformis* (Alcare®), Albac® plus Alcare® or no additives (control group). Faecal samples were taken from them before and during they were given the additives, and the samples were investigated for 6 MACs. These piglets were continuously weighed during the study.

Cows: Three rumen cannulated cows in late lactation were used in a 3 by 3 Latin Square feeding experiment, at Dept of Animal Nutrition and Management, SUAS. The cows were housed in a tie-up stanchion barn and milked twice a day. Periods were three weeks long and sampling from rumen and faeces was done on the third week. When changing diets the cows were gradually adapted to the new concentrate under a 3 day period.

Fishes: Samples of intestinal content were taken from salmons at eight different fresh-water farms, totally 68 samples. From cods caught in a sub-arctic region (Lofoten) 21 samples of intestinal content were taken. These intestinal samples from fishes were investigated for four MAC functions.

6.2. Determination of MACs

At analysis, the sample was mixed and two aliquots were transferred to glass vials for determination of urobilins (1.4 g (horses), 0.7 g (other animals)) and SCFAs (0.5 g) (Fig. 7). A third aliquot (0.5 – 0.7 g) was mixed with saline 1:2 (w/w), homogenized placed at 4 ^{o}C for 2 h and then centrifuged for 30 min at 4 ^{o}C x 5000 rpm. Part of the supernatant was used for determination of mucin, tryptic activity and β-aspartylglycine. The remaining supernatant plus sediment was used for determination of cholesterol conversion. The methods used are described in Papers I and IV.

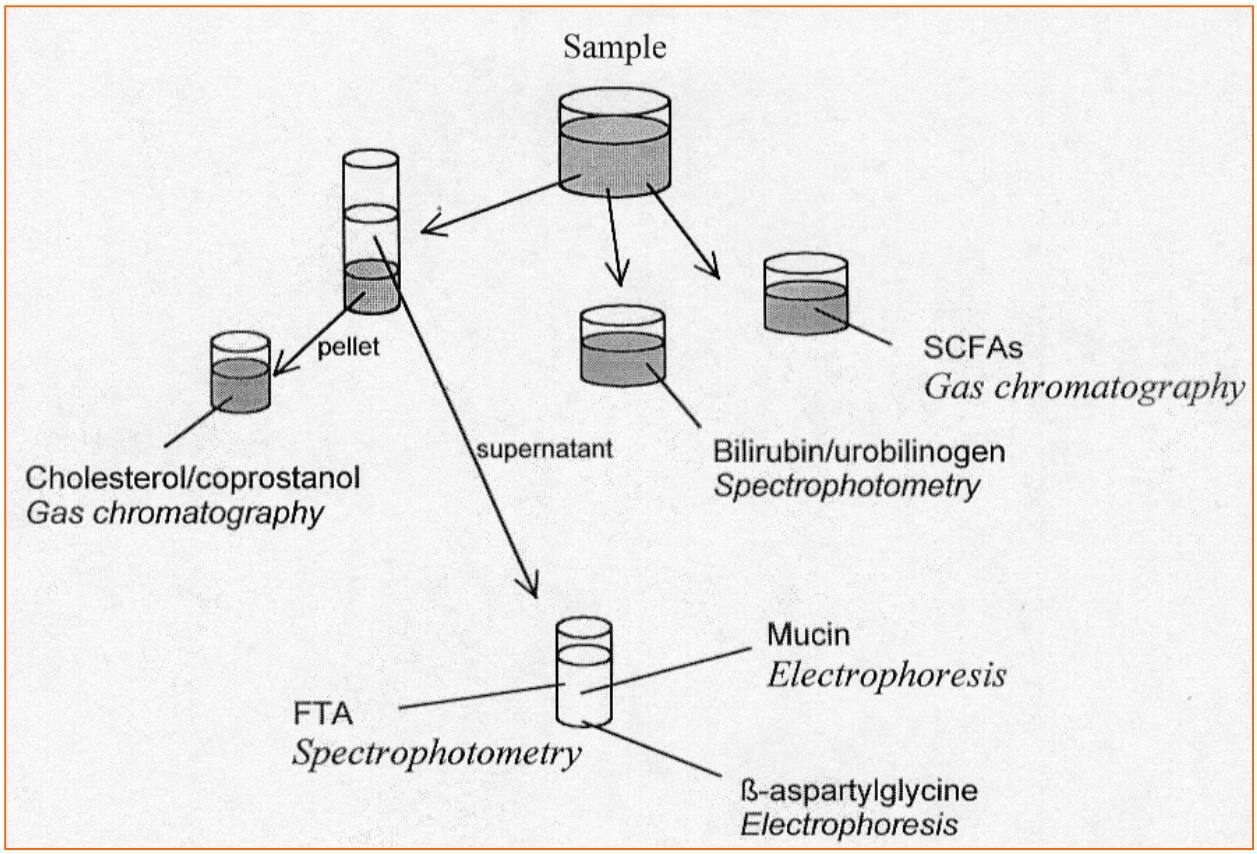


Fig. 7. Scheme of methods used

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6.2.1. Conversion of bilirubin to urobilins

The conversion of bilirubin to urobilins was determined with spectrophotometry (105); the results are presented in mmol/kg intestinal contents/faeces (Papers I-IV).

6.2.2. Conversion of cholesterol to coprostanol

Conversion of cholesterol to coprostanol was determined with gas chromatography (115). The results are presented as an index; the results are presented in percentage coprostanol out of coprostanol + cholesterol (Papers I-IV).

6.2.3. Degradation of mucin

The degradation of mucin was determined using agar-gel electrophoresis (58), and was visualized by three different staining techniques; the results are presented in an arbitrary scale of mucin degradation (Papers 1-IV).

6.2.4. Inactivation of tryptic activity, TA

Evaluation of TA was performed using spectrophotometry (130); the results are presented as mg/kg intestinal content/faeces (Papers I-IV).

6.2.5. Degradation of β-aspartylglycine, β-asp

The degradation of β-asp was determined using paper electrophoresis (179), and β-asp was visualized by staining with heating technique; the results are presented in an arbitrary scale of β-asp degradation (Papers I-IV).

6.2.6. Formation of short-chain fatty acids, SCFAs

SCFAs were determined with gas chromatography (69); the results are presented as total mmol/kg intestinal content/faeces, and presented as proportions of individual acids (out of totals) (Papers I, III, IV).

6.3. Statistical analysis

To evaluate if training vs. resting and/or different diets influence on SCFAs in faeces, analysis of variance was used for differences in the total amount and the proportions of separate SCFAs between the groups (Paper I). Wilcoxon's rank sum test for unpaired groups (160) was used to estimate differences of coprostanol, faecal tryptic activity and urobilins between two groups of horses (Papers I-II). In these studies $p < 0.001$ was considered as significant difference.

In Paper III, the Mann-Whitney *U* test for unpaired observations was used to compare the MAC-values from piglets living outdoors with values from piglets living indoors. Least square means for weight, weight gain, feed conversion and carcass trait were calculated with the GLM procedure in SAS. Regarding both methods in this study, when $p < 0.05$ the difference was considered as significant.

For analyse of differences and contrasts between cohorts and ages, the MAC-values in piglets given different feed additives (Paper IV) were analysed with analysis of variance. The differences were considered significant at $p < 0.05$.

Table 5. Mean values for MACs in faeces or digesta from adult animals of six species, and from three GF animal species. The GF rats received total parental nutrition devoid of SCFAs.

	Herbivores			Omnivore		Other omnivores				
	Colon fermenter	Ruminant		Colon fermenter		Colon fermenter	Caecum fermenters			
	HORSES	**COWS**		**PIGS**		**MAN**	**RATS**		**MICE**	
MAC-parameters		Rumen	Faeces	GF	Conv		GF	Conv	GF	Conv
Conversion of bilirubin to urobilins; mmol/kg	0.06	-	0.08	0	0.1	1.0	0	0.33	0	0.18
Conversion of cholesterol to coprostanol; %	40.0	-	50.2	0	49.9	65.0	0	39.0	0	21
Mucin; degraded	100	100	100	0	100	100	0	100	0	100
TA; mg/kg	8.0	-	10.3	> 800	21.0	37.0	> 700	9.0	> 700	15.0
β-asp; degraded	100	100	100	0	100	100	0	100	0	100
Total SCFAs; mmol/kg	49.5	135.5	54	12.0	129.8	84.5	0.80	129.3	16.4	112.6
acetic %	71.3	63.9	79.1	97.2	56.4	55.0	100	59.6	95.0	50.8
propionic %	21.4	15.9	13.5	0	22.2	16.3	0	10.7	1.5	17.4
iso-butyric %	1.6	0.8	1.3	0	2.9	2.0	0	0.4	0	1.2
butyric %	4.2	16.9	4.6	1.2	10	18.4	0	28.7	3.5	21.9
iso-valeric %	1.4	0.8	0.9	0.4	1.7	2.7	0	0.5	0	4.2
valeric %	0.2	1.3	0.7	0.2	3.5	2.2	0	0.5	0	3.7
iso-caproic %	0	0	0	0.2	0	0	0	0	0	0.6
caproic %	0	0	0	0.8	0.1	0.2	0	0.3	0	0.2
References	*I*	*unpublished*		*III*	*III*	*155*	*109, 115*	*127,150*	*26, 27*	

Table 6. Mean values for 4 MACs in intestinal samples from seven salmon groups in fresh water of different origin; one group split into a control group and one treated with antibiotics, and samples from cods in a sub-arctic region (Lofoten).

	Salmon (S)						Salmon (N)		Cod (N)
Group	A	B	C	D	E	F	control	antibiotic	Lofoten
Num. of samples	(5)	(4)	(3)	(9)	(8)	(9)	(10)	(20)	(21)
Parameters; Conversion of cholesterol to coprostanol; %	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Tryptic activity; mg/kg	**4011**	**458**	**2570**	**23**	**19**	**18**	**1561**	**2547**	**1690**
β-asp; degraded	**100**	**100**	**89**	**51**	**54**	**12**	**100**	**15**	**100**
Mucin; degraded	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Bred in Sweden = S; bred in Norway = N

7. RESULTS AND DISCUSSION

The measured values for each MAC and species in this study are described and discussed below.

7.1. MACs

7.1.1. Conversion of bilirubin to urobilins

The faecal baselines of urobilins in adult horses, cows and pigs were similarly low, and lower than baselines for the rats, mice, and man (Table 5). Values of urobilins were significantly higher in trotters at training, than in horses without training (Paper I). During treatment with ZB, the value of urobilins was significantly decreased in horses (Paper II). High values of urobilins were detected in the youngest piglets (Paper III). At three weeks of age the values of urobilins in the IPs were significantly higher than those in the OPs (Table 7) (Paper III). The values of urobilins in the piglet groups with access to Albac®, Alcare® and with Albac® + Alcare® were significantly lower than in the compared control group (Paper IV), at 7 weeks of age.

The diets to adult horses, cows and pigs are probably more fibre rich than the diet to piglets, rats, mice and man, thereby faecal output increases and thus the amount of urobilins per kg faeces decrease. The low values in older piglets and sows (Paper III) might also be due to a diet-depending increased mass of faeces diluting the constant amount of bilirubin/urobilins.

Figure 8. A thrilling finish of a Trotting race at Solvalla, Stockholm

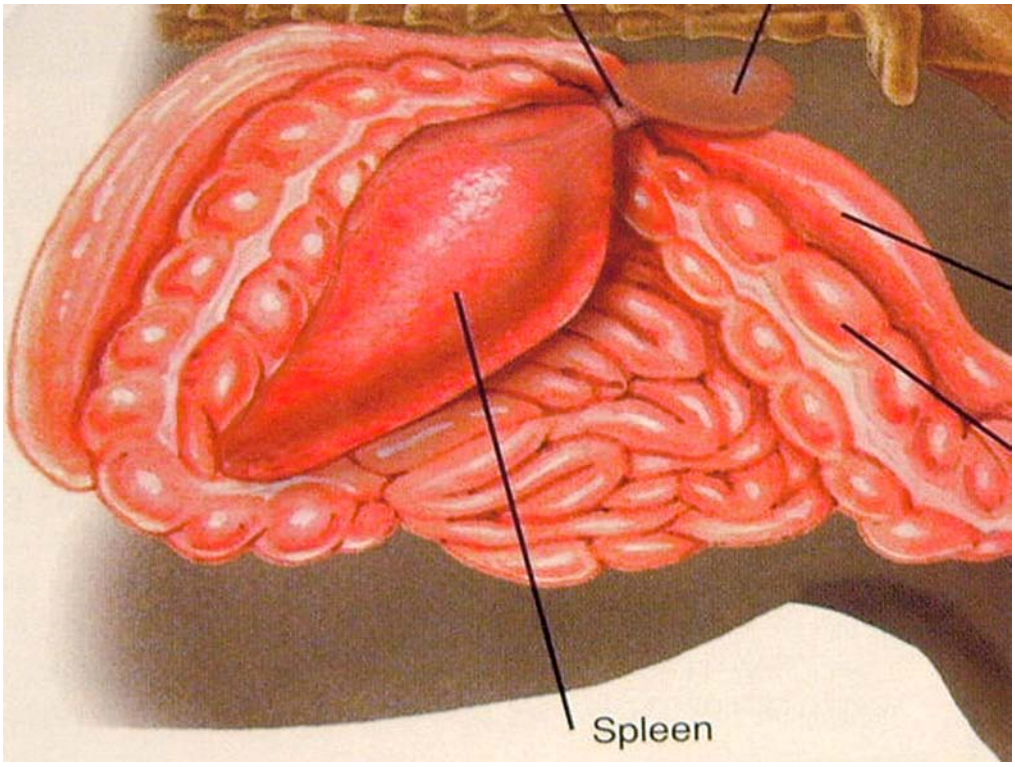


Figure 9. The spleen, the horses' large reservoir of red blood cells.

A dilution might also explain the difference in trotters at training, since fodder to those horses contains less fibre than that for resting horses. The higher value for horses at training may also depend on their higher amount of freely circulating haemoglobin in "fighting" horses (Fig. 8) than in resting horses (158), which makes the red blood cells more available for degradation (36). As the horse is a "flight" animal, its reservoir of red blood cells (~40 %) in the spleen (Fig. 9) is at stress situations rapidly emptied into the blood circulation.

The findings upon influence of ZB, Albac® and Alcare® (Paper II and Paper IV) might reflect direct treatment effects on the intestinal microflora. Similar results have been found in studies on humans (150) and rats (59) treated with ZB, supporting that microbial functions are similar in different mammal species. It seems reasonable to assume that ZB suppresses the microbes capable of transforming bilirubin to urobilins, namely *Clostridium ramosum* (60, 113).

The high amounts of urobilins in young piglets most likely reflect a high excretion of foetal bilirubin into the GI tract, which also appears in several newborn mammals, as well as a relatively early presence of microbes degrading bilirubin.

The significantly high amount of urobilins in IPs may originate from earlier colonization of the rare transforming microbe in the IPs, through more microbes in their environment than in that surrounding for the OPs.

7.1.2. Conversion of cholesterol to coprostanol

The proportion of coprostanol was in adult horses, cows and pigs at the same order of magnitude and similarly as in other species so far investigated (Table 5). It was also found that the conversion to coprostanol started in the small intestine of horse (Fig. 10) (Paper II), and a significantly decreased proportion of coprostanol was found in faeces and intestine contents of horses treated with ZB (Paper II).

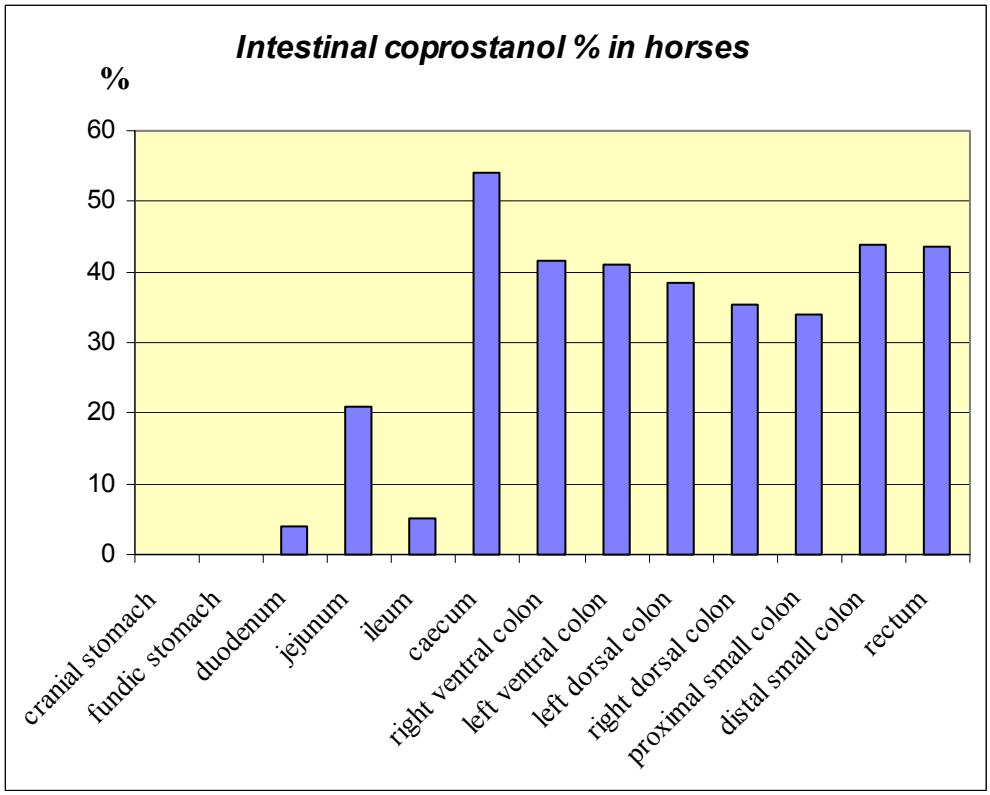


Figure 10. Mean proportions coprostanol in 11 GI sections of five healthy horses.

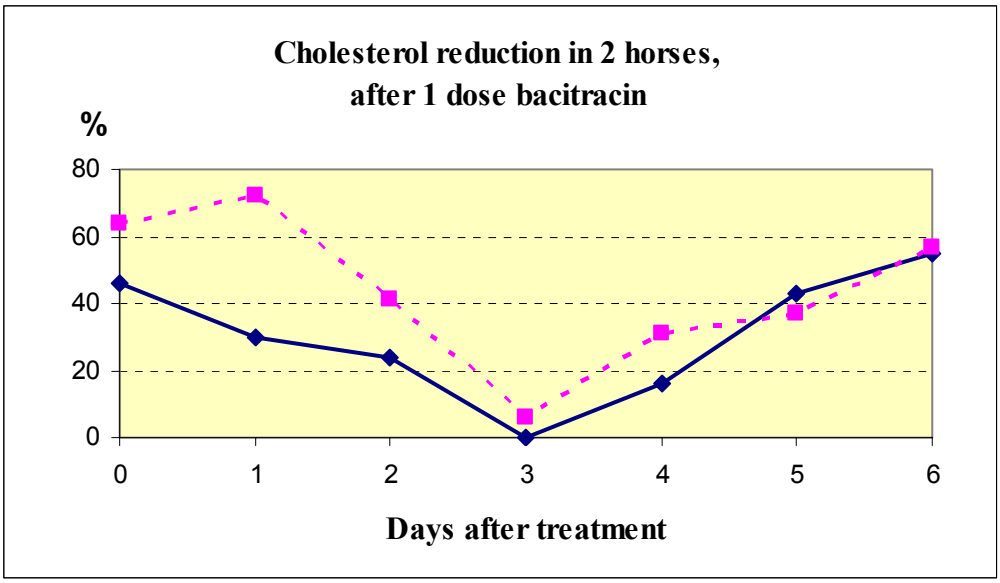


Figure11. Percent coprostanol in samples from two horses treated once with ZB.

In the GF minipigs no coprostanol was found, but it was present in piglets (OPs and IPs) a few days after birth (Paper III); and the concentration of coprostanol in piglets at 20 days and 35 days of age was above adult values (Paper III, Paper IV).

These findings in different species show that microbes capable of converting cholesterol to coprostanol are included in the indigenous flora of those species. It is interesting that cholesterol was converted to coprostanol in the small intestine of horses. In other species so far investigated, the conversion is thought to take place mainly in caecum. This might indicate that various sites of the gut are involved in the conversion of cholesterol in different mammals. To date, the enzymology of cholesterol reduction has not been fully investigated. Obviously, the mechanisms behind these differences should be further elucidated.

Regarding the striking reduction of coprostanol formation in horses treated with ZB, a similar reduction has been found in humans (117) and rats (59). This intestinal conversion of cholesterol to coprostanol was remarkable influenced in horses by just one single dose ZB (Fig. 11) (Paper II), which indicate that bacterial strains with capacity to perform this conversion are extremely sensitive to ZB. A similar decrease has also been found in rats, treated with an extremely low dose of clindamycin (29). Afterwards, the intestinal conversion of cholesterol to coprostanol was normalised much faster in horses than that recognised in humans and rats. The environment surrounding the horse, namely the stable, might give rise to a far more efficient "refill" than clean modern houses. Moreover, the clean surroundings for children might also partly give rise to a comparatively late start of this intestinal conversion to coprostanol, for humans. On the other hand, this parameter did not normalise in one horse that got acute watery foul-smelling diarrhoea with *Clostridium difficile* and its toxins, during the treatment (Fig. 12; horse 4 diseased). This corresponds with other findings of *Clostridium difficile* in humans and horses (12, 24, 96).

The finding of coprostanol in young piglets shows that microbial strains capable of performing conversion of cholesterol to coprostanol are established early in the intestines of pigs. Such rapid establishment of cholesterol converting microbes has also been observed when adult GF rats were exposed to intestinal flora from CONV

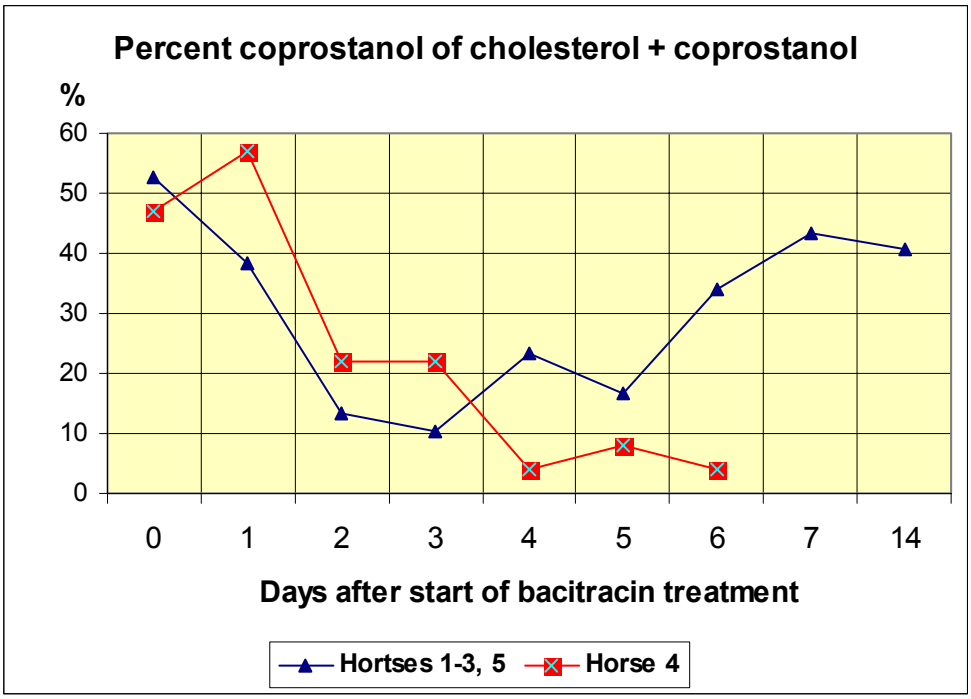


Figure 12. Mean value of coprostanol in four horses treated four days with ZB.

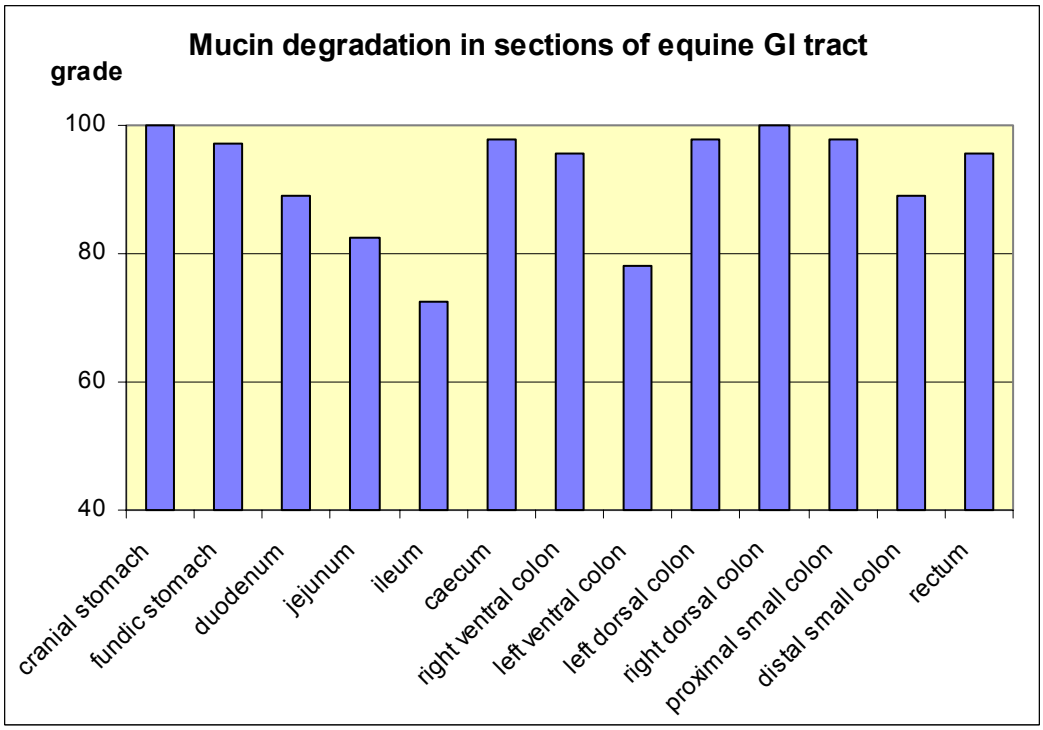


Figure 13. Mean degradation of mucin in 13 GI sections of five healthy horses.

rats (115). In children, this microbial conversion has never been detected before 6 month of age (104).

The high value of coprostanol (above adult values) in young piglets (Paper III, Paper IV) has not previously been registered in man or other mammals. Coprostanol does not even occur in all healthy humans (117), but it occurred in all samples from pigs older than 3 weeks – all adults investigated, except fishes. It was found that ZB as feed additive or the probiotic *Bacillus licheniformis* had no influence on cholesterol reduction in pigs (Paper IV), which indicates obvious differences between the intestinal flora of the species studied. It might be that young pigs have far more cholesterol reducing microbes than is the situation in the flora of adult horses. Resistance against ZB in the pigs can not be excluded.

7.1.3. Degradation of mucin

The mucin pattern in faeces sampled from healthy horses showed no detectable bands in the used staining-methods, namely mucin was totally degraded (Paper I) and the same pattern was found in adult pigs and cows (Table 5). These results are similar as found in samples from man and other mammals.

In some GI sections of the untreated horses (Paper II) the mucin degradation was not complete (Fig. 13), and the degradation of mucin was found to be less complete in faecal samples from horses treated with ZB for some days (Paper II), especially from one horse with diarrhoea. Less degradation was also found in the intestines of the ZB-treated necropsied horse.

When piglets were a few days old, mucin degradation was significantly higher in piglets living indoors than in piglets living outdoors (Paper III), see Table 7. The significantly higher mucin degradation in piglets living indoors, might originate in a higher exposure to active mucin degrading microbes for the piglets that was born in pens than for the piglets born in huts outdoors. An environmental dependent establishment of mucin degradation has been reported in adult ex-germfree rats (115), and a pronounced difference of priming the mucin degradation has been found between breast-fed and formula-fed children (106). After antibiotic treatment a variable reestablishment of mucin degradation has been found in healthy adult humans (30). These findings indicate that mucin-degrading microbes are parts of many ecosystems.

A significant decrease in mucin degradation was found in the piglet group recieving both the antibiotic Albac® and the probiotic Alcare®, at 7 weeks of age, simultaneously with a significant decrease of total amount of SCFAs (Paper IV).

These results show that healthy adults independently of species harbour a flora capable of a complete degradation of mucin. The incomplete and different degradation in intestinal sections of horses is interesting, and may indicate differences of the intestinal flora between compartments and microbial sites of the gut.

The found impaired degradation of mucin in Paper II (during treatment) indicates that ZB influences on microbes in horses, involved in this degradation. This antibiotic influences similarly on mucin degradation in humans (30) and rats (59) as well as in pigs (Paper IV). It influences mostly gram-positive bacteria (30).

The results in Paper IV show that some microbes degrading mucin in pigs are sensitive to ZB, as it influences on mucin degradation in man (30) and rats (128). These results and the faecal values of SCFAs fit well with the assumption that mucin is a major substrate for a microbial production of SCFAs.

7.1.4. Inactivation of tryptic activity, TA

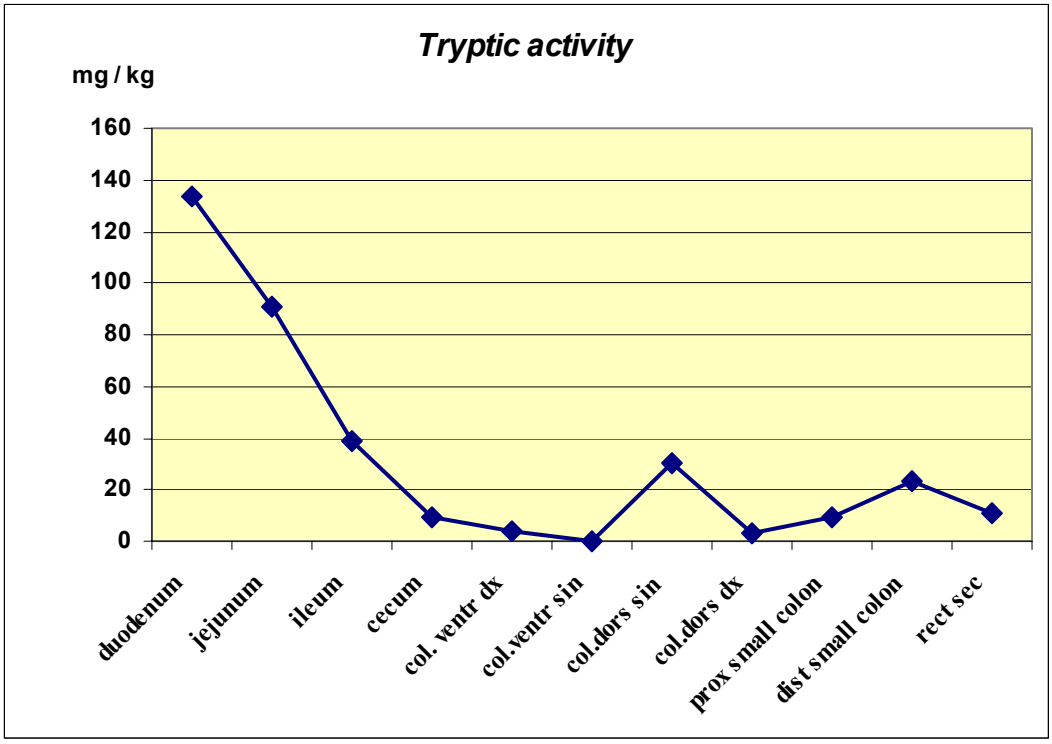
The faecal mean value for TA in adult horses (Paper I, Paper II) corresponds to the values for pigs (Paper III) and cows, and three other mammals (Table 5). TA was found to be much higher in duodenum in the GI tract of horses (Fig. 14), than in the large intestine and in rectum.

It was found that IPs had a significantly lower faecal TA than OPs (Paper III), at three weeks of age (Table 7). In GF pigs, TA was in the same order of magnitude (Table 5), as in GF rats and mice, and this remarkably high TA confirms that the enzyme is inactivated by microbes.

Table 7. Mean values for MACs in samples from GF minipigs, and from piglets reared outdoors (OPs) or indoors (IPs) and their sows.

Ages:	7 days	12 hrs	4 days		20 days		35 days		70 days		160 days		Sows	
Number of samples	(10)	(16)	(16)	(10)	(12)	(10)	(15)	(10)	(25)	(12)	(10)	(9)	(6)	(5)
	GF	IPs	OPs	IPs	OPs	IPs	OPs	IPs	OPs	IPs	OPs	IPs	Out	In
MAC-parameters														
Total SCFAs; mmol/kg	**12**	**29**	**44**	**58**	**22*****	**49*****	**46**	**51**	**74**	**71**	**109**	**120**	**103****	**162****
acetic %	97.2	87.4	58.5	61.7	72**	49.6**	52.2	56.1	52.9	56	53.1	49.4	57.9**	54.4**
propionic %	0	4.7	15.1	17.1	10.4**	18.4**	21.9	22.2	26.7	25.4	27.4	24.7	23.9**	25.1**
iso-butyric %	0	1.4	4.1	4.5	2.9	4.4	3.1	2.9	1.5	1.4	1.7	1.5	1.3	1.5
butyric %	1.2	4.4	14.6*	7.2*	7.9*	16.4*	13.5	10	11.4	12	12	17.6	13.4**	15.4**
iso-valeric %	0.4	1.4	5.1	3.5	4.3	7	5.1	1.7	1.9	1.7	2	1.7	1.3	1.6
valeric %	0.2	0.1	2.5	4.1	2.5	4.1	3.8	3.5	4.8	2.9	3.2	3.7	2.1	1.9
iso-caproic %	0.2	0.2	0.1	1.6	0	0	0	0	0	0	0	0	0	0
caproic %	0.8	0.4	0	0.3	0	0.1	0.4	0.6	0.8	0.6	0.6***	1.4***	0.1	0.1
Conversion of cholesterol to coprostanol; %	**0**	**0**	**22**	**10**	**71**	**74**	**76**	**75**	**64**	**63**	**56**	**58**	**47**	**54**
Conversion of bilirubin to urobilins; mmol/kg	n. e.	n. e.	n. e.	n. e.	**0.4***	**0.7***	**0.22**	**0.19**	**0.05**	**0.07**	**0.03**	**0.05**	**0.11**	**0.08**
TA; mg/kg	**> 800**	**4**	**22**	**16**	**47****	**2****	**6**	**5**	**3**	**9**	**5**	**9**	**21**	**21**
β-asp; degraded	**0**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Mucin; degraded	n. e.	**70**	**46****	**76****	**92**	**87**	**99**	**98**	**100**	**92**	**95**	**96**	**100**	**100**

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ between breeds, by Mann-Whitney U test; n. e. = not evaluated, due to too less samples obtained



Figue 14. Mean TA in 11 GI sections in five healthy horses.

The differences of the TA–values between species might originate from differences in their diet. However, the low values of TA in samples from horses at training or resting (Paper I) may depend on high microbial inactivation, or low production of trypsinogen, which has been claimed to take place (3) or altered passage time. The found TA-values in duodenum, jejunum and ileum indicate that the microbial inactivation of trypsin mainly takes place in the small intestine. Such low TA-values have previously been shown in caecum and colon of CONV rats (130).

Microbes capable of inactivating TA was most likely colonised earlier in piglets living indoors (IPs) than in those living outdoors (OPs), which probably originate from a higher exposure to active trypsin-inactivating microbes indoors, than outdoors. Thus, colonisation of microbes capable to degrade trypsin is dependent on surroundings to the host, and these microorganisms might ensure an intestinal survival of passively delivered immunoglobulins.

The microbes inactivating trypsin were not influenced by ZB (Paper II, Paper IV), on line with most antibiotic studies on rats (16, 29, 59, 114) and man (114, 129). These findings are in accordance with the assumption that ZB is highly active against gram-positive rather than gram-negative bacteria (164), and TA has till now been found to be inactivated by the gram-

negative *Bacteroides distasonis* (143). Interestingly, antibiotics influence upon this parameter in fish (Table 6).

The values of TA were variably in the salmon groups bred at different places, and significantly higher in salmons treated with antibiotics (Table 6). The varying values at different fish breedings might indicate that microbes capable of inactivating TA occur variously frequent. Whether and to what extent the TA-values in fish are influenced by diet and environment should be further elucidated.

7.1.5. Degradation of β-aspartylglycine, β-asp

The results on adult mammalians show that their microbial flora has been capable to degrade this dipeptide. As far as we are aware of, presence/absence of β-asp has never previously been investigated in samples from horses or cows. However, the mere fact that this dipeptide has been found in all mammalian species in which the intestinal flora has been absent or heavily disturbed, makes it reasonable to assume that this dipeptide might be present in horses and cows under similar altered/disturbed condition.

The results (Paper III) indicate that micro-organism(s) capable to degrade this dipeptide most likely were established early, in both OPs and IPs. Thus the piglets' intestines have obtained a good CR within their first 12 hours of life.

In all samples from GF minipigs β-asp was present. β-asp was not found in any sample from adult horses (Papers I-II), pigs (Paper III-IV) or cows (Table 5).

In fish, β-asp was found to a vary considerably (Table 6). Applying the explanation in mammals to fish, it might either depend on dietary factors or absence of microbes lacking specific dipeptidase(s). Whether and to what extent presence of β-asp in fish, especially in farm fish, can be used to express degreeCR, remains to be investigated.

7.1.6. Formation of short-chain fatty acids, SCFAs

The values of SCFAs in faecal samples from healthy horses (Paper I) were approximately similar as those found in faeces from healthy cows, both with regard to total amount and proportions of acids (Table 5). These values were significantly lower than those found in omnivores, namely pigs, humans, rats and mice. The rumen values of SCFAs were significantly different from the faecal values in cows (Table 5).

In Paper II, the proportions of SCFAs were measured in different parts of the GI tract of healthy horses (Fig. 15)

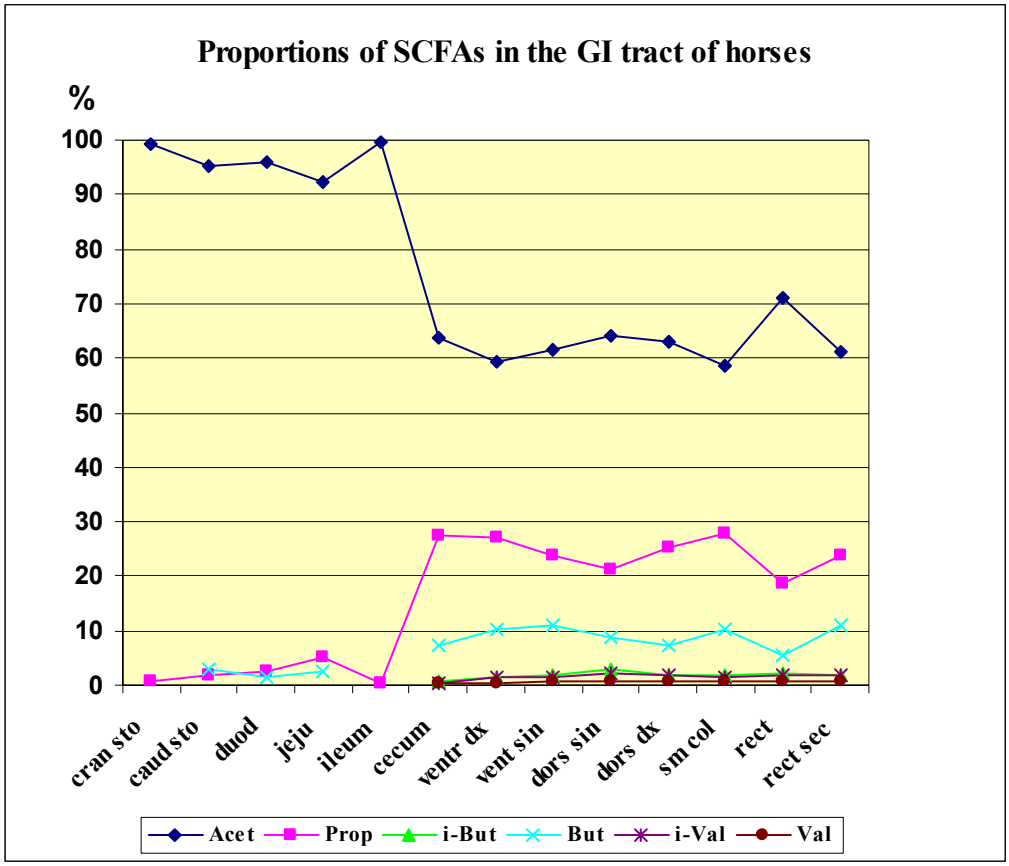


Figure15. Mean proportions of SCFAs in 13 GI sections in five healthy horses.

Horses and cows are included in the same group of herbivores, they are obligate grazers or grass and bulk eaters. However, the reticulo-omasal orifice in ruminants (Fig. 16) restricts passage of the larger-size particulate matter (72), which still might originate from some diet difference between them.

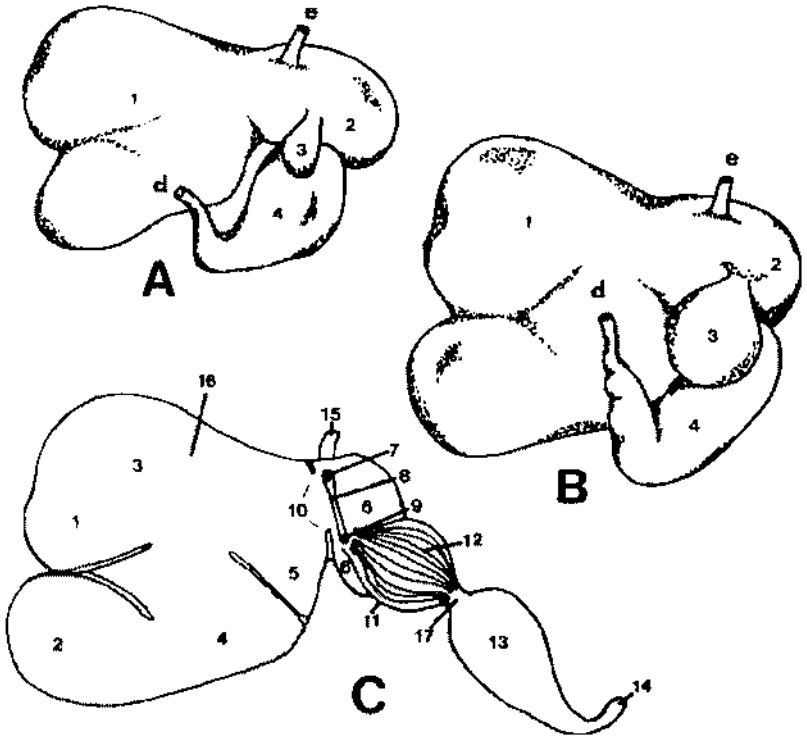


Figure 16. Diagrammatic sketches of the ruminant stomach. (A) Stomach of ruminants in the family Cervidae (deer, moose, elk, etc.). (B) Stomach of ruminants in the family Bovidae (cattle, sheep, goats, etc.).

Legend for A and B: (1) rumen; (2) reticulum; (3) omasum; (4) abomasums; (d) duodenum; (e) esophagus.

Legend for C; Diagrammatic cross section of Bovidae stomach: (1) dorsocaudal blindsac; (2) ventrocaudal blindsac; (3) dorsal sac; (4) ventral sac; (5) cranial sac; (6) reticulum; (7) cardia or esophageal opening; (8) reticular or esophageal groove; (9) reticuloomasal orifice; (10) rumen-retivulum opening; (11) omasum; (12) omasal leaves; (13) abomasum; (14) duodenum; (15) esophagus; (16) rumen; (17) omasal-abomasal orifice. (from 41)

The found values of total SCFAs and proportions of acids in rumen correspond with assumed baselines for cows fed a diet on hay and/or addition of readily fermentable carbohydrates, which usually increases the rate of fermentation and decreases the acetate/propionate ratio (166). The low total values of SCFAs in faeces and high acetic value might depend on that ingesta in colon lacks readily fermentable carbohydrates and pass relatively fast (24 h) through large intestine of cows. The low faecal values in horses might partly be caused by a similar proximal degradation of carbohydrates as found in cows, partly a diluting effect, as indicated in the discussion of urobilins. The high values of SCFAs in CONV adult pigs might be caused by a high colonic digestion of soluble carbohydrates, together with a rapid passage of intestinal digesta (9).

Factors that have to be taken into account, regarding differences of SCFAs-values between species, are variations in diet, intestinal transit time and amount of intestine fluid etc. The lumenal output of fluid, which contributes to the species, and small intestine, specific regulation of microbial growth, is found to be 200 ml/day/kg body weight in horses, 100 in pigs, and 20 in man (10, 92, 101, 141). As SCFAs are present as main ions in the large intestine and are capable of influencing many functions in the host (Table 4), variations in this parameter are of importance for the host. Recently has been claimed that ileal SCFAs in pigs are involved in distal gastric motility by a humoral pathway (38) and motor activities in the small intestine (39), which influence upon these intestinal outputs of fluid. The microbial formation of SCFAs has furthermore been suggested to be important in the pathogenesis of the gastric ulcers in pigs (8). These examples illustrate cross-talks between prokaryotes and eukaryotes and the multitudinous essential functions the microbial flora exerts influence(s) upon.

The measured proportions of SCFAs in GI sections (Fig. 15, horses) are similar to the results by Argenzio *et al.* (11). Values in GF pigs and in GF mice represent the total amounts of SCFAs in their feed, which show that a considerable part of some values in young piglets (Table 7) seems to represent these acids in the feed.

The great decrease of the total of SCFAs in OPs (Paper III) at 20 days of age might depend on different energy demands between OPs and IPs and the different proportions of the acids are probably originated from different colonic floras in the groups. A corresponding proportional difference has been found between breast-fed and formula-fed human children (154). The age-dependent ‘shift to the right’, namely a quantitative increase of longer carbon chains of SCFAs, occurred in the growing piglets, in accordance with previous findings in growing children (73, 116, 154, 169).

The delay of 'shift to the right' found in pigs probably originates from that Albac® and/or Alcare® act as hindrance for establishment of the complex flora needed to form longer carbon chains.

7.2. pH and dry matter

Intestinal pH influences several GI functions, such as production and absorption of SCFAs, NH_3, absorption of water, etc (7), thereby influencing upon the amount of dry matter in the intestinal content. Our results, regarding lumen pH (Fig. 17) and amount of dry matter (Fig. 18) in GI sections of five horses (Paper II), were in accordance with values previously found in healthy horses; pH (11, 79), dry matter (163).

7.3. Fishes

The intestinal flora of fishes (Fig. 19) has another composition and stability than the flora in terrestrial animals, and its role in the fish is still unclear. Some authors have claimed that the intestinal flora mostly reflects the feeding and drinking of the animal and is therefore a function of the surroundings (25, 64, 168). Our findings might support these suggestions.

Temperature (86) and drinking habit might be factors of environmental influence. Since all fish species are poikilothermy, their flora reflects environmental temperature. Additionally, drinking habit in fish, namely around 0.5 % of its body weight per hour (98), may also influence markedly upon the intestinal flora. A high surrounding bacterial load might of course influence upon functions studied of the flora, namely the MACs.

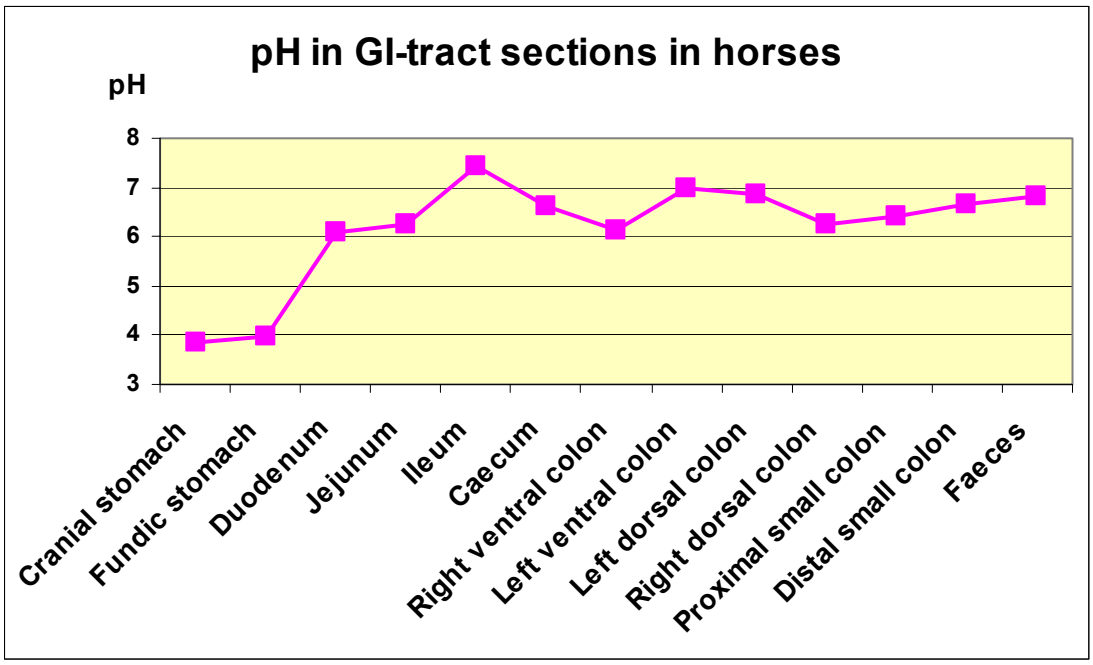


Figure 17. Mean value of pH in 13 GI sections in five healthy horses.

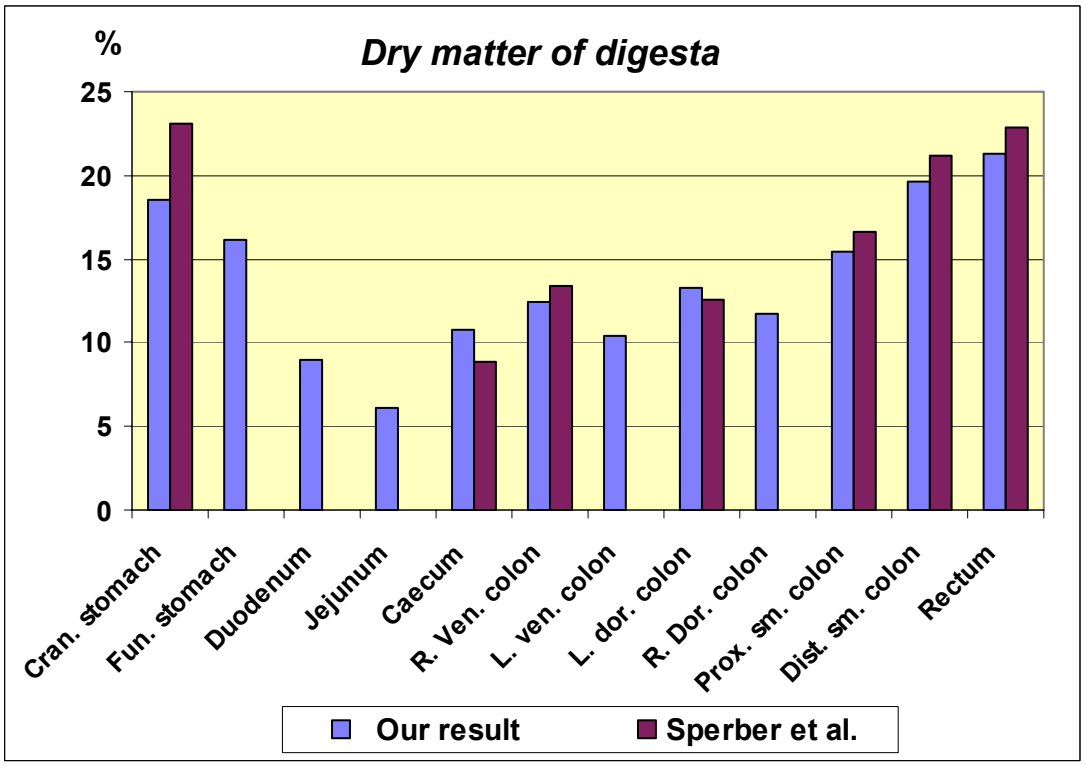


Figure 18. Mean dry matter of intestinal contents in 12 GI sections in five healthy horses, compared with another investigation on healthy horses (163).

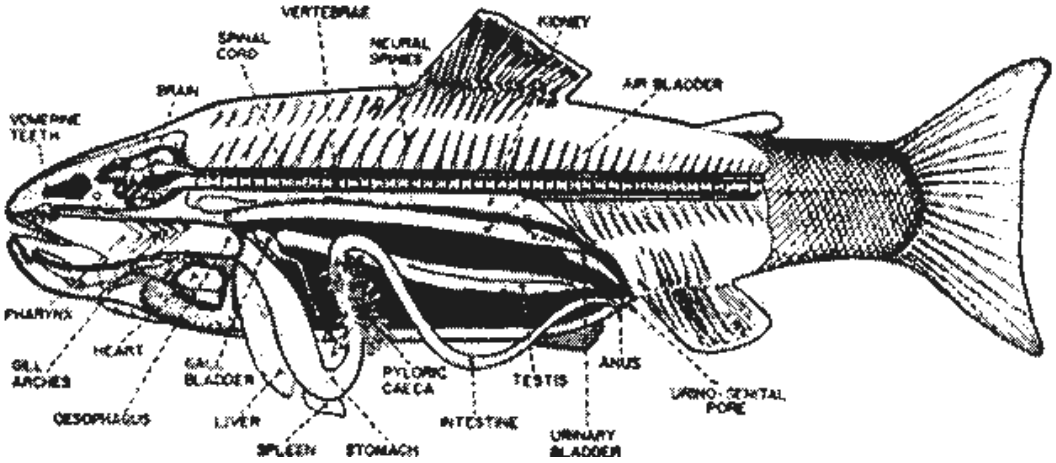


Fig. 19. The intestinal structure of the brown trout, semi-diagrammatic. (Based on Parker and Haswell, 1928. Fig. 876).

8. SUMMARY, CONCLUSIONS AND FUTURE SCOPE

The present results clearly demonstrate that the MAC/GAC concept, irrespectively of anatomical structure of the GI tract and major type of diet, can be used to study GI ecosystem(s).

The present results also demonstrate that:

A. The MAC/GAC concept is suitable for studies of establishment of microbial functions with regard to age (Paper III);

B. Alteration in major exogenous factors, such as environment, physical efforts, diet, stress, temperature, etc, may cause alteration in MACs (Papers I-IV, fishes);

C. Specific exogenous factors, as antibiotic and probiotic, may also cause alterations in MACs (Paper II, Paper IV).

It is well established that a well functioning gastrointestinal ecosystem is a corner-stone for animal health and welfare. It goes without saying that in the future, when more details are known about the establishment and maintenance of intestinal microbe/host interactions in other species, the MAC/GAC concept can be of increasingly importance for studies of short- and long-term effects of dietary and environmental variables in modern animal husbandry. Accepting the assumption that a well functioning GI ecosystem is a front-line defence factor against disease-carrying microorganisms, it seems reasonable to assume that this concept might be a valuable diagnostic system to measurements of prevention and reduction of some disorders in domestic animals – as well as in farmed fish.

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