

From THE MICROBIOLOGY AND TUMOR BIOLOGY
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**INFLUENCE OF PROBIOTICS AND
OTHER EXTERNAL FACTORS ON
INTESTINAL BIOCHEMICAL
MICROFLORA-ASSOCIATED
CHARACTERISTICS**

Studies *in vitro*, and *in vivo* in gnotobiotic
mice and in pigs

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A mi esposo, Jose y

A mis padres, Bernardo y Edelmira

ABSTRACT

The benefit of harboring a balanced intestinal microflora is well recognized. Therefore, in order 'to maintain or improve the intestinal flora', the use of microbial species as feed supplements, i.e. probiotics, has rapidly increased. Additionally, antibiotics and other means for health and growth- promoting purposes are currently used. This thesis addresses the influence of different ways of modulating the microflora, namely probiotics and/or other exogenous factors, on some biochemical Microflora-Associated Characteristics, MACs. The MACs analyzed were: conversion of cholesterol to coprostanol and of bilirubin to urobilinogen, deconjugation of taurodeoxycholic acid (TDCA) and of glycodeoxycholic acid (GDCA), production of short-chain fatty acids (SCFAs), inactivation of fecal tryptic activity (FTA), degradation of mucin and of β -aspartylglycine, and β -glucuronidase activity.

In the first part, seventeen probiotics belonging to the genera *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus* and *Streptococcus* were screened for their capability to alter the MACs *in vitro* and/or *in vivo* in gnotobiotic mice. When tested, seven probiotics were able to deconjugate both TDCA and GDCA and four expressed β -glucuronidase activity, *in vitro*. Nine of the monoassociated groups had significantly higher total SCFA concentrations in their large intestinal contents than the germ-free control. The other parameters tested were not influenced by any of the probiotics. The results show that the probiotics tested only exert minor influences on the investigated MACs. However, absence of a function *per se*, does not exclude an effect upon that function when a probiotic strain is acting within an intestinal ecosystem.

In the second part, the effect of health and/or growth promoters on six MACs was studied in piglets. The environmental influence was investigated by rearing piglets either indoors or outdoors. Alterations were observed in three MACs. Influence of probiotics and/or antibiotics was addressed by supplementing the diet to weaned piglets. Zinc bacitracin (ZB), *B. licheniformis* or both supplements were added to the diet until ten weeks of age. ZB and *B. licheniformis* influenced three MACs. Taken together, these results show that the MACs investigated are rapidly established in piglets and a possible higher exposure to functionally active microorganisms occur in the indoor than in the outdoor environment. Moreover, influences of different rearing environment, ZB and *B. licheniformis* on the MACs investigated were observed mainly at young ages. However, disturbances on biochemical functions might have been overcome by a 'healthy adult microflora' observed at completion of both experiments.

Our results add new insights related to specific functions of probiotics. It seems reasonable to assume that this approach should be used in future selection of microbial strains for health- and growth- promoting purposes. Comparative studies in germ-free and gnotobiotic animals underline the importance of a functionally active microflora in the establishment and maintenance of host-microbe and microbe-microbe 'cross-talks' in any organism.

The investigation of the intestinal ecosystem in terms of functionality, i.e. the MAC concept can be applied on all levels of interactions.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AGUS	A rat strain of uncertain origin inbred and reared at the Laboratory of Medical Microbial Ecology, Karolinska Institutet, Sweden, since 1956.
ATCC	American Type Culture Collection
CB-PY	Calf brain-peptone yeast
CB	Coomassie Brilliant blue
CV	Conventional. A macroorganism with an undefined flora.
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and cell cultures).
FTA	Fecal tryptic activity
GAC	Germ-free Animal Characteristic. The recording of any anatomical structure, biochemical, immunological or physiological function that has not been influenced by the microflora.
GDCA	Glycodeoxycholic acid
GF	Germ-free. A macroorganism free from all demonstrable forms of outer life including bacteria, viruses, fungi and protozoa.
GI	Gastrointestinal
GLC	Gas-liquid chromatography
IPs	Piglets born and raised indoors
MAC	Microflora-Associated Characteristic. The recording of any anatomical structure, biochemical, immunological or physiological function in a macroorganism, which has been influenced by the microflora.
MRS	de Man, Rogosa and Sharpe
NCTC	National Collection of Type Cultures
NMRI-KI	A mouse strain from the Naval Medical Research Institute, USA, transferred to the Laboratory of Medical Microbial Ecology, Karolinska Institutet in 1960.
LAB	Lactic acid bacteria
OPs	Piglets born and raised outdoors
PAS	Periodic Acid Schiff
SCFAs	Short-chain fatty acids
SRI	Stainless-steel rearing isolator
TB	Toluidine blue
TDCA	Taurodeoxycholic acid
TG	Thioglycollate broth
TLC	Thin-layer chromatography
TH	Todd Hewitt
ZB	Zinc bacitracin

1 INTRODUCTION

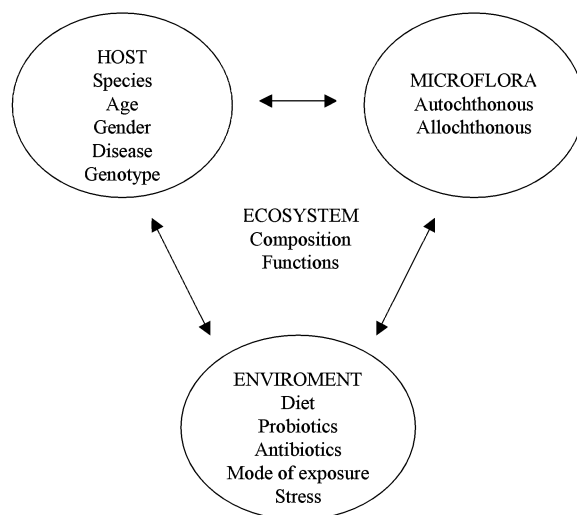
Any newborn animal or human is exposed to microbes immediately at birth. Different microorganisms colonize the skin, respiratory tract, gastrointestinal (GI) tract or any other sites appropriate for bacterial survival. Throughout life, the macroorganism and its flora create several ecosystems; the most complex one is found in the GI tract (Luckey, 1972; Macfarlane and Macfarlane, 1997).

The composition of the GI flora differs between animal species, between individuals within the same species and between different compartments within the GI tract. The GI microflora is influenced by diet (Kleesen et al., 2000), age (Husebye et al., 1992; Mitsuoka, 1992), environmental conditions (Adlerberth et al., 1991) and by host genotype (Zoetendal et al., 2001).

The GI microflora exerts a high impact on its host mainly by i) performing a great variety of metabolic activities, ii) protecting the host against colonization by pathogens and iii) priming and stimulating the gut immune system.

In a healthy state the host and its microflora develop a symbiotic association, i.e. they both benefit from each other. The recognition of this mutual benefit has led to the development of the probiotic concept.

The present investigation addresses aspects related to specific actions of probiotics as well as host-interactions in a complex microflora with regard to exogenous factors that may affect its normal 'balance'. Functionality of the microflora referred to as Microflora Associated-Characteristic /Germ-free Animal Characteristic or MAC/GAC concept, has been used as an approach.



The gastrointestinal ecosystem

2 BACKGROUND

2.1 THE GASTROINTESTINAL MICROFLORA

Soon after delivery the GI tract of a newborn is colonized by large numbers of facultative anaerobic strains such as *Escherichia coli* and streptococci. These bacteria may create a highly reduced environment as they multiply. Then, in a normal successional colonization, and partly influenced by a more complex diet, the obligate anaerobes become the numerically predominant species (Savage, 1977). Quantitatively, the dominant intestinal bacteria in animals and man are the bacteroides. Other bacterial groups present in the GI tract are bifidobacteria, eubacteria, clostridia, streptococci, enterococci and lactobacilli (Murray, 1998; Wilson, 1999).

The genus *Lactobacillus* contains many species that occur in the GI tract of most warm-blooded animals. The ecological significance of lactobacilli in the GI tract of mice has been demonstrated in a large series of investigations, especially in mice with a deprived flora (McConnell and Tannock, 1991; McConnell and Tannock, 1993; Tannock et al., 1988; Tannock et al., 1989; Tannock et al., 1994).

2.2 PROBIOTICS

Although the use of fermented food products for supporting health dates back to Elie Metchnikoff (1845-1916) (Metchnikoff, 1908), consumption of such products has increased during the last decades. Initially, the term “probiotic” was used to describe substances produced by one microorganism, which stimulated the growth of another. Currently, a generally accepted definition of probiotic is “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989).

Many species in particular the lactic acid-producing bacteria, are used in the manufacture of feed supplements. Beside lactobacilli and bifidobacteria, other microorganisms such as *Bacillus* spp (Adami and Cavazzoni, 1999; Alexopoulos et al., 2001), *E coli* (Rembacken et al., 1999) and yeast - *Saccharomyces* species (Filho-Lima et al., 2000; Ortuno et al., 2002) are used as probiotics.

Numerous health-promoting properties have been attributed to probiotic consumption (Andersson et al., 2001; Hove et al., 1999; Salminen et al., 1998). Some of the specific claims are: prevention or recovery from different types of diarrhea, reduction of “lactose maldigestion”, lowering of serum cholesterol levels, prevention and treatment of different kinds of allergic diseases, intestinal immune stimulation and prevention and treatment of inflammatory bowel diseases (Gilliland, 1990; Isolauri, 2001; Marteau et al., 2001; Saavedra, 2001). Some properties in the microorganisms used as probiotics have been described (Lorca et al., 2002) and several mechanisms of action have been proposed (Kaur et al., 2002). However, the claims concerning the role of probiotics on health improvement are still controversial (McFarland, 2000; Salminen, 2001).

2.3 LACTIC ACID BACTERIA

The concept of 'a cluster of lactic acid bacteria' was first applied by Orla-Jensen (Orla-Jensen, 1919) to describe some bacteria whose main end product from glucose fermentation was lactic acid. Today, the lactic acid bacteria (LAB) comprise a taxonomically diverse group of Gram-positive, non-spore forming cocci and rods, which grow under microaerophilic to strictly anaerobic conditions. The most important genera of the LAB group are *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Bifidobacterium* (Klein et al., 1998). The latter genus is generally included in that group because it shares some important features with the other genera. However, it is phylogenetically unrelated and has a unique mode of sugar fermentation (Vries and Stouthamer, 1967).

LAB are divided into homofermentative and heterofermentative according to their end products and mode of glucose fermentation under standard conditions (Axelsson, 1998). The homofermentative species produce almost exclusively lactic acid and the heterofermentative species produce lactic acid along with ethanol and carbon dioxide.

LAB are widespread mainly where high concentrations of carbohydrates are available such as in milk and dairy products, fermented foods and beverages. They are inhabitants of the human oral cavity, the GI tract and the vagina. In humans, the LAB have been claimed to be individually specific and relatively stable, especially in the GI and genital tracts (Mikelsaar et al., 1998).

Since LAB mainly lactobacilli and bifidobacteria, are commonly occurring inhabitants of the GI tract of humans and animals, and only occasionally have been reported as opportunistic pathogens (Antony, 2000; Axelrod et al., 1973; Husni et al., 1997; Uttley et al., 1988), there has been an increased interest in the use of lactic acid-producing bacteria as feed supplements.

2.4 BACILLUS LICHENIFORMIS AND BACITRACIN

B. licheniformis belongs to the family Bacillaceae, rod-shaped Gram-positive bacteria whose most important taxonomic feature is the formation of spores. Within this family, *B. licheniformis*, *B. pumilus* and *B. subtilis* are considered the 'subtilis group'. The species of that group can be isolated from a wide variety of sources; they are prevalent in soils, mainly in those with low-nutrient concentrations. They are also common on straw and cereals. Other common habitat especially for *B. licheniformis* and *B. subtilis* is the marine sediment in which they might be highly predominant (Priest, 1993).

Bacillus spp. may be important members of the rumen ecosystems. Some species including *B. licheniformis* have been implicated in hemicellulose conversion in the rumens of cows, sheep and goats (Williams and Withers, 1983).

Although most members of the *Bacillus* genus are considered non-pathogenic, strains of *B. cereus* (Drobniewski, 1993), *B. licheniformis* (Salkinoja-Salonen et al., 1999) and *B. subtilis* have been implicated in food poisoning (Gilbert, 1983).

B. licheniformis has been used as a probiotic with the purpose of preventing or treating disturbances at weaning, especially post weaning diarrhea, a main concern in pig farming (Kyriakis et al., 1999).

The finding that *B. licheniformis* was able to produce a substance with antibiotic effect, bacitracin (Johnson et al., 1945), made the strain very useful in the pharmacological field. Since then, bacitracin has been used as an antibiotic. Later investigations demonstrated an enhancement of the antibacterial effect of bacitracin with the addition of some divalent metal ions, especially zinc (Adler and Snoke, 1962; Smith and Weinberg, 1962; Weinberg, 1959). Recent studies have also reported enhancement of the anti-parasite action of bacitracin with the addition of zinc ions (Andrews, 1994; Andrews et al., 1995).

2.5 GASTROINTESTINAL MICROFLORA OF MICE

The first microorganisms to appear in the GI tract of the newborn mice are lactobacilli and streptococci (Tannock, 1990). A few days after, lactobacilli predominate in the proximal region of the mouse GI tract, as some strains adhere and colonize the epithelium lining the esophagus and forestomach (Savage, 1970; Savage et al., 1968; Tannock, 1997). The *Lactobacillus* population is retained in the murine GI tract throughout the animal's life. Due to changes in the GI milieu and partly when milk is supplemented to the offspring, the obligate anaerobes begin colonization of the large intestine and become the most numerous bacteria in this region of the tract.

2.6 GASTROINTESTINAL MICROFLORA OF PIGS

The stomach and the small intestine of a newborn piglet are colonized by microorganisms about 3 h after birth and about 12 h after birth bacteria are detected in the large intestine (Swords et al., 1993). In piglets as in other species, the establishment of an adult intestinal microflora is a gradual process in which the complexity increases along with the number of anaerobes. The total bacterial population of the GI tract of the pig has been estimated to be about 500 species, with highly predominance of anaerobic bacteria. *Bacteroides* spp. is the predominant anaerobe present in the adult colon. *Lactobacillus* spp. is present throughout the GI tract of pigs, constituting one of the main groups inhabiting proximal regions of the GI tract (Tannock, 1990).

2.7 THE GNOTOBIOTIC MODEL

Mice are highly used as animal models for gnotobiotic studies. Their wide preference in the microbial ecology field and many other areas lies in their convenient size, fertility, short gestation period, known genetic background and their availability from commercial sources.

Gnotobiotic mice when raised under standardized conditions, offer a great tool for studies of physiological functions associated with the presence of specific microorganisms.

2.8 INVESTIGATION OF THE GASTROINTESTINAL FLORA

The GI microflora can be investigated by three routes:

- a) Enumeration of microorganisms, isolation and identification studies. By using classic and modern molecular methods, the complexity of the GI microflora has been well documented. However, it is also 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, this approach can be applied to some clinical situations (Midtvedt, 1999).
- c) Performance. What have the microflora done? An adequate answer to this question can be given by comparing structures and functions in animals reared under germ-free (GF) conditions with conventional (CV) animals. In such studies, the Microflora-Associated Characteristic/Germ-free Animal Characteristic or MAC/GAC concept (Midtvedt et al., 1985) has been shown to be of considerable value. A MAC is defined as any anatomical structure or biochemical, immunological or physiological function in a macroorganism, which has been influenced by the microflora. A GAC is defined as the same structure or function in the absence of the microorganism(s) functionally active, as in GF animals, newborns or in hosts on antimicrobial therapy.

The following studies are based on the MAC/GAC concept.

3 BIOCHEMICAL MICROFLORA-ASSOCIATED CHARACTERISTICS

3.1 CONVERSION OF CHOLESTEROL TO COPROSTANOL

Coprostanol is an essential component of all mammalian cell membranes and it is the precursor of steroid hormones, vitamin D and primary bile acids. The total cholesterol pool is derived from endogenous and exogenous sources. Endogenous cholesterol is synthesized mainly in the liver and small intestine. The main routes for elimination of cholesterol are biliary excretion and hepatic conversion to bile acids. Cholesterol in the intestine can be absorbed or may be transformed into coprostanol by the action of intestinal microorganisms. The major microbial metabolite in feces of mammals is coprostanol. A few microorganisms capable of converting cholesterol to coprostanol have been isolated from different sources (Eyssen et al., 1973; Freier et al., 1994; Sadzikowski et al., 1977). The species responsible for that function have been mostly classified within the *Eubacterium* genus.

Coprostanol is non-absorbable and consequently excreted in feces. Thus, GF animals do not excrete coprostanol. Danielsson and Gustafsson (Danielsson and Gustafsson, 1959) found higher serum cholesterol levels in GF than in CV animals fed the same standard diet. Other factors such as *de novo* synthesis of cholesterol and conversion to bile acids have also been found to differ in GF and CV animals (van Eldere and Eyssen, 1984). Some studies have shown an association between higher excretion of coprostanol and lower serum cholesterol concentrations after feeding *Eubacterium coprostanoligenes* to rabbits (Li et al., 1995) and to GF mice (Li et al., 1998). However, the same strain fed to laying hens did not lower the plasma cholesterol concentrations, despite a higher coprostanol excretion (Li et al., 1996).

3.2 DECONJUGATION OF BILE ACIDS

Bile acids are synthesized in the liver from cholesterol. The most common primary bile acids occurring in several mammalian species, including man, are cholic acid and chenodeoxycholic acid. Within the liver, they are conjugated mainly with taurine or glycine prior to secretion from the gall bladder into the duodenum. In their conjugated form, they enhance emulsification and digestion of dietary lipids. In the intestinal tract, bile acids undergo several microbial transformations including deconjugation, dehydrogenation and dehydroxylation (Midtvedt, 1974).

Deconjugation of bile acids is carried out exclusively by bacteria. Therefore, bile acids are excreted as conjugates in GF animals but in their free form in CV animals. The deconjugation capability is common among intestinal microorganisms (Midtvedt and Norman, 1967). Under normal conditions, the deconjugation process appears to be restricted to the distal ileum and to the colon (Midtvedt and Norman, 1968) but under some pathological conditions such as blind loop syndrome, the number of

deconjugating microorganisms tends to increase in the proximal parts of the intestinal tract (Midtvedt et al., 1969).

During the intestinal transit, most of the bile acids are reabsorbed via the portal vein and only a small amount is excreted in the stools. Since the body bile acid pool is approximately constant, the liver compensates that loss.

Some of the other microbial bile acid transformations such as 7 α -dehydroxylation has been found to be a very rare capability among intestinal microorganisms (Gustafsson et al., 1966; Takamine and Imamura, 1995).

3.3 PRODUCTION OF SHORT-CHAIN FATTY ACIDS

Short-chain fatty acids (SCFAs) are yielded from GI microbial fermentation. In non-ruminants the concentration of SCFAs is low in the small intestine (Hoverstad et al., 1984a) while in the colon and feces, they are the dominant anions. Quantitatively, the main SCFAs in the GI tract are acetic, propionic and n-butyric acids. They contribute for about 90% to the total fecal SCFA concentrations in man (Cummings, 1981). Other intermediate and anabolic/catabolic end products derived from anaerobic fermentation include lactic, succinic and formic acids together with hydrogen, carbon dioxide and methane (Cummings, 1981).

In non-ruminants production of the major SCFAs takes place mainly in the caecum and the ascending part of the colon (Cummings, 1981). The production of SCFAs is influenced by availability of substrate and composition of the microflora. Several exogenous compounds, mainly complex carbohydrates and some endogenous compounds, such as mucin, are the major sources for the microbial GI production of SCFAs.

Comparative studies between newborn children (Midtvedt et al., 1988) and adults and between GF and CV animals (Hoverstad and Midtvedt, 1986; Hoverstad et al., 1985; Lee and Gemmell, 1972; Maier et al., 1972; Roach and Tannock, 1979), have shown distinct fermentation pattern of SCFAs, supporting their microbial origin, except for acetic acid. Thus, feces from newborns and GF animals contain small amount of SCFAs, comprising mainly of acetic acid, while those of the human adults and CV animals comprise a great variety of SCFAs, including branched-fatty acids. Substantial amounts of SCFAs have also been detected in commercial animal diets (Midtvedt, 1994).

The actions of SCFAs include energy supply to the host, with great contribution to energy requirements mainly in ruminants (70%). In humans, the contribution has been estimated to be about 5-10% (Cummings and Macfarlane, 1991; McNeil, 1984). In addition, the absorption of SCFAs stimulates fluid and electrolyte absorption in the GI tract (Rajendran and Binder, 1994). Butyric acid is a preferred energy source for colonic epithelial cells and is thought to play an important role in maintaining colonic health in humans (Roediger, 1980).

Altered amounts of SCFAs have been found in various colonic disorders such as ulcerative colitis, diversion colitis and pouchitis (Cook and Sellin, 1998; Cummings

and Macfarlane, 1991; Mortensen and Clausen, 1996) and in antibiotic-induced diarrhea (Clausen et al., 1991).

3.4 INACTIVATION OF FECAL TRYPTIC ACTIVITY

Trypsin is synthesized in pancreas as a precursor molecule, trypsinogen, and secreted into the duodenum. Trypsinogen is activated to trypsin, mainly by brush border enzymes and trypsin plays a major role in proteolysis in the small intestine. Fecal tryptic activity (FTA) reflects the net sum of mechanisms involving the secretion and activation of trypsinogen and the intestinal presence of host-, microbial- and diet-derived compounds that inactivate trypsin.

GF rodents always have high levels of FTA, whereas CV rodents have little or no FTA (Borgström et al., 1959; Norin et al., 1986). So far, only one human-derived strain capable of performing the inactivation of tryptic activity, *Bacteroides distasonis*, has been identified (Ramare et al., 1996).

FTA is usually high in feces from infants and this has been assumed to be due to a late establishment of microbes capable of inactivating trypsin (Norin et al., 1985). Excretion of FTA is low in healthy adults (Norin et al., 1988). High levels of FTA have been found in patients with Crohn's disease (Bergstrand et al., 1981; van de Merwe and Mol, 1982).

3.5 DEGRADATION OF MUCIN

Mucin is produced throughout the GI tract by goblet and glandular mucous cells. Mucin comprises a polypeptide core with oligosaccharides side chains linked by *o*-glycosidic bonds. The GI mucin acts as a lubricant, as a barrier and stabilizer for the intestinal microclimate and as a source of energy for the microflora.

Under normal conditions, the mucin is degraded by the intestinal microflora. GF rats excrete large amounts of mucin in feces compared to CV rats (Gustafsson and Carlstedt-Duke, 1984; Hoskins and Zamcheck, 1968) in which the intestinal mucin undergoes microbial degradation. A few strains capable of degrading the carbohydrate chains of mucin have been isolated (Hoskins et al., 1985; Salyers et al., 1977a; Salyers et al., 1977b). Carlstedt-Duke et al. (Carlstedt-Duke et al., 1986b) isolated a *Peptostreptococcus* strain able to obliterate all gel electrophoretic mucin bands completely, *in vitro* and *in vivo*. This strain seems to produce peptidases but lacks glycosidases.

Mucin has been found altered in some GI diseases such as inflammatory bowel disease, colorectal cancer, etc. (Clamp et al., 1981; Corfield et al., 1992). However, as recently reviewed by Corfield et al. (Corfield et al., 2000) the relevance of these alterations to GI disease is far from being elucidated.

3.6 DEGRADATION OF β -ASPARTYLGLYCINE

The biochemical background for the presence of this dipeptide in feces is probably as follows: dietary proteins are the main targets of intestinal proteolytic enzymes. β -aspartylglycine is a member of a group of β -carboxyl dipeptides formed in the intestinal tract when host-derived proteolytic enzymes break down dietary proteins. The β -carboxyl bonds are thought to be broken down only by proteases of bacterial origin.

β -aspartylglycine was originally detected in caecal contents from GF mice, whereas it was absent in the CV controls (Welling and Groen, 1978). Further studies showed that association of GF mice with an increasing number of anaerobic intestinal bacterial strains, gradually reduced β -aspartylglycine in feces (Welling et al., 1980).

The presence of β -aspartylglycine in feces from CV mammals indicates that the intestinal microbial ecosystem is markedly altered.

3.7 CONVERSION OF BILIRRUBIN TO UROLIBINS

Bilirubin is derived from the catabolism of hemoglobin and some heme-containing compounds. In the liver, bilirubin is conjugated to glucuronate and the conjugates, mainly mono- and diglucuronides, are secreted into the bile. Following excretion into the intestine, the glucuronides undergo deconjugation and transformation by microbial enzymes to a series of urobilinogens and related products, collectively termed urobilins (Saxerholt, 1989).

The bilirubin deconjugating capability is very common among intestinal microorganisms, whereas the ability to transform bilirubin to urobilinogen is very rare. A few microorganisms capable of performing the bilirubin conversion have been isolated and classified within the genera *Clostridium* (Gustafsson and Swenander-Lanke, 1960; Midtvedt and Gustafsson, 1981; Vitek et al., 2000) and *Bacteroides* (Fahmy et al., 1972).

3.8 β -GLUCURONIDASE ACTIVITY

Many foreign (xenobiotics) and some endogenous compounds (bilirubin, some steroids, etc.) are eliminated by conjugating them with glucuronic acid. Hydrolysis of glucuronides in the intestine is performed by β -glucuronidases. Some intestinal β -glucuronidases may be derived from endogenous sources, but most are of microbial origin (Rod and Midtvedt, 1977). This enzyme is produced by many bacterial strains (Hawksworth et al., 1971; Kent et al., 1972), though it is not clear which are most active *in vivo* (Cole et al., 1985; Gadelle et al., 1985). Some studies have shown that GF rats excrete fecal bilirubin conjugates and the conjugates were mainly of the glucuronide type (Saxerholt et al., 1984).

The biochemical parameters described above are summarized in Table 1, together with some major anatomical structures and physiological functions that are influenced by the microflora.

Table 1. Influence of the microflora on some intestinal structures, physiological and biochemical parameters

Parameter	MAC ^a	GAC ^b	Microorganism
Anatomical/Physiological			
Caecum size (rodents)	Normal	Enlarged	Partly known
Cell kinetics	Normal	Slower	Mostly unknown
Colloid osmotic pressure	Normal	Increased	Unknown
Electro-potential Eh	Low (<-100)	High (>-100)	Unknown
Intestinal wall	Thick	Thin	Unknown
Migration motor complexes	Normal	Fewer	A few species
Osmolality	Normal	Reduced	Unknown
Oxygen tension	Low	High	Several species
Production of peptides	Normal	Altered	Unknown
Biochemical			
β-aspartylglycine	Absent	Present	Species in concert
β-glucuronidase	High activity	Low activity	Several species
Bile acid metabolism	Deconjugation	No deconjugation	Many species
	Dehydrogenation	No dehydrogenation	Many species
	Dehydroxylation	No dehydroxylation	A few species
Bilirubin metabolism	Deconjugation	Little deconjugation	Many species
	Urobilins	No urobilins	A few species
Cholesterol metabolism	Coprostanol	No coprostanol	A few species
Fecal tryptic activity	Little or absent	High activity	A few species
Intestinal gases	Carbon dioxide	Some carbon dioxide	Many species
	Hydrogen	No hydrogen	Some species
	Methane	No methane	A few species
Mucin	Degradation	No degradation	Several species
Short-chain fatty acids	Large amounts	Far less	Many species
	Several acids	Few acids	

^a Microflora-Associated Characteristic.

^b Germ-free Animal Characteristic.

Modified from Midtvedt T., 1999 (Midtvedt, 1999).

4 AIMS OF THE STUDIES

The aims of these studies were:

- A. To investigate the capability of several probiotics to alter some biochemical microbial functions, i.e. MACs *in vitro* and/or *in vivo* after monoassociation of GF mice. **Papers I-IV.**
- B. To investigate host-microbial interactions in CV animals under the influence of 'health- and/or growth- promoters':
 - a) Establishment and development of some MACs in growing piglets and the effect of different rearing environment. **Paper V.**
 - b) Establishment and development of some MACs in growing piglets and the effect of a probiotic, *Bacillus licheniformis* and/or an antibiotic, zinc bacitracin (ZB). **Paper VI.**

5 MATERIAL AND METHODS

5.1 BACTERIA

Seventeen probiotic strains were investigated. Additionally, *Clostridium ramosum* G62 and *Escherichia coli* X7 were controls for conversion of bilirubin to urobilinogen and β -glucuronidase activity, respectively. The bacteria were purchased from international collections, provided as free gifts from various sources, or were part of the stock at the Laboratory of Medical Microbial Ecology (Table 2).

The bacteria were stored in appropriate basal media. They were re-cultured in 10 ml of their respective media (Table 2), previous to *in vitro* and *in vivo* inoculations. *B. licheniformis* NCTC 13123 and *E. coli* X7 were incubated aerobically at 37°C for 24 h. All the other strains were incubated anaerobically at 37°C for 72 h.

Paper I-IV. The seventeen probiotics strains listed in Table 2 were investigated.

Paper VI. *B. licheniformis* NCTC 13123 was tested as a feed supplement in weaned piglets.

Table 2. Bacterial strains used

Bacterial strain	Label ^a	Source ^b	Medium ^c
<i>Bifidobacterium bifidum</i> B11	0014405	Tine, Norway	MRS
<i>Bifidobacterium bifidum</i> B12	5001151	Ch. Hansen, Denmark	MRS
<i>Lactobacillus acidophilus</i> La5	0014410	Ch. Hansen, Denmark	MRS
<i>Lactobacillus acidophilus</i>	ATCC 4356	Arla, Sweden	MRS
<i>Lactobacillus casei</i> strain Shirota	YIT 9018	MME ^d	MRS
<i>L. delbrückii</i> subsp <i>bulgaricus</i>	DSMZ 20081	Ch. Hansen, Denmark	MRS
<i>Lactobacillus fermentum</i>	ATCC 14931	MME ^d	MRS
<i>Lactobacillus plantarum</i>	Strain 299	Probi, Sweden	MRS
<i>Lactobacillus plantarum</i>	Strain 299v	Probi, Sweden	MRS
<i>Lactobacillus reuteri</i>	Strain 2010	BioGaia, Sweden	MRS
<i>Lactobacillus rhamnosus</i>	Strain 271	Probi, Sweden	MRS
<i>Lactobacillus rhamnosus</i>	ATCC 7469	MME ^d	MRS
<i>Lactobacillus rhamnosus</i> GG	ATCC 53103	Valio, Finland	MRS
<i>Streptococcus thermophilus</i>	ATCC 19258	Ch. Hansen, Denmark	TH
<i>Streptococcus thermophilus</i> B16	1344506-1	Ch. Hansen, Denmark	MRS
<i>Enterococcus faecium</i>		Gaio, Denmark	MRS
<i>Bacillus licheniformis</i>	NCTC 13123	Alpharma, Norway	TG
<i>Escherichia coli</i> (Control)	Strain X7	MME ^d	GI
<i>Clostridium ramosum</i> (Control)	Strain G62	MME ^d	RCM

^a Type strain by the culture collection or labeled by the donor.

^b Companies that provided the strains.

^c The strains were grown in MRS: de Man, Rogosa and Sharpe; TH: Todd Hewitt; TG: Thioglycollate broth, RCM: Reinforced Clostridial Medium and GI: Glucose.

^d Medical Microbial Ecology.

5.2 MEDIA FOR THE *IN VITRO* STUDIES

Paper I. Calf brain-peptone yeast (CB-PY) medium. A peptone yeast extract, prepared as described in the Anaerobe Laboratory Manual (Holdeman and Moore, 1972) supplemented with freeze-dried calf brain, was used to test cholesterol conversion.

Paper II. The probiotic strains were grown in their respective media (Table 2), prior to SCFAs analyses.

Paper III. A medium inoculated with GF AGUS rat feces, described in Paper III, was prepared in order to analyze both FTA and β -aspartylglycine.

5.3 ANIMALS AND HUSBANDRY

5.3.1 Mice

Paper I-IV. A total of 108 NMRI-KI mice, around 3 months old and inbred for more than 35 generations at the Laboratory of Medical Microbial Ecology, were used. Groups of 4-8 GF mice, of both sexes were allotted to seventeen groups and monoassociated with a respective probiotic. Another group was monoassociated with *C. ramosum* G62. Two more groups comprising 14 and 15 mice were the GF and CV controls, respectively. The GF animals were reared in lightweight stainless-steel isolators (Gustafsson, 1959) and the CV mice in an ordinary animal room with artificial light between 06:00 and 18:00, temperature $24\pm 2^{\circ}\text{C}$ and humidity $55\pm 10\%$. All the animals were fed an autoclaved rodent diet R36 (Lactamin, Sweden) and had free access to water.

5.3.2 Piglets

Paper V. Twelve litters of crossbred pigs (Swedish Landrace x Swedish Yorkshire) from the Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala, were included in the study. Half of the litters were born and reared indoors (IPs) and the other half were born and reared outdoors (OPs); they comprised 58 and 57 piglets, respectively. The sows were gilts, i.e. first time farrowing. All the litters were born in April and slaughtered the same year, in autumn. The indoors farrowing took place in individual pens, and that outdoors, in individual huts in a common enclosure. The piglets and the sows had free access to a pelleted standard complete feed for lactating sows. The piglets were weaned at 63 days of age. After weaning, they were given a conventional dry feed. Fecal samples were collected from OPs of 4 days old until 160 days old and from IPs of 12 hours old until 160 days old. Additionally, a group of ten seven-day old GF minipigs from the Department of Immunology and Gnotobiology, Czech Republic, was investigated. The minipigs were delivered by cesarean section and housed in sterile plastic isolators (Travnicek et al., 1975). They were fed a sterilized full-cream condensed cows milk diet.

Paper VI. The study included four litters comprising a total of 47 piglets (Swedish Yorkshire crossbred), raised at the Pig Research Herd, Swedish University of Agricultural Sciences, Uppsala. They were littermates and born within fourteen days. Throughout the study, the animals had access to a standard complete feed for lactating sows. From 3 weeks of age, suckling was supplemented with a measured amount of feed (0.2-0.3 kg/day) in which *B. licheniformis* NCTC 13123, ZB or both additives had been added. The piglets were weaned at 5 weeks of age and thereafter they had free access to feed containing the above-mentioned additives. Fecal samples were collected before giving the additives and at regular intervals afterwards.

5.3.3 Monoassociation

Papers I-IV. Aliquots of 10 ml from each grown culture were dispensed into ampoules, which were sealed, sterilized on the outside with chromsulfuric acid and taken into a respective isolator. Each group of mice was transferred from a large isolator into a small stainless-steel rearing isolator (SRI) together with the bacterial suspension. Inside the SRI, the ampoule was broken and the content spread on the fur and the bedding material of the mice. The animals remained within the SRI for 11-15 days. Thereafter, they were taken out and killed by cervical dislocation. Bacterial establishment was verified by culturing samples from caecum. The large intestinal contents were sampled and stored at -20°C until the biochemical analyses were performed.

5.4 BIOCHEMICAL ANALYSES *IN VITRO*

Except for differences in preparing the aliquots to be tested, the methods were performed similarly in the *in vitro* and in the *in vivo* parts of this investigation.

Paper I. *Conversion of cholesterol to coprostanol.* The samples were hydrolyzed, extracted and further assayed by gas-liquid chromatography (GLC) as previously described (Midtvedt et al., 1990). The results were expressed as percentage of coprostanol from the total amount of cholesterol plus coprostanol present in the sample.

Paper I. *Deconjugation of TDCA and of GDCA.* The media were supplemented either with TDCA or GDCA and thereafter inoculated with the strains. The bile acids were extracted and further assayed by thin-layer chromatography (TLC) as previously described (Ikawa and Goto, 1975). The results were expressed as positive or negative with regard to deconjugation capability.

Paper II. *Production of SCFAs.* The samples were vacuum-distilled and assayed by GLC as previously described (Hoverstad et al., 1984b; Zijlstra et al., 1977). The chromatogram peaks were analyzed using a Turbochrom autoanalyzer system.

Additionally, the content of SCFAs in aliquots of sterile rodents' diet was tested following a similar procedure.

Paper III. *Inactivation of FTA and degradation of β -aspartylglycine.* FTA was assayed by spectrophotometry (Midtvedt et al., 1988). Results were expressed as mg FTA/kg faeces. β -aspartylglycine was assayed by high-voltage paper electrophoresis (Welling et al., 1980). Results were given as present or absent.

Papers III, IV. *β -glucuronidase activity.* The activity of β -glucuronidase was assayed qualitatively using *p*-nitrophenyl β -D-glucuronide as substrate. Liberation of *p*-nitrophenol by the development of a yellow color was recorded as +, ++, +++ (strong yellow) or negative (colorless).

5.5 BIOCHEMICAL ANALYSES *IN VIVO*

The samples -feces or large intestinal content- were thawed, homogenized and aliquots of 0.7 g and of 0.5 g were taken to measure urobilins and SCFAs, respectively. Additional aliquots of 0.5-1.0 g were diluted in saline solution (1:2), placed at 4°C for 2 h and centrifuged at 4,000 x g 4°C for 30 min. Then, the supernatants were separated to assay FTA, mucin and β -aspartylglycine. The remaining supernatant plus the sediment was used to measure coprostanol.

Papers I-VI. *Conversion of cholesterol to coprostanol, excretion of SCFAs, inactivation of FTA and degradation of β -aspartylglycine* were assayed as for the *in vitro* analyses.

Papers III-VI. *Conversion of bilirubin to urobilins.* Excretion of urobilins was determined by applying the Ehrlich's aldehyde reaction and read in spectrophotometer (Dacie, 1956). The values were converted and given as mmol/kg of large intestinal content or feces (wet weight).

Papers III, V, VI. *Degradation of mucin.* The supernatants were assayed by agar gel electrophoresis and the bands visualized with three staining procedures Toluidine blue (TB), Periodic Acid Schiff (PAS) and Coomassie Brilliant blue (CB), as previously described (Gustafsson and Carlstedt-Duke, 1984). The bands were compared with the controls and reported as degraded or undegraded (Paper III), or as an arbitrary scale from 0 to 100 for mucin degradation (Papers V, VI).

5.6 STATISTICAL ANALYSIS

The statistical evaluation of the differences in the amount of SCFAs between monoassociated groups and the GF group was performed using one-way ANOVA and Dunnett's test. The Mann-Whitney *U* test for unpaired observations was used for statistical analysis of MAC values in OPs and IPs. Differences in MACs of piglets fed a

supplemented diet were analyzed with one-way ANOVA. Differences were considered significant at p values <0.05 .

6 RESULTS

6.1 INFLUENCE OF PROBIOTICS ON MACS *IN VITRO*, AND IN GNOTOBIOTIC MICE

Paper I. In this study two microbial functions that may influence directly or indirectly the cholesterol metabolism were investigated. The total probiotics (Table 2) were tested for their capability to convert cholesterol to coprostanol *in vitro*, and *in vivo* after monoassociation with GF mice. The same probiotics, except for *L. casei* strain Shirota, *L. reuteri* strain 2010 and *B. licheniformis* NCTC 13123, were tested for their capability to deconjugate TDCA and/or GDCA *in vitro*.

The gas-liquid chromatograms of the monocultures and those of the monoassociated mice showed presence of cholesterol and absence of coprostanol, i.e. none of the probiotics investigated was able to convert cholesterol to coprostanol *in vitro* or *in vivo*. A chromatogram of a large intestinal content from a monoassociated mouse is presented in Fig 1 (Paper I). Similar chromatograms were observed in all the samples from the monoassociated animals.

Concerning capability of the probiotics to deconjugate bile acids, as expected, most strains were able to deconjugate either TDCA or GDCA (Table I, Paper I). Eight out of thirteen probiotics tested were able to split TDCA and eleven out of fourteen probiotics were able to deconjugate GDCA *in vitro*.

Paper II. This study was carried out in order to screen the capability of the probiotics to produce SCFAs in routine media and/or to influence their amount in the GI tract of GF mice. The probiotics presented in Table 2 except for *B. bifidum* BB11, *L. delbrückii* subsp *bulgaricus* DSMZ 20081 and *L. fermentum* ATCC 14931, were tested for SCFAs as monocultures in their respective growth media, i.e. TH or MRS. Only one species, *L. reuteri* strain 2010, showed a slightly higher amount of SCFAs when compared to the control medium. The other monocultures showed lower amounts than the control. Thus, none of the probiotics tested was able to increase significantly the amount of SCFAs *in vitro*. These results are summarized in Table 3. More details are given in Tables 1 and 2 (Paper II).

Table 3. Total amount of SCFAs in monocultures of probiotics

Group ^a	SCFAs <i>in vitro</i>	
	Growth medium ^b	Monoculture ^c
No bacterium	TH	7.7
<i>S. thermophilus</i> ATCC19258	TH	4.7
No bacterium	MRS	16.6
Other bacterial strains	MRS	(3.9-17.9) ^d

^a Thirteen probiotics were investigated.

^b TH: Todd Hewitt, MRS: de Man, Rogosa and Sharpe.

^c SCFAs are given as mmol/l sample.

^d Figures are maximum and minimum values found in twelve monocultures.

The probiotics presented in Table 2 were monoassociated with GF mice and the total and individual SCFAs were tested in the large intestinal content from the animals. In nine out sixteen groups, the total amount of SCFAs was higher than those of the GF group. They were in ascending order those inoculated with the strains *S. thermophilus* ATCC 19258, *L. reuteri* strain 2010, *L. plantarum* 299, *B. bifidum* BB 11, *L. acidophilus* La5, *S. thermophilus* B16, *L. casei* strain Shirota, *L. rhamnosus* ATCC 7469 and *L. rhamnosus* GG ATCC 53103. The predominant acid in all these groups was acetic acid. The amount of SCFAs in the CV group was however, much higher than that of any of these groups. The GF group showed higher values of SCFAs than expected. Therefore, in order to search for other sources of SCFAs, the animal's diet was tested for SCFAs content. In fact, appreciable amounts of all SCFAs were detected in the sterile pellets. More details are presented in Fig 1 and Tables 2 and 3 (Paper II).

Paper III. In this work, we wanted to investigate the capability of probiotics to influence five biochemical MACs *in vitro* and/or *in vivo*. The probiotics listed in Table 2 were monoinoculated into specific media and tested for β -glucuronidase activity, inactivation of FTA and degradation of β -aspartylglycine. Four out of sixteen strains expressed β -glucuronidase activity. They were *L. reuteri* 2010 and all the *L. rhamnosus* strains tested (Table 1, Paper III). The activity was determined at different incubation times and at pH 6.5 and pH 8.0. In general, when the enzyme activity was first detected, the reaction was weak but turned out stronger along with incubation. No differences in pH were observed. The other two functions were tested in the probiotics listed (Table 2) except for *L. reuteri* strain 2010, *L. casei* strain Shirota and *B. licheniformis* NCTC 13123. Values of FTA in the inoculated media were similar to those of the control. β -aspartylglycine was present in the uninoculated and in the inoculated media (Table 4).

For the *in vivo* investigations the probiotics in Table 2 were monoassociated with GF mice and the biochemical MACs FTA, β -aspartylglycine, urobilins and mucin were

tested in the large intestinal contents from the animals. All the strains were tested for FTA and β -aspartylglycine. High values of FTA were detected in all the monoassociated groups, similar to those of the GF group. β -aspartylglycine was present in all the monoassociated animals (Table 4).

Table 4. MACs *in vitro*, and *in vivo* in mice monoassociated with probiotics^a

Group ^b	Fecal tryptic activity (FTA) ^c		β -aspartylglycine ^d	
	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>
Medium	45 (44-45)		Present	
Probiotics	48 (40-65)	1007 (695-1297)	Present	Present
<i>B. licheniformis</i>		1149 (988-1330)		Present
Germ-free (GF)		824 (696-958)		Present
Conventional (CV)		18 (0-27)		Absent

^a The probiotic strains listed in Table 2 were investigated *in vitro* except for *L. reuteri* strain 2010, *L. casei* strain Shirota and *B. licheniformis* NCTC 13123; all probiotics listed in Table 2 were investigated *in vivo*.

^b Four to five mice were monoassociated with a respective probiotic; 14 mice were investigated in each group control (GF and CV).

^c Figures are medians (maximum and minimum) of FTA given as mg/kg of either feces or large intestinal content.

^d The electrophoretic pattern of the samples was compared with that of the standard and reported as present or absent.

The probiotics listed in Table 2 were monoassociated with GF mice and the presence of urobilins and mucin was tested in the large intestinal contents from the animals. Urobilins were absent in all these samples but present in a group of mice monoassociated with *C. ramosum* G 62 was used as control. The mucin electrophoretic pattern in the samples from the monoassociated mice was similar to that of the GF group.

Paper IV. In this study, we wanted to investigate the influence of the probiotic *B. licheniformis* NCTC 13123 on a similar set of MACs studied in the previous publications. The strain was tested for β -glucuronidase activity, similarly as in Paper III. *B. licheniformis* NCTC 13123 did not express β -glucuronidase activity *in vitro* after 48 h incubation.

Other five biochemical MACs were investigated in the large intestinal contents from ex-GF mice monoassociated with *B. licheniformis* NCTC 13123. The concentrations of total SCFAs in the monoassociated mice were slightly lower than those found in GF mice (Table 5).

Table 5. Total and individual amounts of short-chain fatty acids (SCFAs)^a in the large intestinal contents from mice mono-associated with *Bacillus licheniformis* NCTC 13123 (B lichen)

Group ^b	Total SCFAs	Acetic	Propionic	n-butyric	Other SCFAs ^c
GF (SD) ^d	16.89 (1.51)	16.05 (1.37)	0.25 (0.56)	0.59 (1.01)	ND
CV (SD) ^d	111.73 (17.29)	56.79 (10.70)	19.45 (4.66)	24.43 (6.33)	11.06
B lichen	12.35 (2.47)	11.65 (2.38)	0.15 (0.34)	0.08 (0.01)	0.47

^a Figures are means (SD) of SCFAs given as mmol/kg large intestinal content (wet weight).

^b GF: Germ-free, CV: Conventional, 5, 4 and 8 animals were used, respectively.

^c Other SCFAs included iso-butyric, iso-valeric and capronic acid.

^d Data taken from Cardona et al (Cardona et al., 2001).

Except for the amount of SCFAs, all the biochemical MAC values found in the monoassociated mice in this study, were similar to the findings in the other probiotics tested in this thesis, i.e. coprostanol and urobilins were absent in all the samples, the tryptic activity was high and β - aspartylglycine was present in all the samples. Results of the latter two MACs are shown in Table 4.

6.2 INFLUENCE OF PROBIOTICS AND OTHER EXTERNAL FACTORS ON MACS IN PIGLETS

Paper V. The studies in Papers V and VI were designed in order to investigate the effect of health and/or growth promoters on six biochemical MACs in growing piglets. In Paper

V the influence of rearing piglets in a conventional way, i.e. indoors or in an ecological way, i.e. outdoors on the MACs was studied. In main, the results show statistically significant differences between the groups in three MACs, i.e. excretion of total SCFAs, degradation of mucin and excretion of urobilins. The differences observed were most pronounced at 20 days of age and the MAC values were most often higher in the IPs than in the OPs (Table 6). More details are given in Table 2 (Paper V).

Additionally, four MACs, i.e. conversion of cholesterol to coprostanol, SCFAs excretion, inactivation of FTA and degradation of β -aspartylglycine were tested in fecal samples from GF minipigs. All the MAC values found in the latter group were within the range of a GAC pattern. More details are presented in Table 2 (Paper V).

Table 6. Means of MAC values in fecal samples from piglets reared outdoors (OPs) or indoors (IPs) and their sows

Piglet group ^a	Coprostanol% ^b	Total SCFAs ^c	Mucin ^d	Urobilins ^e
4 days				
OPs (16)	22	44	46 ^f	NT
IPs (10)	10	58	76 ^g	NT
20 days				
OPs (12)	71	22 ^f	92	0.4 ^f
IPs (10)	74	49 ^g	87	0.7 ^g
35 days				
OPs (15)	76	46	99	0.22
IPs (10)	75	51	98	0.19
70 days				
OPs (25)	64	74	100	0.05
IPs (12)	63	71	92	0.07
160 days				
OPs (10)	56	109	95	0.03
IPs (9)	58	120	96	0.05
Sows				
Outdoors (6)	47	103 ^f	100	0.11
Indoors (5)	54	162 ^g	100	0.08

^a Figures in parentheses are number of samples investigated.

^b Figures are percentages of coprostanol of the total amount of coprostanol plus cholesterol present in the sample.

^c Figures are total amount of SCFAs expressed as mmol/kg feces (weight wet).

^d Degradation of mucin is expressed as an arbitrary scale from 0 (no degradation) to 100 (total degradation).

^e Figures are mmol of urobilins excreted/kg feces (wet weight).

^{f, g} Data marked with different letter in groups at the same age differed significantly ($p < 0.05$); comparisons between groups were performed by Mann-Whitney U test.

Paper VI. In this study, the two diet supplements, *B. licheniformis* NCTC 13123 and ZB tested in fecal samples from growing piglets, influenced three MACs: the total amount of SCFAs, degradation of mucin and the excretion of urobilins. These differences were observed mainly at seven weeks of age. The major trends were an increasing amount of coprostanol that decreased along with age, regardless of the diet and an increasing amount of fecal output of SCFAs (Table 7) along with age, too. More details concerning SCFAs are given in Table II, Paper VI.

Table 7. Mean values of MACs in fecal samples from piglets fed a diet containing zinc bacitracin (ZB) and/or *B. licheniformis* NCTC 13123 (B lichen)

Group ^a	Coprostanol% ^b	Total SCFAs ^c	Mucin ^d	Urobilins ^e
3 weeks (29)	85	42	91	1.1
5 weeks				
ZB (10)	85	43.9 ^g	98	0.52
B lichen (10)	83	40.8 ^f	97	0.46
ZB/B lichen (12)	81	69.7	98	0.46
Control (11)	83	61.7	98	0.47
7 weeks				
ZB (11)	69	93.6 ^g	95	0.07 ^f
B lichen (12)	65	96	100 ^g	0.07 ^g
ZB/B lichen (11)	71	88.8 ^f	76 ^f	0.08 ^h
Control (11)	68	110.5	89	0.18
10 weeks				
ZB (10)	67	98.1	99 ^f	0.06
B lichen (13)	61	94.9	89	0.06
ZB/B lichen (11)	61	101.6	92	0.07
Control (10)	66	91	91	0.08

^a Figures in parentheses are number of samples investigated.

^b Figures are percentages of coprostanol of the total amount of coprostanol plus cholesterol present in the sample.

^c Figures are mmol SCFAs/kg feces (weight wet).

^d Degradation of mucin is expressed as an arbitrary scale from 0 (no degradation) till 100 (total degradation).

^e Figures are mmol urobilins/kg fecal samples (wet weight).

^{f, g, h} Figures in the same column at similar age marked with different letter differed significantly from the control ($p < 0.05$); comparisons between each group and the control were performed with ANOVA. The additives were offered to the animals from 3 weeks of age.

7 DISCUSSION

7.1 CONVERSION OF CHOLESTEROL TO COPROSTANOL

The correlation between high concentrations of plasma cholesterol and cardiovascular diseases in humans (Austin, 1994) has stimulated the development of dietary approaches in order to lower blood cholesterol.

Among the three most likely mechanisms by which microorganisms might influence cholesterol metabolism, i.e. conversion of cholesterol to coprostanol, interference with the enterohepatic circulation of bile acids and adsorption of the cholesterol to the microbe, we investigated in Paper I the direct action of each selected probiotic on the cholesterol molecule, i.e. conversion of cholesterol to coprostanol. The results show that none of the probiotics was able to transform cholesterol to coprostanol *in vitro* or *in vivo* in monoassociated mice. So far, no probiotic has been found capable of performing the cholesterol conversion. Therefore, it is reasonable to assume that cholesterol conversion as a mechanism of direct microbial action upon cholesterol metabolism, does not account for the supposed cholesterol-lowering capability of probiotics. Nevertheless, other mechanisms accounting for the cholesterol-lowering effect of probiotics should not be ruled out. In a previous study, Zacconi et al. (Zacconi et al., 1992) showed a decrease in serum cholesterol levels in ex-GF mice monoassociated with *Lactobacillus acidophilus* and with *Enterococcus faecium*. Since according to the authors, both bacteria were able to 'assimilate' cholesterol *in vitro* and to grow in presence of bile salts, it was suggested that the hypocholesterolemic effect of these strains might be related to these properties.

Microbial conversion of cholesterol to coprostanol has been found in all CV adult mammals so far studied. However, a wide range from zero to high levels of coprostanol excretion has been observed in humans (Midtvedt et al., 1990; Wilkins and Hackman, 1974). In our studies on GF minipigs, this microbial function was absent, as expected. However, when we investigated this function in piglets at different ages and under the influence of diverse exogenous factors, i.e. rearing environment and supplementation of diet with an antibiotic or a probiotic (Papers V, VI), coprostanol was found in all the samples through the entire experimental period without difference among the groups. Interestingly, piglets at very young ages excreted amounts of coprostanol above adult values. Since the cholesterol-converting property has been reported only in strict anaerobic microorganisms (Freier et al., 1994), we assume that these microbes are early established in piglets. The high values of coprostanol observed in young piglets suggests presence of high number of cholesterol-reducing species that may overcome the effects of the additives and explain why neither ZB nor *B. licheniformis* influenced the cholesterol conversion property of the intestinal microflora in these studies. On the other hand, the dose of ZB and the inoculum of *B. licheniformis* may have been not high enough to act upon the cholesterol-conversion property of intestinal microorganisms. In contrast to our observations, this microbial function has shown high sensitivity to bacitracin intake in

humans (Midtvedt et al., 1990) and in horses (Collinder et al., 2000a). Resistance to ZB in piglets' bacterial flora should not be excluded.

7.2 DECONJUGATION OF TDCA AND OF GDCA

The main elimination means for plasma cholesterol are direct biliary excretion and conversion of cholesterol to bile acids, which are conjugated in the liver, followed by biliary excretion. Most of the conjugated bile acids are reabsorbed through the enterohepatic circulation. However, in the intestine bile acids undergo microbial deconjugation. Free bile acids are less efficiently reabsorbed and hence excreted in feces. Since the total bile acid pool is constant, that loss is compensated by new synthesis in the liver from cholesterol. On the other hand, deconjugated bile acids have reduced solubility leading in turn to reduction in the cholesterol solubility. The hydrolysis capability of microorganisms may be responsible for the cholesterol-lowering effect. Consequently, ingestion of microorganisms capable of deconjugating bile acids has been suggested as an indirect way of lowering cholesterol.

In Paper I we show that twelve out of fourteen probiotics tested were able to split bile acid conjugates *in vitro*. The results correlate with previous findings of bile salt hydrolysis-activity in *Lactobacillus* and *Bifidobacterium* (Midtvedt and Norman, 1967; Shindo and Fukushima, 1976). Additionally, a correlation was reported in a previous study between caecal bile acid composition in monoassociated mice and microbial bile acid transformation by human intestinal bacteria *in vitro* (Narushima et al., 1999). Thus, it seems reasonable to assume that our strains would be able to deconjugate bile acids *in vivo*. It should be noted that all strains that have been claimed to possess cholesterol-lowering capability (Anderson and Gilliland, 1999; Fukushima and Nakano, 1996; Mott et al., 1973; Zacconi et al., 1992) belong to species that are most often able to deconjugate bile acids. Moreover, lactobacilli have been claimed to be the main contributors to total bile salt hydrolase activity in the murine intestinal tract (Tannock et al., 1989).

In Paper I, we have investigated two microbial functions that are closely linked, conversion of cholesterol to coprostanol and deconjugation of bile acids. Both functions have been associated with the hypocholesterolemic effect of probiotics. We did not find evidences of direct microbial action upon cholesterol metabolism. In main, our results agree with previous findings regarding deconjugation capability of the strains (Klaver and van der Meer, 1993; Midtvedt and Norman, 1967; Tannock et al., 1989). However, the ability of the probiotics to deconjugate bile acids should be confirmed *in vivo*, as a possible mechanism of lowering cholesterol.

A third suggested mechanism of microbial influence on cholesterol metabolism, i.e. adsorption of the cholesterol molecule to the microorganism (probiotic), has been based mainly on *in vitro* investigations (Dambekodi and Gilliland, 1998; Gilliland et al., 1985) and more research *in vivo* is needed. It is important to emphasize that whatever the mechanism is, the action of the bacterium must be exerted in the small intestine, to interfere with cholesterol metabolism.

From the results in Paper I, it is clear that the mechanisms behind the cholesterol-lowering capacity of probiotics are not fully elucidated and extensive research on this field is needed in order to get stronger evidence for their cholesterol-lowering properties that have been claimed.

7.3 PRODUCTION OF SCFAS

Microbial production of SCFAs in the GI tract of mammals represents a clear symbiotic relationship between the host and its microflora. By utilizing exogenous and endogenous substrates, the microflora helps its host gaining maximum nutritional value from the molecules that would otherwise be lost (Hooper et al., 2002). The microorganisms, in turn, obtain nutrients for their growth and proliferation. The importance of SCFAs in a large number of physiological functions such as maintenance of the integrity and welfare of the intestinal mucosa (Roediger, 1980), and the association between SCFA alterations and several colonic diseases (Cook and Sellin, 1998), highlights the importance of investigating these metabolites in relation to ingestion of probiotics.

The studies on SCFAs (Paper II, IV) were undertaken in order to screen for capability of several probiotics (Table 2) to produce SCFAs *in vitro* and/or to induce their formation *in vivo* in monoassociated mice. In the *in vitro* investigations, the production of SCFAs was tested in monocultures of the probiotics (Paper II). None of the probiotics tested was able to increase significantly the amount of SCFAs *in vitro*. Since the concentrations of the monocultures tended to be lower than those of the control media, it is reasonable to assume that the microorganisms have utilized the SCFAs already present in the media. However, neither, utilization of SCFAs nor production of other metabolites, was investigated. Furthermore, since each anaerobic bacterium presents a specific fermentation pattern (Macfarlane et al., 1994) and needs specific substrates, it may be possible that our routine media as well as our experimental conditions did not fulfill the requirements for production of SCFAs. Thus, under other circumstances, some of the probiotics may produce SCFAs.

In our *in vivo* investigations, nine out of sixteen monoassociated groups (Paper II), showed a higher amount of total SCFAs than the GF group. But the amounts of SCFAs in these groups were markedly lower than those of the CV mice. However, these studies were hampered by the fact that the animal's diet contained substantial amount of all SCFAs (Table 2, Paper II). Therefore, a possible alteration in absorption of the dietary-derived SCFAs induced by the probiotics has to be kept in mind.

In the mice monoassociated with *B. licheniformis* (Paper IV) the amount of total SCFAs was lower than that of GF mice. Since we assume that the SCFAs found in the GF animals are derived from the diet, utilization of the acids already present rather than production might have occurred. As mentioned above, this possibility was not investigated. However, it is well known that *B. licheniformis* is able to utilize acetic acid and propionic acid as a sole carbon source (Sneath, 1986).

Some actions attributed to SCFAs such as energy supply, stimulation of absorption of fluid and electrolytes in the GI tract and provision of a major metabolic fuel for

colonic mucosa, are of importance for maintaining the intestinal health of mammals. These functions may be especially important in critical periods of growing piglets such as weaning and post-weaning. In the studies in Paper V in which piglets were reared in two different environments, i.e. outdoors or indoors, the fecal SCFAs were measured at different ages, from 4 days of age to slaughter age. In general, the total fecal amount of SCFAs was higher in the IPs than in the OPs; the difference was significantly higher at 20 days of age, as a consequence of a decrease in the total amount of SCFAs in the OPs. Interestingly, a similar difference was observed in the sows. In addition to the difference in the total amount of SCFAs, differences in the proportion of the individual acids were observed. The proportion of acetic acid with respect to the other acids was significantly higher in the OPs at 20 days of age than that of the IPs in which the proportions of the individual SCFAs were similar to those of the sows indoors. Taken together, these results suggest presence of a higher number of microorganisms active in the fermentation process and probably a higher diversity of species in the indoor- than in the outdoor- environment. Another explanation for the difference in the total SCFAs at 20 days of age may be a higher demand of energy in the OPs. A tendency towards a quantitative increase of longer chain of SCFAs along with age occurred in all of the groups, similar to that previously found in children (Midtvedt et al., 1988) and in piglets (Swords et al., 1993), an indication of a possible increase in the complexity of the GI microflora.

It is important to mention that being aware of the influence that an early weaning may exert on functionality of the intestinal microflora, in this study the piglets were weaned at an older age (63 days old), than in the current farming practice (21-35 days old).

Antibiotics and more recently probiotics have been used in piglets aimed at controlling gastrointestinal health and improving growth. However, previous studies indicate a significant impact of antibiotics intake on the animal intestinal microflora (Gargallo and Zimmerman, 1980; Mikelsaar and Siigur, 1992). Obviously, depending on its profile, antibiotics may either decrease or suppress specific functional active microorganisms.

In piglets fed on a diet supplemented with ZB, *B. licheniformis* or with both supplements, the excretion of total SCFAs was lower than in the control at young ages, i.e. 5 weeks and 7 weeks. These differences may be related to the feed additives. The suppression or decrease in the amount of active microorganisms by the action of the antibiotic may explain the reduction in the excretion of SCFAs in the group fed a diet containing ZB. According to the concentrations of SCFAs observed in the group fed the probiotic supplement, it seems *B. licheniformis* has exerted a similar action on SCFAs. Since bacitracin is actually derived from the *B. licheniformis* strain, production of the antibiotic by *B. licheniformis* might have occurred as a mechanism of action of the probiotic. However, the combination of ZB/*B. licheniformis* did not influence the concentration of total SCFAs in 5 week-old piglets. Differences in intake of solid feed and the limitation of the food during the suckling period should be taken into consideration. In

fact, the findings in total SCFAs in 7 week-old piglets pointed out that the effect on SCFAs was most probably exerted by the antibiotic, though a tendency towards a lower excretion of SCFAs was observed in all the groups when compared to the control.

The usual tendency towards an age-dependent higher production of longer chain-fatty acids seems to be delayed by both additives, suggesting an effect of ZB and *B. licheniformis* on the establishment of a more complex intestinal microflora in growing piglets.

7.4 INACTIVATION OF FTA

The net amount of FTA reflects the pancreatic secretion of trypsinogen and trypsin inactivators, the stimulation of secretion of trypsinogen by the diet, the activation of trypsinogen to trypsin by brush border enzymes and the inactivation of trypsin by the microflora. Since there is a high demand for proteolytic activity at early ages, it is important to know the action of ingested bacteria on this function. An increase in microorganisms capable of inactivating trypsin would interfere with the normal proteolytic activity leading the host to lose essential protein-derived metabolites.

In the studies on FTA presented in Papers III and IV, none of the probiotics investigated (Table 2) *in vitro* and *in vivo* showed any ability to inactivate FTA. Until now, this capability has been observed only in one bacterial strain namely *Bacteroides distasonis* (Ramare et al., 1996).

When investigated in GF minipigs, values of FTA were high and similar to those found in GF rodents reported in the present and other studies.

FTA was not influenced by environmental conditions, i.e. rearing piglets outdoors or indoors (Paper V). These results suggest presence of high number of active trypsin-inactivating microbes in both environments.

FTA was influenced neither by ZB nor *B. licheniformis* when used as dietary supplements. This finding is in accordance with previous studies that showed no action of bacitracin on this MAC (Gustafsson and Norin, 1977; Norin et al., 1988). It also agrees with the assumption that bacitracin may be most active against Gram-positive bacteria.

7.5 DEGRADATION OF MUCIN

Degradation of mucin by the intestinal microflora is a physiological function observed in CV animals and healthy adult humans. In contrast, in GF animals and newborn children mucin is not degraded and excreted in feces. Due to the roles of mucin on several physiological functions and to the association with some pathological conditions with mucin alterations (Corfield et al., 2000), it is of relevance to investigate the influence of probiotics on this microbial function.

When the probiotics listed in Table 2 were investigated for their capability to degrade mucin in monoassociated mice, none of the strains was able to induce any change in the mucin electrophoretic pattern (Paper III). However, our methods do not allow us to conclude that the strains have not removed certain sugar residues from the main molecules. It has been hypothesized that several strains might interact with the mucin

structure in a special sequence to ensure complete degradation (Hoskins et al., 1985). Therefore, in spite of the similarity of the mucin patterns of GF and monoassociated mice, a possible participation of the probiotics in degradation of mucin when acting in concert with other bacteria, should not be excluded.

When the degradation of mucin was studied in piglets reared indoors or outdoors, a higher degradation was observed in IPs than in OPs four days old (Paper V). This difference suggests that colonization by mucin-degrading microorganisms may occur earlier in IPs than OPs.

In piglets fed on a diet supplemented with ZB, *B. licheniformis* or with both supplements (Paper VI), neither ZB nor *B. licheniformis* showed consistent influence on degradation of mucin. However, a clear observation in this study was the early establishment of this microbial function. In contrast, other authors have reported a pronounced effect on this function in man (Carlstedt-Duke et al., 1986a) and in rats (Gustafsson and Norin, 1977).

7.6 DEGRADATION OF β -ASPARTYLGLYCINE

Degradation of β -aspartylglycine is a function of the GI microflora. Welling et al. have demonstrated that appearance of β -aspartylglycine in feces of hosts under antibiotic treatment correlated with the elimination of the anaerobic flora (Welling, 1982; Welling and Groen, 1978). The authors suggested using determination of β -aspartylglycine as a marker of the integrity of the intestinal microflora.

When the probiotics listed in Table 2 were tested for their capability to degrade β -aspartylglycine (Papers III, IV), none of them was able to perform this function *in vitro* or *in vivo*. At present, no single microorganism has been implicated in the degradation of β -aspartylglycine, it appears that this function is influenced by the interactions of increasing amounts of bacteria rather than just one (Welling et al., 1980).

In GF minipigs, β -aspartylglycine was not degraded (Paper V). However, in the comparative studies between OPs and IPs as well as in the study on piglets fed on a diet supplemented with ZB and/or *B. licheniformis* (Papers V, VI), β -aspartylglycine was completely degraded since the beginning of the experiments and was not affected by any of the experimental conditions.

7.7 CONVERSION OF BILIRUBIN TO UROBILINS

Two microbial functions are implicated in the metabolism of bilirubin, deconjugation of bilirubin glucuronide and conversion of bilirubin to urobilins. This formation is likely to be a detoxification step of bilirubin since urobilins may not be toxic whereas unconjugated bilirubin is toxic against nervous tissue. Because of the implication of bilirubin metabolism in the pathogenesis of neonatal jaundice, it is of clear importance to investigate the possible actions on these functions of ingested bacteria in hosts, especially in newborn and children.

In the studies presented in Papers III and IV where urobilins were investigated in mice monoassociated with the probiotics presented in Table 2, urobilins were absent in all the samples, indicating that none of the probiotics was able to perform the conversion of bilirubin to urobilins *in vivo*. However, this function has been found in other different genera of intestinal microflora such as clostridia and bacteroides (Fahmy et al., 1972; Gustafsson and Swenander-Lanke, 1960; Midtvedt and Gustafsson, 1981; Vitek et al., 2000).

In the studies performed in growing piglets under different environmental conditions and fed on a diet supplemented with ZB and/or *B. licheniformis* (Paper V, VI), the amount of urobilins excreted was very high in piglets at young ages. This amount consistently decreased as the piglets age increased. The high values of fecal urobilins in young piglets may reflect a high excretion of bilirubin, as well as the presence of microorganisms capable of forming urobilins. Regarding the low values found in older piglets and sows, a diet-dependence suggested for other species (Collinder et al., 2000b), i.e. an increased mass of feces diluting the fecal sample thus decreasing the amount of urobilins/volume, may also occur in piglets.

When analyzing urobilins in the groups fed on a supplemented diet, lower concentrations were found in all the experimental groups at seven weeks of age, when compared to the control group (Paper VI). This finding may reflect an influence of the additives on the intestinal microflora. Indeed, the concentrations of fecal urobilins in the control group were similar to those of the piglets studied in Paper V. In addition, this finding may also reflect an influence of ZB on urobilins excretion by decreasing the microorganisms responsible for this conversion.

7.8 β -GLUCURONIDASE ACTIVITY

The intestinal microflora is considered as the major source of β -glucuronidases. The glucuronides formed in the liver from endogenous (bilirubin, some steroids, etc) as well as from exogenous (xenobiotics) compounds may be further deconjugated by β -glucuronidases. This leads to enterohepatic circulation of the free compounds and thus retardation in the elimination of potentially toxic compounds. Therefore, it is important to know whether and to what extent strains used as probiotics express this enzyme activity.

In the studies in Papers III and IV, four out of seventeen probiotics expressed β -glucuronidase activity *in vitro*. These results are in agreement with previous investigations showing deconjugating activity in some lactobacilli (Hawksworth et al., 1971; Kent et al., 1972). We would also expect expression of the enzyme *in vivo*, since previous comparative studies have always shown β -glucuronidase activity *in vivo*, when expressed *in vitro* (Cole et al., 1985; Gadelle et al., 1985; Tamura et al., 1996). It has been already established that most β -glucuronidases present in CV mammals is of bacterial origin (Rod and Midtvedt, 1977). Therefore, feeding a probiotic capable of producing β -glucuronidases, may increase the availability of free compounds influencing the excretion of these compounds and thereby affecting the establishment of a well-balanced host-

microbial interactions on the enterohepatic circulation of bilirubin and other compounds (Aziz et al., 2001; Midtvedt, 2001).

Nevertheless, several investigations have reported significant decrease in the levels of some fecal enzymes, including β -glucuronidase, after feeding probiotic products (Abdelali et al., 1995; Goldin et al., 1980; Ling et al., 1994). This effect may be due to bacteria-bacteria interactions within the GI tract. In those studies nonetheless, the enzymatic activity of the probiotic strain itself was not reported. Furthermore, it should be emphasized that the mechanisms of bilirubin transformation by microorganisms involves complex reactions that are not fully understood.

8 CONCLUSIONS AND FUTURE PERSPECTIVES

The results of the present investigations showed the suitability of investigating the intestinal microflora of mammals in terms of functionality, i.e. to evaluate actions of probiotics, antibiotics and/or environmental factors on microbial functions by applying the MAC/GAC concept in experimental studies as well as in animals. We conclude that:

- a. The probiotics tested exert only minor influences on the MACs investigated. However, absence of a function *per se*, does not exclude an effect upon that function when a probiotic strain is acting within the intestinal ecosystem where also microbial-microbial interactions take place.
- b. The MACs investigated are rapidly established in piglets.
- c. A higher exposure to functionally active microorganisms may occur in the indoors than in the outdoors environment.
- d. The MACs investigated are likely to be influenced by the rearing environment, ZB and *B. licheniformis* in piglets, mainly at young ages.
- e. Disturbances on biochemical functions might have been overcome by a 'healthy adult microflora' observed at completion of both experiments.

Our results add new insights related to specific functions of probiotics. This approach should be used in future selection of microbial strains for health- and growth- promoting purposes.

In spite of absence of specific functions of the probiotics investigated in monoassociated animals, they may create an environment suitable for growth of functionally active microorganisms. Gustafsson et al. (Gustafsson et al., 1998) found that *L. delbruekii* subsp. *bulgaricus* DSMZ 20081 and *S. thermophilus* ATCC 19258 given orally to rats, were able to reduce the antibiotic effect on the formation of deoxycholic acid, i.e. the major microbial metabolite of cholic acid. Since those strains could not perform that transformation, it seems reasonable to assume that the probiotics stimulate microbial species already present in the intestine and thus, exert a clear probiotic effect.

Further research focused on clarification of mechanisms for microbe-host and microbe-microbe cross-talks, will lead to a more proper selection process as well as a more selective use of probiotics under physiological and pathophysiological conditions.

Access to GF animals creates possibilities for studies of probiotics in poly-associated models.

The MACs are applicable tools for evaluating the establishment of functionally active ecosystems as well as for following possible alterations caused by exogenous factors.

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