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MICROFLORA ASSOCIATED CHARACTERISTICS: CHARACTERISATION OF THE COMPOSITION OF THE MICROFLORA BY VARIOUS BIOCHEMICAL TESTS CONCERNING BACTERIAL PRODUCTS AND CHEMICAL MODIFICATION OF BILE ACIDS

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SUMMARY

The interplay between the host and his microbes can be followed by studying a set-up of biochemical reactions in intestinal content and faeces. In this review, the interest has been focused on two topics, i. e. presence/absence of β-aspartylglycine and microbial transformation of bile acids.

INTRODUCTION

Normally, humans harbour an intestinal microflora that numbers 10^{13}-10^{14} microbes, which is a population equalling or exceeding the total number of cells comprising the whole human body. From man's cradle to his grave, this population forms dynamic ecosystems, governed by a wide variety of host- and microflora-derived physiochemical conditions, including pH, redox potential, nutrient availability, peristalsis, and transit time.

Several approaches can be made to the task of evaluating the gastrointestinal microflora. In the past, many reports have dealt with the composition of the human gastrointestinal flora, and great efforts have been expended in isolation, identification and enumeration of the hundreds of species constituting this flora. Additionally, nearly as much efforts have been made to follow alterations in the composition and balance of the flora after various external factors, such as changes in dietary habits and oral or parenteral administration of antimicrobial agents. In earlier studies, coliform bacteria most often were used as indication organisms. However, as skills and techniques have improved, the trend has been to study alterations in the numbers of both aerobic and anaerobic microorganisms. And - as underlined by other speakers at this symposium - a qualitative and quantitative evaluation of the gastrointestinal microflora in man is extremely time-consuming and difficult to perform and, to the best of my knowledge, a full-scale evaluation has so far never been carried out.

Another approach is to study the metabolic capacity of the microbial flora ("what can the microbes do"). It goes without saying that most often, these studies are carried out *in vitro*. A long series of biochemical transformations have been studied. Some *in vivo* metabolic capacity tests have been worked out, and some of them are well established in clinical medicine, as the bile acid deconjugation test (see later) and the lactulose test.

A third approach is more directly to

Table 1: Some intestinal structures and functions influenced by the microflora

Parameter	MAC	GAC	Microbes involved
Intestinal wall	Thick lamina propria	Thin lamina propria	Unknown
	Irregular villi	Regular villi	Unknown
	High cell turnover	Low cell turnover	Unknown
Caecum size	Normal	Enlarged	Unknown
Intestinal smooth muscle activity	Vivid spontaneous contractions	Markedly reduced contractions	Unknown
Sensitivity toward biogenic amines	Normal	Markedly reduced	Unknown
Amounts of biogenic amines	MAC=GAC	GAC=MAC	
Bile acid metabolism	Deconjugation	No deconjugation	Several species
	Dehydrogenation	No dehydrogenation	Several species
	Dehydroxylation	No dehydroxylation	Few species, mostly anaerobic, non-spore-forming, Gram + rods
Bilirubin metabolism	Deconjugation	Little deconjugation	Several species
	Urobilinogen formation	No urobilinogen formation	*Clostridium ramosum*
Cholesterol	Mainly coprostanol	Only cholesterol	An *Eubacterium* species
Dipeptidases (such as β-aspartylglycine)	Absent	High amounts	Unknown
Intestinal gases	H_2, CH_4, CO_2	No H_2 or CH_4, reduced CO_2	Unknown
Mucus	Absent in faeces	High amount	Several species, such as*Peptostreptococcus micros*, *Bacteroides ruminococcus*, and *Bifidobacterium*
SCFAs	High amounts, several acids	Small amounts, few acids	Probably several species
Tryptic activity in faeces	Little or no activity	High activity	Unknown

study the functional status of the gastrointestinal flora ("what have the microbes done"). In order to do so, it is necessary to clarify which mechanisms and reactions are related to the host and which to the microflora itself, respectively. With a slight travesty of the well-known terminology introduced by Claude Bernhard, the host himself can be characterised as the milieu interieur, the microflora as the milieu exterieur, and the host and his microflora as the milieu total.

Studies on adult mammals, birds, fishes, insects and reptiles with no microbial flora, i. e. germfree individuals, have established long series of values with regard to anatomical structures, physiological and biochemical functions in a milieu interieur, i.e. the macroorganism itself.

When such baselines are first estab-

Table 2: Microflora-associated characteristics investigated

Parameter	Microbial interaction	Method	Function
Bile acids	Deconjugation 7α-dehydroxylation	Gas chromatography	Entero-hepatic circulation
Bilirubin Urobillogen formation	Deconjugation Spectrophotometry	Spectrophotometry	Entero-hepatic circulation
Cholesterol	Coprostanol formation	Gas chromatography	Entero-hepatic circulation
Mucin	Breakdown	Gel electrophoresis	Mucosal
Tryptic activity	Inactivation	Spectrophotometry	Pancreatic
Short Chain Fatty Acids	Presence	Gas chromatography	Dietary
β-aspartylglycine	Absence	High voltage electrophoresis	Dietary

lished, the normal function(s) of the flora as well as alterations in these functions can be worked out. In such functional studies, two new terms - MAC and GAC - have been shown to be of considerable value. A MAC (i.e. a Microflora-Associated Characteristic) can be defined as the recording of any anatomical structure, or physiological or biochemical function in a macroorganism, which has been influenced by the microflora. When microbes actually influencing the parameter under study are absent - as in germfree animals, newborns, and/or in relation to ingestion of antibiotics - the recording of a MAC can be defined as a GAC (i.e. Germfree Animal Characteristic). Consequently, a set-up of GACs describes the milieu total under germfree conditions whereas a similar set-up of MACs describes the milieu total. A simple equation: Milieu total minus milieu interieur gives milieu exterieur ("what have the microbes done") as an answer. Below, some of the most well known set-ups of MACs/GACs are summarised and some data concerning the most active part (species when known) of the microbial flora is quoted (Table 1).

SOME ACTUAL GAC/MAC PARAMETERS FOR ROUTINE ANALYSIS

The list shown above covers just some few of the GAC/MAC pairs, which exist within the gastrointestinal tract. However, it goes without saying that the list is too "ambitious" to be used in a routine setting. In Table 2 the tests which we have found to be of great value when evaluating the functional part of the intestinal microflora are listed. Most of the tests have been the subject of a Ph.D. thesis (*Høverstad*, 1985; *Norin*, 1985; *Carlstedt-Duke*,

1987; *Saxerholt*, 1990), and are very well standardised. In the following part, the interest will be related to two areas, i.e. colonisation resistance and microbial transformation of bile acids.

PARAMETERS RELATED TO COLONISATION RESISTANCE

Presence/absence of β-aspartylglycine

As underlined and summarised by *van der Waaij* in this volume, the presence in faeces from conventional mammals, including man of some dipeptides, especially β-aspartylglycine, indicates that the normal microbial intestinal ecosystems are seriously altered. In short, the biochemical background for presence of β-aspartylglycine may be as follows. Dietary proteins are the main targets of intestinal proteolytic enzymes. Biochemically, β-aspartylglycine is a member of a group of β-carboxyl dipeptides, formed in the intestinal tract when dietary proteins are broken down by host derived proteolytic enzymes (*Welling* et al., 1985). The β-carboxyl dipeptide bindings are suggested to be broken down only by microbial derived proteolytic enzymes. This is substantiated by findings in germfree rats and mice (*Welling* and *Groen*, 1978; *Norin* and *Midtvedt*, l987). Adult germfree rats and mice always excrete β-aspartylglycine in their faeces, whereas their conventional counterparts never do. Thus, presence/absence of β-aspartylglycine represents a GAC/MAC system. However, the microbe(s) capable of switching this particular GAC to a MAC is (are) now known.

Thus, presence of this particular GAC, i. e. presence of β-aspartylglycine, is depending on (i), the presence of dietary precursor(s); (ii), the presence of host derived proteolytic enzymes, and (iii), the absence of microbial derived proteolytic enzymes.

In some past and on-going studies on GAC/MAC parameters, we have followed the presence/absence of β-aspartylglycine in experimental animals as well as in humans, and some of our results will be reported upon hereunder.

Beta-aspartylglycine/antibiotics/human

The following antibiotics were given for 6 days to groups of healthy volunteers: Ampicillin 500 mg q.i.d., bacitracin 25,000 IU q.i.d., clindamycin 150 mg q.i.d., co-trimoxazole 150/800 mg b.i.d., doxycycline 200 mg day 1, followed by 100 mg daily, erythromycin 250 mg q.i.d., metronidazole 400 mg t.i.d., nalidixic acid 500 mg q.i.d., ofloxacin 200 mg b.i.d., and vancomycin 240 mg q.i.d. Faecal sampling was done before, during and after medications. Drug concentrations were measured in faeces and blood on day 6. All faecal samples were investigated for alterations in the following MAC parameters: Presence of β-aspartylglycine, conversion of cholesterol to coprostanol, bilirubin deconjugation and formation of urobilinogen, 7-alpha-dehydroxylation of bile acids, breakdown of mucin, inactivation of mucin and production of short chain fatty acids. The results are published elsewhere (*Steinbakk*, 1992), and here will be commented upon alterations in the β-aspartylglycine parameter only.

Beta-aspartylglycine was absent in all individuals prior to administration of antibiotics, and present in one individual during the administration. This individual received ampicillin and was the only individual not having detectable amounts of β-lactamases in her faeces on day 6. In that particular sample, high

amount of β-aspartylglycine was found to be present, together with 480 mg/kg of ampicillin. From these series of experiments, we concluded that presence of β-aspartylglycine was a rare event following oral ingestion of antibiotics.

In another study, clindamycin was given to conventional rats (4 mg/kg for 5 days). Additionally, some rats received clindamycin together with 2 strains of lactobacilli (*Carlstedt-Duke*, 1987). Faeces were analysed for variations in some MAC patterns. However, β-aspartylglycine could not be detected in any sample taken prior to, during or after ingestion of clindamycin.

Establishment of a β-aspartyl-degrading flora in ex-germfree rats

A time-course study for the establishment of some biochemical microbial intestinal functions was undertaken in ex-germfree rats conventionalised, i.e. colonised with conventional flora, in three different ways: Untreated (Group 1); contact with visitor rats (Group 2); inoculated with intestinal contents from conventional rats (Group 3). The biochemical parameters studied were the same as mentioned above. The results, which are described in detail elsewhere (*Midtvedt* et al., 1987), showed that the way in which the microbes ware introduced and the biochemical functions themselves were of importance. Concerning the β-aspartylglycine parameter, a significant difference was found between group 1 and group 3. On day 3 after being taken out of their germfree isolators, all the rats in Group 1 showed presence of β-aspartylglycine whereas it was absent in all the rats in Group 3. Fourteen and 21 days after they have been taken out of their isolators, all the rats in Groups 2 and 3 had switched from a GAC to a MAC pattern. Although the specific microbial species involved in this GAC/MAC switch are virtually unknown, it might be reasonable to assume that the capability of performing this reaction is not rare among intestinal microorganisms.

Time-schedule for presence of β-aspartylglycine in young germfree rats

As mentioned, it has been shown that high amount of β-aspartylglycine always is present in faeces from adult germfree rats and mice. In a series of experiments, we intended to follow when β-aspartylglycine starts to be present in young germfree rats and also whether, and to what extent, weaning may influence upon the occurrence of β-aspartylglycine in faeces. The experiments, which are described in greater details elsewhere (*Norin* and *Midtvedt*, 1987), were performed as follows. A litter of germfree AGUS rats was raised together with their mother up to day 17, when the animals were randomly divided into following two groups.

Group I: Six rats, were weaned onto water and a commercially obtained, pelleted rat food *ad libitum* (R3, Ewos, Södertälje, Sweden)

Group II: Five rats, were receiving mother's milk only during the period 17-23 days of age (the mother was taken away from the young rats twice daily, when she was given full access to the diet R3)

From both groups of young rats, individual samples of faeces were obtained every morning by rectal stimulation. The samples were immediately frozen at -20°C and stored until analyses. The results, which are given in Table 3, show that β-aspartylglycine is absent up to day 17 after birth; then a quantitative rather than a qualitative difference is established between Group I and Group II. Several explanations to these findings may be possible. The initial diet, i.e. mother's milk may over the time

Table 3: β-Aspartylglycine in faeces from young germfree rats

β-Aspartylglycine*	Day	17	18	19	20	21	22	23
Group I (R3 diet)		-	+++	+++	+++	+++	+++	+++
Group II (Milk)		-	+	+	++	++	++	+++

*: no β-asp-gly
+ and ++: increasing concentration
+++: adult level of β-asp-gly

vary in its content of precursors for the formation of β-aspartylglycine, the host derived proteolytic activity; i.e. trypsin, may vary in its activity during the same time schedule, the intestinal mucosa in young animals may allow an absorption of β-aspartylglycine, etc. The data obtained in the older animals, i.e. the animals older than 17 days, may give support to an assumption that more than one mechanism is at work.

Presence of β-aspartylglycine in faeces from children 0-24 months of age

In a longitudinal study we have followed the establishment of some biochemical MACs in a cohort of Swedish children from 0-24 months of age. Meconium was collected by the staff at the maternity ward and the parents collected faeces from the children at 1, 3, 6, 9, 12, 15, 18, 21, and 24 months of age. All samples were stored in clean plastic vials at -20°C until analysed. Based on their diet regimens, the children were divided into groups in a manner similar to that of *Cooperstock* and *Zedd* (1983). The MACs listed above have been investigated, and some of the results have already been published (*Midtvedt* et al., 1988, *Midtvedt* and *Midtvedt*, 1992). Concerning the β-aspartylglycine parameter it can briefly be stated that none of the samples taken prior to 6 months and after 9 months of age contained any β-aspartylglycine. In some few samples taken at 6 and 9 months of age, small amounts of β-aspartylglycine could be detected.

General comments on β-aspartylglycine

Our data support the view that presence of β-aspartylglycine is a rare event in adult individuals receiving oral antibiotics.

Our data indicate that in young animals and in children, the β-aspartylglycine parameter might be more controversial than in adults. Obviously, further investigations have to be carried out in order to clarify the value of this parameter in children.

MICROBIAL BILE ACID TRANSFORMATION

Comparative work in germfree and conventional animals have substantiated that microbial bile acid metabolism creates several GAC/MAC systems. The main microbial interactions include deconjugation, oxidation/reduction, dehydroxylation and hydroxylation. In mammals, bile acid metabolism can briefly be described as follows.

The bile acid derives from cholesterol by hepatic transformation. In man, cholic acid and chenodeoxycholic acid are the two most commonly occurring primary bile acids. Within the liver, the primary bile acids are conjugated, mainly with the amino acid taurine or

glycine at C-24 and excreted into the bile. A minor part may be present as sulphate or glucuronide conjugates; the esterification takes place mainly at C-3. In the intestinal tract, the conjugated bile acids are attacked by microbial enzymes and converted to a variety of metabolites. The so-called secondary bile acids thus formed may either be excreted into the faeces, or absorbed and sometimes further metabolised by hepatic enzymes to tertiary bile acids before re-excretion by the bile into the intestine where they can be further attacked by microbial enzymes. The bile acids are undergoing enterohepatic circulation several times each day. Most of the absorption takes place by an active transport in the distal ileum. However, a passive transport over the mucosa of some bile acids may take place in the whole small intestine. In general, microbial converted bile acids have reduced capacity to participate in the normal absorption of fat, and some of the derivatives may have a reduced absorption rate. The bile acids, which are not absorbed, are excreted in the faeces.

To summarise: The final composition of biliary and faecal bile acids are an interplay between liver biosynthetic enzymes and intestinal microbial transformation, both factors may vary with age. The following will be focused upon some main pairs of bile acid GACs/MACs.

Deconjugation of bile acids

As mentioned, bile acids are excreted by the liver in the form of conjugates and the findings are similar in germfree and conventional animals. In conventional animals nearly all bile acids present in faeces are in their free forms, and this is contrary to the findings in germfree animals where all bile acids present in faeces are found as conjugates. The intestinal hydrolysis is, as far as we know, exclusively brought about by the action of microbial enzymes.

The first successful isolation of a bacterium capable of hydrolysing conjugated bile acids was made by *Frankel* (1936). Since then, many reports have been made and several reviews have been written. The capability to split bile acid conjugates, especially glycine and taurine conjugates, is commonly occurring among intestinal microorganisms. Under normal conditions, the deconjugation appears to be restricted to the large bowel and the terminal part of the ileum. However, under pathological conditions the numbers of deconjugating microbes may increase in the proximal part of the small intestine. Deconjugation of radiolabelled bile acids is a test commonly used for diagnosing microbial overgrowth in the small intestine.

Deconjugation may temporarily be reduced following intake of several antibiotics (*Gustafsson* and *Norin*, 1977), but normal levels of deconjugation are usually retained shortly after the intake has been stopped.

In an on-going study on the establishment of microbial function in newborns, we have found that deconjugation is one of the first GAC/MAC switches to be established in infants. One month after birth, nearly all faecal bile acids were present in their unconjugated forms (*Jönsson* et al., unpublished results).

Oxidation-reduction of hydroxyl groups

Bile acids may carry hydroxyl groups at C-3, C-6, C-7, C-12, C-16 and C-23 positions, respectively. The main primary bile acids in most mammalian species, including man, are cholic acid and chenodeoxycholic acid with hydroxyl groups at C-3, C-7, C-12 and C-3, C-7, respectively. Most of the work with microbial bile acid hydroxyl dehydrogenases has been made on these

bile acids. It has been shown that such dehydrogenases are produced by a very wide range of bacterial genera (*Midtvedt* and *Norman*, 1967; *Dickinson* et al., 1971, *Prevot*, 1961; *Midtvedt*, 1974).

In conventional rats, dehydrogenating microorganisms are present in high numbers in caecal contents and faeces, but present in low numbers in the small intestine (*Midtvedt* and *Norman*, 1968). Under pathological conditions, as after the establishment of a blind loop, dehydrogenating microorganisms are present in high numbers in the blind loop and throughout the small intestine (*Midtvedt* et al., 1969).

The reduction of a keto group leading to the formation of a β-hydroxyl group can partly be performed by microbial enzymes but can also partly be performed by the liver. Formation of 3β-derivatives seems to be a sole microbial capacity. Most of the strains so far studied are anaerobes. Unpublished results from my laboratory indicate that some aerobes - as some *Pseudomonas* strains - can perform this reaction. At least in conventional rats, the 3β-forming microbes are lacking or present in low numbers only in the small intestine, but are present in the large intestine.

As for deconjugation, dehydrogenation can also be influenced upon by intake of antibiotics. Similarly, microbial oxidation-reduction of the various hydroxyl groups is established soon after birth.

Dehydroxylation of bile acids

Here, the interest has been focused on the dehydroxylation of the hydroxyl group at C-7 in cholic and chenodeoxycholic acid, leading to the formation of deoxycholic and lithcholic acid, respectively. The first microbial strains capable of performing this reaction were described in 1966 by *Gustafsson* et al. Since then, there have been several reports and most of the authors have found this capability to be a rare one among intestinal microorganisms. Most reports identify Gram-positive, non-sporeforming, anaerobic rods, probably belonging within the *Eubacterium* group (*Gustafsson* et al, 1966; *Hirano* et al., 1981; *Hylemon* et al., 1980; *Midtvedt*, 1967), whereas strains of *Clostridium* (*Aries* and *Hill*, 1970; *Ferrari* and *Beretta*, 1977; *Hayakawa* and *Hattori*, 1970; *Hill*, 1985; *Hill* et al., 1971; *Hirano* et al., 1981; *Stellwag* and *Hylemon*, 1979), *Bacteroides* (*Aries* and *Hill*, 1970; *Bokkenheuser* et al., 1969; *Edenharder* and *Slemrova*, 1976; *Hill* et al., 1971) and *Bifidobacterium* (*Ferrari* et al., 1980; *Hill* et al., 1971) have been described.

As for deconjugating and dehydrogenating microbes, 7-alpha-dehydroxylating microbes are usually absent or present in low numbers only in the small intestine whereas they are present in high numbers in the large intestine.

A significant effect upon 7-alpha-dehyroxylation has been found after intake of several antibiotics, in man (*Canzi* et al., 1985, *Gustafsson* et al., 1977; *Andreasson* et al., 1988) as well as in animals (*Gustafsson* and *Norman*, 1977; *Gustafsson* et al., 1993). Obviously, this is a GAC/MAC parameter worth to be studied in greater detail.

In contrast to deconjugation and dehydrogenation, 7-alpha-dehydroxylation is a rare event to be established in infants. Unpublished results indicate that it may take months until this function is established (*Jönsson* et al., unpublished results).

Hydroxylation of bile acids

In vitro, several microbial strains can hydroxylate bile acids. However, no hydroxylation of bile acids seems to take place in the intestinal tract of mammals (*Midtvedt*, 1974).

To summarise: Deconjugation, dehydrogenation and dehydroxylation are

three main MAC/GAC switches of considerable interest in clinical medicine, Alterations may reflect that the microbes are outside their normal habit (as in the case of the conjugate-breath-test for bacterial overgrowth) or are eradicated (following intake of antibiotics). Whether, and to what extent these alterations reflect alterations in colonisation resistance remains to be settled.

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