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# Establishment of some microflora-associated characteristics in ex-germfree rats and the developmental pattern

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*Introduction*

In healthy mammals, the intestinal tract harbors a wide variety of microbial species, usually described as normal intestinal microflora. The composition varies individually and is depending on both endogenous and exogenous factors (*Savage* 1989). Together with the host, this intestinal microflora constitutes ecosystem(s) that are of benefit to the host as well as to the microbes. The systems are established after birth, when various microbial species find their way into the intestinal tract.

A similar establishment takes place when germfree (GF) animals are conventionalized, i.e. colonized with conventional flora. One approach for studying the establishment of an intestinal microflora in ex-GF animals and in children is related to estimation of its functional interactions with host-influenced parameters. Such interactions have been described as microflora-associated characteristics, MACs. These are defined as the recordings of any anatomical structure or physiological or biochemical function in a macroorganism which have been acted upon by the microflora (*Midtvedt* 1985, *Midtvedt et al.*1985). When the microbes that actually influence the parameter under study are absent, as in GF animals, in children and sometimes in patients receiving antibiotics, these recordings are defined as GACs, germfree animal characteristics.

Previously, we have reported upon a time course study for the establishment of MACs in ex-GF rats conventionalized in three different ways: i, by random, ii, by contact with visitor rats, and iii, by inoculation with intestinal contents from conventional (CONV) rats (*Midtvedt et al.* 1987). Main parameters studied were conversion of cholesterol to coprostanol and of bilirubin to urobilinogen to follow hepatic and intestinal interactions, degradation of mucin to follow an intestinal function of major importance in preserving the integrity of the intestinal mucosa, degradation of β-aspartylglycine reflecting co-functions between the diet and the microflora and the inactivation of intestinal trypsin to follow the co-function between pancreas and intestinal microbes. The results showed that the way in which the microbes were introduced and the microflora related functions themselves were of importance. In several cases, social contacts, i.e. with visitor rats, were just as effective for the functionally adequate establishment of MACs as was the inoculation with intestinal contents from CONV rats.

In the present study we have included one more MAC, i.e. microbial conversion of cholic acid (CA) to deoxycholic acid (DCA), reflecting another parameter to follow hepatic and intestinal interactions. In an attempt to avoid inoculation from other laboratory animals, an enlarged group of rats was placed in clean cages in a room with separate ventilation. Additionally, the experiment with visitor rats was performed twice, with an extended group of rats. The examined parameters in the present study were formation of DCA, coprostanol and urobilinogen, degradation of mucin and of β-aspartylglycine and inactivation of the intestinal trypsin.

*Materials and Methods*

Animals: 35 GF AGUS rats of both sexes, weight 300 - 400 g (*Gustafsson* 1959, *Festing* 1993) were randomly selected to the different groups before

they were taken out of the isolator, Table 1. All rats were reared on steam-sterilised R-36 (Lactamin, Sweden) and water *ad libitum*. The study was approved by the local ethical Committee, Stockholm Nord, Sweden.

Study design. The rats in group A - D were housed individually in clean metabolic cages in a room with conventional animals of the same strain, with a temperature of 24 ± 2 ° C, relative humidity of 55 ±10 % and controlled light. All groups were kept on separate shelves and care was taken to avoid contamination between the groups. The group E rats were kept individually in clean metabolic cages in a separate room without any other animals.

Feces was collected hourly for 12 hours each day during the first week and thereafter once a week every second hour for 12 hours. All samples were immediately frozen and kept at - 20° C until analyses.

The rats were brought in contact with the microflora as follows.

Group A. The rats were kept in their cages until day 29 (only allowed to catch microbes from the environment by random) when they received 1 ml of a homogenate of cecum contents (HCC). HCC was obtained from six CONV AGUS rats, diluted 1 : 10 in Todd-Hewitt broth (Oxoid Ltd, Basingstoke, England) and homogenated. The HCC was given both intragastrically and as an enema.

Group B. Each rat was given 1 ml HCC, intragastrically and as an enema, on the day the rats were taken out of the isolators.

Groups C and D. Each rat was allowed to have a separate CONV rat visitor nightly during the first week. Group D was an extended group of rats. Similar as in group A, all rats were given 1 ml HCC on day 29.

Group E. The rats were kept in a separate room without any further treatment until day 29 when they received 1 ml HCC. Care was taken to avoid transfer of any CONV animal delivered material into the room as well as any transfer between the animals in the group.

Chemicals: 2.2-Dimethoxypropane was obtained from Merck-Schuckardt (Hohenbrunn, GDR), chlorotrimethylsilane and hexamethylsilazane from E Merck (Darmstadt, GDR), and methoxiamine-HCl from Eastman-Kodak Company (Rochester NY, USA). The remaining chemicals were obtained from Kebo AB (Stockholm, Sweden).

Determination of MAC's and GAC's The methods (except for detection of DCA) are presented by *Midtvedt et al.* 1987. The normal values for the parameters are given in Table 2.

Determination of DCA. The fecal samples were homogenized in ethanol with Ultra Turrax and after reflux boiling with ethanol, and with chloroform/ methanol 1:1 for two hours respectively, the samples were treated with methoxyaminehydrochloride and methylated by dimethoxypropane (*Andréasson et al.* 1988). The derivatives were then purified on a glass column with silica gel before trimethylsilylation with hexamethyldisilazane and trimethylchlorosilane in pyridine. Gas-liquid chromatographic analyses were performed

*Table 1.* Study design

| | In an ordinary animal room | | | | In a separate room |
|---|---|---|---|---|---|
| Time (Days) | Group A 5 rats | Group B 5 rats | Group C 5 rats | Group D 10 rats | Group E 10 rats |
| 0 | NT | E | NT | NT | NT |
| 1-7 | NT | NT | V | V | NT |
| 8-28 | NT | NT | NT | NT | NT |
| 29 | E | NT | E | E | E |

NT: No treatment
E: 1 ml HCC, see Material and methods
V: Visitors during 5 nights

in a Pye Unicam GCD with two different columns, DB WAX Megabore fused silica column or a column with 1 % cyklohexanedimethanolsuccinate on Chromosorb, at 220 °C.

*Results and Discussion*

All GF rats demonstrated GACs when reared in the isolators and all the rats used as visitors and suppliers of cecum contents demonstrated MACs when included in the study. During the whole study all animals remained healthy. The number of days until detection of the MACs in fecal samples from all animals are given in Table 3 and as shown in the table, the establishment of functions can be clustered in two groups. Cluster I contains formation of cholic acid to deoxycholic acid and of bilirubin to urobilinogen. Cluster II contains the remaining parameters.

Cluster I is characterized by a rapid, i.e. mostly within four days, establishment of the functions in all the investigated groups and

Cluster II is characterized by a much wider timespan in establishment. The most rapid establishment was found in group B, in the rats that received an enema of HCC. In this group, the median time for establishment of the four parameters were three days or less. In the two groups of rats that were left alone in clean cages, group A and E, the median time for establishment of the four parameters were 14 days or more.

The time course variations mentioned above may to some extent reflect characteristics of the microbial species involved in the particular functions. Intestinal microbial conversion of cholic acid to deoxycholic acid includes deconjugation and 7α-dehydroxylation. The ability to hydrolyse taurine and glycine conjugates of cholic acid is widely distributed among intestinal bacteria, especially among strains belonging to the genera *Clostridium, Bifidobacterium, Lactobacillus* and *Peptostreptococcus* (*Midtvedt* 1974, *Hylemon* 1985). The ability to remove the hydroxyl group at position C-7 in cholic acid has been found among strains belonging to the genera *Clostridium, Eubacterium* and *Lactobacillus* (*Midtvedt* 1974, *Hylemon* 1985). However, this 7α-dehydroxylation is a more rare capability than deconjugation and it requires very specific and strict anaerobic

*Table 2.* Values or pattern for Microflora-Associated Characteristics (MACs) and Germfree Animal Characteristics (GACs).

| MAC-function | Detection technic | M A C value | G A C value |
|---|---|---|---|
| Formation of DCA | Gas chromatography | Presence of DCA | Absence of DCA |
| Formation of urobilinogen | Spectro-photomertry | Presence of urobilinogen | Absence of urobilinogen |
| Formation of coprostanol | Gas chromatography | Presence of coprostanol | Absence of coprostanol |
| Degradation of betal-aspartylglycine | Paper electrophoresis | Absence of beta-aspartylglycine | Presence of beta-aspartylglycine |
| Degradation of mucin | Agar gel electrophoresis | MAC band pattern | GAC band pattern |
| Inactivation of tryptic activity | Spectro-photometry | <700 mg/kg feces | ≥700 mg/kg feces |

conditions (*Hylemon* 1985). It is generally assumed that cholic acid has to be hydrolyzed before the 7α-dehydroxylation occurs *(Gustafsson et al.* 1968). Thus, the variations in 7α-dehydroxylation presented in table 4, may be partly due to a variation in presence of both deconjugating and 7α-dehydroxylating microorganisms and partly to variations in the specific conditions necessary for the reaction to take place.

Transformation of bilirubin to urobilinogen, the other parameter in Cluster I, is also performed in two steps. The capability to hydrolyse the mono- and di-glucuronide conjugates of bilirubin is widely spread among intestinal microorganisms, especially among strains belonging to the genera *Clostridium, Peptostreptococcus* and *Lactobacillus* (*Gadelle et al. 1985, Cole et al. 1985).* Unlike conversion of conjugated cholic acid to deoxycholic acid, it is not known whether - or to what extent - bilirubin has to be deconjugated before formation of urobilinogen can take place. In spite of the fact that intestinal tranformation of bilirubin to urobilinogen is carried out in all mammalian species so far investigated, the capability of performing this reaction seems to be a rare property among intestinal microorganisms. Up to date, only one bacterial strain, probably within the species *Clostridium ramosum,* has been found to be capable of performing this function both *in vivo* and *in vitro* (*Gustafsson & Lanke* 1960, *Midtvedt & Gustafsson* 1981). It goes without saying that these species or strains with similar capability have to be widely spread in mammals.

Among the four parameters included in Cluster II, it is not known which microbial species that are responsible for a GAC/MAC switch in degradation of β-aspartylglycine and inactivation of trypsin. However, unpublished results from our department indicate that the capability of per-

*Table 3.* Days until detection of MAC-functions in feces from ex-Germfree rats.

| MAC-function | | Group A | Group B | Group C | Group D | Group E |
|---|---|---|---|---|---|---|
| *Cluster I* | | *days* | *days* | *days* | *days* | *days* |
| Deoxycholic acid | Median | 4 | 4 | 4 | 3 | 7 |
| | Range | 3-4 | 3-11 | 2-8 | 1-4 | 1-7 |
| Urobilinogen | Median | 3 | 3 | 3 | 3 | 3 |
| | Range | | | | | |
| *Cluster II* | | | | | | |
| Coprostanol | Median | 14 | 3 | 4 | 8 | b), 0/10 c) |
| | Range | 8-37 a) | 3-7 | 3-8 | 3-14 | 43 a) |
| Beta-aspartylglycin | Median | 14 | 3 | 3 | 8 | b), 0/10 c) |
| | Range | 8-28 | | 3-14 | 3-8 | 43 a) |
| Mucin | Median | 28 | 1 | 4 | 8 | 28 |
| | Range | 21-31 a) | | 2-7 | 3-8 | 8-28 |
| Tryptic activity | Median | 21 | 3 | 7 | 14 | b), 0/10 c) |
| | Range | 21-36 a) | | 3-14 | 3-28 | 43 a) |

Analyses of Group A-C and all DCA-determinations were performed after 1, 2, 3, 4, 7 days and weekly thereafter. Samples from Group D and E were analysed on day 3, 8, 14, 21, 28 and on day 43.

Median: When >50% of the ex-GF rats had MAC values.

a) The animals in Group A, C, D and E were given 1 ml of a homogenate of cecum contents from 6 CONV rats on day 29.

b) >50% of the rats had not MAC value before day 29.

c) Rats with MAC value at day 28.

*Table 4.* Detection of Cholic acid (CA) and Deoxycholic acid (DCA) in ex-Germfree rats (7 α-dehydroxylation).

| Animal No | Days after removal from the germfree isolator | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 7 | | 10 | | 14-15 | |
| | CA | DCA | CA | DCA | CA | DCA | CA | DCA | CA | DCA | CA | DCA | CA | DCA |
| Group D "vititor" during 5 nights | | | | | | | | | | | | | | |
| 1 | NP | | + | - | + | + | + | - | - | + | - | + | NP | |
| 2 | + | + | + | + | + | - | + | - | + | - | + | + | - | + |
| 3 | NP | | + | + | + | + | + | - | + | - | - | + | - | + |
| 4 | NP | | + | - | + | + | + | - | + | - | - | + | + | + |
| 5 | - | + | NP | | - | + | + | - | + | - | - | - | + | + |
| 6 | NP | | + | - | + | - | - | + | + | - | - | + | NP | |
| 7 | NP | | + | - | + | + | + | - | - | + | - | + | NP | |
| 8 | NP | | + | - | + | + | + | + | - | + | - | + | - | - |
| 9 | + | + | - | + | + | - | + | - | + | - | - | + | - | + |
| 10 | NP | | + | - | + | + | + | - | + | - | - | + | NP | |
| Group E In a separate room | | | | | | | | | | | | | | |
| 11 | - | + | - | + | - | + | - | + | NP | | + | + | NP | |
| 12 | NP | | NP | | + | - | + | - | - | + | - | - | - | + |
| 13 | NP | | NP | | + | - | + | - | - | + | - | - | - | + |
| 14 | NP | | NP | | + | - | + | - | + | + | - | - | - | + |
| 15 | NP | | NP | | + | - | + | - | + | + | + | + | + | + |
| 16 | NP | | NP | | + | - | + | - | - | + | - | + | NP | |
| 17 | NP | | NP | | + | - | - | + | + | + | + | + | NP | |
| 18 | NP | | NP | | + | - | - | + | + | + | - | - | + | + |
| 19 | NP | | NP | | + | - | - | + | - | + | - | - | - | + |
| 20 | NP | | NP | | + | - | + | - | - | + | + | + | NP | |

\+ = Present in the fecal samples
\- = Absent in the fecal samples
NP= Analysis not performed

forming these two switches are lost when intestinal content is heated. A GAC/MAC switch has never been found to have taken place in ex-GF animals monocontaminated with strains belonging to the genus *Clostridium*.

Microbial intestinal transformation of cholesterol to coprostanol has, so far, been demonstrated to occur only in strains belonging to the genus *Eubacterium*, i.e. non-sporeforming, strict anaerobic, gram-positiv rods (*Sadzikowski et al.* 1977, *Eyssen* 1973). The ability to degrade mucin has been found in strains belonging to the genera *Bifidobacterium, Bacteroides, Peptostreptococcus* and *Ruminococcus,* i.e. anaerobic, non-sporeforming microorganisms (*Carlstedt-Duke et al.* 1986, *Hoskins* 1991).

A prerequisite for a MAC to be established in the gastrointestinal tract is that microbial strain(s) capable to perform the reaction is(are) presented to the animal. A second prerequisite is that these strain(s) can established and can express their function within the tract. Taken together, the present results clearly indicate that characteristics of the microbial species involved

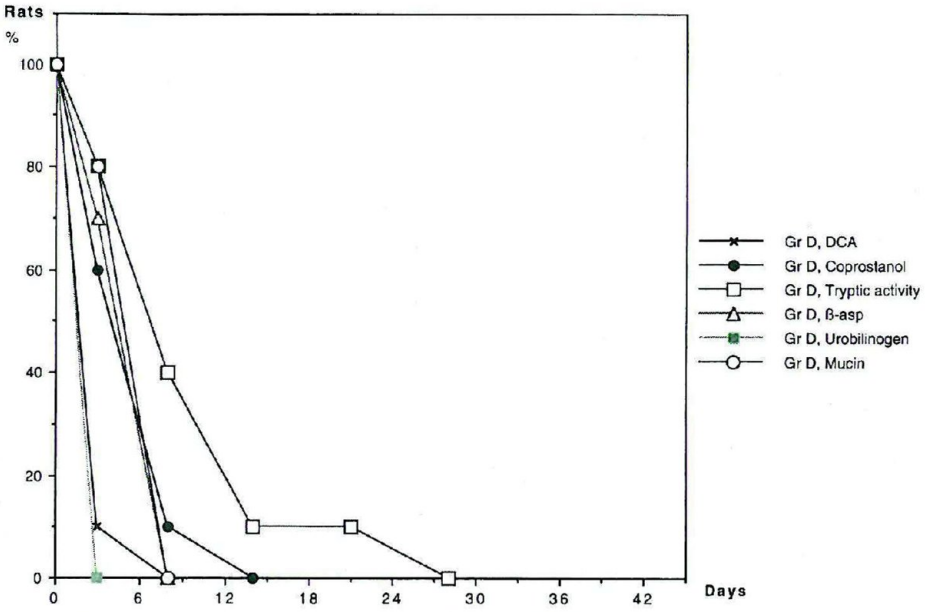


*Figure 1A.* The percentage of rats in Group D with GAC values.
*Figure 1.* The sum of all areas under the curves of Group E vs Group D was approximately 5.

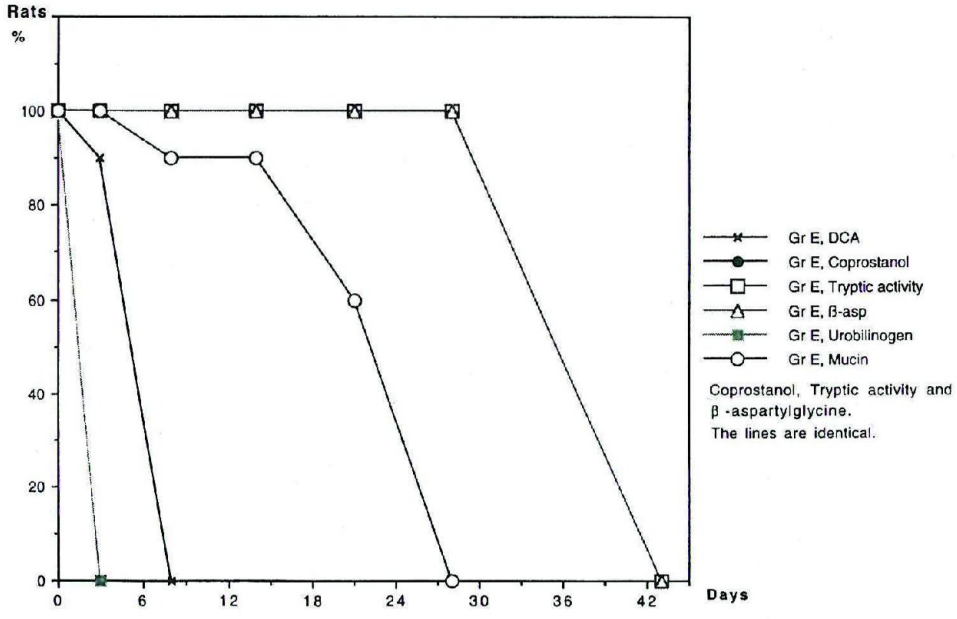


*Figure 1B.* The percentage of rats in Group E with GAC values.
*Figure 1.* The sum of all areas under the curves of Group E vs Group D was approximately 5.

as well as the way the microbes are introduced, are of importance.

Establishment of functions in Cluster I seems mostly to depend on characteristics of the microbial species involved. Both MACs in Cluster I can be carried out by spore-forming microbes. It may be reasonable to assume that spores from these species are widely spread in rooms with animals and cages. The ex-GF animals are rapidly exposed to these spores. Without regard to functions, it is a common experience in laboratories working with GF animals that anaerobic spore-forming microbes are more easily established in the gastrointestinal tract than anaerobic non-sporeformes.

As known so far, the functions in Cluster II are performed by non-sporeforming microorganisms. Concequently, the way by which the microbes are introduced, seems to be of greater importance. A huge loading dose of intestinal contents from conventional rats, i.e. group B, was the most effecient way, closely followed by social contacts, group C and D, to colonize. In rats, in group A and E, which were in no direct contact with conventional animals, the establishment of the parameters were either markedly delayed or completely absent until an enema was given.

In a wider setting, the functional approach described in this report should allow further evaluation of the complicated mechanism behind spreading of microbes within an animal facility as well as in a human society.

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