Micromorphometrical analysis of rodent related (SPF) and unrelated (human) gut microbial flora in germfree mice by digital image processing

D. VEENENDAAL[1], J. DE BOER[2], B. C. MEIJER[3], D. VAN DER WAAIJ[2] AND M. H. F. WILKINSON[2]

[1] *Department of Medical Microbiology, Public Health Laboratory, P.O. Box 21020, 8900 JA Leeuwarden*
[2] *Laboratory for Medical Microbiology, University Hospital Groningen*
[3] *Department of Medical Microbiology, Public Health Laboratory, Groningen, The Netherlands*

SUMMARY

Digital image processing (DIP) of bacterial smears is a new method of analysing the composition of the gut microbial flora. This method provides the opportunity to compare and evaluate differences in the complex highly concentrated anaerobic fraction of gut microbial flora, based on micromorphological differences. There is ample evidence that this fraction can be characterized as related or unrelated to the host organism by its immunogenicity. In this study germfree ND2 mice were associated with either related (rodent) SPF microflora (SPF-MF) or unrelated human MF (HUM-MF). DIP analysis was performed on original SPF-MF and HUM-MF and on the faeces of ex-germfree mice 4 weeks after association. The micromorphological pattern of highly concentrated anaerobic bacteria in faeces of HUM-MF associated ex-germfree mice was significantly different from SPF-MF associated counterparts with regard to the scores for elongation ($P < 0.01$) and morphological variety ($P < 0.05$). Moreover, gross morphological variability was present between individual HUM-MF associated mice but not between individual SPF-MF associated animals. No differences were found between original SPF and HUM-MF. The data are discussed with regard to differences in the presence of (non-)immunogenic bacteria and the ability for related and unrelated flora to colonize the murine gut. This study provides evidence that murine host specificity of microbial flora may not only be reflected in the number of non-immunogenic bacteria but also in the micromorphological pattern of highly concentrated anaerobic bacteria in faeces measured by DIP analysis.

INTRODUCTION

It has become possible to analyse the complex highly concentrated anaerobic bacterial fraction of the gut microbial flora by micromorphometry. This new method called digital image processing (DIP), developed and described by Meijer and colleagues [1–3] and Wilkinson and colleagues [4], enables detection and quantitative as well as qualitative analysis of bacterial objects in faeces, based on their morpho-logical appearances. Analysis is determined by the distribution of bacterial species present in faeces at the highest concentration only; these are obligate anaerobic bacteria which normally reach concentrations of 10^{10}–10^{11} micro-organisms per gram faeces [5, 6].

The composition of the gut microbial flora has been found to be host specific. The highly concentrated anaerobic fraction in rodents is known to be micro-morphologically different compared with those in humans, e.g. stool specimens of mice contain large

numbers of fusiform shaped bacteria whereas small rod shaped and coccoid bacteria are predominant in human faeces. Differences have also been described between rodent animal species [7, 8] as well as between human individuals [3, 9, 10]. In humans, van der Merwe and colleagues [11] showed that genetically identical individuals (i.e. homozygote twins) have an indistinguishable gut microbial flora. These findings support previous theories that the composition of the microbial flora may be defined genetically, perhaps by major histocompatibility complex (MHC) antigens [12, 13].

There is ample evidence that autochthonous (host-related) and allochthonous (animal-species related but host unrelated) intestinal bacteria do not elicit an immune response, whereas xenochthonous (animal-species unrelated) bacteria do [14–16]. In a previous study we have found that unrelated human (xeno-chthonous) microbial flora (HUM-MF) in ex-germ-free mice elicits a high antibody response, whereas SPF rodent-related (allochthonous) MF (SPF-MF) does not. After association in GF mice, gross micromorphological as well as immunological changes were observed in the human flora, whereas only minor changes were found in related (alloch-thonous) SPF-MF before and after association. Highly immunogenic bacteria appeared to be reduced to undetectable levels after association with xenoch-thonous HUM-MF [17].

The present study was carried out in order to evaluate differences in the micromorphological pattern of gut microbial flora of germfree (GF) mice associated with either rodent SPF-MF or human HUM-MF. Slides with smears of faecal bacterial were read microscopically by a video camera and analysed by a DIP system, developed in our laboratory [1–4].

METHODS
Mice

Experiments were carried out with 6–8 weeks old conventional (CONV) and germfree (GF) ND2 (H-2q) mice. All animals were bred at the Central Animal Facility of the University of Groningen.

Housing

GF and microbial flora (MF) associated ex-GF animals were housed in plastic GF isolators in sterilized cages, fed with sterilized rodent pelleted food (SRM-A, Hope Farms B.V., Woerden, The Netherlands) and sterilized tap water *ad libitum*.

Conventional mice were maintained under standard conditions as described previously [8].

Faecal floras

Two faecal microbial floras were used in this study. Fresh stools were collected from homebred specified pathogen free (SPF) Wistar rats (Central Animal Facility, University Groningen), and a healthy adult human male. SPF Wistar rats, which were housed under 'standard' SPF conditions, were the offspring of ex-germfree-animals associated with a faecal flora originally derived from mice; previously called colonization resistance microbial flora (CRF) [18, 19]. Specimens of stools were stored at −70 °C until used for association.

Flora association

Two groups of six GF animals were orally associated three times at 48 h intervals with either rodent-SPF ALLO-MF or human XENO-MF at the age of 6–8 weeks. Stool samples used for association were thawed and suspended in saline (1:20 (w/v)) just before oral contamination. The latter was accomplished by inserting the tip of a 1 ml syringe with the suspension and gently injecting 0·2–0·3 ml of the suspension into the mouth of each animal. Conventional ND2 mice were used as controls.

Sampling and preparation of faeces

Four weeks after association, stools of ex-GF animals were sampled and stored in small aliquots at −20 °C until analysis. The same was carried out in 10–12 weeks old CONV controls. Six separately stored aliquots of SPF-MF and HUM-MF were used for DIP. Preparation of faeces was carried out as described previously [1–3]. In short, faecal aliquots were thawed and suspended (1:9 w/v) in 0·5 % Tween-80 and centrifuged at 12 g for 10 min. Subsequently, 150 μl of the supernatant was pipetted into Eppendorf tubes, and centrifuged for 15 min at 11000 g. Finally, the bacterial pellet was resuspended in 300 μl 5 % nigrosin in 0·5 % Tween-80. Five μl of this suspension was spread on a clean fat-free microscopic slide, dried and imbedded in mounting reagent (Eukitt, O. Kindler GmbH & Co., Freiburg, FRG). Two slides were made of each flora.

Digital image processing (DIP)

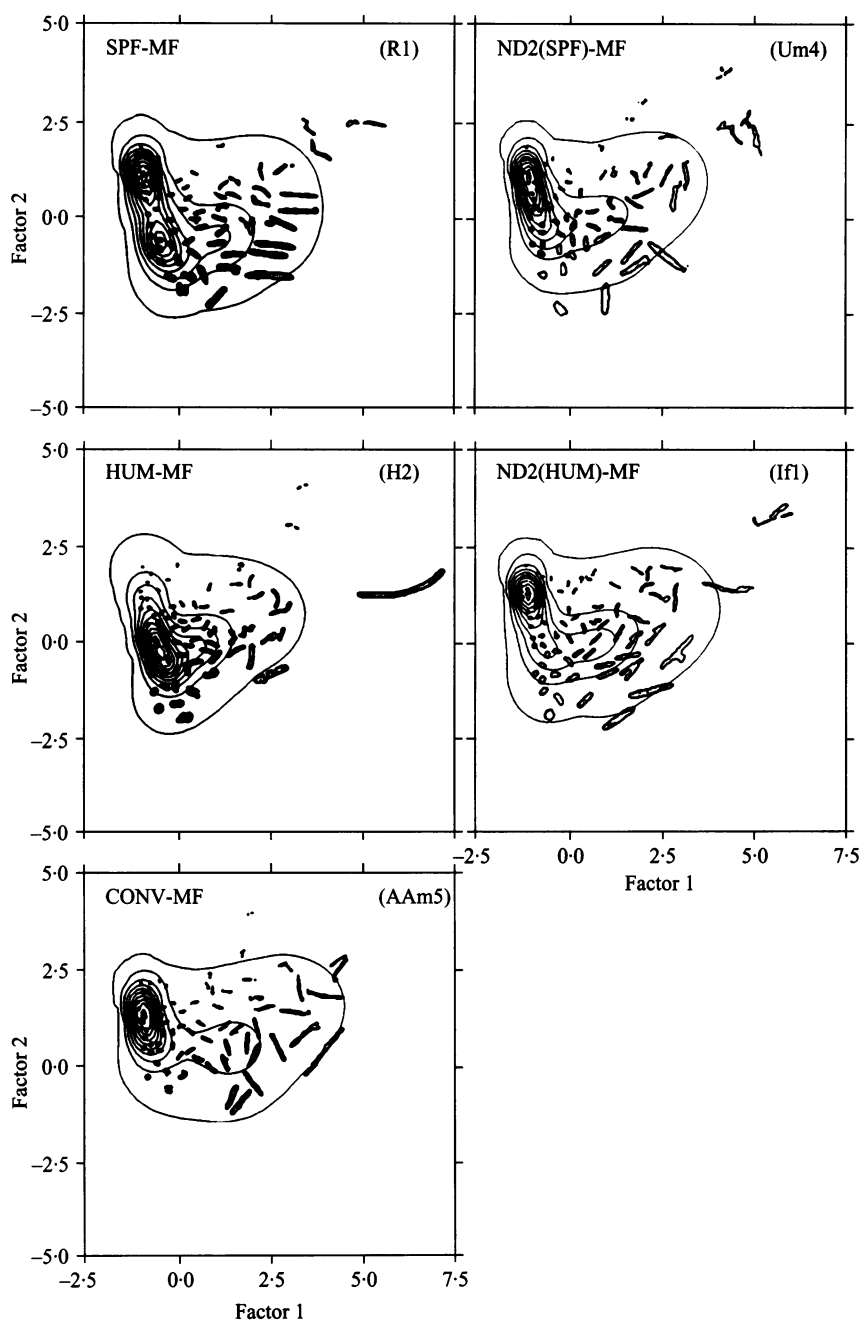The nigrosin counter-stained washed faecal bacterial

Fig. 1. Modified scatter plots of five different faecal samples. Objects, representing bacteria, are drawn on the spot of their component score, i.e. elongation (Factor 1) and width (Factor 2). Altitude lines represent the density of the object within the sample measured.

smears were read microscopically by a videocamera and an image processing system as described by Meijer and colleagues [1–3] and Wilkinson and colleagues [4] In each sample approximately 1000 objects representing bacteria were recorded and measured. Each object was characterized by four parameters according to Meijer and colleagues [2, 3]. Three were obtained by logarithmic and principal component transformation of raw data, thus obtaining three standardized morphometrical components

per object; F1: elongation, F2: (inverse) width, and F3: concavity (degree of irregularity). The medians of the component scores per specimen were used for statistical analysis. The fourth parameter used was entropy, i.e. the degree of morphological diversity within each specimen [2–4]. One of the possibilities provided by the DIP-system, used in this study, is the qualitative and quantitative presentation of different bacterial forms per sample in so-called modified scatter plots.
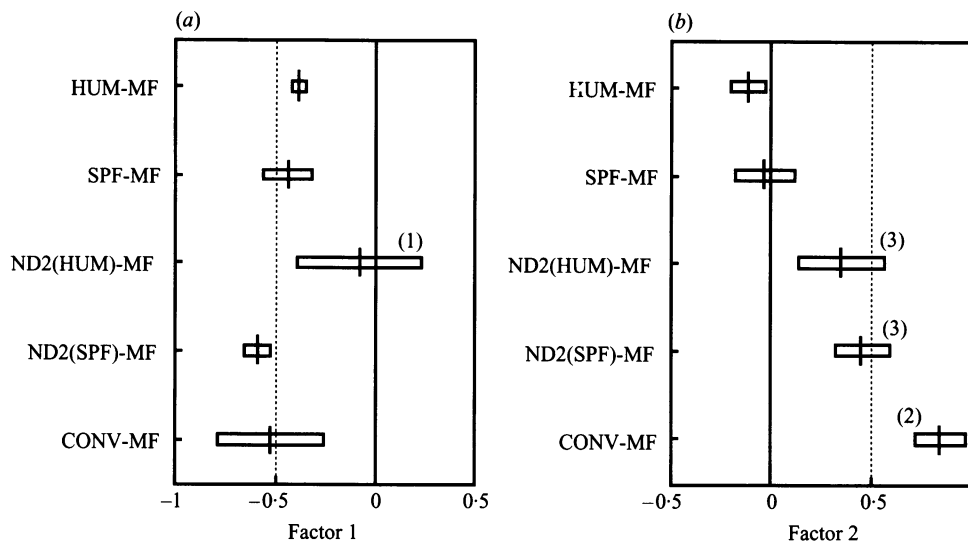
Fig. 2. Means of the median component scores ($\pm$ S.D.) for (a) elongation (Factor 1) and (b) width (Factor 2) estimated in five groups ($n = 6$ per group). (1) $= P < 0\cdot01$ compared with ND2(SPF)-MF and CONV-MF; (2) $= P < 0\cdot01$; (3) $= P < 0\cdot01$ compared with original SPF-MF and HUM-MF.

Numbers of fields and objects

The mean number of objects per faecal specimen in each group was around 1000. These numbers were achieved by counting 10 (range 7–14) fields of view of each specimen.

Statistical evaluation

Statistical evaluation of the medians of morpho-metrical factor scores as well as entropy was carried out by Newmann–Keuls ANOVA. Significance levels were taken at $P < 0\cdot05$.

RESULTS

Qualitative and quantitative analysis per specimen

Figure 1 shows modified scatter plots in which the bacteria themselves are drawn on the spot corresponding their component scores, i.e. elongation (F1) and width (F2). In both figures altitude lines are drawn presenting the density of objects in the sample.

Micromorphometry

The differences between groups were estimated by comparing the means of median component scores ($n = 6$ for each group) for elongation (F1), width (F2) and concavity (F3).

Elongation

The means of the median principal component scores for elongation (F1) are shown in Figure 2a. F1 was

found to be significantly ($P < 0\cdot05$) the highest in stool specimens of (human) HUM-MF associated animals compared with specimens of (rodent) SPF-MF associated animals or the CONV control group. HUM-MF and SPF-MF appeared to have similar component scores for elongation. Large standard deviations (S.D.) for the means of median F1 scores were found in the CONV-MF ($0\cdot260$) and HUM-MF associated mice [ND2(HUM)-MF] ($0\cdot312$). Low S.D.'s were, however, found in the original HUM-MF ($0\cdot033$) and SPF-MF ($0\cdot120$) as well as in stool specimens of ND2 mice associated with SPF-MF [ND2(SPF)-MF] ($0\cdot060$).

Width

Great variability was found in the mean of the median component F2 scores between the groups (Fig. 2b). However, no difference was found between the original HUM-MF and SPF-MF nor between stool specimens obtained from HUM-MF and SPF-MF associated animals, respectively ND2(HUM)-MF and ND2(SPF)-MF. F2 scores in ND2(SPF)-MF were significantly higher ($P < 0\cdot01$) compared to ND2(HUM)-MF. CONV mice were found to have the highest mean of the median F2 score ($0\cdot839$, SD: $0\cdot114$).

Concavity

No significant differences were found between the mean of median F3 scores (results not shown).
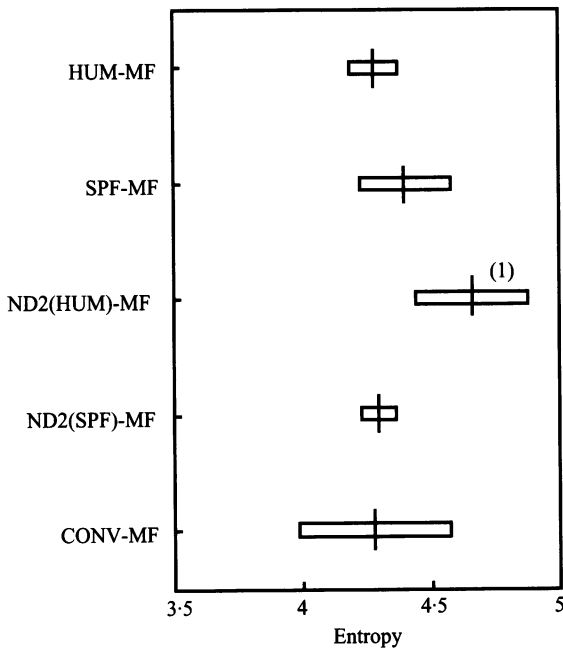
Fig. 3. Means of median score ($\pm$ s.d.) for entropy, i.e. the degree of morphological diversity, estimated in five groups ($n = 6$ per group). (1) $= P < 0.05$ compared with original HUM-MF and ND2(SPF)-MF.

Entropy

The mean of the median scores for entropy are shown in Figure 3. The highest entropy score was found in ND2(HUM)-MF, i.e. stool specimens obtained from HUM-MF associated animals. This suggests a high degree of morphological diversity in bacterial objects in this MF. A significant ($P < 0.05$) difference in entropy was only found between ND2(SPF)-MF and original HUM-MF.

DISCUSSION

This study provides further evidence that highly concentrated anaerobes in the intestinal microbial flora are host specific. Digital image processing of the highly concentrated fraction of intestinal MF is a useful method to detect morphological differences between floras. However, high variability between human individuals or individual animals, e.g. mice, may still be present, even if the animals are housed under strictly isolated GF conditions. Variability in the mean of the median component F1 (elongation) as well as F2 (width), was found to be lowest in allochthonous (rodent) SPF-MF associated mice, whereas variability for both component scores was high in xenochthonous HUM-MF associated counter-

parts. Moreover, a high degree of morphological variety, i.e. entropy, was found in stool specimens of HUM-MF associated mice as compared to SPF-MF associated animals. This suggests that rodent related SPF-MF establishes well within the gut of ND2 mice whereas unrelated MF of human origin does not.

Still, stool samples of SPF-MF associated ex-GF ND2 mice were not comparable with CONV mice in this study. This may be explained by the fact that the CONV animals used had acquired bacteria from the environment for three generations. The first generation was originally GF. The standard deviation (s.d.) for the median F1 scores (elongation) of CONV as well as HUM-MF associated animals [ND2(HUM)-MF] was high; 0.260 and 0.312 respectively (Fig. 2a). On the other hand low s.d. for F1 scores were found in faeces of SPF-MF associated animals [ND2(SPF)-MF] (0.06). Thus, gross micromorphological variability was present between individual SPF-MF associated animals.

Despite the fact that DIP-analysis did not reveal gross micromorphometrical differences between original SPF-MF and original HUM-MF, there is indirect evidence that the floras are different. Firstly, both floras have previously been found to elicit different immune responses in mice [17]. Secondly, after association in mice the mean of the median F1 score (elongation) as well as the entropy were found to be significantly ($P < 0.05$) different between groups of HUM-MF and SPF-MF associated mice.

A possible explanation for the different observations, made for HUM-MF and SPF-MF before and after association, may be that the murine related SPF flora predominantly contained non-immunogenic anaerobes, i.e. bacteria which have been found not to induce an immune response in mice [17]. Low or absent immune responses against bacterial species have previously been found to correlate well with high concentrations in the intestinal tract [14, 15]. If there are high numbers of non-immunogenic bacteria in the implanted flora, the immune system may not play a significant role in the selection process. Instead, other factors may play a prominent role; such as competition for nutrients, mucus, binding to the mucosal surface, and inter-microbial interactions (reviewed by Lee [20]). Non- or low-immunogenic bacteria may rapidly colonize the intestinal tract of ex-GF mice, reach high concentrations in the intestine and form a complex flora. Subsequently, these early colonizers prevent or inhibit other species to colonizing. This phenomenon has been called colonization resistance

(CR). Following association with a species-unrelated (xenochthonous) flora, the immune system may play a dominant role instead. The finding that it takes more than 7 days for HUM-MF associated GF-mice to have a similar CR-level, is in agreement with this hypothesis [17]. As has been noted above, most of the anaerobes of the HUM-MF used in this study, induce a high immune response in mice. These anaerobes may ultimately fail to colonize the intestinal tract at high concentrations. Instead, murine-related rod shaped and fusiform bacteria, which apparently were present at a low (undetectable) level in the original HUM-MF, finally colonized the murine intestinal tract. This relatively slow colonization process explains the high variability in the morphometrical composition of the gut microflora. This is indicated by the high degree of morphological diversity as well as the high S.D. in the mean of the median F1 and F2 scores found in the specimens taken from the HUM-MF associated animals.

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REFERENCES

1. Meijer BC, Kootstra, GJ, Wilkinson MHF. A theoretical and practical investigation into the characterisation of bacterial species by image analysis. Binary 1990; **2**: 21–31.
2. Meijer BC, Kootstra GJ, Wilkinson MHF. Morphometrical parameters of gut microflora in human volunteers. Epidemiol Infect 1991; **107**: 383–91.
3. Meijer BC, Kootstra GJ, Geertsma DG, Wilkinson MHF. Effects of ceftriaxone on faecal flora: analysis by micromorphometry. Epidemiol Infect 1991; **106**: 513–21.
4. Wilkinson MHF, Jansen, GJ, Van der Waaij D. Computer processing of microscopic images of bacteria: morphometry and fluorimetry. Trends Microbiol 1994; **12**: 485–9.
5. Moore WEC, Holdeman LV. Human faecal flora: the normal flora of 20 Japanese Hawaiians. Appl Environ Microbiol 1974; **27**: 961–79.
6. Nord CE, Kager L. The normal flora of the gastrointestinal tract. Neth J Med 1984; **27**: 249–52.
7. Koopman JP, Van den Brink ME, Smits RCTG. Effect on the level and biotypes of Enterobacteriaceae in rat faeces. Micr Ecol Health Dis 1989; **2**: 279–83.
8. Veenendaal D, De Boer F, Van der Waaij D. Effect of selective decontamination of the digestive tract of donor and recipient on the occurrence of murine delayed type graft versus host disease after allogeneic bone marrow transplantation. Med Microbiol Immunol 1988; **177**: 133–44.
9. Holdeman LV, Cato EP, Moore WEC. Human faecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. App Environ Microbiol 1976; **31**: 359–75.
10. Meijer-severs GJ, Van Santen E. Variations in aerobic faecal flora of ten healthy human volunteers with special reference to the Bacteroides fragilis group and Clostridium difficile. Zentralbl Bakteriol Mikrobiol Hyg (A) 1986; **261**: 43–52.
11. Van der Merwe JP, Stegeman JH, Hazenberg MP. The resident faecal flora is determined by genetic characteristics of the host. Implications for Crohn's disease. Antonie van Leeuwenhoek 1983; **49**: 119.
12. Van der Waaij D. The immunoregulation of the intestinal flora; consequences of decreased thymus activity and broad-spectrum antibiotic treatment. Zentralbl Bakteriol Mikrobiol Hyg 1985; Suppl **13**: 73–87.
13. Van der Waaij D. Evidence of immunoregulation of the composition of intestinal microflora and its practical consequences. Eur J Clin Microbiol Infect Dis 1988; **7**: 103–6.
14. Berg RD, Savage DC. Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. Inf Immunol 1975; **11**: 320–9.
15. Foo MC, Lee A. Immunological response of mice to members of the autochthonous intestinal microflora. Inf Immunol 1972; **6**: 525–32.
16. Van der Waaij D, Heidt PJ. Intestinal bacterial ecology in relation to immunological factors and other defence mechanisms. In Hambraeus L, Hanson CA, McFarlane H, eds. Food and immunology. Stockholm: Almquist and Wiksell, 1977: 133–41.
17. Veenendaal D, De Boer F, Van der Waaij D. Immunological selection of related and unrelated microflora by germfree mice. In McDonald TT, Challacombe SJ, Bland PW, Stokes CR, Heatley RV, McMowat A, eds. Advances in mucosal immunology. London: Kluwer academic publisher, 1990: 927–8.
18. Van der Waaij D. The persistent absence of Enterobacteriaceae from the intestinal flora of mice following antibiotic treatment. J Infect Dis 1968; **118**: 32.
19. Van der Waaij D, Berghuis-De Vries JM, Lekkerkerk-Van der Wees JEC. Colonization resistance of the digestive tract in conventional and antibiotic treated mice. J Hyg 1971; **69**: 405–11.
20. Lee A. Neglected niches. The microbial ecology of the gastrointestinal tract. In Marshall KC, ed. Advances in microbial ecology, vol 8. New York: Plenum Publisher, 1985: 115–62.