FLUOROMORPHOMETRICS: A NEW APPROACH IN CHARACTERISING FAECAL FLORA

GIJSBERT J. JANSEN and MICHAEL H.F. WILKINSON

Laboratory for Medical Microbiology, University of Groningen, Groningen, The Netherlands

SUMMARY

Using image analysis and quantitative immunofluorescence, three new methods in characterising faecal microflora are presented. These methods are: Morphological analysis of faecal objects, measurement of titres of circulating Ig against faecal antigens and the combination. Morphological analysis is the least sensitive approach, which is only applicable when large fluctuations in the morphological composition of the faecal microflora have to be monitored. Biological fluctuations in titres of circulating Ig against faecal antigens can be performed at a statistically significant level for IgM and IgG and the distribution of titres of circulating IgG over 144 morphological distinct subsets of faecal bacteria yields a unique pattern in a group of ten volunteers.

INTRODUCTION

Research concerned with the interaction between gutflora and the immune system of the host is a rapidly evolving field since the importance of the gutflora in the aetiology of rheumatoid arthritis (*Hazenberg* et al., 1992), nosocomial infections (*Cerra* et al., 1992), Crohn's disease (*Giaffer*, 1991) and colonic carcinogenesis (*Lidbeck* et al., 1992) is recognised. Thereupon, detailed knowledge of the immunological aspects of the host-gutflora interaction may also explain the decrease in infection risk after the oral intake of viable microorganisms ('probiotics') which is observed on several occasions in different species of vertebrates, including humans (*Metchnikoff*, 1907, *Gorbach*, 1987, *Pollman*, 1986, *Rusch*, 1986).

Earlier research by Perdigon and coworkers in mice has revealed that a change in the bacteriological composition of the gutflora results in a significant response of different immunological systems. For example, the oral intake of viable lactic acid bacteria results in activation of macrophages (*Perdigon*, 1986), a NK-cell response (*Kato*, 1984) and elevation of titres of circulating IgG directed against the latter (*Perdigon*, 1988).

In these experiments, pure cultures of lactic acid bacteria were used as antigenic substrate. As a result of this experimental design, the *in situ* processing of surface antigenic epitopes on the lactic acid bacteria by bacterial enzymes present in the digestive tract, is not taken into account.

In order to overcome this problem a method was developed which enables *in situ* quantification of the total amount of circulating (or mucosal) antibodies bound to faecal bacteria (*Apperloo-Renkema* et al., 1991a). In addition, the morphology of the faecal objects (pre-

dominantly bacteria) can also be quantified (*Meijer*, 1989). Synthesis of these two strategies has - on a semi-quantitative level - been accomplished by *Apperloo-Renkema* and co-workers (1992).

Further quantification of the combined fluorimetrical and morphometrical method, or 'fluoromorphometrical' method, has been performed by *Jansen* and co-workers (1993a).

The morphometrical, the fluorimetrical and the fluoromorphometrical method have been applied in projects concerned with the interaction between host organism and gutflora. Using the morphometrical method, *Meijer* and co-workers (1992) observed a significant decrease in the diversity of shape of faecal bacteria after the host received antimicrobial chemotherapy. Using the fluorimetrical method, *Jansen* and co-workers (1993b) were able to monitor longitudinal fluctuations in titres of circulating Ig against gutflora at a significant level. Using the fluoromorphometrical method, *Apperloo-Renkema* and co-workers (1992) found that the titres of serum Ig directed against 28 morphologically distinct groups of faecal bacteria could be quantified reproducibly and that the pattern of 28 titres is unique per pair of serum and gutflora.

In this paper the morphometrical, fluorimetrical and the fluoromorphometrical method will be presented simultaneously. Furthermore, an overview of previously obtained results using the three methods mentioned above will be given.

METHODS

Hardware

Although both hardware and software have been described previously (*Jansen* et al., 1993b), they will be mentioned here briefly. An ultra-violet microscope equipped with a phase contrast condensor, a 100-watt mercury lamp and a CCD video camera were used. Furthermore, the system comprises of a Compaq deskpro 80486 microcomputer with 8 Mb RAM, a MATROX MVP/AT image-processing board and an exposure-control expansion board interfacing the computer to the CCD. With this board, which was developed in our laboratory (*Wilkinson*, 1993), the integration time of the camera can be increased from one video frame (= 1/30 s) to any integer number of video frames. Analysis of the phasecontrast image of a microscopic slide results in a binary image of 512x512 pixels, containing only background and faecal objects. In addition, under ultra violet illumination, a fluorescence image is generated containing the fluorescence intensities belonging to the objects in the accompanying binary image.

Morphometrical analysis

Description of the faecal objects in the binary image which result from the phase contrast image after digital image processing, requires quantitative morphometrical parameters. We have used four morphometrical parameters derived (*Meijer*, 1991a) which are based upon the area (A), perimeter (P), moment of inertia (I) and area of convex hull (H) of the faecal objects. The moment of inertia is defined as:

$$I = \sum_i [(x_i - x)^2 + (y_i - y)^2] \qquad (1)$$

In which the x_i are the x co-ordinates of the pixels belonging to the object, and x is their mean; likewise for y_i and y. Based on these four measurements, the morphological parameters derived were:

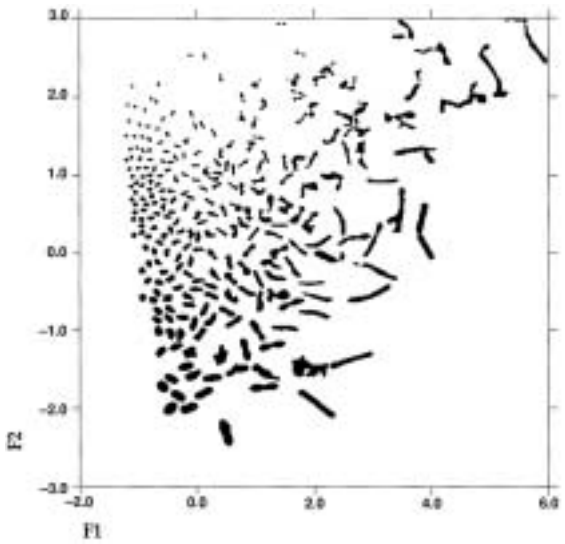

Figure 1: Morphometrical plot. Objects in the morphometrical region are represented as bacteria instead of symbols. For reasons of clarity some bacteria are omitted.

$$a = \log A \quad (2)$$

$$f1 = 2\log P - \log A \quad (3)$$

$$f2 = \log I - 2\log A \quad (4)$$

$$c = \log H - \log A \quad (5)$$

Four principal component scores (F1, F2, F3 and F4) were calculated from these morphological parameters. The matrix of transformation coefficients applicable on the entire dataset was created previously by Meijer et al. using 58 samples of faecal bacteria from nine healthy volunteers. It was decided to use two principal components (F1 and F2) because these two already explain most (i.e. 93.5%) of the original variance. Because principal components F1 and F2 are linearly unrelated they define a morphometrical plane. Although the morphometrical plane is infinite by definition, we focus attention only on the morphometrical region defined by -2<F1<8 and -5<F2<5 because over 99% of the faecal objects fall into this part of the morphometrical plane. The morphology of objects present in a representative faecal sample and their localisation in the morphometrical region is depicted in Figure 1. Then - in order to measure the variety of bacterial form in the morphometrical plane - the morphometrical entropy (S) was calculated. The entropy is computed by dividing each axis of the morphometrical region in sixteen equally sized intervals, so that the morphometrical plane becomes divided into 16x16 fields. For each field we compute:

$$p_i = n_i/N \quad (6)$$

This p-value comprises the probability that a bacterium from the specimen will yield a datapoint falling into morphometrical field i (n_i is the number of bacteria in cell i; N is the total number of bacteria in the dataset). The morphometrical entropy is now estimated by:

$$S = -\sum_i p_i \log p_i \quad (7)$$

When the distribution of datapoints over the morphometrical region is homoge-

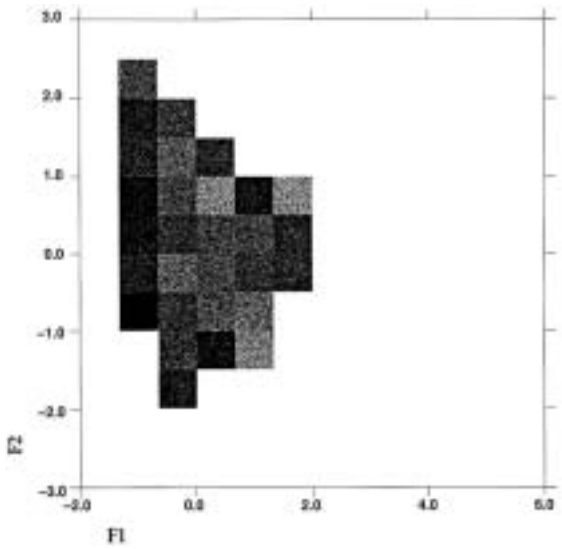

Figure 2: Density plot. Morphometrical region divided into 144 discrete fields or morphotypes. The titre of circulating IgG bound to the bacteria within a morphotype is proportional to the intensity of grey-dithering per field. Grey-levels are relative i.e. the morphotype containing the objects which bind circulating IgG to the most becomes black. Morphotypes containing objects which bind no circulating IgG are white.

nous the value of S is large and when the location of the datapoints is confined to a small area of the morphometrical region, the value of S is small.

Fluorimetrical analysis

For each faecal object in the phasecontrast image, the average grey-value of the corresponding pixels in the fluorescence image is calculated. Then the average grey-level of the pixels in the area which surrounds the objects to a distance of eight pixels is computed, excluding any pixels belonging to other objects. These two averages are then subtracted and the difference, multiplied with the serum dilution factor, is called the fluorescence level. With this method noise due to local differences in background fluorescence is eliminated. For each measurement the median fluorescence level of at least 1000 faecal objects is determined. The median instead of the mean was employed to deal with occasional outliners in the fluorescence distribution.

The performance of this method - using autochthonous polyclonal circulating antibodies directed against faecal flora - has recently been validated (*Jansen* et al., 1993b).

In serological procedures it is common practice to express antibody concentrations as titre. Titre is defined as the inverse of the dilution which is needed to satisfy some arbitrary condition. This arbitrary condition is also designated as the threshold value. In our case, titre may be defined as an arbitrary fluorescence level divided by the actual fluorescence level although we prefer to use the median grey-level because this is a continuous measure of antibody concentration, while the titre depends on a threshold-value.

Fluoromorphometrical analysis

Combining both the morphometrical and the fluorimetrical analysis of faecal flora has been done by *Apperloo-*

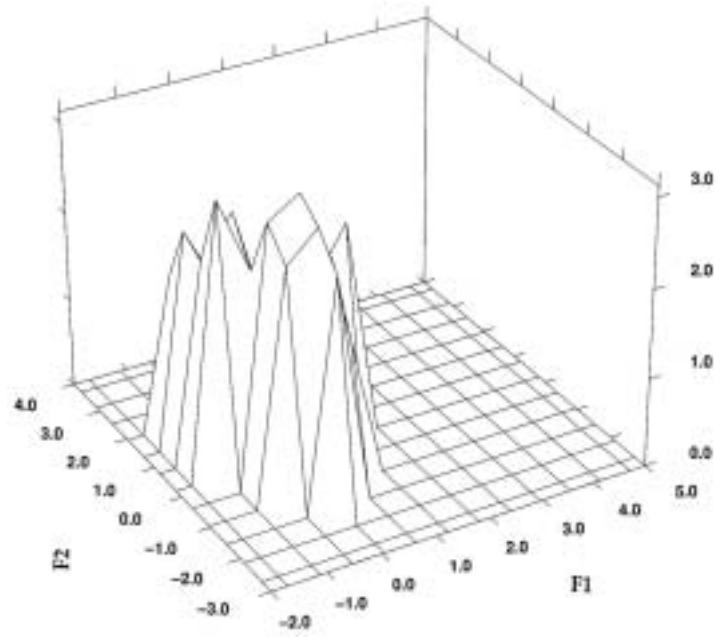

Figure 3: Hiddenline plot. Titres of circulating IgM over bacteria in the morphometrical region represented by the peak height in the third dimension.

Renkema and co-workers (1992). In their study the binding of molecules of circulating Ig over 28 morphologically distinct subsets of faecal bacteria - defined by 28 different, non- overlapping, F1- and F2-ranges - was measured at a semi-quantitative level. *Jansen* and co-workers (1993a) further optimised this fluoromorphometrical method. In their approach, the morphometrical region (i.e. -2<F1<8 and -5<F2<5) is arbitrarily divided into 12x12 equally sized squares. Each square is then called a 'morphotype' because objects within one morphotype appear uniform to the observer. Per morphotype the mean fluorescence of the objects it contains minus the mean fluorescence of the objects in the corresponding morphotype of the negative control is computed, yielding a set of 144 fluorescence intensities. The distribution of circulating IgG over a sample of gutflora is depicted by means of a density plot in Figure 2. Furthermore, using the same faecal sample, the distribution of circulating IgM over the faecal objects present in this gutflora is depicted by means of a hiddenline plot in Figure 3. In Figure 4 the distribution of circulating IgA is depicted by means of a combined contour- and morphometrical plot.

Assuming that these fluorescence intensities - which may be transformed to titre - are independent, they define a 144-dimensional datavector. In 144-dimensional space, the length of a datavector reflects the overall fluorescence intensity and the direction of the datavector reflects the distribution of the fluorescence intensities over 144 morphotypes.

Differences in the binding of molecules of circulating Ig over the 144 morphotypes between two subjects (say: A and B) are reflected by the angle between their corresponding datavectors. Using euclidian metric, the angle (ß) between the datavectors derived from volunteer A and B can be com

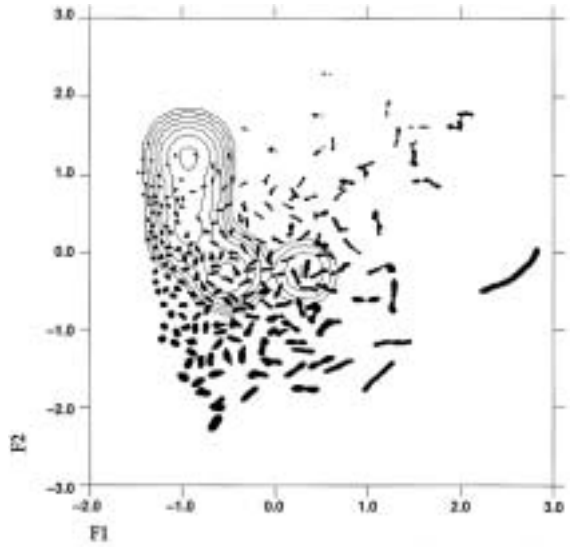

Figure 4: Combined morphometrical and contourplot. Bacteria in the morphometrical regions represented as bacteria instead of symbols. For reasons of clarity some bacteria have been omitted. The contours represent titres of circulating IgA directed against the underlying morphotypes. The outer contour represents objects with a fluorescence level of 2.00. Subsequent contours indicate the location of objects which exhibit a titre twice as large as the contour by which it is enclosed.

puted by means of formula 8.

$$\cos \beta = \frac{AB}{|A| \times |B|} \qquad (8)$$

In this formula, A B do represent the inner product of datavectors A and B, while |A| and |B| represent the euclidian length of datavector A and the euclidian length of datavector B respectively.
The cosine of the angle between two datavectors represents a measure of similarity because when A and B are equidirectional, the cosine of the angle they enclose is one and when A and B are fully independent the cosine of the angle they enclose is zero.

DISCUSSION

The host organism is in direct contact with the mucosal part of the indigenous gutflora. The bacteriological composition of the lower mucosal flora of the gut correlates well with the bacteriological composition of colorectal part of the luminal flora which is present in faeces (*Johansson* et al., 1992). Therefore, the use of faeces as representing the lower mucosal gutflora seems correct.

However, faecal flora may contain as many as 400 species of mainly anaerobic bacteria (*Moore*, 1974). Thus, when the composition of the gutflora is under consideration, culturing and identification techniques are very laborious and time consuming. Also, culturing of bacteria on other media than pure gut mucus may alter the expression of antigenic epitopes on the bacterial cell wall and, furthermore, the absence of intestinal glutamases and proteases (which modificate antigenic epitopes under *in vivo* conditions) adds to the unreliability

of culturing when the interaction with the host's immune system is assessed under *in vitro* conditions (*Osagawara* et al., 1985).

Alternative approaches have been developed e.g. measurement of the activity of bacterial enzymes present in the faeces (*Welling* et al., 1990) or analysis of gutflora-associated metabolites like short chain fatty acids (*Cummings*, 1991). Though the activity of bacterial enzymes can be assessed rapidly and accurately, their value in characterising faecal flora is limited because different species of bacteria may produce the same enzyme. Using the analysis of short chain fatty acids, both genus and species name of a pure culture can be elucidated. However, in a complex ecosystem like faecal flora this method lacks sufficient discriminating power.

Semi-quantitative morphological analysis of - Gram-stained - faecal flora has been performed by *Baquero* and coworkers (1988). In this study faecal objects were classified by eye into 40 morphologically distinct categories. Apart from the error which results from the inherent subjectivity of this method, *Meijer* and co-workers (1991b) have demonstrated that morphological subsets of faecal objects actually do not exist. Instead of that, the distribution of morphometrically defined shapes of faecal objects is a continuum.

The system of Meijer and co-workers has been applied in a hospital environment to quantify the influence of cefalosporins on the morphological composition of faecal microflora. In a group of eleven healthy volunteers a significant decrease of the morphometrical entropy (S) was observed after intramuscular administration of 1 g of ceftriaxon (*Meijer* et al., 1992).

Because the contact between gut flora and host organism is believed to occur predominantly at the mucosal membrane of the digestive tract, research in the field of host-gutflora interaction has so far mainly concerned the mucosal immune system (*McGhee* et al., 1992). In addition to the mucosal immune system, antigens originating from the gut flora may occasionally also be presented to the systemic immune system. Mechanisms responsible for this transfer of antigen comprise bacterial translocation to other lymphoid organs than gut associated lymphoid tissue (*Debure*, 1987; *Wells*, 1987a, 1987b).

The presence and titre of circulating Ig directed against indigenous gut flora has firstly been demonstrated by *Apperloo-Renkema* and co-workers (1991b) using a quantitative immune fluorescence method. With an improved quantitative immune fluorescence method, Jansen and co- workers were able to detect fluctuations in the capacity of faecal bacteria to bind circulating Ig at a statistically significant level. The quantitative immune fluorescence - or fluorimetrical - method has the obvious advantage that objects which are clearly not of bacterial origin can be excluded from the analysis. When measuring levels of circulating Ig directed against intestinal bacteria fluorimetrically, coefficients of variation of 6.1%, 6.4% and 9.8% were obtained for IgG, IgM and IgA respectively. Therefore, the fluorimetrical method can be used when the interaction between a polyclonal antiserum and a complex antigenic substrate like intestinal bacteria is studied.

When the fluoromorphometrical method is employed, the titre of circulating antibodies directed against morphologically identical faecal bacteria can quantified. Morphologically identical bacteria do not necessary belong to the same species, as Meijer and co-workers have demonstrated. Conversely, within a population of bacteria belonging to one species large morphological deviations do occur. Therefore, the division

of the morphometrical region (-2<F1<8 and -5<F2<5) into 144 equally sized fields or morphotypes has no relation with conventional bacteriological nomenclature. Results of this fluoromorphometrical analysis have to be interpreted in terms of humoral reactivity against morphologically identical faecal bacteria.

Despite this restriction, Apperloo-Renkema and co-workers have demonstrated - at a semi-quantitative level - that the distribution of circulating Ig over 28 arbitrarily chosen morphotypes yields a pattern which is unique per gutflora-serum combination. This finding was confirmed by Jansen and co-workers using the previously described method. Performance analysis of the fluoromorphometrical method (unpublished data) reveals that the error of this method is largely due to inaccuracies in the process of slide manufacturing (i.e. the assay error), while the error due to longitudinal fluctuations in the antibody repertoire of the serum and the antigenic composition of the faecal flora is only half the magnitude of the assay error.

The fluoromorphometrical method may be useful when small changes in the composition of the gutflora are to be expected. An especially interesting field of research could be the influence of live bacterial food additives, or probiotics (*Fuller*, 1988) on the composition of the gutflora and the influence of this alteration on the repertoire of circulating Ig. Such a study, using *Enterococcus faecalis* as a probiotic is currently being undertaken.

ACKNOWLEDGEMENTS

The authors are very grateful to the Institute for Microecology, Herborn-Dill, Germany, for the financial support of this study. Furthermore, Dr W. Sluiter is gratefully acknowledged for his statistical advice

LITERATURE

Apperloo-Renkema, H.Z., Wilkinson, M.H.F., Oenema, D.G., and van der Waaij, D.: Objective quantitation of serum antibody titres against *Enterobacteriaceae* using indirect immunofluorescence, read by videocamera and image processing system. Med. Microbiol. Immunol. 180, 93-100 (1991a).

Apperloo-Renkema, H.Z. and van der Waaij, D.: Study of colonization resistance for *Enterobacteriaceae* in man by experimental contamination and biotyping as well as the possible role of antibodies in the clearance of these bacteria from the intestines. Epidemiol. Infect. 107, 619-626, (1991b).

Apperloo-Renkema , H.Z., Wilkinson, M.H.F., and van der Waaij D.: Circulating antibodies against faecal bacteria assessed by immunomorphometry: Combining quantitative immunofluorescence and image analysis. Epidemiol. Infect. 109, 497-506 (1992).

Baquero, F., Fernandez-Jorge, A., Vicente, M.F., Alos, J.I., and Reig, M. Diversity analysis of the human faecal flora: a simple method based on bacterial morphotypes. Microb. Ecol. Health Dis. 1, 101-108 (1988).

Cerra, F.B., Maddaus, M.A., Dunn, D.L., Wells, C.L., Konstantinides, N.N., Lehmann, S.L., and Mann, H.J.: Selective gut decontamination reduces nosocomial infections and length of stay but not mortality or organ failure in surgical intensive care unit patients. Arch. Surg. 127, 163-169 (1992).

Cummings, J.H. and McFarlane, G.T.: The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. 70, 443-459 (1991).

Debure, A., Rambaud, J.C., Ducluzeau, R.,

Yurdusev, N., and Raibaud, P.: Translocation of strictly anaerobic bacteria from th intestinal tract to the mesenteric lymph nodes in gnotobiotic rodents. Ann. Inst. Pasteur/Microbiol. 138, 213-221, (1987).

Fuller, R.: Probiotics in man and animals. J. Appl. Bacteriol. 66, 365-378 (1988).

Giaffer, M.H., Holdsworth, C.D., and Duerden, B.I.: Effects of an elemental diet in the faecal flora in patients with Crohn's disease. Microb. Ecol. Health. Dis. 4, 369-374 (1991).

Gorbach, S.L., Chang, T.W., and Goldin, B.R. Succesful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. Lancet ii, 1519 (1987).

Hazenberg, M.P., Klasen, I.S., Kool. J., Russeler-van Embden. J.G.H., and Severijnen, AJ.: Are faecal bacteria involved in the etiology of reumathoid arthritis ? APMIS 100, 1-9 (1992).

Jansen, G.J., Wilkinson, M.H.F., Deddens, B., and van der Waaij.: Characterization of human faecal flora by means of an improved fluoro-morphometrical method. Epidemiol. Infect. 111, 265-272 (1993a).

Jansen, G.J., Wilkinson, M.H.F., Deddens, B., and van der Waaij, D.: Statistical evaluation of an improved quantitative immunofluorescence method of measuring serum antibody levels directed against faecal bacteria. J. Microbiol. Meth. 17, 137-144 (1993b).

Johansson, M.L., Molin, G., Jeppson, B., Noback, S., Ahrne, S., and Bengmark, S.: Administration of different *Lactobacillus* strains in fermented oatmealsoup: *In vivo* colonization of human intestinal mucosa and effect on the indigenous flora. Appl. Environm. Microbiol. 59, 15-20 (1992).

Kato, I., Yokokura, T., and Mutai, M.: Augmentation of mouse natural killer cell activity by *Lactobacillus casei* in mice. Microbiol. Immunol. 27, 611-618 (1984).

Lidbeck, A., Overvik, E., Rafter, J., Nord, C.E., and Gustafsson, J.A,. Effect of *Lactobacillus acidophilus* supplements on mutagen excretion in faeces and urine in humans. Microb. Ecol. Health. Dis. 5, 59-67 (1992).

McGhee, J.R., Mestecky, J., Dertzbaugh, M.T., Eldridge, J.H., Hirasawa, M., and Kiyono, H.: The mucosal immunesystem: From fundamental concepts to vaccine development. Vaccine 10 , 75-88 (1992).

Meijer, B.C., Kootstra, G.J., and Wilkinson, M.H.F.: A theoretical and practical investigation into the characterization of bacterial species by image analysis. Binary comp. microbiol. 2, 21-31 (1989).

Meijer, B.C., Kootstra, G.J., and Wilkinson, M.H.F. Morphometrical parameters of gut flora in human volunteers. Epidemiol. Infect. 107, 383-92 (1991a).

Meijer, B.C., Kootstra, G.J., and Wilkinson, M.H.F.: Characterization of bacterial species by image analysis. Binary comp. microbiol. 2, 21-31 (1991b).

Meijer, B.C., Kootstra, G.J., Geertsma, D.G., and Wilkinson, M.H.F.: Effects of ceftriaxone on faecal flora: analysis by micromorphometry. Epidemiol. Infect. 106, 513-521 (1992).

Metchnikoff, E.: The prolongation of life. Heinemann Publishers, London 1907.

Moore, W.E.C. and Holdeman, L.V.: Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27, 961-979 (1974).

Perdigon, G., Nader de Marcias, M.E., and Alvarez, S.: Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immun. 53, 404-410 (1986).

Perdigon, G., Nader de Marcias, M.E., and Alvares, S.: Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophillus*. Immunology, 63, 17-23 (1988)

Pollman, D.S.: Probiotics in pig diets. In: Recent advances in animal nutrition (Eds.: Haresign, W. and Cole, D.J.A). Butterworth, London, 193-205 (1986).

Rusch, V., Kalinski, S., and Schneider, H.: *Streptococcus faecalis* vaccine - results of double blind placebo studies. Microecol. Ther. 16, 59-74 (1986).

Welling, G.W., Meijer-Severs, G.J., Helmus, G., van Santen, E., Tonk, R.H.J., de Vries-Hospers, H.G., and van der Waaij, D.: A microflora associated characteristic (MAC) compared with a germfree animal characteristic (GAC) during antimicrobial treatment. Microecol. Ther. 20, 505-509 (1990).

Wells, C.L., Maddaus, M.A., Reynolds, C.M., Jechorek, R.P., and Simmons, R.L.: Role of anaerobic flora in the translocation of aerobic and facultatively anaerobic intestinal

bacteria. Inf. Imm. 55, 2689-2694 (1987a).
Wells, C.L., Maddaus, M.A., and Simmons, R.L.: Role of the macrophage in the translocation of intestinal bacteria. Arch Surg. 122, 48-53, (1987b).