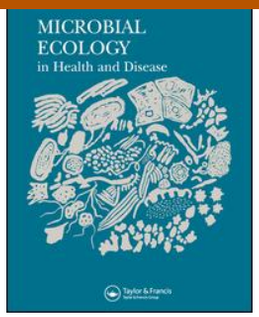

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Bacterial Faecal Flora in Healthy Women of Different Ages

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The composition of the intestinal flora is the result of host physiology, microbial interaction and environmental influences. The possible relationship between faecal flora composition and hormonal modifications in healthy women of different ages was studied. Forty-four normal women were divided into the following groups according to age: group I, 27–47 yr; group II, 50–55 yr, <5 yr after menopause; group III, 56–78 yr, >5 yr after menopause. The subjects received no pharmacological treatment. Samples were collected on the 8th and 23rd day of the cycle; two samples were obtained from each postmenopausal woman. Qualitative and quantitative determination of microorganisms was carried out using slightly modified standard methods. In fertile women (group I), the microflora composition was similar for samples collected on the 8th–10th day and during the premenstrual period (23rd day). In postmenopausal women (group III), an increase in fungi, clostridia and aerobic lactobacilli mean concentrations were observed. *Escherichia coli* mean levels increased and Enterobacteriaceae such as *Enterobacter cloacae* and *Citrobacter freundii* were present in 80 per cent of subjects studied. The length of menopause was found to have only a slight influence on flora: the behaviour of the microflora composition in menopausal women in group II may be considered intermediate between groups I and III. This preliminary study demonstrates that there are fluctuations in the composition of the faecal flora in healthy women. The differences observed between premenopausal and postmenopausal women may be a consequence of modifications of the steroid sex hormone pattern.

KEY WORDS—Intestinal flora; Age; Premenopausal women; Postmenopausal women; Menopause.

INTRODUCTION

The human flora does not vary greatly from individual to individual, and several studies suggest that the intestinal flora in any given person is usually quite stable in the absence of factors such as disease and/or antimicrobial therapy.[11,29,32] Different diets induce no significant changes in bacterial flora composition,[5,15,20] although some enzyme activity may be affected.[26]

The intestinal flora plays a significant role in the metabolism of endogenous and exogenous compounds.[7,31] Microorganisms may degrade cholesterol and bile acids as well as steroid sex hormones, which have a chemical affinity with them.[7,8,20] About 60 per cent of the oestrogen metabolites appear in the bile, exclusively in conjugated forms, but only 7 per cent of the metabolites appear in the faeces, the rest being hydrolysed to the free hormone and reabsorbed in the intestinal tract via the enterohepatic circulation. The hormonal conditions in women before and after menopause are very different. The premenopausal period is characterised by changes in oestrogen-progesterone levels, 17-β oestriol being the main metabolite. In postmenopausal women ovarian function has ceased, and there is thus a constant level of oestrogens produced by the adrenal cortex and adipose tissue, oestrone being the main compound.[6] The oestrogen excretion profile changes in women on a vegetarian diet.[13,16] The intestinal microflora, however, is the result of host physiology, environmental influences and interaction between microbial species. Dubos *et al.*[9] observed that, in mice, considerable microflora variations of both quantitative and qualitative nature can result from environmental and physiological changes. Similarly, the presence of endogenous or exogenous compounds may influence the intestinal microbial population in man.

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There are very few data in the literature on the influence of age on the human intestinal microflora,[19] and such reports as are available do not mention the relationship between the steroid sex hormone profile and the composition of the flora.

Assuming that in a given population the lifestyle, and thus the flora of the colon, is relatively standard, the aim of our preliminary research programme was to study faecal flora composition in different hormonal conditions, i.e. in pre- and post-menopausal healthy women.

MATERIALS AND METHODS

Subjects

The population studied consisted of 44 healthy women (married and unmarried; students, housewives and white-collar workers) admitted to the Padua University Department of Obstetrics and Gynaecology for smear screening (Pap test or endometrial cytology).

The volunteers were assigned to one of the following groups: group I, 27–47 yr of age, mean age $40{\cdot}4 \pm 6{\cdot}5$ yr (18 subjects); group II, 50–55 yr of age, mean age $52{\cdot}2 \pm 2{\cdot}2$ yr, <5 yr after menopause (five subjects); group III, 56–78 yr of age, mean age of $65{\cdot}2 \pm 8{\cdot}4$ yr, >5 yr after menopause (21 subjects).

The blood-chemistry parameters were in the normal range (determined by SMA-6 and SMA-18 Auto-Analyzer, Technicon). The cycle was regular in all the premenopausal subjects, corresponding to 28 ± 3 d (mean $\pm$ SD). None of the volunteers had received pharmacological treatment during the 3 months prior to the investigation (antibiotics, hormones, laxatives, vitamins). The free-choice diet was similar in all subjects and was based mainly on typical Mediterranean food, including pasta, meat, fish, vegetables, fruit, olive-oil, wine, etc.

Habitual consumption of yogurt constituted a criterion for exclusion from the study project. Subjects with slight or mild gastrointestinal and gynaecological pathology (cysts, uterine leiomyoma, etc.) or with neurological disorders (stress, depression, etc.) were also excluded.

Informed consent was obtained from all volunteers.

Collection of specimens

Faeces were collected from each premenopausal subject on the 8th–10th day and on the 23rd day (premenstrual period) of the cycle; two samples at a 15 d interval were obtained from each menopausal woman.

The stool specimens were collected in sterile plastic containers, gassed with CO_2 (95 per cent)–H_2 (5 per cent) and immediately stored at $-80°C$ until assayed. In these conditions, freezing at $-80°C$ yields more reliable results than those obtained at $-24°C$. Many data are presently based on determinations carried out on stool samples frozen at $-80°C$.[10,17]

Sample preparation and assays

One gram of faeces were suspended by manual homogenisation in 9 ml prereduced sterile saline, filtered through gauze; ten-fold serial dilutions were then produced to a dilution of 10^{-12} in an anaerobic box (CO_2, 95 per cent–H_2, 5 per cent and a cold catalyst alumina with palladium 1 per cent). Instead of the usual classic Petri dishes we used trays with 80 wells (the trays were sterile virology plexiglass plates). Each well (diameter 16 mm) was filled with 0·5 ml agar medium, and each tray with a different culture medium. Ten microlitres (Combitips, Eppendorf) of each dilution were seeded simultaneously on the surface of selective and non-selective culture media. Each row (10 wells) comprised all dilutions of each sample. Anaerobic faecal flora was analysed using the following technique: 0·01 ml of the dilutions was transferred into the wells of plexiglass plates in an anaerobic atmosphere. Immediately thereafter, 0·5 ml of oxygen-free media, at 51°C, were seeded into the same wells. The plates were then wrapped in sterile perforated cellophane sheets, and placed in an anerobic chamber (Heraeus) under an 85 per cent nitrogen–10 per cent carbon dioxide–5 per cent hydrogen atmosphere, using carbon with palladium 1 per cent and copper as a cold catalyst and incubated at 37°C for 48–72 h. The aerobic plates were incubated at 37°C for 24 h. Samples were seeded in duplicate. We considered as positive the last well presenting growth of three colonies. Moreover, 0·1 ml samples of the 1st dilution were streaked on blood agar solidified in classic Petri dishes.

This rapid and accurate technique, which is a semimicromethod previously described in detail elsewhere,[28] allows a saving of time and materials and yields qualitative and quantitative results comparable to those obtained with the more expensive standard techniques (Petri dishes, etc.).

After incubation, the different colony types were counted, isolated in pure culture and identified by standard methods[11,21] on the basis of Gram stain and morphological and biochemical analysis (API System S.A., La Balme Les Grottes, France). Enterobacteriaceae were identified by API 20 E, streptococci by API 20 Strep; staphylococci by API Staph; fungi by API C AUX; and anaerobes by the Rap ID ANA System. The lowest detectable number of organisms with this method is 2·0 $\log_{10}$ per gram of fresh faeces.

All media used were purchased from Difco Laboratories (Detroit, USA), unless otherwise specified: blood agar for total aerobic and anaerobic count; Schaedler agar[30] and thioglycollate Medium agar (anaerobic total count); kanamycin (12 mg/l)–vancomycin (20 mg/l)–Schaedler agar (bacteroides); Reinforced Clostridial Agar (clostridia); *Clostridium difficile* selective agar (Oxoid); Phenylethyl Alcohol agar (PEA, Oxoid, gram-positive anaerobic cocci); Mitis Salivarius agar (aerobic and anaerobic cocci); Tomato Juice Agar and Rogosa SL Agar (bifidobacteria, aerobic and anaerobic lactobacilli); Bile Esculine Agar and Azide Agar (enterococci); Mannitol Salt Agar (staphylococci); McConkey Agar and SS Agar (Enterobacteriaceae); Saline Medium Agar (*E. coli* and coliforms; composition: NaCl g 5·0; NH_4Cl g 1·0; KH_2PO_4 g 3·0; $Na_2HPO_4 . 2H_2O$ g 7·52; Mg $SO_4 . 7H_2O$ g 0·205; sodium citrate g 2·0; agar g 18·0; H_2O to 1000 ml; pH = 7·2. Tetrazolium blue (2 per cent) and glucose (40 per cent) were added (1 ml/100 ml medium); Littman Oxgall Agar (yeasts).

The stool specimens of each group were analysed in the same experiment in order to minimise variations attributable to the manual procedure.

RESULTS

In healthy women of different ages the bacterial flora composition showed only slight quantitative changes; however, appreciable changes in certain components of the microflora were observed among the different groups.

Although there were occasional differences on a week-to-week basis, the composition of the faecal flora of menopausal women appeared fairly stable. Considerable variations were observed among subjects of the same age.

The bacterial flora composition of each subject is shown in Figure 1 (aerobic flora) and Figure 2 (anaerobic flora).

To simplify the presentation of the data, only the genus is reported for some organisms (although species identification was performed for most isolates).

Aerobic flora

Gram-negative facultative aerobic rods, primarily *E. coli*, were found in the majority of individuals at levels of over 3×10^5–10^6 per gram of stool. No significant differences (1 log) were noted between the pre- and the post-menopausal period as regards the number of *E. coli*. In the premenopausal group, *Enterobacter cloacae*, *Klebsiella oxytoca* and *Citrobacter freundii* were isolated in 4/18 subjects on the 8th day and in 2/18 in the premenstrual period. These *Enterobacteriaceae* species showed an increase in frequency in the postmenopausal period. *K. oxytoca* and *E. cloacae* were identified in 3/5 women in group II; in 17/21 women in group III, whose menopause had taken place more than 5 years previously, the following microorganisms (potential pathogens) were present: *E. aerogenes*, *E. cloacae*; *Serratia odorifera*, *Acinetobacter calcoaceticus* (two subjects); *C. freundii 1* (three subjects); *Morganella morganii* (two subjects); *K. pneumoniae* (five subjects).

Gram-positive aerobes or fermentative bacteria, namely lactobacilli and cocci, showed slight changes in mean concentrations.

Enterococci were found in all subjects. *Enterococcus faecium* (subspecies 1, 3 and durans) was the prevalent species, being present in 60 per cent of women studied; the presence rates were similar in fertile and menopausal women. *E. faecalis* **2** was present only in a minority (7/44 subjects).

Mean levels of lactobacilli increased slightly in postmenopausal women ($10^{7·3}$, group III). The lactobacilli/enterococci ratio was 1·1 in fertile women, but showed a tendency to decrease (0·84) in menopausal women (group II).

A relatively low count (10^3) of the *Staphylococcus* genus was present in 87·7 per cent of women and consisted of *S. xylosus* (80 per cent), *S. epidermidis*, *S. saprophyticus* and *S. hominis* species. *S. aureus* was found in three subjects.

Low numbers of fungi (range 10^3–10^4) were detected in the majority of specimens from premenopausal women (60 per cent). Fungi and yeasts showed a significant increase (3 log, $P < 0·01$, *t*-test) in the postmenopausal group and were isolated in all subjects (group III). *Candida pseudotropicalis* was detected in 10 per cent of subjects.

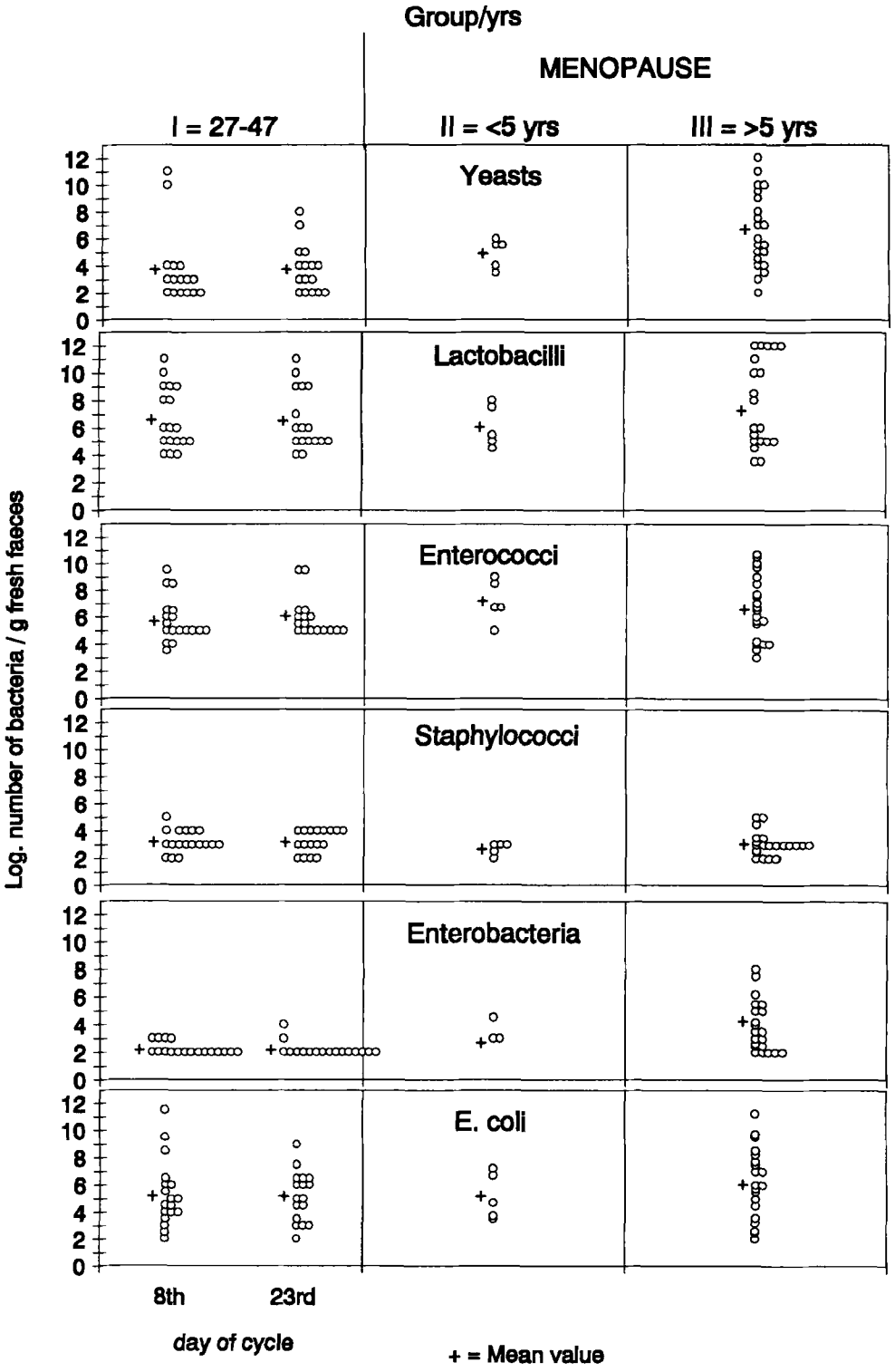

Figure 1. Aerobic faecal microflora in healthy fertile (group I) and postmenopausal women (groups II and III). Samples from fertile women were collected on the 8th and 23rd day (premenstrual period) of the cycle; two samples were obtained from each menopausal woman at a 15 d interval. All concentrations are expressed as $\log_{10} n$ bacteria/g fresh faeces. Crosses represent mean values.

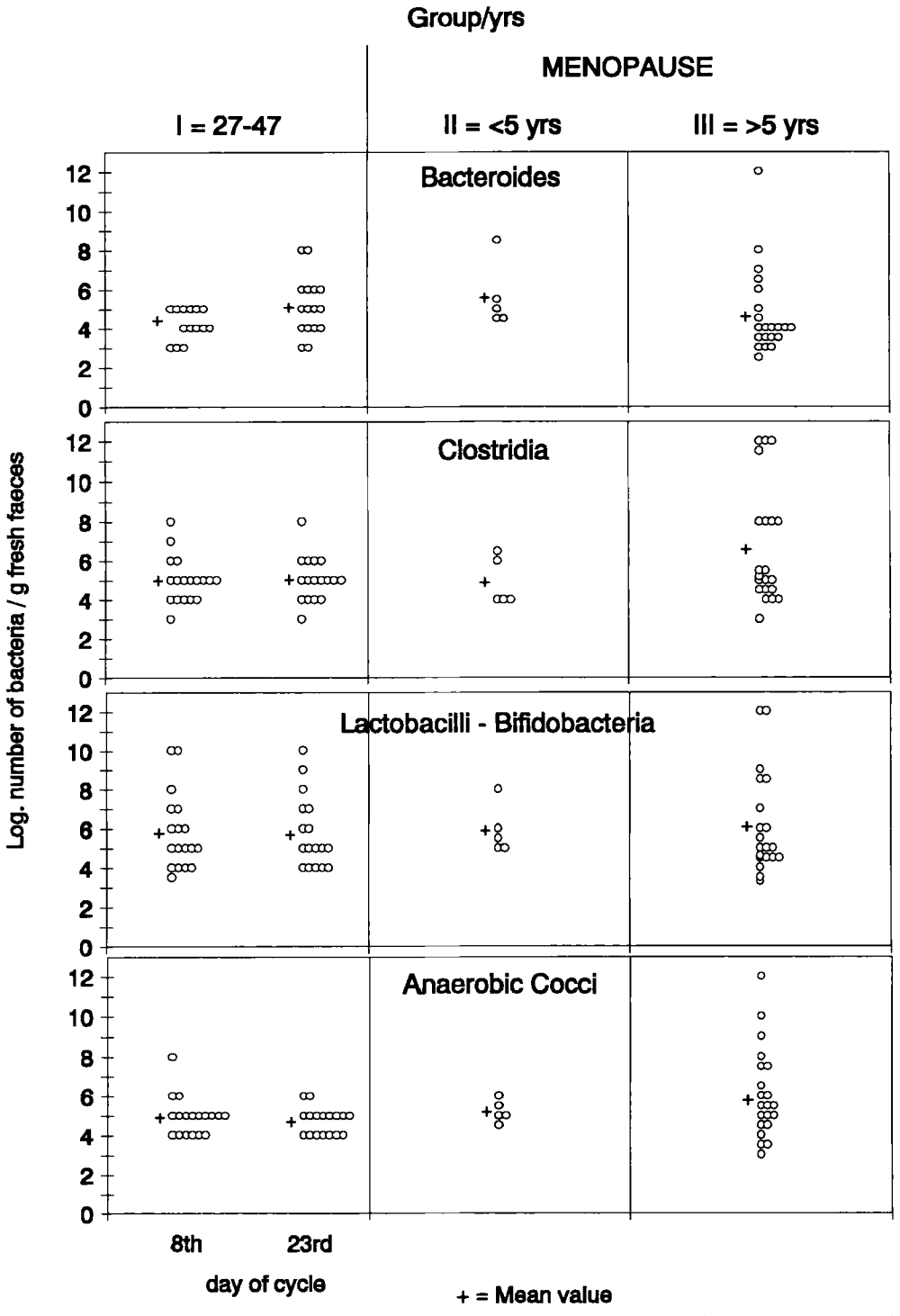

Figure 2. Anaerobic faecal microflora in healthy fertile (group I) and postmenopausal women (groups II and III). Samples from fertile women were collected on the 8th day and 23rd day (premenstrual period) of the cycle; two samples were obtained from each menopausal woman at a 15 d interval. All concentrations are expressed as $\log_{10} n$ bacteria/g fresh faeces. Crosses represent mean values.

Table 1. Composition of anaerobic faecal flora in healthy women of different ages. Premenopausal women: group I; postmenopausal women: group II <5 yr of menopause and group III >5 yr of menopause. The prevalent species identified in the different groups of subjects are summarised.

Group	Gram-positive cocci	Clostridia	Gram-positive non-spore-forming rods	Gram-negative anaerobic rods
I	*S. morbillorum* *Ps. magnus* *Ps. asaccharolyticus* *S. intermedius*	*C. limosum* *C. symbiosum* *C. tetani* *C. subterminale* *C. innocuum*	*E. lentum* *E. limosum* *L. acidophilus* *P. acnes*	*P. gingivalis* *P. asaccharolytica* *F. nucleatum*
II	*S. morbillorum* *Ps. magnus*	*C. symbiosum* *C. subterminale* *C. tetani*	*E. lentum* *E. limosum* *P. acnes* *L. acidophilus*	*P. gingivalis* *B. intermedius* *F. nucleatum*
III	*S. intermedius* *S. morbillorum* *Ps. magnus* *P. saccharolyticus* *Ps. micros* *Ps. prevotii*	*C. symbiosum* *C. tetani* *C. perfringens* *S. sporogenes* *C. beijerinkii* *C. clostridiforme* *C. botulinum* *C. tertium* *C. butyricum*	*E. limosum* *E. lentum* *P. acnes* *L. acidophilus* *Bifidobacterium* spp. *A. israelii*	*P. gingivalis* *P. asaccharolytica* *B. intermedius* *B. fragilis* *F. mortiferum*

Anaerobic flora

Anaerobic faecal flora showed only minor quantitative changes (Figure 2), but presented a number of interesting modifications in qualitative composition, possibly related to the age of the women. In the menopausal period we observed a significant increase ($P<0{\cdot}05$, t-test) in mean concentrations of clostridia ($10^{6{\cdot}6\pm0{\cdot}5}$, mean $\pm$ SEM) in comparison to fertile age ($10^{5{\cdot}0\pm0{\cdot}2}$). Anaerobic cocci also increased. No differences were observed in the anaerobic lactobacilli levels between the pre- and post-menopausal periods.

The most significant variations in the bacterial species among the different groups studied are summarised in Table 1.

Clostridia (C) were found in all subjects studied. The species most frequently encountered in the faeces were *C. limosum*, *C. symbiosum*, *C. tetani* and *C. subterminale*. *C. limosum* was not found in postmenopausal women (groups II and III). *C. difficile* was isolated in only one subject. In the late menopausal period the number of species isolated increased, compared to the fertile group (Table 1).

Among the gram-positive non-spore-forming anaerobic rods, *Eubacterium lentum*, *E. limosum*, *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Propionibacterium acnes* were the most prevalent.

Seven different genera of anaerobic cocci were isolated (Table 1). *Streptococcus morbillorum* and *S. intermedius* were present in all groups; *S. morbillorum* was frequently isolated in group I (80 per cent of fertile women), while *S. intermedius* was isolated in the late menopausal period (39 per cent in group III). *Peptostreptococcus magnus* was also frequently isolated in fertile subjects (45 per cent).

In group III, *Ps. micros* appeared, while the frequency of *Ps. magnus* (one subject) and *Peptococcus saccharolyticus* (two subjects) decreased. *Veillonella* spp. were present in 12 per cent of group I women.

Among the gram-negative anaerobic bacilli *Porphyromonas gingivalis* was isolated in all the subjects studied and *P. asaccharolytica* was frequent. Fusobacteria were isolated in all groups: *Fusobacterium nucleatum* was found in 4/23 women (groups I–II).

The composition of the faecal flora of women in the early phase of menopause (group II) showed a trend similar to that of group III, while the microorganism mean level ranged between the mean values of groups I and III.

For the purpose of comparison, we also analysed the flora composition of three elderly men (mean age 73 ± 9·5 yr) and found high mean levels of *E. coli* (10^{12}), enterococci (10^{11}), aerobic lactobacilli (10^{11}), fungi (10^{10}), bacteroides (10^{7}), clostridia (10^{7}), anaerobic cocci (10^{5}) and anaerobic lactobacilli (10^{10}).

No significant difference in faecal pH was found in the three groups, the values being 6·8 ± 0·5, mean ± SD, in group I, 6·9 ± 0·6 in group II and 6·85 ± 0·5 in group III. In elderly men the mean pH was 7·25.

DISCUSSION

The results presented offer a general picture of faecal flora composition in healthy women on a Mediterranean diet. These data show that the microbial flora composition in the faeces of healthy women was remarkably similar in the three groups studied. However, we observed a number of changes at species level between fertile and menopausal women. Each fertile subject was studied in the two different phases of the cycle. The minimal changes observed (qualitative and quantitative) may also be related to modifications in the endocrine balance. Moreover, we divided the postmenopausal women into two subgroups on the basis of their different hormonal condition. In women < 5 yr after menopause (group II), the adrenal cortex production of androgens and the metabolic conversion of androstenedione to oestrone by adipose tissues may compensate for the reduction in ovarian activity; it would also appear that the ovary conserves some androgenic activity. In women whose menopause dates back more than 5 yr ovarian activity is suppressed.[6,33] The early phase of the menopause is a transitional condition of a woman's life involving a whole series of hormonal, psychological and functional phenomena, possibly including changes in the intestinal ecosystem.

The differences between the premenopausal and postmenopausal period were evident, though not statistically significant, owing to the high inter-subject variability. In the postmenopausal flora increases are observed in clostridia, fungi, enterobacteria and potential pathogens, such as *Acinetobacter calcoaceticus*, *E. aerogenes*, *C. freundii*, etc. Though the mean levels of gram-positive microorganisms, such as enterococci and lactobacilli, were little affected in groups I and III, and the ratio of lactobacilli to enterococci were above 1 for both aerobes and anaerobes, the numbers of species isolated increased. This microflora composition can be ascribed to the hormonal profile, but also to other 'physiological' modifications which are characteristic of elderly subjects, i.e. the decrease in specific and aspecific immunological defences,[25,34] metabolising capacity[27] and intestinal motility.[34] Since the results observed in elderly men were significantly different from those of women of the same age, despite few samples we consider that 'age factors' may have the same influence on the microflora composition of men and women, and that hormonal status may be the source of variability. Our data are in accord with the study of Gorbach and colleagues,[19] in which elderly people (men and women) were found to harbour fewer anaerobic lactobacilli and more coliform organisms and fungi than younger individuals. Therefore, some of the observed changes, i.e. the reduction in the normal bacterial flora of certain species such as anaerobic streptococcus or the imbalance among species, could conceivably lead to a prevalence of gram-negative aerobic bacilli or could be regarded as reduced colonisation resistance, i.e. resistance to colonisation by exogenous microorganisms such as *C. difficile*.[1,35] In healthy women the anaerobic flora showed only minimal changes. The anaerobic microflora in humans, however, would not appear to be the main factor in colonisation resistance.[2]

Changes in bacterial flora composition may induce different metabolic activities of intestinal microorganisms. The enzymes of the microflora are capable of aromatising, dehydroxylating, deconjugating, reducing keto- to hydroxy-groups, and epimerising steroids in the gut.[7,18,23] Many of these enzymes are synthesised by *E. lentum* and phenotypically related organisms.[36] Different reductive reactions are mediated by *Clostridium* spp., *Bacteroides* spp. and *E. lentum*.[14,36] The most common transformation is hydrolysis of conjugated steroids, and oestrogen deconjugation is mediated by the bacterial enzymes, β-glucuronidase and sulphatase.[18] Glucuronidase is synthesised by *E. coli* and *Bacteroides* spp.[36] Oestriol-glucuronide is converted to free oestriol when incubated with human faeces. Mixed human faecal flora is able to interconvert oestrone to oestradiol *in vitro*,[22] but an appreciable reduction to 17-β oestradiol occurs when oestrone is incubated with human faeces. Moreover, reductive reactions have been observed at a high faecal concentration, while the oxidative reactions occur at a low concentration.[24] This activity may lead to an alteration in the quantity but also in the quality of the steroid hormones, which are

metabolised and reabsorbed via the enterohepatic circulation.

Since the intestinal bacterial population is the result of different ecological factors, the flora composition may also depend on the different biochemical substrates present in the gut, i.e. the presence or absence of androgens, oestrogens, progestins and their metabolites. The absence of a particular substrate may be an additional reason for the modifications observed in the intestinal microflora ecosystem of postmenopausal women, the bacterial population also being the result of environmental pressures. While it is well known that the intestinal microflora plays a definite role in the metabolism of certain steroid sex hormones,[7,32,36] the converse has yet to be clarified, i.e. the influence of different chemically related compounds and/or steroid sex hormones on microbial flora composition. A number of reports show that antimicrobial drugs[12,29] and disease[32] can affect the human colonic flora.

In a previous study of ours,[3] we observed substantial changes in the bacterial flora composition of premenopausal women with severe premenstrual syndrome (PMS), aged 23–26 years. In the same subjects a prevalence of gram-positive bacteria was observed on the 11th day of the cycle, while gram-negative bacteria were prevalent on the 23rd day of the cycle. The fluctuations observed in fertile women with severe PMS may be related to hormonal changes as well as to psychosomatic features.

The composition of the intestinal microflora in women with uterine leiomyoma or breast cancer, which are oestrogen-dependent pathologies, shows a significant increase (3–4 log) in mean counts of Enterobacteriaceae, bacteroides, clostridia and anaerobic lactobacilli, as compared to healthy women of the same age.[4]

In conclusion we have observed an increase in gram-negative enterobacteria, yeasts and clostridia in postmenopausal women. It seems that age and the related modifications of the steroid sex hormone pattern during life may induce changes in the faecal microflora of women. Therefore, we suggested that age, or menopausal condition and sex should be well defined in studies on human intestinal flora in order to further minimise sources of variability.

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