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ORIGINAL CONTRIBUTION

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Variation of microbiological and biochemical parameters in the faeces of two healthy people over a 15 day period

Summary *Background* Recent years have seen increasing interest in the complex microbial ecosystem of the human gastrointestinal tract. Knowledge of its microbial colonists and their beneficial/detrimental activities is important. However, generalized assumptions about the microbial composition of the human gut should be taken with caution until more studies in different human communities have been conducted. The capacity of modulating or inhibiting harmful populations through high doses of beneficial microorganisms (probiotics) is now an attractive possibility. *Aims* The aim of this study was to determine the daily variation in the most prominent and representative of the cultivable microbial populations in the faeces of two healthy Spanish persons, with special reference to the lactic acid bacteria (lactobacilli and bifidobacteria). Faecal enzymatic activities caused or modulated by gut microorganisms were also examined. *Methods* Microbial populations were enumerated in selective and differential media. LAB species isolated from MRS agar plates were further identified by phenotypic and genetic techniques. Enzymatic activities were measured by the semi quantitative method of the API ZYM system. *Results* Obligate anaerobes (members of the *Clostridium* clusters and species of bacteroides and bifidobacteria) made up the largest bacterial populations in both individuals (ranging between 10^{10}–10^{11} cfu/g of faeces) and remained constant over time. Lactobacilli species were found at an intermediate level (around 10^8 cfu/g), and yeasts and moulds, staphylococci, enterococci, coliforms and *Enterobacteriaceae* at a lower level (between 10^3 to 10^6 cfu/g). 38 lactic acid bacteria strains identified belonged to *Bifidobacterium bifidum* [19], *Bifidobacterium longum* [7], *Bifidobacterium adolescentis* [7] and *Lactobacillus ruminis* [5] species. Enzymatic profiles and values were shown to be personal and stable, but inter-sample fluctuations were recorded. *Conclusions* The dominant microbial populations in the faeces of the two persons were similar and stable during the sampling period. Large differences were found in the lactic acid bacterial composition of each individual. Biochemistry seemed to be also personal and stable over time.

Key words gastrointestinal microbiology – biochemistry of faeces – probiotics – lactic acid bacteria

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Introduction

The human gastrointestinal tract (GIT) is colonised soon after birth by a complex and diverse collection of microbial species. Among the 400 described, 30 to 40 represent more than 99 % of these microorganisms, forming what has been called the "normal microbiota" [1]. The normal microbiota can vary between communities or individuals due to either host-specific or diet-related differences [2–4]. The constituents of this microbiota influence several biochemical, physiological and immunological

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characteristics of their host [5–9]. In recent decades, classical and molecular studies have revealed that each individual harbors a personal microbial community, which is rather stable over time [10–16]. But as implications of this conclusion are so important some authors think that the GIT microbiota of more human communities should be examined before general assessments are made [17, 18].

Lactic acid bacteria (LAB) species, including bifidobacteria, are one of the outstanding microbial groups, as they are thought to positively affect the host health through metabolic, trophic, and protective functions [9, 17, 19]. In fact, selected LAB are employed as dietary supplements in large numbers, or included in processed food products, for maintaining or recovering the healthy state [6, 7, 20, 21]. Probiotic bacteria must be alive when entering the GIT and must survive its barriers to be able to perform most of their functions. Microorganisms from probiotics are able to colonize the gut of consumers only transiently [22–24], but the possibility still exists for a permanent modification of the "autochthonous" microbiota through the repeated intake of "exogenous" bacteria in the great variety of probiotic products already on the market [3, 17, 21]. Thus, this microbiota examination has to be done urgently.

This paper reports the main GIT microbial populations and some biochemical parameters of the faeces of two healthy persons. The daily changes in these variables were monitored over a 15 day period. Representatives of the most numerous LAB components were then isolated and classified. This culture-based approach will allow us to characterize the isolates for those criteria they have to meet to be used as probiotics [25, 26].

Methods

Sampling and processing of samples

The selection of donors and sampling was performed as recommended by the Regional Ethic Committee of the Principado de Asturias (*Asturian Principality*), Spain. Faeces were collected in sterile containers and transported to the laboratory in anaerobic jars containing Anaerocult A as a reducing agent (Merck, Darmstadt, Germany). Eleven faecal samples were collected from individual A and seven from B over a two-week period. All samples were processed in less than two hours following their deposition in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK) containing an anoxic atmosphere (10 % H_2, 10 % CO_2, 80 % N_2). Serial dilutions were prepared in a reducing medium containing BHI broth (Merck), 0.5 % yeast extract (Merck), 0.02 % cysteine (Merck), 10 µg/L vitamin K1 (Merck) and 0.02 g/L haemin (Sigma; Sigma Chemical Co., St. Louis, MO), and plated on Petri dishes containing agar with the same medium.

Microbial enumeration

Direct microbial counts

Total cell counts were determined by the direct examination of dilutions using an Olympus phase-contrast microscope (Olympus Optical Co., Hamburg, Germany) and a Pretroff-Hausser counting chamber.

Total bacterial counts

Total bacterial counts were determined on Columbia blood agar (CBA) (Merck) and on brain heart infusion (BHI) agar with 0.5 % yeast extract, 0.02 % cysteine, vitamin K1 and haemin, after anaerobic incubation at 37 °C for 48 h.

Clostridia

Clostridia were counted on reinforced clostridium agar (RCA) (Merck) with 20 µg/ml of polymixin B (Sigma) after incubation in anaerobic conditions at 37 °C for 48 h.

Bacteroides

The bacteroides group was enumerated on esculine bile agar (EBA) (Merck) with 100 µg/ml kanamycin (Sigma) and 7.5 µg/ml vancomycin (Sigma). Incubations were performed anaerobically at 37 °C for 48 h.

Lactobacilli and bifidobacteria

Several media described by Payne et al. [27] were tested for counting populations of bifidobacteria and lactobacilli. Finally, counts were made on Man, Rogosa and Sharpe (MRS) agar (Merck) following anaerobic incubation at 37 °C for 72 h.

Enterobacteriaceae and coliforms

Violet red bile glucose (VRBG) and violet red bile lactose (VRBL) agar (Merck) were used to enumerate *Enterobacteriaceae* and coliforms respectively, following aerobic incubation for 24–48 h at 32 °C.

Enterococci

Enterococci were scored after 24–48 h of aerobic incubation at 44 °C in Slanetz and Bartley (S-B) agar (Merck), a medium containing 10 g L^{-1} triphenyltetrazolium chloride (TTC).

Staphylococci

Dilutions were plated on Baird-Parker (B-P) agar (Merck) and aerobically incubated for 24 h at 37 °C.

Black colonies with or without egg yolk clearing were recorded.

Yeasts and moulds

Dilutions of the samples were plated on chloramphenicol glucose agar (CGA) (Merck) and incubated aerobically for 3–5 d at 25 °C.

Classification and characterization of isolates

Carbohydrate fermentation profiles

Carbohydrate fermentation profiles were determined using a commercial kit (PhenePlate™ system, PhP, Stockholm, Sweden).

Sequencing of partially amplified rDNA

PCR primers Y1 (5'-TGG CTC AGG ACG AAC GCT GGC GGC-3') (position 20–43 on 16S rDNA, *Escherichia coli* numbering) and Y2 (5'-CCT ACT GCT GCC TCC CGT AGG AGT-3') (positions 361–338) [28], based on prokaryotic conserved regions of the 16S rRNA gene, were used to amplify a 348-bp stretch of DNA from all microorganisms examined. Cell extracts and PCR conditions were essentially as reported by Ward et al. [29]. Amplicons were purified using Microcon PCR filters (Millipore, Bedford, MA) to remove unincorporated primers and nucleotides, and sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were then compared to others in public databases using the BLAST program [30].

Biochemical analyses

Enzymatic activity in faeces

Enzyme activities were assessed by a semi quantitative method (API ZYM, bioMérieux, Montalieu-Vercieu, France), following the manufacturer's recommendations. Faecal samples were suspended 1/500 in distilled water and 65 µl of the suspension were used to measure the enzymatic activities following the procedures recommended by the supplier.

Results and discussion

Microbial analyses

Total cultivable bacterial counts in CBA and BHI were comparable. This suggests that both media are equally suitable for enumeration of cultivable microorganisms. Counts of the different microbial groups showed the most numerous populations to be species of clostridia, bifidobacteria and bacteroides (Figs. 1 and 2). The dominant populations were rather stable over the study time and only point fluctuations were seen. However, direct counting by microscope observation consistently gave one logarithmic unit more than cultures. Estimations of cultivability of bacteria in the gastrointestinal tract ecosystem vary from 10 to 50 % [12, 13, 15, 16, 31], as a consequence of unknown growth requirements, the selectivity of the media and/or the physiological state of the cells. The bacteroides population we found (around $5x10^8$ cfu/g of faeces) was somewhat smaller than that reported by other authors [2, 11]. The EBA medium with both kanamycin and vancomycin proved to be very selective (morphologically all isolates examined were *Bacteroides*-like bacteria; data not shown), but this selectivity surely affected the percentage of recoveries. In fact, several other isolates from the highest dilutions in BHI were alike to the *Bacteroides* from EBA. Bifidobacterial counts were quite stable at a level near 10^{10} cfu/g. The fall of more than two logarithmic units of counts in sample 6 of subject B is noteworthy (Fig. 2). Gram-positive cocci (peptococci or peptostreptocci) were always found at a similar level. Counting of the lactobacilli was usually hampered by the large numbers of bifidobacteria (and the Gram-positive cocci) which were not inhibited in the *Lactobacillus*-selective media used (even when the pH was adjusted to 5.4) (Figs. 1 and 2).

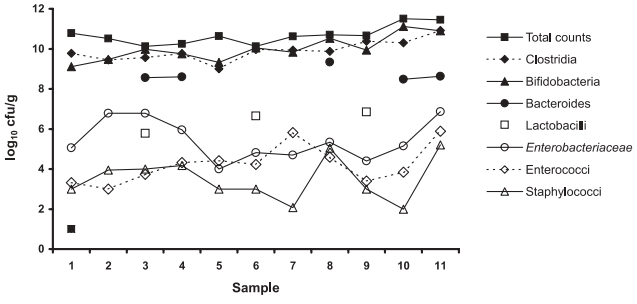

Fig. 1 Evolution of different microbial populations in faeces from individual A

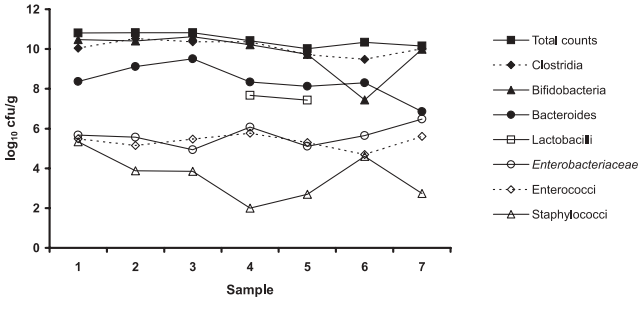

Fig. 2 Evolution of different microbial populations in faeces from individual B

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Larger numerical differences were seen in counts of aerobic and facultative anaerobic microorganisms (staphylococci, enterococci, *Enterobacteriaceae* and coliforms, and yeasts and moulds). These populations were much less numerous in the faeces than the strict anaerobes, as is well known [1–3, 11]. However, these and other populations could be dominant in other parts of the GIT. For reasons of clarity, changes in the yeast and mould populations (usually at the limit of detection, around 10^2–10^3 cfu/g) have not been depicted in Figs. 1 and 2. *Enterobacteriaceae* counts usually matched coliforms counts, therefore only *Enterobacteriaceae* are shown in the figures). As an exception, in samples 6–8 of subject A, coliforms were displaced by a lactose-negative *Enterobacteriaceae* (finally identified as a *Salmonella* spp. strain). This perturbation coincided in time with subject A suffering diarrhoea accompanied by general discomfort and fever which lasted at least three days.

Identity of lactic acid bacterial isolates

A total of 38 representative colonies of the different morphologies seen in the higher dilutions on MRS agar were selected at random from two faecal samples of each individual (10 colonies from sample 1 and 17 from sample 11 of individual A; and 6 colonies from sample 6 and 5 from sample 9 of individual B) and purified in the same medium. The isolates were grouped by their carbohydrate fermentation profile using the PhenePlate™ system. Cell extracts of several strains of the phenotypic groups were used as templates for amplifying a segment of their 16S rRNA gene, which was then purified and sequenced. Sequences were finally compared to others in public databases. In individual A, all 10 isolates of the first sample were shown to be strains of *Bifidobacterium bifidum*. In the second sample, a mixture of *B. bifidum* [8], *Bifidobacterium adolescentis* [7] and *Bifidobacterium longum* [2] strains was encountered. Despite this, the total number of bifidobacteria remained constant over this period (Fig. 1). This may suggest that although the total number of a given population remains constant, the species, subspecies or strains that form this population could be continuously changing. *Lactobacillus* spp. strains did not appear among the most numerous lactic acid bacteria in this person. Although lactobacilli are common inhabitants of the human large intestine, in some cases, they can not be detected in human faecal samples [32]. In individual B, the strains analyzed from sample 6 belonged to *B. bifidum* [1], *B. longum* [1] and *Lactobacillus ruminis* [4], whereas those from sample 9 belonged to *B. longum* [4] and *L. ruminis* [1].

B. bifidum, B. longum and *B. adolescentis* have been reported the most prevalent species in humans from different geographic regions [10, 11, 19, 33]. *L. ruminis* has frequently been isolated in the past from bovine rumen [34], but it has recently been shown by culture independent methods as a regular component of the microbiota of human faeces [35].

Biochemical analyses

Tables 1 and 2 summarize the enzymatic activities of the faeces. Small intersample fluctuations were recorded in both cases, but it would appear that personal profiles can be envisioned. In this way, esterase and esterase-lipase activities were usually higher in individual A. In contrast, alkaline phosphatase, α- and β-galactosidase and β-glucosidase activities were higher in samples from individual B. Similar interpersonal results have been described by Mykkänen et al. [36], who found that daily variations in enzyme activities within individuals were not significant, whilst those between individuals were. The microbial displacement of coliforms by a *Salmonella* spp. mentioned in the preceding paragraph in individual A coincided in time with a clear change in his enzymatic profile (Table 1). A clear enhancement in β-glucosidase activity was seen plus a notable reduction in α-glucosidase activity, as well as other small differences (Table 1, samples 6 and 7). In individual B, an increase of some glycosidic enzyme activities occurred in the last sample, but with no apparent change in the microbial profile. Glycosidases are one of the most important bacterial enzymes in the colon. They hydrolyze poorly absorbed glycosides of plant origin releasing aglycones that could result in toxic, carcinogenic or mutagenic compounds [7].

Conclusion

The gastrointestinal microbiota is composed of many kinds of different microorganisms, which are present at different numbers. Components of this microbiota are probably stabilized, reaching a personal equilibrium through interactions between them and with their host. However, the subtle changes appreciated by the culture methods are probably much more dramatic than ever thought at the species, subspecies and strain level. Furthermore, changes in minority populations of faeces (without disturbing the majority populations) could have a major impact on host health. No clear relationship was found between microbial populations and their biochemical effects. The short-term evolution of microbial and biochemical parameters of this study could be of help to understand variations in ample periods.

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Table 1 Enzymatic activities measured with the API ZYM system in the eleven faecal samples from individual A

ENZYME	Control	Alcaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Lipase (C14)	Leucine arylamidase	Valine arylamidase	Cysteine arilamidase	Trypsin	α-chymotrypsin	Acid phosphatase	Naphtol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	N-acetyl-β-glucosaminidase	α-mannosidase	α-fucosidase
Modal Activity*	0	20	20	10	0	15	0	0	0	0	15	20	5	30	20	30	5	30	0	5
SAMPLE																				
1																				2.5
2			10																	
3						10														
4																				
5		40																		
6							5				10		2.5							
7						5					10	15				15	20			
8											10				15	15	20			2.5
9		40									10							40		
10			10			5														2.5
11			10			10														2.5

* Activity was recorded as the approximate nanomoles of hydrolised substrate. Significant differences to modal activities are underlined

Table 2 Enzymatic activities measured with the API ZYM system in the seven faecal samples from individual B

ENZYME	Control	Alcaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Lipase (C14)	Leucine arylamidase	Valine arylamidase	Cysteine arilamidase	Trypsin	α-chymotrypsin	Acid phosphatase	Naphtol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	N-acetyl-β-glucosaminidase	α-mannosidase	α-fucosidase
Modal Activity*	0	40	7.5	5	0	10	0	0	0	0	10	10	20	40	30	15	25	30	0	5
SAMPLE																				
1			10				2.5								20					
2																		35		
3						7.5														
4												7.5				20	20	35		
5													15		25		20			
6			15	10		15					15	20	25			25				
7			20	10							15	20	40			40	40			

* Activity and indications as in Table 1

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