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# EU project Crownalife: functional foods, gut microflora and healthy ageing
# Isolation and identification of *Lactobacillus* and *Bifidobacterium* strains from faecal samples of elderly subjects for a possible probiotic use in functional foods

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**Abstract**

A new EU-funded project called "Crownalife" has been set up both to assess structural and functional alterations of the intestinal microflora with ageing in Europe, and to validate functional foods that promote health by improving the function of the intestinal microflora in the elderly. Species of bifidobacteria and lactobacilli were isolated from faecal samples of healthy elderly Italian subjects and identified for a possible probiotic use in functional foods. Total anaerobic and aerobic, *Lactobacillus* and *Bifidobacterium* counts were performed on the faecal samples. Colonies grown on selective media were identified using enzymatic tests. *Lactobacillus fermentum* and *Bifidobacterium longum* were the most represented species and could be considered good candidates to be further characterized and then utilized to design appropriate dietary strategies. Some of the identification results related to *Bifidobacterium* species were validated by using PCR procedure based on 16S rRNA gene sequences species-specific primers showing a good concordance rate with the enzymatic method.


*Keywords:* Elderly people; Intestinal microflora; Probiotic; *Bifidobacterium*; *Lactobacillus*

## 1. Introduction

Lactobacilli and bifidobacteria are normal components of the healthy human intestinal microflora. They are commonly used as probiotics added to food products mainly because of their supposed health-promoting activities (Hirayama & Rafter, 2000; Isolauri, 2001; Marteau, de Vrese, Cellier, & Schrezenmeir, 2001; Mattila-Sandholm et al., 1999). These dietary supplements could be used to restore and maintain the intestinal microflora of elderly people that seem to have a decreasing number of bifidobacteria (Mitsuoka & Hayakawa, 1972). The modified microflora composition could be associated with the high incidence and susceptibility to degenerative and infectious diseases of elderly people.

The EU-funded project "CROWNALIFE—Functional foods, gut microflora and healthy ageing" aims to provide information on the structural and functional alterations of the microflora with ageing in Europe and to improve the health status of the ageing population via specific nutritional recommendations that will necessitate the design and provision of functional food ingredients that positively affect the intestinal microbiota.

One task of the project was to isolate and identify species of lactobacilli and bifidobacteria from elderly faecal flora. This has been a preliminary phase enabling us to provide other partners with lactic acid producing bacteria (LAB) for testing their anti-microbial activities against gastro-intestinal pathogens to develop synbiotics to be used as functional food ingredients, and for assessing the phylogenetic specificities of the gastro-intestinal microflora in the elderly.

In the present study, LAB strains were isolated from faecal samples of elderly Italian subjects using selective

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media. Colonies were identified by enzymatic systems to assess the microbial distribution of LAB species in each subject. Some of the identification results have been validated using molecular methods.

## 2. Materials and methods

### 2.1. Subjects

Subjects of both sexes (six males and six females) were recruited from the "Sassatelli" boarding home in Fermo (Central Italy). The elderly people ranged in age from 65 to 87 with a median age of 75. No subject had been on antibiotic treatment for at least four weeks prior to faecal sampling. Each individual gave informed consent.

### 2.2. Sample collection

Fresh faecal specimens were obtained from 12 healthy elderly Italian people. Each sample was taken from a single subject in a sterile container and put in a plastic watertight bag under anaerobic conditions obtained by inserting an AnaeroGen sachet (Unipath Ltd., Basingstoke, Hampshire, England). The samples were stored at 4 °C until processed.

### 2.3. Bacterial counts

A sample of faeces of approximately 1 g (wet weight) was immediately placed in an anaerobic cabinet (Concept 400, Ruskinn Technology Limited, Leeds, West Yorkshire, UK), suspended in 9 ml reducing solution (Holdeman, Cato, & Moore, 1977) and homogenized with Stomacher Lab Blender Model 80-BA 7020 (Seward Medical London, UK). 1 ml of the faecal homogenate was suspended in 9 ml of reducing solution and a series of 10-fold dilution ($10^{-1}$–$10^{-10}$) was prepared. A given amount of each dilution (50 μl) was plated on to a non-selective media, Columbia blood agar (bioMérieux, Marcy-l'Etoile, France) supplemented with vitamin $k_3$ for both aerobic and anaerobic total bacterial counts, and on to four selective media, LAMVAB medium (Hartemink, 1999) and MRS agar (Oxoid) for *Lactobacillus* counts, Beerens' agar (Silvi, Rumney, & Rowland, 1996) and Raffinose Bifidobacterium (RB)-medium (Hartemink, 1999) for *Bifidobacterium* counts. The non-selective inoculated media were incubated aerobically and anaerobically at 37 °C for 72 h and the selective inoculated media were incubated anaerobically under the same conditions.

### 2.4. Identification of isolates by enzymatic method

From the selective media of each sample, isolated colonies with different morphologies were picked. Colonies grown in LAMVAB medium and MRS agar were examined microscopically and all non-spore forming straight rods were tested by API 50 CHL (bioMérieux). The colonies grown in Beerens' agar and in RB medium were examined microscopically and all bifid-shaped rods were characterized for identification by the enzymatic activity which was tested using Rapid ID 32 A system (bioMérieux) combined with API 50 CH (bioMérieux) modified by us. The API CH 50 strip was inoculated with a medium based on that formulated by Hartemink (1999) deprived of agar and sodium thioglycollate and with a concentration of bromocresolpurple of 0.17 g/l. The biochemical profiles obtained for the *Bifidobacterium* strains were identified using a database available on the web (http://kounou.lille.inra.fr/bifidAppl.html).

### 2.5. Identification of isolates by molecular method

PCR procedure based on 16S rRNA gene sequences species-specific primers was used to validate the combined enzymatic method. Nine *Bifidobacterium* strains

Table 1
*Bifidobacterium* species-specific primers based on 16S rRNA gene sequences

| Species | Primer | Sequence (5′ → 3′) | PCR product size (bp) |
|---|---|---|---|
| *Bifidobacterium adolescentis* | BiADO-1[a] | CTCCAGTTGGATGCATGTC | 279 |
| | BiADO-2[a] | CGAAGGCTTGCTCCCAGT | |
| | BIA-1[b] | GGAAAGATTCTATCGGTATGG | 244 |
| | BIA-2[b] | CTCCCAGTCAAAAGCGGTT | |
| *Bifidobacterium bifidum* | BiBIF-1[a] | CCACATGATCGCATGTGATTG | 278 |
| | BiBIF-2[a] | CCGAAGGCTTGCTCCCAAA | |
| *Bifidobacterium longum* | BiLON-1[a] | TTCCAGTTGATCGCATGGTC | 831 |
| | BiLON-2[a] | GGGAAGCCGTATCTCTACGA | |
| | BIL-1[b] | GTTCCCGACGGTCGTAGAG | 153 |
| | BIL-2[b] | GTGAGTTCCCGGCATAATCC | |

[a] Matsuki, Watanabe, Tanaka, Fukuda, and Oyaizu (1999).
[b] Wang, Cao, and Cerniglia (1996).

previously identified by enzymatic tests, were cultured anaerobically at 37 °C in Brain Heart Infusion broth (Oxoid). 1 ml of suspension of each bacterial culture was prelevated and centrifuged at 12,000 rpm (A-12 microcentrifugette rotor n. 6642, ALC-Milano) for 3 min. The pellets were suspended in 200 μl of sterile distilled water, heated at 100 °C for 5 min, immediately cooled in ice water and serially diluted in 1% Triton X-100 ($10^{-1}$–$10^{-2}$ ).

The *Bifidobacterium* species-specific primers used in this study are listed in Table 1. One μl of cell lysate was added to 19 μl of PCR mixture containing 10X buffer (Perkin Elmer, USA), 25 mM $MgCl_2$, 500 ng/μl of each primer (Life Technologies, Italia Srl San Giuliano Milanese, Italy), 2mM mix $dNTP_s$ (Life Technologies), 0.2 μl of Taq DNA polymerase (Perkin Elmer, USA). The reaction mixtures, after incubation at 94 °C for 1 min and 30 s, were cycled through the following temperature profile: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min. Finally, the reactions were incubated at 72 °C for 10 min and at 45 °C for 10 min. The PCR was conducted in a Tpersonal Thermal Cycler (Biometra, Göttingen, Germany). The PCR products (8 μl of each) were separated by electrophoresis in 2% agarose gels containing 0.5 μg/ml (w/v) of ethidium bromide (Life Technologies).

## 3. Results

### 3.1. Bacterial counts

Bacterial counts of faecal bacteria of elderly Italian subjects are shown in Figs. 1–3. The average counts of anaerobes and aerobes were determined as 11.4 and 9.8 log CFU/g of faeces (wet weight) respectively, and the anaerobes/aerobes ratio was 1.6.

The total count of *Lactobacillus* obtained on MRS agar was always exceeding that on LAMVAB medium. The respective mean values of 10.9 and 7.4 log CFU/g of faeces were significantly different according to the Student's *t*-test ($p < 0.001$).

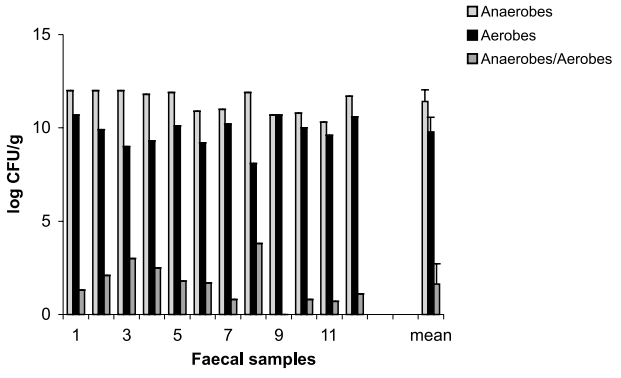


Fig. 1. Total bacterial counts (and ratio) of anaerobes and aerobes.

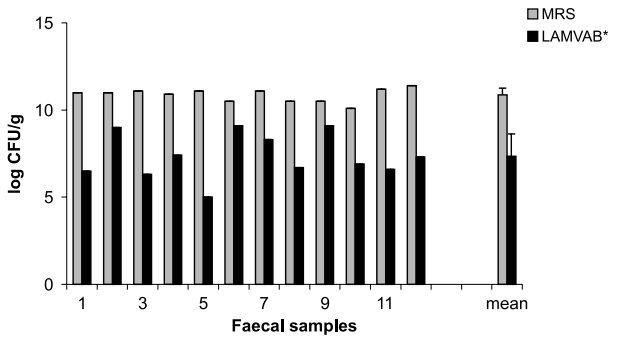


Fig. 2. Counts of *Lactobacillus* on MRS agar and LAMVAB medium. *—Mean value is significantly different from MRS agar, according to the Student's *t*-test ($p < 0.001$).

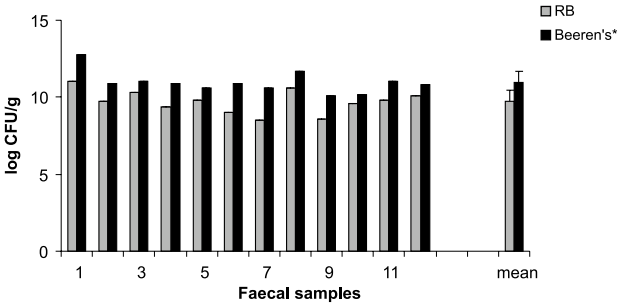


Fig. 3. Counts of *Bifidobacterium* on RB medium and Beerens' agar. *—Mean value is significantly different from RB medium, according to the Student's *t*-test ($p < 0.001$).

The mean value of total *Bifidobacterium* count observed on Beerens'agar was significantly higher ($p < 0.001$, Student's *t*-test) than on RB medium (10.9 log CFU/g and 9.7 log CFU/g respectively).

Table 2
Distribution of species of *Lactobacillus* and *Bifidobacterium* in faecal samples of healthy elderly Italian subjects

| Species | Percentage | Frequency[a] |
|---|---|---|
| *Lactobacillus* | | |
| *L. acidophilus* | 10.0 | 4/12 |
| *L. brevis* | 6.0 | 1/12 |
| *L. buchneri* | 2.0 | 1/12 |
| *L. cellobiosus* | 2.0 | 1/12 |
| *L. delbrueckii* ssp. *bulgaricus* | 2.0 | 1/12 |
| *L. fermentum* | 20.0 | 5/12 |
| *L. paracasei* ssp. *paracasei* | 12.0 | 4/12 |
| *L. plantarum* | 10.0 | 3/12 |
| *L. rhamnosus* | 6.0 | 2/12 |
| *L. salivarius* | 6.0 | 2/12 |
| *L. curvatus* | 4.0 | 1/12 |
| *Lactobacillus* spp. | 20.0 | 6/12 |
| *Bifidobacterium* | | |
| *B. adolescentis* | 9.8 | 6/12 |
| *B. bifidum* | 8.8 | 4/12 |
| *B. longum* | 16.7 | 7/12 |
| *Bifidobacterium* spp. | 64.7 | 2/12 |

[a] Frequency of occurrence is expressed as the number of subjects with the species detected/number of subjects examined.

### 3.2. Bacterial identification by enzymatic method

Eleven species of *Lactobacillus* were detected in the elderly Italian faecal samples as shown in Table 2. *Lactobacillus fermentum* was the most represented (20%), *L. paracasei* spp. *paracasei*, *L. acidophilus* and *L. plantarum* were present with a percentage of 12, 10 and 10 respectively. All the others *Lactobacillus* species were present with a percentage <10%. A good identification of genus has been obtained in 20% of the identified strains, since the enzymatic strip was not able to characterise the species (Table 2). Three species of *Bifidobacterium* were detected in the 12 faecal samples: *Bifidobacterium longum* (16.7%), *B. adolescentis* (9.8%), *B. bifidum* (8.8%). The remaining percentage (64.7%) was labelled as *Bifidobacterium* spp. (Table 2).

Table 3 shows the distribution of *Lactobacillus* and *Bifidobacterium* species for each faecal sample of healthy elderly Italian subjects. Samples 9 and 11 point out a wide microbial diversity regarding the *Lactobacillus* species. A similar microbial composition has been noticed in sample 4 and 10, 3–5 and 7, 9 and 11 as regard *Bifidobacterium* species. It is interesting to observe that each healthy elderly subject presents a typical profile of bacteria species of both genera, even if more evident in *Lactobacillus* species.

Table 3
*Lactobacillus* and *Bifidobacterium* species identified from each faecal sample of healthy elderly Italian subjects

| Sample | *Lactobacillus* | % | *Bifidobacterium* | % |
|---|---|---|---|---|
| 1 | *L. curvatus* | 40 | *Bifidobacterium* spp. | 100 |
| | *L. paracasei* ssp. *paracasei* | 40 | | |
| | *L. plantarum* | 20 | | |
| 2 | *L. fermentum* | 33.3 | *B. longum* | 22.2 |
| | *Lactobacillus* spp. | 66.7 | *B. adolescentis.* | 11.1 |
| | | | *Bifidobacterium* spp. | 66.7 |
| 3 | *L. fermentum* | 20 | *B. longum* | 33.3 |
| | *L. paracasei* ssp. *paracasei* | 40 | *Bifidobacterium* spp. | 66.7 |
| | *L. plantarum* | 40 | | |
| 4 | *L. fermentum* | 25 | *B. adolescentis* | 7.7 |
| | *L. rhamnosus* | 25 | *B. bifidum* | 38.5 |
| | *L. salivarius* | 50 | *B. longum* | 38.5 |
| | | | *Bifidobacterium* spp. | 15.3 |
| 5 | *L. paracasei* ssp. *paracasei* | 66.7 | *B. longum* | 40 |
| | *Lactobacillus* spp. | 33.3 | *Bifidobacterium* spp. | 60 |
| 6 | *L. acidophilus* | 100 | *B. bifidum* | 12.5 |
| | | | *B. longum* | 25 |
| | | | *Bifidobacterium* spp. | 62.5 |
| 7 | *L. acidophilus* | 14.3 | *B. longum* | 12.5 |
| | *L. fermentum* | 57.1 | *Bifidobacterium* spp. | 87.5 |
| | *Lactobacillus* spp. | 28.6 | | |
| 8 | *L. acidophilus* | 33.3 | *Bifidobacterium* spp. | 100 |
| | *L. fermentum* | 66.7 | | |
| 9 | *L. brevis* | 33.3 | *B. adolescentis* | 18.2 |
| | *L. cellobiosus* | 16.7 | *Bifidobacterium* spp. | 81.8 |
| | *L. delbrueckii* ssp. *bulgaricus* | 16.7 | | |
| | *L. plantarum* | 33.3 | | |
| 10 | *Lactobacillus* spp. | 100 | *B. adolescentis* | 30 |
| | | | *B. bifidum* | 20 |
| | | | *B. longum* | 20 |
| | | | *Bifidobacterium* spp. | 30 |
| 11 | *L. buchneri* | 20 | *B. adolescentis* | 22.2 |
| | *L. paracasei* ssp. *paracasei* | 20 | *Bifidobacterium* spp. | 77.8 |
| | *L. rhamnosus* | 40 | | |
| | *Lactobacillus* spp. | 20 | | |
| 12 | *L. salivarius* | 33.3 | *B. adolescentis* | 10 |
| | *L. acidophilus* | 33.3 | *B. bifidum* | 10 |
| | *Lactobacillus* spp. | 33.4 | *Bifidobacterium* spp. | 80 |

### 3.3. Bacterial identification by molecular method

Eight species out of nine analysed were amplified using the respective species-specific primers confirming the results of the enzymatic method. Fig. 4 illustrates the PCR products in a 2% agarose gel for three strains of *Bifidobacterium bifidum* (406, 408, 414). BiBIF1/BiBIF2 primers were able to detect the target species specifically, providing PCR products with the expected size. These primers showed a negative PCR results only for one strain of *B. bifidum* (1020) (Table 4). Positive PCR results were obtained for all of the tested *B. adolescentis* strains using BIA1/BIA2 and BiADO1/BiADO2 primers

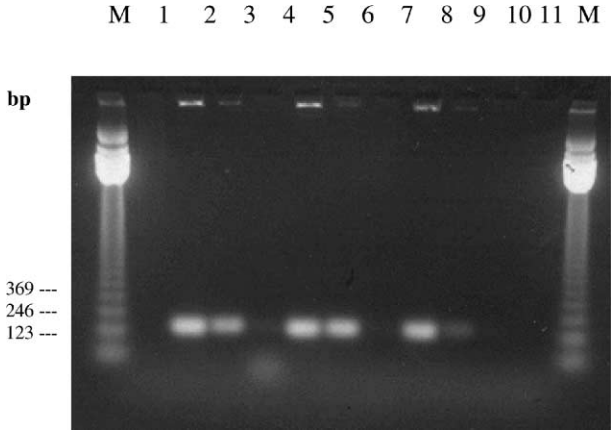


Fig. 4. PCR products in a 2% agarose gel for three species of *Bifidobacterium* with their specific primer BiBIF1/BiBIF2. Lanes: M, DNA size markers (sizes are shown at the left); 1, negative control; 2, *B. bifidum* (strain 406); 3, *B. bifidum* (strain 406) diluted 1:10; 4, *B. bifidum* (strain 406) diluted 1:100; 5, *B. bifidum* (strain 408); 6, *B. bifidum* (strain 408) diluted 1:10; 7, *B. bifidum* (strain 408) diluted 1:100; 8, *B. bifidum* (strain 414); 9, *B. bifidum* (strain 414) diluted 1:10; 10, *B. bifidum* (strain 414) diluted 1:100; 11, negative control.

Table 4
Identification of isolated strains of *Bifidobacterium* through the use of species-specific primers

| Strains no. | Species-specific primers | | | | | Identification by Rapid ID32A combined with API 50 CH |
|---|---|---|---|---|---|---|
| | BIA1/BIA2 | BiADO1/BiADO2 | BiBIF1/BiBIF2 | BIL1/BIL2 | BiLON1/BiLON2 | |
| 213 | + | + | − | − | − | *B. adolescentis* |
| 416 | + | + | − | − | − | *B. adolescentis* |
| 1017 | + | + | − | − | − | *B. adolescentis* |
| 406 | − | − | + | − | − | *B. bifidum* |
| 408 | − | − | + | − | − | *B. bifidum* |
| 414 | − | − | + | − | − | *B. bifidum* |
| 1020 | − | − | − | − | − | *B. bifidum* |
| 305 | − | − | − | + | + | *B. longum* |
| 616 | − | − | − | + | + | *B. longum* |

as well as for all of the tested *B. longum* strains using BIL1/BIL2 and BiLON1/BiLON2 primers (Table 4).

## 4. Discussion

In view of the steady increase in the proportion of Europeans over 60 years old the research community is challenged to contribute to improving the quality of life for this age group. An optimal nutrition for the specific needs of the elderly is an important factor in achieving this objective. In elderly individuals, faecal bifidobacterial counts are thought to show a marked decrease in comparison to those of younger people (Kleesen, Sykura, Zunft, & Blaut, 1997; Mitsuoka, 1990). This may be relevant in the susceptibility of these individuals to pathogenic infection. Confirmation of a decline in bifidobacteria and other LAB in the gut of ageing subjects opens up the possibility of reversing such trends by administration of probiotics (bifidobacteria or lactobacilli), prebiotics that selectively encourage the growth of LAB in the intestine, or a combination thereof called a synbiotic.

This study has contributed to identify *Lactobacillus* and *Bifidobacterium* strains in faeces of healthy elderly Italian people, with the aim of collecting typical strains of elderly microbiota. The mean value of bacterial counts appeared fairly variable as regard the lactobacilli, while it was more stable and on the average higher as regard the bifidobacteria. These results showed a good concordance with those reported by other authors (Hartemink, 1999; Rowland, 1998). The total anaerobes and aerobes bacterial counts reflected normal values and the anaerobes/aerobes ratio was quite low and so could be considered positive if it is seen in relation with the colon cancer risk (Hill et al., 1971).

The identification of *Lactobacillus* and *Bifidobacterium* in each faecal samples showed that there is a typical set of species for each person. However, *L. fermentum* (5 people out of 12) and *B. longum* (7 people out of 12) were the most represented species. These two species could be considered good candidates, upon further characterization and assessment of their anti-pathogen effects, to be utilized for the design of appropriate functional foods to fortify the intestinal microflora of the elderly.

The combined enzymatic method used to characterized the *Bifidobacterium* species was validated using the molecular method. The species-specific PCR technique which was applied to the identification of isolated strains of *Bifidobacterium* yielded similar results to the enzymatic method, with a concordance rate of 88.9%. These results demonstrate that even if the PCR technique is a convenient identification method since it is rapid, accurate and cost effective (Matsuki et al., 1999; Matsuki, Watanabe, Tanaka, & Oyaizu, 1998; Roy & Sirois, 2000), the enzymatic test is reliable in spite of its limits.

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