# Enumeration of bifidobacteria in animal intestinal samples

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**ABSTRACT**: TPY agar supplemented with mupirocin (100 mg/liter) and glacial acetic acid (1 ml/liter) was effective in the enumeration of bifidobacteria in animal (11 species) fecal or intestinal samples. On the other hand, less than 50% of isolates from pig feces were identified as *Bifidobacterium* sp. using this medium. Subsequently TPY agar modified by the addition of mupirocin (100 mg/liter), glacial acetic acid (1 ml/liter), and colistin (25 mg/liter) was developed for the enumeration of bifidobacteria in pig feces. The results suggest that the bifidobacteria selective media should be chosen in respect of the animal species origin of the samples tested.

**Keywords:** bifidobacteria; selective media; intestinal tract; feces

Bifidobacteria are a major component of the human and animal gastrointestinal tract (Sgorbati *et al.*, 1995). The genus *Bifidobacterium* can generally be characterized as Gram-positive, non-sporeforming, non-motile anaerobes that are catalase-negative and saccharolytic (Scardovi, 1986). The selective enumeration of bifidobacteria is of great interest because of the assumed health and nutritional benefits ascribed to these bacteria (Mitsuoka, 1992). Thus, the availability of easy and inexpensive methods for enumeration of *Bifidobacterium* sp. is very important in intestinal microbiology. A lot of media for detection of these bacteria have been described (Teraguchi *et al.*, 1978; Muñoa and Pares, 1988; Beerens, 1991; Hartemink *et al.*, 1996). But none of the proposed media seem to be completely selective. In addition, some of the selective media do not allow the growth of all the species of the genus *Bifidobacterium*. The applicability of the media was usually evaluated by Gram-staining of individual colonies from the selective agars (Hartemink *et al.*, 1996; Silvi *et al.*, 1996). Rarely, the colonies were identified as members of the genus *Bifidobacterium* by the detection of fructose-6-phosphate phosphoketolase (F6PPK) (Muñoa and Pares, 1988). This assay is more time consuming in comparison with Gram-staining but the positive F6PPK test is a direct and reliable characteristic distinguishing bifidobacteria from the related genera (Scardovi, 1986; Biavati *et al.*, 1992; Tannock, 1999).

The aim of the present work was to evaluate a recently described selective medium for hen cecal bifidobacteria (Rada and Petr, 2000) in human and wide range of animal intestinal or fecal samples. The evaluation was performed by Gram-staining, F6PPK-test, and PCR procedure. Subsequently, a new medium for the enumeration of bifidobacteria in pig fecal samples was developed.

## MATERIAL AND METHODS

The first medium under investigation was TPY agar (ADSA, Barcelona, Spain) modified by the addition of mupirocin (100 mg/liter) and glacial acetic acid (1 ml/liter). The medium was prepared as described previously (Rada and Petr, 2000). This medium (MTPY agar) was used for isolation of bifidobacteria from all kinds of tested samples. The second tested medium was TPY agar modified by the addition of mupirocin (100 mg/liter), glacial acetic acid (1 ml/liter), and colistin (25 mg/liter). Colistin (Sigma, Prague, Czech Republic) was filter sterilized and added after autoclaving the medium and cooling to 45°C. This medium (MTPYC agar) was used for isolation of bifidobacteria from pig fecal samples.

Fourteen strains of bifidobacteria listed in Table 1 were tested for the ability to grow on the MTPY agar.

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Table 1. List of bifidobacteria strains used in this study

| Strain | Origin |
|---|---|
| *Bifidobacterium animalis* | fermented milk product |
| *B. asteroides* | honeybee gut |
| *B. bifidum* | ATCC 29 521 |
| *B. bifidum* | CCM 3762 |
| *B. breve* | ATCC 15 700 |
| *B. infantis* | ATCC 17 930 |
| *B. longum* | ATCC 15 707 |
| *B. longum* 1 | infant feces |
| *B. longum* 2 | infant feces |
| *B. pseudolongum* | fermented milk product |
| *B. pseudolongum* | rabbit ceca |
| *Bifidobacterium* sp. | calf feces |
| *Bifidobacterium* sp. | hen ceca |
| *Bifidobacterium* sp. | hen crop |

ATCC = American Type Culture Collection, Rockville, USA
CCM = Czech Collection of Microorganisms, Brno, Czech Republic

All strains were identified to the genus level by Gram-staining, F6PPK test (Scardovi, 1986), and by PCR using genus specific primers (Kok *et al.*, 1996). Some strains were identified to the species level using API 50 CHL and API ID 32A kits (BioMérieux, France). Bifidobacterial cultures were grown for 24 h in TPY broth (ADSA, Barcelona, Spain) under anaerobic conditions. A loopful of culture broth was streaked on the plates with MTPY agar and incubated in $CO_2/H_2/N_2$ (10/10/80%) atmosphere in anaerobic jars equipped with palladium catalysts (Oxoid, Prague, Czech Republic).

Mouse, rat, guinea pig, rabbit, monkey, and hamster samples were obtained from Biotest Ltd. (Konárovice, Czech Republic). Laying hen, pig, and honeybee samples were obtained from an experimental station of the Czech University of Agriculture Prague (Prague, Czech Republic). Fecal or intestinal samples were taken as fresh as possible and aliquots were transferred into Wilkins-Chalgren broth (Oxoid, Prague, Czech Republic). Then the samples were serially diluted. Appropriate dilutions were inoculated into sterile Petri dishes which were immediately poured with selective media and placed into anaerobic jars and incubated at 37°C for

Table 2. Viable counts on TPY agar (ADSA, Barcelona, Spain) modified by the addition of mupirocin (100 mg/liter) and glacial acetic acid (1 ml/liter). Gram-staining[a] and F6PPK activity[b] of cultures isolated from bifidobacteria selective media

| Species (samples from) | Avg logCFU/ml (SD) | No. of animals sampled | Gram-staining | F6PPK |
|---|---|---|---|---|
| Mouse *Mus musculus* (colon) | 7.02 (0.79) | 4 | 38/39 | 25/25 |
| Rat *Rattus norvegicus* (colon) | 5.81 (0.14) | 4 | 41/42 | 10/10 |
| Guinea pig (feces) | 8.33 (1.03) | 2 | 37/38 | 5/5 |
| Rabbit (cecum) | 1.44 (1.86) | 8 | 23/23 | 6/6 |
| Monkey *Macaca mulata* (feces) | 8.19 (0.33) | 3 | 38/42 | 14/14 |
| Mouse *Acomys cahirinus* (feces) | 7.27 (0.27) | 2 | 22/23 | 9/9 |
| Human (feces) | 9.78 (0.92) | 4 | 100/100 | 30/30 |
| Honeybee (rectum) | 9.55 (0.20) | 5 | 10/10 | 10/10 |
| Hen (crop) | 7.47 (0.48) | 14 | 39/42 | 40/42 |
| Hen (ceca) | 9.61 (0.47) | 9 | 41/43 | 52/53 |
| Hamster *Mesocricetus auratus* (feces) | 8.54 (1.10) | 4 | 18/18 | 13/17 |
| Hamster *Cricetulus griseus* (feces) | 8.78 (1.12) | 3 | 8/8 | 14/14 |
| Pig (feces) | 7.62 (0.87) | 6 | 28/134 | 8/17 |
| Pig (feces)[c] | 6.98 (1.44) | 3 | 51/51 | 17/17 |

[a]data are expressed as the number of Gram-positive pleomorphic rods/total number of the colonies tested
[b]data are expressed as a number of positive strains/total number of the strains tested
[c]viable counts on TPY agar modified by the addition of mupirocin (100 mg/liter), glacial acetic acid (1 ml/liter), and colistin (25 mg/liter)

72 h (hen and bee samples) or 48 h (other samples). The colonies arising on plates were counted to determine bifidobacterial counts. Randomly picked off colonies were Gram-stained. All Gram-positive pleomorphic, nonsporeforming rods were tentatively considered as bifidobacteria. Another colonies were transferred to the TPY broth supplemented with 1% of raffinose (Sigma, Prague, Czech Republic) to obtain pure cultures of bifidobacteria. The isolates were cultured at 37ºC for 24 hours. Subsequently all strains were tested for the presence of fructose-6-phosphate phosphoketolase (Biavati *et al.*, 1992). F6PPK-positive cultures were considered as bifidobacteria. Bifidobacterial isolates (10 strains) and non-bifidobacterial isolates (10 strains) originated from pig feces were tested for antimicrobial susceptibility using AN-IDENT kits (Oxoid, Prague, Czech Republic). Subsequently, all isolates were tested for minimal inhibition concentration (MIC) to colistin (Sigma, Prague, Czech Republic) using a standard broth dilution method.

## RESULTS AND DISCUSSION

All pure cultures of bifidobacteria listed in Table 1 were able to grow on MTPY agar. Table 2 shows the plate counts of bifidobacteria in selective media. Absolute counts were highest for the hen cecal contents followed by honeybee rectum contents. On the contrary, the rabbit cecal samples showed the lowest counts ranging from 0 to 4.95 log CFU/g of contents. This result is in line with literary data. Although there were four species of the genus *Bifidobacterium*, which were isolated from the rabbit cecum (Biavati *et al.*, 1992), these bacteria never dominated in the rabbit intestinal tract (Straw, 1989). Bifidobacteria from the hen and bee intestine grew slowly compared to bifidobacteria from other samples. MTPY agar was selective for all the samples tested with the exception of pig fecal samples. Less than 50% of pig fecal isolates showed bifid-like morphology and had F6PPK activity (Table 2). Non-bifidobacterial isolates from pig samples were Gram-negative rods, susceptible to colistin (MICs < 5 mg/liter). On the contrary, pig bifidobacteria strains were resistant to this compound (MICs > 40 mg/liter). Hence, MTPYC agar showed 100% selectivity for bifidobacteria (Table 2). In general, the results of microscopical examination were similar to the results of F6PPK screening (Table 2).

Approximately since the fifties, more than 20 media for the differential enumeration of bifidobacteria have been developed (for review see Biavati *et al.*, 1992; Tamine *et al.*, 1995; Pacher and Kneifel, 1996;). In the present study, The MTPY agar showed high selectivity for *Bifidobacterium* sp. in human and most animal samples tested. However, this medium was not effective in the enumeration of pig fecal bifidobacteria. Addition of colistin enabled to enumerate pig fecal bifidobacteria. Colistin (polymyxin E) is a similar compound to polymyxin B, which was used for isolation of *Bifidobacterium* sp. from water samples (Muñoa and Pares, 1988). It could be concluded that bifidobacteria selective media should be chosen in respect of the animal species origin of the samples tested.

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