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A new medium containing mupirocin, acetic acid, and norfloxacin for the selective cultivation of bifidobacteria

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ABSTRACT

Various culture media have been proposed for the isolation and selective enumeration of bifidobacteria. Mupirocin is widely used as a selective factor along with glacial acetic acid. TOS (transgalactosylated oligosaccharides) medium supplemented with mupirocin is recommended by the International Dairy Federation for the detection of bifidobacteria in fermented milk products. Mupirocin media with acetic acid are also reliable for intestinal samples in which bifidobacteria predominate. However, for complex samples containing more diverse microbiota, the selectivity of mupirocin media is limited. Resistance to mupirocin has been demonstrated by many anaerobic bacteria, especially clostridia. The objective was to identify an antibiotic that inhibits the growth of clostridia and allows the growth of bifidobacteria, and to use the identified substance to develop a selective cultivation medium for bifidobacteria. The susceptibility of bifidobacteria and clostridia to 12 antibiotics was tested on agar using the disk diffusion method. Only norfloxacin inhibited the growth of clostridia and did not affect the growth of bifidobacteria. Using both pure cultures and faecal samples from infants, adults, calves, lambs, and piglets, the optimal concentration of norfloxacin in solid cultivation media was determined to be 200 mg/L. Our results showed that solid medium containing norfloxacin (200 mg/L) in combination with mupirocin (100 mg/L) and glacial acetic acid (1 mL/L) is suitable for the enumeration and isolation of bifidobacteria from faecal samples of different origins.

1. Introduction

The intestinal microbiota is a dynamic population containing a complex combination of microorganisms. Bifidobacteria, which are anaerobic, Gram-positive, acids producing, irregular bacilli, are one of important beneficial genera in gut microbiome of humans and other mammals, dominating especially during the milk-feeding period [1]. In recent years, the metabolism and mechanisms of the probiotic functions of bifidobacteria have been intensively studied [2], and the selection of new probiotic strains is of interest. To isolate bifidobacteria from complex populations such as the faecal microbiota, selective media that allow the growth of the bacteria of interest while inhibiting the growth of other microorganisms present in a sample should be employed. Several media have been developed for the selective enumeration and isolation of bifidobacteria from different types of samples, and the use of some of these media was proposed for quality control analysis of dairy products containing probiotics [3,4].

Selective media for bifidobacteria are typically based on commercially available media such as Man, Rogosa, and Sharpe (MRS); Colombia; Reinforced Clostridial; and Wilkins–Chalgren agars, which are supplemented with different individual antimicrobial compounds or combinations of these compounds. In these media, the growth of non-bifidobacterial strains is usually inhibited by an antibiotic, low pH, or both [5]. Based on the recommendation of Bergey's Manual of Systematic Bacteriology, TPY (trypticase, phytone, yeast extract) medium supplemented with mupirocin (100 mL/L) should be used for the isolation of bifidobacteria. The main components of this medium (trypticase, phytone, and yeast extract) have proven to be satisfactory for the growth of bifidobacteria from all known habitats [6]. Mupirocin (50 mg/L) as a selective factor is also present in the medium intended for the enumeration of bifidobacteria in milk products also containing

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lactic acid bacteria. The base of the medium is TOS agar (trans-galactosylated oligosaccharides; Yakult, Japan). This medium has been recommended by the International Dairy Federation for the enumeration of bifidobacteria in dairy products, milk powders, infant formulas, and starter cultures [7]. Modified TPY medium (MTPY) supplemented with mupirocin (100 mg/L) and glacial acetic acid (1 mL/L) [8], was used for the enumeration of bifidobacteria in infant faecal samples by Vlková et al. [9], who showed that this medium was not effective for faecal samples from bifidobacteria-free infants with high numbers of clostridia, which were able to grow in the presence of mupirocin. Similar results were presented by Rada and Petr [8], who analysed hen caeca samples using the same medium and showed that about 5% of the colonies that grew were non-bifidobacterial. In addition, Lakshminarayanan et al. [10] reported that *Clostridium perfringens* was not inhibited by mupirocin in the dose 100 mg/L in MRS agar when the medium was used for the enumeration of bifidobacteria in faecal samples from elderly volunteers. Ferraris et al. [11] tested different media for the detection of bifidobacteria in faecal samples, and although Wilkins-Chalgren agar supplemented with mupirocin (50 mg/L) was determined to be the most selective medium, clostridia were isolated from 8 of the 15 samples tested.

The development of new selective media for the enumeration of bifidobacteria in intestinal samples may be considered unnecessary since culture-independent methods are used; however, selective media are essential for the isolation of new species. Although media designed for bifidobacteria determination are effective for samples in which only lactic acid bacteria are present along with bifidobacteria, the faecal microbiota is more complex and includes closely related genera that are difficult to separate. For intestinal samples in which bifidobacteria dominate, MTPY agar is reliable for their enumeration. However, mupirocin, even in combination with glacial acetic acid, does not suppress the growth of faecal clostridia. The objective was to find an antibiotic that inhibits the growth of clostridia and allows the growth of bifidobacteria, and to use this substance to develop a selective cultivation medium for bifidobacteria.

2. Material and methods

2.1. Bacterial strains and cultivation media

Bifidobacteria and clostridia from human, calf, lamb, and pig faeces, and hen caeca were used in this study (Tables 1 and 2). The samples were collected from infants (parents of all babies sampled in this study gave informed written consent for the analysis of faecal samples) and adult volunteers. Animals used for the sampling were housed at the farm of the Czech University of Life Sciences Prague. The experiment was carried out under standard regime and farm management procedures and was approved by the Institutional Animal Care and Use Committee (Czech University of Life Sciences Prague). One gram of the sample was aseptically transferred to the tube containing oxygen-free Wilkins-Chalgren broth (Oxoid) and serially diluted in the same medium. Bacteria were isolated using modified Wilkins-Chalgren agar (Oxoid, MWCHmup) supplemented with soya peptone (5 g/L, Oxoid), L-cysteine (0.5 g/L, Sigma), Tween® 80 (1 mL/L, Sigma–Aldrich), mupirocin (100 mg/L, Merck), and glacial acetic acid (1 mL/L) [8] after anaerobic cultivation at 37 °C for 3 days. Anaerobic jars (Anaerobic Plus System, Oxoid) were used for the anaerobic cultivation of plates. The jars were equipped with palladium catalysts (Oxoid) and filled with a CO_2/H_2 (10%/90%) atmosphere. After cultivation, bacterial colonies were picked and transferred to vials with Wilkins-Chalgren broth (Oxoid) prepared by the Hungate technique [12]. This technique was used to prepare all liquid media used in this study. The morphology of the isolates was examined by phase-contrast microscopy. Irregular rods were identified as bifidobacteria by the detection of fructose-6-phosphate phosphoketolase (F6PPK) activity [13]. Rods with regular morphology were classified as clostridia by fluorescence *in situ* hybridisation (FISH) using a fluorescein isothiocyanate (FITC)-labelled probe specific for *Clostridium butyricum* (BioVisible, The Netherlands). Bifidobacteria of human origin were identified to the species level by polymerase chain reaction (PCR) using species-specific primers targeting the 16S rRNA gene [14] and isolates of animal origin were identified by sequencing the 16S rRNA gene according to the method of Killer et al. [15]. Control strains were obtained from American Type Culture Collection (ATTC) and the German Resource Centre for Biological Material (DSM).

Stock cultures of bifidobacteria were maintained at −70 °C in Wilkins-Chalgren broth (Oxoid) containing glycerol (20% v/v). Clostridia were stored in cooked meat medium (Oxoid) at room temperature. Before the assay, bacteria were subcultured twice under anaerobic condition at 37 °C in Wilkins-Chalgren broth (Oxoid) for 24 h. Modified Wilkins-Chalgren agar (Oxoid, MWCH) without mupirocin and acetic acid supplementation was used for antimicrobial susceptibility testing.

2.2. Antimicrobial susceptibility testing

In the first step of this study, the sensitivity of 13 bifidobacterial and 13 clostridial strains (Table 1) to 12 antibiotics was tested. The following antibiotics with activity against Gram-positive anaerobic bacteria in standard concentrations for antibiotic susceptibility evaluation were chosen for the test: (i) cell wall synthesis inhibitors: glycopeptide – vancomycin (30 μg); cephalosporins – ceftazidime (30 μg), and cefoxitin (30 μg); (ii) protein synthesis inhibitors: monoxycarbolic acid – mupirocin (200 μg); aminoglycosides – apramycin (15 μg), kanamycin (30 μg), and neomycin (30 μg); and (iii) nucleic acid synthesis inhibitors: quinolones – ciprofloxacin (5 μg), flumequine (30 μg), and norfloxacin (10 μg); sulphonamide – sulfamethoxazole (25 μg); nitroimidazole – metronidazole (5 μg). Antibiotic discs (diameter = 6 mm) were obtained from Oxoid. An aliquot (1.5 mL) of each bacterial suspension containing 10^7 cells per mL was used as the inoculum, and antimicrobial susceptibility was determined by the disk diffusion method on MWCH agar. Standard discs of the antimicrobial agents were placed onto the seeded plates and incubated anaerobically at 37 °C for 48 h. The diameter of the inhibition zones including the disk diameter was measured in millimetres, and the results were expressed as resistant (≤6 mm), moderately susceptible (6.1 mm–9.9 mm), or susceptible (≥10 mm). All antibiotics were tested in triplicate.

In the second step of this experiment norfloxacin and mupirocin were chosen for more detailed testing, because only norfloxacin inhibited the growth of all clostridia, and did not affect the growth of bifidobacteria, while mupirocin is a selective factor that is widely used in cultivation media for bifidobacteria. Another 74 bifidobacterial strains (11 collection strains, 19 strains from infants, 9 strains from adults, 10 strains from calves, 10 strains from lambs, 9 strains from hens, and 6 strains from pigs) and 62 clostridial strains (9 collection strains, 17 strains from infants, 14 strains from adults, 4 strains from calves, 9 strains from lambs, 3 strains from hens, and 6 strains from piglets) were tested for their sensitivity to norfloxacin and mupirocin by the disk-diffusion method as described above.

The minimal inhibitory concentration (MIC) of norfloxacin for the bacteria listed in Table 2 was determined. Overnight axenic cultures of bifidobacteria and clostridia were inoculated at about 1×10^7 cfu into 10 mL of Wilkins-Chalgren broth supplemented with norfloxacin concentrations 10, 30, 50, 80, 100, 150, 200, 250, or 300 mg/L. Bacterial inhibition was also tested in the presence of