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Comparison of mupirocin-based media for selective enumeration of bifidobacteria in probiotic supplements

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ABSTRACT

An international standard already exists for the selective enumeration of bifidobacteria in milk products. This standard uses Transgalactosylated oligosaccharides (TOS) propionate agar supplemented with mupirocin. However, no such standard method has been described for the selective enumeration of bifidobacteria in probiotic supplements, where the presence of bifidobacteria is much more variable than in milk products. Therefore, we enumerated bifidobacteria by colony count technique in 13 probiotic supplements using three media supplemented with mupirocin (Mup; 100 mg/l): TOS, Bifidobacteria selective medium (BSM) and modified Wilkins-Chalgren anaerobe agar with soya peptone (WSP). Moreover, the potential growth of bifidobacterial strains often used in probiotic products was performed in these media. All 13 products contained members of the genus *Bifidobacterium*, and tested mupirocin media were found to be fully selective for bifidobacteria. However, the type strain *Bifidobacterium bifidum* DSM 20456 and collection strain *B. bifidum* DSM 20239 showed statistically significant lower counts on TOS Mup media, compared to BSM Mup and WSP Mup media. Therefore, the TOS Mup medium recommended by the ISO standard cannot be regarded as a fully selective and suitable medium for the genus *Bifidobacterium*. In contrast, the BSM Mup and WSP Mup media supported the growth of all bifidobacterial species.

1. Introduction

Bifidobacteria are probiotic microorganisms that are widely used in the food industry (Miranda et al., 2011). Probiotic microorganisms are usually available as culture concentrates in dried or deep-freeze form to be added to a food for industrial or home uses (Tripathi and Giri, 2014). In addition to the food probiotics, there are various health products and pharmaceutical preparations containing probiotics on the market (Saad et al., 2013). The most commonly used species of probiotic bacteria in lyophilised form and milk products are *Bifidobacterium adolescentis*, *Bifidobacterium animalis* ssp. *lactis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *infantis*. The amount of probiotic bacteria required for therapeutic effect is considered to be in the range of 10^9 cells of live microorganisms per day. To exert a beneficial effect, the bacteria must remain viable in the product until the time of consumption. Commercially available probiotics are usually in the form of freeze-dried, powdered bacteria or in the capsule-packed forms, which can affect their persistence and viability. According to Makinen et al. (2012) there are three major factors governing the stability of probiotics during manufacture and storage; strain robustness, process and storage conditions. The manufacturer should correctly inform customers about bacteria amounts and species composition in the product. A widely used method for the microbiological control of food quality, including probiotics, is culturing. Different culture media have been proposed for the selective enumeration of bifidobacteria (Ashraf and Shah, 2011; Karimi et al., 2012; Roy, 2001). There also exists an ISO standard for the enumeration of bifidobacteria in food, such as milk products. The ISO standard, denoted by ISO 29981:2010 (IDF 220:2010) use a colony count technique performed at 37 °C under anaerobic conditions on Transgalactosylated oligosaccharides propionate agar (TOS, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) supplemented with mupirocin. This method is applicable for milk products such as fermented and non-fermented milk, milk powders, infant formulas and starter cultures. The TOS-mupirocin agar is selective even when bifidobacteria are present in combination with lactic acid bacteria. The basal medium (TOS) has for many years been commercially produced and marketed exclusively by Yakult-Japan. However, both the TOS and mupirocin are now licenced, produced and marketed by VWR in Europe and around the world (Raeisi et al., 2013). The use of mupirocin as a selective factor for the isolation and quantification of bifidobacteria in fermented dairy products was first described by Rada and Koc (2000). The authors of this study recommended the use of Wilkins-Chalgren agar (Oxoid, ThermoFisher Scientific, Carlsbad, CA, USA) supplemented with mupirocin (100 mg/l). This agar was modified with the addition of

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soya peptone (5 g/l), L-cysteine (0.5 g/l), and Tween 80 (1 ml/l; Bunešová et al., 2012). Soya peptone contains galactooligosaccharides, which promote the growth of bifidobacteria. Another commercial agar for the enumeration of bifidobacteria is available from Fluka (Sigma-Aldrich, St. Louis, MO, USA).

However, no standard method has been described for the selective enumeration of bifidobacteria in probiotic supplements. The use of bifidobacteria in pharmaceutical supplements is becoming increasingly popular, resulting in a wide variety of products being marketed with specific or generic claims of health benefits. These products often contain multispecies probiotic microorganisms indicating the presence of species other than bifidobacteria. The aim of this study was to evaluate different mupirocin selective media for the enumeration of bifidobacteria in probiotic supplements.

2. Material and methods

2.1. Culture media

Three agars (Table 1) supplemented with mupirocin lithium salt at a concentration of 100 mg/l (Mup; Oxoid, ThermoFisher Scientific, Carlsbad, CA, USA) were used in this study for the quantification of the most commonly used bifidobacterial species and bifidobacteria in different probiotic supplements.

2.2. Testing of pure bifidobacterial strains

The growth characteristics of *Bifidobacterium* sp. type strains of species often used in probiotic products were tested on different agars. The tested strains included *B. adolescentis* DSM 20083 (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Leibniz, Germany), *B. animalis* ssp. *lactis* DSM 10140, *B. bifidum* DSM 20456, *B. breve* DSM 20213, *B. longum* ssp. *infantis* DSM 20088 and *B. longum* ssp. *longum* DSM 20219. The *B. bifidum* collection strains DSM 20082, DSM 20215 and DSM 20239 were also added for verification of the results.

Cultures of pure bifidobacterial strains grown anaerobically overnight were serially diluted and inoculated into Petri dishes which were immediately filled with tested agars, and cultivated in anaerobic atmosphere (AnaeroGen Compact System, Oxoid) at 37 °C for 2 days.

2.3. Enumeration of bifidobacteria in probiotic products

A total of 13 different human and animal probiotic supplements (lyophilized capsules, sachets and drops) commercially available in the European market (Table 2) were analysed. All the probiotic products were tested prior to the expiration date indicated on the labels of the product and were stored according to the manufacturer's recommendations.

One gramme or millilitre of each probiotic product was aseptically homogenised in 9 ml of sterile Saline peptone diluent (Oxoid) and serially diluted under anaerobic conditions (roll-tube technique; Hungate, 1969). The appropriate dilutions were transferred to sterile dishes and immediately filled with media listed in Table 1. All the probiotic products were tested in triplicate. The plates were incubated as described in Section 2.2.

2.4. Evaluation of agar selectivity

Twenty colonies per each sample and media were selected for further confirmatory tests. Pure isolates were cultivated in Wilkins-Chalgren broth supplemented with soya peptone (5 g/l, Oxoid). Tests were conducted for morphology, Gram staining, and fructose-6-phosphate phosphoketolase (specific enzyme for Bifidobacteriaceae family) activity (F6PPK-test; Orban and Patterson, 2000) in order to confirm the selectivity of the MUP agar for bifidobacteria.

2.5. Statistical analyses

Bifidobacterial counts were converted to $\log_{10}$ Colony Forming Unit (CFU) per g or ml. The results, based on triplicate analysis of probiotic bacteria in the selective media (TOS Mup, BSM Mup, and WSP Mup) were evaluated by multiple range comparison. Multiple range tests ($p < 0.001$) were perfored using Statistica (Statistica 12.0, Tulsa, USA). The same statistical test was used to compare the growth of pure cultures in the tested media.

3. Results and discussion

All the tested pure cultures of bifidobacterial type strains were able to grow on all sets of tested media, in counts ranging from 5.78 to 10.25 log CFU/ml (Table 3), depending on the primary growth of individual strain in the enrichment media. All tested bifidobacterial type strains except for the type strain *B. bifidum* DSM 20456 showed similar counts on all three tested agars and no significant differences were found (Table 3). On the other hand, the type strain *B. bifidum* DSM 20456 showed a statistically significant lower increase in growth in TOS Mup medium, compared to WSP Mup and BSM Mup media. This increase was detected even in titrations where the order of magnitude of bacterial number was four times less. These results were verified by testing the three collection strains of *B. bifidum* (DSM 20082, DSM 20215 and DSM 20239). We observed that two of the collection strains (DSM 20082 and DSM 20215) showed growth on all tested media with identical counts (Table 3). However, the strain *B. bifidum* DSM 20239 again showed a statistically significant lower increase in TOS Mup media compare to WSP Mup and BSM Mup media. The lower counts of *B. bifidum* DSM 20456 and DSM 20239 may be due to the fact that these strains have limited abilities to utilize transgalactosylated oligosaccharides. TOS agar contains transgalactosylated oligosaccharides obtained by the transformation of lactose by the enzyme β-galactosidase, magnesium sulphate for enhancing recovery and growth of injured bifidobacteria, and sodium propionate as an inhibitor for other adjunct flora (Raeisi et al., 2013). These galacto-oligosaccharides as a specific substrate for bifidobacteria, however, cannot be specific to all bifidobacterial species. According to Miranda et al. (2014), the strain *B. animalis* ssp. *animalis* CIRMBIA 1335 also showed no colony forming in these media. Moreover, many physiological characteristics of bifidobacteria are species- or strain-specific.

Table 1
The media used for the enumeration of bifidobacteria in probiotic products.

	Medium abbrevation	Medium name (producer)	Final concentracion of aditives
Mupirocin media (100 mg/l)	TOS Mup	TOS-propionate agar (Yakult Pharmaceutical Industry)	Acetic acid (final pH 6.3 + 0.2; at 25 °C)
	BSM Mup	Bifidobacteria selective medium (Fluka)	BSM supplement (0.116 g/l)
	WSP Mup	Wilkins-Chalgren Anaerobe Agar (Oxoid)	Soya peptone (5 g/l)
			L-cysteine (0.5 g/l)
			Tween 80 (1 ml/l)