Storage of intestinal bacteria in samples frozen with glycerol

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For clinical studies, it would be useful to store faecal samples before bacteriological analyses. However, information is needed on the effect of freezing on the survival of micro-organisms. In this study, faecal samples were stored in glycerol at −80°C for four months.

Although the concentrations of predominant anaerobic bacterial populations (>8 log(cfu g⁻¹), total anaerobes and bifidobacteria, decreased significantly (p < 0.05) after freezing, this decrease did not exceed interindividual variation. The facultative anaerobes, enterobacteria, enterococci and lactobacilli, were not significantly affected by freezing. Furthermore, the magnitude of individual variability was similar before and after freezing. These results confirm the relevance of our freezing technique for studying the intestinal microflora which involves numerous samples that cannot be analysed simultaneously for practical reasons. Key words: intestinal microflora, faeces freezing, storage.

INTRODUCTION

In the field of human nutrition, many studies deal with the role of autochthonous intestinal bacteria in host physiology. Quantification and identification of intestinal microflora can be performed on fresh faecal samples to ensure the survival of micro-organisms (1–3). However, for practical reasons, the number of fresh samples that can be analysed per day using this procedure is a limiting factor. For these reasons, the conservation of faecal samples during storage appears a topical subject. In many studies, faecal samples were directly frozen without a cryoprotective agent before microbial enumeration of anaerobes (bifidobacteria, Bacteroides) (4, 5) or facultative anaerobes (enterococci, enterobacteria, lactobacilli) (6–8). However, the fate of bacteria during freezing was not measured. Thirty years ago Crowther (9) described the fate of faecal bacteria from one volunteer using several storage methods. This author highlighted the interest of using glycerol to freeze samples. The aim of the present study was to estimate the fate of faecal bacteria (total anaerobes, bifidobacteria, enterobacteria, enterococci and lactobacilli) obtained from 20 infants, after freezing the faeces in glycerol for several months.

MATERIALS AND METHODS

Sample preparation. Faecal samples from 20 infants, aged 9 to 15 months-old, were collected in sterile plastic containers under anaerobic conditions (Anaerocult, Merck, Darmstadt, Germany). The samples were immediately stored in a refrigerated box (below 5°C) until analysis at the laboratory (within 12 h). Serial dilutions (10⁻¹ to 10⁻⁸) were performed from each sample in a pre-reduced Liquid Casein Yeast medium (LCY) [containing (g l⁻¹): casein enzymatic hydrolysate (USBC) 2, yeast extract (Difco) 2, NaCl 5, KH₂PO₄ 1]. Dilutions (10⁻⁴ to 10⁻¹⁰) were plated on different selective media with a spiral platter (Interscience, St Nom la Bretèche, France). Total anaerobes, bifidobacteria, enterobacteria and enterococci were counted on 20 fresh faecal samples. Lactobacilli were counted on 10 fresh samples. Simultaneously, each sample diluted to 10⁻¹ was mixed with sterile glycerol 1/1 (vol./vol.) and kept at −80°C. Four months later, the same bacterial enumerations were performed after the samples were thawed in ice.

The media. Total anaerobes were counted on BHI agar [containing (g l⁻¹): Brain Heart Infusion (Difco) 37, yeast extract (Difco) 5, haemin 0.005]. Bifidobacteria were counted on Beersens medium [containing (g l⁻¹): Brain Heart Infusion (Difco) 37, yeast extract (Difco) 5, cystein chloride 0.5, propionic acid 5]. Enterobacteria were counted on DCA medium [containing (g l⁻¹): peptone 10, lactose (Sigma) 10, sodium desoxycholate (Sigma) 1, sodium chloride 5, dipotassic phosphate 2, iron citrate 1, sodium citrate 1, neutral red 0.03]. Enterococci were counted on GAPTS1 medium [containing (g l⁻¹): K₂HPO₄ yeast (Difco) 10, peptone 15, tryptone 10, liver extract 5, glucose 5, sodium azide 3] and lactobacilli were...
were observed microscopically for their cell morphology. Colonies were counted and bacteria in all cases the decrease from the initial level never exceeded 7% of the fresh count. We calculated the mean difference between bacterial counts from fresh and frozen samples. The ratio \( \Delta m/SD \) indicated that the loss due to freezing was always lower than individual variability. It was 0.7/0.8 for bifidobacteria, 0.4/1.0 for enterobacteria, 0.2/0.8 for enterococci and 0.3/0.6 for lactobacilli. Crowther (9) showed that \( \text{Clostridium}, \text{Bacteroides}, \text{bifidobacteria}, \text{enterococci} \) and \( \text{enterobacteria} \) were well preserved in the suspensions of faeces from one subject in broth containing 10% glycerol, frozen for one month at a temperature lower than \(-25^\circ\text{C}\). After storing frozen faecal samples for four months in 50% glycerol at \(-80^\circ\text{C}\), we observed that facultative anaerobes were well preserved and that the level of dominant anaerobes was only very slightly reduced. The results of our experiment demonstrate that the relative equilibrium between the bacterial populations is preserved, thus confirming the relevance of the technique used. Furthermore, we obtained successful results with this method in a nutritional study involving the bacterial analysis of hundreds of faecal samples from healthy children (10).

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