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Viability and stability of *E. coli* and enterococci populations in fecal samples upon freezing

N Masters¹, M Christie², H Stratton^{2, 3} and M Katouli^{1, 3}

¹Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Sippy Downs; ²School of Biomolecular and Physical Sciences, Griffith University, Nathan, and ³Smart water Research Centre, Griffith University , Gold Coast, Queensland, Australia

N. Masters nmasters@usc.edu.au
M. Christie m.christie@griffith.edu.au
H. Stratton h.stratton@griffith.edu.au
*M. Katouli mkatouli@usc.edu.au

*Corresponding author: A/Prof Mohammad Katouli, ¹Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Sippy Downs
Phone: (+61) 7 5430 2845;
Fax: (+61) 7 5459 4880

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Abstract

We studied the survival of *Escherichia coli* and enterococci populations in fecal samples of seven host species after storage at 20°C and 80°C for 30 days. Composite fecal samples were collected from cows, chickens, horses, pigs, dogs, birds and humans, and bacteria were enumerated before and after storage. Twenty-eight colonies of each bacterial species were typed before and after storage and the strains were assigned to different biochemical phenotypes (BPTs). A significant reduction in the number of *E. coli* was observed in all samples stored at 20°C, but only in three of those samples stored at 80°C. However, the numbers of enterococci were similar in most stored samples (except cow and birds). The number and the distribution of *E. coli* and enterococci BPTs in fresh samples did not vary significantly from those stored at either temperature. Furthermore the population structure of *E. coli* and enterococci did not change significantly after storage at 80°C, this was always the case for those samples stored at 20°C. We conclude that for those studies investigating *E. coli* or enterococci population structure short term storage (≤ 30 days) of fecal samples, in a glycerol broth at 80°C, is a preferable option.

Keywords: deep freezing, PhP typing, bacterial composition, diversity, population structure.

Introduction

The immediate processing of fecal samples after collection and transport to the laboratory is not always technically or economically feasible, particularly in large-scale studies that are challenged temporally and spatially. In these cases fecal samples in question should be appropriately stored for future analyses, this will also accommodate concurrent processing of samples, eliminate inter-assay variations and allow subsequent analysis at a later date. The most common and well established method of preservation is freezing (Achá et al. 2005; Katouli et al. 1990; Nechvatal et al. 2008; Panasci et al. 2011).

In microbiological laboratories, where the focus of the study is on analysing the bacterial constituent of the sample, it is also important to have knowledge of the survival rates of bacterial communities, and in some cases, specific bacterial populations in the feces after freezing of samples. Although techniques for preserving and storing bacterial isolates are well established, changes that may occur in bacterial populations of fecal samples are less reported. Previous studies evaluating the effects of storage conditions on fecal samples have focused on either the survival rates of the bacteria (Sinton et al. 2007), or the preservation of particular genetic material (Murphy et al. 2002; Nechvatal et al. 2008; do Nascimento et al. 2012). Studies that have evaluated the stability of target strain populations after fecal sample storage have either tested only human samples (Wasfy et al. 1995; Ott et al. 2004), or focused on only one bacterial species (Achá et al. 2005). Furthermore these studies have evaluated samples that were collected from individual hosts, not large composite samples and not from diverse host species as targeted as here. The aim of our study was to evaluate the viability and diversity of *E. coli* and enterococci

populations in fecal samples of human and animal origin after storage at either -20°C or -80°C for 30 days.

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69 **Materials and Methods**

70 **Sampling collection and preparation**

71 Fresh fecal samples were collected from domestic animals cow ($n = 40$), chicken (n
72 $= 25$), horse ($n = 29$), pig ($n = 19$), dog ($n = 7$), birds ($n = 15$) and healthy adult
73 volunteers ($n = 7$) aged between 22 and 50 years old. Dog fecal samples were
74 collected from individual domestic houses in three different suburbs, whereas horses,
75 chicken, pigs and cows were collected from between two and five farms within 25 km
76 radius. Bird fecal samples were collected from a wild life sanctuary. From each
77 individual, 5 - 50 grams of wet fecal material were collected and transferred to the
78 laboratory on ice. All samples were processed within 12 hours of collection.
79 On ice, individual samples were combined in equal proportions into a sterile 50 mL
80 plastic container to yield a 45 g wet weight composite sample for each species. To
81 this, 5 mL of sterile phosphate buffered saline (PBS [pH 7.4]) was aseptically added
82 to yield a 50 mL of fecal slurry. Fecal slurries were then homogenised by a wrist-
83 action shaker for 10 minutes, and stored on ice for further preparation.

84 **Storage of composite fecal samples**

85 Four replicate fecal slurries were concurrently prepared for each species as
86 described above, with the addition of 50% /v sterile glycerol to the PBS, and
87 homogenised. Using parafilm tape, replicates were then sealed air-tight in their
88 primary container and placed in two secondary air-tight containers. Each secondary
89 container was then stored at either -20°C or -80°C for a period of 30 days. A fresh

90 slurry sample was used to test the number of *E. coli* and enterococci and their
91 diversity, which gave the time zero point as described below.

92 **Enumeration and isolation of *E. coli* and enterococci**

93 Fresh fecal samples were processed by plating triplicate 100 μ L aliquots of each
94 serial dilution onto mTEC agar (Difco, Australia) and mEI agar (Difco plates. Plates
95 were then incubated at 35°C for 2 h to revive cells and then at 44.5°C $\pm$ 0.5°C for 22
96 h for enumeration of *E. coli*, and at 41°C $\pm$ 0.5°C for 22h for the enumeration of
97 enterococci.

98 Twenty-eight *E. coli* or enterococci spp. were then randomly isolated from each
99 sample, purified twice on MacConkey agar no. 3 (Oxoid, Australia) or m-
100 *Enterococcus* agar (Difco), respectively and stored at -80°C until further testing.
101 Selecting this number of isolates provided >90% chance of detecting minor clones
102 amongst the host *E. coli* fecal population as previously described (Schlager et al.
103 2002). Presumptive identification of the *E. coli* isolates was done on SIM and
104 Simmons Citrate agars (Oxoid), and PCR was used to confirm identification using a
105 highly specific *E. coli* universal stress protein (*uspA*) gene (Gündoğdu et al. 2011).
106 Identification of enterococci was confirmed by observing esculin hydrolysis activity
107 on bile esculin agar (Oxoid) after incubation at 45°C for 1 h (Manero and Blanch
108 1999).

109 A similar procedure was used for samples stored at -20°C and -80°C after 30 days
110 storage except that these samples were initially placed on ice and stored overnight
111 at 4°C to slow the rate of defrost. The samples were then shaken and triplicate serial
112 dilutions were prepared and 100 μ L were spread on the same media used for fresh

samples. Plates and subsequent isolated bacteria were treated as described for fresh samples.

Typing

All *E. coli* and enterococci strains were typed using a high-resolution biochemical fingerprinting method (PhPlate system), specifically developed for *E. coli* i.e. PhP-RE and enterococci spp. i.e. PhP-RF plates (PhPlate AB, Stockholm, Sweden). The fingerprinting method was performed to measure the kinetics of *E. coli* and enterococci isolates as previously described (Kühn et al. 1991; Kühn et al. 2003). Biochemical activity was measured by taking absorbance readings at intervals of 8, 24 and 48 h for *E. coli*, and 16, 40 and 64 h for enterococci; using a HP Scanjet 4890 flatbed scanner. A mean absorbance value was then calculated for each reagent, creating a biochemical fingerprint for each isolate (Cassanovas-Massana and Blanch 2013). Similarity among the isolates was calculated as a correlation coefficient, and clustered according to the unweighted pair-group method with arithmetical averages to yield a dendrogram. Isolates showing similarity above the default value of the system (i.e. 97.5%) were regarded as identical and assigned the same biochemical phenotype (BPT). Isolates with the highest minimum and highest mean similarity to those isolates belonging to the same cluster were selected as the representative strains (Kühn et al. 1991).

Diversity indices, similarity coefficients and clustering analysis

Phenotypic diversity of the isolates in each sample was calculated as Simpson's diversity index (D_i) (Atlas 1984) according to Kühn et al. (1991). The similarity between different bacterial populations in two or more samples was calculated as a population similarity (S_p) coefficient. The S_p coefficient calculates the proportion of

isolates that were identical (i.e. having the same BPT) in two or more compared bacterial populations (Kühn et al. 1991). Clustering of Sp coefficients was performed according to the UPGMA method. All data handling, including optical readings, calculations of population similarity values as well as clustering and printing dendrograms, was performed using the PhPlate software v6.1.

Statistical analysis

One way ANOVA test was used to compare the difference between concentrations of *E. coli* and enterococci derived from each composite species sample before and after storage. Two-tailed t-test was used to compare the Sp-values of *E. coli* and enterococci populations. Both statistical tests were performed using Graph pad Prism 6 (version 6.02 for windows) software.

Results

Bacterial survival after storage

The number of *E. coli* in fresh fecal samples differed among different animal species and ranged between 1×10^6 CFU/gram of wet feces in cow to 1×10^8 CFU/gram of wet feces in pig (Figure 1). The number of *E. coli* in fecal samples preserved at -20°C was significantly lower in all species (except horse) compared to the fresh samples (Figure 1). This however was not the case for samples preserved at -80°C where the number of *E. coli* was significantly lower in fecal samples of only cow, pig and dog. Up to one log increase was observed in fecal samples of chicken and bird preserved at -80°C (Figure 1), which could be partially due to the inter-assay variation that can be seen in tests like this.

The number of enterococci in fresh fecal samples also differed among the animal species and ranged between 1×10^4 CFU/gram of wet feces in cow and human to $1 \times$

10⁸ in chicken (Figure 2). Unlike the changes in the number of *E. coli*, there was no significant difference in the number of enterococci in most samples (except cow and birds) preserved at 20°C and 80°C (Figure 1 and 2). Furthermore, the number of enterococci in fecal samples from horse, dog and bird did not differ between the fresh samples and those preserved at 80°C (Figure 2).

Population structure of *E. coli* and enterococci before and after storage

To identify if there were any changes in the population structure of these bacteria, we typed 28 colonies from the fecal sample of each host before and after storage. In all, 588 *E. coli* and 588 enterococci isolates were collected from the fresh and preserved samples and grouped into biochemical phenotypes (BPTs). The presence of the same common (C) BPTs and the number of isolates in that BPT as well as the number of the same single (S) BPTs in fecal samples before and after storage were investigated.

The stability of dominant clones after preservation was evaluated by comparing the biochemical fingerprints of the typed isolates using the PhP software. Across all host samples dominant clones were found to persist in comparable numbers after preservation. Figure 3 exemplifies this and shows the BPTs of *E. coli* and enterococci isolates screened prior to and after preservation of the human composite fecal sample. Analysis of the PhP fingerprints of strains isolated after storage at 20°C or 80°C indicated these dominant clones persisted after storage. All four *E. coli* clones and 5 out of 6 enterococci clones were found to persist in the samples stored at 20°C or 80°C (Figure 3). A similar pattern, of persistent dominant clones was seen across the other host samples (data not shown).

Diversity of *E. coli* and enterococci before and after storage

The diversity of the two bacterial species in each sample was calculated as Simpson's index of diversity (D_i). The results showed a marked variation in the D_i of *E. coli* strains in preserved samples; ranging between 0.079 – 0.974. The D_i for all -20°C samples decreased except for the bird sample which had a three-fold increase. Despite this variation, the D_i of the fresh samples did not vary significantly from those preserved at -20°C ($P = 0.545$) or -80°C ($P = 0.4061$) (Table 2).

The diversity of enterococci populations remained relatively stable in all preserved samples, and unlike *E. coli*, all species maintained a relatively stable D_i value in spite of storage (Table 2).

Stability of *E. coli* and enterococci populations

The stability of *E. coli* and enterococci populations was measured as population similarity (S_p) coefficient generated by comparing the presence of similar BPTs in fecal samples before and after storage. Generally, there was more similarity among samples stored at -80°C and the fresh fecal samples (Table 1). In some cases there was a higher similarity between the preserved samples than the fresh fecal samples in some hosts. Overall, the *E. coli* populations had a much higher similarity than those of enterococci (Table 1 and Figure 3). These trends were replicated across the samples of each animal screened in this study (data not shown).

Discussion

The gut microbiota offers important clinical, ecological and epidemiological information regarding the host. Resident gut flora typically exhibit particular host-specific characteristics because of the unique environment of the host's gut. This is primarily due to the differences in their gut physiology and dietary composition (Aslan

et al. 2004; Ley et al. 2008). Consequently, genotypic and phenotypic differences exist in the resident bacteria flora of different species. These host-dependent strain-specific characteristics are the pursuit of novel fecal source tracking methods, and underpin the fundamental principle of microbial source tracking (MST) for assessing the microbial quality of water systems. Fecal indicator bacteria such as *E. coli* and enterococci are still a key target species for MST. Accordingly, identifying the composition and profile of these bacteria enable the detection of source contaminants in a water body and is essential for defining the risk and fashioning more efficient and effective management strategies (WHO 2003; Scott et al. 2005; Field and Samadpour 2007; Harwood et al. 2014). However, because of the ubiquitous nature of *E. coli* and enterococci, characterisation of their population structure in each host often requires testing a high number of strains to determine the specificity and sensitivity of the MST method. In some cases this may also require the testing of their prevalence and abundance in the target host community (Shanks et al. 2010). Most often the evaluation of the MST method is limited by the number of sources and hosts that can be sampled. Moreover, often spatial and temporal variations skew the performance of many assays, since samples are collected from a limited geographical region and small number of representative hosts. Increasing the number of samples that could be collected in a sampling regime could improve the robustness and reliability of the method tested. One step that may increase sampling flexibility is freezing the fecal sample.

For methods targeting indicator bacteria such as *E. coli* or enterococci, it is not only important to test a high number of colonies from each fecal sample but also from a diverse number of individuals within each target species. Ideally, fecal samples should also come from locations that are spatially and temporally separate to

accommodate diversity among the species'. Collections of such a high number of samples and testing them within the same day are normally problematic and/or require technical resources which may not be available in many laboratories. Furthermore, where the aim is to perform a blind study to look for specific markers in fecal samples, tests should be done simultaneously using the same reagents and materials. Under these conditions it may be best to collect samples, pool common sources and store them under conditions where the population structures of indicator bacteria in fecal samples are preserved.

In the present study we collected fecal samples from many individuals within each species and from as many diverse farms/sources where possible and pooled them before preserving them at temperatures which are commonly available to most laboratories. It was found that the number of both indicator bacteria was less affected in samples preserved at -80°C. We believe that the small variability seen in some post-storage samples could in part be due to inter-assay differences. There was a log increase in the number of bacteria in two post storage samples which might be the consequence of regrowth upon thawing. The results also showed that despite some variation between the numbers of these bacteria in some samples upon storage, there was not any significant change in the overall population structure of bacteria compared to fresh samples. To evaluate the stability of the *E. coli* and enterococci populations we used a high resolution biochemical fingerprinting methods specifically developed for typing of *E. coli* and enterococci strains. The system has been previously shown to be as powerful as molecular typing methods such as RAPD-PCR (Ramos *et al.* 2010) and ERIC-PCR (Ansaruzzaman *et al.* 2000) for *E. coli* and PFGE (Torell *et al.* 2003) as well as ribotyping (Kuhn *et al.* 1995) for enterococci. The phenotypic diversity of both *E. coli* and enterococci populations

before and after storage remained relatively stable. However the greatest stability was associated with those samples stored at -80°C . This suggests that for those studies investigating *E. coli* or enterococci populations the short term storage of fecal samples may be a feasible option that would not compromise the composition of these bacteria.

Considering the individual variations in resident *E. coli* and enterococci populations between members of the same species we decided to pool fecal samples from each species to provide a more robust representation of these bacteria. We selected 28 isolates to be tested from each species as it has been previously reported to identify >90% minor clones in a fecal sample (Schlager et al. 2002). In this study we pooled fecal samples and assumed that we would have a similar recovery of minor clones if we test 28 colonies from the pooled samples. Furthermore we previously showed that individual members of the same animal species (including humans) may carry identical *E. coli* and enterococci clones (Vollmerhausen et al. 2011; Ahmed et al. 2005). Based on these studies we assumed that pooling samples from individuals of each species will add to the chance of detecting these dominant clones. The results proved that this was a valid assumption as indicated by the presence of persistent dominant clones across all samples. Despite this, the diversity of both bacterial groups was not affected upon storage, further indicating the stability of these populations upon storage.

The number of both C- and S-BPTs in some fecal samples did not change greatly after storage. This is mainly due to the presence and survival of the same dominant clones in the original and the stored samples. Certain S-BPTs however, were found in stored samples which were not detected in the original samples. This is due to the fact that we tested only 28 colonies from pooled faecal samples which contained S-

BPTs from many individuals and therefore the chance of picking the same S-BPTs in the stored samples is slim. We also postulated that this might be partly due to changes that might have occurred in the metabolic fingerprints of these bacteria as S-BPTs, in some samples showed high similarity, but not identity, to some of the BPTs in the original samples. Using the same biochemical fingerprint method, we previously showed that the degree of substrate utilisation by *E. coli* can slightly change upon subculturing, whereas storage at a different temperature did not seem to affect the BPT patterns (Katouli et al. 1990). Therefore, changes in the overall BPTs of some strains may not be the reason for having new S-types in our stored samples. Nonetheless, population similarity analysis between fresh samples and those collected at 20°C and 80°C showed that this change was not significant, and did not affect the overall structure of both indicator bacteria in fecal samples. This can be an advantage of using this method of analysis in conjunction with other methods to identify change that may occur in the population structure of these bacteria upon storage at different temperature.

In conclusion, we propose that in studies evaluating the population structure of *E. coli* and enterococci in fecal samples, storing samples at 80°C for up to 30 days would not change the overall population structure of these bacteria. This will allow researchers to collect as many samples as required and test them simultaneously to minimise the effect of inter assay variation. However, when the aim is to only count the number of these strains, tests should be done on fresh fecal samples as the number of these bacteria in stored feces, of some species, may vary upon storing.

Transparency Declarations

None to declare

Conflict of Interest

The authors declare that there is no conflict of interest with the organization that sponsored this research and publications arising from this research.

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Table 1: Mean population similarity (Sp-value) between *E. coli* or enterococci populations in fresh faecal samples and those preserved at -20°C and -80°C for 30 days.

Host species	Mean Sp-value					
	<i>E. coli</i>			enterococci		
	Fresh/-20°C	Fresh/-80°C	-20°C/-80°C	Fresh/-20°C	Fresh/-80°C	-20°C/-80°C
cow	0.479	0.363	0.319	0.183	0.258	0.312
human	0.301	0.479	0.589	0.490	0.309	0.404
chicken	0.331	0.391	0.338	0.113	0.128	0.309
pig	0.222	0.186	0.201	0.139	0.233	0.344
horse	0.406	0.422	0.545	0.136	0.149	0.452
dog	0.775	0.749	0.849	0.474	0.356	0.348
bird	0.294	0.855	0.272	0.473	0.457	0.423

No significant differences were found between the mean Sp-values of all compared populations; *E. coli* *P* = 0.3421 and enterococci *P* = 0.3161.

Table 2: Variations in the diversity of *E. coli* and enterococci phenotypes identified in the community faecal samples of each host species, before and after preservation for 30 days. *Median is calculated as 50th percentile.

Host	<i>E. coli</i>			enterococci		
	Fresh	-20°C	-80°C	Fresh	-20°C	-80°C
Cow	0.807	0.826	0.336	0.706	0.865	0.913
Human	0.840	0.508	0.635	0.831	0.897	0.944
Chicken	0.878	0.537	0.899	0.949	0.942	0.912
Pig	0.974	0.831	0.929	0.937	0.759	0.907
Horse	0.836	0.706	0.728	0.966	0.847	0.926
Dog	0.392	0.127	0.362	0.706	0.837	0.928
Bird	0.220	0.786	0.079	0.910	0.876	0.910
Median*	0.836	0.706	0.635	0.910	0.865	0.913

Legends to Figures

Figure 1. Mean number of *E. coli* in faecal samples before and after storage at 20°C and 80°C. Significant ($P < 0.001$) differences between the number of *E. coli* in fresh faecal samples and those preserved at 20°C (†), or 80°C (*); and between 20°C and 80°C (X). Significant ($P < 0.05$) differences between the number of *E. coli* in faecal samples preserved at 20°C (††) and 80°C (**).

Figure 2. Mean number of enterococci in fresh faecal samples prior to and after 30 days storage at 20°C and 80°C. Significant ($P < 0.001$) differences in the number of enterococci after storage at 20°C (†), or 80°C (*); and between 20°C and 80°C (X). Significant ($P < 0.05$) differences between the number of enterococci after storage at 20°C (††) and 80°C (**),

Figure 3. Representative dendrograms showing the stability of *E. coli* and enterococci in population structures in fresh human fecal samples (A and D) and after 30 days storage at 20°C (B and E) or 80°C (C and F), respectively. Twenty-eight isolates were typed prior to and after storage using the PhenePlate system™. The x-axis represents the degree of similarity between isolates, and those above the vertical dotted line (97.5%) belong to the same PhP type. Identical clonal groups found in the initial sample and subsequently recovered after storage have been identified by black, grey or white circles or squares.

Figure 1.

1 **Figure 2.**

2

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**Viability and stability of *E. coli* and enterococci populations
in faecal samples upon freezing**

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