Temporal Analysis and Molecular Characterization of Dominant *Enterococcus* Strains Present in Feces from Healthy Human Subjects

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ABSTRACT

The human intestinal microflora is an intricate environment where bacteria, viruses, fungi and the immune system interact. While the Enterococci are described as intestinal commensals, traits like the presence of chromosomal or mobile virulence factors and antibiotic resistance determinants have also lead to their classification as opportunistic pathogens. This group comprises around 1% of the total human intestinal flora and little is known about the population dynamics in terms of species or strain dominance. Although studies have focused on intestinal carriage and survival of specific strains (Sørensen, 2001; Lund, 2002; Sohn, 2013), there is little data on the natural population in healthy humans. Herein the dominant enterococci populations in healthy humans (not medicated for chronic illness nor antibiotic intake one month prior or during the sampling period) are described. These were recovered from stool samples of 3 healthy male subjects aged 18-25 years. A total of 140 isolates per subject were obtained during a sampling period of 7 consecutive days. Isolates were subjected to antibiotic resistance screening, molecular species ID, presence of virulence factors, plasmid families and strain characterization. Analysis showed that all three subjects carried different dominant enterococci but these were specifically limited to *E. faecalis* and *E. faecium*. Subject #1 was dominated by two distinct species, a commensal *E. faecium* or a Vancomycin Resistant *E. faecalis*. Only *E. faecalis* was present in Subject #2 and appears mostly commensal with *esp* as its sole virulence factor. However, for subject #3's isolates, we saw an increase in the pathogenic potential, a Tetracycline Resistant *E. faecalis* with the potential to carry Vancomycin resistance and 2 virulence factors. Data

suggests that the intestinal diversity of enterococci is subject dependent, as there are different dominant strains in each subject; from commensal strains to strains with a higher frequency of antibiotic resistance and virulence factors that are similar to clinical strains. The presence of pathogenic strains in healthy humans reinforces the importance of studying humans as antibiotic resistance reservoirs as well as the prudent use of antibiotics. Intestinal carriage of pathogenic strains and the high turnover rate of strains can be of importance as species of concern may not be detected during culture cased screenings carried out before surgical procedures.

RESUMEN

La microflora intestinal humana es un entorno complejo donde bacterias, virus, hongos y el sistema inmune interactúan directa o indirectamente. Los miembros del género *Enterococcus* son descritos como comensales intestinales, aunque la presencia de factores de virulencia y resistencia a antibióticos cromosómica o en elementos móviles, también los ha clasificado como patógenos oportunistas. Los Enterococos componen alrededor del 1% de la flora intestinal humana total y poco se sabe sobre la dinámica de la población en términos de dominancia de especie o de cepa. Los estudios se han centrado en la supervivencia y potencial colonización de cepas específicas (Sørensen, 2001; Lund, 2002; Sohn, 2013), sin embargo, hay pocos datos sobre la población natural en humanos saludables. Para describir el enterococo dominante en una población humana saludable, se realizó un muestreo de material fecal de 3 sujetos masculinos entre las edades de 19 a 25 años. Se obtuvo 140 aislados por sujeto durante un período de muestreo de 7 días consecutivos. Los seres humanos sanos, para este estudio, se definen como un sujeto sin enfermedad crónica medicada ni consumo de antibióticos 1 mes antes o durante el período de muestreo. El análisis molecular nos permite confirmar el género, determinar la especie, presencia de factores de virulencia, genes de resistencia antibióticos y determinar distintas cepas. Los resultados nos muestran que los tres sujetos poseían diferentes enterococos dominantes, limitados específicamente a *E. faecalis* y *E. faecium*. El sujeto # 1 estaba dominado por dos especies distintas, un *E. faecium* comensal o un *E. faecalis* resistente a Vancomicina. Sólo *E. faecalis* estuvo presente en el sujeto # 2 y semeja una cepa principalmente comensal, ya que no posee resistencia a antibióticos y posee *esp* como único factor de virulencia. Sin embargo, para los aislados

del sujeto # 3, vemos un aumento en el potencial patogénico con 84% de los aislados identificados como *E. faecalis* resistentes a la tetraciclina con el potencial de cargar resistencia a vancomicina y 2 factores de virulencia adicionales. Los datos sugieren que la diversidad intestinal de los enterococos es dependiente del sujeto, ya que vemos diferentes cepas dominantes en cada sujeto; Desde cepas comensales hasta cepas con una mayor frecuencia de resistencia a los antibióticos y factores de virulencia que son similares a las cepas clínicas. La presencia de cepas potencialmente patógenas en humanos sanos refuerza la importancia de estudiar a los humanos como reservorios de resistencia a los antibióticos. El transporte intestinal de cepas patógenas y la rápida sucesión de cepa dominante, pueden ser importantes cuando se someten a procedimientos quirúrgicos, ya que la presencia intestinal de estas cepas puede no salvaguardar la salud del paciente.

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Table of Contents

List of Figures

List of Tables

Introduction

The human gut or digestive system has been known to harbor a plethora of different microorganisms; one group of medical interests is the *Enterococci*. As a group, they are commonly isolated from animal and human feces, and are typically used as fecal indicators in water bodies by the Environmental Protection Agency (USEPA, 2002). Different *Enterococcus* species have been found in both salt and fresh waterways, and in varying concentrations depending on the contamination source. Studies based on animal population have also shown that enterococci composition in the intestine varies between species, for example, in ruminant animals *E. faecalis, E. faecium* and *E. avium* are amongst the most frequently dominant species in ruminants (Devriese, Laurier, Herdt, & Haesebrouck, 1992), while *E. casseliflavus* is found in over 40% of isolates from insects (Martin & Mundt, 1972). Human enterococci population have been described to be dominated by *E. faecalis* and *E. faecium* as determined by Fisher, cited by Klein, 2003. The most Frequently detected enterococci in humans,, *E. faecium* and *E. faecalis* are the two Enterococci that most are most frequently associated with nosocomial infections as these are hosts of various virulence factors and antibiotic resistance determinants (Mccormick, Hirt, Dunny, & Schlievert, 2000). These traits also influence their frequent appearances as antagonistic bacteria in locations other than the human GI tract (Jett, Huycke, & Gilmore, 1994).

Enterococci are members of the Lactobacillus group, which consists of less than 1% of the total human intestinal microflora. Some *Enterococcus* species, like *E. faecium* are of importance in the food industry as they are used as fermentation starters, which may lead to

their dispersal trough the human food chain (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). However, enterococci used should harbor no plasmids, virulence factors, antibiotic resistance or have low pathogenic potential in order to be used in the food industry. This suggests that we may be ingesting enterococci as part of our regular diet. Some researchers have found that enterococci present in food, are able to survive digestion and can be detected in feces afterwards (Lund, Adamsson, & Edlund, 2002; Sørensen et al., 2001). The focus of this study was to determine the range of strains and species stability of Enterococci in the human gut, and as such *E. faecalis* and *E. faecium* are expected as the dominant species in the samples. Our data indicates that both species are present in the human intestinal tract and could have the potential to be pathogenic given the opportunity. However, stability of the *Enterococcus* genus in the intestine has not been completely evaluated; whether this group has a transient or variable composition, or if strains colonize and persist for extended periods of time remains to be determined. To evaluate this stability, an analysis was conducted that focused on the species diversity, virulence factors and antibiotic resistance of isolates obtained from 3 healthy human subjects during a 7-day sampling period.

Literature Review

The Enterococci are gram-positive, catalase-negative, aerotolerant, obligate fermenters, non-sporulating bacteria that belong to the Lactobacillales group, (Fisher & Phillips, 2009). They are capable of growth at 6.5% salt concentrations, pH ranges from 3.5-9.6, temperatures of up to 45°C and capable of aesculin hydrolysis (Manero & Blanch, 1999; Sherman, 1937). Considered a nearly ubiquitous genus, two species, *E. faecium* and *E. faecalis*, are the most frequently detected in the human gastrointestinal tract (Klein, 2003). However, they compose around 1% of the gut microflora (Eckburg et al., 2005)

It is known that *E. faecalis* is found in human feces ranging from 10^5 to 10^7 CFU per gram, and *E. faecium* at a lower concentration, between 10^4 and 10^5 CFU per gram (Franz, Holzapfel, & Stiles, 1999). While this is an average value, *Enterococcus* species concentrations may vary between individuals and geographical location as observed by Kühn (2003). After analyzing around 2,868 *Enterococcus* samples from different sources, including healthy individuals, hospitalized patients, clinical isolates, sewage, the environment and from animals, in Denmark, UK, Spain, and Sweden; they observed different ratios of *E. faecalis* and *E. faecium* depending on geographical location, source and medical condition (Kühn et al., 2003). Nevertheless, studies show that these microorganisms are always present in our bodies, normally in the intestine, and could become an opportunistic pathogen if given the appropriate conditions, for example, a suppressed immune system (Murray, 1990). Due to their constant presence in the human

body, it is important to identify factors that could have an impact on health, such as virulence factors and antibiotic resistance.

The isolation of the first *Enterococcus* came from an endocarditis patient in 1906, and was originally identified as *Streptococcus faecalis* by Andrew and Morder (Fisher & Phillips, 2009). This drew attention to its relevance in infections and virulence potential. Since then, studies have shown that there has been over a 20-fold increase in nosocomial infections caused by *Enterococcus* in the last 15 years (NNIS, 2004*)*. According to the World Health Organization (WHO), a nosocomial infection is defined as, "an infection acquired in hospital by a patient who was admitted for a reason other than that infection". Most enterococcal nosocomial infections are caused by *E. faecium* or *E. faecalis (*WHO, 2002)**.** Members of the *Enterococcus* genus have been shown to possess various genetic markers associated with virulence factors such as, *esp* (extra-cellular surface protein); *asa1* (aggregation substance); *cylA* (a proteolytic activator of the toxin cytolysin; *hyl* (a hyaluronidase) and *gelE* (a gelatinase-collagenase) among others (Borgmann et al., 2004; Vankerchkhoven, 2004). In general, these virulence factors are responsible for facilitating adherence and tissue colonization therefore enhancing pathogenicity (Borgmann et al., 2004). Some factors like *esp,* are in pathogenicity islands and may vary between species, for example, coding sequences and expression of *esp* production on *E. faecium* can be different from that of *E. faecalis* (Leavis et al., 2004). Moreover, studies have shown that *E. faecalis* strains possess a higher probability of carrying virulence factors over *E. faecium* and other enterococci species (Huycke, Sahm, & Gilmore, 1998).

The genus *Enterococcus* is also notorious for its intrinsic resistance to a wide range of antibiotics from β-lactams to low level resistance to aminoglycosides (Murray, 1990). This resistance to most antibiotics has led to the use of Vancomycin as a last resort for treatment against infections. Excessive use of vancomycin has caused the emergence of vancomycin-resistant enterococci (VRE) in both the US and UK (Woodford, Johnson, Morrison, & Speller, 1995). The proportion of VRE from blood isolates has doubled in 5 years from 13% to 26% between 1995 and 2000 in the US (NNIS 2000). Nosocomial infections caused by VRE may have up to 52% mortality, twice more than susceptible strains (Brown et al., 2006). Mortality from bacteremia caused by a vancomycin resistant strain is higher (75%) than if caused by a vancomycin susceptible strain (45%) (Bearman & Wenzel, 2005). Enterococci isolated from clinical settings have different antibiotic resistances than those obtained from food products (Klein, 2003). On a general basis, *E. faecium* strains tend to possess a higher intrinsic antibiotic resistance than other enterococci (Routsi et al., 2000). Because enterococci species inhabit the intestine, some researchers have hypothesized that this intestinal burden is responsible for most nosocomial infections, due to microbial translocation (Berg, 1996). However, there are others that state, as summarized by Gilmore, Clewell, and Ike *et. al.* (2014); 'More commonly, however, infection results from the colonization, overgrowth, and translocation of hospital-adapted antibiotic-resistant strains with enhanced pathogenicity. This suggests that there is not one exclusive method of infection.

Ingested *Enterococci* have been proven to survive digestion and can be detected in feces at varying concentrations depending on the bacterial load ingested. This was observed in Bodil Lund's study (2001) when 20 subjects ingested a known probiotic strain of *E. faecium* for 10 days and that probiotic strain was recovered in feces during ingestion of the product. They selected for *E. faecium* using arabinose agar plates, as *E. faecalis* cannot use it as a substrate. Utilizing a *Sma*I (restriction enzyme) PFGE protocol they analyzed the total population of *E. faecium* from the various subjects and were able to distinguish between the probiotic strain and other strains present in feces. After day 10, which coincided with cessation of ingestion of the probiotic, they were not able to detect the probiotic strain, but other *E. faecium* strains were present, after PFGE analysis 106 distinct strains were detected. While the focus of this study was to observe digestion survival of the probiotic strain, it suggests that their strain did not colonize the intestines for a prolonged time. The presence of around 106 different *E. faecium* strains isolated, between 8 subjects, also suggests a high diversity of recoverable strains from feces limited to *E. faecium*.

Another study that focused on *Enterococci* interactions in the intestine was done by Thomas Lund Sørensen (2001). This study focused on the intestinal carriage of antibiotic resistant *E. faecium* after ingestion. Of the 18 subjects used for the study, 6 ingested a mix of two glycopeptide resistant strains, 6 a streptogramin resistant strain and the remaining ingested a strain susceptible to both vancomycin and streptogramin. Subjects ingested an oral suspension of 10^7 CFU in 250 mL of milk of different *E. faecium* strains to observe persistence in the human gastrointestinal tract. One difference from this study, when

compared to Bodil Lund's methodology, is that subjects were only given the known *Enterococcus* once on day 1. They could detect the different antibiotic-resistant strains in feces for up to 6 days after ingestion, which occurred on day 1 (Sørensen, 2001). *Enterococcus* quantity was diminishing everyday up until day 6, where all but one subject had no detectable amounts of the ingested antibiotic-resistant *Enterococcus* strain. The average amount of *Enterococcus* was between 10^4 to 10^8 CFU on days 2 and 3. Their data shows us that ingested *Enterococcus* could survive ingestion, survive in the gastrointestinal tract and that no long-term colonization occurred since the known *Enterococci* strain was not detected on all but one subject after day six, although they were selecting for the antibiotic resistance strains by giving the appropriate antibiotic to the volunteer and using PFGE to screen for the pattern.

A study done by Sohn *et. al.* (2013), focused on analyzing Vancomycin-resistant *Enterococci* (VRE) in patients from outpatient clinics through stool and anal swab sampling. They could detect VRE for an average of 5.57 weeks after being discharged from the primary care unit and subsequent treatment in the outpatient clinics (Sohn *et al.*, 2013). While their study is directed toward analyzing risk factors associated with increasing the carriage of VRE, they suggest that this median time of 5.57 weeks is indicative of colonization by the VRE. An important aspect is that they do not focus on differentiation between VRE from both the subjects and between sampling events. This raises the question: Do *Enterococci* colonize, could re-inoculation have played a role or did plasmid harboring Vancomycin-resistance genes transfer between strains? Some Vancomycin

resistance genes have been located in mobile plasmids, specifically *VanA* and *VanB* which are most commonly found in *E. faecalis* and *E. faecium*, with *VanA* possessing the highest transferability (French, 1998). In hindsight, analysis of the present VRE strains could have been used to determine whether the VRE strain detected on day 1 was the same one present for 5.57 weeks. A limit to their methodology is that they are using a growth media with antibiotic (Vancomycin) and are not able to determine if the isolated VRE are the dominating strain or if they are present in smaller concentrations.

Enterococcus, as the central focus of scientific research, has been growing in importance over the last decades given the role that they play in nosocomial infections. The main purpose of this study is to characterize the dominant enterococcal strain present in healthy human feces. A complete analysis of the daily dominant strain is necessary, not only because the information available is insufficient, but also because this will help us understand how this genus behaves in the human intestine. The study done by K.M. Sohn (2012) is focused on VRE and after observing their methodology, there is a growth medium bias, selecting for the antibiotic resistant strains utilizing growth media supplemented with the antibiotic in question, as opposed to the dominant strain as this study. Another missing piece of information from their study is that they only analyzed vancomycin resistance as a phenotype, no genetic analysis was mentioned, and there was no method to differentiate between strains observed from day 1 to those from 5 weeks later. Molecular analysis will determine the species of the dominant strain and through BOX PCR differentiate between isolates from the sampling period. This data is important because it can determine the

diversity of strains present during the sampling period. Identification of one or more different strains per sampling episode could suggest a transient organism, one that does not colonize.

One area that's gaining importance is the analysis of plasmids characterized from pathogenic and commensal strains of enterococci. Carriage of transferable antibiotic resistance and virulence factors increases the potential pathogenicity of strains due to the possibility of conjugation events. Jensen et al. (2010) proposed a classification system for plasmids obtained from enterococci utilizing conserved areas of the replication initiation genes. This Rep gene PCR can help identify plasmid family's presence and correlate with antibiotic resistance and virulence factors from similar strains. Rep families may be shared between species, as is the case with Rep-2 which is found on both *E. faecalis* and *E. faecium*, while some like Rep-9 are mostly limited to *E. faecalis* and correspond to a family of pheromone responsive plasmids. Analysis of rep families and subsequent subgroups adds another layer to the differentiation of strains. However, it is important to note that there may be more than one rep family identified per isolate as some strains, such as the infection derived *E. faecalis* V583 which harbors three different plasmids.

Objectives

The focus of our study is to characterize the dominant culturable *Enterococci* population obtained from feces of healthy humans. One focus is to observe the changes in dominant *Enterococci* strains through the sampling period. Molecular analysis was used to determine the species, determine the dominant strains and compare between isolates between subjects. The length of our sampling period allows us to determine how many strains can be recovered and the isolation frequency. Objectives are:

1. To conduct the biochemical and molecular characterization of the dominant *Enterococcus* strains in feces of 3 healthy volunteers.

2. To compare virulence factors and, antibiotic resistance profiles, as well as Box PCR genomic fingerprints across the isolates, in order to identify the dominant strains among the tested individuals during a sampling period of 7 days.

Materials and Methods

1. Biochemical and molecular characterization of the dominant *Enterococcus* strains in feces from healthy volunteers.

Volunteer Requirements

Three healthy volunteers served as stool donors during a sampling period of 7 days. A "healthy volunteer" is defined as a subject that has not undergone antibiotic treatment at least a month prior to the stool sampling. No antibiotic usage during the stool sampling must also be upheld as a bias will be created towards any resistant cells. Any event of loose stools or constipation was also recorded and labelled as such, to determine differences if any event occurs. Information on subjects is presented in **Table 1** with Gender, Age, and Sampling Date. No other personal data was required from the subjects, they were informed how their samples were to be analysed and processed. Protocols were submitted to the IRB and research was exempt from further review.

Table 1. Stool sampling period and Subject Information (Age and Gender).

Subject	Gender	Age	Sampling Date
1	Male	24	March 30 – April 5, 2015
2	Male	19	July 22 – July 28, 2015
3	Male	25	December 10 - December 16, 2015

Sample Collection

To ensure that stool samples were collected aseptically in the home of the voluntary donors, the donors were given instructions and supplied with sterile materials. The donors were instructed to deposit the samples on a sheet of sterile (autoclaved) aluminum foil, which would rest on top of a "hammock" made from newspaper. The newspaper hammock was placed inside the toilet bowl to ensure that the stool sample was only to be in contact with the sterile surface of the aluminum foil, while avoiding contact with the bowl water. Stool samples were aseptically collected utilizing a standard sterile stool sampling cup. Samples were obtained in 24 hour periods from the first sampling date. The study consisted of a continuous 7-day sampling period per subject. Samples were processed on the same day when possible, if not, they were stored in the refrigerator for a maximum of 24 hours, but fresh processing was prioritized. Afterwards, 1g of fresh sample was weighted in a sterile test tube with 10mL of sterile 100mM PBS (pH 7.0, 100mM KH_2PO_4, 100mM NaCl) with five 3.5 mm glass beads (Cat. No. 11079135, Bio Spec Products). Homogenization of the sample consisted of applying "vortex" to the tube using the glass beads (Bead-beating), and manual homogenizing using a sterile wooden applicator. After homogenization, serial dilutions were prepared up to 10^{-7} using the same PBS preparation. Excess material was discarded down the toilet and the container was placed in a biohazard waste and disposed following institutional guidelines.

Sample Processing

Dilutions 10^{-4} through 10^{-7} were used for plating and subsequent tests. Sampling method was used in accordance to US EPA method 1600 (USEPA, 2002), but using mEnterococcus Agar (Difco, BD Scientific) instead of mEI (membrane-Enterococcus Indoxyl-ß-D-Glucoside Agar). This method consisted of filtering a known amount of sample, in our case, using 1 mL of the selected dilution using 25 mL of sterile 1X PBS [pH 7.0, 100mM KH_2PO_4, 100mM NaCl] as buffer, through a 47 mm 0.45µm membrane filter (Cat. No. (28148-399) Pall Life Sciences), and placing the filter on a plate of mEnterococcus Agar. Enumeration was done after 48 hours at 41°C and presumptive enterococci should appear as dark red to pink colonies. Colony quantification data was presented as colony forming units per mL (CFU/mL). Isolate enumeration was averaged between triplicate filters and the standard deviation was calculated. Twenty isolates were selected at random from each sampling day and were used for all subsequent tests, for a total of 140 isolates from each subject per sampling period. All isolates were stored in glycerol stocks at -20˚C for safe keeping and future use.

Enterococci identification

Identification of presumptive enterococci was confirmed utilizing various biochemical tests. Individual and isolated presumptive colonies from the filters were transferred to a brain heart infusion (BHI) agar plate and incubated at 41°C overnight. The

following morning, individual colonies were tested for the presence of catalase using a glass slide and a commercial 3% hydrogen peroxide solution, using *Staphylococcus aureus* as catalase positive control. All negative isolates were then transferred to BHI with 6.5% NaCl, Bile Esculin Agar (BEA), Sulfate Indol Motility media (SIM) and BHI incubated at 45°C. Positive reactions and growth in every medium or condition further confirmed the presumptive status of the isolates. Motility and pigmentation was used to predict possible species, *E. faecalis* and *E. faecium* are both non-motile and non-pigmented, observation of yellow pigmentation and/or motility was used to predict other species, for example *E. casseliflavus*, is both pigmented and motile and may be present in the human intestine, although at very small concentrations (Manero & Blanch, 1999). Molecular characterization was used to identify the species isolated and the diversity of strains within any given species (details in PCR section for characterization of presumptive Enterococci).

DNA Extraction

DNA was extracted using a CTAB based protocol (Doyle, 1987) and the addition of egg white lysozyme (5mg/ml). The cells, obtained from a 3-ml overnight culture grown on BHI, were precipitated by centrifugation (at 13K rpm for 5 min, 1.5ml at a time) and resuspended in 500ul of 2X CTAB Buffer [2% CTAB, 2M NaCl, 10mM EDTA, 50mM Tris, pH 8.0]. The pellet was homogenized and then 150 µl of lysozyme solution was added to each tube and incubated in a water bath for 1 hr at 37°C. Using another set of tubes, supernatant was transferred and added 500 µL of chloroform and mixed by inversion for 10

seconds, then centrifuged for 15 minutes at 13K RPM. Using the last set of tubes transferred the supernatant and the same amount of cold 100% isopropanol was added, finally 0.1 volume of Cellgro® 3M Sodium Acetate (pH 5.2^{+}/- 0.1, Cat. No. 46-033-Cl VWR) was also added. Tubes were inverted slowly and left them for 24 hrs at -20°C to allow proper precipitation. After removing the tubes from the freezer, and centrifuging for 15 minutes at 13k RPM, the supernatant was discarded and added 100 µl of cold 70% ethanol followed by slow inversion and centrifuged for 1 minute at 13k RPM. The ethanol was discarded, and the tubes were left to dry in the biological hood until the alcohol evaporated. Finally, the DNA was suspended in 100 µl of TE Buffer (10mM Tris-Cl, 1.0mM EDTA, VWR, Cat. No. T0225 pH 7.0) and stored at -20°C. DNA concentration was measured with a Nano-Drop Spectrophotometer 1000 (Thermo Scientific).

2. To assess enterococcal population dynamics in the human gut in each timeframe

***Enterococci* species identification through PCR**

Enterococci genus confirmation was performed using a PCR protocol described by Ke *et al*. (1999). *Enterococcus* genus was determined amplifying the Tu Elongation Factor (EF-Tu) gene; a 112-bp PCR product is considered positive. Our PCR mixture was composed of 22.8µL ddH_2O, 10.0 µL 5X Buffer, 5.0µL dNTP's (2.5mM each), 5.0 µL MgCl2 (25mM), 2.5 µL forward primer (20µM), 2.5 µL reverse primer (20µM), 0.2µL Promega Flexi Taq Polymerase (5U/µl) per reaction and 2.0 µL DNA. PCR product size

was confirmed on a 1.8% agarose gel, utilizing a 100-bp DNA ladder, at 100V for 90 minutes, stained with ethidium bromide and documented with a VersaDoc MP4000 Imager system. After confirmation of *Enterococcus* genus using the *tuf* gene PCR, species identification was done utilizing, a species-specific multiplex PCR, which analyzes the *A* segment of the superoxide dismutase gene, *sod*A (Jackson, Fedorka-cray, John, & Barrett, 2004). Specific-specific primers amplify different segments of the *sod*A gene. This multiplex PCR can identify 27 enterococci species, which are separated by groups, by using the previous biochemical methods *Enterococci* are grouped by their pigmentation and SIM results and use that data to select which group our presumptive enterococci would fall into. A similar PCR mixture to the one presented by Jackson, consisting of 22.75 µl of ddH_2O, 10µl of 5x *Taq* polymerase Buffer (Promega), 5 µl of [25mM] $MgCl_2$, 5 µl of [16mM] Ammonium Sulfate, 2 µl of [2.5mM] dNTP's, 0.625 µl of *E. faecalis* primers and 1.25 µl of *E. faecium*, both forward and reverse (20µM), and finally 0.5 µl of *Taq* polymerase (Promega) for a total of 49 µl. DNA template used was 1µl of 50 ng/µl aliquots of DNA per tube from the isolates. Primer amplification of *E. faecalis* corresponds to 360 bp and *E. faecium* to 215-bp compared to the 100-bp DNA standard. Standard PCR product size was confirmed on 2% agarose gel at 100V for 90 minutes, stained with ethidium bromide and documented with a VersaDoc MP 4000 Imager system.

Virulence Factor Identification

After isolate species were determined, the next phase was to determine the presence of virulence factors. This technique allows for the categorization of the samples from different subjects and to observe the frequency and variation of these virulence factors in human fecal enterococci. Virulence factor presence was determined using the method described by Vankerckhoven et al. (2004). Presence of virulence factors is another way of differentiating isolates, while also describing the potential pathogenicity of the isolated strains. Identification was done using a multiplex PCR identifying 5 different virulence genes *asa1* (375-bp), *gelE* (213-bp), *cylA* (688-bp), *esp* (510-bp) and *hyl* (276-bp) using a 100-bp ladder as the DNA standard. Amplicon size was determined on a 1.8 % agarose gel electrophoresis at 100V for 90 minutes, stained with ethidium bromide and documented with a VersaDoc MP 4000 Imager system.

Strain comparison of Enterococci between sampling dates:

To compare between same species samples, Box PCR was used to determine strains and differentiate between isolates (Brownell et. al., 2006). This helped us differentiate between same species isolates, because different strains will yield different amplification patterns, when visualized on the agarose gel. The following recipe was used for 1X reactions, with one difference being the use of Box A1R primer instead of Box A2R, 9.5 µl of ddH_2O, 5.0 µl of 5X *Taq* Polymerase buffer, 3.5 µl [25mM] $MgCl_2$, 2.5 µl of [2.5mM]

dNTP's, 0.25 µl of BSA (Bovine Serum Albumin), 2.0 µl of 10µM BOX A1R primer, and finally 0.25 µl of *Taq* Polymerase from Promega, the master mix total is 23 µl. Isolate DNA per tube was 2 ul, for a better resolution DNA aliquots with a 50 ng/µl concentration were used. PCR product was observed in a 2% agarose gel electrophoresis, stained with ethidium bromide, for 240 minutes at 60V, using a 1-kb ladder (New England Bio-Labs) as the DNA standard. Visualization was done by VersaDoc MP 400 imager system (Bio-Rad Systems).

Plasmid Detection using *rep* gene PCR

Classification of plasmids can help determine source and identify possible genes present in these. *Rep* family classification uses different genes associated with plasmid replication to identify plasmid presence. These *rep* genes could be a topoisomerase, primase or replication proteins. The use of plasmid identification PCR based on *rep* families helps to increase the level of differentiation for isolates that harboured determinants, such as Tetracycline and Vancomycin resistance and mobile virulence factors like *asa1* and *cylA*. Out of the 19 *rep* families described (Jensen et al., 2010), only 4 were selected for isolates analysis, *rep2, rep7, rep17* and *rep9* with its subgroups (pCF10, SubG, and pTEF2). Base PCR mix consisted of 2.5 µl of $MgCl_2$ [25mM], 5X Taq Polymerase Green Buffer (New England Biolabs), 2.5 µl dNTP's [2.5mM], 1.25 µl of Primer [20µM], 0.5 µl of Taq Polymerase [5U/µl] (New England BioLabs) and completed with sterile deionized water for 24 µl per reaction, and 1µl of DNA template was used. PCR amplicon

size was compared to 100-bp ladder standard. PCR product was run on a 1.8% agarose gel for 90 minutes at 100V, stained with ethidium bromide and visualized on a VersaDoc MP400 Imager System.

Rep 9 subgroups were also analysed to determine specific plasmid families. PCR master mix preparation was identical to that used for *rep* genes using primers for identification of SubG (167-bp), pTEF2 (195-bp) and pCF10 (210-bp) subgroups. PCR amplicon size was compared to 100-bp ladder standard. PCR product was run on a 1.8% agarose gel for 90 minutes at 100V, stained with ethidium bromide and visualized on a VersaDoc MP400 Imager System.

Tetracycline resistance genes

Tetracycline resistance genes were identified using the Multiplex PCR protocol and primers described by (Ng, Martin, Alfa, & Mulvey, 2001). Tetracycline resistance genes, L (267-bp), M (406-bp), O (515-bp) and S (667-bp) were analysed. Amplification was compared to a 100-bp DNA standard (New England Biolabs). PCR product was run on a 1.8% agarose gel for 90 minutes at 60V, stained with ethidium bromide and visualized on a VersaDoc MP400 Imager System.

Results

Enterococci Quantification

Quantification of Enterococci obtained from stool samples vary greatly in orders of magnitude between subjects and between samplings of the same subject. A study done by Franz (1999) determined that, on average, *E. faecalis* is found in human feces ranging from 10^5 to 10^7 CFU per gram, and *E. faecium* at a lower concentration between 10^4 and 10^5 CFU per gram. Total enterococci population from subject #1 hover around 9.78E+07 CFU. On average for subject #2, the total population hovered around 9.15E+07, while Subject #3 on the other hand, harboured the lowest number of recoverable enterococci population, at an average of 6.84E+06 CFU (**Figure 1**). Average enterococci population are around 6.17E+07± 4.83E+7 CFU between all three subjects. When analysed by species it was determined that *E. faecium* population was around 2.46E+07 and *E. faecalis* was 1.08E+08 from subject #1. While total population varied between subjects, *Enterococci* were present and recoverable. Recovered species diversity was limited to the dominant species already described.

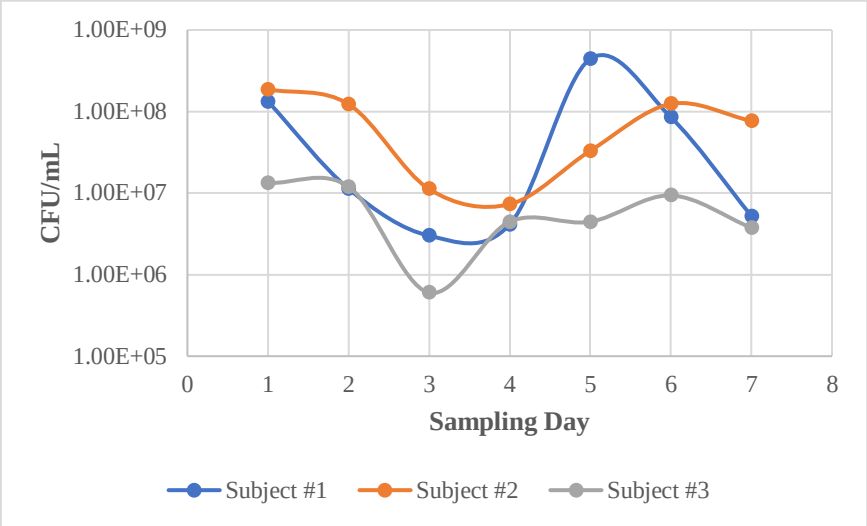


Figure 1. Average most abundant cultivable enterococci population isolated from fecal matter obtained from 3 healthy male subjects. Enumerated from triplicate filters on mE using serial dilutions of the homogenized solid samples.

Enterococci Identification

After biochemical tests and *Tuf* PCR confirmed the genus (Ke et al. 1999), all isolates were analysed using the Multiplex PCR described by Jackson et al. (2004) to determine their species. An overview of both protocols can be seen on **Figure 2** and **Figure 3** respectively. Only two species were recovered during our samplings, *E. faecalis* and *E. faecium*. No other species were present at the highest dilutions, although others could be present at lower concentrations in the human gut (not part of our objectives).

Figure 2 *Tuf* gene PCR for Enterococcus Genus confirmation. Amplification at 112 bp confirms enterococcus genus after biochemical testing was finalized; lane 9, 100 bp DNA standard on 1.8% agarose gel ran at 100V for 60min.

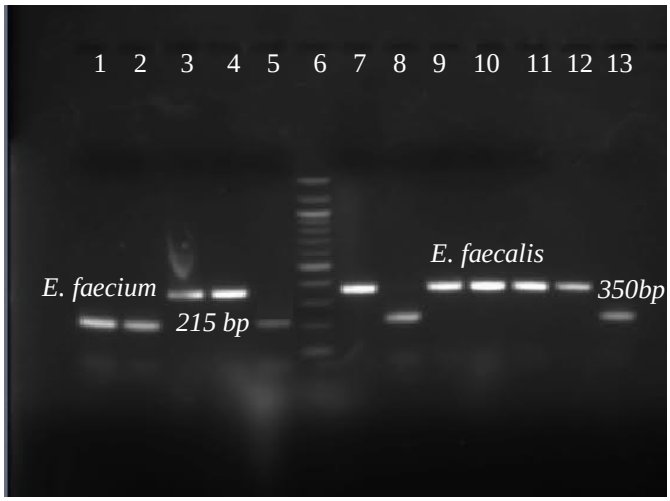


Figure 3 Multiplex PCR for Enterococci Species Detection. Overview of *E. faecalis* and *E. faecium* amplification at 350 and 215 bp respectively; lane 6, 100 bp DNA standard on 1.8% agarose gel ran at 100V for 60min.

A shift in species dominance was observed through the sampling event of subject #1 and #3, which was limited to two species *E. faecalis* and *E faecium*. Enterococci population for subject #2 was dominated by *E. faecalis* for the totality of the event. While subject #3, like subject #1, has a change in dominating species during the sampling event, from *E. faecalis* to *E. faecium.* Changes in species composition by day can be seen in **Figure 4**, however, total species shifts are not seen in any of the subjects.

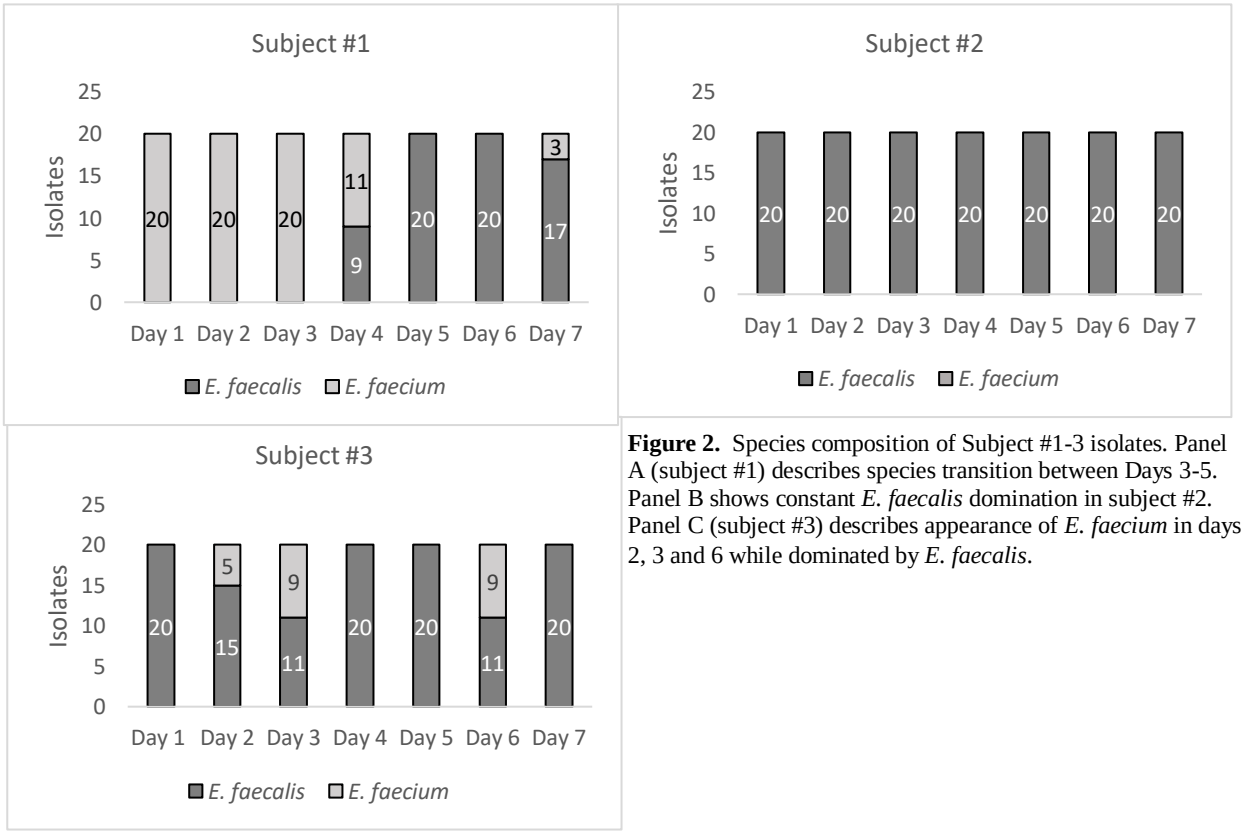


Figure 2. Species composition of Subject #1-3 isolates. Panel A (subject #1) describes species transition between Days 3-5. Panel B shows constant *E. faecalis* domination in subject #2. Panel C (subject #3) describes appearance of *E. faecium* in days 2, 3 and 6 while dominated by *E. faecalis*.

Antibiotic Resistance

Multidrug-resistant enterococci are of great clinical importance given their role in nosocomial infections in health clinics and hospitals around the world. Each sampled Enterococci was tested with four different antibiotics (Rifampicin, Tetracycline, Piperacillin and Vancomycin) using the Kirby-Bauer disk diffusion method (Bauer, 1966). With each of the four antibiotics tested, changes were observed between resistant and susceptible isolates during each sampling event and antibiotic resistance was present in all subjects at different ratios (**Figure 5**). Subject #1 had two isolated species, *E. faecium* and *E. faecalis* during the 7-day sampling period. Of the 70% (98/140) Rifampin resistant isolates, 62% (61/98) were identified as *E. faecium* with the remaining 38% (37/98) identified as *E. faecalis.* Medically significant Vancomycin resistance was found at a higher percentage on *E. faecalis* 73% vs *E. faecium* 27% isolates, out of a total of 64 vancomycin resistant isolates between both species. Tetracycline resistance was also found at a higher percentage in *E. faecalis* than in *E. faecium,* 15 vs 9 resistant isolates. No significant Piperacillin resistance was detected on subject #1's isolates, only present on 2 *E. faecium* isolates. In subject #2, Piperacillin resistance was found on 41 *E. faecalis* isolates, while for subject #3, 17 *E. faecium* and 46 *E. faecalis* harboured this resistance.

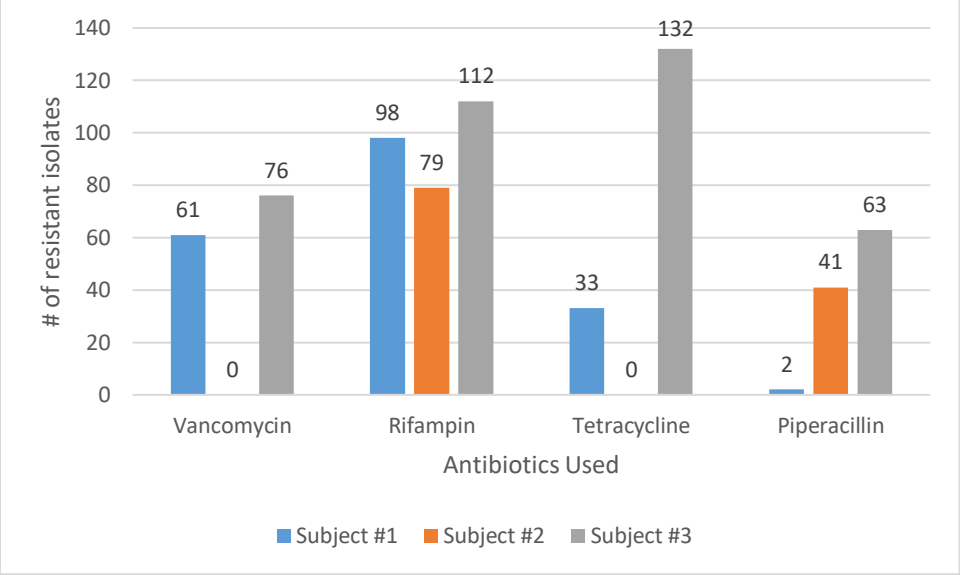


Figure 3. Enumeration of antibiotic resistant isolates obtained from healthy humans using the Kirby-Bauer plate method.

In contrast with subject #1, no isolates from subject #2 harboured vancomycin or tetracycline resistance. Vancomycin and Tetracycline phenotypes for subject #2 were highly consistent across the entire event, with a susceptible classification for both. Another shift was noted from a Piperacillin resistant population towards a susceptible one, while the phenotype for Rifampicin resistance was the most stable of the four. For subject #1 however, there is a very different panorama, since there is change in species, in contrast to subject #2, there is a higher amount of antibiotic resistance patterns. Out of 140 sampled isolates from subject #3, medically significant antibiotic resistance is present at ratios similar to infection derived or clinical isolates, as there are isolates with 3 or 4 resistances. Tetracycline resistance was present on over 90% of the isolates, while over 30% for Vancomycin, around 40 isolates were resistant to all antibiotics used. Subject #3 harboured the highest frequency of antibiotic resistance out of the three volunteers.

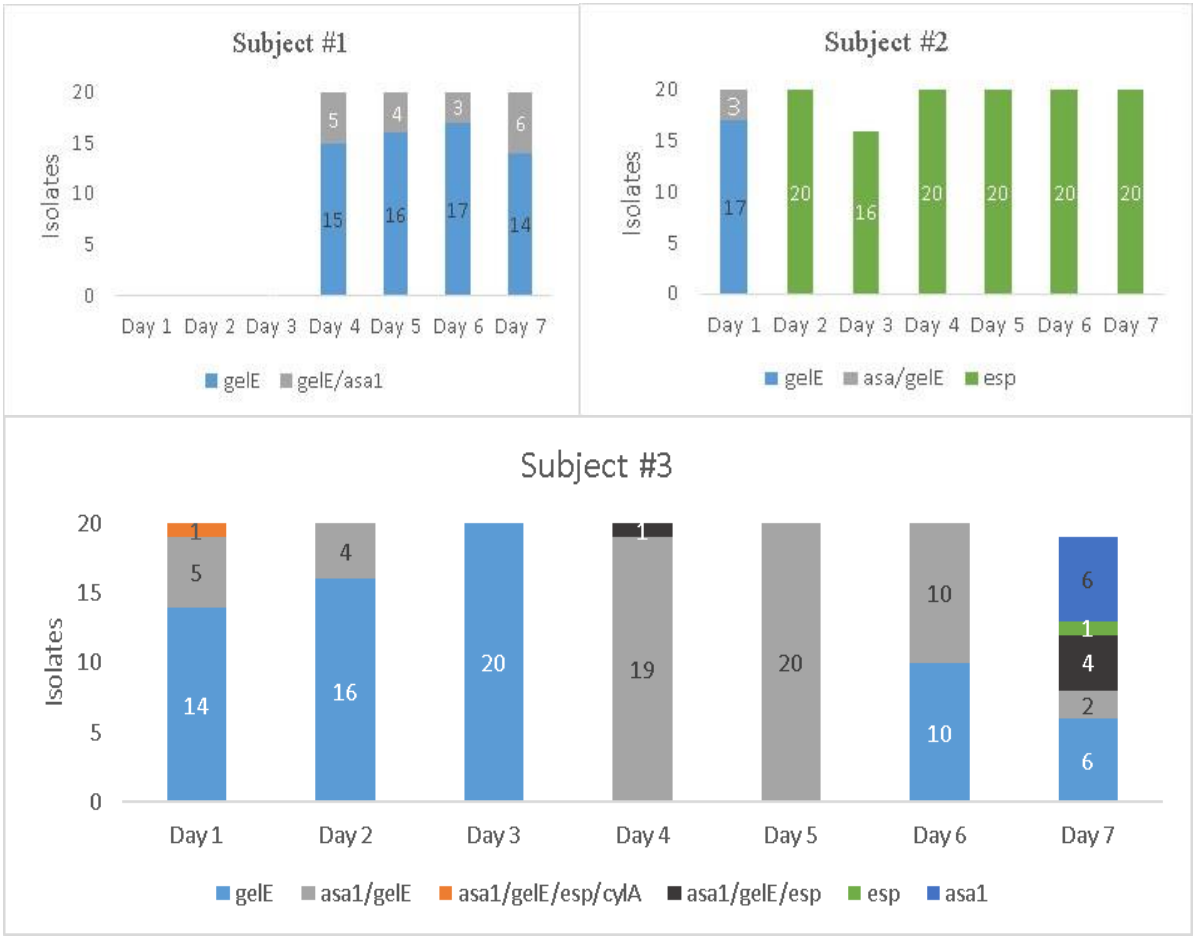
Virulence Factors

Determination of virulence factors from isolates adds another layer of characterization to the investigation. Four of the five virulence factors used for the multiplex PCR in some isolates; *esp, gelE, cylA* and *asa1* were identified, while no isolates were positive for *hyl*. Presence of virulence factors varied between species and subjects. Subject #1 only harboured isolates with at most two virulence factors, *asa1* and *gelE*. Subject #2's isolates harboured at least three different virulence factor patterns, with *gelE*, *asa1/gelE* and *esp* patterns. Patterns observed from subject #3's isolates are similar to those from other subjects with only one new pattern appearing with 4 virulence factors, *asa1/gelE/esp/cylA*, which was determined to be *E. faecalis*. Enumeration of virulence factors obtained from all three subjects and their distribution by species can be found on **Table 2**.

Table 2 Species and virulence factors of isolates obtained from the stool sample of 3 healthy male subjects. N = number of isolates tested.

Subject	Species	N	asa1	gelE	esp	cylA	asa1/gelE	asa1/gelE/esp	asa1/gelE/esp/cylA
1	*E. faecalis*	66/140	0	51	0	0	14	0	0
	E. faecium	74/140	0	8	0	0	4	0	0
2	*E. faecalis*	140/140	0	20	116	0	3	0	0
3	*E. faecalis*	117/140	6	44	1	0	56	5	1
	E. faecium	23/140	0	16	0	0	3	0	0

Virulence factors were not detected on isolates from subject #1 during the first 3 days, which were dominated by *E. faecium*, isolates from days 4 to 7 were mostly *gelE* positive (69/80) while the remaining were *asa1/gelE* positive (11/80), and both *E. faecium* and *E. faecalis* were present. Subject #2's virulence patterns were identical from day 2 onwards, only day 1 harboured *gelE* (18/20) and 3 *asa1/gelE* isolates, day 2 to 7 were *esp* positive (116/120). Patterns obtained from isolates of subject #3 were all *gelE* positive, however different patterns were obtained, such as those harbouring 4 virulence factors (*gelE*, *asa1*, *esp* and *cylA*), as shown on **Figure 6**.



Box PCR Results

Analysis of the isolates using BOX PCR allows a comparison between sampling days and subjects. Subject #1's analysis showed different patterns between species as was expected, no pattern was the same between *E. faecalis* and *E. faecium.* Subject #1 harboured four distinct dominating patterns for *E. faecalis.* These 4 patterns were identified between days 4 through 7, as days 1- to 3 were dominated by *E. faecium.* However, for *E. faecium* isolates, 5 different patterns were present between days 1 to 4 and during day 7.

Subject #2 appears mostly homogenous with no medically significant antibiotic resistance and only one virulence factor (*esp*) present after day 2 through day 7. Box patterns for days 2 to 7 appear similar, with most patterns only differing by one or two bands, which by our standards constitute a different strain. In total, d 4 different *E. faecalis* strains were identified, where Day 1 harboured *asa1*/*gelE* virulence and day two onwards the population was mostly consistent with *esp* as the sole factor.

Box Patterns for subject #3's *E. faecalis* isolates are unique to this subject, although some patterns are shared between days 1 to 6. Patterns of virulence throughout the sampling event where very similar among isolates, which may correlate with the presence of *gelE* on 94% of the isolates. Five different BOX patterns for *E. faecalis* were isolated between days 1 to 7. For *E. faecium*, only 3 different patterns were observed, suggesting less variance than *E. faecalis* patterns compared to other subjects.

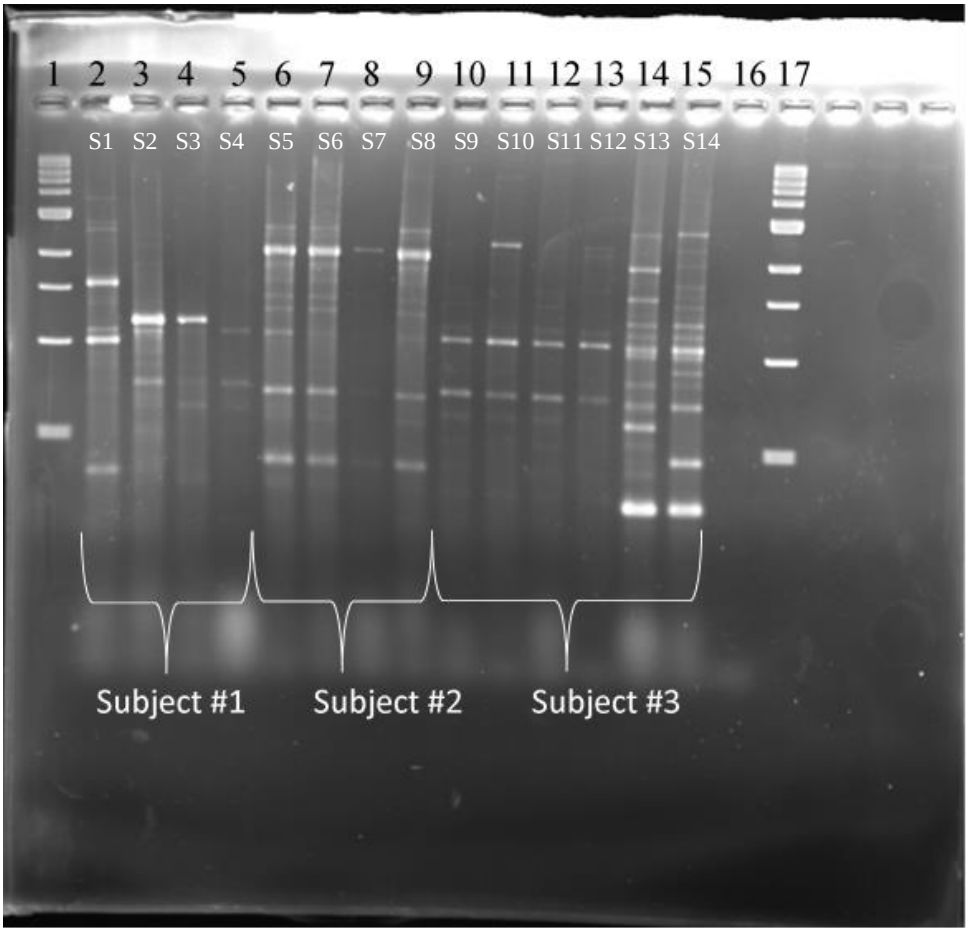


Figure 5 Total Box Patterns of *E. faecalis* isolated from healthy humans. Representative strains that compose the total population of *E. faecalis* isolated from the 3 subjects. Lane 1, 1kb Ladder (New England BioLabs); lane 2-15, Strain S1-S14; Lane 16, Negative Control (No DNA Added); Lane 17, 1kb Ladder (New England BioLabs).

Gel electrophoresis comparing Box patterns by enterococci species can be seen on **Figure 7** and **Figure 8**, for *E. faecalis* and *E. faecium* species respectively.

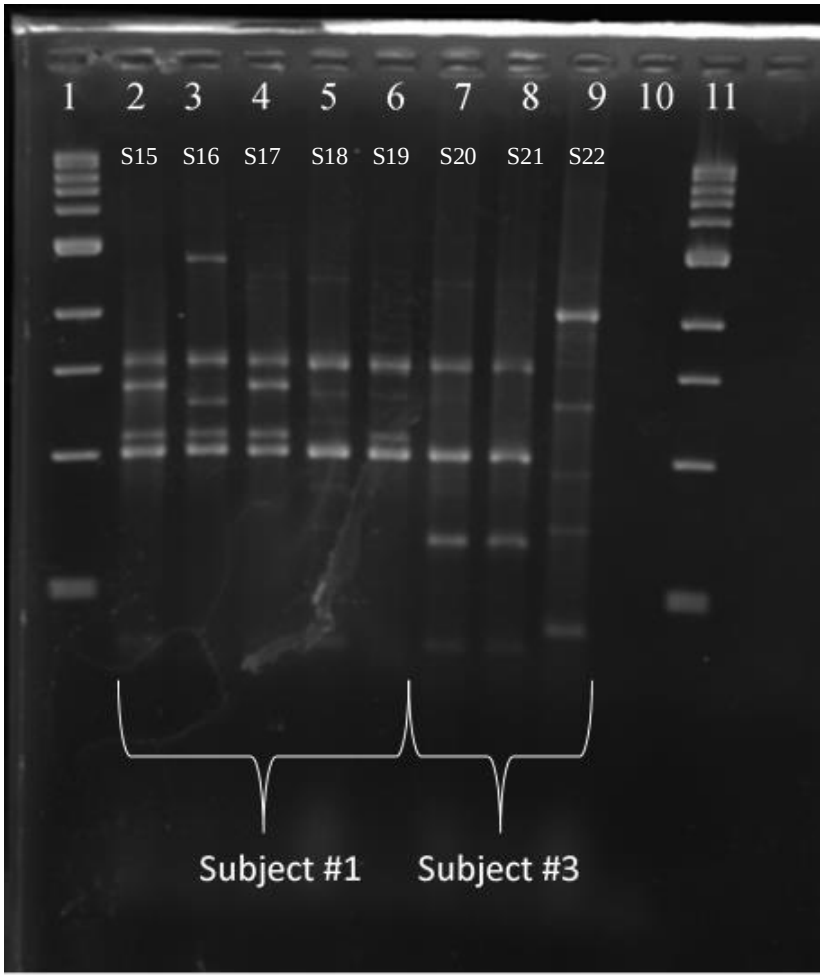


Figure 6 Total Box Patterns of *E. faecium* isolated from healthy humans. Representative strains that compose the total population of *E. faecium* isolated from the 2 subjects. Lane 1, 1kb Ladder (New England BioLabs); lane 2-9, Strain S15-S22; Lane 10, Negative Control (No DNA Added); Lane 11, 1kb Ladder (New England BioLabs).

Rep-Family PCR

Analysis of Rep families was limited to rep 2, 7, 9 and rep-17, as they represented families that were more likely to be present in our samples. Sub-typing for rep-9 subgroups was of importance due to the presence of *asa1* and its relation to pheromone responsive plasmids. Analysis of rep17 was done exclusively on *E. faecium*; however, no presence of the plasmid pRUM was detected. Rep-2 and Rep-7 plasmid families are present on *E. faecalis* and *E. faecium;* however, none were present in the samples tested.

PCR of Rep-9 isolates was done in conjunction with the UV resistance gene, *uvrA* as a multiplex, to determine a correlation between the presence of both genes and Rep-9 plasmid subgroups (**Figure 9**). Presence of Rep-9 was detected on 100% (15/15) of all Tet (M), *gelE* positive *E. faecalis* isolated from subject #1. Subgrouping of these isolates showed presence of subpTEF2 plasmid family, no pCF10 or SubG plasmids were identified. Isolates from subject #2 were shown to harbor no Rep-Families, even from Day 1 where *asa1* positive isolates where present; days 2-7 were *esp* positive with no transferable antibiotic resistance. This was not the case for subject #3, as Rep-9 was the most prevalent Rep family detected.

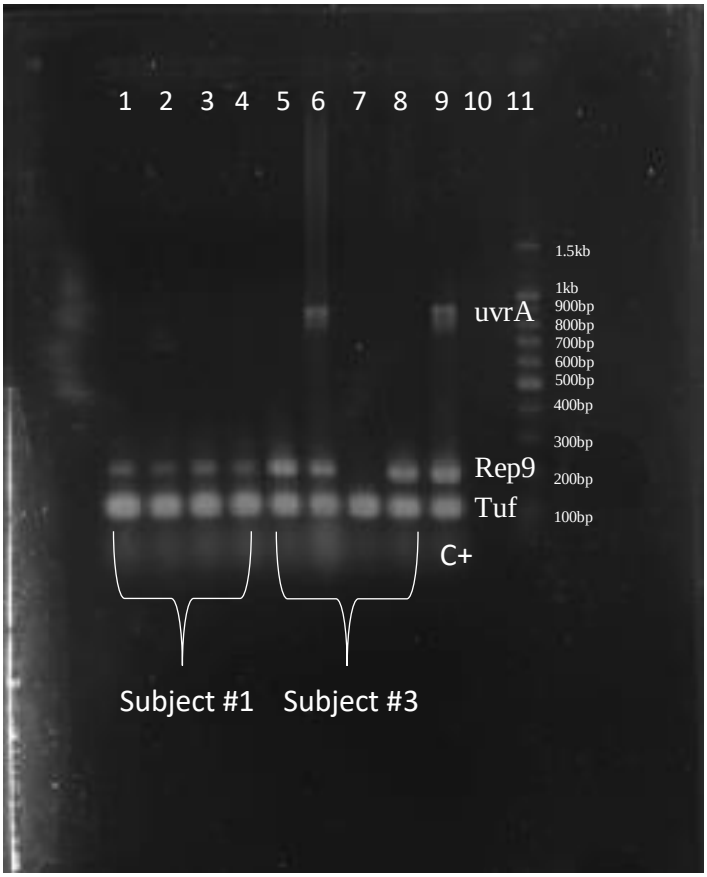


Figure 7. Presence of Rep-9, uvrA and Tuf genes of *Enterococcus faecalis* isolated from subject #1 and #3. Lane 1, Day 4 10; lane 2, Day 4 14; lane 3, Day 5 4; lane 4, Day 6 4; lane 5, Day 1 1; lane 6 Day 1 10; lane 7 Day 2 2; lane 8 Day 3 1; lane 9, C+ *Enterococcus faecalis* clinical isolate 1163-03; lane 10, negative control (no DNA added); lane 11, 100 bp ladder (New England Biolabs)

Presence of urvA genes and Rep-9 positive amplicons were detected on tested *E. faecalis* isolates. Rep-9 family was detected on isolates 53.8% of the time and Rep-9 plus *urvA* around 26% of the time. Subtyping of these isolates resulted in a high prevalence of sub pTEF2 in around 45% and SubG family was also found on 36% of Rep-9 positive *E. faecalis* from subject #3. No SubG positive isolates were detected on subject #1, although pTEF2 was found dominating in both. No pCF10 was detected in any of our isolates, although cytolysin A was detected on one isolate from subject #3. Subtyping was limited to only Sub pTEF2 and SubG pheromone responsive plasmids, while some isolates obtained from subject #3 harbored positives for both.

Discussion

Studies on the diversity of Enterococcus in the human gut has mostly focused on the analysis of species diversity and the identification of virulent strains isolated from clinical studies, environmental samples, and human populations (Klein, 2003; Martin & Mundt, 1972). The dominant species present in all three subjects are consistent with those described by Franz *et al*., 1999; Klein, 2003; Kühn *et al*., 2003; Lleò, Benedetti, & Canepari, 2005; with only *E. faecium* and *E. faecalis* being found in quantities higher than 3 x 10^6 CFU. While other species may be present in lower quantities, our study focused on the dominant strains (meaning higher densities within fecal samples), and were limited to the two species.

On subject #1 and #3, there was a mixed dominance event with both species being present in varying concentrations. Species shift in subject #1 shows that the outgoing *E. faecium* strain has no virulence factors, like *asa1* or *gelE*, while the incoming *E. faecalis* harbors 2 virulence factors, *asa1* and *gelE*. However, the *E. faecium* isolated during the mixed dominance day also harbors *asa1/gelE* (Heaton M., et. al, 1996), which is an unusual genotype given that the *asa1* gene is found on the Rep-9 conjugative plasmid family on *E. faecalis* (Hirt et al., 2005). Positive amplification of Rep-9 was found on the asa1/gelE positive *E. faecium,* similar to that found by Heaton in 1996. This is important given that Rep-9 plasmid families are almost exclusive to *E. faecalis* which could suggest inter-species mating events. There is another mixed dominance event present on day 7, where

asa1/gelE positive *E. faecalis* and *E. faecium* are present, though this was on day 7 and the sampling ended limiting our further observations.

Only *E. faecalis* was isolated in subject #2 during the sampling period. Isolates from day 1 carry 2 virulence factors, *asa1* and *gelE*, while from day 2-7 only *esp* was detected. After Box PCR analysis, two different patterns were detected, one corresponding to isolates from day 1 and another pattern found dominating day 2 onward. All isolates from day 2-7 were dominated by the same pattern, which was not present in any other subject suggesting that one specific strain could dominate for at least 5 straight days.

Composition of subject #3's isolates is very similar to that found in subject #1, two dominant species, *E. faecalis* and *E. faecium,* and a shift in prevalent species with different periods of dominance. Around 60% of the total enterococci isolated were identified as an *asa1/gelE* positive *E. faecalis* with tetracycline resistance, while the *E. faecium* dominant strain is a Tetracycline resistant *gelE* positive strain 84% of the time. Rep-9 was the focus for subject #3, given the high frequency of *asa1* and tetracycline resistance. Rep analysis of *E. faecalis* isolates showed presence of Rep-9 on around 50% of all sampled *E. faecalis*, subgroup analysis showed plasmid family for pTEF2 present on 36% of the isolates. Plasmid family for subG was also identified in around 45% of the isolated *E. faecalis.* Detection of different subgroups between sampling days coupled with other determinants could suggest different strains becoming the dominant population.

Antibiotic resistance also plays an important part in characterizing the dominant enterococci isolated from healthy humans. Rifampin resistance is not plasmid bound and is described as a spontaneous mutation in specific sites of the RNA polymerase beta subunit gene (Kristich et al., 2014). Studies have detected rifampicin resistance in over 70% of clinical isolates (Andrews et al., 2000); Resistance was seen in 67% of infection-derived *E. faecalis* and *E. faecium* obtained from Italy (Busani et al., 2004). Resistance to Rifampin was detected on 289/420 of our isolates across all three subjects, regardless of species, which corresponds to 69% of isolates from our healthy human stool sampling. Although the panorama differs slightly when viewed by subject, subjects #1 and #3 carried Rifampin resistance around 70% and 80% respectively, while subject #2 was below 57%, it is important to note that Rifampin is not an antibiotic used for treatment of enterococcal infections, however, analysis of the mutation site that triggers the resistance might allow us to further characterize our populations, unfortunately this was not part of our objectives and remains to be tested.

Vancomycin resistance can be acquired or intrinsic, VanA and VanB genotypes are described as highly inducible transferable resistance, while VanC has been found in *E. gallinarum* and *E. casseliflavus* (Patel et al., 1997). Vancomycin resistance was encountered in 34% of isolates from subjects #1 and #3, none on #2. Although genotype was not determined, vanA and vanB have been described to be common in *E. faecalis* and

E. faecium, both of which are present on mobile elements. Vancomycin resistance on *E. faecium* that are Rep-17 positive would signal presence of pRUM plasmid, however, none of the 7 VRE *E. faecium* were Rep-17 positive, which may suggest resistance is located on the chromosome.

Tetracycline resistance is a similar case given the presence of around 10 different genes found on *Enterococcus*. Tetracycline resistance was detected on subjects #1 (24%) and subject #3 (94%), while none was found on subject #2. A small sample size of Tetracycline resistant isolates from healthy humans was tested for detection of Tet resistance genes L, M, O, and S; only TetM was found in our isolates. Positive controls for Tet genes were selected according to amplicon length and resistant phenotype; sequencing could determine the identity of the amplicon. Tetracycline resistant *E. faecalis* isolates amplified for TetM exclusively, no Tet genes were detected on resistant *E. faecium*, even though there was a resistant phenotype. On isolates tested from European cheese, the prevalence of TetM was found on over 95% of the antibiotic resistant samples (Huys, D'Haene, Collard, & Swings, 2004), however there is no reported data for tetracycline resistance from human commensal *Enterococcus* populations. Another study describes the antibiotic prevalence in *Enterococcus* used for fermentations in the EU and describes Tetracycline resistance in *E. faecalis* at around 45% of their isolates, although no specific genes are mentioned (Franz et al., 2001).

Box comparison of isolates was divided by species to compare if similar strains were found on more than one subject or present in other sampling days. Based on BOX patterns isolated from subject #2, there is an apparent domination by a single strain between days 2-7, which suggests limited strain diversity and variability in the 7-day sampling period. However, for subject #1 and #3, no *E. faecalis* strains were shared between both subjects even though they share similar virulence factors (*asa1/gelE*). Box PCR patterns were compared between 3 identical isolates from different environments with the patterns obtained from PFGE (SmaI), as well as three different PFGE patterns, and both techniques provided the necessary discriminatory potential for strain identification, as seen on **Figure 10.**

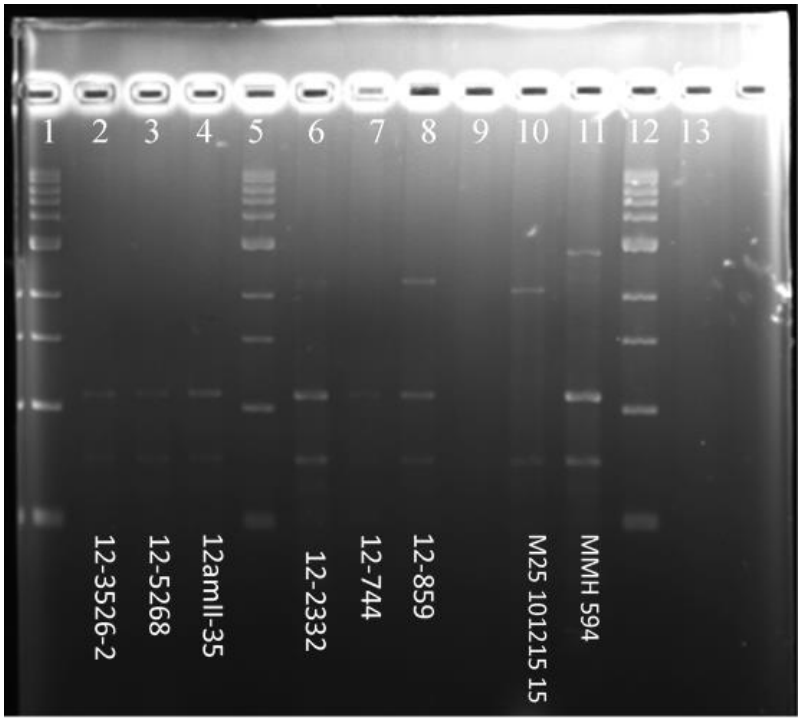


Figure 8. Comparison of PFGE identical isolates through BOX PCR. Isolates from lanes 2-4 were identical through PFGE analysis. Lanes 6-8 harbored different PFGE patterns. Lanes 10 and 11 isolates possessed identical antibiotic resistance patterns and virulence factors. Lanes 1, 5 and 12; 1kb DNA ladder (NEB).

Multiple dominant strains were isolated per day in 2/3 subjects, while one subject was dominated by one strain during 6 days. Different box patters were identified on isolates with identical virulence factors and antibiotic resistance, which suggests that they are different strains. Some isolates that harbored different virulence determinants, which would be expected to be different strains according to BOX PCR, shared BOX patterns with other isolates. Some patterns were identical between isolates that possessed different virulence determinants, specifically those with *asa1/gelE* patterns when compared with *gelE* positive or *esp* positive isolates as these last two are genome bound, while *asa1* is carried on a plasmid. Since BOX PCR patterns were identical, we utilized all other information obtained from the isolates, such as virulence factors and the antibiotic resistance to fully compare strains.

The presence of strains with more than 2 virulence factors and antibiotic resistance, signals the carriage of potentially pathogenic strains in healthy human stool. An isolated *E. faecalis* from subject #3 is genotypically similar to the infection-derived *E. faecalis* MMH594 strain, as they both share *asa1/gelE/esp/cylA* virulence factors and Tetracycline resistance (Vankerckhoven et al., 2004). Comparison of both isolates by BOX PCR can be seen on **Figure 10** on lanes 10 and 11. Different patterns suggest different strains with similar characteristics. Subject #3 appears to carry a more pathogenic intestinal flora than that of subject #1 and #2. Presence of potentially pathogenic strains in healthy humans is important if these strains translocate out of the gut and cause a localized or systemic infection.

A total of 4 different box patterns were identified for *E. faecalis* isolates obtained from subject #1, while for *E. faecium* 5 strains were observed. Since no *E. faecium* strains were isolated from Subject #2, only 4 different strains of *E. faecalis* were detected and these appeared to be subject specific. Subject #3's *E. faecalis* strains were limited to 6 different box patterns, which were different when compared to both other subjects; the same case was seen for *E. faecium*, where only 3 different patterns were identified. All strains identified were subject-exclusive, none were shared between subjects showing a high diversity of strains found dominating in healthy humans. However, on neither study done by Sorensen (2001) or by Lund (2002) did they compare isolates PFGE patterns between subjects, only as a method of detecting the antibiotic resistant or probiotic strain respectively.

Our hypothesis stated that the dominant enterococci strain is constantly changing and that long-term colonization does not occur, our data suggests that there is a change in dominant species, 14 different *E. faecalis* strains and 8 *E. faecium* strains, none which were shared between subjects. A change in dominant species was seen in two of the three subjects, where population fluctuated between *E. faecalis* and *E. faecium* dominance. In conclusion, *Enterococcus* human gut populations are constantly changing in composition and over 22 different strains were isolated between *E. faecalis* and *E. faecium.* Changes between not only dominant strains, but changes in dominant species can occur in a period

of 24 hours, which suggests a dynamic and changing enterococci population. Diversity of the dominant strains was small, even though there were over 400 isolates, this could suggest a limited amount of strains colonizing the intestine that are present at lower levels; depending on the foods eaten, strains may be favored and increase in quantity resulting in the observed dominance.

Determination of human-bound enterococci strains could help us determine if there is a range of strains that are exclusive to humans and isolation in other environments could suggest fecal contamination. However, a much bigger sample size would be needed to determine the range of strains. Since the study only focused on the dominant recoverable enterococci, strains diversity may be limited and analysis of strains and species present below of our dominant threshold could help answer that question. Analysis of total enterococci population present in the human gut could determine if there is a range of strains that are subject-specific and colonize at lower levels or if there is a constant entering and exiting of strains in our system limited to our daily intake.

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