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Genistein-Enriched Pig Gut Microbiota Library as a Potential Probiotic Consortium

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GENISTEIN-ENRICHED PIG GUT MICROBIOTA LIBRARY AS A POTENTIAL PROBIOTIC CONSORTIUM

BY

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this research to my dad, Mr. Stephen Amponsah and my late mom, Madam Lydia Effah-Crentsil.

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ABBREVIATIONS

UV	Ultraviolet
CoA	Coenzyme A
CDC	Centers for Disease Control and Prevention
Er- β	Estrogen receptor beta
G2/M	Second growth/Mitotic phase
ROS	Reactive oxygen species
TGF- β	Transforming growth factor-beta
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Vpu	Viral Protein U
HIV	Human immunodeficiency virus
PBS	Phosphate buffered saline
BHI	Brain heart infusion
MES	4-morpholinoethanesulfonic acid hydrate
QIIME 2 (version 2)	Quantitative Insights into Microbial Ecology Framework
SCFA	Short chain fatty acids
MACs	Microbiota-accessible carbohydrates
DPH	1,6-Diphenyl-1,3,5-hexatriene
FBS	Fetal Bovine Serum
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectometry

MOI	Multiplicity of infection
NGS	Next-generation sequencing

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ABSTRACT

GENISTEIN-ENRICHED PIG GUT MICROBIOTA LIBRARY AS A POTENTIAL PROBIOTIC CONSORTIUM

THERESAH AMPONSAH

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Plant-based diets contain numerous flavonoid compounds that produce antibacterial effects and gut health improvement. Genistein is among the most abundant isoflavones present in a plant-based diet and can be found in high amounts in soy products (up to 1g Kg^{-1}). We describe here a robust method to identify genistein tolerant and metabolizing bacteria in swine gut microbiota and to screen the strains that have antibacterial and immune-stimulatory properties. Such strains could be developed as non-antibiotic alternatives to prevent enteric infections in pigs and improve gut immunity. To this end, a mini bioreactor-based system was used to enrich genistein metabolizing bacteria in swine microbiota from pooled pig fecal samples. Pooled pig fecal samples were supplemented with 0.5mg/mL genistein and run in a mini bioreactor model in six replicates for 21 days. At the end of the run, the six replicates were pooled together and were used for isolating genistein-metabolizing bacteria. Bacterial species were isolated by micro cultivation array and routine anaerobic culture using modified BHI media and were identified by MALDI-TOF MS and 16S rRNA analysis. The genistein biotransformation capacity of the strains was determined using the DPH assay. We also determined their hemolytic and invasive capabilities. Our culture method was able to isolate a large number of strains belonging to 19 species. These include *Streptococcus lutetiensis, Streptococcus equinus, Enterococcus faecalis, Streptococcus alactolyticus, Streptococcus gallolyticus, Acidaminococcus*

fermentans, Lactobacillus salivarius, Peptostreptococcus russellii, Mitsuokella jalaludinii, Faecalicoccus pleomorphus, Lactobacillus agilis, Bacteroides vulgatus, Bacteroides uniformis, Bacteroides fluxus, Enterococcus avium, Sharpea azabuensis, Collinsella phocaeensis, Selenomonas montiformis and *Syntrophococcus sucromutans.* Of the 19 species isolated, *Lactobacillus salivarius* is the only species that was completely hemolytic (*β* hemolysis). Eleven species namely *Streptococcus gallolyticus, Acidaminococcus fermentans, Streptococcus equinus, Streptococcus alactolyticus, Bacteroides vulgatus, Bacteroides fluxus, Mitsuokella jalaludinii, Bacteroides uniformis, Sharpea azabuensis, Selenomonas montiformis* and *Syntrophococcus sucromutans* were non-invasive. *Mitsuokella jalaludinii* and *Peptostreptococcus russellii* were negative for the DPH assay. 16S rRNA analysis revealed the abundance of six phyla namely Firmicutes, Bacteroidota, Actinobacteria, Proteobacteria, Synergistota and Euryarcheota and several genera belonging to Firmicutes. All the genera identified with culturomics were present in the taxa data from the 16S analysis, however, 16S analysis revealed other genera that could not be captured with culturomics. Hence, combining culturomics with 16S analysis is the best shot at trying to cover as much diversity as possible. We have been able to isolate ten non-hemolytic, non-invasive genistein-metabolizing species, which are potential antibiotic alternatives. Future studies will be focused on the species' properties regarding their in vitro adhesion and pathogen exclusion, in vivo colonization and pathogen exclusion and acid and bile tolerance.

Chapter 1: Literature Review

1 General overview of flavonoids

Flavonoids are polyphenols abundant in generally all organs of plants. They give them color, fragrance and flavor characteristics. Flavonoids are involved in regulating plant cell growth, attracting pollinating insects and protecting plants against biotic and abiotic stresses like herbivores, UV irradiation, drought, cold, heat and salinity (1-4). They are also involved in sex determination, regulation of photosynthesis, morphogenesis, regulation of growth factors and energy transfers (5). Plants synthesize these polyphenols in response to microbial infection; they have been reported to be potent antimicrobials against pathogens (6). Flavonoids serve as phytoalexins, which means they protect plants against pathogens (7) as well as allelochemicals that inhibit microorganisms growth around plants(8, 9).

Epidemiological studies indicate that a high dietary intake of flavonoids reduces the risk of chronic diseases including cancers (10-13). Flavonoids have shown anti-inflammatory, anticancer, antidiabetic, antimicrobial, cardio-protective, anti-oxidative, immunostimulatory and neuroprotective properties in humans (14-16). Deficiencies in polyphenol intake do not result in any known deficiency disease (16, 17). It has been estimated that the average daily intake in humans is a few hundred milligrams (18). Prolong intake of flavonoids in the diet barely have side effects as a result of their relatively low bioavailability, lesser intestinal permeability and a higher rate of metabolism by the gut microbes (19). They are non-toxic to humans and animals because of their poor absorption coefficient (20, 21). There is a growing list of different naturally occurring flavonoids, the number exceeding 6000 (22, 23).

Flavonoids are polyphenolic substances with a low molecular weight based on a flavan moiety (2-phenyl-benzo-λ-pyran). The basic structure comprises two benzene rings A and B connected via a heterocyclic oxygen-containing pyran ring C (24). The carbon atoms in rings A and C are numbered from 1 to 8 and those in ring B are numbered from 1' to 6' (Figure 1). Flavonoids are derived from two biosynthetic pathways namely the phenylpropanoid, which synthesizes phenylpropanoid skeleton (C6-C3) and polyketide, which synthesizes blocks for C2 polymer units (25). Chalcone synthase catalyzes 2'-hydroxychalcone scaffold formation from ρ-coumaroyl CoA and malonyl CoA (Figure 2). These are used in a series of enzymatic steps to synthesize other flavonoids (26).

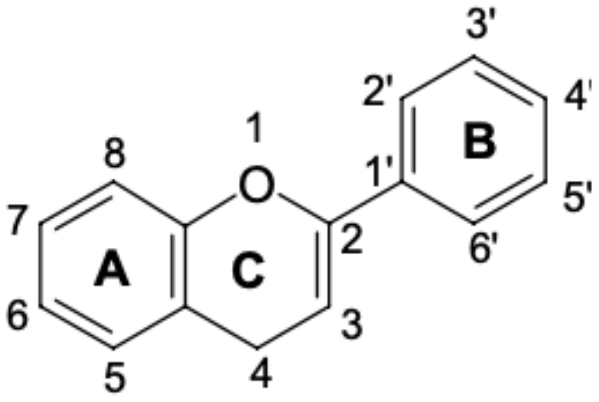


Figure 1: The basic structure of flavonoids. Adapted from Aukje Steensma (2006)

The major classes of flavonoids are flavonols, flavones, flavanones, isoflavones, catechins, anthocyanins and chalcones (Table 1). Flavonoids are classified based on the chemical modifications of the heterocyclic C ring, which could undergo hydrogenation, hydroxylation, methoxylation, malonylation, sulfation and glucuronidation. Flavonoids

naturally exist as glycosylated to sugar moieties and are called glycosides. The cleavage of the sugar results in the formation of aglycones.

Plant extracts were the major means of fighting infections before antibiotics were discovered in the 1930s (27-29). In the last 60 years, antibiotics have been used to treat infections. The common use of antibiotics in agriculture, medicine, and veterinary has resulted in the emergence of antibiotic-resistant bacteria over the years (6). The number of drug-resistant bacteria is on the rise (30, 31) leading to a post-antibiotic era (32). The CDC has estimated that one in five pathogens from nosocomial infections represents a multidrug-resistant strain (33). Hence, there is a need to find antibiotic alternatives to use in these industries.

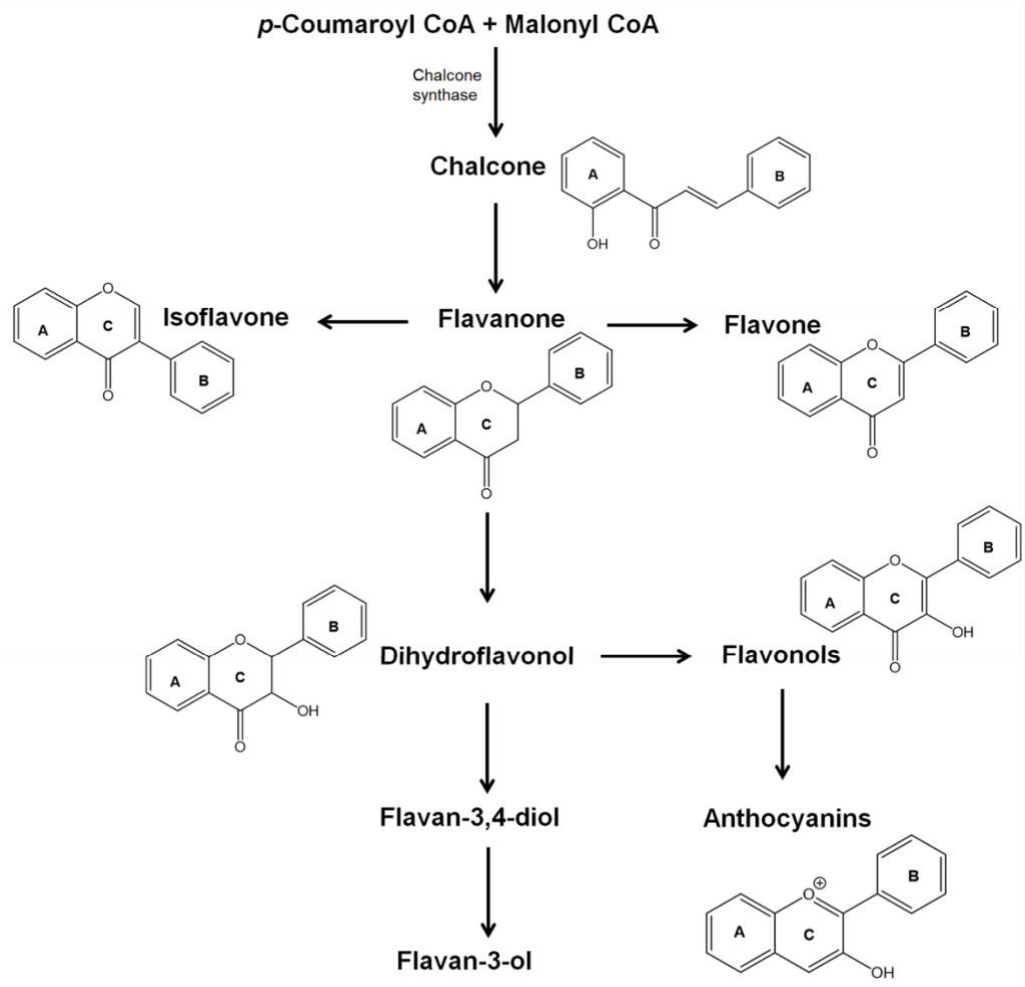


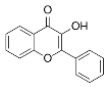
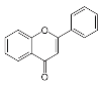
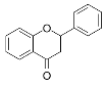
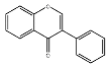
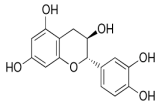
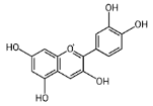
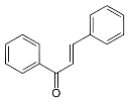
Figure 2: Flavonoid biosynthesis. Adapted from Kazuki Saito KY-S et al. (2013)

2 Isoflavones

Isoflavones are a subclass of flavonoids that have the phenyl ring (B ring) linked at the 3- rather than the 2-position of the heterocyclic ring as shown in table 1.1. They are made from a rearrangement of flavanones resulting in 2,3-aryl migration and dehydrogenation. They are found in the subfamily of Faboideae of the family Fabaceae or Leguminosae and are predominant in soybeans and red clover (34). Soy contains approximately 1 g of genistein/Kg of dry beans (35). They are found in smaller quantities in other food sources which include lupin, fava beans, chicken peas, common beans, kudzu roots and peanuts (36). The main subtypes of isoflavones are genistein, daidzein and glycitein. Their naturally occurring forms are genistin, daidzin and glycitin respectively.

In plants, isoflavones are involved in stress responses and their concentrations depend on the plant and stress type. For instance, some studies have reported that UV-A and UV-B light increase isoflavone concentrations (37, 38). In another study by Swigonska et al. (2014), weather fluctuations and osmotic stress increased isoflavones in the roots of soybean seedlings (39).

Table 1: The flavonoid classes, their chemical structure, major source, common and chemical aglycone names

Flavonoids	Basic chemical structure	Major source	Predominant aglycones	Chemical name
Flavonols		tea, onions.	quercetin	3,5,7,3’,4’-pentahydroxy flavone
		apples	kaempferol	3,5,7,4’-tetrahydroxy flavone
			myricetin	3,5,7,3’,4’,5’-hexahydroxy flavone
Flavones		herbal, celery	apigenin	5,2’,5’-trihydroxy flavone
		green pepper	luteolin	5,7,3’,4’-tetrahydroxy flavone
		and camille tea		
Flavanones		citrus fruits	hesperetin	5,7,3’-trihydroxy-4’-methoxyflavanone
			naringenin	5,7,4’-trihydroxyisoflavanone
Isoflavones		legumes	genistein	5,7,4’-trihydroxy isoflavone
		(soybeans)	daidzein	7,4’-dihydroxy isoflavone
			glycitein	7,4’-dihydroxy-6-methoxyl isoflavone
Catechins		tea, chocolate	catechin	3,5,7,3’4’-pentahydroxyflavan
(Flavane)			epicatechin	3,5,7,3’,4’-pentahydroxyflavan
			gallocatechin	3,5,7,3’,4’,5’-hexahydroflavan
			epigallocatechin	3,5,7,3’,4’,5’-hexahydroflavan
Anthocyanins		berries, cherries	cyanidin	3,5,7,3’,4’-pentahydroxy anthocyanin
		wine		
Chalcones		apples	phloretin	5,7,9,4’-tetrahydroxy chalcone

2.1 History of isoflavones

In the 1940s, isoflavones were discovered in *Trifolium subterraneum* (subterranean clover) being grazed by horses in Australia. The horses manifested dystocia, uterine prolapse and infertility (40). The major isoflavone in clover is the 4'-methoxy derivate of daidzein, formononetin, which can be metabolized eventually to equol (41). Isoflavones and their metabolites exert estrogenic activity. These discoveries set the premise for studies on the estrogenic activity and metabolism of isoflavones in animals (40, 42, 43). In 1984, the interest in isoflavones was rekindled after their discovery in human urine samples (44, 45). The concentrations of isoflavones recorded in human blood and urine samples, upon ingestion of soy-based foods far exceeded concentrations of endogenous estrogens in humans. Diets rich in soybeans have been reported to be associated with reduced incidences of cancers in Asian countries (10, 11). The average daily intake of soybeans by Asians is about 50 mg, whereas less than 1 mg is consumed by people from Western countries per day (46, 47). It appears the quantity of isoflavones in soy products consumed by Asian women does not affect their reproductive capacity as was observed for the sheep in the previous study in Australia (48).

2.2 Mechanisms of action of isoflavones

2.2.1 Cardiovascular Effects

Outcomes of some studies have proved that isoflavones have health-promoting activities including cardiovascular effects (49, 50). This could be due to their antioxidant activity whereby lipoprotein is inhibited, serum cholesterol levels are reduced, tyrosine kinase is inhibited and vascular activity is improved (51, 52).

2.2.2 Estrogenic Action

Isoflavones are structurally similar to 17β-estradiol and are able to bind to the estrogen receptors, mainly ER-β with an 87% affinity (53, 54), decreasing the risk of developing hormonally dependent cancers (55, 56). Hence, isoflavones are termed phytoestrogens as they have estrogenic activity. Genistein has been studied for its ability to reduce hormone-sensitive cancers such as breast, ovarian, and prostate. In the breast cancer cell cycle, genistein halts the G2/M phase and causes a ROS-dependent apoptosis(57).

2.2.3 Anti-inflammatory Action

Isoflavones exert their anti-inflammatory activities by inhibiting angiogenesis (58) and inducing apoptosis in breast cancer (59). Genistein, when combined with exercise, abolishes inflammation associated with high fat diet (60). Availability of genistein was enhanced when delivered with a nanoparticle to exert its anti-inflammatory action on colitis (61).

2.2.4 Antiproliferative Action

Another mechanism of action of isoflavones is inhibition of steroid biosynthesis enzymes which include aromatase (62) and 17 β-hydroxysteroid oxidoreductase (63) probably resulting in a reduction or modification in production and bioavailability of estrogens (64). Kim et al. (1998) reported that genistein interferes with the TGF- β signaling pathway (65) which regulates cell proliferation, hence genistein is antiproliferative.

2.2.5 Antibacterial Action

Isoflavones exert antibacterial properties by interfering with the biofilm formation (6, 14). Bacterial biofilm-associated infections contribute to a significant amount of all microbial

and chronic infections in humans and animals (66, 67). Bacteria that grow as biofilms are about 10 to 1000 times more resistant to antimicrobials than their planktonic cells (68). A study by Lee et al. (2011) reported that isoflavones such as genistein inhibit biofilm formation in *Escherichia coli* O157:H7 (69).

Another antibacterial mechanism of action is the stabilization of the topoisomerase II-DNA cleavage complex. This results in an impairment of cell division and ultimately inhibits the growth of bacteria (70). Ulanowska et al. (2016) showed the inhibitory effects of genistein on *Vibrio harveyi, Bacillus subtilis* and *Escherichia coli* as a result of this mechanism (71). Finally, genistein has the ability to inhibit exotoxins from *Staphylococcus aureus* (72) as well as to potentiate the antibacterial activities of norfloxacin and berberine in wild type *Staphylococcus aureus* and *Mycobacterium smegmatis*, respectively (73, 74).

2.2.6 Antifungal Action

Antifungal mechanisms of isoflavones include plasma membrane disruption, induction of mitochondrial dysfunctions, inhibition of cell wall formation, cell division, RNA and protein synthesis (75). Genistein inhibits DNA, RNA and protein synthesis in *Cochliobolus lunatus* (76). Studies by Lee et al. (2010) and Bitencourt et al. (2013) reported antifungal activities of isoflavones against *Candida albicans* and *Trichophyton rubrum*, respectively (77, 78).

2.2.7 Antiviral Action

Isoflavones can block any of the stages of viruses' life cycle be it attachment, penetration into host cells, replication, translation, assembly or release to inhibit viral growth (79). A study by Sauter et al. (2014) showed that genistein inhibited Vpu protein formation of ion channels in HIV-infected cells and thus inhibiting the assembly and release of HIV (80).

Soy isoflavones inhibit the infectivity of human rotavirus in cultured macrophages (81) and cytopathic effects in herpes simplex virus types-1 and -2 (82).

2.4 Dietary intake of isoflavones

Isoflavones are found in soybeans and soy-based products such as soy milk, isolated soy protein, soy flour, tofu, natto, miso, tempeh, soy supplements and soy hot dog (83-85), where they exist as glycosides (86). Crop variety, geographic location, soil type, crop year and environmental factors affect isoflavone content (87, 88). Processed soy products have different isoflavone contents than unprocessed ones (89). When soy is fermented into products such as tofu and bean paste, the isoflavone content is reduced by a factor of 2 to 3, aglycone forms are increased (89, 90). Boiling also reduces isoflavone content (91). Other means of cooking such as baking and frying do not seem to affect the isoflavone contents of soy products (90). Averagely, cooked soybeans and soymilk powder contain >95% of the total isoflavones as glycosidic forms whereas soybeans in fermented products contain about 20 to 40% of aglycones (92). In the Asian adult population, about 39 to 47 mg of isoflavones are ingested per day (46, 85, 93) whereas less than 1 mg is ingested by the general American population (94, 95). The estimated average daily intake of isoflavones in men and women in Finland is 0.9 and 0.7 mg/day respectively (96). The average Western population and vegetarians consume about 1-2 mg/day and 3-12 mg/day of isoflavones respectively (97, 98).

2.5 Bioavailability of isoflavones

Bioavailability is the proportion of a substance that enters the circulation when introduced into the body and can have an active effect. Isoflavones reach minute concentrations in the blood upon consumption of soy products (95). A single dose of 50 mg of isoflavone results

in a maximum concentration of about 2 μM of total aglycones in the plasma (99). The intestinal microflora determines isoflavone bioavailability, hence, there are differences in concentrations among individuals and species.

2.6 Absorption of isoflavones

The natural states of isoflavones have sugar moieties attached to them, making them hydrophilic (100) while their aglycone forms are lipophilic molecules having octanol/water partition coefficient between 0 and 4 (101). Lipophilic molecules can interact with the proteins in the intestinal cell membrane and are able to passively diffuse through the lipid bilayers. Hence, the cleavage of the glycosidic linkage by glycosidases is a necessary step for isoflavones metabolism to occur in the intestines. Isoflavones have been reported as the most abundant flavonoid absorbed and available in the intestine (16, 102, 103). A proportion of aglycones are absorbed in the small intestine and a significant amount is broken down by colonic microbiota into readily absorbable forms (104). Upon absorption, isoflavones undergo glucuronidation and sulfation and are released into circulation or in the liver (105). Once in circulation, they can either be transported to other tissues or excreted in the urine (106).

3 Isoflavone use in the pig industry

Isoflavone use in the pig industry has conferred some health benefits on pigs. A new study from the University of Illinois shows that pigs that eat soybeans as part of their diet are better protected against porcine reproductive and respiratory syndrome virus as a result of the antiviral and anti-inflammatory properties of the isoflavone present (107). Hongzhi et al. (2021) investigated the effects of soybean isoflavone together with astragalus polysaccharide on hormone levels, colostrum components, immune functions and serum

antioxidant activities in lactating sows. There was an increased yield in lactation as well as improved antioxidant, immune and hormone levels (108). Isoflavones enhance growth performance, protect intestinal morphology and improve antioxidation in pigs (109). Supplementing Chinese boars' diets with about 250 mg/kg of soy isoflavones increased reproductive parameters such as testis index and viable germ cells (110) whereas female Bama miniature pigs fed with 1250 mg/kg experienced delayed onset of puberty (111).

3.1 Pig diet composition

The primary dietary protein source for pigs in the United States is the soybean meal (112). It is produced globally in high quantities and contains about 38% of protein, making them the preferred protein component for the pig diet (113). It provides biologically active components, protein, phosphorus and amino acids (Table 2).

Table 2: Diet composition for starter and finisher pigs

Ingredients (%)	**Starter**	**Finisher**
Corn	52.09	57.43
Soybean meal (CP 44%)	39.4	33.97
Soybean oil	4.8	5.6
Dicalcium phosphate	1.6	1.21
Oyster shell	1.08	0.92
Salt	0.4	0.32
Vitamin premix[1]	0.25	0.25
Vitamin premix[2]	0.25	0.25
DL-methionine	0.13	0.06

3.2 The microbiome of a healthy pig

The gut microbiota plays a significant role in maintaining host health and metabolism. Holman et al. (2017) reported that the core genera of commercial swine worldwide are *Prevotella, Clostridium, Alloprevotella, Rumicococcus* and the RC9 (114).

The abundance of microorganisms differs among the various locations. *Ecsherichia-Shigella, Terrisporobacter, Romboutsia* and *Clostridium sensustricto* are more abundant in the ileum while *Alloprevotella, Lactobacillus* and Prevotellaceae NK3B31 group are most prevalent in the cecum. In the colon, *Clostridium, Streptococcus* and *Lactobacillus* are predominant (115). Firmicutes and Actinobacteria are predominant in cecum and jejunum, respectively (116).

Gender is a factor in shaping the gut microbiota of pigs. In boars, Veillonecallaceae, *Roseburia*, *Bulleidia* and *Escherichia* are abundant whereas *Treponema* and *Bacteroides* are highly abundant in gilts (117).

In a study by Mach et al. (2015), the composition of the fecal microbiomes for 31 healthy piglets across five age strata revealed that the phyla Firmicutes and Bacteroidetes were predominant at each age (118). Wylensek et al. (2020) isolated 110 bacterial species from 19 pigs from Germany, USA and Canada. They belonged to the phyla Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Fibrobacteres, Synergistetes, Fusobacteria, Lentisphaerae and Spirochaetes (119). In another study, 287 fecal samples were collected from pigs from France, Denmark and China. Half of the non-redundant genes identified could be classified taxonomically, 28.73% and 9.28% belonged to Firmicutes and Bacteroidetes, respectively (120). Two breeds of pigs namely Tamworth and Feral showed some variations in their microbiome abundance. Tamworth pigs were highly dominated by Bacteroidetes whereas Feral pigs had about the same proportions of Firmicutes and Bacteroidetes (121).

There is a fecal microbial shift during weaning transition in healthy piglets. Firmicutes and Bacteroidetes are dominant during pre-weaning and post-weaning, respectively (122).

Low-fat pigs have increased abundance of *Bacteroides*, increasing their *Bacteroides*-*Prevotella* ratio as compared to high-fat pigs. These pigs are able to produce short chain fatty acids and lessen lipid accumulation (123).

It can be inferred from these studies that the gut microbiome of healthy pigs are modulated by various factors which include gender, breed, fat content and geographic locations. Generally, the phyla Firmicutes and Bacteroidetes seem to be the most dominant.

3.3 Metabolism of isoflavones by the gut microbiome

Studies on isoflavone metabolism in different human and animal subjects have been reported. The gastrointestinal tract is the site for the metabolism of isoflavones (124), where their sugar moieties are acted on by β-glucosidases and intestinal bacteria. The transformation of genistein starts with a hydrogenation reaction to generate dihydrogenistein by gut microbiota. Dihydrogenistein is further reduced to yield 5-hydroxy equol and 6'-hydroxy-*O*-desmethylangolensin. Selective hydrolysis of 6'-hydroxy-*O*-desmethylangolensin between carbon atoms 1' and 1 yields 4-hydroxyphenyl-2-propionic acid (Figure 3)(125). Daidzein, another isoflavone is degraded by gut bacteria into dihydro-daidzein and further to equol. All ruminants are equol producers because their gut microbiota favors the biosynthesis of this metabolite (126). Bacterial strains SNU NiuO16 from the bovine rumen, SNU Julong732 from the human intestine, *Slackia equolifaciens*, *Slackia isoflavoniconvertens*, *Eggerthella sp*, D-G6, *Eubacterium* strains D1 and D2 from swine, *Hugonella massiliensis* and *Senegalimassilia faecalis* from humans, MT1B8 from mouse, *Lactobacillus delbrueckii-like* strain MF-07 from chicken, *Escherichia coli* HGH21 and strain HGH6 from humans have been reported to convert daidzein and genistein to dihydrodaidzein and dihydrogenistein, respectively. Their individual metabolic conversion was lower than that of a complex community of feces with different bacteria, suggesting the involvement of other species (127-133).

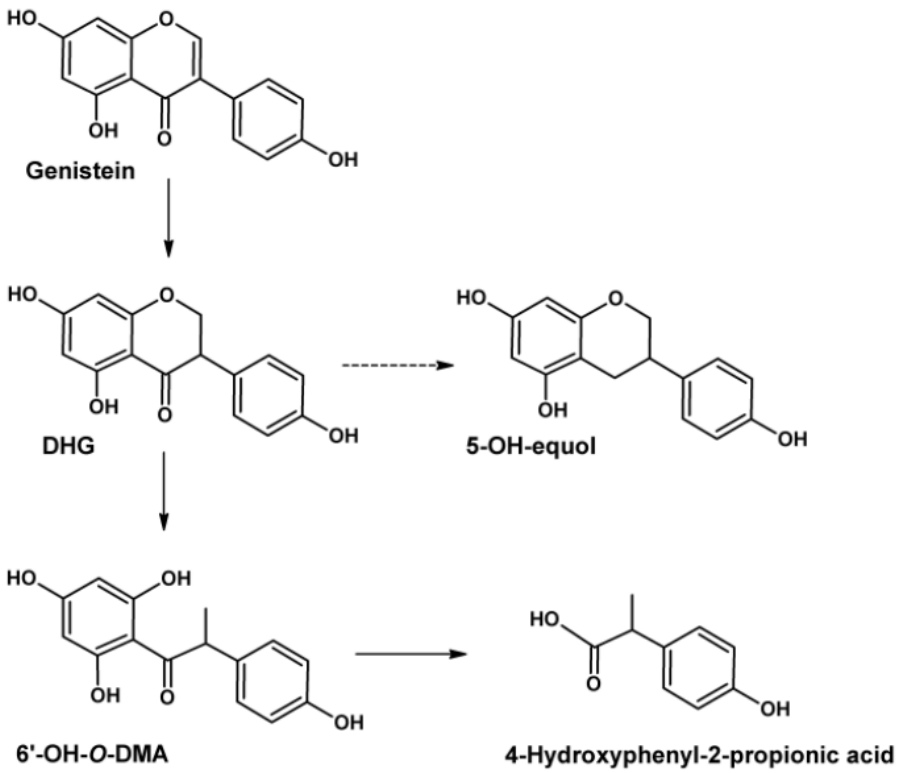


Figure 3: Biotransformation of genistein. Adapted from Rossi et al. (2010).

Chapter 2: Genistein enriched pig gut microbiota library preparation using an in vitro mini bioreactor model

Abstract

Genistein is an isoflavone predominant in soybean (up to 1 g/kg). They have been studied for mainly their estrogenic and some antibacterial properties. Limited studies have explored their metabolism by gut bacteria in swine. We supplemented fecal samples from finisher pigs with 500 mg/L of genistein and cultured them in a mini bioreactor for a period of 21 days. Samples were taken every week (days 7, 14 and 21) for short-chain fatty acids (SCFAs) estimation and 16S rRNA sequencing. 16S data analysis revealed the abundance of the phylum Firmicutes comprising mainly of the genera *Streptococcus, Megasphaera, Acidaminococcus, Mitsuokella, Lactobacillus and Eubacterium.* Other phyla include Bacteroidota, Actinobacteria, Proteobacteria, Synergistota and Euryarcheota. *Streptococcus* was the most abundant genus. Shannon diversity index showed a significant decrease in community richness by day 21 when genistein was supplemented ($p = 0.013806$) and the Bray-Curtis distance calculation showed dissimilarities between the groups ($p < 0.001$). Acetate and propionate were produced as a result of the fermentation of microbiota accessible carbohydrates (MACs) by gut microbes while there was no butyrate production in the genistein-treated group. We have been able to develop a genistein-enriched library from which species identification and characterization can be performed in subsequent experiments.

1 Introduction

Diet is one of the key modulators of gut microbiota composition which influences host homeostasis and biological processes (134). The mutualism relationship between the host and its gut bacterial symbionts can be altered through dietary habits. Recent research has been inclined toward knowing the effects of dietary components on gut microbiota and how their interaction with the gut microbiome impacts human health (134). Fruits and vegetables contain a variety of phenolic compounds that are known to produce beneficial health effects by modulating gut microbiota. Flavonoids are a well-known polyphenol present in almost all fruits and vegetables and certain beverages. Flavonoids are associated with a broad spectrum of health-promoting effects and are an important component of nutraceutical, pharmaceutical, medicinal and cosmetic applications. They play a major role in the prevention of a variety of gastrointestinal diseases like CRC, IBD and Crohn's disease because of their antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme functions. There are six significant groups of flavonoids, including anthocyanins, flavanols, flavones, flavonols, flavonones and isoflavones.

Genistein is one of the most abundant isoflavones in the diet and can be found in high amounts in soy foods (up to 1g Kg^{-1}) (125, 135, 136). Genistein is not freely present in food sources, but it is usually conjugated to a sugar moiety (forming glucosides) that ensures its stability (136). Genistein glucosides are poorly absorbed from the intestine, and hence these linkages are cleaved by enzymes either present in the small intestine or the colon to facilitate absorption. As the enzymes produced by the human body is sometimes incapable of cleaving the glycosidic linkages, microbial enzymes produced have been

implicated in these deconjugation reactions. Studies have highlighted the role of the gut microbiota in the metabolism of isoflavones in the human body, thereby changing their bioavailability or generating metabolites that affect human health in metabolizing the isoflavones in human health are now emerging. There are reports suggesting that genistein supplementation resulted in an altered composition of gut microbiota at different taxonomic levels, including a decrease in relative amounts of *Lactobacillus* spp., *Ruminococcus flavefaciens*, Bacteroidales and Clostridiales in female mice while Rikenaceae, Ruminococcaceae, Lachnospiraceae and *Lactococcus* spp. were elevated. In males, Genistein decreased the relative abundance of *Flexispira* spp., Clostridiales, Bacteroidales, *Odoribacter* spp. and Desulfovibrionaceae but increased the relative abundance of Lachnospiraceae and *Allobaculum* spp (137).

Despite the advances in our knowledge of the physiological importance of these microbe–host interactions, relatively little is known about the specific microbial strains, genes, and enzymes responsible for these activities. Fully understanding the molecular mechanism involved in metabolizing isoflavones and their effects on host health and disease remains a great challenge.

Here in this study, we developed a genistein metabolizing/tolerant gut microbiome library using the mini bioreactor model which replicates the hindgut environment.

2 Materials and methods

2.1 Pig fecal sample collection and preparation

Fecal samples were collected from five healthy finisher pigs with no antibiotic use from the Swine Education and Research Facility, South Dakota State University. The samples were transferred to an anaerobic chamber within 10 minutes of collection, and we performed a 10-fold dilution with phosphate-buffered saline (PBS) (Lot number: SLCJ0944, Sigma). The fecal samples were pooled together in equal ratios for use in the mini bioreactor.

2.2 Mini bioreactor

The modified brain heart infusion (BHI) medium (138) was used as a control medium and it was prepared as follows: 37 g/L brain-heart extract, 5.0 g/L yeast extract, 10.0 g/L inulin, 0.3 g/L L-cysteine, 0.25 mg/L resazurin and 1.0 mg/L menadione were dissolved in 900 ml of milliQ water, pre-reduced and sterilized by autoclaving at 121 ^{o}C for 30 min. Upon autoclaving and cooling, 100 ml of filter-sterilized 1M MES hydrate was added as a buffer and 1.7 ml of acetate (30 mM), 2 ml of propionate (8 mM), 2 ml of butyrate (4 mM), 100 µl of isovalerate (1 mM), 1 ml hemin (0.5 mg/ml), 10 ml of ATCC vitamin and mineral mixtures were added as supplements. Commercially available genistein (LC Laboratories, MA) was dissolved in a modified BHI medium at a concentration of 0.5mg/ml.

Mini bioreactors were sterilized and assembled, and the experiment was performed as described previously (139) with minor modifications (140) in an anaerobic chamber (Coy Lab Products, Grass Lake MI) containing 5% CO_2, 10% H_2 and 85% N_2 maintained at 37 ^{o}C. Briefly, the input and output on Watson Marlow pumps were set at 1 rpm and 2 rpm

respectively. The rotating magnetic stirrer was set at 130 rpm. The media were allowed to flow continuously for 24 hours

each in 6 replicates. Three hundred microliters of the inoculum were introduced into all wells with a retention time of 16 hours. The bioreactor was allowed to run continuously for 21 days (Figure 4). Samples were collected from each reactor well into microcentrifuge tubes and directly frozen at -80^{o}C for later analysis.

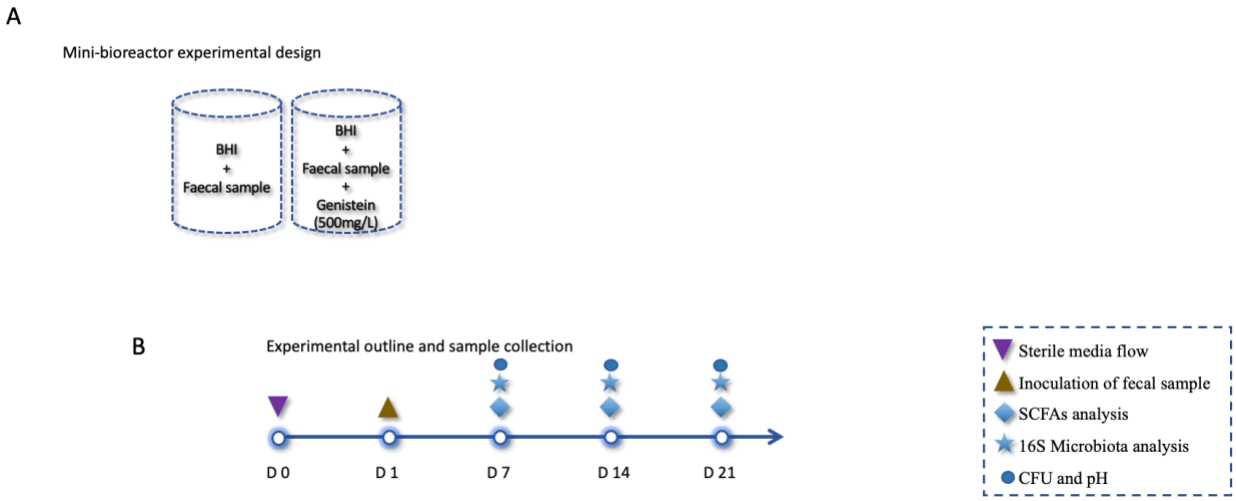


Figure 4: Overview of the study design

A schematic diagram showing the media conditions used in the study. Genistein was used at a final concentration of 500 mg/L in the modified BHI, the control group consisted of modified BHI and fecal samples only. Each condition was run in 6 replicates and inoculated with the same inoculum. **B** Outline of the bioreactor experiment demonstrating the time points for fecal inoculation and sample collection.

2.3 Microbial DNA extraction and sequencing

DNA isolation was performed on 36 samples. The DNA was extracted from 500 µl of the sample using a Powersoil DNA isolation kit (MoBio Laboratories Inc, CA) following the manufacturer's instructions. After extraction, the quality of DNA was measured using NanoDrop^{TM} one (Thermo Fisher Scientific, DE) and quantified using Qubit Fluorometer 3.0 (Invitrogen, CA). The DNA samples were stored at -20^{o}C until further use. To analyze the variation of the microbial composition over time, all samples were amplicon sequenced using an Illumina MiSeq platform with paired-end V3 chemistry. The library was prepared using an Illumina Nextera XT library preparation kit (Illumina Inc, CA) targeting V3-V4 regions of the 16S rRNA. The libraries were bead normalized and multiplexed before loading into the sequencer (Miseq, SY-410-1003, Illumina Inc, CA).

2.4 16S rRNA data analysis

The time-series changes in the microbial communities were analyzed using 16S rRNA community analysis in QIIME, Version 2.0 (141). Briefly, the demultiplexed reads obtained were quality filtered using the q2-demux plugin and denoised applying DADA2. The outputs were then imported into Microbiome Analyst (142) for visualizations. Shannon diversity and Bray Curtis dissimilarity indices were calculated as alpha and beta diversity metrics. The reads were normalized by rarefying to 25,000 and the taxonomy was assigned to amplicon sequence variants using the q2-feature-classifier (143) using Silva as the reference (144).

2.5 Estimation of Short-chain fatty acids

For the estimation of short-chain fatty acids (SCFAs), 800 µl of samples were collected from each mini bioreactor, mixed with 160 µl of 25% m-phosphoric acid (Lot: 188565, Fisher Chemical) and frozen at -80°C until further analysis. Later, the frozen samples were thawed and centrifuged (>15,000 × g) (LEGEND XFR centrifuge, Thermo Scientific) for 20 min. Five hundred microliters of clean supernatant were collected into a 2 ml screw vial kit (Lot: 887040385834, ThermoScientific) before loading into the gas chromatograph (Agilent Technologies, USA) for analysis (138). The SCFA concentrations were compared between the groups using the Two-way ANOVA and visualized using GraphPad Prism 6.0.

3 Results

3.1 Identification and diversity indices of isolates

Fecal samples of pigs cultured in a mini bioreactor and supplemented with or without genistein were collected on days 7, 14 and 21. DNA extraction was performed on these samples and the extracts were sent for 16S rRNA sequencing. QIIME2 analysis and Microbiome Analyst visualization revealed six phyla namely Firmicutes, Bacteroidota, Actinobacteria, Proteobacteria, Synergistota and Euryarcheota. The actual abundances revealed Firmicutes to be abundant in both groups and appeared to be almost the same on day 7. On day 14, genistein supplementation appeared to have increased Firmicutes abundance and decreased by day 21. However, the relative abundances showed that Firmicutes abundance remained stable from day 7 to day 21(Supplementary figure 1). Bacteroidota was the second most abundant phylum which showed a gradual decrease from day 7 to day 21 in both groups. Proteobacteria was increased in the control group and appeared to be highly reduced in the genistein-treated group by day 21. Synergistota

appeared to be present only on day 21 (Figure 5). At the genus level, the large increase in the Firmicutes is a result of the presence of several genera such as *Streptococcus*, *Megasphaera, Acidaminococcus, Mitsuokella, Lactobacillus* and *Eubacterium* (Figure 6, Supplementary figure 2) all belonging to this phylum.

Genistein supplementation appears to have slightly increased the community richness on day 7 and reduced the community richness by day 21 (Figure 7) ($p = 0.013806$). The PCoA plot shows that each group is dissimilar from the other, and this dissimilarity is seen even in the same group with different days ($p < 0.001$) (Figure 7).

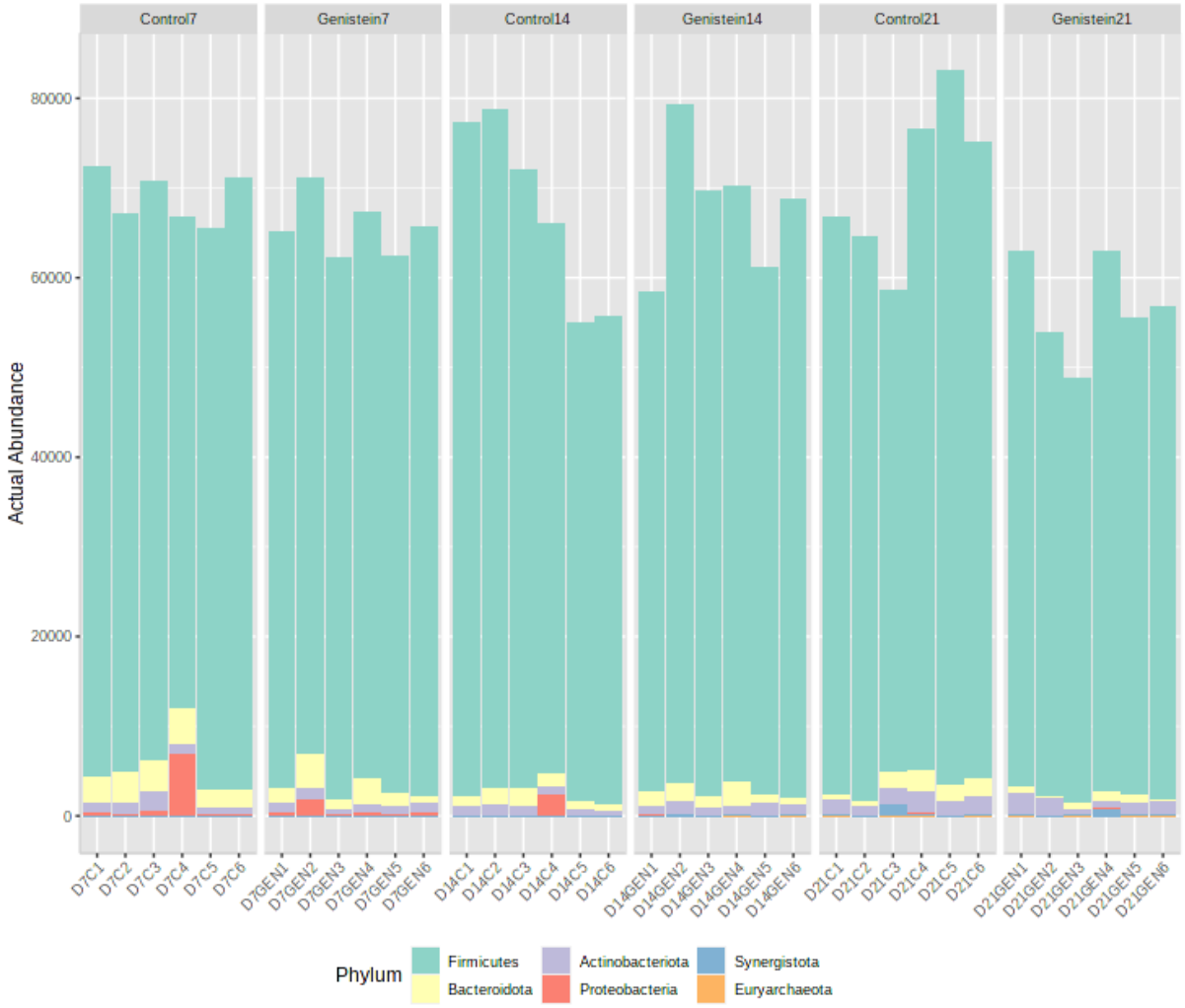


Figure 5: Actual abundances for Phyla for genistein treated and control groups on days 7, 14 and 21

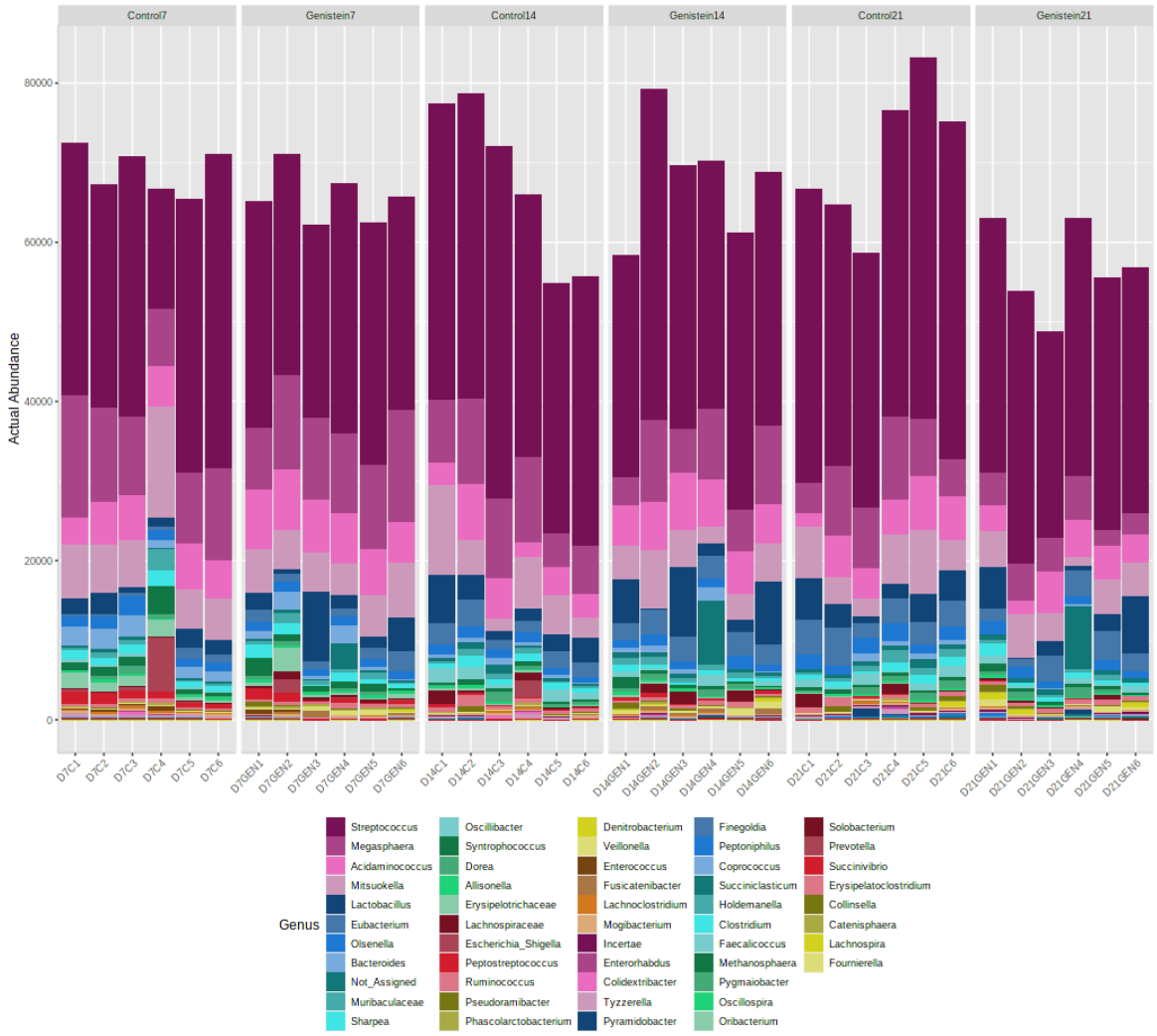


Figure 6: Actual abundances for Genus for genistein treated and control groups on days 7, 14 and 21

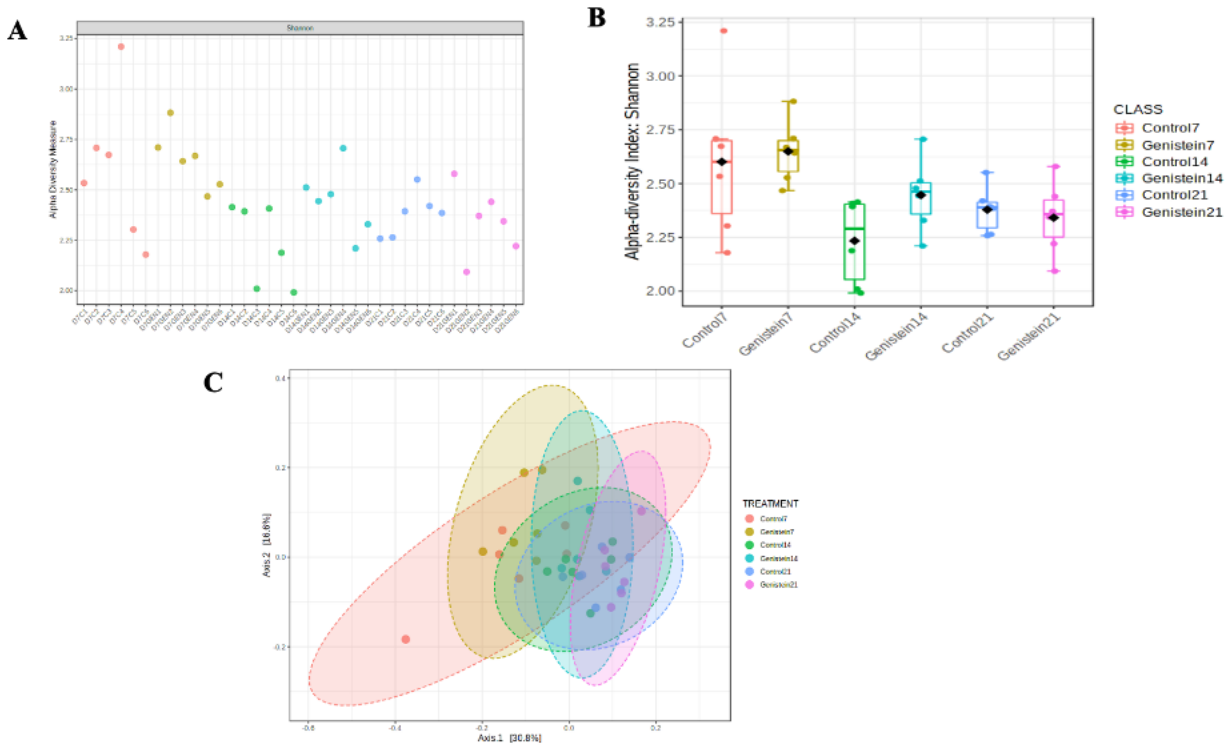


Figure 7: Alpha and beta diversities of microbiota in genistein and control groups.

A and **B** Alpha diversity measure (Shannon diversity) of genistein supplemented and control groups on days 7, 14 and 21. Statistically, ANOVA was calculated on the alpha diversity with $p = 0.013806$, $F = 3.4591$. **C** **Bray**-Curtis distance calculation to visualize differences between groups. PERMANOVA was calculated with $p < 0.001$, $F = 3.2103$ and R-squared = 0.34856.

3.2 Genistein supplementation resulted in no butyrate production

The gut microbiota mediates the impact of diet on the metabolic status of the host. Hosts who ingest meals low in microbiota-associated carbohydrates produce less SCFAs (145). In order to elucidate how genistein has altered the fermentation capacity of the pig microbiota, we measured SCFAs from each mini bioreactor on days 7, 14 and 21. We observed an increase in acetate production from day 7 to 21 in the genistein-treated group although not significantly different compared to the control (Figure 8A) while isobutyrate and propionate levels decreased from day 7 to 21 in the genistein-treated group (Figure 8B and C). There was a significant difference ($p<0.05$) in propionate production between days 7 and 14, where we observe a decrease in the genistein-treated group as compared to the control. There appears to be no butyrate production in the genistein group (Figure 8D).

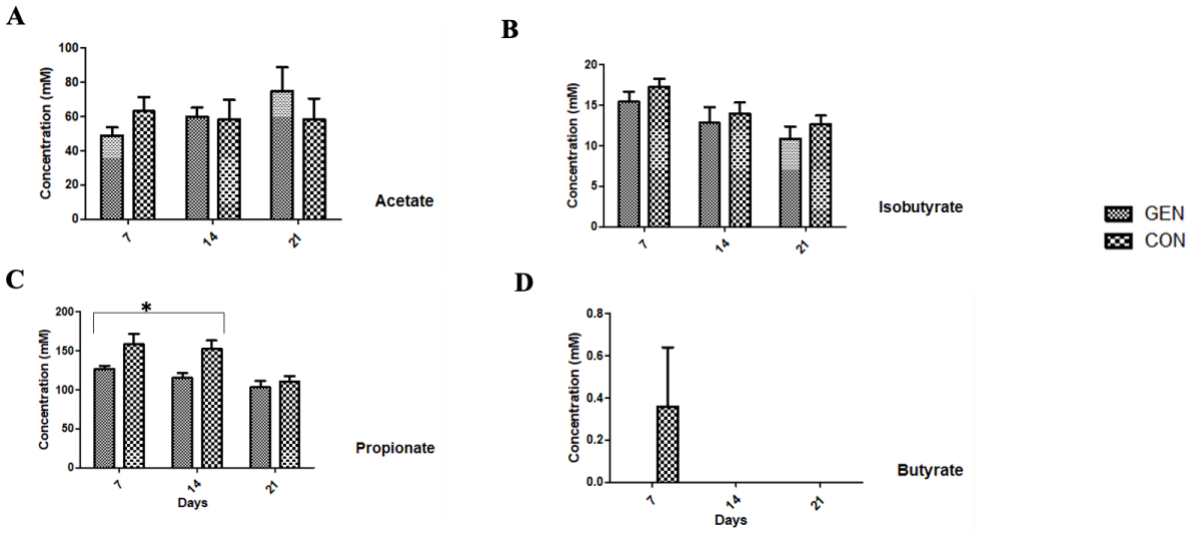


Figure 8: Effect of genistein on short-chain fatty acids production in mini bioreactors.

A to **D** represent the comparison of concentrations of acetate, Isobutyrate, propionate and butyrate respectively from genistein treated (GEN) and control (CON) groups on days 7, 14 and 21. A two-way ANOVA test was performed between the days and groups to identify the different significant groups. '*' represents significance at $p < 0.05$ in propionate production on days 7 and 14 in the genistein-treated group. Error bars represent the standard error of the mean of data obtained from six different bioreactors.

Table 3 : Effect of genistein on short chain fatty acids production in mini bioreactors (mean+/-standard deviation of concentrations)

SCFAs	Mean of GEN group day 7	Mean of GEN group day 14	Mean of GEN group day 21	Mean of CON group day 7	Mean of CON group day 14	Mean of CON group day 21	P-value	Summary
Acetate	49.03±11.40	59.83±13.19	75.15±33.69	63.09±20.37	58.42 ±27.64	58.29±30.30	> 0.05	ns
Isobutyrate	15.46±3.05	12.84±4.74	10.91±3.65	17.30±2.43	14.01 ±3.28	12.69±2.54	> 0.05	ns
Propionate	126.50±10.93	115.60±15.17	103.30±20.81	158.80±32.64	152.40±26.99	110.80±16.86	< 0.05	*
Butyrate	0.00	0.00	0.00	0.36 ±0.69	0.00	0.00	> 0.05	ns

4 Discussion

In vivo, in vitro, and clinical studies have shown changes in microbial communities because of genistein treatment and their different mechanisms of action which include antibacterial and anti-inflammatory (6, 14, 59, 66-69, 146). Host factors probably contributed to the variations in microbiota composition in these studies as most of these experiments were carried out in rodents, cattle, and a few pigs. The metabolism of genistein by pig microbiota is underexplored. In this project, we aimed at exploring the impact of genistein on pig microbiota using an in vitro mini bioreactor model (139). The mini bioreactor model eliminates host factors while studying microbial communities in the gut. 16S rRNA sequencing was performed on DNA extracted from mini bioreactor samples.

Our report on the increased abundance of Firmicutes in the gut microbiota of pigs with and without genistein is consistent with other swine studies carried out in Germany, USA, Canada, Denmark and China (119, 120). A study by Quan et al. (2018) which revealed the predominance of *Streptococcus* and *Lactobacillus* in the colon of pigs (115) agrees with our findings. *Eubacterium sp.* are capable of isoflavone conversion to equol and 5-hydroxy equol (147) hence their presence in the taxonomy output. Proteobacteria comprise of several pathogens causing conditions characterized by inflammation (148). The decrease in taxa abundance by day 21 in the genistein-treated group is possible due to the antibacterial and anti-inflammatory actions of genistein exerted on these pathogens.

Genistein, an isoflavone has been reported to have antibacterial effects on pathogens such as *Staphylococcus sp.* by inhibiting their exotoxins (72) and preventing biofilm formation in *Escherichia coli (69)*. It is also predominant in soybean, a major component of bovine and pigs' diet. *Streptococcus sp* comprise a group known as *Streptococcus bovis/equine*

complex which are used as probiotics for young calves to establish their anaerobic microbiota (149). However, studies on genistein-enriched microbiota from pigs and their potential use as probiotics are limited. The abundance of *Streptococcus sp* from the genistein-enriched pig microbiota makes it promising to supply these species together with genistein as probiotics to piglets to provide beneficial effects.

Microbiota-accessible carbohydrates (MACs) are carbohydrates that are resistant to host digestion and can only be acted on by gut microbes to produce SCFAs. They can be found in fruits, vegetables, and legumes. One would expect that since genistein is predominant in legumes, their supplementation would result in SCFAs production (145). Also, inulin, a starchy substance that can only be acted on by gut microbes formed part of our modified BHI. SCFAs are important metabolites in maintaining intestinal homeostasis (150). Our study shows the production of acetate, isobutyrate and propionate but no butyrate production in the genistein-treated group. Firmicutes are butyrate producers (151), and our study reports an increase in the abundance of this phylum. However, the common species responsible for producing butyrate which include *Faecalibacterium prausnitzii, Eubacterium rectale, Roseburia spp., Clostridium butyricum, Clostridium Beijerinkii, Eubacterium hallii, Anaerostipes spp.* and *Butyricicoccus pullicaecorum* are missing. The absence of these butyrate producers explains why we recorded no butyrate production (152-154).

In conclusion, we report an increase in abundance of the of six genera *including Streptococcus, Eubacterium* and *Lactobacillus* resulting in the abundance of the phylum Firmicutes. Absence of butyrate producing-bacteria yielded no butyrate production. This study was conducted using fecal samples from five pigs from the same facility. A larger

sample size from different facilities could have contributed to a more diverse pig gut microbiota library. In our next study, the cultures from the mini bioreactor would be screened for identification of potential probiotic bacteria.

Chapter 3: A preliminary screen to identify potential probiotic bacteria from the enriched pig gut microbiota library

Abstract

The emergence of multidrug-resistant pathogenic strains has made it necessary to explore other alternatives that are safe and efficient. We identified ~540 isolates and 19 species from our genistein-enriched library using MALDI-TOF MS and 16S Sanger sequencing. We confirmed the ability of representative species to metabolize genistein and conducted hemolysis and invasion assays to rule out potential pathogens. *Lactobacillus salivarius* was completely hemolytic, *Mitsuokella jalaludinii* and *Peptostreptococcus russellii* could not completely metabolize genistein and about 61 % of our species were non-invasive. In conclusion, we have developed a library of potential probiotics that include *Streptococcus gallolyticus, Acidaminococcus fermentans, Streptococcus equinus, Streptococcus alactolyticus, Bacteroides vulgatus, Bacteroides fluxus, Bacteroides uniformis, Sharpea azabuensis, Selenomonas montiformis* and *Syntrophococcus sucromutans.* Experiments would be conducted on these species to assess their in vitro adhesion and pathogen exclusion, in vivo colonization and pathogen exclusion and acid and bile tolerance abilities.

1 Introduction

In our quest to find antibiotic alternatives for use in medicine, agriculture and veterinary, there is the need to ensure that these alternatives are non-pathogenic, capable of exerting a beneficial effect on the host, and are anti-inflammatory and immunostimulatory. Isoflavones such as genistein have been studied for their antibacterial properties in *Vibrio harveyi*, *Bacillus subtilis* and *Escherichia coli* (71). Other microorganisms thrive in the presence of isoflavones and are able to metabolize them.

Bacterial strains SNU NiuO16, SNU Julong732, *Slackia equolifaciens*, *Slackia isoflavoniconvertens*, *Eggerthella sp*, D-G6, *Eubacterium* strains D1 and D2, *Hugonella massiliensis*, *Senegalimassilia faecalis*, MT1B8, *Lactobacillus delbrueckii-like* strain MF-07 and *Escherichia coli* HGH21 and strain HGH6 have been reported to convert daidzein and genistein to equol and 5-hydroxy-equol, respectively. Their individual metabolic conversion was lower than that of a complex community of feces with different bacteria, suggesting the involvement of other species (127-133, 155). It is therefore imperative to isolate and identify other potential metabolizers of isoflavones, especially genistein and study their possible use as alternatives to antibiotics in the pig industry. In the pig industry, the transition to the post-weaning stage results in manifestations of diarrhea, dehydration, significant mortality and loss of body weight in surviving pigs. In order to reduce financial losses, antibiotics are included in their diets to help alleviate these symptoms (156). Probiotics isolated from pig gut microbiota would be ideal alternatives.

We identified over 500 isolates from the fecal samples of finisher pigs supplemented with 500 mg/L of genistein using automated systems and manual anaerobic routine culture.

Nineteen of these isolates were distinct species that were tested for their potential as probiotics.

2 Materials and methods

2.1 Isolation and identification of genistein-metabolizing bacteria

Isolation of species was done using a micro cultivation array (General Automation Lab Technologies Inc) and routine anaerobic culture using modified BHI media supplemented with a combination of ampicillin sodium salt (Lot: 194962, Fisher Scientific) and clindamycin hydrochloride monohydrate (Lot: WHPMD-NQ, Tokyo Chemical Industry) at a concentration of 1 mg/L. MALDI-TOF MS (BRUKER Microflex) was used in species identification after smearing bacterial colonies on the MALDI-TOF target plate and covering with 1 μl of the matrix solution. Bacteria test standard was used as a control.

Species that could not be identified with MALDI-TOF MS were identified with 16S Sanger sequencing. For this, DNA was extracted using a Powersoil DNA isolation kit (MoBio Laboratories Inc, CA) following the manufacturer's instructions. After extraction, the quality of DNA was measured using NanoDrop^{TM} one (Thermo Fisher Scientific, DE) and quantified using Qubit Fluorometer 3.0 (Invitrogen, CA). The DNA samples were stored at -20^{o}C until they were ready to be sent for Sanger Sequencing. FASTA sequences sent to us were identified using EzBioCloud (157).

2.2 DPH assay

The ability of the species to degrade genistein was determined using DPH (Lot: A366360, ACROS Organics) assay following a protocol by Lilian Schoefer et al. (2001)(158). DPH and genistein were dissolved in DMSO at concentrations of 0.5mM and 10mM

respectively. A circular nylon membrane was soaked in a mixture of 350 µl 0.5 mM DPH and 350 µl 10 mM Genistein and transferred onto a modified BHI agar plate. Bacteria colonies were smeared on the membrane and incubated under anaerobic conditions at 37°C for 24 to 48 h. For detection of fluorescence, the plate was kept under a UV lamp (Crystal BioGrow, BG-32-AA) at a wavelength of 365 nm.

2.3 Hemolysis screening

Isolates were grown separately in 3 mL of anaerobic modified BHI broth and incubated at 37°C overnight. Commercially available sheep blood agar plates (Carolina biological supply company, Burlington, North Carolina) were purchased and pre-reduced under anaerobic conditions for 48h. The isolates were streaked on pre-reduced sheep blood agar plates and incubated for 48h at 37°C under anaerobic conditions.

2.4 Invasion assay

Dulbecco's Modified Eagle Medium (Lot: RNBK0461, Sigma Aldrich) supplemented with 10% (v/v) FBS (Lot: 2405706RP, gibco) and 1% Penicillin/ Streptomycin (Lot: 30002345, Corning) was used to maintain and passage Caco-2 cells (#55-65). Non-hemolytic isolates were cultured in 5 mL of modified BHI broth and incubated overnight at 37°C under anaerobic conditions. Gentamicin protection protocol described by Lee et al. was used with slight modifications (159) Bacteria cultures were adjusted to an OD_{600} of 1 and used to infect Caco-2 cells at an MOI of 10 in 24 well plates (Cyto One). The plates were incubated at 37°C in 5% CO_2 for 2 h in an Isotemp Co_2 Incubator (Fisherbrand). Gentamicin (Lot: 3682558, EMD Millipore Corp.), at a concentration of 100 µg/ml was used to kill extracellular non-invading bacteria. The plate was incubated again for 1 h at 37°C in 5% CO_2. Cells were then lysed with 1% Triton X-100 (Sigma) for 10 minutes to release

intracellular bacteria. 100 µl of lysed cell suspension from each well was serially diluted using PBS and plated on modified BHI agar plates. Plate count was taken after 48h.

3 Results

3.1 Identification of genistein-enriched pig microbiome

We identified ~540 isolates from the pig gut across 12 genera using MALDI-TOF MS and EzBioCloud (Supplementary Table 1). Modified BHI was used to culture the isolates received from GALT and MALDI-TOF MS revealed that over 80 % belonged to the genus *Streptococcus* and a total of 10 species were isolated. The species are *Streptococcus lutetiensis, Streptococcus equinus, Streptococcus alactolyticus, Streptococcus gallolyticus, Enterococcus faecalis, Acidaminococcus fermentans, Lactobacillus salivarius, Faecalicoccus pleomorphus, Peptostreptococcus russellii* and *Mitsuokella jalaludinii* (Table 4, Figure 9). DNA was extracted from isolates that could not be identified with MALDI-TOF MS (7.5 %) and sent out for 16S Sanger sequencing. EzBioCloud identification showed four new genera namely Sharpea *azabuensis, Selenomonas montiformis, Syntrophococcus sucromutans* and *Collinsella phocaeensis* (Table 5). An antimicrobial susceptibility test showed that the genus *Streptococcus* was susceptible to either clindamycin or ampicillin at a concentration of 1 mg/L (Supplementary figure 3). Hence, we supplemented the modified BHI with a combination of these two antibiotics at this concentration and manually isolated 5 other species namely *Bacteroides vulgatus, Bacteroides uniformis, Bacteroides fluxus, Lactobacillus agilis* and *Enterococcus avium* (Table 6, Figure 10). In total, 19 distinct species were isolated and identified.

Table 4: Species isolation using a micro cultivation array (GALT)

Name of strain	**Frequency (%)**
Streptococcus lutetiensis	45.3
Streptococcus equinus	26
Streptococcus alactolyticus	5
Streptococcus gallolyticus	7.1
Enterococcus faecalis	3.4
Acidaminococcus fermentans	1.1
Lactobacillus salivarius	0.4
Faecalicoccus pleomorphus	0.4
Peptostreptococcus russellii	0.2
Mitsuokella jalaludinii	0.2
No identification	7.5
No growth	3.6

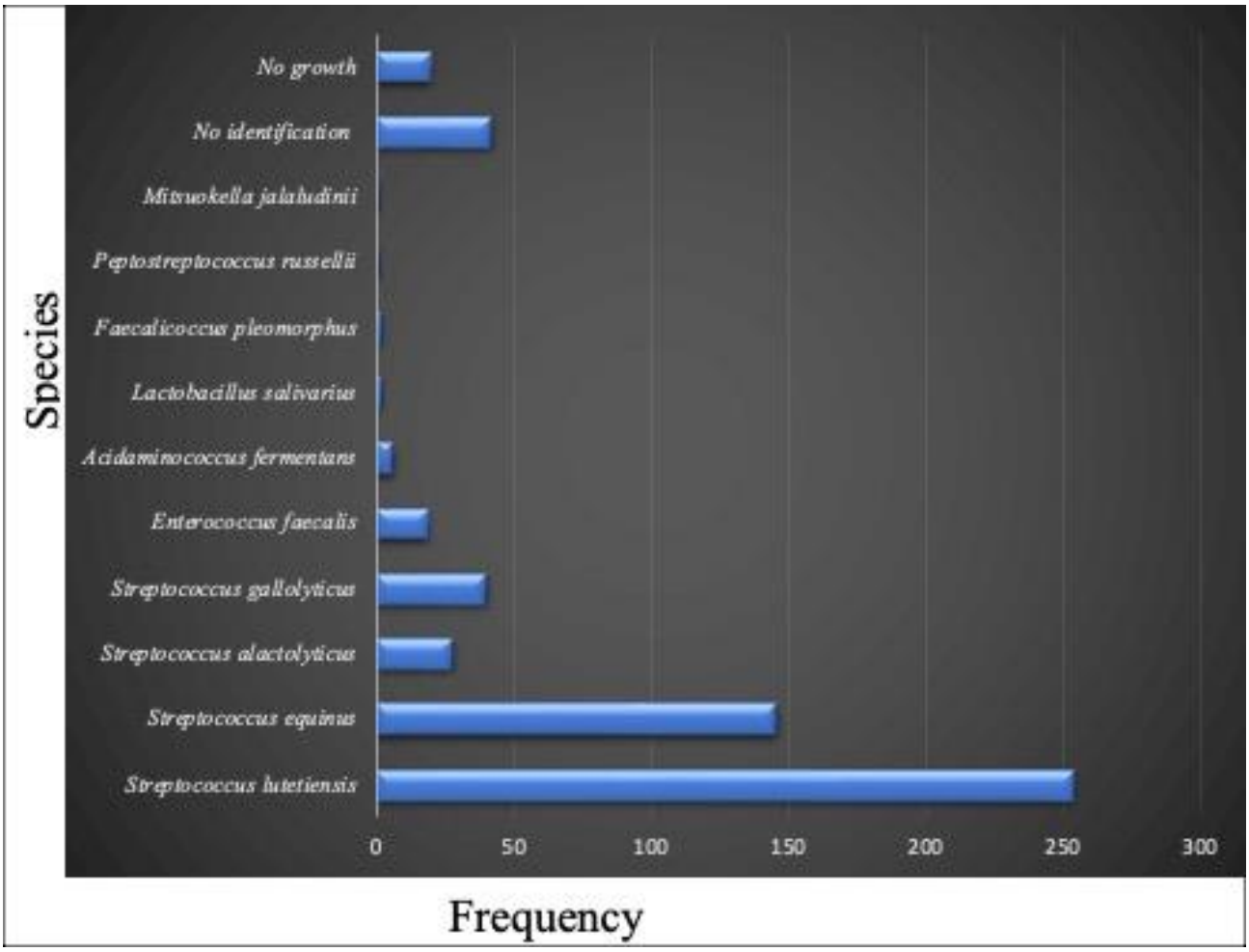


Figure 9: Species isolation using a micro cultivation array (GALT)

A bar plot representing the individual isolates from micro cultivation array (GALT) identified with MALDI-TOF MS.

Table 5 : Species identification with EzBioCloud

Name of species	**Frequency (%)**
Collinsella phocaeensis	2.2
Mitsuokella jalaludinii	6.7
Selenomonas montiformis	2.2
Sharpea azabuensis	2.2
Syntrophococcus sucromutans	2.2
Streptococcus equinus	6.7
Streptococcus lutetiensis	77.8

Table 6: Species isolation using routine culture methods

Name of species	**Frequency**
Enterococcus faecalis	20
Bacteroides vulgatus	5
Lactobacillus salivarius	8
Bacteroides uniformis	6
Bacteroides fluxus	1
Lactobacillus agilis	1
Enterococcus avium	3
No growth	3
No identification	1

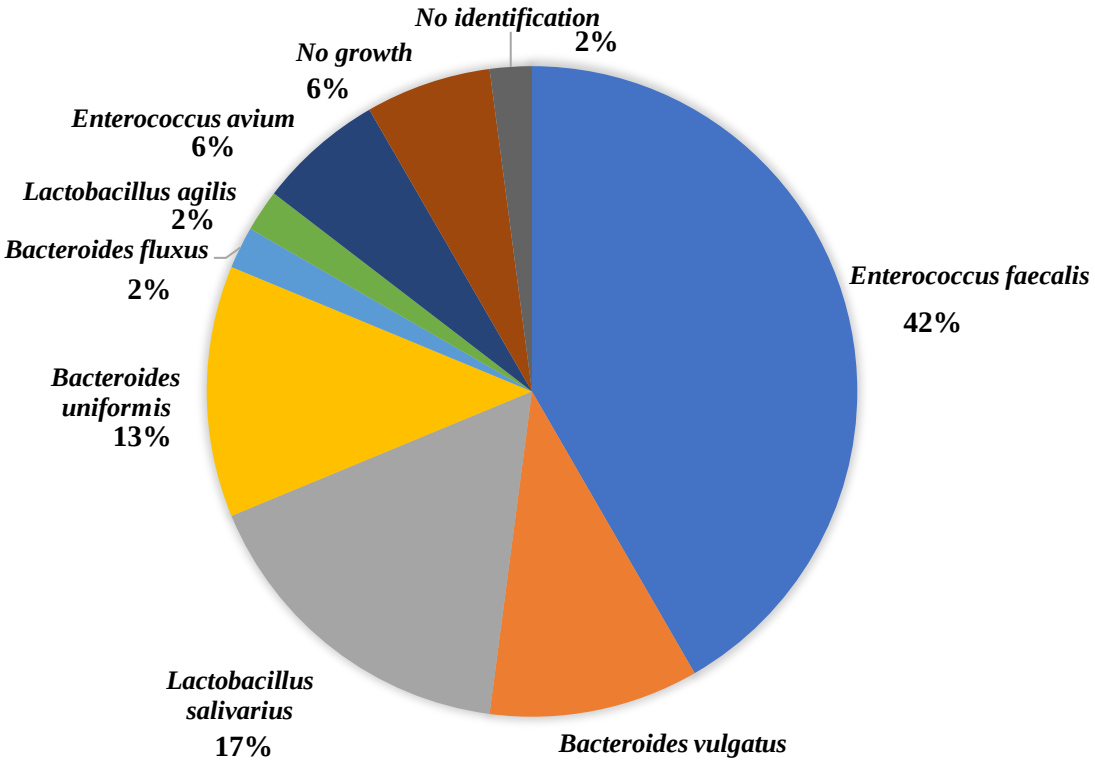


Figure 10: A pie chart representing the species isolated with modified BHI supplemented with antibiotics and identified with MALDI-TOF MS

3.2 Hemolysis screening revealed one species as completely hemolytic

Hemolysis screening is one of a battery of tests recommended by the FAO/WHO in establishing safety guidelines for probiotic species. In this assay, screening is performed on blood agar plates, which are complex media containing 5% sheep red blood cells and tests the ability of an organism to produce hemolysins. These hemolysins damage red blood cells. The blood agar plate is read for complete (β), partial (α) or no (γ) hemolysis. Of the 19 species cultured, *Lactobacillus salivarius GEN 087* was completely hemolytic and the remaining species were non-hemolytic (Figure 11).

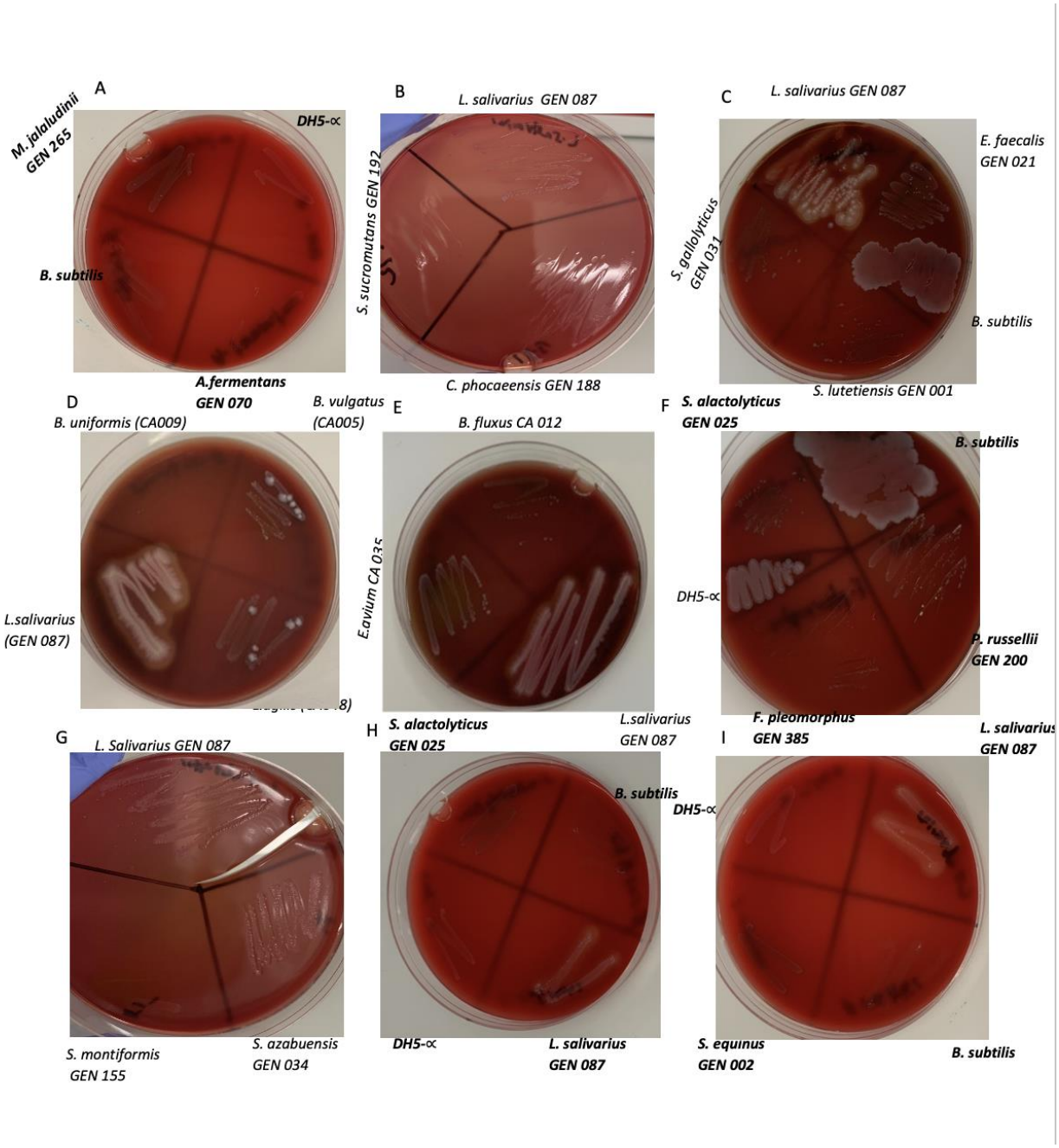


Figure 11: Hemolysis screening of isolated species

A to **I represent** the growth of selected species from the genistein-enriched library on sheep blood agar plates

3.3 Genistein metabolism by isolates

The DPH assay is a qualitative, easy and fast method for the detection of microorganisms with the ability to transform genistein based on the interaction of these compounds with the fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH). The underlying principle of DPH assay is genistein attached to DPH quenches its ability to fluorescence (158). If the bacteria of interest can transform genistein, it will cleave the DPH from genistein and there would be fluorescence (observed as a white spot), otherwise, there is no fluorescence (observed as a brown spot). *E. coli* and *B. glycinifermentans* served as negative and positive controls, respectively. Ideally, all species are expected to metabolize genistein since they were isolated from genistein-enriched samples. However, it appears *M. jalaludinii* and *P. russellii* have light brown spots (Figure 12).

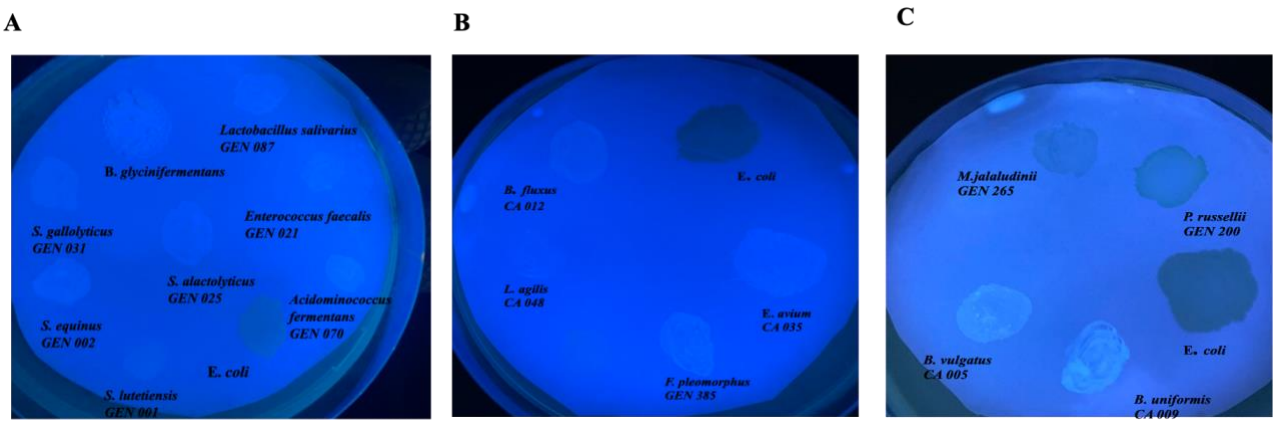


Figure 12: Genistein degradation ability of isolated species

A to **C** represent fluorescence of DPH after cleavage of genistein by selected species from the genistein-enriched library on nylon membranes lined on modified BHI plates.

3.4 61% of species were non-invasive and could qualify as potential probiotics

A good probiotic should be able to attach and colonize the host to exert its function. Any species that is invasive is outrightly ruled out as a possible probiotic. In fact, invasion is a characteristic of pathogenic microorganisms. Eighteen out of the 19 species that were non-hemolytic were investigated for their ability or inability to invade Caco-2 cells at an MOI of 10. *Salmonella enterica* and *Bifidobacterium longum* were positive and negative controls, respectively. Eleven species namely *Streptococcus gallolyticus, Acidaminococcus fermentans, Streptococcus equinus, Streptococcus alactolyticus, Bacteroides vulgatus, Bacteroides fluxus, Mitsuokella jalaludinii, Bacteroides uniformis, Sharpea azabuensis, Selenomonas montiformis* and *Syntrophococcus sucromutans* were non-invasive (Figure 13).

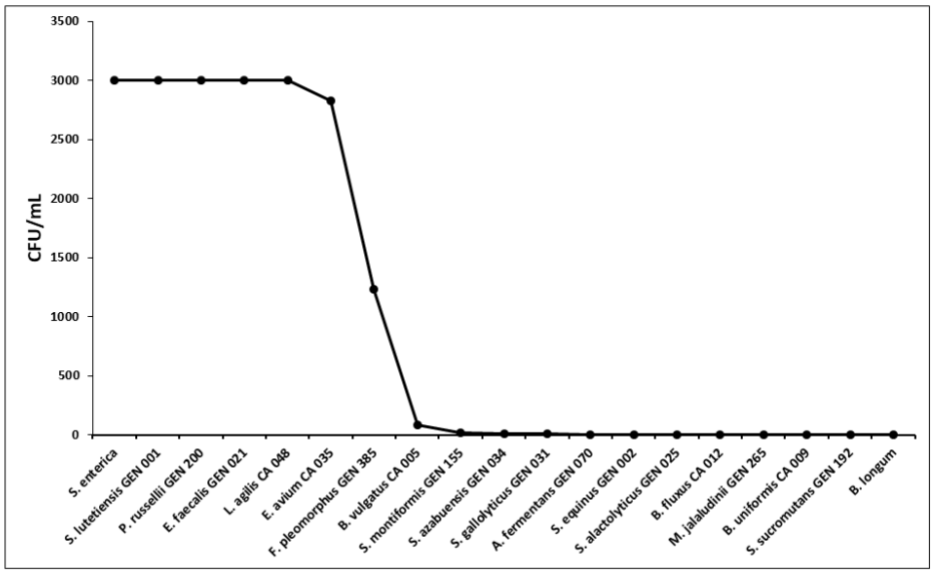


Figure 13: Invasion screening of selected species using in vitro Caco-2 cells

4 Discussion

Routine anaerobic culture methods are fast, easy and relatively cheaper ways to isolate species. However, advances in research have proven that combining culturomics with next-generation sequencing (NGS) ensures that enough diversity is covered (121). Species that are not cultivable could be identified with NGS and vice versa. In this study, we identified 19 distinct species from over 540 isolates that were cultured with modified BHI supplemented with or without 1 mg/L of clindamycin and ampicillin. Identification was done for most of the isolates using MALDI-TOF MS and the unidentified ones with 16S Sanger sequencing. The most abundant of these species belong to the *Streptococcus* genera (83.4%) which is consistent with our 16S rRNA sequencing data. Eight genera were identified with the anaerobic routine culture method (culturomics) namely *Streptococcus, Enterococcus, Acidaminococcus, Lactobacillus, Faecalicoccus, Peptostreptococcus, Mitsuokella* and *Bacteroides*. A study by Wylensek et al. (2020) cultured a collection of bacteria from the gut of 19 pigs and reported about 16 genera (119). Our study reports 7 out of these 16 genera; *Mitsuokella* was not identified. The difference in the diversity is because this group used 26 different enriched and selective culture media which included BHI to isolate bacteria from a larger number of pigs. The four genera identified using 16S Sanger sequencing are *Sharpea, Selenomonas, Collinsella,* and *Syntrophococcus.* All the genera identified with culturomics were present in the taxa data from the 16S analysis, however, the 16S analysis revealed other genera that could not be captured with culturomics.

Hemolysis screening showed that *Lactobacillus salivarius 087* was completely hemolytic and was ruled out as a potential probiotic. Although probiotics are made mainly from lactic

acid-producing bacteria from the genera *Bifidobacterium* and *Lactobacillus* (160-162), some species and strains of *Lactobacillus* are pathogens. For instance, a study by Salminen et al. (2006) isolated some *Lactobacillus sp.* including *Lactobacillus salivarius* from bacteremia patients (163). Our report contradicts a study by Li et al. (2020) which showed that *Lactobacillus salivarius* isolated from wild boar was not hemolytic (161). The differences in habitats of wild boar and finisher pigs as well as the differences in strains of *Lactobacillus salivarius* could contribute to this discrepancy.

The fluorescence quenching test allows quick and easy identification of isoflavone-degrading bacteria circumventing time-consuming analysis to detect degradation (158). Schoefer et al. (2001) tested the ability of *Eubacterium ramulus* to degrade flavonoids using the fluorescent quenching test. Isoflavones such as genistein could strongly quench DPH fluorescent, suggesting that the species metabolized genistein (158). We expected that the species isolated from the genistein-enriched library should degrade genistein. However, two of them namely *M. jalaludinii GEN265* and *P. russellii GEN 200* could not degrade genistein. A possible reason is that they probably partially degrade genistein and hence, a concentration of genistein is still attached to a part of DPH, preventing it from completely fluorescing.

Invasive pathogens are disease-causing microorganisms that disseminate to new locations to pose the risk of disease. Invasive bacterial infections are usually caused by *S. aureus, S. pneumoniae, S. agalactiae, Salmonella sp., H. influenzae, N. meningitidis, and L. monocytogenes(164)*. Our study reports new species that were invasive in an in vitro Caco-2 cell model. They are *Faecalicoccus pleomorphus GEN385, Peptostreptococcus russellii GEN200, Streptococcus lutetiensis GEN 001, Enterococcus faecalis GEN 021,*

Lactobacillus agilis CA 048 and *Enterococcus avium CA 035.* In vivo experiments need to be conducted to verify the pathogenicity of these species.

To sum it all up, we have isolated 19 species from which one is hemolytic, two partially degrade genistein, and six are invasive. The remaining 10 species namely *Streptococcus gallolyticus, Acidaminococcus fermentans, Streptococcus equinus, Streptococcus alactolyticus, Bacteroides vulgatus, Bacteroides fluxus, Bacteroides uniformis, Sharpea azabuensis, Selenomonas montiformis* and *Syntrophococcus sucromutans* are safe at this juncture to consider as potential probiotics. They have not been reported in any study yet. Previously reported swine gut microbiota strains capable of genistein metabolism are *Eubacterium* strains D1 and D2(129). Other strains from other hosts are strains SNU NiuO16 from bovine rumen, SNU Julong732 from human intestine, *Slackia equolifaciens, Slackia isoflavoniconvertens, Eggerthella sp,* D-G6, *Hugonella massiliensis* and *Senegalimassilia faecalis* from humans, MT1B8 from mouse, *Lactobacillus delbrueckii-like* strain MF-07 from chicken, *Escherichia coli* HGH21 and strain HGH6 (127, 128, 130-133).

In this study, modified BHI supplemented with or without ampicillin and clindamycin was the growth media used. This growth media is enriched and can be used to culture a variety of bacteria. However, the inclusion of other enriched and selective media could have helped isolate many other unisolated species.

Chapter 4: Conclusions and future directions

The transition to the post-weaning stage is faced with diarrhea among other symptoms which are of economic significance in the pig industry. Pig farmers incur financial losses when these pigs lose body weight and others eventually die. To minimize these losses, antibiotics are included in the diets and drinking water of pigs. The emergence of multidrug-resistant pathogenic strains is shifting our focus to other alternatives that are effective. Genistein is an isoflavone that has been reported to have antimicrobial properties. It is metabolized by the gut microbiota into metabolites that have beneficial impacts on the host. We have developed a genistein-metabolizing library from pig gut microbiota using culturomics and next-generation sequencing. We identified ~540 bacterium isolates and 19 species. We screened these species for safety properties to rule out hemolytic and invasive ones and to confirm their ability to degrade genistein. *Lactobacillus salivarius GEN 087* was the only completely hemolytic species. All except two species (*Mitsuokella jalaludinii GEN 265* and *Peptostreptococcus russellii GEN 200*) could completely degrade genistein and eleven species were non-invasive. We isolated and identified 10 non-pathogenic species that can completely metabolize genistein, which could be potentially used as probiotics to replace antibiotics. We are the first to report these 10 species isolated from pigs as non-pathogenic genistein-metabolizers which could be used as food supplements in post-weaning pigs to alleviate diarrhea. The limitations of our study are the smaller sample size from the same facility and the use of one growth medium (modified BHI), resulting in less diversity of isolates.

The World Health Organization has established basic selection criteria for probiotics which include epithelium adhesion ability, antimicrobial activity and acid and bile tolerance.

Hence, in vitro adhesion and pathogen exclusion assays, in vivo colonization and pathogen exclusion assays and acid and bile tolerance assays should be considered in the future.

APPENDIX

Supplementary Table 1: Isolates retrieved from genistein enriched microbiome library

I.D.	**Species**	**Identification method**	**Isolation media**	Score value
GEN001	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.03
GEN002	*Streptococcus equinus*	MALDI	Modified BHI	1.90
GEN003	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.74
GEN004	*Streptococcus equinus*	MALDI	Modified BHI	1.98
GEN005	*Streptococcus equinus*	MALDI	Modified BHI	2.00
GEN006	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN007	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN008	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN009	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN010	*Streptococcus equinus*	MALDI	Modified BHI	2.01
GEN011	*Streptococcus equinus*	MALDI	Modified BHI	1.89
GEN012	*Streptococcus infantarius*	MALDI	Modified BHI	1.80
GEN013	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN014	*Streptococcus equinus*	MALDI	Modified BHI	1.72
GEN015	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN016	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN017	*Streptococcus equinus*	MALDI	Modified BHI	2.02
GEN018	*Streptococcus equinus*	MALDI	Modified BHI	2.02
GEN019	*Streptococcus equinus*	MALDI	Modified BHI	1.70

GEN020	*Streptococcus equinus*	MALDI	Modified BHI	1.89
GEN021	*Enterococcus faecalis*	MALDI	Modified BHI	2.29
GEN022	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN023	*Enterococcus faecalis*	MALDI	Modified BHI	2.43
GEN024	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN025	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.91
GEN026	*Streptococcus equinus*	MALDI	Modified BHI	1.88
GEN027	*Streptococcus infantarius*	MALDI	Modified BHI	1.90
GEN028	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN029	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.07
GEN030	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.73
GEN031	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.72
GEN032	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.74
GEN033	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN034	*Sharpea azabuensis*	16S sequencing	Modified BHI	N/A
GEN035	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.77
GEN036	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN037	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.06
GEN038	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.70
GEN039	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN040	*Enterococcus faecalis*	MALDI	Modified BHI	2.43
GEN041	*Streptococcus equinus*	MALDI	Modified BHI	2.00
GEN042	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.79

GEN043	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN044	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.70
GEN045	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.92
GEN046	*Streptococcus equinus*	MALDI	Modified BHI	1.98
GEN047	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN048	*Streptococcus equinus*	MALDI	Modified BHI	2.02
GEN049	*Streptococcus equinus*	MALDI	Modified BHI	1.82
GEN050	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN051	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN052	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN053	*Streptococcus equinus*	MALDI	Modified BHI	2.03
GEN054	*Streptococcus equinus*	MALDI	Modified BHI	1.97
GEN055	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.86
GEN056	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN057	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN058	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.77
GEN059	*Streptococcus equinus*	MALDI	Modified BHI	2.04
GEN060	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.72
GEN061	*Streptococcus equinus*	MALDI	Modified BHI	1.79
GEN062	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN063	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.73
GEN064	*Streptococcus equinus*	MALDI	Modified BHI	1.87

GEN065	*Streptococcus equinus*	MALDI	Modified BHI	1.83
GEN066	*Streptococcus equinus*	MALDI	Modified BHI	1.87
GEN067	*Streptococcus equinus*	MALDI	Modified BHI	1.96
GEN068	*Streptococcus equinus*	MALDI	Modified BHI	1.97
GEN069	*Streptococcus equinus*	MALDI	Modified BHI	2.05
GEN070	*Acidaminococcus fermentans*	MALDI	Modified BHI	2.50
GEN071	*Streptococcus equinus*	MALDI	Modified BHI	1.96
GEN072	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN073	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.84
GEN074	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN075	*Streptococcus equinus*	MALDI	Modified BHI	1.83
GEN076	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN077	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN078	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN079	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.73
GEN080	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN081	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.80
GEN082	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.81
GEN083	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN084	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.76
GEN085	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.96
GEN086	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.90

GEN087	*Lactobacillus salivarius*	MALDI	Modified BHI	2.05
GEN088	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN089	*Mitsuokella jalaludinii*	16S sequencing	Modified BHI	N/A
GEN090	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN091	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.92
GEN092	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.80
GEN093	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN094	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.73
GEN095	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.70
GEN096	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.94
GEN097	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN098	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN099	*Streptococcus equinus*	MALDI	Modified BHI	1.86
GEN100	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN101	*Streptococcus equinus*	MALDI	Modified BHI	1.90
GEN102	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN103	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.74
GEN104	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN105	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN106	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.77
GEN107	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.75
GEN108	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.74

GEN109	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.77
GEN110	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN111	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN112	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.77
GEN113	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.73
GEN114	*Streptococcus equinus*	MALDI	Modified BHI	2.05
GEN115	*Lactobacillus salivarius*	MALDI	Modified BHI	2.21
GEN116	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN117	*Acidaminococcus fermentans*	MALDI	Modified BHI	2.13
GEN118	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.75
GEN119	*Streptococcus equinus*	MALDI	Modified BHI	1.82
GEN120	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.7
GEN121	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.84
GEN122	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.79
GEN123	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.71
GEN124	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN125	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.97
GEN126	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.75
GEN127	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.90
GEN128	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.03
GEN129	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN130	*Streptococcus equinus*	MALDI	Modified BHI	1.73

GEN131	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN132	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN133	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.73
GEN134	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN135	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN136	*Streptococcus equinus*	MALDI	Modified BHI	1.82
GEN137	*Streptococcus equinus*	MALDI	Modified BHI	1.89
GEN138	*Streptococcus equinus*	MALDI	Modified BHI	1.70
GEN139	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.79
GEN140	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.97
GEN141	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.73
GEN142	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.02
GEN143	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN144	*Streptococcus equinus*	MALDI	Modified BHI	1.77
GEN145	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.86
GEN146	*Enterococcus faecalis*	MALDI	Modified BHI	2.37
GEN147	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.84
GEN148	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN149	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN150	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.84
GEN151	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.77
GEN153	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83

GEN154	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN155	*Selemonas montiformis*	16S sequencing	Modified BHI	N/A
GEN157	*Streptococcus equinus*	MALDI	Modified BHI	2.01
GEN158	*Streptococcus infantarius*	16S sequencing	Modified BHI	N/A
GEN159	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN161	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.80
GEN163	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.78
GEN164	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.99
GEN165	*Streptococcus equinus*	MALDI	Modified BHI	1.76
GEN166	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN167	*Streptococcus equinus*	MALDI	Modified BHI	2.00
GEN168	*Acidaminococcus fermentans*	MALDI	Modified BHI	1.87
GEN169	*Acidaminococcus fermentans*	MALDI	Modified BHI	1.74
GEN170	*Enterococcus faecalis*	MALDI	Modified BHI	2.38
GEN171	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.72
GEN172	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN173	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN174	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN175	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN176	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.05
GEN177	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.80
GEN178	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.97

GEN179	*Streptococcus infantarius*	MALDI	Modified BHI	1.94
GEN180	*Streptococcus equinus*	MALDI	Modified BHI	2.06
GEN182	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN184	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN185	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN186	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN187	*Acidaminococcus fermentans*	MALDI	Modified BHI	1.93
GEN188	*Collinsella phocaeensis*	16S sequencing	Modified BHI	N/A
GEN189	*Enterococcus faecalis*	MALDI	Modified BHI	2.41
GEN190	*Enterococcus faecalis*	MALDI	Modified BHI	2.18
GEN192	*Syntrophococcus sucromutans*	16S sequencing	Modified BHI	N/A
GEN193	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN194	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN195	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN196	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN197	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.75
GEN198	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.86
GEN199	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.70
GEN200	*Peptostreptococcus russellii*	MALDI	Modified BHI	2.32
GEN201	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN202	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN203	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN204	*Acidaminococcus fermentans*	MALDI	Modified BHI	2.39

GEN205	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN206	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN207	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.83
GEN208	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN209	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN210	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.73
GEN211	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.84
GEN212	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN213	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.84
GEN214	*Streptococcus equinus*	MALDI	Modified BHI	1.85
GEN215	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN216	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN217	*Enterococcus faecalis*	MALDI	Modified BHI	2.33
GEN218	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.76
GEN219	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN220	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN221	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.77
GEN222	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN223	*Streptococcus equinus*	MALDI	Modified BHI	1.82
GEN224	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN225	*Enterococcus faecalis*	MALDI	Modified BHI	2.37
GEN226	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.71
GEN228	*Streptococcus equinus*	MALDI	Modified BHI	1.90

GEN229	*Enterococcus faecalis*	MALDI	Modified BHI	2.28
GEN230	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.72
GEN231	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN232	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN233	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN234	*Enterococcus faecalis*	MALDI	Modified BHI	2.40
GEN238	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.70
GEN239	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN241	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.02
GEN242	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.84
GEN243	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN246	*Mitsuokella jalaludinii*	16S sequencing	Modified BHI	N/A
GEN247	*Mitsuokella jalaludinii*	16S sequencing	Modified BHI	N/A
GEN248	*Streptococcus equinus*	MALDI	Modified BHI	1.76
GEN249	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN250	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN251	*Streptococcus equinus*	MALDI	Modified BHI	1.87
GEN253	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN254	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN256	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN257	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN258	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN259	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.90

GEN260	*Streptococcus equinus*	MALDI	Modified BHI	1.98
GEN261	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN262	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN263	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN264	*Streptococcus equinus*	MALDI	Modified BHI	2.03
GEN265	*Mitsuokella jalaludinii*	MALDI	Modified BHI	2.21
GEN266	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.01
GEN267	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN268	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN269	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.77
GEN270	*Streptococcus equinus*	MALDI	Modified BHI	1.97
GEN271	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.84
GEN272	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.84
GEN273	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.71
GEN274	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.75
GEN275	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN276	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.78
GEN277	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN278	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN279	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN280	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN281	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.78

GEN282	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN283	*Enterococcus faecalis*	MALDI	Modified BHI	2.46
GEN284	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN285	*Enterococcus faecalis*	MALDI	Modified BHI	2.31
GEN286	*Enterococcus faecalis*	MALDI	Modified BHI	2.44
GEN287	*Enterococcus faecalis*	MALDI	Modified BHI	2.32
GEN288	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.05
GEN289	*Streptococcus equinus*	MALDI	Modified BHI	2.01
GEN290	*Streptococcus equinus*	MALDI	Modified BHI	2.07
GEN291	*Streptococcus equinus*	MALDI	Modified BHI	2.07
GEN292	*Streptococcus equinus*	MALDI	Modified BHI	2.10
GEN293	*Streptococcus equinus*	MALDI	Modified BHI	2.03
GEN294	*Streptococcus equinus*	MALDI	Modified BHI	1.98
GEN295	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN296	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.11
GEN297	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.08
GEN298	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN299	*Streptococcus equinus*	MALDI	Modified BHI	1.99
GEN300	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.99
GEN301	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN302	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN303	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.02
GEN304	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN305	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87

GEN306	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.99
GEN307	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN308	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.73
GEN309	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN310	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN311	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN312	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN313	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN314	*Enterococcus faecalis*	MALDI	Modified BHI	2.29
GEN315	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN316	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN317	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.02
GEN318	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN319	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.91
GEN320	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.90
GEN321	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.83
GEN322	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN323	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.05
GEN324	*Streptococcus equinus*	MALDI	Modified BHI	2.00
GEN325	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN326	*Streptococcus equinus*	MALDI	Modified BHI	2.04
GEN327	*Streptococcus equinus*	MALDI	Modified BHI	1.87

GEN328	*Streptococcus equinus*	MALDI	Modified BHI	1.97
GEN330	*Streptococcus equinus*	MALDI	Modified BHI	2.02
GEN331	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.09
GEN332	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN333	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN334	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.05
GEN335	*Streptococcus equinus*	MALDI	Modified BHI	2.09
GEN337	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN338	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN339	*Streptococcus equinus*	MALDI	Modified BHI	2.13
GEN340	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN341	*Streptococcus equinus*	MALDI	Modified BHI	1.77
GEN343	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN344	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN345	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.04
GEN346	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.8
GEN347	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN348	*Streptococcus equinus*	MALDI	Modified BHI	2.02
GEN349	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN350	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN351	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN352	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.08

GEN353	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.11
GEN355	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.07
GEN356	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.08
GEN357	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN358	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN359	*Streptococcus equinus*	MALDI	Modified BHI	2.08
GEN360	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.09
GEN361	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.09
GEN362	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.97
GEN363	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN364	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.79
GEN365	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN366	*Streptococcus equinus*	MALDI	Modified BHI	1.90
GEN367	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN368	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN369	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.95
GEN370	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN371	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.04
GEN372	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.90
GEN373	*Streptococcus equinus*	MALDI	Modified BHI	1.83
GEN374	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.90
GEN375	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92

GEN376	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN377	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN378	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN379	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.04
GEN380	*Streptococcus equinus*	MALDI	Modified BHI	1.79
GEN381	*Enterococcus faecalis*	MALDI	Modified BHI	2.24
GEN382	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN383	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN384	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.71
GEN385	*Faecalicoccus pleomorphus*	MALDI	Modified BHI	2.11
GEN386	*Streptococcus equinus*	16S sequencing	Modified BHI	N/A
GEN387	*Streptococcus equinus*	MALDI	Modified BHI	1.97
GEN388	*Streptococcus equinus*	MALDI	Modified BHI	1.85
GEN389	*Streptococcus equinus*	MALDI	Modified BHI	2.02
GEN390	*Streptococcus equinus*	MALDI	Modified BHI	1.97
GEN391	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.83
GEN392	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.70
GEN393	*Streptococcus equinus*	MALDI	Modified BHI	1.99
GEN394	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.75
GEN395	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN396	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89
GEN397	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.77

GEN398	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.78
GEN399	*Streptococcus equinus*	MALDI	Modified BHI	1.82
GEN400	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN401	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN402	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN403	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.70
GEN404	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.72
GEN405	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN406	*Streptococcus equinus*	MALDI	Modified BHI	1.81
GEN407	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN408	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN409	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.74
GEN410	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN411	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.75
GEN412	*Streptococcus equinus*	MALDI	Modified BHI	1.81
GEN413	*Streptococcus equinus*	MALDI	Modified BHI	1.89
GEN414	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN415	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.74
GEN416	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.84
GEN417	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.75
GEN418	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.72
GEN419	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85

GEN420	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.81
GEN421	*Streptococcus equinus*	MALDI	Modified BHI	1.75
GEN422	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN423	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.84
GEN424	Streptococcus lutetiensis	MALDI	Modified BHI	1.81
GEN425	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.08
GEN426	*Streptococcus equinus*	MALDI	Modified BHI	2.1
GEN427	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN428	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN429	*Enterococcus faecalis*	MALDI	Modified BHI	2.42
GEN430	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN431	*Streptococcus equinus*	MALDI	Modified BHI	2.00
GEN432	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN433	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN434	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN435	*Streptococcus equinus*	MALDI	Modified BHI	2.05
GEN436	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.84
GEN437	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82
GEN438	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.79
GEN439	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.78
GEN440	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.71
GEN441	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88

GEN442	*Streptococcus equinus*	MALDI	Modified BHI	2.06
GEN443	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN444	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN445	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN446	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.79
GEN447	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.99
GEN448	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN449	*Streptococcus equinus*	MALDI	Modified BHI	2.01
GEN450	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.73
GEN451	*Streptococcus equinus*	MALDI	Modified BHI	1.76
GEN452	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN453	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.76
GEN454	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN455	*Streptococcus equinus*	MALDI	Modified BHI	1.74
GEN456	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.86
GEN457	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.00
GEN458	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.74
GEN459	*Streptococcus equinus*	MALDI	Modified BHI	1.88
GEN460	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN461	*Streptococcus equinus*	MALDI	Modified BHI	1.77
GEN462	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN463	*Streptococcus infantarius*	MALDI	Modified BHI	1.82

GEN464	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.76
GEN465	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.86
GEN466	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN467	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN468	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN469	*Streptococcus equinus*	MALDI	Modified BHI	1.98
GEN470	*Streptococcus equinus*	MALDI	Modified BHI	1.96
GEN471	*Streptococcus infantarius*	MALDI	Modified BHI	1.98
GEN472	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN473	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.86
GEN474	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN475	*Streptococcus infantarius*	MALDI	Modified BHI	1.93
GEN476	*Streptococcus infantarius*	MALDI	Modified BHI	1.99
GEN477	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.86
GEN478	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN479	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.96
GEN480	*Streptococcus equinus*	16S sequencing	Modified BHI	N/A
GEN481	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN482	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN483	*Streptococcus infantarius*	MALDI	Modified BHI	1.76
GEN484	*Streptococcus equinus*	MALDI	Modified BHI	1.88
GEN485	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.89

GEN486	*Streptococcus equinus*	MALDI	Modified BHI	2.10
GEN487	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.82
GEN488	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN489	*Streptococcus equinus*	MALDI	Modified BHI	1.92
GEN490	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.93
GEN491	*Streptococcus infantarius*	MALDI	Modified BHI	1.73
GEN492	*Streptococcus equinus*	MALDI	Modified BHI	1.87
GEN493	*Streptococcus equinus*	MALDI	Modified BHI	1.78
GEN494	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.78
GEN495	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.79
GEN496	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN497	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN498	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.70
GEN499	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN500	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.83
GEN501	*Streptococcus equinus*	MALDI	Modified BHI	1.74
GEN502	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN503	*Streptococcus equinus*	MALDI	Modified BHI	1.75
GEN504	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN505	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.81
GEN506	*Enterococcus faecalis*	MALDI	Modified BHI	2.4
GEN507	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.82

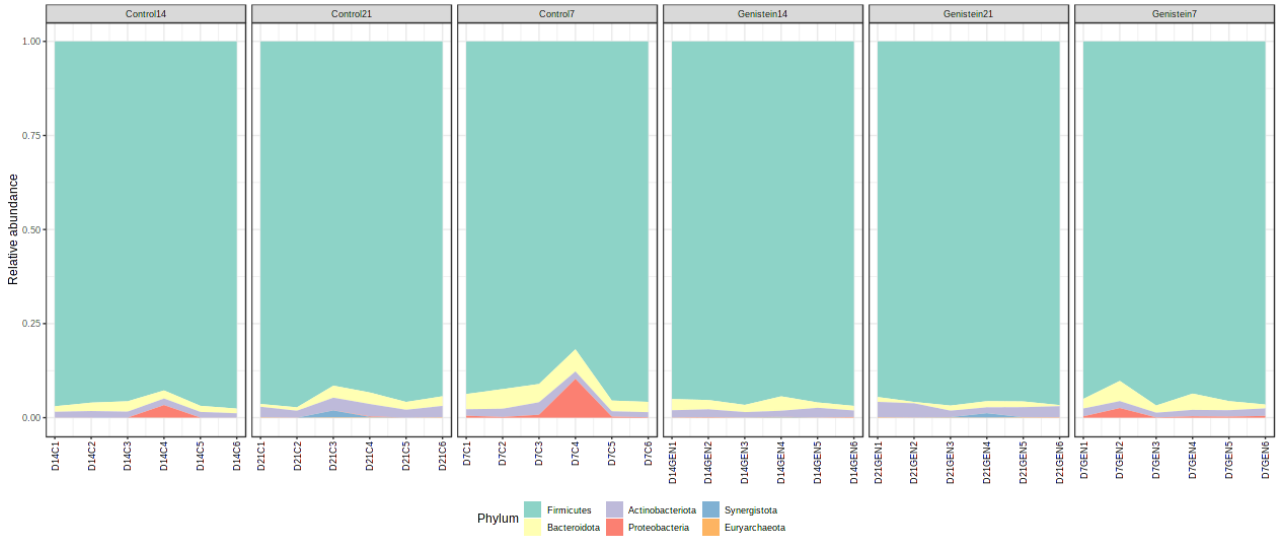
GEN508	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN509	*Streptococcus equinus*	MALDI	Modified BHI	1.90
GEN510	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.74
GEN511	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN512	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN513	*Streptococcus infantarius*	MALDI	Modified BHI	1.76
GEN514	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN516	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN517	*Faecalicoccus pleomorphus*	MALDI	Modified BHI	1.98
GEN518	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN519	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN520	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.79
GEN521	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.88
GEN522	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN523	*Streptococcus equinus*	MALDI	Modified BHI	2.04
GEN524	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN525	*Streptococcus equinus*	MALDI	Modified BHI	1.88
GEN526	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.71
GEN527	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN528	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN529	*Streptococcus equinus*	MALDI	Modified BHI	1.91
GEN530	*Streptococcus infantarius*	MALDI	Modified BHI	1.97

GEN531	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.92
GEN532	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN533	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.94
GEN534	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN535	*Streptococcus equinus*	MALDI	Modified BHI	1.89
GEN536	*Streptococcus equinus*	MALDI	Modified BHI	1.80
GEN537	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.91
GEN538	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN539	*Streptococcus equinus*	MALDI	Modified BHI	1.93
GEN540	*Streptococcus equinus*	MALDI	Modified BHI	1.73
GEN541	*Streptococcus equinus*	MALDI	Modified BHI	1.84
GEN542	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.87
GEN543	*Streptococcus infantarius*	MALDI	Modified BHI	1.76
GEN544	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN545	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.77
GEN546	*Streptococcus infantarius*	MALDI	Modified BHI	1.90
GEN547	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.78
GEN548	*Streptococcus lutetiensis*	16S sequencing	Modified BHI	N/A
GEN549	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.97
GEN550	*Streptococcus equinus*	MALDI	Modified BHI	1.98
GEN551	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.85
GEN552	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.76

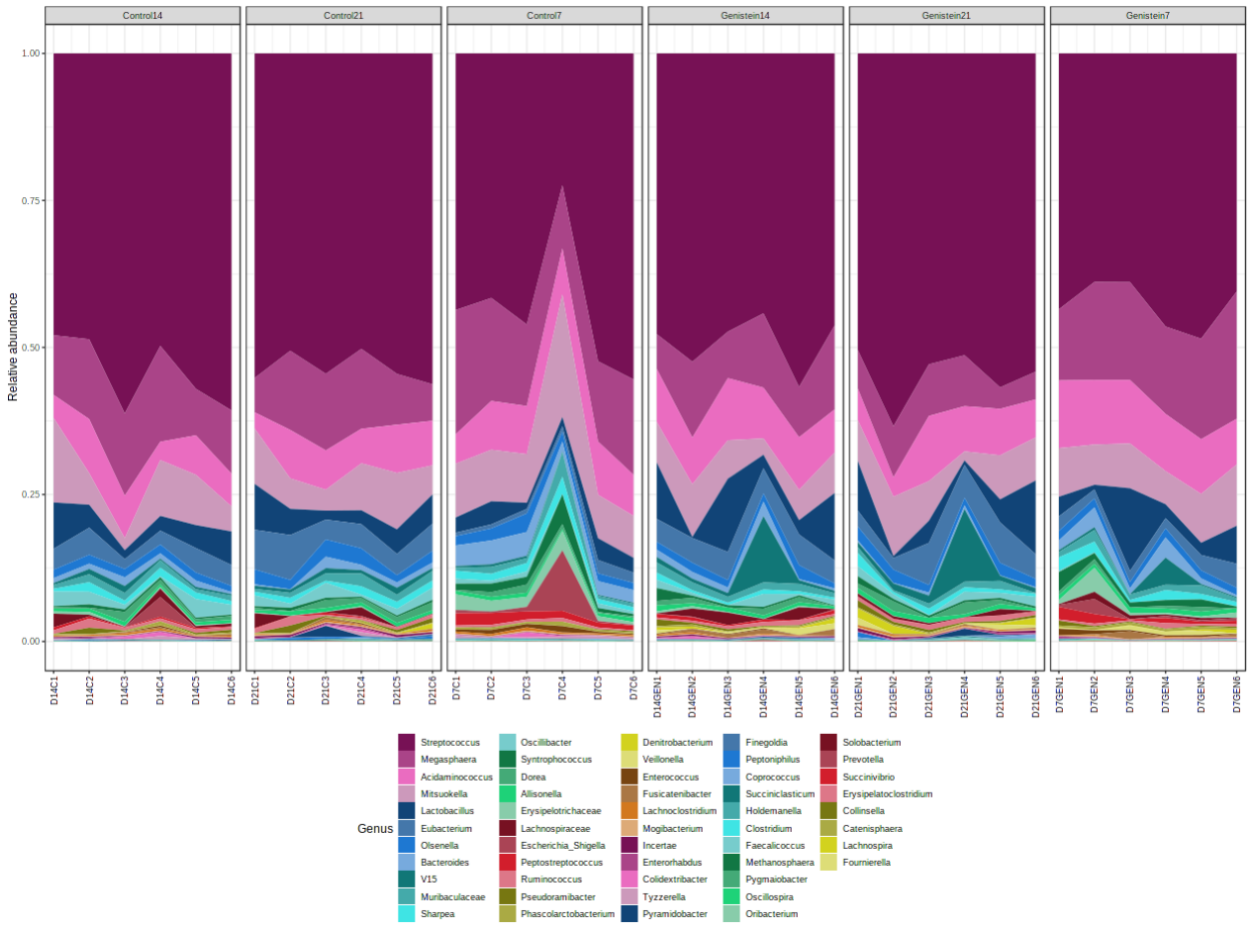
GEN553	*Streptococcus alactolyticus*	MALDI	Modified BHI	1.75
GEN554	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.97
GEN555	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.98
GEN556	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.01
GEN557	*Streptococcus equinus*	MALDI	Modified BHI	1.74
GEN558	*Streptococcus equinus*	MALDI	Modified BHI	1.94
GEN559	*Streptococcus lutetiensis*	MALDI	Modified BHI	2.01
GEN560	*Streptococcus gallolyticus*	MALDI	Modified BHI	1.81
GEN561	*Streptococcus equinus*	MALDI	Modified BHI	1.89
GEN562	*Streptococcus lutetiensis*	MALDI	Modified BHI	1.99
CA002	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	1.92
CA003	*Bacteroides vulgatus*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.33
CA004	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.21
CA005	*Bacteroides vulgatus*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.56
CA006	*Bacteroides vulgatus*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.59
CA007	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.28
CA008	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.35
CA009	*Bacteroides uniformis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.47
CA010	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.13
CA011	*Bacteroides uniformis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.49
CA012	*Bacteroides fluxus*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.53
CA013	*Bacteroides vulgatus*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.43

CA014	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.21
CA015	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.45
CA016	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.26
CA017	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	1.89
CA018	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.13
CA019	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	1.99
CA020	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.23
CA021	*Bacteroides uniformis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.43
CA022	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.19
CA023	*Bacteroides vulgatus*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.55
CA024	*Bacteroides uniformis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.39
CA027	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.10
CA028	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.17
CA029	*Bacteroides uniformis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.40
CA030	*Lactobacillus salivarius*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.10
CA031	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.34
CA032	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.02
CA033	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.41
CA034	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.24
CA035	*Enterococcus avium*	MALDI	Modified BHI (amp+ clin, 1mg/L)	1.86
CA036	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	1.95
CA037	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.31

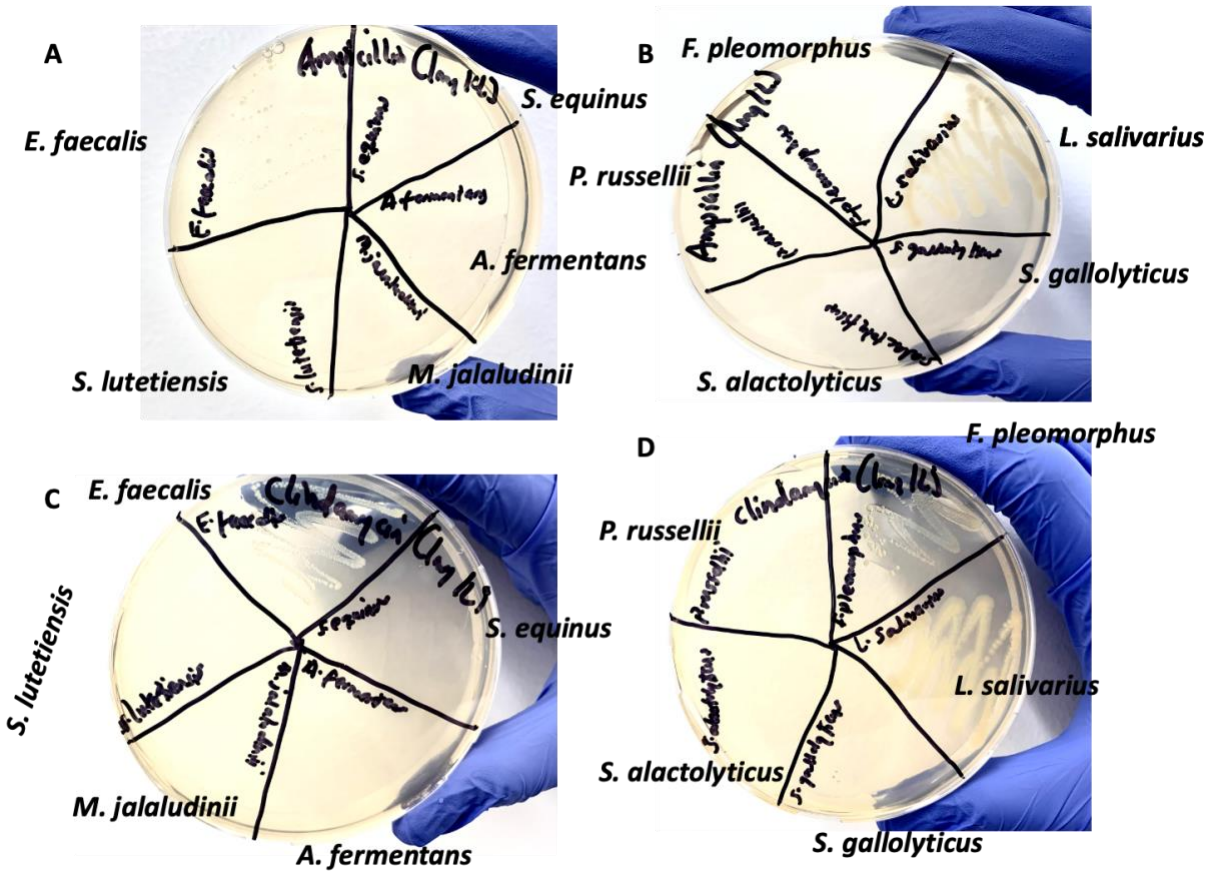
CA038	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.41
CA039	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.27
CA040	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.35
CA041	*Bacteroides uniformis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.22
CA043	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.05
CA044	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.13
CA045	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.15
CA046	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.17
CA047	*Enterococcus faecalis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	2.33
CA048	*Lactobacillus agilis*	MALDI	Modified BHI (amp+ clin, 1mg/L)	1.92



Supplementary figure 1: Relative abundances of Phyla for genistein treated and control groups on days 7, 14 and 21



Supplementary figure 2: Relative abundances of genera for genistein treated and control groups on days 7, 14 and 21



Supplementary figure 3: Antimicrobial susceptibility testing to determine the suitable antibiotics to inhibit *Streptococcus sp.*

A and **B** are modified BHI agar plates supplemented with 1 mg/L ampicillin and streaked with bacteria. **C** and **D** are modified BHI plates supplemented with 1mg/L clindamycin and streaked with bacteria.

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