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An Examination of the Vaginal and Endometrial Microbiota of Heifers and the Effects of Selenium Supplementation on Microbiome Composition

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Abstract

Selenium, an essential trace element, is involved in many biological processes serving catalytic, structural, and regulatory roles through mechanisms that are not entirely understood. Due to its health effects from either deficiency or toxicity, selenium has been well-studied, with an emphasis towards selenium deficiency which has been shown to cause immune and reproductive issues in animals. To address deficiency, different forms of supplementation have been used, yet few studies have examined selenium's effects on the reproductive microbiome based on the type of supplementation (i.e., inorganic or organic forms). By examining bovine vaginal and endometrial mucosal samples via 16S gene sequencing, selenium's effects on overall bacterial abundance and alpha diversity, as well as identification of core vaginal and endometrial microbiomes, were able to be determined. As such, analysis indicate that a mixture of inorganic and organic selenium supplementation results in an increased bacterial diversity in vaginal mucosa, yet a decreased bacterial diversity in endometrial mucosa. Furthermore, treatment with the mixture compared to inorganic selenium alone resulted in the removal of four bacterial groups from the vaginal microbiome, which has potential implications on agricultural, economic, and human and animal health, overall. However, future studies are warranted in order to identify unknown groups and elucidate potential mechanisms of action.

Keywords: selenium; bacteria; microbiome; 16S gene sequencing; reproduction; bovine

Contents

List of Figures

List of Tables

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Introduction

Selenium Biochemistry

Significance of Selenium in Animals

Selenium is a trace element (1) that has been defined as an essential trace element, under both the 1973 World Health Organization (WHO) (2) and 1996 WHO trace element reports (3). Though differing grouping methods have been proposed regarding the classification of trace elements (4,5,6), some scholars claim that these other methods may remain controversial (5), instead grouping based on the amount found in tissues or on subjective interpretations rather than nutritional significance. As such, this project uses the WHO classification of essentiality, defining selenium as an essential trace element (i.e., an essential element being defined as one whose deficiency results in a reduction in physiologically important functions, or if the element is a critical part of an organic structure that performs a vital function within the organism (3)) with its structural role in the enzyme glutathione peroxidase being considered a vital function within organisms.

These physiologically important functions that selenium is known to be involved with include a variety of biological process in animal bodies such as DNA synthesis, detrimental signaling peroxide scavenging, redox signaling control, thyroid hormone metabolism, and protein folding through their involvement with selenoproteins (i.e., a protein containing at least one selenocysteine (Sec) residue) (7). Selenium thereby has been characterized as having the potential for antioxidant (1-11), anticarcinogenic (1, 3, 6, 7-11), and metal detoxification properties (2, 5, 8-11), with most of the antioxidant defense activity being in the form of the selenoprotein glutathione peroxidase - an immunoregulator involved in both thyroid function and in the reproductive system (5).

Selenium also serves in a variety of catalytic, structural, and regulatory components and functions through its interactions with other biological molecules including proteins, enzymes, vitamins, and hormones (9). However, though the exact mechanism remains to be elucidated, it appears that selenium's most commonly accepted role of physiological importance is in the form of selenoproteins, with an emphasis towards glutathione peroxidase (an enzyme that protects against oxidation (i.e., oxidative stress) by free radicals via elimination of hydrogen peroxide from the body, protecting not only fatty acids, red blood cells, and hemoglobin, but also cellular components such as DNA and cell membranes (11)).

Glutathione peroxidase, the first identified selenoenzyme (1), has synergistic effects with other molecules, such as vitamin E, and the combination of these compounds with the selenoprotein effectively protects cellular components (e.g., mitochondria, cytochrome, and microsomal membranes) from the oxidation of fatty acids, helping to determine proper growth and fertility (11). Though glutathione peroxidase, as well as related selenoprotein glutathione reductase, seem to be the most commonly studied and reported selenoproteins, it remains likely that other selenoproteins may exist and remain to be discovered (3,4). With consideration that approximately half of the selenoproteome has yet to be attributed to any known biological process, their role thereby remains to be known (12).

Currently, selenium is important in the regulation of immune functions, playing an essential role in the innate immune response, with low levels being associated with a weakened immune system (1), though its exact mechanism remains unelucidated. However, studies have proposed that selenium may impact the immune response by altering cytokine, or cytokine receptor, expression or by making immune cells more resistant to the effects of oxidative stress (4). Other studies have proposed that the selenium's effect on immune function is a result of the

thyroid gland, since selenium is particularly abundant in the thyroid, reportedly having the highest content of selenium per tissue unit (13). Furthermore, selenium is also known to affect the function of the thyroid gland, as well (11).

However, other studies have focused on the reproductive role of selenium, considering that many selenoproteins are expressed in the uterus. This suggests an important role in reproductive function, with some studies reporting that up-regulated expression and down-regulated expression of certain selenoprotein genes (SELENOP and GPX1/GPX3, respectively) have been reported in small atretic bovine follicles compared to healthy bovine follicles (7). Yet, limited studies have examined the association between selenium and reproduction function despite growing evidence implicating selenium in a variety of adverse pregnancy health conditions (e.g., pre-eclampsia, miscarriage, preterm birth) even in cattle (e.g., decreased fertility rate, placental retention, and reproductive diseases such as metritis) (7). Furthermore, few studies consider the immunological role (i.e., autoimmune thyroid disease) that may impact reproductive efficiency, despite the strong linkage between thyroid hormones and reproductive functions (12).

As such, selenium's effects on the immune and reproductive function in animals remains a significant avenue of interest in the field of reproductive immunology, with its mechanisms not yet clearly defined. Furthermore, there exists few studies currently examining this association, and as a result, there remains scarce information on the significance of selenium in female fertility (12).

Forms of Selenium and Bioavailability

Selenium is accumulated to the largest extent in animals, specifically human and bovine, via ingestion. Plant and animal origins appear to be its main sources, though anthropogenic activity also impacts availability of selenium, with combustion of coal and lignite causing

selenium to penetrate the soil, alongside the use of agricultural processes such as fertilization and liming (11).

The predominant dietary forms of selenium can be divided into the broad categories of inorganic selenium (ISe)and organic selenium (OSe) (11, 14) with the most common forms of inorganic selenium being found as selenate and selenite, whereas organic selenium forms typically include selenocysteine (SeCys) and selenomethionine (SeMet) (11-15).

The form of selenium remains a vital factor influencing bioavailability. The general consensus appears to be that organic selenium compounds are typically more bioavailable for animals (14,15) with some studies proposing that organic selenium is actively absorbed through amino acid transport mechanisms (15) thereby increasing its bioavailability.

Though some scholars hold that the form of selenium is the main determinant of supplementation efficiency (15) and emphasize that the form of selenium for consumption is more important than dosage (having an important influence on whether it is considered harmful or beneficial), other scholars assert that the form and dosage have equal importance in defining the role of selenium in physiological homeostasis (12).

There are also differences in the bioavailability within the classifications themselves. For inorganic selenium, selenite is more commonly transformed into organic metabolites than selenate, suggesting it is more bioavailable in certain tissues, whereas SeCys is more easily digested by the gastrointestinal tract compared to SeMet, suggesting higher absorption. These ideas may explain the reason that SeCys dominates in terms of products of animal origin (11).

Moreover, Se in plant foods is more bioavailable than Se in animal foods (14), though plants typically accumulate selenium in its inorganic form (i.e., selenates) that are then converted to organic forms (particularly selenomethionine and selenocysteine) (11). Some reports have

suggested a higher level of bioavailability of organic forms compared to inorganic (approximately 90 – 95% bioavailability reported for organic forms of selenium, compared to only 80-85% for inorganic forms of selenium) (12), though it is unclear whether these reports consider bioavailability as absorption, since the 1996 WHO report states that absorption of one ISe (i.e., selenite) is greater than 80%, whereas another Ise (i.e., selenate) and the OSe SeMet are reported to have absorption rates greater than 90% (3).

As such, though many studies have shown that organic selenium generally has a higher absorption rate compared to inorganic selenium, there exists other considerations such as the idea of competitive absorption, which leads some scholars to believe a combination of both selenium sources leads to an overall higher absorption efficiency (15). Thus, it appears that conversion to metabolically active forms, rather than absorption, is the main determinant in the overall bioavailability, with scholars proposing that the difference in metabolic pathways may be one of the reasons the forms of selenium ultimately have different effects on the concentration of selenium in various tissues (15). Thus, bioavailability in terms of both forms (i.e., inorganic versus organic selenium) and dosage (i.e., intake levels) affect the functionality of selenium in metabolism, helping to provide some explanation to selenium's various roles in the body.

In highlighting the complexities of these potential metabolic pathways undertaken by selenium and its forms, both inorganic and organic forms (specifically, selenate, selenite, SeMet, and SeCys) can be metabolized to hydrogen selenide, which is a molecule involved in both selenoprotein synthesis and methylation excretion of selenium. (14) In examining the organic forms, SeMet can not only participate in general protein synthesis (being utilized instead of methionine), but it can undergo conversion via trans-sulfurization to SeCys (another organic form) (14); SeCys can then be converted to hydrogen selenide by a beta-lyase (i.e.,

selenocysteine lyase (16)). The inorganic forms are able to be converted to hydrogen selenide via a reductive metabolism (14) mechanism.

In examining this metabolite of both inorganic and organic forms of selenium, hydrogen sulfide is able to be further converted into a crucial transport RNA that synthesizes selenoproteins (Selenocysteinyl-tRNA), or if selenium intake exceeds the need for selenoprotein synthesis (i.e., greater than nutritional requirements), hydrogen selenide is methylated, forming methylselenol (a critical anti-cancer metabolite (14,17)). At further increased intake levels, this metabolite (methylselenol) is again methylated to either dimethylselenide or trimethylselonium ions (14). Furthermore, hydrogen sulfide may be converted to selenosugars, and all three of these metabolites (i.e., dimethylselenide, trimethylselonium ion, and selenosugars) are able to be excreted, either via respiration or urine, (14) helping to maintain selenium homeostasis in the body.

Selenium Deficiency and Toxicity.

Interesting to note is how relatively narrow the intake range between selenium deficiency and toxicity is (with deficiency symptoms appearing at less than 11 micrograms per day, and toxicity symptoms appearing at over 900 micrograms per day) (14) considering the various metabolic pathways that seemingly offer different avenues of utilization or excretion. Within this range, however, between 100-200 micrograms of selenium per day is recommended to reduce DNA damage and cancer progression in humans (14). As such, selenium deficiency, and any trace element deficiency for that matter, may appear as a combination of various clinical features, rather than specific manifestations since trace elements are related to many biological pathways and enzyme systems (4). The difficulty in diagnosing selenium deficiency is due to both this range of clinical features leading to numerous hypotheses such as viral infections,

environmental intoxication, mycotoxins, and nutritional deficiencies (though hypotheses relating to a selenium deficiency remains the most accepted of these hypotheses (5,6)) as well as the limited ability to gauge nutritional status of selenium. Namely, this inability is due to chemical limitations (i.e., there are various chemical forms of selenium) (11) as well as unclear standards in determining levels of selenium (to determine levels of selenium, typical methods include analyzing different tissues such as serum blood levels - which may be a convenient biomarker, yet is susceptible to immediate effects of supplementation - whereas other tissues such as hair and nails may provide a more uniform long-term interpretation, though some studies question the usefulness of these biomarkers due to insufficient evidence (4, 18)).

Broadly speaking, however, selenium deficiency causes a decrease in neutrophil activity (potentially due to selenium's effect on immune cell differentiation and differentiation, with a deficiency leading to a reduction in humoral immune activity (19)), and it also leads to a decrease in glutathione peroxidase enzyme activity (leading to cells being more susceptible to oxidative damage and the respective cascading effects). Both decrease in neutrophil and glutathione peroxidase activity can negatively impact the immune system. Furthermore, some scholars suggesting the absence of selenium's heavy metal antitoxic effects lead to brain mercury retention, further modulating neurotoxicity and oxidative stress in the nervous tissue of animals, causing irreversible brain injury and affecting other nervous system functions (1,5,11).

Selenium deficiency leads to impairment of the immune system overall, in both innate and adapted immunity, with the deficiency manifesting in the form of decreased antiviral defense, decreased immune response, and an increased autoimmune response (5). In using epidemiological studies, researchers have concluded that moderate selenium deficiency affects disease development as a result of reduced immunological function (11).

In addition to the immune system, the reproductive system is negatively impacted by selenium deficiency as well since selenium has an effect on influencing the incidence of metritis and ovarian cysts. Furthermore, a greater expression of a glutathione peroxidase gene (GPX1) is found in the granulosa cells of healthy ovarian follicle, suggesting an antioxidant role during ovarian follicular development (1).

Reactive oxygen species (ROS) and their relationship with antioxidants (such as selenoproteins) are known to be involved in both female and male reproductive processes, with a proper oxidative balance being necessary for optimal function. When an imbalance between these reactive species and antioxidants occurs, it results in oxidative stress; however, there exists a paradoxical situation since a certain degree of ROS are necessary for the continuation of normal cell function, yet too many ROS can create an unsuitable environment for both normal female and male physiological reactions (20). Specifically, ROS, and in particular oxidative stress, can affect female reproductive systems via several processes such as follicular development, fetal development, and ovulation due to oxidant-induced damage (20), whereas ROS are known to cause infertility in male reproduction via two mechanisms: sperm membrane damage caused by reactive species and sperm DNA damage leading to defective paternal DNA transmission to fetus (1).

The effects of selenium deficiency are not limited to humans, however, with selenium deficiency in cattle typically manifesting in the form of increased incidence of placental retention (21), metritis, mastitis, abortion, susceptibility to infections, and overall lower fertility, whereas in other livestock (i.e., lambs, kids, and pigs) disorders such as white muscle disease, yellow fat disease, and VESD (vitamin E /selenium deficiency) syndrome (1) are associated with selenium deficiency. At least one study has reported that selenium deficiency in grazing beef cattle was the

sole cause of abortion in cows (based on negative laboratory testing for other common etiologies) and the researchers offered two potential mechanisms explaining the abortifacient effect: fetal heart failure and progesterone concentration, both likely a result of selenium deficiency (22). Other studies have shown the importance of selenium and cardiovascular health, with selenium deficiency considered to be a causative factor of certain forms of heart failure (23) offering additional support for selenium's proposed abortifacient mechanism due to fetal heart failure. Overall, selenium deficiency has both direct and indirect effects on cattle in terms of growth, reproduction, and general health and well-being, though excessive supplementation can have negative effects leading to toxicity (24).

Selenium toxicity is also known as selenosis, with clinical features such as hair loss, nail brittleness, gastrointestinal tract disorders, skin rashes, irritability, garlic odor, and nervous system disorders such as tremors and muscle contractions (11, 14) characterizing this phenomenon. Though acute selenium poisoning is rarely observed, selenium toxicity as a whole is often less frequent than selenium deficiency, with selenium deficiency remaining more of a geographic problem compared to selenium toxicity (8, 11, 24). This geographical issue is highlighted by the recommended daily dose of selenium being variable depending on the geographic region, with the content of selenium in food products of a particular region being proportional to the amount that is present in the soil of that region, an issue that is further compounded by the risk of symptom occurrence varying in any given individual of any particular organism (11).

Ultimately, either selenium deficiency or selenium toxicity can have harmful effects, with the dosage range between proper function and physiological disruption remaining relatively small (11,14), most likely due to the distortion and dysfunction of proteins and enzymes

ultimately causing biochemical disturbances and affecting proper physiological function. The prevention of these health disorders has been the subject of study for many researchers, with the idea of selenium supplementation becoming the leading cautionary measure due to its incidence compared to selenium toxicity, with emphasis being placed on achieving overall adequate selenium supplementation to the diet (1).

Selenium Supplementation and Agricultural Implications

In considering the goal of proper selenium levels, scholars recognize that adequate selenium intake, specifically in cattle and other livestock, can be achieved through supplementation via the addition of selenium in various forms to drinking water, supplementation with mineral salts, introduction of selenium-enriched yeast, injections, and the use of fertilizers, as long as the toxicity potential is also considered to not harm either animal or consumer (24). To determine an effective supplementation plan, it is recommended that diet content should be based on the efficacy range for a geographic region, with consideration that selenium deficiency, and similarly, selenium toxicity, is related to an organism's respective geographic area (8, 11, 24).

In looking at bovine selenium supplementation specifically, one study reported that, on average, cattle who received feed supplemented with organic selenium (in the form of selenium yeast) produced more selenium in their milk compared to cows supplemented with inorganic forms (in the form of sodium selenite) (25), yet both increased the overall selenium content in milk. With consideration that recent attempts have been made to improve nutritional value of foods for human consumption, selenium supplementation may improve milk quality in terms of selenium content, to help meet nutritional requirements of consumers (i.e., humans) (8) and furthermore, the form of selenium supplementation has been shown to impact reproduction and

fertility in cattle (26). These considerations raise not only agricultural and economic concerns, but human health concerns, as well.

Microbiome and Core Microbiomes

The field of microbiome research has dramatically increased in recent years, with some scholars contributing this rise in interest to both advances in technology and significant cost reductions, which help contribute to the vast wealth of data (27). Of this technology, 16S next generational sequencing in particular has been an avenue of interest, particularly since it does not rely on culturing bacteria (the traditional method of microbial analysis) which allows for advanced understanding of microbiomes by allowing for discovery and subsequent characterization of unculturable microbes, thereby allowing for prediction of function (28).

With consideration that a host lives in symbiosis with its microbiome under healthy conditions, many illnesses can be contributed to microbial dysbiosis (i.e., an imbalance in the host-microbial interaction (29)) and can result in both communicable diseases (e.g., tuberculosis and syphilis) or non-communicable diseases (e.g., inflammatory bowel disease, diabetes, obesity, and cancer) (28). Thus, by being able to identify a core microbiome (i.e., any set of microbial taxa, as well as the genomic or function characteristics of those taxa, that are attributed to a particular environment of interest (30)) the distinction between symbiosis and dysbiosis is able to be better elucidated, allowing for more effective responses to such illnesses and their effects.

Selenium and Microbiome

In particular interest to cattle fertility health (including its subsequent agricultural, economic, and human health effects) has been the mechanisms by which selenium

supplementation affects the health of cattle. Though some scholars propose selenoproteins as the main mediator of a majority of biological functions of selenium, proposing that the microbiome, bacterial pathogens, and host immune system are in competition for a limited supply of selenium (31), other scholars have examined the effects of subsequent depletion and repletion on host microbiome homeostasis (32). However, there appears to be limited literature examining how the form of selenium and its subsequent bioavailability affects the microbiome and microbial host interactions. With consideration that the form of selenium can influence microbial colonization, (and in turn influence the host's selenium levels and expression of selenoproteins) (33), and the fact that microbiome composition has been associated with many health disorders (with microbial composition and stability being essential for ruminant welfare, health and production efficiency (34)), the effects of selenium on the microbiome composition remains an avenue of interest in terms of reproductive immunology.

Objectives and Hypothesis

As such, the overall aim of this study was to address the effects of selenium supplementation on the microbiota composition of heifers in vaginal and endometrial mucosa. The focus of this project was to determine how the composition of bacteria present in the mucosa changes when the heifers are supplemented with differing types of selenium supplementation (i.e., inorganic selenium or organic selenium) and to attempt to determine a core microbiome for each bovine mucosa studied.

In examining these effects on the bacterial microbiome in bovine, three different metrics were analyzed for the bacteria (abundance, alpha diversity, and bacterial composition). As such, it was hypothesized that a comparison between vaginal and endometrial mucosa would reveal a

greater abundance and alpha diversity in vaginal samples compared to endometrial samples. In examining the effects of the different supplementation, it was hypothesized for both the vaginal and endometrial samples that the treatment samples would have an increase in both abundance and alpha diversity due to the increase in bioavailability of selenium. The bacterial composition data will be utilized to attempt to determine core microbiomes for vaginal and endometrial environments in bovine.

Methods

Vaginal and endometrial mucosal samples were collected from two groups of cattle, one group that was supplemented with inorganic selenium only (ISe), and another group supplemented with a 1:1 mixture of inorganic and organic selenium (MIX). Vaginal mucosal samples were collected at three different assessment points from six different cattle, with half of the cattle receiving Ise (n = 3) and the other half receiving MIX (n = 3). Due to logistical constraints, for endometrial samples, only one assessment point was available, and nine samples were collected. Of the endometrial samples, approximately half were supplemented with Ise (n = 5), and the other half received the MIX supplementation (n = 4).

After collection, the samples were analyzed with the ZymoBIOMICS® Targeted Sequencing Service (Zymo Research, Irvine, CA) which used one of three differing DNA extraction kits depending on sample type and sample volume (either the ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA), ZymoBIOMICS® DNA Miniprep Kit (Zymo Research, Irvine, CA) or ZymoBIOMICS® DNA Microprep Kit (Zymo Research, Irvine, CA). The analysis involved bacterial 16S ribosomal RNA gene targeting sequencing using the *Quick*-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA) with bacterial 16S primers typically

amplifying the V3-V4 region of the 16S rRNA gene. Bioinformatics analysis was obtained via the DADA2 pipeline with specific taxonomy assignment being performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database (an internally designed and curated 16S database).

In obtaining absolute abundance data, a quantitative real-time PCR was set up with a standard curve with a PCR input volume being used to calculate the number of gene copies per microliter in each DNA sample. This value was calculated by dividing the gene copy number by an assumed number of gene copies per genome (with the assumed number being four in terms of 16S sequencing). To obtain alpha-diversity data, analyses were performed with Qiime v.1.9.1.

Results

Abundance and Alpha Diversity

Vaginal vs. Endometrial Mucosa

In first contrasting the absolute abundance (i.e., the absolute abundance of bacterial (16S) DNA measured in the samples) between vaginal mucosa and endometrial mucosa, it appears that there is a large difference in both measures of central tendency (Figure 1). There appears to be a greater absolute abundance of bacteria (measured as gene copies per microliter (copies/μL) in the vaginal mucosa compared to endometrial mucosa, with a median of 45697.5 copies/μL and mean of 307340 copies/μL (compared to the endometrial mucosa having 163 copies/μL and 363 copies/μL, respectively) (p = 0.0695; Table 1). However, according to Figure 1, the vaginal mucosa also presents with a greater variance compared to the endometrial mucosa.

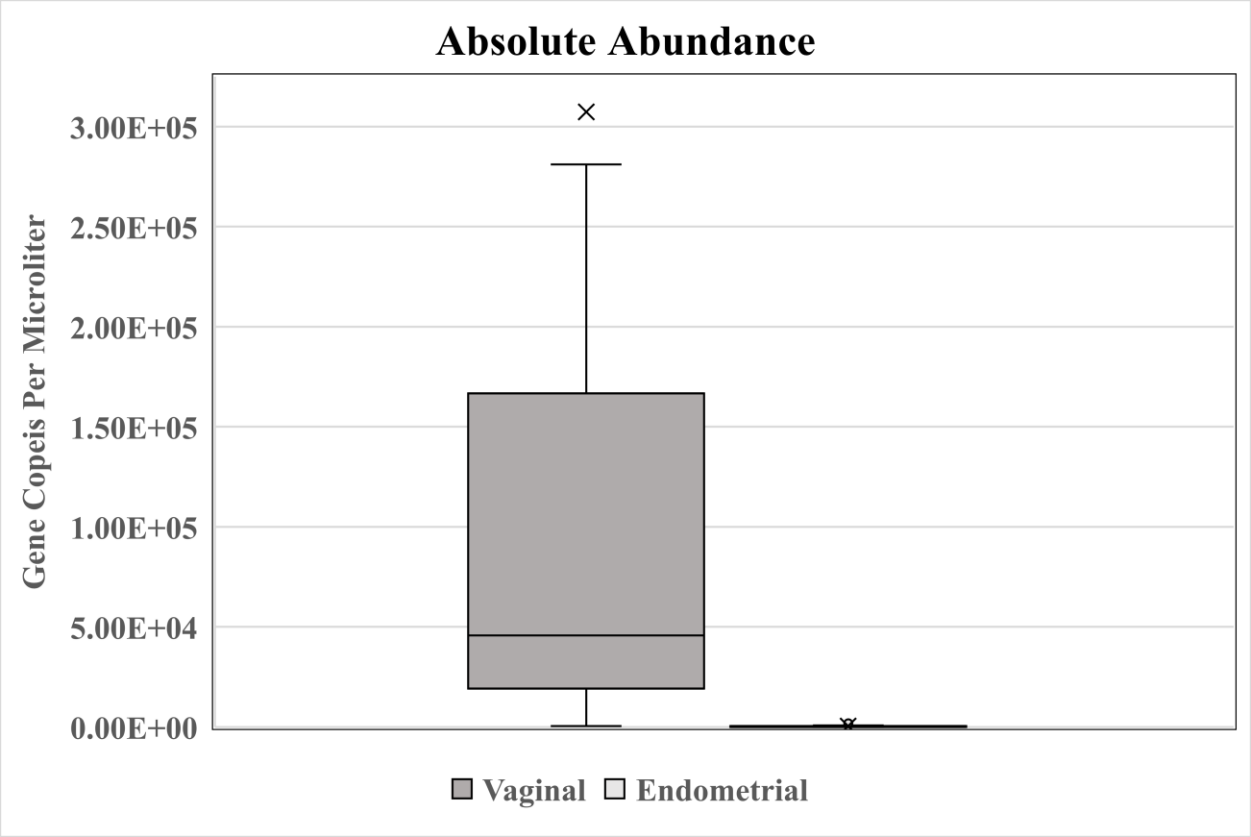

Figure 1. The absolute abundance of bacterial (16S) DNA measured in the samples from vaginal and endometrial mucosa via 16S gene sequencing.

Table 1. Descriptive statistics for the absolute abundance of bacterial (16S) DNA measured in the samples from vaginal and endometrial mucosa via 16S gene sequencing.

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Vaginal	18	466	19178.25	45697.50	166622.25	281166	307340	-
Endometrial	9	3	8.5	163	519	692	363	-
								0.0695

In evaluating alpha diversity in terms of both the Shannon diversity index (H) (SDIH) (Figure 2; Table 2) and the Simpson diversity index (E) (SDIE) (Figure 3; Table 3), a similar trend emerges in both. In terms of both median and mean, the vaginal mucosa appears to have a greater SDIH (7.87 and 7.30) and a greater SDIE (0.990 and 0.974) compared to the endometrial mucosa (SDIH of 4.32 and 4.09; SDIE of 0.911 and 0.893) ($p = 0.0000$; $p = 0.0000$) (Table 2; Table 3). Figure 2 suggests a similar variance in the two samples for SDIH, whereas Figure 3 is dissimilar, suggesting a higher variance in the endometrial mucosal sample.

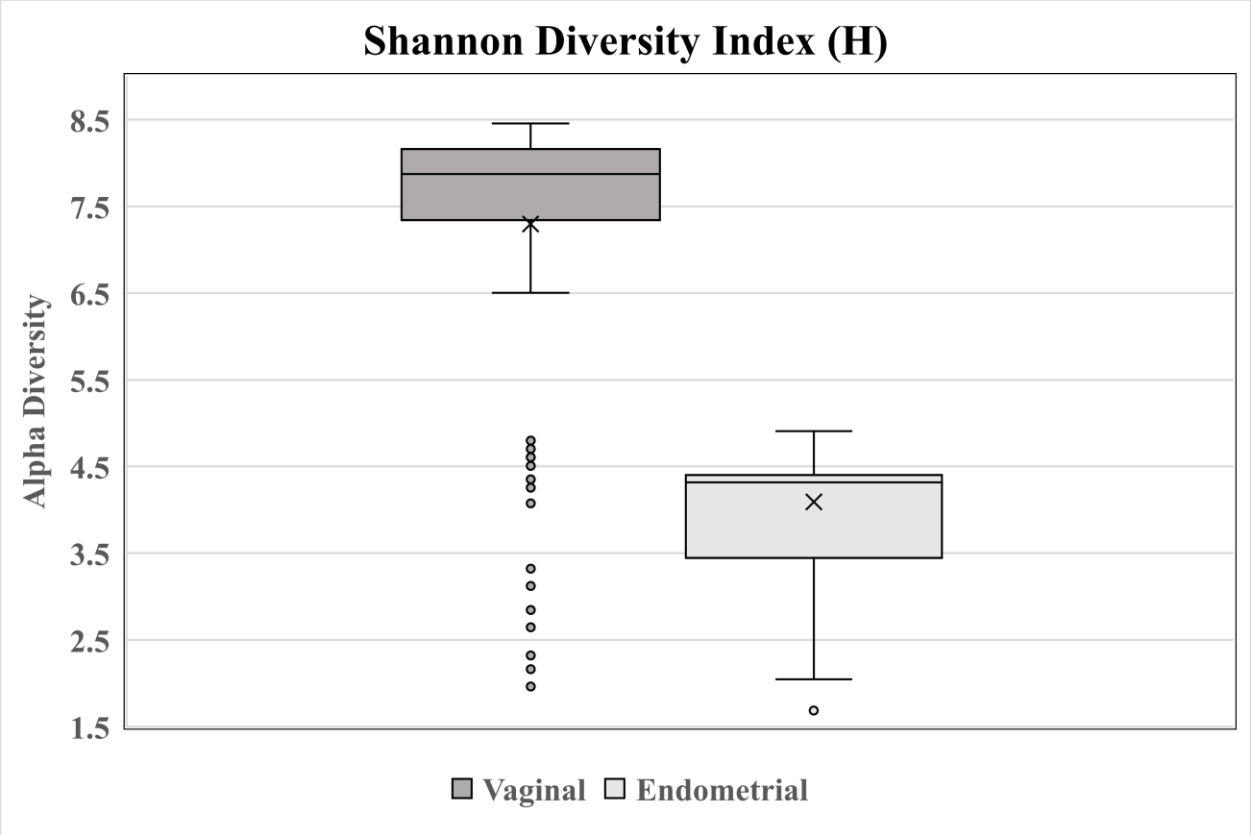

Figure 2. The alpha diversity of bacterial (16S) DNA measured in the samples from vaginal and endometrial mucosa via 16S gene sequencing in terms of the Shannon diversity index (H).

Table 2. Descriptive statistics for the alpha diversity of bacterial (16S) DNA measured in the samples from vaginal and endometrial mucosa via 16S gene sequencing in terms of the Shannon diversity index (H).

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Vaginal	1980	6.50	7.34	7.87	8.16	8.45	7.30	-
Endometrial	550	2.05	3.45	4.32	4.40	4.91	4.09	-
								0.0000

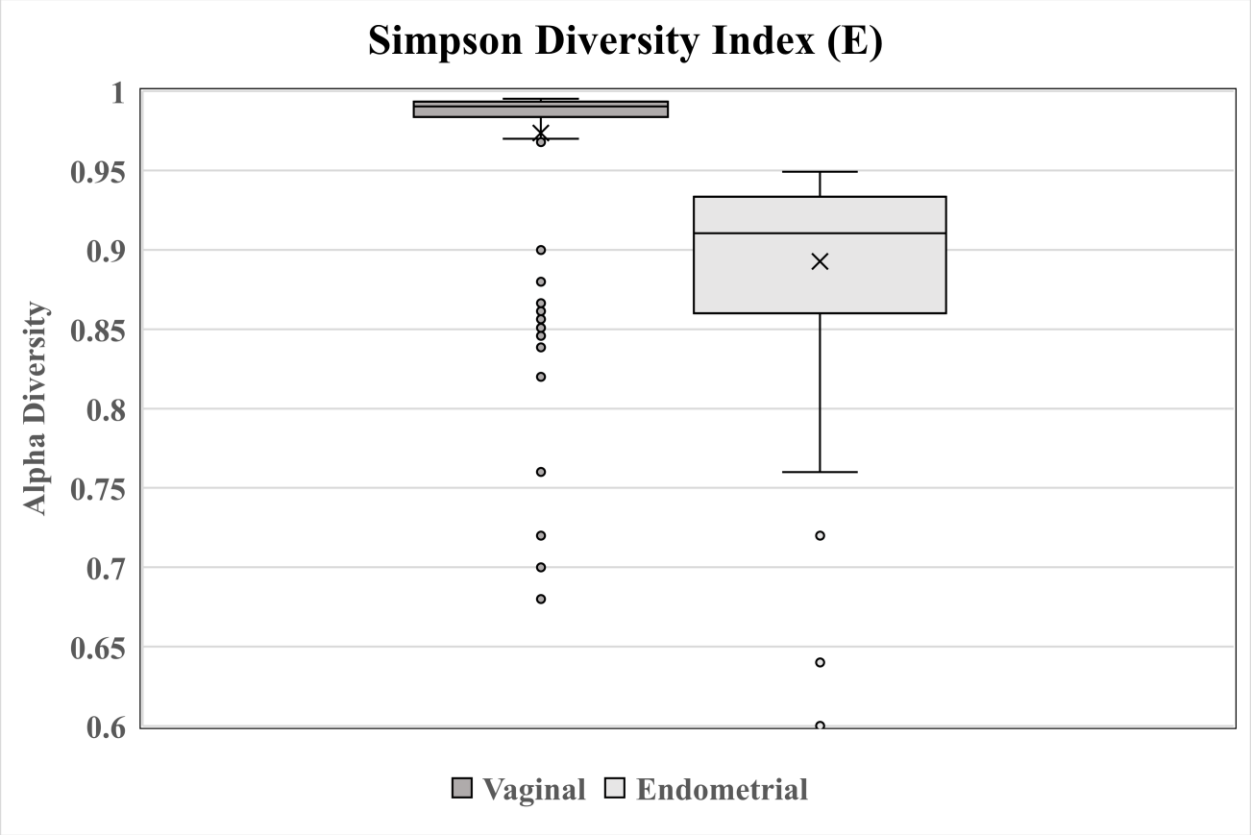

Figure 3. The alpha diversity of bacterial (16S) DNA measured in the samples from vaginal and endometrial mucosa via 16S gene sequencing in terms of the Simpson diversity index (E).

Table 3. Descriptive statistics for the alpha diversity of bacterial (16S) DNA measured in the samples from vaginal and endometrial mucosa via 16S gene sequencing in terms of the Simpson diversity index (E).

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Vaginal	1980	0.970	0.984	0.990	0.993	0.995	0.974	-
Endometrial	550	0.760	0.860	0.911	0.933	0.949	0.893	- 0.0000

Vaginal Control vs. Vaginal Treatment

When looking individually at the sample mucosa, beginning with vaginal mucosa, the absolute abundance and alpha diversity can also be compared when examining control samples (i.e., samples only supplemented with ISe) and treatment samples (i.e., samples supplemented with the 1:1 MIX of ISe and OSe). For the absolute abundance, it appears that the treated samples boasted a greater median and mean (281166 copies/μL and 293950 copies/μL) relative to the control (29096 copies/μL and 33203 copies/μL) (p = 0.1349) though the treated samples also appear to boast a greater variance (Figure 4; Table 4).

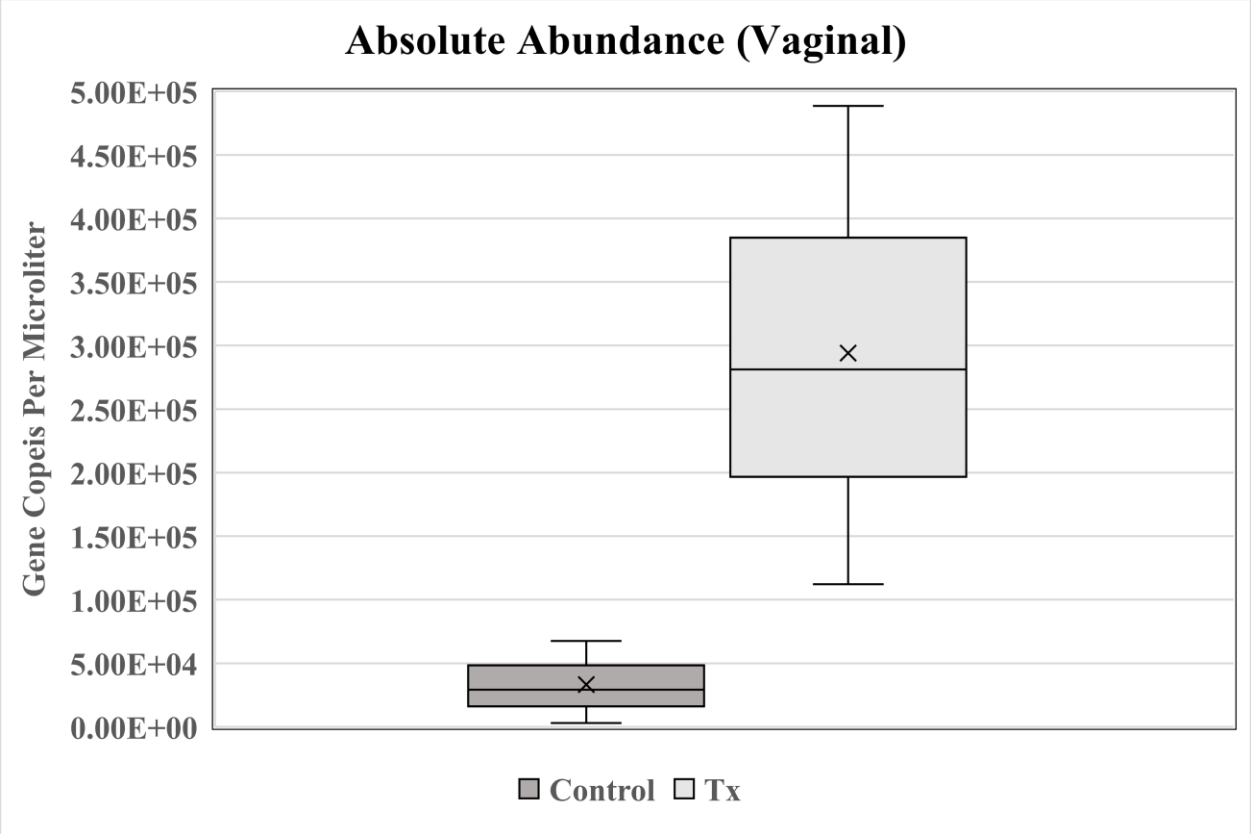

Figure 4. The absolute abundance of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing.

Table 4. Descriptive statistics for the absolute abundance of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing.

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Vaginal Ct	3	112299	196733	281166	384776	488386	293950	-
Vaginal Tx	3	2901	15999	29096	48354	67611	33203	-
								0.1349

A similar trend can be noted in the measures of alpha diversity, with the treated vaginal mucosal samples seemingly having a greater median and mean for both SDIH (7.93 and 7.88) and SDIE (0.990 and 0.989) (though relatively fairly similar variances (Figure 5; Figure 6)) compared to the control (SDIH of 7.77 and 7.62; SDIE of 0.984 and 0.984, respectively) (p = 0.0000; p = 0.0000) (Table 5; Table 6).

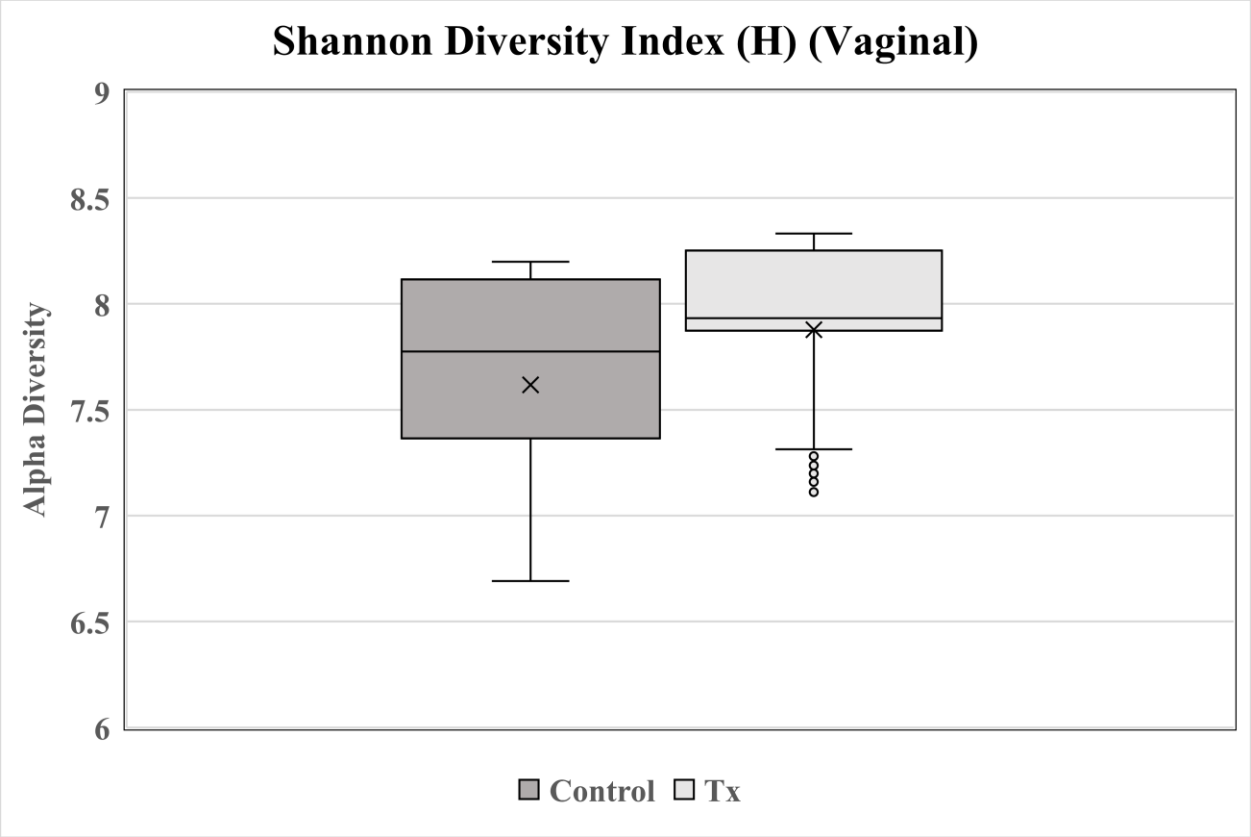

Figure 5. The alpha diversity of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing in terms of the Shannon diversity index (H).

Table 5. Descriptive statistics for the alpha diversity of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing in terms of the Shannon diversity index (H).

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Vaginal Ct	1230	6.69	7.36	7.77	8.11	8.20	7.62	-
Vaginal Tx	1230	7.31	7.87	7.93	8.25	8.33	7.88	-
								7.3986E-17

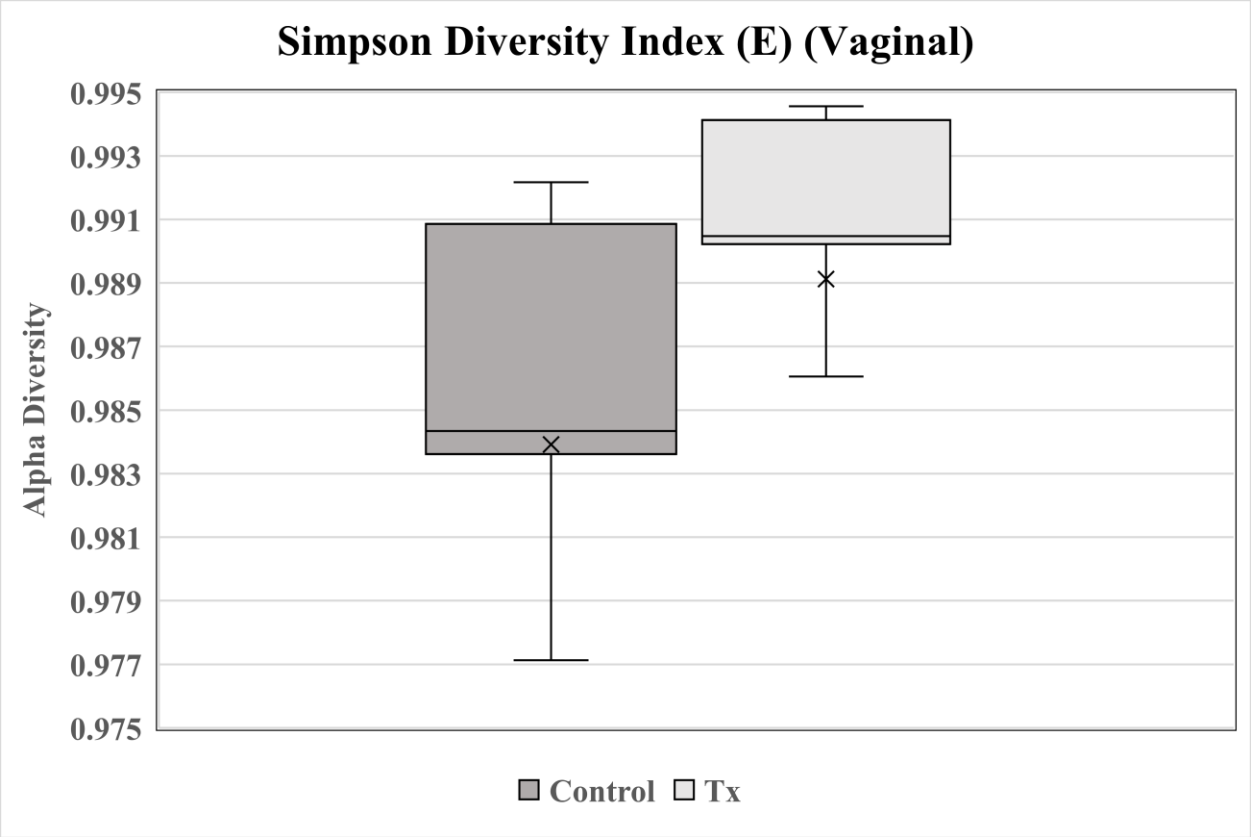

Figure 6. The alpha diversity of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing in terms of the Simpson diversity index (E).

Table 6. Descriptive statistics for the alpha diversity of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing in terms of the Simpson diversity index (E).

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Vaginal Ct	1230	0.977	0.984	0.984	0.991	0.992	0.984	-
Vaginal Tx	1230	0.986	0.990	0.990	0.994	0.995	0.989	-
								4.922E-17

Endometrial Control vs. Endometrial Treatment

In looking at the endometrial control and treated sample mucosa and their respective absolute abundance values, analysis reveals a similar trend compared to the vaginal mucosal samples: the treated endometrial samples possess a greater median and mean (445 copies/μL and 671 copies/μL) ($p = 0.2952$) with the treated samples appearing to also have a greater variance (Figure 7; Table 7).

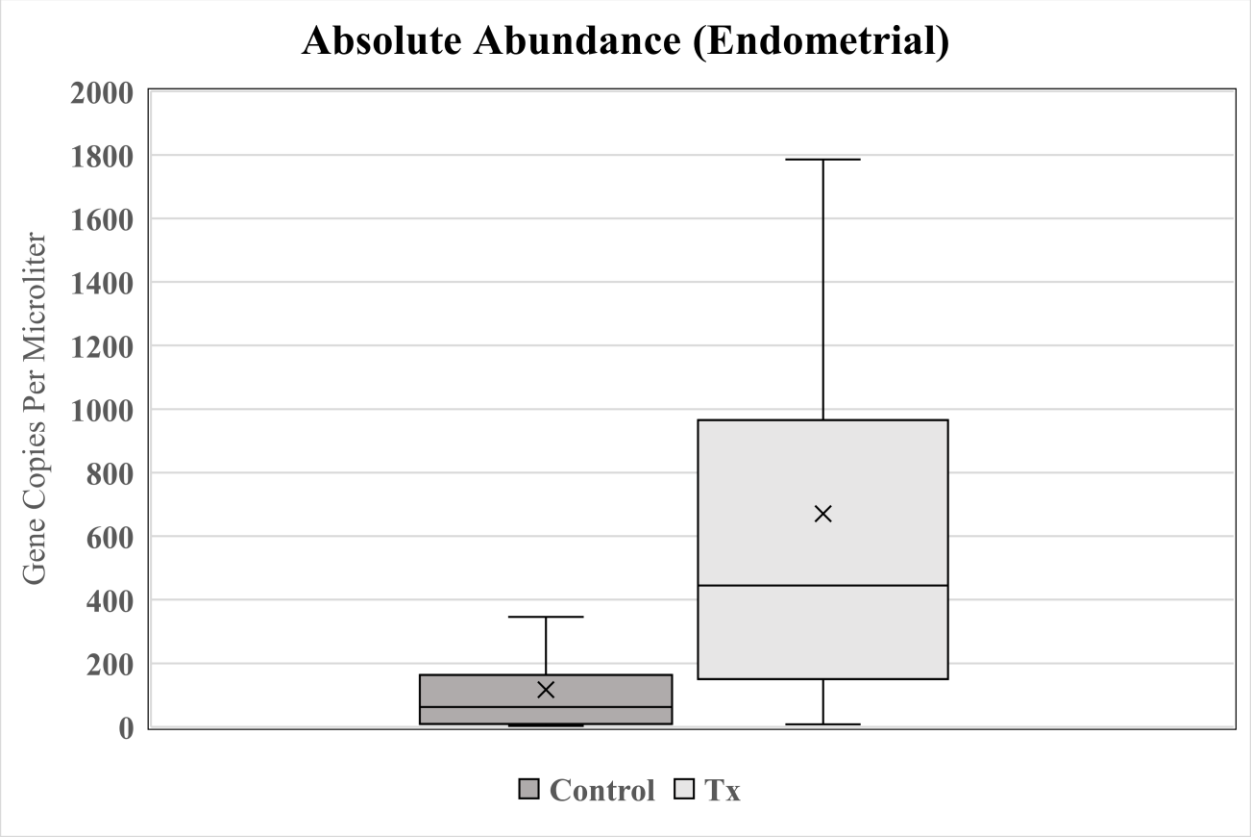

Figure 7. The absolute abundance of bacterial (16S) DNA measured in the control and treated samples from endometrial mucosa via 16S gene sequencing.

Table 7. Descriptive statistics for the absolute abundance of bacterial (16S) DNA measured in the control and treated samples from endometrial mucosa via 16S gene sequencing.

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Endometrial Ct	3	3	9	62	163	346	116.6	-
Endometrial Tx	3	8	150.5	445	965.5	1786	671	-
								0.2592

However, in comparing the alpha diversity of endometrial control and treated samples and the vaginal control and treated samples, there appears to be a dissimilarity to the trends noted in the vaginal mucosal samples. Namely, the opposite trend is noted, with the treated endometrial samples having a lower median and mean in both SDIH (3.44 and 3.96) and SDIE (0.85 and 0.88) compared to the control endometrial samples (SDIH of 4.32 and 4.17; SDIE of 0.91 and 0.90) (p = 0.0005 (Table 8); p = 0.0000 (Table 9)) though the treated samples appear to have a greater variance in both, as well (Figure 8; Figure 9).

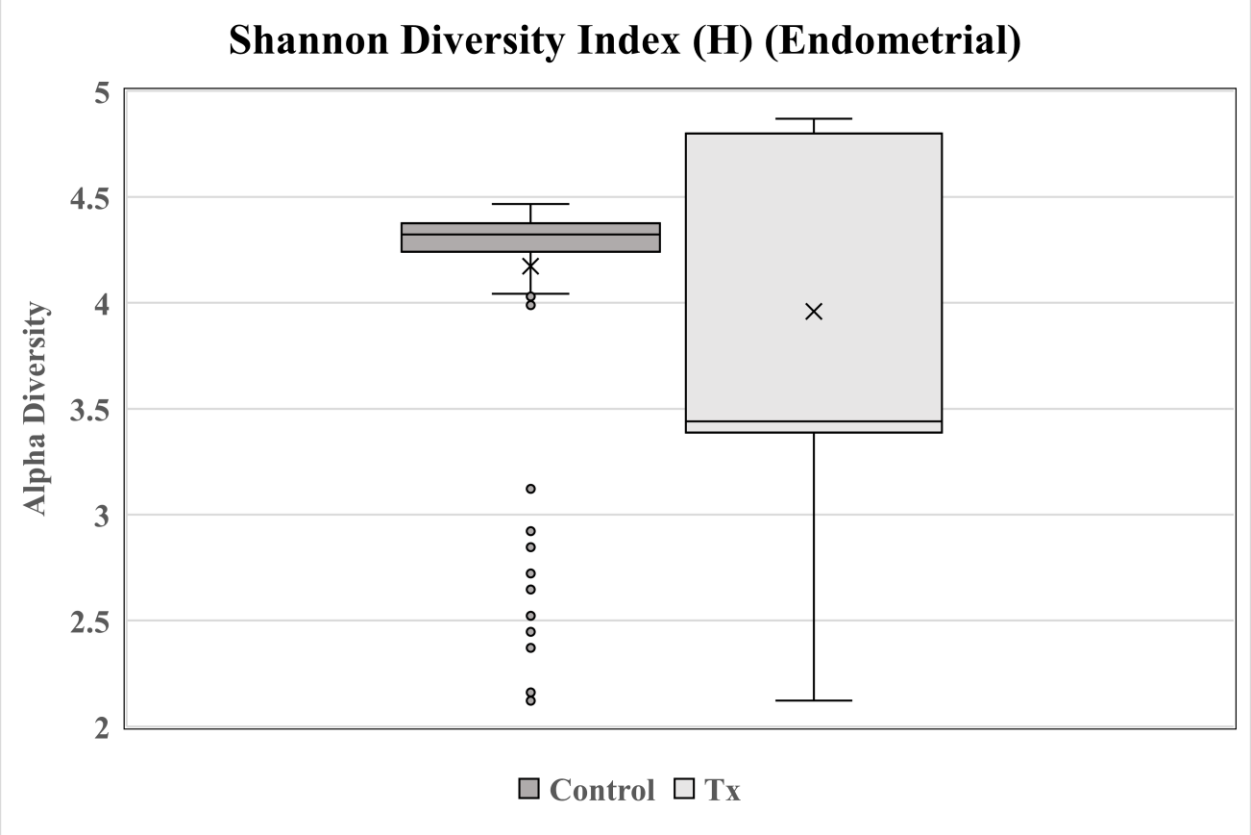

Figure 8. The alpha diversity of bacterial (16S) DNA measured in the control and treated samples from endometrial mucosa via 16S gene sequencing in terms of the Shannon diversity index (H).

Table 8. Descriptive statistics for the alpha diversity of bacterial (16S) DNA measured in the control and treated samples from endometrial mucosa via 16S gene sequencing in terms of the Shannon diversity index (H).

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Endometrial Ct	330	4.04	4.24	4.32	4.37	4.47	4.17	-
Endometrial Tx	220	2.12	3.39	3.44	4.80	4.87	3.96	-
								0.0005

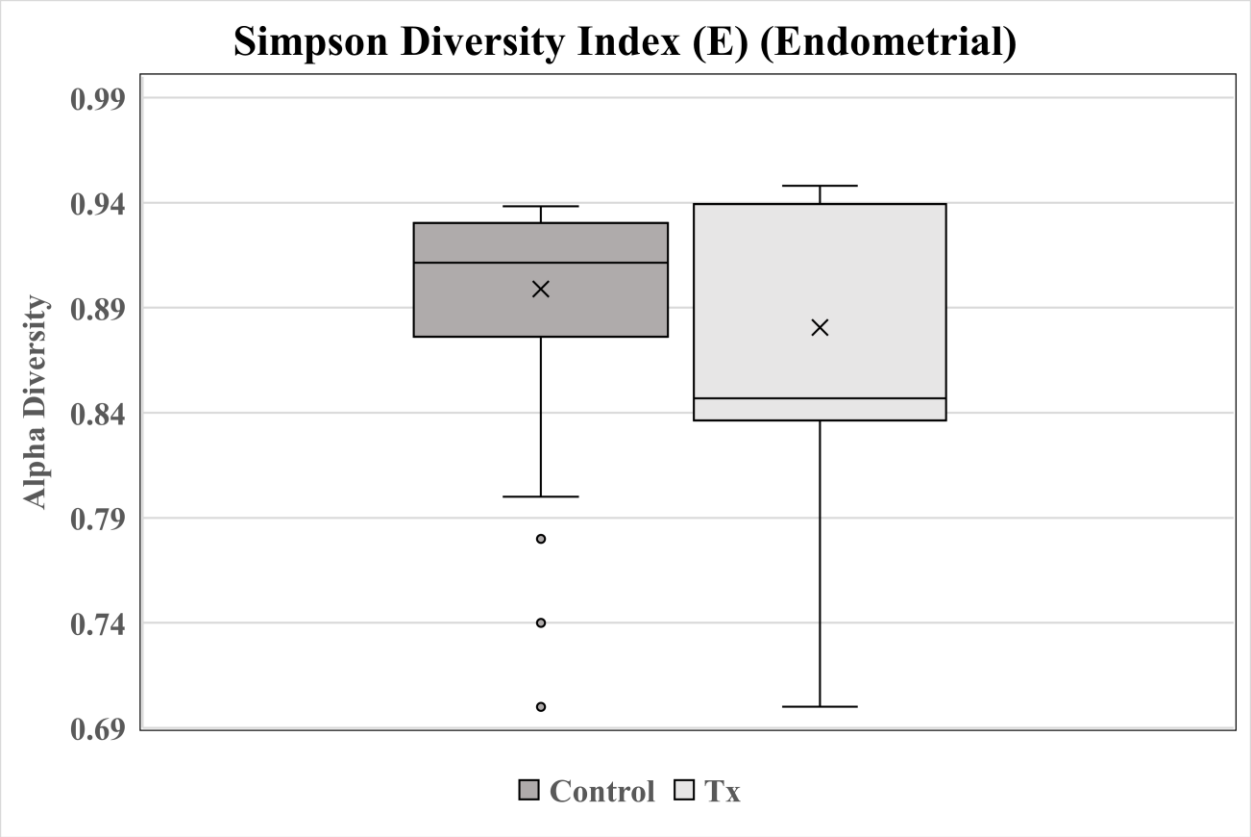

Figure 9. The alpha diversity of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing in terms of the Simpson diversity index (E).

Table 9. Descriptive statistics for the alpha diversity of bacterial (16S) DNA measured in the control and treated samples from vaginal mucosa via 16S gene sequencing in terms of the Simpson diversity index (E).

	n	minimum	Q1	median	Q3	maximum	mean	p-value
Endometrial Ct	330	0.80	0.88	0.91	0.93	0.94	0.90	-
Endometrial Tx	220	0.70	0.84	0.85	0.94	0.95	0.88	-
								3.9308E-05

Microbial Composition

Vaginal vs. Endometrial Mucosa

In examining the core microbiome (i.e., taxa that were present in all samples at all time points) of both vaginal and endometrial mucosa in bovine, consideration was given to attempt to identify the taxon to its lowest taxonomic level possible, ending at the familial level. In cases in which no lower taxonomic level was available, the most specific classification was used. Analysis of the vaginal mucosal core microbiome reveals 17 different taxa, as well as one classification of unknown. With consideration that the data were obtained via 16S gene sequencing, the unknown could represent either a single individual taxon, or it could be representative of multiple, and due the presence in all vaginal mucosal samples, the classification was included in this analysis, though further identification at this time appears virtually impossible. In evaluating the remaining 17 taxa, 16 were able to be identified to familial level, whereas one was identified only to class level. In order of greatest to least contribution to both total sample composition (calculated via averaging the composition in all samples) and core microbiome composition (its relative contribution to the core microbiome), the respective taxa are as follows: Ruminococcaceae, Lachnospiraceae, Rikenellaceae, Prevotellaceae,

Bacteroidaceae, Christensenellaceae, Peptostreptococcaceae, Coriobacteriaceae, Familly XIII, Methanobacteriaceae, Erysipelotrichaceae, Porphyromonadacaeae, Mollicutes, Acidaminococcaceae, Clostridiaceae, Alcaligenaceae, and Peptococcaceae. From this list, all are familial taxa, except for the class of Mollicutes. Of the familial level identifications, all are either classified into the Bacteriodetes phylum or Firmicutes phylum, except for three which are currently classified in either Euryarcaeota (Methanobacteriaceae), Actinobacteria (Coriobacteriaceae), or Proteobacteria (Alcaligenaceae) phyla.

Of the vaginal core microbiome taxa, the top five in terms of relative core microbiome percent composition are Ruminococcaceae (37.20%), Lachnospiraceae (18.98%), Rikenellaceae (8.70%), Prevotellaceae (5.35%), and Bacteroidaceae (4.65%). The remaining taxa fell into either a range between 2.00% and 4.50% of the core microbiome composition (Christensenellaceae (4.36%), Peptostreptococcaceae (4.25%), Coriobacteriaceae (3.54%), Familly XIII (3.00%), Methanobacteriaceae (2.59%), and Erysipelotrichaceae (2.14%)) or between 0.25% and 1.00% (Porphyromonadacaeae (0.85%), Mollicutes (0.78%), Acidaminococcaceae (0.76%), Clostridiaceae (0.43%), Alcaligenaceae (0.35%), and Peptococcaceae (0.29%). The unknown taxa compromise a total of 1.81% of the vaginal core microbiome. (Table 10; Figure 10).

Despite the unknown taxa (“unknown”) comprising only 1.81% of the core microbiome of vaginal mucosa, the unknown comprises the entirety (i.e., 100%) of the core microbiome of endometrial mucosa, with this unknown taxa/taxon being the only group found in all of the samples of the endometrial mucosal samples. These results are summarized in Table 10.

Table 10. Core microbiome of both vaginal and endometrial mucosal samples in bovine with both the total percent composition in the samples as well as the core percent composition (i.e., the relative contribution that the taxa make towards defining the core microbiome). Note that the identification was performed to the lowest taxonomic level possible, ending at the familial level.

Source	Level	Identification	Total %	Core %
Vaginal	Class	Mollicutes	0.58%	0.78%
	Family	Ruminococcaceae	27.55%	37.20%
		Lachnospiraceae	14.06%	18.98%
		Rikenellaceae	6.44%	8.70%
		Prevotellaceae	3.96%	5.35%
		Bacteroidaceae	3.44%	4.65%
		Christensenellaceae	3.23%	4.36%
		Peptostreptococcaceae	3.14%	4.25%
		Coriobacteriaceae	2.62%	3.54%
		Familly XIII	2.22%	3.00%
		Methanobacteriaceae	1.92%	2.59%
		Erysipelotrichaceae	1.58%	2.14%
		Porphyromonadacaeae	0.63%	0.85%
		Acidaminococcaceae	0.56%	0.76%
		Clostridiaceae	0.32%	0.43%
		Alcaligenaceae	0.26%	0.35%
		Peptococcaceae	0.21%	0.29%
	Unknown	Unknown*	1.34%	1.81%
Endometrial	Unknown	Unknown*	26.48%	100%

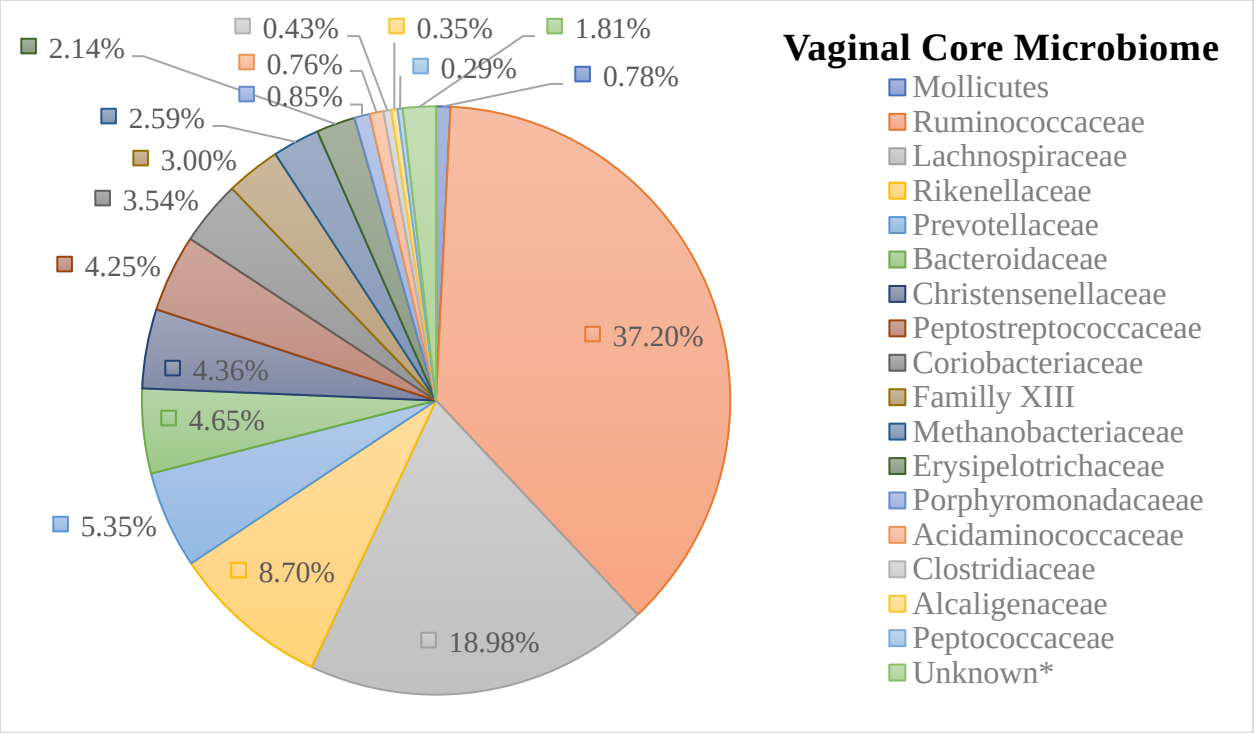

Figure 10. Pie chart depicting the core percent composition (i.e., the relative contribution that the taxa make towards defining the core microbiome) of each taxon towards the vaginal core microbiome in bovine.

Vaginal Control vs. Vaginal Treatment

When evaluating the vaginal mucosal core microbiome respective to either the control or treated vaginal mucosal samples, there is a difference between the representative taxa. The vaginal control mucosal samples boast four more taxa compared to the aforementioned vaginal core microbiome (which essentially represents the vaginal treated core microbiome, since only those taxa present in all samples at all time points were included in calculations). Of these four additional groups, one was identified at the phylum level (Saccharibacteria; 0.85% core composition), one at the order level (Gastranaerophilales; 0.36% core composition), and two at

the familial level (Streptococcaceae (2.15% core composition) and Defluviitalaeaceae (0.13% core composition) (Table 11).

Table 11. Core microbiome of control and treated vaginal mucosal samples in bovine with both the total percent composition in the samples as well as the core percent composition (i.e., the relative contribution that the taxa make towards defining the core microbiome). Note that the identification was performed to the lowest taxonomic level possible, ending at the familial level.

Source	Level	Identification	Total %	Core %
Vaginal Control	Phylum	Saccharibacteria	0.70%	0.85%
	Class	Mollicutes	0.70%	0.85%
	Order	Gastranaerophilales	0.30%	0.36%
	Family	Ruminococcaceae	30.93%	37.44%
		Lachnospiraceae	14.57%	17.63%
		Rikenellaceae	7.69%	9.31%
		Prevotellaceae	4.12%	4.99%
		Bacteroidaceae	4.01%	4.85%
		Peptostreptococcaceae	3.26%	3.94%
		Coriobacteriaceae	3.06%	3.70%
		Christensenellaceae	2.98%	3.60%
		Familly XIII	2.23%	2.70%
		Methanobacteriaceae	1.81%	2.19%
		Streptococcaceae	1.78%	2.15%
		Erysipelotrichaceae	1.60%	1.94%
		Porphyromonadacaeae	0.72%	0.87%
		Acidaminococcaceae	0.56%	0.67%
		Alcaligenaceae	0.24%	0.30%
		Clostridiaceae	0.19%	0.23%
		Peptococcaceae	0.18%	0.22%
		Defluviitaleaceae	0.11%	0.13%
	Unknown	Unknown*	0.90%	1.09%
Vaginal Treatment	Class	Mollicutes	0.48%	0.70%
	Family	Ruminococcaceae	24.17%	35.35%
		Lachnospiraceae	13.54%	19.81%
		Rikenellaceae	5.20%	7.61%
		Prevotellaceae	3.80%	5.56%
		Christensenellaceae	3.48%	5.09%
		Peptostreptococcaceae	3.03%	4.44%
		Bacteroidaceae	2.88%	4.21%
		Familly XIII	2.21%	3.23%
		Coriobacteriaceae	2.19%	3.20%
		Methanobacteriaceae	2.02%	2.96%
		Erysipelotrichaceae	1.57%	2.29%
		Acidaminococcaceae	0.57%	0.83%
		Porphyromonadacaeae	0.53%	0.78%
		Clostridiaceae	0.44%	0.65%
		Alcaligenaceae	0.27%	0.39%
		Peptococcaceae	0.24%	0.36%
	Unknown	Unknown*	1.74%	2.55%

The remaining taxa stayed consistent between control and treatment (i.e., the bacteria were present when inorganic and organic selenium were present) at fairly similar levels, with the greatest difference being approximately 2% between control and treatment. These results are summarized in Table 11, and the vaginal control core microbiome and the vaginal treatment core microbiome relative core percent composition are illustrated in Figures 11 and 12, respectively.

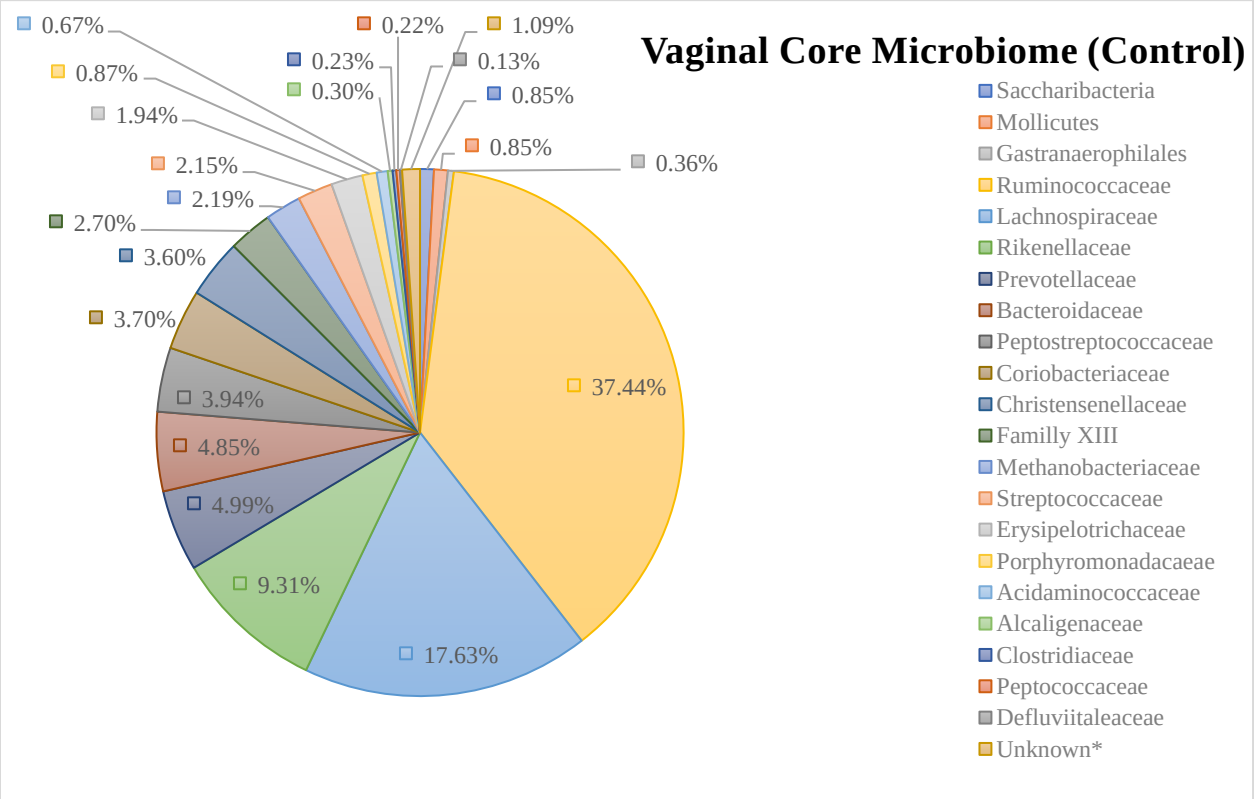

Figure 11. Pie chart depicting the core percent composition (i.e., the relative contribution that the taxa make towards defining the core microbiome) of each taxon towards the vaginal core microbiome of control samples in bovine.

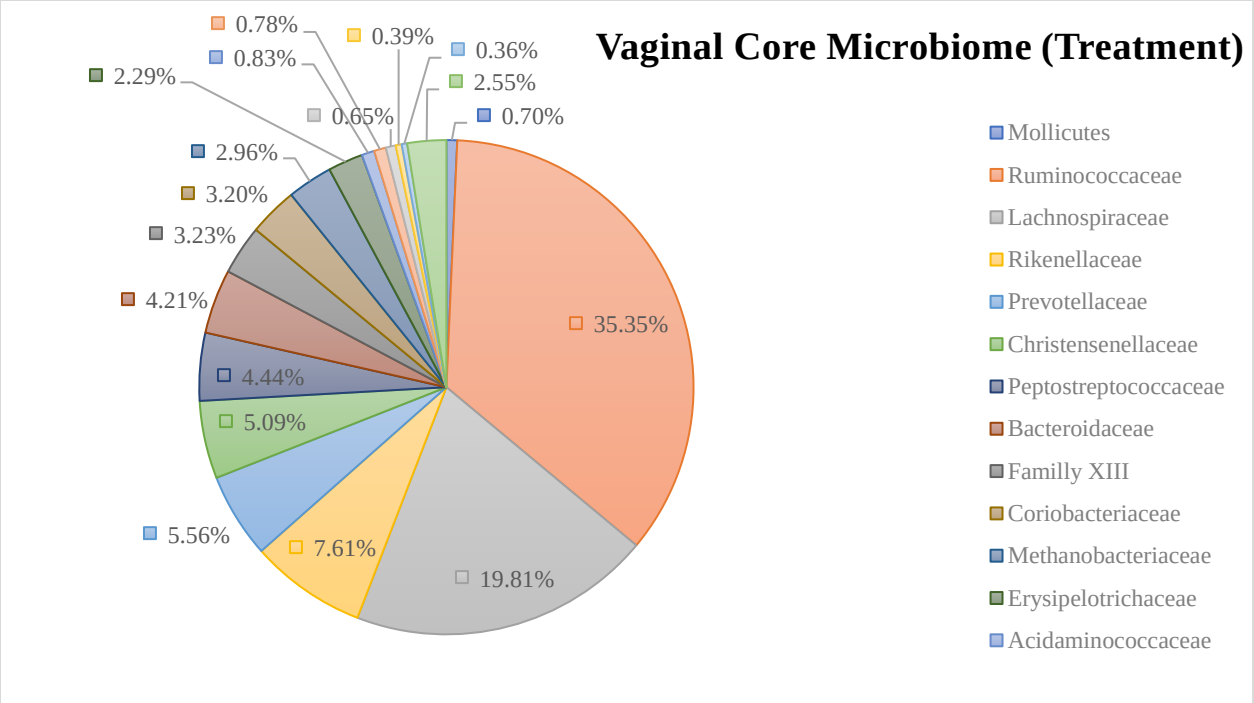

Figure 12. Pie chart depicting the core percent composition (i.e., the relative contribution that the taxa make towards defining the core microbiome) of each taxon towards the vaginal core microbiome of treated samples in bovine.

Endometrial Control vs. Endometrial Treatment

Though no direct identification is possible due to the nature of the unknown, this group still comprises the entirety of the endometrial core microbiome, and thus a difference between the total percent composition in the samples was determined. Specifically, the endometrial control samples had an unknown group comprising 20.95% of the total bacterial population in the endometrial samples, yet in the endometrial treatment samples, a total bacterial population composition was determined to be 34.78% (Table 12). This represents an almost 14% increase in the unknown population in the treated samples compared to the control samples of endometrial mucosa.

Table 12. Core microbiome of control and treated endometrial mucosal samples in bovine with both the total percent composition in the samples as well as the core percent composition (i.e., the relative contribution that the taxa make towards defining the core microbiome). Note that the identification was performed to the lowest taxonomic level possible, ending at the familial level.

Source	Level	Identification	Total %	Core %
Endometrial Control	Unknown	Unknown*	20.95%	100%
Endometrial Treatment	Unknown	Unknown*	34.78%	100%

Discussion

Abundance and Alpha Diversity

Vaginal vs. Endometrial Mucosa

To both avoid controversy and minimize statistical misconduct, the classic *p* value historic 0.05 threshold has been lowered to 0.005 (proposed by some statisticians) for the purposes of this study (rather than being abandoned entirely, as other scholars have suggested) (35).

As such, in evaluating the results with respect to the proposed hypotheses, though the absolute abundance is higher in vaginal mucosal samples compared to endometrial samples (in both measures of central tendency) the results are not statistically significant (*p* value = 0.0695 > 0.005), meaning that null hypothesis is not rejected, and though there appears to be a trend, further statistical analyses will have to be conducted to confirm significance. A potential explanation of the *p* value would be the low sample sizes used in this analysis from the vaginal and endometrial mucosa (n = 18, n = 9, respectively).

Though no conclusions can be made regarding the absolute abundance, the alpha diversity results appear statistically significant, both for the SDIH (p value = 0.0000 < 0.005) and for the SDIE (p value = 0.0000 < 0.005). As such, the results suggest that vaginal mucosa has a higher alpha diversity compared to endometrial mucosa, in terms of the Shannon diversity index (which focuses on the population as a whole) and in terms of the Simpson diversity index (another diversity index, but it also takes into account species richness as well as evenness). Therefore, the hypothesis is supported in this aspect, and it can be concluded that vaginal mucosal samples have a greater alpha diversity compared to endometrial mucosal samples.

Vaginal Control vs. Vaginal Treatment

In a similar trend to analyzing the vaginal mucosa in comparison with the endometrial mucosa, it appears that the absolute abundance shows a difference (i.e., the vaginal treated samples had a greater absolute abundance of bacteria compared to the vaginal control samples), yet a definite conclusion cannot be made since the results are not statistically significant (i.e., the null hypothesis cannot be rejected) (p value = 0.1349 > 0.005). A similar explanation for this phenomenon can be due to the low sample sizes (n = 3 for both), yet further studies must still be conducted in order to confirm this apparent trend.

However, the alpha diversity results are able to be confidently interpreted, with the SDIH (p value = 0.0000 < 0.005) and SDIE (p value = 0.0000 < 0.005) having results that are statistically significant. As such, it appears that the vaginal treated mucosal samples have a greater alpha diversity as a whole (SDIH) and with respect to species evenness and richness (SDIE). These results suggest that selenium supplementation affects the alpha diversity of vaginal microbiomes. Specifically, these results indicate that using a 1:1 mixture of ISe and OSe

results in a greater diversity compared to using Ise supplementation alone, thereby supporting the hypothesis.

Endometrial Control vs. Endometrial Treatment

A similar trend is also apparent for the measurement of absolute abundance of endometrial control mucosal samples and endometrial treated mucosal samples, with the trend seemingly pointing to an increase in overall abundance for the treated samples, yet the results also remain not statistically significant (p value = 0.2592 > 0.005). A small sample size was also utilized in this analysis, yet the relatively high p value compared to the other abundance analyses suggest that this relationship (p value = 0.2592) is less likely to be explained by sample size compared to the vaginal treated and control musical samples (p value = 0.1349) and the vaginal/endometrial mucosal sample comparison (p value = 0.0695).

Yet, the alpha diversity indices show promising results, having statistically significant SDIH and SDIE results (p value = 0.0004 < 0.005 and p value = 0.0000 < 0.005, respectively). Despite the similar abundance trend, the results indicate that selenium supplementation of a mixture of ISe and OSe actually results in a decrease in alpha diversity in both SDIH and SDIE measurements, which does not support the hypothesis, nor was the hypothesis supported in terms of absolute abundance of endometrial control and treated mucosal samples.

Support for Hypothesis

Due to the lack of statistical significance regarding the absolute abundance results, it cannot be concluded that the vaginal mucosa has a greater abundance of bacteria compared to endometrial mucosa, nor can it be concluded that treatment with a mixture of inorganic selenium and organic selenium impacts the bacterial abundance. However, it can be concluded that the vaginal mucosa has a greater diversity compared to endometrial mucosa, and it also may be

concluded that the form of selenium supplementation impacts the diversity of the vaginal and endometrial mucosa (albeit, in differing ways, with an increase in vaginal bacterial diversity and a decrease in endometrial bacterial diversity when treated with a mixture of inorganic and organic selenium). As such, the abundance aspect of the hypothesis was not supported by this study, whereas the alpha diversity aspect was supported, suggesting that the type of selenium supplementation impacts the bacterial diversity of vaginal and endometrial mucosa.

Microbial Composition

Vaginal Mucosa

In examining the results of the vaginal mucosa, it appears that the core microbiome may be impacted by the form selenium supplementation since treatment with the mixture of inorganic and organic selenium resulted in the removal of four groups (Saccharibacteria, Gastranaerophilales, Streptococcaceae, and Defluviitaleaceae) from the vaginal control microbiome (Figure 13). These results suggest that the bioavailability of selenium has an impact on microbiome composition within vaginal mucosa, though future studies need to be conducted to elucidate the mechanism by which this phenomenon occurs.

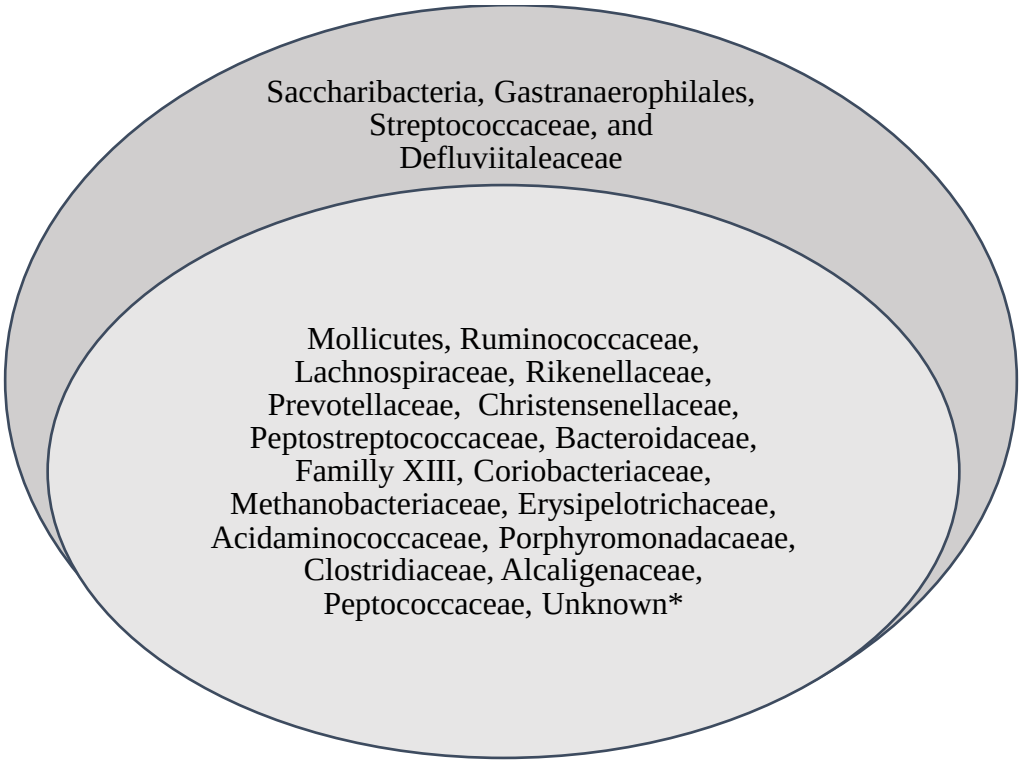

Figure 13. Diagram illustrating the difference between the bovine vaginal mucosal control core microbiome (dark and light areas) and the vaginal mucosal treated core microbiome (light area only).

Despite limited knowledge regarding the exact mechanism by which these groups are essentially removed as a result of the type of selenium supplementation, the fact remains that these groups still respond accordingly. In looking at these individually, it appears that limited knowledge exists regarding Saccharibacteria, though accumulating evidence associates this phylum with periodontal disease (36). Similarly, limited knowledge currently exists regarding Gastranaerophilales, which are found in animal guts and are thought to have a beneficial effect as a source of vitamins B and K as well as aiding digestion, though their exact role is unknown (37) (nor is it known the effect of these bacterial in the vaginal or reproductive tract). There also is limited scholarship regarding the Defluviitaleaceae family, being represented by only one genus

with two valid species names and one invalid species name (38). However, Streptococcaceae is a well-studied family, and one genus in particular tends to be pathogenic (*Streptococcus*). One species of this specific genus *Streptococcus agalactiae* (also known as group B Streptococcus) is reported as a major cause of neonatal disease in humans, and in an emerging pathogen in both humans and cattle (39). As such, these results suggest that the form of selenium supplementation as a mixture of both inorganic and organic could be beneficial in removing potentially pathogenic bacteria (i.e., Streptococcaceae) from the reproductive tract, though current limited knowledge prevents indications for the remaining three taxa.

In looking at the core vaginal microbiome as a whole, the top five microbiome composition contributors (Ruminococcaceae, Lachnospiraceae, Rikenellaceae, Prevotellaceae, and Bacteroidaceae) are commonly found within the gastrointestinal tract of animals (40) and typically are involved in normal metabolic processes within cattle (41). Furthermore, these five groups are prominent bacterial families in healthy human gastrointestinal tracts (with the tract being dominated by Firmicutes, Bacteriodetes, Actinobacteria, and Proteobactera) (40). With consideration that all of the known groups fall within these phyla (except for Methanobacteriaceae, which are involved in healthy cattle metabolism (42)), these results suggest that the gastrointestinal microbiome impacts the vaginal microbiome in cattle through an unknown mechanism. Despite the unknown mechanism, these results may be explained by the anatomical location of the anus and vagina, with the end of the gastrointestinal tract being located above the vaginal tract opening.

Endometrial Mucosa

Due to the limited nature of the results in elucidating the core microbiome of the endometrial mucosa in cattle, no conclusions can be made regarding the effects of selenium

supplementation on the endometrium microbiome. The results merely suggest that selenium supplementation type may increase the percent composition of these unknown within the endometrial microbiome, but without knowing any specific classification of this group, determination of beneficial or negative effects is impossible with the current knowledge.

Implications

Overall, the results of this study suggest that biodiversity of the reproductive tract may be impacted based on the form of selenium supplementation (i.e., inorganic and organic selenium forms). Furthermore, these results indicate that the form of selenium supplementation impacts the microbiome composition of vaginal and endometrial mucosa.

Future studies need to be conducted in order to address the limitations of this study. Namely, the lack of significant statistical data regarding the absolute abundance of bacteria in the reproductive tract, and the elucidation of both the mechanisms by which selenium supplementation (in essence, selenium bioavailability) impacts diversity, as well and the identification of unknown groups found in the vaginal and endometrial mucosa in cattle. Since symbiosis and dysbiosis are reliant on the idea of balance (and imbalance, respectively) of the microbiome, it is virtually impossible to conclude whether the form of selenium supplementation has a positive or negative effect on the reproductive potential of cattle based on this data alone. However, this study contributes to the growing literature surrounding selenium supplementation and its impacts on microbial composition in the reproductive tract, and it is sincerely hoped that this data may help contribute to a greater understanding of the reproductive microbiome.

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