Review

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*Review*

# Elucidation of the Vaginal Microbiome During Gestation and Its Involvement with the Fraternal Birth Order Effect and Male Homosexuality

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**Abstract**

The vaginal microbiome plays a crucial role in protecting its host from bacterial, viral, and fungal pathogens and serves as a first line of immune system defense for the lower reproductive tract. The composition of the microbiome is relatively understudied despite its integral role in women's reproductive health. Maintenance of the correct abundances of bacteria can help prevent many different infections. Additionally, the microbiota also serve to initiate and support the immune system of a newborn in the case of vaginal birth. The introduction of these organisms can mediate many interactions in the developing brain and nervous system of the infant (Günther, V. et al. 2022). Similarly, research on biological causes of sexual orientation is a new field that severely lacks extensive research. There are a few theories related to the development of homosexuality that have been backed by research. Synthesizing what we know from previous research on these two fields, we aim to bridge the gap between these two areas of study by postulating a relationship between the composition of the female vaginal microbiome during pregnancy and the fraternal birth order effect leading to male homosexuality. We propose that during pregnancy, changes in the maternal vaginal microbiome result in a change in the vaginal microbiome that makes them more susceptible to the development of anti-NLGN4Y antibodies responsible for the immune attack on the Y-linked NLGN4Y protein responsible for male brain development (Bogaert, A. et al. 2017), specifically in the anterior hypothalamus. We propose that mothers who have already given birth to males are especially susceptible to this antibody development, providing a possible explanation for the Fraternal Birth Order effect.

**Keywords:** sexual orientation; maternal immune activation; vaginal microbiome; fraternal birth order effect; sexually dimorphic nuclei; NLGN4Y

## 1. Introduction

The vaginal microbiome is crucial to female reproductive health, yet there is not nearly enough research exploring its intricacies to further discover its full importance in the human body. Past research in rodents has shown that the vaginal microbiome has the capacity to influence the mental health of offspring as well as the maternal mental health (Jašarevic, E. et al. 2018). Conversely, perceived stress can also influence changes in the vaginal microbiome. Research (Turpin, R. et al. 2021) has shown that participants with an increase in perceived stress had greater risks of developing bacterial vaginosis, which is characterized by the imbalance of the abundance of *Lactobacillus* species in the microbiome. This alone has direct effects on reproductive health, but the further causes of shifts in vaginal flora and their greater effects on reproductive and general health remain uncharacterized.

The interplay between the vaginal microbiome and the maternal immune system during and after pregnancy contributes to the initial development of the fetal immune system. It has been shown

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that, in addition to the maternal vaginal microbiome and immune system influencing the body systems of the infant, genes expressed on the Y chromosome of a male fetus lead to the development of maternal antibodies against a specific protein, NLGN4Y. This protein is responsible for some parts of male brain development (Bogaert, A. et al. 2017). It has been proposed that with the development of these antibodies following the gestation and birth of a male infant, subsequent male births have an increased likelihood of being homosexual males. This phenomenon has been named the Fraternal Birth Order Effect (FBO) and was coined in 1996, when two psychologists, Ray Blanchard and Anthony Bogaert, found that, on average, gay men have a larger number of older brothers than heterosexual men. Despite this finding, the FBO effect only accounts for a small number of men who are gay, who also have older brothers.

We hypothesize that there is a relationship between the makeup of the maternal vaginal microbiome during gestation and the development of homosexuality in male offspring, by means of fluctuating levels of *Lactobacilli* species influencing the accumulation of anti-NLGN4Y proteins, therefore increasing the chance of male offspring being homosexual as outlined by the fraternal birth order effect. While this phenomenon seems promising in discovering mechanisms that lead to the development of homosexuality, there are likely numerous variables that come together to influence human sexuality, especially when considering gender expressions and sexualities outside of the binary. The goal of this study is to further delineate how the maternal vaginal microbiome and immune system potentially contribute to the development of homosexuality in male offspring, including any other potential interactions between these three variables and their impact on both sexual orientation and female reproductive health.

## 2. Literature Review

### *2.1. Impact of the Vaginal Microbiota*

The dynamics of the vaginal microbiome have remained in the shadows of research until recently. Early research has explored the effects of exposure to the vagina microbiome on offspring gut health, immune function (Hooper, L.V. et al. 2012), and brain development (Jašarević, E, et al. 2018). In 2018, Dr. Tracy Bale published a study evaluating how early prenatal stress disrupts the transmission of maternal vaginal microbiota to the offspring, leading to lasting effects on offspring gut microbiota establishment, immune function, and brain development. The conclusions from this study suggest that prenatal stress on the maternal vaginal microbiome causes a shift of microbiome composition (which varies between women), often called vaginal dysbiosis, that is detrimental to proper hypothalamic development in offspring. This suggests that if contact with a healthy maternal vaginal microbiome during birth is essential for brain development, then perhaps the absence of vaginal birth, or the presence of prenatal stress (on the mother), could mean that an offspring is more susceptible to poor brain development. A study done earlier this year reported that 6-month-old vaginally delivered infants displayed significantly greater neurodevelopment, as measured by the Ages and Stages Questionnaire, compared to infants delivered through C-section (Zhou, L., et al. 2023). They also concluded that vaginal microbiota transfer (VBT) is essential for offspring gut microbiota maturation within 42 days after birth. These findings were consistent with previous studies (Jost, T., et al., 2012).

Moving on to the topic of how the vaginal microbiome can influence offspring gut health, previous studies have shown that exposure to the maternal microbiome increased Bacteroides species concentrations in newborns within the first week of life, which was essential for the establishment of proper gut microbiota. Without this interaction, offspring can develop dysbiotic gut microbiota, which has been increasingly associated with short and long-term immunological disorders, including Crohn’s disease and ulcerative colitis (Greer, J. B. & O’Keefe, S.J., 2011).

Finally, the vaginal microbiome has a great influence on both offspring and maternal immune function. This is attributed to a leading species of a stable vaginal microbiome called Lactobacillus. Lactobacillus species flourish in anaerobic environments and convert glycogen into glucose and lactic

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acid, while also producing hydrogen peroxide and other antimicrobial compounds, thereby contributing to combat invading pathogens present in the vagina (Chen, X., et al. 2021) but also in the GI tract, oral cavity, and epidermal layer (Chee, W.J.Y., et al. 2020). In fact, Lactobacillus has been proven to produce bacteriocin through fermentation, which inhibits the growth of pathogens that can invade the urogenital tract, such as G.vaginalis, S. agalactiae, and P. aeruginosa (Chee, W.J.Y., et al. 2020). Lactic acid produced by lactobacillus is responsible for giving the vaginal microbiome a slightly acidic pH of 3.8-4.5 (Gajer, P., et al. 2012). Many previous studies have shown that a dysregulation of lactobacillus can lead to vaginal dysbiosis, which is characterized by a change in microbiome pH or symptoms including pregnancy loss, preterm labor, and other gynecological issues (Chee, W.J.Y., et al. 2020). Other than lactobacillus, the vaginal microbiome consists of many other species that are often difficult to predict. It is well known now that the composition of the vaginal microbiome is highly variable among women, and especially throughout an individual's lifespan. Thus, researchers have organized the compositions of these microbiomes into five general classes: I, II, III, IVC, IVD, and V (Gajer, P., et al. 2012). Longitudinal studies that have shown how the vaginal microbiome changes over time provide a more realistic view of the complexities of the female reproductive system (Gajer, P., et al. 2012). This study followed 32 women over 16 weeks and demonstrated the fluctuation of various microbial species during the menstrual cycle, generally showing high levels of Streptococcus and Peptostreptococcus and low levels of Lactobacillus species. All of these findings provide us with a stable foundation on which we will further explore, with emphasis on the maternal immune system during pregnancy and after successive births.

### *2.2. Maternal Antibody Development*

During pregnancy, it has been shown that microbiome profiles become less diverse during the second trimester, usually consisting of higher lactobacillus abundance in women with lactobacillus-dominated community state types (Freitas, A.C., et al. 2017) and accompanied by lower prevalence of mycoplasma and ureaplasma. These changes in microbiome composition were significant in pregnant women compared to non-pregnant women (Romero, R., et al. 2014). Microbiome composition was quantified in these studies through metagenomic characterization using DNA isolated from the vagina, and the 16S RNA was sequenced (Aagaard K. 2012). Synthesizing these ideas along with what we know about lactobacillus and how it aids in immune function in the vaginal microbiome, we can further elucidate how low levels of lactobacilli during pregnancy could relate to maternal antibody production.

Understanding the mechanisms of antibody transfer from the maternal immune system to the fetus will be essential to understanding the NLGN4Y attack theorized for the FBO effect. During pregnancy, the fetus is protected by the placenta, which forms around the fetus on the interior of the uterine lining. The uterus's blood supply during pregnancy comes from the uterine and ovarian arteries, which supply the baby with oxygen and nutrients required for growth (Chaudhry R., et al., 2023). Usually, the placenta acts as a barrier to separate fetal and maternal blood, which is a state of high immune function during pregnancy. Recent research has shown that pregnant women with preeclampsia (elevated blood pressure) display higher levels of pro-inflammatory IL-6 (immune response) compared to normal pregnant women (Bernsen RM. 2006). These researchers concluded that this indicated a lower number of T regulatory (Treg) cells (which are another component of the maternal immune system) present in women with preeclampsia. Treg cells are found in high concentrations at the maternal-fetal interface and play a critical role in maintaining the maternal tolerance to fetal antigens during pregnancy. In a study examining how Treg cells influence genital inflammation, it was shown that a lactobacillus species called *Lactobacillus crispatus* induced the expression of an anti-inflammatory phenotype that included the increase of Treg cells in the female reproductive tract (Ssemaganda, A. 2021). These studies could provide insight into how the maternal immune system displays increased vigilance during pregnancy (due to increased *Lactobacillus crispatus* and Treg cells) and can potentially start producing antibodies against the NLGN-4Y protein in male fetuses.

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The development of NLGN-4Y antibodies has a similar mechanism to the development of Rh factors, which increases in concentration for every successive pregnancy (Dumitru, A., 2021). Rhesus factor (Rh) protein is used to determine blood type as either positive (presence of Rh) or "negative" (absence of Rh) (Dumitru, A. 2021). Rh-negative individuals do not possess the Rh "antigen" and thus will display an immune response against a Rh-positive fetus. Although the placenta is normally able to prevent the mixing of maternal and fetal blood, it has been shown that childbirth often induces bleeding that can make maternal immune cells more sensitive to foreign factors and proteins (Pollock, J.M & Bowman, J.M., 1982). In our studies, we postulate that these findings about Rh antibody development are similar to those of NLGN-4Y in that these anti-male antibodies produced by the mother can cross the blood-brain barrier of the immature fetal brain and ultimately interfere with brain development of the fetus (Bogaert A.F. & Skorska M. 2011).

After understanding how anti-NLGN4Y could be developed, we also need to understand how the changes in anti-NLGN4Y expression in mothers increase after successive births. In a study following women postpartum, researchers found a significant increase in microbiome diversity postpartum (MacIntyre, D.A., et al. 2015). They found that the prevalence of Community State Type (CST) IV in postpartum women was drastically changed compared to microbial compositions during pregnancy. This state involves very low levels of the lactobacillus species. Tying into the involvement of hormones, since it has been previously shown that estrogen plays an important role in shaping the composition of the vaginal microbiome during pregnancy, and increased levels of estrogen are thought to increase the proportion of Lactobacillus species in the vagina, these researchers postulated that the reduction of estrogen levels in the postpartum period would reduce microbiota community stability of the vaginal microbiome (Boskey, E. R., et al. 2001) which is also consistent with decreased immune function. These mechanisms have been elucidated as estrogen-driven maturation of the vaginal epithelium leads to glycogen accumulation, which is broken down to produce maltose, maltotriose, and maltotetraose, all of which support lactobacillus species colonization. This information provides insight into how hormonal changes postpartum can influence the production of lactobacillus species and vaginal microbiome health during pregnancy and during postpartum.

A major contribution to the fields of human sexuality and maternal immune function was made by Boegart. A.F et al. in 2017. They showed that there was a greater likelihood of male homosexuality with each successive male birth. They found that during successive male births, there was an increase in anti-NLGN-4Y production by the maternal immune system, which led to decreased activity of the NLDN-4Y protein, a Y-linked brain development factor (Boegart, A.F., et al. 2017, Blanchard, R. et al., 2006). The groundbreaking work by Bogaert and colleagues significantly enhances our understanding of the connections between maternal immune function and human sexuality.

### *2.3. Fetal Brain Development*

Upon crossing the fetal blood-brain barrier, anti-NLDN-4Y antibodies influence fetal neural development (Bogaert A.F. & Skorska M. 2011) specifically in the anterior hypothalamus. Previous lesion experiments with rats showed that the medial preoptic area/anterior hypothalamus (MPOA/AH) is crucial for the expression of male sexual behavior (Paredes, R. G., Tzschentke, T., & Nakach, N. 1998). Both human and animal studies have converged on sexually dimorphic hypothalamic structures that are developmentally sensitive to prenatal conditions. In humans, the third volume of the interstitial nuclei of the anterior hypothalamus (INAH3) was reported to be more than twice as large in heterosexual men compared with homosexual men (LeVay 1991). Likewise, an experimental study done in 2004 showed that in rams, the ovine sexually dimorphic nucleus (oSDN) was also twice as large in female-oriented rams (preferring female partners) as opposed to male-oriented rams (Roselli C.E. et al., 2004). The oSDN was identified as a cluster of neurons within the MPOA/AH that express cytochrome P450 aromatase, an enzyme that converts androgens into estrogens. These researchers found that the oSDN in female-oriented rams contained significantly higher aromatase mRNA levels compared to male-oriented rams, suggesting that variation in sexual partner preference in rams may not only be influenced by brain anatomy, but also could be mediated

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by the capacity for estrogen synthesis. Such similarities across mammals highlight plausible developmental targets for upstream biological mechanisms. If maternal antibodies to Y-linked neural adhesion molecules alter fetal neurodevelopment, their effect would likely be most visible in brain regions undergoing sexually differentiating processes during mid-gestation. Both the INAH3 in humans and the oSDN in rams meet this criterion: they are hormonally sensitive, variably sized across individuals, and strongly associated with adult sexual partner preference.

The gut and vaginal microbiota are now recognized as major regulators of maternal immune tone, particularly through effects on Treg expansion and inflammatory cytokine balance (Ssemaganda, A. 2021). Dysbiosis during pregnancy increases inflammatory signaling and reduces maternal tolerance to fetal antigens, potentially intensifying maternal immune responses to Y-linked proteins such as NLGN4Y (Bogaert et al., 2017). Since these maternal antibodies cross the immature fetal blood–brain barrier during the same mid-gestation window when sexually dimorphic hypothalamic nuclei, including INAH3 in humans and the oSDN-POA in rams, undergo androgen-dependent structural differentiation (LeVay S., 1991, and Roselli C.E., et al., 2004), microbial effects on maternal immunity may indirectly alter neural development. Thus, the microbiome–immune–brain axis provides a biologically plausible pathway through which maternal vaginal microbial states could influence the development of neural substrates linked to adult sexual partner preference (**Table 1**).

**Table 1.** Proposed mechanisms by which maternal factors may shape fetal brain development and the FBO effect.

| | Mechanism | References |
|---|---|---|
| **1** | Maternal vaginal microbiome during gestation: increased *Lactobacillus crispatus* and Treg cells | Hooper, L.V. et al. 2012, Ssemaganda, A. 2021 |
| **2** | Maternal immune activation and development of anti-NLGN4Y antibodies (higher with each successive male fetus) | Blanchard, R. et al., 2006, Boegart, A.F., et al. 2017 |
| **3** | Anti-NLGN4Y antibodies cross the fetal blood brain barrier | Bogaert A.F. & Skorska M. 2011 |
| **4** | Prenatal exposure to anti-NLGN4Y antibodies influences fetal brain development, possibly modifying the structure (and/or capacity for estrogen synthesis) of sexually dimorphic hypothalamic nuclei such as INAH3 | LeVay S., 1991<br>Roselli C.E. et al., 2004 |
| **5** | Increased probability of exclusive same-sex orientation phenotype in adulthood (Fraternal Birth Order Effect) | Bogaert, A.F., 2006, Blanchard, R et al., 2006, Bogaert, A.F. and Skorska, M., 2011 |

### *2.4. Current Focus on Factors Contributing to Homosexuality*

Homosexuality and other sexual and gender minorities have been thought to arise from active choices individuals make for themselves. Naturally, science supports that this is not the case, but that

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leaves a large knowledge gap lacking in explanations about how deviations from heterosexuality and the gender binary occur in humans. A sizable portion of the research that is currently published regarding male homosexuality is outdated and does not provide much direction for future research. That being said, there is published research that supports a few suggested factors of male homosexuality.

The study of sexual orientation is fairly new, starting with early studies on sexual dimorphism. In 1980, Breedlove and colleagues found a sexually dimorphic nucleus located in the fifth and sixth lumbar segments of the spinal cord (Breedlove S. M., & Arnold, A. P., 1980). They found that this nucleus was dependent on the action of androgens and was termed the spinal nucleus of the bulbocavernosus (SNB). They showed that the motor neurons of the SNB accumulate more androgens (testosterone and dihydrotestosterone) than other motor neurons in other segments of the spinal cord. Their findings supported their hypothesis that androgen was a contributing factor for sexually dimorphic SNB and copulatory behavior after their results showed that in females, androgen injections, but not estrogen injections, induced sensitivity of the target muscles of the SNB. This study was the first of many that looked into morphological sex differences in motor neurons that suggest involvement in sexual dimorphic copulatory behavior in rats.

More current studies on sexual orientation have focused on biological causes. As previously mentioned, one of the currently suggested biological mechanisms for the development of homosexuality in males is the prenatal androgen theory, which states that there may be a relationship between the levels of androgens fetuses are exposed to in utero and the development of homosexuality in males. While it is impossible to retrospectively measure in utero hormone levels, some physiologic markers that are measurable in adults can be correlated to these levels. One of the markers used to measure developmental androgen levels is the ratio of the 2nd to 4th digit lengths of the hand. The rationale behind studies exploring prenatal hormone levels is that sex hormones play a role in developing the sexual dimorphisms and sex characteristics observed in cisgender individuals, and it is thought that gay males would exhibit different developmental characteristics than heterosexual men. In 2000, Robinson, S.J., and Manning, J.T. showed that homosexual men had a lower digit ratio with respect to the left-hand ratio ($0.96\pm0.03$) and the mean value of both hands ($0.97\pm0.03$) than the left hand ($0.98\pm0.04$) and the mean of both hands ($0.98\pm0.03$) of the control group. The homosexual group had a digit ratio that was significantly less than the control group only for the left hand ($t=3.82$, $p=0.0002$) and the mean of both hands ($t=3.01$, $p=0.003$), but not with respect to the right hand ($t=1.38$, $p=0.17$). (Robinson, S.J. and Manning, J.T. 2000). They concluded that homosexual men's left hand 2D:4D ratio is less than the normal value for the population. The same investigators also performed a study involving the number of older brothers that the homosexual men had. The results showed that there was a non-significant positive relation between homosexual men and the number of older brothers they had. They concluded that the 2D:4D ratio is negatively related to prenatal testosterone and positively related to prenatal estrogen. A notable limitation to this study is that the participants were almost exclusively Caucasian.

In contrast to Williams et al. (2000), this study did not find a negative relationship between 2D:4D ratio and fraternal birth order (FBO), however, they found a non-significant positive relation between the same two variables. The validity of these measurements, in these studies and others, is contested. The true relationship between FBO, 2D:4D, and male homosexuality may be weak, and more research is needed to discover more compelling evidence.

Common limitations in this research include low or unpublished effect sizes, lack of accurate representative samples, and difficulty isolating distinct genetic causes. Filling this knowledge gap would be extremely beneficial to current and future research surrounding male homosexuality, and could also prove useful in further study of the maternal immune system and vaginal microbiome, and how they may be factors contributing to sexual orientation.

## 3. Historical Perspectives

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Homophobia is a very broad term that can be used to describe negative attitudes towards anyone part of the greater LGBTQIA2+ community. The term 'homophobia' will mostly be used in the context of discrimination against cisgender gay men in this analysis, but it is applicable to any discrimination against any sexual or gender minority.

The history of homosexual culture spans nearly the entirety of the time the human race has been on Earth. Contrary to the common thought, homosexuality was not nearly as accepted in ancient civilizations such as Greece and the Roman Empire, but it also was not established as strongly as an identity as it is in the present day. In ancient Greece, the primary same sex relationships were between adult men and pubescent or adolescent boys. Putting current world views aside, this was relatively accepted for the time period. It was prohibited in some city-states or was not addressed publicly as right or wrong. Despite its unclear acceptance, archeological evidence has shown that relationships between older men and younger boys existed. The most accepted stance on these relationships during this time is that during penetrative sex between two males, the partner assuming the passive role would be labeled as no longer a man. This point is where discrimination towards individuals who participated in same-sex interactions came from. In a slight contradiction, intercourse between same-sex individuals was not necessarily considered a homosexual act, but rather as a rite of passage for young boys, and for them to have an opportunity to be mentored by an older individual. Obviously, ancient Greeks engaged in what today would be considered homosexual behavior, but during that time, sexual orientation was not used as a social identity as it is today (Wikipedia 2023).

Ancient Romans adopted similar ideals towards same-sex behavior as the societies before them did. The patriarchal society of Rome established that freeborn males possessed political liberty and held the right to rule over themselves and their households. Similar to the Greeks, Roman men were able to engage in sexual activities with other men as long as they did not take the passive role in penetrative sex. Acceptable sexual partners included former or current slaves or prostitutes. To the Romans, gender was not the factor that determined whether or not a sexual act was appropriate, but rather any interaction was admissible so long as the man taking part in the act did not infringe on another man's integrity. This also comes across as a contradiction. Careless exercise of this societal allowance would demonstrate a lack of the ability to adequately rule over himself and his family, as well as lower his identity as a cultured individual in society (Wikipedia 2023). With the consequence of lowering social status, more negative attitudes towards homosexual behavior were established.

Jumping ahead many years to the 1900s, homophobia as a named concept is characterized. Today, homophobia stems from sexism and male dominance, which are not necessarily related to sexual orientation, but they have observably become associated with sexual orientation. Heterosexism, the idea that heterosexuality is the accepted norm, developed closely with homophobia, providing an institutional ideology that creates a hierarchy of sexual identities. This institutional development brings two different problems to the table: 1. Heterosexuality is established as normal, and individuals are assumed to be heterosexual and 2. Individuals who deviate from this set point are considered diseased or exhibiting unnatural characteristics. Both issues fuel the two previously mentioned characteristics that homosexuality supposedly contests: male dominance and male superiority over women. Sexism and dominance over women are two phenomena that primarily come from the influence of men, meaning that one of the strongest displays of homophobia comes from ideas relating to masculinity. This train of thought is what leads to the societal and clinical, in some cases, classification of homosexuality as a disease rather than a phenotypical variation in humans. The establishment of heterosexism and heteronormativity serves as the cornerstone of modern-day homophobia and discrimination against sexual minorities (Fraïsse, C. and Barrientos, J. 2016).

Moving on from the psychosocial context of homophobia, the scientific lens of homophobia has grown in interest in the past 40 years. It is challenging to find appropriate measures for variables to measure attributes of sexual minorities, and this struggle is evident in the current research regarding sexual minority health and well-being. A systematic review published in the Journal of Sex Research in 2016 by Jeremy Grey and collaborators explores the different methods employed to measure attitudes towards homosexual men. The turning point for the view of homosexuality was in 1973, when it was no longer considered a mental disorder in the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders*. With the removal of clinically backed, institutional homophobia, hostile attitudes shifted to causing distress in a gay person's life via negative influences towards their physical and mental wellbeing, rather than the argument that they have a mental disorder. This review identified 23 different methods to measure homophobia in research studying sexual minorities (Fraïsse, C. and Barrientos, J. 2016). The most common indices used were iterations of a Likert scale, and had measurements from one to five, often with one representing less homophobic attitudes and with five representing more. They summarized the compiled results of these studies, which included measurements for both homophobia and internalized homophobia; however, this section of the paper will focus on general homophobia rather than internalized homophobia.

The first result discussed was homophobia related to face-to-face interactions with gay men. It was found that heterosexual individuals who had less social or professional contact with gay men scored higher on the measurements, representing more attitudes of homophobia. Conversely, heterosexual individuals who had more social or professional contact with gay men scored lower, meaning they held less homophobic attitudes (Grey, J. et al. 2013). It was also found that individuals, heterosexual and homosexual, with negative attitudes about AIDS were more likely to hold negative homophobic attitudes. Holding conservative religious or political beliefs was also positively correlated with increasing homophobic attitudes. Lastly, individuals who already held negative attitudes towards any other minority group (e.g., Muslims, overweight people) were more likely to also score higher on homophobia measurements (Grey, J. et al. 2013).

Most of the results discussed were related to personal biases and opinions, not necessarily backed by science or evidence. There is no way to categorize individual biases as right or wrong without including personal bias, but the effect these attitudes have on the targeted minorities is what research on sexual minorities looks for. Many of these biases take influence from the idea that sexual orientation is an active choice individuals make, rather than a result of many environmental and genetic factors. The discourse of the acceptance of sexual minorities in the context of politics and religion is beyond the scope of this paper, but they are factors in the development of homophobic attitudes, nonetheless.

Overall, the development of discrimination against sexual minorities originates from the heteronormative structure of sexual orientation in society. A lack of research exploring the biological origins of human sexual orientation leads to misperceptions and differing societal opinions on sexual minorities. A similar area of societal polarization is women's reproductive health. The intersection of the vaginal microbiome and the development of homosexuality in this review takes stereotypes and negative attitudes from both sides. The biological origins of homosexuality and its relation to the vaginal microbiome and reproductive health go hand in hand when aiming to scientifically eradicate both homophobia and other minority discrimination.

An equally important area of historical context to discuss is the acceptance and attitudes towards female reproductive health. The definition of female reproductive health discussed will pertain to individuals who have ovaries, a uterus, and a vagina. The data from the studied time period does not include all individuals with this anatomy, but rather cisgender females. Everyone remembers sex education in middle school health class; students who could not take the class seriously, teachers who were observably uncomfortable discussing this information with the students, and also the individuals who were eager to learn yet too embarrassed to ask in front of their peers. This class likely gave a very broad and shallow introduction to sexual health and wellness, and left out pieces of information that may have been pertinent to sexual minorities. The data that are about to be presented are dated, but the message they deliver is still relevant. In 1999, a nationally representative survey collected data on United States teachers of 7th-12th grade who were also responsible for teaching sexual education curricula. The interesting points of data collection were topics of abstinence, sexual orientation, and birth control. 23% of teachers taught that abstinence was the single most important

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method for preventing pregnancy. Additionally, 41% of teachers expressed that abstinence was the idea they intended on getting across to students the most (Darroch, J., et al. 2000). Shockingly, only 69% of school district superintendents had policies in place mandating sexual education be taught, and within that subset, 35% required abstinence be the only contraceptive method to be taught (Darroch, J., et al. 2000). Granted these data are over 20 years old, they represent the foundation of this current generation of individuals who were not given a comprehensive sexual education class to set them up for their sexual experiences in the future.

Without the basis of this education established in secondary school, individuals can be left with more questions than answers. This missing information plays a role in the spreading of misperceptions regarding sexual and reproductive health and wellness, especially in the case of female sexual health. A study published in 2024 explored the origins of these sexual and reproductive health (SRH) misperceptions via a survey of 1000 Chinese women. They found that stigmatic perceptions positively predicted information avoidance ( $ß= 0.207$, $p<0.001$), and such information avoidance led to the development of misperceptions ($ß=0.195$, $p<0.001$)(Dong, Y. et al. 2024). The same level of significance was found in similar relations between misinformation exposure and resulting development, and also information overload and its positive relation to both information avoidance and misperception development. This represents that with stigma surrounding a topic, people are more likely to avoid searching for correct information. Continuing, avoiding the truth will lead to the establishment of misperceptions. Misperceptions present risks to the sexual health of young people when they assume truth in rumors they accept as fact. A study published in 2016 followed questions submitted to a health Q&A, text-based platform employed in Nigeria. These data are by no means representative of the adolescent population of the world, but they provide a window into what sexual education could look like with the removal of stigma and misperceptions. The majority of the queries from adolescents in this population were related to HIV and pregnancy. Frequent keywords in questions related to HIV were 'AIDS', 'contract', and 'infect' (Blanc, A.K, et al. 2016). In the questions concerning pregnancy, words like 'sex', 'menstruation', and 'prevent' were common (Blanc, A.K. et al. 2016). To put these searches into context, approximately one in three 18-year-old women in Nigeria has at least one child or is currently pregnant; however, Nigeria is one of the few developing countries with a nationwide sexual health and education program (Blanc, A.K. et al. 2016). In both the US and Nigeria, it would be expected that mandated sexual education would lead to fewer misperceptions and knowledge gaps regarding sexual health, but that is evidently not the case. These attitudes translate into misinformation regarding the vaginal microbiome. Consequences of this include individuals purchasing cleansing products that are harmful to the vagina or leading them to think that the normal attributes of the vagina, like smell or discharge, are not normal. Research directed towards the vaginal microbiome and maternal immune system would improve the sexual education and knowledge of the coming generations of young people. The most effective way to combat misinformation is to establish more clarifying research.

## 4. Methodology

### *4.1. Tracking Birth Sex*

In mammals, the fate of biological sex relies on the X and Y chromosomes in male and female gametes during fertilization. The sexual phenotype of the Y chromosome is controlled by the presence of the SRY gene. Preimplantation "sexing" of embryos is thus difficult to visualize. Green Fluorescent Protein (GFP) is a common method to tag transcription proteins to visualize expression levels through light activation. Regular mice expressing X and Y chromosomes face difficulties due to X-inactivation or Y-chromosome loss when crossed with wild-type (WT) female mice (Hirata, W., et al., 2022). We plan to conventionally microinject fertilized mouse oocytes with GFP prior to implantation to develop a transgenic mouse line with Y-chromosome-linked GFP tagging (Yamamoto, S., et al., 2014). Fluorescent embryonic cells that show the presence of the SRY gene (and thus the Y-linked NLGN4Y protein) will be collected and implanted into female wildtype mice. Pregnancy with male

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birth sex will be induced this way and repeated 3-4 times. Tagging the Y chromosome will help to track male offspring and induce all male offspring to mimic the fraternal birth order effect.

### *4.2. Measurement Techniques*

To quantify NLGN4Y antibody protein plasma concentrations, we will perform ELISA assays, which are commercially available in kits. Samples of maternal blood will be collected during each successive pregnancy. Blood samples will be tested using ELISA assays to determine NLGN4Y antibody blood contents, which will be compared between successive male births. As done by Bogaert and colleagues, 96-well ELISA microplates were coated with recombinant proteins of NLGN4Y isoforms (Bogaert A.F., et al., 2017). Plasma from each participant (and for our study, participants would be the mouse mothers and offspring) was titrated and detected with a secondary anti-human IgG antibody labeled with peroxidase. Then, another substrate, called chromogen, was added to reveal the immune reaction and thus quantify the NLGN4Y protein, as measured using a Biotek microplate reader. Standard curves were generated for each ELISA plate to provide a normalized comparison. Cohen's D, one-way ANOVA, and T-tests for statistical analysis can be used to determine the statistical significance of our findings.

### *4.3. Behavioral Studies*

While the FBO effect has been shown in humans by Bogaert A.F. and colleagues in 2017, the addition of animal model behavioral studies will help to validate these findings and further aid in the understanding of sexual orientation. Previous studies have assessed mounting behaviors to measure same-sex partner preference in male sheep. This particular study was able to determine that kisspeptin-GnRH signaling plays a critical role in regulating stable fetal testosterone levels that are necessary for the masculinization of behavior and the brain (Roselli C.E., 2020). Other studies have shown same-sex sexual behavior (SSSB) in primates in response to pheromone smell. Similar studies in mice were completed, where disruption of the transient receptor potential cation channel 2 (TRPC2) gene, crucial for the transduction of chemosensory signals from pheromones, led to increased likelihood of SSSB (Pfau, D., et al., 2021). Based on these previous studies, we plan to assess SSSB as a measurement for homosexuality in male mouse offspring that we control for using GFP tagging as described above. We will define SSSB as same-sex mounting. We expect that if the FBO effect is consistent in these mice, then the mice that display the most SSSB will have more older brothers on average and correspond to higher levels of anti-NLGN4Y in the mother's serum. These animal behavioral studies could also help elucidate an evolutionary explanation for SSSB in humans.

## 5. Expected Contributions

We aim to explore the relationship between the vaginal microbiome, the maternal immune system, and the development of homosexuality in males. Anticipated contributions and application of the information that could be obtained through the study of these topics have been elaborated on throughout this review, but they will again be summarized here.

Current knowledge concerning the vaginal microbiome is limited yet increasing in depth and understanding. There are still unanswered questions regarding how changes in the vaginal microbiome can further affect female reproductive health beyond bacterial infections and similar transient complications. Research suggests that changes in the vaginal microbiota can be felt both in the maternal immune system during pregnancy and in the colonizing microbiota in an infant. Additionally, research shows that reactions of the maternal immune system to Y chromosome components may play a further role in the brain development of fetuses, especially in the case of subsequent male births following previous male pregnancies. There are some accepted mechanisms on how these systems deal with these situations independently, but the interplay between them is still uncertain.

The scientific benefits of a more thorough understanding of these topics extend beyond maternal and female reproductive health. Advancements made in this area of study would benefit current understandings of microbiology, physiology, immunology, and developmental psychology, as well as sociological and psychological attitudes surrounding reproductive health and the societal security of sexual minorities. Health policy, current and future healthcare practice, prenatal care, and healthcare of members of the LGBTQIA2+ community are also areas that this research could translate into. This analysis intends to serve as a small starting point for further research to expand upon. The vaginal microbiome, maternal immune system, and fraternal birth order effect, and their interactions, are a crucial starting point for the development of a greater understanding of reproductive and sexual minority health.

## 6. Conclusions

### *6.1. Biological Basis of Sexual Orientation*

By providing a biological basis of sexual orientation, we hope that our study will help to provide a step forward in advancing tolerance and acceptance for the gay community in society. We hope that eventually, studies similar to this one can eradicate false perceptions of the cause for sexual orientation, as well as deepen our knowledge about these biological phenomena. As further studies shed more light on how sexual orientation is not a choice, we hope to contribute to the potential abolishment of social stigma and discrimination against homosexuals, as well as challenge harmful beliefs and misconceptions of sexual orientation. A more thorough scientific understanding of sexual orientation is also essential for fostering an inclusive educational curriculum. We hope that understanding the biological basis of sexual orientation can also contribute to society recognizing sexual orientation as a fundamental aspect of human diversity, which is crucial for the advancement of human rights policies and inclusive attitudes for promoting equal treatment. Reinforcing these ideas will put our society one step closer to developing laws and policies that value the rights of individuals regardless of their sexual orientation and address issues such as hate crimes, discrimination, and access to health care.

### *6.2. Study Limitations and Future Research*

While these studies provide further background and insight into exploring the realm of sexual orientations as biologically derived rather than voluntary choice, we must acknowledge some limitations. Firstly, the FBO effect only applies to 5% of homosexual men. Although this is low, FBO is still an important piece to the many possible biological causes for sexual orientation, and other studies have elucidated some other biological factors, such as genetics or hormones (Breedlove S. M., & Arnold, A. P., 1980). Future studies could examine why this 5% of homosexual men could be more susceptible to the FBO effect by investigating how different cultures, ethnicities, or socioeconomic factors could interact with or influence the FBO effect.

Secondly, although our study involves using controlled female dams and offspring sex controlled by GFP visualization, our study is not well controlled for environmental factors during gestation that could potentially alter the outcome of the offspring. For instance, environmental factors such as external stress could be detrimental to both the maternal microbiome and offspring development. To combat this, we plan to ensure that dams are in low-stress environments that are consistent with each other. We hope this will help limit confounding factors that could possibly interfere with successive births.

Thirdly, the female vaginal microbiome among women is highly variable, despite the broad classification of the various Community State Types discussed earlier. Furthermore, little research has been done to assess more diverse populations of women, especially women of minority ethnicities and women in postpartum periods (MacIntyre, D.A., et al. 2015). To combat these limitations, we propose to compile data from previous studies measuring microbiome composition

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to hopefully provide a more diverse repository of Community State Types from a greater sample size of women of different ethnicities.

Another future direction could involve studying the sexual orientation of female offspring based on their exposure to the vaginal microbiome during birth. Female sexual orientation has been shown to be more flexible than male sexuality. A study has shown that compared to heterosexual men and gay men, lesbian and heterosexual women displayed greater self-reported changes in the dimensions of sexual orientation (Kinnish, K., et al. 2005). This adds a deeper level of knowledge that can be explored when investigating determinants of female sexuality, as it seems to be more complicated than male sexuality. As discussed before, improving knowledge of the biological bases of greater LGBTQ2IA+ community populations will help improve societal misconceptions and promote inclusivity.

### *6.3. Final Thoughts and Summary*

The vaginal microbiome, maternal immune system, and the fraternal birth order effect are very complex and seemingly distinct concepts. However, there is likely an uncharacterized relationship between the three ideas that has not yet been fully illuminated. The maternal immune system and the fraternal birth order effect have already been shown to be related to one another in the literature, but the true extent to how widespread the relationship is, has yet to be determined. The vaginal microbiome and the maternal immune system also work in unison in female reproductive health, and have also been shown to have effects on neonatal and infant gut microbiota and brain development. The factors that unite this physiologic triad are yet to be fully elucidated.

In this review paper, we summarize how a decrease in lactobacillus species during pregnancy can lead to increased Treg cell activity and immune function. Decreased lactobacillus is also seen in women postpartum and is attributed to increased sensitivity of the placental barrier, making it easier for maternal antibodies such as anti-NLGN4Y to cross the fetal blood-brain barrier and lead to increased likelihood of homosexuality in successive male offspring (**Table 1**). Through GFP tagging for visualization of SRY in embryos, quantification of anti-NLGN4Y proteins through ELISA assays, and behavioral studies of SSSB in mice, we will bridge the missing gap between the vaginal microbiome, maternal immune system, and the fraternal birth order effect.

Access to female reproductive healthcare, including but not limited to contraception, prenatal care, and gynecological care, is a crucial part of the healthcare system. It has roles in family planning and can help encourage healthy families and informed decisions. Public health has a large burden on the healthcare system, and any advancements in this field are beneficial to humans as a whole. The effects of female reproductive health can even be felt in the economic sphere of the world. Comprehensive reproductive healthcare to those who benefit allows them to become healthy and prosperous individuals in society.

Diversity is what makes humans stand out as a species. Homosexuality is a contributing factor to the generation of that diversity. Research that continues to further define homosexuality as a naturally occurring variation within the population will foster acceptance and inclusivity. Along the same lines, the development of information regarding the origins of homosexuality strengthens human rights and gives more security to sexual minorities. The same can be said about female reproductive health. In addition to the literature previously outlined, defined factors that influence homosexuality will help decrease negative social attitudes around the LGBTQIA2+ community. Lastly, the mental health of the greater queer community could be greatly improved by advancements in both diversity and human rights.

The three outlined topics this review explores are all part of a greater conversation about human rights, diversity, and inclusivity. Further research into the vaginal microbiome, maternal immune system, and the fraternal birth order effect will help inform public opinion, education, and healthcare in order to reshape our world into a more informed and empathetic society.

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