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Investigating the Microbiome of the Eye and the Potential of Probiotic Use in Optometry

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Honors Thesis

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Abstract

While the eye was once considered free of resident bacteria due to its efficient immunity mechanisms, recent studies have determined that most healthy eyes contain a variety of microbiota. Many studies have been aimed at classifying bacteria that are part of the core microbiome of the eye and the conditions under which they differ. As with gut health, a dysbiosis of ocular bacteria could correlate to disease, which presents the idea of treatment with probiotics to help regulate the microbiota of the eye. This study utilized growth assays to determine a common probiotic's effect on bacteria that can be found on the ocular surface. Also, it used a survey to investigate current ocular experts' outlook toward the use of probiotics in optometry. Results have showed a mixed perspective, but with a commonality of the desire to cut back antibiotic use and an optimism toward probiotic treatment in the next 10 years. While the growth assay technique continues to be refined, it has supported the growth inhibition of *E. coli* due to the presence of *L. acidophilus*. Additional research should focus efforts on dry eye disease.

Keywords: probiotic, *Lactobacillus acidophilus*, microbiota, microbiome, eye, dry eye disease, ocular health

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This experiment is two-fold. The first part utilizes a growth assay to provide additional evidence of probiotic interaction while the second administers surveys to gauge current stances toward probiotic use among experts.

Growth Assays

The surveillance of bacterial growth has been used in a myriad of studies over many years, and the technique of doing so continues to evolve. The growth assays performed in this experiment utilize newer high-throughput techniques. This experiment's goal was to observe the effect of a probiotic on two strains of bacteria that have been associated with ocular disease. Certain strains of *Escherichia colitis* (*E. coli*) have been found to cause eye diseases such as conjunctivitis (Nunes et al., 2022). Additionally, *Bacillus subtilis* (*B. subtilis*) has been one bacterium associated with dry eye disease (Narayanan et al., 2013; Willis et al., 2020). Because of the limits of a biosafety level 1 lab, an *in vitro* experiment was performed to observe the *in vitro* effects of probiotics on potentially pathogenic bacteria. Previously, commensal bacteria have been found to have *in vitro* effects on various pathogenic bacteria, including *E. coli* (Gao et al., 2022; Fooks & Gibson, 2002). Additionally, *in vivo* studies have uncovered some interaction between commensal bacteria and the activation of immune responses such as the release of antimicrobial products into the tears (Leger et al., 2017). Prior studies have also determined that micro assays with fluorescent readings have been a high throughput and quantifiable method of measuring bacterial growth (Kurokawa & Ying, 2017; Ross et al., 2022). Thus, this study utilized growth assays in 96-well plates and fluorescent tagging to quantify the effect of *L. acidophilus* on the two bacteria, *E. coli* and *B. subtilis*. Because *S. epidermidis* is another bacterium found as a commensal bacterium on the eye, this study also aimed examine its growth

to observe any selectivity in growth inhibition, as it would be relevant to the eye (Willcox, 2013).

Survey

Surveys have long been used to obtain a consensus among a particular group or population. Additionally, the use of technology and free online survey-making services have made surveys easier and more efficient than ever. This survey utilizes the Qualtrics^{XM} online survey generator and targets experts in the optometric field, including optometrists and ophthalmologists. The aim of this survey is to gauge optometrist's interest in probiotics by gathering input from doctors around the Omaha area, focusing on their exposure to and view of probiotics as a potential treatment in various forms. It is clear that probiotics have found a place in gut health (Wang et al., 2021). Moreover, this treatment is growing in popularity in areas outside of the gut, such as the integumentary system (Guan et al., 2022). Many studies have made connections directly from a probiotic eye-drop and ocular health (Iovieno et al., 2008). Others have connected ocular health to probiotics via the gut-eye axis (Floyd & Grant, 2020). While ocular-focused probiotics seem to be growing in popularity in the laboratory setting, there is a need to collect data on the clinical practitioner's view. If clinical experts have a knowledge and openness toward probiotic use in optometry, the outlook for such use can be considered promising, and research efforts conducted in the lab and clinic should proceed.

Literature Review

Probiotics' Necessity and Function

Probiotics are becoming an established form of treatment and preventative health care, most commonly for gut health, but with expanding horizons (Day et al., 2019). One reason for this is the observed negative impacts of antibiotics. The hesitation to use antibiotics grows as negative impacts have been discovered. Yet, they continue to be over-prescribed by practitioners

in the field of optometry. Shekhawat et al. analyzed the occurrence of topical antibiotic prescriptions and found that while antibiotics are rarely crucial to treat various diseases, such as conjunctivitis, they are often prescribed (Shekhawat et al., 2017). It is common knowledge that antibiotic overuse can lead to antibiotic resistant strains of bacteria, but this is not the only risk. It can also have effects by altering the microbiome of the body. This can be seen in the phenomenon of antibiotic associated diarrhea, which has been treated for by the use of probiotics, in an effort to restore the commensal bacteria (“Probiotics”, 2023).

Evidence supporting the general positive impact of probiotics comes from many studies. For example, Fooks and Gibson used disk growth assays to study the effect of two probiotics, *Lactobacillus plantarum* and *Bifidobacterium bifidum*, on several pathogenic bacteria, including *E. coli*. They also used these probiotics along with prebiotics, including fructo-oligosaccharides (FOS), xylo-oligosaccharide (XOS), and inulin. They found that the prebiotic FOS was the most useful and was used selectively by the probiotic, promoting its growth and consequently inhibiting the growth of the pathogenic bacteria. According to this study, one of the main mechanisms through which this occurs, apart from simple competition, is the products the probiotics produce, acetate and lactate (Fooks & Gibson, 2002). Another study proposes that the lactic acid produced by probiotics such as *L. acidophilus* can lower the pH, which inhibits the pathogenic bacterial growth (Gao et al., 2022). The interaction of probiotics and the immune system is another possibility. Wang and company found a strong immune component at play when they noticed gut microbiota produced products such as short-chain fatty acids that create a stronger mucosal barrier and reduce the amount of inflammation (Wang et al., 2021). Both of these effects could potentially have an impact on immunity and general health in areas beyond the gut and may provide relevance to the ocular system. Studies have even begun testing genetically engineered bacteria in order to treat pathogenic bacteria. Guan and company found

that genetically engineered *S. epidermidis* can limit the growth of *S. aureus* (Guan et al., 2022). This could lead to safer and more precise treatment in the future.

The Microbiome of the Eye

In the past, the idea that the eye could harbor a stable variety of bacteria was controversial at best. The eyes are immune privileged so that the local immune response and inflammation is limited, ensuring unobstructed vision on a day-to-day basis (Zhou & Caspi, 2020). A major part of this immune privilege is the physical barriers the eye has in order to prevent any lingering potentially pathogenic microbes. One of the greatest barriers to the external environment is the tear film, which not only washes away the pathogens through tear production and blinking, but also contains antimicrobial compounds that inhibit bacterial growth (McDermott, 2013). Because of this effective barrier, it was thought that the surface of the eye was unfit for hosting a microbiome. Additionally, traditional techniques in identifying bacteria in the body tend to consist of cultivating a growth culture using a swab from tissue, either from the conjunctiva, cornea, or eyelids. These methods yielded some varying results. Coagulase-negative staphylococci were the most commonly found bacteria from the ocular surface, but even so it has about a 50% frequency in conjunctival swabs (Willcox, 2013). The proposed reason for such inconsistency is that some types of bacteria are difficult to grow in lab conditions. Various incubation conditions may have also led to discrepancies across studies. Still, there were some overarching patterns. For one, the gram-positive bacteria were more commonly found than gram-negative. Furthermore, the most common bacteria type was negative-coagulase staphylococci (including *Staphylococcus epidermidis*), which was followed by *Propionibacterium sp.* and *Corynebacterium sp.*

A more recent study by Li and company criticizes the reliability of culture cultivation as many of the real bacteria found in the microbiome may not be easily cultivable. They propose

newer molecular techniques such as immunoassays, which identify peptides and antigens using antibodies (Li et al., 2020). Additionally, metagenomic sequencing, which identifies DNA or RNA, is often now used to identify bacteria more reliably and efficiently. For this, 16S rRNA is often used for identification. These newer methods do not rely on growing colonies on a plate, isolating the colonies and running pure colonies through a variety of tests, which can lead to biased and inconsistent results. With these DNA/RNA sequencing techniques, several studies were able to contribute more data to the proposed microbiome. Graham and company found that the molecular methods identified more bacteria than the prior culture growth methods. The common “core” bacteria genera were expanded from those found in culture methods and included *Staphylococcus*, *Rhodococcus*, *Corynebacterium*, *Propionibacterium*, *Klebsiella*, *Bacillus*, and *Erwinia* (Graham et al., 2007). Dong and company expanded it even more to include *Pseudomonas*, *Bradyrhizobium*, *Acinetobacter*, *Brevundimonas*, *Aquabacterium*, *Sphingomonas*, *Streptococcus*, *Streptophyta*, and *Methylobacterium* (Dong et al., 2011). Other studies supported evidence for a variety of these, with the genera appearing repeatedly being *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, *Pseudomonas*, and *Acinetobacteria* (Doan et al., 2016; Ozkan et al., 2017; Li et al., 2020; Huang et al., 2016; Schabereiter-Gurtner et al., 2001).

While bacteria have been found on the majority of subjects’ ocular surface tissue, it was uncertain whether it was there transiently or stably. Leger and company performed an experiment in which they grew *C. mast* on the surface of the eye with no need for reinfection, providing evidence that the eye can host commensal bacteria for a long duration of time. They also found that this bacterium was passed down from mother to offspring in mice (Leger et al., 2017). This discovery is of particular importance as the eye has the potential to support a probiotic long enough for it to have any beneficial impact. Additionally, Graham’s 3-month study showed healthy patients to have relatively stable species of bacteria (Graham et al., 2007).

Lastly, while the core microbiome for healthy individuals is debated, Ozkan and company's data supports that an individualized core microbiome may be possible (2017). This would explain inconsistencies in sample-wide data while the individual microbiome stays considerably stable without outside forces acting upon it.

Evidence of bacteria existing on the eye would be given further significance if the bacterium types show correlations for different diseases or conditions. Thus, many studies' next move was to find some of the conditions under which the microbiota makeup is different. Some of these differences have been related to non-disease factors such as age, ocular area, proximity to sleep, and contact lens wear. Studies have found that young adults had a greater diversity in their ocular microbiome than the elderly subjects, specifically in the meibum and the conjunctiva (Suzuki et al., 2020; Zou et al., 2014). Additionally, Ramachadran was able to show a statistically significant increase in gram-positive bacterial growth immediately after the eyes have been closed for 8 hours of sleep as opposed to during the day (Ramachadran et al., 1995). Another study compared the location of the eye in which swabs were being taken to compare the microbiota makeup between the conjunctiva and the cornea. Using RNA sequencing data, they discovered that *Proteobacteria* was the main phyla in the corneal microbiota while *Firmicutes* were the dominant phyla in the conjunctival microbiota. In fact, the top two corneal genera, *Paenibacillus* and *Lactobacillus*, were not found at all in the conjunctiva (Matysiak et al., 2021). Unsurprisingly, contact lenses have been found to alter the ocular surface microbiota, providing a better surface and environment for opportunistic bacteria (McDermott, 2013). Hence, this alteration is often for the worse. Stapleton found that (hydroxyethyl)methacrylate (HEMA) contact lens wearers had greater chances of hosting harmful gram-negative microbes. This was especially true for extended-use wearers, but even daily-use contacts were linked with an increase in coagulase-negative staphylococci (Stapleton et al., 1995). Another study found that

contact lens use has been linked with infections from *P. aeruginosa*, *acanthamoeba*, and *F. solani* (McDermott, 2013). The reason for such differences in microbiota and an increased disease risk is multifactorial, but the contact lens changing the natural microbiota of the eye may be at play.

Along with these factors, some direct associations have been found between microbiota and ocular disease. The most prevalent of which relates to dry eye disease. In a high throughput sequencing of rRNA, Li and company found that the ocular surface microbiota differed between dry eye patients and non-dry eye patients (Li et al., 2019). They discovered that *Bacteroidia* and *Bacteroidetes* were more abundant in dry eye patients while the *Pseudomonas* population was greater in healthy controls. Additionally, they analyzed any microbiota difference in meibomian gland dysfunction, which is a common cause of dry eye disease. While there was not a major difference found here, they did notice that *Bacilli* was the dominant microbe for patients with meibomian gland disorder. Another study by Willis and company also associated bacillus as one type of bacteria linked with dry eye disease, among other unique microbiota (Willis et al., 2020). Graham et al. further supports the *Bacillus* association as they identified 3 bacteria found only in the dry eye samples: *Bacillus sp.*, *Propionibacterium acnes*, and *K. oxytoca* (Graham et al., 2007). Apart from dry eye disease, other studies correlated additional diseases with a unique microbiota makeup (Petrillo et al., 2020). For example, Lee and company discovered that patients with blepharitis had a greater amount of *Streptophyta*, *Corynebacterium*, and *Enhydrobacter* than healthy patients (Lee et al., 2012). Additionally, a loss of microbiota diversity existed for patients with trachomatous disease (Zhou et al. 2014). The determination of a pattern of change in microbiota for a given disease supports that probiotics could help in the prevention or treatment of several ocular diseases.

Eyes and probiotics

Probiotics are widely gaining recognition for their benefits to the body, and the popularity of their use only grows. Various studies have been performed to analyze the effect of probiotics on the eyes, either directly or indirectly through the gut-eye axis. The mechanisms by which probiotics affect the eye are multifaceted and still not well understood. However, a few propositions have been made and supported. Willcox proposed that similar to gut health probiotics, ocular probiotics could stimulate an immune response that produces antimicrobial tears as a preventative measure (Willcox, 2013). Leger and company contributed toward this idea as they experimented with the commensal bacteria *Corynebacterium mastitidis* (*C. mast*) and its immune effect in mice. Their findings indicated that this bacterium triggered the immune response producing interleukin 17. As a result, the mice had more neutrophils present in their conjunctiva. *C. mast* also led to an increase in antimicrobials in the tears. Both of these outcomes seemed to offer protection against pathogens, including *Candida albicans* and *Pseudomonas aeruginosa*. (Leger et al., 2017). Using a reversed technique, Kugadas et al. removed microbiota completely from the eyes of mice and found that doing so increased mice's susceptibility to keratitis as opposed to mice with normal microbiota. They attributed microbiota's protective activity to increasing innate immunity molecules such as secretory Immunoglobulin A and complement proteins (Kugadas et al., 2016). Iovieno and company used the probiotic *L. acidophilus* as an eye drop to treat vernal keratoconjunctivitis, with some success (Iovieno et al., 2008; Iovieno et al., 2006). They propose an anti-inflammatory mechanism is responsible for this effect.

The mechanism of probiotic effect could even originate from beyond the isolated visual system. The gut-eye axis is a studied area where gut dysbiosis may increase the risk or effects of multiple ocular diseases. One review identified various ocular diseases with a correlation to an

unhealthy gut, including diabetic retinopathy, age-related macular degeneration, choroidal neovascularization, and uveitis. (Floyd & Grant, 2020). A study on inflammatory bowel disease's effects that occur outside the intestines revealed a frequency of ocular damage such as inflammation (Thomas & Lin, 2016). Both Sjögren's syndrome and dry eye severity have been associated with gut dysbiosis as well (Moon). With a correlation being found, the proposed solution involves oral administration of probiotics and prebiotics to restore a healthy gut microbiota (Napolitano et al., 2021). Supporting this idea, a few studies have used oral probiotics to improve ocular health. Such use in mice has helped to maintain the density of goblet cells as well as improves the function of the corneal barrier, both effects having a positive impact on ocular health and dry eye disease (Schafer et al., 2023). Additionally, the effect of oral probiotics on human patients with dry eye disease has resulted in some improvement in their symptoms after 4 months of treatment (Tavakoli et al., 2022).

Materials and Methods

Growth assays

Materials

- *Lactobacillus acidophilus* Obtained from American Type Culture Collection (ATCC) in Manassas, VA
- *Escherichia colitis* Obtained from ATCC
- 50mM CaCl₂ solution
- Disposable spreader
- NanoDrop 2000c Spectrophotometer
- Pipettes P200, P20, etc.
- Multi-channel pipette
- Multi-channel pipette troughs

- Sterile pipette tips
- Waste Container for tips
- Ice bucket
- pGlo DNA plasmid in solution
- LB broth Media (600 μ l)
- 200 proof ethanol
- LB agar plate
- 96 well plate
- Synergy LX multimode reader
- Parafilm
- Incubator at 37°C

Methods

In order to observe the effect of the probiotic, *L. acidophilus* on the bacteria *E. coli*, *B. subtilis*, and *S. epidermidis*, separate growth assays were created and observed at various time increments. Each of these are considered facultative anaerobes and grow best in similar temperatures, with 37°C fitting well into each range (Errington & Aart, 2020; Son & Taylor, 2021; Klaenhammer & Russel, 1999; Supragingival Microbes, 2015). The first bacteria tested was *E. coli*. Because 2 different bacteria would be present in each well, the *E. coli* was transformed with a pGLO DNA plasmid beforehand. This was done by first incubating them with CaCl₂ on ice to make them competent. The bacterial solution was then treated with heat in order to take up the introduced plasmids and incubated on ice again to promote membrane recovery. Lastly, they were transferred to agar plates and allowed several nights to grow. Once growth appeared on the agar plate, the fluorescent bacteria were transferred into approximately 20 mL of liquid nutrient broth and grown in an incubator overnight at 37°C. The probiotic was

also grown overnight in the same conditions. The next day, the initial concentrations of each bacterium were estimated using a NanoDrop 2000c Spectrophotometer. 2 μL of each bacterium were individually placed into the spectrophotometer. Using the measured absorbencies, a dilution was made so that the absorbance would be about equivalent, resulting in a 20mL stock solution of each bacterium. Using the dilution stock solutions, a 96-well plate was prepared so that each cell was filled with a 200 μL volume. A multi-channel pipette was used to transfer the directed volumes of both bacteria as well as additional nutrient broth. 4 different controls were also created to serve different purposes. The no-bacteria control in column 1 served as a comparison for absorbency. The column 2 control with only the probiotic would add insight into whether there was any potential fluorescence given off by the *L. acidophilus*. Column 11 served as a negative control, assuming that the ethanol would prevent any bacterial growth so the *E. coli* would have a baseline fluorescence to normalize fluorescence values. Lastly, column 12 served as a positive control as it had unhindered *E. coli* growth. The solutions were prepared as laid out in Table 1.

Table 1*The Preparation of Various Mixtures for the 12 Columns of the 96-well Plate.*

Column	Probiotic to Bacteria ratio	<i>E. coli</i> (μL)	<i>L. acidophilus</i> (μL)	Nutrient broth (μL)	Ethanol (μL)
1*	N/A	0	0	200	0
2**	1:0	0	10	190	0
3	2:1	10	20	160	0
4	8:5	10	16	164	0
5	6:5	10	12	168	0
6	1:1	10	10	170	0
7	4:5	10	8	172	0
8	3:5	10	6	174	0
9	2:5	10	4	176	0
10	1:5	10	2	178	0
11***	N/A	10	0	180	10
12****	0:1	10	0	190	0
*no bacteria control (negative control)					
**only probiotic control (negative control)					
***no growth control (negative control)					
****only <i>E. coli</i> control (positive control)					

Once the 96-well plate was prepared, a Synergy LX multimode Reader was used to read the absorbance of each well and the fluorescence of each well. This was stored in an excel spreadsheet. Readings of both absorbance and fluorescence were recorded at 0 hours, 2 hours, 4 hours, and 16-20hours (overnight). Between readings, the plate was stored in an incubator at 37°C with a parafilm seal to prevent evaporation. This entire process was intended to be repeated with *S. epidermidis* and *B. subtilis*. However, schedule difficulties and time restrictions resulted in the omission of these two bacteria. Instead, two separate experimental runs were executed with two different *E. coli* populations.

Surveys

To gauge current optometrists' and ophthalmologists' opinions toward probiotic use in the field of optometry, a 12-question survey was created using the online survey generator, Qualtrics^{XM}. This survey utilized multiple choice and short answer questions in order to provide

the most information while still remaining efficient for the participants. Additionally, the appearance of several questions depended on the answers to previous questions. For example, if the participant selected that they hadn't done any prior research regarding this topic, they would not be asked the follow-up question regarding what that research consisted of. The survey was sent electronically via a link to doctors at 10 different eye clinics in Nebraska. The questions created can be found in the Appendix.

Results

Growth Assays

The growth assay was performed twice with green fluorescence-tagged *E. coli* and the probiotic *L. acidophilus* in order to observe the effect *L. acidophilus* has on *E. coli*'s growth.

Trial 1 began on November 8th, 2023. *Trial 2* began on November 28th, 2023.

Trial 1

Trial 1 readings were taken at 0 hours, 2 hours, 4 hours, and 20 hours, as planned. The readings of the absorbance level and fluorescence level did not produce the expected results. The absorbance mostly increased as would be predicted since it represented the collective growth of both *E. coli* and *L. acidophilus*. Each reading showed an increase in absorbance as time in the incubator accumulated. However, as the incubation time increased, the readings started to show some signs of contamination. Column 1 increased in absorbance despite having been inoculated with no bacteria. Additionally, Column 11 in row D displayed abnormally increased absorbance while it contained ethanol, which should have ceased any bacterial growth (Table 2). It was also noted that some splashing occurred during the initial application of the parafilm, which may explain some contamination. Additionally, the readings were taken with the parafilm removed and the plastic lid placed loosely on top. Some condensation and fogging occurred, which could

have impacted the absorbance reading or caused contamination. This could have also affected the fluorescence readings. The fluorescence level fluctuated without any observable pattern for the first 3 readings. However, the last reading showed more fluorescence in the positive control column with only *E. coli* as seen in Table 3. While this increase for the positive control is expected, the presence of increased fluorescence in the first column without any inoculation questions the accuracy of the readings. Because the data was rather inconsistent, the raw data was shown in a visual depiction of the 96-well plate with conditional formatting (Table 2 and Table 3). This was generated as opposed to a graph in order to avoid the risk of showing biased data for the first run.

Table 2

Raw Absorbance Level of the 96-well Plate. This data is visually depicted with conditional formatting where the greater the value is, the darker the blue hue is. This shows the last reading at 20 hours, when the most growth had occurred.

Absorbance Level of 10 μ L E. coli with Various Amounts of <i>L. acidophilus</i> (LA) at 20 hrs												
	No Bacteria control	No <i>E. coli</i>	20 μ L LA	16 μ L LA	12 μ L LA	10 μ L LA	8 μ L LA	6 μ L LA	4 μ L LA	2 μ L LA	Ethanol	0 μ LA
A	0.584	0.813	0.696	0.773	0.778	0.771	0.792	0.730	0.745	0.716	0.116	0.731
B	0.615	0.789	0.800	0.716	0.723	0.742	0.748	0.724	0.700	0.715	0.115	0.759
C	0.640	0.706	0.669	0.732	0.750	0.795	0.731	0.741	0.713	0.717	0.110	0.716
D	0.555	0.749	0.715	0.715	0.714	0.777	0.763	0.742	0.733	0.732	0.740	0.752
E	0.581	0.728	0.737	0.755	0.747	0.775	0.756	0.742	0.757	0.751	0.112	0.731
F	0.532	0.804	0.761	0.747	0.747	0.744	0.716	0.752	0.720	0.748	0.115	0.728
G	0.643	0.764	0.749	0.764	0.744	0.739	0.726	0.722	0.768	0.751	0.117	0.685
H	0.735	0.752	0.764	0.613	0.836	0.722	0.724	0.709	0.714	0.708	0.119	0.784
Average	0.611	0.763	0.736	0.727	0.755	0.758	0.745	0.733	0.731	0.730	0.193	0.736

Table 3

Raw Fluorescence Level of the 96-well Plate. This data is visually depicted with conditional formatting where the greater the value is, the darker the blue hue is. This shows the last reading at 20 hours, when the most observable difference in fluorescence had occurred.

Fluorescence Level of 10 μ L E. coli with Various Amounts of <i>L. acidophilus</i> (LA) at 20 hrs												
	No Bacteria control	No <i>E. coli</i>	20 μ L LA	16 μ L LA	12 μ L LA	10 μ L LA	8 μ L LA	6 μ L LA	4 μ L LA	2 μ L LA	Ethanol	0 μ LA
A	141	99	100	101	122	126	121	114	126	128	101	155
B	95	87	112	115	120	120	116	110	110	113	90	158
C	104	94	106	118	112	117	120	110	117	116	90	151
D	100	97	118	120	114	120	121	120	126	123	131	158
E	184	100	120	114	117	111	121	119	109	117	106	144
F	117	105	116	118	117	118	119	119	118	105	94	151
G	92	110	127	130	119	124	121	121	118	114	104	165
H	173	127	123	134	76	130	126	130	115	129	112	219
Average	125.75	102.375	115.25	118.75	112.125	120.75	120.625	117.875	117.375	118.125	103.5	162.625

Trial 2

Trial 2 produced results that were more congruent with the expected outcome than trial 1. A different batch of *E. coli* was used and grown on an ampicillin media plate in an effort to add selective pressure and increase the fluorescence expression. Additionally, the plate readings were taken without lids to try to limit any potential noise in the readings created from fog or condensation. Another adjustment made was the time and number of readings. A few more readings were taken, specifically at 0 hours, 2 hours, 11.5 hours, 22.5 hours, 26 hours, and 50 hours (about 2 days). The absorbance readings showed less signs of contamination as the column one remained fairly stable throughout both days, with the exception of one well, which showed signs of contamination at the 11.5 reading. It was thrown out of the calculated average from that point on in order to keep the control at the baseline level. The relative absorbance readings increased as expected (Figure 2). The pattern of fluorescence strength was much more distinct in trial 2 than in trial 1, especially around the 22.5- and 26-hour marks. The amount of *L. acidophilus* appeared to have a linear effect when the line of best fit was graphed for the 22.5-hour reading (Figure 1). At 50 hours, the difference isn't as distinct, which could be due to a limiting capacity. Still, when tracking the fluorescence throughout the entire incubation process at each probiotic amount, there is a consistent gradient with the greater amount of *L. acidophilus* resulting in less *E. coli* growth (Figure 3).

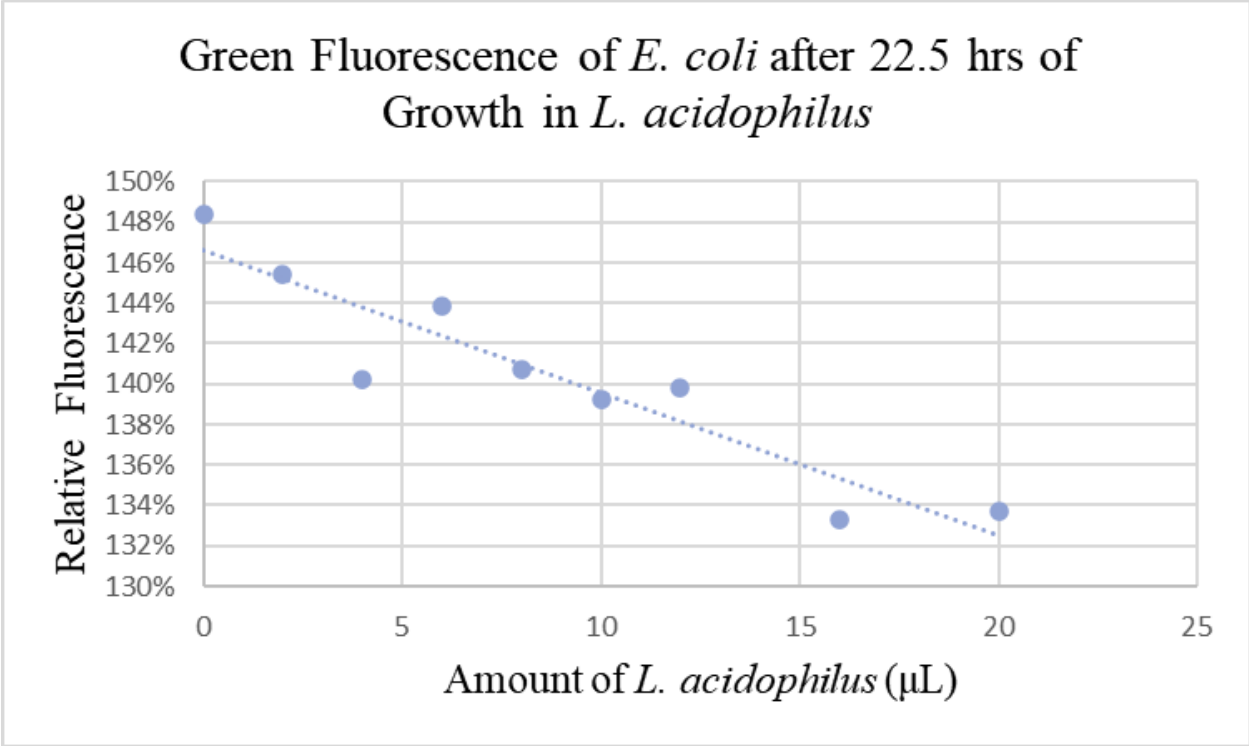


Figure 1

Growth Assay of E. coli as Observed by the Fluorescence Readings. The fluorescent percent values have been normalized to the control well 11, where 10 μL of E. coli was immediately killed by ethanol.

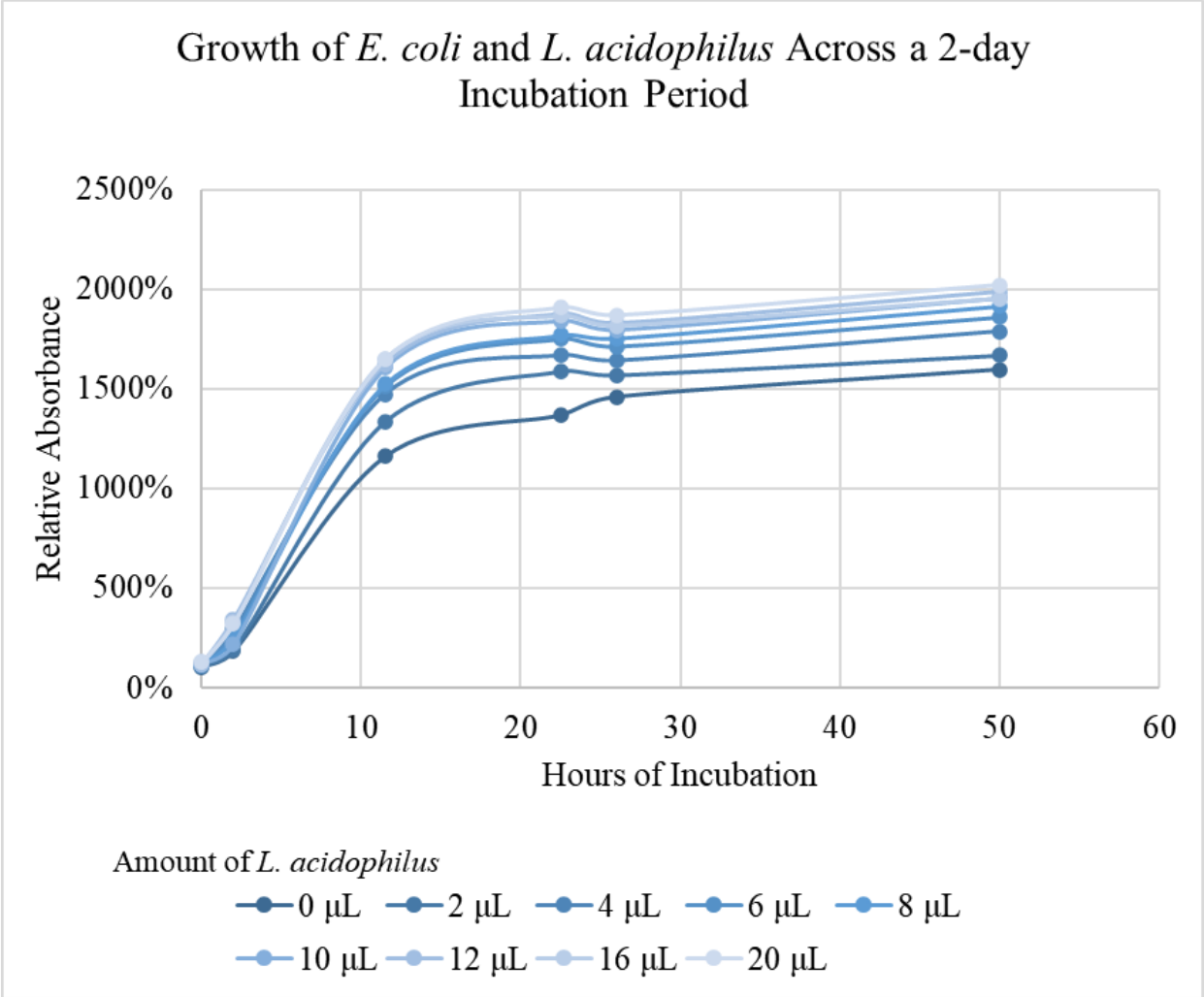


Figure 2
Comparative Growth of Both E. coli and L. acidophilus as Observed by its Relative Fluorescence. Relative absorbance was normalized to Column 1 of the 96-well plate, which contained no bacteria.

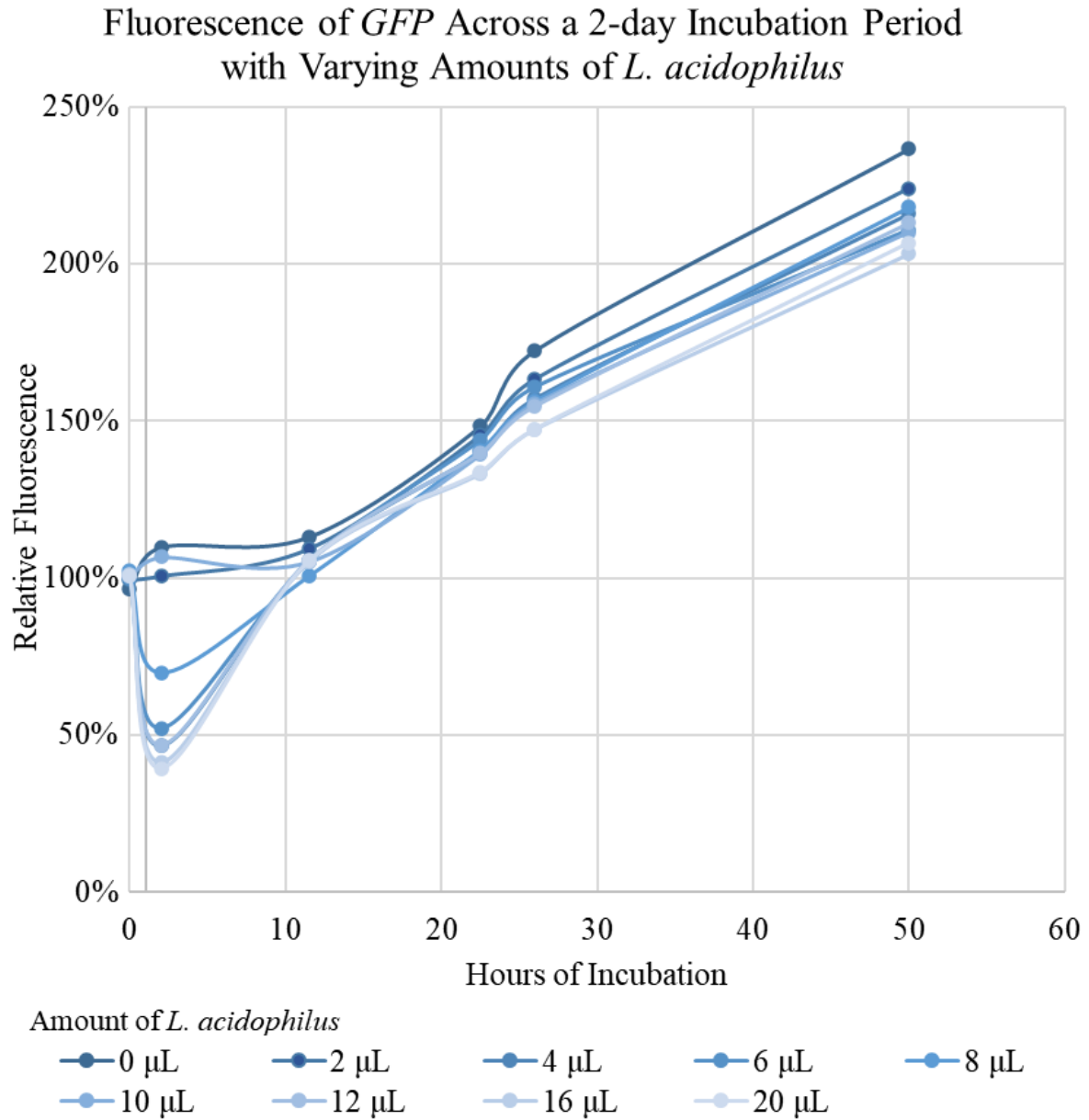


Figure 3

Comparative Growth of E. coli as Observed by its Relative Fluorescence. Relative Fluorescence was normalized to Column 11 of the 96-well plate, which contained the same initial amount of E. coli (10 μL) with 200 proof ethanol.

Survey

The survey was sent out via a link to Qualtrics^{XM} and responses were gathered anonymously. 11 responses were gathered in total. Out of the 11, 6 participants were optometrists, and 5 participants were ophthalmologists. The experience in their field ranged from 1 to 40 years, with the majority having spent 1-5 years practicing. Two questions focused on the current treatment for two common ocular conditions, bacterial conjunctivitis and dry eye disease. When asked about their most used treatments, “antibiotics” was the most common response regarding bacterial conjunctivitis, with a 91% frequency. The only varied response still included antibiotics, but in combination with a steroid. Dry eye disease was most commonly treated with artificial tears at a 55% frequency. Steroids were the second most common at 27%. When asked about their perspective on antibiotic use, 9 out of 11 respondents responded with at least some degree of concern toward the use of them. 2 responses expressed no need to sway from antibiotic use, both of which were given by ophthalmologists. As for prior knowledge of probiotic use in optometry, only 1 respondent has explored the subject, and they reported conducting their own study as well as reading the current literature. Out of the remaining 10, only half had heard of the idea previously. The next portion of questions focused on the prospective outlook on when, if, and how probiotics could be used for the ocular system. Respondents showed a general optimism toward probiotic use in the next 10 years and beyond (Figure 4). The most common disease that respondents viewed to be treatable by probiotics was dry eye disease (Figure 5). Lastly, there is the most optimism toward oral administration to preventatively treat ocular conditions or as a post-treatment therapy. Using probiotics as a treatment gained the least positive outlook (Figure 6).

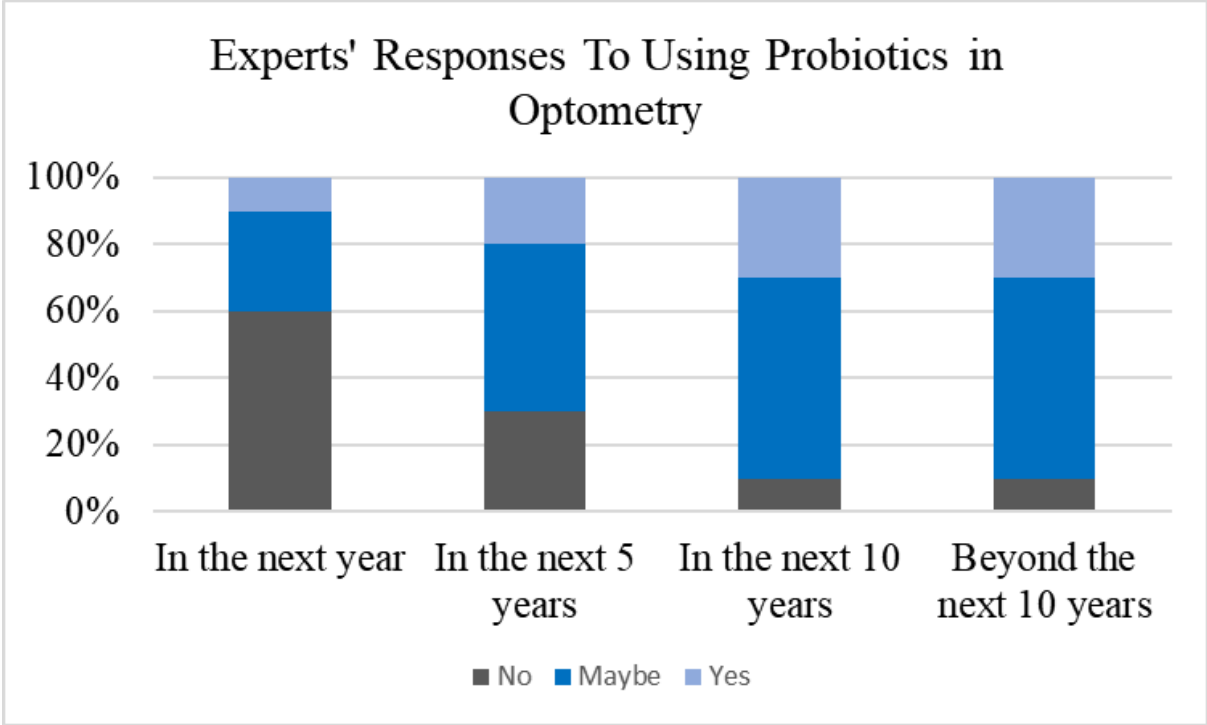


Figure 4

Survey Responses to Question 9. Each respondent was required to respond with a yes, no or maybe to whether they think probiotics will be used in optometry based on the given criteria of time.

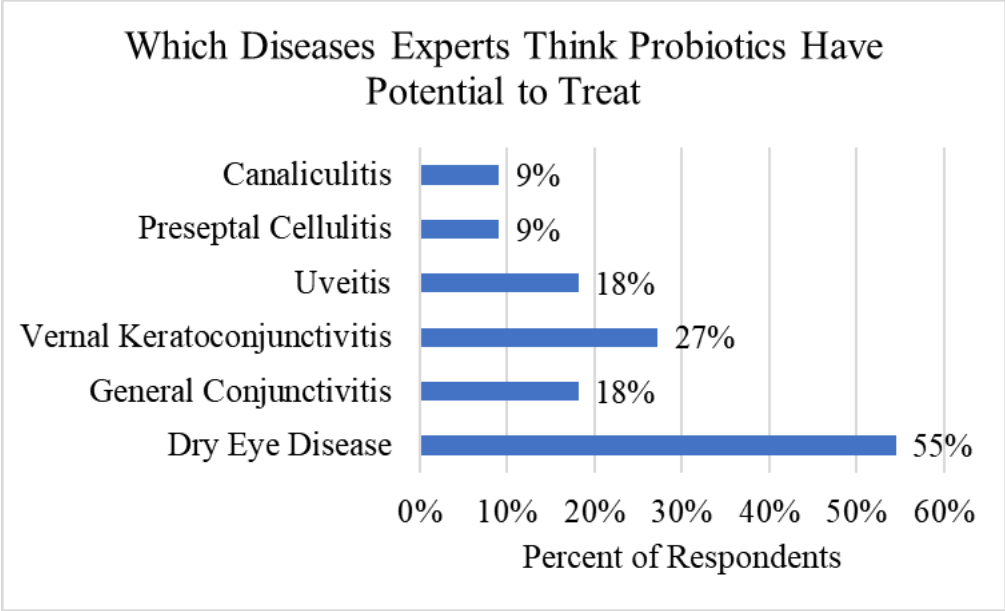


Figure 5

Survey Responses to Question 10. Respondents were able to select as many as they saw fit and also write in their own. Canaliculitis and Preseptal Cellulitis were written in responses

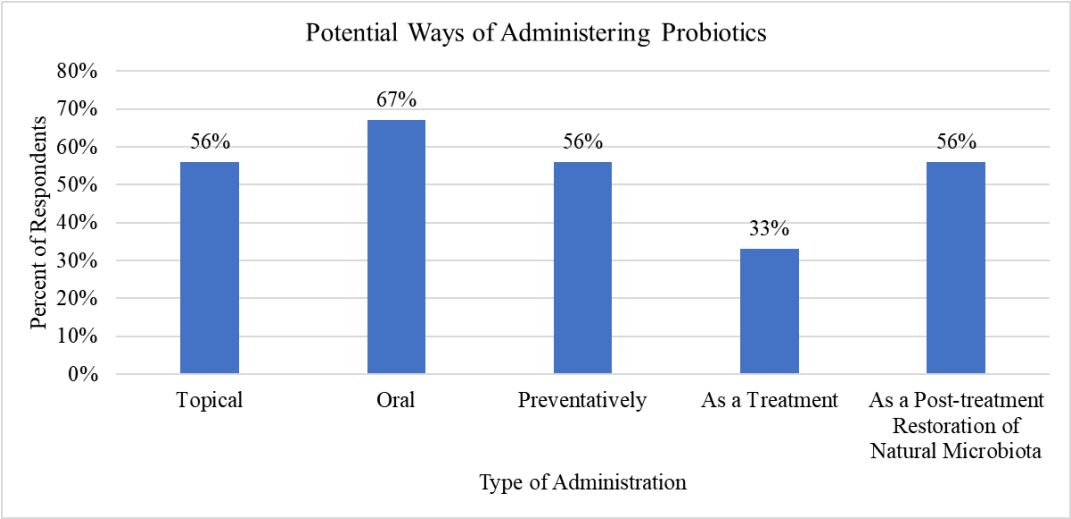


Figure 6

Survey Responses to Question 11. Respondents were able to select as many as they saw fit.

Discussion

Growth Assays

The results from the first 96-well growth assay had some unexpected results that demanded repetition of trials, but ultimately, trial 1 and 2 showed support in the probiotic's effectiveness in limiting *E. coli* growth. While in trial 2, the *E. coli* fluorescence continued to increase, it did so at a lower rate. This was observed by the lesser fluorescence in the presence of more *L. acidophilus*. The experimental procedure has undergone some troubleshooting, and it continues to have room for fine-tuning. For example, the challenge of growing bacteria in such small amount of broth brought about the fear of the cultures drying out overnight. This was successfully adjusted for by the use of parafilm during incubation period. Additionally, some contamination was observed and could be due to condensation and the close proximity between each cell. One way trial 2 attempted to limit this was by removing the lid during the readings, which seemed to help create less visual hindrance and more consistency in the results. As with any experiment, human error may have also been at play. Especially with amounts as small as 2 μL , even slightly imprecise multichannel pipettes or technique could greatly impact the results. One previous study proposed using larger wells to account for this effect, using 1mL wells instead (Ross et al., 2022). This study attempted to overcome human error by simple repetition of trial, but it would have been beneficial to do even more trials had time allowed.

Another point to consider is the factors that could have affected growth and fluorescence. With the growth assay being *in vitro*, it limits the possibilities of how a probiotic could hinder pathogenic microbe growth. The primary mechanism *in vitro* would be competition for resources or the byproducts of one microbe altering the environment, such as pH, to hinder the growth of another. The competition factor could potentially increase both *in vitro* and *in vivo* with the use of pre-biotics. In this experiment, the difference in growth rates between the two bacteria may

have affected the results. *L. acidophilus* has a doubling time of 30 minutes while *E. coli* doubles every 20 minutes (Klaenhammer & Russell, 1999; Gibson et al., 2018). Future experiments could add the use of a prebiotic that selectively nurtures the probiotic to see if it exacerbates the probiotic's effect *in vitro*. While the immunity related mechanism and systemic inflammation correlation has been found in prior studies, this experiment offers a glimpse at how important isolated factors could be, which would promote more topical treatments in the eye, as opposed to orally administered probiotics.

Survey

The survey helped offer insight into how feasible probiotic use could be in the field of eyecare. It also challenged whether the laboratory experiment findings are lining up with clinical practitioners' opinions. It is worth noting that the exposure respondents had to this topic prior to completing the survey was very limited. Almost half of them had never heard of it at all. This brings attention to the necessity of increased communication between the lab and the clinic. Despite the lack of foreknowledge, the survey responses do, in fact, show some correlation to findings from the literature review. For example, the most optimistic uses for probiotics include dry eye disease and Sjogren's disease, which involve inflammation and/or autoimmune components. This lines up with the majority of ocular-focused probiotic research emphasizing the immune response and anti-inflammatory effects. The openness respondents showed particularly toward orally administered probiotics adds to these diseases' potential for probiotic treatment. Additionally, most doctors in the survey have some hesitation toward antibiotics, which matches with the general caution from clinical and laboratory studies. Still, antibiotics are highly prescribed by local doctors, especially for conjunctivitis. Conjunctivitis is the most common type of eye infection, and it is usually viral, which means antibiotics would be ineffective (Watson et al., 2018). This fact combined with the overuse of antibiotics found by

Shekhawat and company and the willingness of survey respondents to use probiotics for conjunctivitis points to a promising possibility in the future.

Conclusion

Overall, this field of ocular-focused probiotic treatment is still quite new and emerging. Of course, any new form of treatment always has risks. Leger and company bring up the risk of a pathobiont infection, where previously commensal bacteria become pathogenic (Leger et al., 2017). This would be most risky for immunocompromised patients but is considered low risk for others. Another challenge in the field of probiotics is the unpredictability of using live organisms as a treatment. However, with the rise of genetic engineering, a more precise form of probiotic use could be made possible in the future. In conclusion, the potential of probiotics in optometry is not ruled out. Optometrists and ophthalmologists seem to have an openness and even optimism about their use. Additionally, the growth assays display a small and simple growth hindrance, that could be bolstered with *in vivo* trials and the addition of selective prebiotics. While this study was not able to repeat the trial with *S. epidermidis* and *B. subtilis*, it would make a good second *in vitro* experiment, especially with the repetition of *B. subtilis* in dry eye literature. The results of this experiment all point to a worthwhile pursuit of continuing ocular probiotic studies. Considering the effective mechanisms and expert perspectives discovered, the most promising future efforts seem to be toward dry eye disease, Sjogren's disease and conjunctivitis.

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Appendix

Survey Questions

Question 1

I am an...

- Optometrist
- Ophthalmologist

Question 2

How many years have you practiced as an Optometrists or Ophthalmologist?

- 1-5
- 6-10
- 11-20
- 21-30
- 31-40
- 41+ years

Question 3

What is your current most-used treatment for bacterial conjunctivitis?

- Antibiotic
- Warm compress
- Supplements
- Other _____

Question 4

What is your current most-used treatment for dry eye disease?

- Antibiotic
- Steroid
- Heat therapy
- Light therapy
- Supplements
- Other _____

Question 5

What best describes your view of antibiotic use in the field of optometry/ophthalmology?

- It works well and there is no reason to sway from treatment as we use it.
- They are effective and I use them, but I am concerned about the long-term effects.
- I generally avoid prescribing antibiotics, when possible, but I think they will always serve a purpose.
- I hope that eventually, the role of antibiotics as we currently use them will be obsolete.
- Other _____

Question 6

What is your current level of knowledge about the use of probiotics in optometric practice?

- I've never heard of this idea
- I've heard of this idea but have not explored it
- I've done some research/exploration in this topic

Question 7

Select all types of probiotic exploration that apply to you

- Attending a lecture on this topic
- Conducting my own study
- Reading the current literature

Question 8

Please describe your exploration of probiotics and/or the microbiome of the eye.

TEXT BOX ENTRY

Question 9

Do you foresee the use of probiotics as a form of treatment/therapy for eyes in the future?

	Yes	No	Maybe
In the next year	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In the next 5 years	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In the next 10 years	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Beyond the next 10 years	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Question 10

What disease/conditions would you consider using probiotics to alleviate, either alone or accompanied by another treatment (Check all that apply)

- Dry eye disease
- General conjunctivitis
- Vernal Keratoconjunctivitis
- Uveitis
- Glaucoma
- Age-related Macular Degeneration
- Diabetic retinopathy
- Sjogren's Disease
- Other _____

Question 11

In what way(s) could you see probiotics being administered to improve ocular health?

(Check all that apply)

- Topical treatment onto the surface of the eye
- Orally administered

- As a preventative treatment
- As a treatment
- As a post-treatment restoration of natural microbiota
- Other _____

Question 12

In what area do you see the *most* potential for using probiotics in the optometry/ophthalmology field?

TEXT BOX ENTRY