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Technical Bulletin – *Limosilactobacillus reuteri* 3613-1

Introduction

Women's health has become a large area of concern for the general consumer, growing by 385% in 2020 alone. Within the area of women's health, a large focus is on vaginal and urogenital health. Urogenital tract infections are a common, often recurring health problem in women of reproductive age and can include acute or chronic urinary tract infection (UTI) and genital tract infections. Various microbial pathogens are responsible for urogenital tract infections, including Gram-negative bacteria (*e.g.*, *Escherichia coli*), Gram-variable bacteria (*e.g.*, *Gardnerella vaginalis*), and yeasts (*e.g.*, *Candida albicans*).

A healthy genital tract is populated by a normal microflora, which largely consists of *Lactobacillus* species. When a shift occurs in the normal flora and *Lactobacillus* populations decrease, an abnormal growth of pathogens can occur leading to a urogenital tract infection. The most common treatment for urogenital tract infection is oral or topical administration of an antibiotic or anti-fungal medication to the affected subject. However, use of an antibiotic or anti-fungal medication can lead to a reduction of the normal flora in the urogenital tract, recurrent infections, and generation of drug-resistant pathogens. Moreover, the antibiotic or anti-fungal treatment may cause undesired side effects in the subject. Therefore, there is a need for more effective, less harmful methods for treating and preventing urogenital tract infection, such as probiotic strain *Limosilactobacillus reuteri* 3613-1.

The healthy human vagina is populated by a variety of *Lactobacillus* species, which play an essential role in protecting women from urogenital infections. *Lactobacilli* have the ability to inhibit the growth of pathogens by reduction of the vaginal pH through lactic acid production, production of H2O2, and production of bacteriocins such as reuterin all creating a hostile environment for the growth of organisms such as *E. coli, G. vaginalis,* and *C. albicans*. In addition, Lactobacilli can adhere to vaginal epithelia, deplete nutrients otherwise available to pathogens, and modulate the host immune response and microenvironment.

Limosilactobacillus reuteri strain 3613-1 was identified from a library of over 6000 lactic acid bacteria as a unique strain for its ability to produce antimicrobial metabolites. *L. reuteri* is naturally found in the intestine and vagina of humans and animals and has the ability to produce lactic acid. Arm & Hammer's proprietary strain *L. reuteri* 3613-1 also can produce reuterin, a compound that has potent anti-microbial properties against some pathogens as well as H2O2.

Scope of Investigation

In vitro studies performed at Arm & Hammer were conducted to substantiate the effect of *L. reuteri* 3613-1 on the growth and survival of common pathogens associated with urogenital tract infections including vaginally isolated *E. coli, G. vaginalis,* and *C. albicans*.

Urinary Tract Infections.

Urinary Pathogenic *Escherichia coli* (UPEC) accounts for 75% of all urinary tract infections. UPEC's primary reservoir is in the gastrointestinal tract. It is hypothesized that the *E. coli* is excreted in feces and is able to make its way into the urethra and bladder indicating the importance of *L. reuteri* 3613-1 inhibiting both vaginal and stool isolated *E. coli.*

Inhibition studies were performed to test the potential of *Limosilactobacillus reuteri* 3613-1 to inhibit the growth of four *E. coli* isolates, three isolated from the human vaginal tract and one isolated from human stool.

L. reuteri 3613-1 was grown overnight and conditioned supernatant was used to assess inhibition vs. the *E. coli* isolates (Fig. 1).

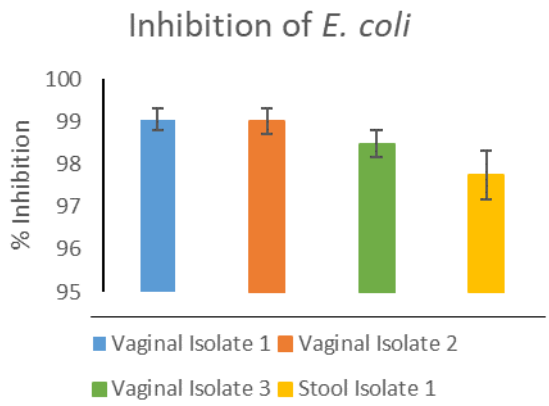

Figure 1. Inhibition of vaginal and stool *E. coli* isolates by *L. reuteri* 3613-1 conditioned bacteriocin supernatant (n=3).

Overall, *L. reuteri* 3613-1 bacteriocin supernatant demonstrated an ability to inhibit the growth of all four *E. coli* strains. The effectiveness of reuterin inhibiting the growth of the *E. coli* strains is indicated by a greater than 97% reduction of growth of all four isolates.

A follow up, proof of principle trial, was performed in human participants to demonstrate the ability of the probiotic strain to reach the site of infection during UTI when the probiotic is taken orally. Four college aged females plagued with recurring urinary tract infections began taking *L. reuteri* 3613-1 by mouth once daily for 60 days. Analysis of vaginal swabs determined that oral administration of *L. reuteri* 3613-1 survives passage through the gastrointestinal tract and can access and potentially populate the vagina. The participants reported no UTIs during the 60 days of treatment and tolerated the probiotic well with no adverse effects recorded.

Bacterial Vaginosis

Increased levels of *G. vaginalis* can lead to bacterial vaginosis. Bacterial vaginosis is a polymicrobial disorder of the vaginal microflora strongly associated with *G. vaginalis. G. vaginalis* produces a cytolysin, vaginolysin, which assists in the initial colonization in host epithelial cells. Once attached, *G. vaginalis* can form a biofilm which allows for increased pathogen survival against host immune mechanisms as well as antibiotic treatments. Bacterial vaginosis can lead to reproductive tract infections, preterm labor, and has been shown to lead to an increase in sexually transmitted infections indicating the need for a method of preventing *G. vaginalis* colonization in the vaginal tract. *In vitro* studies were conducted to substantiate the effect of *L. reuteri* 3613-1 on *G. vaginalis* growth and survival.

Inhibition studies of *G. vaginalis* compared the effect of *L. reuteri* 3613-1 and a competitor *L. reuteri*. Both probiotic strains were grown overnight in MRS broth, which was filter sterilized to remove bacterial cells. Overnight filter sterilized supernatant was diluted with sterile broth, to make a 25% supernatant solution. In duplicate, supernatant solution was added to a 48-well microtiter plate containing overnight *G. vaginalis* culture. Sterile broth was inoculated with *G. vaginalis* as a positive control and un-inoculated sterile broth was used as a negative control.

To account for the effects of pH on pathogen reduction, an aliquot of the overnight filter-sterilized supernatant was brought to neutral (pH of 6.2) using 1M NaOH. The neutralized supernatant was then used to set up the inhibition assay as described above. Neither pH neutralized probiotic culture was effective at inhibiting *G. vaginalis* growth.

In a third assay, production of the bacteriocin-like molecule reuterin was induced in media. The bacteriocin supernatant was filter sterilized and used to set up the inhibition assay as described above.

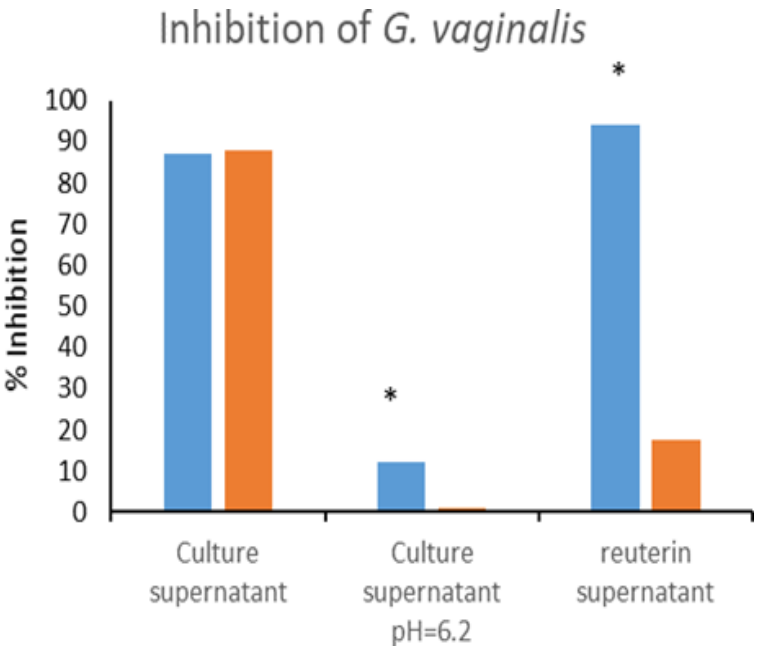

Figure 2. Inhibition of *Gardnerella vaginalis* by *L. reuteri* 3613-1 and *L. reuteri* (competitor) MRS supernatant, neutralized MRS supernatant, and reuterin supernatant (*p ≤ 0.05).

Yeast Infections

Candida albicans is a fungus that is commonly found on the skin or in mucous membranes such as the vagina or mouth on the human body. While *C. albicans* is considered normal flora, it can overgrow and cause disease known as candidiasis. Candidiasis often occurs in people who have a weakened immune system, are taking antibiotics which cause disruption of the microbiota, or are taking medications that cause a reduction in mucus production or drying of mucus membrane areas.

One of the most common types of candidiasis occurs in the vagina, more commonly known as a yeast infection. Vaginal candidiasis is the second leading cause of vaginal infection, affecting more than 200,000 women per year in the United States. According to the CDC, about 75% of women will have a vaginal yeast infection in their lifetime While anti-fungal medication is given to treat the disease, some women experience recurring infections and need treatment up to three times per year, indicating an important need for prevention of vaginal infection.

In vitro studies were conducted to substantiate the effect of *L. reuteri* 3613-1 on *C. albicans* growth and survival.

Briefly, inhibition studies of *C. albicans* compared the effect of *L. reuteri* 3613-1 and a competitor strain. Both bacterial strains were grown overnight, and broth was filter sterilized to remove bacterial cells.

Overnight filter sterilized supernatant was diluted with sterile broth to make a 25% supernatant solution. *C. albicans* culture was seeded into bacterial conditioned supernatant in each well. Sterile MRS broth was inoculated with *C. albicans* as a positive control and inoculated sterile MRS broth was used as a negative control.

In a separate iteration to test the effects of pH, an aliquot of the overnight filter-sterilized supernatant was brought up to a neutral MRS pH of 6.2. The neutralized supernatant was then used to set up the inhibition assay as described above.

Finally, production of the bacteriocin-like molecule reuterin was induced in media. The bacteriocin supernatant was filter sterilized and used to set up the inhibition assay as described above. *In vitro* assays demonstrated that the reuterin bacteriocin supernatant from *L. reuteri* 3613-1 significantly inhibited the growth of *C. albicans* compared to a competitor (Figure 3).

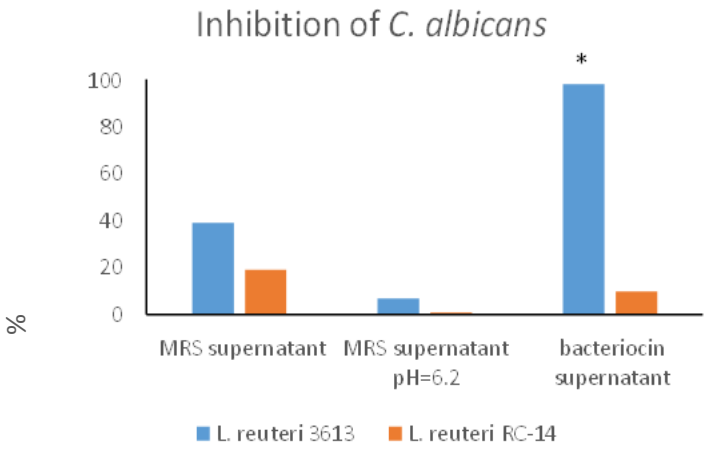

Figure 3. Inhibition of *Candida albicans* by *L. reuteri* 3613-1 bacteriocin supernatant. (*p ≤ 0.05).

Conclusion

Overall, *L. reuteri* 3613-1 bacteriocin supernatant demonstrated an ability to inhibit the growth of the potential urogenital pathogens. A common competitor strain did not produce reuterin, which is demonstrated by the difference in growth reduction with the bacteriocin supernatant. The effectiveness of reuterin to inhibit the growth of the *E. coli* was illustrated by a greater than 97% reduction of growth of all four isolates. Furthermore, *L. reuteri* 3613-1 demonstrated an ability to inhibit *G. vaginalis* growth by over 90% as well as greater than a 98% reduction in growth of *C. albicans. L. reuteri* 3613-1 outperformed the competitor *L. reuteri* strain in the potential prevention of urinary tract infections, bacterial vaginosis and vaginal candidiasis *in vitro*.

A proof of principle study demonstrated that *L. reuteri* 3613-1 is well tolerated and can be detected in the vagina after oral treatment. In conclusion, *L. reuteri* 3613-1 demonstrates great potential as a probiotic for women's urogenital health.

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