Technical Bulletin

Probiotic strain *Lactobacillus reuteri* 3613-1 provides a benefit to women's health

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 infections are 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 *Lactobacillus reuteri* 3613.

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 H_2O_2, 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.

Lactobacillus reuteri strain 3613 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 also has the ability to produce reuterin, a compound that has potent anti-microbial properties against some pathogens as well as H_2O_2.

Scope of Investigation

In vitro studies performed at Arm & Hammer were conducted to substantiate the effect of *L. reuteri* 3613 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 inhibiting both vaginal and stool isolated *E. coli.*

Inhibition studies were performed to test the effects of *Lactobacillus reuteri* 3613 on the potential 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 was grown overnight and conditioned supernatant was used to assess inhibition assays against the *E. coli* isolates (Fig. 1).

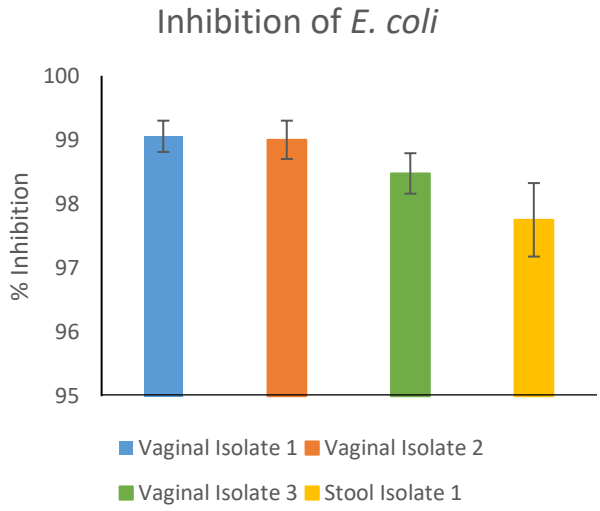

Figure 1. Inhibition of vaginal and stool *E. coli* isolates by *L. reuteri* strain conditioned bacteriocin supernatant (n=3)

Overall, *L. reuteri* 3613 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 by mouth once daily for 60 days. Analysis of vaginal swabs determined that oral administration of *L. reuteri* 3613 survives passage through the gastrointestinal tract and can access and potentially populate the vagina, preventing the onset of UTI. The participants reported no UTIs during the time of treatment and tolerated the probiotic well.

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 performed at Arm & Hammer were conducted to substantiate the effect of L. reuteri 3613 on *G. vaginalis* growth and survival.

Inhibition studies of *G. vaginalis* compared the effect of *L. reuteri* 3613 and a competitor *L. reuteri*. Both probiotic strains were grown overnight in MRS broth, which was filter sterilized to remove bacterial cells. Briefly, 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 MRS (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. Overnight *L. reuteri* cultures were centrifuged and the bacterial pellet was washed with sterile PBS three times. The bacteriocin supernatant was filter sterilized and used to set up the inhibition assay as described above.

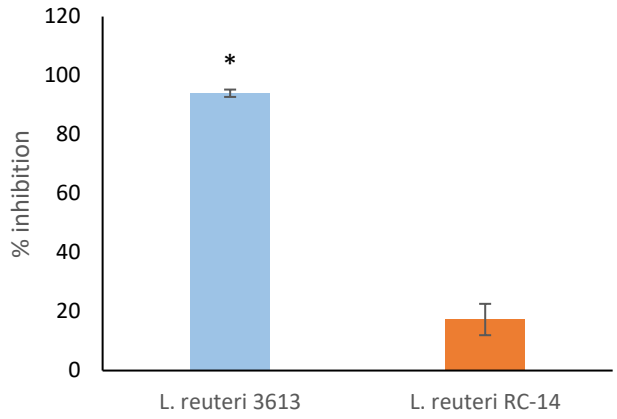

Figure 2. Inhibition of *Gardnerella vaginalis* by *L. reuteri* 3613 and *L. reuteri* (competitor) MRS supernatant, neutralized MRS supernatant, and reuterin supernatant ($p \leq 0.05$).

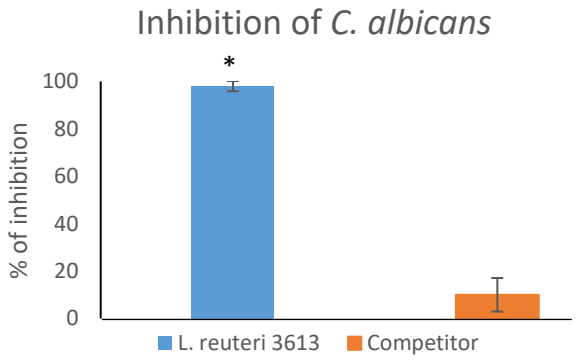

Figure 3. Inhibition of *Candida albicans* by *L. reuteri* 3613 bacteriocin supernatant.

While *Candida* species are often considered normal flora, they have the ability to overgrow and cause disease, candidiasis. According the CDC, about 75% of women will have a vaginal yeast infection in their lifetime. Vaginal candidiasis is the second leading cause of vaginal infection, affecting more than 200,000 women per year in the United States.

Initial testing of *L. reuteri* 3613 demonstrates the ability of the probiotic strain to control pathogens *in vitro.* A proof of principle trial was performed 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 by mouth once daily for 60 days. Analysis of vaginal swabs determined that oral administration of *L. reuteri* 3613 survives passage through the gastrointestinal tract and is able to access and potentially populate the vagina, preventing the onset of UTI. The participants reported no UTIs during the time of treatment and tolerated the probiotic well.

Conclusion

Overall, *L. reuteri* 3613 bacteriocin supernatant demonstrated an ability to inhibit the growth of the potential urogenital pathogens. The competitor strains do not have the ability to produce reuterin, which is demonstrated by the difference in growth reduction with the bacteriocin supernatant. 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. *L. reuteri* 3613 derived reuterin 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 outperforms the competitor *Lactobacillus reuteri* strains 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 is well tolerated and can be detected in the vagina after oral treatment. In conclusion, *L. reuteri* 3613 demonstrates great potential as a probiotic for women's urogenital health.