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Spring 2025 – VTPB 405
Critical “Peer” Review of:

**Competitive fitness of asymptomatic bacteriuria *E. coli* strain 83972 against uropathogens in human urine**

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## Identify and discuss the gap in knowledge being fulfilled by the research and/or impetus for the research (10 points)

This research addresses the unclear understanding of how ABU 83972 suppresses UPEC and other uropathogens in the urinary tract, specifically in terms of mechanisms of competition and environmental conditions that affect its ability to dominate. Previous studies have shown that ABU 83972 can reduce UTI incidence by outcompeting UPEC in pooled human urine; however the specific factors impacting this competitive advantage, such as nutrient availability, cell density, and physiological relevance in vivo, were not well not understood or addressed. This research seeks to investigate the conditions such as growth media (human urine vs lab media), oxygen conditions, and bacterial starting densities, impact on ABU 83972 dominance. Another key research motivator is the need for non-antibiotic-based UTI prevention strategies, especially as antibiotic resistance is rising. In addition, the research explores the use of engineered living materials (ELMs) to overcome poor colonization in the bladder.

Overall, this research fills the research gap related to how and why ABU 83972 outcompetes uropathogens in urine, while proposing a potential way to enhance its use as a UTI preventative measure.

## Identify and discuss the significance of the problem or disease they are studying (10 points)

The researchers are studying urinary tract infections (UTIs). UTIs are a significant global health problem, impacting about 150 million people per year. UTIs are especially prevalent among women, with approximately 40% experiencing a clinical episode at some point in their life. The infection begins with bacteria entering into the urethra and moving into the bladder, leading to bladder infection, known as cystitis. However, these bacteria can spread beyond the bladder, leading to more serious complications including kidney damage through inflammation, known as pyelonephritis. These can ultimately lead to more systemic infections including bacteremia and sepsis by bacterial dissemination in the blood. UTIs can be classified from uncomplicated to complicated; complicated UTIs most commonly occur in individuals with underlying health issues and are therefore particularly challenging to treat. It is important to note that recurrent UTIs often lead to repeated and extended use of antibiotics without preventing a recurrent infection. This excessive and extended use can lead to other complications contributing to future UTIs. UTIs can be caused by many clinically significant uropathogencs, however the most common causative agent is uropathogenic Escherichia coli (UPEC). UPEC contains virulence factors, such as adhesins and toxin, that allow them to colonize and damage the urinary tract. The increase in antibiotic resistance among uropathogens is a significant factor in recurrent infections and leads to limited treatment options and extended use of the antibiotics that can be

used, further contributing to the antibiotic resistance. An interesting finding was found related to the presence of asymptomatic bacteriuria (ABU). The presence of ABU E. coli, specifically the ABU 83972 strain of E. coli, can be present in the urinary tract system for extended periods of time, months to years, without showing any evidence of symptoms. Furthermore, ,the ABU 83972 strain has been found to decrease the colonization of other uropathoens in the urinary tract. This highlights two key points about UTI health: UTIs are extremely common and are becoming increasingly difficult to manage with traditional, current treatments. This underscores the need for new, non-antibiotic approach to prevent and control UTIs – such as the use of asymptomatic bacteriuria (ABU) strains like E. coli 83972, which can naturally suppress pathogenic bacteria without causing disease themselves.

**Identify the hypothesis or major objective of the study (5 points)**

The objective of this study is to evaluate the competitive fitness of the E. coli strain ABU 83972 against uropathogenic E. coli (UPEC) in human urine and investigate the use of engineered living materials (ELMs) ability to integrate into the system to deliver competitive strains such as ABU 83972. ELMs are living microorganisms, typically bacteria or yeast, that can support the survival and growth of cells. The study also investages whether the ELMs embedded with ABU 83972 could increase the competitive fitness of UPEC. This strain (ABU 83972) causes an asymptomatic bacteriuria which can prevent urinary tract infections (UTIs) by suppressing colonization of uropathogenic E. coli. The nature of competition and growth repression of uropathogenic E. coli by ABU 83972 is not understood, so this study explores how this competition occurs. Better understanding of this process can be used for creating more effective UTI prevention treatments. Currently, typical treatment of UTIs includes antibiotics, however because UTIs can be recurring, this has led to increased antibiotic use, contributing to increased antibiotic resistance and increasing potential for more serious health issues. Harnessing this competitive strain could decrease the need for antibiotics when treating UTIs and help decrease contributions to antibiotic resistance. The hypothesis is that ABU 83972 can consistently outcompete UPEC and other pathogens in human urine, and ELMs can enhance its delivery and colonization.

**Identify and discuss the methods used to test the hypothesis or address the objective (10 points)**

The researchers used several laboratory and animal-based methods to test their hypothesis that E. coli ABU 83972 can outcompete harmful UPEC bacteria and potentially prevent UTIs. First, they created a rafampin-resistant version of ABU 83972 so they could easily track it throughout the experiments. They confirmed this strain was genetically similar and behaved the same as the original ABU 83972 through Sanger sequencing. Then, the researchers performed competition experiences where ABU 83972 and UPEC were mixed in equal amounts (1:1 ratio) and grown together in different environments, including pooled human urine (from multiple distinct sources), LB broth with and without agar, M9 minimal media, and artificial urine medium. The strains were grown in the media for 24 hrs at 37°C. The researchers then diluted and resuspended the medium to count how many bacteria survived and see which strain outgrew the other. In addition, they also tested different starting concentrations, ran experiments under low-oxygen (anaerobic) conditions, and used spent culture fluids to understand how growth environment affected competition.

To mimic more realistic physiological conditions, the researchers set up a continuous flow system where fresh human urine constantly flowed over the bacteria for 10 hours. This tested

whether ABU 83972 could still dominate when the nutrients were constantly being refreshed. The researchers used a mouse model, where they introduced ABU 83972, UPEC, or both into the bladders of mice to see how well each strain colonized the urinary tract. Urine and organs were collected from the mice to count bacterial levels and compare how well ABU 83972 survived in the living system.

To explore a potential treatment method, the researchers developed Engineered Living Materials (ELM): hydrogel structures to hold and slowly release ABU 83972. The ELM was used to test if ABU 83972 released from these materials could still prevent UPEC from growing in human urine. These ELMs were designed to mimic long-term, non-antibiotic approach to preventing UTIs.

Overall, the researchers used a combination of lab experiments, controlled competition studies, a mouse infection model, and bioengineering tools like ELMs to test how well ABU 83972 could fight off harmful bacteria and evaluate its potential as a preventative therapy.

**Summarize the major results and conclusions of the article (25 points)**

ABU 83972 and UPEC exhibit comparable growth in pooled human urine

The E. coli strain, ABU 83972, and UPEC strains showed similar growth patterns and generation times in both pooled human urine and LB at a100-fold dilution. At the dilution of 1:1000, the growth curves were still comparable. Demonstrating that both thrive in the same environments, at least when grown alone. Other significant uropathogens, including K. pneumoniae, P. aeruginosa, S. aureus, and A. baumannii, grew similarly to the E. coli strains. The generation times showed no significant difference between ABU 83972, ABU 83972 RifR, and UPEC in either medium. ABU 83972 RifR was used in further experiments as a selective marker to obtain more detailed results. It is also important to note that K. pneumoniae RifR grew significantly faster than ABU 83972 RifR in urine but not in LB. A. baumannii grew slower than ABU 83972 RifR in both media, while P. aeruginosa had a longer generation time in LB but not in urine.

ABU 83972 outcompetes UPEC during growth in pooled human urine

When ABU 83972 was co-cultured with UPEC, or other common urinary tract pathogens like Klebsiella, pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, and Staphylococcus aureus, ABU 83972 consistently became the dominant strain after 24 hours in pooled human urine. This competitive edge did not depend on antibiotics used as markers or on oxygen availability. The ABU 83972 was mixed with UPEC strains at equal amounts and grown together in urine. The researchers used antibiotic markers to identify the strains and confirmed that ABU 83972 consistently outcompeted UPEC, regardless of which strain carried the antibiotic resistance. The experiment was extended to 96 hours, where ABU 83972 continued to dominate over time. Even when grown in anaerobic conditions, ABU 83972 still significantly outcompeted UPEC, just like under normal aerobic conditions.

ABU 83972 competition with UPEC is affected by inoculum ratio and cell density

When ABU 83972 started at 75%, it grew to almost 100% dominance after 24 hours, while UPEC dropped to less than 10%. ABU 83972 still outcompeted UPEC even when starting at a lower ratio (25%) or at lower total number of bacteria (cell density). When UPEC started higher at a 1:3 ratio, ABU 83972 still increased its share from 25% to 35%, while UPEC fell from 75%

to 65%. However, at very low cell densities (100 CFU/mL or less), UPEC could outcompete ABU 83972. When testing total bacterial concentrations, using the 1:1 ratio, at higher intensities, ABU 83971 consistently outcompeted UPEC, at 10^3 CFU/mL, both strains showed equal growth; at very low desnities, less than 100 CFU/mL, UPEC outcompeted ABU 83972. ABU 83972's ability to outcompete UPEC depends on both its initial ratio and the overall number of bacteria present. The researchers found it can still outcompete even when starting at a disadvantage, but only if the total cell density is high enough.

ABU 83972 outcompetes a diverse range of uropathogens

Competition assays were performed between ABU 83972 and uropathogens such as *K. pneumoniae* $Rif^R$*, A. baumannii, P. aeruginosa,* and *S. aureus* in pooled human urine to explore the interactions. Results indicated a significant decrease in all tested pathogens compared to ABU 83972. ABU 83972 has a significant competitive advantage over many clinically significant uropathogens.

UPEC outcompetes ABU 83972 in laboratory media but not in artificial urine medium

The researchers tested whether nutrient composition affects the competition between ABU 83972 and UPEC. They found that UPEC dominates in nutrient-rich lab media, but ABU 83972 thrives in urine-like environments. In nutrient-rich lab media like LB, UPEC had the advantage and outgrew ABU 83972, growing from 50% to 75%. In artificial urine medium (AUM) and diluted or spent human urine, ABU 83972 maintained its competitive advantage, reaching 65% after 24 hours. This suggests ABU 83972 is better adapted to the nutrient composition of urine than UPEC.

Addition of nutrients has minimal impact on the extent of ABU 83972 competition against UPEC in pooled human urine

A significant increase in the relative abundance of ABU 83972 was noted in 2-, and 10-fold-diluted human urine. To stimulate real urinary conditions, researched mimicked urine flow and tested bacterial competition in pooled human urine. ABUC 83972 dominated UPEC within 4 hours, reached 80% by 10 hours. Even in diluted human urine, 2-fold and 10-fold, ABU 83972 maintained a competitive edge. Both strains had similar growth in diluted urine, suggesting nutrient limitation doesn't weaken ABU 83972's advantage. These findings demonstrated that the concentration of nutrients does not affect the competitive fitness of ABU 83972 relative to UPEC.

ABU 83972 outcompetes UPEC in spent pooled human urine

ABUC 83972 outcompeted UPEC in all cases using spent urine, even when nutrients were scarce, reaching up to 84%. Interestingly, UPEC grew just as well in ABU 83972 spent urine as in its own, showing no toxic or inhibitory substances were secreted by ABU 83972. These findings suggest that ABU 83972's competitive advantages likely come from more efficient nutrient usage, not from producing compounds that kill or suppress UPEC.

ABU 83972 does not compete with UPEC in the murine urinary tract

In mouse models, ABU 83972 was less competitive. UPEC had higher bacterial loads in the bladder, urine, and other tissues. Over 5 days, UPEC levels remained high, while ABU 83972 levels dropped significantly. Even in mouse urine in vitro, UPEC outgrew ABU 83972, reaching

97% of the population.  This shows that ABU 83972's advantage is may be specific to human urine and not seen in the mouse urinary tract.

ABU 83972 released from engineered living materials prevents UPEC expansion

When ABU 83972 was embedded in engineered hydrogels (ELMs) and released over time, it still successfully outcompeted UPEC in human urine, especially after longer incubation periods (up to 72 hours). This supports the idea that ELMs could be used to deliver beneficial bacteria like ABU 83972 into the bladder. In pooled human urine, ABU 83972 released ELMs grew to 74% (1:1 start ratio) or 82% (10:1 start ratio) by 24 hours. By 72 hours, ABU 83972 reached up to 95%, strongly dominating UPEC. Mock ELMs (without bacteria) had no effect. ELMs are a promising way to deliver ABU 83972 and suppress UPEC, especially in urine-like environments.

Conclusion

ABU 83972 is a non-pathogenic E. coli strain originally isolated from a girl with long-term asymptomatic bacteriuria. ABU 83972 shows strong potential as a non-antibiotic alternative to prevent recurrent UTIs. In this study, ABU 83972 was shown to outcompete UPEC and other antibiotic-resistant uropathogens in pooled human urine, due to its rapid growth and efficient nutrient use. Using ABU 83972 would provide an antibiotic-independent approach to prevent recurrent UTIs, with the potential to reduce the adverse consequences with increased antibiotic use.

Even though ABU 83972 lacks virulence factors, it has a rapid generation time of under 20 mins, allowing it to continue to dominate despite decreased urine flow. ABU 83972 does not produce toxins or require direct contact to dominate, suggesting its competitive advantage lies in its metabolic efficiency.

The researchers found the competition between ABU 89372 and UPEC to be consistent with other published reports. The competition between them was dependent on the environment. In lab media, including L8 and M9, UPEC dominated, but in human urine, ABU 89372 consistently outperformed it, even in nutrient-limited or continuous urine flow conditions (mimic the conditions in an adult human), and in anaerobic and aerobic conditions. Low bacterial densities (less than 1000 CFU/mL) did result in ABU 89372 being unable to compete; this highlights the need for sustained high concentrations of densities of ABU 83972 in the bladder.

In mouse models, ABU 83972 did not colonize well and was outcompeted by UPEC, likely due to differences in urine composition and host factors.

The study also tested Engineered Living Materials (ELMs) that slowly released ABU 83972. This tool allowed ABU 83972 to successfully dominate UPEC in vitro, even at lower initial ratios. This approach may provide an effective approach to help maintain effective ABU levels in the bladder.

Overall, the study found a superior competitive fitness of ABU 83972 against UPEC strains, which provides promising new treatments for preventing recurring UTI infections without the use of antibiotics.

**Give constructive criticism on the primary research critiquing basic reporting, the experimental design, validity of findings, whether the conclusions were supported, and strengths and weaknesses of the paper. (20 points)**

This research paper is organized well and is clearly structured, making it easy follow from the introduction to the results and discussion. The figures used are easy to read and support the key findings. However, the figure captions do not provide a good interpretation of the results, so the reader has to dig into the main text to understand the figure significance. Clearer explanations directly below the figures would be beneficial to reader understanding.

One of the significant limitations of this study is that only one ABU strain (83972) was tested. Other strains may perform even better and be safer or could have adverse effects. Overall, these results support further development and investigation of ABU 83972 and other strains, especially in combination with the use of ELMs, as a promising non-antibiotic strategy or UTI prevention. Future studies should test more ABU and UPEC strains. In addition, future studies should further explore the mechanisms behind the competition between them for the process of nutrient use differences. Investigations in ELMs in live animal models or human trials are also needed.

Another significant limitation was the use of only female mice. In future studies, male mice and evaluation of male urinary tract systems should be investigated.

The findings from the in vitro models contrast sharply with the urine models. This raises questions about the real-world application of this research. The article does a good job discussing these differences, however more extensive research or research in alternative in vivo systems, such as humans or other animal models, is necessary to strengthen the applicability of these findings.

Throughout the study, the nutrient uptake is highlighted as an underlying factor to ABU 83972's success, however the specific nutrients or transport systems involved are not listed. More details on this aspect of the methodology and mechanism should be included in the future.

Another key element of this research that should have been improved is the reproducibility factor. Researchers should provide more detailed methodology to enable other researchers to recreate these experiments to test if the results are reproducible in other laboratories.

Overall, the findings of this research are very interesting and relevant to future healthcare treatment and prevention strategies for UTIs.