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Rational development and evaluation of novel formulations for urinary health

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ABSTRACT

Objective: Urinary tract infections (UTI) among women form a substantial part of medical practice and both patients and medical professionals have an interest in non-antibiotic treatments and preventative measures. This research provides preliminary data on a multi-functional composition, DAPAD, which explored several biologic activities of relevance to UTI.
Study Design: This formulation included D-mannose, citric acid, three prebiotic compounds, and extracts of dandelion and astragalus. Studies performed employed 4 bacterial strains that have relevance to UTI including *E. coli, Proteus mirabilis, Streptococcus agalactiae* and *Enterococcus faecalis.*
Results: Key findings from in vitro studies included: DAPAD at full- and half-strength inhibited growth of all UTI bacteria. Evidence for D-mannose agglutination of *E. coli* was demonstrated. D-mannose also showed unexpected effects on bacterial membrane integrity with vital staining and modest growth restriction. We did not demonstrate growth inhibition by dandelion or astragalus extracts but the latter showed diminished cytokine elaboration by bladder epithelial cells.
Conclusion: DAPAD is a multifunctional composition that may warrant further development as a UTI treatment or preventive if supported by clinical evaluation.
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Introduction

Among the conditions that are persistently a part of the practice of obstetrics and gynecology are vaginitis and urinary tract infections. Both conditions are of concern because they may be chronic or recurrent and are associated with more serious conditions, especially when occurring during pregnancy. Acute pyelonephritis is associated with asymptomatic bacteriuria in pregnancy [1] and vaginal and urinary tract infection has been associated with preeclampsia [2] and low birth weight, intrauterine growth restriction and preterm birth [3]. Additional interactions between vaginal and urinary microbiology is evidenced by the finding that introital colonization with *E. coli* is associated with urinary infection [4] and the finding that ingested *Lactobacillus* can be beneficial for urinary infections [5,6] implies a further connection between intestinal and vaginal colonization.

The pharmacologic approach to urinary tract infection – both symptomatic and asymptomatic - raises concerns about frequent or prolonged use of antibiotics which may select for resistant strains and constrain future use of antibiotics in serious infections, especially when considering the frequency with which urinary infections require treatment. In addition to consideration of antibiotic stewardship to obviate development of resistance, many patients are interested in non-pharmaceutical or natural products for management of urinary symptoms. The problem of recurrent infections suggests a role for options that are preventive along with those that address acute urinary infections. Support for non-pharmaceutical approaches also comes from the European Association of Urology which states non-antibiotic treatments for urinary infection should be employed before resorting to antibiotics [7].

Numerous compounds have been proposed and tested for use as urinary tract therapeutics and many of the studies done, have compared the activity and efficacy of a single compound to one or more antibiotics. These non-antibiotic approaches have been reviewed in the literature [8–10]. Cranberries (usually as cranberry juice) have been one of the more studied compounds in the pre-

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vention of UTIs for many years. The mechanism of action has not been completely elucidated and the different trials report contradictory results. Other substances like Vitamin C (ascorbic acid), D-Mannose and have been used and some findings are promising, but further clinical trials are needed.

Decades of research on non-antibiotic compounds for urinary infection has generated a long list of compounds that focus on one of many aspects of pathogenesis. For example, approaches include but are not limited to natural compounds or chemicals with antibacterial effect, compounds that block microbial adherence to uroepithelium, compounds that alter the gut microbiome, vaginal or urobiome, compounds affecting urine physiochemical properties (eg. pH), immune modulation or anti-inflammatory and probiotic or prebiotic approaches, not to mention hygiene and hydration in concert with other treatments.

In a previously published study [11], we examined several compounds that were under consideration for their intrinsic potential as supportive of urinary health with the intention of approaching urinary health from multiple directions including antimicrobial, prebiotic and immunologic, with the central hypothesis that combining these may produce a more useful product, while achieving the goal of natural origin and non-antibiotic. Two multi-component formulations were studied in our previous work [11] with one composition with more antimicrobial and anti-inflammatory properties being relevant to acute therapy of urinary infection and a second formulation with less antimicrobial activity but more probiotic potential as a candidate for prevention of recurrence.

In the research reported here, we built on the previous observations to compose and evaluate a combination, designated as DAPAD of non-antibiotic compounds that may have theoretical and objectively demonstrated biological activity that would support moving to clinical studies in the future. This report summarizes preliminary in vitro observations on DAPAD in relation to UTI- related bacteria, probiotic microorganisms, and bladder epithelial cells.

Material and methods

Test Articles: Individual compounds employed in this study were selected for anticipated antimicrobial activity, blocking tissue adherence, immune modulating activity and prebiotic activity to potentially support endogenous or exogenous probiotic organisms. Except for standard laboratory chemicals (citric acid) all the following (including lot designation) were supplied by Giellepi SpA, Milan, Italy. DAPAD included these components: arabinogalactan (lot FA16186), astragalus CHNQ.A (Lot 813475), dandelion, dry 4:1 (Lot N6243701), acacia gum B (Batch 180728), D-mannose (Lot DM180521), and Polydextrose IP (Lot 1922415008M).

The mixture identified with the palindromic designation of DAPAD, consisted of D-mannose (2000 mg/dL), 1500 mg/dL prebiotic mixture (1000 mg polydextrose plus 300 mg arabinogalactan plus 200 mg acacia gum), citric acid (100 mg/dL), astragalus (100 mg/dL) and dandelion (50 mg/dL) in aqueous solution. This test material was autoclaved to assure no contamination from natural products interfered with microbiology experiments. The mixture of polydextrose, arabinogalactan and acacia gum were combined in the proportions above and unless otherwise indicated used together and are referred to as prebiotic mixture and each of these compounds have precedence in the literature [12–14].

Microorganisms: To demonstrate antimicrobial activity of compounds of interest, four bacterial species relevant to urinary tract infection (challenge organisms) were *Escherichia coli* (ATCC 11775), *Proteus mirabilis* (ATCC 7002), *Streptococcus agalactiae* (ATCC 13813) and *Enterococcus faecalis* (ATCC 19433). Our laboratory also maintains stocks of *Lactobacillus* including *L. crispatus, L. gasseri, L. jensenii,* and *L. rhamnosus* and a strain of *Saccharomyces boulardiae*. Microbial strains were maintained by growing in brain heart infusion (BHI) broth and mixing with an equal volume of sterile glycerol and frozen at −80 °C. Prior to use, frozen cultures were thawed and inoculated into BHI and incubated overnight as starter cultures and diluted 1:1000 for inoculation of experiments unless otherwise noted. For convenience and space consideration, the 4 UTI-relevant strains are referred to as the uropathogen panel and the Lactobacilli and *Saccharomyces* are referred to as the probiotic panel.

Microbiology Methods: The antibacterial activity of DAPD and its components was addressed by placing 90 μL of DAPAD or one of its components in a 96 well microtiter plate and supplementing each well with 10 μL of BHI and inoculating with 10 μL or a 1:1000 dilution of starter cultures of the challenge organisms listed above. The growth control contained 10% BHI. At the time of inoculation, the absorbance (abs 450) of each well was read at 450 nm and then read again after overnight incubation at 37 °C. The increase in absorbance indicated growth and due to differences in bacterial strains and background absorbance of the test articles, data was normalized by reporting the percent increase in absorbance compared to the BHI control (ending abs 450-starting abs 450/BHI ending abs 450 – BHI starting abs 450). In addition, a 10 μL aliquot was plated on BHI agar to determine if viable organisms were present. This method will detect bacterial counts above 100/mL

Viability of test organisms was determined using flow cytometry in which propidium iodide (concentration) was used to stain bacteria with compromised membrane integrity, a surrogate measure of non-viability. Cultures were stained for at least 5 min with propidium iodide and analyzed in the Fl3 channel of a Becton-Dickenson Accuri C6 cytometer. Fresh cultures with a high percentage of bacteria being viable was used to establish the level of fluorescence associated with viable cells, a marker was set and the percent of cells in each experimental culture with fluorescence above the marker were considered the non-viable population.

Probiotic activity was demonstrated by growing *Lactobacillus* (*L. crispatus, L. gasseri, L. jensenii* and *L. rhamnosus*) and *Saccharomyces boulardiae* in media consisting of BHI with or without substances that may elaborate inhibitory activity which was demonstrated by culturing uropathogens in spent culture medium and comparing to growth in BHI alone. Results are normalized to allow comparison between cultures by using the fold-increase or decrease of spent cultures relative to appropriate controls.

Biofilm was demonstrated by growth for 10 days performed in 2 mL cultures in 24 well styrene plates with test compounds in BHI broth. After incubation, the wells were washed three times with distilled water, stained with 1% safranin, and again washed 3 times with water. Plates were allowed to dry and the retained safranin was mobilized into reagent alcohol and read spectrophotometrically at 560 nm.

Cell culture: Bladder epithelial cells (T24) were purchased from ATCC and cultivated in McCoy's 5a medium supplemented with 10% fetal bovine serum. Cultures were refed approximately twice weekly and split when 80–90% confluent. For bladder culture infection and cytokine responses, confluent cultures were exposed to bacteria, with and without DAPAD and with and without infection of the cell culture with the uropathogen panel.

Cytokine Profiling: Experiments were conducted on supernatants from cell cultures described above with analysis of cytokine concentration using the flow cytometric bead array for 6 cytokines obtained from Becton-Dickenson. Samples were obtained directly from culture flasks exposed to bacteria with and without addition of DAPD for 4 h. The BD bead array system included standards which allowed quantitation of cytokines up

to 5,000 pg/mL. Samples exceeding this level were diluted to remain within the standard curve.

Results

In this study, nine compounds were considered for their potential role in treating or preventing urinary tract infection. D-mannose has been studied previously, is a component of many non-antibiotic treatment or preventive for recurrent infection [15] and is considered to block uropathogenic *E. coli* to bladder epithelium but its unsuitability as a carbon source may also contribute to its mechanism of action [16]. Citric acid [17], dandelion [18–20] and astragalus [21] have been reported to indirectly elicit antimicrobial mediators. Likewise, botanical extracts such as astragalus may also have immune modulating activities [22,23]. Finally, arabinogalactan, polydextrose and acacia gum may serve as prebiotic enhancers of bacteria-mediated probiotic activity [12–14]. The overall hypothesis for this work posits that the above compounds in combination can simultaneously achieve multifunctional biological activity to support urinary tract health.

Given the fact that the antimicrobial activity is one of the most important attributes of a non-antibiotic urinary tract product, we confirmed that activity was present in DAPAD and compared it to the individual components. These were each prepared at double concentration (relative to the complete formulation) and mixed with an equal volume of BHI broth. Each condition was inoculated with the test organisms. The complete formulation, and citric acid failed to show growth and the other conditions developed turbidity visually similar the BHI control, except D- mannose which was clearly less turbid than the control.

These results were followed up with the same experimental setup in triplicate in 96-well plates. Because each condition had a different background absorbance, the starting (at the time of inoculation) and ending absorbance at 450 nm (after overnight incubation at 37 °C) was read and results averaged and normalized for the growth in the BHI control (Fig. 1).

Fig. 1 DAPAD (complete formulation) and components of DAPAD were mixed with BHI such that the final concentration of each was the same as in the formulated DAPAD (see methods for formulation). Tests were performed in 96 well plates. Uropathogens were inoculated and incubated overnight at 37 °C and absorbance at 450 nm measured and divided by the absorbance of the BHI control for the relevant test organism. Values above 100 indicated growth greater than control cultures.

As expected, the complete DAPAD and citric acid showed the least growth of the test organisms. D-mannose consistently grew

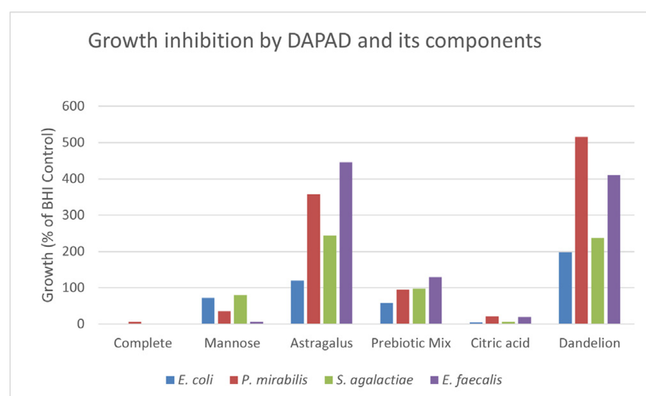

Fig. 1. Growth of test organisms in components of DAPAD and DAPD versus BHI Control

only slightly less than the controls. Also of interest was the increase in growth with the two botanicals but this might be explained by added nutrients furnished as part of these compounds and may play a role in differences in inhibitory capacity of the complete DAPAD formulation.

Additional information was gained by determining the minimal inhibitory concentration of citric acid and DAPAD as shown in Fig. 2. The results for DAPAD indicate that it is sufficiently inhibitory to restrict bacterial growth at a two-fold dilution, however, neither DAPAD nor citric acid was inhibitory at the 1:4 dilution.

Fig. 2 Broth dilution assay of Citric acid and the complete (“C”) DAPAD formulation. Concentration of 1 indicates undiluted test article. DAPAD and citric acid were prepared at double strength and mixed with equal parts BHI. The fold increase compares the absorbance at the time of inoculation with that after overnight incubation.

An additional insight into the interaction of the test organisms with DAPAD and its components was provided by propidium iodide staining of bacteria. *E. coli* and *E. faecalis* were incubated with 90 μL of DAPAD or astragalus, citric acid, dandelion extract, prebiotic mixture, and D- mannose all at the same concentration present in DAPAD and inoculated with 10 μL of a 1:1000 dilution of the test organisms and incubated at 37 °C overnight. Cell counts were recorded in the forward scatter channel (FSC) of the flow cytometer and fluorescence channel 3 (Fl3) was used to quantify the PI-stained bacteria, which serves as a surrogate marker of non-viability. Data from this experiment are found in Table 1.

It was useful to evaluate the effects of DAPAD and its components using flow cytometry because there is a possibility that some of the bacteria which may have been able to replicate, could after prolonged exposure to components of DAPAD may have undergone membrane damage. These results led to several observations, the first of which was for both organisms DAPAD and citric acid showed low counts and substantial evidence of membrane damage among the cytometrically counted organisms. Less than 10% of bacteria in the BHI controls stained with PI which comports with the expectation of growth in standard media, based on cell counts while demonstrating a high degree of viability. Nevertheless, *Enterococcus* appeared more fragile than *E. coli*, and as observed previously, astragalus supported growth in numbers for both organisms, while dandelion extract supported high counts in *E. coli*, but did not support high counts for *Enterococcus*.

D-mannose is of interest as well because in the data from Table 1, it yielded a substantial population of PI staining organisms. As a result of these findings as well as the extensive use of D-mannose in the urinary infection literature, we considered another aspect attributed to D- mannose, namely autoagglutination. A method has been proposed in which a suspension of bacteria will show diminished turbidity (measured at 1 cm from the surface of fluid culture) as organisms in the culture clump together [24]. In our hands, the decrease in turbidity required a long incubation, but at 4 h *E. coli* turbidity decreased by 2% in 4 h, but over 7 days the change was readily observed as shown in Fig. 3. Cultures with DAPAD or citric acid did not develop turbidity due to growth inhibition. Of note is the tube with D-mannose, which has decreased turbidity above the base of the tube but visible aggregations along the side of the tube and a non-homogenous pellet at the base of the tube. This phenomenon did not appear in tubes with the other additives. The botanical products, prebiotic mixture, as well as the growth control have substantial turbidity with some settling of the organisms in the base of their respective tubes.

Fig. 3 Seven-day cultures of DAPAD and components of DAPAD in BHI. Starter cultures (100 μL) were added without dilution to 2 mL of BHI and DAPAD and components (2 mL). Additives in each tube are: 1 DAPAD, 2 astragalus, 3 citric acid, 4 prebiotic mixture, 5 D- mannose, 6 dandelion extract, and 7 *E. coli* in BHI.

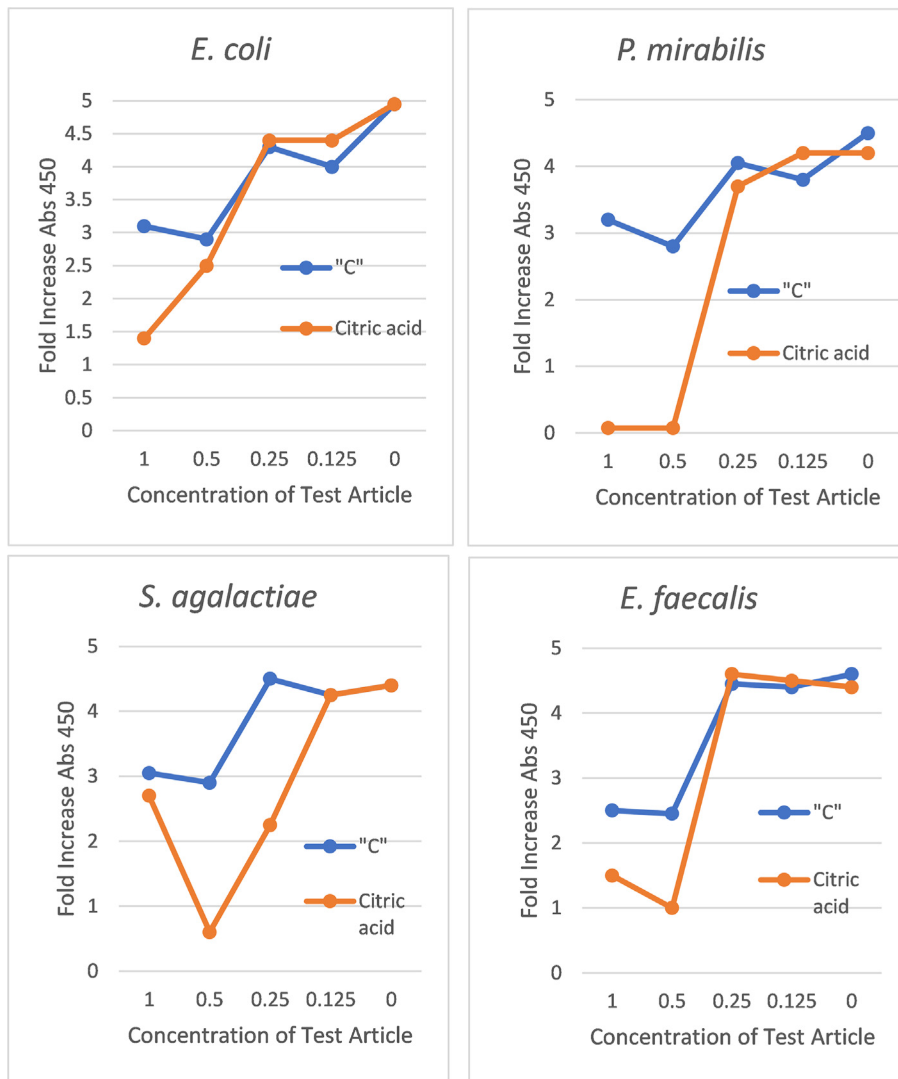

Fig. 2. MIC determination for DAPAD and citric acid.

Table 1
Microorganism viability as determined by propidium iodide exclusion measured by flow cytometry.

	DAPAD	Astragalus	Citric acid	Dandelion	Prebiotic	D-mannose	BHI 10%
E. coli							
Cell Count	78/mL	12,500	12	9,900	3,500	12,600	8,700
PI Stained %	49.9	26.6	1.9	7.5	6.4	36.6	8.6
E. faecalis							
Cell Count	824/m	7,193	48	360	100	290	5,637
PI Stained %	72.8	59.1	50.5	32.9	23.9	89.5	9.5

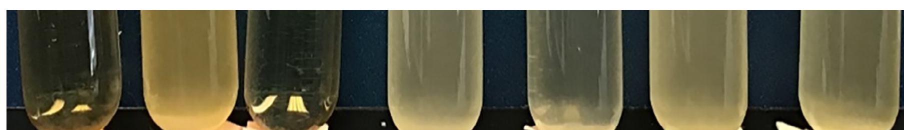

Fig. 3. Turbidity and settling of DAPAD or its components in BHI inoculated with E. coli.

Autoaggregation has been claimed to have a role in biofilm formation and although biofilm is frequently discussed in the context of microbial virulence, it has also been considered advantageous in the establishment of probiotic microorganisms [25]. Since support for biofilm formation by putatively probiotic organisms may be beneficial, we examined biofilm production by a panel of probiotic organisms in relation to DAPAD or its components, summarized in Fig. 4.

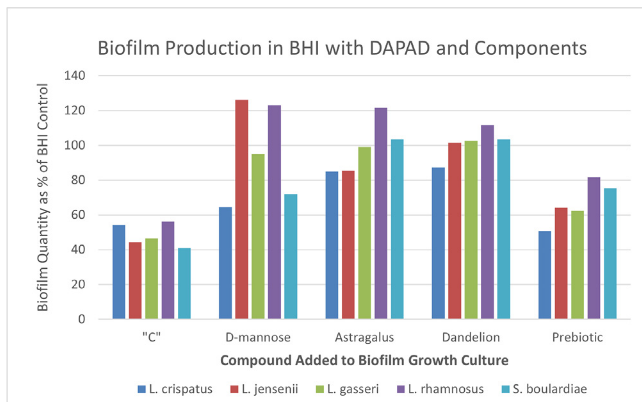

Fig. 4. Biofilm production when grown with DAPAD or DAPAD components.

Fig. 4 Biofilm produced by each of five probiotic organisms in BHI supplemented with complete ("C") DAPAD or its components was measured as absorbance of safranin retained by biofilm. The absorbance from BHI alone represented the baseline and values below 100 represent diminished biofilm production.

The key results that flow from this experiment represent data normalized for the biofilm detected in BHI controls, revealed only 8 conditions were at or slightly above control levels. This study also found DAPAD was less supportive of biofilm formation by *Lactobacillus* and *Saccharomyces* than prebiotic mix; D-mannose and botanical extracts appeared supportive of control or slightly above control levels of biofilm, suggesting that diminished biofilm in the presence of DAPAD could relate to diminished growth of the test organism.

Probiotic potential was explored by culturing probiotic panel strains in BHI with DAPAD, D-mannose, astragalus, prebiotic, citric acid or dandelion (totaling 35 conditions). Absorbance values aver-

Fig. 5. Comparison of preconditioned media supernatants from *Lactobacillus* cultures supplemented with DAPAD ("C") and its components challenged with uropathogens.

aged for the 5 probiotic organisms after 7 days incubation with prebiotic supplement was 146% of BHI alone and in most (14 of 15) pairings of probiotic organisms with BHI supplemented with D-mannose, astragalus or dandelion yielded absorbance at or above the level for BHI alone. All 35 of these cultures were centrifuged to remove probiotic organisms and the spent culture supernatants were supplemented with 10% (final concentration), aliquoted and inoculated with each of the 4 uropathogen test organisms. Absorbance at 450 nm was recorded at the time of inoculation and after overnight culture at 37 °C and shown graphically in Fig. 5.

From this experiment the main observations were that most of the urinary tract organisms responded similarly to the spent cultures containing additives though it must be noted that variations were present and there were outliers among the 140 observations in this experiment.

Fig. 5 Growth medium was preconditioned by allowing probiotic organisms to grow for 7 days in BHI or BHI supplemented with DAPAD or its components and centrifuged to remove probiotic bacteria. Cultures thus conditioned were then inoculated with each of the uropathogens and growth was measured by absorbance after overnight incubation at 37 °C and divided by starting absorbance (fold increase).

The least growth of test organisms occurred with DAPAD and citric acid which was expected since the additives were carried over from the pre-conditioning of media with *Lactobacillus*. But somewhat unexpectedly, D-mannose also demonstrated limited growth in this experiment and prebiotic also showed less growth than the BHI control.

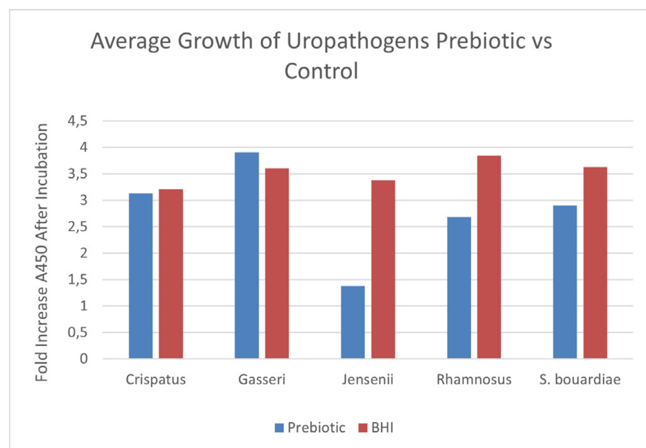

Fig. 6. *Lactobacillus* species cultured with prebiotic 7 days, challenged with uropathogens (*E. coli, P. mirabilis, S. agalactiae* and *E. faecalis*) compared to BHI control

Although the spent cultures with prebiotic during *Lactobacillus* culture did not appear to restrict growth as much as DAPAD, further evaluation highlighting the species of *Lactobacillus* with prebiotic used to condition the medium for 7 days. The average (for the 4 uropathogens) growth yield (expressed as the difference between starting and ending cultures) is presented in Fig. 6 which summarizes this evaluation. *L. jensenii* provided the greatest difference followed by *L. gasseri* and *L. rhamnosus.*

Fig. 6 aggregated results of spent media experiment are presented by probiotic organism compared to BHI growth control. Data for uropathogens were derived from the data in Fig. 5 and presented as averages for the 4 test organisms response to probiotic organism-conditioned medium. Comparisons using paired T-test showed differences to be significant for *L. jensenii* ($p = 0.006$), *L. rhamnosus* ($p = 0.012$) and *S. boulardiae* ($p = 0.002$).

In our previous work [11], we used a cytokine bead array method to determine if inflammatory markers from a panel of 6 human proinflammatory cytokines were elicited from bladder cancer epithelial cells when exposed to uropathogens and if any alteration in cytokine expression occurred when exposed to DAPAD or some of its components. Table 2 below summarizes the findings for four cytokines. The bead array allows quantitation of Il10 and Il12-70p but these were not detected and are not included in the table. All assays were conducted with 0.5 mL samples taken from culture supernatants from confluent 6-well plates containing 5 mL of growth medium per well.

Throughout this experiment, high levels of Il6 and Il8 were elaborated into the cell culture medium. And as indicated by the standard deviations reported, substantial variability was seen between strains of bacteria. Because some of the compounds present in DAPAD are purported to have anti-inflammatory activity, support for this concept was sought. Of note, astragalus alone and astragalus added to infected cell cultures show lesser amounts of Il6 and Il8 than infected and untreated cultures, however these observations were too few in number and suffered from wide variations among bacterial strains.

Discussion

The attractiveness of non-antibiotic measures to prevent and treat UTI and their more consequential sequelae pertains to the goal of the medical community which has concern for medical treatments that increase the risk of promoting antibiotic resistance and extends to patients who have a positive image of natural substances with medicinal properties. Against this backdrop, many non-antibiotic substances are available and given prior experience [11] DAPAD was conceived as a potential means of addressing UTI.

Combining active compounds, as in the case of DAPAD allows for addressing UTI from several of the pathophysiologic attributes which include viable bacterial pathogens that can multiply and spread to adjacent or distant tissue sites, microbial adherence to

Table 2
Cytokine concentration in supernatants from T24 bladder epithelial cell cultures with or without infection; with or without treatment with DAPAD, astragalus or dandelion extract.

Condition	TNF	Il-6	Il-1β	Il-8
Infected without treatment	2.87 + 1.54	3328 + 2062	3.52 + 3.6	3087 + 1692
Infected + DAPAD	2.61 ± 2.23	3422 ± 1491	30.4 ± 38.5	3611 ± 1154
DAPAD only*	2.45	4359	248	3462
Infected + Astragalus	3.66 + 4.84	2207 + 1917	63.6 + 61.7	2613 + 1881
Astragalus only*	0	995	0	2381
Infected + Dandelion	2.78 + 1.97	3300 + 2057	36.1 + 36.7	3374 + 1579
Dandelion only*	0.81	0	0	1709

Data from the cytokine bead array assay is presented as pg/ml and the highest standard was 5000 pg/mL and samples above that level were diluted ten-fold and re-assayed (see Methods).
* Standard deviations are shown only for conditions where all 4 uropathogens were analyzed. For samples which were uninfected only one value is available.

host tissues, inflammatory processes involved in UTI and interaction with protective microbial species. Components of DAPAD individually may address these elements of pathogenesis.

For example, citric acid, a weak organic acid, may modify pH to provide an environment inimical to pathogenic organisms, but perhaps not harmful to acidophilic probiotic microorganisms. Citric acid also known to have chelating properties which may contribute biological interactions with microorganisms and host cells. A detailed study of citric acid along with other organic acids and flavones [26] reported citric acid was inhibitory toward several bacterial species at 500 μg/mL and another study [27] reported 0.5% citric acid inhibited *Mycobacterium bovis*. Citric acid was found to penetrate biofilm and kill bacteria at pH less than pKa 1 of citric acid which is pH 3.1 [28]. Our findings indicated that citric acid and DAPAD were inhibitory with a citric acid concentration of 1 mg/mL.

D-mannose is a major component of DAPAD and has had a great deal of citation in the literature but notably, a recent meta analysis [15] of recurrent UTI with and without D-mannose treatment, found only 8 reports that met their evaluation criteria despite the presence of 776 unique citations of D-mannose. This data suggests that well-constructed clinical studies are rare compared to the number of literature citations. However, this group found that D-mannose was efficacious in the studies ultimately evaluated. D-mannose has been of interest because it has been reputed to cover mannose receptors on uropathogenic *E. coli*, potentially blocking pathogen adherence to the bladder wall [29]. A recent report extended information about D-mannose interaction with uropathogenic *E. coli*, indicating that mannose was least preferred by *E. coli* as a carbon source among several tested and did not inhibit growth and that prolonged growth of *E. coli* with D-mannose did not select for Fim1 (the fimbrial structure that binds mannose and uroepithelial cells) variants that lose their binding capacity [16]. The majority of research on adherence of bacteria to uroepithelium has focused on *E. coli*. In our experience, several experiments (see Figs. 1 and 5, Table 1) indicated the D-mannose was not conducive to bacterial growth and this extended to species other that *E. coli*. Coupled with affirming literature, the presence of D-mannose in DAPAD is supported.

The prebiotic mix employed in DAPAD has relevance because introital colonization by urinary tract bacteria may be the precursor to bladder infection. Probiotic lactobacilli from the gut or urobiome may be supported by prebiotics that stabilize benign colonizers and suppress uropathogens. First, we showed that the prebiotic mix was less supportive of growth of uropathogens than other components of DAPAD (Fig. 1) but we did not find the method used for demonstrating probiotic activity with spent cultures from the probiotic bacteria and yeast had substantial probiotic activity, however when compared to BHI controls, demonstrable activity was present (see Fig. 6). Our more recent work with co-cultures suggested that probiotic effects may be seen with co-culture where the probiotic organism is in contact with the pathogen throughout the growth phases in both organisms [30]. As mentioned with uropathogens, follow on research should evaluate a larger panel of probiotic microbial strains to determine which are most potent in suppressing uropathogens and which probiotic strains are best supported by prebiotic compounds.

DAPAD also contained two botanicals which have been reported in the literature to have antimicrobial and immune supportive activities as well as additional biological activities relevant to UTI. The ability of a number of botanical substances to modify cytokine expression was reviewed by Spelman et al. [22] who mentioned 18 plant species that affect cytokine expression. Astragalus has commonly been cited as having immune modulating activities and was studied in RAW 264.7 macrophage-like cell lines treated with astragalus polysaccharides in a study by Zhou et al. [23] in which TNF-α, Il-1β, Il-6 and nitric oxide were increased, and also provided evidence that these polysaccharides engaged TLR-4. Adesso et al. [31] found that intestinal epithelial cells exposed to LPS responded to *Astragalus membranicus* with anti- inflammatory effects that included decreased TNF-a, decreased reactive oxygen species with NF-κB. We also concluded that among the cytokines we measured, the effects of our preparation of astragalus appeared to affect Il-6 and Il-8. However, a recent paper [32] emphasizes that different extracts of astragalus can differ in the content of various compounds and hence, careful characterization of the extract is essential.

In our hands, both the dandelion and astragalus extracts did not inhibit growth when cultured with uropathogens (Fig. 1). However, dandelion contains substances with antibacterial properties, but key references have depended on processing and extraction process to obtain purified and well-characterized substances [18–20]. These reports of specific compounds of interest may be useful to establish how to exploit some of these for use in UTI.

While this study was focused on preliminary characterization of antimicrobial and immunologic properties of DAPAD and its components, some limitations should be noted before proceeding with additional studies. We observed high levels of cytokines in some of our test conditions and if further studies of immune modulation are undertaken, the test materials should be evaluated for the presence of endotoxin, especially when natural products are involved, as results may be substantially impacted. This is not an issue for microbiologic studies.

In this initial evaluation of the antimicrobial activity of DAPAD and its components we elected to use bacterial strains characterized by ATCC. It would be useful to have several strains from the species we employed, and if they were obtained from individuals with UTI. This would enhance the external validity of further studies on DAPAD.

Further to the relevance to clinical use, it will be important to consider details of the intended use of DAPAD or a derivative composition, and it should be emphasized that the DAPAD composition used in this study was prepared in our laboratory. To allow use in patients, DAPAD composition will require the addition of functional compounds and excipients able to guarantee a good palatability and preserve stability over time. In any use in patients, the formulation would probably undergo changes from the DAPAD we used.

Formulation is an exacting and demanding process that has elements and processes beyond what is typical of a research laboratory. Route of administration, dosage schedule and specific population (acute, asymptomatic, or recurrent UTI) would be important to consider both in formulation as well as mode of use. Likewise, clinical value will only be established with human subjects. Finally, the laboratory observations suggest through results of factorial experiments which components of DAPAD might be increased or decreased in formulations to rationally adjust anticipated biological activities in future clinical studies.

Conclusions

This in vitro study investigated the effect of the multi-component and its components on UTI bacteria, probiotic bacteria and cytokine elaboration from T24 bladder epithelial cells. DAPAD and citric acid showed antimicrobial activity against UTI-relevant organisms. Mixed results were obtained with probiotic bacterial strains with a mix of prebiotics failing to enhance probiotic activity, but DAPAD and D-mannose limited growth of uropathogens in spent cultures of probiotic organisms. D-mannose showed expected aggregation of E. coli and some restriction of growth among other uropathogens. At the concentrations employed,

botanicals including astragalus and dandelion extracts did not inhibit microbial growth but astragalus showed a tendency to reduce IL-6 elaborated by T24 cells. DAPAD may benefit from its multifunctional composition and merits continued studies with larger panels of urinary pathogens derived from clinical sources.

Declarations

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Availability of data and Material: All data generated or analysed during this study are included in this published article.

Code availability: Not applicable. **Ethics approval**: Not applicable. **Consent to participate**: Not applicable. **Consent for publication**: Not applicable.

Declaration of Competing Interest

The authors declare Z.J., G.S., A.M. and B.L. have no conflict of interest. F.D. serves as scientific advisor to Giellepi S.p.A and was instrumental in providing DAPAD components as stated in the methods section, to our laboratory. F.D. had no direct involvement in data development or interpretation. Employees of Giellepi S.p. A. were not provided a copy of this manuscript prior to submission and had no role in reviewing or interpreting the data.

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