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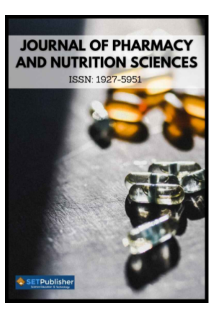

In Vitro Efficacy Test on a Food Supplement for the Treatment of Urinary Tract Infections (UTIs)

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Abstract:

Urinary tract infections (UTIs) are among the most common bacterial infections, representing a massive burden on healthcare systems and accounting for significant morbidity and healthcare expenditure. As bacteria generally cause UTIs, the primary therapy is defined by antibiotics, whose wide use can cause antibiotic resistance. In this scenario, using nutraceuticals to manage UTIs is an attractive alternative.

This study aims to test the *in vitro* efficacy of a new food supplement for treating UTIs containing D-mannose, palmitoylethanolamide, HibCyn® (hibiscus extract), N-acetylcysteine and microencapsulated *Lactobacillus rhamnosus* LR04.

The activity of the product was evaluated in terms of antimicrobial, antibiofilm, cytotoxic, antiinflammatory, and synergic activity with fosfomycin with respect to the main microorganisms responsible for UTIs, specifically *Klebsiella pneumoniae*, *Enterococcus faecium*, and *Escherichia coli.*

“UTIVAL” food supplement has an action on both the inhibition and eradication of the biofilms formed by all the microorganisms. In the *in vitro* tests performed in this study, the activity of the product was effective at a concentration of 0.78 g/l.

It was also possible to affirm that the product has an inhibiting effect on inflammation mediated by tumor necrosis factor αlfa (TNFα) in a concentration range of 0.04 to 0.625 mg/ml.

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1. INTRODUCTION

Urinary tract infections (UTIs) are among the most prevalent community-acquired and hospital-acquired infections, affecting almost 50% of the population at least once in their lifetime. With a significant social and economic impact, UTIs constitute a considerable burden on healthcare systems and account for substantial morbidity and healthcare expenditure with an estimated annual cost of US$3.5 billion in the USA [1,2].

As UTIs are generally caused by bacteria, most patients undergo long-term antibiotic treatment that severely impairs the normal microbiota and increases the risk of developing multidrug-resistant microorganisms. However, the type of medication and length of treatment depends on the type of bacteria, its level of susceptibility, history, symptoms, and the patient's immune status.

Although most UTIs have a benign course, they show various clinical scenarios whose diagnosis and characterization are often not precise and timely, with clinical consequences and risks that vary considerably by type of patient, associated clinical conditions, and environment in which the infection has developed [3].

Considering the expected few availabilities of new antibiotics in the coming years and the further increase of bacterial resistance [4,5] to the current families of antibiotics [6], the identification of new therapies for the treatment of UTIs becomes an urgent need [7].

In this scenario, using nutraceuticals or phytotherapy to alleviate symptoms related to UTIs and decrease the rate of symptomatic recurrences represents a promising alternative.

The use of dietary supplements with positive and synergistic effects on the urinary tract prevents the onset of UTIs and lightens the economic and social burden on the national health system caused by these types of infections.

Currently, the main non-antibiotic approaches to managing UTIs are represented by therapeutic botanicals, which have shown lower side effects, more patient approval, and lower cost. Diuretics such as Solidago spp herb, the root of Levisticum officinale, the fruit of Petroselinum *Crispus*, and Urtica dioica can increase urine volume, thus favoring the excretion of potential threats. Antiseptic and anti-adhesive herbs such as Arctostaphylos uva-ursi (ursi grape), Juniperus spp (Juniper) leaves, and Vaccinium macrocarpon (cranberry) fruits have shown positive effects in protecting against acute and chronic UTIs, thanks to their capacity of facilitating the excretion of antimicrobial compounds, which can directly kill microbes or interfere with their adhesion to epithelial cells [8]. Furthermore, the antimicrobial properties of berberine can be found in the roots of Mahonia aquifolium (Pursh) Nutt. (Oregon grape) (Berberidaceae) and Hydrastis *Canadensis* L. (Goldenseal) (Ranunculaceae) suggest the powerful role of this compound in fighting bacterial infections, thanks to its ability to prevent the adhesion of bacteria to the host cell [9].

Moreover, the use of probiotics has also been considered for the prevention of UTIs. A beneficial effect has been observed for Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus reuteri, Bifidobacterium bifidum, and Bifidobacterium lactis [10,11].

It has been shown that Lactobacilli are able to prevent the adherence, growth, and colonization of uropathogenic bacteria [12] and have a strong inhibitory effect on E. coli [13], responsible for causing the majority of UTIs [14].

In this context of increasing interest in antibiotic-free protective approaches to manage UTIs [15], the present study explores the development of a supplement with unique characteristics and undoubted efficiency and effectiveness, which would fill the gap between the drug and the totally natural treatment [16].

As part of the study, the determination of a food supplement's *in vitro* activities to treat UTIs was investigated [17]. “UTIVAL” contains D-mannose [18] whose anti-adhesive properties on the urinary tract are well-known in the literature [19], palmitoylethanolamide [20], an endocannabinoid-like lipid mediator with proven antiinflammatory, analgesic, antimicrobial, and immunomodulatory properties, HibCyn® (hibiscus extract), a unique composition of Hibiscus flower derived anthocyanins and hydroxyl acids with anti-hypertensive and antioxidant properties able to prevent UTIs [14] N-acetylcysteine [21], known as an for its antioxidant, mucolytic and antiinflammatory properties [22] and microencapsulated Lactobacillus rhamnosus LR04 already known for its properties on gastrointestinal disorders [23].

2. MATERIALS AND METHODS

The activity of **the "UTIVAL"** product was evaluated in terms of:

- **Antimicrobial activity**, according to the CLSI M07 method "Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically" [24]. For this purpose, the MIC (Minimum Inhibitory Concentration) [25] values of the compound as a whole, of D-mannose alone, and of fosfomycin were determined, compared to the main microorganisms responsible for UTIs, specifically *Escherichia coli, Klebsiella pneumoniae* [26] and *Enterococcus faecium.*

- **Antibiofilm activity**, in which the ability of the product as a whole and fosfomycin, to inhibit the formation of biofilms [27] produced by the three microorganisms responsible for UTIs was evaluated. The test was performed by treating the microorganisms with 8 scalar concentrations of compounds chosen to be lower than the MIC value determined in the previous test. Biofilm formation was visualized by staining with Crystal Violet dye, penetrating bacterial cells, and staining them purple.

- **Cytotoxic and antiinflammatory activity** [28]: the effect of the product as a whole was evaluated on the viability of a cell line derived from the human bladder (HT1376) [29] by exploiting the redox reactions of compound 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), which forms purple crystals in the presence of viable cells. The ability of the product to inhibit or reduce the inflammatory state induced by the proinflammatory cytokine TNFα in the same cells [30,31] was also evaluated. This treatment leads to the production of Interleukin 8 (IL-8), which is responsible for the "recruitment" of white blood cells to the inflammation site. The levels of IL-8 are directly proportional to the intensity of the inflammation itself. The six product concentrations used in the antiinflammatory test were chosen based on the results of the cell viability test.

Since the effectiveness of these molecules is widely demonstrated in the literature, the synergy between them is also important. Just as more and more supplements are used in medicine for preventive purposes, obtaining effective results, and as adjuvant therapy [18].

For this purpose, the synergistic activity, in which the potential synergy between the product as a whole and fosfomycin was evaluated.

The test was performed according to the protocol called "broth microdilution checkerboard method" [32], or "checkerboard method," which allows evaluation of the inhibiting effect of two agents simultaneously by crossing serial dilutions of the same. Specifically, eight concentrations of product and six concentrations of fosfomycin were crossed. Biofilm formation was visualized by staining with Crystal Violet dye. The synergistic effect was evaluated in both inhibition and eradication.

RNAse-free water was obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). iScript cDNA synthesis kit and iQ SYBR Green supermix (2X) kit were obtained from Bio-Rad (Hercules, California, USA). NQO1, GSTA3, GSTM1, GSTM3, GSTP, SOD3, HO-1, GCLC, and GAPDH primers for qPCR were synthesized by Vivantis Technologies (Oceanside, CA, USA). BHA and isopropyl alcohol were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Chloroform was purchased from Merck KGaA (Darmstadt, Germany). Ethanol was purchased from BDH chemicals (Radnor, PA, USA).

2.1. Microbial Strains

The microorganisms used in the tests were the following:

- *Klebsiella pneumoniae* DSM 30104
- *Enterococcus faecium* DSM 20477
- *Escherichia coli* DSM 1576

The bacteria listed above [33–35] were revitalized starting from the working cell bank stored at -80° C for each experiment performed. The microorganisms were inoculated at 4-7 x 10^5 CFU/ml concentrations. The tryptic soy broth (Condalab) was used for *Escherichia coli* and the tryptic soy broth supplemented with yeast extract (Condalab) 3 g/l was used for *Enterococcus faecium,* and *klebsiella pneumoniae*. The cultures were incubated at 37°C for 24 hours.

2.2. Food Supplement (Product as a Whole)

The food supplement (produced in its entirety) was formulated as follows:

- 1500 mg of D-mannose
- 200 mg of hibiscus
- 300 mg of N-acetylcysteine (NAC)
- 300 mg of palmitoylethanolamide (PEA)

The product was dissolved in TSB/TSYEM sterile medium and then used to measure MIC, MBIC, and MBEC.

2.3. Minimum Inhibitory Concentration (MIC)

The assay for determining the minimum inhibitory concentration [36] was carried out in 96-well plates by testing 12 concentrations (serial dilutions) of the compound. A volume of 200 µL of the sample prepared at 2x concentration of the maximum test concentration was dispensed into the first well, and 100 µL of the medium was added into the subsequent wells by performing serial 1: 2 dilutions up to the 12th well. Finally, 100 µL of each organism was inoculated into each well at a concentration of 10^5 CFU/ml. Two antibiotics were used as controls: Fosfomycin (Sigma) [37] and Ceftriaxone (Sigma) [38], at different final concentrations based on the microorganism, used. Ceftriaxone against *E. coli* and *K. pneumoniae* was tested in the concentration range of 5.12-0.0025 µg/ml and from 512 to 0.25 µg/ml against *E. faecium*. Fosfomycin was tested between 1000 and 4.8 µg/ml for *E.coli* and *E. faecium* and between 20000 and 0.7 µg/ml for *K. pneumoniae*. Both antibiotics were dissolved in sterile water and sterilized with 0.22 µm filters. A growth and sterility check of the medium and product were performed. The antimicrobial activity of D-mannose was also evaluated individually. The plates thus prepared were incubated at 37° C for 24h, and then the results were observed.

2.4. Minimum Inhibitory Concentration of Biofilm (MBIC)

Forty-eight-well plates were used for the determination of MBIC [39]. A volume of 200 µL of the sample prepared at a concentration 2x of the maximum test concentration was dispensed into the first well and 100 µL of medium was added into the subsequent wells by performing serial 1: 2 dilutions up to the 8th well. Finally, 100 µL of each organism at a concentration of 10^5 was inoculated into each well. The inhibition activity of the fosfomycin biofilm at a concentration lower than the MIC value was also evaluated, and sterility and growth controls were performed for each microorganism. The plates were incubated at 37° C for 24 hours, then the supernatant was removed together with the planktonic bacteria not adhering to the plate.

The biofilm was then washed three times with 1x PBS and incubated at 60° C for 2 hours to fix the cells before staining crystal violet. Staining with crystal violet was performed to evaluate the product's and fosfomycin's effect on the biofilm's formation. In each well, 50 µL of crystal violet dissolved in deionized water was dispensed, after 5 minutes of incubation at room temperature each well was finally washed three times with 1x PBS. The biofilm inhibition activity was visualized under a stereomicroscope and a phase contrast optical microscope. The final concentrations for fosfomycin are 156,3 µg/ml for *Escherichia coli*, 322 µg/ml for *Enterococcus faecium*, and 2500 µg/ml for *Klebsiella pneumoniae*. The final concentration of the product is 100 g/l for all microorganisms in the test.

2.5. Minimum Biofilm Eradication Concentration (MBEC)

Forty-eight-well plates were used to determine the minimum eradication concentration MBEC of the biofilm [40]. The microorganisms were incubated for 72 h at 37° C to allow the formation of biofilms. Once the biofilms were formed they were treated with fosfomycin and the product to eradicate the biofilm. In particular, after the formation of biofilm, the supernatant was aspirated and replaced with solutions with a scalar concentration of product or fosfomycin. The plates thus prepared were incubated for 24 h at 37° C. At the end of the incubation time, the supernatant present in each well was removed together with the non-adherent bacteria to the plate. After washing, the plates were incubated at 60° C for 2 hours to fix the cells before staining with crystal violet. Staining with crystal violet was performed to evaluate the product's and fosfomycin's effect on the biofilm's removal. In each well, 50 µL of crystal violet dissolved in deionized water was dispensed, after 5 minutes of incubation at room temperature each well was washed three times with 1x PBS. The eradication activity of the biofilm was visualized through microscopic observation.

The fosformycin starting concentration was 30000 µg/ml for Escherichia coli and 3000 µg/ml for the other two microrganismis. The product was used starting from 50 g/l for all microorganisms.

2.6. Synergy in Inhibition of the Biofilm

Forty-eight-well plates were used to determine the synergy [41]. A volume of 200 µL of the total product

prepared at a concentration 2x of the maximum test concentration was dispensed into the eighth column and 200 µL of medium added into the subsequent wells by performing serial 1: 2 dilutions up to the 2nd well of each row. Subsequently, 200 µL of fosfomycin at a concentration 2x the MIC value (different for each organism) was dispensed in the first row, and serial dilutions were performed on the columns up to the penultimate row.

Finally, 200 µL of each microorganism at a concentration of 10^5 CFU/ml was inoculated into each well.

The plates were incubated at 37° C for 24 hours, then the supernatant was removed together with the planktonic bacteria not adhering to the plate. The biofilm was then washed three times with 1x PBS and incubated at 60° C for 2 hours to fix the cells before staining crystal violet. Staining with crystal violet was performed to evaluate the products' effect on the biofilm formation. In each well, 100 µL of crystal violet dissolved in deionized water was dispensed, after 5 minutes of incubation at room temperature each well was finally washed three times with 1x PBS.

The biofilm inhibition activity was visualized under a stereomicroscope and a phase contrast optical microscope. The product was tested from 100 g/l to 0,78 g/l and the D-mannose from 65 to 0,51 g/l.

2.7. Synergy in Biofilm Eradication

Forty-eight-well plates were used to determine the synergy in eradication [42]. A volume of 200 µL of organism was inoculated into all wells at a concentration of 10^5 CFU/ml. Microorganisms were incubated for 24 h at 37° C for biofilm formation. Once formed, the biofilm was treated with fosfomycin and the product for its eradication. In particular, the supernatant was aspirated and replaced with product and fosfomycin scalar solutions. Combinations of product and fosfomycin were prepared to start from 50 g/l for the whole product and 6000 µg/ml for fosfomycin. In particular, a volume of 200 µL of product in full 100 g/l was dispensed in the eighth column and 200 µL of medium added in the subsequent wells by performing serial dilutions 1: 2 up to the 2nd well of each row. Subsequently, 200 µL of fosfomycin at a concentration of 6000 µg/ml was dispensed in the first row, and 1: 2 serial dilutions were performed on the columns up to the penultimate row. At the end of the incubation time with the compounds, the supernatant present in each well was removed along with the non-adherent bacteria, and after washing, the plates were incubated at 60° C for cell fixation before staining with crystal violet. Staining with crystal violet was performed to evaluate the product's and fosfomycin's effect on the biofilm's removal. In each well, 100 µL of crystal violet dissolved in deionized water were dispensed, after 5 minutes of incubation at room temperature each well was washed three times with PBS. The eradication activity of the biofilm was visualized by microscopic observation of the colored biofilm.

2.8. Cell Lines and Culture Conditions

Eukaryotic cell experiments were performed on HT1376 human bladder epithelial cells. Unless specified, cells were cultured at 37° C and 5% CO2 in complete Eagle's Minimum Essential Medium (EMEM with 1% penicillin / streptomycin, 1% non-essential amino acids, 2 mM L-glutamine), supplemented with 10% fetal bovine serum (FBS).

2.9. Cell Viability Assay

HT1376 cells at >90% confluence were collected and diluted to 10^5 cells/ml concentration. 100 µL of this dilution (10^4 cells) was aliquoted into each well of a sterile 96-well plate, which was incubated overnight at 37° C and 5% CO2. At the end of the incubation, the medium was removed and replaced with 100 µL of complete EMEM supplemented with 1% FBS and containing the product at the following concentrations (in mg/ml): 5 - 2.5 - 1.25 - 0.625 - 0.313 - 0.156 - 0.078 - 0.039 - 0.02 - 0.01 - 0.005 - 0.0024. Sodium dodecyl sulfate (SDS) at a concentration of 1 mg/ml was used as a positive control. Cells were incubated for 24 hours at 37° C and 5% CO2. At the end of the treatment, MTT was added [43] to each well at the final concentration of 0.5 mg/ml, and the incubation was prolonged for another two hours. The culture medium was then removed from the wells, and the reduced MTT crystals were solubilized with the addition of 100 µL of dimethyl sulfoxide (DMSO). After an incubation of 30 minutes at room temperature, each sample's absorbance at 595 nm was measured with an Infinite M NANO + plate reader (Tecan) [43]. Cell viability is expressed as a percentage of untreated cells' absorbance at 595 nm (NT).

2.10. Determination of the Optimal Amount of TNFα

HT1376 cells at >90% confluence were collected and diluted to 10^4 cells/ml concentration. 1 ml of this dilution (10^4 cells) was aliquoted into each well of a sterile 48-

well plate, which was incubated at 37° C and 5% CO_2 for 48h. At the end of the incubation, the medium was removed and replaced with 1 ml of complete EMEM without FBS, containing TNFα at the following concentrations [44] (in µg/ml): 0.2 - 0.1 - 0.05 - 0.025. The treatment was carried out at 37° C and 5% CO_2 for a total of 6 hours; aliquots of 200 µL of the culture medium were taken at the beginning of the treatment (T0) and after 1, 2, 4, and 6 hours, and then analyzed by ELISA test.

2.11. Antiinflammatory Treatment

HT1376 cells at >90% confluence were collected and diluted to 10^4 cells/ml concentration. 1 ml of this dilution (10^4 cells) was aliquoted into each well of a sterile 48-well plate, which was incubated at 37° C and 5% CO_2 for 48h. At the end of the incubation, the medium was removed and replaced with 500 µL of complete EMEM without FBS, containing 0.2 µg/ml of TNFα alone or with the product at the following concentrations (in mg/ml): 0.625 - 0.313 - 0.156 - 0.078 - 0.039 - 0.02. The antiinflammatory drug Dexamethasone [45] was used as a positive control at a concentration of 0.1 mg/ml. The treatment was carried out for 6 hours, at the end of which 200 µL aliquots of the culture medium were taken and then analyzed by ELISA test.

2.12. ELISA [46]

IL-8 detection was performed on 100 µL of each stored aliquot using the "Human IL-8 Uncoated ELISA Kit" (Invitrogen), according to the protocol provided by the manufacturer. The quantification was carried out by setting up a calibration line with different concentrations of a standard solution of IL-8 supplied with the kit. The antiinflammatory effect is expressed as a percentage change in the concentration of IL-8 compared to cells treated with TNFα alone.

3. RESULTS AND DISCUSSION

3.1. MIC

As shown in Figure **1**, the product at the concentrations under test does not appear to have a complete inhibition effect on the growth of the microorganisms tested, as it is possible to see cell growth (pellets and increased turbidity) in all wells. This result is not unexpected as the main activity of the compounds contained in the product is to inhibit the formation of biofilms and not bacterial replication.

MIC values were determined for fosfomycin, which was different for each microorganism. *E. coli* was the most sensitive microorganism to this type of antibiotic; the MIC value is 19.53 µg/ml. *K. pneumoniae* was the most resistant microorganism, having a MIC value of 5000 µg/ml. For *E. faecium* the MIC value for fosfomycin was 78.13 µg/ml.

The antimicrobial activity was also evaluated on D-mannose. As with the product, even D-mannose in the range of concentrations tested did not exhibit antimicrobial activity, as cell growth is visible in all wells.

3.2. MBIC

To evaluate the inhibition effect of biofilm formation, the microorganisms were incubated with scalar concentrations of the product; following the incubation time, the results obtained by staining with crystal violet were analyzed.

As shown in Figure **2**, the product has an inhibitory activity on the formation of biofilm.

As highlighted by the colors, the biofilm increased when the concentration of the product decreased.

At a concentration of 1.56 g/l of the product, it is possible to appreciate the reduction of the biofilm already with a macroscopic visualization.

Subsequently, photos were acquired under the optical microscope with a 400x magnification to better evaluate the status of the biofilm.

In the following image (Figure **3**), it is possible to observe in microscopic detail the state of the biofilm following the treatments and, in particular, the form of the bacterial structures.

The destructuring effect and non-presence of the bacterial biofilm reticulum following the crystal violet coloring.

For all microorganisms at a concentration of 1.56 g/l of product the biofilm was inhibited entirely; only in *E. coli* was a minimal number of attached cells present. At a concentration of 0.78 g/l, the biofilm inhibiting effect was evident, especially on *E. faecium* and *K. pneumoniae*.

Fosfomycin was tested for each organism at different concentrations based on the MIC value.

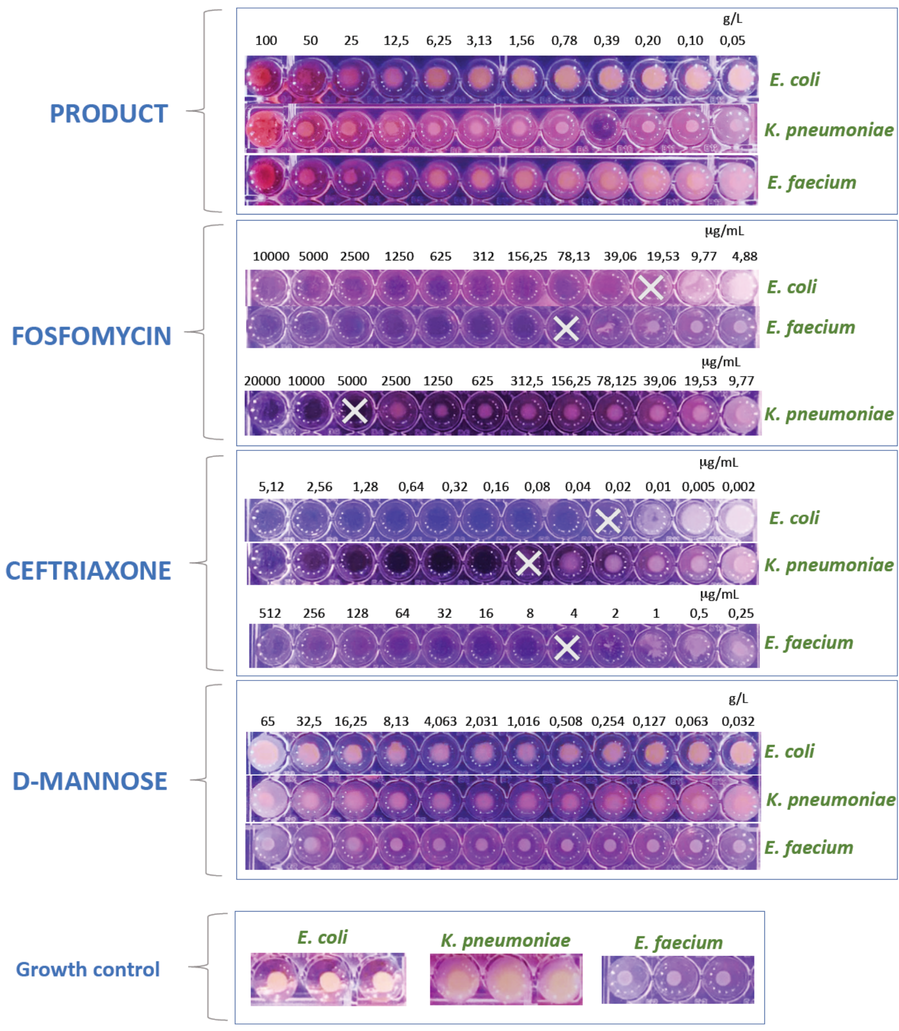

Figure 1: MIC test results. Tests were performed on 96-well plates. Photos show the microbial biomass pellet, marked with "X" at the first concentration where no growth is visible.

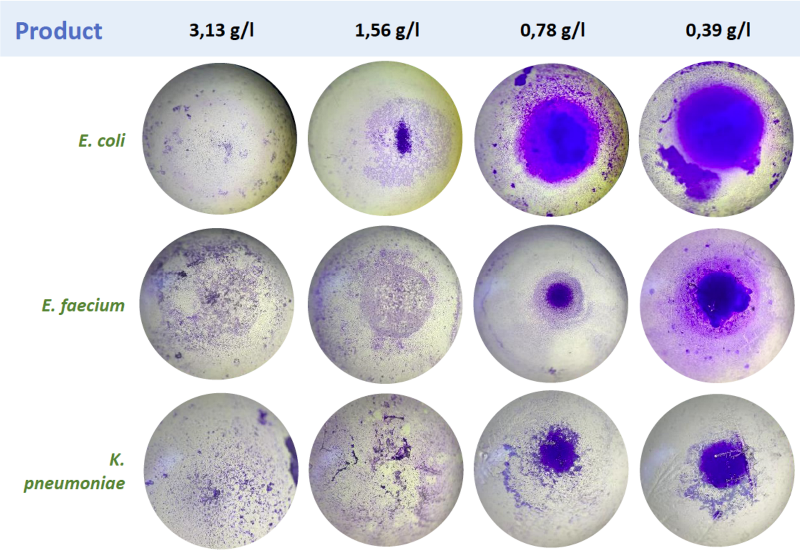

Figure 2: Minimum inhibitory concentration of biofilm (MBIC) - Observation under the stereomicroscope. Overall view of the biofilm of the various microorganisms treated with the product following staining.

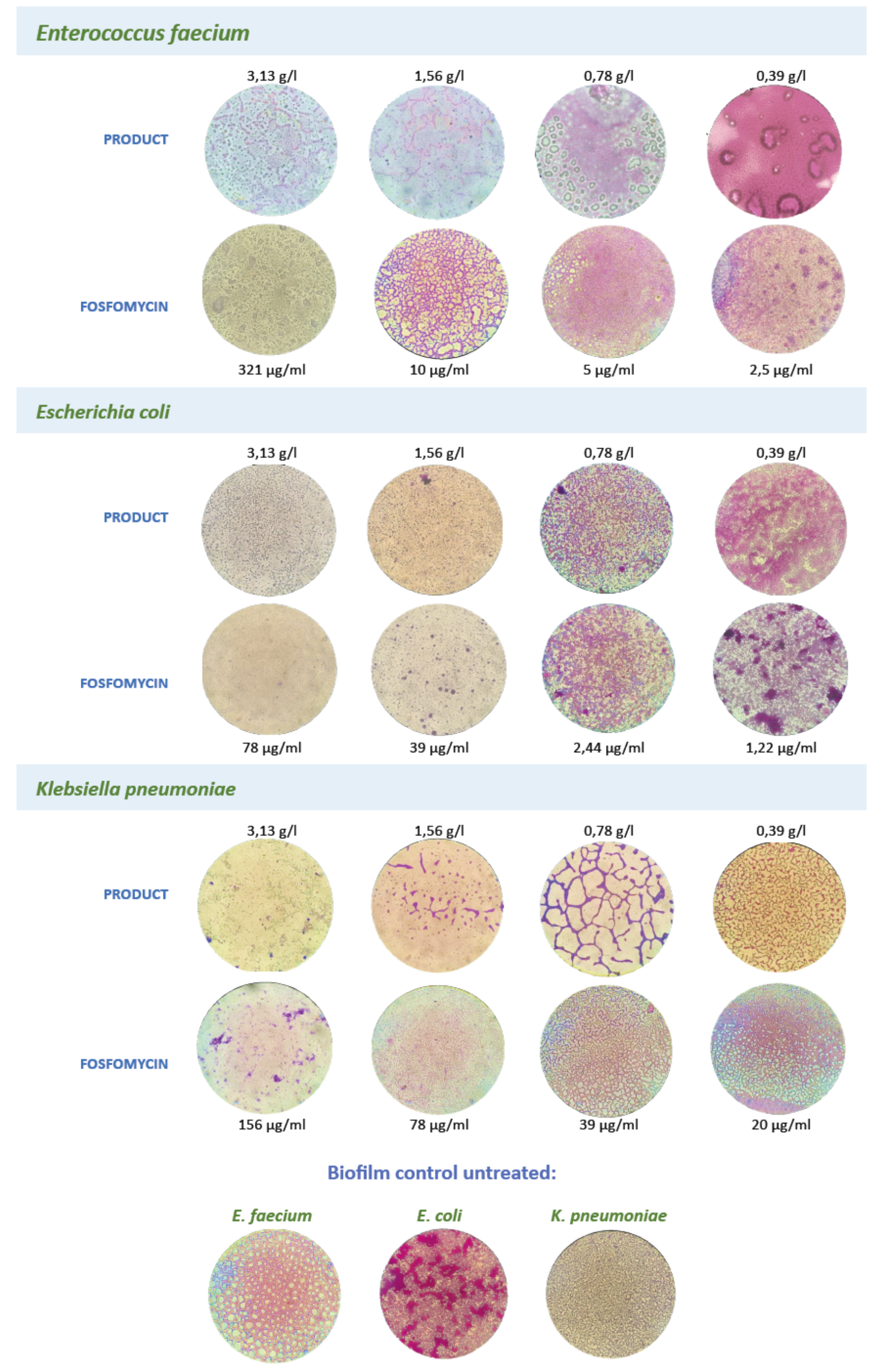

Figure 3: *E. faecium, E.coli, K. pneumoniae* MBIC test, magnification 400x, whole product, and fosfomycin.

On *K. pneumoniae* and *E. coli*, fosfomycin had a biofilm inhibiting effect at concentrations greater than or equal to 78 µg/ml, while on *E. faecium*, a concentration of at least 321 µg/ml was required.

3.3. MBEC

The following image (Figure **4**) shows the results of the biofilm eradication test by microscopic observation. Also, in this case, it is evident that as the concentration of the product increased, the bacterial biofilm structure decreased for the three microorganisms tested.

The product as a whole had an action on the eradication of the biofilm formed by *K. pneumoniae* and *E. faecium* at a concentration of 0.78 g/l and on *E. coli* at a concentration of 6.25 g/l. Fosfomycin did not appear to have a biofilm eradicating effect at any of the

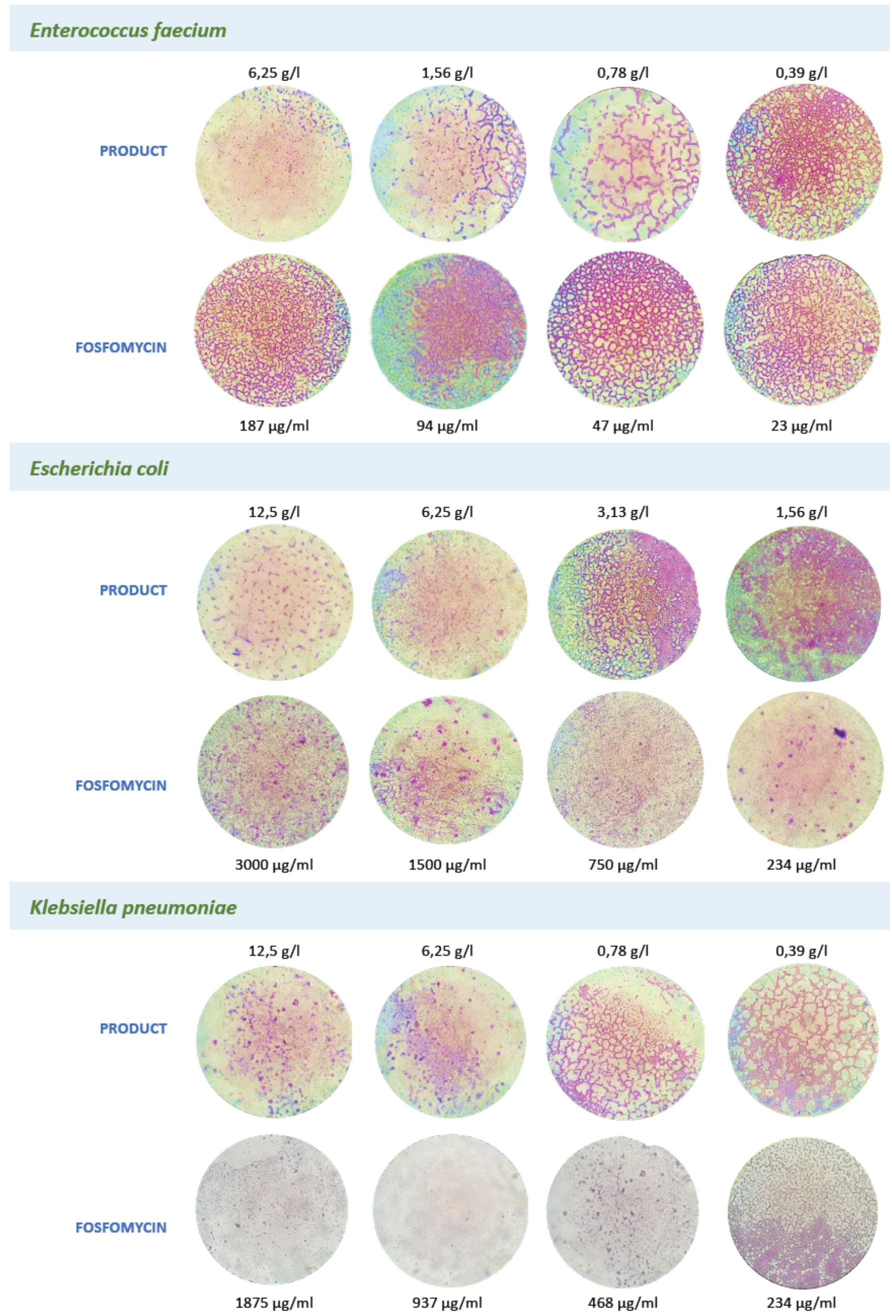

Figure 4: *E. faecium, E. coli, K. pneumoniae* MBEC test, magnification 400x, full product, and fosfomycin.

concentrations tested for *E. faecium* and *E. coli* while it had activity on *K. pneumoniae* at concentrations greater than 468 µg/ml.

Based on the data obtained, it is possible to conclude that the "UTIVAL" food supplement has an action on both the inhibition and eradication of the biofilm formed by the microorganisms *K. pneumoniae*, *E. faecium*, and *E. coli*. In the *in vitro* tests performed in this project, the activity of the product was already effective at a concentration of 0.78 g/l.

3.4. Cell Viability

As shown in Figure **5**, the product did not appear to have a toxic effect on cell viability, at least up to the concentration of 0.625 mg/ml. The data obtained at higher concentrations showed an increase in cell viability as the product itself leads to the reduction, and therefore the coloration, of MTT.

Therefore, six decreasing concentrations of product starting from 0.625 mg/ml were used for the antiinflammatory assay.

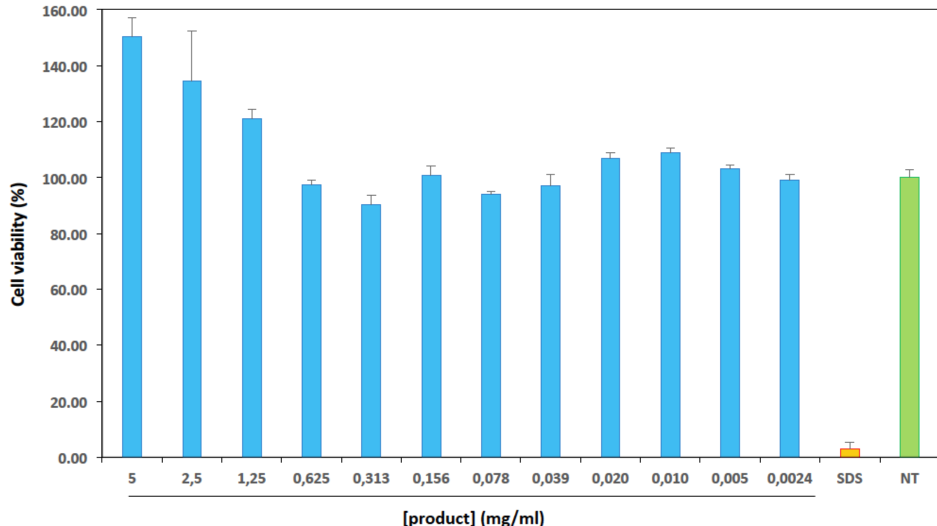

Figure 5: Viability assay. HT1376 cells were treated with different concentrations of product for 24 h. Cell viability was determined by measuring the absorbance at 595 nm after reaction with MTT. The viability values are expressed as a percentage of the absorbance of untreated cells (NT). Sodium dodecyl sulfate (SDS) 1 mg/ml was used as a positive control.

The cell viability was determined by measuring the absorbance at 595 nm after reaction with MTT. The vitality values are expressed as a percentage of the absorbance of untreated cells. The SDS 1 mg/ml was used as a positive control.

3.5. Synergy in Inhibition of the Biofilm

The data collected show that the biofilms of the microorganisms *E. coli* and *E. faecium* in some product / fosfomycin combinations were reduced compared to the corresponding condition with the product alone at the same concentration.

The FIC indexes were then calculated for these wells according to the following formula:

$$FiC\ index = \frac{MBIC\ fosfomycin\ in\ combination}{MBIC\ fosfomycin} + \frac{MBIC\ product\ in\ combination}{MBIC\ product}$$

The association is synergistic if the FIC index values are <1. When the FIC index assumes a value = 1, the compounds do not interact with each other, while if the value is> 1, the association of the compounds is antagonistic.

Figure **6** compares the status of the biofilm treated with the product alone and at the same concentration in combination with fosfomycin.

Figure **7** represents the matrices of concentrations performed for the determination of anti-biofilm synergism in which the combinations of compounds considered for the synergistic effect are highlighted.

The corresponding FIC index was calculated for the combinations considered most significant.

For the microorganism *E. coli*, there are 2 combinations with a synergistic effect, while for *E. faecium*, there are three combinations.

No combinations of product and fosfomycin relevant for a synergistic effect of the compounds were observed for the microorganism *K. pneumoniae*.

3.6. Synergy in Biofilm Eradication

The data collected showed that the product has a synergy effect with fosfomycin in eradicating the biofilm of *E. faecium*.

In particular, for this microorganism, a combination of product and fosfomycin resulted synergy as the biofilm treated with the two products blended together is more eradicated than the treatments with the single products.

This combination is considered synergistic since the FIC index is less than 1 according to the following formula:

$$FiC\ index = \frac{MBEC\ fosfomycin\ in\ combination}{MBEC\ fosfomycin} + \frac{MBEC\ product\ in\ combination}{MBEC\ product}$$

The association is synergistic if the FIC index values are <1. When the FIC index assumes a value = 1, the compounds do not interact with each other, while if the value is> 1, the association of the compounds is antagonistic.

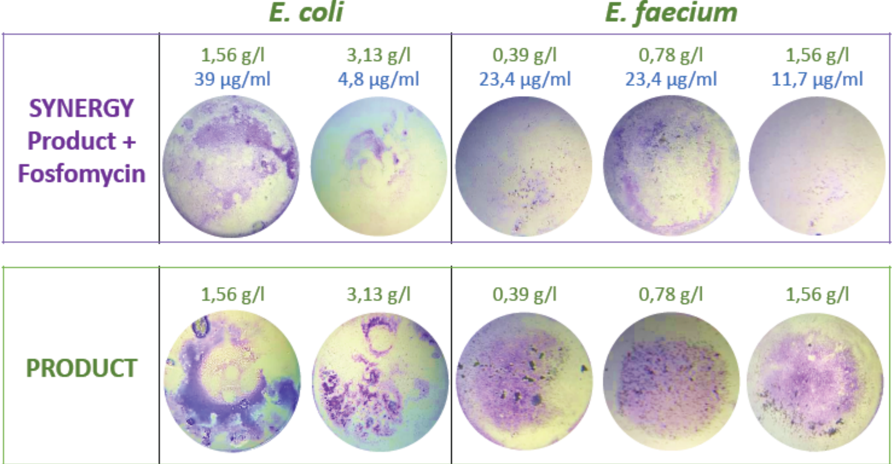

Figure 6: On the left: *E. coli* biofilm inhibition with the product in single-use at 1.56 and 3.13 g/l (green Square) and in combination with fosfomycin respectively at 39 and 4.8 µg/ml (purple Square). On the right: *E. faecium* biofilm inhibition with the product in single-use at 0.39, 0.78, and 1.56 g/l (green Square) and combined with fosfomycin, respectively at 23.4, 23.4, and 11.7 µg/ml (purple Square).

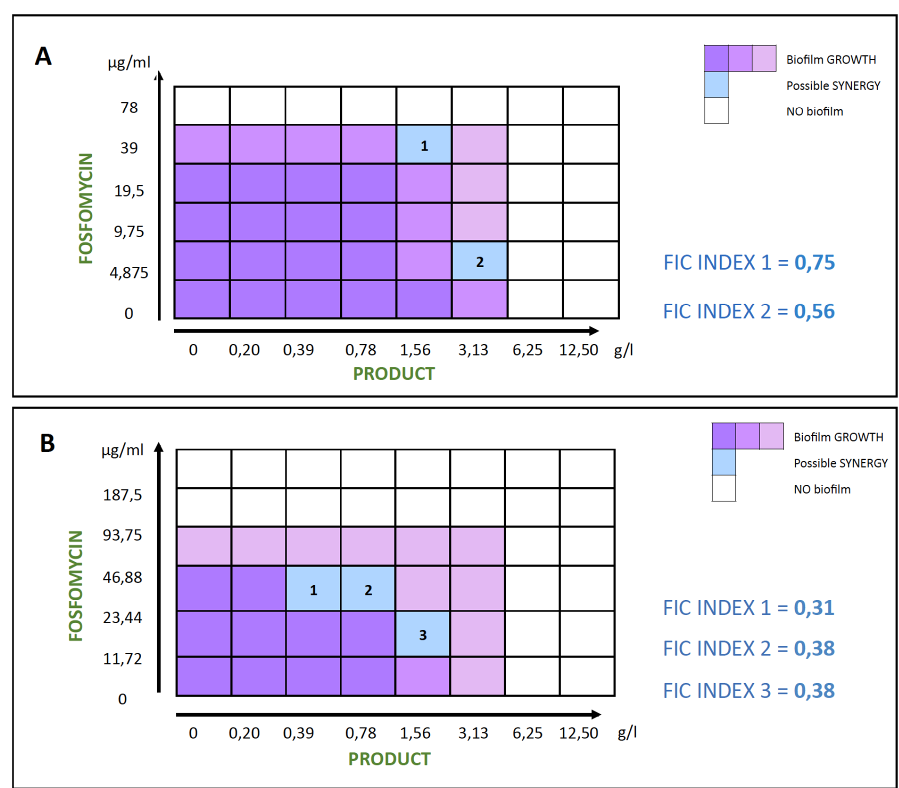

Figure 7: Schematic representation of the "chessboard method" used for the evaluation of the inhibition synergy against *E. coli* (**A**) and *E. faecium* (**B**).

Figure **8** on the left reports the state of the *E. faecium* biofilm treated with the product alone, fosfomycin alone, and with both compounds in combination at the same concentrations.

Figure **8** on the right shows graphically the matrix of concentrations performed for the determination of the eradication synergism of the biofilm in which the combination of compounds considered for the synergistic effect is highlighted. The FIC index value is also reported for this combination.

3.7. Determination of Treatment with TNFα

The optimal TNFα concentration was determined by monitoring the amount of IL-8 produced by HT1376

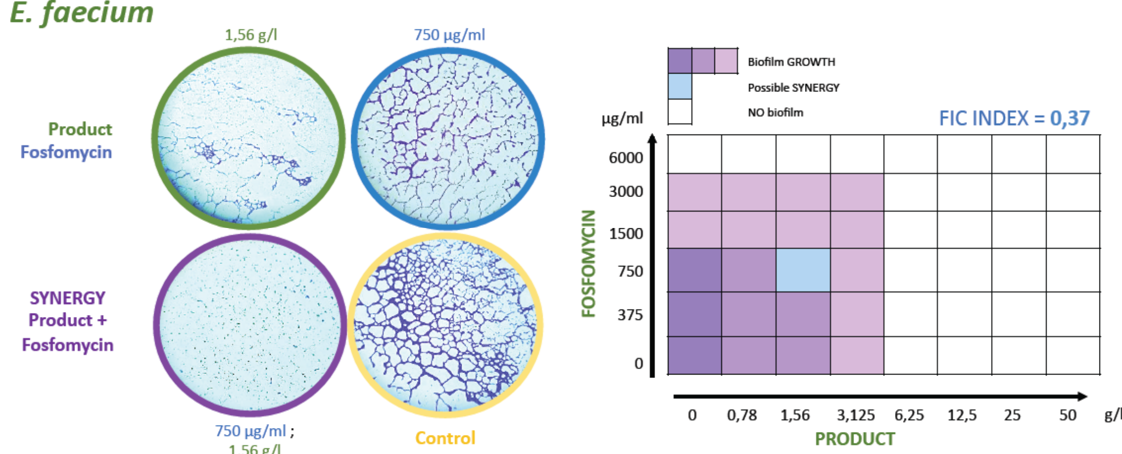

Figure 8: On the left: synergistic efficacy of the product and fosfomycin in eradicating the *E. faecium* biofilm. Microscopic observation at 400x magnification. In green, the activity of product alone at a concentration of 1.56 g/l, in blue that of fosfomycin alone at 750 µg/ml; in purple, the synergistic combination of product and fosfomycin at the same concentrations; and in yellow the untreated control. On the right is a schematic representation of the "chessboard method" used to evaluate the eradication synergy against *E. faecium.*

cells. For this, the cells were treated with different amounts of TNFα, and the production of the chemokine in each treatment was followed over time (Figure **9**).

As can be seen, treatment with any amount of TNFα causes overexpression of IL-8 compared to untreated cells (NT). In general, it seems that at least 4 hours of treatment are required to observe appreciable differences in the production of IL-8, but the greatest effect was observed after 6 hours. Treatment with 0.025 - 0.05 or 0.1 µg/ml of TNFα seems to give similar results, with a production of 40 - 45 pg/ml of IL-8 after 6 hours of treatment.

Increasing TNFα to 0.2 µg/ml leads to greater interleukin production, with a maximum of approximately 63.5 pg/ml after 6 hours of treatment.

To be able to appreciate as accurately as possible the variations in IL-8 production due to treatment with the product, based on these data, it was decided to treat the cells with 0.2 µg/ml TNFα for 6 hours.

3.8. Antiinflammatory Effect

The antiinflammatory effect of the product was evaluated by measuring its ability to reduce the amount of IL-8 produced by HT1376 cells following treatment with TNFα.

For this purpose, the cells were treated with the proinflammatory cytokine TNFα under the previously developed conditions, in the product's absence or presence. Then, the quantity of IL8 expressed was measured. The antiinflammatory drug Dexamethasone

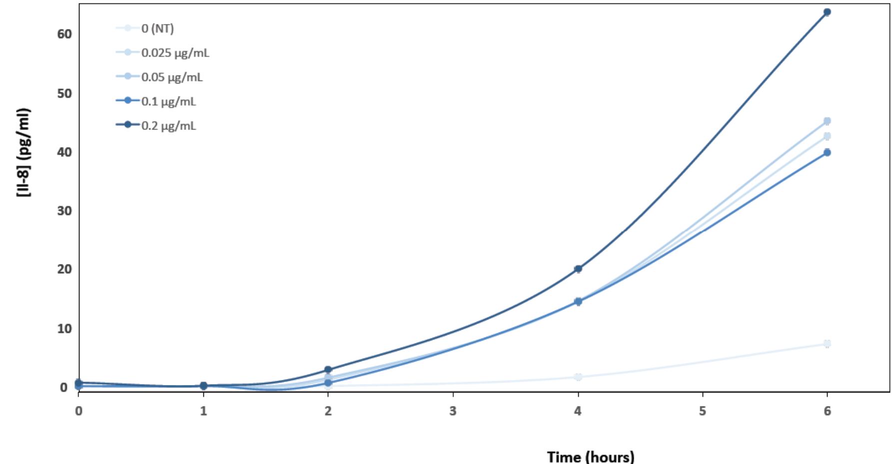

Figure 9: Treatment with different amounts of TNFα. HT1376 cells were treated with varying concentrations of TNFα for up to 6 h. ELISA quantified IL-8 production over time.

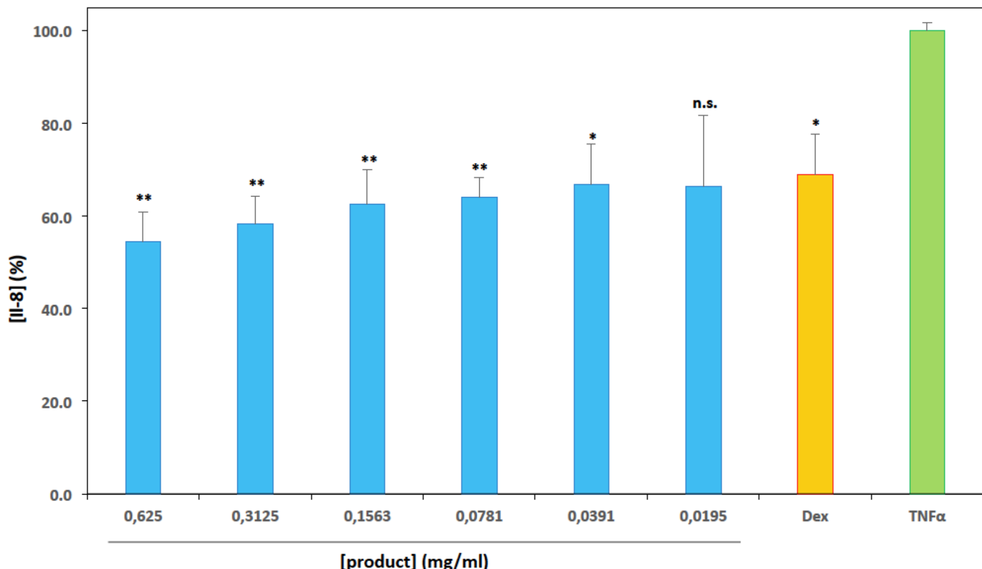

Figure 10: Antiinflammatory effect. HT1376 cells were treated with 0.2 µg/ml TNFα in the absence or presence of the product. The values are expressed as a percentage of IL-8 produced by cells treated with TNFα alone. Dexamethasone was used as a positive control at a 0.1 mg/ml concentration. Statistical analysis was carried out using two-tailed t-tests: n.s. = not significant; * = p <0.05; ** = p <0.01.

Table 1: Antiinflammatory Effect. The Values are Expressed as a Percentage of IL-8 Produced by cells Treated with TNFα Alone

Controls	Product (mg/ml)						
TNFα (0.2 µh/ml	100.00	0.625	0.313	0.156	0.078	0.039	0.02
Dex (0.1 mg/ml)	69.0	54.5	58.4	62.6	64.2	66.9	66.4

at a concentration of 0.1 mg/ml was used as a positive control. As shown in Figure **10** and Table **1**, the product appears to be able to reduce the overexpression of IL-8 induced by TNFα, in a dose-dependent manner, up to a concentration of 0.04 mg/ml: at this value, IL-8 produced is about 33% less than that produced by cells treated with TNFα alone, an effect comparable to that obtained with Dexamethasone at a concentration of 0.1 mg/ml (31%).

The greatest effect was obtained with a product concentration of 0.625 mg/ml, with a 45.5% reduction in the production of IL-8.

As shown in the results, it can be stated that the product has an inhibiting effect on inflammation mediated by TNFα, in a range of concentrations between 0.04 and 0.625 mg/ml.

4. CONCLUSIONS

Although prophylactic antibiotics remain the preferred preventive treatment for recurrent UTIs, the increasing antimicrobial resistance and the demand for antibiotic-free protective approaches drive the development of non-antibiotic strategies. As previously mentioned, this study was aimed at determining the *in vitro* activity and effectiveness of a food supplement for the treatment of UTIs, a widespread condition caused by bacteria or occasionally fungus that can affect any part of the urinary tract, with a huge socio-economic impact both on patients and on healthcare structures.

Based on the results obtained, it is possible to affirm that the food supplement "UTIVAL" owns an inhibiting effect on the inflammation mediated by TNFα and has an action on both inhibition and eradication of the biofilm formed by the microorganisms *K. pneumoniae*, *E. faecium*, and *E. coli*.

Moreover, the food supplement as a whole and fosfomycin have a synergistic effect against the inhibition of the biofilm of microorganisms *E. coli* and *E. faecium*.

In particular, the synergistic effect in inhibition was most evidently observed in the following combinations:

- *E. coli*:

Total product 1.56 g / L + fosfomycin 39 µg/ml

Total product 3.13 g / L + fosfomycin 4.8 µg/ml

- *E. faecium*:

Total product 0.39 g / L + fosfomycin 23.4 µg/ml

Total product 0.78 g / L + fosfomycin 23.4 µg/ml

Total product 1.56 g / L + fosfomycin 11.7 µg/ml

Finally, it can be concluded that the whole product and fosfomycin have a synergistic effect in biofilm eradication against the microorganism *E. faecium* in the following combination of concentrations:

- *E. faecium*:

Total product 1.56 g/l + fosfomycin 750 µg/ml

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REFERENCES

[1] Foxman B. The epidemiology of urinary tract infection. Nature Reviews Urology 2010; 7: 653-60. https://doi.org/10.1038/nrurol.2010.190

[2] Griebling TL. Urologic diseases in america project: trends in resource use for urinary tract infections in women. Journal of Urology 2005; 173: 1281-7. https://doi.org/10.1097/01.ju.0000155596.98780.82

[3] Foxman B, Brown P. Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. Infectious Disease Clinics of North America 2003; 17: 227-41. https://doi.org/10.1016/S0891-5520(03)00005-9

[4] Sannathimmappa MB, Nambiar V, Aravindakshan R. Antibiotics at the crossroads - Do we have any therapeutic alternatives to control the emergence and spread of antimicrobial resistance? Journal of Education and Health Promotion 2021; 10: 438.

[5] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P & T : A Peer-Reviewed Journal for Formulary Management 2015; 40: 277-83.

[6] Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. The American Journal of Medicine 2002; 113: 5-13. https://doi.org/10.1016/S0002-9343(02)01054-9

[7] Mazzulli T. Resistance trends in urinary tract pathogens and impact on management. The Journal of Urology 2002; 168: 1720-2. https://doi.org/10.1016/S0022-5347(05)64397-2

[8] Yarnell E. Botanical medicines for the urinary tract. World Journal of Urology 2002; 20: 285-93. https://doi.org/10.1007/s00345-002-0293-0

[9] Das S. Natural therapeutics for urinary tract infections—a review. Future Journal of Pharmaceutical Sciences 2020; 6: 64. https://doi.org/10.1186/s43094-020-00086-2

[10] Forster CS, Hsieh MH, Cabana MD. Perspectives from the Society for Pediatric Research: Probiotic use in urinary tract infections, atopic dermatitis, and antibiotic-associated diarrhea: an overview. Pediatric Research 2021; 90: 315-27. https://doi.org/10.1038/s41390-020-01298-1

[11] Aragón IM, Herrera-Imbroda B, Queipo-Ortuño MI, Castillo E, del Moral JS-G, Gómez-Millán J, *et al*. The Urinary Tract Microbiome in Health and Disease. European Urology Focus 2018; 4: 128-38. https://doi.org/10.1016/j.euf.2016.11.001

[12] Hanson L, VandeVusse L, Jermé M, Abad CL, Safdar N. Probiotics for Treatment and Prevention of Urogenital Infections in Women: A Systematic Review. Journal of Midwifery & Women's Health 2016; 61: 339-55. https://doi.org/10.1111/jmwh.12472

[13] Beerepoot MAJ, Geerlings SE, van Haarst EP, van Charante NM, ter Riet G. Nonantibiotic Prophylaxis for Recurrent Urinary Tract Infections: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Journal of Urology 2013; 190: 1981-9. https://doi.org/10.1016/j.juro.2013.04.142

[14] Fraile B, Alcover J, Royuela M, Rodríguez D, Chaves C, Palacios R, *et al*. Xyloglucan, hibiscus and propolis for the prevention of urinary tract infections: results of *in vitro* studies. Future Microbiology 2017; 12: 721-31. https://doi.org/10.2217/fmb-2017-0015

[15] Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiology Spectrum 2016; 4. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

[16] Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature Reviews Microbiology 2015; 13: 269-84. https://doi.org/10.1038/nrmicro3432

[17] Stamm WE, Norrby SR. Urinary Tract Infections: Disease Panorama and Challenges. The Journal of Infectious Diseases 2001; 183: S1-4. https://doi.org/10.1086/318850

[18] Xia J, Yang C, Xu D, Xia H, Yang L, Sun G. Consumption of cranberry as adjuvant therapy for urinary tract infections in susceptible populations: A systematic review and meta-analysis with trial sequential analysis. PLOS ONE 2021; 16: e0256992. https://doi.org/10.1371/journal.pone.0256992

[19] Altarac S, Papeš D. Use of d-mannose in prophylaxis of recurrent urinary tract infections (UTIs) in women. BJU International 2014; 113: 9-10. https://doi.org/10.1111/bju.12492

[20] LoVerme J, la Rana G, Russo R, Calignano A, Piomelli D. The search for the palmitoylethanolamide receptor. Life Sciences 2005; 77: 1685-98. https://doi.org/10.1016/j.lfs.2005.05.012

[21] Kelly GS. Clinical applications of N-acetylcysteine. Alternative Medicine Review : A Journal of Clinical Therapeutic 1998; 3: 114-27.

[22] Allameh Z, Salamzadeh J. Use of antioxidants in urinary tract infection. Journal of Research in Pharmacy Practice n.d.; 5: 79-85.
https://doi.org/10.4103/2279-042X.179567

[23] Margiotta G, Ferretti S, Graglia B, Gatto A, Capossela L, Bersani G, *et al*. Effect of Lactobacillus reuteri LRE02-Lactobacillus rhamnosus LR04 combination and gastrointestinal functional disorders in an Emergency Department pediatric population. European Review for Medical and Pharmacological Sciences 2021; 25: 3097-104.

[24] Giacometti A, Cirioni O, Barchiesi F, del Prete MS, Fortuna M, Caselli F, *et al*. *In vitro* Susceptibility Tests for Cationic Peptides: Comparison of Broth Microdilution Methods for Bacteria That Grow Aerobically. Antimicrobial Agents and Chemotherapy 2000; 44: 1694-6.
https://doi.org/10.1128/AAC.44.6.1694-1696.2000

[25] Mann CM, Markham JL. A new method for determining the minimum inhibitory concentration of essential oils. Journal of Applied Microbiology 1998; 84: 538-44.
https://doi.org/10.1046/j.1365-2672.1998.00379.x

[26] Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, *et al*. Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. The Lancet Infectious Diseases 2013; 13: 785-96.
https://doi.org/10.1016/S1473-3099(13)70190-7

[27] Mitsuya Y, Kawai S, Kobayashi H. Influence of macrolides on guanosine diphospho-d-mannose dehydrogenase activity in Pseudomonas biofilm. Journal of Infection and Chemotherapy 2000; 6: 45-50.
https://doi.org/10.1007/s101560050049

[28] Al Zuhairi JJMJ, Jookar Kashi F, Rahimi-Moghaddam A, Yazdani M. Antioxidant, cytotoxic and antibacterial activity of Rosmarinus officinalis L. essential oil against bacteria isolated from urinary tract infection. European Journal of Integrative Medicine 2020; 38: 101192.
https://doi.org/10.1016/j.eujim.2020.101192

[29] Lee S-J, Park S-S, Cho Y-H, Park K, Kim E-J, Jung K-H, *et al*. Activation of matrix metalloproteinase-9 by TNF-α in human urinary bladder cancer HT1376 cells: The role of MAP kinase signaling pathways. Oncology Reports 2008.
https://doi.org/10.3892/or.19.4.1007

[30] Moazzezy N, Asadi Karam MR, Rafati S, Bouzari S, Oloomi M. A Synthetic Peptide 2Abz23S29 Reduces Bacterial Titer and Induces Proinflammatory Cytokines in a Murine Model of Urinary Tract Infection. Drug Design, Development and Therapy 2020; 14: 2797-807.
https://doi.org/10.2147/DDDT.S259937

[31] Gonzalez S. TNF-$alpha; $minus; 308A promoter polymorphism is associated with enhanced TNF-$alpha; production and inflammatory activity in Crohn?s patients with fistulizing disease. The American Journal of Gastroenterology 2003; 98: 1101-6.
https://doi.org/10.1016/S0002-9270(03)00134-5

[32] Rand KH, Houck HJ, Brown P, Bennett D. Reproducibility of the microdilution checkerboard method for antibiotic synergy. Antimicrobial Agents and Chemotherapy 1993; 37: 613-5.
https://doi.org/10.1128/AAC.37.3.613

[33] Chen M, Li Y, Li S, Tang L, Zheng J, An Q. Genomic identification of nitrogen-fixing *Klebsiella variicola*, *K. pneumoniae* and *K. quasipneumoniae*. Journal of Basic Microbiology 2016; 56: 78-84.
https://doi.org/10.1002/jobm.201500415

[34] Ng ZJ, Zarin MA, Lee CK, Phapugrangkul P, Tan JS. Isolation and characterization of Enterococcus faecium DSM 20477 with ability to secrete antimicrobial substance for the inhibition of oral pathogen Streptococcus mutans UKMCC 1019. Archives of Oral Biology 2020; 110: 104617.
https://doi.org/10.1016/j.archoralbio.2019.104617

[35] Pinto L, Poeta P, Radhouani H, Coelho C, Carvalho C, Rodrigues J, *et al*. Proteomic evaluation of Escherichia coli isolates from human clinical strains. Journal of Integrated OMICS 2011; 1.
https://doi.org/10.5584/jiomics.v1i1.20

[36] Ellner PD. The Inhibitory Quotient. JAMA 1981; 246: 1575.
https://doi.org/10.1001/jama.1981.03320140063033

[37] Falagas ME, Vouloumanou EK, Samonis G, Vardakas KZ. Fosfomycin. Clinical Microbiology Reviews 2016; 29: 321-47.
https://doi.org/10.1128/CMR.00068-15

[38] Richards DM, Heel RC, Brogden RN, Speight TM, Avery GS. Ceftriaxone A Review of its Antibacterial Activity, Pharmacological Properties and Therapeutic Use. Drugs 1984; 27: 469-527.
https://doi.org/10.2165/00003495-198427060-00001

[39] Gupta A. Original Research Article Biofilm Quantification and Comparative Analysis of MIC (Minimum Inhibitory Concentration) & MBIC (Minimum Biofilm Inhibitory Concentration) Value for Different Antibiotics against E. coli. vol. 4. 2015.

[40] Verma P. Minimum Biofilm Eradication Concentration (MBEC) Assay of Silver and Selenium Nanoparticles against Biofilm forming Staphylococcus aureus. Journal of Medical Science And Clinical Research 2017; 05.
https://doi.org/10.18535/jmscr/v5i4.77

[41] Okuda T, Kokubu E, Kawana T, Saito A, Okuda K, Ishihara K. Synergy in biofilm formation between Fusobacterium nucleatum and Prevotella species. Anaerobe 2012; 18: 110-6.
https://doi.org/10.1016/j.anaerobe.2011.09.003

[42] Namivandi-Zangeneh R, Sadrearhami Z, Bagheri A, Sauvage-Nguyen M, Ho KKK, Kumar N, *et al*. Nitric Oxide-Loaded Antimicrobial Polymer for the Synergistic Eradication of Bacterial Biofilm. ACS Macro Letters 2018; 7: 592-7.
https://doi.org/10.1021/acsmacrolett.8b00190

[43] Marks DC, Belov L, Davey MW, Davey RA, Kidman AD. The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. Leukemia Research 1992; 16: 1165-73.
https://doi.org/10.1016/0145-2126(92)90114-M

[44] Palin K, Bluthé R-M, McCusker RH, Levade T, Moos F, Dantzer R, *et al*. The type 1 TNF receptor and its associated adapter protein, FAN, are required for TNFα-induced sickness behavior. Psychopharmacology 2009; 201: 549-56.
https://doi.org/10.1007/s00213-008-1331-4

[45] Tsurufuji S, Sugio K, Takemasa F. The role of glucocorticoid receptor and gene expression in the antiinflammatory action of Dexamethasone. Nature 1979; 280: 408-10.
https://doi.org/10.1038/280408a0

[46] Clark MF, Lister RM, Bar-Joseph M. ELISA techniques, 1986; pp. 742-66.
https://doi.org/10.1016/0076-6879(86)18114-6