



Evaluation of reticulated gelatin-hibiscus-propolis against intestinal commensal species commonly associated with urinary tract infections

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from commensalism to pathogenicity of potentially uropathogenic strains are not well understood. With regard to *E. coli*, its structure is clonal and the strains are distributed among five main phylogroups (A, B1, B2, D and E) [8,9]. Strains of the various phylogroups differ in their phenotypic characteristics, in their ecological niches as well as in their propensity to cause disease [10]. Uropathogenic isolates are predominantly from the phylogenetic group B2 and take advantage of host behavior and susceptibility by employing virulence factors or fitness factors that facilitate bacterial growth, biofilm formation and persistence in the urinary tract [3,11–13]. These factors include mainly adhesins (e.g., type 1 fimbriae), immune-evasion-related determinants (e.g., polysaccharide capsules K1 and K5), iron- and zinc-acquisition systems (e.g., siderophores, ZupT), biofilm formation components and toxins (e.g., endotoxin, hemolysin) [3,13]. Factors with similar functions are found among other uropathogens [3,14].

Reticulated gelatin hibiscus and propolis (RGHP) is an oral class III medical device recently approved in the EU to control and prevent UTIs [15]. RGHP contains a combination of natural products: reticulated gelatin (RG), *Hibiscus sabdariffa* and propolis. The RG forms a protective biofilm on the intestinal wall and is commonly used to control and reduce the symptoms associated with acute diarrhea by reducing the adhesion and proliferation of bacterial pathogens in the mucosa [16,17]. It has also been reported that RG reduces the immune response of Caco-2 cells stimulated with Gram-negative bacterial lipopolysaccharide [18] and decreases the severity of colitis in mice exposed to dextran sodium sulfate [19]. In this chemically induced colitis model, treatment with RG preserved the appearance and elasticity of the intestinal mucus layer, and it partially reduced colitis-induced overgrowth of Enterobacteriaceae and *Enterococcus* spp. [19]. *In vitro*, RG protected intestinal epithelial cells against the adhesion and invasion of the pathogens *Salmonella typhimurium* and *E. coli* (ATCC 8739), probably through physical protection of the intestinal epithelium [20].

Considering that UTIs may result from extra-intestinal colonization of opportunistic gut commensals [7], and that the antimicrobial properties attributed to the basal components of RGHP are essentially based on *in vitro* studies, the first aim of this study was to evaluate the

protective properties of RGHP *in vivo* against fecal species whose strains are known to be involved in the etiology of UTIs. The focus of the evaluation is the key bacteria *E. coli*, a species within which opportunist uropathogens have currently acquired resistance mechanisms against β -lactams and fluoroquinolones [21,22]. The overall objective of the study was also to provide further insight into a potential new nonantibiotic-based strategy able to limit the source of fecal contamination leading to UTIs.

Material & methods

• Compound

RGHP was kindly provided by Novintethical Pharma SAGL (Lugano, Switzerland).

• Bacterial strain

E. coli M2/29Str^R strain isolated from the feces of a healthy human was kindly provided by Ulrich Dobrindt (Münster, Germany). The mutation on the ribosomal subunit protein S12 (K42R) conferred the streptomycin-resistance phenotype (Str^R) used *in vivo* to facilitate gut colonization of the streptomycin-treated rats as previously described [23]. This commensal *E. coli* strain shares some common phylogenetic characteristics of the archetypal UPEC strains: B2 phylogenetic group, serotype (O1, K1), genes encoding adhesins such as *fimH* (subunit of adhesin type 1 fimbriae) *sfafoc* (S fimbriae, F1C fimbriae) and *papC* (subunit of adhesin P-pili) and iron-uptake-related genes (*aer*). The *E. coli* M2/29Str^R strain inoculated to mice (10⁸ CFU subcutaneously according to the mouse model developed by Picard *et al.* [11]) was able to colonize urine, blood (heart), kidney and spleen 6 h after the inoculation.

• Animals

Female Wistar rats (Janvier S.A., Le Genest St. Isle, France) of 175–200 g were housed at the Animal Care Facility of Toxalim (Experimental Zootechnique Platform) with free access to food and water throughout the study. All animal experiments were conducted in accordance with the guidelines laid out in the ‘Guide for the Care and Use of Laboratory Animals’ [24] and complied with EU regulations. The Toxalim animal facility, named EZOP (INRA, UMR 1331, Toulouse), is licensed by the French Ministry of Agriculture (agreement no. B31.555.13). All animal experiments were also conducted with approval from the Regional

Animal Care Committee (CNREEA no. 1; MP/03/62/11/11).

• Experimental design

The impact of RGHP treatment on intestinal bacterial species commonly associated with UTIs was investigated in two experiments. The first involved female rats with intestinal microbiota not altered by streptomycin (**Figure 1A**). After a 7-day period of acclimatization into animal facility in order to stabilize rat intestinal microbiota, the control group (vehicle, $n = 6$) was treated with water supplemented with Na_2CO_3 (5%) and the treated groups ($n = 6$) with RGHP or RG twice daily, from day 7 to day 10, in other words, usual adult human posology adapted to the rat metabolism. The second involved female rats highly colonized with M2/29Str^R *E. coli* strain (**Figure 1B**); the same design was applied with three groups of ten rats except that drinking water was supplemented with streptomycin sulfate (5 g/l, Sigma-Aldrich) from day 0 to day 5, and two oral administrations of M2/29Str^R *E. coli* (10^9 CFU) dissolved in phosphate-buffered saline were performed at days 3 and 7 of the experiment for all rats. This previously described streptomycin-pretreatment animal model [23] opens intestinal niche for *E. coli* transient colonization (4–6 weeks) by disrupting colonization resistance while preserving dense and diverse anaerobic community present in conventional animals as closely as possible.

In these experiments, treatment with RGHP and RG is shown in **Figure 1A & B**:

- RGHP 770 mg per rat twice daily orally with supplemental Na_2CO_3 (5%) for 4 days (containing approximately 500 mg/day/rat of RG);
- RG 250 mg per rat twice daily orally with supplemental Na_2CO_3 (5%);
- Oral vehicle twice daily with supplemental Na_2CO_3 (5%).

• Bacterial analyses

Bacterial analyses were performed in samples recovered from feces on days 7 and 11 as scheduled in the experimental procedures. Fecal samples were collected throughout the course of the experiment. *E. coli* and other Enterobacteriaceae such as *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp. (named collectively KESC), *Enterococcus* spp. and *Proteaeae* spp. were monitored by using selective chromogenic

agar plates (ChromID CPS, bioMérieux, Marcy-l'Etoile, France). The M2/29Str^R *E. coli* strain was analyzed by using ChromID *E. coli* agar plates (bioMérieux) supplemented with streptomycin sulfate (100 $\mu\text{g}/\text{ml}$). After 24–48 h incubation at 37°C, colonies were counted. The LOD was $2\log_{10}$ CFU/g.

• Statistical analysis

Bacterial counts were \log_{10} -transformed; data were expressed as medians and interquartile ranges (25–75% percentiles) and individual data points were displayed on graphics for each experiment. Significance for effective pairing (before and after treatment) was first verified by calculating the non parametric Spearman correlation coefficient, r_s , before testing statistical significance of differences linked to treatment by performing a Wilcoxon matched-paired signed rank test. (Prism 6, Graph Pad Software). Comparisons between vehicle–streptomycin-treated rats and RGHP–streptomycin-treated or RG–streptomycin-treated rats were performed using nonparametric one-way analysis of variance (Kruskal–Wallis test) with Dunn's multiple comparison post-test (Prism 6, Graph Pad Software). Two-tail analyses were used throughout and p -values of <0.05 were considered statistically significant.

Results

The impact of RGHP treatment on fecal bacterial species commonly associated with UTIs was analyzed in conventional Wistar rats and in rats highly colonized with a human gut commensal M2/29Str^R *E. coli* strain sharing some common genetic traits of archetypal UPEC strains (**Figure 1**). Comparison with RG alone (at a dosage equivalent to that found in RGHP) was also performed in these two animal experiments (**Figure 1**).

• Impact of RGHP on commensal *E. coli* populations in conventional rats

The overall median count for rat fecal *E. coli* populations before treatment was 3.87 (3.65 – 4.48) \log_{10} CFU/g feces. Oral administration of vehicle did not impact initial median fecal load ($p = 0.16$, 3.91 [3.04 – 4.95] after vs 3.68 [2.98 – 4.1] before \log_{10} CFU/g feces), whereas RGHP significantly reduced the initial median fecal count of *E. coli* ($p = 0.031$, 2.45 [1.5 – 4.58] after vs 4.36 [3.55 – 4.74] before \log_{10} CFU/g feces, **Figure 2**). *E. coli* was no longer detectable in the feces of two of six rats treated

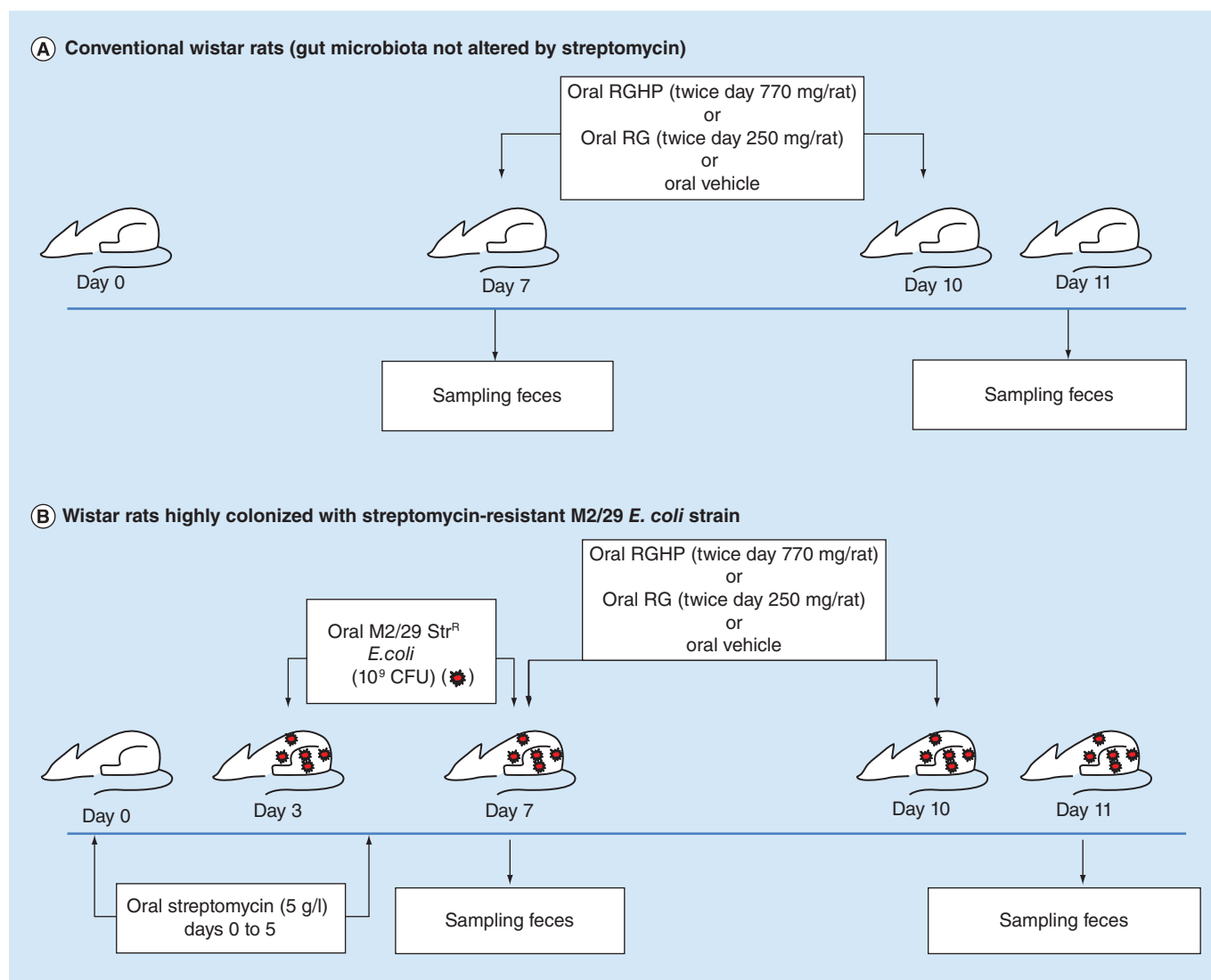


Figure 1. Experimental design. The impact of RGHP treatment and RG on selected fecal commensals was investigated on two groups of female Wistar rats. **(A)** Conventional Wistar rats with gut microbiota not altered by streptomycin ($n = 6$ rats/group, vehicle-control, RGHP-treated and RG-treated groups). **(B)** Streptomycin-treated Wistar rats highly colonized with a streptomycin-resistant *Escherichia coli* strain (M2/29Str^R strain, $n = 10$ rats/group, before treatment, vehicle-control, RGHP-treated and RG-treated groups). Oral treatments: RGHP (2×770 mg/day/rat), RG (2×250 mg/day) and vehicle ($2 \times$ water supplemented with Na_2CO_3) and streptomycin sulfate (5 g/l). Bacterial monitoring of feces ($\text{LOD} = 2\log_{10}$ CFU/g). RG: Reticulated gelatin; RGHP: Reticulated gelatin hibiscus and propolis.

with RGHP. Administration of an equivalent dose of RG alone resulted in a similar decrease of the fecal *E. coli* count as compared with initial levels before treatment ($p = 0.031$, 2.49 [1.5–3.8] after vs 3.89 [3.56–4.49] before \log_{10} CFU/g feces, **Figure 2**). Rat fecal *E. coli* populations were no longer detectable in the feces of three of six rats treated with RG. This suggests that the RG component is the main contributor to the antagonistic activity of RGHP.

• Impact of RGHP in conventional Wistar rats on other commensal species commonly associated with UTIs

The fecal samples collected before and after treatment with RGHP, RG or vehicle were also used to estimate levels of fecal *Enterococcus* spp. (**Figure 3A**) and KESC (**Figure 3B**). *Proteaeae* spp. (*Proteus* spp., *Providencia* spp. and *Morganella* spp.) were not detected in Wistar rat feces.

Compared with initial counts of rat commensal bacteria before treatment, RGHP-treated

animals showed reduced fecal median counts of *Enterococci* ($p = 0.03$, 3.76 [3.37–4.42] after vs 5.39 [4.67–6.07] before \log_{10} CFU/g feces), whereas in the vehicle group a non-significant increase in the median *Enterococci* count ($p = 0.16$, 5.81 [4.73–7.19] after vs 4.9 [4.73–5.32] before \log_{10} CFU/g feces) was observed in half of rats (**Figure 3A**). A similar but smaller median decrease in fecal *Enterococci* levels was observed in the RG treatment group as compared with initial level ($p = 0.03$, 4.4 [3.81–4.95] after vs 5.62 [5.18–5.9] before \log_{10} CFU/g feces, **Figure 3A**).

Contrary to the antagonistic activities observed against *E. coli* and *Enterococcus* spp., RGHP and RG did not significantly alter the levels of colonization with KESC (**Figure 3B**).

• Impact of RGHP on fecal *E. coli* in streptomycin-pretreated rats highly colonized with the M2/29Str^R *E. coli* strain

Using this model, the median overall initial M2/29Str^R *E. coli* fecal count before treatment was 7.18 [6.93–7.37] \log_{10} CFU/g feces. A decrease in the fecal *E. coli* level was observed in the vehicle group (6.45 [6.01–6.8] after vs 7.11 [6.95–7.45] before \log_{10} CFU/g feces; $n = 8$ pairs due to the absence of data for two

rats, **Figure 4**). This decrease due to the use of the streptomycin-pretreatment transient colonization model was not considered significant as compared with the paired initial levels ($p = 0.11$, **Figure 4**). This colonization process thus remained sufficiently robust to evaluate changes during 4 days of treatment with RGHP.

Compared with the initial levels of M2/29Str^R *E. coli* strain before treatment, RGHP significantly reduced median fecal counts ($p = 0.002$, 4.94 [4.41–5.72] after vs 7.15 [6.66–7.49] before \log_{10} CFU/g feces; **Figure 4**). Compared to vehicle-treated rats, RGHP treatment reduced the fecal counts of *E. coli* ($p = 0.034$, 4.94 [4.41–5.72] in RGHP-treated rats as compared with 6.45 [6.01–6.8] in vehicle-treated rats; **Figure 4**). A similar but less pronounced decrease in initial fecal *E. coli* counts was observed for rats treated with RG alone ($p = 0.002$, 5.81 [5.29–6.18] after treatment vs 7.2 [7.05–7.3] before \log_{10} CFU/g feces; **Figure 4**).

Discussion

In this study, we evaluated the ability of orally administered RGHP to target and impact intestinal commensal bacteria in female Wistar rats. The focus of our study involved the most frequently isolated bacteria implicated in UTIs, in

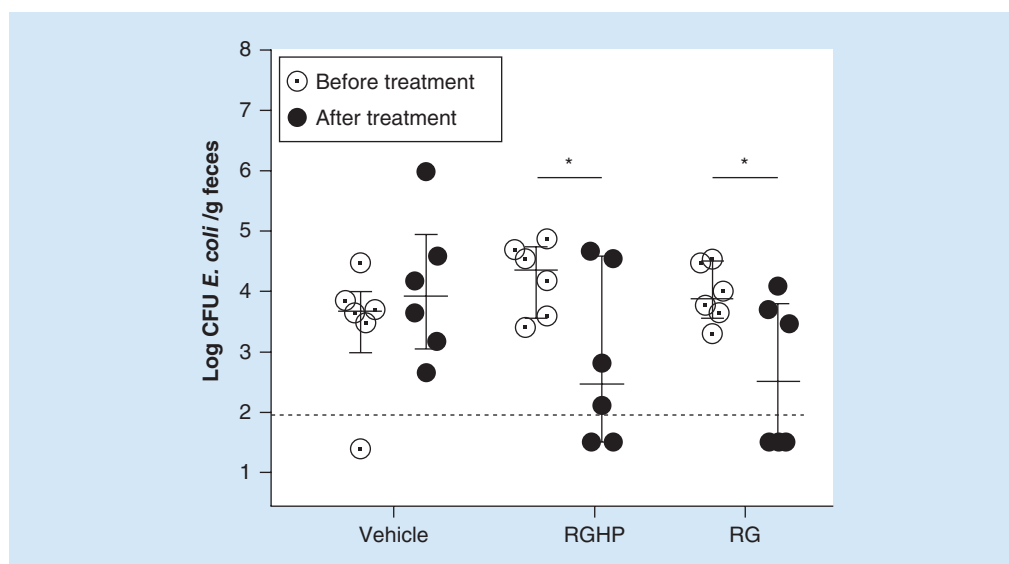


Figure 2. Impact of reticulated gelatin hibiscus and propolis and reticulated gelatin on fecal commensal *Escherichia coli* population of conventional female Wistar rats ($n = 6$ per group).

Medians with interquartile ranges are represented and data are representative of one experiment. Levels of endogenous *E. coli* (\log_{10} CFU/g feces) were compared before and after treatments.

* $p < 0.05$; the Wilcoxon matched-pairs signed rank test. LOD = $2 \log_{10}$ CFU/g (dotted line).

RG: Reticulated gelatin; RGHP: Reticulated gelatin hibiscus and propolis.

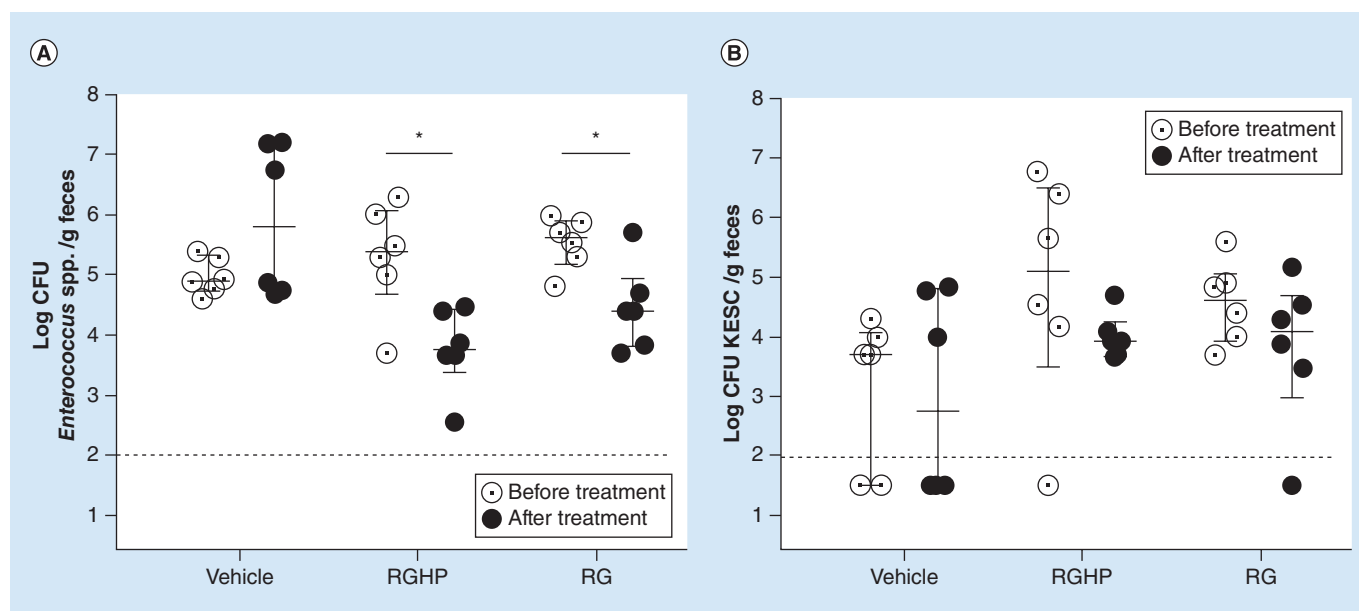


Figure 3. Impact of reticulated gelatin hibiscus and propolis and reticulated gelatin on other bacterial species commonly associated with urinary tract infections in feces of conventional female Wistar rats (n = 6 per group). Medians with interquartile ranges are represented, and data are representative of one experiment. LOD = 2 log₁₀ CFU/g (dotted line). **(A)** Levels of *Enterococcus* spp. (log₁₀ CFU/g feces) were compared before and after treatments; *p < 0.05, the Wilcoxon matched-pairs signed rank test. **(B)** Levels of KESC (log₁₀ CFU/g feces) were compared before and after treatments.

KESC: *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp; RG: Reticulated gelatin; RGHP: Reticulated gelatin hibiscus and propolis.

other words, *E. coli*, *Enterococcus* spp., *Proteaeae* spp. and other Enterobacteriaceae such as KESC. The primary niche of these commensal microorganisms is the lower intestinal tract of mammals. Some species appear to be well equipped, in terms of their metabolic characteristics and fitness, to colonize extra-intestinal niches such as the urinary tract [25].

The results of the study showed that RGHP selectively reduced the fecal level of some commensal bacteria found in the conventional rat intestinal microbiota. Fecal colonization by *E. coli* and *Enterococcus* spp. was reduced following RGHP treatment and eradicated in some animals, whereas colonization by other Enterobacteriaceae (e.g., KESC) remained relatively unaffected. Unexpected presumptive *Staphylococci* colonies were detected in some fecal samples, but the low numbers involved and the variable response to treatment preclude any assessment of RGHP efficacy for this genus. No presumptive *Proteaeae* spp. colonies were detected in Wistar rat feces in this study.

Mechanisms underlying the genus-dependent protective activity displayed by RGHP treatment have not been further explored in this study, and

that issue still needs to be addressed. Considering that RG compound found in RGHP may preserve the appearance and elasticity of the intestinal mucus layer of colitic mice [19], we can presume that this compound may affect more specifically microbial communities occupying ecological niches such as mucus layer. Spatial distribution of bacteria such as *E. coli*, *Enterococci* from other KESC within the gut is not known and it would be of interest to localize their respective transverse niches. More specifically, the persistence of these bacteria within the outer mucus layer after RGHP treatment still needs to be evaluated. Indeed, in the case of *E. coli*, Li *et al.* recently showed that despite the fact that this facultative anaerobe has a restricted glycoside hydrolase repertoire, thus limiting its mucus metabolism, it developed adaptive strategies for population persistence specifically in the mucus as compared with another mucolytic symbiont *Bacteroides thetaiotaomicron* [26]. By increasing the expression of the ferric iron uptake pathway in colonic mucus, as compared with intestinal luminal uptake, *E. coli* improved its capability to proliferate more quickly in the outer layer of mucus (estimated half-life of about 3 h) than in

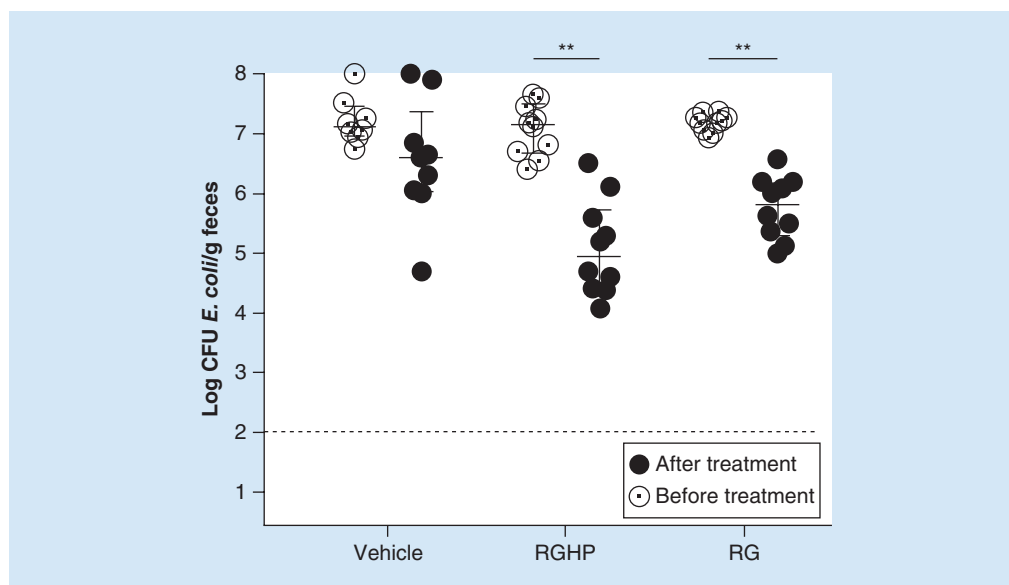


Figure 4. Impact of reticulated gelatin hibiscus and propolis and reticulated gelatin on fecal M2/29Str^R *Escherichia coli* strain orally administered in female streptomycin-treated Wistar rats (n = 10 per group). Medians with interquartile ranges are represented, and data are representative of one experiment. Levels of M2/29Str^R *E. coli* strain (log₁₀ CFU/g feces) were compared before and after treatments.

**p < 0.01, the Wilcoxon matched-pairs signed rank test. LOD 2 log₁₀ CFU/g (dotted line).

RG: Reticulated gelatin; RGHP: Reticulated gelatin hibiscus and propolis.

the lumen (estimated half-life of about 8 h) [26].

Following manipulation of the intestinal microbiota to encourage colonization with the M2/29Str^R *E. coli* strain from the phylogenetic group B2, we showed that RGHP still retained antagonistic activity against this opportunistic uropathogenic strain. This animal model has the advantage that streptomycin pretreatment promotes both the pauciclonality and clonal dominance in feces of the orally inoculated strain of phylogroup B2, in other words, some criteria considered as favoring an enabling gut environment for increased risk of UTI [7,11].

Treatment with RG alone led to a similar spectrum of activity against both rat gut commensals and an exogenous human clone of *E. coli* strain sharing some common characteristics of the archetypal UPEC strains. Taken together, these data strongly support the concept that the RG component is the main contributor to the antagonistic activity of RGHP.

RG has been shown to exert protective effects against dextran sodium sulfate induced mucosal damage and, interestingly, confocal microscopy showed that the polymeric protective layer that RG forms on ulcerated mucosa limits the contact or invasion of bacteria into the intestinal

epithelial layer. Taking into account the ability of *E. coli* to proliferate more quickly in the outer layer of mucus as mentioned above, we hypothesize that the protective effect of RGHP may be attributed to the formation of polymeric protective layer leading to an overall decrease in *E. coli* load.

Conclusion

This *in vivo* study clearly demonstrated that RGHP reduced fecal colonization by gut bacterial commensals such as *E. coli* and *Enterococci* or by a dominant human *E. coli* strain able to colonize extra-intestinal niches such as the urinary tract. Collectively, these results provide important new data confirming the preventive potential of RGHP to reduce the risk of contamination of the urinary tract by opportunistic bacteria of fecal origin.

Future perspective

The ascendant pathway for UTIs is well established and eradicating the uropathogen in the infected urinary tract is the primary focus of antibacterial-based therapy. Due to increased resistance in the community and in hospitals, antibiotics must be used with caution.

Consequently, alternative methodologies for reducing the intestinal bacterial burden with a device such as RGHP is an interesting approach for the future control of UTIs and their recurrences. Indeed, if such devices can help lower the demand for antibiotics it will be an important step forward.

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Financial & competing interests disclosure

The study was sponsored by Novintethical Pharma SAGL, Lugano, Switzerland. The authors have no other relevant

affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

- Forty to sixty percent of adult women experience at least one urinary tract infection (UTI) episode during their lifetime. In a global manner, UTIs represent every year more than 10 million cases in Western Europe.
- Several recurrent UTIs case reports illustrate that the intestinal microbiota is the most common immediate source of uropathogens. The uropathogenic *E. coli* (UPEC) strain responsible for the UTI is also present in the intestinal microbiota of 78% of women.
- Herein, we evaluated the ability of an oral class III medical device i.e., reticulated gelatin hibiscus and propolis (RGHP) recently approved in the European Union to control and prevent UTIs to limit the source of fecal contamination leading to UTIs in rat, by reducing the intestinal burden of intestinal commensals commonly involved in UTIs.
- RGHP significantly reduced fecal levels of commensal *E. coli* and *Enterococcus* spp. levels without affecting other targeted Enterobacteriaceae. Interestingly, RGHP also reduced the fecal level of a human commensal *E. coli* strain with uropathogenic potential.
- In conclusion, in order to limit antibacterial treatments largely used against UTIs with the associated risk of bacterial antibiotic resistance development, RGHP represents a therapeutic option through the reduction of intestinal colonization by opportunistic uropathogens.

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