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Pre-biotic Effects of Sobo Drink on Colonization Resistance to Experimental Infection with *Staphylococcus aureus* 8588 in Rats

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Abstract: This study investigated the effects of the continuous consumption of Sobo on colonization resistance to experimental infection in rats. Four matched groups of male and female adult Wister rats were orally administered *H. sabdariffa* calyx infusion at 37.6 and 100.0 mg mL^{-1} concentrations while the four control groups received distilled water once daily for 15 days. All rats were challenged on day 16 with 3.0-3.5X10^5 viable *S. aureus* 8588 CFU in 0.5 mL phosphate buffer and sacrificed on day 21. Faecal samples were collected six times (days 0-21) for dry mass index, coliform and *S. aureus* load. Blood was analysed for *S. aureus* antibody and lymphocyte count. Data were subject to statistical analyses. Faecal coliform counts were significantly depressed in Sobo administered groups with increased *S. aureus* shedding. Lymphocyte counts were however not significantly different and *S. aureus* antibody was not detected in the serum of rats. Sobo seems to possess pre-biotic properties and its continuous consumption may contribute to the elevation of colonization resistance to infection in the gastrointestinal tract.

Key words: *H. sabdariffa* calyx, Sobo, *S. aureus*, colonization resistance

INTRODUCTION

The immunomodulatory properties of pro-biotics and their possible place in the management of some enteric microbial infections have been documented in many recent studies[1-5]. Both pre- and pro-biotics have been shown to be effective in improving outcome in certain gastrointestinal diseases of man[6-9] and out of these studies, the delicate microbial-host-pathogen relationship has emerged as a major determinant of outcome in the complex microbial ecology of the gut[10]. In this respect, the type of microflora and the equilibrium position of its constituent micro organisms seem to determine possible benefits to the host.

Some of the most important benefits to the host gastrointestinal health include the maintenance of adequate gut colonization resistance through the regulation of microbial pool size and composition, the generation of appropriate antigen recognition repertoire and enhanced effector function of immune cells[11]. Also, microflora-host interaction in the gut influences the effect of other luminal content, such as Short Chain Fatty Acids (SCFA) and flavonoids, on balance determinants such as the secretory pattern of the gut glyco-conjugate in the overall maintenance of mucosal health[12].

The consumption of pre- and pro-biotics, such as drinks and beverages, appears to enhance indices of immune performance such as lymphocyte counts, antibody response profile to experimental infection, phagocytosis by macrophages and aggregation of neutrophil polymorphs[13,14]. Humoral immune response to rotavirus vaccination, for instance, has been shown to be increased in subjects given milk fermented with *Lactobacillus cases* or with *Lactobacillus acidophilus* compared to controls[15]. Inulin and oligofructose have been similarly demonstrated to have good gastrointestinal effects in human and animal studies[16]. In children, the critically ill and the elderly, in whom gut mucosal immune response is compromised, colonization is often followed by infection[17] but the continuous consumption of pre- and pro-biotic drinks may offer significant health benefits[18].

Pre-biotics, such as yoghurt, are fermented by a complex mixture of pro-biotic bacteria among which are lactobacilli, lactococci, leuconostocus, acid acetobacteria and yeasts[19]. Kefir drink contains small clusters of micro organisms held together in a polysaccharide matrix[19]. Sobo, a water extract of the *Hibiscus sabdariffa* calyx, contains organic acids including citric, lactic, malic and tartaric acids[20] and flavonoids such as anthocyanin manoxide, cyanidin-3, 5-diglucoside and cyanidin-3-(2-glucose)-rutinoside[21]. Sobo is rich in iron[22] and has been reported to lower serum cholesterol and exhibit remarkable diuretic properties in rats after continuous

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consumption[23]. Little is, however, known of the pre-biotic effects of a continuous consumption of Sobo particularly on the gut mucosa, its microflora and antimicrobial defenses.

This study investigated the possible pre-biotic effects of the continuous consumption of Sobo on selected microflora of the host and on some indices of gut antimicrobial defense against experimental infection.

MATERIALS AND METHODS

Source of materials: The dry *Hibiscus sabdariffa* calyx was bought in the local market in Ile-Ife.

Sobo preparation: The Sobo drink was prepared in two strengths to assess the effects of concentration. Briefly 3.75 and 10.0 g of the diced dry hibiscus calyx were weighed, added to 50 mL distilled water and boiled for 30 min and then sieved. The residues were washed thrice with distilled water and the supernatant pooled and made up to the 100 mL mark with distilled water.

Animals: Male and female adult Wister rats of the same parental stock and between 7 and 9 mon old were used in the study. The rats were individually code-marked, assigned a unique group serial number and housed in eight cages of five rats each. Commercial diet (Guinea Feeds Ltd, Ewu, Nigeria) and water were provided *ad libidum*. The rats were kept and maintained under hygienic conditions following essentially the Organisation for Economic Cooperation and Development (OECD) guidelines.

Experimental design: Four groups of five male rats each were given the codes MZ1, MC1, MZ2 and MC2 and another four groups of five female rats each were coded FZ1, FC1, FZ2 and FC2. The groups were age and weight matched. MC and FC rats were the control groups and the MZ and FZ rats were Sobo treated rats.

Administration of Sobo: Male and female group one rats received 0.1 mL of the 37.5 mg mL^{-1} Sobo orally through the use of canulla. Male and female group two received 0.1 mL of the 100.0 mg mL^{-1} preparation while control rats received equal volumes of distilled water as appropriate for the group being controlled. The regime was given once daily for 15 days.

***Staph aureus* 8588 challenge:** Typed culture *Staph aureus* 8588 was grown in nutrient broth for 18 hours. The cells were harvested and washed twice in phosphate buffer pH 7.02. They were re-suspended in peptone water at a concentration of 6.0-7.0x10^6 cells mL^{-1} 0.5 mL of this was given once to each experimental and control rat 24 h after the withdrawal of Sobo. The animals were kept for seven days after which they were sacrificed. The suspension (0.1 mL) was also plated out on Mannitol Salt Agar (MSA) to determine the viability of the cells.

Lymphocyte count and polyclonal antibody production: Blood samples were collected via cardiac puncture while the rats were under anaesthesia with diethyl ether. Between 3.0 and 5.0 mL of blood was collected per rat. EDTA was added to 1.5 to 2.0 mL of the fresh blood as anticoagulant while the rest was allowed to clot for the expression of serum. Leishmann-stained thin films were made on slides from the EDTA-treated blood to obtain the leukocyte differential counts. Using a Thoma pipette, a 1: 20 dilution of the EDTA-treated blood was also made in 1% acetic acid to lyse the red cells and for the filling of the counting chambers of an improved Neuber haemocytometer to obtain the Total Leukocyte Count (TLC). The total lymphocyte count was obtained numerically from both total and differential counts. Serum was obtained from the coagulated sample after overnight chilling at 4°C to permit maximal serum expression from the clot.

Faecal coliform, *E. coli* and *S. aureus*: Changes in gut microflora due to Sobo administration were assessed using some indicator organisms. Briefly 0.25 g faecal samples were collected aseptically into sterile specimen bottles for total coliform and *E. coli* on days 0, 5 and 15 while samples for *S. aureus* colony counts were obtained on day 15 just prior to *S. aureus* challenge and on day 21.

The faecal materials were emulsified immediately after collection in 1.0 mL phosphate buffer pH 7.0 and serial dilutions made in phosphate buffer. Appropriate dilutions for plating were obtained from our earlier pilot study. Dilutions were plated out in duplicate on Eosin Methylene Blue (EMB) and Mannitol Salt Agar (MSA) differential media for coliforms, *E. coli* (EMB) and *S. aureus* (MSA). All plates were incubated at 37°C for 72 h and then the colonies were counted.

Dry mass index: The effect of Sobo on the consistency of the gut luminal content was assessed using the faecal dry mass index. 0.5 g of faecal material was collected from all the rats on days 0, 5 and 15. The samples were put in the oven at 60°C for 48 h after which the samples were weighed again for changes in weight (dry mass). The ratio of the dry to wet mass of the faecal sample was taken as the dry mass index.

Serum polyclonal antibody production: Polyclonal antibody production to oral challenge with the test organism was determined by gel diffusion. Gel diffusion plates were prepared in triplicate by dissolving 1% immunological agar in glass distilled water and autoclaving the solution. The agar was poured into sterile petri dishes and allowed to set. A central well was bored in the middle of the dish followed by six peripheral wells 1 cm radius from the central well. 0.1 mL of a two-fold serial dilution of *S. aureus* 8588 culture filtrates, obtained after 18-20 h culture at 37°C, was put in each of the peripheral wells while 0.1 mL of serum was put in the central well. The plates were incubated at 37°C in a moist chamber for 72 h and then examined for the presence of precipitin lines at the end of incubation.

Statistical analyses: Data were analysed using GraphPad InStat Version 3.06 for Windows 2003. One way ANOVA was used to assess the overall significance of the variation in the data means/median. The Kruskal-Wallis Test was performed on non-parametric data. Dunn's Multiple Comparison test and the Student-Newman-Keuls test were used for post tests.

RESULTS

Table 1-3 present the mean values for the five rats in each group with respect to the stated parameters and the period of sampling. The Kruskal-Wallis test indicated a significant variation in the means though the differences are not significant ($p>0.05$).

Faecal coliform and *S. aureus* counts are reported pair-wise at the two concentrations studied pre- and post-treatment (treatment refers to Sobo and distilled water intake for experimental and control rats respectively) for ease of assessment (Fig. 1-8). Pre- and post-treatment faecal *S. aureus* counts in control and experimental rats which received 37.5 and 100.0 mg mL^{-1} of Sobo are presented in Fig. 1, 2, 5 and 6, respectively. Post-treatment experimental rats shed relatively more *S. aureus* (CFU) in their faeces when compared with the pre-treatment levels. Post-treatment faecal *S. aureus* counts were, however, significantly higher ($p<0.05$) for the Sobo-fed rats at both concentrations when compared with the pre-treatment values. Faecal coliform counts were significantly lower ($p<0.05$) in the experimental groups after treatment at both concentrations when compared with the pre-treatment levels (Fig. 3 and 7) When compared with controls, experimental rats showed significantly higher ($p<0.05$) *S. aureus* shedding at both concentrations after treatment while coliform counts were significantly lower only at the 100.0 mg mL^{-1} Sobo concentration. Pre- and post-treatment coliform counts for control rats were, however, not significantly different ($p<0.05$) at all levels (Fig. 4 and 8).

Sex-related effects analysed statistically indicated that coliform counts were significantly different ($p<0.05$)

Table 1: Mean dry mass index of stool for the different groups

Grouping	Before treatment Mean	Before treatment SD	After treatment Mean	After treatment SD
MC1	0.92	0.023	0.94	0.028
MC2	0.85	0.029	0.82	0.032
MZ1	0.93	0.023	0.65	0.083
MZ2	0.90	0.018	0.58	0.055
FC1	0.88	0.013	0.93	0.019
FC2	0.96	0.020	0.96	0.034
FZ1	0.89	0.013	0.66	0.083
FZ2	0.91	0.019	0.75	0.088

SD-Standard deviation

Table 2: *E. coli* load/0.25 g faeces in *E. coli*-positive rats

Grouping	Rat serial No.	Day 0	Day 15	Day 21
MC 2	1	4.9X10^4	4.4X10^4	2.75X10^4
MZ 1	4	2.5X10^6	-	-
FC 2	4	7.5X10^4	-	-
FZ 2	2	4.5X10^4	4.9X10^4	3.2X10^4
FZ 2	4	2.3X10^6	1.12X10^2	-
FZ 1	4	1.8X10^6	1.08X10^2	-
FZ 2	2	7.5X10^5	1.57X10^2	-
FZ 1	2	2.5X10^4	1.18X10^2	-
FZ 2	2	4.5X10^6	1.66X10^3	-

Table 3: Mean peripheral blood neutrophil and lymphocyte count

Grouping	Neutrophil count mL^{-1} Mean	Neutrophil count mL^{-1} SD	Lymphocyte count mL^{-1} Mean	Lymphocyte count mL^{-1} SD
MC 1	4.3X10^6	9.0X10^4	5.2X10^6	8.9X10^4
MC 2	3.9X10^6	7.1X10^4	5.4X10^6	7.0X10^4
MZ 1	5.5X10^6	1.2X10^5	6.7X10^6	1.0X10^5
MZ 2	5.0X10^6	1.0X10^5	7.6X10^6	1.3X10^4
FC 1	4.4X10^6	8.7X10^4	5.8X10^6	8.5X10^4
FC 2	4.1X10^6	7.5X10^4	5.0X10^6	8.7X10^4
FZ 1	4.9X10^6	9.9X10^4	6.2X10^6	1.3X10^5
FZ 2	5.7X10^6	1.2X10^5	6.9X10^6	9.9X10^4

SD-Standard deviation

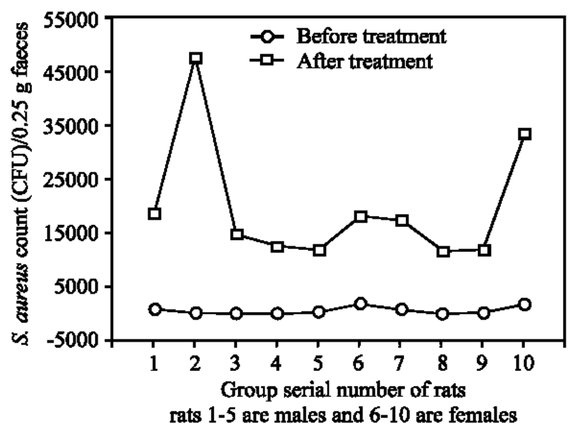

Fig. 1: Pre- and post-treatment *S. aureus* count in MZ1 and FZ1 rats fed 37.5 mg mL^{-1} Sobo

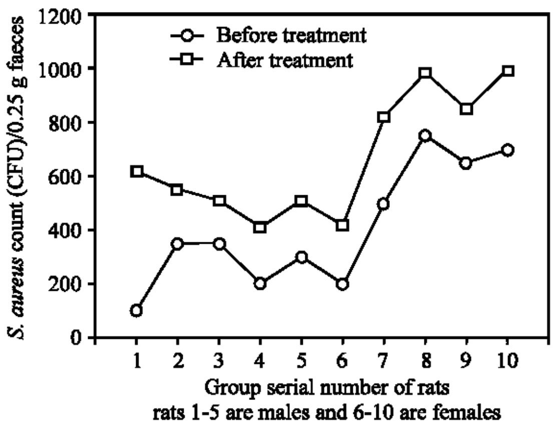

Fig. 2: Pre- and post-treatment *S. aureus* count in control MC1 and FC1 rats fed (37.5 mg mL^{-1} Sobo controls)

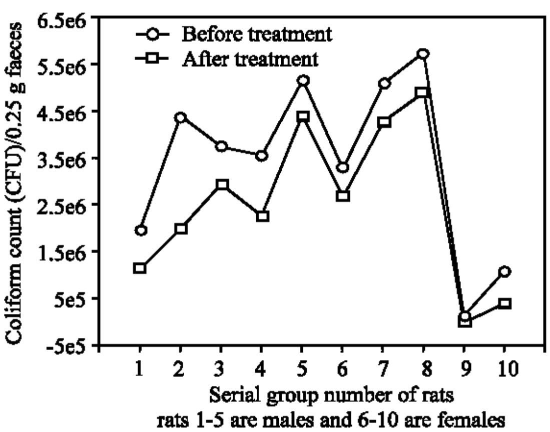

Fig. 3: Pre- and post-treatment coliform count in MZ1 and FZ1 rats fed 37.5 mg mL^{-1} Sobo

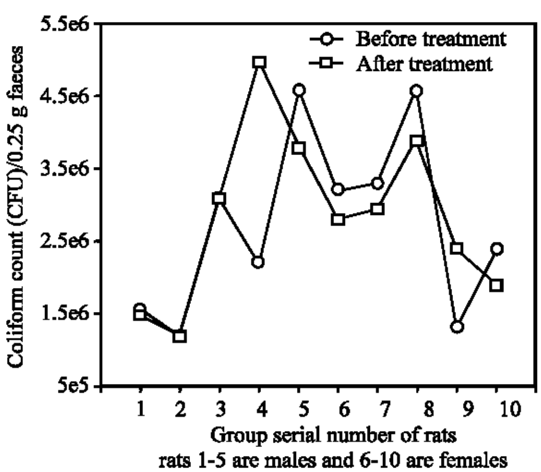

Fig. 4: Pre- and post-treatment coliform count in MC1 and FC1 rats(37.5 mg mL^{-1} Sobo controls)

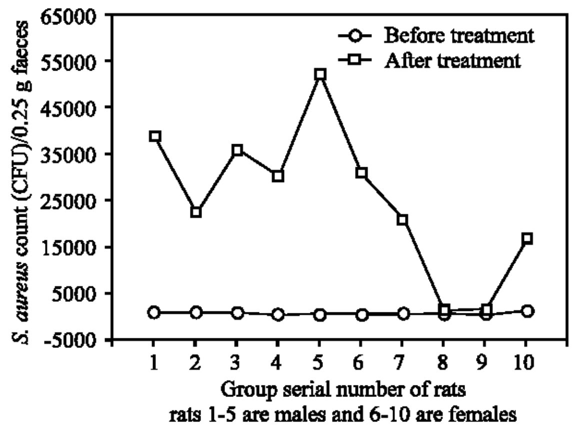

Fig. 5: Pre- and post-treatment *S. aureus* count in control MZ2 and FZ2 rats fed 100.0 mg mL^{-1} Sobo

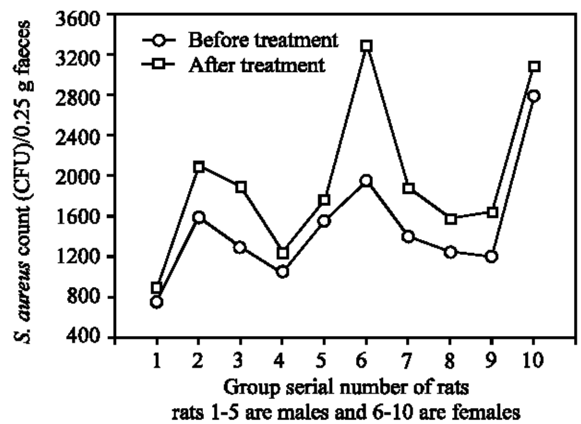

Fig. 6: Pre- and post-treatment *S. aureus* count in control MC2 and FC2 rats (100.0 mg mL^{-1} controls)

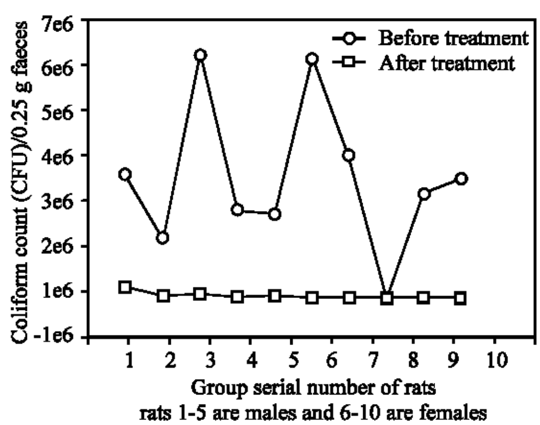

Fig. 7: Pre- and post-treatment coliform count in MZ2 and FZ2 rats fed 100.0 mg mL^{-1} Sobo

within groups before and after treatment at the two Sobo concentrations in male but only at the higher

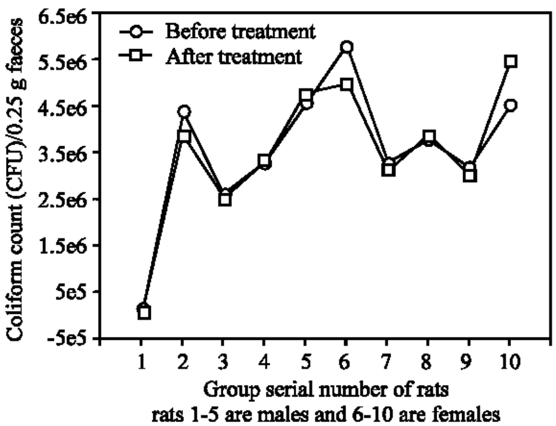

Fig. 8: Pre- and post-treatment faecal coloiform count in MC2 and FC2 rats (100.0 mg mL^{-1} Sobo controls)

concentration in female. Also, within groups, *S. aureus* counts were significantly different ($p<0.05$) before and after treatment at both concentrations of Sobo in female but only at the higher concentration in the male. When compared with controls (between group independent t- test) coliform count in the experimental group was not significantly different ($p>0.05$) after treatment at both concentrations of Sobo in the male but is significantly different at the higher concentration in the female groups. Similarly, *S. aureus* count was significantly different between groups only at the higher Sobo concentration in the male.

DISCUSSION

The consumption of pre-biotics has generally been reported to depress coliform count in the gastrointestinal tract[24]. Present results showed a decline in total coliform count and an increase in faecal *S. aureus* count of the experimental rats as the study progressed however the limiting values for coliform counts were not reached. In contrast, *E. coli* has disappeared by the 21st day from the faeces of *E. coli*-positive rats receiving Sobo. This apparently selective reduction in coliform count and increase in *Staph aureus* count from the gastrointestinal tract is probably linked to pre-biotics-epithelial cell interaction with the consequent modulation of epithelial cell expression and function[25]. This modulation may affect specific interactions of coliform with appropriate epithelial cell surface receptors and ligands. Mukai *et al.*[26] reported the inhibition of binding of *Helicobacter pylori* to epithelial glycolipid receptors by the pro-biotic *Lactobacillus reoteri*. Rasta-Lenert and Barrett[27] have also reported that live pro-biotics could inhibit the adhesion and invasion of enteropathogenic *E. coli* into human intestinal cell lines. Though the actual mechanism(s) of action of pro-biotics and pre-biotics are not well known, these may include alterations of the metabolic activities of gut microflora, alteration of physicochemical conditions in the gut and a modulation of host immune response among others.

Although there was no change in diet, Sobo-fed rats ate more and gained more weight. Present results showed a change in the dry mass index of stool from the experimental rats in comparison to controls though this change was not significant. However, physical examination of the faeces of Sobo-fed rats (data not included) revealed lower mucus content and a more brittle mass. The lower mucus content of the faeces may reflect a conservation of the epithelial mucin layer or an increased binding to the epithelial cell by the gut microflora which makes less mucin available in the luminal content. Mucins are produced by goblet cells and are the first line of host defense against microbial invasion. They do this through the binding capacity of their carbohydrate side chains to microbial adhesions. When gut microflora bind to mucin, invading organisms are unable to attach and so are swept out during peristalsis and bowel movement. It has been established that conspicuous qualitative and quantitative changes in the synthesis of mucins in goblet cells do occur at different phases in drug-induced mucosal atrophy[28]. Such changes may lower gut microflora population while at the same time increasing the faecal throughput of the challenge organism[29] as present results seem to confirm.

The leukocytosis observed in the Sobo-fed rats (Table 3) was insignificant and was essentially lymphocytic and not neutrophilic. Gel diffusion of rat sera against a serial dilution of the cell-free supernatant of the exponential phase culture of the *S. aureus* 8588 in glucose- supplemented minimal medium did not reveal any precipitin lines. This seems to imply that *S. aureus* 8588 antigens were not present in the systemic circulation. Sobo has been reported to possess remarkable diuretic properties[24], a factor that could account for the insignificant lymphocytosis. It is suggested that the lymphocytosis may be inductive and not responsive to any translocation of the challenge organism beyond the mesenteric lymph nodes to the systemic circulation as earlier observed[30].

Sex and the concentration of Sobo consumed seem to affect the outcome in a number of significant ways. The picture is, however, unclear. Though dose-dependent relationship between pro-biotics and beneficial intestinal effects has not always been established[31], present results showed a significant increase in *S. aureus* shedding and a superior reduction in faecal coliform at the higher Sobo concentration. Further work will be required to establish dose response profiles and saturation conditions vis-à-vis attendant benefits to the consumer. Also, the results revealed significant differences between male and female rats in respect of the levels of reduction in coliform count achieved after Sobo intake at the two concentrations ($p<0.05$). Similarly, the *S. aureus* counts differed significantly ($p<0.05$) sex-wise. These differences were, however, significant mainly at the higher concentration of Sobo. The reasons for these sex-related differences are not clear. However, neuro-immuno-endocrinologic influences are suspect. It is concluded that the water extract of *Hibiscus sabdariffa* calyx (Sobo) possesses pre-biotic properties with potentials for raising gut colonization resistance and the prevention of gut bacterial infection.

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