THE EFFECT OF BODO SALTANS BACTERIAL GRAZING IN ENHANCEMENT OF E. COLI O157:H7 POLLUTED TIGRIS RIVER WATER QUALITY

By: dr. Talib Hassan Ali*

ABSTRACT

We studied the influence of pathogenic bacteria grazing by bactreiovorous nanoflagellates in a bacterial model community under defined experimental conditions to obtain further insights to understand the ability of using these species as biological control approach against pathogenic bacteria polluted fresh water. By another word, to investigate the ability of genera of (*Bodo saltans*) bacteriovorous nanoflagellates to graze allochthonous pathogenic, diarrhea causing bacteria strain (*Escherichia coli* O157:H7) in Tigris River. The investigation was done by monitoring and quantifying the removal (clearance rate) of artificially concentrated bacteria from experimental river water ecosystem at eight selected time points. Our result indicated that pathogenic bacteria consuming as food was greatly sustain the studied nanoflagellate life. *Bodo saltans* grazing rates of different enteric bacteria species combinations (*E. coli* + *Salmonella typhi*) or (*E. coli* + *Shigella flexneri*) in mixed culture suggested that *Bodo saltans* flagellate do have differential feeding behavior among different species of Enterobacteriaceae family. Key words: nanoflagellate, bactreiovory, *Bodo saltans*

INTRODUCTION

Predation by bacterivorous protists, particularly by the ubiquitous bacterivorous nanoflagellates, is a major mortality factor for aquatic bacteria [1, 2, 3] in marine and fresh water [4, 5, 6]. The nanoflagellates are the primary consumers of bacteria, cyanobacteria and microalgae [7], and their grazing influence on the composition of bacterial communities is based on a complex interplay of several factors such as differences in cell size distribution of bacterial species [8], and different abilities of bacterial species in grazing defense [9, 10] or individually depends on bacterial cell size, bacterial motility and bacterial surface characteristics [11, 12, 13]. Furthermore, grazing may influence the growth conditions of individual bacterial species through the regeneration of substrates or by reduction of competitors. Diverse physical and chemical factors, such as light, temperature, pH, toxicity of heavy metals, salinity and antibiotic substances produced by other bacteria and algae, have been reported to result in a decrease in the number of enteric bacteria in natural aquatic media [14, 15, 16, 17, 18, 19]; but no decrease in the total number of enteric bacteria has been reported [20]. Therefore those physical and chemical factors do not contribute to the decrease in the total number of enteric bacteria in natural waters. Protist predation has been shown to

*Ph.D in Molecular immunology, University of ThiQar, College of Medicine, Department of microbiology

be a major factor responsible for the decay in the CFU of enteric bacteria in natural samples [21, 22, 23, 24, 25, 26, 19] especially in warm freshwater pond (> 25°C) (like Tigris water) because of prolonged survival of pure *E. coli* in such environment [27]. Many experimental studies have shown that protistan predation yields distinct changes in bacterioplankton communities [28, 29, 30, 31, 32], but the impact of protistan grazing on the structure and function of the prokaryotic community rather pathogenic bacteria in fresh water, has not been examined deeply yet. Several techniques were developed to quantify of the grazing rate of bacteria by bateriovorous protista such as gene profile analysis, denaturing gradient gel electrophoresis (DGGE), cloning and sequencing of 16S rDNA genes, fluorescence in situ hybridization (FISH). Here we used bacterial colony color and appearance on selective solid medium (makonkey agar) to evaluate the clearance rate of pathogenic *E. coli* O157:H7 from artificial river fresh water (ARW) (Tigris River). Our method based on the differential lactose containing medium fermentation of this pathogenic species as precise and reliable approach.

Bodo saltans sp. a cosmopolitan and one of the most commonly reported heterotrophic nanoflagellate on freshwater. It is small (largest diameter around 5μm), have two flagella, member of the Kinetoplastida order Bodonidae family. It is chosen here as experimental organisms because of their common presence in fresh river water environments such as Tigris River.

Fecal coliforms are an operationally defined grouping of enteric bacteria whose presence in natural waters is used as an indicator of fecal contamination and therefore the possible presence of pathogenic microorganisms [33] in natural and drinking waters [34, 35, 10].

Escherichia coli O157:H7 first identified as a human pathogen in 1982 by its association with two outbreaks of haemorrhagic colitis [27]. Now it is considered a serious threat to public health, with some forms of infection having very severe clinical implications. The consumption of *E. coli* O157: H7 contaminated water is considered as major route of infection dissemination. Their spreading from person to person, suggesting that the infective dose is low [36, 37, 38]. This study attempts to provide more quantitative evidence about the feeding mechanisms of bacteriovorous nanoflagellates with respect to their grazing of pathogenic bacteria. We analyzed whether flagellates showed variable grazing on bacteria belonged to the same bacterial family (Enterobactereacea), and whether flagellates preferentially grazed some enteric bacteria species compared with the others.

MATERIAL & METHODS

The predator samples: The nanoflagellate species used in these experiments was established by using single-cell isolations from water samples taken from 0.5 m depth in 10 L polypropylene bottles precleaned with diluted HCl, then processed within 2 h of sampling. Fresh Tigris water samples were collected in June 2002 within the pelagic zone of an oligotrophic Tigris River, located in Jaderyah beach, Baghdad, Iraq. In all cases the water temperature was 13°C. After nano- flagellate isolation by dilution method [39] and their classification according to [51, 52], culturing of the

nanoflagellates occurred in covered, sterile 500 ml flask containing 300 ml of sterile (15 min, 121°C, 15 lb/in^2) autoclaved river water. Nanoflagellates used in the grazing experiments were taken from 4- to 10 days old cultures grown at room temperature (around 20 °C)

Ptotozoan maintenance bacteria:

Natural bacteria that isolated directly from Tigris river water was grew as a suspension primarily as 0.5 to 2µm-long rods and cocci, and maintained by their culturing on (Yeast extract agar) and incubating at 25 °C for 48 hrs to reach exponential phase. They used to maintain flagellate culture by the inoculation in protozoan media before the experimental setting with pathogenic bacteria.

Isolation of pathogenic strain of E .Coli

The bloody diarrhea samples of infants were processed to isolate *E. coli*, *Salmonella typhi* and *shigella flexneri* in the central child hospital laboratories in Baghdad in May to October 2002. O157:H7 Strain was diagnosed in the central health laboratory by using physical characteristics, Epi20E system, biochemical and serological tests, while *s. typhi* and *sh. flexneri* species, were identified by their biochemical characteristics and using Epi20E detection system.

Experimental design*:*

Artificial River Water (ARW) was used in all laboratory experiments. A pathogenic *E. coli* bacterium was prepared from a late-exponential-phase culture of a diarrhea causing strain, grown in nutrient broth (Difco Laboratories) at 37°C. Total bacteria Cells were harvested (5,900 x g, 20 min), washed, and suspended in 0.45 µm filtered RAW, (200 ml; initial average bacterial concentration in all flasks, 3 x 10^6 to 6 x 10^6 cells ml^{-1}). Autoclaved and roughly filtered river water was compared with 0.45 µm-filtered water samples to exclude the putative effect of endogenous coliform. No significant differences were obtained (data not shown). Each preparation was inoculated with 10 ml of a mid-exponential-phase culture of *Bodo saltans* sp. and incubated at 22°C. The prey (bacteria) and predator (nanoflagellate protozoan) count at 0, 24, 48, 72, 120, 144, 168, 192, and 384 hours time course. Aliquots were collected daily, and numbers of remaining living bacteria were monitored with time by using (MacConkey agar) culturing under experimental condition.

Determination of nonspecific and specific grazing rate: In samples inoculated with *E.coli*, CFU numbers were determined both in absence and presence of nanoflagellate organisms. However, direct counts of bacteria were determined by count of lactose fermented bacteria colonies on the Enterobacteriacea selective medium (MacConkey agar), incubated at 37°C for 24h. The percentage of grazing rate was calculated as the equation:

% grazing rate = CFU on control plate (without the predator) – CFU on test plate (with the predator) / CFU on control plate (without the predator) x 100

Assay for species-selective grazing: we exposed two certain concentrations of bacteria (*E. coli* + *Salmonella typhi*) or (*E. coli* + *Shigella flexneri*) mixtures, to grazing by the flagellates *Bodo saltans.* The mixed bacteria were added to bacteriovorous nanoflagellate suspension vessel; incubate at 22°C for the aforementioned time course. At each time point, a particular culture volume was removed for direct protozoan count by cytometer slide counting method and mixed enteric bacteria enumerating by their differential lactose fermentation on MacConkey agar (*E. coli* is lactose

fermented species has pink color colonies, while *Salmonella typhi* or *Shigella flexneri* are non lactose fermented ones that have white color colonies). The percentage of grazing rate was evaluated by using the same equation mentioned above.

Statistical analysis: Statistical comparisons for each bacterial species were performed by using two-way analysis of variance with replication (n = 3) and unplanned comparisons.

THE RESULT

Bacterial and protistan abundances in pure batch culture

Initial bacterial numbers were about (9.37 $\times$ 10^7 cell ml^{-1}) in all treatments. Our experiments showed that the number of living cells of *E. coli* decreased sharply, after the introduction of *B. saltans*. On the second day for each experiment, the elevating of protozoan count and the dropping of bacterial abundance was observed. Bacterial numbers reach maximal values ($1.35 \times 10^8 \pm 0.8$ cells ml^{-1}) in the absence of bacteriovourous nanoflagellate at first day, but decline 20-40% of their initial value after 48 hrs. The significant increasing of *E. coli* CFU at the beginning of the experiment of control state (without *B. saltans*) may be a result of suitable environmental condition that reversed by the protozoan predator's introducing after 48 hrs. We noted that the time required for 50% reduction of the initial *E. coli* number lasted under two days, however, after 16 days of the experiment, residual *E. coli* cells were also observed. *B. saltans* increased from 0.38 $\times$ 10^3 to 640 $\times$ 10^3 cells ml^{-1} within 2 days after inoculation. Afterwards, abundances stagnated and decreased to 16.5 $\times$ 10^3 ml^{-1} at the last sampling date. The experiments have demonstrated also the close coupling between *E. coli* clearance time and the number of heterotrophic flagellates (Figure 1A-B).

Within 48 hrs, the total grazing rate of the *B. saltans* population increased from 0.67 x 10^6 to about 82 x 10^6 bacteria ml^{-1} day^{-1}. Uptake rates ranged from 8.6 to 73.5 bacteria $flagellate^{-1}$ h^{-1} (Data not shown). So a characteristic feature of the findings is the apparently coupled bacteria consumption and flagellate numbers, may suggest that flagellate grazing of *E. coli* is more effective than on smaller autochtonic bacterial cells.

Bacterial and protistan abundances in mixed culture

B. saltans count increases dramatically, begining 24 till 72 hrs in equally pattern in both *(E. coli + Salmonella typhi)* or *(E. coli + Shigella flexneri)* mixtures, before *(E. coli + Salmonella typhi)* specific significantly protozoan sustaining in comparison with the another bacterial mixture after 120 hrs till the end of studied time course (see fig. 2). *(E. coli + Salmonella typhi)* mixture greatly enhanced protozoan growth when compare with the second mixture which means that *S. typhi* species was essentially required for the optimal flagellate organism duplication may be due to their higher nutritional value.

The clearance rates of two distinct enteric bacterial mixtures by flagellated protozoan were observed clearly. The constant bacterial multiplication all the experiment time (0-384 hrs), for all studied enteric bacteria species (fig 3A and C), was reduced sharply at first 48 hrs, when protozoan predator was introduced in manner (fig 3B and D). In *B. saltans* free culture, *E. coli* kept its constant multiplication rate all the time either when shared the environment with *S. typhi* or with *shi. flexneri* that grow sporadically in

mixed cultures, to suggest their higher susceptibility toward water conditions in comparison with pollution indicative species (*E. coli).* Nano flagellate food selection may reform by Chemotactic orientation [49] and/or physiological state of the grazers [50], so *B. saltans* preferred up taking of *S. typhi* from their mixture with *shi. flexneri* and it preferably ingested *E. coli* efficiently than *shi. flexneri* in the second mixture (fig 3B and D).

DISCUSSION

The experimental conditions used in our experiments mimicked the growth rates and abundances of bacteria [40], the growth rates and viability of bacterivorous flagellates [41, 2, 4] and the mean cell size of bacteria that reported for mesotrophic to eutrophic lakes [42, 43, 44]. Medium-size bacterial cells are most susceptible to predation by flagellates and ciliates, whereas smaller cells and large filamentous forms may be partly resistant to grazing [29, 30]. In spite of the intense biogeochemical, microbiological, and molecular work done on sea water field, the impact of protistan grazing on the structure and function of the prokaryotic community was largely unknown previously in fresh water. Aggregates of bacteria represent high concentrations of prey and as such could be an important nanoflagellate food source [8], in spite of well demonstrated fact those nanoflagellates were able to efficiently prey even on low concentrations of Fluorescent labeled bacteria [45]. *Escherichia coli* are the most popular indicator organism used for routine control of drinking water quality. This fact in addition to that *Escherichia coli* O157 strain contamination of water has emerged as an important public health concern, makes *E. coli* O157 present in the drinking water offered to livestock may contribute to the prevalence of infection in cattle, a factor directly related to the contamination of beef products and the environment [46, 47, 48]. This study shows quantitatively the impact of bacteriovory process, through the use of defined bacteriovores nanoflagellate, that have feeding mechanism based on grazing of pathogenic bacteria as well as natural bacteria. The grazing rates may be dependent on the predator species and whether the prey is aggregatible such as natural bacteria or not as pathogenic ones. Further, the results imply that human pathogenic bacteria can play a major role in determining which nanoflagellate species predominate in an environment. The importance of the heterotrophic nano-flagellates as bacterial consumers may be assessed by increasing their numbers in experimentally natural circumstances by determined values of clearance of bacteria.

The present study suggests how bacteria can be removed and explains the nanoflagellate feeding behavior that usually found in polluted river fresh water. It also suggests that these nano-predators represent the 'nano bio-filter tools' between larger suspension feeders. The peak of bacterial density which stimulated protozoan growth was leading to a decline in bacterial count after predator introducing. So we observed an inherent tendency for cyclic behavior if constant bacterial supplement is maintained in some satiations such heavy continuous bacterial supply (data not shown). However, it is most likely that the cycle was initiated by human pathogenic bacteria carrying waste discharge into water body tend to decrease over time by the affect of such bacteriovorous nanoflagellates control. We concluded that this must be explained as an

example of a prey/predator relationship. Our experiments showed that exposure to an aquatic environment and nanoflagellate grazing was the major factors responsible for the disappearance of *Escherichia coli* in the studied artificial ecosystem. We noted that the time required for 50% reduction of initial *E. coli* numbers was less than two days. the result presented here may paved the way toward using these creatures as biological control approaches come instead of chemical and physical dangerous and expensive techniques for bacterial polluted water purification.

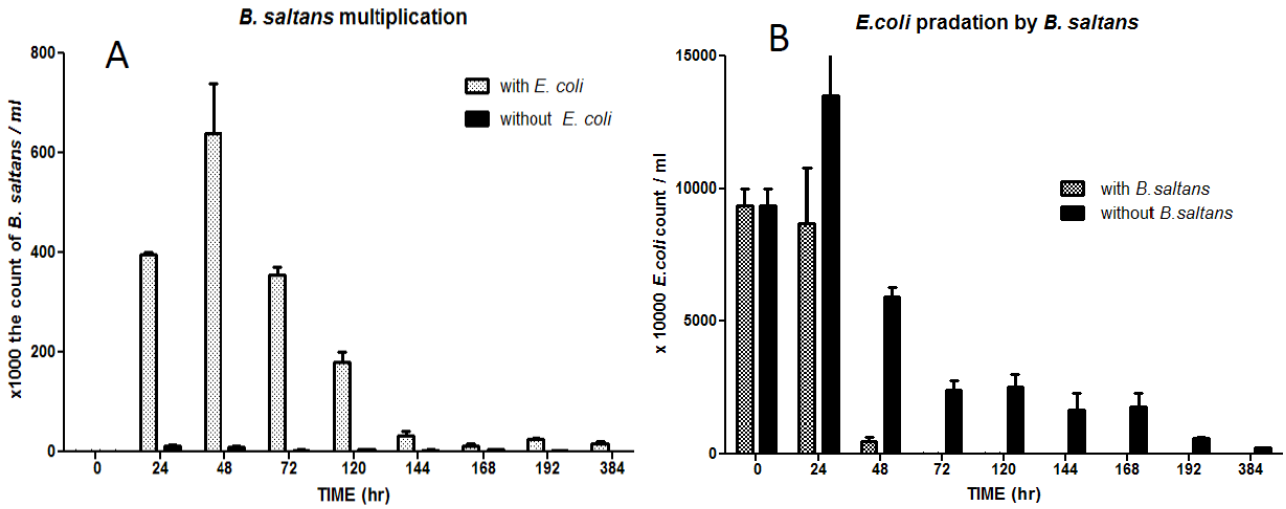

Figure-1: The nanoflagellate multiplication rate and E. coli consumption rate. *A- Represents B. saltans reproduction during experimental time course. The grazing of pathogenic bacteria clearly sustain the predator growth note the significant changes of bacteriovourous nanoflagellates species population density with pathogenic E. coli especially after 48 hrs .B- represents E. coli density during the previous time course with and without B. saltans, note the significant reduction of pathogenic E. coli beginning from 48 hrs in comparison with control state (without B. saltans). (The results presented as mean value ± SD)*

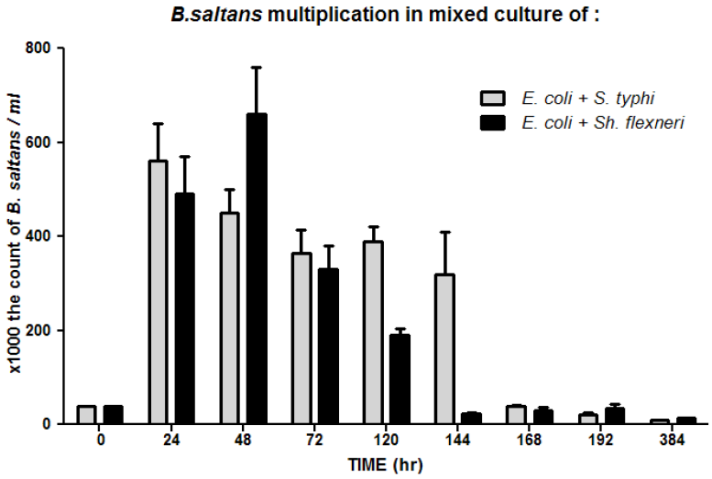

Figure-2: The nanoflagellate multiplication rate in E. coli + S. typhi and E.coli +Sh. Flexneri mixtures: *Represents B .saltans reproduction during experimental time course. The grazing of pathogenic bacteria clearly sustains the predator growth like in E. coli culture here there are no significant differences in B. saltans population density when cultured in (E. coli /S. typhi) or (E.coli/ Sh. Flexneri) bacterial mixtures. (The results presented as mean value ± SD)*

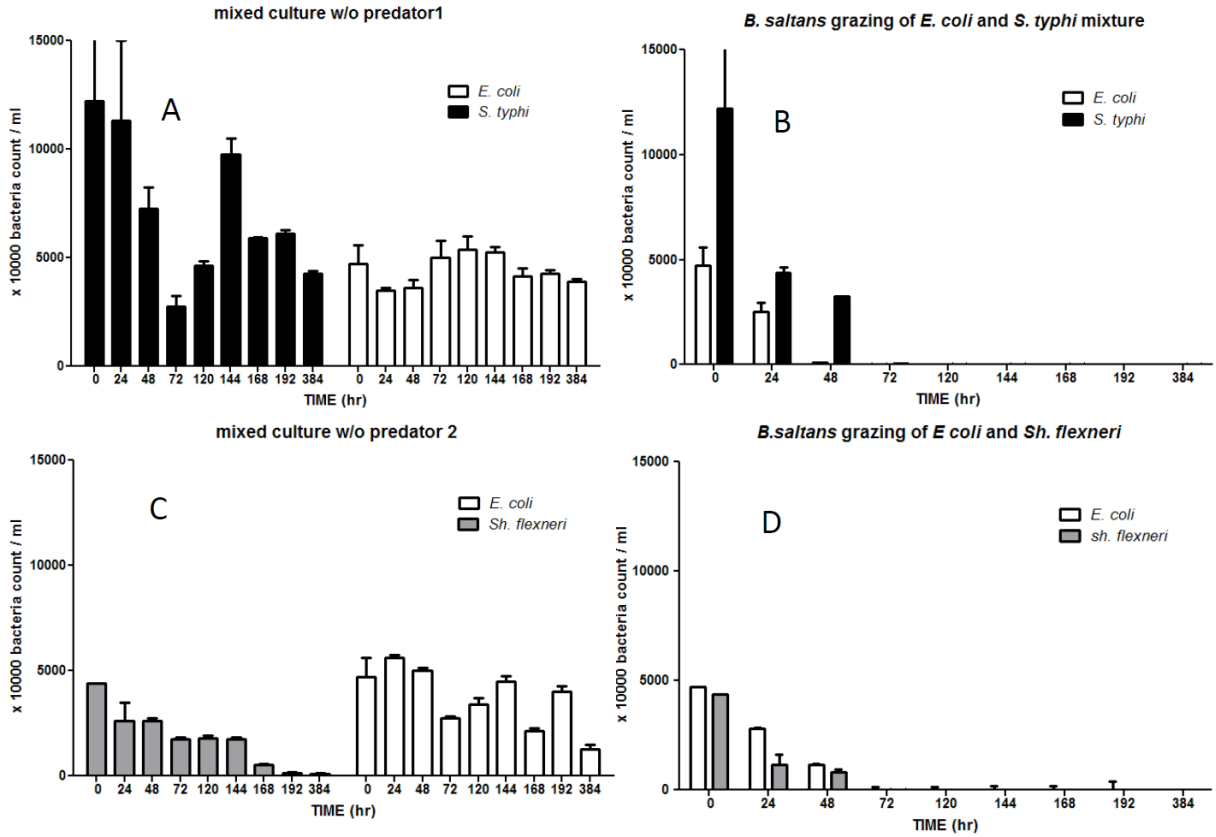

Figure-3: Bacteriovorous predatory of Bodo saltans in E. coli + S. typhi and E.coli + Sh. Flexneri mixtures. *The time course of (E. coli + S. typhi) bacterial mixture multiplication, calculated without (A) or with (B) presence of B. saltans bacteriovory nanoflagellate. (C) Represent the multiplication rate of (E. coli + Sh. Flexneri) bacterial mixtures in B. saltans*

free culture (D) pervious bacterial mixtures when cultured with B. saltans. (The results presented as mean value ± SD)

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تأثير الرعي الجرثومي للكائن الابتدائي السوطي *Bodo saltans* في تحسين نوعية مياه نهر دجلة الملوثة بالبكتيريا القولونية من النمط المصلي O157 : H7

د. طالب حسن علي*

الملخّص

درسنا تأثير رعي البكتيريا المسبّبة للمرض من قبل نوع من السوطيات الدقيقة جدا الآكلة للبكتيريا في نموذج لمجتمع تحت ظروف تجريبية محددة وقياسية، للحصول على دلائل أخرى لفهم قدرة إستعمال هذه الانواع الابتدائية للسيطرة الحياتية على تلوث الماء العذب بالمسبّبات المرضية. بكلمة أخرى جرى التحرّي عن قدرة النوع السوطي الدقيق الآكل للبكتيريا *Bodo saltans* لرعي البكتيريا المتنوعة المسبّبه للاسهال في نهر دجلة، كبكتيريا الاشريشيا القولونية (*Escherichia coli* O157:H7). التحقيق جرى بمراقبة وتحديد نسب الإزالة (clearance rate) للبكتيريا المركّزة بشكل إصطناعي في نظام ماء البيئة النهرية التجريبي. لقد وجدنا ان البكتيريا المعوية تدعم نمو وتضاعف الكائن الابتدائي المدروس وان السوطيات الدقيقة جدا ترعى انواع البكتيريا المعويّ بمعدلات مختلفة عند استعمالنا للمزارع البكتيرية المختلطة (*E. coli* + *Salmonella*)او (*E. coli* + *Shigella flexneri*) عند مقارنة نسب تناولها لتلك الانواع البكتيرية المعوية المختلطة. فخلصت نتائجنا الى أنّ الكائن السوطي *Bodo saltans* يمتاز باستجابة غذائية إنتقائية تجاه الانواع المختلفه لعائلة البكتيريا المعوية Enterobacteriaceae.

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* قسم علم الأحياء المجهرية - كلية الطب - جامعة ذي قار