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Assessment of survival of pathogenic bacteria in fresh vegetables through in vitro challenge test

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Abstract

Background: Being led by the previous observation of bacterial growth and survival in the fresh-cut tomato, carrot, lettuce and cucumber, current investigation further attempted to emphasize on the growth and consequent endurance of the pathogenic bacteria within chili (*Capsicum frutescens*), onion (*Allium cepa*), capsicum (*Capsicum annuum*) and coriander (*Coriander sativum*) collected from local markets.

Results: Samples were primarily made free of contaminating bacteria and then subjected to inoculation by eight (8) test bacteria; i.e., *Eshecrichia coli*, *Klebseilla* spp., *Vibrio* spp., *Bacillus* spp., *Salmonella* spp., *Listeria* spp., *Pseudomonas* spp. and *Staphylococuus* spp. and kept at room temperature. Bacterial growth pattern was observed up to 15 days and in general, the bioburden was noticed to be reduced up to 3 log from the initial load of the inoculam in all the samples used.

Conclusion: The chilli and onion samples were found to reduce the bacterial load more effectively than the capsicum and coriander samples. The survival of the pathogens in the vegetable samples raises the necessity of maintaining proper sanitary condition during handling and storage of fresh vegetables.

Keywords: Chili, Onion, Capsicum, Coriander, Challenge test, Bacterial survival, Consumer safety

Background

The widened understanding of the nutritional benefits of vegetables made them to be consumed in daily meals around the world almost every day (Gomez and Ricketts 2013; Southon 2000; Wargovich 2000). Eating a lot of vegetables as part of a low fat and high fiber diet may help reduce blood pressure, risk of heart disease, stroke, diabetes, cancer and manage weight (Gomez and Ricketts 2013). Conversely, raw fresh vegetables like other food items have been reported to come in contact with an array of harmful microorganisms in agricultural land where indusrial and domestic wastes are disposed, during harvesting, transportation and storage and through water bodies that result in the onset of various diseases (Burnett and Beuchat 2001; Guo et al. 2002; Solomon et al. 2002; Wachtel et al. 2002; Nipa et al. 2011; Rahman and Noor 2012; Ahmed et al. 2014; Feroz et al. 2014; Noor et al. 2014; Acharjee et al. 2015; Alam et al. 2015; Noor et al. 2015). Furthermore, contamination and growth of spoilage microorganisms which are usually likely to limit the shelf life of vegetables (King and Bolin 1989; Robbs et al. 1996; Uyttendaele et al. 2009; Fatema et al. 2013; Feroz et al. 2013, 2014; Noor and Feroz 2015). Moreover, the survival and growth of pathogens in fresh vegetables are of paramount importance in perspective of spreading and transmitting diseases in humans and animals. However, survival and/or growth of pathogens on fresh vegetables and fruits are known to be potent factor for causing human/animal illness, and are influenced by the types of resident microorganisms (Fatema et al. 2013; Feroz et al. 2013, 2014).

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Public health and hygiene are of prime concern of a nation as it needs healthy and sound workforce for its development. Like other developed countries, the people of Bangladesh are also leaning towards healthy and hygienic diets as the life style is changing. Intermittent and chronic illness may occur due to consumption of contaminated /poorly sanitized raw vegetables. Despite human casualities,economical issues such as the hospital costs, the cost of sick leave are also important to take into account.

Previously we found the fresh vegetables to be contaminated by an array of microorganisms, of which most were resistant against the commonly used antibiotics (Rahman and Noor 2012; Ahmed et al. 2014; Alam et al. 2015; Noor and Feroz 2015). Conversely, an antibacterial activity of lettuce and cucumber samples against potential pathogenic bacteria was also notable (Ahmed et al. 2014). Our earlier research on the bacterial survival within carrot, lettuce, cucumber and tomato samples employed the microbiological challenge test which is actually known to facilitate the observation of microbial growth and survival pattern within the food and pharmaceutical products (Fatema et al. 2013; Feroz et al. 2013).

Microbial challenge test aids in estimating the survival and/or growth potential of microorganisms in food products which in turn focuses on the product stability. In Bangladesh, such microbial growth simulating research on fresh vegetables is still in its infancy and data are scanty except a few reports (Rahman and Noor 2012; Fatema et al. 2013; Ahmed et al. 2014; Senjuti et al. 2014; Tahera et al. 2014; Alam et al. 2015). However, in the current study we have further extended our research on the bacterial survival within capsicum, chili, coriander and onion samples.

Methods

Sampling and sample processing

Samples of chilli, onion, capsicum and coriander were aseptically collected from different super markets, local markets and from street vendors early in the morning and transported to the laboratory in a way as described earlier (Feroz et al. 2013; APHA 1998). Prior to the challenge test, the initial identification and enumeration of pathogenic bacteria and fungi in the vegetable samples were done previously as already published by our group (Rahman and Noor 2012; Feroz et al. 2013; Ahmed et al. 2014).

The collected fresh Vegetable samples were processed as described by Feroz et al. (2013). Briefly, samples were initially washed with distilled water and with 90 % alcohol, and after chopping, 10 g of each sample were blended along with 90 ml buffered peptone water (BPW). After centrifugation at 5000 rpm for 5 min, supernatants were removed and rinsed in BPW, following the next round of centrifugation. After repeating the process for 5 times, the resulting pellets were washed twice with 90 % alcohol followed by the final treatment with 70 % alcohol (Feroz et al. 2013). Finaly, the samples were completely washed twice with distilled water to remove the remain part of the BPW and alcohol from the samples even from the surface of the falcon tubes. To notify the complete elimination of intrinsic contaminating microorganisms within the samples, an aliquot of 100 μl of each sample was placed on to Luria Baurtoni (LB) agar media (Manufactured by Oxoid Ltd. Wade road Basingstoke, Hant, UK), and the absence of colony forming units (CFUs) on the agar media confirmed the samples ready to be inoculated by the test bacteria to initiate the microbial challenge test.

Microbial challenge test

One loop full (~10^8 cells) of the each pure culture of *E. coli*, *Klebsiella* spp., *Salmonella* spp., *Pseudomonas* spp., *Bacillus* spp., *Staphylococcus* spp., *Listeria* spp. and *Vibrio* spp. were transferred into 9 ml sterile normal saline containing test tubes separately (Feroz et al. 2013). Each of the vegetable sample suspensions (10 ml) were inoculated (by means of dipping) with 100 μl of bacterial suspension resulting in the initial load of ~10^5 cfu/g (Fatema et al. 2013; Feroz et al. 2013). The inoculum versus vegetable suspension (serving as the the challenge test media) ratio (1:1000 v/v) was thus balanced in a way so that the final water activity (a_w, the water required for microorganisms) was sufficient enough to support the inoculated microbial growth and survival (Jay 2000). Control samples were kept un-inoculated. All the suspensions kept at room temperature at which vegetables are stored and handled. The pH of the suspension of chili was recorded to be 6.3, for onion the pH was 6.8, for capsicum the pH was 6.0, and for the coriander suspension, the pH was recorded to be 6.5. The pH of the control suspension was 5.5 which was measured by the pH-25 permission pH/mV meter (Manufactured by BSK technologies, Hyderabad, India). The water activity of the suspensions of processed chili, onion, capsicum and coriander was estimated to be 0.88, 0.86, 0.90 and 0.87, consecutively (by using standerd machine) . The inoculated samples were enumerated up to 15 days at every 24-h by means of standard spread plate methods (Cappuccino and Sherman 1996; Feroz et al. 2013). The growth potential is defined as difference between $\log_{10}$ cfu/g of final and initial concentrations of inoculated microorganisms (Feroz et al. 2013). In order to assess the growth potential of the artificially inoculated bacterial species in the samples used for the challenge test, the log reduction and

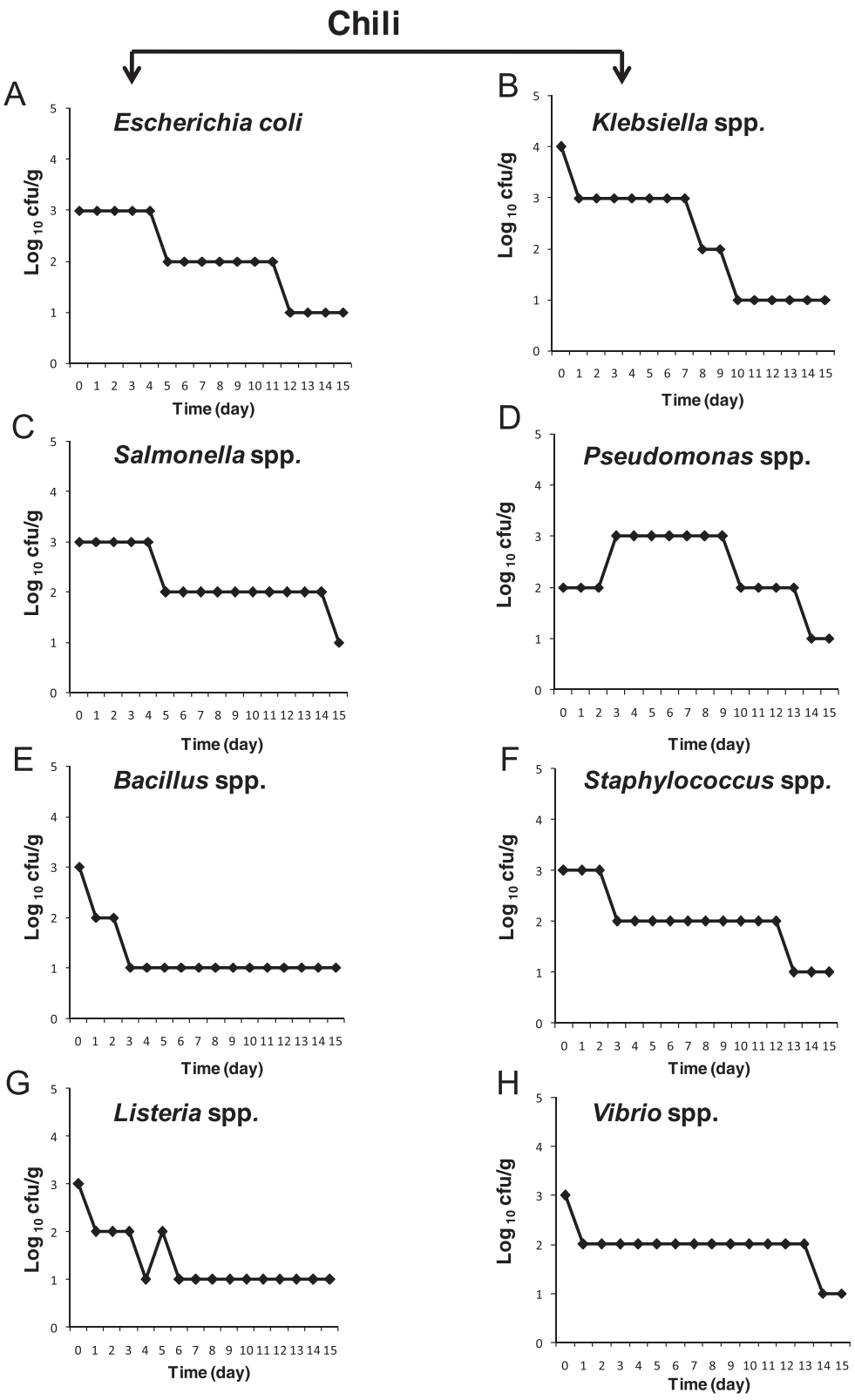

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Bacterial survival assay in chili samples: **a** *Escherichia coli,* **b** *Klebsiella* spp., **c** *Salmonella* spp., **d** *Pseudomonas* spp., **e** *Bacillus* spp., **f** *Staphylococcus* spp., **g** *Listeria* spp. and **h** *Vibrio* spp. Vegetable samples were processed and the bacterial cells were grown as stated in Materials and Methods. The turbidity of the bacterial culture to be introduced within the samples were adjusted with the standard solution of McFarland (OD_{600} 0.5). After inoculation, the culturable bacterial populations within the chili samples were estimated by measuring the colony forming units per gram of sample (cfu/g) on LB plates at every 24 h up to 15 days

percent reduction was calculated using the following formulas given earlier by Feroz et al. (2013):

$$\begin{aligned} \mathrm{Log_{10}reduction} &= \mathrm{Log_{10}(initial\ load) - Log_{10}(final\ load)} \\ \%\ \mathrm{Reduction} &= \{[\mathrm{Log_{10}(initial\ load) - Log_{10}(final\ load)}]/ \\ &\quad \mathrm{Log_{10}(initial\ load)}\} \times 100 \end{aligned}$$

Since a growth potential of >0.5 log(10) cfu/g within food products is known to potentially endanger human health, the chosen inloculum size of approximately 5 log cfu/g (i.e., around 10^5 cells/g) in the current study may reflect the fatal degree of microbial contamination in real (Skalina and Nikolajeva 2010). The maximal growth points (with the highest bioburden along with incubation) were considered in order to compare the initial load with that of the final load after 15 days. The experiments were performed 3 times independently and the data were statistically analyzed through *t* test.

Results and discussion

The study of microbial challenge test of food is used to simulate the microbial growth and survival or simply the increase or decrease in the number of microorganisms during food preparation, processing, handling and distribution. A microbiological challenge study determines the potential of microorganisms to utilize the food as substrate and consequently reflect the possible health hazard or food spoilage risk. Knowledge of conducting the challenge test with precise interpretation of the results may assist the food production units to ensure the food quality and food safety in accordance to the recommended microbiological specifications. The earlier detection of a vast amount of microorganisms (from 10^5 to 10^8 cfu/g) within the raw vegetables led the possibility of those samples to serve as substrate to enhance the growth and replication of the spoilage microbes (Abadias et al. 2008; Cordano and Jacquet 2009; Rahman and Noor 2012). Therefore, the increased consumption of raw and fresh vegetables has triggered the necessity of research on how microenvironment of these kinds of foods affects produce's safety. As per our recent studies relating the microbial challenge tests, decay in the growth and proliferation of bacteria has been observed (Fatema et al. 2013; Feroz et al. 2013). Another point is to ponder that our recent studies found the samples studied here to be naturally contaminated with heterotrophic bacteria with an average load of 10^6 cfu/g (Ahmed et al. 2014). Additionally, all samples were found to be contaminated with fungi (Ahmed et al. 2014). Prevalence of such huge number of heterotrophic bacteria and fungi indicated the vegetable items to be good substrates for microorganisms. To further demonstrate whether the vegetables can support the pathogenic microorganisms upon artificial introduction, the current study with the approach of microbial challenge test (MCT) was conducted.

After inoculation, the bacterial load in chilli samples was found to be reduced more than 2 log for *E. coli*, *Klebseilla* spp., *Vibrio* spp., *Bacillus* spp., *Salmonella* spp., *Listeria* spp., and *Pseudomonas* spp. and *Staphylococuus* spp. (Fig. 1, Additional file 1). However, a sudden increase by approximately 1-log in the growth of *Klebsiella* spp., *Salmonella* spp. and *Listeria* spp. was noticed at 4–6, 12, 6–10 and 12–14 days, consecutively. After 15 days of observation the microbial growth was declined significantly from the initial load of the isolates.

In the onion samples, nearly 3-log reduction was observed for *Salmonella* spp. and *Vibrio* spp., while *Staphylococcus* spp., *Pseudomonas* spp., *Listeria* spp., *Bacillus* spp., *E. coli*, and *Klebseilla* spp. were found to be reduced nearlly as 2-log (Fig. 2, Additional file 1). However, bacterial culture of *Salmonella* spp., *Bacillus* spp., *Staphylococcus* spp and *Vibrio* spp. were found to exhibit enhanced culturable cells by 1-log increase till 6 days of incubation which afeterward exhibied 1 log reduction.

In capsicum samples, more than 2-log reduction was observed for *Salmonella* spp. and *Vibrio* spp. whereas the growth of *Klebseilla* spp., *E. coli* and *Staphylococcus* spp. were found to be reduced by nearly 2-log after 15 days of incubation. Only *Bacillus* spp, *Listeria* spp., and *pseudomonas* spp were found to be reduced as 1-log reduction. The isolates was sponteously reduced up to 15 days (Fig. 3, Additional file 1).

In case of the coriander samples, nearly 2-log reduction was detected for *Klebseilla* spp., *E. coli, Listeria* spp., and *Pseudomonas* spp. while more than 2-log reduction was observed for *salmonella* spp., *vibrio* spp. *Staphylococccus* spp., and *Bacillus* spp. (Fig. 4, Additional file 1). Overall the log reduction of the tested bacterial species, especially of *Vibrio* spp. was noticed to be higher in chilli and onion

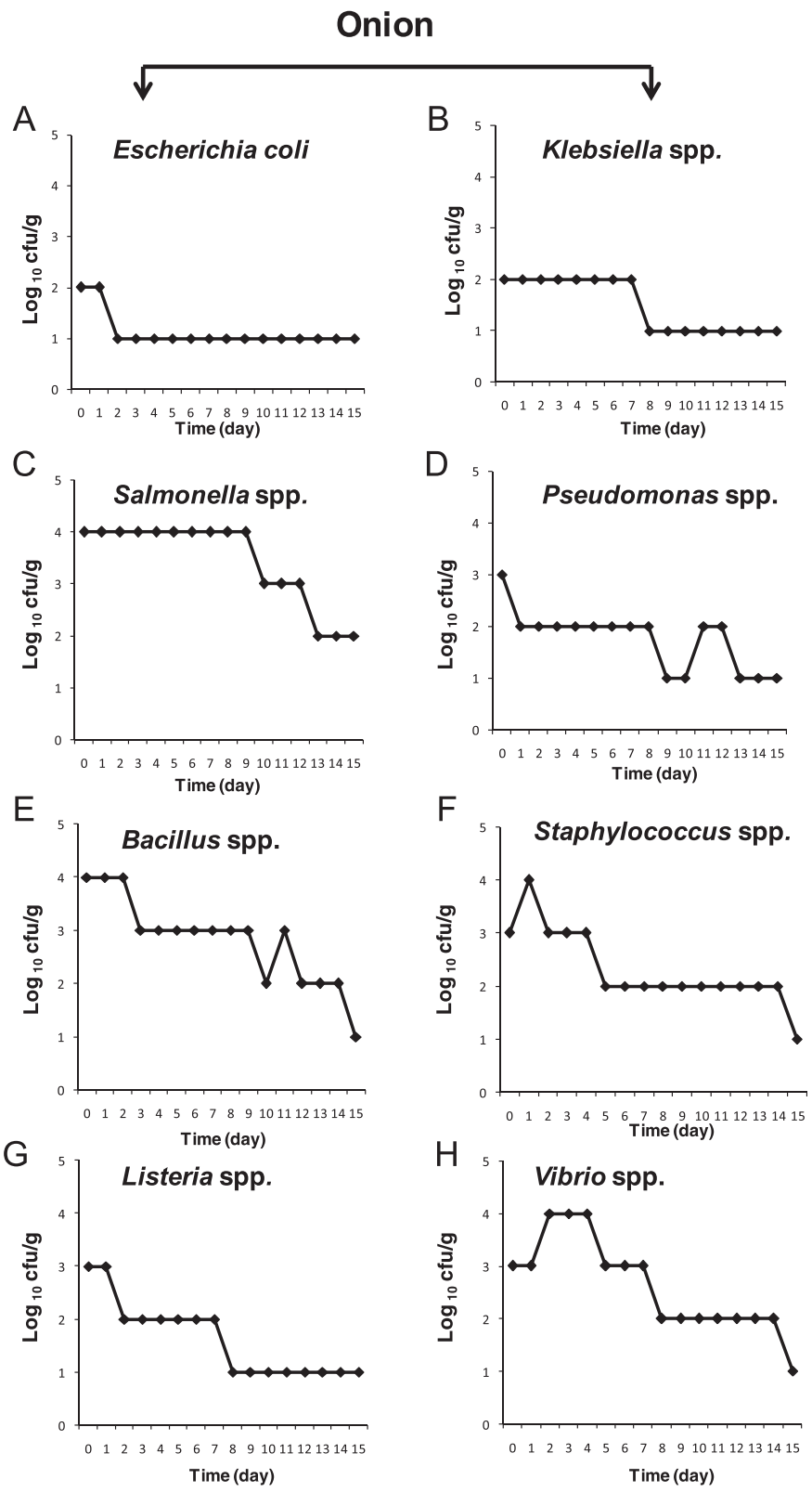

Fig. 2 Bacterial survival assay in onion samples: **a** *Escherichia coli,* **b** *Klebsiella* spp., **c** *Salmonella* spp., **d** *Pseudomonas* spp., **e** *Bacillus* spp., **f** *Staphylococcus* spp., **g** *Listeria* spp. and **h** *Vibrio* spp. Vegetable samples were processed and the bacterial cells were grown as described earlier. After inoculation, the culturable bacterial populations within the onion samples were estimated at every 24 h up to 15 days

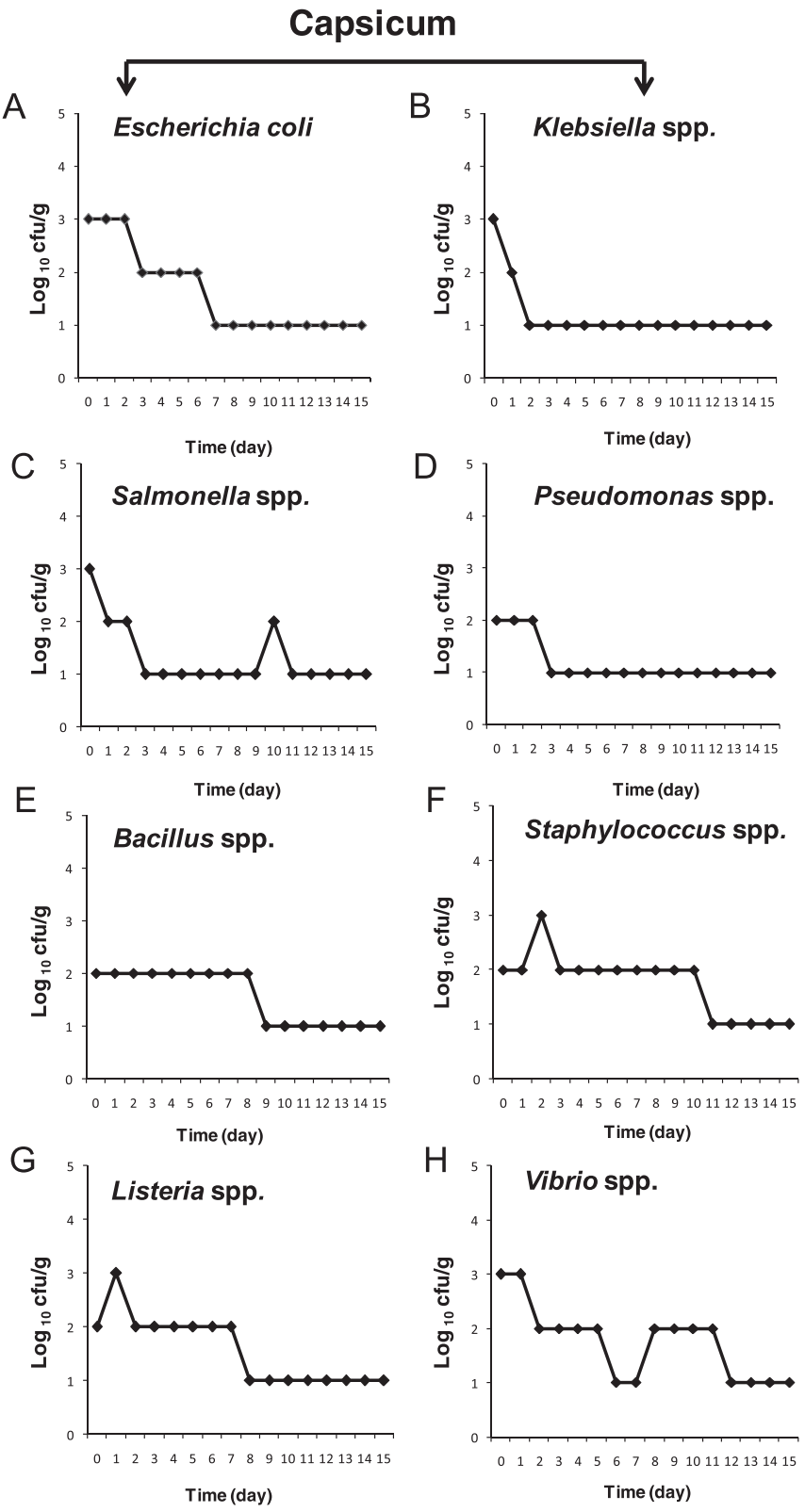

Fig. 3 Bacterial survival assay in capsicum samples: **a** *Escherichia coli,* **b** *Klebsiella* spp., **c** *Salmonella* spp., **d** *Pseudomonas* spp., **e** *Bacillus* spp., **f** *Staphylococcus* spp., **g** *Listeria* spp. and **h** *Vibrio* spp. Vegetable samples were processed and the bacterial cells were grown as described earlier. After inoculation, the culturable bacterial populations within the samples were estimated at every 24 h up to 15 days

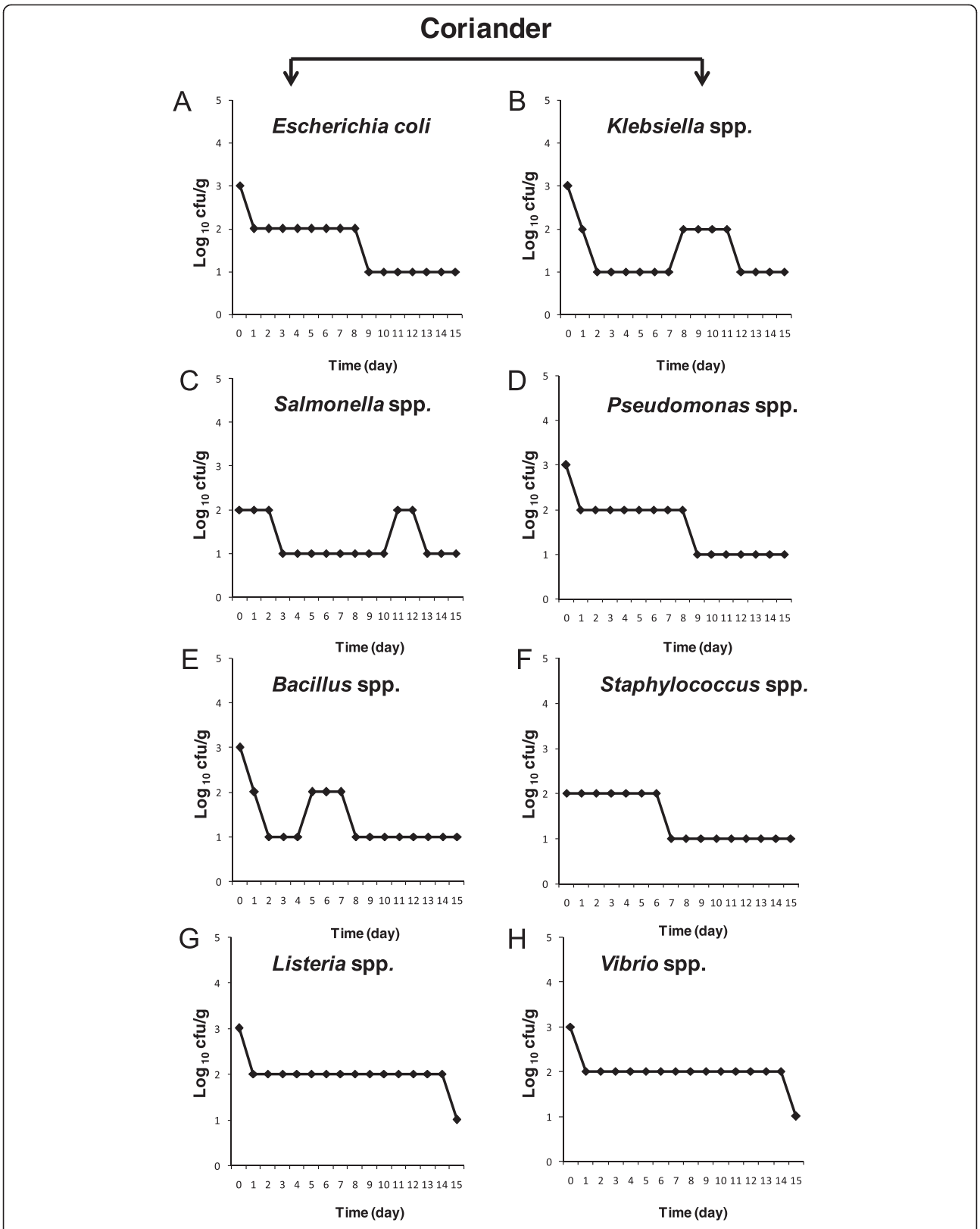

Fig. 4 Bacterial survival assay in coriander samples: **a** *Escherichia coli*, **b** *Klebsiella* spp., **c** *Salmonella* spp., **d** *Pseudomonas* spp., **e** *Bacillus* spp., **f** *Staphylococcus* spp., **g** *Listeria* spp. and **h** *Vibrio* spp. Vegetable samples were processed and the bacterial cells were grown as described earlier. After inoculation, the culturable bacterial populations within the samples were estimated at every 24 h up to 15 days

samples than those challenged within the capsicum and corainder samples (Additional file 1).

In consistent to our earlier studies with tomato, carrot, lettuce and cucumber samples, in the present work, after inoculating capsicum, coriander, chili and onion samples, a steady increase in the bacterial culturable cells was observed which firmly ponder the fresh vegetables sources for nutrition to support bacterial growth (Feroz et al. 2013). However, the afterward decrease in bacterial growth in the vegetables might be due to the nutrient depletion or because of the malfunction of the bacterial house-keeping genes (Gould 2000; Bibek 2005; Kabir et al. 2005; Noor et al. 2009a; Fatema et al. 2013; Feroz et al. 2013). Moreover, as discussed by Feroz et al. 2013, the increase in bacterial burden after the reduction was not unlikely due to the expression of certain stress responsive genes (Noor et al. 2009a; Noor et al. 2009b). However, the ultimate reduction in bacterial burden was indicative of the subsequent utilization of substrates to the limiting concentrations by the tested bacterial population (Feroz et al. 2013).

Together with our earlier investigations, the microbiological challenge tests conducted in this study may reveal how inoculum size, type of vegetables and other physicochemical factors influence growth and survival of pathogens in the surface of vegetables (Fatema et al. 2013; Feroz et al. 2013). The limitations of the study leans against the lack of molecular study to detect the expression of bacterial genes under the stress regulons, which might unveil the actual mechanism of bacterial reduction within the vegetable samples studied. Another limitation of our study is the lack of using real food for challenge test which could allow us to determine the role of resident microbiota on the potential colonization and survival of pathogenic bacteria. Nevertheless, the current study revealed the potential effects of food components on contaminating bacterial survival and their response to sublethal injury. The knowledge on such bacterial survival might be helpful in assessing food safety, food stability and finally the consumer safety. The practical implementation of challenge tests lies on the assurance of longer self life of product through maintaining recommended microbiological practices in product processing and handling. Present work imparted a model of challenge study design comprising the applicability of models to different fresh vegetables studied here, concerned pathogens in accordance with their growth rates, challenge test conditions, inoculation methods, inoculum and sample sizes, etc. comparable with the un-inoculated controls. Thus, the present work portrayed the survivality of the pathogens in fresh vegetables which are often contaminated with the bacterial pathogens.

Conclusions

Our research on the survival of pathogenic bacteria within the fresh vegetables revealed the necessity of maintaining proper sanitary condition during processing, storage and handling of the vegetables that we regularly consume and also notified the importance of searching the way how we can minimize the risk of getting different diseases. The findings of this research may also help form a policy guideline for safe consumption of raw vegetables based on the capacity of the particular vegetable to resist against contaminating pathogens and also contribute to ensure food safety and security. After successful completion of several practical trials using other vegetables and properly validating the experimental data in real fresh vegetables, the results are expected to provide support and service to different sectors of national economy such as agriculture, health, food and environmental sciences.

Additional file

Additional file 1: Growth decay and survival capacity of the test isolates in "Chilli, Onion, Capsicum and Coriander". (DOC 69 kb)

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

RN and MMR participated in the study conception and design, RN participated in revising the manuscript critically for important intellectual content and has given the final approval of the version to be submitted. MM (2nd author), MSR and MM (4th author) conducted the experiment and acquisition of the data. MA performed data acquisition. All authors read and approved the final manuscript.

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RN is the Head of the Department of Microbiology, Stamford University Bangladesh. MM, MSR and MM are the MS students of the same Department while MA is working as a Lecturer there. MMR is a Professor of the Department of Microbiology, University of Dhaka.

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