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Biocontrol of *Listeria monocytogenes ATCC* 7644 in Fresh Tomato with Probiotics

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The effectiveness of probiotics *Lactobacillus bulgarius* and *Streptococcus salivarius* as biocontrol agents against *Listeria monocytogenes* ATCC 7644 in fresh tomato throughout storage and their effect on the physicochemical properties of tomato was evaluated. Tomato samples were cut into wedges and inoculated with 10^8 CFU/ml of *Listeria monocytogenes* ATCC 7644, thereafter inoculated with *L.bulgaricus* and *S.salivarius* separately. Tomato was also inoculated with probiotics *L.bulgaricus* and *S.salivarius* without inoculation of *L.monocytogenes*. Nutrient broth was prepared and inoculated with 10^8 CFU/ml of *L.monocytogenes* ATCC 7644 and thereafter inoculated with *L.bulgaricus* and *S.salivarius* separately after which all treatments were stored at 4°C for 72 hours. Chlorine was used as a control and compared against probiotics. *L. monocytogenes* counts taken during storage period in nutrient broth showed that *L. bulgaricus* had a 2.19 log reduction and *S. salivarius* had a 1.65 log reduction. The tomato study showed that *L. bulgaricus had a 3.15* log reduction and *S.salivarius had a 3.01* log reduction. Physicochemical properties of tomato were not affected ($p > 0.05$) by treatment with probiotics when compared to control. Statistical analysis showed a significant difference between both probiotics and chlorine in tomato. This research indicated that *L.bulgaricus* and *S. salivarius* could potentially be used as eco-friendly biocontrol agents in the produce industry.

Key words: Tomato, *Listeria monocytogenes*, Probiotics, *Streptococcus salivarius* and *Lactobacillus bulgaricus*.

Fresh minimally processed vegetables provide a convenient fresh product for food services and consumers (Maistro and Corrêa 2012). A high level of quality and safety are crucial in minimally processed vegetables. Vegetables require more attention than other food groups due to the large number of enzymic, respiratory and microbiological factors which impact on the safety (Maistro and Corrêa 2012). Minimally processed vegetables are a rapidly developing genre of foods which have attracted food sectors such as, food manufacturers, restaurants and retail food stores. However, during growth, harvesting, handling and transportation fresh produce can become contaminated with pathogenic bacteria from different sources (Berger*et al.*, 2010). It is for this reason that there have been numerous produce outbreaks due to the contaminated produce being consumed by humans and even animals (Beuchat , 2002). *Listeria monocytogenes* and other persistent pathogens such as *E. coli* O157:H7 and *Salmonella* spp remain leading cause of food borne outbreaks and food recalls. *L. monocytogenes* is commonly recovered from fresh produce and may cause listeriosis and other complications in children, aged, pregnant women and immunocompromised individuals (Farbera and Peterkin 1991, CDC, 2006.

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According to Beuchat (2006), fresh produce may be contaminated during pre- harvest and post- harvest stages. The sources of pre harvesting include pathogenic microorganisms in the soil due to droppings of animals (Olaimat and Holley, 2012). Water is also a source of contamination as water from streams and lakes may be contaminated with pathogenic microorganisms (Beuchat, 2006). Organic fertilisers used for vegetable production may also contaminate the produce. Post- harvest sources include the handling, storing, and transportation of the vegetables from farms to stores and during cleaning. Environmental factors such as dust and insects also play a role in post- harvest contamination (Beuchat, 2006).

Chlorine is the most commonly used sanitizer in the fresh produce industry (Parish *et al*., 1997). Chlorine rinses are frequently used with concentrations varying from 50 to 200 ppm and with typical contact times of less than 5 min (Parish *et al*., 1997). The beneûts of chlorine use for the produce industry outweigh the concerns of potential formation of harmful by-products. Studies have shown that chlorine rinses can decrease the bacterial load by values ranging from a 1 log CFU/ g to 3.15 log CFU/g, depending on inoculation method, chlorine concentration, contact time, and the target bacteria (Ramos *et al.*, 2013). Chemicals that are chlorine based are often used to sanitize produce and surfaces within produce processing facilities, as well as to reduce microbial populations in water used during cleaning and packing operations. Chlorine however are not one hundred percent effective.

Numerous other control measures have been implemented to reduce the number of pathogenic microorganisms in vegetables. Such measures include temperature, physical removal of microorganisms, chlorine, quaternary ammonium compounds, irradiation, ozone, hydrogen peroxide and bio-control agents (Beuchat, 2002). Bio-control agents such probiotics and bacteriophage are of interest in recent time because consumers have become interested sustainable methods.

Bio-control is the application of microorganisms to prevent pathogenic contamination during post harvesting (Stiling and Peter 2005). This method may be preferred to others because it is not harmful to humans and to the environment unlike the other control measures. Bio-control agents include the use of probiotics and bacteriophages.

Probiotics are live microorganisms which offer a health benefit on the host (Marco and Kleerebezem 2006). Lactic acid bacteria are generally used as probiotics and the most common probiotics are *Lactobacillus sp* and *Bifidobacteria sp*.It is therefore important to determine the effectiveness of using probiotics, an environmental friendly alternative. Furthermore there is little or no reported work on the use of this agent for the control of pathogens in fresh produce.The aim of this study is consequently to use probiotics to control food borne pathogen in fresh or minimally processed vegetable and determine its effect on the quality of the vegetables.

MATERIALS AND METHODS

Two types of probiotics were used namely *Lactobacillus bulgaricus*ATCC 53103 and *Streptococcus salivarius*ATCC 13419. Both probiotics were used individually.

Preparation of probiotics:

*Lactobacillus bulgaricus*ATCC 53103 was grown in MRS broth for 15 ± 2h at 37ÚC. The cells were obtained by sub culturing on MRS agar while *Streptococcus salivarius*ATCC 13419. The probiotic strain was grown on MSA agar for 24 hours at 37ÚC. After incubation the colonies were sub cultured into BHI broth.

Preparation of inoculums:

***Listeria monocytogenes* ATCC 7644**

Listeria monocytogenes (ATCC 7644) was used as the food borne pathogen. *L.monocytogenes*ATCC 7644 was bought in the form of a swab. *L.monocytogenes* ATCC 7644 was swabbed on *Listeria* agar (Oxoid, UK) and incubated for 24 hours at 37ÚC. After incubation the *Listeria* colonies were inoculated in ½ Fraser broths (Oxoid, UK) for 24 hours at 37ÚC. The ½ Fraser broths were prepared according to manufacturer's instructions. After incubation 1ml of the ½ Fraser broths were inoculated into 5-10 full Fraser broths and incubated for 24 hours at 37ÚC. The full Fraser broths (Oxoid, UK) were prepared according to manufacturer's instructions. After incubation slants were prepared using nutrient agar, contents from the full Fraser broth

were inoculated here and stored at 4ÚC.

The concentration of probiotics and pathogen used were determined using a McFarland's standard which gave a concentration of 1.5×10^8cfu/ml (Algere*et al.*, 2011).

***Lactobacillus bulgaricus*ATCC 53103**

A spread technique was used. 1ml of probiotic from MRS broth was plated onto MRS agar plates. The plates were then incubated at 37ÚC for 24hours. After incubation a McFarland standard was prepared using 1% barium chloride and 1%sulphuric acid. In a test tube 9.95ml of sulphuric acid was pipetted and 0.05ml of barium chloride was pipetted this gave a concentration of 1.5×10^8cfu/ml. In another test tube 10ml of saline water was prepared, colonies of *Lactobacillus bulgaricus* were inoculated into the test tube until the concentration of the probiotic test tube matched the concentration of the McFarland standard.

***Streptococcus salivarius*ATCC 13419**

A spread technique was used. 1ml of probiotic from BHI broth was plated onto BHI agar plates. The plates were then incubated at 37ÚC for 24hours. After incubation a McFarland standard was prepared using 1% barium chloride and 1%sulphuric acid. In a test tube 9.95ml of sulphuric acid was pipetted and 0.05ml of barium chloride was pipetted this will give a concentration of 1.5×10^8cfu/ml. In another test tube 10ml of saline water were prepared, colonies of *Streptococcus salivarius* was inoculated into the test tube until the concentration of the probiotic test tube matched the concentration of the McFarland standard.

Effect of probiotics on the survival of inoculated *Listeria monocytogenes*in broth cultures under storage

Nutrient broths were prepared aseptically following manufacturer's instructions. Two sets of nutrient broths were prepared. The first set of nutrient broths were inoculated with 1ml of *Listeria monocytogenes* ATCC 7644 from the McFarland standard and thereafter inoculated with 10^8cfu/ml of probiotic *Lactobacillubulgaricus* from the Mcfarland standard. The second set of nutrient broths were inoculated with 1ml of *Listeria monocytogenes* ATCC 7644 followed by inoculation of 10^8cfu/ml of probiotic *Streptococcus salivarius*ATCC 13419. Both set of broths were stored at 4ÚC for 72 hours. On each day serial dilutions were done thereafter a spread plate technique was carried out and plates were incubated at 37ÚC for 24 hours. After incubation there was a *Listeria monocytogenes* ATCC 7644 count.

The control for this objective was 200ppm of chlorine.

Effect of probiotics on the survival of inoculated *Listeria monocytogenes*in fresh tomato under storage

Tomatoes were washed with distilled water and 70% ethanol and then sliced into 10g. The first step was to ensure that the tomato was free from *Listeria monocytogenes*ATCC 7644. A streak plate technique was used and *Listeria* agar was used. The plates were then incubated at 37ÚC for 24 hours. After incubation a count was done.

Once there was certainty that the tomatoes were free from *Listeria monocytogenes* objective 2 commenced.

Tomatoes were washed with distilled water and 70% ethanol thereafter sliced into 10g. For this objective 10×10g of tomato were used. All of the 10×10g of tomato was placed in stomacher bags thereafter10^8cfu/ml of *Listeria monocytogenes* ATCC 7644 were inoculated from the McFarland's standard into each bag, in 5 of the bags 10^8cfu/ml of probiotic *L.bulgaricus* were inoculated from the McFarland's standard and the other 5 bags were inoculated with 10^8cfu/ml of probiotic *S.salivarius.* All 10 bags were then placed in the stomacher for maceration and stored at 4ÚC for 72 hours. Each day the respective bags were removed and 90ml of sterile water were added. Dilutions were done followed by enumeration. Plates were incubated at 37ÚC for 24 hours thereafter a *Listeria monocytogenes* ATCC 7644 count were done. The control for this objective was 200ppm of chlorine

Effect of probiotics on the physicochemical properties(pH, soluble solids content and titratable acidity) of fresh tomato under storage.

For this objective no pathogen was used, only probiotics and tomato were used. The tomatoes were washed and sliced into 10g. 10×10g of tomato were used and placed into stomacher bags. Five of the bags were inoculated with 10^8cfu/ml of probiotic *Lactobacillus* from the McFarland standard. The other 5 bags were inoculated with10^8cfu/ml of probiotic *Streptococcus* from the

McFarland standard. The bags were then stored at 4ÚC for 72 hours. On each day physicochemical tests such as pH, soluble solid content and titratable acidity were performed on the respective bags. 90ml of distilled water were added before the physicochemical tests were performed.

pH

The pH of the tomato was determined using a pH meter. 50ml of sample were used(Algere*et al*., 2011).

Soluble solid content

The soluble solid content was determined using a Brix refractometer at 20ÚC. 20ml of tomato juice were filtered using filter paper. Using a plastic dropper 2 drops of the filtrate were applied to the refractomer (Algere*et al*., 2011).

Titratable acidity

The tomato juice was filtered and 10ml of filtered tomato juice were diluted with 50ml of distilled water. This mixture was then titrated against 0.1 N NaOH. The volume of the NaOH added to the solution was then multiplied by a correction factor of 0.064 to estimate the titratable acidity (Algere*et al*., 2011).

The control for this objective was only tomato.

Statistical analysis

Analysis of variance (ANOVA), p*d"*0.05, (Tulsa, Oklohama, USA, 2003) was used to determine whether there were significant differences between growth of *L. monocytogenes*ATCC 7644 after treatment with the two probiotics and to also determine differences between the quality of tomato treated with probiotics and control. The experiments were repeated three times

RESULTS AND DISCUSSION

Figure 1 shows the growth of *L. monocytogenes* ATCC 7644 in nutrient broth after application of probiotics *Lactobacillus bulgaricus* and *Streptococcus salivarius*. While the control chlorine resulted in a 2.15 log reduction of *L.monocytogenes*. *L.bulgaricus* resulted in a 2.19 log reduction of *L.monocytogenes* and *S.salivarius* resulted in a 1.65 log reduction of *L.monocytogenes*. Biocontrol with *L.bulgaricus* was more effective in reducing *L.monocytogenes* in nutrient broth as compared to *S.salivarius.*

Table 1 and Figure 2 show the Survival of *L.monocytogenes* ATCC 7644 in fresh tomato after biocontrol with probiotics *Lactobacillus bulgaricus*and *Streptococcus salivarius*.The results from the above graph show that chlorine

Table 1. Survival *of L.monocytogenes* ATCC 7644 in fresh tomato after biocontrol with probiotics *Lactobacillus bulgaricus* and *Streptococcus salivarius*[1]

Treatment time	*L.bulgaricus* in tomato(log CFU/ml)	*S.salivarius*in tomato(log CFU/ml)	Control (log CFU/ml chlorine)
1 h	$5.10^{a} \pm 0.11$	$5.77^{b} \pm 0.03$	$3.18^{c} \pm 0.02$
24 h	$4.96^{a} \pm 0.09$	$4.75^{b} \pm 0.02$	$3.14^{c} \pm 0.02$
48 h	$4.71^{a} \pm 0.09$	$4.75^{b} \pm 0.02$	$2.97^{c} \pm 0.04$
72 h	$4.62^{a} \pm 0.05$	$4.69^{b} \pm 0.10$	$2.76^{c} \pm 0.03$

1 Means ± SD (n=3)

2 Means with different superscript letters in columns are significantly different ($p \leq 0.05$)

Table 2. Changes in pH of fresh tomato inoculated with probiotics *L.bulgaricus* and *S.salivarius* during storage at 4°C for 72 hours [1]

Treatment time	*L.bulgaricus* in tomato	*S.salivarius* in tomato	Control (tomato)
1 h	$4.42^{a} \pm 0.09$	$4.55^{a} \pm 0.11$	$4.55^{a} \pm 0.04$
24 h	$4.55^{a} \pm 0.03$	$4.50^{a} \pm 0.05$	$4.58^{a} \pm 0.01$
48 h	$4.55^{a} \pm 0.07$	$4.42^{a} \pm 0.02$	$4.52^{a} \pm 0.06$
72 h	$4.47^{a} \pm 0.05$	$4.52^{a} \pm 0.01$	$4.55^{a} \pm 0.09$

1 Means ± SD (n=3)

Table 3. Changes in soluble solids of fresh tomato inoculated with probiotics *L.bulgaricus* and *S.salivarius* during storage at 4°C for 72 hours 1

Treatment time	*L.bulgaricus* in tomato (°Brix)	S.salivarius in tomato (°Brix)	Control Tomato (°Brix)
1 h	0.4a ± 0.1	0.4a ± 0.1	0.4a ± 0.1
24 h	0.4a ± 0.1	0.4a ± 0.1	0.4a ± 0.1
48 h	0.4a ± 0.0	0.4a ± 0.0	0.5a ± 0.1
72 h	0.5a ± 0.1	0.4a ± 0.1	0.5a ± 0.1

1 Means ± SD (n=3)

Table 4. Changes in titratable acidity of fresh tomato inoculated with probiotics *L.bulgaricus* and *S.salivarius* during storage at 4°C for 72 hours 1

Treatment time	*L.bulgaricus* in tomato (%malic acid)	*S.salivarius* in tomato (%malic acid)	Control Tomato (%malic acid)
1 h	0.09a ± 0.0	0.08a ± 0.0	0.08a ± 0.0
24 h	0.08a ± 0.0	0.08a ± 0.0	0.08a ± 0.0
48 h	0.09a ± 0.0	0.08a ± 0.0	0.08a ± 0.0
72 h	0.09a ± 0.0	0.09a ± 0.0	0.09a ± 0.0

1 Means ± SD (n=3)

Table 5. P values of effect of chlorine, *L. bulgaricus*and *S. salivarius* on survival of inoculated *Listeria monocytogenes* ATCC 7644 in nutrient broth and tomato

Treatment	P value for nutrient broth	P value for tomato
Chlorine	0.001*	0.001*
L. bulgaricus	0.001*	0.001*
S. salivarius	0.008*	0.001*

*P<0.05 indicating significant difference

resulted in a 5 log reduction of *L. monocytogenes*over 72 hours. Biocontrol with *L.bulgaricus* resulted in a 3.15 log reduction of *L.monocytogenes* and biocontrol with *S.salivarius* resulted in a 3 log reduction of *L.monocytogenes*. All 3 treatments showed to have significant difference in reducing *L.monocytogenes* in tomato. However *L.bulgaricus* was more effective in reducing *L. monocytogenes* in fresh tomato compared to *S. salivarius* for the following reasons. This may be because the inhibitory bacteriocin produced by *S. salivarius may not be* as effective

Table 6. P values of effect of pH, soluble solids and titratable acidity on *L. bulgaricus*and *S. salivarius* in fresh tomato

Treatment	pH P value	Soluble solids P value	Titratable acidity P value
Tomato (Control)	0.652*	0.217*	0.059*
L. bulgaricus + Tomato	0.068*	0.389*	0.193*
S. salivarius + Tomato	0.113*	0.848*	0.637*

*P>0.05 indicating no significant difference

as the one produced by *L. bulgaricus*since there are different forms of bacteriocin (Settanni and Corsetti, 2008).

Figure 3 shows the log reduction of *L. monocytogenes* ATCC 7644 in nutrient broth and tomato using chlorine, *L. bulgaricus* and *S. salivarius*over a period of 72 hours.The above graph shows that there were higher log reductions of *L.monocytogenes* in tomato compared to nutrient broth. The overall log reduction of *L.monocytogenes* in tomato using chlorine was 5 logs, *L.bulgaricus* 3.15 logs and *S.salivarius* 3 logs respectively. This reason for this may be because the way microorganisms grow in vivo might not necessarily be the same way they grow in vitro. In addition, specific understanding of behaviour of pathogens especially on vegetable or fruit surfaces is limited (Brackett, 1999). The result also showed that the two probiotics were not able to effectively remove all the inoculated pathogens. Combination of probiotics with other control measures such as low pH, low temperature and perhaps safe chemical such as SDS may prove more effective. Randazzo*et al.* (2009) concluded in their work with bacteriocins that they may provide a novel, safe alternative and effective hurdle when they are combined with other control measures.

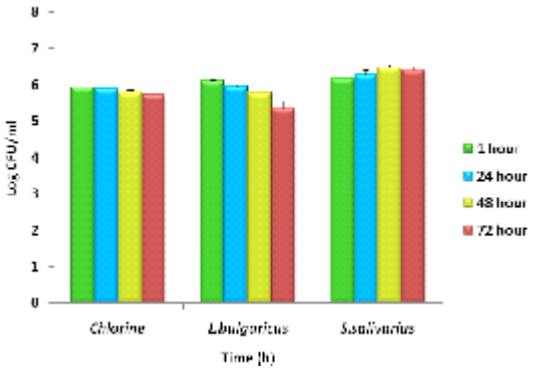

Fig 1. Survival of *L.monocytogenes* ATCC 7644 in nutrient broth after biocontrol with probiotics *Lactobacillus bulgaricus* and *Streptococcus salivarius*

Table 2, 3 4 show that the quality parameters (pH, soluble solids and titratable

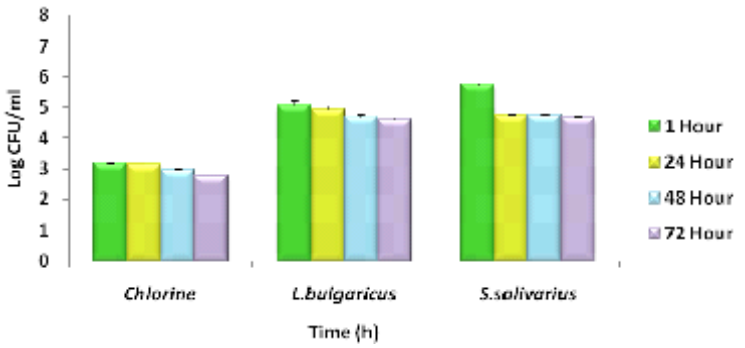

Fig 2. Survival of *L.monocytogenes* ATCC 7644 in fresh tomato after biocontrol with probiotics *Lactobacillus bulgaricus*and *Streptococcus salivarius*

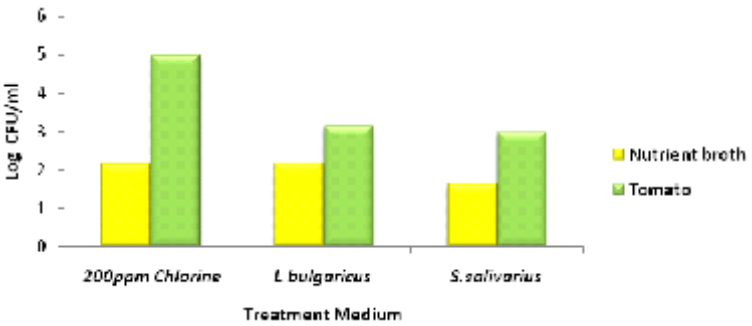

Fig 3. Log reduction of *L. monocytogenes* ATCC 7644 in nutrient broth and tomato using chlorine, *L.bulgaricus* and *S.salivarius* over a period of 72 hours

acidity) of both tomato treated with both probiotics and the control were similar. Signifying that these parameters were not influenced by treatment with probiotics *L.bulgaricus* and *S.salivarius* and storage time. This result was also shown statistically in Table 5 and 6.

The physicochemical results indicate that the use of probiotics in fresh tomato does not negatively affect the physicochemical properties of tomato as probiotics are generally regarded as safe. The use *of L. bulgaricus* and *S. salivarius*in fresh tomato did not affect the food matrix of the tomato hence maintaining stability and resulting in no significant change in physicochemical properties (Saarela*et al*., 2000). The result also signify that tomato supplemented with probiotics may be acceptable to consumers since the sensory and organoleptic integrity of the produce was not comprise.

CONCLUSION

This work indicated that *L. bulgaricus* and *S. salivarius* may be used as potential eco-friendly biocontrol agents in the fresh produce industry without any change in physicochemical properties. Chlorine is still a more effective sanitizer when compared to the use of probiotics. Probiotics have a promising potential for exploitation as functional supplements in fruit products due to their impressive tolerance to acidic environments (Algere*et al*., 2011).In addition to using probiotics as biocontrol agents in the fresh produce industry they may also add possible health benefits to produce such as, immunomodulation, control of diarrhoea, anticancer effects and possible improvement of Crohns disease. Probiotics have become an attractive treatment option because the rate of antibiotic resistance among pathogens continues to increase (Willey*et al*., 2011). Furthermore, they may provide a novel, safe alternative and effective hurdle when combined with other control mechanisms such as low pH, low temperature and modified atmosphere packaging.

Future studies can be done by increasing the concentration of the probiotics and by combining different biocontrol methods such as bacteriophages and probiotics for possible greater log reduction of *L. monocytogenes* in fresh produce.

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REFERENCES

1. Alegre I., InmaculadaVinas, JosepUsall, Marina Angurera and Maribel Abadias. Microbiological and physicochemical quality of fresh cut apple enriched with the probiotic strain *Lactobacillus rhamnosus GG. Journal of food microbiology,* 2011; **28**, 59-66.
2. Berger, C. N., Sodha S V, Shaw R. K, Griffin P M and Frankel, G, Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Journal of environmental microbiology,* 2010; **12**, 2385 – 2397.
3. Beuchat, L. R. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables.*Microbes and Infection,* 2002; **4**, *4*13 -423.
4. Beuchat, L. R. Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal,* 2006; **108**, 38-53.
5. Brackett, R.E. Incidence, contributing factors, and control of bacterial pathogens in produce. *Postharvest Biology and Technology,* 1999; **15**, 305–311
7. CDC. 2006. Surveillance for foodborne-disease outbreaks in US, 1998–2002.
8. Http://www.cdc.gov/mmwr/preview/mmwrhtml/ss5510a1.htm?_cid=ss5510a1_e.
9. Accessed 17 November 2009.
10. Farbera, J. M. and P. I. Peterkin. *Listeria monocytogenes,* a Food-Borne Pathogen. *Journal of microbiological reviews,* 1991; **55**, 476-511.
11. Maistro and L. Corrêa . Microbiological quality and safety of minimally processed vegetables . *Journal of food control,* 2012; **28**, 258-270.
12. Marco, M. L. and Kleerebezem S. P . Towards understanding molecular modes of probiotic action.*Journal of food biotechnology,* 2006; **17**, 204-210.
13. Olaimat A . N and Holley R. A . Factors influencing the microbial safety of fresh produce: A review. *Food Microbiology,* 2012; **32**, 1 – 19

14. Parish, M.E. Public health and nonpasteurized fruit juices. *Critical Review of Microbiology*, 1997; **23**, 109–119.
16. Willey J . M., Sherwood L . M and Woolverton C. J., Prescott's Microbiology. Eight Edition. McGraw-Hil, New York. Pg 1070, 2011.
17. Saarela M., Mogensen G, Fonden R, Matto J and Mattila- Sandhom. Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology*, 2000; **84**, 197 – 215
18. Stiling and Peter, What makes a successful biocontrol agent? A meta-analysis of biological control agent performance. *Journal of Biological control*, 2005; **34**: 236-250.
19. RandazzoC . L., Pitino L, Scifo G O and Caggia C., Biopreservation of minimally processed iceberg lettuces using a bacteriocin produced by *Lactococcuslactis* wild strain. *Food Control*, 2009l; **20**, 756 - 763
20. Ramos, B., Miller F. A, Branao T.S, Teixeira P and Silva C. M., Fresh fruits and vegetables - An overview on applied methodologies to improve itsquality and safety.*Innovative Food Science and Emerging Technologies*, 2013; **20**, *1* - 15.
21. Settanni, L. and Corsetti, A. Application of bacteriocins in vegetable food biopreservation. *International Journal of Food Microbiology*, 2008; **121**, 123–138