

Prevalence of High Risk Bacterial Pathogens in Indian Fresh Vegetables and Novel strategies for their inactivation

*A thesis
submitted in fulfillment of the requirement
for the award of the degree of*

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**

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2011**

CERTIFICATE



Certified that the thesis entitled “Prevalence of high risk bacterial pathogens in Indian fresh vegetables and novel strategies for their inactivation” which is submitted by **Ms. Richu Singla**, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate’s own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis “**Prevalence of high risk bacterial pathogens in Indian fresh vegetables and novel strategies for their inactivation**” submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Abhijit Ganguli**, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

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“ONE WHO DIRECT THE PATH OF PROGRESS IS ANGELIC”

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List of Abbreviations

| | |
|-------|--|
| ABTS | 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| APC | Aerobic plate count |
| BHIA | Brain heart infusion agar |
| BHIB | Brain heart infusion broth |
| BHT | butylated hydroxytoluene |
| BLAST | Basic Local Alignment Search Tool |
| BSE | Bovine spongiform encephalopathy |
| CFR | Code of Federal Regulations |
| CPC | Cetylpyridinium chloride |
| DFI | Druggan-Forsythe-Iversen formulation |
| DNA | Deoxyribonucleic acid |
| dNTP | 2'-deoxynucleoside-5'-triphosphate |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| EDTA | Ethylenediamine-tetra acetic acid |
| EEB | Enterobacteriaceae enrichment broth |
| EPA | Environment protection agency |
| EPS | Extracellular polymeric substances |
| FAO | Food agricultural organization |
| HEA | Hektoen Enteric agar |
| HPLC | High performance liquid chromatography |
| IDF | Insoluble dietary fiber |
| IFPA | International Fresh-cut Produce Association |
| KOH | Potassium hydroxide |
| MAP | Modified atmospheric packaging |
| MRD | Maximum recovery diluents |
| NCBI | National centre for biotechnology information |
| OD | Optical Density |
| PCA | Plate count agar |
| PCR | Polymerase chain reaction |
| ppm | Parts per million |
| rDNA | Ribosomal deoxyribonucleic acid |
| rpm | Revolution per minute |
| rRNA | Ribosomal ribonucleic acid |
| RVS | Rappaport-Vassiliadis Soya Peptone |
| SDF | Soluble dietary fiber |
| SEM | Scanning electron micrography |
| SOD | Superoxide dismutase |
| SPS | Sanitary and Phytosanitary |
| TAL | Thin Agar Layer |
| Td | Degradation temperature |
| TDF | Total dietary fiber |
| TSA | Tryptone soya agar |
| TSB | Tryptone soya broth |
| UPB | Universal Pre-enrichment broth |
| VP | Vogues – Proskauer |
| WTO | World Trade Organization |
| XLD | Xylose Lysine Deoxycholate medium |

List of Symbols

| | |
|-------------------------------|-------------------|
| % | Percentage |
| μ | Micron |
| μg | Microgram |
| μl | Microlitre |
| a _w | Water activity |
| bp | Base pair |
| C | Carbon |
| d | Days |
| Da | Dalton |
| g | Gram |
| H | Hydrogen |
| H ₂ O ₂ | Hydrogen peroxide |
| hr | Hours |
| Hz | Hertz |
| kb | Kilo base |
| KDa | Kilo Dalton |
| kV | Kilo volt |
| L | Litre |
| M | Molar |
| mA | Milli ampere |
| mg | Milligram |
| Mg | Magnesium |
| Min | Minutes |
| mL | Milliliter |
| N | Nitrogen |
| ng | Nanogram |
| sec | Seconds |
| U | Unit |
| UV | Ultraviolet |
| V | Volt |
| v/v | volume by volume |
| w/v | Weight by volume |
| α | Alpha |
| β | Beta |

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INTRODUCTION

Fresh Produce is globally recognized as an important component of a healthy diet because it is a source of vitamins, minerals, fiber, and antioxidants. Because most produce is grown in a natural environment, it is vulnerable to contamination with pathogens. Factors that may affect the occurrence of such contamination include agricultural water quality, the use of manure as fertilizer, the presence of animals in fields or packing areas, and the health and hygiene of workers handling the produce during production, packing, processing, transportation, distribution, or preparation. The above reasons as well as the fact that produce is often consumed raw without any type of intervention (that would reduce, control, or eliminate pathogens) prior to consumption contributes to its potential as a source of food borne diseases. Over the last decade, the epidemiology of food borne diseases in the developing as well as developed countries has changed due to the emergence of newer pathogens believed to be due to major changes in global economy (facilitating the rapid transport of perishable foods, increasing the potential for exposure to food borne pathogens from other parts of the world) as well as other factors (types of food that people eat, the sources of those foods, a possible decline in public awareness of safe food preparation practices as well as acculturation).

Food safety is a growing concern across the world. There is increasing need to provide greater assurance about the safety and quality of food to consumers. The increase in world food trade and the advent of the Sanitary and Phytosanitary (SPS) Agreement under the World Trade Organization (WTO) have led to increasing recognition and adoption of food safety measures. The capacity of India to penetrate world markets depends on its ability to meet increasingly stringent food safety standards imposed in developed countries. Indian

Food processing industry has an important role to play in linking the farmers to the final consumers in the domestic as well as the international markets. Food processing combined with marketing has the potential of solving the basic problems of agricultural surpluses, wastages, rural jobs, and better remuneration to the growers. In the next ten years, food production is expected to double. Food standards are expected to acquire greater importance given. Increasing concerns on food safety on the back of breakout of diseases such as BSE (mad cow disease), Avian Influenza, Bird Flu etc on the one hand, and growing consumer demand for products which are healthy on the other. These produces, if processed and marketed smartly, can make India a leading food supplier of the world.

Thus, the evolving epidemiology of food borne diseases, especially in developing countries needs to be monitored and understood so as to implement appropriate prevention technologies. An important, much sought after technology is 'Minimal processing' wherein, non-thermal technologies are used to process food in a manner for food safety and preservation as well as to maintain fresh-like characteristics of fruits and vegetables as much as possible. Among these, visual properties of fresh-produce (vegetables and fruits) commodities are one of the most important parameter to evaluate the total quality of the product by consumers. In addition to washing and disinfection of fresh produces (fruits and vegetables) currently, other treatments such as antioxidants, UV-C, antimicrobial preparations, heat shocks, non-ionizing radiations, organic acids, modified atmospheric packaging (MAP) alone or in different combinations have proved useful in controlling microbial growth, inactivation of several pathogens and maintaining quality during storage of fresh-cut produce. A few studies have examined the potential of wash additives including sodium hypochlorite (Beuchat et al., 1998), trisodium phosphate (Liao and Sapers, 2000), hydrogen peroxide (Sapers et al., 1999), and chlorine dioxide (Wisniewsky et al., 2000) sodium hypochlorite, hydrogen peroxide, potassium sorbate, and sodium salts of benzoate,

EDTA and phosphoric acids. Diluted potassium permanganate is useful for a wide variety of applications in food industry (Sukul and Sheth, 2012). But, its capacity to react with organic matter makes it less effective as sanitizer and also forms toxic substances during packaging. Although chlorine is a fairly widely used sanitizer for minimal processing, chlorination causes the formation of hazardous by-products, such as carcinogenic trihalomethane (THM), in food. Consequently, the use of chlorine is restricted legally in several countries. Therefore, the development of alternative working methods with different sanitizers is of great interest. Ozone may be suitable as an alternative sanitizer. It is a highly effective oxidant and is approximately four times more reactive than chlorine. Ozone has been in use for over 100 years as a preservative for foods and food ingredients, for purification in the brewing industry, for odor control and for medical therapy. Its reputation as a gaseous decontaminant has been well documented over recent years; however, research into its use as an aqueous sanitizer for produce washes is limited. However, studies with different produces as well as important pathogens are warranted for commercially prospecting the candidature of ozone alone or in combination as a novel minimal intervention strategy.

Increased numbers of foodborne outbreaks have been associated with fresh produce during the past decade likely in large part because of better outbreak surveillance systems, but also because of increased consumption of potentially more risky fresh-cut prepackaged products. Concerns with produce as a vehicle of foodborne infections, however, have been documented for more than a decade. Increased number of food borne outbreaks due to consumption of fresh and fresh-cut fruits and vegetables contaminated with a variety of human bacterial pathogens such as *Salmonella* spp., (Haeghebaert et al., 2003; Samadpour et al., 2006) and *Shigella* spp. (Davis et al., 1988) as well as the emerging bacterial pathogen *Cronobacter* (*Enterobacter*) *sakazakii*. For instance, *E. coli* O157: H7, *Salmonella* spp. and *Listeria monocytogenes* have been isolated from sprouted seeds, including alfalfa, moong

bean (Taormina et al., 1999). Although the prevalence of these key pathogens has been extensively studied elsewhere, few studies have been undertaken to determine their prevalence and survival in Indian fresh produces. Lack of appropriate scientific information in these lines has thus far, impeded the development of novel and effective intervention strategies indigenously.

The fresh-cut fruit and vegetable industry is constantly growing mainly due to the consumer's tendency of health consciousness and their increasing interest in the role of food for maintaining and improving human well-being (Gilbert, 2000; Ragaert et al., 2004). In fact, fruits and vegetables are basic ingredients of the highly demanded Mediterranean diet, associated with a beneficial and healthy function against numerous diseases (Flood et al., 2002; IFIC, 2001). This beneficial effect has been attributed to non-essential food constituents, phytonutrients, that possess a relevant bioactivity when frequently consumed as a part of regular diet (Steinmetz and Potter, 1996). This also corresponds to one of the traditional claims in proper dietary habits which aims for an increasing intake of fruits and vegetables (Liu et al., 2000). However, it is well-known that modern ways of life usually trend to a reduction of suitable intake of rich sources of antioxidant compounds, such as fruit and vegetables, being more emphasized in some parts of the population, especially children. It is known that a food which meets nutritional requirements is unlikely to be accepted by consumers if they do not like the flavor or other quality attributes (Da Costa et al., 2000). Additionally, it has been shown that consumer's needs for convenience are correlated with food choice (Ragaert et al., 2004; Verlegh and Candel, 1999).

Therefore, the fresh-cut fruit and vegetable industry is still working to increase the assortment of minimally processed vegetable products that meets consumer's needs for 'quick' and convenient products that preserve their nutritional value, retain a natural and

fresh color, flavor and texture, and contain fewer additives such as preservatives (Jongen, 2002).

Objectives of the present study

Therefore, both in-depth understanding of the ubiquity, behavior (survival kinetics and attachment) and data on inactivation kinetics (using organic acids and ozone) of pathogens, the thesis entitled “Prevalence of high risk bacterial pathogens in Indian fresh vegetables and novel strategies for their inactivation” was targeted; the thesis attempts to identify some prevalent key bacterial pathogens and evaluate novel minimal intervention strategies to reduce the incidence of food borne illness associated with the consumption of selected fresh vegetables contaminated with these pathogens.

Thus, the research objectives were designed as follows:

- (i) Characterization of selected microbial pathogens in selected Indian fresh produce and identify the possible entry points in their farm-to-plate chain.
- (ii) Determine the suitability of organic acids, ozone treatments or combined sanitizer treatments for inactivation of key pathogens in selected fresh produce through well-designed simulation studies.
- (iii) Understand and elucidate the behavior of acid resistant pathogens in fresh produce and consequently establish appropriate treatment regime for intervention.
- (iv) Determination of the final combined effect of treatment process on pathogens, resident microbes and on essential quality characteristics of produce.

REVIEW OF LITERATURE

Fruit and vegetable consumption is growing rapidly in recent years. Associated with the new consumer's profile "rich in cash/poor in time", there is a demand for ready to eat products. For this reason, the market of minimally processed fruits and vegetables has grown rapidly in recent decades as a result of changes in consumer attitudes. There is mounting evidence to support the alleviation of many degenerative diseases including cardiovascular disease, cancer and ageing by the consumption of fruit and vegetables. These beneficial health effects of fruit and vegetables have been attributed to the presence of antioxidants that act as receptors of free radicals. Ascorbic acid and beta-carotene are the antioxidants present in the greatest quantities in fruit and vegetables. However, increase in consumption has led to an increase frequency of food borne illnesses associated with raw fruits and vegetables. Minimal processing techniques have emerged to meet the challenge of replacing traditional methods of preservation while retaining nutritional and sensory quality.

Minimally processed fruits and vegetables, also called ready-to-use, fresh-cut or pre-cut produce, are raw fruits and vegetables that have been washed, peeled, sliced, chopped or shredded into 100% usable product that is bagged or packaged to offer consumers high nutrition, convenience, and flavor while still maintaining its freshness. Minimal processing of raw fruit and vegetables has two purposes. First, it is important to keep the produce fresh, yet supply it in a convenient form without losing its nutritional quality. Second, the product should have a shelf life sufficient to make its distribution feasible to its intended consumers. The microbiological, sensory and nutritional shelf life of minimally processed vegetables or fruit should be at least 4-7 days, but preferably even longer, up to 21 days depending upon the market (Ahvenainen, 1996).

As predicted by Food Agricultural Organization of the United Nations (FAO) a paramount increase in the global population (by 2030) coupled with an increasing consumer demand of functional foods would significantly increase the need for fresh fruits and vegetables (or RTE vegetables/fruits) (O'Brien, 2000). The increasing threat of emerging pathogens coupled with a reduced economic barrier for produce across countries requires mandatory information regarding prevalence of pathogens, their persistence and consequent inactivation strategies. Whereas, India is currently the world's second largest producer of food and anticipated to be the biggest in fresh produce, microbiological safety of produce has been poorly emphasized. For instance, the occurrence of *Salmonella* spp., *Shigella* spp. on fresh produce is well documented (outbreak in past as well as current) in both developed and developing countries whereas few reports (Frantamico et al., 2007) of outbreaks or prevalence are available from India, hospitalized cases indicate *Salmonella* to be endemic in India (Nair et al., 2006) and rising cases of *Shigella* (especially in pregnant women and immune-compromised patients) (Gupta et al., 2003; Taneja, 2007).

Furthermore, the emerging pathogen *E. sakazakii* implicated in outbreaks through infant formula (Forsythe, 2005) and now believed to prevalent in fresh produce, is yet to be characterized from the Indian subcontinent, especially from fresh produce, infant formula as well as other settings, although reports of *E. sakazakii* mediated illnesses (neonatal meningitis) in clinical settings have been documented (Ray et al., 2007). An impressive gamut of non-thermal (physical, chemical, biological) process have been advocated recently, for inactivating the above pathogens and these also include, a number of natural products with broad-spectrum antimicrobial activities, newer sanitizers such as electrolyzed water, organic acids, edible coatings with or without food additives and preservatives, novel microbial antagonists, short-term, minimal exposure to high hydrostatic pressure, UV-C as well as ionizing irradiations. Applications incorporating ozone have been currently sought

after for its superior inactivation effects, simplicity and economy. Therefore, in conjunction with appropriate organic acid(s) ozone treatments may be a powerful strategy for minimally processing fresh produces.

2.1 Food Safety and Hygiene

The fresh fruit and vegetable industry has witnessed exponential growth in the past decade. This trend is the result of a combination of many factors, such as globalization and increased demand by consumers for healthy, ready-to-eat products. Globalization has made it possible for the average consumer to purchase their favorite seasonal fresh fruits and vegetables year round in neighborhood retail outlets and foodservice operations. The fresh-cut segment of the produce industry, in turn, continues to fill the niche for value-added, conveniently packaged fruit and vegetables based on growing consumer demand. Compliance with international food standards is a prerequisite to gain a higher share of world trade.

Inevitably, with the growing demand for fresh fruits and vegetables the Centers for Disease Control and Prevention (CDC, 2002) reported an increase in the frequency of produce associated foodborne disease outbreaks (Bean et al., 1997; Mead et al., 1999). Common foodborne pathogens associated with fresh produce include *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., and *Escherichia coli* O157:H7 (Beuchat, 1996b; FDA, 2001a). Recent studies (Liao and Sapers, 2000; Takeuchi and Franks, 2000; Ukuku and Sapers, 2001) have showed that the surface structure of lettuce can protect *E. coli* O157:H7 cells from disinfection by chlorine, the most widely used chemical disinfectant (Beuchat and Ryu, 1997; Brackett, 1987; WHO, 1998).

Ackers et al. (1998) indicated that *E. coli* O157:H7 as a confirmed agent in an outbreak involving consumption of lettuce. The potential for contamination increases as the fresh produce moves from farm to table (i.e. irrigation water, improperly composted manure,

wash water systems, soiled equipment, unsanitary practices, etc.). Noticeably, the attention given to minimally processed produce by academia, government, and industry has increased. The focus has been on the microbiological safety through interventions strategies aimed at eliminating or reducing microbial hazards (i.e. human pathogens), mainly by using chemical disinfectants. The addition of a chemical disinfectant to the wash water has proven to reduce the microbial load (Beuchat and Ryu, 1997; Sapers, 2001). Consequently, the need for chemical sanitizers becomes evident to reduce microbial contamination in wash water systems. However, it is important to recognize that such reductions, although important, are not sufficient to assure microbiological safety of minimally processed fresh-cut vegetables (Sapers, 2001).

Kaufman et al. (2000) attributed the increase in consumption to: “First, Federal agencies, the private sector, and voluntary organizations stepped up efforts to improve the nutritional health of Americans through informed food choices. For example, to reduce the risk of cancer, the Food Guide Pyramid advises 5-9 daily servings of fruits and vegetables. The Produce for Better Health Foundation’s 5-A-Day program has raised consumer awareness of produce’s benefits. Improved quality, increased variety, and year-round availability via world trade have also boosted consumption of fresh fruits and vegetables (Kaufman et al., 2000).

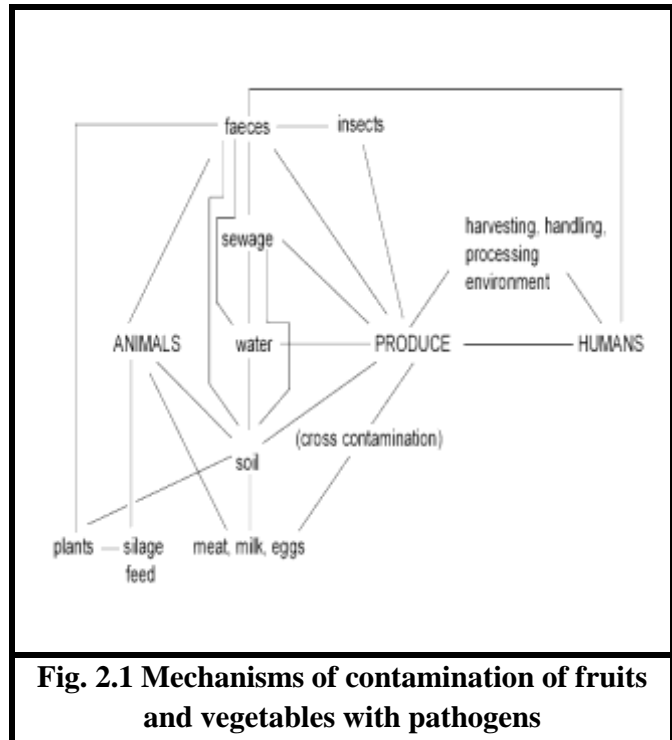
2.2 Source of Contamination

Earlier studies have identified and listed the natural total aerobic counts found in minimally processed fruits and vegetables, including whole and shredded lettuce (Nguyen-The and Carlin, 1994; Beuchat, 1996b). Beuchat (1996b) reported that aerobic mesophilic counts can range from 3 to 8 log CFU/g, in fresh produce. Other studies reported that the total mesophilic counts present on lettuce or packaged salads to be between 1.84 and 8.9 log

CFU/g. However, even within an individual product the populations of microorganisms are often not uniformly distributed. For example, Maxie (1978) isolated $> 4 \log$ CFU/g of mesophilic aerobic bacteria on the external lettuce leaves but only 32 CFU/g on the innermost leaves. The widespread occurrence and use of un-composted or improperly composted animal manure as fertilizer is a growing environmental concern, because it contaminates: water for drinking, irrigation, aquaculture and recreation; the hides, coats, and feathers of farm animals; and farm equipment and buildings. In the U.S., cattle, hogs, chickens and turkeys produce an estimated 1.36 billion tons of manure annually (EPA, 2000), with greater than 90% attributed to cattle.

Recent evidence of foodborne disease outbreaks associated with the consumption of fresh produce has prompted some to consider the role of contaminated irrigation and surface runoff waters. Irrigation water containing raw or improperly treated human sewage can be the source of many pathogens, with *Shigella* and the enteric viruses (hepatitis A virus, Norwalk-like viruses, rotaviruses) being perhaps the most significant (Beuchat and Ryu, 1997; Beuchat, 1996b). Irrigation water contaminated with animal fecal matter can also be a source of pathogens on fresh produce (Tauxe et al., 1997). The proximity of domestic (or wild) animals to irrigation water may serve as a vehicle for *E. coli* O157:H7 to gain access to produce during pre-harvest operations (Wachtel et al., 2002). The researchers suggested that preharvest crop contamination via infected irrigation water can occur through lettuce plant roots. Solomon et al. (2002) reported that *E. coli* O157:H7 was transmitted to lettuce plants through spray and surface irrigation. After harvest, contamination may occur as a result of using contaminated water or ice, improper handling by workers or consumers, transport containers, presence of wild or domestic animals in processing environment, cross contamination and improper storage or handling (FDA, 2001a; Tauxe et al., 1997). Garg et al. (1990) established that the shredders and slicers were a major source of contamination during

the processing of lettuce and other vegetables. For example, the aerobic plate count of lettuce increased from 4.25 log CFU/g to 6.14 log CFU/g after shredding. Chen et al. (2001) reported that even if proper hand-washing methods are followed, microorganisms may still be present and can be transferred from washed hands to lettuce during chopping. Fig. 2.1 illustrates potential mechanisms by which pathogens may contaminate produce.



2.3 Cross-contamination during Fresh-cut Processing

Fresh-cut processing operations involve the application of several unit operations, including trimming, cutting, shredding, washing, dewatering, and packaging. For the most part, these operations provide opportunities for cross-contamination whereby a small lot of contaminated product may be responsible for contamination of a large lot. Such incidents may involve direct contact between contaminated and uncontaminated product or may involve an intermediary source such as contaminated equipment surfaces or contaminated water.

For example, key contamination points identified by Kaneko et al. (1999) at two fresh-cut produce facilities included the trimming knives, wash water, the blades and interior surfaces of a mechanical shredder, and the interior of a centrifuge used for dewatering. Reinforcing the importance of equipment surfaces as a point of contamination, 41 cases of *Salmonella* Bovismorbificans infection in Australia were traced to the cutting wheel of a

lettuce shredder (Stafford et al., 2002). In the preparation of orange juice, processing equipment has also been linked to cross contamination events with mean *E. coli* O157:H7 counts on cutting boards, knives, and extractors ranging from 0.4 to 1.8 log CFU/cm² after exposure to inoculated fresh oranges (Martínez-Gonzales et al., 2003).

Likewise, large cell numbers of *Campylobacter jejuni* or *Salmonella* have been transferred from contaminated stainless steel surfaces to wet or dry lettuce even 1-2 h after surface contamination (Moore et al., 2000). Extended transfer during the processing of leafy greens would be of concern due to the reported production of biofilm by *E. coli* O157:H7 that would protect the pathogen from the effects of sanitizer (Ryu et al., 2004). Water is an excellent medium for distributing pathogens from one site to another. To illustrate this point, 100% of lettuce leaves were contaminated with *E. coli* O157:H7 when one inoculated dry lettuce piece was mixed with a large volume of dry lettuce and stored in water in the refrigerator (Wachtel and Charkowski, 2002). Consequently, sanitizers such as chlorine are added to the process water to circumvent cross-contamination events (Brackett, 1999). For example, sanitizing agents prevented cross-contamination of un-inoculated fuji apples that were mixed with *E. coli* inoculated golden delicious apples in a dump tank (Annous et al., 2001). At the same time, these sanitizing agents were ineffective in reducing the *E. coli* populations on the inoculated apples might be due to short exposure time (25 sec) and adherence of bacteria to the calyx and stem.

2.4 Survival and Growth of Pathogens on Produce during Storage

Transmission of enteric pathogens by produce is dependent on the survival of that pathogen during storage. Enteric pathogens are often capable of surviving on produce for the periods that the product is typically in distribution. Hence, for those pathogens having a low infectious dose, their presence is unacceptable. In general, the fate of enteric pathogens on produce during storage is dependent on a number of factors, including storage temperature,

relative humidity, and gaseous composition of the atmosphere, nutrient availability, and presence of competitive bacteria or antimicrobial compounds. In addition, damage to the product often enhances survival and growth of contaminating pathogens.

For example, lettuce tissue from heads dropped 6 feet incurred survival or growth of ~ 0.5 log *E. coli* O157:H7 greater than in undamaged tissue when stored at ambient temperature for 4 h followed by 4°C storage for 48 h (Wachtel et al., 2002). Slicing methods that shear or tear the tissue also led to consistently higher *E. coli* and *L. innocua* counts on packaged vegetables (carrots, iceberg and butterhead lettuce) during storage than slicing manually with a razor (Gleeson and O’Beirne, 2005). Biological damage is also of concern as it often leads to enhanced survival or growth of enteric pathogens. For example, produce that has been affected by soft rot is more conducive to growth of *Salmonella* than non-diseased produce (Wells and Butterfield, 1997).

2.5 Bacterial Pathogens of Concern and Associated Outbreaks

The potential for contamination increases as the fresh produce moves from farm to table (i.e. irrigation water, improperly composted manure, wash water systems, soiled equipment, unsanitary practices, etc.). The Centers for Disease Control and Prevention (CDC, 2002) reported that the mean number of produce related foodborne outbreaks more than doubled from 1973 to 1987 (4.3 per year) and again from 1988 to 1991 (9.75 per year) (Tauxe et al., 1997). Sivapalasingam et al. (2004) detailed that from 1973 through 1997 in the U.S., 32 states reported 190 produce-associated outbreaks, 16,058 reported illnesses, 598 hospitalizations, and eight deaths. In addition, the researchers reported that among the 190 outbreaks, 25 were associated with lettuce causing 2,078 reported illnesses, 181 hospitalizations, and six deaths. Common foodborne pathogens associated with fresh produce include *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Cronobacter sakazakii* and *Escherichia coli* O157:H7 (Beuchat, 1996b; FDA, 2001a). A brief description of pathogens

that have been isolated from minimally processed leafy vegetables and salads with emphasis on their association with foodborne outbreaks are given below.

Salmonella is a member of the family Enterobacteriaceae which comprises a large and diverse group of Gram-negative rod-shaped bacteria. *Salmonella* are facultative anaerobic, non-lactose fermenting, non-spore forming, and most are motile. There are currently over 2400 serotypes. Complete inhibition of growth occurs at pH < 3.8 and > 9.0, temperature < 7°C, or water activity < 0.94 (Gray and Fedorka-Cray, 2002; Jay, 2000; Ray, 1996). Optimum growth occurs at pH near neutrality and temperatures between 35°C and 37°C (Ray, 1996). *Salmonella* gastroenteritis usually follows the ingestion of contaminated food or drinking water. Typically, gastroenteritis in humans begins 24-28 h after ingestion and normally consists of fever, chills, headache, nausea, and vomiting, followed or concomitant with, abdominal cramps and diarrhea. These symptoms are usually accompanied by prostration, muscular weakness, faintness, and drowsiness. Ingestion of contaminated food with 5 log CFU/g to 6 log CFU/g cells is sufficient to trigger symptoms (Ray, 1996). The spectrum of disease ranges from loose stools to severe dysentery-like syndrome. *Salmonella* grow readily in many foods, as well as water contaminated with feed or feces. The primary habitat of the bacteria is the intestinal tract of animals, humans, and on insects (Jay, 2000; Ray, 1996). In addition, *Salmonella* has been isolated from soil, water, and sewage contaminated with fecal matter (Ray, 1996). Salmonellae were isolated from fresh produce, many of which have been linked to outbreaks of salmonellosis (Hedburg and Olsterhol, 1993; WHO, 1998). The incidence (survival and growth) of this pathogen in fresh lettuce is well documented (Ercolani, 1976; FDA, 2001c; Lin et al., 1996; Little et al., 1999).

The genus *Shigella* belongs to the family Enterobacteriaceae. There are four serological subgroups under the genus *Shigella*: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. *Shigella* species are Gram-negative, facultative anaerobic, non-motile, rod-shaped

bacteria. The strain can grow between 7°C and 46°C, with optimum at 37°C (Jay, 2000; Ray, 1996). The infective dose of this microorganism is as low as 200 cells, although, Lampel and Maurelli (2002) reported 10 cells are sufficient to cause disease. Most cases of shigellosis result from the ingestion of food or water contaminated with human feces. Shigellosis is characterized by an incubation period of 1 to 7 d and by signs and symptoms of diarrhea, abdominal pain, fever, and often vomiting. Stools may contain blood, mucus, or pus (Bryan, 1979). Shigellae are transmitted by personal contact, flies, and water, as well as by food. Fresh produce can become tainted through the use of contaminated irrigation water, the use of raw sewage as fertilizer, insect transfer, or human contact (WHO, 1998). Studies have showed that lettuce has been implicated as a vehicle for shigellosis (Davis et al., 1988; Frost et al., 1995). *Shigella* species can survive on shredded lettuce under refrigeration for up to 3 d without populations decreasing and can survive on raw fruits, including watermelon and raw papaya (Escartin et al., 1989; Satchell et al., 1990). In 1994, an outbreak of *Shigella sonnei* was traced back to infected lettuce in several European countries (Kapperud et al., 1995) and in 1995, another *Shigella* outbreak was traced back to lettuce in the United States (Tauxe et al., 1997).

Cronobacter sakazakii is a member of the family Enterobacteriaceae, genus *Enterobacter*, and is a motile peritrichous, Gram-negative bacillus. The organism, which was initially referred to as “yellow-pigmented cloacae,” was reclassified as “*E. sakazakii*” in 1980 on the basis of differences in DNA-DNA hybridization, biochemical reactions, pigment production, and antibiotic susceptibility, compared with *Enterobacter cloacae* (Farmer et al., 1980; Iversen et al., 2007). Recent studies have demonstrated that *Cronobacter sakazakii* is a genomically heterogeneous and, therefore, poorly defined species (Iversen et al., 2004; Lehner et al., 2004). *Cronobacter sakazakii* is regarded as an emerging opportunistic human pathogen and the etiological agent of life-threatening bacterial infections in infants (Bar-Oz et

al., 2001; Block et al., 2002). *Cronobacter sakazakii* was first implicated in a case of neonatal meningitis in 1958, when an outbreak in England resulted in the deaths of 2 infants (Urmenyi and Franklin, 1961).

Although the incidence of *Cronobacter sakazakii* infection is low, the prognosis is poor, and infection is associated with significant morbidity and mortality. Powdered infant formula (PIF) products have been shown to contain *Cronobacter sakazakii* and have been epidemiologically linked to several clinical cases (Himmelright et al., 2002; Simmons et al., 1989; van Acker et al., 2001). Related coliforms, such as *Citrobacter diversus*, have also been isolated from PIF (Thurm and Gericke, 1994). Like *Cronobacter sakazakii*, these organisms can cause invasive infections. There is also evidence from surveillance activities that low-level contamination of PIF with *Salmonella* species has led to cases of disease in infants (Usera et al., 1996).

Recalls of infant formula contaminated with *Cronobacter sakazakii* have occurred in the United States and Europe (Simmons et al., 1989; van Acker et al., 2001). This has resulted in increased efforts to implement appropriate strategies to reduce the health risks associated with the use of PIF. Furthermore, this pathogen is implicated in outbreaks through infant formula (Forsythe, 2005) and now believed to be prevalent in fresh produce, although the pathogen is yet to be characterized from the Indian subcontinent, especially from fresh produce, infant formula as well as other settings, reports of *Cronobacter sakazakii* mediated illnesses (neonatal meningitis) in clinical settings have been documented (Ray et al., 2007; Singla et al., 2010).

2.6 Interventions to Inactivate Pathogens on Produce

To minimize the risk of distributing a contaminated product, several studies have explored the efficacy of both physical and chemical interventions applied to produce.

Fonseca (2006) recently provided a review of those interventions. In general, treatment produces must be safe for humans. They should eliminate/inactivate at least 3 log of enteric pathogens and ideally provide at least a 5 log reduction (FDA, 1998). Acceptable quality characteristics of produce should also be retained following intervention application. Finally, the pathogens should not survive in any antimicrobial treatment solutions to avoid subsequent cross-contamination of treated produce or environmental pollution. While these risk factors are noteworthy, they still do not provide sufficient information on how the product could have been contaminated in the field and whether postharvest practices could have accentuated the contamination.

The FDA Guide to Minimize Microbial Food Safety Hazards for fresh fruits and vegetables defines “sanitize” as “to treat clean produce by a process that is effective in destroying or substantially reducing the numbers of microorganisms of public health concern, as well as other undesirable microorganisms, without adversely affecting the quality of the product or its safety for the consumer” (FDA, 1998). Thus, effective sanitization requires a delicate balance between quality and microbiological safety of produce. However, it is well-known that processing of vegetables promotes a faster physiological deterioration, biochemical changes and microbial degradation of the product even when only slight processing operations can be used (O’Beirne and Francis, 2003), which may result in degradation of the color, texture and flavor (Kabir, 1994; Varoquaux and Wiley, 1994). Active and intelligent packaging systems are emerging and promising technologies that will increasingly be applied in the years to come to extend shelf- life and improve the quality, safety and integrity of packaged foods. In recent years, many active and intelligent systems have been developed and it is expected that new concepts will become commercially available in the near future. However, for innovative food- packaging technologies to be successful, they must comply with regulations.

The simple step of thorough washing minimally processed salads reduces indigenous microflora and pathogens that may be present as a result of contamination at any point in the processing chain. Therefore, produce wash water systems are of great concern, in particular, if the water is recycled. Water recirculation can increase the potential for foodborne illness by distributing the source of contamination to product already in the wash water system and not previously contaminated or by contaminating newly introduced product. Product wash water, not treated with a chemical disinfectant, can become a source of microbial contamination if reused (IFPA, 2001), highlighting the need for chemical disinfectants in wash water systems. Washing lettuce leaves with tap water alone was reported to reduce indigenous microflora by approximately 1 log CFU/g (Adams et al., 1989; Nguyen-The and Carlin, 1994). Similarly, Singh et al. (2002) reported that deionized water achieved close to 1 log and 0.22 CFU/g reductions in populations of *E. coli* O157:H7 inoculated on shredded lettuce after multiple washing and single wash, respectively.

The addition of a chemical disinfectant to the wash water further reduces the microbial load (Beuchat and Ryu, 1997; Sapers, 2001). The use of a chemical disinfectant in wash water provides a barrier to cross contamination of produce and is effective in removing disease-causing organisms from the surface minimally processed produce (FDA, 2001a). These chemical sanitizers can therefore reduce microbial contamination of subsequent batches processed in the same recirculated wash water system. However, it is important to recognize that such reductions, although important, are not sufficient to assure microbiological safety of minimally processed fresh-cut vegetables (Sapers, 2001).

Currently, sodium hypochlorite is the most commonly used chemical sanitizer in produce wash water (Brackett, 1987; Beuchat and Ryu, 1997; WHO, 1998). There is a growing number of alternative water sanitizing compounds which are used to reduce microbial populations in fresh-cut produce, including chlorine dioxide (Reina et al., 1995;

Zhang and Farber, 1996), ozone (Kim et al., 1999), electrolyzed water (Park et al., 2002), hydrogen peroxide (Sapers, 2001), organic acids (Venkitanarayana et al., 2002), peroxyacetic acid (Rodgers et al., 2004), trisodium phosphate (Zhang and Farber, 1996), and radiation (Prakash et al., 2000a, b). None of the previously mentioned chemical disinfectants were able to completely eliminate the pathogens from fresh produce when used at acceptable levels (WHO, 1998).

2.6.1 Chlorine

Chlorine is the most widely used sanitizer in reducing microbial load in fresh fruit and vegetable wash water; (IFPA, 2001; WHO, 1998). Chlorine is very reactive and combines with any oxidizable substrate to form secondary compounds, such as trihalomethanes (IFPA, 2001). For chlorine to disinfect produce the recommended usage level is 50-200 ppm, at a pH below 8.0, and with a contact time of 1-2 min (FDA, 2001a; WHO, 1998). The most common forms of free chlorine include liquid chlorine and hypochlorites. However, chlorine has a limited effect on reducing microorganisms on fresh fruit and vegetable surfaces (Beuchat, 2000; Sapers, 2001).

The inhibitory or antimicrobial activity of chlorine depends on the amount of hypochlorous acid (free chlorine) present in the water that comes into contact with the microbial cells. Hypochlorous acid is the form of available free chlorine that has the highest bactericidal activity against microorganisms commonly found in fresh fruits and vegetables (Sapers, 2003). Besides hypochlorous acid the bactericidal activity of chlorine is dependent on water pH, temperature, presence of organic matter, contact time, light, air, or metals (FDA, 2001a; IFPA, 2001; WHO, 1998).

The effects of pH on chlorine dissociation indicate that at pH 7.5 or greater the quantity of chlorine available as active hypochlorous acid (HOCl) is limited; rather, chlorine exists mainly as inactive hypochlorites (OCl⁻). If the pH of the wash water decreases below

4.0, then chlorine gas may be formed which is a health hazard for employees (IFPA, 2001). Therefore, the pH of the water should be maintained between 6.0 and 7.5 to ensure adequate and safe chlorine activity. The percentages of chlorine as HOCl at pH 6.0 and 8.0 are about 97% and 23%, respectively (WHO, 1998).

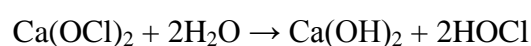
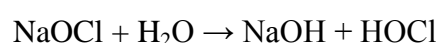
The effects of temperature on HOCl indicate that as the temperature of the water decreases; HOCl is in favor regardless of the pH. For example the proportion of chlorine as hypochlorous acid is slightly lower at 20°C than at 0°C, especially when the pH falls between 6 and 9 (Eifert and Sanglay, 2002). The maximum solubility of chlorine is achieved in water at approximately 4°C. However, the temperature of the water should be ideally at least 10°C higher than the fruit or vegetable to achieve a positive temperature differential, thereby minimizing the uptake of wash-water through stem tissue and open areas in the skin or leaves, whether due to mechanical assault or naturally present (e.g. lenticel and stomata) (WHO, 1998). Beuchat (2000) described the reactions that occur when chlorine is added to water using the following reactions:

1) The addition of chlorine gas to wash water:



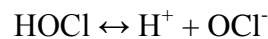
In this reaction, chlorine is hydrolyzed to produce hypochlorous acid (HOCl), hydrogen ion (H⁺), and chlorine ions (Cl⁻). The resulting hypochlorous acid is the primary reason for the antimicrobial properties of chlorine (Sapers, 2003).

2) The addition of liquid chlorine, such as, sodium hypochlorite (NaOCl) and calcium hypochlorite [Ca(OCl)₂] to the wash water can be expressed as follows:



In these reactions, both sodium and calcium hypochlorite are hydrolyzed to produce hypochlorous acid and sodium hydroxide (NaOH) and calcium hydroxide [Ca(OH)₂].

3) Thus, whether the addition of chlorine in a liquid or gas form hypochlorous acid is the most effective antimicrobial of the entire chlorine residual fraction. The hypochlorous acid that is formed in the water may further dissociate to produce hydrogen ion (H⁺) and hypochlorite ion (Cl⁻), as demonstrated in the following reaction:



The dissociation of hypochlorous acid to hypochlorite and hydrogen ions is dependent on the pH of the wash water. Most of the HOCl will remain undissociated at pH < 7. The proportion of undissociated HOCl is greatest at pH > 5. If the pH falls < 4, the proportion of potentially hazardous chlorine gas increases. As the pH > 4.0 the ratio of HOCl to OCl⁻ decreases. At pH 8, the proportion of HOCl that remains undissociated will be less than 25%. Since OCl₂ is less germicidal than HOCl, a chlorine sanitizer solution with a pH range of 6.5 to 7.5 may have greater antimicrobial efficacy. Beside pH and temperature, organic load can have a significant detrimental effect on chlorine efficacy (Li et al., 2001; Takeuchi and Frank, 2001). Taormina and Beuchat (1999a, b) reported that free chlorine in a 200 ppm solution decreased to about 20 ppm (about 90% decrease), within 15 min after treating alfalfa seeds in varying foreign organic loads.

In another study, Beuchat et al. (2004a, b) attributed the rapid decrease in chlorine concentration to the release of tissue juices from shredded lettuce, which increased the concentration of organic materials accessible for reaction with and neutralization of chlorine. In addition, chlorine is readily inactivated upon contact with organic matter, and the effectiveness depends on direct contact with cells. Takeuchi and Frank (2001) concluded that cells located 30 to 40 μm from the cut surface were the most protected from chlorine

disinfection. Thus, disinfectants that can be inactivated by organic material are unlikely to be effective in eliminating viable *E. coli* O157:H7 cells that have penetrated into tissue. Zhang and Farber (1996) reported reduction of *Listeria monocytogenes* on shredded lettuce and cabbage. The researchers achieved a 1.3 and 1.7 log CFU/g reduction on lettuce and 0.9 and 1.2 log CFU/g reduction on cabbage after treatment with 200 ppm chlorine for 10 min at 4°C and 22°C, respectively. The study demonstrated that the bactericidal effect on *L. monocytogenes* was higher at 22°C than at 4°C, and was more effective on lettuce than on cabbage. Delaquis et al. (1999) reported similar results when treating shredded lettuce and cabbage at 47 and 4°C for 3 min. Rodgers et al. (2004), reported that treatment with 100 ppm chlorine for 5 min reduced *L. monocytogenes* and *E. coli* O157:H7 to non-detectable levels on whole apples, whole lettuce, strawberries, and cantaloupe, whereas approximately 1 log CFU/g of *L. monocytogenes* and *E. coli* O157:H7 remained on sliced apples and shredded lettuce. The increased reduction on whole versus shredded lettuce can be attributed to the attachment of microorganisms to the cut edges of lettuce and increase organic materials in water due to the release of tissue juices in shredded lettuce.

2.6.2 Ozone (O₃)

In 1982, the Food and Drug Administration (FDA) affirmed ozone as generally recognized as safe (GRAS) with specific limitations, for use as a disinfectant in bottled water (EPRI, 1997; FDA, 1982). In 1997, Ozone was approved by the U.S. Department of Agriculture (USDA) for reconditioning recycled poultry chilling water, and received GRAS status, by an expert panel.

This assertion brought about a broader use of this gas in the food industry, particularly the minimally processed fresh produce segment (CFR, 2005b; Graham, 1997). Ozone is permitted by the FDA for treatment of drinking water (CFR, 2005a). In 2001, the FDA approved the use of ozone as an antimicrobial agent for the treatment, storage, and

processing of foods in gas and aqueous phase in direct contact with foods, including raw and minimally processed fruits and vegetables (FDA, 2001b). Ozone has to be generated on site because of its instability. Ozone is highly unstable in water and decomposes to oxygen in a very short time. The half-life of ozone in distilled water at 20°C is generally considered to be 20 to 30 min (Khadre et al., 2001). The stability of ozone in aqueous solutions depends on the source of water. Water used in food processing or beverages generally contains readily oxidizable organic and inorganic substances. These substances may react rapidly with ozone, considerably decreasing the shelf-life.

Ozone results from the rearrangement of atoms when oxygen molecules are subjected to high-voltage electric discharge (Khadre et al., 2001). Ozone is a blue gas at ordinary temperature, but at the concentrations at which is normally produced the color is not noticeable. However, at -112°C, ozone condenses to a dark blue liquid (Guzel-Seydim et al., 2003). The oxidizing power of ozone is up to 3000 times faster than chlorine (EPRI, 1997). Unfortunately, this oxidizing power has the negative effect of causing deterioration and corrosion on metal and other types of surfaces. Ozone can react with contaminants directly as molecular ozone (O₃) or indirectly as ozone-derived free radicals such as OH and H₂O (Koseki et al., 2001). Ozone is readily detectable by human smell at 0.01 to 0.04 ppm; increased concentration to 1 ppm produces a pungent, disagreeable odor and irritation to the eyes and throat; and can be lethal to humans with prolonged exposure at concentrations above 4 ppm (Guzel-Seydim et al., 2003).

Restaino et al. (1995) suggested that the bactericidal cell surface is the primary target of ozone activity. Khadre et al. (2001), described the inactivation of bacteria by ozone as a complex process because ozone attacks numerous cellular constituents including proteins, unsaturated lipids and respiratory enzymes and nucleic acids in the cytoplasm, and proteins and peptidoglycan in spore coats and virus capsids. Guzel-Seydim et al. (2003) confirmed

previous assumptions and offered that ozone destroys microorganisms by the progressive oxidation of vital cellular components. According to Restaino et al. (1995), pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Salmonella typhimurium*, and *Yersinia enterocolitica*, are sensitive to treatment with 20 ppm ozone in water. Kim et al. (1999) explained that the decontamination of produce by ozone depended, among other factors, on the number and kind of contaminating microorganisms, physiology of vegetables, reactor design, water quality, temperature, and pH. In this study, when ozonated water, without turbulence, was used on lettuce treatment, minimal elimination of contaminants was observed. It was apparent that ozone causes damage to membranes of Gram-negative bacteria which results in loss of its cellular components (Fig. 2.2).

However, bubbling ozone (1.3 ppm) in water lettuce mixture for 3 min inactivated 1.2 log CFU/g and 1.8 log CFU/g mesophilic and psychrotrophic microorganisms, respectively. Hence, bubbles and agitation likely enhanced the efficacy of ozone by breaking cell clusters (Rodgers et al., 2004). When the duration of the treatment was extended to 5 min, populations of mesophilic and psychrotrophic microorganisms were

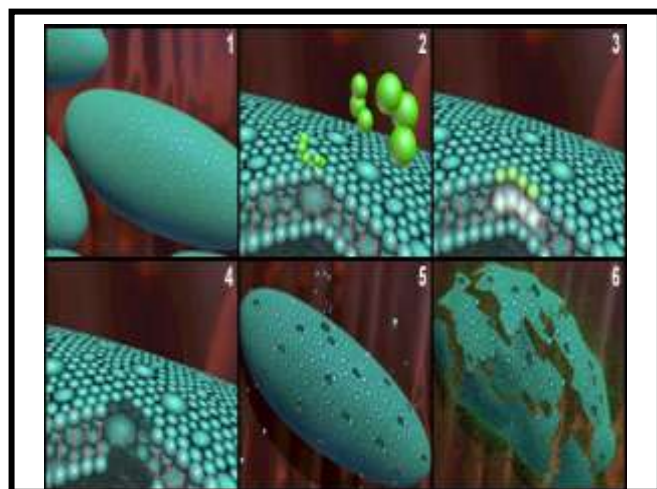


Fig. 2.2 Mechanism of ozone on microbial cell. 1. Ozone molecules, 2. Attachment of ozone molecule with cell. 3. Ozone penetration, 4. Bacterial cell following contact with a few ozone molecules, 5. Pore formation, 6. Complete destruction of cell

reported to decrease 3.9 log CFU/g and 4.6 log CFU/g, respectively. Unfortunately, this longer exposure time is likely to be impractical in food applications. Moreover, in the same study, ozone treatment (~3 to 10 ppm) was ineffective in reducing *Pseudomonas fluorescens*

inoculated (24 h prior to treatment) on lettuce, resulting in <1 log reduction. Koseki et al. (2001) that the number of aerobic organisms on lettuce decreased only 1.5 log following a 10-min exposure at 5 ppm ozone. More recently, Garcia et al. (2003) conducted a study to determine the effectiveness of ozone (2.5, 5.0, and 7.5 ppm) on the microbiological attributes of shredded lettuce and reported a 0.6 to 0.8 log reduction in aerobic plate count after a 10 min treatment.

Table 2.1 summarizes several studies using ozone as the chemical disinfectant at varying treatments and its efficacy at reducing populations of natural microflora and pathogens on vegetables. The results demonstrate that despite the ozone concentrations, the maximum reduction at 3 min was ≤ 1.8 logs in minimally processed vegetable products when agitated (bubbling). Higher reductions were achieved with longer treatment times, however, they are impractical in food applications.

Table 2.1 Effects of Ozone treatments on Minimally Processed Vegetables and Salads

| Disinfecting Treatment | Product | Microbial Reduction | Reference |
|--|---------|---|-----------------------|
| 1.3 ppm for 3 min (bubbling) | Lettuce | 1.2 and 1.8 log reduction of mesophilic and psychrotrophic microorganisms, respectively | Kim et al., (1999) |
| 1.3 ppm for 5 min (bubbling) | Lettuce | 3.9 and 4.6 log reduction of mesophilic and psychrotrophic microorganisms, respectively | Kim et al., (1999) |
| 10 ppm for 1 min (bubbling) | Lettuce | <1 log reduction of <i>Pseudomonas fluorescens</i> | Kim et al., (1999) |
| 5 ppm for 10 min | Lettuce | 1.5 log reduction | Koseki et al., (2001) |
| 2.5, 5.0, 7.5 ppm, stirred for 10 min at ~20°C | Lettuce | 0.6 to 0.8 log reduction in APC (compared to untreated) | Garcia et al., (2003) |
| 3 ppm for 5 min (bubbling) | Lettuce | 4 to 5 log reduction of mesophilic bacteria | Rodgers et al., 2004 |

2.6.3 Organic acids

The antimicrobial activity of organic acids is a function of reduction of pH in the microbial environment leading to disruption of membrane transport and permeability,

accumulation of anions and lowering of intracellular pH within the cell by dissociation of hydrogen ions from the acid (FDA, 2001). The most commonly studied organic acids are lactic acid, citric acid, acetic acid, tartaric acid, *p*-aminobenzoic acid (PABA) and orthophosphoric acid (FDA, 2001; Richards et al., 1995). Lactic acid has been used extensively as sanitizer on food animal carcasses (Castillo et al., 2002) and is a potential sanitizer for produce. It is generally recognized as safe (GRAS) for use in food products and has been found to be an effective antimicrobial sanitizer at elevated temperatures alone, or in combination with other chemicals (Materon, 2003; Sorrells et al., 1989). Table 2.2 summarizes several studies using different organic acids as the chemical disinfectant at varying treatments and its efficacy at reducing populations of natural microflora and pathogens on vegetables.

Lactic acid has been found to be more effective than chlorinated water in reducing pathogens on the surface of cantaloupes. Materon (2003) found that lactic acid at 35°C drastically reduced *E. coli* O157:H7, by more than 7 log cfu/cm² on the surface of cantaloupes. Also, cantaloupe decontamination with (1.5%) lactic acid was found to be more effective than decontamination with chlorine or deionized water. A study conducted by Ibarra-Sanchez et al. (2004) on the effect of chemical sanitizers on pathogen reduction in tomatoes showed that 2% lactic acid spray at 55°C reduced surface as well as internalized *S. typhimurium* and *E. coli* O157:H7. Citric acid was capable of reducing populations of *S. typhi* on papaya cubes (Escartin et al., 1989) and *Campylobacter jejuni* on watermelon and papaya (Castillo and Escartin, 1994). PABA was found to be a more potent antimicrobial compared to lactic, acetic, propionic, citric and formic acids against *L. monocytogenes*, *S. enteritidis*, and *E. coli* (Richards et al., 1995). Acids are either naturally present as constituents of the food or are added to the product through formulation. The undissociated form of the acid is responsible for the antimicrobial activity, which is highly dependable on pH.

Table 2.2 Effects of Organic acid Treatments on Minimally Processed Vegetables and Salads

| Disinfecting Treatment | Product | Microbial Reduction | References |
|---|------------------------------------|---|---------------------------|
| Peroxyacetic acid (Chlorine 100), 80 ppm at 3 to 4°C for 15 sec | lettuce and romaine lettuce pieces | ~ 1 log reduction of <i>L. monocytogenes</i> | Beuchat et al., (2004a) |
| Acetic acid (2%) for 15 min | Lettuce leaves | 3.37 and >2.25 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Acetic acid (4%) for 15 min | Lettuce leaves | 3.91 and >2.25 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Peracetic acid (80 ppm) for 15 min | Lettuce leaves | 1.85 and 1.44 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Vinegar (6%) for 15 min | Lettuce leaves | 1.83 and 1.58 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Vinegar (25%) for 15 min | Lettuce leaves | 2.42 and >1.99 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Vinegar (50%) for 15 min | Lettuce leaves | 2.89 and >2.21 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Acetic acid (2%) for 15 min | Lettuce leaves | 3.37 and >2.25 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Acetic acid (4%) for 15 min | Lettuce leaves | 3.91 and >2.25 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Peracetic acid (80 ppm) for 15 min | Lettuce leaves | 1.85 and 1.44 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |
| Vinegar (6%) for 15 min | Lettuce leaves | 1.83 and 1.58 log reduction in aerobic mesophilic and total coliform populations, respectively (compared to untreated) | Nascimento et al., (2003) |

Nascimento et al. (2003) compared the results of sodium hypochlorite with seven different sanitizing solutions (vinegar at 6, 25, and 50%; acetic acid at 2 and 4%; peroxyacetic acid at 80 ppm; and sodium dichloroisocyanurate at 200 ppm). The statistical analysis of the results demonstrated that the effectiveness levels of all the sanitizing agents tested were equivalent to or higher than that for sodium hypochlorite at 200 ppm. The best results were achieved with 4% acetic acid, which reduced the initial aerobic mesophilic population by 3.93 log CFU/g and reduced the mold and yeast population by 3.58 log CFU/g. Nascimento et al. (2003) concluded that the results of the study demonstrated the effectiveness of acetic acid and vinegar as alternative sanitizing agents for the disinfection of fresh produce.

2.6.4 Other Chemical and non-chemical Disinfection Methods

In addition to the chemical disinfectants there are a number of other sanitizing agents (chemical and non-chemical) that are approved that have been evaluated in laboratory-scale investigations for fresh-cut vegetables. For instance, hydrogen peroxide is currently classified as GRAS for use in food products. Lin et al. (2002) reported that the treatment of lettuce with 2% H₂O₂ at 50°C reduced *E. coli* O157:H7 and *L. monocytogenes* by ≤ 4 and 3 log. In 1986, the FDA approved the use of peroxyacetic acid as a food-grade sanitizer at concentrations not to exceed 100 ppm (Rodgers et al., 2004). Moreover, unlike chlorine and ozone, peroxyacetic acid is non-corrosive, unaffected by changes in temperatures, and remains effective in the presence of organic matter (Rodgers et al., 2004). Peroxyacetic acid is a strong oxidizer formed from H₂O₂ and acetic acid. Essential oils increase the permeability of cytoplasmic membrane and lead to the loss of cellular constituents (Sikkema et al., 1994). Wan et al. (1998) reported washing lettuce with 0.1% (v/v) and 1.0% (v/v) suspensions of basil essential oil resulted in 2.0 and 2.3 log reduction of viable bacteria on fresh cut lettuce, respectively.

Cetylpyridinium chloride (CPC) is a quaternary ammonium compound with the potential application in disinfection of fresh-cut leafy vegetables. Reductions of 2.4 to 3.2 log CFU/g of *S. typhimurium* and 1.0 to 1.6 log CFU/g of *E. coli* following a 5 min immersion treatment of vegetables using 0.1% to 0.5% CPC (Wang et al., 2001). Yang et al. (2003) reported that 0.3% CPC reduced *S. typhimurium* and *E. coli* O157:H7 by 0.96 and 1.21 log CFU/g, respectively, at a spray pressure of 0.7 kg/cm². When spray pressure increased from 0.7 to 2.1 kg/cm², *S. typhimurium* and *E. coli* O157:H7 were further reduced by 1.5 and 0.5 log CFU/g, respectively. However, approximately 300 ppm of CPC persisted on the lettuce after 2 min water rinse.

In the U.S., the FDA has authorized the use of irradiation for pork, poultry, red meats, fruits, vegetables, herbs, spices, grains, seeds for sprouting, and shell eggs. Current U.S. regulations limit the use of irradiation for fresh fruits and vegetables up to 1 kGy and specifically for disinfestations and inhibition of produce growth and maturation (CFR, 2005c). However, dry or dehydrated vegetables derived spices, seasonings, flavorings and coloring agents may be irradiated to 30.0 kGy (CFR, 2005c). In a study by Farkas et al. (1997), *L. monocytogenes* and spoilage bacteria were reduced by approximately 4 and 5 log on pre-cut bell peppers and carrot cubes, respectively, when treated with 1.0 kGy. Hagenmaier and Baker (1997) reported a significant reduce of the normal microflora and moderately increased respiration on commercially prepared fresh-cut lettuce by treating the produce with a radiation dose of 0.19 kGy; demonstrated the unirradiated lettuce had 5.34 log CFU/g, while the irradiated lettuce had 2.46 log CFU/g. Prakash et al. (2000a, b) reported the treatment on cut romaine lettuce with irradiation at 0.35 kGy decreased the aerobic plate counts by 1.5 log and yeast and molds by 1 log; these differences were maintained through 22 d storage at 4°C. A summary of different chemical disinfectants at varying treatments and efficacy at reducing populations of natural microflora and pathogens on vegetables is

presented in Table 2.3. A careful observation reveals that although higher reductions were achieved with acetic acids, the treatment required 5 min. Unfortunately, this extended treatment period is impractical in food applications. Irradiation, demonstrated significant reductions on a variety of vegetables inoculated with *L. monocytogens*, however, the cost of treatment may outweigh the benefits. Currently, new washing technologies using chemical sanitizing agents, vacuum infiltration, vapor-phase treatments, surface pasteurization, bacteriophage control, high hydrostatic pressure, ultraviolet light, and pulse electric are needed to overcome failures of conventional methods by targeted treatment of microbial attachment or internalization sites are being developed (FDA, 2001a; Greer, 2005; Sapers, 2001).

2.6.5 Hurdle Technology and Synergistic Effects of Chemical Disinfectants

According to Leistner (1994), in food preserved by hurdle technology, the possibility exists that different hurdles in a food will not just have an additive effect on stability, but could act synergistically. A synergist effect, achieved when the combination of two compounds is more effective than each compound alone, could work if the hurdle in a food hits different targets (e.g., cell membrane, DNA, enzyme systems, pH, a_w) within the microbial cell, and thus disturbs the homeostasis of the microorganisms present in several aspects. The physiological responses of microorganisms during food preservation such as homeostasis, metabolic exhaustion, and stress reaction are the basis for the application of hurdle technology.

Therefore, deliberately disturbing several homeostasis mechanisms simultaneously by using multiple hurdles in the preservation of a particular food should be an advantage, because microbial stability could be achieved with a combination of gentle hurdles. Since different hurdles have different spectra of antimicrobial action, the combined hurdles could

attack microorganisms in different ways and may increase synergistically the effectiveness of preservation. In practical terms, the use of different preservatives in small amounts may be more effective than only one preservative in a large amount. The reason for the efficacy is that different preservatives might affect different targets within the bacterial cell, and thus act synergistically (Leistner, 1994).

Table 2.3 Effects of Different treatments on contradictory Processed Vegetables and Salads

| Disinfecting Treatment | Product | Microbial Reduction | References |
|--|--|---|------------------------|
| 2% H ₂ O ₂ at 50°C | Lettuce | 4 and 3 log reduction of <i>E. coli</i> O157:H7 and <i>L. monocytogenes</i> , respectively | Lin et al., (2002) |
| Basil essential oil (0.1 and 1.0% (v/v)) | Fresh-cut lettuce | 2.0 and 2.3 log reduction of viable bacteria, respectively. | Wan et al., (1998) |
| CPC (0.1 to 0.5%) for 5 min | Vegetables | 2.4 to 3.2 log reduction of <i>S. typhimurium</i> and 1.0 to 1.6 log reduction for <i>E. coli</i> | Wang et al., (2001) |
| CPC (0.3%), spray pressure at 0.7 kg/cm ² | Lettuce | 0.96 and 1.21 log reduction of <i>S. typhimurium</i> and <i>E. coli</i> O157:H7, respectively | Yang et al., (2003) |
| CPC (0.3%), spray pressure at 2.1kg/cm ² | Lettuce | 1.5 and 0.5 log reduction of <i>S. typhimurium</i> and <i>E. coli</i> O157:H7, respectively | Yang et al., (2003) |
| Gamma irradiation (1 kGy) | Pre-cut bell peppers | 4 log reduction of <i>L. monocytogenes</i> | Farkas et al., (1997) |
| Gamma irradiation (1 kGy) | Carrot cubes | 5 log reduction of <i>L. monocytogenes</i> | Farkas et al., (1997) |
| Gamma irradiation (0.35 kGy) | Cut romaine lettuce | 1.5 log reduction of aerobic plate count | Prakash et al., (2000) |
| Irradiation (1 kGy) | Broccoli, moong beans, cabbage, and tomato | 4.14 to 5.25 log reduction of <i>L. monocytogenes</i> | Bari et al., (2005) |

Hagenmaier and Baker (1997) found that refrigerated cut iceberg lettuce irradiated at 0.2 kGy after a chlorine wash and modified atmosphere packaging (MAP) had only 2.5 log CFU/g reduction after 8 d. At the same time the control had 5.29 log CFU/g. Thus, irradiation in combination with chlorine can significantly reduce microbial levels. Foley et al. (2002)

reported that chlorination plus irradiation (0.55 kGy) resulted in a 5.4 log reduction of *E. coli* O157:H7 on shredded iceberg lettuce with little significant effect on quality. A mixture of 1.5% lactic acid and 1.5% H₂O₂ on apples, oranges and tomatoes reduced counts of *Salmonella* and *E. coli* O157:H7 by >5 log per fruit without damage to the sensory quality of the fruit (Venkitanarayana et al., 2002). The combination of acids with other chemical sanitizers provided more hurdles for bacteria to clear, thus increasing the chances of a lethal effect or at least an inhibition of growth. Garcia et al. (2003) conducted a study to determine the effectiveness of ozone in combination with chlorine on the microbiological and sensory attributes of lettuce as well as the quality of water used for processing commercial lettuce. In their study, iceberg lettuce was inoculated with 8.0 log CFU/g microorganisms isolated from spoiling lettuce, treated with combinations of chlorine and ozone, and analyzed microbiologically. They reported that chlorine, ozone, and chlorine-ozone reduced aerobic plate count by 1.4, 1.1, and 2.5 log, respectively. The use of combination of ozone and chlorine resulted in notable microbial reduction. The un-intentional benefit is that using a reduced chlorine treatment (by adding ozone) may reduce the formation of trihalomethane compounds, which are carcinogenic. Table 2.4 summarizes several studies using a combined mixture of two or more chemical and non-chemical disinfectant and efficacy at reducing populations of natural microflora and pathogens on vegetables. The results demonstrate that combining chlorine and irradiation or lactic acid and hydrogen peroxide had greater reduction than using chlorine alone.

2.7 Alternation in Nutritional status following Minimal processing

Vegetables are a good source of dietary fiber, phytonutrients, provitamins, antioxidants, polyphenols and minerals. Antioxidants in beets and green beans (Cardador-Martinez et al., 2002; Jiratanan and Liu, 2004), prebiotics and immune protecting

phytochemicals of asparagus (Diwanay et al., 2004; Gautam et al., 2004; Gibson, 1998), hydroxycinnamic acid of eggplant (Whitaker and Stommel, 2003), glucosinolates of cauliflower (Kushad et al., 1999; Tian et al., 2005) have been associated with health promoting effects. Besides, phytonutrients in vegetables have been shown to stimulate natural detoxifying enzymes in the body and lower the risk of atherosclerosis and cancer (Ames et al., 1993; Hecht, 1999).

Raw, fresh vegetables are consumed in salads, sandwiches and/or with dips and are believed to be more nutritious than those consumed after cooking. A number of chemical and biochemical processes that take place during the storage of minimally processed vegetables contribute to the deterioration of their overall quality. Plants age during long-term storage, and their physiological activity and adaptability to adverse environmental conditions is lowered. Therefore, it becomes necessary to compare the overall antioxidant activity of the minimally processed vegetable samples.

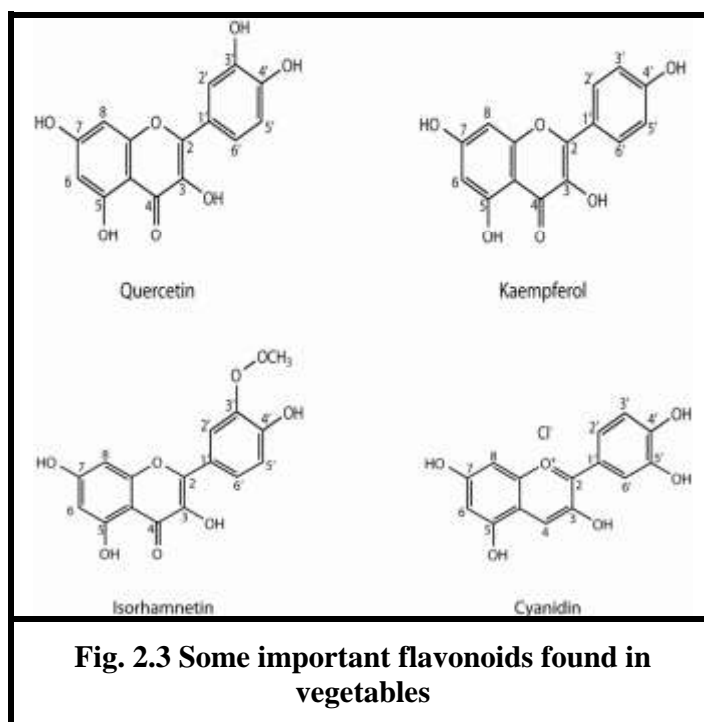
Table 2.4 Effects of Combination treatments on Vegetables and Salads

| Disinfecting Treatment | Product | Microbial Reduction | References |
|---|-------------------------------|--|--------------------------------|
| Chlorine wash, irradiation (0.2 kGy) and MAP | Fresh-cut iceberg lettuce | 2.5 log CFU/g 8 d after irradiation | Hagenmair and Baker, (1997) |
| Chlorination plus irradiation (0.55 kGy) | Shredded iceberg lettuce | 5.4 log reduction of <i>E. coli</i> O157:H7 | Foley et al., (2002) |
| Mixture of 1.5% lactic acid and 1.5% H ₂ O ₂ | Apples, oranges, and tomatoes | > 5 log reduction of <i>Salmonella</i> and <i>E. coli</i> O157:H7 | Venkitanarayana et al., (2002) |
| Combined 7.5 ppm ozone and 150 ppm chlorine | Shredded lettuce | 1.4 log reduction in APC (compared to untreated) | Garcia et al., (2003) |
| Mixture of ClO ₂ (85 ppm) and Fit powder* product (0.5%), pH 3.5 for 5 min | Water | >5.3 and >6.0 log CFU/ml reduction of <i>Bacillus cereus</i> vegetative cells and spores, respectively (compared to untreated) | Beuchat et al., (2004a, b) |

*Fit Powder Procter and Gamble, Cincinnati, Ohio

2.7.1 Flavonoids

Flavonoids are compounds comprising fifteen carbons with two aromatic rings connected by a three-carbon bridge, hence C6-C3-C6 (Fig. 2.3). They are the most numerous of the phenolics and are found throughout the plant kingdom. They are present in high concentrations in the epidermis of leaves and fruits and have important and varied roles as secondary metabolites, being involved in processes like UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance (Crozier et al., 2006; Pereira et al., 2009). Flavonols are the most widespread of the flavonoids.



2.7.2 Phenolics

The beneficial effects of vegetables on health improvement have been partly attributed to their complex mixture of phytochemicals possessing antioxidant activity. In recent years, considerable attention has been directed towards the identification of natural antioxidants, namely those plant derived that may be used for human consumption regarding health promotion and disease prevention. Among phytochemicals possessing antioxidant capacity, phenolic compounds are one of the most important groups (Jahangir et al., 2009). “Phenolic compounds” is a generic term that refers to a large number of compounds (more than 8,000) widely dispersed throughout the plant kingdom and characterized by having at least one aromatic ring with one or more hydroxyl groups attached. Phenolics are produced in

plants as secondary metabolites via the shikimic acid pathway. Phenylalanine ammonia-lyase (PAL) is the key enzyme catalyzing the biosynthesis of phenolics from the aromatic amino acid phenylalanine. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols (Crozier et al., 2006; Pereira et al., 2009). They can be classified based on the number and arrangement of their carbon atoms in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) (Crozier et al., 2006).

2.7.3 Antioxidants of Carrots

The fresh-cut fruit and vegetable industry is constantly growing, mainly due to the consumer's tendency of health consciousness and their increasing interest in the role of food for maintaining and improving human well-being (Allende et al., 2006). Carrots contain not only nutritional antioxidants such as vitamins A, C, and E, but also a great quantity of non-nutritional antioxidants, such as β -carotene, carotenoids, flavonoids, flavones, phenolics compounds, etc. (Yen et al., 2008). Studies of β -carotene indicate that its antioxidant activities and health benefits only occur when it is derived from food and not supplements. β -carotene has been used as a so-called oral sun protectant due to its antioxidant properties, and its efficacy has been proven in studies (Stahl et al., 2000). In recent years, worldwide consumption in carrots has been steadily increasing (Marquez, 2010). Prices of carrots depend on a target market. The average selling price of carrot to be sold on fresh vegetable markets is much higher than that of carrot for food processing (Szwejkowska et al., 2009). After harvesting and processing however, carrots have generally been considered to have lower nutritional value than when they are fresh. This is mainly due to the loss of nutritional compounds such as vitamins and carotenoids (Alasalvar et al., 2005). The fresh-cut fruit and

vegetable industry is still working to increase the assortment of minimally processed vegetables that meets consumer's needs for quick and convenient products that preserve their nutritional value, retain a natural and fresh color, flavor, texture, and contain fewer additives as preservatives (Allende et al., 2006).

2.7.4 Antioxidants of Turnip

It has been noticed that increasing the consumption of fruits and vegetables is a practical strategy for consumers to optimize their health and to reduce the risk of chronic diseases. In fact, plant-based foods contain significant amounts of bioactive non-nutritive compounds, which provide desirable health benefits beyond basic nutrition. This association is often attributed to the antioxidant phytochemicals, namely phenolic compounds and organic acids (Liu, 2003; Pulido et al., 2000; Silva et al., 2004). These compounds are also known to contribute to the organoleptic characteristics of fruits and vegetables (Vaughan and Geissler, 1997) and have been widely applied for their quality control (Ferrerres et al., 2005; Sousa et al., 2005). The phenolic (Ferrerres et al., 2005; Sousa et al., 2005; Romani et al., 2006; Vrchovska et al., 2006) and organic acid (Ferrerres et al., 2006; Sousa et al., 2005; Vrchovska et al., 2006) composition of *Brassica* vegetables or byproducts has been widely investigated. In what concerns *B. rapa*, only the flavonoids in var. *sylvestris* (Romani et al., 2006) were characterized. Turnip (*Brassica rapa* var. *rapa* L.) is one of the oldest cultivated vegetables, being used for human consumption all over the world especially in Asian countries like India (Liang et al., 2006). This species is notably popular in Europe, particularly in its colder regions. It grows well in cold climates and can be stored several months after harvest.). Phenolic compounds, several phenolic acids and flavonoids were previously determined namely, hydroxycinnamic acids, kaempferol, and isorhamnetin derivatives in turnip (Fernandes et al., 2007).

2.7.5 Antioxidants of Sprouts

Raw sprouts comprise of an important segment of fresh produces routinely consumed by Indian and other Asian population. Radish and moong bean sprouts in particular is a delicacy which is consumed either raw or slightly cooked form as side dishes. Raw sprouts are rich in several antioxidant compounds, capable of protecting against reactive oxygen species (ROS) mediated damage (Cuzzocrea et al., 2001). Radish (*Raphanus sativa*) sprouts are rich in antioxidants due to presence of flavonoids (Takashi et al., 2006), polyphenols, vitamins and activity to scavenge free radicals (Michie and Kazuko, 2004). Moong bean (*Phaseolus aureous*) sprouts are rich sources of antioxidants, polyphenols and also exhibit antimicrobial activities (Randhir et al., 2004).

Despite these important nutritional characteristics, consumption of raw sprouts has been associated with numerous outbreaks of food-borne illness (Taormina et al., 1999a, b). Several microbiological surveys have shown the presence of foodborne pathogens in sprouts (Andrenne et al., 2001). For instance, *E. coli* O157: H7, *Salmonella* spp. and *Listeria monocytogenes* have been isolated from sprouted seeds, including alfalfa, moong bean (Taormina et al., 1999a). Sprouting seeds are usually susceptible to contamination by human pathogens, including *Shigella* due to the high temperature (25-35°C) and humidity typically used in production (Hara-Kudo et al., 1997). Although, the reported incidence of food borne shigellosis is lower than that of salmonellosis or other enteric pathogens, each year there are a significant number of outbreaks, mainly associated with consumption of raw sprouts (Harris et al., 2003). Some strains of *Shigella* are able to tolerate adverse conditions such as acidic environment (pH 2-3) and low O₂ conditions (Gil and Selma, 2006). In fact, salads with an acidic pH (commercial carrot salad pH 2.7–2.9, potato salad pH 3.3–4.4, coleslaw pH 4.1–4.2, and crab salad pH 4.4–4.5) have been implicated in shigellosis outbreaks (Rafii and

Lunsford, 1997); hence require some alternate processing technique to overcome these associated problems. Furthermore, the infective dose of *Shigella* may be as low as 10–500 organisms (DuPont et al., 1989). To reduce the risk associated with *Shigella* spp. on fresh produce such as sprouts, intervention strategies namely, UV-C (Rajkowski, 2007) and chlorine has been attempted (Wu et al., 2000). Effective removal was however not achieved during such treatments; moreover the use of sanitizers such as organic acids or ozone has not been reported elsewhere. Although treatment with sanitizers ensures both organoleptic and nutritional properties, depletion of key nutritional factors to some extent has been noted in fresh produce (Vandekinderen et al., 2008), therefore the effect of sanitizer on the antioxidant status of the produce need to be addressed while designing minimal treatment processes.

2.7.6 Antioxidants of Mushrooms

Mushrooms have been consumed throughout the world for many centuries, not only for nutritional value but also for functional purpose. Mushrooms provide key nutrients and bioactive components such as high quality proteins, vitamins (riboflavin, niacin and folates), minerals (potassium, phosphorus, magnesium, zinc, copper, and selenium), unsaturated fatty acids and dietary fibers (Mattila et al., 2001). Moreover, mushrooms contain various polyphenolics and flavonoids which are recognized as excellent antioxidants. Several studies have indicated role of mushrooms in improving immunity (Sia and Candish, 1999), lowering blood cholesterol and lipid (Cheng et al., 2002), as a chemoprotectant (Grube et al., 2001) having antibiotic activity (Van et al., 2003). However, on account of the highly perishable nature of mushroom (short shelf life), freshly harvested mushrooms usually have to be processed for manufacture of pickles, chutneys, soups, etc. Despite the functional properties of mushrooms, these might become contaminated with pathogens due to improper processing techniques (Doyle and Schoeni, 1986; Samadpour et al., 2006; Van et al., 1989).

Washing mushrooms has recently gained commercial popularity as a means of improving appearance by removing casing soil particles and to increase shelf life. Significant research has been conducted to develop washing processes to achieve the objective to use. A few studies have examined the potential of wash additives including sodium hypochlorite (Beuchat et al., 1998), trisodium phosphate (Liao and Sapers, 2000), hydrogen peroxide (Sapers et al., 1999), and chlorine dioxide (Wisniewsky et al., 2000) sodium hypochlorite, hydrogen peroxide, potassium sorbate, and sodium salts of benzoate, EDTA and phosphoric acids respectively for mushrooms.

Although, there have been no reported outbreaks associated with consumption of mushrooms several reports have established human pathogens in fresh mushrooms. In a survey in the Pacific Northwest, 1% of retail samples tested positive for *L. monocytogenes* and 5% tested positive for *Salmonella* spp. (Samadpour et al., 2006). In the Netherlands, *L. monocytogenes* was recovered in 10% of mushrooms purchased at a grocery store (Van et al., 1989). Doyle and Schoeni (1986) isolated *C. jejuni* in three of 200 (1.5%) retail mushroom packages from several Midwestern U.S. grocery stores. Recently, threat due to the evolution of new microbes (highly virulent, antibiotic resistant, ability to survive in adverse conditions) has led to increased concerns of safety. Contamination with such pathogens increases the chances to contaminate other raw vegetables and also affects the health and hygiene of personnel, also hinders export options. Doyle and Schoeni (1986) summarized that the mushrooms could be contaminated by pickers or packagers, who may lack good personal hygiene and sanitary practice and these pathogenic bacteria, could survive on the surface of mushrooms during post harvest storage and retail display.

None of above discussed treatments was sufficiently effective to improve the shelf life and remove the targeted pathogen from fresh produce to an acceptable level. Sanitizer

treatment may also damage the eating quality and overall antioxidant status of the fresh produce/fungus. Recently, treatment of fruits or seeds with organic acid or vinegar has also been shown to be effective in reducing fungal decays and food borne pathogens (Sholberg and Gaunce, 1995). Basically, antimicrobial effect of organic acids has been attributed to undissociated acid molecules that enter inside the cell, releases a proton and ultimately lowers the intracellular pH which interfere with cellular metabolism or a decrease in biological activity as a result of pH change in the cell's environment takes place (Booth, 1985).

2.7.7 Antioxidants of Betel leaves

Piper betle Linn. (Family: Piperaceae) is a common plant cultivated in Asian Countries; the plant has alternate, heart-shaped, smooth, shining and long-stalked leaves, with pointed apex, the betel leaves are known to possess antioxidant action due to presence of polyphenols, flavonoids, alkaloids (Arambewela et al., 2006). Furthermore, betel oil present in betel leaves contains several phenols including hydroxychavicol, eugenol, betel phenol and chavicol; vitamin C (1.9 mg/g) and a large amount of carotenes (80.5 mg/g) have also been reported (Wang and Wu, 1996). Betel leaves form an important part of East Asian diet, (consumed/ chewed after meals) as well as an important produce item that is exported. Compounds, especially from natural sources, capable of protecting against reactive oxygen species (ROS) mediated damage may have potential application in the prevention and/or cure of the disease (Cuzzocrea et al., 2001; Meydonni et al., 1995). The beneficial effects have been credited to several non-essential food constituents, phytonutrients; pose a relevant bioactivity when frequently consumed as a part of regular diet (Steinmetz and Potter, 1996). To this end, the exploration of dietary plants with medicinal properties is promising and might provide functional foods. Despite the excellent medicinal properties and commercial significance, betel leaves have been attributed to typhoid outbreak in betel-eating areas

(Taylor and Francis Health Sciences, 2004). Several multidrug resistant strains of *Salmonella* have been reported in betel leaves (Singh et al., 2006). A suitable minimal process which retains the nutritional value, natural and fresh color, flavor and texture, and contain fewer additives (such as preservatives) (Jongen, 2002) in addition to inactivating the bacterial pathogens in betel leaves, for consumer safety, has not been reported to date.

MATERIALS AND METHODS

3.1 Chemicals and Media

All chemicals and reagents used were of the highest analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (St. Louis, MO, USA) and Hi-Media (Mumbai, India). Chemicals of molecular biology grade were procured from New England Biolabs, UK.

3.2 Collection of the Fresh vegetable Samples

A total two hundred and thirty six selected fresh vegetable samples especially eaten as raw or with slight processing (spinach leaves, capsicum, coriander, turnip, radish, betel leaves, mushroom, cucumber, cabbage, tomato, carrot, moong bean sprouts and radish sprouts) were collected from different locations of Patiala city covering three main sites i.e. directly from farm, local mandis and street vendors (Table 3.1). All samples were collected in sterile ziplock bags, transported to the laboratory on ice and analyzed within 2 hours of receipt (Ghosh et al., 2004).

3.3 Microbiological analysis of Vegetable samples

Aerobic plate count method is used to determine the number of viable bacterial cells per gram of the sample using agar plate media. Aerobic plate count was observed for all the collected vegetable samples and investigates the microbiological quality of the sample. Aerobic plate count was determined by surface rinsed method as described by Food and Drug Administration, (Andrews and Hammack, 2003). Twenty five gram of the all individual vegetable samples were mixed with 225 ml (1:9 w/v) of 0.1% peptone water and number of

aerobic counts were determined by plating on to plate count agar and incubated at 37°C for 18-24 h.

Table 3.1 A total of 236 vegetable samples covering different locations of Patiala city

| S. No. | Common name | Botanical name | Number of samples examined (N=236) |
|--------|--------------------|--------------------------------|------------------------------------|
| 1 | Spinach leaves | <i>Basella alba</i> | 18 |
| 2 | Capsicum | <i>Capsicum annuum</i> | 18 |
| 3 | Coriander | <i>Coriandrum sativum</i> | 15 |
| 4 | Turnip | <i>Brassica rapa</i> | 18 |
| 5 | Radish | <i>Raphanus sativus</i> | 20 |
| 6 | Betel leaves | <i>Piper betle</i> | 19 |
| 7 | Mushroom | <i>Agaricus bisporous</i> | 18 |
| 8 | Cucumber | <i>Cucumis sativus</i> | 18 |
| 9 | Cabbage | <i>Brassica oleracea</i> | 18 |
| 10 | Tomato | <i>Lycopersicon esculentum</i> | 17 |
| 11 | Carrot | <i>Daucus carota</i> | 18 |
| 12 | Moong bean sprouts | <i>Phaseolus aureus</i> | 20 |
| 13 | Radish sprouts | <i>Raphanus Sativa</i> | 19 |

3.4 Isolation and Enumeration of food borne pathogens

3.4.1 *Salmonella* spp.

Twenty five gram of each vegetable sample was mixed with 225 ml of pre-enriched in Universal pre-enrichment (UPB) broth (37°C for 24±2 h) followed by enrichment in rappaport-vassiliadis soya peptone (RVS) broth (CM866, Oxoid Ltd.) at 37°C for 24±2 h respectively. The enriched samples were streaked on xylose lysine deoxycholate medium (XLD) (CM469, Oxoid Ltd), incubated at 37°C for 24±2 h and observed for pink colonies with black center. *Salmonella typhimurium* strain ATCC 23564 was used as a positive control for all the experiments.

3.4.2 *Shigella* spp.

Twenty five gram of each vegetable sample was mixed with 225 ml of *Shigella* broth for enrichment and incubated overnight at 37°C for 18±2 h. The incubated *Shigella* broth samples were streaked on hektoen enteric agar (HEA) to obtain well isolated colonies and incubated at 35°C for 24±2 h. Presumptive *Shigella* spp. colonies appear entirely green after 24 h incubation. *Shigella flexneri* Type 2(a) was used as a positive control for all the experiments.

3.4.3 *Cronobacter* spp.

Twenty five gram of each vegetable sample was mixed with 225 ml of Enterobacteriaceae enrichment broth (EE) (pH 8.0) for enrichment and incubated in a rotary shaker (Labcon, 5081U, USA, 120 rpm/min), overnight at 37°C for 18±2 h. The incubated EE broth samples were streaked on DFI (an *E. sakazakii* specific chromogenic medium, Oxoid Ltd., UK, pH 7.3) and incubated at 37°C. Presumptive *Cronobacter* spp. colonies appear entirely blue-green after 24 h incubation on DFI agar. The *Cronobacter sakazakii* strain ATCC 29004 was used as a positive control for all the experiments (Iversen and Forsythe, 2004).

3.5 Morphological and Biochemical characterization of Isolates

All the isolates were morphologically and biochemically characterized according to *Bergey's Manual of Systematic Bacteriology* (Holt, 1994) and are briefly described below.

Gram staining

Bacterial smear from actively growing cells were spread on a glass slide, heat fixed and flooded with filtered crystal violet for 30 sec. Water washing was done briefly to remove excess crystal violet, flooded with Gram's iodine for 10 sec and washed in water. Decolourization was done with acetone until the moving dye front passed the lower edge of the

section and washed immediately in tap water and counter stained with safranin for 15 sec. Again water washing was done to remove the excessive stain. Glass slide was air dried and then observed under the microscope at 100 X resolution.

Oxidase test

Cultures were spread onto TSA for 24 h and oxidase discs were placed on plates. Development of purple color within 60 sec indicated positive test.

Catalase test

The growth in TSA slant was used for catalase test. 3% hydrogen peroxide was added on the TSA slant and observed for the production of gas bubbles.

Indole test

Indole reagent was added to culture in tryptone broth. A positive test was indicated by the appearance of red color.

Simmons citrate test

Simmons citrate agar was streaked with the presumptive isolate and incubated at 37°C for 24 h. Development of initial green to deep blue indicated positive test.

Bile Esculin test

Bile esculin agar was streaked and incubated overnight at 37°C. Development of black coffee color indicated positive test.

Voges – Proskauer test

VP broth was inoculated and incubated at 37°C for three days. Then reagents (0.6 ml of 5% α -Naphthol and 0.2 ml of 40% KOH) were added and tubes were allowed to stand for 30 min. Development of pink color indicated positive test.

Nitrate reduction test

Nitrate broth was inoculated for 5 d and then reagents (6 drops of Sulfanilic acid and 6 drops of Naphthylamine) were added. Development of red color indicated positive test.

Urease test

Urea agar was streaked and incubated at 37°C for 48 h. Development of pink color indicated positive test.

Ornithine decarboxylase test

Moeller Ornithine decarboxylase broth was inoculated and overlaid with paraffin oil and incubated at 37°C for 48 h. Development of purple color indicates positive test.

Lysine decarboxylase test

Moeller Lysine Decarboxylase broth was inoculated and overlaid with paraffin oil and incubated at 37°C for 48 h. Development of purple color indicated positive test.

Sugar tests

Phenol red broth was prepared with different sugars (adonitol, arabinose, cellobiose, glucose, lactose, mellibiose, saccharose, raffinose, rhamnose, trehalose, xylose). All combinations were incubated overnight. Development of yellow color indicated positive test.

3.6 Molecular identification of Isolates

3.6.1 DNA Isolation and PCR amplification

All molecular biology techniques were performed as outlined in Sambrook et al. (1989) unless otherwise noted. All enzymatic reactions and routine purification of nucleic acid involving kits were performed as indicated in the manufacturer instructions (Promega, Madison, WI, USA). All DNA modifying enzymes were obtained from Promega or Fisher Scientific (USA). All PCR reactions were conducted using universal primers P0 (5'

GAGAGTTTGATCCTGGCTCAG-3') and P6 (5' CTACGGCTACCTTGTTACGA-3'). The PCR mixture contained 1 µl *Taq* (10 X) commercial buffer, 5 µl purified DNA (50-100 ng), 150 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase. Total reaction volume was 100 µl. The program for 16S rDNA was as follows: The reaction mixtures were first incubated for 5 min at 95°C, and then cycled for 36 cycles according to the following temperature profiles: 1 min at 94°C, 1 min at 55°C 2 min at 72°C, followed by a final extension for 10 min at 72°C, unless otherwise specified, on a Bio Rad thermal cycler (Bio Rad, MyCycler, USA).

3.6.2 DNA Sequencing and Analysis of sequence data

All DNA sequencing was performed by the Geneii Molecular Biology Resource Facility at Bangalore India, using an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with Big Dye Terminator Cycle Sequencing v 2.0 (PE Applied Biosystems). The related sequences showing similarity in BLAST were retrieved from GenBank and aligned using the program CLUSTALW (Thompson et al., 1997). Phylogenetic dendogram was constructed by neighbour-joining method using MEGA 4 software (Tamura et al., 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses.

3.7 Storage of Isolated pathogens

All the isolated pathogens were inoculated in 5 ml of TSB (Tryptone soya broth) in a test tube at 37°C for 24±2 h in a rotary shaker (120 rpm). The cultures were centrifuged and the pellet were washed twice with 0.85% saline, resuspended in fresh TSB and stored in cryovial containing 40% glycerol at -80°C. Working cultures were revived in either tryptone soya broth (TSB) or brain heart infusion broth (BHI) at least three times prior to any experiment.

3.8 Ozone generation and Measurement

Aqueous ozone was produced on site by an electrochemical process using an ozone generator (Oz-Air, Creative Oz-Air Pvt. Ltd., Noida, India) in the laboratory. Ozone concentration was measured before immersing samples using an ozone concentration sensor (Dissolved Ozone Monitor, Model A15/64, Analytical Technology, Inc., Collegeville, PA, USA) which is an on-line monitoring system designed for the continuous measurement of ozone gas in solution.

3.9 Sanitizer treatment on Planktonic bacterial cells

3.9.1 Ozonated water

All the isolated pathogens (ST1, ST2, SF1, SF2, CS1 and CS2) were inoculated in 5 ml of TSB (Tryptone soya broth) in a test tube and incubated at 37°C for 18±2 h in a rotary shaker (120 rpm). Overnight cultures in TSB were transferred (0.1 ml) to 10 ml of fresh TSB and vortexed for 5 min. After vortexing, 200 µl volumes were transferred into 100 well microtitre plate. Microbial growth was analyzed in automatic growth analyzer (Bioscreen C, Helsinki, Finland) after adding various concentrations of ozonated water (0 - 2 ppm) to observe the effective concentration of ozonated water against the growth of bacterial pathogens. The pH of ozonated water was checked by digital pH meter at 25°C. The cell turbidity was monitored using a microtitre plate reader (Bioscreen C) at an optical density at 580 nm (Djordjevic et al., 2002).

3.9.2 Organic acids

All the isolated pathogens (ST1, ST2, SF1, SF2, CS1 and CS2) were inoculated in 5 ml of TSB (Tryptone soya broth) in a test tube and incubated at 37°C for 18±2 h in a rotary shaker (120 rpm). Overnight cultures in TSB were transferred (0.1 ml) to 10 ml of fresh TSB and vortexed for 5 min. After vortexing, 200 µl volumes were transferred into microtitre plate. Microbial growth was analyzed in automatic growth analyzer (Bioscreen C, Helsinki,

Finland) after adding various concentrations of organic acids (malic acid, citric acid, acetic acid, lactic acid) (0 - 2%) in 100 well microtitre plate to observe the effective concentration of organic acid against the growth of bacterial pathogens. pH of acidic water was checked by digital pH meter at 25°C The cell turbidity was monitored using a microtitre plate reader (Bioscreen C) at an optical density at 580 nm (Djordjevic et al., 2002).

3.10 Preparation of bacterial suspension

All the isolated cultures (ST1, ST2, SF1, SF2, CS1 and CS2) were maintained in brain heart infusion broth (BHI) at 4°C. Overnight cultures were prepared by inoculating 0.1 ml of culture into 50 ml BHI broth contained in a 250 ml Erlenmeyer flask and incubated at 37°C for 18±2 h in a rotary shaker (120 rpm). The cells were harvested by centrifugation (10,000 g for 5 min, 28°C) washed once with phosphate buffered saline (PBS, pH 7.2) and finally resuspended in maximum recovery diluent (MRD) (CM733, Oxoid Ltd.) to give a cell density of 4 log or 5 log CFU/ml (A_{600}) (Warriner et al., 2003). Bacterial suspension for each experiment was prepared using this protocol.

3.11 Ozone treatments onto artificially inoculated vegetable samples

3.11.1 *Daucus carota* (carrot) and *Brassica rapa* (turnip) with ST1 and ST2

3.11.1.1 Preparation of sample disks and inactivation

A pre-processing was done in carrot and turnip to remove tops and bottoms of the sample. Sample disks of carrot and turnip (5 mm) were prepared by using a sterile blade washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Carrot disks were immersed in bacterial suspension of ST1 (1:2 w/v) and turnip disks were immersed in bacterial suspension of ST2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow. Both types of inoculated disks were completely immersed (1:2 w/v) and sprayed

(1:2 w/v) in 1 ppm, 2 ppm, 3 ppm of ozonated water and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Disks were packed in sterile ziplock bags and stored at 28°C for ten days.

3.11.1.2 Microbiological analysis

During storage at 28°C, carrot and turnip disks were analyzed for the bacterial growth of ST1 and ST2 each with 2 day interval. Disks were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media, xylose lysine deoxycholate agar for *Salmonella typhimurium* and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.11.2 *Raphanus sativa* (radish) and *Phaseolus aureus* (moong bean sprouts) with SF1 and SF2

3.11.2.1 Preparation of sample and inactivation

Radish and moong bean sprouts were washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Moong bean sprouts were immersed in bacterial suspension of SF1 (1:2 w/v) and radish sprouts were immersed in bacterial suspension of SF2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow. Both types of inoculated sprouts were completely immersed (1:2 w/v) and sprayed (1:2 w/v) in 1 ppm, 2 ppm, 3 ppm of ozonated water and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Both types of sprouts were packed in sterile ziplock bags and stored at 28°C for ten days.

3.11.2.2 Microbiological analysis

During storage at 28°C, radish and moong bean sprouts were analyzed for the bacterial growth of SF1 and SF2 each with 2 day interval. Sprouts were washed in PBS (1:9

w/v) and then the number of pathogen was determined by plating on to appropriate selective media hektoen enteric agar for *Shigella flexneri*, and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.11.3 *Agaricus bisporous* (mushroom) and *Piper betle* (betel leaves) with CS1 and CS2

3.11.3.1 Preparation of sample disks and inactivation

Sample disks of mushroom (5 mm) and betel leaves (1x1 cm) were prepared by using a sterile blade washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Mushroom disks were immersed in bacterial suspension of CS1 (1:2 w/v) and betel leaves disks were immersed in bacterial suspension of CS2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow according. Both types of inoculated disks were completely immersed (1:2 w/v) and sprayed (1:2 w/v) in 1 ppm, 2 ppm, 3 ppm of ozonated water and untreated (control) for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Mushroom disks were packed in sterile ziplock bags and stored at 15°C for five days and betel leaves disks were stored at 28°C for ten days.

3.11.3.2 Microbiological analysis

During storage at 15°C, mushroom disks were analyzed for the bacterial growth of CS1 after 1 day interval. Betel leaves disks were analyzed for the bacterial growth of CS1 after 2 day interval. Disks were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media tryptone soya agar for *Cronobacter sakazakii*, and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.12 Organic acid treatment onto artificially inoculated vegetable samples

3.12.1 *Daucus carota* (carrot) and *Brassica rapa* (turnip) with ST1 and ST2

3.12.1.1 Preparation of sample disks and inactivation

Sample disks of carrot and turnip (5 mm) were prepared by using a sterile blade washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Carrot disks were immersed in bacterial suspension of ST1 (1:2 w/v) and turnip disks were immersed in bacterial suspension of ST2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow. Both types of inoculated disks were completely immersed (1:2 w/v) and sprayed (1:2 w/v) in 1%, 2%, 3% malic acid, acetic acid, lactic acid, citric acid and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Disks were packed in sterile ziplock bags and stored at 28°C for ten days.

3.12.1.2 Microbiological analysis

During storage at 28°C, carrot and turnip disks were analyzed for the bacterial growth of ST1 and ST2 each with 2 day interval. Disks were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media, xylose lysine deoxycholate agar for *Salmonella typhimurium* and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.12.2 *Raphanus Sativa* (radish) and *Phaseolus aureus* (moong bean sprouts) with SF1 and SF2

3.12.2.1 Preparation of sample and inactivation

Moong bean and radish sprouts were washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to

remove traces of ethanol. Moong bean sprouts were immersed in bacterial suspension of SF1 (1:2 w/v) and radish sprouts were immersed in bacterial suspension of SF2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow. Both types of inoculated sprouts were completely immersed (1:2 w/v), sprayed (1:2 w/v) in 1%, 2%, 3% malic acid, acetic acid, lactic acid, citric acid and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Both types of sprouts were packed in sterile ziplock bags and stored at 28°C for ten days.

3.12.2.2 Microbiological analysis

During storage at 28°C, moong bean and radish sprouts were analyzed for the bacterial growth of SF1 and SF2 each with 2 day interval. Sprouts were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media hektoen enteric agar for *Shigella flexneri*, and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.12.3 *Agaricus bisporous* (mushroom) and *Piper betle* (betel leaves) with CS1 and CS2

3.12.3.1 Preparation of sample disks and inactivation

Sample disks of mushroom (5 mm) and betel leaves (1x1 cm) were prepared by using a sterile blade washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Mushroom disks were immersed in bacterial suspension of CS1 (1:2 w/v) and betel leaves disks were immersed in bacterial suspension of CS2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow according. Both types of inoculated disks were completely immersed (1:2 w/v), sprayed (1:2 w/v) in 1%, 2%, 3% malic acid, acetic acid, lactic acid, citric acid and untreated (control) for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Mushroom disks were packed in sterile ziplock

bags and stored at 4°C and 15°C for five days and betel leaves disks were stored at 28°C for ten days.

3.12.3.2 Microbiological analysis

During storage 4°C at 15°C, mushroom disks were analyzed for the bacterial growth of CS1 after 1 day interval. Betel leaves disks were analyzed for the bacterial growth of CS1 after 2 day interval. Disks were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media tryptone soya agar for *Cronobacter sakazakii*, and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.13 Combined sanitizer treatment onto artificially inoculated vegetables

3.13.1 *Daucus carota* (carrot) and *Brassica rapa* (turnip) with ST1 and ST2

3.13.1.1 Preparation of sample disks and inactivation

Sample disks of carrot and turnip (5 mm) were prepared by using a sterile blade washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Carrot disks were completely immersed in bacterial suspension of ST1 (1:2 w/v) and turnip disks were immersed in bacterial suspension of ST2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow. Both types of inoculated disks were completely immersed (1:2 w/v), sprayed (1:2 w/v) in 2% of organic acid (malic acid, acetic acid) combined with 2 ppm ozone and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Disks were packed in sterile ziplock bags and stored at 28°C for ten days.

3.13.1.2 Microbiological analysis

During storage at 28°C, carrot and turnip disks were analyzed for the bacterial growth of ST1 and ST2 each with 2 day interval. Disks were washed in PBS (1:9 w/v) and then the

number of pathogen was determined by plating on to appropriate selective media, xylose lysine deoxycholate agar for *Salmonella typhimurium* and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.13.2 *Raphanus Sativa* (radish) and *Phaseolus aureus* (moong bean sprouts) with SF1 and SF2

3.13.2.1 Preparation of sample and inactivation

Moong bean and radish sprouts were washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Moong bean sprouts were immersed in bacterial suspension of SF1 (1:2 w/v) and radish sprouts were immersed in bacterial suspension of SF2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow. Both types of inoculated sprouts were completely immersed (1:2 w/v), sprayed (1:2 w/v) in 2% of organic acid (malic acid, acetic acid) combined with 2 ppm ozone (1:2 w/v) and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Both types of sprouts were packed in sterile ziplock bags and stored at 28°C for ten days.

3.13.2.2 Microbiological analysis

During storage at 28°C, moong bean and radish sprouts were analyzed for the bacterial growth of SF1 and SF2 each with 2 day interval. Sprouts were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media hektoen enteric agar for *Shigella flexneri*, and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.13.3 *Agaricus bisporus* (mushroom) and *Piper betle* (betel leaves) with CS1 and CS2

3.13.3.1 Preparation of sample and inactivation

Sample disks of mushroom (5 mm) and betel leaves (1x1 cm) were prepared by using a sterile blade washed thrice with tap water and surface sterilized with 70% ethanol followed by three times washing with sterile distilled water to remove traces of ethanol. Mushroom disks were immersed in bacterial suspension of CS1 (1:2 w/v) and betel leaves disks were immersed in bacterial suspension of CS2 (1:2 w/v) at room temperature for 5 min and dried for one hour in laminar air flow according. Both types of inoculated disks were completely immersed (1:2 w/v), sprayed (1:2 w/v) in 2% of organic acid (malic acid, acetic acid) combined with 2 ppm ozone (1:2 w/v) and untreated (control) at room temperature for 5 min to determine the effectiveness of sanitizer for removing or inactivating attached bacteria. Mushroom disks were packed in sterile ziplock bags and stored at 15°C for five days and betel leaves disks were stored at 28°C for ten days.

3.13.3.2 Microbiological analysis

During storage at 15°C, mushroom disks were analyzed for the bacterial growth of CS1 after 1 day interval. Betel leaves disks were analyzed for the bacterial growth of CS1 after 2 day interval. Disks were washed in PBS (1:9 w/v) and then the number of pathogen was determined by plating on to appropriate selective media tryptone soya agar for *Cronobacter sakazakii*, and incubated at 37°C for 18-24 h (Liao and Sapers, 2000).

3.14 Recovery of acid resistant pathogens ST1 and ST2

In this study, it was observed that both ST1 and ST2 were resistant to higher concentrations of organic acids in comparison to other pathogens, therefore, these acid resistant pathogens were recovered using thin agar layer plates (TAL) (recovery medium for stressed cells) (Wu et al., 2001) and incubated at 37°C for 24 hour for the growth of

respective pathogen. Biofilm forming *Salmonella typhimurium* has been reported to form biofilms which makes the organism resistant to such sanitizer treatments (Niemira et al., 2005). Therefore, acid resistant strains of *Salmonella*, ST1 and ST2 were further studied to form biofilms onto respective vegetable samples as well as other food contact surfaces.

3.14.1 Presumptive identification of biofilm formation by calcofluor assay

An 10x stock solution of calcofluor fluorescent brightener (4,4'-bis[4-aminophenyl]-2,2'-bis(2-hydroxyethyl)amino-stilbenedisulfonic acid) (Sigma Chemical Co., St. Louis, MO, USA) was prepared at a final concentration of 800 µg/ml (de Rezende et al., 2005). Polyvinyl chloride (PVC) pipe used for the water distribution system and polyethylene bags used for the packaging with surface areas of 1x1 cm² were used. Each test surface was acid washed and placed inside test tubes containing 5 ml of broth. 2% malic acid and combined with 2 ppm ozone was added to the test tubes, inoculated with both strains of ST1 and ST2, incubated and biofilms were allowed to accrue for 3 days at 25°C with broth replacement every 24 h. The fourth and last broth contained 80 µg/ml of working concentration of calcofluor was used and were reincubated for an additional 18 h. Pieces were removed using sterile forceps and rinsed with 50 ml of PBS to remove unattached cell and unbound dye. A hand-held UV-lamp Long wave (UV-366nm, Blak-Ray™ Lamp model UVL-21, San Gabriel, CA) was used to detect the presence of biofilm on each surface. The extent of biofilm formation was evaluated based on extent and intensity of fluorescence on the plastic surface.

3.14.2 Identification of biofilm formation by ST1 and ST2 using alcian blue

Light microscopy was used in conjunction with alcian blue staining to confirm the presence of biofilm matrix by acid resistant pathogens. Alcian blue stains acidic polysaccharides often present in the biofilm matrix (exopolymeric substance-EPS) (Fassel and Edmiston, 1999). Staining was carried out by the direct addition of an aqueous solution

of 0.1% alcian blue 8GX (Sigma) onto the surface of the sample. Polyethylene bags used for the packaging and transportation of food samples were used for this study. Polyethylene bags were cut in 1x1cm square films and inoculated in 5 ml *Salmonella* ST1 and ST2 and incubated for 3 days at 25°C to provide sufficient time to develop biofilms. 2% malic acid and combined with 2 ppm ozone was added to test tubes except control. After incubation time, thin films were taken out, were gently washed with PBS and kept in 0.1% alcian blue dye for 20 min followed by washing with sterilized distilled water to remove excess dye. The stained thin films were then mounted in two to three drops of sterile water, and a cover-slip was applied and observed under microscope. All microscopic examinations were carried out using an Eclipse 55i (Nikon, NY, USA) microscope and photographs were taken with camera attached with the microscope.

3.14.3 Biofilm production assay using microtitre plates

Both *Salmonella* strains ST1 and ST2 were grown in 10 ml of tryptone soya broth supplemented with 0.5% (w/v) yeast extract (TSBYE), at 37°C overnight (Premaratne et al., 1991). Overnight cultures in TSBYE were transferred (0.1 ml) to 10 ml of fresh TSBYE and vortexed. After vortexing, 100 µl volumes were transferred into sterile polystyrene microtitre plate (Bioscreen C) wells. 2% malic acid and combined with 2 ppm ozone was added in the test wells. Plates were made in duplicate and incubated at 32°C for 20h and 40 h. The cell turbidity was monitored using a automatic growth analyser (Bioscreen C) at an optical density at 580 nm, and were recorded at different time intervals. The first set of plates was used for biofilm formation measurement after 20 h and the duplicate set was used to determine the 40 h biofilm formation. After a 20 h or 40 h incubation period, medium was removed from wells and microtitre plate wells were washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air dried for 45 min and each well was stained with 150 µl of 1% freshly prepared crystal violet solution in water for 45 min.

After staining, plates were washed with sterile distilled water five times. At this point, biofilms were visible as purple rings formed on the side of each well. The quantitative analysis of biofilm production was performed by adding 200 μ l of 95% ethanol to destain the wells. One hundred microliters from each well was transferred to a new microtitre plate and the level (OD) of the crystal violet present in the destaining solution was measured at 580 nm.

3.14.4 Biofilm identification on carrot and turnip using scanning electron microscopy (SEM)

3.14.4.1 Inoculation of vegetable samples with ST1 and ST2

Both *Salmonella* strains ST1 and ST2 were grown in 10 ml of tryptone soya broth and incubated in a rotary shaker (120 rpm) at 37°C for 24 \pm 2 h. The cells were harvested by centrifugation (10,000 g for 5 min, 28°C) washed thrice in 0.1% peptone water and then resuspended in equal volume of 0.1% peptone water. Vegetable samples (carrot and turnip) were excised in 1x1 cm² small pieces, washed with tap water and surface sterilized with 70% ethanol. Fifty microliter of a 7 log CFU/ml suspension of cells was inoculated in 10 ml of TSB containing excised pieces of turnip and carrot individually. Two more batches were prepared in the same way and one batch treated with 2% malic acid and other with 2% malic acid combined with 2 ppm ozone to see the effect on biofilm formation. The samples were allowed to stand for 3 days at 28°C to allow the formation of biofilms.

3.14.4.2 Processing of vegetable sample for scanning electron microscopy

The protocol for sample processing followed the one used by Pao et al. (2001) for imaging pathogens on turnip and carrot was fine tuned at the National Microscopy Imaging Center (All India Institute of Medical Sciences, New Delhi, India). Following incubation, samples were washed in PBS and fixed overnight in 3% glutaraldehyde and 2.5% paraformaldehyde (Sigma, MO, USA) in 0.1 M potassium phosphate buffer, pH 6.8 at 4°C to

maintain the structural integrity and secure bacteria in the tissue. After washing in buffer, samples were post fixed 1% OsO₄ for 2 hour at 4°C. The samples were dehydrated in an ascending grade of acetone, critical-point dried and mounted on aluminium stubs. These were sputter-coated with colloidal gold and observed under a leo 435 VP scanning electron microscope (Warwickshire, UK) at an operating voltage 15kV. Images were digitally acquired by using a CCD camera attached to the microscope.

3.15 Effect of sanitizer treatment on antioxidant levels

Although treatment with sanitizers ensures both organoleptic and nutritional properties, depletion of key nutritional factors to some extent has been noted in fresh produce (Vandekinderen et al., 2008), the effect of sanitizer on the antioxidant status of the produce need to be addressed while designing minimal treatment processes. Therefore, physicochemical properties of raw and treated vegetable samples with special reference to color, sensorial and antioxidants properties like DPPH, ABTS, reducing power, flavonoids, polyphenols and sodium oxide dismutase was compared to ensure the nutritional content of the fresh produce.

3.15.1 Antioxidant levels of all the vegetable samples

3.15.1.1 Extraction to determine antioxidant activity of vegetables

Extraction of raw and processed vegetable samples was done as described by Ganguli et al. (2007) with some modifications for the determination of antioxidant activities. Ten gram of the vegetable samples was homogenized with 200 ml of 99% ethanol in a rotary shaker (120 rpm) at 37°C for 24 h. The mixture was further extracted with a homogenizer for 15 min at a medium speed and filtered. The extraction solvent (ethanol) was removed using a rotary vacuum evaporator (R205B, Shanghai, China) at 40°C under vacuum and redissolved into 50 mL of ethanol to be tested; the extracts were stored in cryovials at -20°C until analysis.

3.15.1.2 Determination of DPPH radical scavenging activity

The scavenging activity on DPPH radical was measured according to the method of Cheung et al. (2003) with some modifications. Ethanolic solution of DPPH, 0.8 ml of 0.2 mM was mixed with 0.2 ml of the sample extracts. The mixture was vigorously shaken and allowed to stand for 10 min in dark at room temperature. The absorbance was measured at 520 nm. The DPPH radical scavenging activity (%) was calculated by the following equation:

$$\text{Radical scavenging activity (\%)} = 1 - (A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where A_{sample} is the absorbance in the presence of sample and A_{control} is the absorbance in the absence of sample, respectively. All extracts were analyzed in triplicate.

3.15.1.3 Determination of ABTS radical scavenging activity

The scavenging activity on ABTS radical cation was measured according to the method of Re et al. (1999) with some modifications. ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate solution and the mixture was left to stand overnight in the dark at room temperature. The ABTS radical cation solution was diluted with distilled water to obtain absorbance of 1.4-1.5 at 414 nm (molar extinction coefficient, $\epsilon = 6 \cdot 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$). Diluted ABTS radical cation solution (1 ml) was added to 50 μl of the extract or ascorbic acid standard solution or distilled water. After 90 min, the absorbance was measured at 414 nm using spectrophotometer (U-2800, Hitachi, Japan). The ABTS radical cation scavenging activity was expressed as ascorbic acid equivalent antioxidant activity (AEAC) and defined as mg of ascorbic acid equivalents per 100 g of vegetable sample. The AEAC was calculated by the following equation:

$$\text{AEAC} = (\Delta A_{\text{sample}} / \Delta A_{\text{aa}}) \times C_{\text{aa}} \times V \times (100 / W_{\text{sample}})$$

Where ΔA_{sample} is the change of absorbance in the presence of extract, ΔA_{aa} is the change of absorbance after addition of ascorbic acid standard solution, C_{aa} is the concentration of ascorbic acid standard solution (mg/ml), V is the volume of extract (ml) and W_{sample} is the weight of sample used for extraction (g). All extracts were analyzed in triplicate.

3.15.1.4 Determination of flavonoid content

Flavonoids content were determined by colorimetric method described by Zhishen et al. (1999) with some modifications and results were expressed as mg catechin equivalents per 100 g of vegetable sample. Sample extract (250 μl) was mixed with 1.25 ml of distilled water and 75 μl of a 5% NaNO_2 solution. After 5 min, 150 μl of a 10% $\text{AlCl}_3 \cdot \text{H}_2\text{O}$ was added followed by addition of 500 μl of 1 M NaOH and 275 μl of distilled water after 6 min, mixed well and intensity of pink color was measured at 510 nm (Hitachi U-2800, Japan). Catechin was used as standard. All extracts were analyzed in triplicate.

3.15.1.5 Determination of polyphenolics

Polyphenolic contents were determined using the Folin-Ciocalteu method as described by Turkoglu et al. (2007) with some modifications and results were expressed as mg gallic acid equivalents per 100 g of sample. Sample extract (200 μl) was mixed with 2 ml of 2% sodium carbonate solution and 100 μl of a 50% Folin-Ciocalteu reagent. After incubation for 30 min at room temperature, the absorbance was measured at 750 nm. Gallic acid was used as standard. All extracts were analyzed in triplicate.

3.15.1.6 Determination of reducing power

The determination of the reducing power was conducted according to the method developed by Oyaizu (1986) with some modifications. A 2.5 ml of sample extract was added to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then placed in a 50°C water bath for 20 min. Then, samples were kept at room

temperature and 2.5 ml of 10% trichloroacetic acid was added to the mixture. Finally, 0.125 ml of the mixture mixed with 0.125 ml distilled water and 1 ml of 0.1% ferric chloride and incubated for 10 min. The absorbance of the samples was measured at 700 nm. BHT (butylated hydroxytoluene) and α -tocopherol were used as standards. All extracts were analyzed in triplicate.

3.15.1.7 Determination of β -carotene of carrots

The β -carotene content of the tubers was determined by AOAC method (1997). Carrot samples (0.2 g) were hydrolyzed with 25 ml of 5% alcoholic KOH and extracted thrice with 50 ml petroleum ether. The petroleum ether fraction was evaporated to dryness and residue taken up in 10 ml chloroform. The absorbance was measured, using spectrophotometer at wavelength of 440 nm. The concentrations of β - carotenoids in test samples were read from the standard curve.

3.15.1.8 Determination of superoxide dismutase (SOD) activity of mushroom

The determination of the sodium oxide dismutase activity was conducted according to the method developed by Kim et al. (2001) with some modifications. Sample (mushroom) extract 0.2 ml was mixed with 3 ml of 50 mM Tris-HCL buffer (50 mM Tris (hydroxymethyl) amino-methane + 10 mM EDTA, pH 8.5) and 0.2 ml of 7.2 mM pyrogallo, and the mixture was incubated at 25°C for 10 min. The reaction was stopped by the addition of 1 ml of 1 N HCL. The absorbance of the mixture was determined at 420 nm against blank.

3.15.1.9 Measurement of dietary fiber content of mushroom

α -Amylase and protease were used to remove the starch and protein respectively, and 95% ethanol was added to precipitate the dietary fibre; then, the residue was obtained by drawing the later filter under reducing pressure. The total dietary fibre was expressed by subtracting the protein and ash contents from the residue. The insoluble dietary fiber (IDF)

content was measured using the method of Prosky et al. (1988). Sample protein was removed by using enzymes such as α -amylase, amyloglucosidase and protease and filtered (4°C). The residue was treated with 95% and 78% solutions of ethanol and acetone, and dried in an oven. After analyzing the protein and ash contents, IDF was determined as the residual value from which the amounts of protein and ash were subtracted. The soluble dietary fiber (SDF) content was determined by subtracting Insoluble dietary fiber (IDF) from total dietary fiber (TDF).

3.15.1.10 Color measurements of mushroom

Color analysis was made on raw and treated mushroom samples stored upto 5 days at 15°C. The changes in color during the storage were monitored by colorimetric measurements using a Benchtop Spectrophotometer (Color i5 Gretag, Macbeth, USA) (O'Neill et al., 1998). The instrument was standardized against a white tile before each determination. The color of mushroom was determined as L* values; which corresponds to the whiteness of mushroom.

3.16 Sensory evaluation of all the vegetable samples

The sensory characteristics of all the vegetable samples were evaluated in terms of texture/freshness, taste/flavor, aroma/smell, color/appearance and overall acceptability scores using a nine point Hedonic scale according to Larmond (1977) by a panel consisting of ten trained evaluators. The panelists were served with all the vegetable samples at (25°C). Between evaluations of the samples the panelists would rinse their mouths with deionized water to remove any lingering taste. Subjects refrained from drinking, smoking, and eating anything except water for 30 min prior to each panel session. This method does not, of course, reflect actual consumer perception, but it does strongly indicate attributes which a good quality product should possess (George et al., 2010). Panelists were scientific staffs of the laboratory who were trained in attributing rating for the characteristics examined. The

scores were assigned from extremely liked (9) to disliked extremely (1). Samples were randomly drawn from each experimental block, coded, and served to the panelists randomly in a room illuminated with white light and maintained at 25°C. The panelists were asked to drink some water to cleanse the palate between tastes.

3.17 Statistical analysis

The statistical analysis software package SPSS (version 11.5) was used to carry out the statistical testes (Kinnear, 1999). The aim of the statistical treatment was to establish that a result is robust to repetition (or replication) of the study. For every experiment, the average and standard deviation (SD) were calculated as measure of central tendency and dispersion of the values acquired with the experiment. However, the screening and rejection of atypical values (outliers) was always done. For confirmatory statistical analysis was used the paired samples Student *t*-test, in order to ascertain the significance of a difference between two means. The model underlying a *t*-test assumes that the data have been derived from the normal distributions with equal variance, being considered a parametric test. The homogeneity of variance was assessed by the Levene's test for equality of variance. The output of the statistical test is the *P*- value. When the *P*- value was larger than the significance level, the assumption was accepted and *vice-versa*. The statistical calculations were based on a significance level equal or higher than 95%. The quantitative characters were statistically analyzed. The mean, standard deviation (SD) and standard error (SE) were calculated for each of the characters using the following formula.

$$\text{Mean, } \bar{x} = \Sigma x/N$$

Where x = measured characters

N = number of observations

$$\text{Standard error (SE)} = \text{SD}/\sqrt{N}$$

Where N = number of observations and SD = Standard deviation

Each bar in the graph represents mean \pm SD of triplicate data

RESULTS

4.1 Collection and microbial evaluation of vegetable samples

Increased numbers of foodborne outbreaks have been associated with fresh vegetables during the past decade due to increased consumption of potentially more risky fresh-cut vegetables and fruits. Fresh fruits and vegetables for human consumption are normally altered in form by: peeling, slicing, chopping, shredding, coring and trimming. These artificial alterations may introduce bacteria from external sources within the produce matrix and can enhance the growth of pathogenic bacteria due to the components leaching out from the vegetables, and infect people following consumption. Therefore, a minimum intervention process is required to eliminate /reduce bacterial pathogens to ensure adequacy in terms of consumer safety. In the present study, a total two hundred and thirty six selected fresh vegetable samples especially eaten as raw (spinach leaves, capsicum, coriander, cabbage, turnip, radish, betel leaves, mushroom, cucumber, tomato, carrot, moong bean sprouts and radish sprouts) were collected from different locations of Patiala city and subjected to microbiological analysis. All samples were collected were collected from three main sites namely from farm, local mandis and street vendors.

In order to understand the overall microbiological status, aerobic plate count (APC) was performed. Aerobic plate count is intended to indicate the level of microorganism in a product. Aerobic plate count was observed in the range from 10^2 - 10^8 CFU/g. In vegetable samples; analyzed for APC, highest counts was observed in those obtained directly from farm i.e. 7.8×10^8 CFU/g from carrot and 6.8×10^8 CFU/g from turnip (Table 4.1). Subsequent analysis for pathogens indicated the presence of *Salmonella* spp., *Shigella* spp., *Cronobacter* spp. from the samples collected from street vendors namely; *Daucus carota*, *Brassica rapa*, *Raphanus sativa*, *Phaseolus aureus*, *Agaricus bisporous* and *Piper betle*.

Table 4.1 A total of 236 vegetable samples covering different locations of Patiala city and aerobic plate count of different vegetable samples

| S. No. | Samples | Number of samples examined (N=236) | Botanical name | Total aerobic bacterial count (CFU/g) | |
|--------|--------------------|------------------------------------|--------------------------------|---------------------------------------|-------------------|
| | | | | Range | Mean |
| 1 | Spinach leaves | 18 | <i>Basella alba</i> | $1.5 \times 10^6 - 5.8 \times 10^8$ | 3.5×10^8 |
| 2 | Capsicum | 18 | <i>Capsicum annuum</i> | $2.7 \times 10^3 - 2.3 \times 10^7$ | 1.5×10^7 |
| 3 | Coriander | 15 | <i>Coriandrum sativum</i> | $2.5 \times 10^7 - 2.8 \times 10^9$ | 1.8×10^9 |
| 4 | Turnip | 18 | <i>Brassica rapa</i> | $2.3 \times 10^6 - 3.4 \times 10^7$ | 2.9×10^7 |
| 5 | Radish | 20 | <i>Raphanus sativus</i> | $4.4 \times 10^5 - 5.2 \times 10^7$ | 3.5×10^7 |
| 6 | Betel leaves | 19 | <i>Piper betle</i> | $2.1 \times 10^3 - 2.6 \times 10^6$ | 2.5×10^6 |
| 7 | Mushroom | 18 | <i>Agaricus bisporous</i> | $2.9 \times 10^5 - 3.2 \times 10^8$ | 3.1×10^8 |
| 8 | Cucumber | 18 | <i>Cucumis sativus</i> | $3.5 \times 10^2 - 4.8 \times 10^4$ | 4.5×10^4 |
| 9 | Cabbage | 18 | <i>Brassica oleracea</i> | $2.5 \times 10^4 - 2.8 \times 10^5$ | 2.5×10^5 |
| 10 | Tomato | 17 | <i>Lycopersicon esculentum</i> | $1.5 \times 10^4 - 1.8 \times 10^6$ | 1.9×10^6 |
| 11 | Carrot | 18 | <i>Daucus carota</i> | $4.5 \times 10^7 - 7.8 \times 10^8$ | 6.5×10^8 |
| 12 | Moong bean sprouts | 20 | <i>Phaseolus aureus</i> | $3.5 \times 10^6 - 6.8 \times 10^8$ | 5.8×10^8 |
| 13 | Radish sprouts | 19 | <i>Raphanus Sativa</i> | $4.5 \times 10^6 - 6.3 \times 10^8$ | 6.1×10^8 |

4.2 Isolation and characterization of pathogens from vegetable samples

Among all the two hundred and thirty six different vegetable samples analyzed for the presence of three main food borne pathogens of concern namely *Salmonella* spp., *Shigella* spp. and *Cronobacter* (*Enterobacter sakazakii*); six different types of vegetables were found to harbor these pathogens respectively from *Daucus carota* (carrot), *Brassica rapa* (turnip), *Raphanus sativa* (radish sprouts), *Phaseolus aureus* (moong bean sprouts), *Agaricus bisporous* (button mushroom) and *Piper betle* (betel leaves).

4.2.1. *Daucus carota* (carrot) and *Brassica rapa* (turnip)

Preliminary isolation of *Salmonella* spp. was done from *Daucus carota* (carrot) and *Brassica rapa* (turnip). Pink colonies with black center were observed in XLD medium. The pathogen was biochemically characterized and designated as ST1 and ST2 respectively. Morphological and biochemical properties of the isolated colonies of ST1 and ST2 were analyzed; bacterial colonies were pink colored, circular and smooth. The isolate was facultative anaerobe, Gram negative, rod shaped, motile, catalase positive, oxidase negative and non endospore forming, its other physiological and biochemical properties are summarized in Table 4.2.

4.2.2 *Raphanus sativa* (radish sprouts) and *Phaseolus aureus* (moong bean sprouts)

Preliminary isolation of *Shigella* spp. was carried out from *Raphanus sativa* (radish sprouts) and *Phaseolus aureus* (moong bean sprouts). Entirely green colonies were observed in HEA medium. The pathogen was biochemically characterized and designated as SF1 and SF2. Morphological and biochemical properties of the isolated colonies of both SF1 and SF2 were characterized. The bacterial colonies were green colored, circular, smooth. The isolate was facultative anaerobe, Gram negative, rod shaped, non motile, catalase positive and non endospore forming; other physiological and biochemical properties are summarized in Table 4.2.

4.2.3 *Agaricus bisporus* (mushroom) and *Piper betle* (betel leaves)

Preliminary isolation of all the pathogens was done from *Agaricus bisporus* and *Piper betle* (betel leaves). Both *Salmonella* spp., *Shigella* spp. were absent in the sample, but, *Cronobacter* spp. was presumptively isolated in DFI medium forming blue green colonies and designated as CS1 and CS2. Morphological and biochemical properties of the isolated colonies of CS1 and CS2 were characterized and identified. A yellow colored non diffusible pigment was produced by both CS1 and CS2, upon grown in TSA plates, which is typical characteristic of *Cronobacter* spp. The bacterial colonies were blue colored, mucoid. The isolates were facultative anaerobe, Gram negative, rod shaped, non motile, catalase positive, oxidase negative and non endospore forming. The other physiological and biochemical properties are summarized in Table 4.2.

Table 4.2 Characterization of isolated foodborne pathogens

Table 4.2a Morphological tests

| Tests | ST1 | ST2 | CS1 | CS2 | SF1 | SF2 |
|-------------------|--------------------|--------------------|------------|------------|---------------------|---------------------|
| Configuration | Circular | Circular | Circular | Circular | Circular | Circular |
| Size | 1-2 mm | 1-2 mm | 1-2 mm | 1-2 mm | 1-2 mm | 1-2 mm |
| Margin | Entire | Entire | Entire | Entire | Entire | Entire |
| Elevation | Convex | Convex | Convex | Convex | Convex | Convex |
| Surface | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth |
| Pigment | Pink, black centre | Pink, black centre | Blue green | Blue green | Green, black centre | Green, black centre |
| Gram's reaction | -ve | -ve | -ve | -ve | -ve | -ve |
| Cell shape | Rod | Rod | Rod | Rod | Rod | Rod |
| Size | 0.7-1.5 µm | 0.7-1.5 µm | 0.6-1.2 µm | 0.6-1.2 µm | 0.4-1.6 µm | 0.4-1.6 µm |
| Spore(s) | -ve | -ve | -ve | -ve | -ve | -ve |
| Endospore | - | - | - | - | - | - |
| Motility | + | + | - | - | - | - |
| Fluorescence (UV) | - | - | - | - | - | - |

Table 4.2b Biochemical tests

| Tests | ST1 | ST2 | CS1 | CS2 | SF1 | SF2 |
|-----------------------------|-----|-----|-----|-----|-----|-----|
| Indole test | - | - | - | - | + | + |
| Methyl red test | + | + | - | - | + | + |
| Voges Proskauer test | - | - | + | + | - | - |
| Citrate utilization | + | + | + | + | - | - |
| Esculin hydrolysis | - | - | + | + | - | - |
| ONPG | - | - | + | + | - | - |
| Nitrate reduction | + | + | + | + | + | + |
| Urea hydrolysis | - | - | - | - | - | - |
| Lysine decarboxylation | + | + | - | - | - | - |
| H ₂ S production | + | + | - | - | - | - |
| Catalase test | + | + | + | + | - | - |
| Oxidase test | - | - | - | - | - | - |
| Ornithine decarboxylase | + | + | + | + | - | - |

Table 4.2c Utilization of sugar

| Tests | ST1 | ST2 | CS1 | CS2 | SF1 | SF2 |
|------------|-----|-----|-----|-----|-----|-----|
| Adonitol | - | - | - | - | - | - |
| Arabinose | + | + | + | + | - | - |
| Cellobiose | - | - | + | + | - | - |
| Glucose | + | + | + | + | + | + |
| Lactose | - | - | + | + | - | - |
| Mellibiose | + | + | + | + | - | - |
| Saccharose | - | - | + | + | + | + |
| Raffinose | - | - | + | + | - | - |
| Rhamnose | + | + | + | + | - | - |
| Trehalose | - | - | + | + | - | - |
| Xylose | + | + | + | + | - | - |

4.3 Molecular characterization and identification of the isolated bacterial pathogens

Sequences of all the respective cultures were deposited in the GenBank database with accession numbers (Table 4.3). The related sequences showing similarity in BLAST were retrieved from GenBank and aligned using the program CLUSTALW (Thompson et al., 1997). Phylogenetic dendogram was constructed by neighbor-joining method using MEGA 4 software (Tamura et al., 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses.

Table 4.3 Identity of pathogens isolated from respective vegetable samples

| Samples | Pathogen | Pathogen designation | Accession number |
|---------------------------|-------------------------------|-----------------------------|-------------------------|
| Carrot | <i>Salmonella typhimurium</i> | ST1 | JN254641 |
| Turnip | <i>Salmonella typhimurium</i> | ST2 | JN254642 |
| Moong bean sprouts | <i>Shigella flexneri</i> | SF1 | JN254645 |
| Radish sprouts | <i>Shigella flexneri</i> | SF2 | JN254646 |
| Mushroom | <i>Cronobacter sakazakii</i> | CS1 | JN254643 |
| Betel leaves | <i>Cronobacter sakazakii</i> | CS2 | JN254644 |

4.3.1 *Salmonella* spp. ST1 and ST2

The aligned sequence data of ST1 (1538 bp) and ST2 (1532 bp) is shown in Table 4.4a, b. The phylogenetic tree of ST1 and ST2 revealed that both strains formed evolutionary lineage within the radiation cluster comprising the *Salmonella* spp. and the strain type ST1 and ST2 phylogenetically was most closely related to the type strain *Salmonella typhimurium* (98%) (Fig. 4.1). Thus, based on its morphological, physiological, biochemical properties and 16S rDNA sequence results, the isolated pathogens ST1 and ST2 were assigned as *Salmonella typhimurium*.

4.3.2 *Shigella* spp. SF1 and SF2

Table 4.5a, b shows the aligned sequence data of SF1 (1523 bp) and SF2 (1492 bp). The phylogenetic tree of SF1 and SF2 revealed that both strains formed evolutionary lineage within the radiation cluster comprising the *Shigella* spp. and the strain type SF1 and SF2 phylogenetically was most closely related to the type strain *Shigella flexneri* (98%) (Fig. 4.2). Thus, based on its morphological, physiological, biochemical properties and 16S rDNA sequence results, the isolated pathogens SF1 and SF2 were assigned as *Shigella flexneri*.

4.3.3 *Cronobacter* spp. CS1 and CS2

The aligned sequence data of CS1 (1402 bp) and CS2 (1470 bp) is shown in Table 4.6a, b. The phylogenetic tree of CS1 and CS2 revealed that both strains formed evolutionary lineage within the radiation cluster comprising the *Cronobacter* spp. and the strain type CS1 and CS2 phylogenetically was most closely related to the type strain *Cronobacter sakazakii* (98%) (Fig. 4.3). Morphological, physiological, biochemical properties and 16S rDNA sequence results suggested, the isolated pathogens CS1 and CS2 were assigned as *Cronobacter sakazakii*.

Table 4.4a Aligned Sequence Data of *Salmonella typhimurium* ST1 (1538 bp)

| | | | | | | | |
|--------|------|-------------|------------|------------|-------------|-------------|------------|
| ORIGIN | 1 | TAATTGAAGA | GTTTGATCAT | GGCTCAGATT | GAACGCTGGC | GGCAGGCCTA | ACACATGCAA |
| | 61 | GTCGAACGGT | AAGAGGAAGC | AGCTTGCTCT | TCGCTGACGA | GTGCCGGACG | GTTGAGTAAT |
| | 121 | GTCTGGGAAA | CTGCCTGATG | GAGGGGGATA | ACTACTGGAA | ACGGTAGCTA | ATACCGCATA |
| | 181 | ACGTCGCAAG | ACCAAAGAGG | GGGACCTTCG | GGCCTCTTGC | CATCGCATGT | GCCCAGATCG |
| | 241 | GATTAGCTAG | TTGGTGGGGT | AACGGCTCAC | CAAGGCGACG | ATCCCTAGCT | GGTCTGAGAG |
| | 301 | GATGACCAGC | CACACTGGAA | CTGAGACACG | GTCAGACTC | CTACGGGAGG | CACCAGTGGG |
| | 361 | GAATATTGCA | CAATGGGCGC | AAGCGTGATG | CAGCCATGCC | GCGTGTATGA | AGAAGGCCTT |
| | 421 | CGTGTGTAA | AGTACTTTCA | GCGGGGAGGA | AGGTGTTGTG | GTTAATACCT | CAGCACATTG |
| | 481 | ACGTTACCCG | CAGAAGAAGC | ACCGGCTAAC | TCCGTGCCAG | CAGCCGCGGT | AATACGGAGG |
| | 541 | GTGCAAGCGT | TAATCGGAAT | TACTGGGCGT | AAAGCGCACG | CAGGCGGTCT | GTCAAGTCAG |
| | 601 | GTGTGAAATC | CCCAGGCTCA | ACCTGGGAAC | TGCATCTGAA | ACTGGCAGGC | TTGAGTCTTG |
| | 661 | TAGAGGGGGG | TAGAATTCCA | GGTGTAGCGG | TGAAATGCGT | AGAGATCTGG | AGGAATACCG |
| | 721 | GTGGCGAAGG | CGGCCCCCTG | GACGAAGAGT | GACGCTCAGG | TGCGAAAGCG | TGGGGAGCAA |
| | 781 | ACAGGATTAG | ATACCCTGGT | AGTCCACGCC | GTAAACGATG | TCTACTTGGA | GGTTGTGCCC |
| | 841 | TTGAGGCGTG | GCTTCCGGAG | CTAACGCGAT | AAGTAGACCG | CCTGGGGAGT | ACGGCCGCAA |
| | 901 | GGTAAAACCT | CAAATGAATT | GACGGGGGCC | CGCACAAAGC | GTGGAGCTTG | TGGTTTAATT |
| | 961 | CGATGCAACG | CGAAGAACCT | TACCTGGTCT | TGCATCCAC | GGAAGTTTTT | AGAGATGAGA |
| | 1021 | ATGTGCCTTC | GGGAACCGTG | AGACAGGTGC | TGCATGGCTG | TCGTCAGCTC | GTGTTGTGAA |
| | 1081 | ATGTTGGGTT | AAGTCCCGCA | ACGAGCGCAA | CCCTTATCCT | TTGTTGCCAG | CGTCCGGCC |
| | 1141 | GGGAAC TCAA | AGGAGACTGC | CAGTGATAAA | CTGGAGGAAG | GTGGGGATGA | CGTCAAGTCA |
| | 1201 | TCATGGCCCT | TACGACCAGG | GCTACACACG | TGCTACAATG | GCGCATACAA | AGAGAAGCGA |
| | 1261 | CCTCGCGAGA | GCAAGCGGAC | CTCATAATGT | GAGTCGTAGT | CCGGATTGGA | GTCTGCAACT |
| | 1321 | CGACTCCATG | AAGTCGGAAT | CGCTAGTAAT | CGTGGATCAG | AATGCCACGG | TGAATACGTT |
| | 1381 | CCCGGGCCTT | GTACACACCG | CCCGTCACAC | CATGGGAGTG | GGTTGCAAAA | GAAGAAGGTA |
| | 1441 | GCTAAACCTT | CGGGAGGGCG | CTTACCACCT | TGTGATTTCAT | GA CTGGTGAA | GTCGTAACAA |
| | 1501 | GGTAACCGTA | GGGAACCTG | CGGTTGGATC | ACCTCCTT | | |

Table 4.4b Aligned Sequence Data of *Salmonella typhimurium* ST2 (1532 bp)

| | | | | | | | |
|--------|------|-------------|------------|-------------|------------|------------|-------------|
| ORIGIN | 1 | AGAGTTTGAA | CCTGGCTCAG | ATTGAACGCT | GGCGGCAGGC | CTAACACATG | CAAGTCGAAC |
| | 61 | GGTAACAGGA | AGCAGCTTGG | TGCTTTGCTG | ACGAGTGGCG | GACGGGTGAG | TAATGTCTGG |
| | 121 | GAAACTGCCT | GATGGAGGGG | GATAACTACA | GGAAACGGTG | GCTAATACCG | CATAACGTCG |
| | 181 | CAAGACAAA | GAGGGGGACC | TTCGCGCCTC | TTGCCATCAC | ATGTGCCAG | ATGGGATTAG |
| | 241 | CTTGTTGGTG | AGGTAACGGC | TCACCAAGGC | GACGATCCCT | AGCTGGTCTC | AGAGGATGAC |
| | 301 | CAGCCACACT | GGAAGTGAAG | CACGGTCCAG | ACTCCTACGG | GAGGCAGCAG | TGGGGAATAA |
| | 361 | TGCACAATGG | GCGCAAGCCT | GATGCAGCCA | TGCCGCGTGT | ATGAAGAAGG | CCTTCGGGTT |
| | 421 | GTAAGTACA | TTCAGCGGGG | AGGAAGGTGT | TGTGGTTAAT | AACCGCAGCA | ATTGACGTTA |
| | 481 | CCCGCAGAAG | AAGCACCGGG | TAACCTCCGTG | CCAGCAGCCG | CGGTAATACG | GAGGGTGCAA |
| | 541 | GCGTTAATCG | GAATTACTGG | GCGTAAAGCC | CACGCAGGCG | CTCTGTCAAG | TCCGATGTGA |
| | 601 | AATCCCCGGG | CTCAACCTGG | GAACCTGCAT | CGAACTGGG | AGGCTTGAGT | CTTGTAGAGC |
| | 661 | CGGGTAGAAT | TCCAGGTGTA | GCGGTGAAAT | GCGTAGAGAT | CTGGAGGAAA | ACCGGTGGCG |
| | 721 | AAGGCGGCC | CCTGGACAAA | GACTGACGCT | CAGGTGCGAA | AGCGTGGGGA | GCAAACAGGT |
| | 781 | TTAGATACCC | TGGTAGTCCA | CGCCGTAAAC | GATGTCTACT | TGGAGGTTGT | GCCCTTGAGG |
| | 841 | CGTGGCTTCG | GGAGCTAACG | CGTTAAGTAG | ACCGCCTGGG | GAGTACGGCC | GCAAGGTTAA |
| | 901 | AACTCAAATG | AATTGACGGC | GGCCCGCACA | AGCGGTGGAG | CATGTGGTTT | AATTCGATGC |
| | 961 | AACGCGAAGA | ACCTTACCTG | GTCTTGACAA | CCACAGAACT | TTCCAGAGAT | GGATTGGTTC |
| | 1021 | CTTCGGGAAC | TGTGAGACAG | GTGCTGCATG | GCTGTGCTCT | GCTCGTGTTC | TGAAATGTCG |
| | 1081 | GGTTAAGTCC | CGCAACGAGC | GCAACCCTTA | TCCTTTGTTG | CCAGCGGTTT | GGCCGGGAAC |
| | 1141 | TCAAAGGAGA | CTGCCAGTGA | TAAACTGGAG | GAAGGTGGGG | AAGACGTCAA | GTCATCATGC |
| | 1201 | CCCTTACGAC | CAGGGCTACA | CACGTGCTAC | AATGGCGCAT | ACAAAGAGAA | GCGACCTCGC |
| | 1261 | GAGAGCAAGC | GGACCTCATA | AAGTGCGTGC | TAGTCCGGAT | TGGAGTCTGC | AACCTCGACTC |
| | 1321 | CATGAAGTCG | GAATCGCTAG | TAATCGTGGA | TCAGAATGCC | ACGGTGAATA | CGTTCCCGGG |
| | 1381 | CCTTGATACAC | ACCGCCCCTG | ACACCATGGG | AGTGGGTTGC | AAAAGAAGTA | GGTAGCTTAA |
| | 1441 | CCTTCGGGAG | GGCGCTTACC | ACTTTGTGAT | TCATGACTGG | GGTGAAGTCG | TAACAAGGTA |
| | 1501 | ACCGTAGGGG | AACCTGCGGC | TGGATCACCT | CC | | |

Table 4.5a Aligned Sequence Data of *Shigella flexneri* SF1 (1523 bp)

| | | | | | | | |
|--------|------|-------------|------------|-------------|------------|------------|-------------|
| ORIGIN | 1 | ACTCCAGAGT | TTGATCATGG | CTCAGATTGA | TCGCTGGCGG | CAGGCCTAAC | ACATGCAAGT |
| | 61 | CGAACGGTAA | CAGGAATCAG | CTTGCTGTTT | TGCTGACGAG | TGGCGGACGG | GTGAGTAATG |
| | 121 | TCTGGGAAAC | TGCCTGATGG | AGCGGGATAA | CTACTGGAAA | CGGTAGCTAA | TACCGCATAA |
| | 181 | CGTCGCAAGA | CCAAAGAGGG | GGACCTTCGG | GCCTCTTGCC | ATCGGATGTG | CCCAGATGGG |
| | 241 | AATAGCTAGT | AGGTGGGGTA | ACGGCTCACC | TAGGCGACGA | TCGCTAGCTG | GTCTGAGAGG |
| | 301 | ATGACCAGCC | ACACTGGAAC | TGAGACACGG | TCCAGACTCC | TACGGGAGGC | AGCAGTGGGG |
| | 361 | AATATTGCAC | AATGGGCGCA | AGCCTGATGC | AGCCATGCCG | CGTGTATGAA | GAAGGCC TTC |
| | 421 | GGGATGTAAA | GTACTTTCAG | CGGGGAGGAA | CCGAGTAAAG | TTAATACCTT | TGCTCATTTGA |
| | 481 | CGTTACCCGC | AGAAGAAGCA | CCGGCTAACT | CCGTGCCAGC | AGCCGCGGTA | ATACGGAGGG |
| | 541 | TGCAAGCGTT | AATCGGAATT | ACTGCGCGTA | AAGCGCACGC | AGGCGGTTTG | TTAAGTCAGA |
| | 601 | TGTGAAATCC | CCGGGCTCAA | CCTGGGAACT | GCATCTGATA | CTGGCAAGCT | TGAGTCTCGT |
| | 661 | AGAGGGGGGT | AGAATTCCAG | GTGTAGCGGT | GAAATGCGTA | GAGATCTGGA | GGAATACCGG |
| | 721 | TGGCGAAGGC | GGCCCCCTGG | ACGAAGACTG | ACGCTCAGGT | GCGAAAGCGT | GGGGAGCAAA |
| | 781 | CAGGATTAGA | TACCCTGGTA | GTCCACGCCG | TAAACGATGT | CGACTTGGAG | GTTGTGCCCT |
| | 841 | TAGGCCGTGG | CTTCCGGAGC | TAACGCGTTA | AGTCGACCGC | CTGGGAGTA | CGGCCGCAAG |
| | 901 | GTTAAAACCTC | AAATGTATTG | ACGGGGCCCC | GCACAAGCGG | TGGAGCATGT | GGTTTAATTC |
| | 961 | GATGCAACGC | GAAGAACCAA | ACCTGGTCTT | GACATCCACG | GAAGTTTCCA | GAGATGAAAT |
| | 1021 | GGTGCCCTTCG | GGAACCGTGA | GACAGGTGCT | GCATGGCTGT | CGTCAGCTCG | TGTTGTGAAA |
| | 1081 | TGTTGGGTTA | AGTCCCGCAA | CGAGCGCAAC | GGTTATCCTT | TGTTGCCAGC | GGTCCGGCCG |
| | 1141 | GGAAC TAAA | GGAGACTGCC | AGTGATAAAC | TGGAGGAAGG | TGGGGATGAC | GTCAAGTCAT |
| | 1201 | CATGGCCCTT | ACGACCAGGG | CTACACACGT | GCTACAATGG | CGCATACAAA | GAGAAGCGAC |
| | 1261 | CTCGCGTGAG | CAAGCGGACC | TCATAAAGTG | CGTCGTAGTC | CGGATTGGAG | TCTGCAACTC |
| | 1321 | GACTCCATGA | AGTCGGTATC | GCTAGTAATC | GTGGATCAGA | ATGCCACGGT | GAATACG TTC |
| | 1381 | CCGGGCCTTG | TACACACCGC | CCGTCACACC | ATGCGAGTGG | GTTGCAAAAG | AAGTAGGTAG |
| | 1441 | CTTAACCTTC | GGGAGCGCGC | TTACC ACTAT | GTGATTCATG | ACTGGGGTGA | AGTCGTAACA |
| | 1501 | AGGTAACCGT | AGGGGAACCT | GCG | | | |

Table 4.5b Aligned Sequence Data of *Shigella flexneri* SF2 (1492 bp)

| | | | | | | | |
|--------|------|--------------|------------|-------------|-------------|-------------|------------|
| ORIGIN | 1 | GGCTCAGATT | GAACGCTCGC | GGCAGGCC TA | ACACATGCAA | GTGCGAACGG | TAACAGGAAA |
| | 61 | CAGCTTGCTT | GTCTTCGCTG | ACGAGTGGCG | GACGGGTGAG | TAATGTCTGG | GAAACTGCCT |
| | 121 | GATGGAGGGG | GATAACTACT | GGAAACGGTA | GCTAATAGCG | CATAACGTCG | CAAGACCAAA |
| | 181 | GAGGGGGACC | TTCCGGCCTC | TTGCCATCGG | ATGTGCCCCAG | ATGGGATAAG | CTAGTAGGTG |
| | 241 | GGGTAACGGC | TCACCTAGGC | GACGATCCCT | AGCTGGTCTG | AGAGGATGAC | CAGCCACTCT |
| | 301 | GGAAC T GAGA | CACGGTCCAG | ACTCCTACGG | GAGGCAGCAG | TGGGGAAATAT | TGCACAATGG |
| | 361 | GCGCAAGGCT | GATGCAGCCA | TGCCCGGTGT | CACTGAAGAA | GGCCTTCGCG | TTGTAAAGTA |
| | 421 | CTTTCAGCCC | GGAGGAAGGG | AGTGAAAGTT | AATACCTTTG | CTCATTTGACG | TTACCCGCAG |
| | 481 | AAGAAGTCAC | CGGCTAACTC | CGTGCCAGCA | GCCGCGGTAA | TACGGAGGGT | GCAAGCGTTA |
| | 541 | ATCGGAATTA | CTGGGCGTAA | AGCGCACGCA | GCGGTTTGT | TAAGTCAGAT | GTGAAATCCC |
| | 601 | CGGGCTCAAC | CTGGGAACTG | CATCTGATAC | TGGCAAGCTA | GAGTCTCGTA | GAGGGGGTAA |
| | 661 | GAATTCCAGG | TGTAGCGGTG | AAATGCGTAG | AGATCTGGAG | GAATACCGGT | GGCGAAGGCG |
| | 721 | GCCCCCTGGA | CGAAGACTGA | CGCTCAGGTG | CGAAAGCGTG | GGCAGCAAAC | AGGATTAGAT |
| | 781 | ACCCTGGTAG | TCCACGCCGT | AAACGATGTC | GACTTGGAGG | TTGTGCCCTT | GAGGCGTGCC |
| | 841 | TTCCGGAGCT | AACGTCGTGT | AAGTCGACCG | CCTGGGGAGT | ACGGCCGCAA | GGTTAATACT |
| | 901 | CAAATGAAGT | GTGACGGGGG | CCCGCACAAAG | CGGTGGAGCA | TGTGGTTTAA | TTCGATGCAA |
| | 961 | GCGGAAGAAC | CTTACCTGGT | CTTGACATCC | ACGGAAGTTA | ATCAGAGATG | AGAATGTGCC |
| | 1021 | TTCCGGGAACC | GTGAGACAGG | TGCTGCATGG | CTGTCTGTCAG | CTCGTGTGT | GAAATGTTGG |
| | 1081 | GTTAAGTCCC | GCAACGAGCG | CAACCTTAT | CCTTTGTTGC | CAGCGTCCG | GCCGGGAACT |
| | 1141 | CATAGGAGAC | TGCCAGTGAT | AAACTGGAGG | AAGGTGGGGA | TGACGTCAAG | TCATCATGGC |
| | 1201 | CCTTACGACC | AGGGCTACAC | ACGTGCTACA | ATGGCGCATA | CAAAGAGAAG | CGACCTCGCG |
| | 1261 | AGAGCAAGCG | GACCTCATAA | AGTGGGTCTG | AGTCCGGATT | GGAGTCTGCA | ACTCGACTCC |
| | 1321 | ATGAAGTCGG | AATCGCTAGT | AATCGTGGAT | CAGAA TGCCA | CGGTGAATAC | GTTCCCGGGC |
| | 1381 | CTTGTTACAC | ACCGTCCCGT | CACACCATGG | GAGTGGGTTG | CAATTGAAGT | AGGTAGTCTT |
| | 1441 | AACCTTCGGG | ATCGGCGCCT | ACACTACTGC | TGATACGTGG | ACTGGCCGGT | CA |

Table 4.6a Aligned Sequence Data of *Cronobacter sakazakii* CS1 (1402 bp)

| | | | | | | | |
|--------|------|------------|-------------|------------|------------|-------------|-------------|
| ORIGIN | 1 | TGCAGTCGAA | GGTAACAGGG | AGCAGCTTGC | TGCTCTGCTG | ACGAGTGGCG | GACGGGTGAG |
| | 61 | TAATGTCTGG | GAAACTGCCT | GATGGAGGGG | GATAACTACT | GGTTACGGTA | GCTAATACCG |
| | 121 | CATAACGTCT | ACGGACCAAA | GTGGGGGACC | TTCGGGCCTC | ATGCCATCAG | ATGTGCCCAG |
| | 181 | ATGGGATTAG | CTAGTAGGTG | GGGTAACGGC | TCACCTAGGC | GACGATCCCT | AGCTGGTCTG |
| | 241 | AGAGGATGAC | CAGCCACACT | GGAAGTGAAG | CACGGTCCAG | ACTCCTACGG | GAGGCAGCAG |
| | 301 | TGGGGAATAT | TGCACTATGG | GCGCAAGCCT | GATGCAGCCA | TGCCGCGTGT | ATGAGAAGGC |
| | 361 | CTTCGGGTTG | TAAAGTACTT | TCAGCGGGGA | GGAGGGCGTT | GTGGTTAATA | ACCGCAGCGA |
| | 421 | TTGACGTTAC | CCGCAGAAAGT | AGCACCGGCT | AACTCCGTGC | CAGCAGCCGC | GGTAATACCG |
| | 481 | AGGGTGCAAG | CGTTAATCGG | AATTACTGGG | CGAAAAGCGC | ACGCAGGCGG | TCTGTTAAGT |
| | 541 | CAGATGTGAA | ATCCCCGGGC | TCAACCTGGG | AACTGCATAT | GAAACTGGCA | GGCTTGAGTC |
| | 601 | TCGTAGAGGG | GGGTAGAATT | CCAGGGTAGC | GGTGAAATGC | GTAGAGATCT | GGAGGAATAC |
| | 661 | CGGTGGCGAA | GGCGGCCGGC | TGGACGAAGA | CTGACGCTCA | GGTGCGAAAG | CGTGGGGAGC |
| | 721 | AAACAGGATT | AGATACCCTG | GTAGTCCACG | CCGTAAACGA | TGTCGACTTG | GAGGTTGTGC |
| | 781 | CCTTGAGGCG | TGGCCCGGAG | CTAACCGGCT | AAAGTCGACC | GCCTGGGGAG | TACGGCCGCA |
| | 841 | AGGTTAAAAC | TCAAATGAAT | TGACGGGGG | CCGCACAAGC | CGTGGAGCAT | GTTGGTTAAT |
| | 901 | TCGATGCAAC | CGGAAGAACC | TTACCTGGTC | TTGACATCCA | GAGAATCCTG | CAGAGATCGC |
| | 961 | GGAGTGCCCT | CGGGAACCTC | GAGACAGGTG | CTGCATGGCT | GTCGTCAGCT | CGTGTGTGTA |
| | 1021 | AATGTTGGGT | TAAGTCCCGC | AACGAGCGCA | ACCCTTATCC | TTTGTGTGCCA | GCGGTTCCGGC |
| | 1081 | CGGGAACTCA | AAGGAGACTG | CCGGTGATAA | ACCGGAGGAA | GGTTGGGGAT | GACGTCATGT |
| | 1141 | CATCATGGCC | CTTACGACCA | GGGCTACACA | CGTGCTACAA | TGGCGCATAAC | ATAGAGAAGC |
| | 1201 | GACCTCGCGA | GAGCAAGCGG | ACCTCATAAA | GTGCGTCGTA | GTCCGGATTG | GAGTCTGCAA |
| | 1261 | CTCGACTCCA | TGAAGTCGGA | ATCGCTAGTA | ATCGTGGATC | AGAATGCCAC | GGTGAATACG |
| | 1321 | TTCCCGGGCC | TTGTACACAC | CGCCCGTCAC | ACCATGGGAG | TGGGTGCAAA | AGAGTAGCTT |
| | 1381 | AACCTTCGGG | AGGGCGCTAC | AT | | | |

Table 4.6b Aligned Sequence Data of *Cronobacter sakazakii* CS2 (1470 bp)

| | | | | | | | |
|--------|------|------------|------------|-------------|------------|------------|-------------|
| ORIGIN | 1 | AGCAGCTTGC | TGCTCTGCTG | ACGAGTGGCG | GACGCGTGAG | TAATGTCTGG | GAAACTGCCT |
| | 61 | GATGGAGGGG | GATAACTACT | GGAAACGGTA | GCTAATACCG | CATAACGTCT | ACGGACCAAA |
| | 121 | GTGGGGGACC | TTCGGGCCTC | ATGCCATCAG | ATGTGCCCAG | ATGGGATTAG | CTAGAAGGTG |
| | 181 | GGGTAACGGC | TCACCTAGGC | GACGATCCCT | AGCTGGTCTG | AGAGGATGAC | CAGCCACACT |
| | 241 | GGAAGTGAAG | CACGGTCCAG | ACTCCTACGG | GAGGCAGCAG | TGGGGAATAT | TGCATAATCC |
| | 301 | GCGCAAGCCT | GATGCAGCCA | TGCCGCGTGT | ATGAAGAAGG | CCTTCGGGTT | GTAAAGTACT |
| | 361 | TTCAGCGGGG | AGGAAGGCGT | TGTGGTTAAT | AACCACAGCG | ATTGACGTTA | CCCGCAGAAG |
| | 421 | AAGCACCGGC | TAACTCCGTG | CCAGCAGCCG | CGGTAATACG | GAGGGTGCAA | GCGTTAATCG |
| | 481 | GAATTACTGG | GCGTAAAGCG | CACGCAGGGG | GTTGATTAAG | TCAGATGTGA | AATCCCCGGG |
| | 541 | CTCAACCTGG | GAACTGCATT | TGAAACTGGT | CAGCTTGAGT | CTCGTAGAGG | GGGTAGAAT |
| | 601 | TGGAGGTGTA | GCGGTGAAAT | GCGTAGAGAT | CTGGAGGAAT | ACCGGTGGCG | AAGGCAGCCC |
| | 661 | CCTGGACGAA | GACTGACGCT | CAGGTGCGAA | AGCGTGGGGA | GCAAACAGGA | TTAGATACCC |
| | 721 | TGGTAGTCCA | CGCCGTAAAC | GATGTCGACT | TGGAGGTTGT | GCCCTTGAGG | CGTGGCTTCC |
| | 781 | GGAGCTAACG | CGTTAAGTCG | ACCGCCTGGG | GAGTACGGCC | GCAAGGTTAA | AATCCTAAATG |
| | 841 | AATTGACGGG | GGCCCGCACA | AGCGGTGGAG | CATGTGGATT | AAATCGATGC | AACGCGAAGA |
| | 901 | ACCTTACCTG | GTCTTGACAT | CCAGAGAATC | CTGCAGAGAT | GCGGGAGTGC | CTTCGGGAAC |
| | 961 | TCTGAGACAG | GTGCTGCATG | GCTGTGCTCA | GCTCGTCTTG | TGAAATGTTG | GGTTAAGTCC |
| | 1021 | CGCAACGAGC | GCAACCCTTA | TCCTTTGTTG | CCAGCGGTTT | GGCCGGGAAC | TCAAAGGAGA |
| | 1081 | CTGCCGGTGA | TAAACCGGAG | GAAGGTGGGG | ATGACGTCAA | GTCATCATGG | CCCTTAGGAC |
| | 1141 | CAGGGCTACA | CACGTGCTAC | AATGGCGCAT | ACAAAGAGAA | GCGACCTCGC | GAGACCAAGC |
| | 1201 | GGACCTCATA | AAGTGCCTCG | TAGTCCGGAT | TGGAGTCTGC | AACTCGACTC | CATGAAGTCC |
| | 1261 | GAATGGCTAG | TAATCGTGGA | TCAGAAATGCC | ACGGTGAATA | CGTTCCCGGG | CCTTGTACAC |
| | 1321 | ACCGGCCGTC | ACACGATGGG | AGTGGGTTGC | AAAAGAAGTA | GGTAGCTTAA | CCTTCGGGGAG |
| | 1381 | GGCGCTTACC | ACTTAGTGAT | | | | |

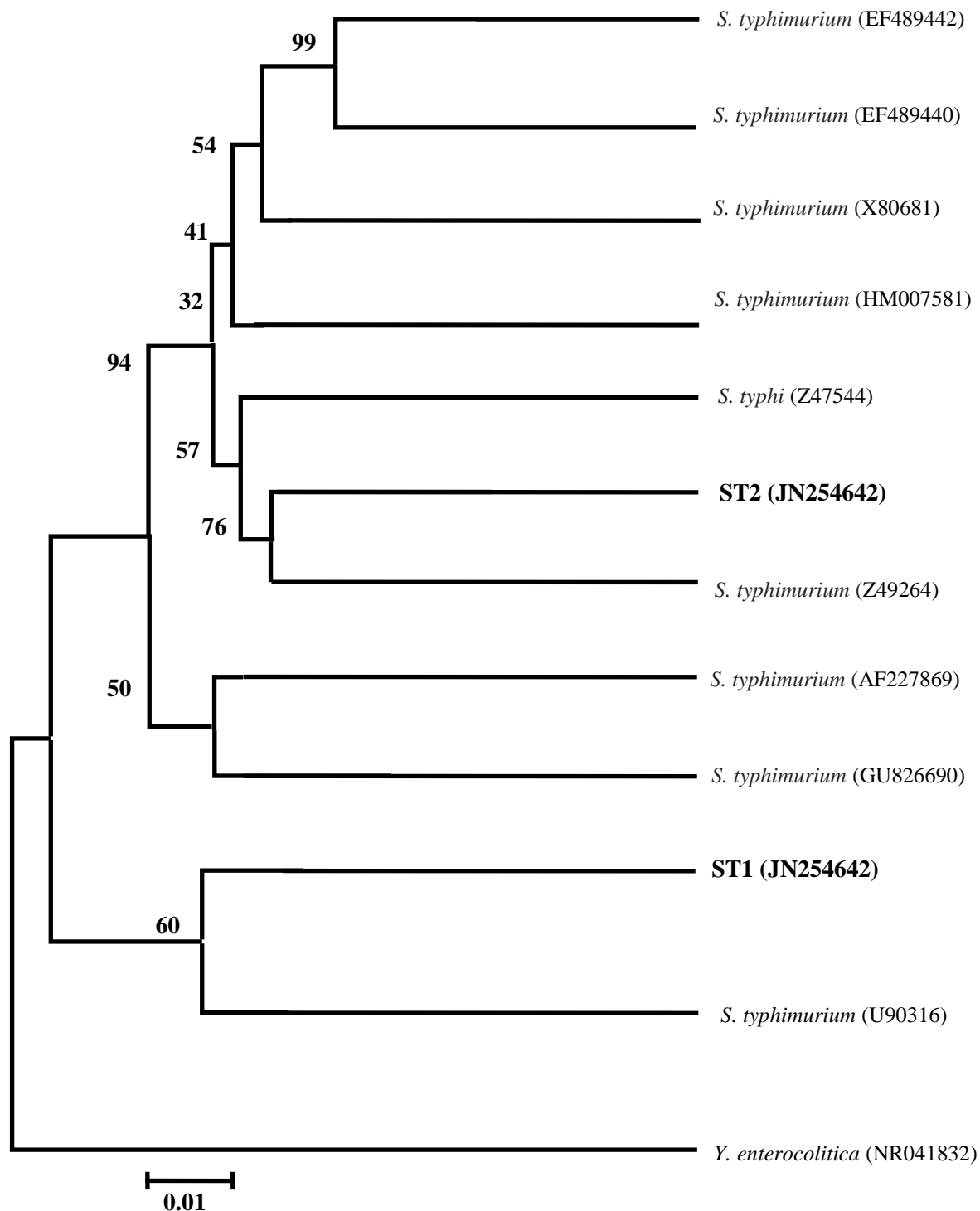


Fig. 4.1 Neighbour-joining tree based on bacterial 16S rDNA sequence of ST1 and ST2. A neighbor joining tree was constructed by aligning the sequences with other selected members from the prokaryotic domain. Listed beside each organism or strain name is the GenBank accession number; numbers at branch-points represents confidence values obtained after bootstrap analysis of the neighbor joining tree

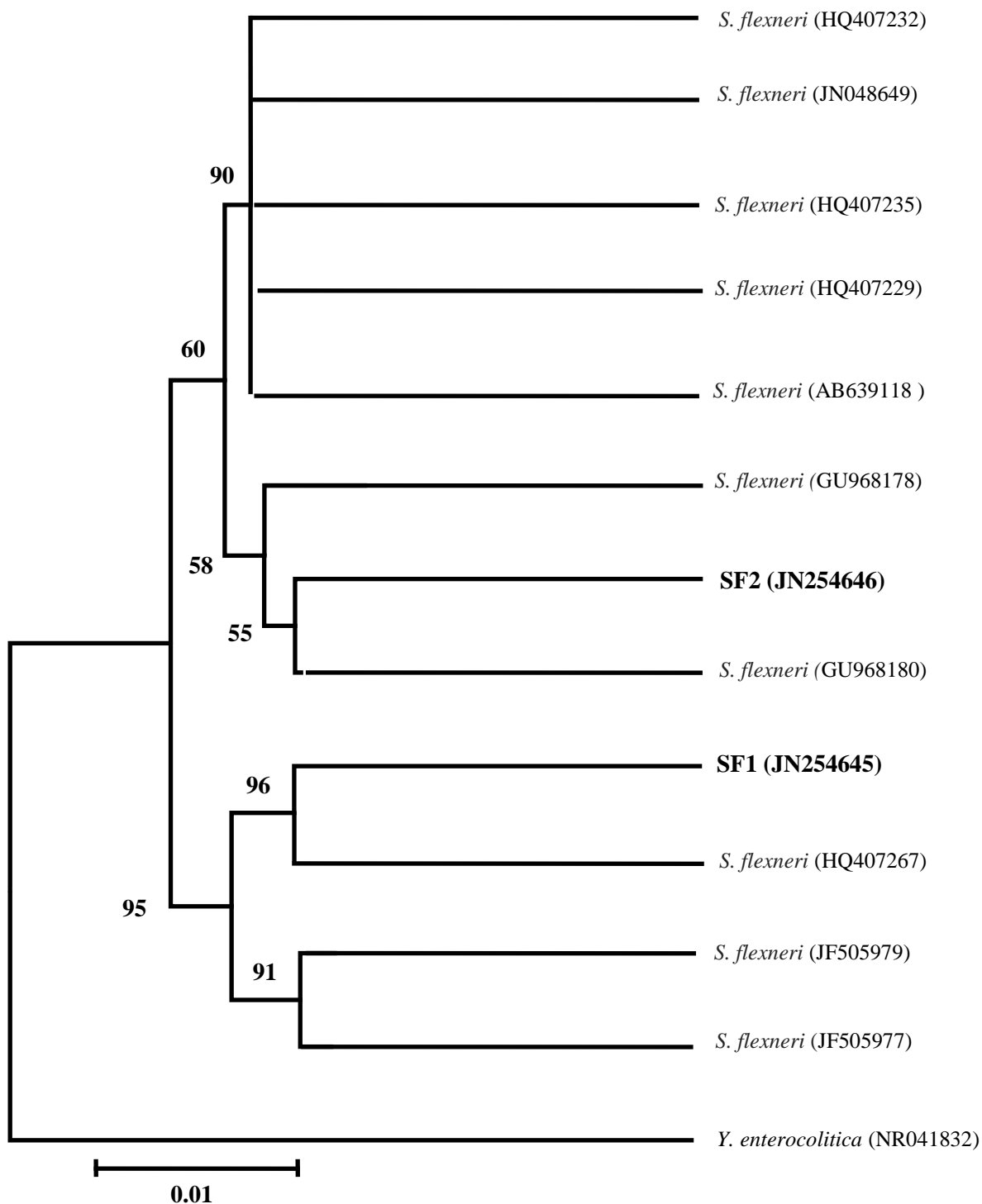


Fig. 4.2 Neighbour-joining tree based on bacterial 16S rDNA sequence of SF1 and SF2. A neighbor joining tree was constructed by aligning the sequences with other selected members from the prokaryotic domain. Listed beside each organism or strain name is the GenBank accession number; numbers at branch-points represents confidence values obtained after bootstrap analysis of the neighbor joining tree

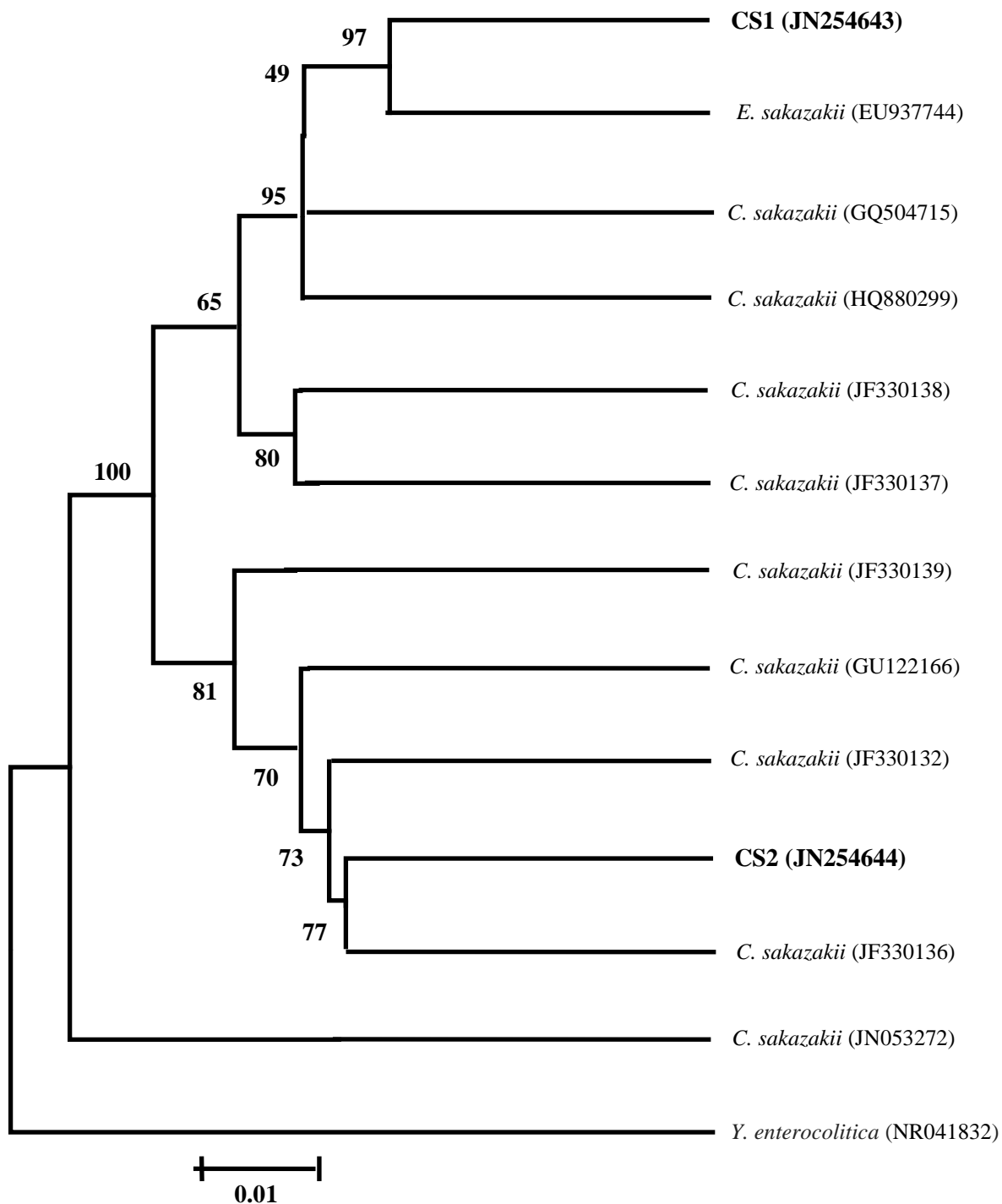
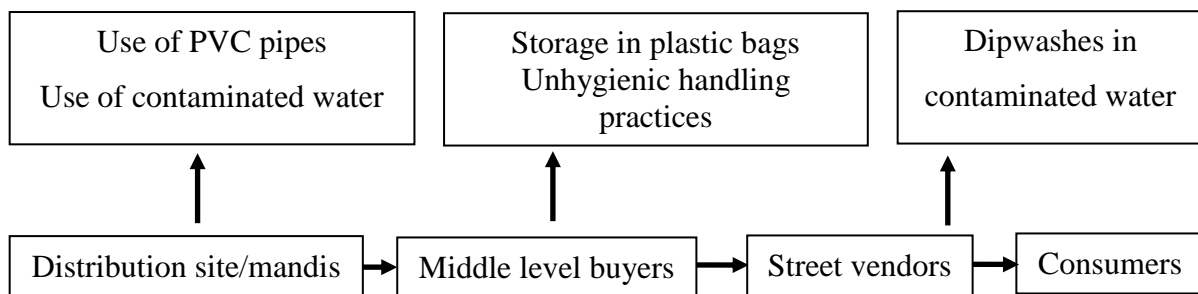


Fig. 4.3 Neighbour-joining tree based on bacterial 16S rDNA sequence of CS1 and CS2. A neighbor joining tree was constructed by aligning the sequences with other selected members from the prokaryotic domain. Listed beside each organism or strain name is the GenBank accession number; numbers at branch-points represents confidence values obtained after boots trap analysis of the neighbor joining tree

4.4 Study of farm to plate chain

It has been mentioned before that sample collection was carried out from three main sites i.e. directly from farm, local mandis and street vendors. During this study, it was observed that maximum aerobic plate count occurred in the samples collected directly from farm. The fresh vegetable samples are usually transported to a centralized trading location commonly known as mandi. There after produce samples are washed by the vendors and transported to local mandis/whole sale market/retailers (Chen et al., 2001). Street vendors are last in the chain of distribution of vegetables and fruits. The practices adopted at each level by the vendors are briefly indicated the schematic diagram shown below.



The pathogens *Salmonella typhimurium* (ST1 and ST2), *Shigella flexneri* (SF1 and SF2) and *Cronobacter sakazakii* (CS1 and CS2); were detected specifically in the vegetables; carrot, turnip, radish sprouts, moong bean sprouts, button mushroom and betel leaves; collected directly from street vendors. Therefore, it may be inferred that pathogens might have entered during their course packaging/transportation or storage. The farm to fork chain has been viewed as an extremely viable route for entry and subsequent proliferation of pathogens. In fact several studies have demonstrated the presence of pathogens in vegetable samples collected from street vendors as well as from retailers in the farm to fork chain in Indian cities. The presence of pathogens in samples analyzed in this study may be attributed to totally unhygienic handling by vendors. The observed resistance to organic acids in two isolates may be attributed to their ability to form biofilms. Biofilms can be an important

source of cross contamination during storage. It is evident that *Salmonella* lodge themselves on surface and can harbor to form biofilms during post harvest storage. A more important consequence of biofilm growth, however, with profound clinical implications is the markedly enhanced resistance to antimicrobial agents where biofilm-associated microorganisms are estimated to be 50 to 500 times more resistant to sanitizing chemicals (Stewart, 2002) which also enhances the chances of cross contamination.

4.5 Effect of ozonated water and organic acids onto the planktonic pathogens

Optimization of effective concentration of organic acids namely malic acid, acetic acid, lactic acid and citric acid onto isolated pathogens (ST1, ST2, CS1, CS2, SF1 and SF2) in TSB (Tryptone soya broth, 37°C, overnight) broth were carried out. Accuracy in kinetic data was ensured by performing the experiments in automatic growth analyzer (Bioscreen C, Helsinki, Finland) where incubation time, temperature shaking as well as absorbance was monitored automatically. Organic acids were studied individually initially in broth followed by trials using respective fresh vegetables (complete immersion, spraying) using different concentrations: 0% - 2.5% (Liao and Cooke, 2001; Liao and Sapers, 2000). Among all the organic acids and concentrations used, 1.2% of malic acid and 1% acetic acid, was found to be most effective to completely control the growth of planktonic cells (Table 4.7), But, both of *Salmonella* strains ST1 and ST2 were resistant to higher concentrations of organic acids; as 2% of malic acid and 2% of acetic acid were effective against the pathogens. 2% of citric acid and 2% of lactic acid was most suitable to inhibit the growth of other pathogens except ST1 and ST2 (Table 4.7). Therefore, in this study, malic acid and acetic acid were selected for further studies onto fresh vegetable samples artificially spiked with respective pathogens.

Ozone has been used as an antimicrobial agent against *Salmonella typhimurium*, *Staphylococcus aureus*, *Yersinia enterocolitica* and *Listeria monocytogenes* for treatment of water (Korich et al., 1990; Peeters et al., 1989; Restaino et al., 1995). Susceptibility of

bacteria to ozone varies among genera and species. Optimization of effective concentration of ozonated water onto reported pathogens (ST1, ST2, CS1, CS2, SF1 and SF2) in TSB (Tryptone soya broth, 37°C, overnight) was carried out. The effect of ozonated water was studied individually firstly in broth; this was followed by trials on vegetable samples (complete immersion, spraying) using different concentrations: 0 ppm - 2.5 ppm) (Liao and Cooke, 2001; Liao and Sapers, 2000). Among all the concentrations used, 1 ppm of ozonated water was found to be most effective for the complete inhibition of growth (Table 4.7) against all the pathogens.

4.6 Effect of ozonated water onto the pathogens in artificially inoculated vegetables

Recently, ozonated water has been applied to fresh-cut vegetables for sanitation purposes reducing microbial populations and extending the shelf-life of some of these products (Beltran et al., 2005). Only 1 log reduction in population of *Salmonella enteritidis* was reported when the pathogen was inoculated on poultry skin and treated with gaseous ozone at 8% (w/w) for 15 s (Ramirez et al., 1994). Therefore, use of aqueous ozone is preferred to enhance the effect onto pathogen.

4.6.1 *Salmonella typhimurium* (ST1) in carrot and (ST2) in turnip

The treated and artificially inoculated carrot and turnip samples with ST1 and ST2 was subsequently stored for 10 days at 28°C in order to study the effects of sanitizing treatments on microbial counts and product quality. The efficacy of 2 ppm ozone on carrot stored at 28°C for 10 days is shown in Fig. 4.4. Complete immersion in 2 ppm ozonated water inhibited pathogen growth by 1 log and 0.6 log after spraying (1:2, w/v) in carrot. Fig. 4.5 shows the efficacy of 2 ppm ozone on turnip stored at 28°C for 10 days. On the other hand, complete immersion in 2 ppm ozonated water inhibited pathogen growth by 1.2 log and 0.8 log after spraying in turnip without any residual antimicrobial affect.

Table 4.7 Effective concentrations of organic acids and ozonated water against respective planktonic pathogens

| Strain | Malic acid* | Acetic acid* | Citric acid* | Lactic acid* | Ozonated water** |
|--------|-------------|--------------|--------------|--------------|------------------|
| ST1 | 2.1±0.03 | 2.0±0.05 | 1.8±0.04 | 2.2±0.01 | 1.0±0.07 |
| ST2 | 2.0±0.02 | 2.0±0.05 | 1.8±0.09 | 2.0±0.04 | 1.1±0.50 |
| SF1 | 1.0±0.04 | 1.0±0.08 | 2.0±0.03 | 2.4±0.05 | 1.2±0.05 |
| SF2 | 1.2±0.01 | 1.0±0.03 | 1.8±0.05 | 2.4±0.09 | 1.1±0.06 |
| CS1 | 1.0±0.02 | 1.0±0.04 | 1.6±0.04 | 2.0±0.03 | 1.0±0.04 |
| CS2 | 1.2±0.05 | 0.8±0.03 | 1.8±0.03 | 2.2±0.01 | 1.1±0.07 |

Note: Average of three mean values; values with ± represent standard deviation (SD)

*% ± SD,

** ppm ± SD

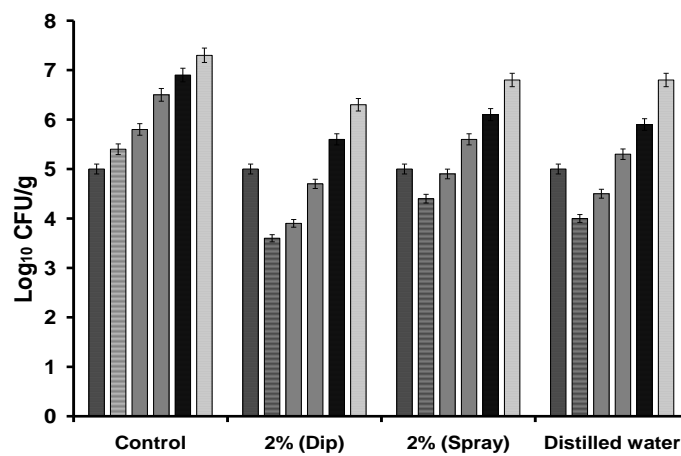


Fig. 4.4 Survival kinetics and lethal action of 2 ppm ozone on ST1 inoculated in carrot, incubated at 28°C for 10 days

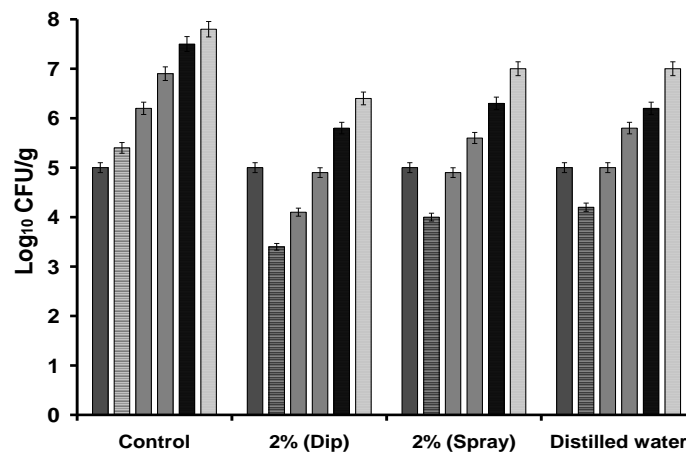


Fig. 4.5 Survival kinetics and lethal action of 2 ppm ozone on ST2 inoculated in turnip, incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.6.2 *Shigella flexneri* (SF1) in radish sprouts and (SF2) in moong bean sprouts

The efficacy of 2 ppm ozone on radish sprouts stores at 28°C was evaluated. Complete immersion (1:2, w/v) in 2 ppm ozonated water inhibited pathogen growth by 1.5 log and 1 log after spraying in radish sprouts (Fig. 4.6). Growth was observed during storage following sanitizer treatment. Similarly, absence of antimicrobial effect during storage was reported in a previous section. Variable bacterial re-growth was observed on treated and pathogen contaminated vegetables depending on the sanitizing treatment using selective media (XLD). Complete immersion of moong bean sprouts reduced *Shigella* counts significantly ($p < 0.05$) by 1.8 log₁₀CFU/g, whereas spraying reduced the counts by 0.9 log₁₀CFU/g in moong bean sprouts (Fig. 4.7). Some growth was observed during storage following sanitizer treatment.

Similar to these results, Li et al. (2001) reported that chlorine treatment did not affect growth of *E. coli* O157:H7 on lettuce during 18 day storage at 15°C. Currently, use of chlorine as sanitizer is prohibited since chlorine reacts with organic matter, components leaching from tissues of cut vegetable surfaces may neutralize some of the chlorine before it reaches microbial cells, thereby reducing its effectiveness. The limited efficacy of chlorine-based agents and production of chlorinated organic compounds with potential carcinogenic action have prompted the investigation for some novel, effective decontamination techniques. Ozone possesses a clean history of applications. ozone has been used synergistically with other antimicrobial agents (Colberg et al., 1978). Foegeding (1985) reported that acidic ozonated water was able to inactivate *Bacillus* and *Clostridium* spores.

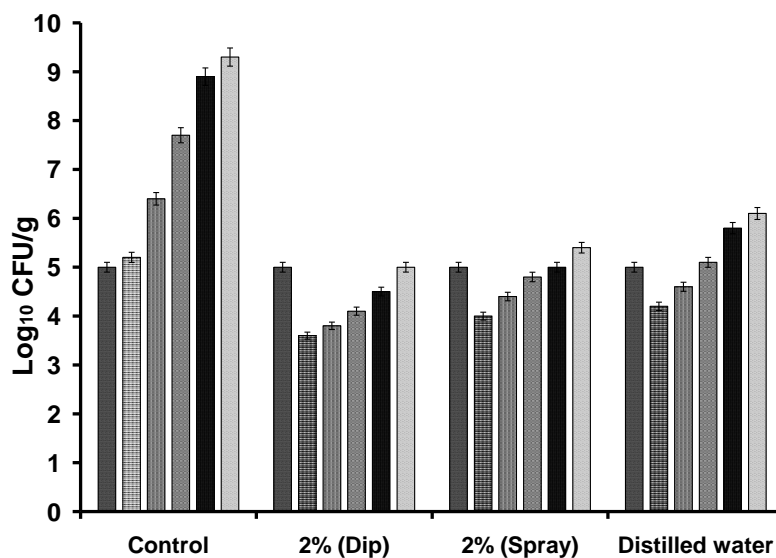


Fig 4.6 Survival kinetics and lethal action of 2 ppm ozone on SF1 inoculated in radish sprouts, incubated at 28°C for 10 days

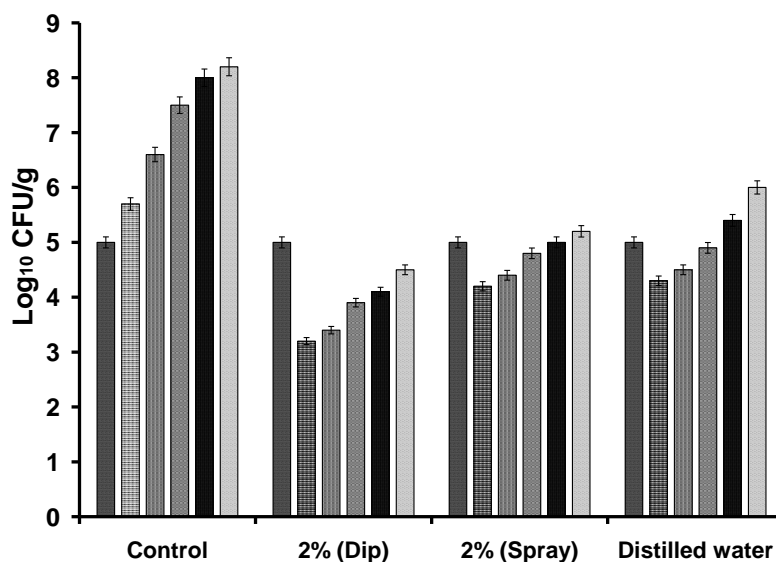


Fig. 4.7 Survival kinetics and lethal action of 2 ppm ozone on SF2 inoculated in moong bean sprouts, incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 6 d; 10 d

4.6.3 *Cronobacter sakazakii* (CS1) in mushroom and (CS2) in betel leaves

Efficacy of 2 ppm ozone on mushroom stored at 15°C for 5 days is shown in Fig. 4.8. Complete immersion in 2 ppm ozonated water inhibited pathogen growth by 1.4 log and 0.9 log after spraying (1:2, w/v) in mushroom. In contaminated mushroom which was untreated bacterial counts grew up to 8 log at 15°C was observed after 5 day-storage depending on the sanitizing treatment. Fig. 4.9 depicts the efficacy of 2 ppm ozone on betel leaves stored at 28°C for 10 days. Complete immersion in 2 ppm ozonated water inhibited pathogen growth by 1.3 log and 1 log after spraying (1:2, w/v) in betel leaves. Reactivity, solubility and disinfection efficacy of ozone are affected by several factors such as temperature, pH, humidity. In addition, microbiocidal effect of ozone is highly dependent on its accessibility to target microorganisms without interacting with the food components.

The inability of sanitizers to totally remove or eliminate the pathogens from vegetable surfaces as evident by the subsequent microbial growth after storage, in the present work, suggests the possibility of persisting pathogens. The persisting pathogens might have survived due to the protection afforded by the produce matrix. Alternatively, a possible “stress” tolerance mechanism might have been responsible for the observed survival and growth of the pathogen. Acid-sensitive bacteria could adapt to acid stress by induction of an acid tolerance response. This response essentially involves growth of the acid sensitive microorganism in a moderately low pH environment that subsequently leads to survival when suddenly exposed to what would normally be considered lethal acidic conditions (Foster, 1995). This adaptation permits the induction of genes involved in an acid-tolerance response and synthesis of a series of acid shock proteins that are protective for extreme acidic conditions (Foster, 1999).

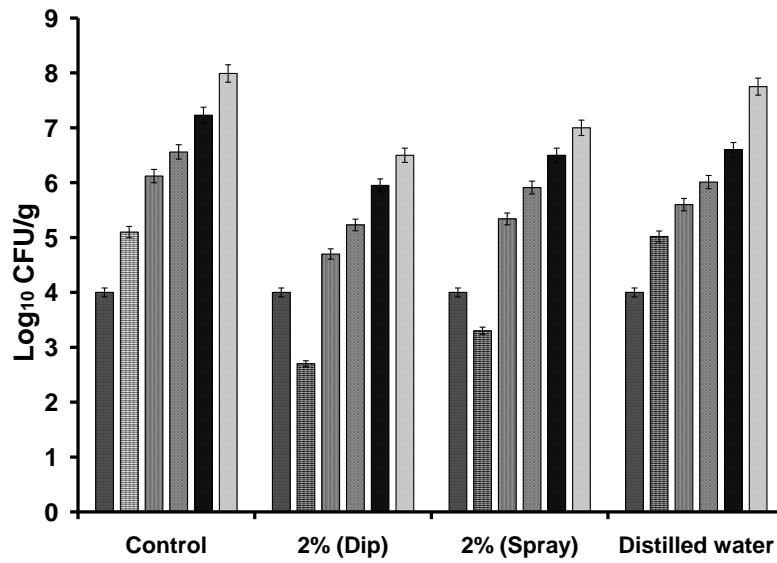


Fig. 4.8 Survival kinetics and lethal action of 2 ppm ozone on CS1 inoculated in mushroom, incubated at 15°C for 5 days

The sequence encodes 0 d; 1 d; 2 d; 3 d; 4 d; 5 d

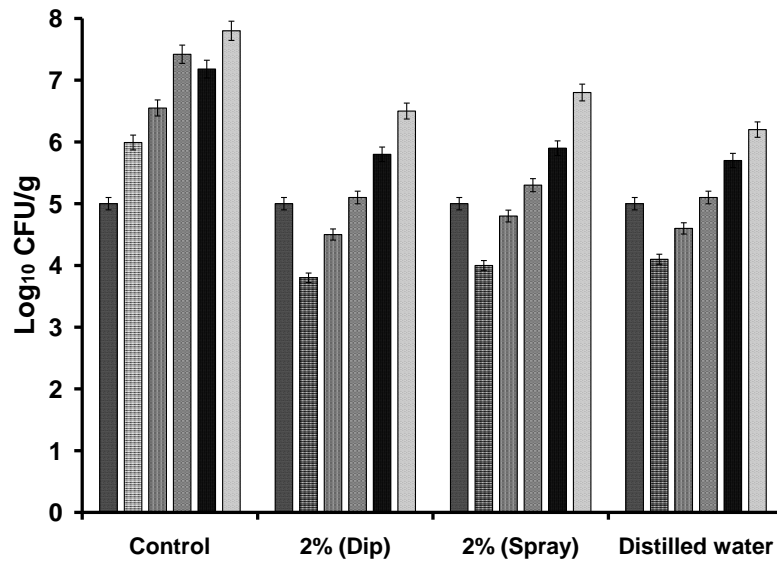


Fig. 4.9 Survival kinetics and lethal action of 2 ppm ozone on CS2 inoculated in betel leaves, incubated at 28°C for ten days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.7 Effect of malic acid and acetic acid onto the pathogens in artificially inoculated fresh vegetables

The potential of organic acids to reduce populations of microorganisms on fresh vegetables has been previously emphasized. Organic acids are commonly used as antimicrobial acidulants to preserve foods either by direct addition or through microbiological fermentation (Foegeding and Busta, 1991). Since many pathogens generally cannot grow at pH values much below 4.5, acidification may act to prevent microbial proliferation. The antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by the dissociation of hydrogen ions from the acid. Acidification of foods with short-chain organic acids, either by fermentation or by fortification, is an important and widespread mechanism for controlling foodborne pathogens in a variety of foods (Barker and Park, 2001).

4.7.1 *Salmonella typhimurium* (ST1) in carrot and (ST2) in turnip

The counts of ST1 and ST2 recovered from the fresh cut pieces of carrot and turnip after 10 days of storage at 28°C is revealed in Fig. 4.10 and Fig. 4.11. The initial load of ST1 used for the study was found to be 5 log₁₀CFU/g. A reduction of 1.5 log was observed in carrot against ST1 following complete immersion, 0.8 log following spraying with 2% malic acid and acetic acid in comparison to control. Whereas, distilled water only reduced the counts upto 0.5 log₁₀CFU/g. Only 2 log reduction was observed in turnip against ST2 after treatment with 2% malic acid and acetic acid after complete immersion and 1.2 log after spraying. Distilled water was used as control to see the effect onto pathogen, but was found ineffective. Similar results were found with both the organic acids.

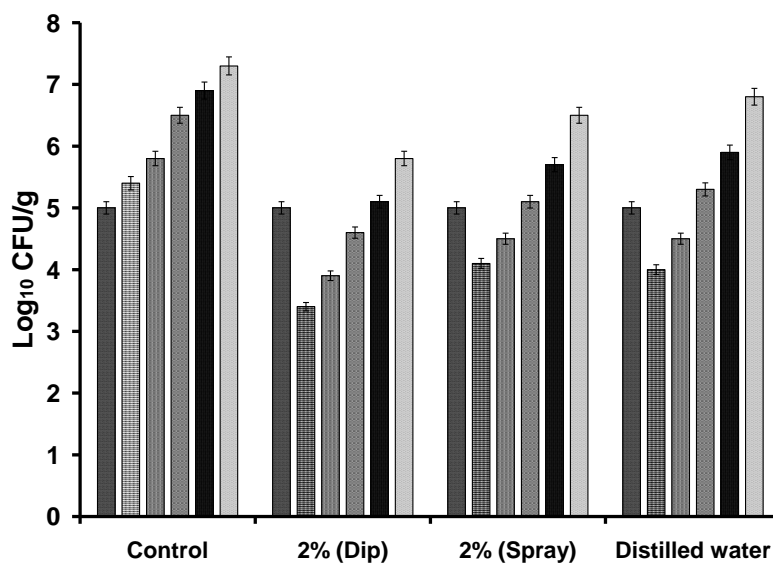


Fig. 4.10 Inactivation of ST1 in carrot by malic acid treatment incubated at 28°C for 10 days

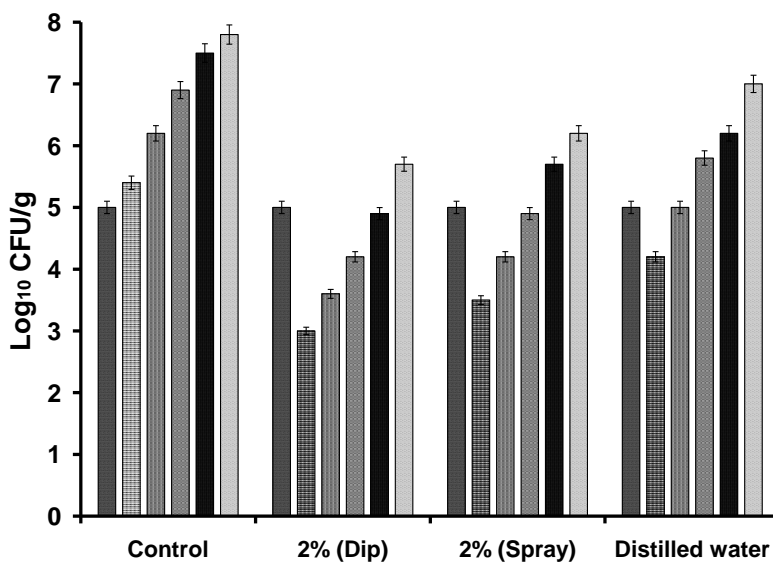


Fig. 4.11 Inactivation of ST2 in turnip by malic acid treatment incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.7.2 *Shigella flexneri* (SF1) in radish sprouts and (SF2) in moong bean sprouts

The results of microbiological analysis of radish sprouts artificially inoculated with *Shigella flexneri* Type 2(a) (SF1) followed by malic acid (1%, 2%, 3%, 4%) treatment after storage at 28°C for 10 days are presented in Fig. 4.12. 2% malic acid significantly ($p < 0.05$) reduced the counts by 3 log₁₀CFU/g (complete immersion), 2 log₁₀CFU/g (spraying) in radish sprouts. Distilled water did not reduce the counts significantly ($p > 0.05$).

The results of microbiological analysis of moong bean sprouts artificially inoculated with *Shigella flexneri* Type 2(a) (SF1) followed by malic acid (1%, 2%, 3%, 4%) treatment after storage at 28°C for 10 days are presented in Fig. 4.13. Malic acid (2%) significantly ($p < 0.05$) reduced the counts by 3 log₁₀CFU/g (complete immersion), 1 log₁₀CFU/g (spraying) in moong bean sprouts. Distilled water did not reduce the counts significantly ($p > 0.05$). Inhibitory effects of organic acids can be explained by acidic pH and their undissociated forms resulting in cytoplasmic acidification of microbial cells. The extent of this process is being largely determined by the trans-membrane pH difference (Brul and Coote, 1999; Davidson, 2001).

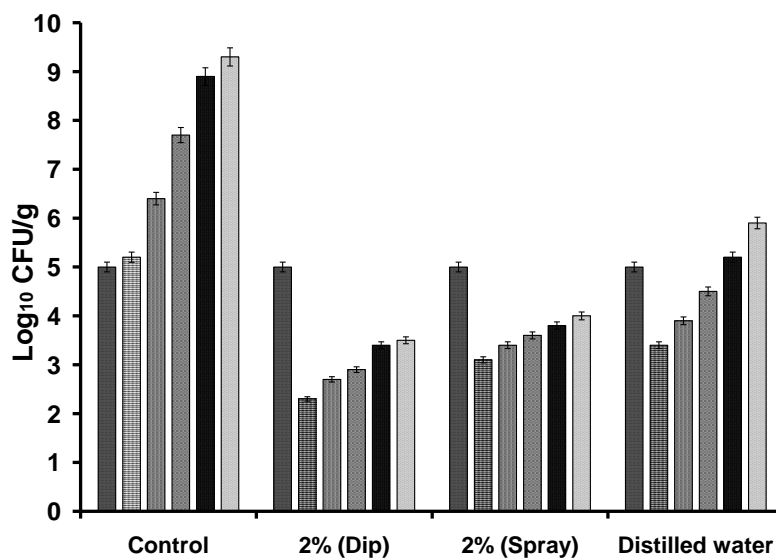


Fig. 4.12 Inactivation of SF1 in radish sprouts by malic acid treatment incubated at 28°C for 10 days

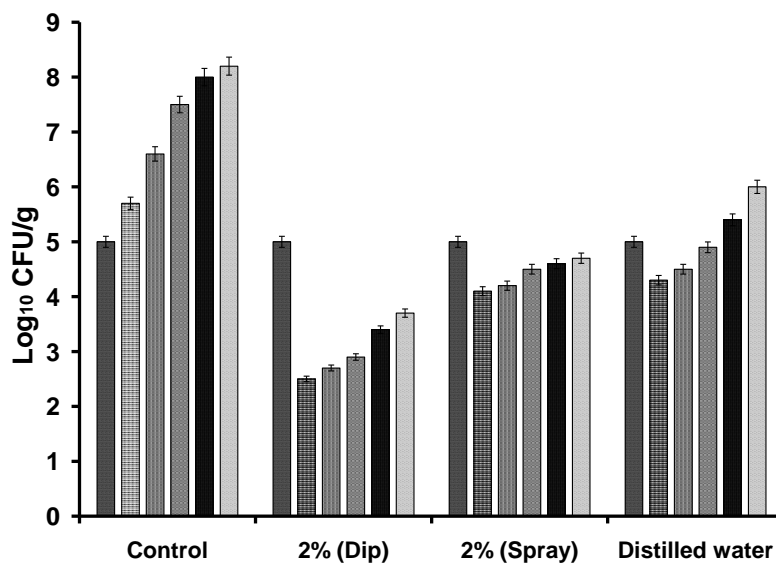


Fig. 4.13 Inactivation of SF2 in moong bean sprouts by malic acid treatment incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.7.3 *Cronobacter sakazakii* (CS1) in mushroom and (CS2) in betel leaves

Results of CS1 contaminated mushroom samples treated with malic acid for exposure time of 5 min are shown in Fig. 4.14 reduced the counts by 2 log and 1.2 log. Following treatment, the samples were stored at 4°C for 5 days. Whereas, 2.3 log₁₀CFU/g and 1.8 log₁₀CFU/g log reduction was observed after complete immersion and spraying in the samples stored at 15°C for 5 days compared with the control (Fig. 4.15). Although, distilled water was also used to evaluate its effect; but, this was unable to control the growth of pathogen. Results of the storage study for up to 5 days showed that the treatment had a residual antimicrobial effect to control the growth of the pathogens during given period when held at 15°C after complete immersion, but was absent after spraying. The insignificant ($p>0.05$) growth observed at 4°C may be attributed to sub-lethal action of malic acid at low temperature and also may be due to the inability of some pathogens to grow at 4°C/ low temperature. This suggests that there is a risk of high population densities of such pathogens on button mushrooms stored at ambient temperature for sufficient time. Citric acid and lactic acid was not able to inactivate the pathogen population to a sufficient level and was thus considered inappropriate as treatment agents.

In the present study, both dipping and spraying betel leaves by 2% acetic acid for (1:2, w/v; 5 min) reduced CS2 by 2.8 log and 2.5 log (Fig. 4.16) following incubation at 28°C for 20 hours, Distilled water was able to reduce the pathogen growth by 1.6 log. Other organic acids (citric acid, lactic acid) were also used in our study as well, but results were not encouraging. An appreciable volume of studies have reported that food borne pathogens have acid resistance characteristics against various kinds of acids although the sensitivity of the organisms to organic acids varies with the nature of the acidulant used (Barker and Park, 2001).

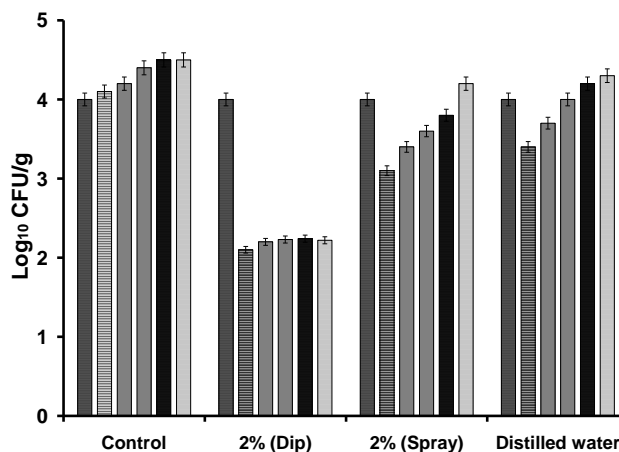


Fig. 4.14 Inactivation of CS1 in button mushroom by malic acid treatment incubated at 4°C for 5 days

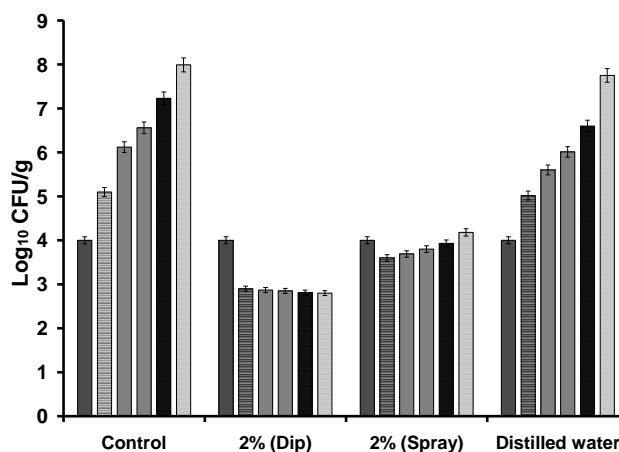


Fig. 4.15 Inactivation of CS1 in button mushroom by malic acid treatment incubated at 15°C for 5 days

The sequence encodes 0 d; 1 d; 2 d; 3 d; 4 d; 5 d

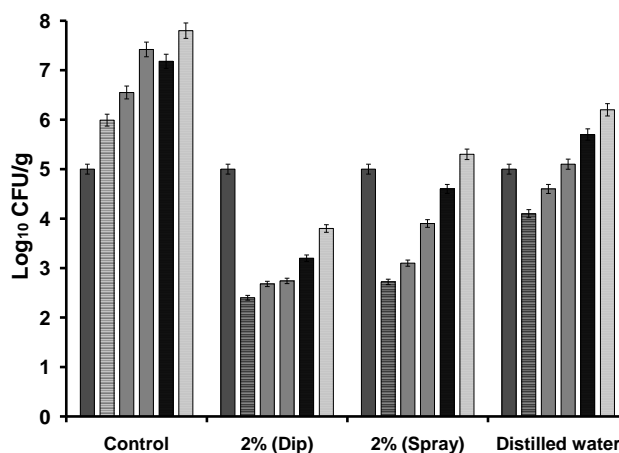


Fig. 4.16 Inactivation of CS2 in betel leaves by acetic acid treatment incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.8 Effect of combined treatment of ozonated water and organic acids onto the pathogens in artificially inoculated fresh vegetables

Combinations of ozone with other technologies have been extensively investigated in order to enhance the treatment's microbicidal efficacy. Ozone-based advanced oxidation processes is a developing technology that utilizes powerful oxidizing intermediates such as hydroxyl radicals, resulting in disinfection efficacy greater than that of ozone alone (Kim et al., 2003). It could be expected that combinations of sanitizers and/or other intervention methods such as organic acid along with ozone, would have additive, synergistic interactions. Since individual treatment with either ozone or organic acids was not effective against the pathogens tested, therefore, ozonated water along with organic acid was tested for the synergetic effect.

4.8.1 *Salmonella typhimurium* (ST1) in carrot and (ST2) in turnip

Combined treatment of 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 6.0 \log_{10} CFU/g in carrot after complete immersion and 5.5 \log_{10} CFU/g after spraying suggesting a residual antimicrobial effect as compared to individual treatments (Fig. 4.17). Ultraviolet radiation followed by gaseous ozone treatment on externally contaminated shell eggs was synergistic against *Salmonella* Enteritidis as reported by Rodriguez-Romo and Yousef (2005). Besides this, reduction in populations of microflora on whole and fresh-cut produce also depends upon the type of produce and the type of natural microflora present.

Combined treatment of 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 7 \log_{10} CFU/g in turnip after complete immersion and 6.2 \log_{10} CFU/g after spraying suggesting a residual antimicrobial effect as compared to individual treatments (Fig. 4.18).

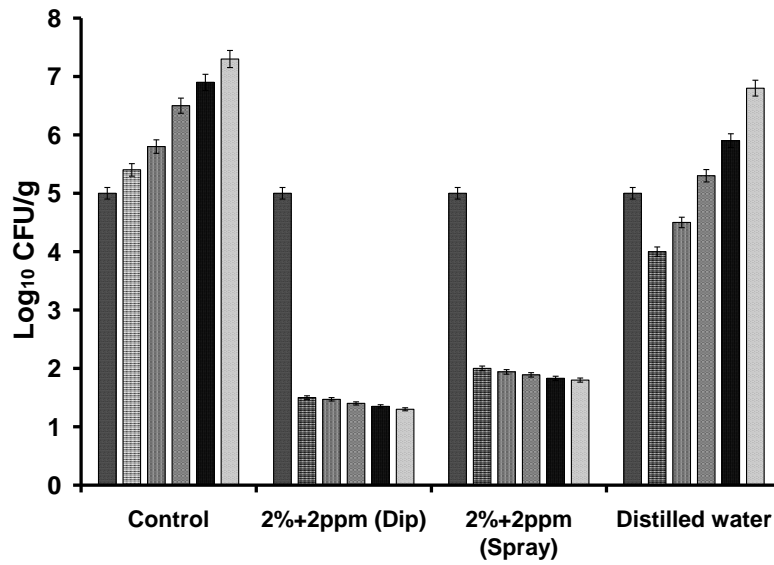


Fig. 4.17 Survival studies and lethal action of combined treatment of 2% malic acid and 2 ppm ozone on ST1 inoculated in carrot incubated at 28°C for 10 days

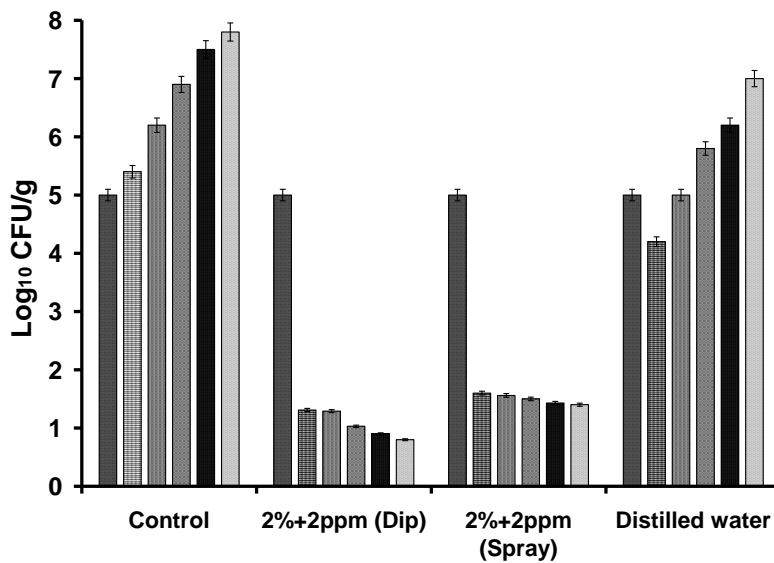


Fig. 4.18 Survival studies and lethal action of combined treatment of 2% malic acid and 2 ppm ozone on ST2 inoculated in turnip incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.8.2 *Shigella flexneri* (SF1) in radish sprouts and (SF2) in moong bean sprouts

Treatment of 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 7.6 \log_{10} CFU/g in radish sprouts this observation suggested a residual antimicrobial effect as compared to individual treatments (Fig. 4.19). Similar to this study, ozone (3 ppm) combined with 1% citric acid significantly reduced *L. monocytogenes* counts by 1.33 log compared to individual treatments (Yuk et al., 2007). Hydrogen peroxide (3%) alone or in combination with 2 or 5% acetic acid sprayed onto green peppers, reduced *Shigella* populations approximately by 5 log units (compared to less than a 1 log reduction by water) (Peters, 1995).

Combined treatment of 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 7.3 \log_{10} CFU/g in moong bean sprouts, suggesting a residual antimicrobial effect as compared to individual treatments (Fig. 4.20). Tetteh and Beuchat (2003) reported that exposure of *S. flexneri* cells, unadapted to an acidic environment, to a mild heat shock renders them more tolerant to acidic conditions and may enhance their survival and ability to grow in high acid foods.

4.8.3 *Cronobacter sakazakii* (CS1) in mushroom and (CS2) in betel leaves

Complete immersion in 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 6.2 \log_{10} CFU/g. Spraying resulted in a pathogen reduction of 4.8 \log_{10} CFU/g suggesting a residual antimicrobial effect as compared to individual treatments (Fig. 4.21).

Combined treatment of 2% acetic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 6.8 \log_{10} CFU/g in betel leaves after complete immersion and 5.4 \log_{10} CFU/g after spraying signifying a residual antimicrobial effect as compared to individual treatments (Fig. 4.22).

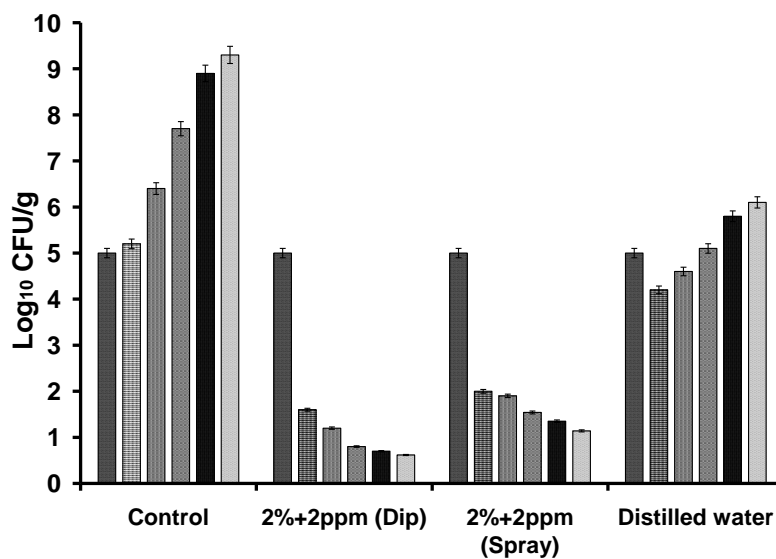


Fig. 4.19 Survival studies and lethal action of combined treatment of 2% malic acid and 2 ppm ozone on SF1 inoculated in radish sprouts incubated at 28°C for 10 days

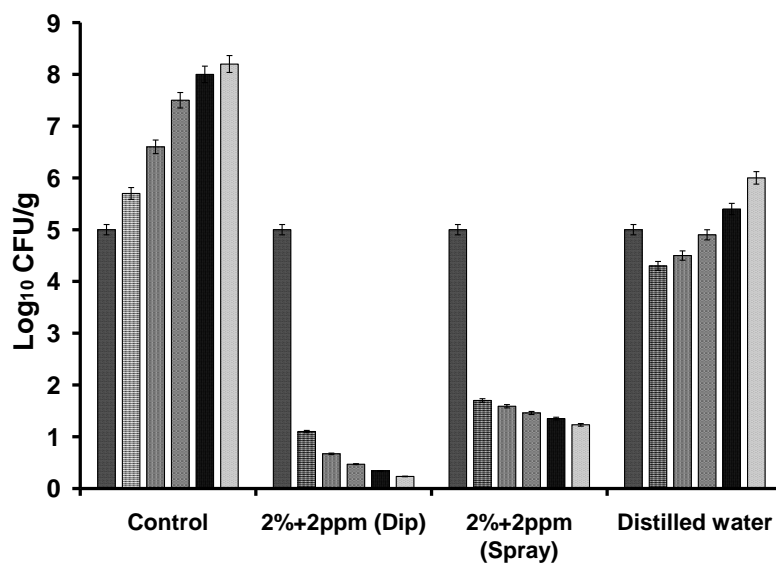


Fig. 4.20 Survival studies and lethal action of combined treatment of 2% malic acid and 2 ppm ozone on SF2 inoculated in moong bean sprouts incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

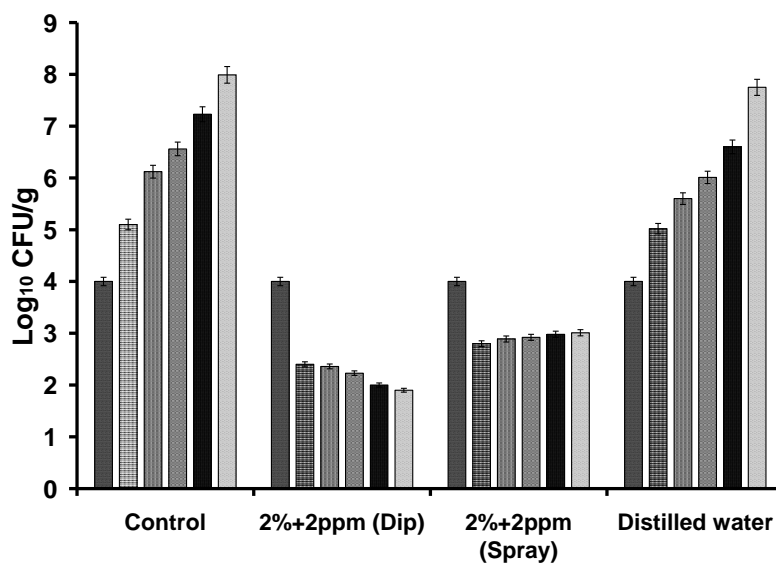


Fig. 4.21 Survival studies and lethal action of combined treatment of 2% malic acid and 2 ppm ozone on CS1 inoculated in mushroom incubated at 15°C for 5 days

The sequence encodes 0 d; 1 d; 2 d; 3 d; 4 d; 5 d

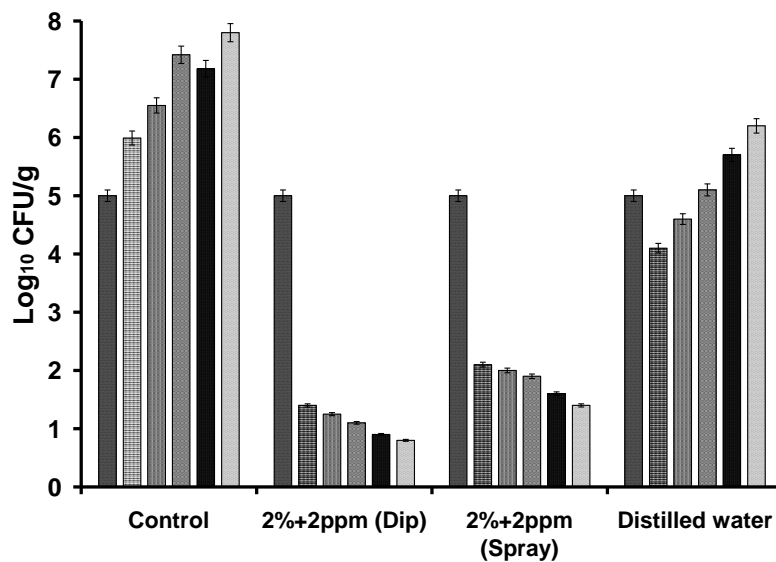


Fig. 4.22 Survival studies and lethal action of combined treatment of 2% malic acid and 2 ppm ozone on CS2 inoculated in betel leaves incubated at 28°C for 10 days

The sequence encodes 0 d; 2 d; 4 d; 6 d; 8 d; 10 d

4.9 Characterization of acid tolerant pathogens ST1 and ST2

A biofilm has been defined as a “community of bacteria living in organized structures at a liquid interface” (Davies, 2003). Microscopic investigations of biofilm structure have demonstrated that bacteria exist in microcolonies that are encapsulated in a matrix of extracellular polymeric material (Starkey et al., 2004). The initial attachment sites for plant pathogens are often protected areas on produce such as the stomata, broken trichomes, and wounds or cracks in the cuticle layer. The role of factors such as hydrophobic interactions, bacterial surface charge, the presence/absence of fimbriae and exocellular polysaccharides as well as duration of exposure and inoculum level in bacterial attachment to the vegetable surface has been investigated with contradictory results (Solomon and Matthews, 2006). A multilayer hydrophobic cuticle composed of cutin and amorphous wax molecules which covers the epidermis of fruits and vegetables is considered responsible for the highly water-repellent nature of plant surfaces. It has been suggested that hydrophobic interactions between this epidermal layer and bacteria play a major role (Burnett and Beuchat, 2001). A number of reports have been published on the persistence of several foodborne pathogens such as *Y. enterocolitica* and *E. coli* O157:H7 in biofilms. In contrast to planktonic cells, bacterial biofilms on plant surfaces exhibit an increased resistance to sanitizers and detergents. This resistance has been attributed to various properties associated with the biofilm such as reduced diffusion, physiological changes due to reduced growth rates and the production of enzymes degrading antimicrobial substances (Ganesh and Anand, 1998). Given adequate environmental conditions, sub-lethally injured cells can grow if provided (Romanova et al., 2007).

Although a low level of pathogens such as *Salmonella typhimurium* may be initially found on naturally contaminated vegetables, sufficient time and appropriate environmental conditions may allow pathogens to grow to sufficient populations to achieve an effective

dose. Organic acid dose was optimized using various concentrations of malic acid (0.5%, 1%, 1.5%, 2%). As, already discussed, among all the pathogens tested, both ST1 and ST2 were resistant to high concentrations of organic acid in comparison to other pathogens. Factors affecting attachment and biofilm formation by microorganisms include nutrient availability, the pH of the surrounding medium, and the nature of the cell and abiotic surfaces (Frank, 2001). Castillo et al. (2004) isolated biofilm forming *Salmonella* from walls and floors of cooling rooms at a packing plant in Texas, indicating the significance of sanitation within packaging sheds. To seek an explanation on the observed resistance of *S. typhimurium* isolates ST1 and ST2. It was deemed necessary to evaluate their biofilm forming ability onto respective vegetables (carrot and turnip) along with other food contact surfaces.

4.9.1 Presumptive identification of biofilms by calcofluor assay

The suitability of calcofluor as a general presumptive probe for biofilm detection was tested more extensively using 3-day-old biofilms grown on PVC pipes (Fig. 4.23a) and polyethylene bags (Fig. 4.23b). Results showed that calcofluor detected biofilms, and can, indeed, be used as a nonspecific test for biofilm contamination both for ST1 and ST2. However, it is important to note that the amount of fluorescence is a function of the affinity of calcofluor for the CPS and the quantity of adherent CPS. Thus, a negative or weak result could denote minimal biofilms formation, which is exactly what the probe is intended to detect. PVC pipes and polybags exhibited considerable amounts of biofilm present on the entire surface (1A, 2A) which depict the intensity of biofilm without disinfectant. The combined effect of malic acid (1B, 2B) along with ozone (1C, 2C), which clearly demarcates the significant inhibition of biofilm as intensity of fluorescent is minimum in this figure. Calcofluor was an extremely effective probe of *Salmonella* biofilms on both tested surfaces (PVC pipes & bags). Both the strains were able to form biofilms on both types of surfaces.

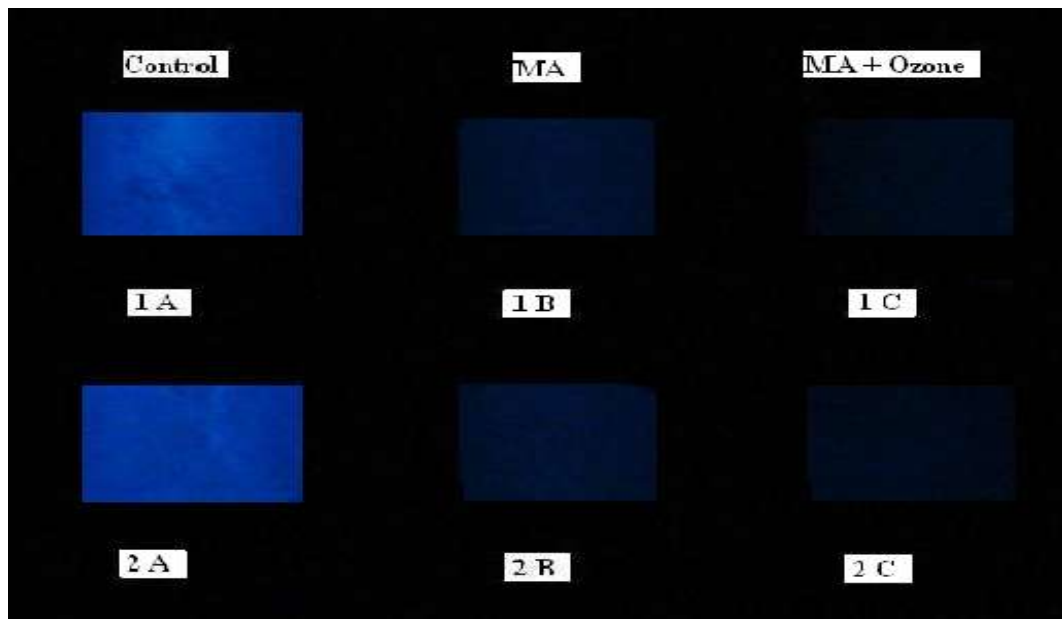


Fig. 4.23a Biofilm of *Salmonella* stained with fluorescent agent calcofluor (80 µg/ml) on PVC pipes. 1A and 2A presents intensity of biofilm formation by ST1 and ST2 without using any washing agent. 1B and 2B shows the lesser biofilm after using organic acid and 1C and 2C shows absence of biofilm after combined treatment with ozone and organic acid

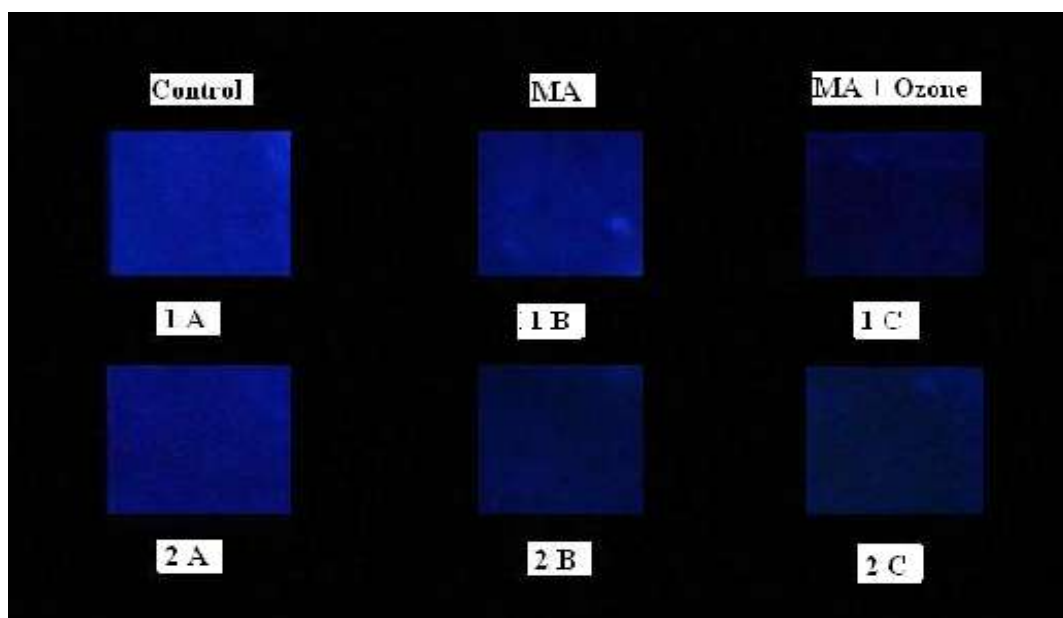


Fig. 4.23b Biofilm of *Salmonella* stained with fluorescent agent calcofluor (80 µg/ml) on plastic bags. 1A and 2A presents intensity of biofilm formation by ST1 and ST2 without using any washing agent. 1B and 2B shows the lesser biofilm after using organic acid and 1C and 2C shows absence of biofilm after combined treatment with ozone and organic acid

4.9.2 Presumptive identification of biofilm by alcian blue microscopy

Alcian blue staining indicated the presence of extracellular polysaccharides in the biofilms of *Salmonella typhimurium* on to the surfaces of polyethylene bags. Large amounts of alcian blue-stained EPS were observed, indicating that massive amounts of biofilm formation (Fig. 4.24). Minor amounts of blue-stained EPS were noted for the treated surface with malic acid (1B, 2B), in-comparison to control (1A, 2A) however malic acid combined with ozone treated surface did not confirm the presence of EPS (1C, 2C).

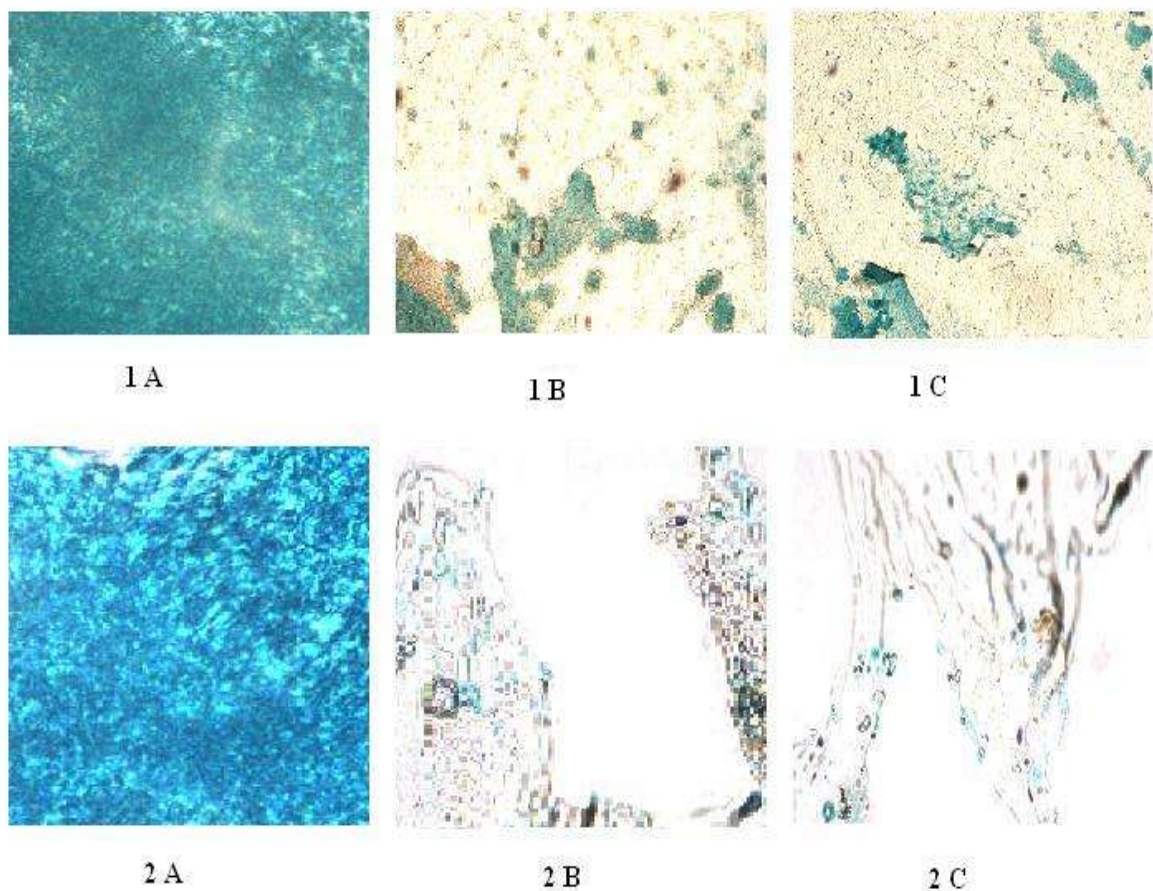


Fig. 4.24 Images of *Salmonella* stained with alcian blue on plastic bags. 1A and 2A presents biofilm formation by ST1 and ST2 without using any washing agent (100 X). 1B and 2B shows the lesser biofilm after using organic acid and 1C and 2C shows absence of biofilm after combined treatment with ozone and organic acid (100 X)

4.9.3 Estimation of biofilm formation by Microtitre plate assay

The activity of the ozone and organic acids on biofilm formation by ST1 and ST2 was screened using a 100 well microtitre plate assay with crystal violet staining. The planktonic growth in TSBYE medium was monitored after 20 h and 40 h. Results showed that *Salmonella enterica* serovar Typhimurium ST1 (Fig. 4.25a) and ST2 (Fig. 4.25b) has potential to form biofilm at 20 h and 40 h in TSBYE medium. However; under stress conditions (malic acid and ozone) biofilm formation significantly ($p < 0.05$) reduced upto 5 fold at 20 h and upto 6 fold at 40 h in both ST1 and ST2.

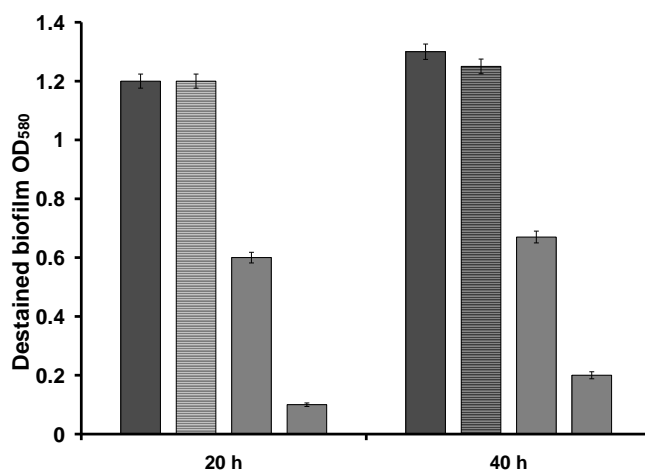


Fig. 4.25a Biofilm production by ST1 in microtitre plate after 20 h and 40 h incubation at 32°C

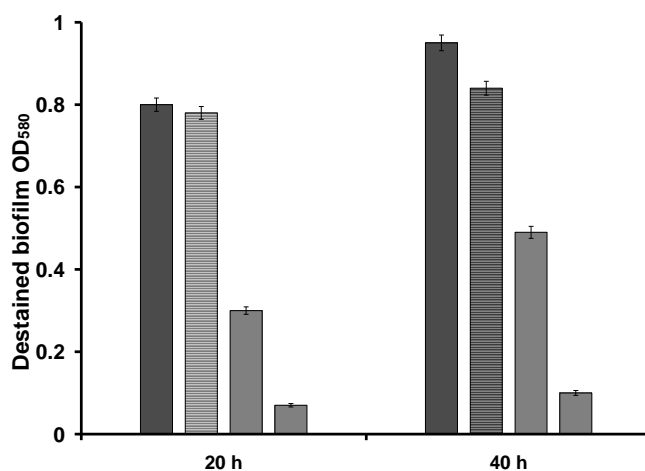


Fig. 4.25b Biofilm production by ST2 in microtitre plate after 20 h and 40 h incubation at 32°C

The sequence encodes Control; Distilled water; 2%; 2%+2 ppm

4.9.4 Scanning electron microscopy

Scanning electron microscopy was the most accurate way to confirm the formation/inhibition of biofilms on to the surfaces of fresh vegetables (carrot and turnip). SEM images provided fine structural details of the biofilms of *salmonellae*. Fig. 4.26 reveals the distinct biofilm formation difference between untreated (Control) (1A, 2A), malic acid (1B, 2B) and combined treated samples (1C, 2C) of carrot and turnip. The netting of biofilm appeared to be extremely compact that harbored a much greater amount of bacteria in its complex structures in both the untreated samples. Malic acid was effective to completely inhibit the biofilm formation in carrot (1B), but not in turnip (2B). On the other hand, ozone combined with malic acid proved to be completely effective sanitizer against biofilm formation in both fresh vegetable samples (1C, 2C). *Salmonellae* were clearly visible over the entire sample showing the presence of peritrichous flagella (white arrow), but the treated sample images (red arrow) showing the absence of flagella. The standard culture, *Salmonella typhimurium* (ATCC 23564) was able to form biofilms on all the surfaces (data not shown). The bacteria colonizing the netting have the potential of forming microcolonies and escape removal by chemical decontaminants.

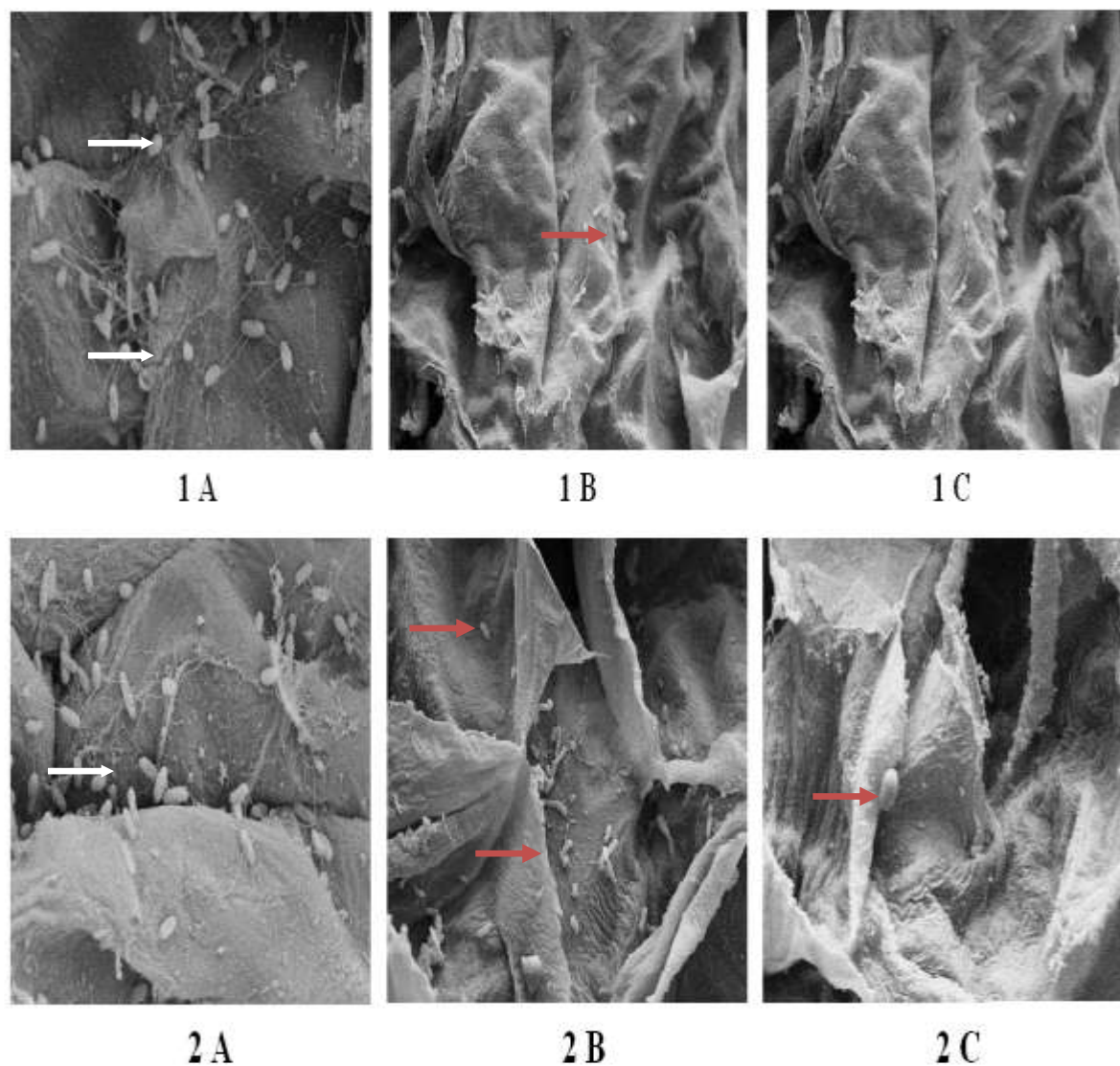


Fig. 4.26 Scanning electron micrographs of carrot and turnip biofilms by ST1 and ST2. 1A excised piece of carrot harbors and more bacteria than the treated one with organic acid 1B showing absence of any bacteria/biofilm after combined treatment. In turnip, 2A harbor maximum bacterial biofilm and fewer bacterial biofilm was found in malic acid (2B) and no biofilm in combined treated samples (2C). The white arrow points to a cluster of *Salmonellae* colonized to form biofilm having peritrichous flagella. Red arrow points to the *Salmonella* lacking flagella

4.10 Essential quality characteristics of fresh vegetables

Since little is known about nutritive value content of minimally processed plant foods (Beltran et al., 2005; Qiang et al., 2005), it was deemed necessary to analyze the overall effect of processing on total antioxidant profile of minimally processed vegetable samples. A number of natural antioxidants have already been demonstrated from different kinds of plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Cullen et al., 1997; Devasagayam et al., 2001; Kamat et al., 2000; Nigris et al., 2003; Packer and Ong, 1997; Ramarathnam et al., 1995).

4.10.1 Antioxidant profile of carrot

4.10.1.1 Sensory properties

The sensory quality of treated, minimally processed carrot was evaluated by a panel of ten trained judges by grading for color, flavour, consistency and overall acceptability score on a nine-point hedonic scale (Table 4.8). Samples were randomly drawn from each experimental block, coded, and served to the panelists. The analysis of the sensory scores revealed that raw carrot as well as the processing carrot samples retained texture, taste, smell, color, appearance (Fig. 4.27) and sensorially acceptable up to 10 days of storage.

Table 4.8 Mean sensory score of malic acid treated carrot on nine point hedonic scale ($p>0.05$)

| Attributes | Untreated carrot | 2%+2 ppm treated |
|---------------------------|------------------|------------------|
| Texture/freshness (9) | 7.8±0.05 | 7.2±0.05 |
| Taste/flavor (9) | 7.7±0.10 | 7.3±0.08 |
| Aroma/smell (9) | 7.7±0.09 | 7.3±0.06 |
| Color/appearance (9) | 7.5±0.70 | 7.8±0.06 |
| Overall acceptability (9) | 7.8±0.08 | 7.7±0.05 |

Note: Average of three mean values; values with ± represent standard deviation

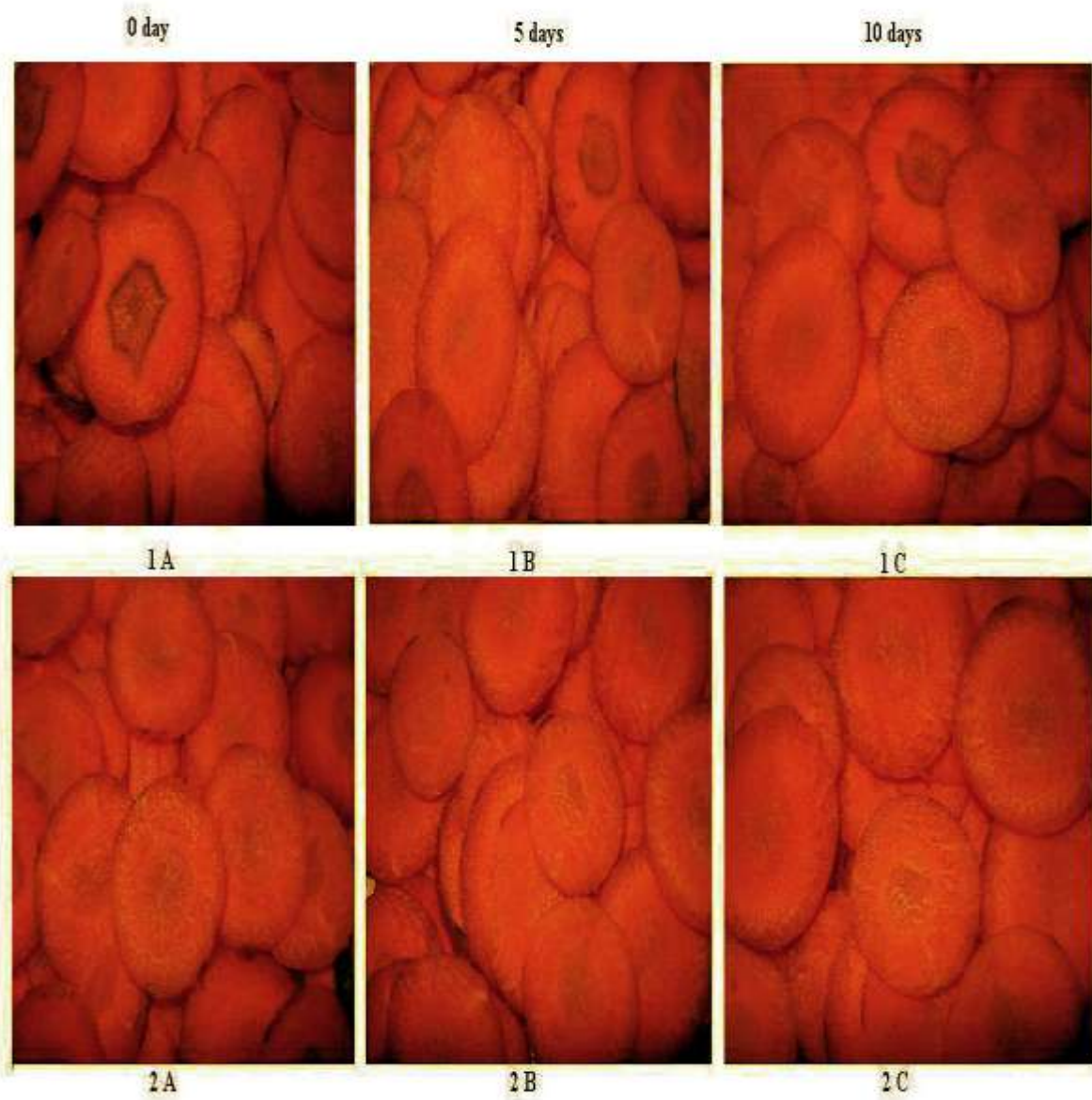


Fig. 4.27 Appearance of carrot before and after treatment. Images 1A, 1B and 1C represents without treatment after storage for 10 days. Images 2A, 2B and 2C showing images after combined treatment of 2% malic acid along with 2 ppm ozone after storage for 10 days

4.10.1.2 Antioxidant properties of carrot

The ABTS radical scavenging activity of the extracts of the raw sample, expressed as ascorbic acid equivalent antioxidant activity (AEAC) and defined as the mg of ascorbic acid equivalents per 100 g of the carrot. ABTS activity of raw carrot was found to be 0.23 ascorbic acid equivalents /100 g of sample, malic acid, ozone and combination treatments were 0.235, 0.24 and 0.23 respectively. Similarly, no significant changes ($p>0.05$) in ABTS activity of untreated and processed carrot was observed (Fig. 4.28a).

The antioxidant activities of the raw and treated carrot as determined by scavenging DPPH radical are presented in Fig. 4.28b. DPPH radical scavenging activity of untreated raw carrot extract was 45.66%, ozone treated 45.7% malic acid treated was 45% and combined treated was 48%, did not differ significantly ($p>0.05$).

Flavonoid content of raw and processed carrot is shown in Fig. 4.28c. Flavonoids of untreated carrot (expressed as mg of (+)-catechin equivalents per 100 g sample), were 25.56 mg/100 g sample, 23.44 mg /100 g sample after acid treatment, 24.78 mg/100 g after ozone treatment and 28.1 mg/100 g after combined treatment. No Significant ($p>0.05$) change was observed in flavonoids of processed carrot.

Effects of treatment on the total polyphenolics of sample extracts are presented in Fig. 4.28d. Polyphenolics of untreated carrot (expressed as mg of gallic acid equivalents per 100 g of sample), were 5.78 mg gallic acid /100 g sample respectively. After acid, ozone and combined treatment polyphenolics were 4.95, 4.92 and 4.72 mg gallic acid/100 g of sample. Therefore, no significant ($p>0.05$) changes in polyphenolics content were observed in carrot.

Reducing powers obtained for all the extracts are presented in Fig. 4.28e. Reducing power ability of untreated carrot was 0.543, whereas after acid, ozone and combined treatment was 0.453, 0.465 and 0.456. Slight insignificant ($p>0.05$) change was observed in reducing power of processed carrot. No significant change was observed in β -carotene content in treated carrots (Fig. 4.28f).

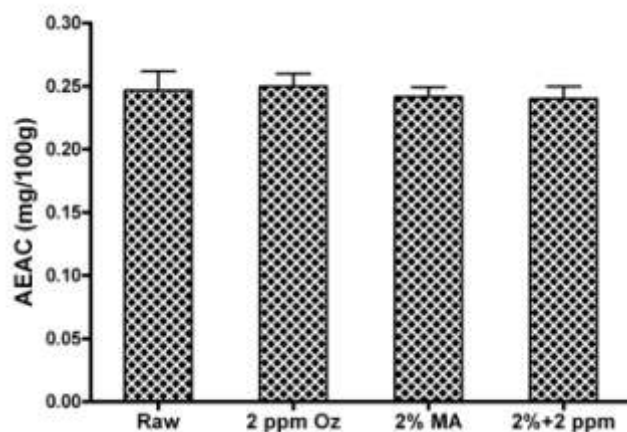


Fig. 4.28a Effect of processing on ascorbic acid equivalent antioxidant activity (AEAC) of raw, malic acid and ozone treated carrot

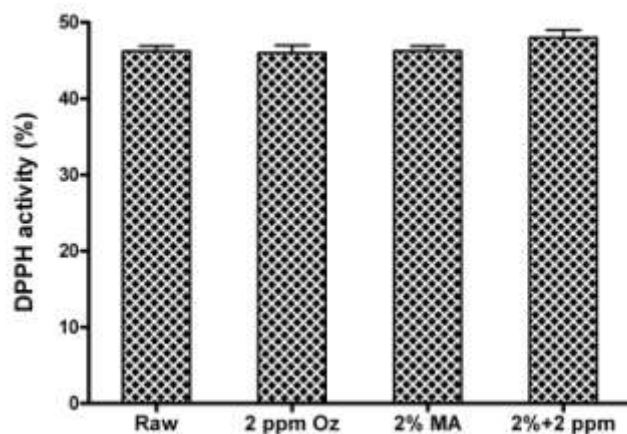


Fig. 4.28b Effect of processing on DPPH radical scavenging activity (%) of raw, malic acid and ozone treated carrot

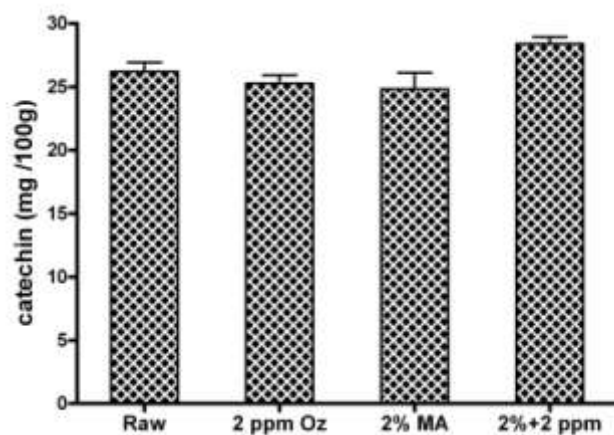


Fig. 4.28c Effect of processing on flavonoid content of raw, malic acid and ozone treated carrot

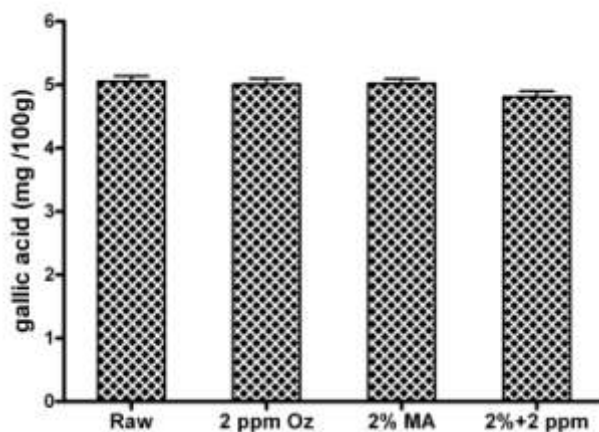


Fig. 4.28d Effect of processing on polyphenolic content of raw, malic acid and ozone treated carrot

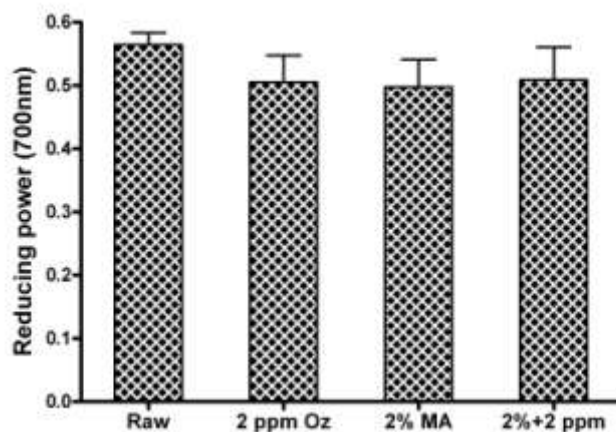


Fig. 4.28e Effect of processing on reducing power of raw, malic acid and ozone treated carrot

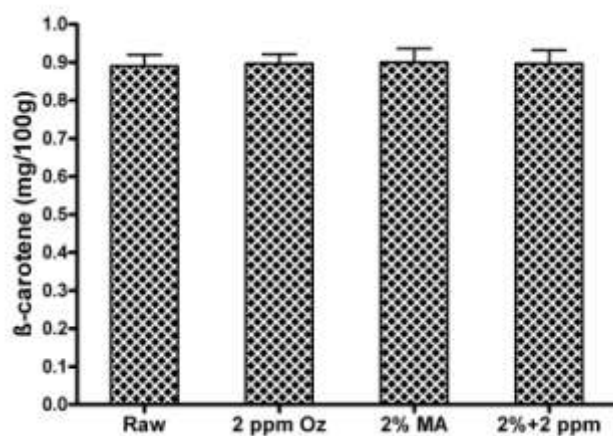


Fig. 4.28f Effect of processing on β-carotene content of raw, malic acid and ozone treated carrot

4.10.2 Antioxidant profile of turnip

Turnip is a highly nutritious starchy, root vegetable is one of the oldest cultivated vegetables that has been used for human consumption since prehistoric times. Turnip is found to be rich in antioxidants and is a vegetable preferred to be consumed raw in salads.

4.10.2.1 Sensory properties

The sensory quality of treated, minimally processed turnip was evaluated by a panel of ten trained judges by grading for texture, taste, smell, color and overall acceptability score on a nine-point hedonic scale (Table 4.9). All the treated samples of turnip were sensorially acceptable in terms of texture, taste, smell color, visual appearance (Fig. 4.29) and overall acceptance.

Table 4.9 Mean sensory score of malic acid treated turnip on nine point hedonic scale (p>0.05)

| Attributes | Untreated turnip | 2%+2 ppm treated |
|---------------------------|------------------|------------------|
| Texture/freshness (9) | 7.4±0.02 | 7.3±0.12 |
| Taste/flavor (9) | 7.5±0.05 | 7.5±0.04 |
| Aroma/smell (9) | 7.5±0.09 | 7.4±0.08 |
| Color/appearance (9) | 7.6±0.03 | 7.8±0.09 |
| Overall acceptability (9) | 7.5±0.05 | 7.5±0.08 |

Note: Average of three mean values; values with ± represent standard deviation

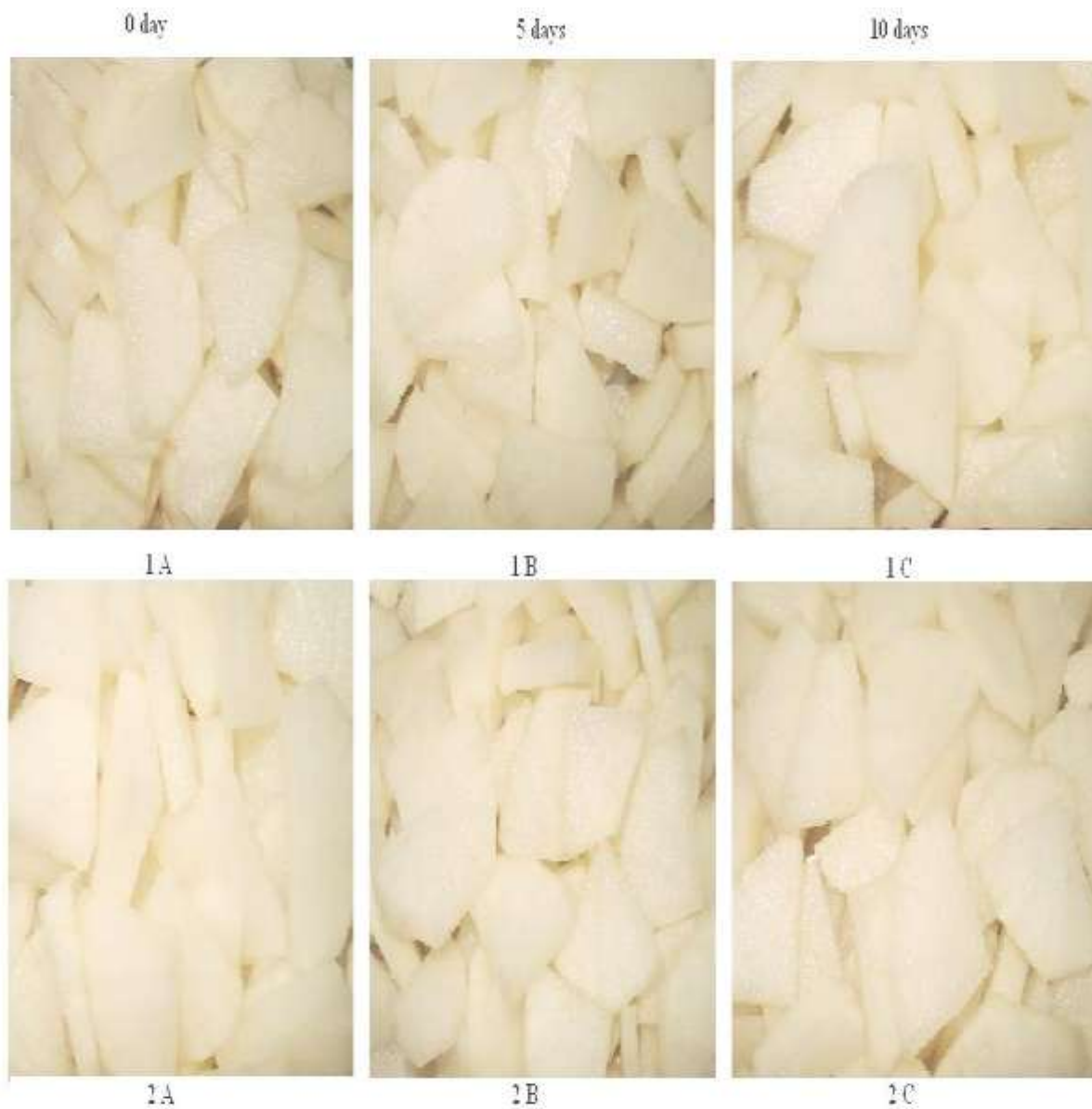


Fig. 4.29 Appearance of turnip before and after treatment. Images 1A, 1B and 1C represents without treatment after storage for 10 days. Images 2A, 2B and 2C showing images after combined treatment of 2% malic acid along with 2 ppm ozone after storage for 10 days

4.10.2.2 Effect of treatment on total antioxidant properties of turnip

The ABTS radical scavenging activity of the extracts of the raw turnip, expressed as ascorbic acid equivalent antioxidant activity (AEAC). ABTS activity expressed as ascorbic acid equivalent activity (AEAC) of raw carrot was found to be 0.15 ascorbic acid equivalents /100 g of sample, malic acid, ozone and combination treatments were 0.156, 0.16 and 0.163 respectively. Similarly, no significant changes ($p>0.05$) in ABTS activity of untreated and processed turnip was observed (Fig. 4.30a).

The antioxidant activities of the raw and treated turnip as determined by scavenging DPPH radical are presented in Fig. 4.30b. DPPH radical scavenging activity of untreated raw turnip extract was 23%, ozone treated 25% malic acid treated was 22.5% and combined treated was 24%, did not differ significantly ($p>0.05$).

Flavonoid content of raw and processed turnip is shown in Fig. 4.30c. Flavonoids of untreated turnip (expressed as mg of (+)-catechin equivalents per 100 g sample), were 12.43 mg/100 g sample, 12.78 mg /100 g sample after acid treatment, 12.65 mg/100 g after ozone treatment and 12.9 mg/100 g after combined treatment. No Significant ($p>0.05$) change was not observed in flavonoids of processed turnip.

Effects of treatment on the total polyphenolics of sample are shown in Fig. 4.30d. Polyphenolics of raw turnip (expressed as mg of gallic acid equivalents per 100 g of sample), were 4.23 mg gallic acid /100 g sample respectively. After acid, ozone and combined treatment polyphenolics were 4.35, 4.78 and 4.65 mg gallic acid/100 g of sample. Therefore, no significant ($p>0.05$) changes in polyphenolics content were observed in turnip.

Reducing powers obtained for all the sample extracts are presented in Fig. 4.30e. Reducing power ability of untreated turnip extract was 0.234, whereas after acid, ozone and combined treatment was 0.256, 0.265 and 0.289. Slight insignificant ($p>0.05$) change was observed in reducing power of processed turnip.

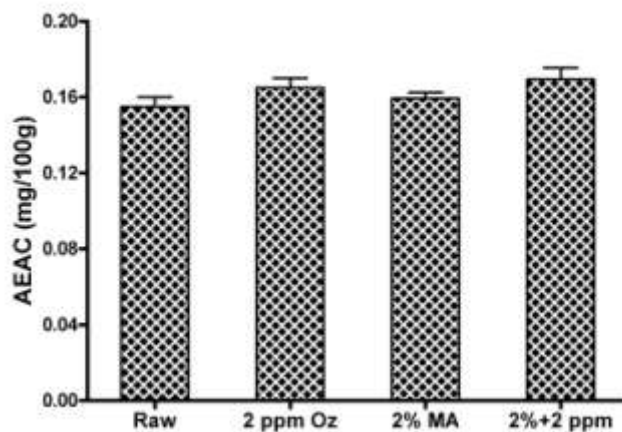


Fig. 4.30a Effect of processing on ascorbic acid equivalent antioxidant activity (AEAC) of raw, malic acid and ozone treated turnip

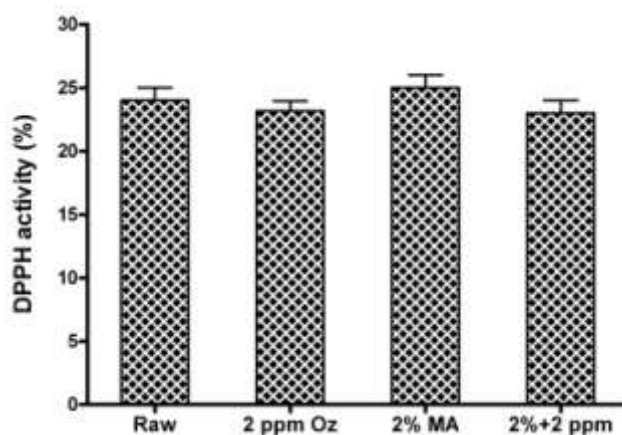


Fig. 4. 30b Effect of processing on DPPH radical scavenging activity (%) of raw, malic acid and ozone treated turnip

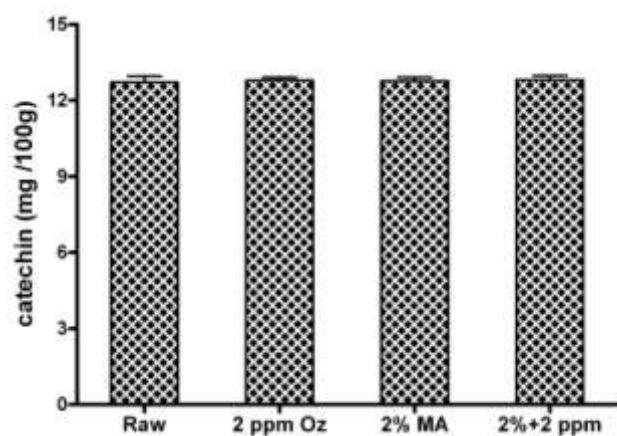


Fig. 4.30c Effect of processing on flavonoid content of raw, malic acid and ozone treated turnip

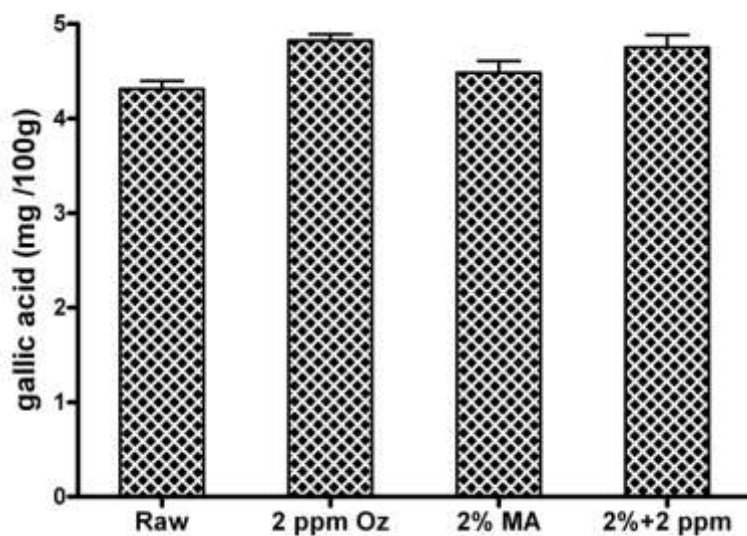


Fig. 4. 30d Effect of processing on polyphenolic content of raw, malic acid and ozone treated turnip

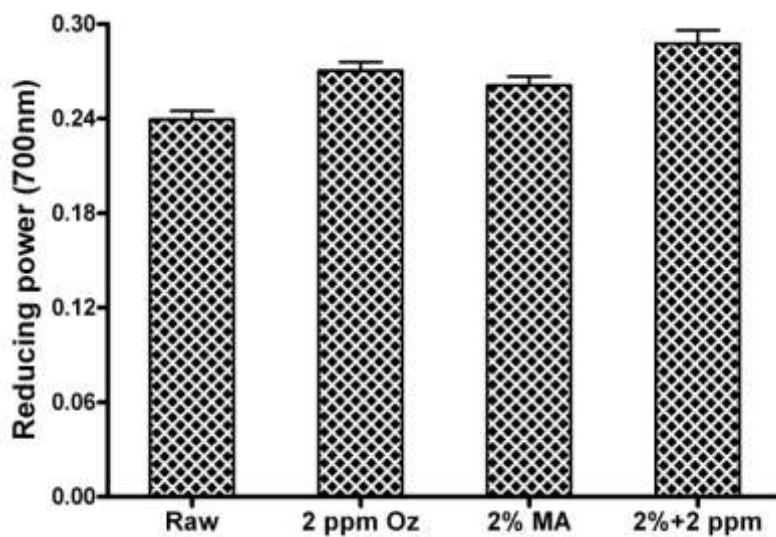


Fig. 4.30e Effect of processing on reducing power of raw, malic acid and ozone treated turnip

4.10.3 Antioxidant profile of radish sprouts

Seed sprouts have long been used in the diet as health food and recent research shows that in addition to being a good source of basic nutrients, they also have important phytochemicals with disease preventive and health promoting properties. Industrial processing such as blanching, canning, sterilization and freezing, as well as domestic cooking affect the content, composition, antioxidant activity and bioavailability of antioxidants present in sprouts and effective yet mild treatment process is thus much sought after.

4.10.3.1 Sensory properties

The sensory quality of treated, minimally processed sprouts was evaluated by a panel of ten trained judges by grading for texture, taste, smell, color, visual appearance and overall acceptability score on a nine-point hedonic scale (Table 4.10). All the treated samples of radish sprouts were sensorially acceptable in terms of color and appearance (Fig. 4.31), taste, texture and consistency.

Table 4.10 Mean sensory score of malic acid treated radish sprouts on nine point hedonic scale ($p>0.05$)

| Attributes | Untreated sprouts | 2%+2 ppm treated |
|---------------------------|-------------------|------------------|
| Texture/freshness (9) | 7.2±0.03 | 7.2±0.06 |
| Taste/flavor (9) | 7.4±0.04 | 7.3±0.07 |
| Aroma/smell (9) | 7.8±0.02 | 7.7±0.07 |
| Color/appearance (9) | 7.9±0.03 | 7.8±0.02 |
| Overall acceptability (9) | 7.6±0.04 | 7.5±0.07 |

Note: Average of three mean values; values with ± represent standard deviation

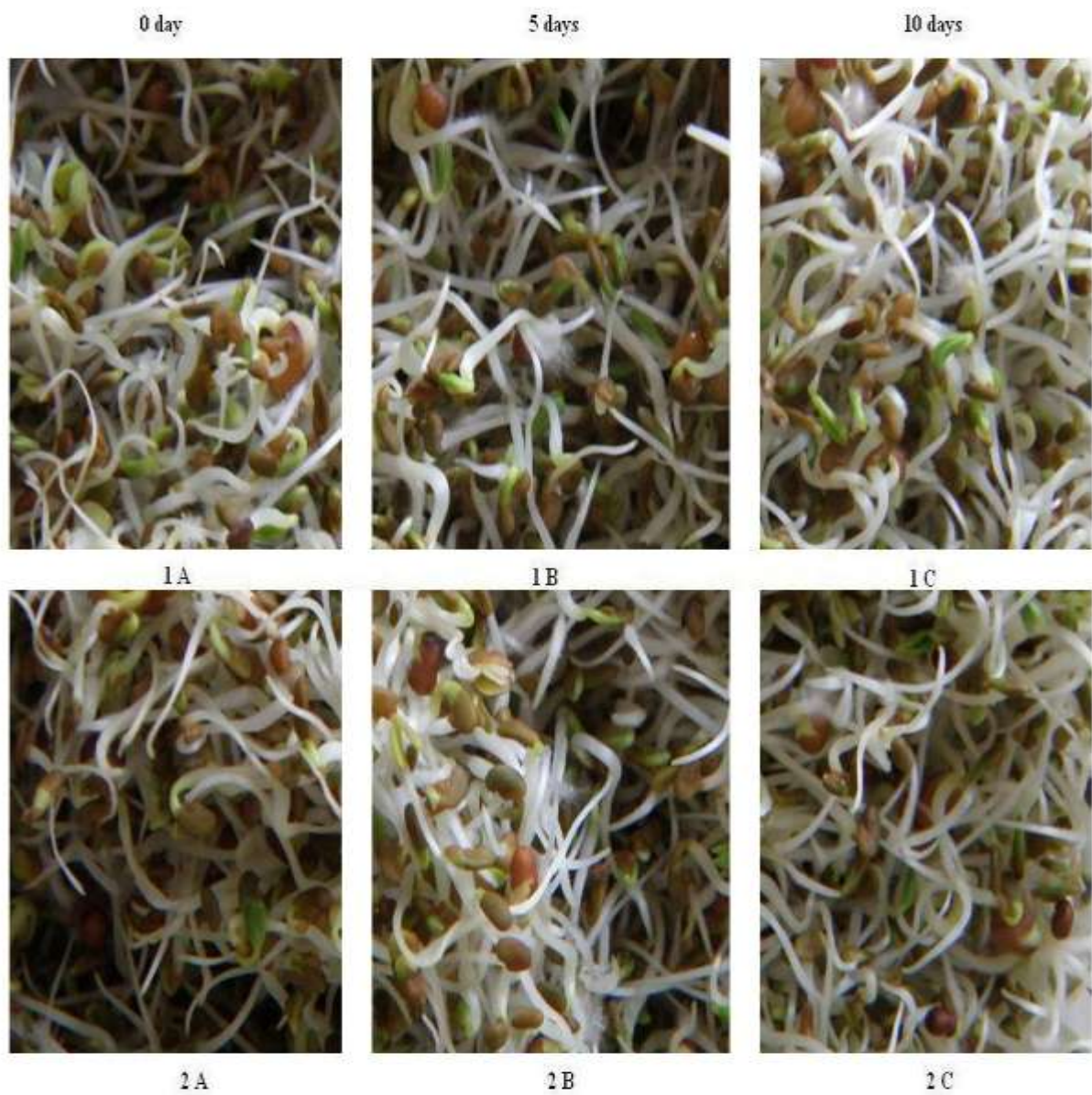


Fig. 4.31 Visual appearance of radish sprouts before and after treatment. Images 1A, 1B and 1C represents without treatment after storage for 10 days. Images 2A, 2B and 2C showing images after combined treatment of 2% malic acid along with 2 ppm ozone after storage for 10 days

4.10.3.2 Effect of treatment on total antioxidant properties of radish sprouts

An important factor of plant food is their antioxidant properties therefore it was necessary to consider the overall effect of processing on total antioxidant profile of sprouts. ABTS activity expressed as ascorbic acid equivalent activity (AEAC) of raw radish sprouts was found to be 0.35 ascorbic acid equivalents /100g of sample, malic acid, ozone and combination treatments were 0.33, 0.35 and 0.34 respectively (Fig. 4.32a).

The antioxidant activities of the raw and treated radish sprouts as determined by scavenging DPPH radical are presented in Fig. 4.32b. DPPH radical scavenging activity of untreated radish sprouts extract was 11.66%, malic acid treated ozone treated 14% and did not differ significantly ($p>0.05$). The methods using ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) or DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging are among the most popular spectrophotometric methods for determining the antioxidant capacity of food samples. These two simple stable radical chromogens (the violet DPPH radicals and the blue-green ABTS radical anions) are easy to use, have a high level of sensitivity, and allow for analysis of a large number of samples.

The flavonoid content of radish sprouts is shown in Fig. 4.32c. The flavonoids of raw radish sprouts (expressed as mg of (+)-catechin equivalents per 100 g sample), were 32.55 mg/100 g sample, 15.84 mg /100 g sample after acid treatment, 25.78 mg/100 g after ozone treatment and 30.1 mg/100 g after combined treatment showed insignificant change ($p>0.05$).

Plant phenolic metabolites are gaining interest due to their potential role in human disease prevention and treatment. These metabolites are mainly produced to protect plants from biotic/abiotic stresses such as photooxidation stress, reactive oxygen species, wounds, UV light, disease and herbivores. Effects of treatment on the total polyphenolics of sample

extracts are shown in Fig. 4.32d. The polyphenolics of raw radish sprouts (expressed as mg of gallic acid equivalents per 100 g of sample), was 2.93 mg gallic acid /100 g sample respectively. After acid, ozone and combined treatment polyphenolics were 2.85, 2.92 and 2.82 mg gallic acid/100 g of sample. Therefore, no significant ($p>0.05$) change in polyphenolic contents were observed in radish sprouts.

In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Reducing powers obtained for all the extracts are presented in Fig. 4.32e. The reducing power ability of raw radish sprouts was 0.649, whereas acid and ozone treated were 0.634 and 0.698 respectively; following combined treatment, reducing activity (0.628) did not change significantly ($p>0.05$).

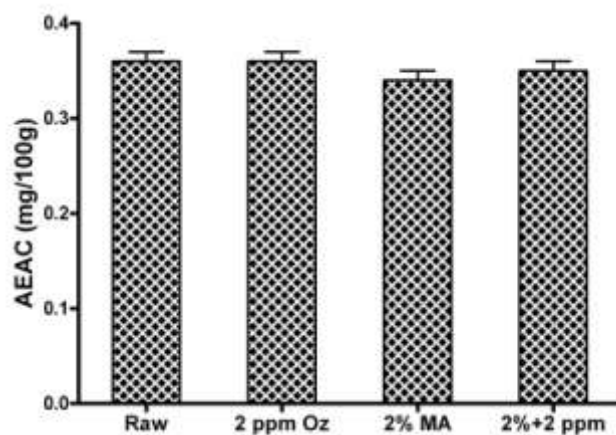


Fig. 4.32a Effect of processing on ascorbic acid equivalent antioxidant activity (AEAC) of raw, malic acid and ozone treated radish sprouts

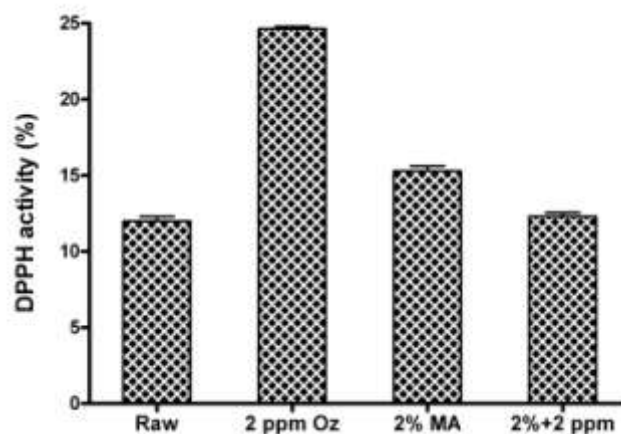


Fig. 4. 32b Effect of processing on DPPH radical scavenging activity (%) of raw, malic acid and ozone treated radish sprouts

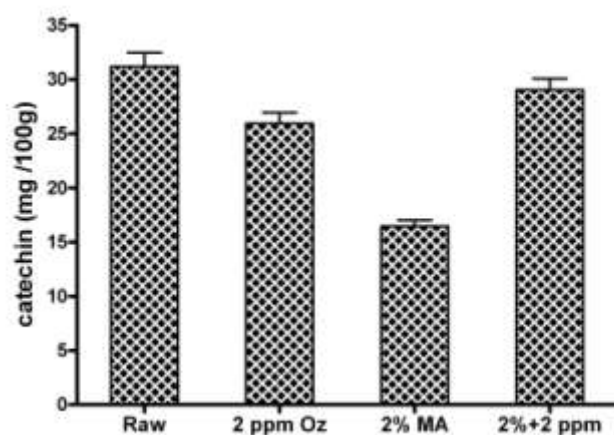


Fig. 4. 32c Effect of processing on flavonoid content of raw, malic acid and ozone treated radish sprouts

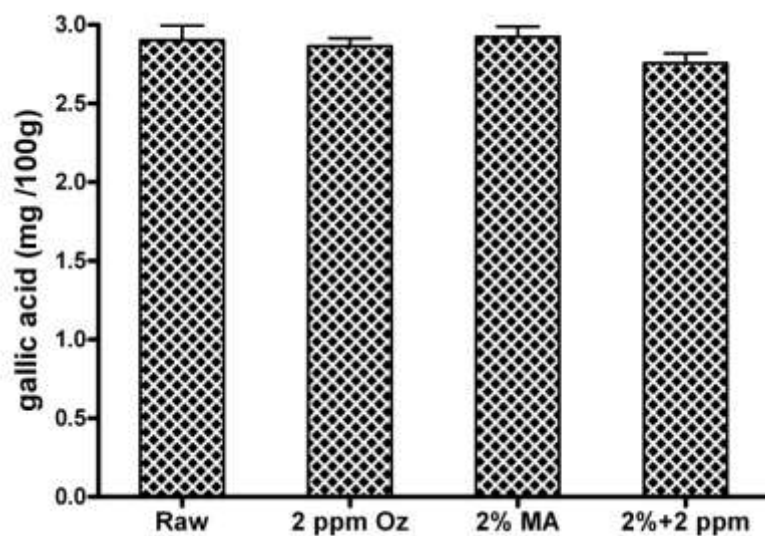


Fig. 4. 32d Effect of processing on polyphenolic content of raw, malic acid and ozone treated radish sprouts

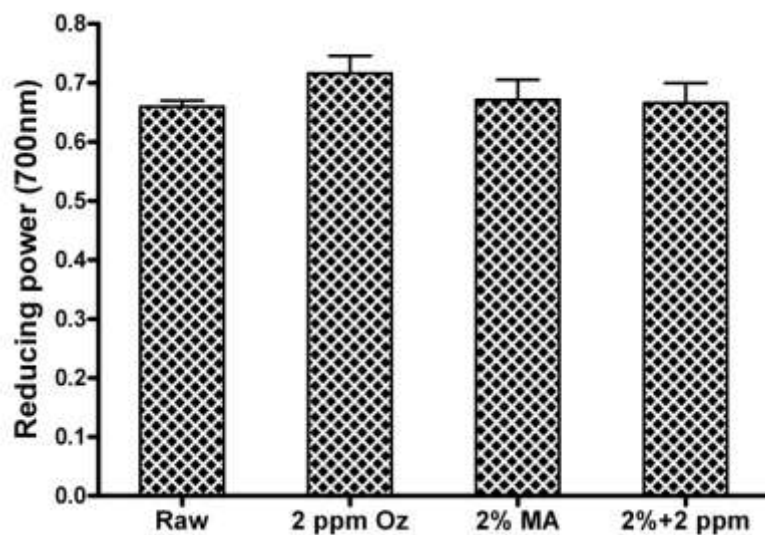


Fig. 4. 32e Effect of processing on reducing power of raw, malic acid and ozone treated radish sprouts

4.10.4 Antioxidant profile of moong bean sprouts

Sprouts have substantial nutritional benefit on the human body because of their high concentration of proteins and essential nutrients in mobilized forms, which can be used readily by the body. Sprouts like moong bean contain concentrated amounts of phenolics. Moong bean sprouts, popular in Asian cuisine are rich in vitamins and minerals. The losses during moong bean sprouts processing need to be taken into account when calculating the dietary intake of dietary antioxidants from processed food.

4.10.4.1 Sensory properties

The sensory quality of treated, minimally processed sprouts was evaluated by a panel of ten trained judges by grading for texture, taste, smell, color, visual appearance (Fig. 4.33) and overall acceptability score on a nine-point hedonic scale (Table 4.11).

Table 4.11 Mean sensory score of malic acid treated moong bean sprouts on nine point hedonic scale ($p>0.05$)

| Attributes | Untreated | 2%+2 ppm treated |
|---------------------------|-----------|------------------|
| Texture/freshness (9) | 7.9±0.05 | 7.8±0.06 |
| Taste/flavor (9) | 7.5±0.09 | 7.6±0.02 |
| Aroma/smell (9) | 7.6±0.08 | 7.7±0.07 |
| Color/appearance (9) | 7.9±0.04 | 7.7±0.02 |
| Overall acceptability (9) | 7.7±0.08 | 7.7±0.05 |

Note: Average of three mean values; values with ± represent standard deviation

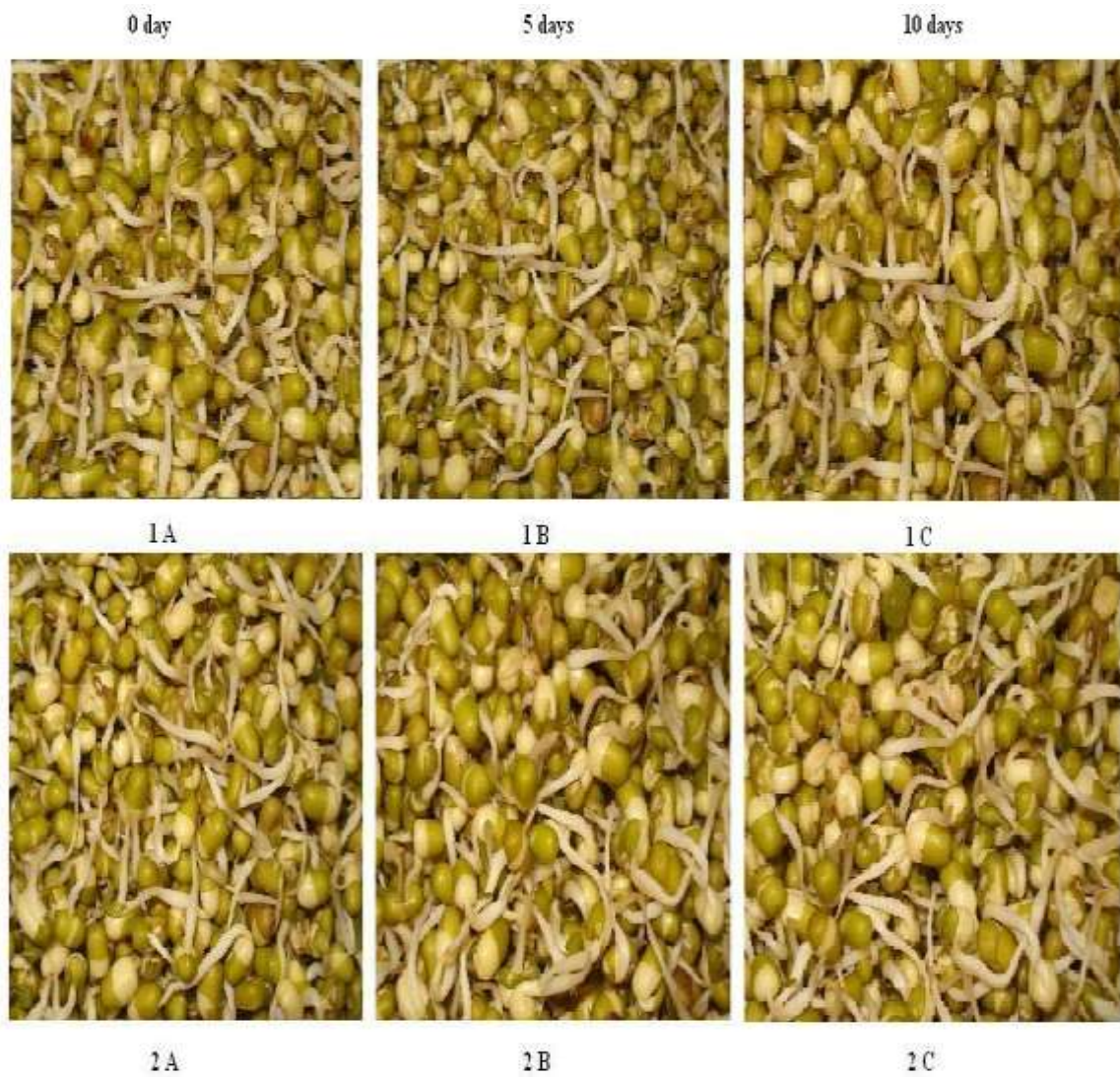


Fig. 4.33 Appearance of moong bean sprouts before and after treatment. Images 1A, 1B and 1C represents without treatment after storage for 10 days. Images 2A, 2B and 2C showing images after combined treatment of 2% malic acid along with 2 ppm ozone after storage for 10 days

4.10.4.2 Effect of treatment on total antioxidant properties of moong bean sprouts

An important factor of plant food is their antioxidant properties. ABTS activity expressed as ascorbic acid equivalent activity (AEAC) of raw moong bean sprouts. Significant changes ($p < 0.05$) in ABTS activity of raw moong bean sprouts (2.34 mg/100 g) following acid treatment (2.01 mg/100 g), ozone treatment (2.02 mg/100 g) and combined treatment (2.14 mg/100 g) was not observed (Fig. 4.34a).

The antioxidant activities of the raw and treated moong bean sprouts as determined by scavenging DPPH radical are presented in Fig. 4.34b. Ozone treated sprouts exhibited a non significant ($p < 0.05$) change (28%), no change was observed in moong bean sprouts following the combined treatment (23%) in comparison to control (24%).

The flavonoid content of and moong bean sprouts is shown in Fig. 4.34c. For moong bean sprouts flavonoid content (expressed as mg of (+)-catechin equivalents per 100 g sample), of raw sprouts was 37.46 mg/100 g, for acid treated 35.26 mg/100 g, after ozonation it was 38.66 mg/100 g and after combined treatment it was 38.45 mg/100 g. No significant ($p > 0.05$) change was observed in flavonoids of moong bean sprouts.

Effects of treatment on the total polyphenolics of moong bean sprouts are shown in Fig. 4.34d. The polyphenolics of raw moong bean sprouts (expressed as mg of gallic acid equivalents per 100 g of sample), 9.53 mg gallic acid /100 g, acid treated 8.90 mg gallic acid/100 g, ozone treatment 9.0 mg gallic acid/100 g and combined treated was 10.12 mg gallic acid /100 g of the sample respectively. Therefore, no significant ($p > 0.05$) change in polyphenolic contents were observed in moong bean sprouts.

Reducing powers obtained for all the extracts are presented in Fig. 4.34e. The reducing power ability of raw moong bean sprouts was 0.99, following acid and ozone treatment these values were 0.96 and 0.978 respectively. A notable departure from the previous values was observed following the combined treatment (1.04).

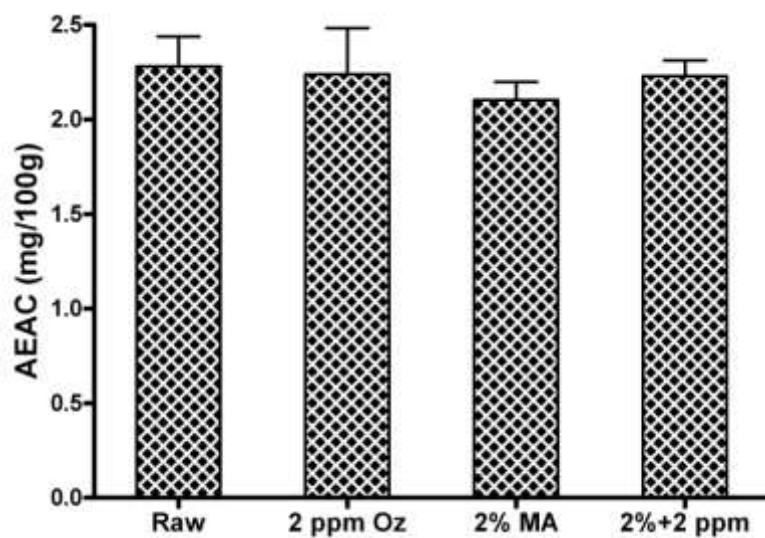


Fig. 4.34a Effect of processing on ascorbic acid equivalent antioxidant activity (AEAC) of raw, malic acid and ozone treated moong bean sprouts

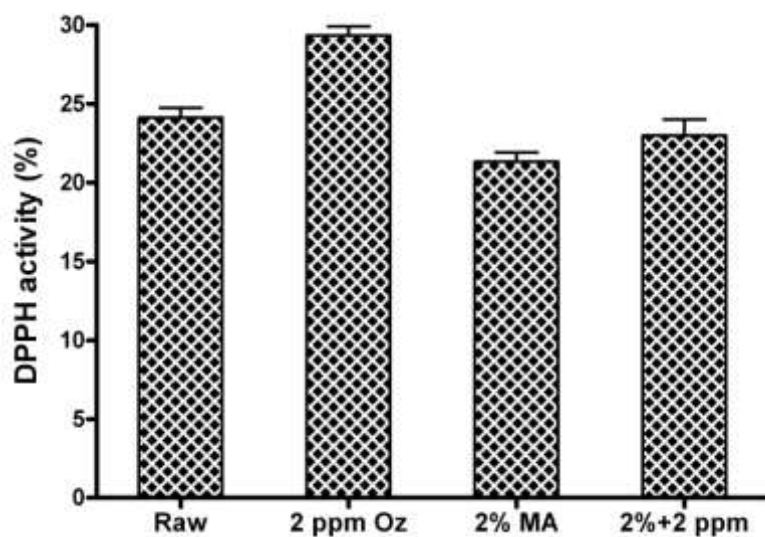


Fig. 4.34b Effect of processing on DPPH radical scavenging activity (%) of raw, malic acid and ozone treated moong bean sprouts

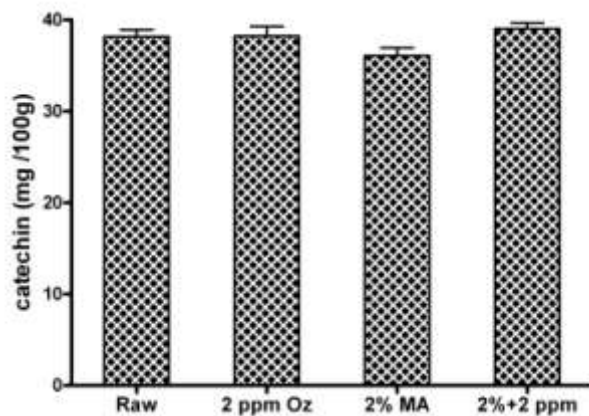


Fig. 4.34c Effect of processing on flavonoid content of raw, malic acid and ozone treated moong bean sprouts

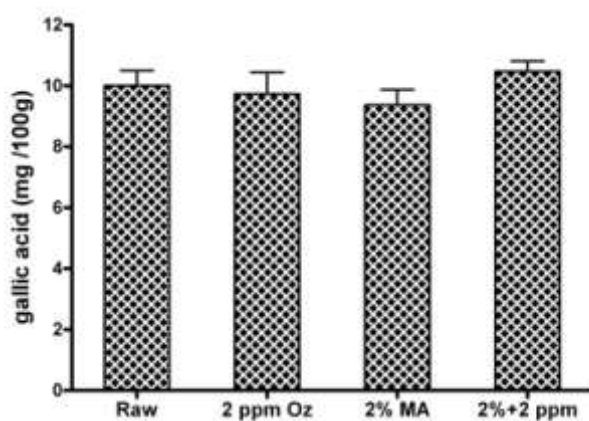


Fig. 4.34d Effect of processing on polyphenolic content of raw, malic acid and ozone treated moong bean sprouts

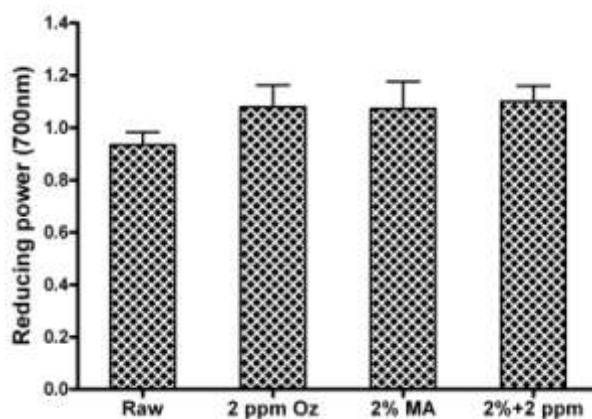


Fig. 4.34e Effect of processing on reducing power of raw, malic acid and ozone treated moong bean sprouts

4.10.5 Antioxidant profile of Mushroom

Edible mushrooms are widely utilized as human food. Mushrooms are appreciated, not only for texture and flavor but also for their chemical and nutritional properties. Mushrooms have also been reported as therapeutic foods that are useful in preventing diseases such as hypertension, hypercholesterolemia, and cancer. These functional characteristics are mainly due to their chemical composition. It becomes pertinent therefore to evaluate the nutritional levels of mushroom after a treatment process. Therefore, nutritional level of ozone and organic acid treated mushroom samples were evaluated and compared against control or untreated mushrooms.

4.10.5.1 Sensory properties

As indicated in Table 4.12, the malic acid treated mushrooms could not be differentiated than the untreated (control) counterparts on the basis of sensory parameters used ($p > 0.05$). Mushrooms treated with acetic acid had slight odour which distinguishable from raw (untreated) mushrooms and was not acceptable. All the malic acid and ozone treated samples of mushroom were sensorially acceptable in terms of texture, consistency, smell, color and appearance (Fig. 4.35) and overall acceptability.

4.10.5.2 Effect of treatment on chemical components and dietary fibers of mushroom

Chemical compositions of the treated samples are shown in Table 4.12. None of the parameters viz moisture, protein, carbohydrate, fat and ash changed following treatment with 2% and 4% malic acid. No significant ($p > 0.05$) differences were observed among these samples. These results were similar to those of Jin et al. (2006) who observed that the chemical composition of cereals showed no differences following treatment with electrolyzed water. Total dietary fiber (TDF), Soluble dietary fiber (SDF) and Insoluble dietary fiber (IDF) contents in treated sample were comparable to each other and were not significantly ($p > 0.05$) different.

Table 4.12 Mean sensory score of malic acid treated button mushroom on nine point hedonic scale ($p>0.05$). Effect of treatment on moisture, protein, carbohydrate, fat, ash and dietary fibers content on malic acid treated mushroom. Effect processing on antioxidant activity (AEAC) in raw and malic acid treated mushroom extracts (n = 3)

| | | Untreated | | Treated | |
|----------------------------------|--------------------------|------------|------------|------------|--|
| Attributes | | Raw | MA | MA+O | |
| Sensory Analysis | Texture/freshness (9) | 7.4±0.05 | 7.4±0.05 | 7.2±0.06 | |
| | Consistency (9) | 7.4±0.05 | 7.0±0.08 | 7.3±0.07 | |
| | Aroma/smell (9) | 7.1±0.07 | 7.4±0.05 | 7.2±0.05 | |
| | Color and Appearance (9) | 7.4±0.05 | 7.5±0.16 | 7.8±0.02 | |
| Overall acceptability (9) | | 7.3±0.05 | 7.4±0.08 | 7.5±0.05 | |
| Chemical Components | Moisture | 8.31±0.09 | 8.34±0.040 | 8.03±0.060 | |
| | Protein | 22.34±0.04 | 23.76±0.08 | 23.12±0.03 | |
| | Carbohydrate | 61.94±0.02 | 61.12±0.06 | 62.23±0.08 | |
| | Fat | 2.57±0.090 | 1.31±0.880 | 2.28±0.120 | |
| | Ash | 4.84±0.120 | 4.78±0.090 | 4.34±0.110 | |
| Dietary Fibers | IDF | 12.12±0.03 | 12.78±0.04 | 11.04±0.05 | |
| | SDF | 1.56±0.080 | 1.78±0.190 | 1.45±0.060 | |
| | TDF | 13.78±0.02 | 13.65±0.07 | 12.40±0.08 | |
| AEAC | mg/100 g of Mushroom | 2.76±0.050 | 2.57±0.060 | 2.56±0.030 | |

Note: Average of three mean values; values with ± represent standard deviation

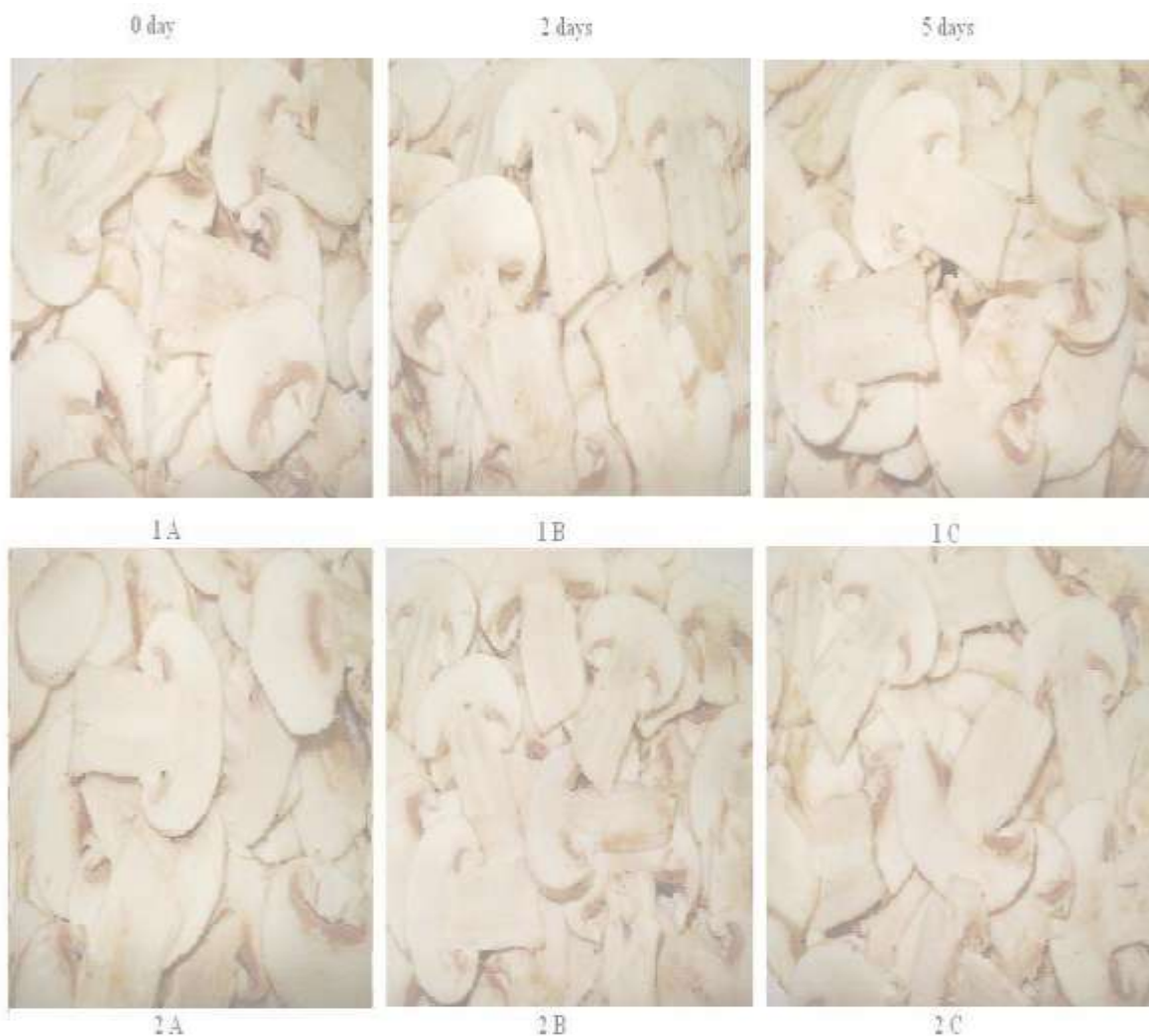


Fig. 4.35 Appearance of mushroom before and after treatment. Images 1A, 1B and 1C represents without treatment (control) after storage upto 5 days. Images 2A, 2B and 2C showing images after combined treatment of 2% malic acid along with 2 ppm ozone after storage for 5 days

4.10.5.3 Effect of treatment on total antioxidant properties of mushroom

The total antioxidant activities of the raw and treated mushroom, as determined by scavenging ABTS radical, are presented in Table 4.9. The ABTS radical scavenging activity of raw mushroom extract expressed as ascorbic acid equivalent antioxidant activity (AEAC) showed 2.76 mg ascorbic acid equivalents/100 g sample. After treatment with 2% and 4% malic acid, the AEAC values did not change significantly ($p>0.05$) and were 2.57 mg ascorbic and 2.56 mg ascorbic acid equivalents/100 g sample respectively.

The antioxidant activities of the raw and treated mushroom as determined by scavenging DPPH radical are presented in Fig. 4.36a. The DPPH radical scavenging activity of raw mushroom extract showed 76%. After processing, the DPPH radical scavenging activities of treated mushroom with 2% malic acid was 69% and mushroom treated with 4% malic acid was 71%. No significant change ($p>0.05$) was observed.

The total flavonoid content of raw and treated mushrooms is shown in Fig. 4. 36b. Flavonoids of raw mushroom were 37.12 mg/100 g mushroom and following treatment with 2% and 4% malic acid flavonoids were 31.54 mg and 31.53 mg /100 g mushroom. It has been suggested polyphenols directly contributes to antioxidant activity since compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily is ingested from a diet rich in fruits and vegetables (Tanaka et al., 1998).

Effects of treatment on the total polyphenolics of mushroom extracts are shown in Fig. 4. 36c. The polyphenolics of raw mushroom was 10.09 mg gallic acid /100 g mushroom respectively. Following treatment with 2% and 4% malic acid polyphenolics decreased to 9.88 mg and 9.9 mg gallic acid /100 g mushroom, this decrease was however not significant ($p>0.05$). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al., 1998).

SOD is concerned with the elimination of O_2^- that develops from natural substances and is retained in the body. SOD is one of the most important factors in oxidative defense systems (Smith and Doolittle, 1999). As shown in Fig. 4. 36d, SOD activity of malic acid treated mushroom (2%, 4%) extract was almost same as that of raw mushroom with no significant change ($p>0.05$).

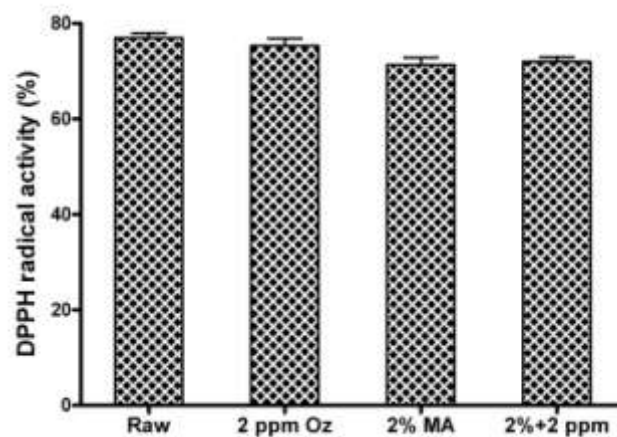


Fig. 4.36a Effect of processing on DPPH radical scavenging activity (%) of raw, malic acid and ozone treated mushroom

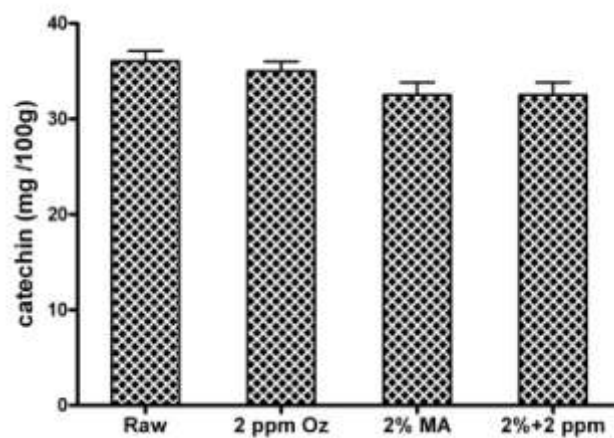


Fig. 4.36b Effect of processing on flavonoid content of raw, malic acid and ozone treated mushroom

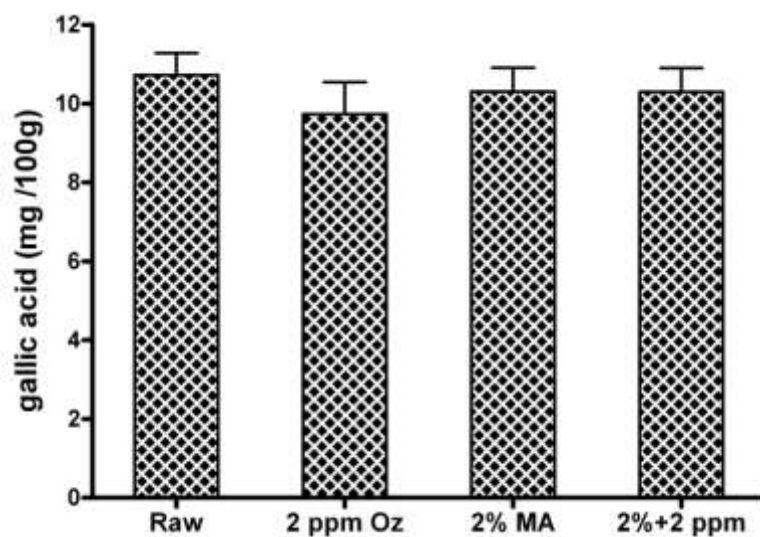


Fig. 4.36c Effect of processing on polyphenolic content of raw, malic acid and ozone treated mushroom

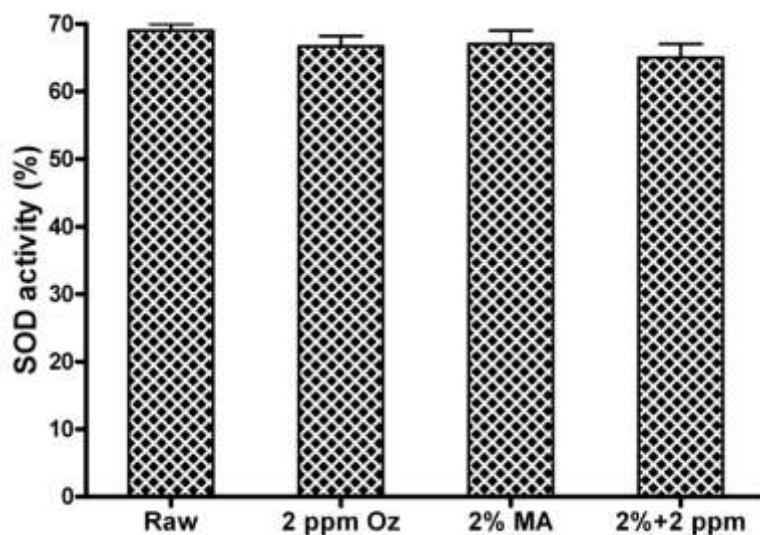


Fig. 4.36d Effect of processing on sodium oxide dismutase activity (%) of raw, malic acid and ozone treated mushroom

4.10.5.4 Effect of treatment on color/whiteness of mushroom

A time dependent study was conducted for 5 days followed by storage at 15°C and 4°C to examine the visual changes. There was a subtle visible change in the surface appearance of the mushrooms from whiteness to a brownish tinge, as visual assessment is considered to be unreliable; a more accurate measurement of color change was conducted using a color spectrophotometer. Fig. 4.37 presents, a color change from whiteness to blackness on a scale of a 100 expressed as L^* values of raw and organic acid treated mushrooms. There was a gradual decrease in L^* values with color change from whiteness to blackness in acetic acid and citric acid treated mushrooms. In malic acid treated mushrooms, when L^* value has increased in comparison to raw mushroom with a highly significant difference between treatment groups ($p < 0.05$). The color change was more pronounced when the mushrooms were stored at 15°C compared to those stored at 4°C.

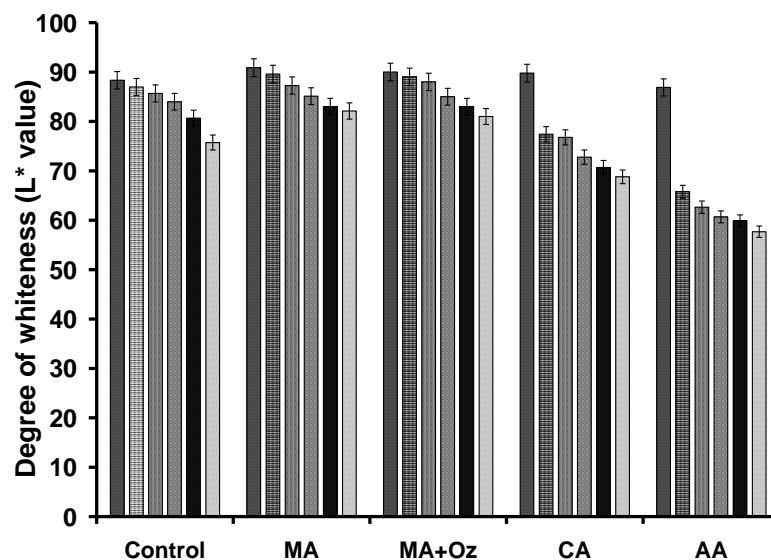


Fig. 4.37 Degree of whiteness of mushroom after treatment with 2% malic acid, 2% acetic acid, 2% citric acid and combined treatment with 2% malic acid along with 2 ppm ozonated water stored at 15°C for 5 days (n = 3)

The sequence encodes 0 d; 1 d; 2 d; 3 d; 4 d; 5 d

4.10.6 Antioxidant profile of betel leaves

Besides being chewed usually after meals, betel leaves are extensively used in folk medicine specially in the Indian subcontinent. Earlier, studies have demonstrated the antiulcerogenic property of the leaf extract. In addition, its antimicrobial, antifungal, and anti-inflammatory, as well as radioprotective, antioxidative, and immunomodulatory properties have been reported.

4.10.6.1 Sensory properties

The sensory quality of treated, minimally processed betel leaves was evaluated by a panel of ten trained judges by grading for color, flavor, taste and aroma and overall acceptability score on a nine-point hedonic scale (Table 4.13). All the treated samples of betel leaves were sensorially acceptable in terms of color and appearance (Fig. 4.38), taste, texture and consistency. The analysis of the sensory scores revealed that control betel leaves as well as the minimally processed betel leaves retained their flavor, aroma, and overall acceptability up to 5 days of storage.

Table 4.13 Mean sensory score of malic acid treated betel leaves on nine point hedonic scale (p>0.05)

| Attributes | Untreated betel leaves | 2%+2 ppm treated |
|---------------------------|------------------------|------------------|
| Texture/freshness (9) | 7.5±0.05 | 7.4±0.06 |
| Taste/flavor (9) | 7.7±0.08 | 7.6±0.18 |
| Aroma/smell (9) | 7.6±0.08 | 7.5±0.07 |
| Color/appearance (9) | 7.8±0.17 | 7.9±0.02 |
| Overall acceptability (9) | 7.7±0.09 | 7.6±0.08 |

Note: Average of three mean values; values with ± represent standard deviation

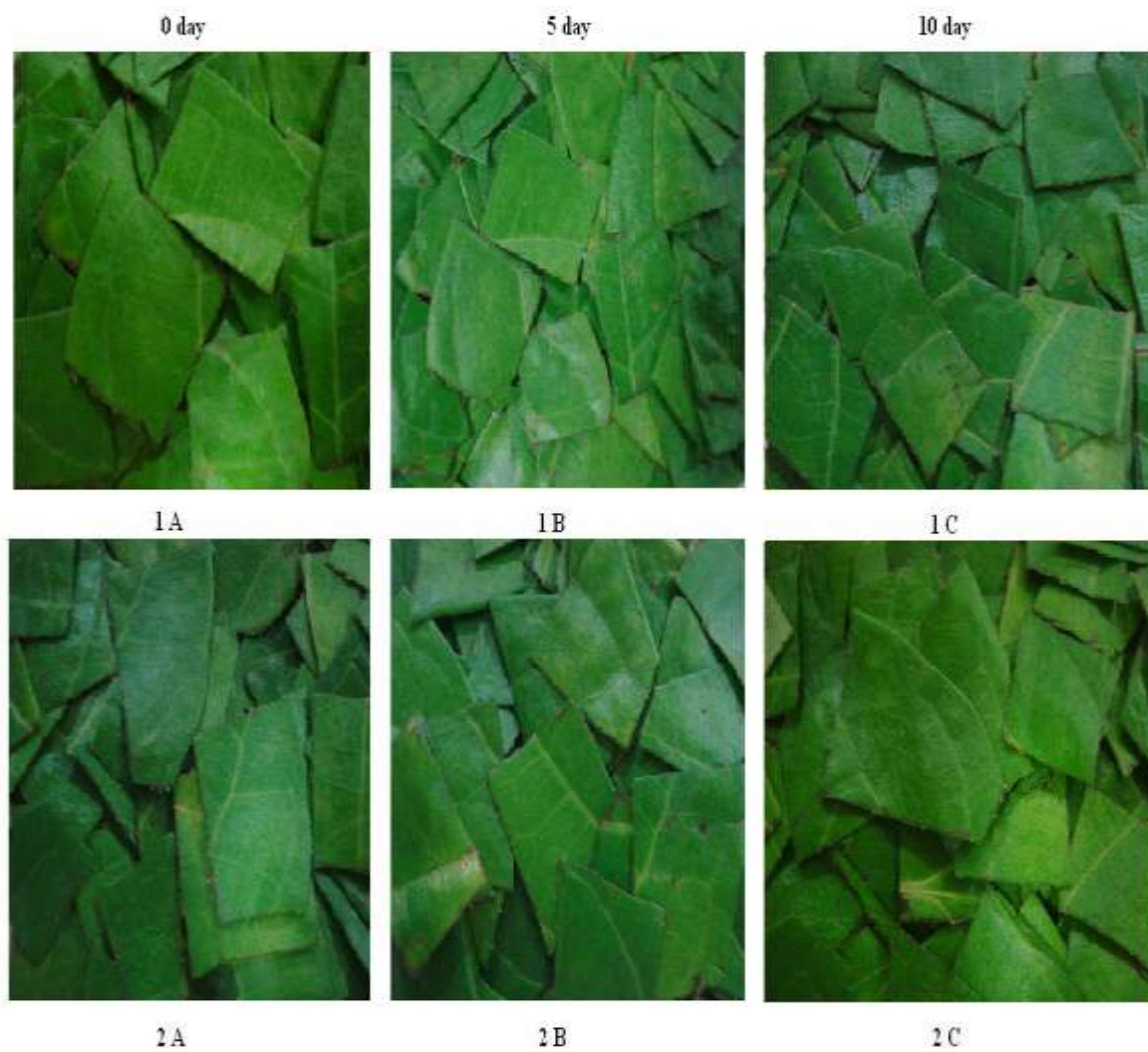


Fig. 4.38 Images of visual appearance of betel leaves before and after treatment. Images 1A, 1B and 1C represents without treatment after storage for 10 days. Images 2A, 2B and 2C showing images after combined treatment of 2% malic acid along with 2 ppm ozone after storage for 10 days

4.10.6.2 Effect of treatment on total antioxidant properties of betel leaves

ABTS activity expressed as ascorbic acid equivalent activity (AEAC) of raw betel leaves was found to be 0.23 ascorbic acid equivalents /100 g of sample, malic acid and combination treatments were 0.24 and 0.25 respectively. Similarly, no significant changes ($p>0.05$) in ABTS activity of untreated and minimally processed betel leaves was observed (Fig. 4.39a).

The antioxidant activities of the raw and treated betel leaves as determined by scavenging DPPH radical are presented in Fig. 4.39b. The DPPH radical scavenging activity of raw betel leaf extract found to be 53% and significantly ($p<0.05$) increased to 57% after processing.

The flavonoid content of raw and treated betel leaves sample is shown in Fig. 4.39c. The flavonoids of raw betel leaves (expressed as mg of (+)-catechin equivalents per 100 g sample), were 32.14 mg/100 g sample and did not change significantly (32.18 mg /100 g; $p>0.05$) following treatment with 2% acetic acid.

Effects of treatment on the total polyphenolics of sample extracts are shown in Fig. 4.39d. The polyphenolics of raw sample (expressed as mg of gallic acid equivalents per 100 g of sample), was 1.155 mg gallic acid /100 g sample respectively. After treatment polyphenolics were insignificantly ($p>0.05$) changed to 1.156 mg gallic acid /100 g sample.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing powers obtained for all the extracts were excellent and did not change significantly ($p>0.05$) after the treatment (Fig. 4.39e). The reducing ability of raw sample showed activity of 0.798 and treated sample with acetic acid showed activity of 0.806 which is significantly ($p<0.05$) higher as that in case of raw sample extract.

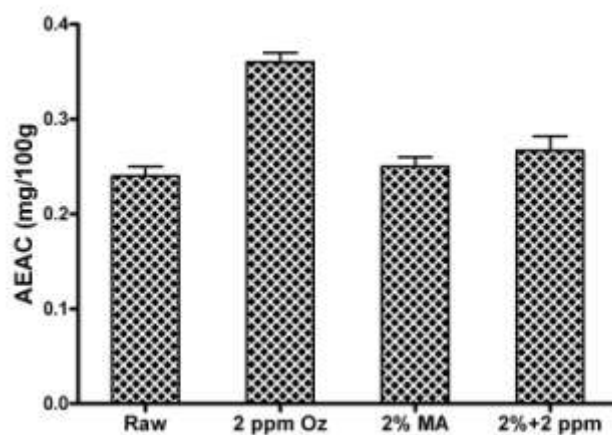


Fig. 4.39a Effect of processing on ascorbic acid equivalent antioxidant activity (AEAC) of raw, malic acid and ozone treated betel leaves

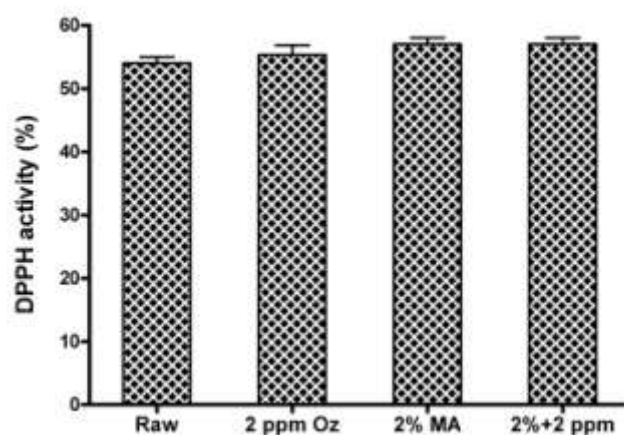


Fig. 4.39b Effect of processing on DPPH radical scavenging activity (%) of raw, malic acid and ozone treated betel leaves

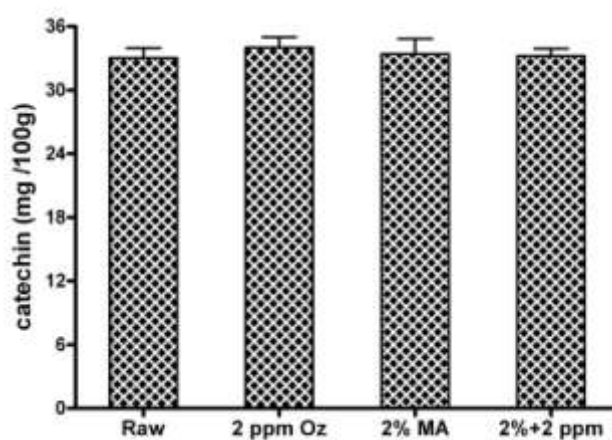


Fig. 4.39c Effect of processing on flavonoid content of raw, malic acid and ozone treated betel leaves

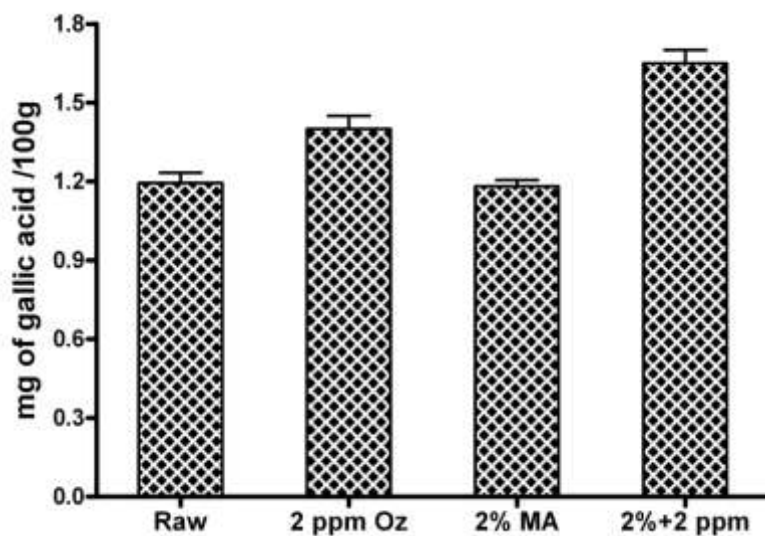


Fig. 4.39d Effect of processing on polyphenolic content of raw, malic acid and ozone treated betel leaves

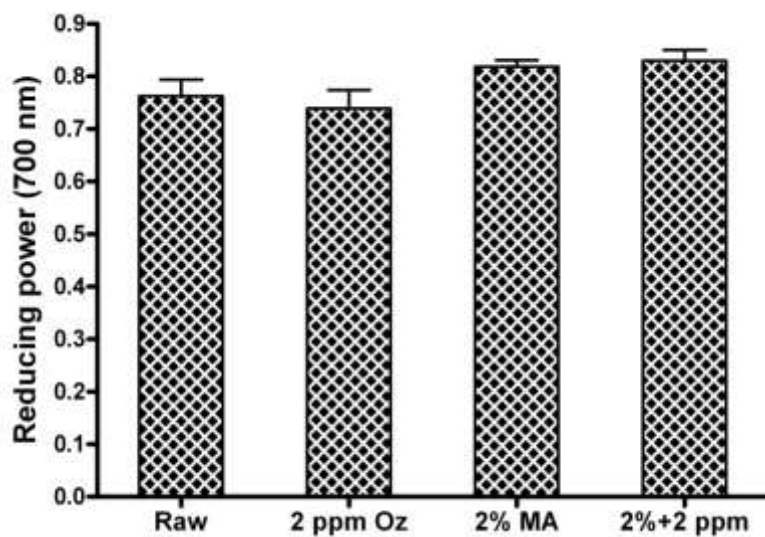


Fig. 4.39e Effect of processing on reducing power of raw, malic acid and ozone treated betel leaves

4.11 Effect on resident flora and spoilage microorganisms of vegetables

During harvesting, processing, and handling operations food might become contaminated with a wide range of microorganisms. One outcome of food spoilage results from the undesirable growth of microorganisms in food is the production of volatile compounds during their metabolism, which the human nose and mouth detect. Given favorable conditions, the microorganisms will multiply and cause spoilage during the distribution and storage of foods. Spoilage is not only due to growth of microorganisms, but also to the production of end metabolites which result in off odors, gas, and slime (Forsythe, 2000). Berrang et al. (1989) observed that modified atmosphere storage extended the shelf life of asparagus long enough to allow *L. monocytogenes* to reach higher populations than would have occurred under ambient-air storage. Wells and Butterfield (1999) reported that *Salmonella* serovar Typhimurium grew better on tomatoes, potatoes, and onions in the presence of spoilage molds *Botrytis* spp. or *Rhizopus* spp. than when the molds were absent. However, absence of resident flora was observed on the treatment samples subjected to combined treatment following storage implying a reduction in possible spoilage mediated by these microorganisms. Our study demonstrates that after combined treatment on all the vegetables tested completely removed resident flora and other food spoiling organisms (Table 4.14). Thus, it is very much obvious that shelf-life and loss of antioxidants and nutrients from fresh vegetables may decline to some extent following treatment. Similarly, the growth of *E. coli* O157:H7 is known to occur in bruised areas of apples (Dingman, 2000). Therefore, food microbiologists and technologists should always consider whether the benefits of reduced spoilage outweigh safety concerns (Brackett, 2001). Nonpathogenic bacteria may also constitute biofilm communities on common salad vegetables (including tomatoes and lettuce) and then, potentially pathogenic microorganisms might become sequestered and protected within these biofilms (Donlan, 2002; Rayner et al., 2004).

Table 4.14 Resident flora in treated vegetable samples during 10 days storage after combined treatment

| Sample | Days | Fungus | APC |
|--------------------|------|--------|-----|
| Carrot | 5 d | ND | ND |
| | 10 d | ND | ND |
| Turnip | 5 d | ND | ND |
| | 10 d | ND | ND |
| Radish sprouts | 5 d | ND | ND |
| | 10 d | ND | ND |
| Moong bean sprouts | 5 d | ND | ND |
| | 10 d | ND | ND |
| Mushroom | 5 d | ND | ND |
| | 10 d | ND | ND |
| Betel leaves | 5 d | ND | ND |
| | 10 d | ND | ND |

Note: ND means not detected

DISCUSSION

Over the decade, compelling evidences of health beneficial effects of fresh produces or vegetables consumed raw have been documented. Consumer awareness of functional attributes of fresh produce and shift towards nature like attributes has prompted the food industry for a safe and consistent supply of fresh produce items. With the increasing demand for produce, developing countries with diverse and abundant agro resources have assumed importance as prospective suppliers. However, the predilection of 'raw consumption' has been marred by the recurrence of food borne pathogens and outbreaks. The pathogenic microorganisms usually enter the produce during irrigation, harvesting, processing and distribution. In fact high aerobic bacterial counts have been traced to irrigation water, ponds water or pollution by human and animals. Further invasive operations such as cutting and subsequent storage at ambient temperature, lead to multiplication of the surviving microbes which have gained entry during harvesting or prior to harvesting.

Several studies have established the routes of entry, survival and growth of pathogens in fresh produce destined to be consumed as salads (Charles et al., 1976). Research in occurrence of pathogenic bacteria in fresh produce in developing countries are scanty, therefore export options remain restricted. Though fragmentary, microbiological studies of fresh produce in developing countries have indicated high disease outbreaks resulted from fresh cut produce as well as pathogens (Harris et al., 2003). The Guideline of Hazard Analysis and Critical Control Points- Total Quality Management (HACCPTQM, 1998) has laid down the parameters of raw food. Raw foods containing $<10^2$ CFU/g of organisms (total aerobic counts) is rated "Good" and those containing $>10^8$ CFU/g as "Spoiled food". According to HACCPTQM guideline, such samples require to be considered as spoiled grade

food and unsafe for consumer. In most cases reported in the above studies, fresh cut vegetables were found to be unfit for consumption. Intervention measures, systematic microbiological analysis of commonly consumed fresh vegetables may enable better understanding and generation of a profile of high risk pathogens. Such findings may be important in devising appropriate intervention strategies for vegetables consumed raw and assuring consumer safety. Carrots, turnips, sprouts and mushrooms are widely consumed in India in raw form; betel leaves are chewed.

Carrot enjoys a favorable status in terms of popularity. In fact during recent years, worldwide consumption of carrots has increased steadily (Marquez, 2010). Pathogens from carrot have been documented earlier like *Salmonella typhimuirum* and *E. coli* O157:H7 (Islam et al., 2004; Abdul et al., 1993). Turnip is one of the oldest cultivated vegetables, being used for human consumption all over the world. Turnips are popular and consumed in cut form in India. Presence of food borne pathogens such as *Aeromonas* spp. has been documented earlier in turnip (McMahon and Wilson, 2001).

Raw sprouts comprise of an important segment of fresh produces routinely consumed by Indian and other Asian population. Radish and moong bean sprouts in particular is a delicacy which is consumed either raw or slightly cooked form as side dishes. Most outbreaks of human disease due to the consumption of sprouts have been attributed to contamination of the seeds used for sprouting. It has been suggested that moist, warm conditions of sprouting may allow small numbers of pathogens present on seeds to multiply by several orders of magnitude during the sprouting period. However, other potential contamination routes exist in the production process. These include contaminated irrigation water and wash water, contaminated growth media such as compost (when used), contaminated production equipment or infected food handlers. These routes of contamination must be controlled by good agricultural practice and good hygiene practice. Despite the important nutritional

characteristics, consumption of raw sprouts has been associated with numerous outbreaks of food-borne illness (Taormina et al., 1999). Several microbiological surveys have shown the presence of food borne pathogens in sprouts (Andrenne et al., 2001). For instance, *E. coli* O157: H7, *Salmonella* spp. and *Listeria monocytogenes* have been isolated from sprouted seeds, including alfalfa, moong bean (Taormina et al., 1999). In the present study, *Shigella flexneri* were detected and subsequently characterized further from radish and moong bean sprouts. Sprouting seeds are usually susceptible to contamination by human pathogens, like *Shigella* due to the high temperature (25-35°C) and humidity typically used in production (Hara-Kudo et al., 1997). The quality of seeds and water used for sprouting also affects the microbial load (Dale and Greg, 1991).

Mushrooms have been consumed throughout the world for many centuries, not only for nutritional value but also for functional purpose. Although, there have been no reported outbreaks associated with consumption of mushrooms, several reports have established human pathogens in fresh mushrooms. In a survey in the Pacific Northwest, 1% of retail samples tested positive for *L. monocytogenes* and 5% tested positive for *Salmonella* spp. (Samadpour et al., 2006). In the Netherlands, *L. monocytogenes* was recovered in 10% of mushrooms purchased at a grocery store (Van et al., 1989). Doyle and Schoeni (1986) isolated *C. jejuni* in three of 200 (1.5%) retail mushroom packages from several Midwestern U.S. grocery stores. Recently, threat due to the evolution of new microbes (highly virulent, antibiotic resistant, ability to survive in adverse conditions) has led to increased concerns of safety. Contamination with such pathogens increases the chances to contaminate other raw vegetables, affects the health and hygiene of personnel and hinders export options. *Cronobacter sakazakii* an emerging pathogen was isolated from mushroom and betel leaves in this study. Earlier, *Cronobacter sakazakii* from infant formula milk has been documented in India but not yet reported in fresh produce. Although reports of *Cronobacter sakazakii*

mediated illnesses (neonatal meningitis) in clinical settings have been documented (Ray et al., 2007) in India. Betel leaves (*Piper betle*) is a common plant cultivated in Asian Countries. Despite the excellent medicinal properties and commercial significance, betel leaves have been attributed to typhoid outbreak in betel-eating areas (Taylor & Francis Health sciences, 2004). Several multi-drug resistant strains of *Salmonella* have been reported in betel leaves (Singh et al. 2006). More recently, betel leaves were shown to harbour *Yersinia* spp. and *Aeromonas* spp. (Singla et al., 2009). Based on these results it was envisaged appropriate to conduct inactivation studies on fresh vegetables using the detected pathogens in simulation studies.

In this study, *Salmonella typhimurium* in *Daucus carota* (carrot) *Brassica rapa* (turnip), *Shigella flexneri* in *Daucus carota* (carrot) *Brassica rapa* (turnip), *Raphanus sativa* (radish sprouts), *Phaseolus aureus* (moong bean sprouts) and *Cronobacter sakazakii* in *Agaricus bisporous* (button mushroom) and *Piper betle* (betel leaves) were the predominant pathogens. It was thus envisaged that appropriately designed simulation studies with effect of ozone and organic acids on planktonic cells of these pathogens in culture media as well on fresh produce itself would be pertinent for understanding the effects of sanitizers.

Survival of *Cronobacter sakazakii* under acidic pH has been reported by other researchers. Edelson-Mammel et al. (2006) investigated the acid resistance of 12 strains of *Cronobacter sakazakii* in TSB. They observed that 10 of the 12 strains showed less than a 1 log reduction at pH 3.5 following a 5 h incubation at 36°C whereas levels of *Cronobacter sakazakii* were decreased by 4.9 to >6.3 log at pH 3.0 following 5 h incubation at 36°C. No growth of *Cronobacter sakazakii* was observed in TSB containing 31-63 mM of acetic acid or 16-31 mM of propionic acid; therefore these acids were the most effective ones for inhibiting growth of *Cronobacter sakazakii*. *Cronobacter sakazakii* did not grow in 125-500 mM malic acid or in 63-250 mM citric acid, respectively (Back et al., 2008). In this study,

among five different concentrations used 1.2% of malic acid and 1% of acetic acid was effective for the control of complete inhibition of *Shigella flexneri* (SF1 and SF2) and *Cronobacter sakazakii* (CS1 and CS2). But, a higher concentration of 2% of organic acid was able to control the growth of *Salmonella typhimurium* (ST1 and ST2). Ozone 1.2% in gaseous and aqueous states has been explored for surface disinfection of packages and equipment (Pascual et al., 2007). Gaseous ozone has been studied for inactivation of microorganisms on various fresh fruits and vegetables during storage. For instance, Komanapalli and Lau (1996) examined the effect of gaseous ozone at 600 mg/L on *Escherichia coli* K-12 for up to 30 min. Kim and Yousef (2000) studied the inactivation kinetics of ozone treated Gram-positive and Gram-negative bacteria in a batch-type reaction system. Ozone was very effective in inactivating *E. coli* O157:H7, *Pseudomonas fluorescense*, *Leuconostoc mesenteroides*, and *Listeria monocytogens* and among these microorganisms *E. coli* O157:H7 was the most resistant while *L. monocytogens* was the most sensitive.

In another study, Gram-negative bacteria were found to be more sensitive to aqueous ozone than were the Gram-positives among tested microorganisms (Restaino et al., 1995). Similarly, Sobsey (1989) reported that Gram-positives were more resistant to ozone treatments than were the Gram-negatives. When *Salmonella* Enteritidis was treated with 1.5 ppm ozone in distilled water, population of the pathogen decreased by 6 log units (Kim et al., 1999). Wright et al. (2000), who reported that water treatment of *E. coli* O157:H7 inoculated apples decreased bacterial populations by 1.1 log and by Sapers and Jones (2006), where, *E. coli* NRRL B-766 population reduced by <1 log in tomatoes after immersion in water. The study provided valuable evidence of the efficacy of ozonated water against tested pathogenic strains. Treatment of *S. sonnei* with 1.6 and 2.2 ppm aqueous ozone decreased the population of the pathogen by 3.7 and 5.6 log units, respectively (Selma et al., 2007).

Fresh fruits and vegetables are susceptible to contamination with pathogenic and spoilage microorganisms, beginning from the pre-harvesting stage through post processing. Ozone as a disinfectant probably enjoys a clean history of application. First introduced as a disinfectant in the treatment of drinking water in 1893 at Oudshoorn, Netherlands (Rice et al., 1981), ozone has been used for water disinfection in many European countries (Bryant et al., 1992). Ozone may be used in the gaseous or aqueous state in food processing. In general, gaseous ozone is applied for storage applications whereas the aqueous form is used for surface decontamination of foods, equipment or packaging material. Ozone has been explored also for treating agricultural commodities because it provides more disinfecting power than other sanitizers (e.g., chlorine) and it removes a myriad of contaminants including microorganisms resistant to chlorine treatment (Graham, 1997).

Furthermore, ozonated water has been applied to fresh-cut vegetables for sanitation purposes reducing microbial populations and extending the shelf-life of some of these products (Beltran et al., 2005). An often-cited disadvantage of using ozone as a disinfectant is its high instability, making it difficult to predict how ozone reacts in the presence of organic matter (Cho et al., 2003). The efficacy of the wash treatments seems to be related to surface characteristics of vegetables and the antimicrobial susceptibility of each species. Readily available high ozone demand compounds may compete with microorganisms for ozone. High fat containing foods such as meat require higher ozone concentration than low fat foods such as fruits and vegetables (Kim et al., 2003). Beuchat (1998) found reduction in microbial levels of produce such as apples, grapes, oranges, raspberries, strawberries and pears on treatment with ozonated water. In general, the advantages of ozone outweigh its disadvantages making it a prospective sanitizer of fresh produce/vegetables suitable for commercial use.

Restaino et al. (1995) observed that bacterial pathogens such as *Salmonella typhimurium*, *Y. enterocolitica*, *S. aureus* and *L. monocytogenes* are sensitive to treatment with

20 ppm ozone in water. Treatment with ozonated water extended the shelf life of apples, grapes, oranges, pears, raspberries and strawberries by reducing microbial populations and by oxidation of ethylene to retard ripening (Beuchat, 1998). Microbial populations on berries and oranges were reduced by treatment with 2-3 ppm and 40 ppm respectively. Kim et al. (1999) reported a 2 log/g reduction in total counts for shredded lettuce suspended in water ozonated with 1.3 mM ozone. However, its application is limited by certain aspects such as strong oxidizing activity, which may cause physiological injury to produce and corrosion of metals in the processing equipment. In this study, only 1 log and 0.6 log reduction was observed after complete immersion of carrot and turnip in ozonated water. The analysis of ozone treated samples revealed the presence of survivors of *Salmonella typhimurium* (ST1). Environment where bacteria are present is equally important for the inactivation efficacy of sanitizer treatment. Though higher concentration of ozone may be effective in several instances, the strong oxidizing activity may affect produce quality (Horvath et al., 1985). Carrots for example upon exposure to ozone gas during storage lost its original hue and had a lighter, less intense color their untreated counterparts (Liew and Prange, 1994).

Scarce information is currently available about inactivation of food borne pathogens such as *S. sonnei* by ozone. To reduce the risk associated with *Shigella* spp. on fresh produce such as sprouts, intervention strategies namely, UV-C (Rajkowski, 2007) and chlorine has been attempted (Wu et al., 2000). Effective removal was however not achieved during these treatments; moreover the use of sanitizers such as organic acids or ozone has not been reported elsewhere. Treatment of alfalfa sprouts with 23.2 mg/ml of ozone for 2 min resulted in 0.91 log reduction of *L. monocytogenes* (Wade et al., 2003). In this study, treatment of SF1 and SF2 with 2 ppm ozonated water could not overcome the growth of these pathogens in radish and moong bean sprouts and resulted in 1.5 and 1.8 log reduction of SF1 and SF2 following complete immersion for 5 min. Similarly, application of ozone (1.6 and 2.2 ppm)

for 1 min decreased *S. sonnei* population in water by 3.7 and 5.6 log₁₀ CFU/ml, respectively and by 0.9 and 1.4 log units with 2 ppm ozonated water in shredded lettuce (Selma et al., 2007). Achen and Yousef (2001) reported that washing with aqueous ozone was more effective in inactivating microorganisms on the surface of apples than in decontaminating the calyx and stem areas. Aqueous ozone treatments at 1.4 and 1.9 ppm for 1 min decreased *Y. enterocolitica* count in water (10⁸ CFU/ml, initially) by 4.6 and 6.2 log units, respectively. When this pathogen was inoculated on potato surface (~2 x 10⁵ CFU/g), ozonated water treatment at 5 ppm for 1 min decreased the population by 1.6 log only (Selma et al., 2007).

Doyle and Schoeni (1986) summarized that the mushrooms could be contaminated by pickers or packagers, who may lack good personal hygiene and sanitary practice may enable the pathogenic bacteria to survive on the surface of mushrooms during post harvest storage and retail display. No work has been done for the control of *Cronobacter sakazakii* using ozonated water in fresh vegetables. The observations of this study indicate reduction of *Cronobacter sakazakii* by 1.4 log of in mushroom after complete immersion and storage at 15°C for 5 days. Similarly, complete immersion in 2 ppm ozonated water inhibited pathogen growth by 1.3 log following complete immersion and storage at 28°C in betel leaves. Contrary to the present results, chlorine treatment did not affect growth of *E. coli* O157:H7 on lettuce during 18-day storage at 15°C (Li et al., 2001). However, another storage study reported complete inactivation of *E. coli* O157:H7 and *L. monocytogenes* on fruits and vegetables treated with 3 ppm ozone for 3 min, with both pathogens remaining undetectable on lettuce during 9 days of storage at 4°C (Rodgers et al., 2004). These contradictory results may be explained by combining the differences in storage temperature, storage time; type of the organism used and cell population number at day 0. In addition, cut mushrooms, which could release nutrients needed for microbial growth, may explain these contrasting results.

The popularity of organic acids as disinfecting agents in fresh produce down the ages, probably remains unrivaled. Several organic acids have been used, albeit with differential results for decontaminating fresh produces. One common organic acid effective against high risk pathogens is lactic acid. Lactic acid at concentrations between 0.3% and 4% was demonstrated to be effective in reducing *Y. enterocolitica* (Virto et al., 2005). Akbas and Olmez (2007) obtained maximal reductions of about 2 log CFU/g and 1.5 log CFU/g for *E. coli* and *L. monocytogenes* respectively, following treatment of fresh-cut iceberg lettuce dipped in 0.5% lactic acid. A concentration of lactic acid (0.2%) resulted in 5.08 log reductions of *Y. enterocolitica* on tomatoes. In contrast, 1% lactic acid produced only 1.71 log cfu reductions of *E. coli* O157:H7 on lettuce (Velazquez et al., 2009). During this study, lactic acid concentration was initially assayed to avoid alterations of vegetables appearance, smell or odour. However, a high concentration of organic acids (1-2%) was effective against all the pathogens studied. Therefore, malic acid and acetic acid was opted for studying their effects onto selected vegetables spiked with respective pathogens.

Minimally processed fruits and vegetables often provide a good substrate for microbial growth (Nguyen-the and Carlin, 1994). Readily available nutrients enable survival and subsequent proliferation of the pathogenic microorganisms. Escartin et al. (1989) reported that application of lemon juice to the surface of papaya inoculated with *Salmonella* Typhi reduced populations compared with control, but growth resumed after several hours. A reduction of 1.5 log was observed after complete immersion in carrot both with 2% malic acid and acetic acid for five min. Similarly, 2 log reduction was observed in turnip following treatment with 2% acetic acid for 5 min followed by storage at 28°C for ten days. Similar results were found with other organic acids. The treatment of raw cabbage with acidified sodium chloride for 5 and 10 min was not found to be effective in reducing the population of *E. coli* O157:H7 (Inatsu et al., 2005). Results reported by Liao and Shollenberger (2003)

were in the same line and indicated that wash with 2.4% acetic acid greatly improved the removal of *Salmonella* from apple slices. Sanitizers using per acetic acid at 40-80 ppm significantly reduced populations of *Salmonella* and *E. coli* O157:H7 on cantaloupe and honey dew melon (Park and Beuchat, 1999).

Sprouts have unique attributes and microbiological issues that have required investigations of non-traditional sanitation regimens. Based on the results of existing studies, chlorine and hydrogen peroxide were quoted to be effective for decontaminating sprouts. In fact alfalfa seeds and sprouts treated with chlorine demonstrated reduced microbial loads as well as inactivation of pathogenic microbes. Treatment of alfalfa seeds and sprouts with chlorine to control salmonellae and *E. coli* O157:H7 has been studied (Jaquette et al., 1996; Beuchat and Ryu, 1997; Taormina and Beuchat, 1999a, b). Chlorine concentrations for up to 100 ppm reduced *Salmonella* populations of pathogens on alfalfa seeds; however, concentrations between 100 and 1000 ppm were not more effective (Jaquette et al., 1996). Treatment of alfalfa sprouts for 2 min with a 500 ppm chlorine dip reduced salmonellae populations by 3.4 log per gram, and, after treatment with 2000 ppm chlorine, salmonellae populations were undetectable (<1 CFU/g) (Beuchat and Ryu, 1997).

The observed differences in reduction of pathogens in most cases cited above may not be illogical and may be justified by the fact that reduction in populations of microflora on whole and fresh produce is a function of the type of produce and the type of natural microflora present (Beuchat, 1996a, b). The results of this study demonstrated no residual antimicrobial effect following organic acid treatment. A reduction of 2 log and 3 log of *Shigella flexneri* (SF1 and SF2) was observed after treatment with 2% acetic acid for five min in both radish and moong bean sprouts following storage at 28°C for ten days. There was no apparent difference in sensory properties as well as visual appearance of the treated sprouts.

Removal of pathogenic bacteria from leafy salad greens by treating with acetic acid has been previously reported. Yuk et al. (2006) found that treatment with 1% acetic, citric, or lactic acid resulted in *L. monocytogenes* population reductions of 0.59, 1.03, or 0.93-log CFU/g, in lettuce respectively. Our observations revealed that *Cronobacter sakazakii* failed to multiply in mushroom samples treated with 2% malic acid followed by storage at 4°C for five days. But, growth was observed in the samples stored at 15°C for five days, although 5.7 log reduction was observed in mushroom samples showing a residual antimicrobial effect. *Cronobacter sakazakii* counts reduced by 4 log in betel leaves with 2% acetic acid treatment for five min followed by storage at 28°C for ten days. No promising results were found with other organic acids in betel leaves.

The present study shows that neither ozonated water nor organic acids could effectively control food-borne pathogens (*Salmonella typhimurium*: ST1 and ST2, *Shigella flexneri*: SF1 and SF2, *Cronobacter sakazakii*: CS1 and CS2). The sensitivity of the gram-negative pathogens and ozonated water and organic acids might be especially applicable for killing these organisms on food surfaces such as fruits and vegetables where interference by organics may be minimal. Alternately, tolerance mechanisms may enable survival of these pathogens. Therefore, combination of ozone along with organic acids was optimized against the above pathogens in the selected vegetables.

Combination of ozone with hydrogen peroxide, UV, and electron beam facilitates the hydroxyl radicals generation (Sommer et al., 2004). Water quality, temperature, pH and the ratio of hydrogen peroxide to ozone are also important factors which require due consideration for achieving optimum oxidative performance (Kim et al., 2003). Antimicrobial effectiveness of ozone and hydrogen peroxide combination was evaluated against viruses, spores of *B. subtilis*, and *E. coli* in water. Application of dosages of 2.5 mg/L ozone and 1.5 mg/L hydrogen peroxide in combination inactivated 6 log units of viruses and *E. coli*

following a 4 min treatment. However, only 0.4 log inactivation of *B. subtilis* spore was achieved with the individual same treatment (Sommer et al., 2004). Although, Ozone in combination with organic acids has not been extensively studied anywhere, to date, combined use of ozone with organic acids to treat these fresh produce has not been documented.

Synergistic action of ozone and chlorine, in sequential applications, against various microorganisms has been reported (Cho et al., 2003; Li et al., 2001; Rennecker et al., 2000). Antimicrobial effectiveness of ozone and hydrogen peroxide combination was evaluated against viruses, spores of *B. subtilis*, and *E. coli* in water (Sommer et al., 2004). A combination of ozone with acetic acid resulted in residual antimicrobial effect in both carrot and turnip. Combined treatment of 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 6.0 log in carrot and by 7 log in turnip following complete immersion for five min and storage at 28°C for ten days. Combined treatments significantly ($p < 0.05$) decreased populations of *E. coli* O157:H7 compared with alone treatments, showing that 3 ppm ozone with 1% citric acid had the greatest efficiency, with a 2.31-log reduction during 1-min exposure (Yuk et al., 2007).

Tetteh and Beuchat (2003) indicate that exposure of *S. flexneri* cells, unadapted to an acidic environment, to a mild heat shock renders them more tolerant to acidic conditions and may enhance their survival and ability to grow in high acid foods. Therefore, it is mandatory to use some combination treatment which may result in some synergistic affect to inactivate these pathogens. In this study, combined treatment of 2% malic acid along with 2 ppm ozone for five min significantly ($p < 0.05$) reduced pathogen populations by 4.4 log in radish sprouts and by 4.8 log in moong bean sprouts following storage at 28°C for ten days, suggesting a residual antimicrobial effect as compared to individual treatments. Similar to these results, ozone (3 ppm) combined with 1% citric acid significantly reduced *L. monocytogenes* counts by 1.33 logs compared to individual treatments (Yuk et al., 2007).

Few works have previously reported synergistic effects of inactivating agents in food. Mitsuda et al. (1990) reported the synergistic effect of ozone and carbon dioxide on microbial inactivation in foods. The synergy was believed to be due to the quenching action of carbon dioxide on the chain decomposition reaction of ozone; such an effect increases the stability and bactericidal effectiveness of the ozone in the treatment environment. Combined treatment of 2% malic acid along with 2 ppm ozone significantly ($p < 0.05$) reduced pathogen populations by 6.2 log in mushroom after storage at 15°C for five days. Similarly, *Cronobacter sakazakii* reduced by 6.8 log in betel leaves treating with 2% acetic acid 2 ppm ozone followed by storage at 28°C for ten days. The combined treatments of 3 ppm ozone and 1% organic acids were more effective than individual treatments, giving approximately 1.80-log reductions of *Listeria monocytogenes* in shredded lettuce during one min exposure (Yuk et al., 2006).

Microorganisms that are strongly attached, internalized, or organized as a biofilm on food surfaces or those embedded in the food matrix are not readily inactivated by ozone treatments. The antimicrobial activity of organic acids is a function of reduction of pH in the microbial environment leading to disruption of membrane transport and permeability, accumulation of anions and lowering of intracellular pH within the cell by dissociation of hydrogen ions from the acid (FDA, 2001). Liu et al. (2004) predicted that when both bacterial and support surfaces are hydrophobic, microbial adhesion is highly facilitated. If both bacterial and support surfaces are hydrophilic, microbial adhesion would proceed with difficulty. Thus, an increase in cell surface hydrophobicity would favor cell adhesion on both hydrophilic and hydrophobic supports surface. Detergents may offer a way to disrupt such interactions and rinse pathogens off surfaces more readily from vegetable surfaces (Raiden et al., 2003). The sanitizing activity of benzalkonium chloride on planktonic cells and biofilms has been previously studied (Houari and Di Martino, 2007; Romanova et al., 2007). But, due

to the protection afforded to cells enclosed within this matrix, chemical sanitizers are generally unable to eliminate most biofilm-associated bacteria. Several reports have documented the increased resistance of biofilm-associated (attached) bacteria to various antimicrobial agents compared to their planktonic (suspended) counterparts. For example, Luppens et al. (2002) demonstrated that a 600-fold increase in chlorine concentration was required to kill biofilm-associated cells of *Staphylococcus aureus* compared with their planktonic counterparts. Biofilm-associated *Salmonella* bacteria were reduced by less than 1 log₁₀ unit after treatment for 10 min using 10 ppm of chlorine whereas 10⁶ CFU/ml of planktonic cells were completely killed using the same treatment (Joseph et al., 2001). Other chemical sanitizers such as trisodium phosphate (Somers et al., 1994) and iodophor (Joseph et al., 2001) have also been shown to have similarly reduced efficacy. Biofilm-associated bacteria have enhanced resistance to heat treatment (Frank and Koffi, 1990).

A majority of the *Salmonella* strains can grow on surfaces and interfaces to form biofilms composed of self-secreted exopolysaccharide or exopolymeric material (Solano et al., 2002), including on the food processing and food contact surfaces. Biofilms pose a potent threat to the safety of food by being a constant source of contamination leading to serious hygienic problems and economic losses (Korber et al., 1997; Forsythe, 2000). Although a low level of pathogens such as *Salmonella typhimurium* may be initially found on naturally contaminated vegetables, sufficient time and appropriate environmental conditions may allow pathogens to grow to sufficient populations to an effective dose and can also form biofilms. Therefore, it is of utmost importance that fresh produce samples, food contact surfaces and food processing equipment should be cleaned and disinfected regularly and sufficiently with an effective disinfectant of appropriate concentration (Luppens et al., 2002).

Calcofluor was selected as a nonspecific probe of biofilms because of its capacity to bind to a wide range of polysaccharides, especially those containing (1-3) and (1-4) β-D-

glucan linkages (Wood, 1980). For example, Jann and Jann (1990) reported such linkages in the CPS (Capsular polysaccharide) of several strains of *E. coli*. Cells in biofilms are more resistant to heat, chemicals, and sanitizers a phenomenon largely attributed to the diffusional barrier created by the biomatrix. Later, the increased resistance is now attributed at least in part, to the very slow growth rate of cells in biofilms (Donlan and Costerton, 1995). Recently, a physiologic or phenotypic adaptation resulting in “biocide tolerance” has been credited to biofilms (Sheldon, 2005). The results obtained in this study demonstrated that calcofluor detected biofilms, produced by *Salmonella typhimurium* strains ST1 and ST2 on PVC pipes and polyethylene bags. PVC pipes are used extensively for supplying water to carrots prior to harvesting whereas plastic bags are used for storing the vegetables during its transit to the consumer. Farmers are ignorant in majority of the cases about Good agricultural Practices (GAP) and hygienic standards are not maintained both in terms of irrigation water and cleanliness of agricultural equipments. Plastic bags on the other hand are extensively used storing the vegetables both for short and long period. Plastic bags are usually kept unhygienic, in several instances the bags are washed with stagnant used water (used for sprinkling /dipping vegetables). Evidence of biofilm formation raises concern and warrants appropriate preventive measures.

Alcian blue stains acidic polysaccharides often present in the biofilm matrix (exopolymeric substance-EPS) (Fassel and Edmiston, 1999). This is especially relevant when the proposed product is to be used on fresh produce that will be incorporated into foods with minimal or no further processing or cooking, such as salads. It is however possible that potentially pathogenic microorganism may sometime become sequestered within the endemic/preexisting biofilms, and can afford some protection by these biofilms. Such sequestered pathogens may vary in their vulnerability to disinfection, and due to translocation of biofilm clumps during food preparation, could result in other domestic surfaces, foods or

utensils also becoming contaminated with biofilm-protected pathogens. The prevalence of biofilm on domestic household surfaces (polyethylene bags and PVC pipes) has significant implications regarding the accurate assessment of antibacterial and cleansing efficacy of commercial sanitizing products. A small number of microbial contaminants are usually present on food packaging materials. Several treatments are currently utilized to achieve sterility of packaging materials including hydrogen peroxide (H₂O₂) alone or in combination with other sanitizers, heat, and UV radiation (Gardner and Shama, 1998; Stefanovic and Dickerson, 1986; Yokoyama, 1990). Sterilization of packaging materials by H₂O₂ is a common practice, yet the process has several disadvantages (Yokoyama, 1990). Unacceptable amount of this sanitizer may remain after the treatment, and this residue may interact with some compounds in the package material (Castle et al., 1995).

Salmonella typhimurium strains were observed to differ in the amount of biofilm produced at 20 h and 40 h, and the microtiter plate assay appeared to detect differences in biofilm forming ability among *Salmonella* strains. It has been suggested that motility and flagella formation can play a role in initial phases of biofilm formation (O'Toole and Kolter, 1998; Vatanyoopaisarn et al., 2000). Both ST1 and ST2 were able to form biofilms at 20 h and 40 h. However, biofilm formation reduced up to 5 fold at 20 hours and up to 6 fold at 40 hours in both ST1 and ST2. It is important to note that the assay presented here measured biofilm production under a minimal nutrient environment. It has been suggested that *Salmonella* spp. growing in a food-processing environment may be exposed to fluctuating levels of nutrients, depending upon location in the plant. For example, populations existing in floor drains will be exposed to high levels of nutrients during pre-rinsing of equipment prior to cleaning and sanitizing. On the other hand, if living on walls, ceilings, or within condensate (on the outside of cooling equipment or pipes), it is likely that these bacteria are surviving under reduced nutrient conditions.

Biofilms are assemblages of microorganism's adherent to each other and/or to a surface and embedded in a matrix of exopolymers (Costerton et al., 1999). Due to the protection afforded to cells enclosed within this matrix, chemical sanitizers cannot effectively eliminate most biofilm-associated bacteria. For example, *Salmonella typhimurium* embedded in the biofilm matrix resisted sodium hypochlorite at concentrations above 500 mg/L, while planktonic cells were sensitive to less than 50 mg/L (Scher et al., 2005). Most isolates of *Salmonella* spp. originating from produce are able to synthesize the main components of the biofilm matrix (curli and cellulose) (Solano et al., 2002; Romling et al., 2003; Zogaj et al., 2003; Solomon et al., 2005), thus it may be hypothesized that *Salmonella* cells in the form of biofilms survive and resist disinfection treatments on vegetables. The motility function as well as chemotaxis appears to be important for biofilm formation (as was found for the cellular adhesion experiments (Kirov et al., 2004).

Bacterial surface structures that could assist their adhesion include outer membrane proteins, capsular polysaccharides, lipopolysaccharides, curli, non hemagglutinating pili, fibrillae, and flagella (de Rezende et al., 2005). Presence of these extracellular substances can influence the physicochemical properties of bacterial cells, such as hydrophobicity and cell charge (Morris et al., 1997). Among these, fibrillae production by *Salmonella* could enhance its ability to form biofilms. Several physiological factors that influence the ability of cells to withstand acid challenge, the role of an r-transcription factor, which is abundant in the stationary growth phase, is well documented (Fang et al., 1992; Lee et al., 1994a; Foster and Spector, 1995; Bearson et al., 1997).

The present study suggested that *Salmonella* could form biofilm on plastic bags, PVC pipes and vegetable surface; a probable explanation to high acid tolerance could be the biofilm formation which was inhibited using a combination of malic acid and ozone. Imaging techniques may provide key evidence of both bacterial pathogens and biofilm formation on

vegetable surfaces; in fact biofilms on vegetable surfaces have been imaged. Annous et al. (2004) for example, observed the development of biofilms within the netting using SEM images of the cantaloupe rind. Most of the attention has been focused on the structural aspects of the netting and the skin but not on surface structures such as trichomes or lenticels that are abundant on the rind. In comparison, SEM images of *S. rubislaw* on oranges presented by Pao et al. (2001) were better understandable than their stereomicroscopy images which did not provide clear structural details of the orange stem scar region. However, few studies have imaged the surface of carrot and turnip to elucidate attachment sites of bacteria. From the present findings, it is evident that *Salmonella* can lodge themselves on surface and can form biofilms prior to harvest or during storage (during the farm-to-plate chain) of carrots and turnips. SEM images of ozone and organic acid treated carrot and turnip clearly indicated the loss of flagella which might be due to the incapacitation of bacteria to form biofilms. However, this proposition needs further study. Therefore, a combination of both can be an effective intervention method for eliminating biofilm forming *Salmonella* in fresh vegetables.

Vegetables are a good source of dietary fibre, phytonutrients, provitamins, antioxidants, polyphenols and minerals. Antioxidants in beets and green beans (Cardador-Martinez et al., 2002; Jiratanan and Liu, 2004), prebiotics and immune protecting phytochemicals of asparagus (Diwanay et al., 2004; Gautam et al., 2004; Gibson, 1998), hydroxycinnamic acid of eggplant (Whitaker and Stommel, 2003), glucosinolates of cauliflower (Kushad et al., 1999; Tian et al., 2005) have been associated with health promoting effects. Phytonutrients in vegetables have been shown to stimulate natural detoxifying enzymes in the body and lower the risk of atherosclerosis and cancer (Ames et al., 1993; Hecht, 1999). Carrots contain not only nutritional antioxidants such as vitamins A, C, and E, but also notable quantities of non-nutritional antioxidants, such as β -carotene,

carotenoids, flavonoids, flavones, phenolics compounds, etc. (Yen et al., 2008). Carrots have been ranked tenth in terms of their nutritional value among 38 other fruits and vegetables, and seventh for their contribution to nutrition (Alasalvar et al., 2005). Raw sprouts are rich in several antioxidant compounds, capable of protecting against reactive oxygen species (ROS) mediated damage (Cuzzocrea et al., 2001).

Radish (*Raphanus sativa*) sprouts are rich in antioxidants due to presence of flavonoids (Takashi et al., 2006), polyphenols, vitamins and activity to scavenge free radicals (Michie and Kazuko, 2004). Moong bean (*Phaseolus aureous*) sprouts are rich sources of antioxidants, polyphenols and also exhibit antimicrobial activities (Randhir et al., 2004). Mushrooms provide key nutrients and bioactive components such as high quality proteins, vitamins (riboflavin, niacin and folates), minerals (potassium, phosphorus, magnesium, zinc, copper, and selenium), unsaturated fatty acids and dietary fibers (Mattila et al., 2001). Betel leaves are known to possess antioxidant action due to presence of polyphenols, flavonoids, alkaloids (Arambewela et al., 2006). Furthermore, betel oil present in betel leaves contains several phenols including hydroxychavicol, eugenol, betel phenol and chavicol; vitamin C (1.9 mg/g) and a large amount of carotenes (80.5 mg/g) have also been reported (Wang and Wu, 1996). Betel leaves form an important part of East Asian diet, (consumed/ chewed after meals) as well as an important produce item that is exported.

Although, a span of several days occurs between processing and consumption of fresh produce, consumers still desire fresh or fresh-like characteristics of vegetables (Go´mez-Lo´pez et al., 2005). However, it is well-known that processing of vegetables promotes a faster physiological deterioration, biochemical changes and microbial degradation of the product even when only slight processing operations can be used (O’Beirne and Francis, 2003), which may result in degradation of the color, texture and flavor (Kabir, 1994; Varoquaux and Wiley, 1994). Thus sensorial analysis following treatment as well as

recommended storage is important for consumer acceptability. An important factor of plant food is their antioxidant properties therefore it is necessary to consider the overall effect of processing on total antioxidant profile. Following organic acid and ozone treatments therefore all vegetable samples were subjected to sensory, visual analysis and as well as for the antioxidants.

DPPH is a free radical compound and has been widely used to test the free radical-scavenging ability of various samples (Hatano et al., 1997). DPPH is one of the compounds that possess a proton free radical with characteristic absorption maxima at 517 nm, which decreases significantly on exposure to proton radical scavengers. The free radical scavenging by the antioxidant samples are credited to their hydrogen-donating ability (Yamaguchi et al., 1998). Total of six vegetable samples tested no significant ($p>0.05$) was observed in DPPH radical scavenging activity after treatment with ozone and organic acids.

Moreover, it has been suggested that a chronic imbalance between formation of reactive oxygen species (ROS) and antioxidant defenses has been implicated in the development of chronic diseases, such as cancer, atherosclerosis, nephritis, diabetes mellitus, rheumatism, ischemic, cardiovascular diseases, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Rekha et al., 2010). AAPH, a thermolabile water-soluble radical initiator, oxidized $ABTS^{2-}$ to ABTS radical anion (Scott et al., 1993). The generation of the ABTS radical anion before the addition of antioxidants was reported to eliminate the interference of compounds affecting radical production (van den Berg et al., 1999).

There are credible epidemiological evidences that the consumption of fruits and vegetables are beneficial for health, due to the protection provided by the antioxidant phytonutrients contained in them (Avila-Sosa et al., 2010). Phenols are important components of plants. They can eliminate free radicals due to their hydroxyl groups (Hatano et al., 1989),

and they contribute directly to antioxidant effect of system (Duh et al., 1999). Polyphenols include other subclasses besides such as phenolic acids, stilbenes, lignans, tannins, and oxidized polyphenols. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Komali et al., 1999; Moller et al., 1999). Polyphenolic compounds have an important role in stabilizing lipid oxidation as well associated with antioxidant activity for lipids (Cakir et al., 2003, Wagner et al., 1992).

Therefore, it is important to consider the effect of treatment on the total polyphenolic content of the processed sample. In this study, no change in polyphenols and flavonoids were observed in the fresh vegetables following treatment with ozonated water and organic acids. Phenols such as BHT and gallate are known to be effective antioxidants (Madhavi et al., 1996). Because of their scavenging abilities on free radicals and chelating abilities on ferrous ions, phenols might possess good antioxidant, antimutagenic, and anticancer properties (Lotito and Fraga, 1998; Ahmad and Mukhtar, 1999). It has been suggested polyphenolics directly contributes to antioxidant activity since compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0g daily ingested from a diet rich in fruits and vegetables (Tanaka et al., 1998). Besides the radical scavenging activities of polyphenolics possess metal chelating properties also. Polyvalent phenolics allow some metal ions to chelate (Yilmaz and Toledo, 2004) which are involved in the formation of metal-catalysed free radicals particularly iron and copper (Rice-Evans et al., 1997).

Superoxide anion plays an important role in the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induces oxidative damage in lipids, protein, and DNA (Halliwell and Gutteridge, 1989; Pietta, 2000). Superoxides are also known to indirectly initiate lipid peroxidation as a result of H₂O₂ formation, creating precursors of hydroxyl radicals (Meyer and Isaksen, 1995). It was reported that the reducing properties are generally associated with the presence of reductones,

which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Shimada et al., 1992).

Sensory evaluation of treated vegetable samples is a crucial parameter in assessing acceptance by the consumer. Sanitizer treatments may cause deterioration of sensory quality, thereby limiting their usefulness to applications just prior to consumption (Zhang and Farber, 1996; Adams et al., 1989). Treatment by dipping in H₂O₂ solution reduced microbial populations on fresh-cut bell peppers, cucumber, zucchini, cantaloupe, and honeydew melon, but did not alter sensory characteristics (Sapers and Simmons, 1998). Vikram et al. (2005) studied the thermal degradation kinetics of nutrients in orange juice heated by conventional methods. Similarly, in this study, treated vegetable samples (*Daucus carota* (carrot), *Brassica rapa* (turnip), *Raphanus sativa* (radish sprouts), *Phaseolus aureus* (moong bean sprouts) and *Piper betle* (betel leaves) were sensorially acceptable in terms of color, overall visual appearances, taste and flavor. An important result was improvement of the inherent whiteness in treated *Agaricus bisporous* following malic acid treatment.

Color is an important organoleptic characteristic used to establish the acceptance of various food products (Hutchings, 1999). The aspect and color of the food surface is the first quality parameter evaluated by consumers, and it is critical to product acceptance. Furthermore, food appearance (determined mostly by surface color) is the first sensation that the consumer perceives and uses as a tool either to accept or reject food (Leon et al., 2006). A principle criteria used to evaluate the freshness of mushrooms is color, which may change upon exposure to the sanitizer. Acetic acid treated mushrooms were not acceptable in terms of color, since treated mushrooms turned brown upon storage. However, malic acid treated button mushroom significantly ($p < 0.05$) retained its white color and freshness following treatment in comparison to acetic acid treated and control. Sensorial properties in terms of

color, taste, appearance, texture, taste did not change significantly following the individual as well as combined treatment.

This study demonstrated the applicability of combined treatment of organic acids and ozone in raw nutritive fresh produces such as sprouts, carrot, turnip and betel leaves and in button mushroom. High risk pathogens such as *Salmonella typhimurium*, *Shigella flexneri* and *Cronobacter sakazakii* isolated from the selected vegetables were initially used to create simulated survival and growth data. Minimal inactivation protocols were designed to assess survival of the pathogens in vegetable samples. Combinatorial treatments with malic acid and ozone significantly enhanced pathogen inactivation by 4-7 log units. The US FDA guide to minimize microbial safety hazards for fruits and vegetables defined sanitizer to treat the produce by a process that is effective in destroying or substantially reducing of number of microorganisms of public health concern, as well as other undesirable microorganisms without adversely affecting the quality of the product or its safety of the consumer (FDA, 1998). The nutritional properties, in particular antioxidant levels as well as sensory attributes of the selected vegetables in this study remained unaltered. Lastly, the treatment method reported in this study is simple, economical and reliable and thus offer a viable strategy for assuring safe, nutritionally adequate fresh vegetables to consumers and confirm the existing regulatory norms.

CONCLUSIONS

1. A total two hundred and thirty six selected vegetable samples were collected from different locations of Patiala city mainly focusing on three main distribution sites directly from farm, local mandis and street vendors.
2. Collected samples were analyzed for total aerobic count and three main pathogens of concern: *Salmonella* spp., *Shigella* spp., *Cronobacter* spp.
3. Highest aerobic plate count was observed in the samples collected directly from farm.
4. Subsequent analysis for pathogens indicated *Salmonella typhimurium*, *Shigella flexneri*, *Cronobacter sakazakii* from the samples collected from street vendors respectively from *Daucus carota* (carrot) *Brassica rapa* (turnip), *Raphanus sativa* (radish sprouts), *Phaseolus aureus* (moong bean sprouts), *Agaricus bisporous* (button mushroom) and *Piper betle* (betel leaves).
5. It was concluded that pathogens might have entered during packaging/transportation and storage.
6. Effective concentration of organic acids namely malic acid, acetic acid, lactic acid and citric acid and ozone against the planktonic cells of isolated/designated pathogens was optimized. Among all the organic acids and concentrations used, 1.2% of malic acid and 1% acetic acid and 1 ppm of ozone was found to be most effective to completely control the growth of pathogens in broth.
7. Both the *Salmonella typhimurium* isolates were resistant to high concentrations of organic acids tested.
8. Effect of ozonated water and effective organic acids individually onto the pathogens in artificially inoculated respective vegetables were examined. Results indicated that 2% of

malic acid, acetic acid, 2 ppm of ozonated water were not able to control the growth of pathogens in vegetables.

9. Therefore, combined treatment of 2% organic acids and 2 ppm ozonated water was tested against the pathogens in respective vegetable samples. As a result, the combined treatment was significantly effective for the control of pathogens as described by Food and Drug Administration.
10. It was observed that both the *Salmonella* isolates were able to form biofilms. Therefore, their capacity to biofilm by these pathogens on polyethylene bags, PVC pipes and microtitre plates was tested and both the isolates were able to form biofilms.
11. Both the pathogens were able to form biofilms on respective vegetable samples i.e carrot and turnip.
12. Combined treatment of 2 ppm ozonated water and 2% of malic acid was able to completely control the biofilm formation in poly bags, PVC pipes, microtitre plates as well as vegetable samples.
13. *Salmonella typhimurium* cells were clearly visible over the entire vegetable sample showing the presence of peritrichous flagella in control, but the cells in treated samples were showing the absence of flagella.
14. The sensory quality of treated, minimally processed vegetable samples was evaluated by a panel of ten trained judges by grading for color, flavour, consistency and overall acceptability score on a nine-point hedonic scale. No changes were observed in any of the treated vegetable and were sensorially acceptable.
15. No change in ABTS radical scavenging activity and DPPH radical scavenging activity was observed in the treated samples.
16. No change in flavonoids and polyphenols were observed in any of the six treated vegetable samples.

17. Mushroom samples treated with acetic acid turned brown upon storage, treatment with malic acid improved the whiteness of the mushroom in comparison to control.

In conclusion, present study carried out primarily:

- a. In this study, *Salmonella typhimurium*, *Shigella flexneri* and *Cronobacter sakazakii* were predominant in carrot, turnip, sprouts, mushroom and betel leaves.
- b. The present study supports the use of malic acid (limits acetic acid because of slight odor) as sanitizer which proved to be an economical minimal processing strategy for the control of pathogens.
- c. No significant change in the essential nutritional parameters (Antioxidant status, polyphenols, flavonoids) and sensory properties of the selected fresh produce was observed following sanitizer treatment.
- d. Although reduction in microflora on whole and fresh-cut produce is dependent upon the type of produce and the type of natural microflora present, the proposed combined technology for inhibiting high risk pathogens may be applied to carrot, turnip, mushroom, betel leaves and sprouts. The minimal processing technology may be extended as well to other vegetable commodities.

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Appendix I

MEDIUM COMPOSITION

1. *Tryptone Soya agar (TSA)**

| <i>Composition</i> | <i>Quantity (gL⁻¹)</i> |
|-----------------------|-----------------------------------|
| Casein peptone | 17.0 |
| Soya peptone | 3.0 |
| NaCl | 5.0 |
| Dipotassium phosphate | 2.5 |
| Dextrose | 2.5 |
| Agar | 15.0 |

2. *Enterobacteriaceae Enrichment (EE) broth*

| <i>Composition</i> | <i>Quantity (gL⁻¹)</i> |
|---|-----------------------------------|
| Peptone | 6.0 |
| Bile salt mixture | 1.5 |
| 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside | 0.1 |
| NaCl | 5.0 |

3. *DFI medium**

| <i>Composition</i> | <i>Quantity (gL⁻¹)</i> |
|---|-----------------------------------|
| Sodium thiosulphate | 1.0 |
| Sodium deoxycholate | 1.0 |
| Ammonium iron(III) citrate | 1.0 |
| 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside | 0.1 |
| Tryptone soya agar | 40.0 |

4. *Rappaport-Vassiliadis Soya Peptone (RVS) broth*

| <i>Composition</i> | <i>Quantity (gL⁻¹)</i> |
|---------------------------------|-----------------------------------|
| Soya peptone | 4.5 |
| Malachite green oxalate | 0.036 |
| NaCl | 7.2 |
| MgSO ₄ | 13.4 |
| KH ₂ PO ₄ | 1.26 |
| Dipotassium phosphate | 0.18 |

5. Hektoen Enteric agar (HEA)*

| <i>Composition</i> | <i>Quantity (g^{L⁻¹})</i> |
|-------------------------------|--|
| Peptone | 15.0 |
| Sucrose | 14.0 |
| Yeast extract | 3.0 |
| Lactose | 14.0 |
| Salicin | 2.0 |
| Sodium thisulfate | 5.0 |
| Ammonium iron(III) citrate | 1.5 |
| Bile salt mixture | 2.0 |
| Bromothymol blue | 0.05 |
| Acedic fuchsin | 0.08 |
| Agar | 13 .5 |

6. Universal Pre-enrichment broth (UPB)

| <i>Composition</i> | <i>Quantity (g^{L⁻¹})</i> |
|---------------------------------------|--|
| Casein peptone | 5.0 |
| Proteose peptone | 5.0 |
| Monopotassium phosphate | 15.0 |
| Disodium phosphate | 7.0 |
| NaCl | 5.0 |
| MgSO ₄ . 7H ₂ O | 0.25 |
| K ₂ HPO ₄ | 1.0 |
| Dextrose | 0.5 |
| Ferric ammonium citrate | 0.1 |
| Sodium pyruvate | 0 .2 |

7. Brain heart infusion agar (BHI)*

| <i>Composition</i> | <i>Quantity (g^{L⁻¹})</i> |
|--------------------|--|
| Protease peptone | 10.0 |
| Calf brain | 200.0 |
| Beef heart | 250.0 |
| Dextrose | 2.0 |
| NaCl | 5.0 |
| Disodium phosphate | 5.0 |
| Agar | 15 .0 |

8. Maximum recovery diluent (MRD)*

| <i>Composition</i> | <i>Quantity (g^{L⁻¹})</i> |
|--------------------|--|
| Peptone | 1.0 |
| NaCl | 8.5 |

9. *Xylose Lysine Deoxycholate medium (XLD)*

| <i>Composition</i> | <i>Quantity (gL⁻¹)</i> |
|----------------------------|-----------------------------------|
| Yeast extract | 3.0 |
| L-Lysine | 5.0 |
| Lactose | 7.5 |
| Sucrose | 7.5 |
| Xylose | 3.5 |
| NaCl | 5.0 |
| Sodium deoxycholate | 2.5 |
| Sodium thiosulphate | 6.8 |
| Ferric ammonium citrate | 0.8 |
| Phenol red | 0.08 |
| Agar | 15.0 |

10. *Plate count agar (PCA)**

| <i>Composition</i> | <i>Quantity (gL⁻¹)</i> |
|--------------------|-----------------------------------|
| Peptone | 5.0 |
| Yeast Extract | 2.5 |
| Dextrose | 1.0 |
| Agar | 15 .0 |

*pH adjusted to neutral at 25°C

*Media were sterilized by autoclaving at 121°C and 15 psi for 20 min and were allowed to cool below 50°C before use

BUFFERS AND SOLUTIONS

1. *TBE buffer (10X)*

| | |
|------------|---------------|
| Tris-HCl | 0.09 M (pH 8) |
| Boric acid | 0.9 M |
| EDTA | 0.02 M (pH 8) |

2. *0.1M Phosphate buffer*

| | |
|---|---------|
| Monobasic sodium phosphate, monohydrate (1 M) | 61.5 mL |
| Dibasic sodium phosphate, monohydrate (1 M) | 38.5 mL |
| Dilute to 1 L with distilled water | |

3. *Agarose gel loading dye (6X)*

| | |
|-------------------|-------|
| Bromophenol blue | 0.25% |
| Xylene cyanol FF | 0.25% |
| Glycerol in water | 30.0% |

4. *Ethidium Bromide*

0.5 µgmL⁻¹

STAINING SOLUTIONS

| | |
|-------------|------------------------|
| Alcian Blue | 0.1% |
| Calcofluor | 800 µgmL ⁻¹ |

Appendix II

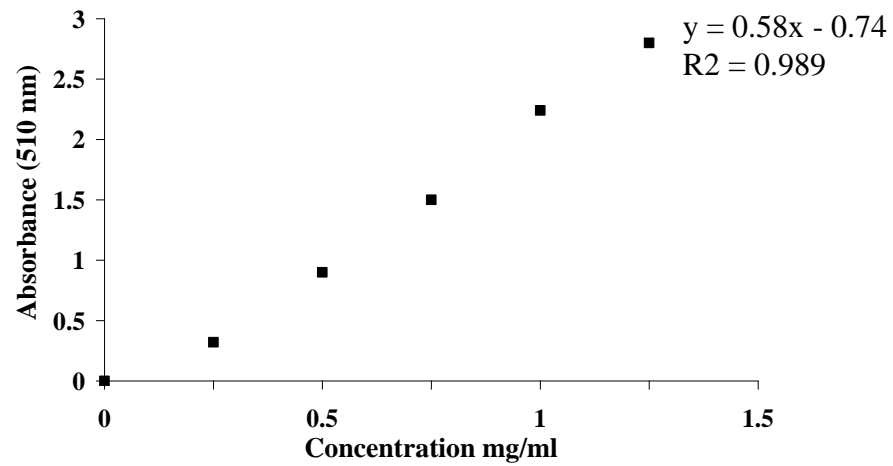


Fig. 3.1 Standard curve of flavonoids assay. Relationship between flavonoids (as mg catechin) and absorbance using colorimetric method assay. $R^2 = 0.989$

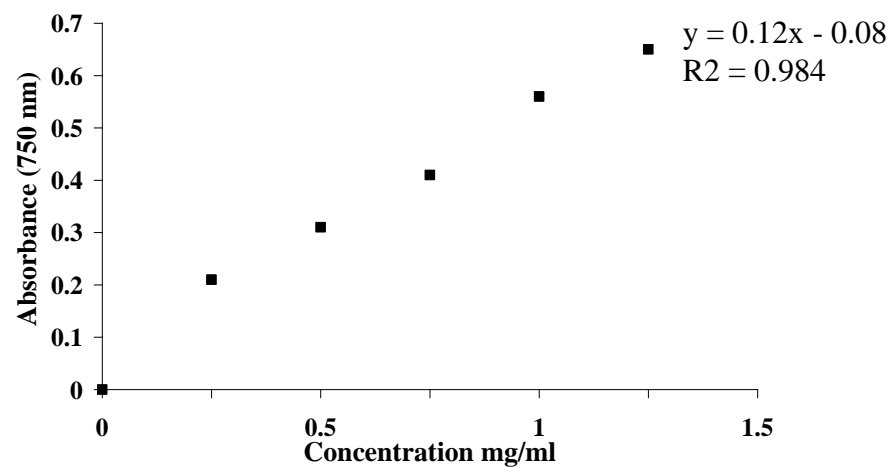


Fig. 3.2 Standard curve of polyphenols assay. Relationship between polyphenols (as mg gallic acid) and absorbance using Folin-Ciocalteu method assay. $R^2 = 0.984$

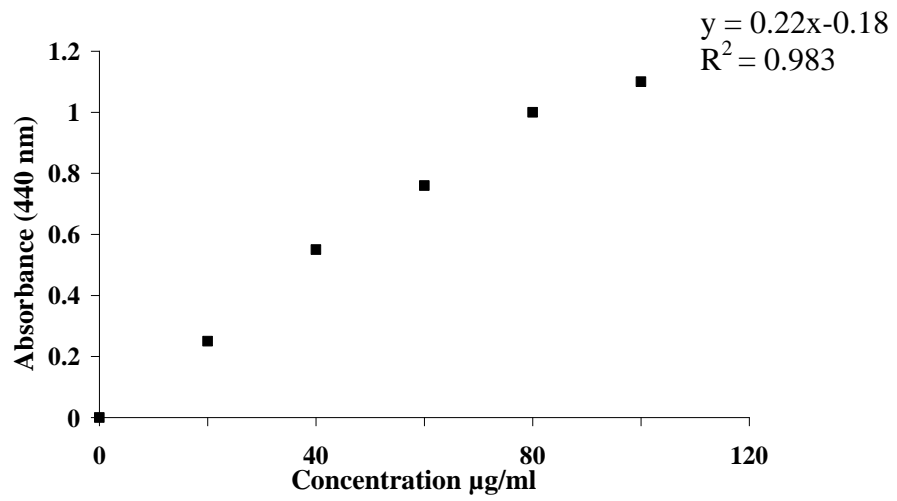


Fig. 3.3 Standard curve of β - carotene. Relationship between β - carotene and absorbance using AOAC method. $R^2 = 0.983$

Publications in Peer-Reviewed Journals

2011

1. **Singla, R.,** Ganguli A., and Ghosh M. 2011. Combined organic acid and ozone inactivates *Shigella* spp. in sprouts but retains antioxidant activity. *Food Control*, 22(7), 1032-1039.

2010

2. **Singla, R.,** Ganguli A., and Ghosh M. 2010. Antioxidant activities and polyphenolic properties of raw and osmotically dehydrated dried mushroom (*Agaricus bisporous*) snack food. *Int. J. Food Properties*, 13(6), 1290-1299.
3. **Singla, R.,** Ganguli A., and Ghosh M. 2010. Physicochemical and nutritional characteristics of organic acid treated button mushrooms (*Agaricus bisporous*). *Food Bioprocess Technol.*, DOI: <http://dx.doi.org/10.1007/s11947-010-0457-7>

2009

4. **Singla, R.,** Ganguli A., Ghosh M. and Sohal, S., 2009. Evaluation of sanitizing efficacy of acetic acid on *piper betle* leaves and its effect on antioxidant properties. *Int. J. Food Sci. Nutr.*, 60(s7), 297-307.
5. **Singla, R.,** Ghosh M., and Ganguli A. 2009. Phenolics and Antioxidant Activity of a ready-to-eat snack food prepared from the edible mushroom, *Agaricus bisporous*. *Nutr. Food Sci.*, 39(3), 227-234.

Oral Presentations

1. **Singla, R.,** Ghosh, M., and Ganguli, A. Survival, growth and transmission of *Cronobacter (Enterobacter) sakazakii* in rice and cereal based weaning foods. International conference on frontiers in biological sciences (InCoFIBS-2010). 01-03 Oct. 2010. NIT Roukela, Orisa
2. **Singla, R.,** Mittal, P., Ghosh, M., and Ganguli, A. Prevalence of *Aeromonas* and *Yersinia* spp. in some commonly consumed selected Indian fresh produce. National symposium on Emerging trends in Biomedical Sciences. 27-28 Feb. 2009. SBSPGI Dehradun

3. **Singla, R.,** Ganguli, A., and Ghosh, M. Antioxidant activities and polyphenolic properties of raw and vacuum dried button mushroom (*Agaricus bisporous*) snack food. 16th International drying symposium 2008. 9-12 Nov. 2008. Sitara Hotel, Ramoji Film City Hyderabad

Poster Presentations

1. **Singla, R.,** and Ganguli., A. Microbial safety, quality and nutritional status of minimally processed turnip and carrot. 9th International Food Data Conference, 14-16 Sept. 2011. Institute of Food Research, Norwich, UK
2. **Singla, R.,** Ganguli, A., and Ghosh, M. Microbial safety, shelf life and nutritional quality of organic acid treated Button Mushrooms (*Agaricus Bisporous*). International Conference on Food Research (ICFR 2010), 22-24 Nov. 2010. Universiti Putra Malaysia, Putrajaya, Malaysia
3. **Singla, R.,** Ganguli, A. Evaluation of acetic acid as sanitizer for reducing *Shigella flexneri* in sprouts and its effect on antioxidant properties. 50th Annual AMI conference. 15-18 Dec. 2009. NCL, Pune.

Workshops attended

1. Participated in International conference on post harvest management and valorization Agri-Horticultural produce and exhibition. 19-20 Feb. 2010. NASC Auditorium, New Delhi
2. Short course on “Yeast molecular biology and biochemical analysis” organized by department of biotechnology and bioinformatics. 23-27 July, 2009. Jaypee university of information & technology, Wagnaghat, HP in association with Institute of Molecular Sciences, Frankfurt University, Germany, Solan, India
3. CME/Workshop in “VIROCON 2008” XVIII National Conference of Indian Virological Society. 11 Dec. 2008. Department of Virology, Post Graduate Institute of Medical Education & Research, Chandigarh, India