Microbial Risk Assessment Of Citrus And Carrot Juices

A PROJECT
SUBMITTED IN PARTIAL FULFILLMENT OF
REQIREMENT FOR THE AWARD OF DEGREE OF
M.Sc BIOTECHNOLOGY

BY
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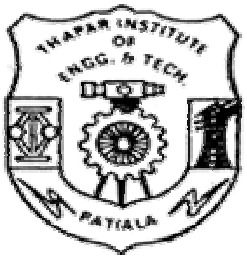

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THAPAR INSTITUTE OF ENGINEERING & TECHNOLOGY,
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PATIALA –147004
MAY, 2003

CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled **"Microbial Risk Assessment Of Citrus And Carrot Juices"** in partial fulfillment of the requirements for the award of the degree of MASTER OF SCIENCE IN BIOTECHNOLOGY, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala is an authentic record to my own work during a period of five months from January 2003 to May 2003, under the supervision of Dr. Abhijit Ganguli, Department of Biotechnology & Environmental Sciences, Thapar Institute of Engineering & Technology.

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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ACKNOWLEDGEMENT

I thank the almighty whose blessings have enabled me to accomplish my dissertation work successfully.

It is my pride privilege to express my sincere thanks and deep sense of gratitude to Dr. A. Ganguli., Lecturer, Department of Biotechnology and Environmental Sciences, for his valuable advice, splendid supervision and constant patience through which this work was able to take the shape in which it has been presented.It was his valuable discussions and endless endeavors through which I have gained a lot. His constant encouragement and confidence-imbibing attitude has always been a moral support for me.

My sincere thanks to Dr. Sunil Khanna, Head .Department of Biotechnology and Environmental Sciences,for his immense concern throughout the project work.

A special word of thanks to all the faculty members for their constant encouragement and support throughout tthis duration.

I feel lacunae of words to express my most heartfelt and cordial thanks to my friends Sunita Bansal, Diwakar Aggarwal, Anita Bansal, who have always been a source of inspiration for me, stood by my side at the toughest times.Finally, I wish to extend a warm thanks to everybody involved directly or indirectly with my work.

The whole credit of my achievements goes to my parents, who were always there for me in my difficulties. It was their unshakable faith in me that has always helped me to proceed further.

Date: May 15, 2003 SANDEEP MUDGIL

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ABSTRACT

The microbiological profile of fresh squeezed kinnow and carrot juices sold through street vended outlets in Patiala, revealed significant loads of total aerobic bacteria, faecal coliforms and staphylococcus. Analysis of same lots of carrots and kinnows throughout the transport chain showed similar loads. Coagulase positve *S.aureus* (13% of samples of carrot juices) was detected kinnow and carrots samples whereas *Salmonella enterica* (5% of carrot and kinnow samples) and *E.Coli O157:H7* was detected (presumptively) in carrot juice samples from three areas. The non -pathogenic variant of the *Salmonella* (LT2, Rif nal) survived well both at 4^0C and 8^oC till 72 hours in kinnow juice samples. The *E.coli O157:H7* surrogate strain was unable to grow in carrot juice samples for extended periods of time viz, beyond 48hours and showed significant decline in counts after this time period- in kinnow juice samples the decline was less as compared to that in carrot juice. No significant decrease in counts of *E.Coli O157:H7* surrogate strain was observed in carrot juices supplemented with additives after 48 hours. Acid stressed *E.coli O157:H7* was able to survive better in kinnow juice samples than its unstressed counterpart till 10 hours. Growth kinetics of inherent spoilage microorganisms in both carrot and kinnow juices indicated that both juices were completely spoiled after 6 hours at 28^0C. The results of this study highlight the potential risk of fresh squeezed carrot and kinnow juices contaminated with either *Salmonella* or *E.Coli O157:H7* to the consumers within the time frame of spoilage at 28^0C.

CONTENTS

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INTRODUCTION

Outbreaks of human diseases associated with the consumption of raw fruits and vegetables often occur in developing countries and have become more frequent in developed countries over the past decade. Factors thought to influence the occurrence and epidemiology of these diseases include irrigation and other agronomic practices, the general level of hygiene in handling fruits and vegetables, international travel, globalization of the supply and distribution of raw produce, the introduction of pathogens into a new geographical areas, change in the virulence and environmental resistance of pathogens, decrease in immunity among certain parts of the population, and changing in the eating habits. In developing countries, foodborne illnesses caused by contaminated fruits and vegetables are frequent and in some areas they cause a large proportion of illness. However due to lack of foodborne disease investigation and surveillance in most of these countries, most outbreaks go undetected and the scientific literature reports only on very few outbreaks. The impact of agronomic practices, handling, processing, distribution and marketing on risk associated with microbiological hazards of raw fruits and vegetables is not well understood.

Contamination, infiltration, and survival of microorganisms in fruits and vegetables have been documented, although most reported data concern plant pathogen and spoilage organisms .One potential source of entry of microorganisms into fruits and vegetable is by environmental exposure with uptake occurring through either specific morphological structures in the plant and /or through breaks in tissue that occur as a result of punctures ,wounds, cuts and splits. These insults to the fruit can occur during growing or harvesting. Hill and Favile(1951)postulated that citrus on tree could become infected by insect punctures ,thorn ,injury or hail damage .Also it was found that 35% of the dropped and frost damaged oranges had microholes too small for visual detection ,and half of the fruit having microholes were contaminated with

microbes. Almed et al (1973) found that citrus fruits could be damaged in numerous ways during harvesting, and that the damage provides opportunities for decay organism to gain entrance to fruit. Other modes of entry are created during harvesting, for example tearing off the portion of the peel around the stem end of the fruit during the manual harvest. Mechanically harvested fruit is subjected to splits, punctures and bruises. Some of this damage occurs because of the number of fruit with attached stems that are firm enough to puncture other fruit.

Additionally, processing conditions may promote opportunities for microorganism to infiltrate fruit. For example, citrus fruit may suffer peel damage and microbial penetration as a result of creasing during washing or packing, which may result in splits in the peel; when warm fruits and vegetables are put under cold ambient temperatures, the gases within fruits and vegetables contract during cooling, and there is an inward hydrostatic potential that draws in fluid from the exterior of fruit. This inward movement of fluid has a potential to draw in microorganism. The peel of citrus fruits serves as natural protectants that prevent microbiological contamination of the interior flesh. Removing the peel eliminates this protective layer and subjects the edible portion to potential microbial invasion and spoilage. The primary concern with undesirable microorganisms in citrus products has historically been with their capability to generate off flavors. The growth of human pathogens in citrus products has been presumed to be prevented because of the acidity of the juice. The safety of the fresh squeezed unpasteurized juice however relies mainly on processing sanitation and juice pH, which usually ranges from 2.8-4.0. All the above factors responsible for microbiological contamination of fruits are supposed to contribute either in combination or alone when they are juiced; unpasteurized juices are therefore likely to harbor such microbes which may be either spoilage causing or pathogenic in nature.

Unpasteurized juices either from vegetables or fruits are also more susceptible to microbial deterioration then juices pasteurized under normal time or temperature regimens. Since the last decade fresh juices has increasingly been

the source of serious food poisoning outbreaks and fatalities. Unpasteurized juice has been implicated in outbreaks of *salmonella* and emerging pathogens such as *E. coli* O157:H7. These incidents have resulted in far stricter sanitary and labeling requirements for commercial fresh juice producers.

Reasons for food poisoning outbreaks have been described as follows:

1. Greater use manure as fertilizer for fruit crops, a particular problem for organically grown produce.
2. Increased demand for and consumption of fresh juice as compared to pasteurized and frozen concentrates.
3. A large number of individuals are immuno-compromised and quite sensitive to low number of pathogens .The very young, very old, pregnant women, transplant patients and those with chronic disease conditions are extremely susceptible to food poisoning, compared to healthy individuals .The consequences of infection are correspondingly more serious.
4. The pathogens are also becoming more robust and resistant to preservation techniques
5. Efficient manufacturing and distribution systems

Microbiological safety of juices and fresh cut produce:

Over past couple of years, questions regarding the microbiological safety of juices have been on forefront. United States and Europe were forced to recall juices on account of microbial safety. Although citrus fruit juices are highly acidic, results indicate the presence of certain bacterial pathogens e.g *E.coli 0157:H7* and *Salmonella* sp Also there is need to establish likelihood and the levels of human pathogens on or within the fruits to provide a rationale basis for designing technologies needed to assure the microbiological safety of juices.

In India, a significant proportion of fresh fruits and vegetables are channelized for the production of fresh squeezed juices, prominent among these are carrots and citrus fruits: kinnows (hybrid of *Citrus nobilis* and *Citrus delicosa*) are more popular in the Northern parts of India on account of its cheapness and nutritional properties, carrots likewise enjoy good popularity. Most of these juices are sold through street vended outlets and a large section of the population (all age groups, young, old etc) consume such juices. Although incidents of microbial infections through such juices do not exist in records, several cases especially diarrhea, typhoid etc can be traced to the consumption of such juices. At present, neither any systematic research has been done regarding the safety of these juices nor do microbiological standards exist for fresh fruit juice produced in India. Although the micro flora of fresh produce varies widely and it is unlikely that a uniform set of standards could be developed, there is an urgent need to develop microbiological standards in order to prevent outbreaks in fresh produce and products obtained from these, especially those, which do not undergo any processing prior to consumption.

Therefore, studies which would correlate the entry of bacterial or other pathogens into the juices must be established; once such safety aspects are determined suitable processing techniques/ hygienic measures could be suggested for various food industries this will not only improve human health by curtailing food borne diseases but also open up export options of these juices.

SCOPE OF THE DISSERTATION:

The present work investigates the profile of selected bacterial pathogens in fresh pressed /squeezed carrot and citrus juices sold through street vended outlets and their possibility of entry into such juices through a farm-to – fork route: one common citrus fruit and vegetable viz: kinnow and carrot was chosen for the study. - Kinnow (hybrid of *Citrus nobilis* and *Citrus delicosa*) is preferably used

for juicing in Punjab due to its taste, nutritional qualities and less price, the consumer preference for carrot juice is high due to the same reasons- the sale of these juices by street vended outlets cannot be stopped as it is a source of livelihood. The behavior of the predominant bacterial pathogens in such juices is also attempted; it is expected that these data would help to understand the risk borne out by such microbes to the consumer in unpasteurized citrus fruits and juices.

REVIEW OF LITERATURE

The total production of fruits in the world is around 370 million MT. India ranks first in the world with an annual output of 32 million MT., citrus fruits constitute around 20% of world's total fruit production. India with its current production of around 32 million MT accounts for about 8% of the world's fruit production. The diverse agro climatic zones the country makes it possible to grow almost all varieties of fruits and vegetables in India.

Although India is the largest producer of fruits in the world, the production per capital is only about 100 gms per day. It is estimated that more than 20-22% of the total production of fruits is lost due to spoilage at various post harvest stages. Thus the per capita availability of fruits is further reduced to around 80 gms per day, which is almost half the requirement for a balanced diet. Total consumption of all fresh fruit in India has increased considerably during the past twenty years in both rural and urban areas. According to the National Sample Survey Organization, 64 percent of rural households reported fresh fruit consumption in 1999 compared to 84 percent of households in urban areas. Kerala, Tamil Nadu and Goa have the highest level of fruit consumption, in both rural and urban areas. Punjab, Haryana, Delhi and the Union Territories of Chandigarh in the north are the next largest consuming regions. Consumption levels are much lower in Bihar and Orissa in the east and Rajasthan and Madhya Pradesh in central India.

Vegetables

India is the second largest producer of vegetables in the world (ranks next to China) and accounts for about 15% of the world's production of vegetables. The current production level is over 71 million MT and the total area under vegetable cultivation is around 6.2 million hectares, which is about 3% of the total area under cultivation in the country.

It is estimated that around 20-25% of the total vegetables is lost due to poor post harvesting practices. Less than 2% of the total vegetables produced in the country is commercially processed as compared to 70% in Brazil and 65% in USA. Around 1.5 lakh MT of vegetables is sold as processed products. Though India ranks second in the vegetable production in the world, the average yield for various vegetables are low compared to those experienced in other countries of the world.

Fresh fruits, vegetables and their juices as a source of nutrition:

Fruits and vegetables contain substantial amounts of carbohydrates, vitamin, fibers and minerals They can protect the body from such major diseases as cancer and heart disease. Some are high in substances called anti-oxidants, such as beta-carotene, Vitamins C and E and selenium, which are nutrients that protect cell membranes from the damage of free radicals. Some are rich in other anti-cancer compounds, such as indoles.

Fresh juices either from fruits or vegetables have been demonstrated to be perfect food supplements. They are far more potent than the isolated nutrients found in vitamin pills. Part of this influence is a synergistic effect among nutrients, which means that nutrients combined naturally in foods work together more effectively than when they are separated as a single supplement. The nutrient profile of carrot and a commonly consumed citrus fruit-orange, is described below:

Nutrient profile of carrot juice (per glass)	Nutrient profile of fresh squeezed orange juice,(per glass)
• Calories: 74 • Protein: 1.5 grams • Fats: 0 grams • Carbohydrates: 17 grams • Fibre :2.3 grams	• Calories: 83 • Protein: 1.5 grams • Fats: 0 grams • Carbohydrates: 20 grams • Fibre: 1 grams

Orange juice	Vitamin C level
Fresh squeezed	93 mg
Reconstituted frozen concentrate	73 mg
Canned, unsweetened	65 mg
Chilled juice	62 mg

However, most of these properties are presumed to be lost through processing operations, although there is controversy to this fact it can be argued that fresh squeezed or pressed juices are both acceptable in taste and nutritional properties.

The Role of Fruits and Vegetables in food borne diseases: Global scenario

The consumption of fresh fruits and vegetables is increasing as consumer strives to eat healthy diets. Fruits have been associated with outbreaks of food borne disease in many countries. Organisms involved include bacteria ,viruses and parasites .These outbreaks vary in size from a few person affected to many thousands .The number of foodborne illness outbreaks linked to fresh produce and reported to the United States Centers for Disease Control and prevention (CDC) has increased in the last years. Some of these increase is due to improved surveillance, but other factors may also come into play .A number of reason have been proposed for this increased association of food borne illness with fresh produce. Since the early 1970's, a significant increase in the consumption of fresh produce has been observed in the United States ,presumably due, in part, to active promotion of fruits as an important of healthy diet .From 1982 to 1997 ,per capita consumption of fresh fruits increased from 91.6 to 121.1 Kg ,an increase of 32% (from table) If contamination levels were consistent ,increased consumption of these foods should be expected to lead to greater numbers of illnesses over this time .Greater volumes of intact and chopped ,sliced or prepared fruits are being shipped from central locations and distributed over much larger geographical areas to many more people .This, coupled with increased global trade ,potentially increases human exposure to a wide variety of foodborne pathogens and also increases the chances that an outbreak will be detected .Reasons for increases in foodborne illness in the summer time are not fully understood ,although abusive temperatures and higher consumption of fresh produce during the summer months are likely to play role.

The debilating costs incurred on a countries economy from food borne diseases may be assumed from the consumption patterns of fruits. Fig: shows the typical consumption pattern in the united States.

TABLE; PER CAPITA (Kg) CONSUMPTION OF RAW FRUITS IN US.(SOURCE:FRUIT AND TREE NUT SITUATION AND OUTLOOK REPORT

YEAR	FRUITS
1982	38.7
1983	41.0
1984	40.2
1985	39.3
1986	42.1
1987	44.1
1988	44.1
1989	43.7
1990	41.6
1991	40.7
1992	44.5
1993	45.3
1994	45.6
1995	44.4
1996	44.8
1997	46.7

Routes of Entry of pathogens in fresh produce (vegetables and fruits):

Produce can become contaminated with microbial pathogens by a wide variety of mechanisms contamination leading to foodborne illness has been shown to occur during production, harvest processing and transporting, as well as in retail and food service establishments and in the home kitchen. Contamination at any point

in the food handling chain can be exacerbated by improper handling and storage of produce prior to consumption .The point of contamination is important because control measures will be most effective if geared towards reducing contamination at the source. Contamination of raw fruits with pathogenic organisms of human health significance can occur directly or indirectly via animals or insects, soil, water, dirty equipment, and human handling.

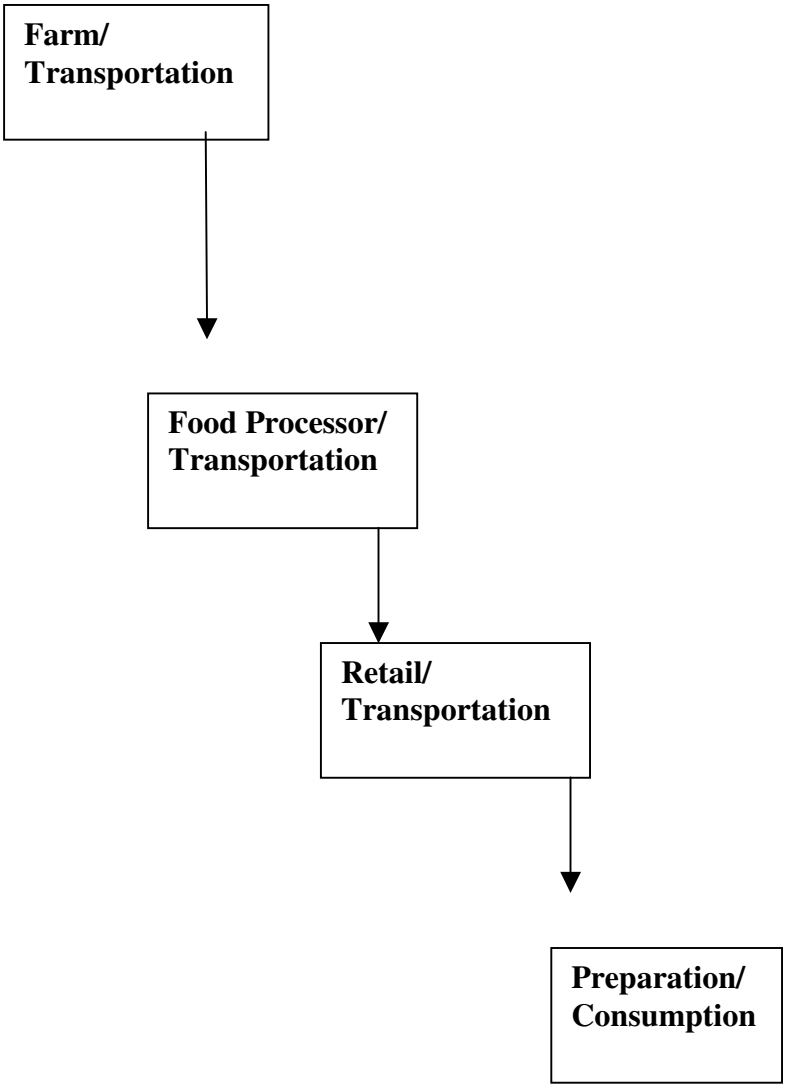
Reasons contributing to outbreaks

Outbreaks of human infections associated with consumption of raw fruits and vegetables have occurred with increased frequency during the past decade. Factors contributing to this increase may include changes in agronomic and processing practices, an increase in per capita consumption of raw or minimally processed fruits and vegetables, increased international trade and distribution, and an increase in the number of immuno-compromised consumers. A general lack of efficacy of sanitizers in removing or killing pathogens on raw fruits and vegetables has been attributed, in part, to their inaccessibility to locations within structures and tissues that may harbor pathogens.

Entry route mapping: Farm to fork studies

Understanding the ecology of pathogens and naturally occurring microorganisms is essential before interventions for elimination or control of growth can be devised, farm –to –fork studies have immense importance in describing the or mapping the entry routes for pathogens during the transport of the produce from farm to the consumer. Throughout this chain of transit and storage, several conditions may actually allow survival and growth of the microorganisms. Results from existing studies indicate, the survival and /or growth of pathogens on fresh fruit is influenced by the type of organism, produce item, and environmental conditions in the field and thereafter, including storage conditions.

A schematic theme of transportation of produce (Fruits and vegetables) From farm to consumers is shown below:



In general pathogens will survive but not grow on the uninjured outer surface of fresh fruits, due to, in part the protective character of the plant's natural barriers(for example cell walls and wax layers).In some cases pathogen levels has been shown to decline on the outer surfaces.

Foodborne diseases: implications on national health:

Number of cases of food-borne illness in a highly industrialized country like the United States reach as high as 80 million per year, with perhaps as many as 7,000 deaths. The causes of these illnesses have changed dramatically during the past decade. Foodborne diseases have a major public health impact (Table 1).In the United States, each year foodborne illnesses affect 6 to 80 million persons, cause 9,000 deaths, and cost an estimated 5 billion U.S. dollars . The epidemiology of foodborne diseases is rapidly changing as newly recognized pathogens emerge and well-recognized pathogens increase in prevalence or become associated with new food vehicles (Table 2). In addition to acute gastroenteritis, many emerging foodborne diseases may cause chronic sequelae or disability. Listeriosis, for example, can cause miscarriages or result in meningitis in patients with chronic diseases. Toxoplasmosis is an important cause of congenital malformation, and *Escherichia coli* O157:H7 infection is a leading cause of hemolytic uremic syndrome, the most common cause of acute kidney failure in children in the United States. Salmonellosis can cause invasive disease or reactive arthritis, and campylobacteriosis can lead to Guillain-Barré syndrome, one of the most common causes of flaccid paralysis in the United States in the last 50 years.

Microbial safety of immediately prepared fresh juices for consumption.

Approximately 2% of all juices sold in United States are unpasteurized , unpasteurized juices are usually made from fruits and vegetables that are ground and /or pressed or squeezed to extract the juice.

Unpasteurized juices are not thermally processed and an evaluation of outbreaks associated with these products might contribute to an understanding of risk factors for contamination of the raw fruits. There have been very few surveys of retail juices for the presence of pathogens, probably because of very low probability of finding pathogens in these products. Still certain rapid test kits to survey retail juices for the presence of *L.monocytogenes, E.coli* 0157:H7, *Salmonella*, coliforms, and fecal coliforms. Only *L.monocytogenes* was isolated from two of 50 juices, an apple juice (pH 3.78) and an apple raspberry blend (pH 3.75) Although there is a long history of juice related outbreaks, they have been relatively infrequent and until 1995,were generally associated with very small commercial processors or home prepared products, while the acidity of most fruit juices prevents the multiplication of pathogens, survival is much better than has been traditionally assumed. Pathogen viability decreases with increasing temperature due to the rapid growth of yeast and other spoilage organism at the higher temperature, this also leads to the decrease in shelf life. While pathogen contamination routes have not been definitively confirmed in any juice outbreak, the use of dropped fruit, the use of non potable water, and the presence of cattle, deer, or, in one case, amphibians, in or near the orchards or groves does appear to be a reoccurring theme.Of five documented outbreaks associated with reconstituted orange juice, three have been the result of contamination by an infected handler preparing the juice .In the other outbreak the water source used to reconstitute the juice was thought to be a factor

The factors contributing to the emergence of foodborne diseases are changes in human demographics and behavior, technology and industry, and international travel and commerce; microbial adaptation; economic development and land use; and the breakdown of public health measures. The following table shows some selected foodborne pathogens, factors influencing their emergence, and possible controls.

Table 1. Estimated number of illnesses and deaths per year caused by infection with selected foodborne bacterial pathogens, United States.

Foodborne pathogen	Estimated cases (10^3)	Estimated deaths (10^3)	Commonly implicated foods
Campylobacter jejuni	4,000	0.2-1	Poultry, raw milk, untreated water
Salmonella (nontyphoid)	2,000	0.5-2	Eggs, poultry, meat, fresh produce, other raw foods
Escherichia coli O157:H7	25	0.1-0.2	Ground beef, raw milk, lettuce, untreated water, unpasteurized cider/apple juice
Listeria monocytogenes	1.5	0.25-0.5	Ready-to-eat foods (e.g. soft cheese, deli foods, pâté)
Vibrio species	10	0.05-0.1	Seafood (e.g. molluscan, crustacean shellfish) raw, undercooked, cross- contaminated.

Table 2. Selected outbreaks in the United States 1988-1997 associated with emerging foodborne pathogens and factors for their emergence.

Pathogen/outbreak	Location()	Year	Factors in emergence	Reference
Hepatitis A Frozen strawberries	MI	1997	international travel and commerce technology and industry	28
Salmonella Typhimurium DT 104 Farm visit	NE	1996	microbial adaptation	48
Cyclospora cayetanensis Guatemalan raspberries	Multistate, Canada	1996	international travel and commerce	25
Salmonella Enteritidis PT 4 Egg-containing foods	CA	1995	international travel and commerce technology and industry	44
Salmonella Enteritidis Mass-distributed ice cream	Multistate	1994	technology and industry	34
Norwalk-like virus Gulf Coast oysters	LA	1994	economic development and land use technology and industry	53
Escherichia coli O157:H7 Fast-food chain hamburgers	Multistate	1993	breakdown of public health measures	54
Escherichia coli O157:H7 Raw apple cider	MA	1991	human demographics and behavior technology and industry	15
Vibrio cholerae O1, El Tor Thai coconut milk	MD	1991	international travel and commerce human demographics and	39

			behavior international travel and commerce	
Trichinella spiralis Undercooked pork	IA	1990	human demographics and behavior	40
			international travel and commerce	
Salmonella Chester Sliced cantaloupe	Multistate	1989	human demographics and behavior	22
Yersinia enterocolitica Pork chitterlings	GA	1988	human demographics and behavior	41

Based upon prevalance, persistence and outbreaks (frequency), pathogens in food have been classed as of 'concern' and 'most concern'. WHO have classed the following as food borne pathogens of most concern. A brief description of some of them is as follows:

***Salmonella* Serotype Enteritidis**

Nontyphoidal salmonellosis is one of the most commonly reported infections in the United States. The doubling of salmonellosis incidence in the last two decades has accompanied modern food industries' centralized production and large-scale distribution. The most prevalent serotypes, *Salmonella* serotype Enteritidis (SE), *Salmonella* Typhimurium, and *Salmonella* Heidelberg.

***E. Coli* O157:H7**

E. coli O157:H7 was first recognized as a human pathogen in 1982 when two outbreaks in the United States were associated with consumption of undercooked hamburgers from a fast-food restaurant chain. The pathogen has

since emerged as a major cause of bloody and nonbloody diarrhea, causing as many as 20,000 cases and 250 deaths per year in the United States. Outbreaks have been reported in Canada, Japan, Africa, the United Kingdom, and elsewhere. In addition to causing bloody diarrhea, *E. coli* O157:H7 infection is the most common cause of the hemolytic uremic syndrome, the leading cause of acute kidney failure in children in the United States. The syndrome is associated with long-term complications; 3% to 5% of patients with hemolytic uremic syndrome die, and approximately 12% have sequelae including end stage renal disease, hypertension, and neurologic injury. Consumption of ground beef, lettuce, raw cider, raw milk, and untreated water have been implicated in outbreaks, and person-to-person transmission is well documented.

Listeria monocytogenes

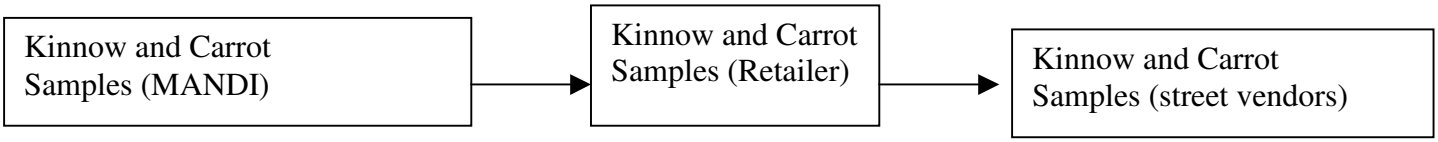
Since the early 1980s, foodborne transmission has been recognized as a major source of human listeriosis. Listeriosis can cause stillbirths, miscarriages, meningitis, or sepsis in immunocompromised hosts. Case-fatality rates as high as 40% have been reported during outbreaks. Outbreaks have been associated with ready-to-eat foods, including cole slaw, milk probably contaminated after pasteurization, pâté, pork tongue in jelly, and soft cheese made with inadequately pasteurized milk. The U.S. Department of Agriculture and U.S. Food and Drug Administration established zero tolerance policies for *L. monocytogenes* in foods in 1989. From 1989 to 1993, the food industry launched efforts to reduce *Listeria* contamination in processed foods, and dietary recommendations were established and publicized for persons at increased risk for invasive listeriosis.

MATERIALS AND METHODS

1. Selection of Collection sites & Collection of the Samples

Microbiological analysis of citrus and carrots during transport :

For this study, the main market where fruits and vegetables are brought from fields was selected (sabji mandi). Fruits and vegetables are usually brought from this area to retail shops , the latter shops sell fruits and vegetables to street vendors . Usually undamaged whole citrus fruits and carrots were used for preparing juices which are sold as fresh pressed /squeezed juices. Samples approximately 1kg of each of the above were purchased from each point in the transport chain, transported to the laboratory in sterile bags and analyzed within 2 hours. The samples were rinsed/rubbed by hand massaging in 500ml of sterile Butterfield's Phosphate Buffer, the latter was serially diluted tenfold in the same and used for the further assessment of microbes.



SCHEME FOR MICROBIOLOGICAL ANALYSISOF CARROT AND KINNOW DURING TRANSPORT.

While in the case of juice samples,Patiala city was divided into 5 major areas, which represented almost whole of the city. Samples were selected from mandi

and from street shops located in the above areas, which sold at least 100 glasses of juice per day were selected ,and also from street vendors of different localities of Patiala City. Juice samples were collected from the vendors in pre-sterilized 250ml bottles.

In the laboratory these samples were analyzed with in 2 hours of procurement for the microbiological analysis. Since both kinnows and carrots are maximally available during the months of October -march analysis of juice samples were carried during this period.

Usually in both of the above studies, both fruit and vegetable samples and juice samples were obtained from the same locations.

Isolation, enumeration & identification of bacterial pathogens from juices

25 ml of the juice sample each of carrot juice and kinnow juice was diluted tenfold with 250ml Butterfield's Phosphate Buffer the latter was diluted tenfold in the same and appropriate dilutions were then enumerated for the following

- **Total aerobic plate counts using Tryptone Soya agar**:

serially diluted the juice samples in Butterfield's Phosphate Buffer, appropriate dilutions were spread on Tryptone Soya Agar plates in triplicates.All plates were incubated at 37^0C for 24-48 hrs. Colonies in each plate were counted, averaged and expressed as CFU/g and converted to log units. Graph Pad Prism software were used for statistical analysis.

- **Total faecal coliforms using Violet Red Bile agar**:

For enumerating the total coliforms, appropriate dilutions, prepared from samples as above, were spread plated on to Violet Red Bile Agar plates in triplicates; all plates were Incubated the plates at 37 ^{0}C for 24-48 hrs.Typical colonies were counted and results expressed as described above.

- **Detection of *E.coli* O157:H7 on Sorbitol-MacConkey (SMAC) agar:**

For enumerating ***E.coli*** O157:H7, appropriate dilutions, prepared from samples as above, were spread plated on to Sorbitol-MacConkey (SMAC) agar plates in triplicates; all plates were Incubated at 37 ^{0}C for 24-48 hrs.Typical colonies were counted and results expressed as described above.

θ **Total *Staphylococcus aureus:***

Appropritae dilutions of samples, obtained as above, were spread plated on to Baird Parker's Agar plates in triplicates, all plates were incubated at 37 ^{0}C for 24-48 hrs. Typical colonies -greyish or black, with clear zones were counted and results reported as described above.

θ **Detection of *Salmonella***:

Qualitative detection of *Salmonella* was carried out as described in the USFDA bacteriological manual; briefly 1ml of juice samples were pre-enriched with 10 ml Universal Preenrichment Broth for 24 hrs at 37^{0}C, 0.1ml of the preenriched culture was inoculated in semisolid Rappaport Vassilidis Agar and incubated at 42^{0}C for 24 hrs, loopfull of the latter were then streaked on Xylose –Lysine Deoxycholate agar and incubated at 37^{0}C fo 24 – 48 hrs**.** Plates were examined for typical colonies

2. Identification of predominant bacterial isolates

θ ***Staphylococcus aureus:***

♣ **Direct plate count**: Typical colonies of *S.aureus* which were circular, smooth, convex, moist, grey to jet black , frequently with light colored margin, surrounded by opaque zone and frequently with an outer clear zone were selected , such colonies had buttery to creamy consistency when touched with inoculating needle.

♣ **Coagulase test:** Suspected colonies of *S.aureus* colonies were transferred into small tubes containing 0.2 –0.3 ml BHI broth and emulsified thoroughly. Inoculated agar slants of suitable maintenance medium, e.g. TSA with loopful

of BHI suspension. Incubated BHI culture suspension and slants 18 –24 hr at 37^0C, 0.5 ml reconstituted coagulase plasma with EDTA was added to the BHI culture and mixed thoroughly. The latter was incubated at 37^0C and examined periodically over 6h period for the clot formation . Only firm and complete clot that stayed in place when tube was tilted or inverted was considered positive for *S.aureus.*

- **Catalase test :** The growth from TSA slant was used for Catalase test on glass slide and illuminated properly to observed production of gas bubbles
- **Thermostable nuclease production:** Micro slides were prepared by spreading 3ml-toluidine blue- deoxyribonucleic acid agar on the surface of each microscope slide. When agar had solidified, 2m diameter wells in agar was cut and agar plug removed, about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures was added to the well on prepared slide. The slides were incubated in moist chamber 4h at 37 ^{0}C .Bright pink halo extending at least 1mm from periphery of well indicated a positive reaction.

θ ***Escherichia coli:***

- **Direct plate count:** For presumptive identification of *E.coli* colonies, which were purple red with 0.5-mm diameter were counted and recorded.
- **Confirmation in Brilliant Green Lactose Bile (BGLB) Broth:** Transferred purple- red colonies from VRBA plates to tube of BGLB broth. Incubated tubes at 35^0C. Examined at 24 and 48 hr for gas production.
- **Confirmation on Eosin methylene blue agar (EMBA):** One loopful was streaked from BGLB broth on to the EMBA plates. *E.coli* showed colonies with metallic sheen.

Biochemical Tests

The IMViC Test - 4 tests (indole, methyl red, Voges-Proskauer, citrate) used to differentiate *E. coli* from the other two coliforms *(Klebsiella* and *Enterobacter*).

Indole Test - Spot indole testing was performed by adding a drop of indole reagent (**refer to annexure**) to the culture of presumptive

E.coli in tryptophan broth. A positive test was indicated by the appearance of a red color.

MRVP Test (methyl red and Voges-Proskauer) -. Methyl red test was performed by inoculating 10ml portion of sugar broth with presumptive colonies of *E.coli*, then incubating at 35^0Cfor 48 hrs. A few drops of methyl red indicator was added to the culture that turned red.

For the Voges-Proskauer test, Several drops of alpha-naphthol were first added to the MRVP broth culture **(refer to annexure)** followed by an equal number of KOH drops. No red color formation occurred

CitrateTest: Simmons citrate agar **(refer to annexure)**, with bromothymol blue (pH indicator), changes from green to blue if citrate is hydrolyzed. For citrate test, Simmon's citrate agar slants were streaked with presumptive colonies of *E.coli* & incubated at 35^0C for 48 hrs. Absence of blue coloration was taken to be positive.

Urease Test:. For Urease test, Urea broth was inoculated **(refer to annexure)** with the colonies from VRBA plate & incubated at 37^0C. Absence of pink color indicated +ve reaction for *E.coli.*

Identification of *E.coli* O157: H7

- **4-methylumbelliferyl-B-D-glucuronide (MUG) medium:** Tested *E. coli* O157 strains for the enzyme B-glucuronidase using broth or agar medium containing the substrate 4-methylumbelliferyl-B-D-glucuronide (MUG). When this enzyme cleaves MUG, a fluorescent product is produced that is detectable with long-wave ultraviolet light. *E. coli* O157: H7 and nonmotile *E. coli* O157 strains that produce Shiga-like toxins lack the enzyme and are MUG negative. For this reason the MUG assay used in conjunction with

testing for Sorbitol fermentation was a useful screening test for toxigenic strains of O157.

- **Confirmation of *E.coli* O157: H7**: Presumptive isolates of *E.coli* O157: H7 were sent to the National Centre for *Escherichia Coli* and *Salmonella*, Central Research Institute, Kasauli, India for further confirmation.

- ***Salmonella Sp.***

- **Direct plate count**: On XLD, enumerated & recorded the presence of *Salmonella* that produced pink colonies with or without black centers or colonies with large, glossy black centers or that appeared almost completely black. Atypically a few *salmonella* cultures produced yellow colonies with or without black centers.
- **IMVIC Test & Urease Test**: IMVIC test & Urease Test for *salmonella* were performed as were done for *E.coli* (described above)
- **Confirmation of Salmonella sp**. Presumptive isolates of *Salmonella* were sent to the National Centre for *Escherichia Coli* and *Salmonella*, Central Research Institute, Kasauli, India for serotyping and further confirmation.

3. Survival at different storage temperatures of predominant bacterial isolates

In order to evaluate the effect of juice on the survival and growth of bacterial pathogens, appropriate surrogates of the predominant isolates were selected.The survival & growth kinetics were carried out at different storage temperatures viz.: 4^0C, 8^0C,21^0C & 28^0C as described below

Selection of pathogens :

E.Coli O157:H7 NCTC12900, a non pathogenic strain and *Salmonella* LT2(rif nal) was used for all subsequent studies. All strains were stored in cryoprotective beads at –200c. Before each study, they were activated on either BHI (Brain Heart Infusion agar) or TSA(Tryptic soya agar) slants.

Culture preparation

Cultures of ***E.Coli O157:H7*** surrogate strain and ***Salmonella*** surrogate strain were grown in 100 ml tryptic soy broth & incubated at 37^{o}C for 24 hours with shaking (150rpm**).** Mid log phase cultures were harvested by centrifugation (12000 rpm, 2 minutes) washed twice with 0.1% sterile peptone water and resuspended in 2 ml of the same in duplicates, one part was used for measuring the absorbance at 600nm, the other were used to inolculate samples of juices.

25 ml of juice samples (carrot& kinnow juice) were distributed in sterile bottles which were then inoculated with culture of bacterial surrogates, all combinations were incubated at 4^{0}C, 8^{0}C , 21^{0}C &28^{0}C for 24 hrs, 48 hrs,& 72 hrs . After the required time of incubation, 10ml of inoculated sample was serially diluted in Butterfield's Phosphate Buffer. Appropriate dilutions were then spread plated on BP & SMAC plates in triplicates. All plates were incubated at 37^{0}C for 24 – 48 hrs, after requisite time , colonies were counted, averaged and expressed as LogCFU/ml.

4. Effect of storage temperatures and stress on the survival of bacterial isolates

Cultures of *E.Coli O157:H7* surrogate strain were grown in 100ml tryptic soy broth & incubated at 37^{o}C for 24 hr. Overnight grown cells in the mid log phase were taken & then washed with 0.1% peptone water & then resuspended in of peptone water to adjust the numbers to approximately 8 Log Cfu/ml.

These culture preparations were then used to inoculate the juice samples sterilized through 0.22µm sterile membrane, which were incubated at 4^{0}C, 8^{0}C ,21^{0}C & 28^{0}C for 24 hrs, 48 hrs, 72 hrs & 144 hours . 1ml of the samples withdrawn after each time period were serially diluted in Butterfield's Phosphate Buffer. Appropriate dilutions were then spread plated on BP & SMAC plates. The plates were incubated at 37^{0}C for 24 – 48 hrs. Results were expressed as described above.

Effect of stress: From the surveillance studies it was found that the vendors usually do not wash the juicers or the storage containers, each time the fresh lot of juice gets mixed with a small amount of previous lot of juice. The latter may contain some pathogens which can serve as inoculum for the fresh lot since the pH of the juice is approx. 3.5, the pathogen would be under acid stress may show a differential growth behavior. In order to see the effect of acid stress on the survival of pathogens, a simulated time course study for 10 hrs was carried out as follows:

Culture preparation: Culture of surrogate strain of *E.coli O157:H7* was grown in tryptic soy broth & incubated at 37^{o}C for 24 hr. Overnight grown cells in the stationery phase of growth were taken. Lowered the pH of culture with10 N HCl, incubated the acid challenged culture at 37^{o}C for 90 min. then the cells were washed with 0.1 % peptone water & resuspended in peptone water and used as inoculum for the juice sample. Control was also taken which was not treated with acid.

Acid treated & normal cultures of *E.coli O157:H7* were inoculated in juices (brought under aseptic conditions)**.** Incubated at 28 ^{o}C for 0hr, 2hr, 4 hr 6 hr, 8 hr, and 10 hrs. After the completion of each incubation interval, 1ml of the sample was taken and serial dilutions were made in Butterfield's Phosphate Buffer. Appropriate dilutions were then spread plated on SMAC plates overlaid with thin agar layer (SMAC-TAL plates) (as described below in pt.**7**). The plates were incubated at 37^{0}C for 24 – 48 hrs in triplicates, after the requisite time conies were counted, averaged and expressed as Log CFU/ml.

Survival of *E.coli* O157: H7 in blended and unblended kinnow and carrot juices (25^{0}C)

Culture preparation: The culture was prepared as mentioned above.

Carrot and Kinnow Juice samples were brought in a sterile 250ml bottles, the foam portion was discarded and juices were sterilized by passing through 0.22ì m sterile membranes. Thereafter, different concentrations of the latter were

prepared with sterile water as follows: 100% concentrated (undiluted), 50% diluted, 25% diluted sterile saline (0.8%), was used as control. From the above dilutions 25ml from each of the sample was taken and serially diluted in 250ml Butterfield' s Phosphate Buffer and blended; the latter was diluted tenfold in the same buffer and then surface plated on SMAC agar plates in triplicates. All plates were incubated at 37^0C for 24 – 48 hrs. Colonies were counted, averaged and expressed as LogCfU/ml as described before.

Survival of *Salmonella* sp in kinnow and carrot juices at different storage temperatures

Culture preparation.

Culture was prepared as described above..

The effect of different storage temperatures on the survival and growth of *Salmonella.* Was ascertained by inoculating cultures as prepared above in 25 ml of kinnow juice (filter sterilized), the latter were incubated at.: 4^0C, 8^0C, 21^0C& 28^0C, 1ml samples were withdrawn after 24 hr,48hr,72hr, diluted in Butterfield Phosphate buffer and spread on XLD plates in triplicates, which were incubated at 37^0C for 24-48 hours, after the requisite time colonies were counted , averaged and expressed as logCFU/ml as described before

Recovery methods for the recovery of injured pathogens

Culture preparation: Cultures of *E.coli O157:H7* and *Salmonella typhimurium* were grown in 100ml tryptic soy broth & incubated at 37^oC for 24 hr. Overnight grown cells were used as inoculum for juice samples.

Enhanced recovery methods

Thin Agar Layer Method: The Thin Agar Layer method of Kang & Fung 1998 was used to enumerate injured pathogens from juices. This method involved overlaying 14 ml of non-selective medium on to the prepoured, pathogen specific, selective medium. To enumerate both injured & uninjured pathogens,

TSA was used as a non-selective medium & BP, SMAC, XLD were used as selective med. for different pathogens. After solidification of the sterilized selective agar in a petridish, 7ml of melted TSA was overlaid. After the solidification of the first layer of TSA, a second layer of 7ml of TSA was overlaid.
100 ml of Juice was inoculated with cell suspension of surrogates of pathogenic bacteria & incubated at 37^{o}C for 2hr. After 2hr of incubation,1ml of each sample was serially diluted in Butterfield's phosphate buffer and were spread plated on to the TAL plates, selective media plates & TSA plates. Finally the recovery of pathogens on different plates was compared.

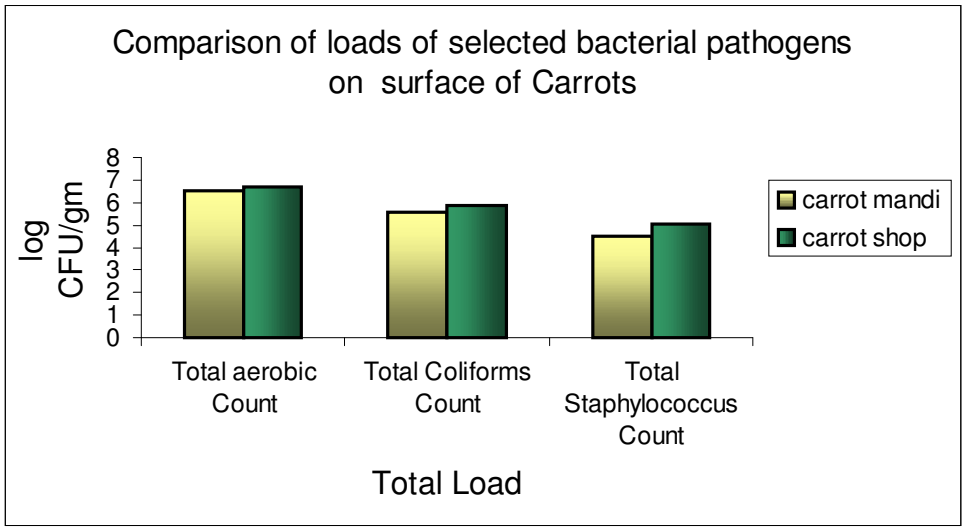
5. Spoilage and survival

Since fresh juices are generally consumed within 4 to 6 hours of its preparation it is essential to know the growth characteristics of inherent spoilage microbes present in the juices; a growth kinetic study of these spoilage microorganism were therefore carried out. For this, samples from different shops, vendors were collected, kept at 28^{o}C. At different time intervals(3, 6,and 9 hours respectively) 25ml from each of the samples was blended and serially diluted in Butterfield's phosphate buffer(250ml). Appropriate dilutions were then spread on TSA & Yeast chloroamphenicol agar media for total microbial load & yeast/molds count, in triplicate, the incubation period in case of yeast and moulds was 5 days at 28 ^{0}C,whereas in case of total microbial load incubation period was 24to 48 Hours at 37 ^{0}C.After the requisite time, colonies were counted and converted to LogCFU/ml as described before.
After each time interval samples of both carrot and kinnow juice were examined for change in total soluble solids and titrable acidity as described below:
Total Soluble Solids: Hand Refract meter of range 0-32 measured TSS of Kinnow juice and carrot juice sample.
% Titrable Acidity: the % titrable acidity of Kinnow and carrot juice sample was estimated by the method described by AOAC (1984). The samples were diluted 5 times with glass-distilled water. The known volume of the diluted sample was

titrated with 0.1N NaOH with phenolphthalein as indicator to faint pink end point. The % titrable acidity was calculated by the following formulae:

$$\% \text{ TA} = \frac{\text{Titre} \times \text{Normality of alkali} \times \text{volume made up} \times \text{Eq.wt of acid}}{\text{Volume of sample} \times \text{wt/vol taken for estimation} \times 1000}$$

Results and Discussion



Fig(1) shows the total load of selected bacterial pathogens on the surface of Carrots. Total aerobic count, total coliform and staphylococcal count were approximately in the range of Log 6-7CFU/g, Log 5.5-6 CFU/g and Log 5-6 CFU/gm respectively. Carrots purchased from mandi or the central market had relatively less counts of all three types in comparison to those obtained from shops.

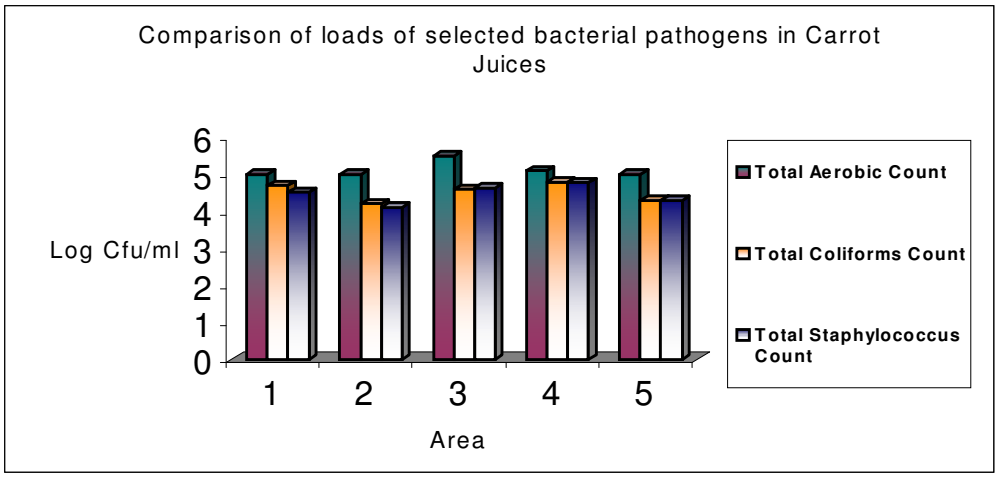


Fig 2

Fig (2) shows the total load of the above bacterial types in carrot juices obtained from different areas; a high coliform , aerobic and staphylococcal count was evident. Carrots are usually brought initially to mandi, which is the central stocking point in Patiala for fruits and vegetables; thereafter it is transported to various shops which either sells them or uses them to prepare juices. We analyzed those shops which purchased the same lot of carrots, which we initially purchased from the mandi. Qualitative analysis of carrot samples showed the presence of *Salmonella enterica* in about 5 % of the samples, *E.Coli O157:H7* was detected from juice samples presumptively in three areas, 13% of Staphylococcus were coagulase positive *S.aureus* It is clear from the results that the load of pathogens increased at each point of the transport process of carrots, the latter may have been introduced into juices prepared from carrots, irrigation practices using contaminated water or unhygienic practices, alternately their entry through handlers or contaminated water cannot be ruled out.

Fig(3)and Fig (4) Show the total aerobic load , total coliforms and total staphylococcal count on the surface of kinnow fruits and in juice samples obtained from the same lots of kinnow fruit.

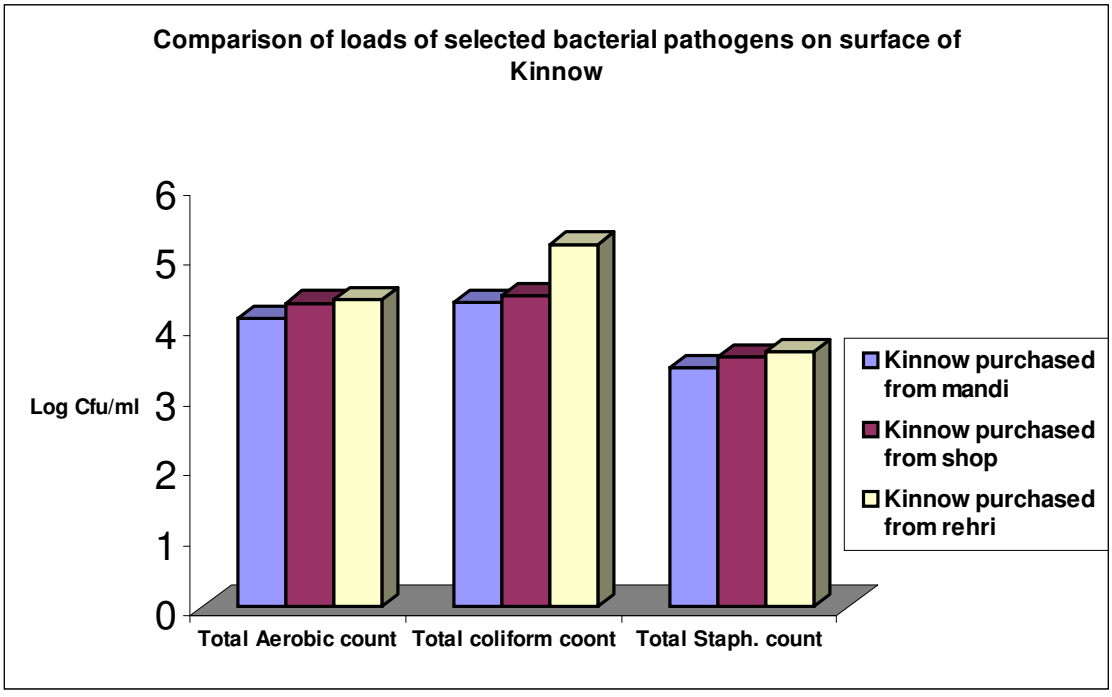


Fig 3

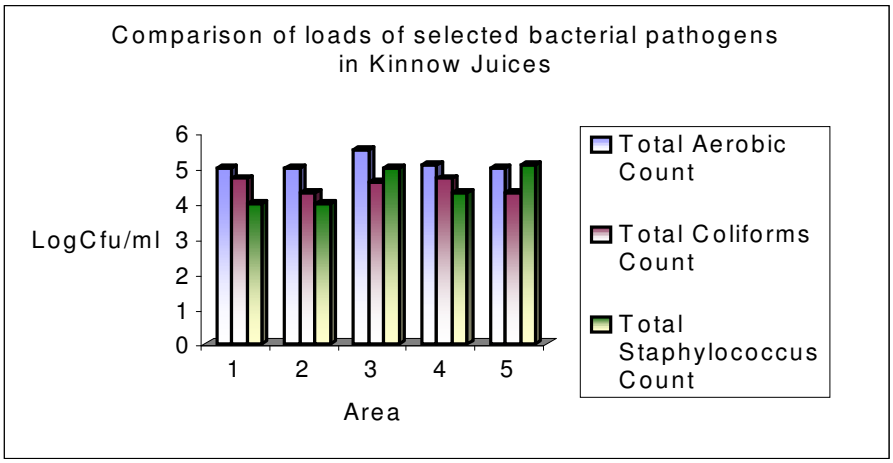


Fig 4

For kinnow fruit samples, the total aerobic counts and coliform counts lay between Log 4-4.5 CFU/g whereas Staphylococcal counts ranged from Log3.5-4CFU/g; an increase in the loads of each were evident although not very significant at each point of transport; the coliform counts were surprisingly high in case of samples purchased from carts or rehris. For juice samples however, there was an increase in all the three counts. Qualitative analysis showed the presence of *Salmonella enteritica* in juice samples obtained from two areas. *E.Coli O157*:H7 was however not detected from any samples, about 3.5% of Staphylococcus isolates were coagulase positive *S.aureus*.The low pH of kinnow juices maybe attributed to the lower loads, however the results indicate unhygienic practices and contaminated fruit through improper handling.

The presence of *Salmonella enteritica* and *E.coli O157:H7* is of concern: both *E.Coli*O157:H7 and *Salmonella* have assumed great importance in food borne diseases and has been shown to survive and persist in unpasteurized juices, fresh produce and fruits, consequently we chose surrogates/non pathogenic variants of these pathogens for further studies.

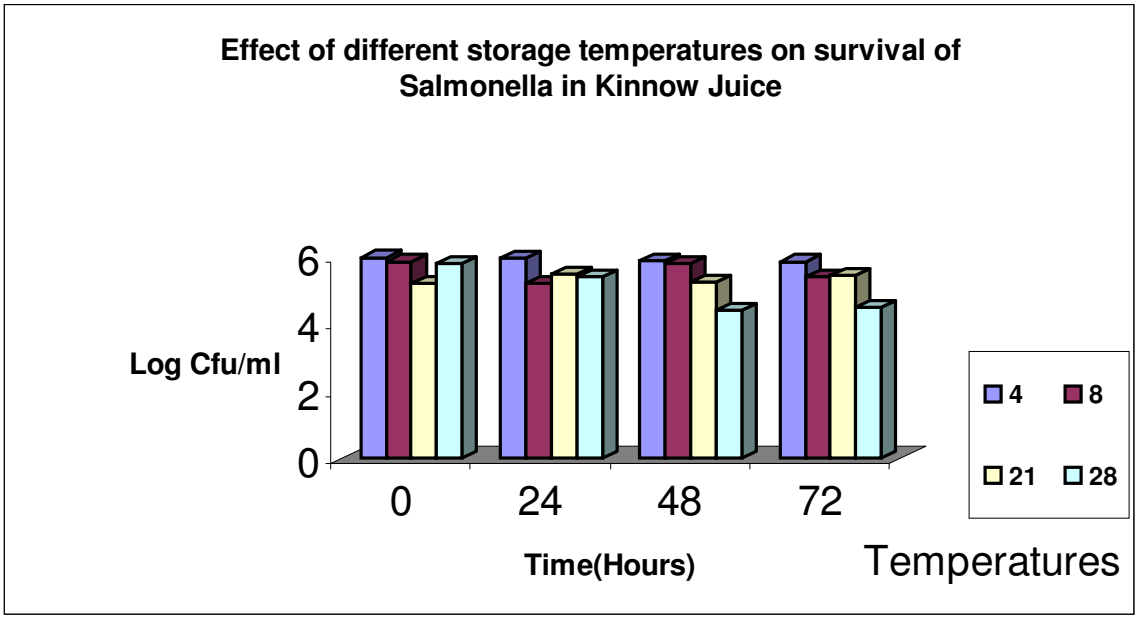


Fig 5

Fig (5) shows the survival kinetics of a non pathogenic strain of *Salmonella* in Kinnow juices stored at different abusive temperatures. It is evident from the results that no significant decrease from the initial inoculum of Log 6 CFU/ml occurred in any of the storage temperatures except at 28^0C, where a slight decrease was noted. Survival of *Salmonella* has been demonstrated in citrus and apple juices, their presence without decline poses a risk on consumers, since they are usually prepared and stored (although not for extended periods) at prevailing temperatures during the months of October – January, challenged juice samples were therefore incubated at these temperatures , an extended incubation time of 72hrs was chosen as it was thought to provide a true estimate of the extent of survival.

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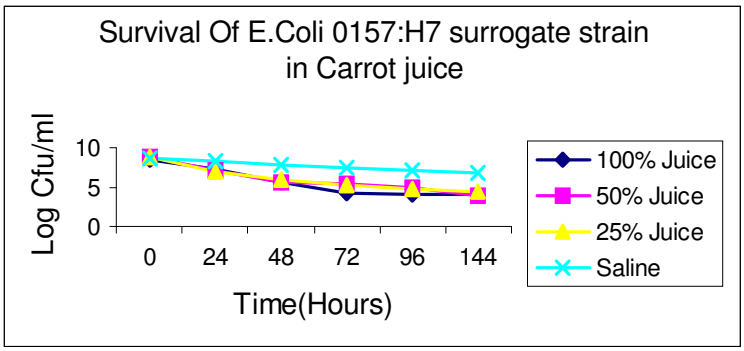

Fig 6

Fig (6) shows the survival of *E.coli O157:H7* surrogate strain in carrot juices at 28^0C; both diluted and undiluted juices were used since carrot juices are usually diluted to these proportions by vendors before serving

A sharp decline in numbers was observed with undiluted juice, significant decline also occurred for 50 and 25% concentrations till 48 hours, however in all the three combinations , constancy was observed after 72 hours till termination of the experiment. In saline there was no notable decrease of initial counts . Inhibition due to pH effect was ruled out as the pH even after dilution did not fall below 4.However the possibility of insufficient nutrients required for sustaining the high levels of *E.coli O157:H7* cannot be ruled out .

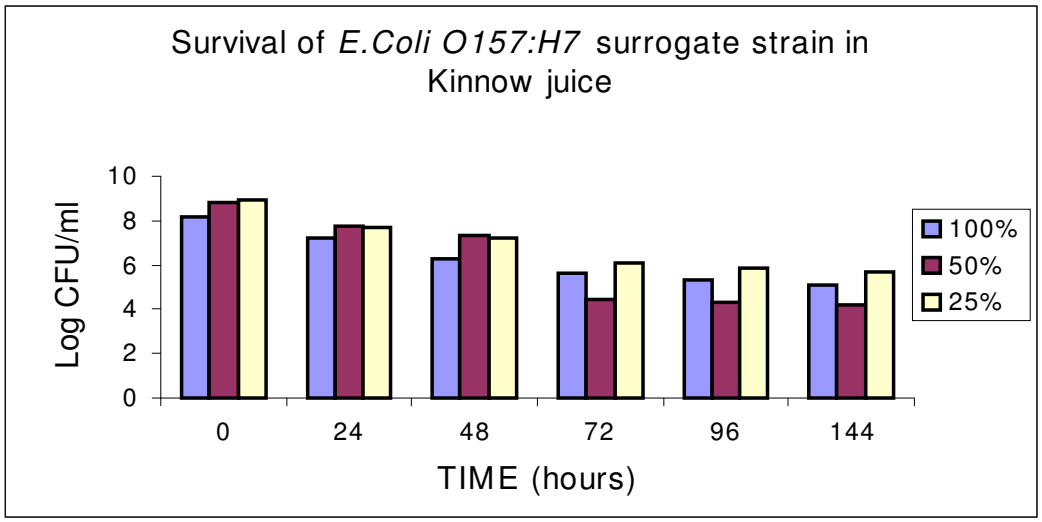

Fig 7

Fig (7) shows the survival of *E.coli O157:H7* surrogate strain in kinnow juice at 28^0C.

A sharp decrease in the initial counts were observed for undiluted and diluted juice samples, which was more evident till 48 hours, however neither juices samples diluted to 25% nor the undiluted juice showed decrease lower than Log5CFU/ml; counts remained constant in juice samples diluted to 50%. The low pH of kinnow juice (3.5) may have been responsible for the decrease in population of *E.Coli O157:H7*; in order to test this hypothesis, the kinetics of survival of the acid stressed and unstressed *E.Coli O157:H7* surrogate strain was carried out in undiluted juice sample.

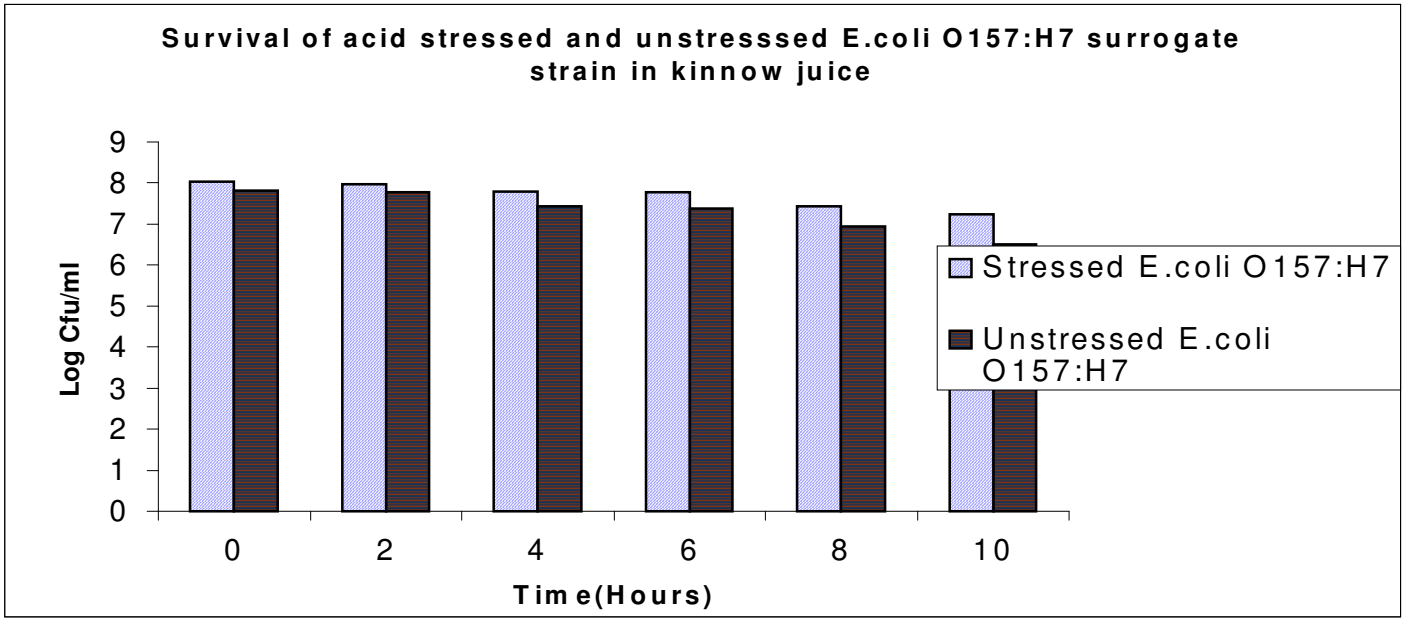

Fig 8

Fig (8) compares the profile of both stressed and unstressed *E.Coli O157:H7* surrogate strain is undiluted (100%) Kinnow juice within a period of 10 hours, no significant difference in decline pattern was observed was till the termination of the experiment. The unstressed strain however exhibited a decrease of about 1 LogCFU/ml after a period of hours. The results indicate that within 6 hours the survival patterns do not change appreciably, enhanced survival of acid stressed *E.Coli O157:H7* have been demonstrated by several workers, especially in high acid foods or juices. It is clear that although nutrient dependency may be responsible for lower survival the latter can survive well at low pH values in such samples containing *E.Coli O157:H7*, therefore would be definitely risky

Fig(9) shows the survival of *E.Coli O157:H7* surrogate strain in carrot juices supplemented with various additives at 28^0C

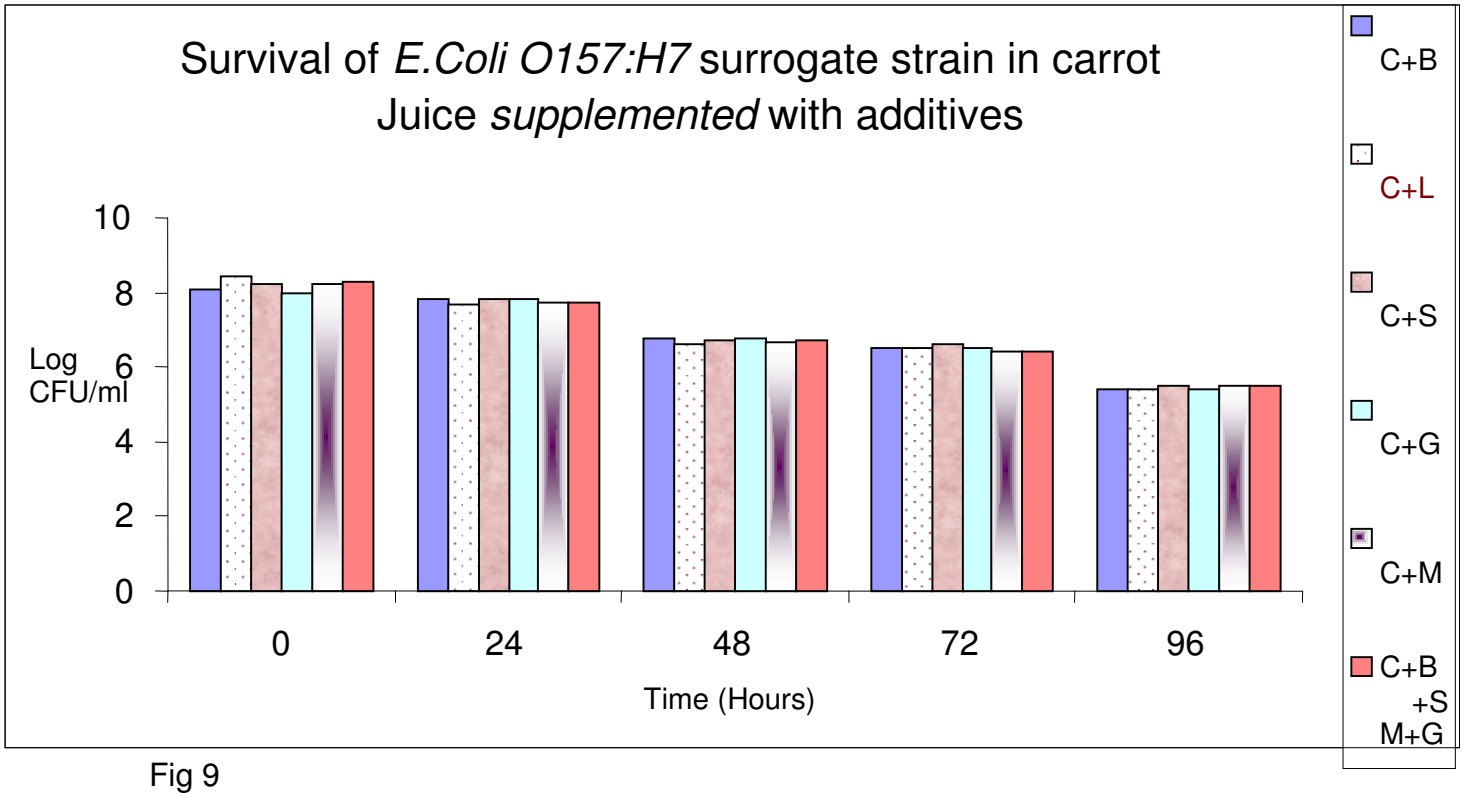

Fig 9

Such additives (beet root (B), mint (M), lemon (L), ginger (G) and salt (s) are incorporated in carrot juices by vendors to render juices . more palatable to consumers; survival experiments with the latter alone and in combination was anticipated to be useful in analyzing the alterations ((increase or decrease) in growth, if any, in juice samples. No appreciable decrease of the initial inoculum was observed in any of the combinations, after 96 hours the average decline was about 2LogCFU/ml. However a slight reduction in counts with samples supplemented with Lemon juice was observed after 48 hours. The latter may be attributed to the presence of organic acids present in lemon juices.

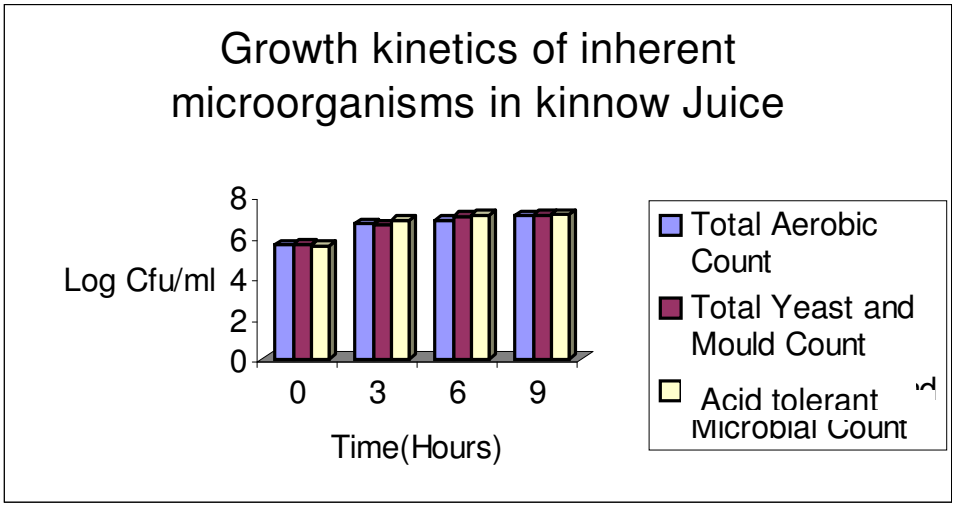

Fig 10

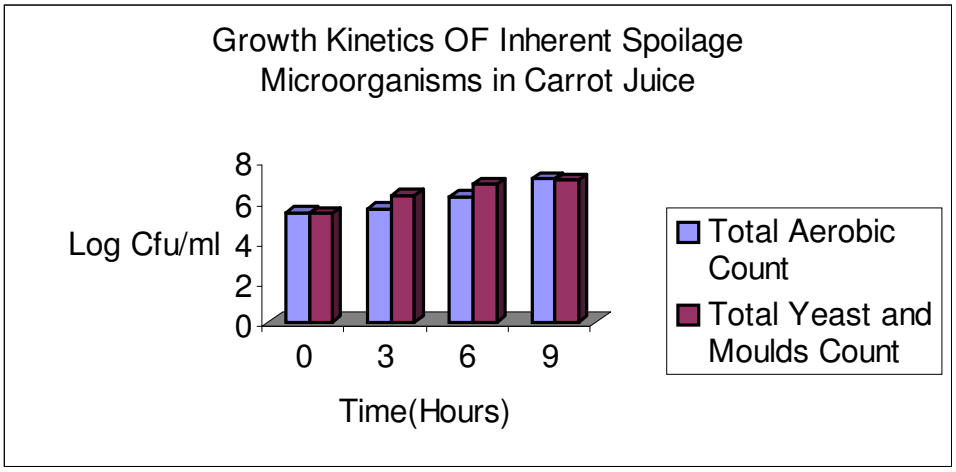

Fig 11

Fig(10) and Fig (11) Shows the growth kinetics of the inherent spoilage microorganisms in carrot and kinnow juices.

The total aerobic counts increased till 9 hours at 28^0C , followed by moulds and yeasts in carrot juices. After approximately 6 hours carrot juices were completely spoiled on account of a loss of sensorial properties, the total aerobic count was

approximately Log 6.2 CFU/ml whereas total mould and yeast counts were Log 6.9CFU/ml. In kinnow juices, the total yeast counts were approximately Log6.8CFU/ml, initially and continued to increase over time, the total aerobic counts, yeast and mould count and counts for acid tolerant microbes also increased. Juice samples were completely spoiled after 6 hours as evidenced by complete loss of sensorial properties. Yeasts and moulds are common spoilage microorganisms in fruit juices, also acid tolerant microbes would be expected in juices having high acidity. Results of spoilage studies were expected to provide an approximate time frame for consumption of fresh pressed juices, the survival of bacterial pathogens or growth within this period could be important in actually ascertaining the risk posed by bacterial pathogens to consumers during this period of time. Our results indicate that an approximate time period of 5 to 6 hrs (28^0C), must be selected for actually assessing the consumer risk of *E.Coli* 0157:H7 and salmonella in freshly pressed carrot and kinnow juice samples.

PERCENTAGE TITRABLE ACIDITY AND DEGREE BRIX IN CARROT JUICE

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Time(Hours)	TSS(Degree Brix)	% Titrable Acidity
0	10.4	0.176
3	10.4	0.176
6	10.8	0.24

PERCENTAGE TITRABLE ACIDITY AND DEGREE BRIX IN KINNOW JUICE

.

Time(Hours)	TSS(Degree Brix)	% Titrable Acidity
0	12.6	0.112
3	11.4	0.176
6	10.8	0.256

Table 1 and 2 shows the corresponding profile of titrable acidity and total soluble sugars in freshly squeezed carrot and kinnow juices with time, at 28^0C.No change was observed in carrot juices while in kinnow juice a steady decrease in total soluble sugars and increase in acidity was observed – the latter results corroborate the increase in yeasts and moulds and acid tolerant microbes during the course of spoilage of the Kinnow juice samples.

CONCLUSION

- Samples of fresh pressed (unpasteurized) carrot and kinnow juices obtained from street vended shops throughout Patiala city, showed significant Total aerobic counts, total faecal Coliform count and staphylococcal counts ranging from: 5-6, 4-5 and 4-5 Log CFU/ml respectively.
- Microbiological analysis of carrot and kinnows supplied to such street venders for juicing showed loads of bacteria (Total Aerobic, Total Faecal Coliforms and staphylococcus counts) similar to those of corresponding juice samples obtained from same vendors. The microbial load on both kinnow and carrots at each point of the supply chain was found to increase.
- Qualitative analysis revealed the presence of coagulase positive *S.aureus*, *Salmonella enterica* and *E.coli O157:H7* in juice samples.
- The nonpathogenic variant of *Salmonella* survived with minimal decrease in counts till 48 hours at 4, 8 and 28^0C in Kinnow juice samples.
- The *E.Coli O157:H7* surrogate strain showed lesser efficiency to survive, both in carrot and kinnow juice samples at 28^0C.
- Carrot juice samples supplemented with various additives, either alone or in combination, failed to bring about significant decline in survival of the *E.Coli O157:H7* surrogate strain till at least 24 hours at 28^0C.
- Acid stressed *E.Coli O157:H7* surrogate strain exhibited better survival in kinnow juice samples at 28^0C till a period of 10 hours in comparison to the survival of its unstressed counterpart.
- Carrot juice samples were completely spoiled after approximately

4 hours at 28^0C as judged by increase in total viable counts, total yeast and mould count and concomitant loss of sensorial properties. The total soluble sugars and total titrable acidity showed no appreciable decrease.

- Kinnow juice samples were completely spoiled after 5 hours at 28^0C, as evidenced by increase in yeast and mould count, total aerobic count and acid tolerant microbes; the total titrable acidity increased with a parallel decrease in total soluble sugars. A complete loss of sensorial properties was evident after 5 hours.
- Established dose response data indicate that very low inoculum sizes are necessary for initial of diseases by S*almonella* and *E.coli O157:H7* strains, our results indicate that both these strains could survive within the time frame required for spoilage -all simulated survival experiments were done assuming a "worst possible scenario" –a high initial inoculum was therefore chosen. Although not shown, a similar trend of survival was also observed in experiments where approximately 4 log CFU/ml of starting inoculum of both strains were used to judge the survival of the latter in kinnow and carrot juices. It may be concluded that unpasteurized kinnow or carrot juices contaminated with either *Salmonella* or *E.Coli O157:H7* strains could pose a potential risk to the consume

Annexure

1. **Butterfield's Phosphate Buffer** :

Stock solution

KH_2PO_4 = 34g

Distilled water = 500ml

Adjust the pH = 7.2 with 1N NaOH. Bring the volume to 1 liter with distilled water. Sterilize 15 min at 121°C. Store in refrigerator.

Dilution Blanks

Take 1.25 ml of the stock solution and bring the volume to 1 liter with distilled water. Dispense into bottles/ test tubes. Sterilize 15 min at 121°C.

2. **Tryptone Soya Agar (TSA**

Composition: Ingredients g/L

Casein Enzymic hydrolysate	17.00
Papaic digest of soyabean meal	3.00
NaCl	5.00
Dipotassium Phosphate	2.50
Dextrose	2.50
Agar	15.00
Distilled Water	1L

Final pH (at 25 °C) = 7.3

3. **Baird Parker's Agar**

Composition:	Ingredients	g/L
	Casein Enzymic hydrolysate	10.00
	Meat Extract	5.00
	Yeast Extract	1.00
	Glycine	12.00
	Sodium Pyruvate	12.00
	Lithium Chloride	5.00
	Agar	20.00
	Distilled Water	1L
	Final pH (at 25 °C) = 7.0	

4. Violet Red Bile Agar :

Composition:	Ingredients	g/L
	Yeast Extract	3.00
	Peptone	7.00
	Sodium Chloride	5.00
	Bile Salts	1.5
	Lactose	10.00
	Neutral Red	0.03
	Crystal Violet	0.002
	Agar	20.00
	Distilled Water	1L

Suspend ingredients in distilled water & let stand for a few min , mix thoroughly & adjust the pH=7.4 (before adding agar). Heat with agitation & boil for 2min. Don't sterilize. Before use cool to 45 °C .

5. **Universal Pre-enrichment broth**:

Composition:

Ingredients	g/L
Casein Enzymic hydrolysate	5.00
Protease Peptone	5.00
Monopotassium Phosphate	15.00
Disodium phosphate	7.00
Sodium chloride	5.00
Dextrose	0.50
Magnesium sulphate	0.25
Ferric ammon. Citrate	0.10
Sodium pyruvate	0.20

Final pH =6.2 (at 25 ^{o}C

6. **Semisolid Rappaport Vassilliadis Medium :**

Composition:

Ingredients	g/L
Tryptose	4.59
Casein enzymic Hydrolysate	4.59
Potassium dihydrogen Phosphate	1.47
Sodium chloride	7.34
Magnesium chloride	10.93
Malachite green	0.037

Agar	2.70

Final pH =5.2 (at 25 ^{o}C)

7. **Xylose Lysine Deoxycholate Agar (XLD):**

Composition:

Ingredients	g/L
Yeast extract	3.00
L-Lysine	5.00
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.00
Sod. Deoxycholate	2.50
Sod. Thiosulphate	6.80
Ferric Ammon. Citrate	0.80
Phenol Red	0.08
Agar	15.00

Final pH =7.4 (at 25 ^{o}C)

8. **Sorbitol Mac Conkey's Agar (SMAC):**

Composition:

Ingredients	g/L

Peptic Digest of animal tissue	17.00
Proteose Peptone	3.00
Sorbitol	10.00
Bile salts	1.50
Sod. Chloride	5.00
Neutral Red	0.030
Crystal Violet	0.001
Agar	13.50

Final pH =7.1 (at 25 ^{o}C)

9. **MUG EC O157: H7 Agar**:

Composition:

Ingredients	G/L
Casein Peptone	20.00
Meat Extract	2.00
Yeast Extract	1.00
Sorbitol	10.00
Ferric Ammon. Citrate	0.50
4-Methyl umbelliferyl â-D-glucuronide	0.10
Sodium chloride	5.00
Bromothymol blue	0.025
Sod. Thiosulphate	2.00
Sod. Deoxycholate	1.12

Agar	13.00

Final pH =7.4 (at 25 ^{o}C)

10. **Tryptophan Broth for Indole test** :

Dissolve 10.0g tryptone in I liter of reagent grade water. Dispense 5ml portion in test
tubes & sterilize 15 min at 121^{o}C.

11. **Test reagent for Indole test**:

Dissolve 5g p-dimethylaminobenzaldehyde in 75ml isoamyl alcohol, and add 25ml conc. HCl.

12. Methyl Red Voges Proskauer Broth:

Dissolve 7.0-g proteose peptone, 5.0g Glucose, and 5.0g dipotassium hydrogen phosphate in 1L reagent grade water. Dispense 5ml portion of medium in test tubes sterilize by autoclaving at 121^{o}C for 15 min.

13. Test reagent for Voges Proskauer Test:

Naphthol solution: Dissolve 5g purified á – naphthol in 100ml absolute ethyl alcohol.

Potassium Hydroxide (7N): dissolve 40g KOH in 100ml-reagent grade water.

14. Simmon's Citrate Agar:

Add 0.2g $MgSO_4.7H_2O$, 1.0g K_2HPO_4 1.0-g ammonium dihydrogen phosphate, 2.0 g sodium dihydrate, 5.0 g sodium chloride, 15.0 g agar and 0.08g bromothymol blue to 1L reagent grade water. Autoclave & Put in test tubes to make slants.

15. **Urea Broth : Urea Base (950ml) + Urea solution (50 ml)**

Urea Base:

Ingredients	g/L
Peptone	1.00
D+ Glucose monohydrate	1.00
Sodium chloride	5.00
KH_2PO_4	2.00
Phenol Red (0.2%)	6ml
Water	1L

Dissolve the dehydrated base in water by heating. Adjust the pH=6.8 after sterilization & sterilize at 121^oC for 20 min.

Urea Solution:

Urea	20g
Water	100ml

Dissolve the Urea in water, sterilize by filtrat

16. Yeast Glucose Chloroamphenicol agar:

Ingredients	g/L
Yeast Extract	5.00
D+ Glucose	20.00
Chloroampenicol	0.10
Agar	14.90

Final pH =6.6 (at 25 ^{0}C)

BIBILIOGRAPHY.

- Ackers M, Mahon B, Leahy E, Damrow T, Hutwagner L, Barrett T, et al. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption, Western Montana [abstract]. Proceedings of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy; 1996 Sep 15-18; New Orleans, LA. Washington (DC): American Society for Microbiology; 1996:257. Abstract No. K43.
- Almed, E.M., Martin, F.G., and Fluck, R.C. 1973. Damaging stresses to fresh and irradiated citrus fruit. J. Food Sci. 38:230-233
- Altekruse SF, Hyman FH, Klontz KC, Timbo BT, Tollefson LK. Foodborne bacterial infections in individuals with the human immunodeficiency virus. South Med J 1994;87:169-73.
- American Public Health Association. 1970. Recommended Procedures for the Examination of Seawater and Shellfish, 4th ed. APHA, Washington, DC.
- American Public Health Association. 1992. In: Marshall, R.T. (ed). Standard Methods for the Examination of Dairy Products, 16th ed. APHA. Washington, DC.
- American Public Health Association. 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. APHA, Washington, DC.
- Brenner, K. P., C. C. Rankin, M. Sivaganesan, and P.V. Scarpino. 1996. Comparison of the recoveries of *Escherichia coli* and total coliforms from drinking water by the MI agar method and the U.S. Environmental protection agency-approved membrane filter method. Appl. Environ. Microbiol. 62:203-208.
- Bartz JA. 1999. Washing fresh fruits and vegetables: lessons from treatment of tomatoes and potatoes with water. Dairy Food Environ Sanit 19(12):853-64.

- Bean NH, Griffin PM. 1990. Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. J Food Prot 53(9): 804-17.
- Bender JB, Smith KE, Hedberg C, et al. Food-borne disease in the 21st century: what challenges await us? Postgrad Med 1999; 106(2):109-19
- Besser RE, Lett SM, Weber T, Doyle MP, Barrett TJ, Wells JG, et al. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh pressed apple cider. JAMA 1993; 269:2217-20.
- Beuchat, L. R. 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59:204-216).
- Beuchat, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes Infect. 4:413-423.
- Blakeman JP, editor. 1981. Microbial ecology of the phylloplane. London: Academic Press. 502 p.
- Boyce TG, Swerdlow DL, Griffin PM. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N Engl J Med 1995;333:364-8.
- Buchanan, R.L., Edelson, S.G. 1999. pH-dependent stationary-phase acid resistance response of enterohemorrhagic Escherichia coli in the presence of various acidulants. J. Food Prot. 62:211-218.
- Buck, J. W., Walcott, R. R., Beuchat, L. R. 2003. Recent trends in microbiological safety of fruits and vegetables. Online. Plant Health Progress doi: 10.1094/PHP-2003-0121-01-RV
- Burnett, S. L., and Beuchat, L. R. 2001. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in contamination. J. Indust. Microbiol. Biotechnol. 27:104-110)
- Caplenas, N.R. and M.S. Kanarek. 1984. Thermotolerant non-fecal source *Klebsiella pneumoniae*: validity of the fecal coliform test in recreational waters. Am. J. Public Health. 74:1273-1275

- Center for Food Safety and Department of Food Science and Technology, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797, USA).
- Chang, G.W., J. Brill, and R. Lum. 1989. Proportion of beta-glucuronidase-negative *Escherichia coli* in human fecal samples. Appl. Environ. Microbiol. 55:335-339.
- Ciebin, B.W., M.H. Brodsky, R. Eddington, G. Horsnell, A. Choney, G. Palmateer, A. Ley, R. Joshi, and G. Shears. 1995. Comparative evaluationof modified m-FC and m-TEC media for membrane filter enumeration of *Escherichia coli* in water. Appl. Environ. Microbiol. 61:3940-3942.
- Conway, P.L. 1995. Microbial ecology of the human large intestine. In: G.R. Gibson and G.T. Macfarlane, eds. p.1-24. Human colonic bacteria: role in nutrition, physiology, and pathology. CRC Press, Boca Raton, FL.
- Council for Agricultural Science and Technology. Foodborne pathogens: risks and consequences. Ames (IA): The Council; 1994. Task Force Report No. 122.
- Dege, N.J. 1998. Categories of bottled water. Chapter 3, In: D.A.G. Senior and P. R. Ashurst (ed). Technology of Bottled Water. CRC Press, Boca Raton, Florida.
- Dickinson CH. 1986. Adaptations of micro-organisms to climatic conditions affecting aerial plant surfaces. In: Fokkema NJ, van den Heuvel J, editors. Microbiology of the phyllosphere. New York: Cambridge Univ. p 77-100.
- Doyle, M.P. and J.L. Schoeni. 1987. Isolation of *Escherichia coli* O157:H7 from retail meats and poultry. Appl. Environ. Microbiol. 53:2394-2396.
- ERS* Updates Foodborne Illness Costs Food Safety v.23, i.3, USDA Sep/Dec00

- Essential Safety Requirements for street-vended foods(revised edition) Food Safety Unit Division of Food and Nutrition, World Health Organization 1996.
- Food safety and food borne illness , Fact sheet N°237, Revised January 2002
- Food Safety Guidelines for the Food Handlers, World Health Organization 2001
- Helmick CG, Griffin PM, Addiss DG, Tauxe RV, Juranek DD. Infectious diarrheas. In: Everhart JE, editor. Digestive diseases in the United States: epidemiology and impact. Bethesda (MD): National Institutes of Health, 1994; Publication No. 94-1447. p. 85-120.
- Hill, E.C. and Faville, L.W. 1951. Studies on the artificial infection of oranges with acid tolerant bacteria. Proc. Fla. State Hort. Soc. 64:174-177
- Institute of Medicine. Emerging infections: microbial threats to health in the United States. Washington (DC): National Academy Press; 1992.
- Jagger J. 1981. Near-UV radiation effects on microorganisms. Photochemistry and Photobiology 34:761-8.
- Kang DH, Fung DYC. 2000.Application of Thin Agar Layer method for the recovery of injured Salmonella typhimurium. Int J Food Microbiology 54: 127-132.
- Kapperud G, Jenum PA, Stray-Pedersen B, Melby KK, Eskild A, Eng J. Risk factors for *Toxoplasma gondii* infection in pregnancy. Am J Epidemiol 1996;144:405-12.
- Lettle GJ, Emmett PM, Heaton KW. Glucose and insulin responses to manufacture and whole-food snacks. Am J Clin Nutr 1987; 45:86–91)
- (Ministry of Health & welfare of Japan, 1997)
- Mishu B, Ilyas AA, Koski CL, Vriesendorp F, Cook SD, Mithen FA, et al. Serologic evidence of previous *Campylobacter jejuni* infection in patients with the Guillain-Barré syndrome. Ann Intern Med 1993;118:947-53.

- Mishu B, Koehler J, Lee LA, Rodrigue D, Hickman-Brenner F, Blake P, et al. Outbreaks of *Salmonella enteritidis* infections in the United States, 1985-1991. J Infect Dis 1994;169:547-52.
- National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on fresh produce. Food Control 10:117-143.
- National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Int. J. Food Microbiol. 52:123-153.
- Nguyen-the, C., and Carlin, F. 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. 34:371-401.
- Parish M.E. 1997. Public health and nonpasteurized fruit juices. *Crit. Rev. Microbiol.* 23:109-119.
- Patience Mensah, DorothyYeboah-Manu, Kwaku Owusu-Darko & Anthony Ablordey, Street foods in Accra, Ghanna: How safe are they? Bulletin of World health Organization 2002, 80 (7)
- (Press release WHO/58, 13 August 1997)
- Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JB, Davis BR, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983;308:681-5.
- Ryu J.H., and Beuchat L.R. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of Escherichia coli O157:H7 in acidified media and fruit juices. Int. J. Food Microbiol. 45:185-193.
- Schuchat A, Swaminathan B, Broome CV. Epidemiology of human listeriosis. Clin Microbiol Rev 1991;4:169-83.

- (Wachtel, M. R., Whitehand, L. C., and Mandrell, R. E. 2002. Association of *Escherichia coli* O157: H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. J. Food Prot. 65:18-25).
- (Solomon, E. B., Yaron, S., Matthews, K. R. 2002. Transmission of *Escherichia coli* O157: H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68:397-400.)
- Snoeyenbos GH, Smyzer CF, Van Roekel H. *Salmonella* infections of the ovary and peritoneum of chickens. Avian Dis 1969;13:668-70.
- Swerdlow DL, Lee LA, Tauxe RV, Bean NH, Jarvis JQ. Reactive arthropathy following a multistate outbreak of *Salmonella typhimurium* infections [abstract]. Proceedings of the 30th Interscience Conference on Antimicrobial Agents and Chemotherapy; 1990 Oct 21-24; Atlanta, GA. Washington (DC): American Society for Microbiology; 1990. Abstract No. 916.
- Tauxe RV. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In: Nachamkin I, Tompkins S, Blaser M, editors. *Campylobacter jejuni*: Current Status and Future Trends. Washington (DC): American Society for Microbiology; 1992.
- U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition (September 30, 2001) Outbreaks Associated with Fresh and Fresh-Cut Produce. Incidence, Growth, & Survival of Pathogens in Fresh and Fresh-Cut Produce
- Uljas, H.E., and Ingham, S.C. 1998. Survival of Escherichia coli O157:H7 in synthetic gastric fluid after cold and acid habituation in apple juice or trypticase soy broth acidified with hydrochloric acid or organic acids. J. Food Prot. 61:939-947.
- USFDA, CFSAN, 2002,Bacteriological Manual Online

- WU V.C.H., Fung DYC. Evaluation of Thin Agar Layer method for Recovery of Heat Injured Foodborne pathogens .J of Food Science vol 66, no.4, 2001
- Zhuang, R.Y., Beuchat, L.R., and Angulo, F.J. 1995. Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine *ApplEnvironMicrobiol.* 61:2127-2131