MICROBIOLOGY OF INDIAN CHUTNEYS

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

BY
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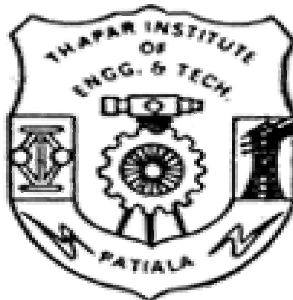

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled **"Microbiology of Indian Chutneys"** 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.

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Date: May 15, 2003

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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Dated: May 15 2003 SUNITA BANSAL

Place: Patiala.

ABSTRACT

Green chutneys served with ready- to- eat foods in Patiala city were analyzed for their microbiological quality.Quantitative analysis showed significant loads of total viable bacteria,faecal coliforms and staphylococcus. Qualitative analysis revealed the presence of coagulase positive *S.aureus*, *Salmonella enteriditis* and *E.coli O157:H7*;non pathogenic (surrogate)strains of *S.aureus* and *E.coli O157:H7* were used to study the behavior of the latter in coriander base and green chutneys (prepared from this base) at different abusive storage temperatures, for extended as well as short time periods.None of the strains were found to grow but *S.aureus* continued to survive without decline in numbers especially at refrigeration temperatures, for 24hours; the trend of survival observed with both high and low inoculum sizes of *S.aureus* was similar. Acid stressed *E.coli O157:H7* exhibited a longer sustainence than its unstressed counterpart in both coriander base and green chutneys. Based on the short term survival data of both these bacterial pathogens and those available from dose response studies of these pathogens, the risk calculated for average serving sizes was significantly high for consumers.

CONTENTS

CHAPTER	TITLE	PAGE NO.
CHAPTER 1	INTRODUCTION	1
CHAPTER 2	REVIEW OF THE LITERATURE	3
CHAPTER 3	MATERIALS & METHODS	17
CHAPTER 4	RESULTS & DISCUSSION	27
CHAPTER 5	CONCLUSIONS	41
BIBLIOGRAPHY		42
ANNEXURE		46

INTRODUCTION

Being ubiquitous in distribution, microorganisms may contaminate and grow in many food products. The entry of Microorganisms into the food chain can occur from various sources during different stages of processing, storage and serving. Additionally, by providing a suitable nutritional and physical environment for growth and multiplication of microorganisms, the foods possess an inherent capacity to sustain them in large numbers. The possible routes of contamination of foods include environmental sources such as soil, dust and air & water, through food handlers or unhygienic practices, raw materials and materials used in preparing the food and also utensils or other articles used during serving of foods.

In developing countries like India, with the changing life styles, a huge section of people, including those in the metropolis have started eating Ready – to-eat foods, especially those sold on the streets; Street-vended foods are ready-to-eat foods prepared and sold by vendors on streets and similar public places. Street foods provide a source of readily available, inexpensive, nutritional meals, while providing a source of income for the vendors. Street-vended foods are prepared and sold in the streets for immediate consumption or for consumption at a later time without further processing or preparation.

In contrast to these potential benefits, concerns over the safety and quality of street-vended foods have been raised, because the vendors lack appreciation of basic food safety issues They often use stands and carts of crude and inefficient construction, running water is not easily accessible, and hand and dish washing is done in the same bucket, sometimes without soap. Wastewater is usually discarded in streets and garbage is discarded nearby providing attraction, food and harborage for insects and rodents. Such conditions and practices are likely to lead to cross-contamination of foods. Furthermore, safe food storage temperatures are difficult to maintain since foods are often displayed over long periods. In other cases vendors buy raw materials from dubious sources which

may either already be contaminated with foodborne pathogens or be unfit for consumption due to other reasons. In countries where street food vending is prevalent, there is commonly a lack of information on the incidence of foodborne diseases related to street-vended foods. However, microbiological studies on street-vended foods in American, Asian and African countries have revealed high bacterial counts and a high incidence of foodborne bacterial pathogens in the food. In some cases street-vended foods have been implicated in outbreaks of foodborne diseases. In Northern, Western and Eastern parts of India,most of these ready –to- eat foods exemplified by samosas, pakoras etc are accompanied with green chutneys**.**

Green chutneys are traditional "Indian culinary aids" and are usually made from standard or seasonal fruits & vegetable, herbs, which are ground to a paste or to a pulpy mash, requisite consistency is obtained by addition of water, vinegar, and lime or tamarind juice. Chutneys are made to be eaten fresh or with in a few days at the most, they do not undergo further processing nor are preservatives added. Green chutneys like all street foods are exposed to the abundant sources of contamination, since none of the raw materials of this preparation are sourced , it is likely that microorganisms would be carried over to the product, this is of concern if these microorganisms are pathogenic since the product is consumed raw; additionally When sold by street vendors several sources may contribute to the level and type of contammination. Surprisingly, little work has been done on the microbiological qualities of green chutneys in India.

Scope of the dissertation

The present study attempts to identify some important bacterial pathogens, study the behavior of the predominant bacterial pathogens in chutneys at different storage temperatures and stress conditions assumed to be near realistic to those actually encountered by street vendors; based on the results the risk borne to a consumer for this product is proposed. The princely city of Patiala (Punjab), known for its taste for exquisite foods and tourist attraction was chosen as a model for our study.

REVIEW OF THE LITERATURE

Food Borne Diseases: Global Scenario

It is believed that hundreds and millions of people worldwide suffer from diseases caused by contaminated food. Developing countries suffer the most from a wide range of diseases including cholera, campilobacteriosis, *Escherichia coli* infections, salmonellosis, shigellosis, brucellosis and hepatitis. The annual incidence of some 1.5 billion episodes of diarrhoea in children under five years of age, resulting in over three million deaths is an indication of the scale of the problem, since a significant proportion of diarrhoeal disease cases are of foodborne origin. According to the latest edition of the World Health statistics, surveys indicate that foodborne diseases may be 300-350 times more frequent than the reported cases tend to indicate.

Paradoxically, in spite of safe water supplies, sound standards of hygiene and application of technologies such as pasteurization, a no. of industrialized countries have experienced an increase in the incidence of food borne diseases in recent years. Surveys indicate that no less than 5-10% of the population is involved annually. On the top of that, the emergence of *Listeria monocytogenes, Escherichia coli* O157: H7and multiantibiotic resistant *Salmonella typhimurium* are justifiably perceived as new significant threats to public health. (Press release WHO/58, 1997)

Food borne illnesses are believed to be a growing problem

Foodborne diseases are a widespread and growing public health problem, both in developed and developing countries.

- The global incidence of foodborne disease is difficult to estimate, but it has been reported that in 1998 alone 2.2 million people, including 1.8 million

children, died from diarrhoeal diseases. A great proportion of these cases can be attributed to contamination of food and drinking water. Additionally, diarrhoea is a major cause of malnutrition in infants and young children.

- In industrialized countries, the percentage of people suffering from foodborne diseases each year has been reported to be up to 30%. In the United States of America (USA), for example, around 76 million cases of foodborne diseases, resulting in 325 000 hospitalizations and 5 000 deaths, are estimated to occur each year.
- While less well documented, developing countries bear the brunt of the problem due to the presence of a wide range of foodborne diseases, including those caused by parasites. The high prevalence of diarrhoeal diseases in many developing countries suggests major underlying food safety problems.
- While most foodborne diseases are sporadic and often not reported, foodborne disease outbreaks may take on massive proportions. For example, in 1994, an outbreak of salmonellosis due to contaminated ice cream occurred in the USA, affecting an estimated 224 000 persons. In 1988, an outbreak of hepatitis A, resulting from the consumption of contaminated clams, affected some 300 000 individuals in China
(Food safety and food borne illness, WHO Press Release, January 2002)

The existing data on the extent of foodborne illnesses have weaknesses and may not fully depict the extent of the problem. Public health experts believe that the majority of cases of food borne illness are not reported because the initial symptoms of most foodborne illnesses are not severe enough to warrant medical attention, the medical facility or state does not report such cases, or the illness is not recognized as foodborne. However, according to the best available estimates, based largely on CDC's data, millions of people become sick from contaminated food each year and several thousands die. In addition, public health and food safety officials believe that the risk of foodborne illness is increasing for several reasons. For e.g., as a result of large-scale food production

and broad distribution of products, those products that may be contaminated can reach a great no. of people in many locations. Furthermore, new and more virulent strains of previously identified harmful bacteria have been identified in the past several decades. Also mishandling or improper preparation can further increase the risk.

Statistics of food borne illnesses and their consequent economic losses to countries:

Between 6.5 million and 81 million cases of food borne illness and as many as 9100 related deaths occur each year, according to the estimates provided by several studies conducted over the past 10 years.

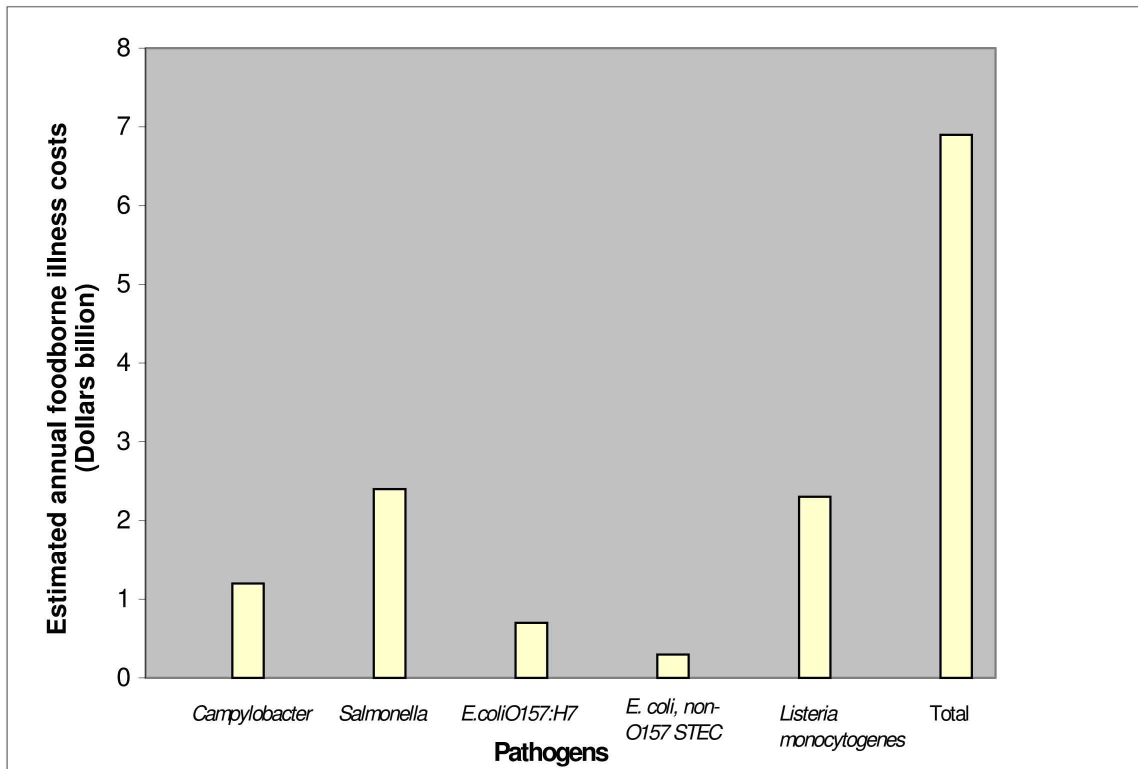
Estimates of foodborne illness and related deaths can be summarized as below:

Study or report/ authors	Estimated no. of Illnesses per year	Estimated no. of deaths per year	Basis of estimation
The incidence and cost of food borne diarrhoeal diseases in the united states, Archer and Kvenberg (1985)	24 million to 81 million	No estimate provided	National survey of physicians conducted by National Center for Health Statistics (1977-78), supplemented with 1983 data on illnesses from specific pathogens.

Closing the Gap: The burden of unnecessary illness, Bennett et. al.(1987)	6.5 million annually	9,100	Published and survey data from National Center for Health Statistics, Center for Infectious Diseases, and the center for Prevention Services
Preliminary Estimates of Costs of foodborne Diseases in the united states, Todd (1989)	12.6 million	523	Median of four estimates based on (1) CDC outbreaks data; (2) 1987 Bennett study data; (3) Salmonella underreporting; and (4) Canadian disease rates extrapolated to US population.
Food borne Pathogens: Risk and Consequences, Council for Agricultural Science and Technology (1994)	6.5 million to 33 million	Up to 9,000	Review

US, GAO, Washington DC. 20548, Resources, Community, and Economic Development Division.B-270753, May8, 1996.

The consequent losses to the economy on account of foodborne diseases are shown below for United States. In developing countries like India and underdeveloped countries, this is anticipated to be more by at least tenfolds, however due to of proper surveillance and documentation, obtaining a true estimate is a problem.



Courtesy: ERS, USDA

Outbreaks associated with fresh & fresh-cut produce.

Vegetables & Fruits have been associated with outbreaks of food borne disease in many countries. Organisms involved include bacteria, viruses & parasites(De Roever, 1998) These outbreaks vary in size from a few persons affected to many thousands. However they represent only a small proportion of the total number of cases reported. For e.g. in the US between 1993 and 1997 fruits and vegetables

were associated with only 1.4% to 3% of outbreaks. However, according to the Centers for Disease Control and Prevention, the number of produce- associated outbreaks per year in the US doubled between the periods 1973-1987 & 1988-1992 (Olsen et al, 2000*)*

The world's largest reported vegetable borne outbreak to date occurred in Japan in 1996 and of the over 11000 people affected, about 6000 were culture confirmed. The outbreak involved the death of three school children and was caused by O157: H7 (Ministry of Health & welfare of Japan, 1997)

The frequency of produce associated outbreaks in Europe appears to be similar to the US. Between 1992 and 1999, 60 outbreaks of foodborne infectious intestinal disease associated with the consumption of salad items, fruits and vegetables were reported from England & Wales. 2170 people (12%) were affected (at risk =19650) and 27 were admitted to hospital. No deaths were reported. In 17 of outbreaks more than 50 people were affected. Fruits and vegetables associated outbreaks represent 4.3% of the total no. of outbreaks of food borne disease reported during that period. In Sweden salad containing one or more cooked ingredients accounted for 4.3% of 252 of reported incidents of illness between 1992 and 1997 (Lindquist et al., 2000). In an overview by ICMSF (1998) they reported that animal wastes used as fertilizer in the Orient may contribute to much as 20% of infections by *Shigella*, *Salmonella*, *Vibrio cholerae* & other protozoa's.

Survival and multiplication of pathogens on raw produce

The survival and/or growth of pathogens on fresh produce is influenced by the organism, produce item, and environmental conditions in the field and thereafter, including storage conditions. In general, pathogens will survive but not grow on the uninjured outer surface of fresh fruits or vegetables, due in part to the protective character of the plant' s natural barriers(for example, cell walls and wax layers). In some cases pathogen levels will decline on the outer surface.

In the field, the physical environment of leaf surfaces is considered to be inhospitable for the growth and survival of bacteria (for example, lack of nutrients and free moisture, temperature and humidity fluctuations, and ultraviolet light)

(Dickinson 1986). Environmental conditions, however, can greatly influence bacterial populations; the presence of free moisture on leaves from precipitation, dew, or irrigation may promote survival and growth of bacterial populations (Blakeman 1981; Andrews 1992; Beattie and Lindow 1995, 1999). Certain conditions, such as sunlight, particularly the shorter ultraviolet wavelengths, can damage bacterial cells (Webb 1976; Jagger 1981;Sundin and Jacobs etal 1999). Consequently, nature may select for bacteria with adaptations to these stressful conditions.

Similarly, after harvest, pathogens will survive but not grow on the outer surface of fresh fruits and vegetables, especially if the humidity is high. In some cases, pathogen levels will decline on the outer surface. The rate of decline is dependent upon the produce type, humidity, and temperature, as well as the atmosphere and type of packaging used. Growth on intact surfaces is not common because foodborne pathogens do not produce the enzymes necessary to break down the protective outer barriers on most produce. This restricts the availability of nutrients and moisture. One exception is the reported growth of *E. coli* O157:H7 on the surface of watermelon and cantaloupe rinds.

Survival of foodborne pathogens on produce is significantly enhanced once the protective epidermal barrier has been broken either by physical damage, such as punctures or bruising, or by degradation by plant pathogens (bacteria or fungi). These conditions can also promote the multiplication of pathogens, especially at nonrefrigerated temperatures. Microorganisms often survive at refrigerated temperatures even though these conditions reduce or eliminate the ability of the organisms to multiply. Exceptions to this are the psychrotrophic pathogens including non-proteolytic *C. botulinum*, *L. monocytogenes*, *Y. enterocolitica*, and the presumptive pathogen *Aeromonas hydrophila*. Various enteric pathogens have been shown to multiply on the surface of cut melons, on shredded lettuce, and on chopped parsley and under acidic conditions, such chopped tomatoes and wounded apple tissue. Temperature control becomes critical for preventing bacterial reproduction on any cut produce item. Fresh-cut produce, by definition, are usually those, which are injured through peeling, cutting, slicing, or

shredding. These same operations can transfer pathogenic microorganisms, if present, from the surface of the intact fruit or vegetable to the internal tissues. Injured cells and released cell fluids provide a nourishing environment for microbial growth.

Most pathogens do not cause produce to spoil, even at relatively high populations. In the absence of spoilage, high populations of pathogens may be achieved and the item may be consumed because it is not perceived as spoiled. For this reason, specifications requiring very low microbial counts may, in some cases, compromise produce safety.

Infiltration of wash-water into intact fruit has been demonstrated with several fruits and vegetables, and is thought to have contributed to an outbreak of salmonellosis associated with fresh market tomatoes. Wash-water contaminated with microorganisms, including pathogens, can infiltrate the intercellular spaces through pores when conditions are right. Internal gas pressures and surface hydrophobicity usually prevents uptake of water. However, when the produce temperature is much higher than the water temperature, the pressure difference created may be sufficient to draw water into the fruit (Bartz 1999).

The following table summarizes predominant bacterial pathogens and the vegetables from which they were isolated

Pathogen	**Product**
Aeromonas	alfalfa sprouts, asparagus, broccoli, cauliflower, celery, lettuce, pepper, spinach
Bacillus cereus	alfalfa sprouts, cress sprouts, cucumbers, mustard sprouts, soybean sprouts
Campylobacter jejuni	green onions, lettuce, mushroom, potato, parsley, pepper, spinach
Clostridium botulinum	cabbage, mushrooms, pepper
E. coli O157:H7	alfalfa sprouts, apple juice, cabbage, celery, cilantro, coriander, cress sprouts, lettuce
Listeria monocytogenes	bean sprouts, cabbage, chicory, cucumber, eggplant, lettuce, mushrooms, potatoes, radish, salad vegetables, tomato
Salmonella	alfalfa sprouts, artichokes, beet leaves, celery, cabbage, cantaloupe, cauliflower, chili, cilantro, eggplant, endive, fennel, green onions, lettuce, mungbean sprouts, mustard cress, orange juice, parsley, pepper, salad greens, spinach, strawberries, tomato, watermelon
Shigella	celery, cantaloupe, lettuce, parsley, scallions
Staphylococcus	alfalfa sprouts, carrot, lettuce, onions sprouts, parsley, radish
Vibrio cholerae	cabbage, coconut milk, lettuce

NACMCF (1999) and Beuchat (1996)& Nguyen-the (1994) etal

Produce related infections in humans:

Several reasons for the increase in produce-related human infections have been proposed. These include changes in dietary habits, including a higher per capita consumption of fresh or minimally processed fruits and vegetables, and the increased use of salad bars and meals eaten outside the home. Yearly consumption of fresh fruits and vegetables in the U.S. has increased by almost 20 pounds per person from 1988 to 1996. This has been attributed to both

consumer desire to maintain a healthier diet and the year-round importation of high-quality produce into the U.S. In addition, changes in production and processing methods, sources of produce, and the emergence of pathogens not previously associated with raw produce have enhanced the potential for foodborne illness outbreaks associated with raw fruits and vegetables. The end result of these changes is an increased exposure of the general public to fruits and vegetables, which has exacerbated potential problems with contamination by human pathogens.

Sources of Contamination

Bacterial pathogens may contaminate fruits and vegetables at any point throughout the production system. Potential pre-harvest sources of contamination include soil, feces, irrigation water, water used to apply fungicides and insecticides, dust, insects, inadequately composted manure, wild and domestic animals, and human handling (Beuchat, L. R. etal1996). In the production of seeds intended for sprout production, the practice of animal grazing to initiate flowering of alfalfa may result in the introduction of enteric bacteria in feces. Similar consequences may result from allowing wild animals access to seed fields. Non-composted or improperly composted manure can contaminate fruits and vegetables through uses such as a fertilizer or soil amendment, or in irrigation water. *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* can be found in animal feces. Transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants, and its migration throughout the plant were recently reported. (Solomon etal 2002). (Wachtel, M.R etal. 2002). Post-harvest sources of contamination include feces, human handling, harvesting equipment, transport containers, wild and domestic animals, insects, dust, rinse water, ice, transport vehicles, and processing equipment (Burnett, S. L etal 2001). Handling of food can introduce and spread pathogenic microorganisms. Food handlers may carry pathogens without experiencing any serious ill-effects them-selves. *Staphylococcus aureus* is commonly associated with the skin, nose, throat and infected skin lesions, particularly in higher primates such as humans where 20-50% of healthy individuals can carry the

organism. The organism is difficult to re-move from the skin where it "hides" in pores and hair follicles. If the hands are damp it can be drawn to the surface and transferred to foods. In one recent example, restaurant workers in Kuwait city were tested and in a sample of 500 people 26.6% were found to carry the organism. Infected food handlers are also a common source of foodborne viruses such as the Hepatitis A virus
(Food Safety Guidelines for the Food Handle, World Health Organization 2001)
All this is likely to be proliferated through street vended foods , which are having a very high consumer preference all over the industrialized as well as developing countries. So there is need to know the risk associated with such foods.

Street vended foods : The Benefits & the Risks Associated

"Street foods" are defined as foods prepared and/or sold by vendors in streets and other public places for immediate consumption or consumption at a later time without further processing or preparation. This definition includes fresh fruits and vegetables, which are, sold outside authorized market areas for immediate consumption. Because of socioeconomic changes in many countries, this sector has experienced significant growth during the past few decades.

According to WHO survey of street-vended foods it was found that 74% of countries reported street-vended foods to be a significant part of the urban food supply; these street-vended foods included foods as diverse as meat, fish, fruits, vegetables, grains, cereals, frozen produce and beverages. The types of preparation included foods without any preparation (65%), ready-to-eat food (97%) and food cooked on site (82%); Vending facilities varied from mobile carts to fixed stalls and food centres. Also it was found that Infrastructure developments were relatively limited with restricted access to potable water (47%), toilets (15%), refrigeration (43%) and washing and waste disposal facilities. The majority of countries reported contamination of food (from raw food, infected handlers and inadequately cleaned equipment) and time and temperature abuse to be the major factors contributing to foodborne disease and

most countries reported insufficient inspection personnel, insufficient application of the HACCP concept and noted that registration, training and medical examinations were not amongst selected management strategies.

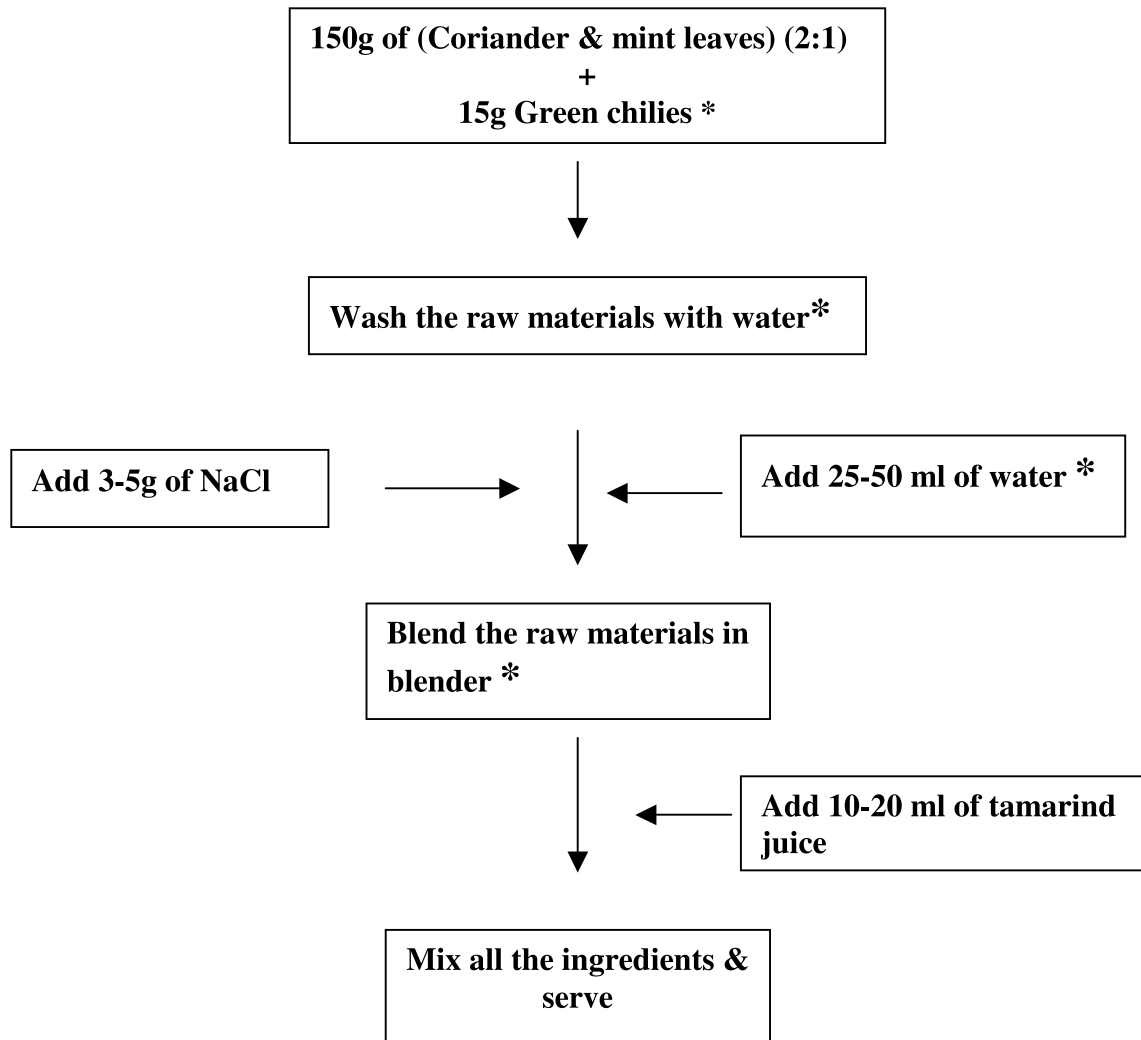
Street-vended foods provide a source of inexpensive, convenient and often nutritious food for urban and rural poor. These are a source of attractive and varied food for tourists and the economically advantaged. Street foods contribute to • a major source of income for a vast number of persons, particularly women, and a chance for self-employment and the opportunity to develop business skills with low capital investment.

In contrast to these potential benefits, it is also recognized that street-food vendors are often poor and uneducated and lack appreciation for safe food handling. Consequently, street foods are perceived to be a major public health risk.

Street-vended foods may pose significant public health problems due to lack of basic infrastructure and services, such as potable water supplies. It is difficult to control the large numbers of street food vending operation because of their diversity, mobility and temporary nature. The risk is higher due to insufficient resources for inspection and laboratory analysis and general lack of factual knowledge about the microbiological status or the precise epidemiological significance of many street-vended foods. Poor knowledge of street vendors in basic food safety measures & inadequate public awareness of hazards posed by certain street foods is another factor contributing to the risks posed by such foods.

In developing countries like India, with the changing life styles, a huge section of people, including those in the metropolis have started eating Ready – to-eat foods such as samosas, pakoras, tikki etc, especially those sold on the streets. Such street vended foods are accompanied with Green chutneys & like all other street vended foods chutneys are also exposed to a higher risk of contamination, there is a need to assess the risk associated with such kind of food which is being served raw along with the cooked foods.

The standard method for preparation of Green chutneys can be schematically represented as below; asterisks indicate the possible routes of contamination.



Green chutneys have a very good nutritional value, which is being summarized in the following table

	% Daily values*		%Daily values*
Total Fat	2 %	**Saturated Fat**	< 1 %
Cholesterol	0 %	**Sodium**	< 1 %
Potassium	4 %	**Total Carbohydrates**	1 %
Dietary Fiber	3 %	**Protein**.	2 %
Sugars		**Vitamin A**	21 %
Vitamin C	58 %	**Calcium**	2 %
Iron	5 %	**Thiamin**	3 %
Niacin	5 %	**Vitamin B6**	5 %
Magnesium	4 %	**Folate**	9 %

* Percent Daily Values are based on a 2,000-calorie diet. (Vegetarian Recipe Green Chutney Detailed Nutritional Information.htm)

Green Chutneys & Street foods

Despite their great nutritional value, just like other street vended foods Green Chutneys have been reported to be contaminated. Viswanathan Poorna etal 2001 shown the prevalence and growth of pathogens on the raw salad vegetables, fruits and sprouts, which are being used as the raw material for the preparation of Green Chutneys. In another study by Kakkar etal, it has been shown that the microbiological quality of chutneys sold on the streets in metropolitan cities is very poor. The poor quality of these chutneys could pose serious health hazard to the consumer. Since there are no standards laid down for such products regarding the permissible limits of different microorganisms it is very difficult to have a check on their quality of production.

MATERIALS AND METHODS

1. Selection of collection sites & Collection of the Samples

Patiala city was divided into 5 major areas, which represented almost whole of the city and comprised of shops preparing ready –to -eat foods (e.g. samosas, bread- pakoras, kachouris, fish fries, meat chops or parathas) and served at least 100 customers (comprising of upper, middle and low-income groups) per day. Sample collection was conducted during the months of October to March.

Each area was designated as 1, 2, 3, 4 and 5 respectively; approximately 30 samples of green chutneys were collected from each area. All samples were collected from the vendors in pre-sterilized 250ml bottles. In the laboratory these samples were analyzed with in1- 2 hours of procurement for the microbiological analysis. It was decided that the names of the vendors would be kept undisclosed.

2. Isolation, enumeration & identification of bacterial pathogens from chutneys

Portions of chutneys weighing 25g were diluted as 1: 10 with 250ml of Sterile Butterfields phosphate buffer which were subsequently diluted tenfold with the same. Appropriate dilutions were then enumerated for the following :

- Total aerobic plate counts using Tryptone Soya agar:

25 g of the chutney sample were added to 250 ml Butterfield's Phosphate Buffer, blended for about 15 minutes further serially diluted the sample in Butterfield's Phosphate Buffer, appropriate dilutions were spread on Tryptone Soya Agar plates in triplicates.All plates were incubated at 37 ^{0}C for 24-48 hrs. Colonies in each plate were counted, averaged and expressed as CFU/g and converted to log . 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 the plates at 37 ^{0}C for 24-48 hrs.Typical colonies were counted and results expressed as described above.

- 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.

- Detection of *Salmonella*:

Qualitative detection of *Salmonella* was carried out as described in the USFDA bacteriological manual; briefly 10g portions of chutneys were pre-enriched with 100 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

3. 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 (Brain Heart Infusion) 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 for 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: Microslides 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 – consists of indole, methyl red, Voges-Proskauer and citrate utilization, test was performed for confirmation of *E.coli*, essentially as follows:

- ♣ 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, red coloration was considered positive for *E.Coli*.

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

- ♣ CitrateTest: For citrate test, Simmon's citrate agar slants were streaked with presumptive colonies of *E.coli* & incubated at 35^0C for 48 hrs. The absence of blue coloration was taken to be positive for *E.Coli*.

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

θ **Identification of *E.coli* O157: H7**

♣ Sorbitol-MacConkey (SMAC) agar: Sorbitol-negative colonies appeared colorless on SMAC & were assumed to be positive for the presence 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 non-motile *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.

θ ***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.

4. Survival at different storage temperatures of predominant bacterial isolates

In order to evaluate the effect of the chutney 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 & 28^0C as follows:

Selection of pathogens :

E.coli O157:H7 NCTC 12900(non pathogenic strain) and a non pathogenic strain of *S.aureus* was used for survival studies. The strains were stored on cryoprotective beads at -20^0C; prior to use they were revived on either BHI (Brain Heart Infusion agar) or TSA (tryptic soy agar) slants.

Culture preparation: Cultures of surrogates of *Staphylococcus*, *E.coli* O157:H7 were grown in 5ml tryptic soy broth & incubated at 37^oC 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 inoculate samples of chutneys.

Preparation of chutney: for preparation of green chutneys coriander, mint leaves were washed with 70% ethanol, twice with sterile distilled water and dried they were then blended together along with green chilies (washed as above) in a household blender, sterilized by applications of soap water, 70% ethanol and sterile distilled water. Requisite consistency of the chutney was obtained by addition of filter sterilized tamarind juice & sterile water. Required taste of the chutney was maintained by the addition of table salt.

After preparing the chutney, it was distributed in sterile containers with 10 g of chutney in each container, it was inoculated with culture of bacterial surrogates & incubated at 4^0C, 8^0C & 28^0C for 24 hrs, 48 hrs, 72 hrs & 96hrs. After the required time of incubation, 100 ml of Butterfield's Phosphate Buffer was added and blended, the latter was diluted tenfold in the same. Appropriate dilutions were spread plated on BP & SMAC plates in triplicates, all plates were incubated

at 37^0C for 24 – 48 hrs. Colonies were counted after the requisite time, averaged and converted to log Cfu/g as described before.

5. Short time Survival at ambient storage temperatures of predominant bacterial isolates:

Since, chutneys are usually consumed within 10 hrs of their preparation, it was of interest to investigate the survival of the bacterial pathogens within this short duration of time. In this case, mid log phase culture of appropriate bacterial surrogates was harvested by centrifugation (12000rpm, 2minutes), washed twice with 0.1% peptone water, resuspunded in 2ml of the same. The latter were inoculated into:

1.Coriander paste (which was prepared under aseptic conditions by blending together previously washed & decontaminated leaves of coriander and green chilies along with requisite amount of table salt & sterile water, as described before for chutneys)

2.Coriander-mint- tamarind paste (prepared under aseptic conditions as described above in pt.**4)**

All samples were incubated at (28^0C). 10g samples were removed after 2hr, 4hr, 6hr, 8hr & 10 hours, dissolved in 100 ml of Butterfield's Phosphate Buffer, blended for 3 minutes and further diluted tenfold in the same. Appropriate dilutions were then spread plated on BP & SMAC plates in triplicates. All plates were incubated at 37^0C for 24 – 48 hours; colonies were counted averaged and converted to logCFu/g as described before.

6. Effect of inoculum size & stress on the survival of bacterial isolates

<u>Inoculum Size</u>:

The initial level of contamination especially for *S.aureus* was anticipated to vary according to differences in handling, therefore both lower and medium amounts of inoculum were used, in addition to high levels of inoculum, to investigate the behavior of such bacterial loads in Green chutneys, as follows:

Cultures of surrogates of *S.aureus* & *E.coli O157:H7*were grown in 5ml tryptic soy broth & incubated at 37^oC for 24 hr. Overnight grown cells in the mid log

phase were harvested by centrifugation (12000rpm, 2minutes), washed with 0.1% peptone water & then resuspended in different amounts of peptone water and adjusted so as to log cfu to be 4log, 6 log & 8 log CFU/ml respectively.

These culture preparations were then used to inoculate the chutney (prepared under aseptic conditions as described above in pt.4) then incubated at 4^0C, 8^0C & 28^0C for 24 hrs, 48 hrs, 72 hrs & 96hrs**.** after each time interval, 25g were removed and dissolved in 250 ml of Butterfield's Phosphate Buffer and blended for about 3minutes, further serial dilutions from this were made in the same. Appropriate dilutions were spread plated on BP & SMAC plates in triplicates. All plates were incubated at 37^0C for 24 – 48 hrs, after the requisite time, colonies were counted, averaged and expressed as logCFU/g.

Effect of stress:

From the surveillance studies it was found that the vendors usually add the fresh lot of chutney into the container already having some amount of previous lot. This little amount of chutney that may contain some pathogen can serve as inoculum for the fresh lot. Also it was anticipated that as the pH of the chutney is approx. 3.5, the pathogen might be under stress for several hrs till the fresh lot is not added & it may show a differential growth behavior. In order to see the effect of stress on the survival of pathogens a simulated study for 10 –24 hrs was carried out as follows:

Culture preparation:

Culture of *E.coli O157:H7* surrogate strain was grown in tryptic soy broth & incubated at 37^oC for 24 hr. Overnight grown cells in the stationery phase of growth were used. The culture pH was lowered with10 N HCl, and the acid challenged culture was incubated at 37^oC for 90 minutes. The cells were harvested by centrifugation (12000rpm, 2minutes) washed with 0.1 % peptone water & resuspended in peptone water –the latter was used to inoculate the chutney samples. Suitable controls where the cells were not acid challenged were used as controls.

Acid treated & normal cultures of *E.coli* were inoculated in chutney (prepared under aseptic conditions as described above in pt.**4). I**ncubated at 28 ^{o}C for 0hr, 2hr, 4 hr 6 hr, 8 hr, 10 hr & 24hrs.After the completion of each incubation interval, dissolved in 100 ml of Butterfield's Phosphate Buffer & then from this further serial dilutions were made in Butterfield's Phosphate Buffer. Appropriate dilutions were then spread plated on SMAC plates(in triplicates) overlaid with thin agar layer (SMAC-TAL plates) (as described below in pt.**7**). All plates were incubated at 37^{0}C for 24 – 48 hrs, after the requisite interval of time colonies were counted, averaged and converted into log CFU/g as described before.

7. **Recovery methods for the recovery of injured pathogens:** Bacterial populations undergoing stress recover slowly or poorly on selective medium, therefore it was decided that for enhanced recovery appropriate method would be optimized.

Culture preparation: Cultures of surrogates of *S.aureus*, *E.coli* and *S.typhimurium* were grown in 5ml tryptic soy broth & incubated at 37°C for 24 hr. Overnight grown cells were used as inoculum for chutney.

Recovery studies:

Thin Agar Layer Method: The Thin Agar Layer method of Kang & Fung 1998 was used to enumerate injured pathogens from chutney 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 g of Chutney was inoculated with cell suspension of surrogates of pathogenic bacteria & incubated at 28°C for 2hr. After 2hr of incubation it was diluted in 100ml of Butterfield's phosphate buffer. Further serial dilutions of chutney were made which were spread plated on to the TAL plates, selective

media plates & TSA plates, in triplicates the latter were incubated at 370c for 24-48 hours - pathogens on different plates was counted (and expressed as described before0 and compared .

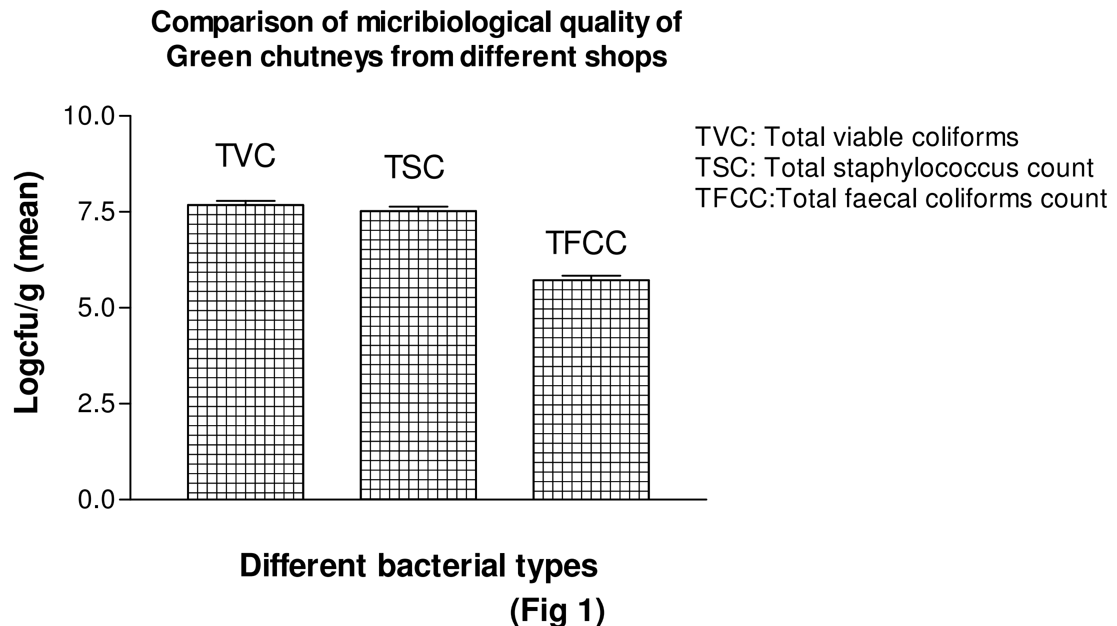
8. Spoilage and survival

Since Green chutneys are generally consumed within 10 hours of its preparation it is essential to know the growth characteristics of inherent spoilage microbes present in the chutney; a growth kinetic study of these spoilage microorganism were therefore carried out.For this samples from different vendors were collected, kept at 28°C. At different time intervals (3, 6, 9 and 12 hours respectively) 25g portions of chutney were removed from the original sample dissolved in 250ml Butterfield's phosphate buffer, blended and diluted tenfold in the same buffer. Appropriate dilutions were then spread on TSA & Yeast chloroamphenicol agar (YGC) media for total microbial load & yeast/molds count, in triplicate, YGC plates were incubated at 28 ^{o}C for five days and TSA plates were incubated at 37 ^{o}C for 24-48 hours. After the requiste time , colonies were counted and converted to LogCFU/g as described before.

9. Risk assessment :

The risk assessment was done based on the serving size, prevalent microorganism & the dose response data. From surveys it was found that the serving size of green chutneys varied from 15-20 g per plate of the ready to eat foods with an average of 18g /plate & from the short term survival studies, the number of *E.Coli O157:H7* and *S.aureus* existing in green chutneys after 2 hours were multiplied with the serving size of green chutneys, the resultant values were matched with the existing dose response data of these organisms.

RESULTS AND DISCUSSION

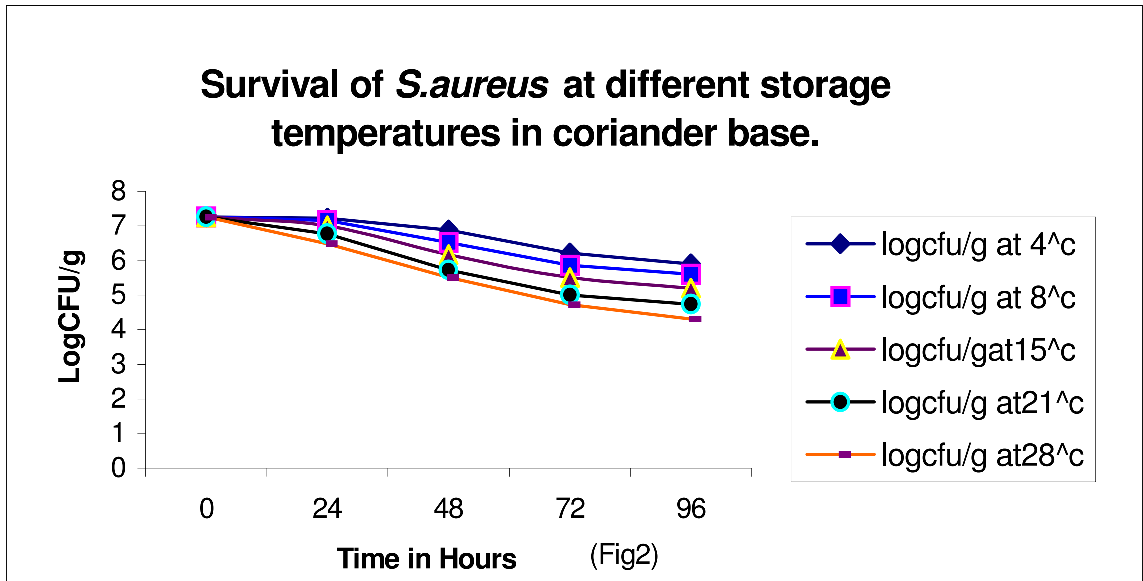


(Fig 1)

Fig1 shows the load of selected bacterial pathogens viz: total coliforms, total staphylococcus in green chutneys obtained from street vended shops throughout Patiala city. The total viable counts, total coliform count and total staphylococcal counts were 7.6, 7.5, 5.5 respectively.

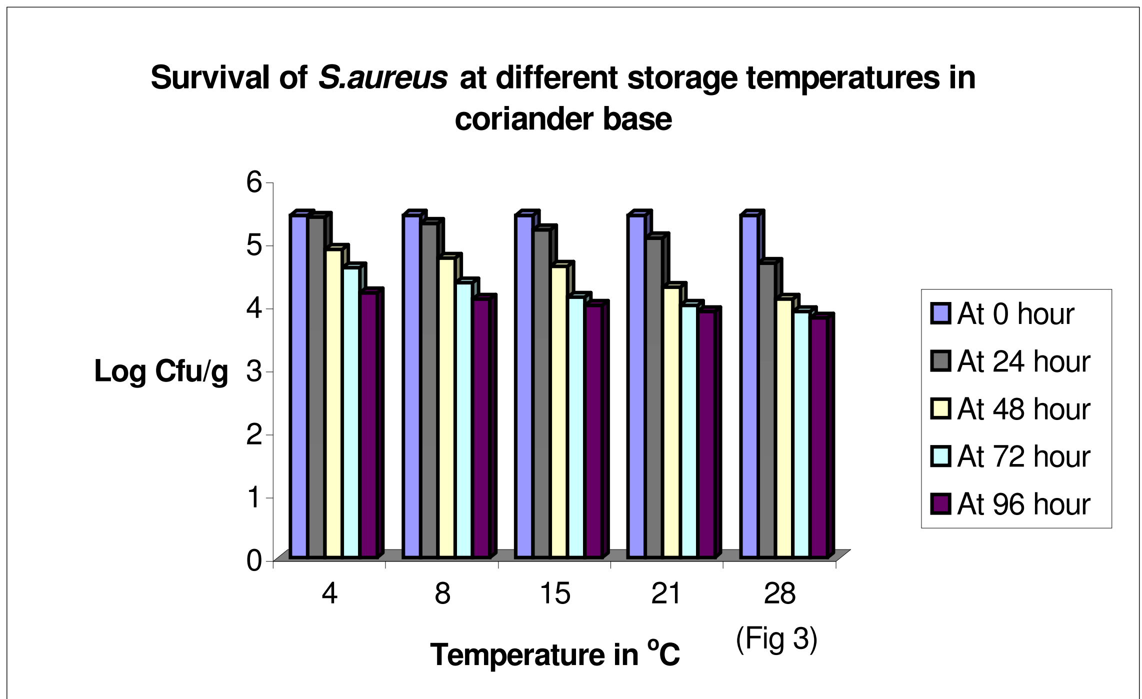
Additionally, *E.Coli* O157:H7 was identified presumptively in two locations,*Salmonella enteritidis* was detected qualitatively in samples obtained from three areas; 43% of staphylococcus isolated were identified to be coagulase positive *S.aureus* . A high total aerobic count and coliform count indicated poor hygienic practice during preparation of the chutneys, alternately, the role of contaminated water or raw materials cannot be ruled out. The presence of coagulase positive *S.aureus* in significant numbers in the samples indicates severe contamination predominantly due to handling. The importance of *E.Coli O157:H7* in food borne diseases is well documented , and the high

occurrence of *S.aureus* prompted us to use their respective surrogates for further studies.



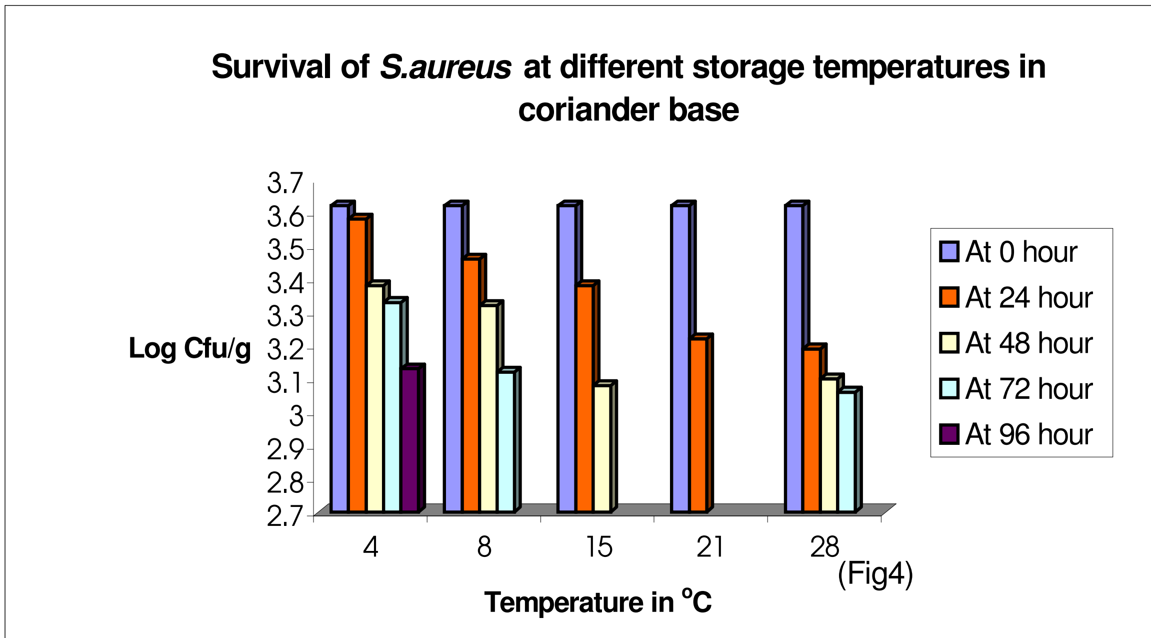
(Fig2)

Fig (2) shows the survival pattern of non toxigenic *S.aureus* at different storage temperatures in coriander base; at 4°C there was no decline of the initial inoculum with in 24 hours after which a gradual decline of approx 1.5 log CFU/gm was observed . If we look at series of 8°C, 15°C, 21°C & 28°C, similar trend is being followed but the values at 28°C are showing a more prominent decline from 0 hour to 96 hour. A comparison of cell counts after 96hrs in samples incubated at 4°C & 28°C showed a significant difference. A high initial inoculum level was chosen, since we found such levels existing during the analysis of samples. *S.aureus* is not known to grow at temperatures below 6.5^{0}C, the pH range for this organism lies usually within 4.5-11, coriander base is usually prepared by retailers and stored- refrigerated - the pH of this is approximately 4.5, the presence of *S.aureus* in significant levels within 24 hours in such material may allow elaboration of toxins, in sufficient amounts to cause disease if consumed or used for preparation.



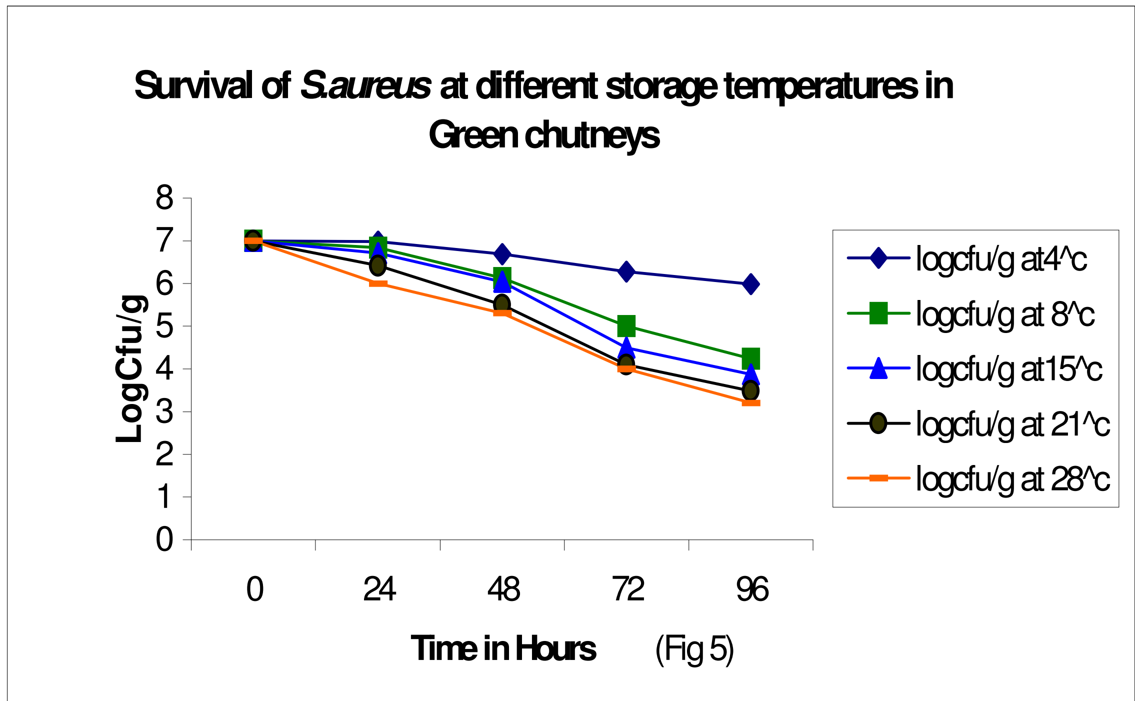
(Fig 3)

Fig (3) shows the Survival of *S.aureus* at different storage temp. in coriander base with initial inoculum of 6log. At all the storage temperatures a gradual decline of the initial value was evident with in a period of 96hours.



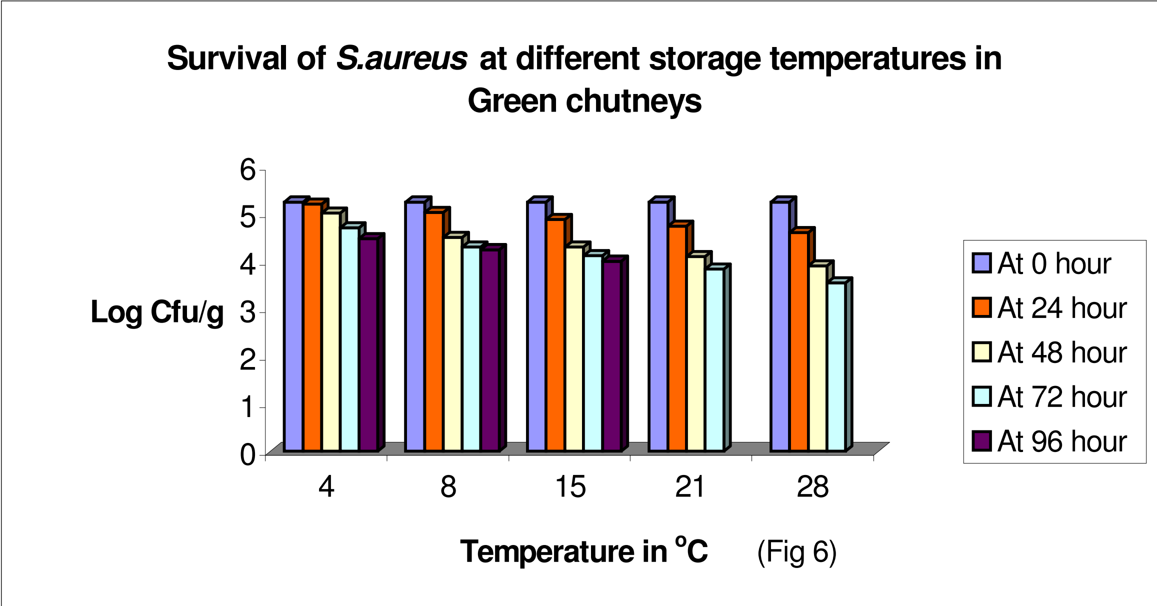
(Fig4)

Fig (4): shows the Survival of *S.aureus* at different storage temperatures in coriander base with initial inoculum of 4log. At 4°C & 8°C , values gradually decreased with in a period of 72hours .A sharp decline was observed in samples incubated at 15°C, 21°C & 28°C with in 24 hours.



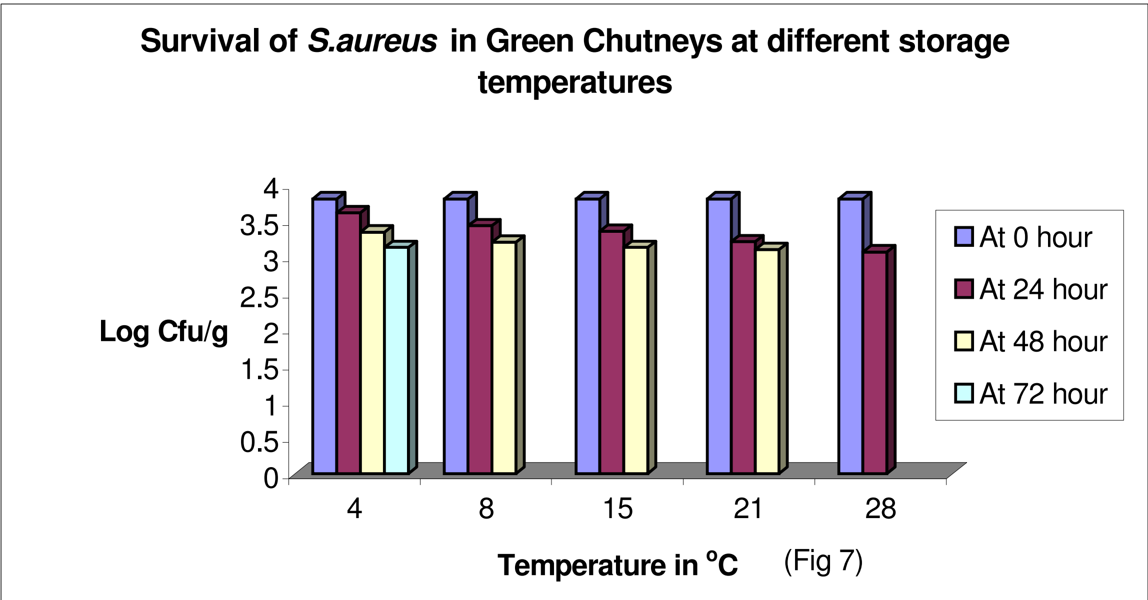
(Fig 5)

Fig (5) shows the survival of non pathogenic *S. aureus* at different storage temperatures in Green chutneys, from an initial inoculum level of approx. 8log , the values showed a decline in a gradual fashion in case of 8°C, 15°C, 21°C, & 28°C. At a storage temperature of 4°c there was no significant difference after 24 hours. Storage studies at abusive temperatures were conducted so as to judge the survival of *S.aureus* at these temperatures; prepared green chutneys are stored at least for 24 hours by several retailers, during october through December such material are stored at the prevailing temperature since the spoilage rate is slow. Survival of *S.aureus* in high numbers could be dangerous since toxins may be elaborated by the bacteria, toxins produced by 10^{6}CFU/g of *S.aureus* has been shown to initiate disease symptoms.



(Fig 6)

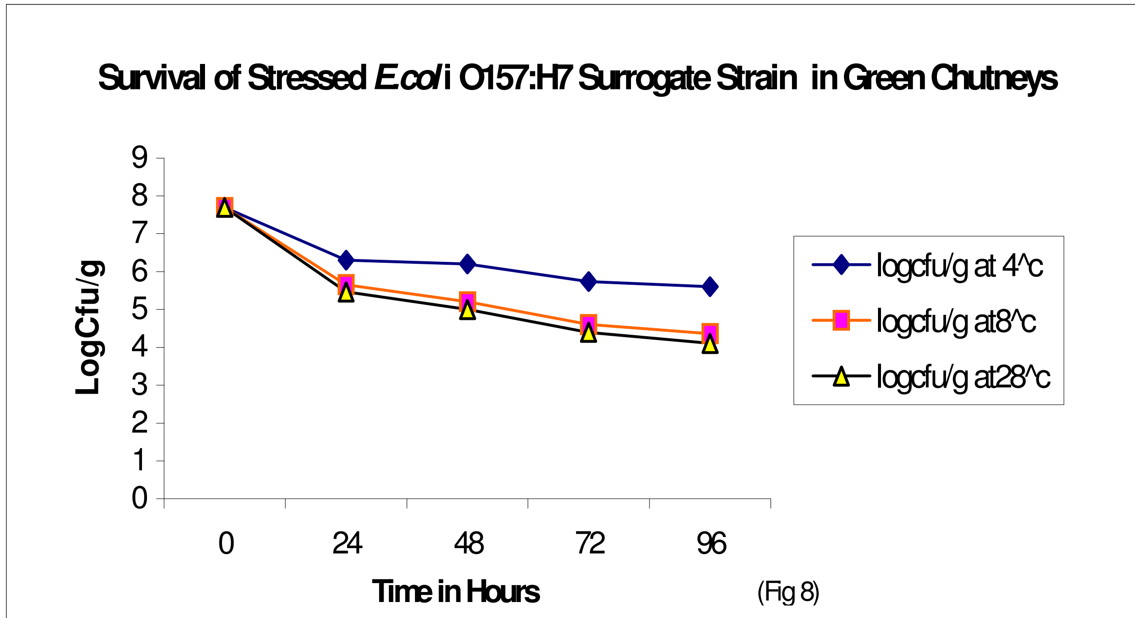
Fig 6 shows the survival of *S.aureus* in Green chutneys at different storage temperatures with initial inoculum of 6log. A gradual decrease at the storage temperatures viz. 4°c,8°C, 15°C, was observed with in a period of 96 hours . However no *S.aureus* fell below the detection limit after 72 hours.



(Fig 7)

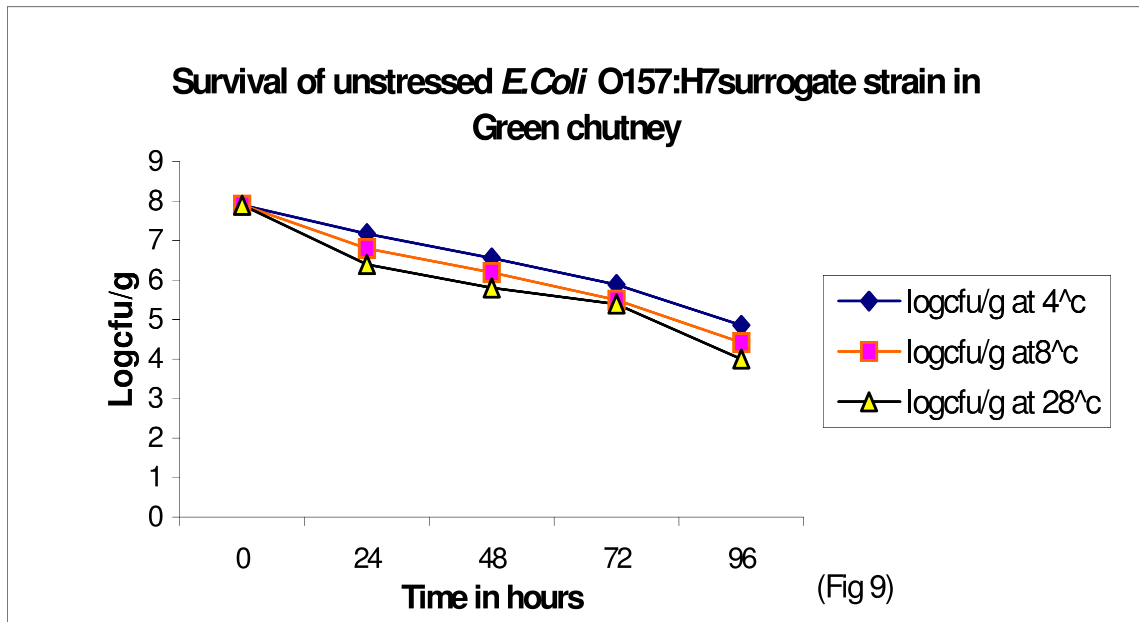
Fig (7) shows the Survival of *S.aureus* in green chutneys at different storage temp. with initial inoculum of 4log. A gradual decrease from the initial inoculum of

Log4CFU/g at all the storage temperatures viz. 4°c,8°C, 15°C, 21°c,& 28°C was evident with in a period of 72 hours . This decrease was lesser at 4°C. No *S.aureus* could be detected in samples incubated at 8°C, 15°C,& 21°C after 48hours.



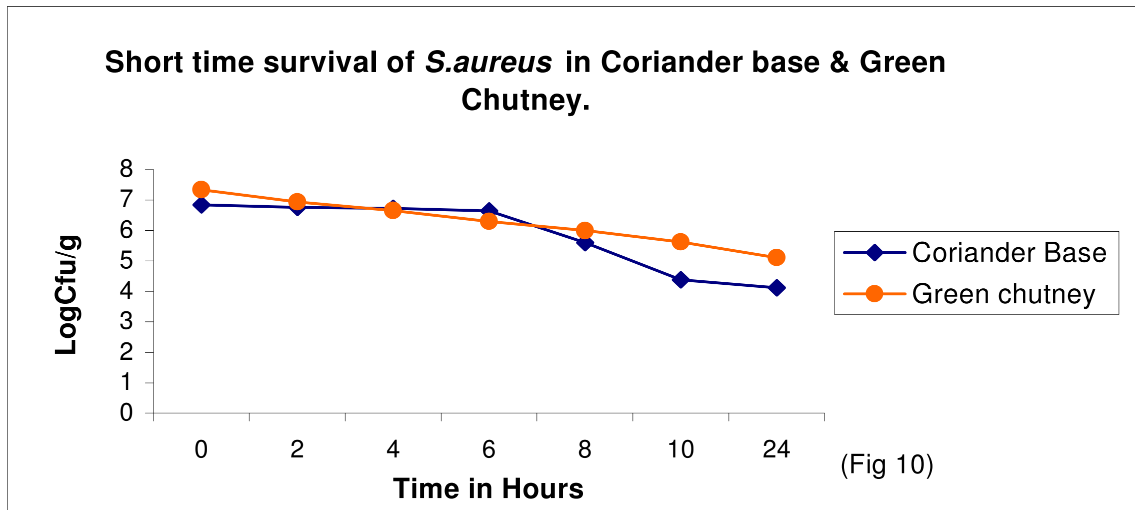
(Fig 8)

Fig (8) shows the survival of acid stressed *E.coli O157: H7* surrogate strain in Green Chutneys at different storage temperatures. There was a sharp decline from the starting inoculum of 8log CFU/g at 4°C till 24 hours. The counts remained constant up to 48hours followed by a gradual decline. Where as in case of 8°C & 28°C, a sharp decline up to 24hours was observed followed by gradual decline till the termination of the experiment, a difference of approx. 1.5 log CFU /g was observed between samples incubated in temperatures viz:4° C and 28°C. In all cases however, the counts remained sufficiently high for purposes of consumption, it was apparent that E.coli *O157: H7* failed to grow in any of the samples at the temperatures used for incubation.



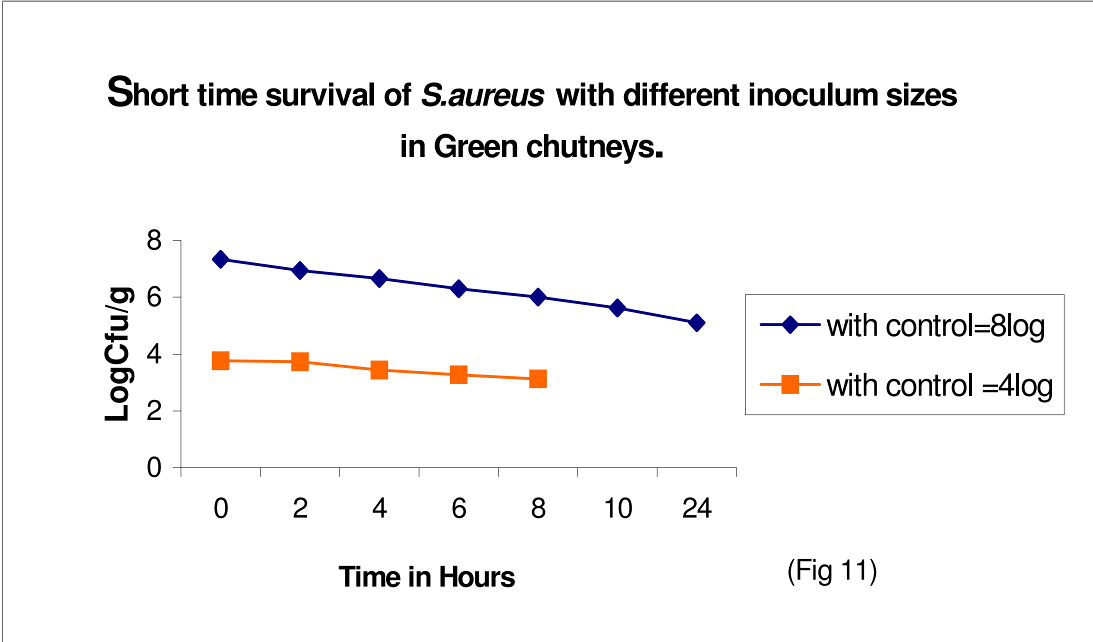
(Fig 9)

Fig (9) shows the survival of unstressed *E.coli O157: H7* surrogate strain in Green Chutney at different storage temperatures. A gradual decline in the values of initial inoculum was observed at temperatures ranging -.4°C, 8°C, & 28°C till the termination of the experiment. A decline of about 50% was observed in all cases after 96 hours. A comparison of survival potential of both stressed and unstressed *E.coli O157: H7* in green chutneys at different temperatures was thought important since prepared green chutneys are stored both refrigerated or at prevailing temperatures especially during the winter; the vendors usually do not clean the vessels after initially preparing the chutneys, assuming that this may be contaminated with *E.coli O157: H7* , the latter lots would have the initial stressed *E.coli O157: H7* as inoculum , we therefore compared the potential for survival of both acid stressed and unstressed *E.coli O157: H7.*



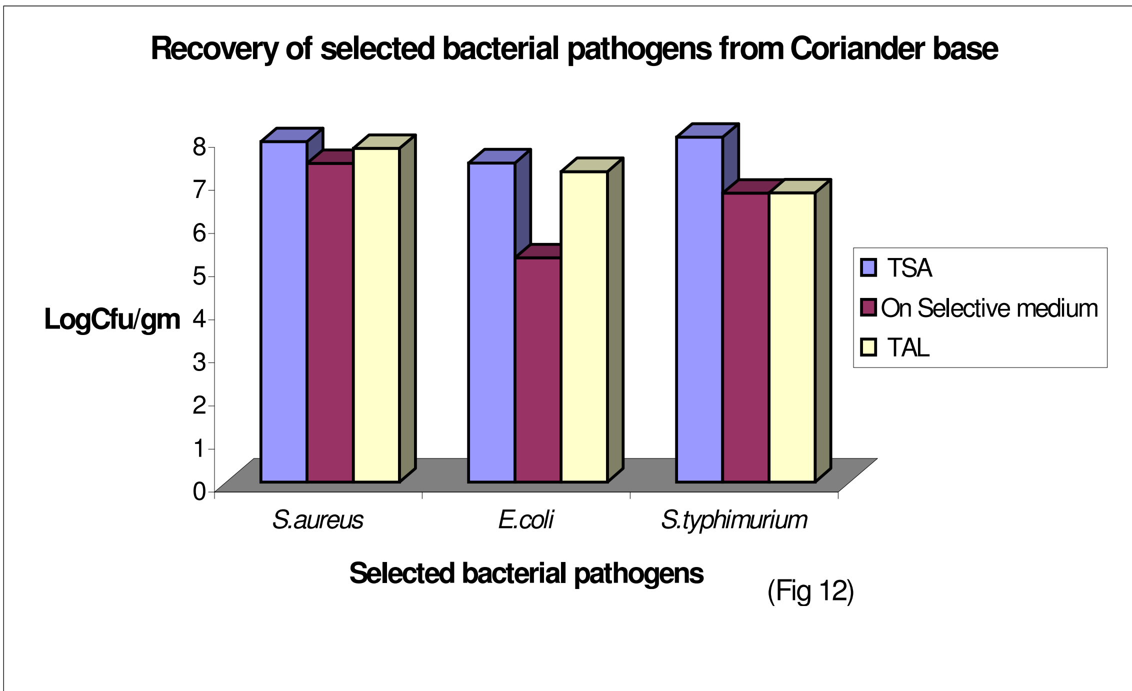
(Fig 10)

Fig (10) compares the survival of *S.aureus* in Green chutneys and coriander base inoculated with Log8CFU/g of non pathogenic *S.aureus*. In Green chutneys a gradual decline of about 2 log CFU /g was observed with in 24 hours whereas in coriander base this decline achieved a constancy till 6hours. After 6hrs a sharp decrease till 10 hours after which this quantity remained constant. The display period of green chutneys range approximately from 8-10 hours, therefore we attempted a short term survival kinetics of *S.aureus*. Since handling processes would introduce varying loads of *S.aureus*, survival studies incorporating both high and low initial inoculum levels were used, the results are shown below in Fig (11)



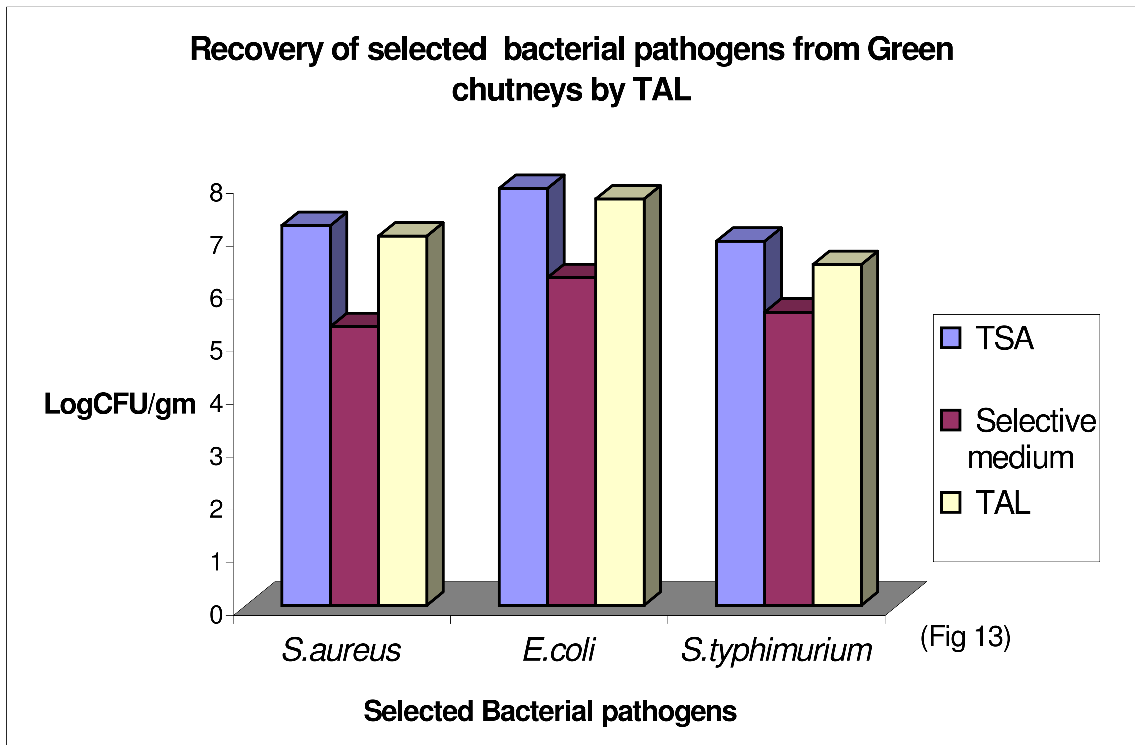
(Fig 11)

Fig (11) shows the survival of nonpathogenic *S.aureus* with different inoculum sizes in green chutneys . Graph showed a sharp decline of about 2log units till 24hrs with higher inoculum level (8logCFU/g), however the decline was not as sharp with a lower inoculum level of 4log CFU/g. No *S.aureus* could be detected after 8hours with low inoculum .



(Fig 12)

Fig(12): shows the recovery of the selected bacterial pathogens from cor base. Recovery of *S.aureus* on TSA & TAL was almost similar & there was no significant difference between the two (Kang DH, Fung DYC. 2000**)**. Similar was the case with *E.coli* & S. *typhimurium,*also the recovery on TSA & TAL was significantly higher than on the selective medium. In case of *S.typhimurium* recovery on selective medium & TAL is almost same & is less than that on TSA. Bacterial populations, which are stressed, are isolated less efficiently on selective medium; in order to obtain true estimates of the behavior of stressed surrogates in coriander base, the thin layer agar method was chosen. Individual bacterial pathogens spiked and recovered using this method, in general showed good recovery when compared to the respective selective medium.



(Fig 13)

Fig (13) shows the recovery of selected bacterial pathogens from Green Chutneys by TAL. In all the three cases recovery in TSA & TAL was higher when compared to that with selective medium. A significant difference in the recovery

values of selective medium was noted when compared to TAL & TSA, in TAL and TSA itself there was no significant difference. Since coriander bases are usually made into green chutneys, it was of interest to look at the recovery of bacterial pathogens from green chutneys; a good recovery of individual bacterial pathogens were evident from these results.

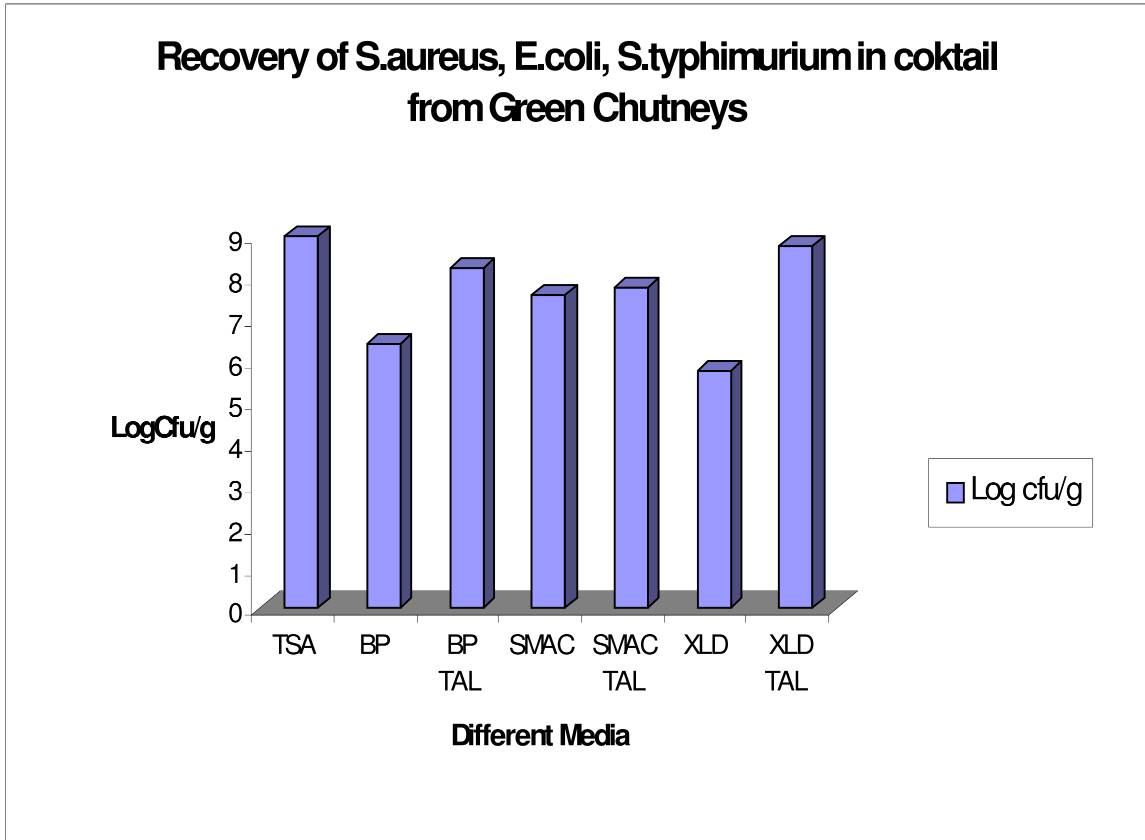
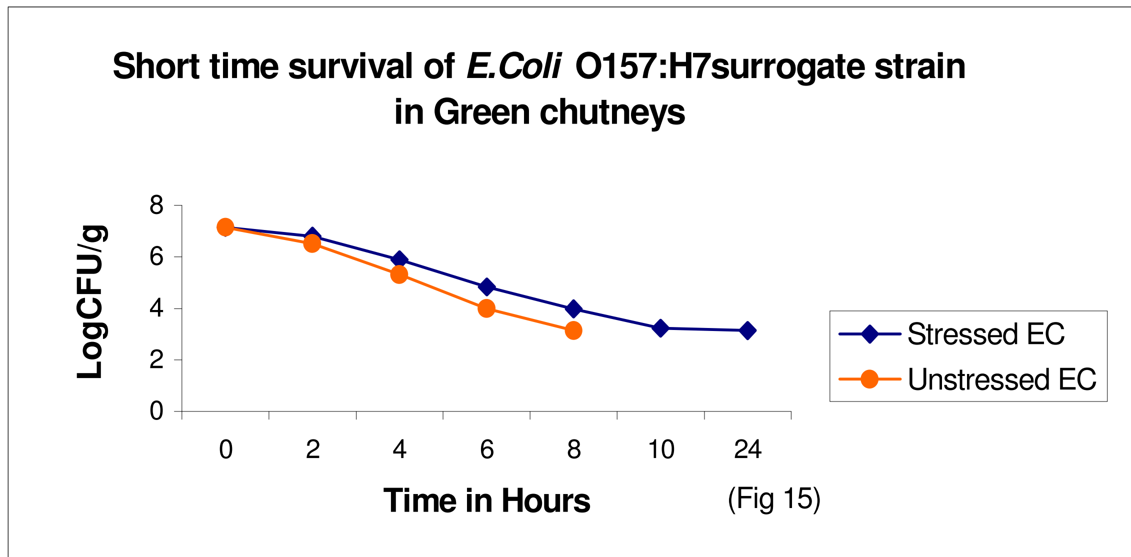


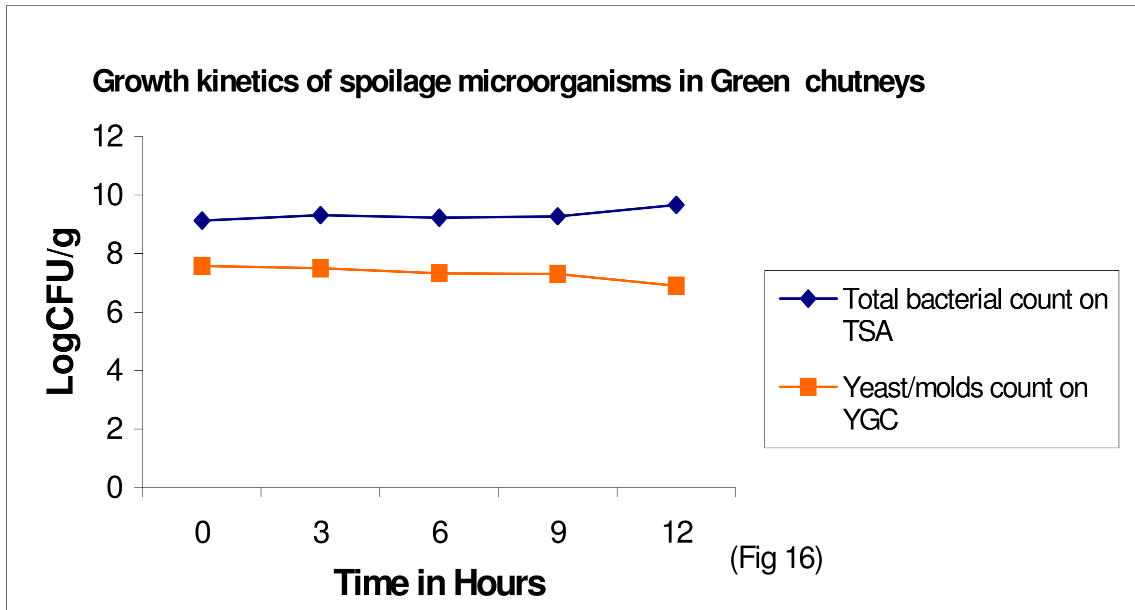
Fig (14) shows the recovery of *S.aureus*, *E.coli*, and *S.typhimurium* in cocktail from Green Chutneys using TAL .An initial inoculum of approx. Log 9.2 CFU/ml was used in this study; all bacterial pathogens could be isolated on their respective selective medium by this method. We assumed situations where two or more different bacterial pathogens may contaminate the green chutney samples, therefore a mixed inoculum comprising of all three types used initially were used as starting inoculum, the results indicate that under such conditions

TAL could both recover the individual types with efficacy when compared with individual selective medium.



(Fig 15)

Fig (15) shows the short time survival of surrogate strain of *E.coli O157:H7* in green Chutneys. Both unstressed and acid stressed bacteria showed a decline from the initial inoculated load within a period of 10 hours . For stressed *E.coli O157:H7* a gradual decline up to 10 hours was evident but counts remained constant till 24 hours, unstressed *E.coli O157:H7* was not detected after 8hours. The short term survival study performed with *E.coli O157:H7*indicated better survival of the latter when acid stressed. Green chutneys usually have an acidic pH of about 3-3.5, *E.Coli* normally has a short generation time, the normal display period of green chutneys are usually 8-10 hours, an estimate of the survival of the latter would be important in terms of the risk of the product.



(Fig 16)

Fig(16), shows the profile of growth of the inherent spoilage microorganisms in green chutneys at 28^0C. Initially a total bacterial count of Log 9.3CFU/g was obtained on TSA, the latter remained almost constant till 9 hours & after which there was an increase in the value at 12 hours. In case of yeast/ molds count on YGC medium the trend was opposite to that of bacterial count, an initial value of Log7.58 CFU/g was obtained, the growth was almost constant till 9hours following which there was a decrease to Log 6.9 CFU/g at 12 hrs. The pH of chutneys remained almost constant during this period. Usually the chutneys were considered completely spoiled based on complete loss of sensorial properties after a period of 9 hours. The spoilage study is essential since chutney samples are likely to be consumed within this period. The risk on account of growth or survival of bacterial pathogens within this period may be actually important.

out.

The Risk assessment of Green chutneys - surveys based on personal communication with vendors revealed that green chutneys are usually sold after about 2 hours after preparation. We assumed both these organisms to be present in high numbers initially during preparation; an ambient temperature of 28^0C was also assumed. Based on these assumptions and on the average serving size the risks were calculated as:

Total number of *S. aureus* per serving $8.5x10^6$ x18 = $1.5x10^8$

Total number of *E.coli* O157: H7 per serving $6.3x10^6$ x18 = 1.13×10^8

It has been shown that 10^6 cells/g of *S. aureus* & 10 cells/g *of E.coli* O157: H7 are enough to cause sickness in a human being (Bergdoll et al, 1989; USDA, FSIS, 1990) It is evident from the above results that green chutneys contaminate with the above at high initial levels could be a potential source of risk to the consumer .

CONCLUSIONS

1. Green chutneys sold by street vended shops were analyzed for their microbiological quality throughout Patiala city;

2. Total viable counts, coliform and staphylococcal counts were significantly high , qualitative analysis showed the presence of *Salmonella entertidis*, *E.Coli O157:H7*(presumptive) and coagulase posiitive *S.aureus*.

3. Non pathogenic isolates or surrogates of *S.aureus* and *E.coli O157:H7* were used for simulated short time and storage studies at different abusive temperatures.

4. *S.aureus* survived well within 24 hours at 4°C and 28° C showing very less decline from initial numbers. The trend of survival of *S.aureus* followed with both high and low inoculum sizes were generally similar.

5. Survival studies at different temperature with *E.Coli O157:H7* showed that the initial inoculum declined appreciably within 24 hours and therefater remained constant.

6. Acid stressed *E.coli o157:H7* sustained for longer periods when compared to the survival of unstressed *E.coli* in green chutneys

7. In order to faciliate recovery of stressed *E.coli* a thin agar layer method was standardized, this method was found to efficiently recover stressed bacterial pathogens from green chutneys

8. Growth kinetics with inherent spoilage microorganisms of 0green chutneys indicated that green chutneys are rendered completely spoiled after 9 hours at 28°C.

9. Based on these observations and average serving size of green chutneys, the consumer risk was calculated assuming high initial pathogen load and survival kinetics within a short time. Estimated data indicate that both bacterial pathogens posed significant risk to consumers.

BIBLIOGRAPHY

1. Altekruse, S. F., and Swerdlow, D. L. 1996. The changing epidemiology of foodborne diseases. Am. J. Med. Sci. 311:23-29.

2. 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.

3. Beuchat, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes Infect. 4:413-423.

4. Beuchat, L. R. 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59:204-216).

5. Blakeman JP, editor. 1981. Microbial ecology of the phylloplane. London: Academic Press. 502 p.

6. 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

7. 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)

8. Cheng Yi-Hsin, Yang Yi- Hsin, Chou Chun-Cheng, Influence of Acid Adaptation on the Tolerance of Escherichia coli O157: H7 to some subsequent Stresses, J. Food Protection, Vol.65, No. 2, 2002, p 260-265.

9. 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.

10. Duffy Geraldine, Riordan CR Denise, Sheridan J. James , Development of a critical control step for E.coli O157: H7 in Pepperoni, ISBN July 1999.

11. ERS* Updates Foodborne Illness Costs Food Safety v.23, i.3, USDA Sep/Dec00

*Economic Research Service, United States Department of Agriculture

12. Essential Safety Requirements for street-vended foods(revised edition) Food Safety Unit Division of Food and Nutrition, World Health Organization 1996.

13. Food safety and food borne illness , Fact sheet N°237, Revised January 2002

14. Food Safety Guidelines for the Food Handlers, World Health Organization 2001

15. Hedberg, C. W., MacDonald, K. L., and Osterholm, M. T. 1994. Changing epidemiology of foodborne disease: A Minnesota perspective. Clin. Infect. Dis. 18:671-682

16. Jagger J. 1981. Near-UV radiation effects on microorganisms. Photochemistry and Photobiology 34:761-8

17. Jordan N.Kieran, Oxford Lynn, Byrne O'P. Conor, Survival of Low-pH stress by Escherichia coli O157: H7 : Correlation between Alterations in the Cell Envelope and Increased Acid Tolerance ,Applied &Environmental Microbiology , July 1999,p.3048-3055, Vol.65.No.7.

18. 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.

19. Kakar DA, Udipi SA , Microbiological Qualities of Different Varieties of Chutneys sold in Mumbai City, J. Food. Sci. Technol. 2000. Vol.87, no. 5. 509-511.

20. Mahopatra Das A, Rath CC, Dash SK, Mishra RK , Microbiological Evaluation of Street Foods in Bhubaneswar, J.Food. Sci. Technol., 2002, Vol.39, No.1, 59-61

21. Ministry of Health & welfare of Japan, 1997

22. National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on fresh produce. Food Control 10:117-143.

23. National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Int. J. Food Microbiol. 52:123-153.

24. Nguyen-the, C., and Carlin, F. 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. 34:371-401.

25. 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)

26. Press release WHO/58, 13 August 1997

27. 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. 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.

28. USFDA, CFSAN , Bacteriological Manual Online

29. 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

30. US, GAO, Washington DC. 20548, Resources, Community, and Economic Development Division.B-270753, May8, 1996.

31. Viswanathan Poorna, Kaur Randhir, Prevalence and growth of pathogens on salad vegetables, fruits and sprouts , Int.J. Hyg.Environ. Health 203, 205-213 (2001)

32. 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

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 ^{o}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 ^{o}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 ^{o}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°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°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°C for 20 min.

Urea Solution:

Urea	20g
Water	100ml

Dissolve the Urea in water, sterilize by filtration.

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 ^{o}C)

PUBLICATIONS

1. Ganguli, A and Bansal, S., 2003, Microbiological qualities of Green chutneys sold in Patiala City, India. African Journal of Food and Nutritional Sciences (*Accepted for Publication*).

2. Bansal, S and Ganguli, A. Hygienic quality of traditional Indian culinary aids: “Green Chutneys”. Journal of Environmental Health (*Communicated*).