UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS
DEPARTAMENTO DE TECNOLOGIA DE ALIMENTOS

Incidência e Desenvolvimento de *Salmonella* spp. e *Listeria* spp. em Frutas de Baixa Acidez

PARECER

Este exemplar corresponde à redação final da tese defendida por **Ana Lúcia Penteado** aprovada pela Comissão Julgadora em 11 de março de 2003.

Campinas, 11 de março de 2003.

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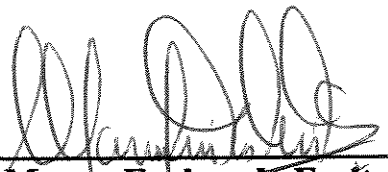
Errata

Pgs. xix, xxi, 75, 85, 86, 89, onde lê-se *Listeria grayii* corrigir para *Listeria grayi*.

Pg. 87 Tabela 3, onde lê-se números totais de *Listeria* spp. em "wholesale" e "street market" igual a 0, alterar para 3 e 6 respectivamente.

Pg. 96 Figura 1. Desconsiderar as passagens dos meios de enriquecimentos seletivos (TT, RV) para caldo M e subsequente análise por TECRA VIA para *Salmonella.* Na análise para *Listeria* desconsiderar a passagem do caldo MFB para análise pelo método TECRA VIA.

BANCA EXAMINADORA



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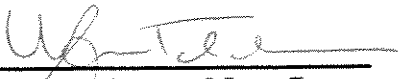

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VIVER EM AÇÃO

É melhor tentar e falhar,

Que preocupar-se a ver a vida passar.

É melhor tentar, ainda que em vão,

Que sentar-se fazendo nada até o final.

Eu prefiro na chuva caminhar,

Que em dias tristes em casa me esconder.

Prefiro ser feliz, embora louco,

Que em conformidade viver.

Martin Luther King

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RESUMO GERAL

Numa etapa inicial, foi estudada a incidência de *Salmonella* spp. e *Listeria* spp. em superfície de frutas de baixa acidez (melão, melancia e mamão). De um total de 120 amostras de frutas, 42 foram analisadas simultaneamente por um método imunoenzimático (TECRA VIA) e outro clássico, de referência (BAM modificado) para *Salmonella* e por um método canadense "Health Protection Branch" e TECRA VIA para *Listeria.* As 78 amostras de frutas restantes foram analisadas somente pelos métodos de cultura.

Salmonella spp. não foi detectada em nenhuma das amostras analisadas pelos dois métodos utilizados, o mesmo ocorrendo em relação ao isolamento de *L. monocytogenes.* No entanto, *Listeria innocua* e *Listeria grayii* foram isoladas a partir de três amostras de melancia, *L. ivanovii* em cinco amostras de mamão e *L. welshimeri* em uma amostra de melão, quando utilizado o método canadense "Health Protection Branch". Observou-se, também, que as amostras coletadas em feiras livres mostraram uma maior incidência de *Listeria* spp. quando comparadas com aquelas obtidas em centrais de abastecimento (CEASA).

Numa segunda etapa do projeto, estudou-se a capacidade de multiplicação de *Salmonella* Enteritidis e *Listeria monocytogenes* em polpas de frutas (mamão, melão e melancia) incubadas em diferentes condições de tempo e temperatura. Os respectivos tempos de geração (g) para *Salmonella* Enteritidis, nas temperaturas de 10 °C, 20 °C e 30 °C foram de 7,31, 1,69 e 0,69 horas em melão de 7,47, 1,60 e 0,51 horas em melancia e de 16,61, 1,74 e 0,66 horas em mamão. Já para *Listeria monocytogenes* os tempos de geração em melão, melancia e mamão foram, respectivamente, de 7,12, 13,03 e 15,05 horas a 10 °C; 1,74, 2,17 e 6,42 horas a 20 °C e 0,84, 1,00 e 1,16 horas a 30 °C.

Os resultados mostraram que tanto *S.* Enteritidis como *L. monocytogenes* podem multiplicar-se em frutas de baixa acidez e que a temperatura de 10 °C,

apesar de reduzir a velocidade de crescimento destes microrganismos não garante a inibição dos mesmos.

Numa terceira etapa, estudou-se a possibilidade de infiltração de *Salmonella* Enteritidis sorotipo S 132 fluorescente em mangas após serem submetidas a tratamento hidrotérmico para eliminar larvas de moscas das frutas. Este estudo foi conduzido na Food and Drug Administration – FDA/USA. Os resultados evidenciaram, indiretamente, a capacidade de infiltração microbiana nas frutas, utilizando-se o corante "Brilliant blue FCF/Sigma", que foi detectada em 67% das frutas submetidas ao tratamento; nos ensaios efetuados diretamente com *Salmonella* Enteritidis fluorescente os níveis de infiltração foram elevados, de 87%, tanto para mangas verdes como maduras. Constatou-se, também, que a infiltração da bactéria foi muito mais acentuada na região do cálice da manga do que na sua porção lateral ou na base.

GENERAL ABSTRACT

In the first part of the project, the incidence of *Salmonella* spp. and *Listeria* spp. on the surface of low acid fruits (melon, watermelon and papaya) was studied. From the total of 120 fruit samples 42 were simultaneously analyzed by the TECRA Visual Immunoassay (TECRA VIA) and the modified BAM for *Salmonella* and by the Health Protection Branch, Canada, and TECRA VIA for *Listeria*; the remaining 78 fruit samples were analyzed only by the cultural procedures.

Salmonella spp. was absent in all the samples analyzed using both methods as was *L. monocytogenes*. However, *L. innocua* and *L. grayii* were detected in watermelon samples, *L. ivanovii* in papaya samples and *L.* welshimeri in melon samples when using the Health Protection Branch method. It was also observed that the samples collected in street markets showed a higher incidence of *Listeria* spp. when compared to those collected in wholesale markets.

In the second part of the project, the ability of *Salmonella* Enteritidis and *Listeria monocytogenes* to grow in fruit pulp (melon, watermelon and papaya) incubated under different conditions of time and temperature, was studied. The generation times (g) for *S.* Enteritidis in melon at 10 °C, 20 °C and 30 °C were 7.31, 1.69 and 0.69 h respectively; for watermelon 7.47, 1.60 and 0.51 h respectively and for papaya 16.61, 1.74 and 0.66 h respectively. For *L. monocytogenes* the generation times for melon, watermelon and papaya were 7.12, 13.03 and 15.05 h at 10 °C respectively, 1.74, 2.17 and 6.42 h at 20 °C and 0.84, 1.00 and 1.16 h at 30 °C.

The results showed that both *S.* Enteritidis and *L. monocytogens* could grow in low acid fruits and that a temperature of 10 °C, although capable of reducing the rate of growth of these microorganisms, could not guarantee their inhibition.

In the third part of this project the possibility of the infiltration of *S.* Enteritidis into mangoes (after being submitted to hot water treatment to eliminate fly larvae),

was studied. This project was carried out at the Food and Drug Administration-FDA in the USA.

Using a dye (Brilliant blue FCF/Sigma), the results showed the ability of the microorganism to infiltrate the mangoes, which was detected in 67% of the fruits submitted to the treatment. In the experiments performed directly with *S.* Enteritidis fluorescent serotype S 132, the infiltration levels were higher (87%) both for green and early-ripened mangoes. It was also observed that bacterial infiltration was more evident in the stem portion when compared to the bottom and middle portions.

INTRODUÇÃO GERAL

Levantamentos epidemiológicos a nível internacional normalmente apontam produtos de origem animal (carnes e produtos cárneos, leite e derivados, etc.) como os veículos mais frequentes dos casos e surtos de doenças de origem alimentar. No entanto, nos últimos anos, particularmente nos Estados Unidos, tem sido descrita, com frequência crescente, a participação de alimentos de origem vegetal, principalmente frutas e hortaliças em número significativo destes eventos.

A este respeito, os dados relatados pelo CDC (2000) na Tabela 1, confirmam esta afirmativa.

Tabela 1. Principais surtos de doenças de origem alimentar de acordo com veículos de transmissão nos EUA de 1993 a 1997 (CDC, 2000).

Veículo de transmissão	Surtos (%)				
	1993	1994	1995	1996	1997
Carne bovina	3,3	3,4	2,2	1,5	1,4
Moluscos	1,4	1,8	1,9	1,0	2,2
Outros peixes	4,9	5,4	4,9	5,0	5,2
Salada de Batata	0,2	1,2	0,2	0,2	0,6
Outras saladas	3,7	2,9	3,3	3,8	4,2
Frutas e hortaliças	2,5	2,6	1,4	2,7	3,0
Alimentos cozidos	0,8	1,8	1,4	1,3	0,8

Diversos agentes etiológicos têm sido relatados como responsáveis pelos surtos, destacando-se, entre eles, *Salmonella* spp, *E. coli*, *Clostridium perfringens*, *Shigella* spp., *S. Aureus* e *Campylobacter* spp. *Salmonella* aparece como o principal agente etiológico de surtos de origem alimentar nos USA, no período de 1993-1997, com 32.610 casos e 13 mortes. *Listeria monocytogenes,* apesar de não estar entre os dez principais microrganismos responsáveis, aparece em

terceiro lugar em relação ao número de mortes (6.9%), superada apenas por *Salmonella* e *E. coli,* que foram responsáveis por 44,8% e 27,6 % respectivamente, do número total de surtos (CDC, 2000).

Estas constatações têm levado as autoridades sanitárias de diferentes países e, de maneira particular, a Food and Drug Administration -FDA dos Estados Unidos da América, a desenvolver uma série de estudos visando melhor avaliar e dimensionar a importância das frutas no aspecto de saúde pública (Tamplin, 1997). Entre os temas a serem melhor estudados destacam-se uma avaliação dos patógenos mais frequentes nestas matérias primas, estudo de seu desenvolvimento e de mecanismos que permitam seu melhor controle.

No Brasil, são praticamente inexistentes estudos desta natureza. No entanto, é um fato reconhecido que as condições higiênico-sanitárias vigentes na produção, colheita, armazenamento, transporte e distribuição nem sempre são as mais adequadas. Além disso, muitas destas frutas são comumente expostas e servidas já fatiadas, o que acentua os riscos de contaminação em função do manuseio e contacto com utensílios não adequadamente sanificados. Como agravante final, muitas das frutas assim manuseadas e consumidas são de baixa acidez (caso do melão, mamão e melancia) o que teoricamente possibilitaria a proliferação dos contaminantes eventualmente presentes.

Com base nestas considerações, foi desenvolvida a presente pesquisa visando a um melhor conhecimento da natureza e intensidade de contaminação de melão, mamão e melancia por algumas bactérias patogênicas (*Salmonella* Enteritidis e *Listeria monocytogenes*) bem como procurando estudar a eventual proliferação das mesmas, na polpa destas frutas mantidas sob diferentes condições de incubação. Procurou-se, também, avaliar a possibilidade de infiltração de *Salmonella* no interior de frutas (mangas) o que aumentaria os riscos destes alimentos como veículos de doenças.

A presente tese está sendo apresentada na forma de artigos (de revisão e de pesquisa) que serão submetidos à publicação.

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CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

Incidence, Outbreaks, Growth and Survival of *Salmonella* spp. and *Listeria* spp. in Fruits and Fruit juices

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ABSTRACT

Over the last few years an increase in the consumption of natural products, among them fruits and fruit juices has been observed, mainly due to an increased concern among the population about health and the benefits which arise from natural food. In this present review, the incidence, outbreaks, growth and survival of *Salmonella* spp. and *Listeria* spp. in fruits and fruit juices are described. As the consumption of these products with no kind of thermal treatment is increasing, except for pasteurized juices, and as *Listeria* spp. and *Salmonella* spp. can survive and grow in fruit pulp and also be present on the surface of some of them, the manipulation of these foods from harvest to consumption should follow good hygienic practice recommendations, trying to minimize food-borne diseases.

Key words: Incidence, growth, survival, fruits, *Listeria*, *Salmonella*

INTRODUCTION

In recent years, fresh produce has been detected as the vehicle of transmission in several food-borne outbreaks. According to the Centers for Disease Control and Prevention (CDC), the number of reported produce–related outbreaks per year doubled between the period 1973-1987 and 1988-1992 with most outbreaks of identified etiology being of bacterial origin; *Salmonella* spp. was most commonly reported in both time periods (21). However, *Listeria monocytogenes* is of special concern because it can grow at refrigeration temperatures, persists as an environmental contaminant in the processing environment, and has the potential to cause mortalities associated with the outbreaks (25). The potential for the microbial contamination of fruits and vegetables is high due to the wide variety of contamination conditions to which the produce is exposed during growth, harvest, and distribution (43).

Incidence of *Salmonella* and *Listeria* in fruits.

An important consideration when addressing safety issues is the incidence of pathogens and outbreaks associated with particular food products (27).

Although practically any bacterial pathogen could potentially be a problem, only a few are of greater concern for fresh produce, particularly *Listeria monocytogenes, Clostridium botulinum, Shigella* spp., *Salmonella* spp., parasites and viruses. Of these, *Shigella*, *Salmonella* and *Listeria monocytogenes* have probably received the most attention, particularly in recent years (9).

Salmonella spp. Incidence

Velaudapillai *et al.* (71) from Apr. to Aug. 1967 reported the incidence of salmonelas, shigelas and enteropathogenic *Escherichia coli* in uncooked food in Colombo, Sri Lanka. Of a total of 392 fruits, only one (wild olive) was *Salmonella* positive.

Al-Hindawi and Rished (2) in 1979 analyzed 353 local foods in the city of Baghdad, Iraq, for *Salmonella* species, among them olives, raw vegetables (tomatoes, celery, lettuce, green salad) and fruits (fresh grapes and dates), showing contamination levels of 1.4%, 0.85% and 0% of *Salmonella* spp. respectively.

Goverd *et al.* (33) during the autumns of 1975, 1976 and 1977, examined apples and juices from large and small cider makers located in the southwest of England, for the presence of coliform organisms and *Salmonella* spp. Coliforms were found both on the fruit and in the juice, and salmonellae were isolated on more than one occasion from the flume water but not from the apple juice.

Papadakis *et al.* (49) between Nov. 1978 and March 1980 in Athens, Greece, investigated the presence of salmonellae in fresh vegetables eaten without any cooking in salads, and compared the efficiency of thee enrichment media. From a total of 423 samples examined and collected from green groceries (tomatoes 142, lettuces 146, green peppers 124, parsley 11), only thee (0.7%) samples of lettuce yielded salmonellae. These belonged to the serotype *S.* Montevideo.

Rude *et al.* (57) in the United States estimated the contamination by nematodes, amoebae, and *Salmonella* in a two-year survey, from 1979 to 1981, of salad vegetables (cucumbers, cabbage, lettuce, celery, carrots, radishes, tomatoes, mushrooms, cauliflower, and spinach) obtained from wholesale and retail sources. *Salmonella* was found in 4 out of 50 samples but not in tomatoes.

Saddik *et al.* (59) in 1985 collected two hundred and fifty samples of raw vegetables and salads from hotels, restaurants, small foodservice shops, markets

and street vendors in Egypt, and tested for *Salmonella, Shigella* and the aerobic colony (30°C) count. *Salmonella* was isolated from two samples of green leafy vegetables (greens) and one sample of mixed salad that most likely contained greens. The tomatoes were *Salmonella* negative.

Ruiz *et al.* (58) in 1987 conducted a comparative study of the incidence of *Salmonella* isolated from irrigation waters, vegetables and human infections. A total of 181 samples of irrigation water from the farmlands of Granada, Spain, were examined for the presence of *Salmonella* spp. Eight hundred and forty - nine samples of the vegetables from these farmlands and from local commercial establishments and centers of distribution were studied. Sampling was done regularly over the period of study, which ran from March 1981 to February 1983. Ten out of 299 (5.4%) samples of fruits (eggplant, marrow, pumpkin, broad bean, bean, cucumber, pepper and tomato) were shown to be *Salmonella* positive.

The Food and Drug Administration (FDA) sampled 1,440 imported melons from March 26 to April 13, 1990, and the results of this sampling revealed that only 11 melons (0.76% of those sampled) had *Salmonella* species on their surfaces. A second survey, carried out from Nov 19. to Jan 3. 1991, showed that 24 (1.06%) of the 2,220 analyzed melons contained *Salmonella* species (43).

Monge *et al.* (46) evaluated the sanitary quality of street-sold fruits during the period from March 1990 to March 1993 in San José, Costa Rica. They looked for the presence of *Salmonella* spp., *Shigella* spp., *Escherichia coli* as well as fecal coliforms in natural refreshments, fruit salads and fruits, usually cut into slices and exposed for selling in the streets (like pineapple, papaya, non-ripe mango and watermelon) and those that could be eaten without peeling, like nances and jocotes. They analyzed 25 samples of each fruit, 50 natural refreshments and 50 fruit salads. *Salmonella* spp. was not isolated from any of the examined samples.

In 1995 Parish (51) collected 70 samples from a Florida citrus processing facility (equipment swabs, fruit surface swabs, juice, and miscellaneous environmental samples) before, during and after processing runs on two different dates. Bottled juice samples from eight previous extraction dates were also collected. Analyses for *Salmonella* cells were conducted on all juice samples, fruit

surface swabs, environmental samples, and selected equipment swabs. *Salmonella* serovars Hartford, Rubislaw, Saintpaul and Newport were detected from either juice, unwashed fruit surfaces or amphibians captured outside the processing building.

Wells and Butterfield (75) analyzed enriched wash water from healthy and decayed portions of 341 fruits and vegetables collected in local supermarket in New Jersey from 1995 to 1997, and affected by fungal rots, for the presence of *Salmonella*. Suspected *Salmonella* was isolated from 20.2% of healthy and from 26.4% of decayed portions, two-thirds of which were caused by *Alternaria* sp. and *Botrytis* sp. In a similar analysis of 121 samples with mechanical injuries, there were no significant differences in *Salmonella* incidence between injured and uninjured portions. Of 332 suspected *Salmonella* randomly isolated from healthy and decayed or injured portions, including tomato and cantaloupe, 17 (5.1%) were confirmed as *Salmonella*.

In 2001 Viswanathan and Kaur (72) analyzed a total of 120 samples, comprising different types of raw vegetables, fruits and sprouts collected from street vendors in Mumbai (India), for their aerobic plate count, coliform count and the presence of various food-borne pathogens. *Salmonella* was present in 33.3%, 37.5% and 4.2% of vegetables, fruit samples and sprouts respectively.

Parish (51) analyzed orange fruit surface and juice (1/3 oranges were graded hulls, 1/3 were washed and graded, and 1/3 were ungraded) for the presence of *Salmonella*. The fruits originated from various sources (orchard to juice plant) in USA. *Salmonella* was absent in all samples (375).

In a FDA survey in 1999 of imported fresh produce, *Salmonella* was isolated from one of 143 analyzed strawberry samples (0.7%), from eight (5.3%) of 151 analyzed cantaloupe samples and was absent in 20 analyzed tomatoes (29).

Listeria spp. incidence

Brackett (8) in 1999 reported that unlike raw vegetables, information concerning the incidence of *Listeria* spp. on raw fruit is virtually nonexistent. The same author mentioned (a) infrequent association between consumption of fruit and listeriosis and (b) most fruits grow well above ground, and are therefore not subject to frequent contact with *Listeria* –contaminated soil or feces. This suggests the incidence of listeriae on fruit may well be as low as or lower than that observed for raw vegetables.

Sizmur and Walker (66) in 1988 examined 60 samples of salads of ten different varieties. The salads tested included beansprouts alone, mixed vegetable salads, and salads containing nuts and fruit. *Listeria* sp. was not isolated from the plain beansprout salads or from those which contained nuts, perhaps because of the acidic pH.

Heisick *et al.* (35) conducted a *Listeria* spp. survey from October 1987 to August 1988 on 10 types of fresh produce from two Minneapolis area supermarkets. The produce included broccoli, cabbage, carrots, cauliflower, cucumbers, lettuce, mushrooms, potatoes, radishes, and tomatoes. No *Listeria* spp. were isolated from broccoli, carrots, cauliflower, or tomatoes.

Farber *et al.* (26) in 1989 analyzed various retail foods in Ottawa (Canada) for the presence of *Listeria* spp. One hundred and ten samples of vegetables including lettuce, celery, tomatoes, and radishes, and 14 samples of pasteurized milk, were found to be free of *L. monocytogenes*.

Parish and Higgins (52) in 1989 failed to detect any *Listeria* spp. in 100 retail samples of reconstituted single-strength orange juice (pH 3.63-3.84) that were pasteurized at 30 geographically distinct dairy and non-dairy facilities located across the United States and Canada.

In 1990 Tiwari and Aldenrath (67) analyzed 598 samples of food products and environmental swabs for *Listeria monocytogenes* in Alberta, Canada. Listeriae were not detected in any of the 20 fresh vegetables or the three prepared salad

samples. The vegetables analyzed included lettuce, broccoli, tomatoes, carrots, cabbage, cauliflower, brussel sprouts and radish.

In 1992 Vahidy *et al.* (70) screened one hundred and fifty samples of fresh fruits and vegetables collected over a period of 12 months from various localities in the city of Karachi (Pakistan), for the presence of *Listeria monocytogenes*. Of 30 samples each of papaya, watermelon and cantaloupe, and 15 each of cucumber, tomato, radish and carrot, *Listeria monocytogenes* was isolated from two samples of papaya and tomato each and one sample of watermelon and cucumber.

A study on the incidence of *Listeria* spp. was carried out by Simón *et al.* (64), on 311 samples of raw foodstuffs from markets and other establishments in the city of Barcelona, from September 1989 to March 1990. These foodstuffs included vegetables (103 samples), minced meats from pork, beef and poultry (168 samples) and bivalve mollusks (40 samples). *L. monocytogenes* was isolated from two samples of leek (20%) and two samples of potato (16.6%), and appeared on one occasion in lettuce, chards, celery and cabbage. *L. innocua* was isolated from tomatoes, asparagus, leeks, fresh garlic and potatoes. *L. welshimeri* was found in lettuce, potatoes and mushrooms, and *L. seeligeri* only in potatoes.

From Dec. 1990 to May 1991, Orsini *et al.* (47) analyzed 246 samples of raw and cooked vegetables, roast meats and fruit drinks served both to hospitalized patients and staff in Italy, for the presence of *Salmonella*, *S. aureus*, *Listeria monocytogenes*, *Clostridium perfringens* and fecal contaminants. No pathogens were isolated.

MacGowan *et al.* (42) analyzed 822 shop-bought food specimens, 136 soil and 692 fecal specimens for *Listeria* spp. in a regular, year round survey, from June 1991 to May 1992, from 4 supermarkets in North Bristol, England. *Listeria* was not isolated in 8 fruit samples.

In 1994 Casolari *et al.* (12) reported in Italy that a *L. monocytogenes* strain of serogroup ½ was isolated from a sample of pickled olives eaten by a woman during the last months of her pregnancy.

In 1995 Doris and Seah (22) investigated a total of 606 food samples from a variety of sources over a 1.5 year period from January 1992 to June 1993 in Singapore. A total of 14 samples (2.3%) were found to contain *L. monocytogenes*. Of 4 fruit juice samples tested, none was positive for *Listeria monocytogenes*.

Gohil *et al.* (31) carried out a survey over a period of 6 months of 1,101 samples of retail food items, imported and local, in the United Arab Emirates, covering dairy products, fresh vegetables, fresh/frozen meat and poultry and a range of "ready-to-eat" meals. Fresh vegetables included sweet potatoes, bean sprouts, cabbages and tomatoes. No isolates of *L. monocytogenes* were found in any of the 183 vegetables samples tested, and only four positives for *L. innocua* were recorded.

Lin *et al.* (41) from Nov. 1995 to Jan. 1996 examined the occurrence of *L. monocytogenes*, *Salmonella* spp., *Escherichia coli* and *E. coli* O157:H7 in 63 vegetable salads served at 31 food service facilities in Gainesville, Florida. Eight were found to be contaminated with *E. coli*, one with *L. monocytogenes* and none with *Salmonella* or *E. coli* O157:H7. The vegetable salad from which *L. monocytogenes* was detected contained iceberg lettuce, red cabbage, carrots, cucumbers and tomatoes.

Sado *et al.* (60) conducted a microbiological survey of 50 retail juices in the Autumn of 1996 in the USA. These juices were analyzed for *L. monocytogenes*, *Escherichia coli* O157:H7, *Salmonella*, coliforms, fecal coliforms and pH. Two unpasteurized juices were positive for *L. monocytogenes*: an apple juice and an apple raspberry blend, with a pH of 3.78 and 3.75, respectively.

From May 1999 to February 2000 and from April 2000 to January 2001, a total of 890 samples of fresh produce, among them strawberries, were obtained from Norwegian markets and examined for the presence of thermotolerant coliform bacteria, *E.coli* O157, *Salmonella* spp., *L. monocytogenes*, *Staphylococcus* spp.. and *Yersinia enterocolitica*. Neither *Salmonella* spp. nor *E. coli* O 157 was isolated. *L. monocytogenes* was detected in one sample each of lettuce, mushrooms and strawberries (37).

Salmonella spp. outbreaks associated with fruits and juices

Salmonellae are by far the most frequently reported cause of food-borne illness in the United States (40). Some of the largest outbreaks have been traced to melons, which are common items in fresh salads (25).

In 1999 De Roever (21), mentioned fresh produce as an emerging and significant commodity group which can and does transmit pathogens. In Minnesota between 1990 and 1996 fresh produce was the first in the number of outbreaks (30%), meat or poultry (16%) being the second. Concerning etiologic agents associated with fresh fruit and vegetable outbreaks in Minnesota during the same period, the Norwalk like virus was the first in the number of outbreaks (54%), *Salmonella* spp. coming next with 16%, but considering the numbers of salad bars, *Salmonella* spp. accounts for 50% and Norwalk like virus 25%. Table 1 shows a summary of the following outbreaks.

In 1922 an outbreak of typhoid fever from the consumption of apple cider was described by Paquet (50).

The 1944 outbreak from orange juice occurred in a Cleveland hotel with 18 cases and one death reported between December 31 and January 28. An asymptomatic restaurant worker who prepared orange juice at the hotel was implicated in the outbreak (23).

In 1950 an outbreak occurred in Rochester (Minnesota) and involved six persons in two families who had consumed *Salmonella* Bareilly contaminated watermelon. In 1954, another *Salmonella* outbreak in Massachusetts involved 17 individuals in five families who consumed watermelon contaminated by *S.* Miami (30).

In 1961, an outbreak in England was associated with eating marshmallow cookies. The source of the *Salmonella* was desiccated coconut sprinkled on the cookies. Subsequent analysis of shipments of desiccated coconut from old Ceylon revealed that 479 (4.8%) of 9,265 samples were contaminated with *Salmonella* (62).

In 1974, a salmonellosis outbreak in New Jersey, USA, involving about 300 people having consumed apple cider is of interest because the use of animal manure as fertilizer was indicated as a possible source of the pathogen (18).

In 1979 in Illinois, USA, 18 individuals in seven families were stricken after eating precut watermelon contaminated with *S.* Oranienburg (17).

In 1989 a typhoid fever outbreak occurred as a result of orange juice consumption at a New York hotel restaurant. There were 43 confirmed and 24 probable cases of typhoid fever with 21 hospitalizations among hotel guests and employees. The outbreak most likely occurred due to contamination by an asymptomatic food handler during reconstitution of concentrated orange juice (6).

In January and February 1990 an outbreak spanned at least 30 states in the USA and involved an estimated 25,000 persons. Two deaths were reported. Imported cantaloupes from Mexico contaminated with *S.* Chester were implicated (55).

In 1990, an outbreak of *Salmonella* Javiana infection involving 176 cases in the USA was epidemiologically linked to the consumption of fresh tomatoes (76).

In 1991, more than 400 cases of *Salmonella* Poona infections in the USA and Canada were linked to the consumption of pre-sliced cantaloupe originated in Texas and/or Mexico (14).

Blostein (7) described a *Salmonella* Javiana outbreak among school children in Michigan as associated with the consumption of watermelon in 1991.

In 1993, 100 outbreak-associated cases of *Salmonella* Montevideo infections were identified in Illinois, Michigan, Minnesota, and Wisconsin, and tomatoes were again implicated as the likely vehicle (34).

In 1995 a salmonellosis outbreak occurred among individuals who consumed non-pasteurized orange juice from a Florida citrus processing facility. The causative agents of the disease isolated from the patients were *S.* Hartford, *S.* Gaminara, and *S.* Rubislaw (15).

In 1999 Mohle-Boetani *et al.* (45), described an outbreak of *Salmonella* Sapha in the USA. Twenty- four patients showed the onset of illness between 23

February and 15 May 1997. Cantaloupes from Mexico were the source of the outbreak.

In 1998, twenty cases of *Salmonella* Oranienburg in Ontario were traced to cantaloupe consumption (20).

A *Salmonella enterica* serotype Baildon multistate outbreak occurred between December 1998 and March 1, 1999, with 86 cases and 3 deaths in the USA, associated with eating raw, domestic tomatoes (13).

In 1999 a mustistate outbreak of *Salmonella* Newport occurred in the USA due to the consumption of imported mangoes with 78 infections (65).

In 1999, a *S.* Munchen outbreak in the USA and Canada was associated with commercially distributed unpasteurized orange juice traced to a single processor (16). Another outbreak in the same year due to unpasteurized orange juice occurred in Australia and was caused by *S.* Typhimurium (3).

A *Salmonella* Enteritidis outbreak affecting 14 people was linked to unpasteurized citrus juice products in Colorado, California and Nevada (11).

From April to May 2000 a *Salmonella* Poona outbreak occurred in the USA and Canada involving 43 cases, due to the consumption of cantaloupe (28).

Listeria spp. outbreaks associated with fruits and fruit juices

During September and October 1979, 23 patients admitted to hospitals in the Boston area had systemic *Listeria monocytogenes* infection. Thee foods were preferred by case patients more frequently than by control patients: tuna fish, chicken salad, and cheese. The only common feature appeared to be the serving of these foods with raw celery, tomatoes and lettuce. However, pasteurized milk could not be excluded as a vehicle of this outbreak (36).

Schlech (61) in 1996, mentioned that blueberries, strawberries and nectarines were implicated in outbreaks of listeriosis.

Growth and survival of *Salmonella* spp. and *Listeria* spp. in fruits and fruit juices

Currently, little is known about the survival and growth characteristics of *Salmonella* and *Listeria* in fresh produce and the sources and routes by which fruits and vegetables become contaminated (19,40).

Some reports related to *Salmonella* and *Listeria* growth and survival in fruits are mentioned in this review.

In 1975 Lee *et al.* (38) studied the growth of *Staphylococcus aureus*, *S.* Typhimurium and *Escherichia coli* and variation of pH in watermelon and muskmelon juice at 4 °C, room temperature (24-35 °C) and 37 °C for 4, 8, 14, 20, 24, 36 and 48 h. At 37 °C, the maximum cell numbers of *S. aureus*, *S.* Typhimurium and *E. coli* were attained after 14, 14 and 20 h. respectively in watermelon juice and after 14, 8 and 14 h in muskmelon juice, and at room temperature, after 24, 20 and 24 h in watermelon juice and 14, 14 and 20 h in muskmelon juice. The pH of watermelon (initial pH 6.21) and muskmelon (initial pH 6.10) juices fell, as the numbers of these 3 organisms increased. At 37 °C and room temperature respectively, the pH of the watermelon juice inoculated with *S.* Typhimurium was 4.71 and 4.70 respectively and the pH of muskmelon juice was 4.14 and 3.98 after 48 h incubation. For the 3 organisms, growth was better in watermelon juice than in muskmelon juice.

In 1979 Goverd *et al.* (33) revealed that salmonellae could survive in apple juice for 30 days at pH 3.68.

Abbey *et al.* (1) conducted experiments to determine the major genera and groups of microorganisms growing on unwrapped and wrapped slices of watermelon, stored at 5 and 25 °C for up to 8 days. Growth of bacterial isolates (*Pseudomonas* sp., *Escherichia coli* and *Staphylococcus aureus*) and *Crytococcus laurentii*, *Listeria monocytogenes* were evaluated in sterilized watermelon. *Pseudomonas* sp., *E. coli* and *L. monocytogenes* entered their logarithmic growth phase in watermelon juice within 28-32 h after inoculation. Only *Pseudomonas* sp.

grew in juice at 5 °C, reaching ca. 10^8 CFU/ml within 5 days. *L. monocytogenes* did not grow in juice incubated at 5 °C and viable cells were not detected after 21 days.

Escartin *et al.* (24) in 1989 found that *Salmonella* could grow on papaya cubes and suspensions of watermelon in distilled water held at temperatures of 22–27 °C.

Parish and Higgins (53) reported the survival of *Listeria monocytogenes* in sterile orange serum in which pH was adjusted from 3.6 to 5.0. Growth was observed prior to the reduction in viable cell numbers at pH 4.8 and 5.0 for storage temperatures of 4 °C and 30 °C.

Beuchat and Brackett (5) studied the rates of growth and death of *L. monocytogenes* inoculated onto raw whole tomatoes and into chopped tomatoes. Growth of the pathogen occurred on whole tomatoes held at 21 °C but not at 10 °C, while death occurred in chopped tomatoes stored at these temperatures.

Salmonella Enteritidis, *S.* Infantis, and *S.* Typhimurium were reported to be capable of growth in chopped cherry tomatoes (pH 3.99 to 4.37) at 22 and 30 °C (4).

Golden *et al.* (32) reported that a *Salmonella* mixture containing *S.* Anatum, *S.* Chester, *S.* Havana, *S.* Poona and *S.* Senftenberg could have rapid and profilic growth on cantaloupes, watermelons, honeydew melons and in TSB at 23 °C.

The survival on tomato of a rifampicin – resistant strain of *Salmonella* Montevideo, the alleged source of the 1993 multistate outbreak of salmonellosis, was affected by inoculum dose and inoculation site as well as by the medium used to deliver the bacterium (73). The survival of *Salmonella* Montevideo G 4639 on and in tomatoes during storage and the efficacy of chlorine treatment on inactivation of the pathogen were studied by Zhuang *et al.* (77) in 1995.

Parish *et al.* (54) in 1997 studied the survival of salmonellae in orange juice S. Gaminara, Hartford, Rubislaw and Typhimurium were inoculated into pasteurized orange juice adjusted to pH 3.5, 3.8, 4.1 and 4.4 and incubated at 0 and 4°C. Inoculated salmonellae survived in detectable numbers up to 27 days at pH 3.5, 46 days at pH 3.8, 60 days at pH 4.1, and 73 days at pH 4.4. Lag times

before initial cell populations began to decline were directly correlated with pH and ranged from < 1day at pH 3.5 up to 27 days at pH 4.4. Death rates were inversely correlated with pH.

Bradford *et al.* (10) reported the ability of two strains of *Salmonella* Enteritidis PT4 to cross-contaminate and survive on sterile and non-sterile foodstuffs (melon or beef). Neither strain of PT4 grew on melon stored at 4 °C for 24 h. At 20 °C growth was rapid with numbers of PT4 isolate E increasing from 10^4 per melon piece to approximately 10^6 per piece within 6 h.

Pao *et al.* (48) studied the survival and growth of *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* on peeled Hamlin orange, with an average pH of 6.0-6.5 at the surface and 3.8 in the juice. Growth was observed with all tested pathogens only at the abusive storage temperature of 24 °C. Refrigeration (4 or 8 °C) effectively inhibited the growth of all pathogens and caused a population reduction of *Salmonella* spp. and *S. aureus*.

In 1999 Roering *et al.* (56) compared the survival of thee-strain mixtures (ca. 10^7 CFU/ml each) of *S.* Typhimurium DT104, *L. monocytogenes* and *E. coli* O157:H7 in pasteurized and unpasteurized preservative-free apple cider (pH 3.3-3.5) during storage at 4 and 10 °C for up to 21 days. *S.* Typhimurium DT 104 populations decreased during 14 days storage at 4 and 10 °C in pasteurized and in unpasteurized cider. *L. monocytogenes* populations decreased below the plating detection limit (10 CFU/ml) within 2 days under all conditions tested.

Wells and Butterfield (75) in 1999, inoculated *Salmonella* Typhimurium into, tomato, potato and onion tissues. The populations of that bacterium increased by one to two logs over 48 h incubation at room temperature. Coinoculation of tissues with *S.* Typhimurium and *Botrytis* or *Rhyzopus* caused a statistically significant increase in populations of *Salmonella* as compared to controls.

Conway *et al.* (19) related that *Listeria monocytogenes* survived and its populations increased on cv Delicious apple slices at 10 or 20 °C in air or controlled atmosphere of 0.5% and 15% CO_2, but did not grow at 5 °C. The pathogen populations declined with time when grown in various concentrations of

apple juice, and the decline was greater as the concentration of the juice decreased.

Weissinger *et al.* (74) in 2000 reported that initial populations of *Salmonella* Baildon of 3.17 log CFU/g in tomatoes were reduced by 1.37 log CFU/g during storage for 12 days at 4 °C. The number of viable cells decreased during storage at 4 °C, initial populations of 3.17 log CFU/g of diced tomatoes were not reduced to undetectable levels during storage at 4°C for 12 days. The pathogen grew in diced tomatoes (pH 4.40 ±0.01) from an initial population of 0.79 log CFU/g to 5.32 and 7.00 log CFU/g within 24 h at 21 and 30 °C, respectively.

Liao and Sapers (40) reported the growth of *Salmonella* Chester on apple disks (pH 4.1) at 8 and 20 °C; results showed that *Salmonella* Chester failed to grow on apple disks at 8°C but grew well on the disks at 20 °C

Viswanathan (72) in 2001 also studied the growth patterns of organisms on vegetables (cucumbers and carrots) and fruits (watermelon and pineapple) at room temperature (32 °C) to assess the growth in the actual food environment. Cucumber and watermelon supported the growth of *S. aureus* and *Salmonella* Typhi, carrot retarded their growth while pineapple did not support the growth.

Leverentz *et al.* (39) reported that *Salmonella* Enteritidis populations can survive on fresh-cut melons stored at 5 °C, increase up to 2 log units at 10 °C and 5 log units at 20°C during a storage period of 168 h.

Ukuku and Sapers (68) in 2001 determined the ability of fresh–cut cantaloupe cubes directly inoculated with *Salmonella* Stanley and stored at different temperatures to support bacterial growth. In 2002 Ukuku and Fett (69) studied the behavior of *L. monocytogenes* inoculated onto cantaloupe surfaces and the efficacy of washing treatments to reduce the transfer from rind to fresh cut pieces. Direct inoculation of *L. monocytogenes* onto fresh cut pieces did not result in growth during 15 days of storage at 4 °C, but survived. Growth was evident by 4 h of storage at 8 and 20 °C.

Sharma *et al.* (63) in 2001 evaluated the effect of various calcium salt supplements on the survival of salmonellae in orange juice stored at 4 °C for up to

32 days, and determined if *Salmonella* Muenchen had unique survival characteristics in orange juice when compared with isolates of *Salmonella* originating from sources other than orange juices. Fortification of orange juice with thee of the four calcium salts affected the survival of *Salmonella* increasing or reducing the rate of inactivation.

In 2001 Medrano *et al.* (44) studied the behavior of *Salmonella* spp. in avocado pulp. At 22 °C *Salmonella* showed a generation time of 54 and 61 min. for low and high inocula, respectively. A population of 6.36 log CFU/g was reached after 18h of incubation. No growth of the pathogen was observed at temperature of refrigeration (4-7 °C).

RESUMO

Um aumento no consumo de produtos naturais, entre eles frutas e sucos de frutas, tem sido observado nos últimos anos principalmente devido a uma maior preocupação da população em relação a saúde e os benefícios decorrentes da alimentação natural. Nesta revisão bibliográfica, são analisadas a incidência, surtos, crescimento e sobrevivência de *Salmonella* spp. e *Listeria* spp. em frutas e sucos de frutas. Como estes produtos na sua maior parte, são consumidos sem nenhum tratamento térmico, exceção aos sucos pasteurizados e como *Listeria* spp. e *Salmonella* spp. podem sobreviver e crescer em polpas de frutas e também estar presentes na superfície de algumas delas, o manuseio adequado desde a colheita até o consumo deveria ser feito conforme as recomendações de boas práticas de higiene, procurando assim minimizar o impacto destes alimentos como veículos de doenças de origem alimentar.

Palavras-chaves: Incidência, crescimento, sobrevivência, frutas, *Listeria*, *Salmonella*

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Table 1. Outbreaks of *Salmonella* with fruits and fruit juices as vehicles.

Year	Disease vehicle	Causative microorganism	Reference
1922	Sweet cider	*S.* Typhi	50
1944	Orange juice	*S.* Typhi	23
1950	Watermelon	*S.* Bareilly	30
1954	Watermelon	*S.* Miami	30
1961	Desiccated coconut	*Salmonella*	62
1974	Apple cider	*S.* Typhimurium	18
1979	Watermelon	*S.* Oranienburg	17
1989-1990	Cantaloupe	*S.* Chester	55
1989	Orange juice	*S.* Typhi	6
1990	Tomatoes	*S.* Javiana	76
1991	Cantaloupe	*S.* Poona	14
1991	Watermelon	*S.* Javiana	7
1993	Tomatoes	*S.* Montevideo	34
1995	Orange juice	*S.* Hartford, *S.* Gaminara, *S.* Rubislaw	15
1997	Cantaloupe	*S.* Sapha	45
1998	Cantaloupe	*S.* Oranienburg	20
1999	Mangoes	*S.* Newport	65
1999	Tomatoes	*S.* Baildon	13
1999	Unpasteurized Orange Juice	*S.* Muenchen	16
1999	Unpasteurized Orange Juice	*S.* Typhimurium	3
2000	Unpasteurized Citrus Juice	*S.* Enteritidis	11
2000	Cantaloupe	*S.* Poona	28

CAPÍTULO II

Growth of *Salmonella* Enteritidis in Melon, Watermelon and Papaya Pulp Stored at Different Times and Temperatures

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Abstract

The ability of *Salmonella* Enteritidis to grow on melon (*Cucumis melo*), watermelon (*Citrullus vulgaris*) and papaya (*Carica papaya*) pulp stored at different times and temperatures was investigated. Fruit pulp portions with an average pH of 5.87, 5.50 and 4.87 for melon, watermelon and papaya, respectively, were obtained aseptically, homogenized, weighed and inoculated with suspensions (approximately 10^2 CFU/g) of *Salmonella* Enteritidis. Viable populations of *Salmonella* were determined by the pour plate technique using of test portions on TSA agar. The test organism increased in numbers at all tested temperatures. The generation times for melon at 10°C, 20°C and 30 °C were respectively 7.31, 1.69 and 0.69 h, for watermelon were 7.47, 1.60 and 0.51 h and for papaya 16.61, 1.74 and 0.66 h. The results showed that *Salmonella* Enteritidis can grow on low acid fruit pulp, and that refrigeration at 10 °C, although reducing the generation rate, does not inhibit its growth.

Keywords: *Salmonella* Enteritidis, low acid fruits, growth

1. Introduction

Preliminary FoodNet data on the incidence of food-borne illness show *Salmonella* at the top of the overall incidence in the United States (Vugia et al., 2002). Reflecting a worldwide trend in the United States, the proportion of *Salmonella* isolates that were *Salmonella* Enteritidis (SE) increased from 6% in 1980 to 25% in 1995 (Altekruse, Cohen, & Swerdlow, 1997).

The risk of acquiring a food borne disease has increased greatly. This is particularly important when the infective dose is low (Escartin, Ayala, & Lozano, 1989). Pathogen survival depends on many factors, including the physical and chemical characteristics of the fruit or vegetable, the post harvest processes applied and consumer handling practices (FDA/CFSAN, 1999).

Watermelon, melon and papaya are highly popular fruits in Brazil. These fruits are low acid with an average pH above 4.5, and often served sliced in food establishments in fresh pieces in mixes for salad bars, at deli counters and as a pulp juice. *Salmonella* spp. can survive and grow in these fruits. Golden, Rhodehamel, & Kautter (1993) reported that a *Salmonella* mixture containing *S.* Anatum, *Salmonella* Chester, *Salmonella* Havana, *Salmonella* Poona and *Salmonella* Senftenberg could grow rapidly on cantaloupes, watermelons, honeydew melons and in TSB at 23 °C. Escartin et al. (1989) found that *Salmonella* could grow on papaya cubes and watermelon suspensions in distilled water kept at temperatures of 22–27 °C. Viswanathan & Kaur (2001) reported the growth patterns of organisms on vegetables and fruits at room temperature (32 °C) to assess the growth in the actual food environment. Cucumber and watermelon supported the growth of *S. aureus* and *S.* Typhi.

According to the United States Centers for Disease Control and Prevention (CDC), the number of reported produce-associated food borne outbreaks per year has increased in the last few years in the USA and doubled between the periods

1973-1987 and 1988-1991 (Tauxe, Kruse, Hedberg, Potter, Madden, & Wachsmuth, 1997).

Several outbreaks of salmonellosis have been associated with the consumption of cut cantaloupe and watermelon. In 1991 a *Salmonella* Javiana outbreak among school children revealed a strong association with the consumption of watermelon (Blostein, 1993). During June and July 1991, more than 400 laboratory-confirmed infections with *Salmonella* Poona occurred in 23 USA states and in Canada, related to the consumption of cantaloupes (Francis et al., 1991). In 1997 an outbreak of *Salmonella* serogroup Saphra was related to cantaloupe from Mexico. Twenty–four consumers showed the onset of illness (Mohle-Boetani, et. al, 1999). Deeks, Ellis, Ciebin, Khakhria, Naus, & Hockin (1998) reported an *S.* Oranienburg outbreak in Canada due to the consumption of imported cantaloupes. In 2000 cantaloupe from Mexico was the food involved in a multistate *Salmonella* Poona outbreak in USA (FDA/CFSAN 2001).

This study was undertaken to examine the ability of *Salmonella* Enteritidis to grow in pulp of local varieties of low acid fruits such as melon, watermelon and papaya stored at temperatures of 10, 20 and 30 °C during different incubation periods.

2. Material and methods

2.1. Fruits.

Ripe, damage-free melons (*Cucumis melo* L. cv. "valenciano amarelo"), watermelons (*Citrullus vulgaris* Schard cv. Crimson Sweet) and papayas (*Carica papaya* L.cv. Sunrise Solo) were obtained from supermarkets in Campinas, State of São Paulo, Brazil.

2.2. Bacterial culture

A strain of *Salmonella* Enteritidis (SE), from the culture collection of the "Laboratório de Higiene e Legislação, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, SP", Brazil was used in this study. This strain was isolated from poultry. The culture was maintained in tryptone soya agar slants (TSA; Oxoid, Oxoid Ltd. Basingstoke, Hampshire, England) at 5 °C, after having its identity confirmed by biochemical and serological tests.

2.3. Inoculum preparation

The organism was cultured in TSA slants at 35 °C. The inocula were transferred to TSA at three consecutive 24 h intervals immediately before their use in the experiment. Cells were collected from TSA and transferred to 5 ml saline solution (NaCl 0.85%) to adjust the suspension to a concentration of 2 x 10^8/ml according to the MacFarland turbidity scale and using the equipment Densimat (bioMerieux). The bacterial suspension was serially diluted (1:10) in 0.1% peptone water, and 1ml aliquots of each dilution were pour plated in TSA agar, followed by incubation at 35 °C for 24 h to determine the viable cell concentration. A dilution concentration of 10^4 CFU/ml was used to inoculate the fruit pulp.

2.4. Sample pulp preparation

After being washed and scrubbed the external surfaces of the fruit were cotton scrubbed with an alcoholic solution of iodine (2%) (FDA, Bacteriological Analytical Manual 1995), and allowed to air dry inside a laminar airflow cabinet (VLFS-12, VECO). Defined areas (100 cm^2) of the fruit skin, and the inner fruit pulp

without seeds, were aseptically removed with sterilized spoons and the fruit portions transferred to a sterilized shaker. After mixing, 50g portions of the pulp were carefully removed with a spoon and transferred to sterilized Erlenmeyers flasks (250 ml). Before the inoculation tests, the pulp was checked for sterility and then frozen. When analyzing papaya pulp, it was necessary to pasteurize at 80 °C for 1min to eliminate the background microflora, although this treatment was not necessary when melon and watermelon pulp was analyzed. Due to the viscosity of papaya, sterilized magnets were placed inside the Erlenmeyers to allow for proper homogenization in a vortex mixer (FANEM, model 251) at the sampling time.

2.5. Pulp inoculation and enumeration of *Salmonella* Enteritidis

Triplicate test portions (50g) of homogenized pulp were inoculated with 1 ml (10^4 CFU) suspensions of *Salmonella* Enteritidis and incubated for 0, 24, 48, 72, 96, 120 and 144 h at 10 °C; 0, 12, 18, 24, 36, 42 and 48 h at 20 °C and 0, 2, 4, 6, 8, 10, 12 and 24 h, at 30 °C. At each sampling time 1 ml of fruit pulp was collected, serially diluted (1:10) in peptone water (0.1%) and pour plate dispersed in TSA (45 °C). The plates were incubated at 35 °C for 24 h followed by counting, using a colony counter (Phoenix, CP 600), with the results being expressed in CFU/g. Uninoculated pulp controls were carried out to assure the absence of any background micro flora before and after the incubation time.

2.6. Generation time

The generation time (g) was calculated from the slope of the line obtained in the semi logarithmic plot of exponential growth of the mean of three repetitions for each evaluated pulp fruit incubated at different temperatures and times in this

study. The following equation was used g = 0.0301/slope as described by Madigan, Martinko & Parker (1997).

2.7. Chemical and physical-chemical analyses.

The pH values of the uninoculated fruit pulp were determined using a calibrated pH meter (model B374, Micronal). The pH was not monitored during the incubation period.

Brix was determined using a Carl Zeiss (Jena) refratometer, model 32-G 110d.

Titratable acidity was determined using the method described in section 37.1.37 of the AOAC (1997).

Sugars (Total and Reducing) were analyzed as described by Lara et al. (1976).

3. Statistical analyses

Data (mean, standard deviation, R^2 and slopes of the lines) from each experiment, repeated three times for each fruit evaluated at the different temperatures and incubation periods were analyzed by the SAS (Statistical Analyses System, Institute, Cary, N.C., USA, Version 8.0, 2000).

4. Results and discussion

Table 1 shows the results of pH, Brix, titratable acidity and sugars (total and reducing) of the different fruit pulp.

The physical and chemical results show that fruit pulp is an adequate substrate for *Salmonella* Enteritidis to grow in, at least concerning their carbohydrate contents. In addition, the average pH cannot be considered as inhibitory for Enterobacteriaceae, especially in melon and watermelon, while papaya pulp, being even more acidic, is typically a low acid fruit (pH >4.6). So the composition of the analyzed fruit pulp is not a barrier to *Salmonella* growth.

The procedure applied for the aseptic removal of the fruit pulp, was adequate. All the analyses of uninoculated samples performed initially and during the incubation period, revealed the absence of salmonellae or other endogenous micro flora in the internal tissues of the fruits.

The results shown in Fig. 1, 2 and 3 confirm the adequacy of the fruit pulp as a substrate for *Salmonella* Enteritidis growth at different temperatures and incubation times.

It can be noticed that at 10 °C there was a lag time of around 24 h for melon and watermelon, before a more intensive growth was observed with lower growth in papaya pulp (pH 4.87) when compared to melon and watermelon where *Salmonella* Enteritidis showed a very similar growth rate. At 20 °C there was not a defined lag time and at 30 °C a lag time of about 2 h for all analyzed fruit pulp. As the temperature increased the growth rate was higher, showing that the main barrier to *Salmonella* Enteritidis growth was the incubation temperature and not the inoculated substrate. Maximum populations of between 10^8-10^9 CFU/g were reached for melon and watermelon pulp after different incubation periods according to the temperature as shown in Figs. 1, 2 and 3. These results confirm that all the analyzed samples of fruit pulp were good substrates for *S.* Enteritidis growth.

Based on these experiments it was possible to calculate the average generation time (g) of *Salmonella* Enteritidis in the different fruit pulp incubated at different temperatures, as shown in Table 2.

The results show that *S.* Enteritidis growth is rapid at 20 °C and 30 °C, while at 10°C it is lower when compared to the other temperatures, although it is important to notice that growth is still observed at this temperature. Francis, Thomas, & O'Beirne (1999) described salmonellae as typical mesophiles, with optimum temperatures for growth between 35-43 °C, the growth rate being substantially reduced at < 15 °C, and prevented at < 7 °C.

Escartin *et al.* (1989) investigated the ability of five strains of enteropathogenic bacteria (*Shigella sonnei, S. flexneri, S. dysenteriae, Salmonella* Derby and *S.* Typhi) to survive and grow on sliced jicama, papaya and watermelon. Suspensions of watermelon in sterile distilled water (up to 20%wt/vol) supported the growth of *S.* Typhi, with a generation time of 1.36 h and 1.32 h at 22 °C for watermelon and papaya suspensions respectively (Snyder, 1999). Golden *et al.* (1993) also reported the ability of *Salmonella* ssp. to grow on the inside of cantaloupe, watermelon and honeydew melons. The fruits were inoculated with a pool of 5 species of *Salmonella* (*S.* Anatum, *S.* Chester, *S.* Havana, *S.* Poona and *S.* Senftenberg). The generation time detected at 23 °C was 1.2 h, 1.1 h and 1.0 h for cantaloupe, honeydew and watermelon respectively (Snyder, 1999). Leverentz *et al.* (2001) found that *Salmonella* Enteritidis populations could survive on fresh-cut melons (honeydew, pH 5.8) stored at 5 °C, increased up to 2 log units on fresh cut fruits stored at 10 °C, and up to 5 log units at 20 °C during a storage period of 168 h.

As shown in Fig. 1 *Salmonella* populations on melon and watermelon pulp increased more than 5 log units after 168 h at 10 °C. However, on papaya pulp the increase was only 1.8 log units during the same period and temperature, probably due to the lower pH. Ukuku & Sapers (2001) reported that *Salmonella* Stanley populations remained unchanged throughout the storage period, when the initial inoculum on the fresh-cut cantaloupe cubes stored at 4 or 8 °C was 10^2 CFU/g; a 0.5-log CFU/g increase in populations was observed for an initial inoculum of 10^3

CFU/g in samples stored at 8 °C for 6 h but remained unchanged thereafter. Leverentz et al. (2001), observed an increase of about 3 log units on honeydew melon cut slices inoculated with *S.* Enteritidis at 10 °C during a 168 h incubation.

At 20 °C the three analyzed fruits presented similar results, with up to 5 log units increase after 42 h incubation (Fig. 2). At 30 °C the final *Salmonella* Enteritidis population in melon, and papaya pulp showed an increase of up to 4 log units after 12 h incubation, while in watermelon pulp under the same conditions the counts were approximately 1 log unit higher than those observed for melon and papaya (Fig. 3). Golden et al. (1993), reported that final *Salmonella* populations in watermelons were approximately 1.0 log greater than those observed on cantaloupe and honeydew, with approximately 5 to 6 log units increases in populations being noticed after a 24 h storage period at 23 °C. Ukuku et al., (2001) reported that *Salmonella* Stanley growth in fresh-cut cantaloupe melons stored at 20 or 30 °C was evident after 6 h incubation and reached 4 to 6 log CFU/g (2.0 to 4.0-log CFU/g increase) when the initial inoculum on the fresh fruits was 10^2 CFU/g. When the initial inoculum was 10^3 CFU/g and the samples were stored at 20 or 30 °C, growth was evident after 4 h, and increased by 3.8 or 5.2 log CFU/g at the end of the storage (10 h) period, respectively.

5. Conclusions

The results confirmed that low acid fruits such as melon, watermelon and papaya are good substrates for the survival and growth of *Salmonella* Enteritidis, and that low temperature (10 °C) retards but do not stop the growth of this bacterium. Considering that these fruits are usually served sliced or as a pulp juice, are highly manipulated and remains exposed for hours on restaurant tables, normally at room temperature, they can be considered as a potential risk as vehicles for food-borne diseases.

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Key to Figure 1:

Time	Fruits/pulp		
	Melon	Watermelon	Papaya
0	1.93 ±0.04	2.27 ±0.06	2.58 ±0.03
24	2.35 ±0.14	2.42 ±0.08	2.65 ±0.07
48	3.34 ±0.16	3.25 ±0.20	2.52 ±0.06
72	4.34 ±0.20	4.07 ±0.12	2.61 ±0.06
96	5.18 ±0.28	5.03 ±0.12	3.20 ±0.15
120	6.46 ±0.28	5.76 ±0.39	3.50 ±0.11
144	7.37 ±0.23	7.18 ±0.16	4.07 ±0.18
168	8.18 ±0.62	8.02 ±0.02	4.46 ±0.09

[a] Results expressed as a mean and standard deviation (S.D.) of three repetitions of the experiments for each evaluated fruit pulp at 10 °C.

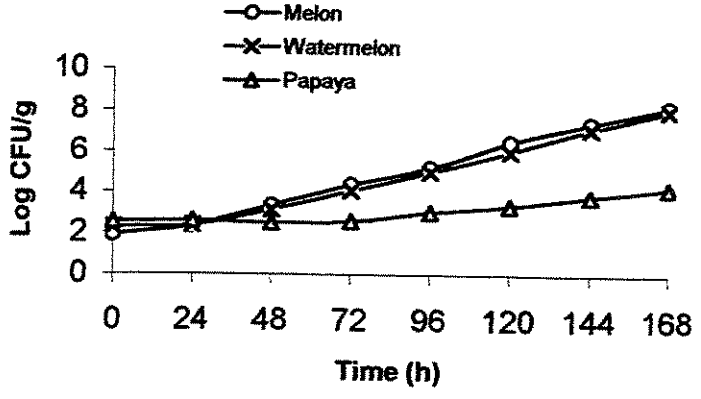

Figure 1. Growth of *Salmonella* Enteritidis (log CFU/g ± S.D.) in melon, watermelon and papaya pulp at 10 °C [a]

Key to Figure 2:

Time	Fruits/pulp		
	Melon	Watermelon	Papaya
0	2.47 ±0.34	2.33 ±0.15	2.42 ±0.01
12	3.63 ±1.03	3.02 ±0.10	3.39 ±0.04
18	4.54 ±1.18	4.40 ±0.18	4.58 ±0.03
24	5.93 ±1.60	5.79 ±0.08	5.80 ±0.14
36	8.24 ±1.40	7.56 ±0.23	7.92 ±0.12
42	8.71 ±0.57	7.77 ±0.05	8.47 ±0.10
48	8.92 ±0.21	7.97 ±0.21	8.68 ±0.19

[a] Results expressed as a mean and standard deviation (S.D.) of three repetitions of the experiments for each evaluated fruit pulp at 20 °C.

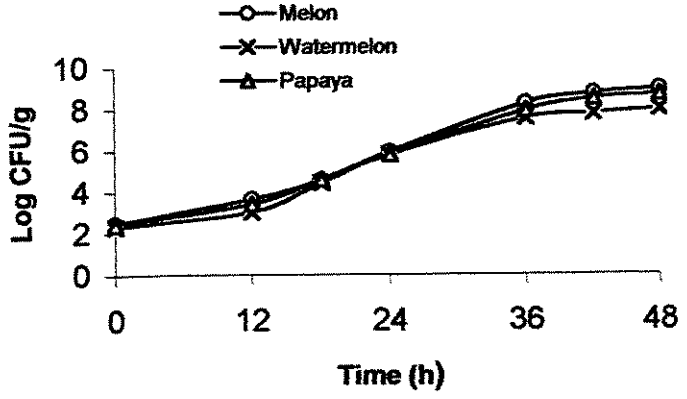

Figure 2. Growth of *Salmonella* Enteritidis (log CFU/g ± S.D.) in melon, watermelon and papaya pulp at 20 °C [a]

Key to Figure 3:

Time	Fruits/pulp		
	Melon	Watermelon	Papaya
0	2.36 ±0.18	2.56 ±0.05	2.54 ±0.29
2	2.58 ±0.22	2.64 ±0.12	2.57 ±0.09
4	3.28 ±0.24	3.30 ±0.04	3.24 ±0.06
6	3.88 ±0.84	4.47 ±0.09	4.10 ±0.16
8	4.57 ±1.35	5.62 ±0.04	4.90 ±0.11
10	5.71 ±1.30	6.94 ±0.19	5.92 ±0.16
12	6.74 ±1.67	7.93 ±0.16	6.90 ±0.18
24	8.90 ±0.20	7.89 ±0.11	8.81 ±0.06

[a] Results expressed as a mean and standard deviation (S.D.) of three repetitions of the experiments for each evaluated fruit pulp at 30 °C.

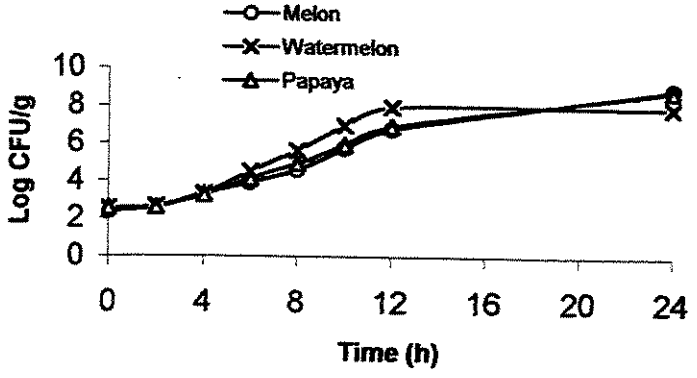

Figure 3. Growth of *Salmonella* Enteritidis (log CFU/g ± S.D.) in melon, watermelon and papaya pulp at 30 °C. [a]

Table 1. Chemical and physical-chemical analyses of melon, watermelon and papaya pulp.

	Fruit Pulp		
	Melon	**Watermelon**	**Papaya**
pH	5.87 ±0.13[a]	5.50 ±0.06	4.87 ±0.01
Brix (°B)	10.25 ±2.33	11.25 ±0.35	13.50 ±0.87
Acidity (%)	1.99 ±0.27	1.55 ±0.10	1.83 ±0.58
Total sugars (%)	7.76 ±1.40	8.20 ±0.56	10.60 ±0.45
Reducing sugar (%)	4.63 ±0.51	4.62 ±0.32	10.43 ±0.43

[a] Mean of three repetitions of each experiment and standard deviation (S.D.).

Table 2. Generation times, in hours, for *Salmonella* Enteritidis in melon, watermelon and papaya pulp stored at different temperatures.

Fruit pulp	Storage Temperature (° C)		
	10	**20**	**30**
Melon	7.31[a] (0.98)[b]	1.69 (0.79)	0.69 (0.67)
Watermelon	7.47 (0.98)	1.60 (0.98)	0.51 (0.99)
Papaya	16.61 (0.95)	1.74 (0.99)	0.66 (0.99)

[a] generation time

[b] (R^2)

CAPÍTULO III

Growth of *Listeria monocytogenes* in Melon, Watermelon and Papaya Pulp Stored at Different Times and Temperatures

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Abstract

Growth of *Listeria monocytogenes* in low acid fruits melon (*Cucumis melo*), watermelon (*Citrullus vulgaris*) and papaya (*Carica papaya*) at different times of incubation and at temperatures of 10 °C, 20 °C and 30 °C was studied. Fruit pulp portions with an average pH of 5.87, 5.50 and 4.87 for melon, watermelon and papaya, respectively, were obtained aseptically, homogenized, weighed and inoculated with suspensions (approximately 10^2 CFU/g) of *Listeria monocytogenes.* Generation times of 7.12, 13.03 and 15.05 h at 10 °C, 1.74, 2.17 and 6.42 h at 20 °C and 0.84, 1.00 and 1.16 h at 30 °C were obtained, respectively, for melon, watermelon and papaya. The results showed that *L. monocytogenes* could grow at all the temperatures analyzed and the microorganism showed diminished growth rate but was not inhibited at 10 °C.

Keywords: *Listeria monocytogenes*, Growth, Low acid fruits

1. Introduction

Microorganisms represent two important problems for the vegetable and fruit industries: the spoilage or reduction of shelf life and human health problems, due to the presence and/or growth of microbial pathogens that result in cases or outbreaks of food-borne diseases (Doyle, 1990).

Listeria monocytogenes is of special concern because it can grow at refrigeration temperatures, persists as an environmental contaminant in the processing environment, and has the potential to cause mortalities associated with outbreaks (Fain, 1996).

Scientific data related to *Listeria monocytogenes* in raw fruits are extremely limited. As of January 1997, Brackett mentioned that only two studies dealing with the viability of listeriae in orange serum and juice have appeared in the scientific literature (Brackett, 1999).

Abbey et al., (1988) reported the growth behaviour of selected bacterial isolates from watermelon and a laboratory stock culture of *Listeria monocytogenes* in watermelon stored at 5 and 25 °C.

Parish and Higgins (1989) reported the survival of *Listeria monocytogenes* in sterile orange serum in which the pH was adjusted from 3.6 to 5.0. Growth was observed prior to the reduction in viable cell numbers at pH 4.8 and 5.0, with storage temperatures of 4 °C and 30 °C.

Beuchat and Brackett (1991) studied the rates of growth and death of *L. monocytogenes* inoculated onto whole raw tomatoes and into chopped tomatoes. Growth of the pathogen occurred on whole tomatoes held at 21 °C but not at 10 °C, while death occurred in chopped tomatoes stored at these temperatures.

Pao et al. (1998) studied the survival and growth of *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* on peeled Hamlin orange, with an average pH of 6.0-6.5 at the surface and 3.8 in the

juice. Growth was only observed with all tested pathogens at the abusive storage temperature (24 °C). Refrigeration (4 or 8 °C) effectively inhibited the growth of all pathogens and caused a population reduction of *Salmonella* spp. and *S. aureus*.

Conway et al. (2000) reported the growth of *Listeria monocytogenes* in apple slices at 10 or 20 °C. The pathogen populations declined with time when grown in various concentrations of apple juice and the decline was greater as the concentration of the juice decreased.

Ukuku and Fett (2002) studied the behaviour of *L. monocytogenes* inoculated onto cantaloupe surfaces and the efficiency of washing treatments to reduce transfer from the rind to fresh cut pieces. Direct inoculation of *Listeria* onto fresh cut pieces showed survival but not growth of the microorganism during 15 days of storage at 4°C. Growth was evident after 4 h of storage at 8 and 20 °C.

In this paper we studied the growth of *L. monocytogenes* in low acid fruits highly consumed in Brazil (melon, watermelon and papaya) at different temperatures and incubation times. The generation time was calculated in order to contribute to a better knowledge of the behavior of the microorganism studied, in fruit pulp.

2. Material and methods

2.1. Fruits.

Ripe damage-free melons (*Cucumis melo* L. cv. "valenciano amarelo"), watermelons (*Citrullus vulgaris* Schard cv. Crimson Sweet) and papayas (*Carica papaya* L.cv. Sunrise Solo) were obtained from supermarkets in Campinas, State of São Paulo, Brazil.

2.2. Bacterial culture

A strain of *Listeria monocytogenes* Scott A (serotype 4b) from the culture collection of the "Laboratório de Higiene e Legislação, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, SP", Brazil was used in this study. The culture was maintained in tryptone soya agar slants with 0.6% yeast extract (TSAYE); (TSA, Oxoid Ltd. Basingstoke, Hampshire, England; YE, Difco) at 5 °C, after having its identity confirmed by biochemical and serological tests.

2.3. Inoculum preparation

The organism was cultured in TSA-YE slants at 35 °C. Inocula were transferred to TSA-YE at three consecutive 24 h intervals immediately before their use in the experiment. Cells were collected from TSA-YE and transferred to 5 ml saline solution (NaCl 0.85%) to adjust the suspension to a concentration of 2 x 10^8/ml according to the MacFarland turbidity scale and using the equipment Densimat (bioMerieux). The bacterial suspension was serially diluted (1:10) in 0.1% peptone water, and 1 ml aliquots of each dilution were pour plated in TSA agar, followed by incubation at 35 °C for 24 h to determine the viable cell concentration. Dilutions of 10^4 CFU/ml were used to inoculate the fruit pulp.

2.4. Sample pulp preparation

After being washed and scrubbed the external surfaces of the fruit were cotton scrubbed with an alcoholic solution of iodine (2%) (FDA, Bacteriological Analytical Manual, 1995), and allowed to air dry inside a laminar airflow cabinet (VLFS-12, VECO). Defined areas (100 cm^2) of the fruit skin and the inner fruit pulp, without seeds, were aseptically removed with sterilized spoons and the fruit

portions transferred to a sterilized shaker. After mixing, 50g pulp portions were carefully spooned from the shaker and transferred to sterilized Erlenmeyers flasks (250 ml). Before the inoculation tests the pulp was checked for sterility and frozen. When analyzing papaya pulp a pasteurization treatment at 80 °C for 1min was necessary in order to eliminate the background microflora while this treatment was not necessary when melon and watermelon pulp were analyzed. Due to the viscosity of papaya, sterilized magnets were placed inside the Erlenmeyers to allow for proper homogenization in a vortex mixer (FANEM, model 251) at the sampling time.

2.5. Pulp inoculation and enumeration of *Listeria monocytogenes*

Triplicate test portions (50g) of homogenized pulp were inoculated with 1 ml (10^4 CFU) suspensions of *Listeria monocytogenes* and incubated for 0, 24, 48, 72, 96, 120, 144 and 168h at 10 °C; 0, 12, 18, 24, 36, 42 and 48 h at 20 °C and 0, 2, 4, 6, 8, 10, 12 and 24 h, at 30 °C. At each sampling time, 1ml of fruit pulp was collected, serially diluted (1:10) in peptone water (0.1%) and pour plate dispersed in TSA-YE (45 °C). The plates were incubated at 35 °C for 24 h followed by counting, using a colony counter (Phoenix, CP 600), with the results being expressed in CFU/g. Uninoculated pulp controls were also analyzed to ensure the absence of any background microflora before and after the incubation time.

2.6. Generation time

The generation time (g) was calculated from the slope of the line obtained in the semi logarithmic plot of exponential growth of the mean of three repetitions for each evaluated pulp fruit incubated at different temperatures and times in this

study. The following equation was used g = 0.0301/slope as described by Madigan, Martinko & Parker (1997).

2.7. Chemical and physical-chemical analyses

The pH values of the fruit pulp were determined using a calibrated pH meter (model B374, Micronal). The pH was not monitored during the incubation period.

Brix was determined using a Carl Zeiss (Jena) refratometer model 32-G 110d

Titratable acidity was determined using the method described in section 37.1.37 of the AOAC (1997).

Sugars (Total and Reducing) were analyzed as described by Lara et al., 1976.

3. Statistical analyses

Mean, standard deviation and R^2 from each experiment, repeated three times for each fruit evaluated at the different temperatures and incubation periods, were subject to SAS (Statistical Analyses System, Institute, Cary, N.C., USA, Version 8.0, 2000).

4. Results and discussion

Table 1 shows the results for pH, Brix, titratable acidity and sugars (total and reducing) of the different fruit pulp.

The physical and chemical results show that fruit pulp is an adequate substrate for *Listeria monocytogenes* to grow in, at least concerning their

carbohydrate contents. In addition, the average pH cannot be considered inhibitory for *L. monocytogenes*, especially in melon and watermelon, while papaya pulp, being even more acidic, is typically a low acid fruit (pH >4.6). So the fruit pulp composition of the analyzed fruits is not a barrier to *Listeria* growth

The procedure applied for the aseptic removal of fruit pulp was adequate. All the analyses of uninoculated samples performed initially and during the incubation period revealed the absence of *Listeria* or other endogenous microflora in the internal tissues of the fruits. Samish et al. (1963) mentioned that in healthy fruits the bacterial flora is assumed to be limited to the surface, while the inner tissue remains sterile.

The results shown in Figs. 1, 2 and 3 confirm the adequacy of fruit pulp as a substrate for *Listeria monocytogenes* growth at different temperatures and incubation times. Nguyen and Carlin (1994) mentioned that minimally processed fresh fruits and vegetables are good media for the growth of microorganisms.

There was a lag time of about 24 h, 6 h and 4 h at 10 °C, 20 °C and 30 °C respectively for the growth of *L. monocytogenes* in melon (Figs. 1, 2 and 3 respectively). The results confirm that low temperature is not a barrier to *Listeria* growth; it can retard the growth but cannot inhibit it. Maximum populations of about 10^9 CFU/g were reached after the end of all the incubation times studied in melon pulp.

Ukuku et al., (2002) reported the growth and survival of *L. monocytogenes* on fresh cut pieces of cantaloupe. Growth was evident at higher storage temperatures, a lag time of 4 h and 6 h being observed for fresh cut pieces stored at 20 °C and 8 °C respectively. The populations in fresh cut pieces stored at 8 or 20 °C increased by 1 log unit up to the end of storage.

When analyzing the growth of *Listeria* in watermelon, lag times of about 24, 12 and 4 h and maximum populations of 10^6, 10^7 and 10^9 CFU/g at 10 °C, 20 °C and 30 °C respectively, were observed (Figs. 1, 2 and 3 respectively)..

Abbey et al., (1988) conducted experiments to determine the major genera and groups of microorganisms which grew on unwrapped and wrapped slices of

watermelon stored at 5 and 25 °C for up to 8 days. *L. monocytogenes* entered the logarithmic growth phase in watermelon juice within 18 h after inoculation and did not grow in juice incubated at 5 °C. Viable cells were not detected after 21 days.

For papaya, *Listeria* presented a lag time similar to that presented for watermelon. The maximum populations of about 5, 4 and 7 log units were reached at temperatures of 10 °C, 20 °C and 30 °C at the end of the incubation period (Figs. 1, 2 and 3 respectively). Growth of *Listeria* in papaya was lower when compared to the other pulp studied, which may be due to the fact that the pH of papaya is 4.87, lower than the values for melon and watermelon.

Table 2 shows the generation times for *Listeria* for the fruit pulp studied. The generation time, calculated using the data provided by growth curves analyzed, decreased as the temperature increased, which is consistent with the literature, since the optimum temperature growth for *Listeria* is 30-35 °C.

5. Conclusion

The results confirmed that papaya, melon and watermelon pulp were good substrates for *Listeria monocytogenes* growth. In Brazil, these fruits are frequently manipulated and served in slices in restaurants, hotels, at home, alone or mixed with other foods, so people must be careful when manipulating and storing these low acid fruits, since although refrigeration temperatures reducing *L. monocytogenes* rate growth they did not inhibit it.

Acknowledgements

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Key to Figure 1:

Time	Fruits/pulp		
	Melon	Watermelon	Papaya
0	2.56 ±0.15	2.44 ±0.05	2.45 ±0.05
24	3.00 ±0.20	2.80 ±0.12	2.82 ±0.05
48	3.96 ±0.19	3.16 ±0.19	2.84 ±0.03
72	5.05 ±0.35	3.73 ±0.37	3.10 ±0.14
96	6.07 ±0.49	4.23 ±0.82	3.37 ±0.09
120	7.16 ±0.84	4.96 ±0.84	3.84 ±0.04
144	7.99 ±0.58	5.51 ±1.10	4.24 ±0.01
168	8.87 ±0.29	6.10 ±1.43	4.84 ±0.17

[a] Results expressed as a mean and standard deviation (S.D.) of three repetitions of the experiments for each evaluated fruit pulp at 10°C.

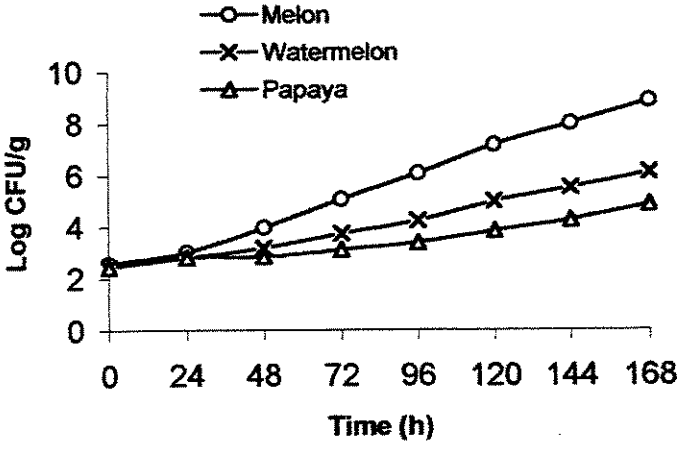

Figure 1. Growth of *L. monocytogenes* (log CFU/g ± S.D.) in melon, watermelon and papaya pulp at 10 °C. [a]

Key to Figure 2:

Time	Fruits/pulp		
	Melon	Watermelon	Papaya
0	2.73 ±0.07	2.45 ±0.03	2.59 ±0.13
12	4.15 ±0.14	2.54 ±0.09	2.59 ±0.08
18	5.21 ±0.16	3.26 ±0.08	2.83 ±0.05
24	6.37 ±0.31	4.11 ±0.20	3.23 ±0.13
36	8.30 ±0.26	5.87 ±0.03	3.67 ±0.04
42	8.97 ±0.19	6.77 ±0.01	3.95 ±0.12
48	9.24 ±0.03	7.30 ±0.03	4.43 ±0.16

[a] Results expressed as a mean and standard deviation (S.D.) of three repetitions of the experiments for each evaluated fruit pulp at 20 °C.

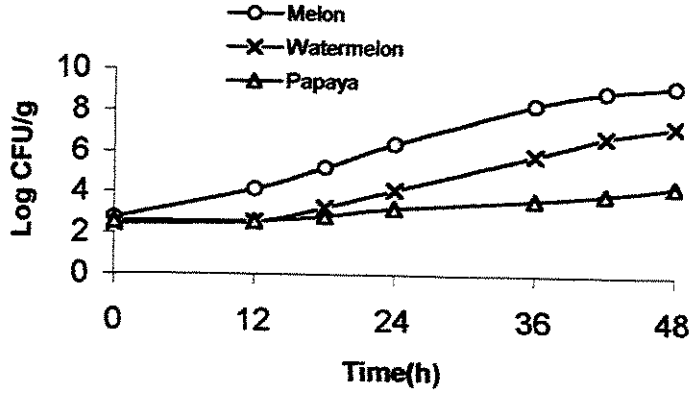

Figure 2. Growth of *L. monocytogenes* (log CFU/g ± S.D.) in melon, watermelon and papaya pulp at 20°C. [a]

Key to Figure 3:

Time	Fruits/pulp		
	Melon	Watermelon	Papaya
0	2.44 ±0.14	2.59 ±0.04	2.61 ±0.02
2	2.65 ±0.12	2.71 ±0.05	2.66 ±0.09
4	3.08 ±0.15	3.04 ±0.07	2.74 ±0.09
6	3.76 ±0.20	3.58 ±0.18	2.90 ±0.09
8	4.49 ±0.26	4.25 ±0.08	3.24 ±0.04
10	5.30 ±0.31	4.80 ±0.14	3.73 ±0.16
12	5.88 ±0.34	5.52 ±0.06	4.13 ±0.07
24	9.18 ±0.28	9.01 ±0.14	7.36 ±0.08

[a] Results expressed as a mean and standard deviation (S.D.) of three repetitions of the experiments for each evaluated fruit pulp at 30 °C.

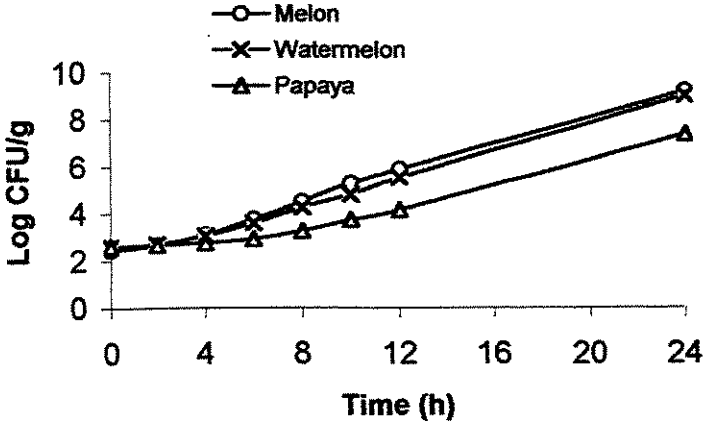

Figure 3. Growth of *L. monocytogenes* (log CFU/g ± S.D) in melon, watermelon and papaya pulp at 30 °C. [a]

Table 1. Chemical and physical-chemical analyses of melon, watermelon and papaya pulp.

	Fruit Pulp		
	Melon	**Watermelon**	**Papaya**
pH	5.87 ±0.13[a]	5.50 ±0.06	4.87 ±0.01
Brix (°B)	10.25 ±2.33	11.25 ±0.35	13.50 ±0.87
Acidity (%)	1.99 ±0.27	1.55 ±0.10	1.83 ±0.58
Total sugars (%)	7.76 ±1.40	8.20 ±0.56	10.60 ±0.45
Reducing sugar (%)	4.63 ±0.51	4.62 ±0.32	10.43 ±0.43

[a] Mean of three repetitions of each experiment and standard deviation (S.D.).

Table 2. Generation times, in hours, for *Listeria monocytogenes* in melon, watermelon and papaya pulp stored at different temperatures.

Fruit pulp	Storage Temperature (° C)		
	10	**20**	**30**
Melon	7.12[a] (0.95)[b]	1.74 (0.98)	0.84 (0.95)
Watermelon	13.03 (0.74)	2.17 (0.99)	1.00 (0.95)
Papaya	15.05 (0.98)	6.42 (0.92)	1.16 (0.99)

[a] Generation time

[b] (R^2)

CAPÍTULO IV

Incidence of *Listeria* spp. and *Salmonella* spp. on the Surface of Fresh Melons, Watermelons and Papayas, Using the TECRA Visual Immunoassay and Cultural Procedures for their Detection

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ABSTRACT

In this work, the incidence of *Salmonella* spp. and *Listeria* spp. on melon (*Cucumis melo*), watermelon (*Citrullus vulgaris*) and papaya (*Carica papaya*) surfaces from fruits collected in wholesale (CEASA) and street market in Campinas, São Paulo, Brazil, were evaluated. From the total of 120 fruit samples, 42 were simultaneously analyzed by the TECRA Visual Immunoassay (TECRA-VIA) method and the modified BAM for *Salmonella* and by the Health Protection Branch, Canada, and the TECRA-VIA for *Listeria*; the remaining 78 fruit samples were analyzed only by the cultural procedures. The results showed that *Salmonella* spp. was absent in all 42 samples analyzed by both methodologies, with one false positive by TECRA-VIA. However, *Listeria* spp. was detected in one sample (2.38%) of those analyzed, with 2 false positive and 3 false negative results using the TECRA-VIA method.

Salmonella spp. was also absent from 78 samples analyzed only by the modified BAM method. However, *Listeria* spp. was detected in 9 (7.50%) of the analyzed samples, with *L. innocua* and *L. grayii* being isolated from watermelons, *L. ivanovii* from papayas and *L. welshimeri* from melons, without any detection of *L. monocytogenes* when using the Health Protection Branch method. The samples

collected from the street market showed a higher frequency of *Listeria* spp. when compared with the ones collected wholesale.

Keywords: *Salmonella*, *Listeria*, Melon, Watermelon, Papaya, Incidence, Detection

1. INTRODUCTION

The potential for the microbial contamination of fruits and vegetables is high, due to the wide variety of conditions to which the produce is exposed during growth, harvest, and distribution (Madden, 1992). *Salmonella* spp. and *Listeria* spp. on the surface of melons and watermelons can contaminate the inner parts during cutting, and multiply or survive in the interior of these fruits (Gayler 1955; Ukuku 2001, 2002). In most outbreaks, it was assumed that *Salmonella* was present on the rind, presumably having contaminated the fruit in the field or during washing in a packinghouse, and that the edible surface became contaminated during the final preparation. Improper storage temperature combined with favorable conditions for growth on the surface of cut melons, were also factors that probably contributed to the outbreaks (FDA, 2001).

A study on the incidence of *Salmonella* spp. and *Listeria* spp. in fruits were reported by Al-Hindawi and Rished, 1979; Goverd *et al.*, 1979; Papadakis *et al.*, 1980; Rude *et al.*, 1984; Saddih *et al.*, 1985; Farber *et al.*, 1989; Heisick *et al.*, 1989; Tiwari and Aldenrath, 1990; Orsini *et al.*, 1992; MacGowan *et al.*, 1994 Monge *et al.*, 1995; Pao *et al.*, 1998. These authors did not find *Salmonella* in the samples analyzed. Meanwhile, incidence and presence of *Salmonella* spp. and *Listeria* spp. in fruits was mentioned by Ruiz *et al.*, 1987; Madden, 1992; Simon *et al.*, 1992; Vahidy *et al.*, 1992.; Casolari *et al.*, 1993; Lin *et al.*, 1996; Parish, 1998; FDA, 2001; Viswanathan and Kaur, 2001.

Research activities as well as epidemiological and regulatory efforts could be supported and accelerated by the use of more rapid, unequivocal and sensitive

pathogen detection methods when compared to the conventional culture-based detection methods (Shearer *et al.*, 2001).

The TECRA Salmonella and Listeria Visual Immunoassay are enzyme-linked immunosorbent assays performed in the sandwich configuration. High affinity "capture" antibodies specific for *Salmonella* spp. or *Listeria* are adsorbed onto the surface of removawell strips. If *Salmonella* or *Listeria* antigens are present in the added sample, they are captured by the antibodies. After incubation, the wells are washed and all other materials in the sample are washed away. The sandwich is completed by the addition of enzyme-labeled antibodies (conjugate) specific for *Salmonella* spp. or *Listeria* spp. Following a second incubation step the wells are washed and a specific substrate is added. The presence of salmonellae or *Listeria* is indicated when the bound conjugate converts the substrate to a green color (Knight *et al.*, 1996 and Hughes *et al.*, 1999).

A comparative study was undertaken of the TECRA Salmonella VIA and cultural procedures based on the Australian Standard methods for the detection of salmonellae in food. A total of 173 samples were examined; 75 foods artificially contaminated at high and low levels with 5 common serotypes of *Salmonella* spp. and 98 uninoculated foods and animal feeds, including 28 naturally contaminated samples. The comparative results between the methods were in close agreement. No false negative results were reported and the incidence of false positive reactions was low (6.8%), (Hughes, 1987).

The performance of the TECRA Salmonella Visual Immunoassay was compared to Australian standard cultural methods for the detection of *Salmonella* spp. in 572 food samples (446 processed and 126 unprocessed). Sixty *Salmonella* positive samples were detected by enzyme immunoassay and confirmed by culture, while cultural methods alone detected 59 positive samples (Jay and Comar, 1988).

Flowers *et al.* 1988 did a collaborative study with 13 laboratories in the USA in which the TECRA method was compared to the standard BAM/AOAC culture method for the detection of *Salmonella* in six food types using uninoculated and

inoculated samples. The study found no significant difference between the two methods at the 5% level.

Lambiri *et al.*, 1990 compared the TECRA Salmonella Immunoassay with a conventional culture method for the detection of *Salmonella* spp. in 41 naturally-contaminated foods and animal feed samples. The overall agreement between the two methods was 95%.

The efficiency of 2 commercial enzyme-linked immunosorbent assay (ELISA) kits [Listeria-Tek ™ and TECRA ™] for detecting *Listeria* in naturally contaminated foods was evaluated and compared with that of the culture method described in the Bacteriological Analytical Manual (BAM). Of the 178 food samples examined, the presence of *Listeria* was detected and culturally confirmed in 38, 37 and 40 samples by the BAM, Listeria –Tek and TECRA methods, respectively. Differences in the results of the ELISAs as compared with those of the BAM method were not statistically significant (Noah *et al.*, 1991).

Burnett and Beuchat 2001 mentioned that methods utilized for preparing raw fruits, vegetables and herbs for enrichment or direct plating to determine the presence and populations of pathogenic bacteria vary greatly. These authors compared three sample processing methods (washing in 0.1% peptone, stomaching, and homogenizing) for recovering *Salmonella* spp. inoculated onto 26 types of raw produce. They concluded that the influence of sample size, diluent composition, and processing time on efficiency of recovery of *Salmonella* spp. and other pathogens needed to be better evaluated before a method(s) for processing samples of raw produce could be recommended.

Based on these comments, the purpose of this study was to investigate the presence of *Listeria* spp. and *Salmonella* spp. on the surface of melons, watermelons and papayas using the TECRA Visual Immunoassay and cultural procedures for their detection.

2. MATERIAL and METHODS

2.1. Evaluation of the washing methodology for recovering *Salmonella* Enteritidis and *Listeria monocytogenes* inoculated onto the surface of papaya.

2.1.1. Cultures utilized and inocula preparation.

Salmonella Enteritidis (SE) and *Listeria monocytogenes* Scott A (serotype 4b) from the culture collection of the "Laboratório de Higiene, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, SP", Brazil were used in this study. *S.* Enteritidis was activated consecutively in tryptone soya agar (TSA, Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 35 °C. *Listeria monocytogenes* was activated consecutively in tryptone soy agar yeast extract (0.6%) supplemented (TSA-YE); (TSA, Oxoid Ltd. Basingstoke, Hampshire, England; yeast extract, Difco) and incubated at 30 °C. Separated microbial suspensions for each studied microorganism were prepared with standard turbidity (densimat bioMerieux), relating to counts in agar plates (TSA and TSA-YE plates incubated at 35 °C and 30 °C for 24 h, respectively). Cultures diluted to 10^{-7} corresponding to 50 – 300 CFU/ml were used for fruit surface inoculation.

2.1.2. Inoculation procedures

Previous washing of the papayas used as negative controls was performed, in order to certify a total lack of *Salmonella* spp. or *Listeria* spp. in these fruits. Nineteen damage-free papayas (*Carica papaya* L. cv. Sunrise Solo) were water - washed and allowed to air-dry in the laminar airflow cabinet (VLFS-12, VECO).

After completely dry, the fruits were put individually inside sterilized plastic bags and 100 ml of sterile saline peptone water was added. After careful massaging for 20 minutes, the washing solution was divided into two 50 ml portions and each portion added to double concentrated pre enrichment medium (Buffered Peptone Water-BPW, Oxoid Ltd, for *Salmonella* and *Listeria* Enrichment Broth Base- LEB, Oxoid Ltd, Basingstoke, Hampshire, England, for *Listeria*) and analyzed for the presence of *Salmonella* and *Listeria*. The same nineteen fruit samples previously used as negative controls were used for the inoculation studies, after being carefully withdrawn from the bags used for washing, and left to air-dry again in the laminar airflow cabinet. After completely dry, surface inoculation was conducted with an automatic pipette (Wheaton – Socorex) (0.1ml per drop) at a rate of 1 ml of inoculum per fruit, and left to dry again in the laminar airflow cabinet. After drying completely, the fruits were washed again inside sterile plastic bags, adding a further 100ml of sterile saline water followed by massaging for 15 minutes. After this period two 50 ml portions were again separated and added to the double concentrated pre-enrichment media, BPW for *Salmonella* spp. and LEB, for *Listeria* spp. (Figure 1).

2.1.2.1. Recovery of *Salmonella* Enteritidis from experimentally inoculated papayas.

The 50 ml portions of both the negative control and the inoculated samples, described in 2.1.2., were separately added to Erlenmeyer flasks containing 50 ml of double concentrated BPW, and incubated at 35 °C for 24 h. After this, 0.1 ml of the overnight culture in BPW was added to 10 ml of Rappaport –Vassiliadis (RV) broth (Oxoid) and incubated at 42.5 °C for 24 h in a water bath (FANEM, São Paulo-Brazil, Model 100). A 1 ml aliquot of the overnight culture was also added to 10 ml of tetrathionate broth base (TT, Difco Lab. Ltda.) and incubated at 37 °C for 24 h. Following the incubation, loopfuls of the RV and TT broths were streak-plated

on Brilliant Green agar (BG, Oxoid Ltd, Basingstoke, Hampshire, England) and Hektoen-Enteric agar (HE, Oxoid Ltd, Basingstoke, Hampshire, England) and incubated overnight at 37 °C. After the incubation period the plates were observed for typical *Salmonella* spp. colonies, and the suspected colonies were isolated, submitted to preliminary biochemical screening in Triple Sugar Iron agar-TSI (Difco, Becton Dickinson, USA) and Lysine Iron agar-LIA (Difco, Lab Ltda) and further biochemical tests according to Andrews *et al.* (1998) and also the use of the BBL Kit Crystal RS/E (Becton Dickinson, USA).

2.1.2.2. Recovery of *Listeria monocytogenes* from experimentally inoculated papayas.

The other 50ml portions of the negative control and the inoculated samples of the wash solution described in 2.1.2. were separately added to an Erlenmeyer flask containing 50 ml of LEB broth in double concentration followed by incubation at 30°C for 24 and 48 h. After this, a 0.1 ml aliquot of the overnight culture was added to 10ml of modified Fraser Broth and incubated at 35°C for 24/48 h. Loopfuls of the LEB after 48 h and modified Fraser Broth after 24 h if esculin-positive or 48 h even if esculin-negative were streaked on modified Oxford agar (MOX) and Lithium Chloride-Phenylethanol Moxalactam supplemented agar (LPM, Difco Laboratories Detroit MI, USA) with incubation at 35°C and 30°C for 48 and 24 h, respectively. Suspected colonies were isolated from the LPM and MOX agar plates and confirmed biochemically according to the procedures described by Farber *et al.* (1994), and using the API Listeria kit (BioMerieux).

2.2. Evaluation of the incidence of *Listeria* spp. and *Salmonella* spp. on the surface of fresh melon, watermelon and papaya fruits using the modified BAM and TECRA VIA for *Salmonella* spp. and the Health Protection Branch and TECRA VIA for *Listeria* spp. detection.

2.2.1. Sampling

Ripe, damage-free melons (*Cucumis melo* L. cv. "valenciano amarelo"), watermelons (*Citrullus vulgaris* Schard cv. Crimson Sweet) and papayas (*Carica papaya* L. cv. Sunrise Solo), were obtained from wholesale (CEASA) and street market in the city of Campinas, State of São Paulo, Brazil.

A total of 120 fruit samples were collected and analyzed during an 8 month period, with 5 repetitions at each sampling time and locality, with the collection of 4 fruit units (at each sampling time) that were packed individually in sterilized plastic bags and immediately shipped to the laboratory for analysis.

2.2.2. Experimental procedure

The surface wash methodology previously described in 2.1.2. was used in this study; however, due to the watermelon size and difficulty in washing it, these fruits were analyzed by the scrubbed surface technique, that was conducted using a sterile sponge (Sveum *et al.*, 1992), instead of the methodologies applied to melon and papaya. In this procedure, the sponge, moistened in sterile peptone water, was scrubbed over the surface, and then put into a sterile plastic bag containing 100 ml of peptone saline solution. After sponge massaging, 50 ml portions were split and added to 50ml of double concentrated BPW for *Salmonella* and LEB for *Listeria*.

From the total of 120 fruit samples, 42 were simultaneously analyzed by the modified BAM and TECRA VIA (Bioenterprises Pty. Ltd., Roseville NSW, Australia) methods for *Salmonella* and by the Health Protection Branch and TECRA VIA for *Listeria* and 78 fruits only by the cultural procedures methods for both microorganisms (Figure 2). The methodologies for isolation of *Salmonella* spp. and *Listeria* spp. were previously described in 2.1.2.1. and 2.1.2.2., respectively. In the TECRA VIA method for *Salmonella*, 1 ml aliquots of each selective enrichment broth (RV and TT) were transferred to 10 ml M broth and incubated at 35°C for 24 h. After incubation, 1 ml portions of each M broth were combined in a clean screw-cap tube and heated in boiling water for 15 min. The M-broth was then cooled to 25-37 °C, and the Enzyme-linked Immunosorbent Assay (ELISA) procedure was performed as described by the manufacturer.

For the *Listeria* VIA test a 0.1 ml aliquot from LEB broth incubated at 30°C/24h was added to 10 ml modified Fraser broth and incubated overnight at 30 °C. After incubation a 1 ml aliquot was added to a closed tube and the test further performed as described by TECRA VIA.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the washing methodology for recovering *Salmonella* Enteritidis and *Listeria monocytogenes* inoculated onto papaya surfaces.

From a total of nineteen papayas analyzed for recovery of *Salmonella* Enteritidis and *Listeria monocytogenes* inoculated onto papaya surfaces, eight fruit samples were analyzed for SE using the modified BAM method and eleven for *L. monocytogenes* using the Protection Health Branch, the washing methodology was applied for both microorganisms (Table 1). The results showed a recovery of only 62.5% for SE from the papaya surface. Different factors should be considered trying to explain these results; among them the low efficiency of the washing

methodology for recovering the inoculated bacteria on the fruit surface, competitive effect of the natural microflora originally present on the fruit surface and problems due to possible deficiencies of the methodology applied. Another possibility could be the bacterial adhesion to the fruit surface, making recovery of the microorganism more difficult. Based on these deficiencies and the low natural occurrence of *Salmonella* on fruit surfaces as reported by different authors, a low prevalence of *Salmonella* on fruit samples would be expected.

Shearer *et al.*, 2001 when using the BAM cultural method and an inoculation level of 1 CFU/25g of *S.* Enteritidis, found 25 positive out of 36 inoculated samples of apple, orange, mango, cantaloupe, strawberry and tomato. When the inoculum size varied from 10 to 100 CFU/25g, there was 100% recovery. For *Listeria monocytogenes* the results were similar to the ones observed for *Salmonella* Enteritidis.

Using the washing methodology, Table 1 shows the recovery of *L. monocytogenes* was higher than that of SE but a 100% recovery was not reached, which could be partially explained by the same reasons mentioned before for explaining the low SE recovery.

Table 1. Evaluation of the washing methodology used for detecting experimentally inoculated *S.* Enteritidis and *L. monocytogenes* on papaya surface.

Assays	Inocula (CFU/Fruit)	Inoculated samples	Positive samples	Negative control samples
1- *S.* Enteritidis				
1	$1,0 \times 10^2$	2	2	0
2	$5,2 \times 10^1$	2	1	0
3	$2,6 \times 10^1$	2	1	0
4	$1,1 \times 10^2$	2	1	0
Total		**8**	**5 (62.5%)**	
2- *L. monocytogenes*				
1	$7,0 \times 10^1$	1	1	0
2	$2,5 \times 10^1$	2	1	0
3	$1,9 \times 10^2$	2	2	0
4	$1,8 \times 10^2$	2	1	0
5	$3,3 \times 10^2$	4	4	0
Total		**11**	**9 (81.8%)**	

3.2. Evaluation of the incidence of *Salmonella* spp. and *Listeria* spp. on the surface of fresh melon, watermelon and papaya fruits using the modified BAM and TECRA VIA for *Salmonella* spp. and the Health Protection Branch and TECRA VIA for *Listeria* spp.

A total of 42 fruit samples collected from wholesale and street market were analyzed for *Salmonella* spp. using the modified BAM and TECRA VIA and for *Listeria* spp. using the Health Protection Brach and TECRA VIA methods.

The results showed the absence of *Salmonella* spp. in all samples with one false positive result by TECRA-VIA in watermelon.

Table 2 shows the *Listeria* results based on the methodologies tested: There were 2 false positive results (one sample of melon and one of watermelon) by TECRA-VIA and three false negatives (one sample of melon and 2 of watermelons). *Listeria grayii* was found by both methodologies in one sample of watermelon

Table 2. Comparison of the TECRA Listeria Visual Immunoassay and Health Protection Branch methods for the detection of *Listeria* spp. on the surface of melon, watermelon and papaya

Fruit	N tested	Positive by both methods	Negative by both methods	Tecra positive Health Protection Branch negative	TECRA negative, Health Protection Branch positive
Melon	14	0	12	1	1
Watermelon	14	1	10	1	2
Papaya	14	0	14	0	0
Total	42	1	36	2	3

Holbrook *et al.*, 1989 determined the efficacy of four commercially available rapid *Salmonella* detection kits (Oxoid Salmonella Rapid Test, Tecra Elisa, Equate Elisa and Bio Control 1-2) and two conventional methods, with naturally contaminated or spiked food samples (meats, sea foods, dairy products, spices and herbs and various dried food materials). Ninety six food samples were examined by all methods except the Bio Control 1-2 where 76 tests were done.

TECRA detected 96% of positive samples, one false positive and three false negatives.

Knight *et al.*, 1996 reported a collaborative study involving 26 laboratories and 5 food types to compare the TECRA Listeria Visual Immunoassay (TLVIA) with the standard culture method. Eleven laboratories analyzed lettuce with *Listeria* levels of 0.15cells/g (low level) and 0.93 cells/g (high level). The traditional culture methodology (BAM/AOAC) detected *Listeria* in 49 out of 55 of the low level samples and 52 out of 55 of the high level samples as positive. TLVIA with visual reading detected 46 out of 55 low level samples and 51 out of 55 high level samples as positive the agreement between the traditional culture method and the TLVIA visual method was 94.6% for the low level and 96.4% for the high level. The uninoculated controls showed no false positives for the 55 TLVIA known negative samples by visual reading.

Blanco *et al.*, 1998 compared the efficiency of two commercial enzyme-linked immunosorbent assays (ELISAs), the Oxoid Listeria rapid test and the TECRA Listeria visual immunoassay for the detection of *Listeria* spp. with the culture method. Of the 60 samples examined by ELISA and the culture procedure, *Listeria* was detected and confirmed by culture in 20 and 44 samples by the TECRA and microbiological methods respectively. The overall sensitivity of the TECRA was 50% with 100% specificity. The efficiency of the TECRA assay was 67%, while the differences between the results of the Tecra assay and the culture procedure were considered significant.

In the current survey the total results of evaluation of the incidence of *Salmonella* spp. and *Listeria* spp. in 120 fruit samples showed the absence of *Salmonella* and *L. monocytogenes* from the surface of all examined fruit samples collected both from wholesale and street market, as shown in Table 3. However, *Listeria innocua* and *L. grayii* were isolated from watermelon (10% of the samples) and *Listeria ivanovii* from papaya (5% of the samples) collected wholesale. Concerning the samples collected in street markets, *Listeria* spp. was isolated with higher frequency, particularly *Listeria grayii*, detected in 5% of the watermelon

samples, *Listeria welshimeri* in 5% of melon samples and *L. ivanovii* in 20% of the papaya samples.

Table 3. Incidence of *Salmonella* spp. and *Listeria* spp. in melon, watermelon and papaya samples collected in wholesale and street market, using the modified BAM methodology for *Salmonella* spp. and the Health Protection Branch method for *Listeria* spp.

Fruits	Wholesale					Street market				
	n- of samples	*Salmonella* spp.		*Listeria* spp.		n- of samples	*Salmonella* spp.		*Listeria* spp.	
		n-	%	n-	%		n-	%	n-	%
Melon	20	0	-	0	-	20	0	-	1	5
Watermelon	20	0	-	2	10	20	0	-	1	5
Papaya	20	0	-	1	5	20	0	-	4	20
Total	60	0	-	0	5	60	0	-	0	10

Vahidy *et al.*, (1992) screened one hundred and fifty samples of fresh fruits and vegetables collected over a period of 12 months from various localities of Karachi, (Pakistan), for the presence of *Listeria monocytogenes*. Of 30 samples each of papaya, watermelon and cantaloupe, and 15 each of cucumber, tomato, radish and carrot, *Listeria monocytogenes* was isolated from two samples of papaya and tomato and one sample of water melon and cucumber.

Monge *et al.* (1995) evaluated the sanitary quality of street sold fruit during the period from March 1990 to March 1993 in San Jose, Costa Rica. They looked for the presence of *Salmonella* spp., *Shigella* spp., *Escherichia coli* as well as fecal coliforms in natural refreshments, fruit salads and fruits usually exposed for selling in the streets, in slices (like pineapple, papaya, non ripe mango and watermelon) or those that could be eaten without peeling, like nances and jocotes. They analyzed 25 samples of each fruit, 50 natural refreshments and 50 fruit salads. *Salmonella* spp. was not isolated from any of the examined samples.

The Food and Drug Administration sampled 1,440 imported melons from March 26 to April 13, 1990, and the results of this sampling revealed that only 11 melons (0.76% of the total sampled) had *Salmonella* spp. on their surfaces. A second survey was conducted from Nov 19 to Jan. 3, 1991, and the results

showed that 24 (1.06%) of the 2,220 analyzed melons contained *Salmonella* spp. (Madden, 1992).

In the period from 1992 to 1993, 211 cantaloupes imported to Canada from USA, Central America, Mexico and Caribbean were sampled for *Salmonella* spp. and only four samples (1.9%) were positive for the microorganism analyzed (Madden, 1992).

More recently, the FDA isolated *Salmonella* spp. from eight (5.3%) of 151 analyzed cantaloupe samples coming from nine countries exporting to the United States (FDA 2001).

In conclusion, the reported results, plus the obtained in this survey show that both *Salmonella* spp. and *L. monocytogenes* are not usually present on the fruit surface. However, depending on the sanitary conditions during growth in the field, harvesting and handling of the fruits, contamination can occur, with the application of Good Agricultural Practices – GAP really becoming important during all the steps from growth in the field up to final consumption (from farm to fork).

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RESUMO

Neste trabalho, foram avaliadas a incidência de *Salmonella* spp. e *Listeria* spp. em superficie de frutas, melões (*Cucumis melo*), melancias (*Citrullus vulgaris*) e mamões (*Carica papaya*), coletadas em feira livre e na central de abastecimento (CEASA) em Campinas-São Paulo, Brazil. De um total de 120 frutas 42 amostras foram analisadas simultaneamente pelo método imunoenzimático TECRA-VIA e

BAM modificado para presença de *Salmonella* e por um método canadense "Health Protection Branch" e TECRA-VIA para detecção de *Listeria*. As 78 amostras restantes foram analisadas somentes pelos métodos de cultura. *Salmonella* spp. não foi encontrada em nenhuma das 42 amostras analisadas por ambas metodologias sendo que o método TECRA VIA apresentou um falso positivo. Contudo *Listeria* spp. foi detectada em 1 amostra (2,38%) das amostras analisadas e apresentou 2 resultados falsos positivos e 3 resultados falsos negativos quando utilizado o método TECRA – VIA.

Salmonella spp. também não foi constatada nas 78 amostras analisadas apenas pelo método BAM modificado. Contudo *Listeria* spp. foi detectada em 9 (7,50%) das amostras analisadas, sendo que *L. innocua* e *L. grayii* foram isoladas de melancia, *L. ivanovii* de papaya e *L. welshimeri* de melão quando utilizado o método canadense "Health Protection Branch". As amostras coletadas em feira livre mostraram uma frequência maior de *Listeria* quando comparadas com as obtidas no CEASA.

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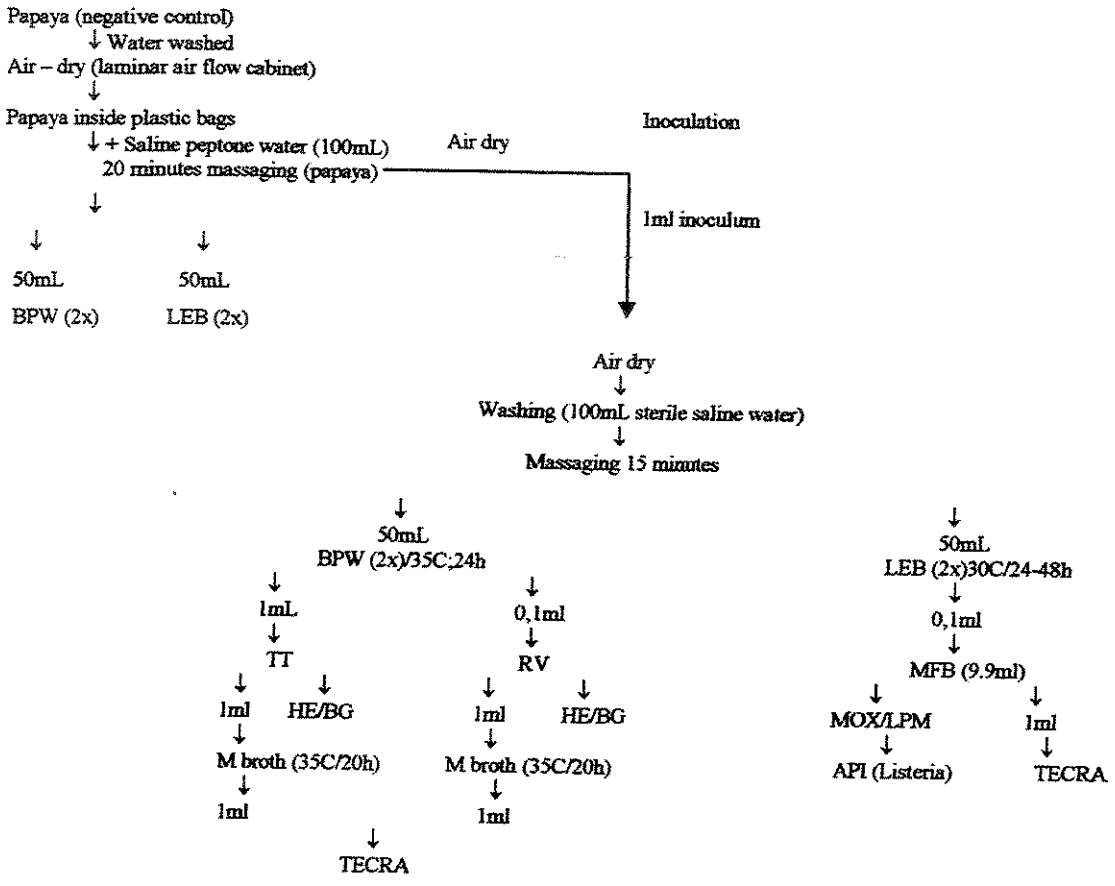

Figure 1. Inoculation procedures

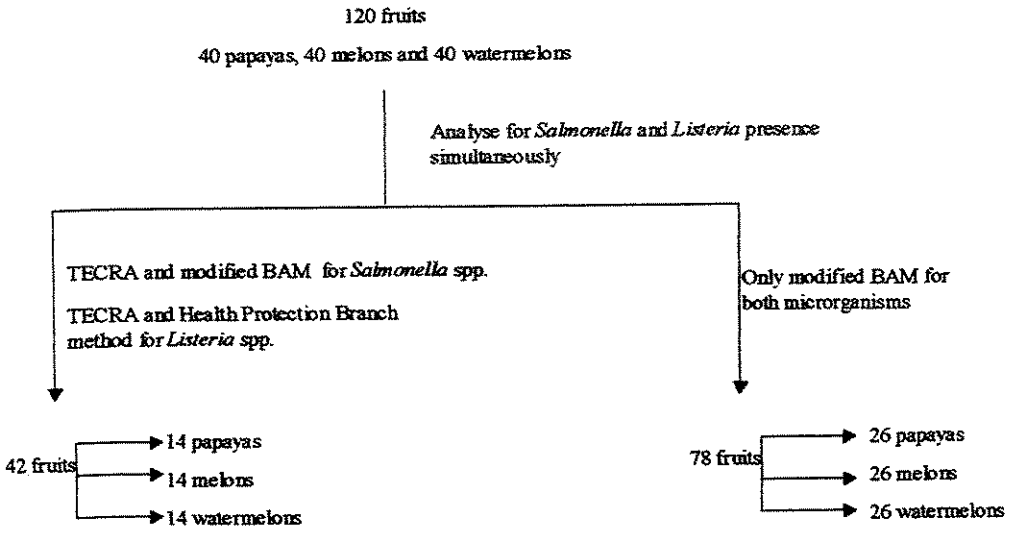

Figure 2. Methodologies applied for the detection of *Salmonella* spp. and *Listeria* spp. on the surfaces of papaya, melon and watermelon.

ANEXO

Evidence of *Salmonella* Internalization into Fresh Mangos During Simulated Post-Harvest Processing Procedures.

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Abstract

A recent U.S. salmonellosis outbreak was epidemiologically associated with consumption of imported fresh mangoes. Here, studies were conducted to simulate the commercial heat disinfestation method used to eliminate tephritid fly larvae, and subsequent product cooling procedures, to assess the potential to promote the infiltration of *Salmonella* into the mangoes. The conditions modeled were used by the outbreak implicated foreign producer/packer. Untreated domestically grown green and ripened Tommy Atkins variety mangoes (n=60) were immersed in water at 46°C for 90 min, followed by immersion at 22°C for 10 min into water containing 10^7 CFU/ml green fluorescent protein labeled *Salmonella* Enteriditis. The fruits were then stored at 10, 20 or 30°C for up to 1 wk. Green and ripened mangoes were positive for *Salmonella* infiltration at a rate of 80% and 87%, respectively. Infiltration frequency into the stem portion (83%) was significantly higher ($P<0.05$) than infiltration into the middle (19%) or bottom (9%) segments. The degree of fruit ripeness, post treatment holding temperature, or duration of storage had no significant effect on infiltration frequency or survival of the *Salmonella* inside the mangoes. This study illustrates the high potential for pathogen internalization if heat-disinfested mangoes are cooled using contaminated water. The results

demonstrated the need for further research to determine fruit processing parameters that eliminate fruit fly larvae while concurrently protecting public health.

Running Title: *Salmonella* internalization into mangos

Introduction

Mango (*Mangifera indica* Linn.), a fruit native to India and surrounding islands, was introduced to the West Indies, Central and South America in the early sixteenth century. U.S. mango imports increased more than 162,000 metric tons (159%) from 1991 to 2000 (USDA, ERS 2001). Mexico is the primary exporter to the United States, comprising 71% of all mango imports. Other countries, including Ecuador, Brazil and Peru, supply the U.S. with mangoes between December and March before the beginning of the harvesting season in Mexico.

Since imported mangoes raise concerns over the possible introduction of tephritid fruit flies (especially the Mediterranean fruit fly) inside the U.S. borders (Jacobi 2001), the USDA Animal Plant Health Inspection Service (APHIS) requires mangoes from South America, the West Indies and Central America (including Mexico) to be exposed to specific temperatures to ensure the destruction of tephritid larvae (7CFR 319.56-2i). All mango fruits exported to the U.S. and Japan are treated with hot water (the only authorized treatment), at 46.1°C for 65, 75 or 90 min depending on the fruit weight (Yahia, 2000). Although not required by regulation, mango growers exporting to American markets frequently cool the fruit in room temperature water after the required heating step to prevent quality losses.

Salmonella is a bacterium of public health concern for fresh fruits and vegetables (Beuchat, 1996). In December 1999 a nationwide increase in *Salmonella* serotype Newport infections, with clinical isolates exhibiting identical pulsed-field gel electrophoresis patterns, was detected by the Center for Disease Control and Prevention (CDC). In total, 78 patients from 13 states were infected by the strain. Fifteen patients were hospitalized and two died. Case control study findings revealed that the consumption of raw mangos was strongly associated

with the outbreak (matched odds ratio=21.6; 95% confidence internval=3.53-infinity, p=0.0001). A trace back investigation was performed by the Food and Drug Administration (FDA), by contacting grocery stores and restaurants where patients reported purchasing or eating mangoes during the five days before the onset of the illness. Trace back investigation information, obtained from four patients living in three states, indicated no common store, restaurant, distributor, importer or shipment to the U.S. However, consumption of mangoes produced at one South American farm was associated with all of the cases. A CDC, FDA, and APHIS environmental investigation team sent to the implicated farm, after the growing season, learned that mangoes shipped to the U.S. were disinfested by dipping crates of mangoes into 116 F water for 75 to 90 min, followed by cool water immersion at 70 F for 6-10 min. Chlorine was added only to the cool water tanks; the target level was 100 mg/L. Cool water was reused for up to 1 wk and chlorine was charged only at the initial water replacement. The implicated farm had also shipped mangoes to other South American countries and Europe. However those fruit were not subjected to the disinfestation procedure, and were never implicated in a food-borne illness outbreak by European surveillance systems. The conclusions of the environmental investigation suggested that the water used for disinfestation treatment and subsequent cooling may have been contaminated from environmental sources. The observed conditions included: no covers over the water tanks, presence of birds and their feces and presence of amphibians and lizards near the water tanks. Laboratory findings showed that water samples collected at the farm had total and fecal coliforms and *Salmonella*. One toad cloacal sample yielded *Salmonella* (Sivapalasingam et al., 2002).

Previous research on tomatoes (Bartz and Showalter, 1981), apples (Buchanan et al., 1999) and oranges (Eblen et al, 2003) has indicated that subjecting warm fruit to a cooler environment can allow internal gases inside the fruit to contract, with the resulting hydrostatic pressure causing internalization of water into the fruit. Therefore, we hypothesize that this phenomenon may have occurred with the imported mango outbreak. The purpose of this study, therefore, was to study the infiltration potential of *Salmonella* into mangoes after hot water

and subsequent cooling treatment. The mangoes used in this study (Tommy Atkins) are a popular variety that is grown both domestically and outside of the US.

MATERIAL AND METHODS

Fruits

Green or early-ripened mangoes (variety Tommy Atkins) were shipped immediately after harvest from a commercial producer in Homestead, FL. Fruits were unwaxed and did not receive a hot water decontamination procedure. Upon receipt, all fruits were maintained at 10°C with 90% humidity until used (maximum of 2 weeks). One day before each experiment, the appropriate number of fruits were transferred to room temperature (21°C). All fruits were individually inspected for defects (e.g. breaks in the peel, bruised areas), and any defective mangoes were discarded.

Microorganisms

A green fluorescent protein (GFP) labeled *Salmonella* Enteritidis serotype S 132, a gift from Dr. Tom Oscar (U.S. Department of Agriculture, Agricultural Research Service, University of Maryland Eastern Shore) was used. The strain was transformed with plasmid pGFPuv (Clontech, Palo Alto, CA) and stably expressed green fluorescent protein. The permanent culture was maintained at -70°C in brain heart infusion (BHI) broth (Difco, Detroit, MI.) with 15% glycerol (Sigma, St. Louis, MO.).

Inoculum

Overnight cultures were started by inoculating 10 ml BHI medium with 0.1 ml of the thawed culture and then incubating at 37°C for 48 h. A 1ml aliquot of this culture was then added to 500 ml of BHI and incubated at 37°C for 18 h.

Dye uptake study

Fifteen mangoes were immersed for 90 min in a plastic container (20.5cm x 34cm x 55 cm) containing 15 L of water (46°C). Following hot water treatment, the mangoes were immersed for 10 min in 15 L of 22°C water containing 0.1% Brilliant Blue FCF (Sigma). After removal from the dye solution, the mangoes were rinsed under tap water and then dried with absorbent paper. The mangoes were then cut in 3 different segments (stem, middle and bottom), as shown in Figure 1. The slices were examined individually for uptake of dye into the flesh.

Pathogen Infiltration Studies

Two trials, performed on different days, using 30 green mangoes and 23 ripe mangoes separately, were conducted. Both ripe and green mangoes, including 1 negative control mango, were completely submerged in a plastic container (20.5cm x 34cm x 55cm) filled with 15 liters of tap water maintained at 46°C, with a constant temperature control and allowed to equilibrate for 90 min. The mangoes were then transferred to another plastic bin of the same dimensions, and completely submerged for 10 min in water at 22°C inoculated with 10^7CFU/ml GFP labeled *Salmonella* Enteritidis. Mangoes were individually removed using gloved hands, and air-dried in a class II Type A/B3 laminar flow hood (Nuaire, Plymouth,

MN) for 1 h. All mangoes were surface sanitized by immersion in 1L of a 2g /L sodium hypochlorite solution (22°C) for 1 min, and air-dried in a laminar flow hood. Each mango was again surface sanitized using 70% ethanol and allowed to dry. Mangoes were incubated at 10°, 20° or 30°C and removed for testing after 0, 24, 48 or 168 hr incubation. Non-inoculated mangoes were evaluated at each time/temperature combination for *Salmonella* uptake.

Before cutting, each mango was surface swabbed, to ensure that cutting did not spread *Salmonella* GFP contamination to the interior of the fruit. Using 3 sterilized knives and cutting boards for each mango, the fruit was sectioned, producing stem, middle and bottom segments (Fig. 1). Using individual sterilized spoons, flesh samples were collected from each segment and placed into individual filtered stomacher bags (Spiral Biotech, Bethesda, MD.). The stem and bottom segments were diluted to 1:5 (wt/vol.). The middle portion was undiluted, because the availability of expressed mango juice eliminated the need for diluents. All samples were homogenized using a Colworth 400 (Seward, London, UK) stomacher for 5 minutes. After diluting as needed with sterile 0.1% peptone (Difco) water (pH 6.8), samples were surfaced plated (50 λl) onto duplicate BHI agar dishes using an Autoplate 4000 spiral plater (Spiral Biotech). All plates were incubated at 37°C for 18-24 h. The plates were screened with a long wave (366 nm) UV light using a model UVGL-58 UV light source (UVP, San Gabriel, CA.) and counted using a model 500A automatic plate counter (Spiral Biotech).

pH and Data Analysis

Fruit pH was measured by inserting a gel epoxy-body combination electrode attached to a VWR model 8005 pH meter (VWR Scientific, West Chester, PA.) into the fruit homogenate. Since the purpose of this study was to determine infiltration potential, quantitative microbiological data were classified as binary (0 = negative, 1 = positive) values. Differences between the proportion of positive samples for

the fruit sections were then analyzed using the Fisher's Exact test (1) option in SAS Proc Freq, version 8.2 (2). P-values greater than 0.05 were considered non-significant. Standard errors for the proportions were calculated using the normal approximation to the binomial distribution.

RESULTS

Dye uptake

Ten of fifteen (67%) mangoes were positive for dye infiltration. Infiltration occurred through the stem scar and penetrated into the middle side section as shown in Figure 2. There was no evidence of direct dye penetration through the side or bottom portion of the fruit.

Pathogen Infiltration

A high rate of pathogen infiltration was observed for green (80%) and ripened (87%) mangoes. The degree of ripeness of the fruit had no significant effect ($P>0.05$) on infiltration frequency. The frequency of infiltration of pooled green and ripened fruit into the stem portion of the mango (83%, 44/53) was significantly higher ($P<0.05$) than infiltration into the side (19%, 10/53) or bottom (9%, 4/53) portions (Table 1).

Post treatment holding temperatures and duration of incubation was also shown to have no significant effect ($P>0.05$) on infiltration (Table 1). Pathogen levels found inside the mango pulp varied greatly (<20 to >Log_{10} 6 CFU/ml) between and within the different treatments (data not shown). *Salmonella* was

detected in mango pulp after one week of incubation at all temperatures tested (Table 1).

DISCUSSION

This study was conducted in response to a 1999 multistate salmonellosis outbreak that was associated with fresh mangoes that were imported from South America. While 78 cases were identified by the CDC, Mead et al. (1999) estimated that under reporting of *Salmonella* infections typically exceed 97%. The present study aimed at determining the potential for internalization of a human pathogen after the fruit was subjected to a simulation model system of the insect disinfestation procedure used by the implicated producer. The principal finding of this study was the demonstration that post-harvest processing conditions could result in the internalization of *Salmonella* into the mango interior. Pathogen internalization occurred in intact fruit, principally through the stem portion of the mango. A significantly lower internalization frequency was observed in the middle side and bottom fruit portions. Other studies assessing the infiltration potential of pathogens into fruit interiors (Buchanan et al.1999; Bartz and Showalter 1981) also found the area around the stem to be the most susceptible to entry. The high *Salmonella* infiltration rates into warm green and ripe mangos (83%) indicate that this fruit is very susceptible to pathogen infiltration from water with a temperature differential.

The frequency of pathogen infiltration into mangoes was observed to be higher than the infiltration studies with other fruit. However, the data in the present study is consistent with that of other produce commodities, in that warm fruit submerged in cool water permits pathogens to infiltrate into the inside of the product flesh. In addition, similar findings from this and other studies demonstrated that dye uptake studies were accurate in predicting the infiltration potential by human pathogens (Merker et al.1999; Eblen et al.2002 and Buchanan et al. 1999).

In the present study the degree of ripeness tested showed no effect on the infiltration frequency of the mangos. Furthermore, post treatment storage temperatures or the duration of incubation did not significantly affect the qualitative survivability of *Salmonella* in mangos for up to 7 days, despite the acidic pH (average pH=3.65). These data showed that *Salmonella* could infiltrate mangos and then survive on the inside for at least 1 week, increasing the potential for food-borne illnesses. If pathogens are internalized, successful surface decontamination treatments will not reduce or eliminate the potential food-borne illness hazard when the product is consumed.

While the present study investigated only the potential for pathogen internalization when the microbial hazard was in the cooler water, other unpublished FDA research demonstrated that *Salmonella* levels are only minimally reduced at 46°C. In that limited study (data not presented) *Salmonella* inoculated into mango wash water obtained from the implicated foreign packer, exhibited only a 100-fold reduction (2 logs) after exposure for 4 hours at 46°C (T. Hammack, FDA, personal communication). This suggests that pathogens introduced into heating tanks could survive for long periods. If present in the tanks, pathogens may become attached to the fruit and then get transported by the fruit or packing crates to the cooling water, where they may internalize during hydro cooling. The possibility of internalization during the heating step was not examined in the present study. However, other studies on apples (Buchanan et al. 1999) did not observe infiltration when the water was warmer than the fruit.

Water that is of poor initial quality, not properly chlorinated, or becomes contaminated during processing may serve as a vector for the contamination of mangoes and for pathogen internalization if the fruit receives the heat disinfestation and cooling. The present study demonstrates the potential for this mechanism to have resulted in the 1999 outbreak, but it does not establish the certainty. Yet, the high pathogen internalization frequency into the fruit observed here, emphasizes the necessity for the use of a high quality water supply, prevention of wild or domestic animals near or inside all processing areas, and the need for adequate water treatment and monitoring during processing.

Due to the economic impact of tephritid flies on agriculture, decontamination procedures are necessary for the importation of mangoes into the United States. Although treatment regimes other than hot water immersion are being considered by APHIS as acceptable for importation, at present, hydro cooling procedures are used to maintain commercial viability. Future studies should be conducted into alternative methods for insect disinfestation including: air cooling and ionizing irradiation. Furthermore, additional studies are needed to establish processing guidelines to ensure that existing disinfestation procedures prevent human pathogen internalization into the fruit.

Consumers may reduce or eliminate surface contamination on mangoes by washing fruit before consumption or by removing the outer surface. However, there is no treatment available to consumers to reduce or eliminate internalized microbial hazards without a loss of fresh product quality. Therefore, prevention of contamination and internalization is critical to ensure highly acceptable yet safe fresh fruit.

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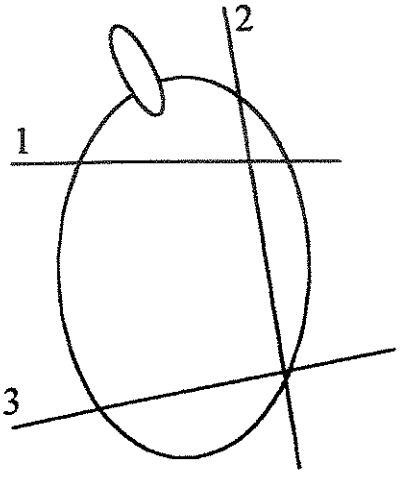

Figure 1. Regions of mango examined for the presence of *Salmonella* Enteritidis 1. Stem 2. Middle Side 3. Bottom

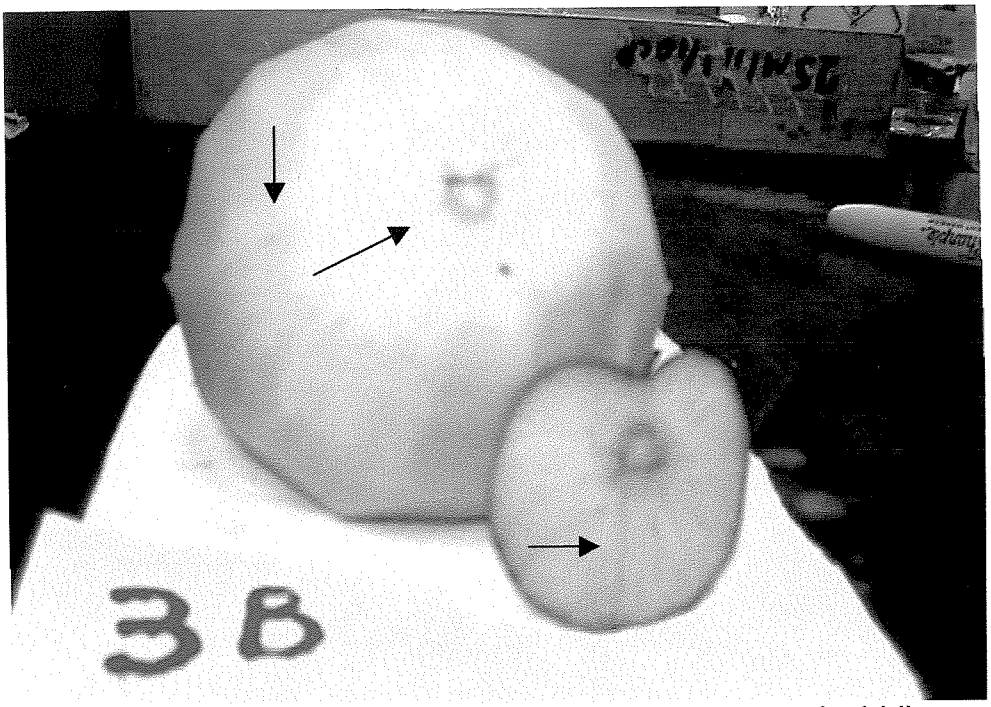

Figure 2. Infiltration of dye (arrows) through the stem and middle side of mangoes (variety Tommy Atkins).

Table 1. Infiltration potential of Green Fluorescent Protein labeled *Salmonella* serotype Enteriditis inside mangos as a function of post challenge incubation time and temperature. Data from green and ripened mangos were pooled.

Fruit Section	Incubation Temp°C	Incubation Time (h) 0	24	48	168	% Infiltrated
Stem	10		5/5[a]	5/6	5/6	
	20	5/6	5/6	5/6	2/4	**83**^{Ab}
	30		6/6	5/5	1/3	
Middle Side	10		2/5	1/6	0/6	
	20	0/6	2/6	1/6	0/4	**19**^{B}
	30		1/6	2/5	0/3	
Bottom	10		1/5	0/6	0/6	
	20	1/6	1/6	0/6	04	**9**^{B}
	30		1/6	0/5	0/3	
Totals (%)		**33**^{cA}	**47**^{A}	**37**^{A}	**21**^{A}	

[a] number of fruit *Salmonella* positive/ total number tested.

[b] Numbers having different superscript capital letters within this column are significantly different ($P<0.05$)

c Numbers having different superscript capital letters within this row are significant ($P<0.05$)