UNIVERSITI PUTRA MALAYSIA

EFFECTS OF GRILLING OF BEEF AND CHICKEN SATAY ON THE SURVIVAL OF *LISTERIA MONOCYTOGENES*

SITI ZULAIHA BINTI HANAPI

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By

SITI ZULAIHA BINTI HANAPI

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EFFECTS OF GRILLING OF BEEF AND CHICKEN SATAY ON THE SURVIVAL OF *LISTERIA MONOCYTOGENES*

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April 2006

Chairman : Associate Professor Zaiton bt Hassan, PhD

Faculty : Science and Food Technology

The isolation and enumeration of heat-injured and non-injured of *Listeria monocytogenes* and other *Listeria* species was studied in samples of chicken (40 samples) and beef (50 samples) satay isolated from 3 different vendors around Kajang and Bangi, Selangor. Samples of chicken satay were grilled by the vendors for 180 seconds while beef satay was grilled for 270 seconds. The internal temperature obtained was 64 ± 4^{0}C measured by probe. This resulted to reduce total viable count of chicken satay and beef satay by 3 log reduction. Three different methods namely, U.S. FDA (Canadian Version), Overlay method (Compendium Method for the Microbiological Examination of Food (1995) and Thin Agar Layer (TAL) (Kang and Fung, 1999) were evaluated for the recovery of *Listeria* species from raw and grilled chicken satay and beef satay. TAL method gives higher recovery rates compared with OV while U.S FDA method unable to allow the recovery of *Listeria* species in raw and grilled satay. *Listeria* species isolated from raw chicken and beef satay using TAL method were *L. monocytogenes*, *L. innocua*, *L ivanovii* and *L. grayi*, respectively. Similar *Listeria* species were also detected in grilled chicken and beef satay after grilled to 180 s.. *L. welshimeri* is the only

species was isolated in raw beef satay. On contrary, in OV agar, three species namely, *L. monocytogenes*,, *L. innocua* and *L. ivanovii* were recovered in chicken satay with grilling time 150 seconds. However, prolonged heating to 180 seconds resulted in destruction of all *Listeria* species. Three different internal temperature (60^0C, 70^0C and 80^0C) and 3 different storage temperature (4^0C, 30^0C and -20^0C) were studied in chicken satay inoculated with *L. monocytogenes* (~10^{6-7} CFU/g). The population of *L. monocytogenes* increased rapidly on chicken satay stored at 30^0C for 24 hours. *L. monocytogenes* was also shown the ability to survive at refrigeration temperature for up to 7 days. In temperature storage at -20^0C, the population of *L. monocytogenes* remained 14 days only. A study of hemolysin gene using specific Polymerase Chain Reaction (PCR) showed that *L. monocytogenes* was found resistance to internal temperature of 60 and 70^0C but not at 80^0C.

Results clearly demonstrated that the detection and enumeration of injured *Listeria* species was influenced by the medium used for the isolation. Microorganism that have been treated with a sub-lethal stress are considered injured if they fail to grow and produce colonies when they are plated on agar media containing selective agents. The interpretation results found that the pattern of growth of *Listeria* in satay (raw and grilled) was strongly influenced by the storage and internal grilling temperatures. Higher heating temperature resulted in destruction of *L. monocytogenes* in grilled satay.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KESAN PEMBAKARAN SATE LEMBU DAN AYAM KE ATAS KEUPAYAAN *LISTERIA MONOCYTOGENES*

Oleh

SITI ZULAIHA BINTI HANAPI

April 2006

Pengerusi : Profesor Madya Zaiton bt Hassan, PhD

Fakulti : Sains dan Tekologi Makanan

Bilangan *Listeria monocytogenes* dan spesies *Listeria* yang lain, cedera dan tidak tercedera oleh haba telah dipencilkan dan dikira daripada sate ayam (40 sampel) dan sate lembu (30 sampel). Sampel ini telah diambil daripada tiga peniaga sate berlainan di sekitar Kajang dan Bangi, Selangor. Kajian ini telah tertumpu kepada penilaian kaedah yang digunakan untuk pemencilan, pengiraan dan pengenalpastian bukan sahaja *Listeria monocytogenes* tetapi juga *Listeria* yang lain daripada sate ayam dan lembu mentah. Sampel sate ayam telah dibakar selama 180 saat manakala sate lembu selama 270 saat sehingga suhu mencapai 64 ± 4^0C menggunakan probe suhu. Pembakaran sate sehingga suhu 64 ± 4^0C telah menurunkan jumlah mikrob sebanyak 3 log. Tiga kaedah berbeza iaitu U.S. FDA (Canadian Version), Overlay (Compendium Method for the Microbiological Examination of Food, 1995) dan TAL (Kang and Fung, 1999) telah digunakan untuk mengkaji keupayaan setiap kaedah itu memulihkan sel-sel *Listeria* daripada sate ayam dan lembu mentah dan telah masak. Kaedah TAL telah memberi kadar pemulihan yang tinggi berbanding kaedah OV manakala kaedah U.S FDA pula tidak berupaya untuk membantu proses pemulihan sel-sel yang tersedera sama ada didalam sate mentah atau masak. Spesis *Listeria*

yang berjaya dipencilkan daripada sate ayam dan lembu mentah atau masak adalah *L. monocytogenes, L. innocua, L. ivanovii* dan *L. grayi.* Hanya *L. welshimeri* satu-satunya spesis yang dipencilkan daripada sate lembu mentah. Berbeza dengan menggunakan kaedah OV, tiga spesis *Listeria* berjaya dipencilkan dalam sate ayam iaitu *L. monocytogenes, L. innocua* dan *L. ivanovii* selepas 150 saat dibakar. Apabila masa pembakaran dipanjangkan kepada 180 saat, semua spesis *Listeria* telah dimatikan. Satu kajian bagi mengkaji keupayaan dan pertumbuhan *L. monocytogenes* telah dilakukan. Sampel ayam yang telah inokulasikan dengan *L. monocytogenes* (10^{6-7} CFU/g) telah disimpan pada 3 suhu peyimpanan iaitu pada suhu 30^0C (24 jam), -4^0C (7 hari) dan -24^0C (4 minggu). Kemudian sate yang telah disimpan ini dibakar menggunakan bara sehingga mencecah suhu iaitu 60, 70 dan 80^0C. Keputusan yang diperolehi menunjukkan bakteria *L. monocytogenes* kekal dan membiak dengan cepat pada suhu ambient (30^0C) selama 24 jam. Populasi *L. monocytogenes* juga tidak berubah apabila disimpan di dalam suhu peti sejuk selama 7 hari. Pada suhu sejukbeku -20^0C, populasi *L. monocytogenes* kekal sehingga 14 hari sahaja. Kajian dibuat keatas gen hemolisis menggunakan specific PCR menunjukkan *L. monocytogenes* berupaya untuk hidup pada suhu dalaman pembakaran sehingga 60 dan 70^0C tetapi tidak pada suhu 80^0C.

Hasil kajian menunjukkan kaedah dan media pemencilan mempengaruhi pengiraan dan pemencilan organisma. Mikroorganisma yang telah diberi tekanan separa kuat dikatakan tercedera sekiranya mereka gagal untuk hidup dan menghasilkan koloni apabila di tumbuhkan diatas media yang mengandungi agen-agen selektif. Keputusan yang diperolehi menunjukkan pertumbuhan *Listeria* didalam sate (mentah dan masak) dipengaruhi kuat oleh faktor penyimpanan dan suhu dalaman semasa

pembakaran. Pembakaran pada suhu tinggi juga menyebabkan kemusnahan *L. monocytogenes* didalam sate yang telah dimasak.

This thesis submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Zaiton Bt Hassan, PhD
Associate Professor
Faculty of Science and Food Technology
Universiti Putra Malaysia
(Chairman)

Gulam Rusul Rahmat Ali, PhD
Professor
Faculty of Science and Food Technology
Universiti Putra Malaysia
(Member)

Russly Abd Rahman, PhD
Professor
Faculty of Science and Food Technology
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 14th SEPTEMBER 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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SITI ZULAIHA BINTI HANAPI

Date: 25th AUGUST 2006

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LIST OF ABBREVIATIONS

AOAC	Association Official Analytical Chemistry
AP-PCR	Arbitrary Primered-Polymerase Chain Reaction
CAMP	Christie-Atkins-Munch-Peterson
CDC	Centers for Disease Control
DNA	Deoxyribonucleic acid
d	day (s)
dNTPs	deoxyribonucleotide triphosphates
EDTA	Ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assays
EtBr	Ethidium bromide
FDA	Food and Drug Administration
g	Gram
HCl	Hydrochloric acid
H	hour (s)
LLO	Listeriolysin O
LEB	*Listeria* enrichment broth
L-PALCAMY	Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk Broth
min	minute (s)
ml	mililiter
µg	microgram
NaCl	Sodium Chloride
µl	microliter
PCR	Polymerase Chain Reaction

PFGE	Pulsed-field gel electrophoresis
RTE	Ready to eat food
RAPD	Random Amplification of Polymorphic DNA
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
s	second
sp	Species
Taq	Thermus aquaticus DNA (polymerase)
TAL	Thin Agar Layer
TBE	Tri-Borate EDTA electrophoresis buffer
Tris	Tris (hydroxymethyl) methylamine
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
OV	Overlay
U.S	United States
USDA	United States Food and Drug Administration
UV	Ultraviolet
UVM	University of Vermont
WHO	World Health Organisation
w/v	weight/volume
>	more than
^{0}C	degree Celcius

CHAPTER 1

INTRODUCTION

L. monocytogenes is a highly adaptable, asporogenous, motile, Gram-positive bacterium and appreciably more heat resistant than many other non-sporing pathogens associated with foods such as *Salmonella* and *Camphylobacter* (Mackey and Bratchell, 1989; Farber and Peterkin, 1991). Its ability to grow at wide range of temperature especially at refrigerator temperature makes it particularly troublesome to the food industry (Knabel *et al.,* 1990; Dykes, 1999; Fung *et al.,* 2004; Kim *et al.,* 2005; Matagaras *et al.,* 2006).

L. monocytogenes can be isolated from many sources in the environment including soil, water, sewage and many animal species (Patel *et al.,* 1994; Kang and Fung, 1999; Diane *et al.,* 1991). *L. monocytogenes* was also been isolated from a wide range of foods, including dairy products, seafood, raw fruits and vegetables, poultry and raw and processed meats (Schlech *et al*., 1983; Bula *et al.,* 1995; Linnan *et al.,* 1988). Foodborne transmission of listeriosis has been recognized since 1981 when an outbreak in Canada was associated with consumption of coleslaw made from contaminated cabbages (Schlech *et al*., 1983). Since then, epidemologic investigation have implicated whole and 2% milk (Flemming *et al.,* 1985), Mexican–style cheese (Linnan *et al*., 1988), liver pate (McLauchin *et al.,* 1991), jellied pork tongue (Jacquet *et al.,* 1995), smooked mussels (Brett *et al.,* 1998), frankfurters (CDC, 1999) and pigs tongue in aspic (FDA, 2001).

The first reported case of human listeriosis was made by Nyfeldt in 1929, where the blood of patients with an infections mononucleosis-like disease was positive with *L. monocytogenes* (Gray *et al.,* 1966). Following the year in 1936, *L. monocytogenes* was a cause of perinatal infection in humans (McLauchlin, 1987). Subsequently, *Listeria* was found to cause abortion in cattle and sheep, septicemia in fowl, encephalitis in sheep and mastitis in cows (Wehr, 1987). Potel provided the first description of foodborne listeriosis in humans with a direct-link to animal in 1953 (Hird, 1987). In this instance, *L. monocytogenes* was isolated from a cow with listerial mastitis and from stillborn twins of a woman who had ingested raw milk from the same animal.

The Centres of Disease Control (CDC) in 1999(a), reported that all the foodborne pathogens tracked by CDC, *L. monocytogenes* had the second highest case fatality rate (20%) and the highest hospitalisation (90%) with approximately 2500 cases of listeriosis occur annually in the United States (Mead *et al.,* 1999). Individuals at highest risk of listeriosis include those with decreased immune function caused by disease or medications; pregnant woman who usually have clinically mild illness; and their infants who may be infected before or during delivery, resulting in stillbirth or neonatal infection. The LA country outbreak, which occurred between January 1 and August 15, 1985, resulted in 142 cases and 48 (34%) deaths. Ninety-three (65.5%) of the infections were in pregnant women or their neonates. Thirty-two (32%) of those cases ended in death. At least, 6% of human maybe asymptomatic gastrointestinal carries of *L. monocytogenes* without ill effects (Rocourt, 1996). The substance that is responsible for hemolysis of erythrocytes and the destruction of

phagocytic cells has been designated as Listeriolysin O (LLO). This hemolysin gene can be detected using the specific Polymerase Chain Reaction (PCR).

Early work by the Food and Drug Administration, which attempted to resolve the *Listeria* heat resistance issue as a result of the 1983 Massachusetts outbreak, determined that *L. monocytogenes* had a $D_{71.7^{o}C}$ value of 0.9 s (Bradshaw *et al.*, 1985). Thus, in order to survive high-temperature, short time pasteurisation ($17.1^{0}C$, 15 sec) populations of $>10^{15}$ *Listeria* /ml would need to be present in milk. The maximum population to which *Listeria* can grow in milk is 10^{9}/ml (Donnelly and Brigss, 1986). Data for other food are less comprehensive but there have been several reports that *L. monocytogenes* can survive in red meat or poultry cooked to temperature as high as $82^{0}C$ (Karaioannoglou and Xenos, 1980; Carpenter and Harrison, 1989). These results suggest that *L. monocytogenes* is much more resistant in meat than in milk.

Listeria can be sublethally injured by a variety of processing treatments such as heating, drying and freezing (Martin and Katz, 1993). Micoorganism that have been treated with a sublethal stress are considered to be injured if they fail to grow and produce colonies when they are plated on agar media containing selective agents such as bile salts, Sodium chloride, antibiotics and others (Smith, 1990). Such treatment would cause the loss of membrane integrity, degrading RNA, reducing enzymatic activity and changing transport kinetics of cells. The temporary loss of tolerance for specific condition by microorganism needs a procedure called resuscitation to allow the organism to recover and regain growth and virulence potential (Hill and Gahan, 2000). If the injured microorganisms can escape the

stressed environment at if the stress is removed, they may, under the proper conditions, repair the damage induced by the stress, divide and potentially as dangerous as their uninjured counterparts (Alex *et al.*, 1999; Jung *et al.*, 2003).

Although many culturing methods have been developed or proposed for *L. monocytogenes*, most are time consuming and not ideal for use by the food industry. Frequently, perishable foods are consumed before analysis for *L. monocytogenes* is completed. Many plating methods have been developed for this purpose and no one medium has emerged as being optimal for all foods. Efficacy of medium to allow recovery and enumeration of *L. monocytogenes* depends on the physical state of the cells, the type of food being analysed and the presence of contaminating microflora (Brackett *et al.*, 1990). Other factors such as incubation temperature, composition of recovery medium and the incubation atmosphere also play important roles (Taormina and Beachaut, 2002). Less inhibitory media are necessary when analysing foods, which contain injured *L. monocytogenes* cells. Kang and Fung (2000) reported that the recovery of injured cells is superior in the absence of oxygen.

This present study will focused on the efficiency of 3 different methods; Thin Agar Layer (TAL), Overlay (OV) and USDA methods to isolate and enumerate *L. monocytogenes* and other *Listeria* species in raw and grilled chicken and beef satay samples. In this study, the survival of *L. monocytogenes* was evaluated at three different temperatures storage (4^0C, 30^0C and -20^0C) and grilling at temperatures of 60^0C, 70^0C and 80^0C. A storage of 4^0C was selected because most of foods are kept in refrigeration temperature. A storage of 30^0C was selected because many foods are handled at this temperature, and often *L. monocytogenes* maybe present on surfaces

and utensils and be a major source of contamination. A storage of -20^0C was selected because many foods are stored at this temperature as a stock for a period of time. Satay was choosen as a model because satay is the favourite foods not only in South East Asia but also in Europe and can be easily found in the market. The customers who eat satay always come in a group with different ages including the young and elderly personal. Nowadays, satay has been sold commercially in a market including ready to eat or in frozen pack. Therefore, the monitoring program and research should be carried out to provide knowledge on the source of contamination and the presence of *L. monocytogenes* and other pathogenic bacteria in satay. Thereby, the control option can be recommended.

The objectives of this study are

1. To compare the selectivity and efficiency of Thin Agar Layer Method (TAL), Overlay Method (OV) and USDA for the enumeration of injured and non-injured *Listeria monocytogenes* in chicken satay and beef satay.
2. Identification of *Listeria monocytogenes* isolated from raw and grilled chicken and beef satay.
3. To study the survival of *L. monocytogenes* inoculated in raw chicken satay stored at 4^0C, 30^0C and -20^0C and the resistance to grilling at internal temperatures of 60^0 C, 70^0 C and 80^0 C.

CHAPTER II

LITERATURE REVIEW

2.1 General Characteristics of *L. monocytogenes*

L. monocytogenes is a member of the genus *Listeria* and is characterized as a gram positive, non-sporeforming bacterium with regular rods 0.4-0.5 μm with rounded ends (Rocourt, 1999). They are both anaerobic and facultative anaerobic bacteria, belong to low G+C content gram-positive bacteria and is closely related to the *Bacillus* and *Streptococcus* genera (Miller *et al.,* 1992). They are motile with tumbling or slightly rotating fashion, best expressed at 20-25^{0}C. The cells are sometimes almost coccoid, occurring singly or in short chains, often presenting 'V' shape. In old and rough cultures, 6-20 μ filaments may develop (Sutherland, and Porrit, 1997). When cultures grown on a clear medium, such as tryptic soy agar, are viewed under magnification using obliquely transmitted light as described by Henry (1933), colonies of *L. monocytogenes* appear bluish-green and have a finely textured surface. Colonies will vary in diameter between 0.3 and 1.5mm, depending on the number of colonies present on the agar surface (Ryser and Marth, 1991).

2.2 Biochemical Characteristics of *Listeria*

Listeria spp. can be identified according to the biochemical test listed in Tables 2.1. The identification of *Listeria* species based on a limited number of biochemical test

Table 2.1: Differentiation of *L. monocytogenes* from other species of *Listeria.*

	Listeria					
	monocytogenes	*ivanovii*	*seeligeri*	*innocua*	*grayi*	*welshimeri*
B-haemolysis	+	+	+	-	-	-
CAMP test						
S. aureus	+	-	+[1]	-	-	-
R.. equi	-	+	-	-	-	-
Nitrate reduction	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+
Acid from						
Mannitol	-	-	-	-	+	-
Rhamnose	+	-	-	+/-	-	+/-
Xylose	-	+	+	-	-	+
Hippurate hydrolysis	+	+		+	-	

[1] Weak reaction only

(Source: Varnam and Evans, 1991)