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THE INCIDENCE OF PROTEOLYTIC PSEUDOMONAS SPECIES ASSOCIATED WITH GROUND BEEF WITH REGARDS TO THEIR SPOILAGE EFFECT

(With 5 Tables)

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(Received at 10/9/2008)

نسبة تواجد ميكروبات السودوموناس المحللة للبروتين في اللحم البقري المفروم مع الإشارة إلي تأثيرها للفساد

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في دراسة لتحديد مدي وجود ميكروبات السودوموناس في اللحم البقري المفروم تم فحص خمسون عينة طازجة والتي تم جمعها عشوائيا من محلات الجزارة بمدينة بورسعيد بهدف عد وعزل وتصنيف بكتريا السودوموناس المحبة للبرودة بالاضافة لتحديد العترات التي لها القدرة علي تحلل البروتين. اظهرت النتائج ان نسبة العينات الايجابية لبكتريا السودوموناس المحبة للبرودة والمحللة للبروتين كانت ١٠٠% (٥٠) لكل منهما, بينما كان متوسط العد الكلي لبكتريا السودوموناس المحبة للبرودة والمحللة للبروتين ٧,٢ X ١٠^{٤} ± ١,٧ X ١٠^{٤} و ١,٣ X ١٠^{٤} ± ١,٨ X ١٠^{٣} خلية/ جرام في اللحم البقري المفروم علي التوالي. تم عد وتصنيف عترات بكتريا السودوموناس المحبة للبرودة المعزولة فوجد أن عدد العترات كان ١٣٩ في اللحم البقري المفروم, بينما كانت الانواع المعزولة من جميع العينات هي سودوموناس فراجاي, سودوموناس ليوندينسز, سودوموناس فلوريسينز وسودوموناس بوتيدا بنسبة ٤٢,٤٥ % (٥٩)، ٢٣,٧٤ % (٣٣)، ١٧,٩٩ % (٢٥)، ١٥,٨٣ % (٢٢) علي التوالي, بينتما العترات القادرة علي تحلل البروتين كانت نسبة ٧١,٢٢% (٩٩). وبدراسة قدرة العترات علي تحلل البروتين وجد ان متوسط النشاط الانزيمي لكل من سودوموناس فراجاي, سودوموناس ليوندينسز, سودوموناس فلوريسينز كان ٥,٥٠ ± ٠,٧٠٠, ١,٦٠ ± ٠,٣٦١ , ٣,٦٩ ± ٠,٨٥٥ علي التوالي , بينما لم تظهر قدرة لميكروب سودوموناس بوتيدا. تم مناقشة الاهمية الصحية والاقتصادية لهذه العترات المعزولة في العينات موضع الدراسة.

SUMMARY

Fifty fresh samples of ground beef were randomly purchased from butchers shops from Port-Said city. The samples were examined for enumeration of the total psychrotrophic and proteolytic *Pseudomonas* counts with studying the proteolytic activity of the isolated strains. The incidence of positive samples for total psychrotrophic and proteolytic *Pseudomonas* were 100% (n=50) for each, while the mean values of the total psychrotrophic and proteolytic *Pseudomonas* counts were $7.2 \times 10^4 \pm 1.7 \times 10^4$ and $1.3 \times 10^4 \pm 1.8 \times 10^3$CFU/g of ground beef respectively. Most of total psychrotrophic and proteolytic *Pseudomonas* counts ranged from 10^3 to $<10^6$ CFU/g of ground beef. 139 psychrotrophic *Pseudomonas* strains isolated from the examined samples were identified as *Pseudomonas fragi* (Cluster II), *Pseudomonas lundensis* (Cluster IV), *Pseudomonas fluorescens* (Cluster II) and *Pseudomonas putida* (Cluster II) with an incidence of 59 (42.45%), 33 (23.74%), 25 (17.99%) and 22 (15.83%) respectively while the 99 proteolytic strains constituted 50 (50.51%), 29 (29.29%), 20 (20.20%) and 0.0 (0.00%) for the aforesaid strains respectively. The proteolytic activity of the isolates in the examined samples were 5.50 ± 0.700, 1.60 ± 0.361 and 3.69 ± 0.855 for *Pseudomonas fragi* (Cluster II), *Pseudomonas lundensis* (Cluster IV) and *Pseudomonas fluorescens* (Cluster II) while that of *Pseudomonas putida* (Cluster II) could not be detected. The public health and economic significance of isolated strains were discussed.

Key words: *Pseudomonas, meat products, ground beef.*

INTRODUCTION

Meat is one of the most perishable foods owing to its palatability, abundance of important nutrients of highly nutritive value and high water content therefore its composition is ideal for the growth and activity of a wide range of spoilage bacteria (Labadie, 1999; Gram *et al.*, 2002; Mayer *et al.*, 2003).

A very heterogeneous bacterial flora can colonize the meat surface through different stages involving adsorption to the meat surface (Chung *et al.*, 1989) by glycocalex (Costerson *et al.*, 1981). Thus meat tissue surface carry considerable bacterial loads (Upmann *et al.*, 2000). These bacteria are distributed throughout the entire product during grinding and mixing processes used in fabrication of ground meat (Rice *et al.*, 1997). The composition of the minced meat spoilage flora is

greatly influenced by the storage conditions such as temperature and type of packaging (Tsigarida and Nychas, 2001; Ercolini, 2004).

The principle causative agents responsible for spoilage of fresh meat products during aerobic storage are several *Pseudomonas* species (Widders *et al.,* 1995; Borch *et al.,* 1996; Gill, 2003) which represent the most psychrotrophic bacteria (Gill and Newton, 1982) and involve members of *Pseudomonas fluorescent* group and psychrotrophic *P. fragi, P. lundensis and P. putida* (Gill, 2003):

Genus *Pseudomonas* is ubiquitous microorganism widely distributed in nature and can use a variety of non-carbohydrates compounds for energy nature. They are Gram –ve, catalse +ve, rods shaped, non-spore forming and motile by one or several polar flagella (Krieg, 1984; Gennari and Dragotto, 1992).

They have a metabolic diversity and ability to grow to high number during refrigerated storage (Gennari and Dragotto, 1992), depend upon extracellular and/or cell associated heat-stable extracellular proteases (highly proteolytic) enzyme systems to liberate protein bound amino acids for assimilation and metabolic processes (Cousin, 1982; Chróst, 1991) leading to biological changes in the composition of meat and its products (Gill and Newton, 1982). These enzymes remain active even following thermal processing steps that can destroy the organisms producing theses enzymes (Cousin, 1982; Sorhaug and Stepaniak, 1997).

These types of bacteria cannot assimilate proteins directly (Payne and Smith, 1994) but firstly has the capability for glucose and amino acid degradation and utilization for its activity even at refrigerated temperature until the release of undesired volatile nitrogenous metabolites such as ammonia and dimethylsulfide (Lerke *et al.,* 1967; Stanbridge and Davies, 1998; Koutsoumanis *et al.,* 2006).

Some species of *Pseudomonas* such as *P. fragi* cause an increase in the water soluble and non protein nitrogen content of muscle and a decrease in both the salt-soluble and insoluble protein content (Borton, *et al.,* 1970) causing certain metabolic changes (Tarrant, *et al.,* 1971) and deterioration in quality of meat consequently organoleptic changes in appearance and odor during prolonged storage (Farber and Idziak, 1984). Some species like *P. fragi and P. fluorescence* cause fruity and putrid odorous beef (Dainty *et al.,* 1989; Ryan and Ray, 2004). Also *P. fragi* have deleterious effect on the color of meat stored at 1°C resulting in a green and slimy appearance (Bala *et al.,* 1977). Also a significant number of *Pseudomonas* species can produce exopolysaccharides that are known as slime layered which contribute to the surface make the products undesirable for human consumption (Jay,

2000) leading to lowering the shelf-life of refrigerated meat (Tarrant, *et al.*, 1971) as a result of the growth of microorganisms to unacceptable levels (Jay, 2000).

Besides these bacterial spoilage cause a significant economic losses for the meat industry (Cousin, 1982). Some *Pseudomonas* species has been recognized as an infectious agent transmitted by food and water affecting primarily immunecompromised people and those suffering from cystic fibrosis (Morais, *et al.*, 1997) leads to several outbreaks of food poisoning (Pererra *et al.*, 1977). On the other hand *Pseudomonas fluorescence* can also enhance the growth of nonpathogenic and pathogenic bacteria (Gabriel-Piette and Idziak, 1991).

The purpose of this study aimed to determine psychrophilic *Pseudomonas* count and its proteolytic activity in ground beef as an indication for pre-spoilage of ground meat as well as its keeping quality.

MATERIALS and METHODS

1: Samples collection:

A total of 50 random samples of fresh ground meat (200 g each) were purchased from Port-Said butcher's shops. Each individual sample was placed separately into sealed sterile plastic bag, thoroughly identified and delivered to the laboratory in a refrigerated container. All specimens were processed within 4 hours of collection for counting, isolation and identification of psychrotrophic *Pseudomonas* with studying of their proteolytic activity.

2: Bacteriological examination:

2-1: Enumeration of psychrotrophic *Pseudomonas* species:

2-1-1: Preparation and enrichment of samples:

A representative 25 g of each ground meat sample were taken aseptically and homogenized by blending at 10,000-12,000 rpm for 2 minutes in 225 ml of 0.1 % peptone water with salt (Na Cl, 0.85% "wt/vol"). Then tenfold serial dilution was prepared using 0.1 % peptone water till dilution 10^6 according to Mead and Adams (1977).

2-1-2: Isolation and enumeration of psychrotrophic *Pseudomonas* species:

0.1 ml of each dilution of meat homogenate was spread on the surface of a separate, marked petri plate of CFC (cetramid, fucidin cephaloridine) agar media. Immediately sample dilutions were spread thoroughly and uniformly allover the solid media. All plates were inverted and incubated at 20°C for 2 days for screening of psychrotrophic *Pseudomonas* species. Plates containing 30-300 colonies

were counted and the *Pseudomonas* count as number of organisms/g of ground meat was calculated according to Harrigan and McCance (1966) and Mead and Adams (1977).

2-1-3: Purification of the isolates:

Suspected colonies were purified by streaking onto slope of nutrient agar and incubated at 4°C for 3-5 days according to Harrigan and McCance (1966) and Mead and Adams (1977) for morphological and biochemical identification of the isolates and proteolytic activity screening of each isolates

3: Morphological and biochemical identification of the isolates:

The isolates were morphologically and biochemically identified by Gram stain, oxidase test, catalase test, motility, carbohydrates fermentation and other biochemical tests according to Stanier *et al.* (1966), Sneath and Sokal (1973), Molin and Ternstrom (1982) Cowan, *et al.* (1993) and Palleroni (1993).

4: Enumeration of proteolytic *Pseudomonas* species:

Thoroughly and uniformly another 0.1 ml of each dilution of meat homogenate was spread on the surface of a separate, marked petri plates of plate count agar "PCA" containing 1% skim milk powder (skim milk agar). All plates were inverted and incubated at 30°C for 72 h. Colonies showing proteolytic activity were counted and recorded and the proteolytic *Pseudomonas* count as number of organisms/g of ground meat was calculated according to Marshall (1993).

5: Screening of proteolytic activity:

Single *Pseudomonas* colonies were streaked on plate count agar "PCA" containing 1% skim milk powder (skim milk agar) for screening the production of extracellular proteolytic enzymes. Then the plates were incubation at 30°C for 72 h. Plates were flooded by 1 N HCl for observation of clearance zones formed by protease positive strains. For each isolate, clearance zones diameter to the colony diameter was calculated. The assay was repeated at least three times and the mean ratios and standard deviation were reported according to Mead and Adams (1977), Frank *et al.* (1992) and Vanderzant and Splittstoesser (1992).

6- Statistical methods:

Minimum, maximum, mean, standard deviation and standard error of mean were used to describe data.

These tests were analysed on a compatible personal computer using the Statistical Package for Social scientists (SPSS) for windows 12.0 (SPSS Inc., Chicago, IL, and USA).

RESULTS

Table 1: Statistical analytical results of the total psychrotrophic and proteolytic *Pseudomonas* counts (CFU/g) recovered from ground beef samples.

		Total psychrotrophic *Pseudomonas*	Proteolytic *Pseudomonas*
No. of samples		50	50
Positive samples	No.	50	50
	%	100	100
Statistics of count	Min.	4.0×10^2	3.0×10^2
	Max.	5.2×10^5	4.5×10^4
	Mean	7.2×10^4	1.3×10^4
	S.E.	1.7×10^4	1.8×10^3
	S.D.	1.2×10^5	1.3×10^4

Min. = Minimum. Max. =Maximum. SE=Standard Error SD = Standard Deviation
*Significant at $P < 0.05$ and $P < 0.01$ using t-test

Table 2: Frequency distribution of the examined ground beef based on their total psychrotrophic and proteolytic *Pseudomonas* counts (n=50)

Count range	Total psychrotrophic Pseudomonas		Proteolytic Pseudomonas	
	No.	%	No.	%
$<10^3$	2	4.00	3	6.00
10^3 - $<10^4$	19	38.00	24	48.00
10^4 - $<10^5$	18	36.00	23	46.00
10^5 - $<10^6$	11	22.00	0.00	0.00
Total	50.00	100.00	50.00	50.00

Table 3: Incidence of psychrotrophic and proteolytic *Pseudomonas* species recovered from ground beef.

Pseudomonas species	Psychrotrophic *Pseudomonas*		Proteolytic *Pseudomonas*	
	No.	%	No.	%
Pseudomonas fragi (Cluster II)	59	42.45	50	50.51
Pseudomonas lundensis (Cluster IV)	33	23.74	29	29.29
Pseudomonas fluorescens (Cluster I)	25	17.99	20	20.20
Pseudomonas putida (Cluster II)	22	15.83	0	00.00
Total	139	100.00	99	100.00

Table 4: Incidence of proteolytic activity grade of *Pseudomonas* species recovered from ground beef.

Pseudomonas species	Proteolytic Activity Grade						Total	
	-ve		+ve					
			Grade I		Grade II			
	No.	%	No.	%	No.	%	No.	%
1-*Pseudomonas fragi* (Cluster II)	-----	-----	-----	-----	59	42.45	59	42.45
2-*Pseudomonas lundensis* (Cluster IV)	-----	-----	33	23.74	-----	-----	33	23.74
3-*Pseudomonas fluorescens* (Cluster II)	-----	-----	14	10.07	11	7.91	25	17.99
4-*Pseudomonas putida* (Cluster II)	22	15.83	-----	-----	-----	-----	22	15.83
Total	22	15.83	47	33.81	70	50.36	139	100.0

-ve = No visible halo, + = 1-2 mm visible proteolysis,
++ = More than 2 mm visible proteolysis from the margin of colony.

Table 5: Statistical analytical results of the proteases activity of the isolated *Pseudomonas* species recovered from ground beef samples.

	Isolated strain			
	Pseudomonas fragi (Cluster II)	*Pseudomonas lundensis* (Cluster IV)	*Pseudomonas fluorescens* (Cluster II)	*Pseudomonas putida* (Cluster II)
No. of samples	59	33	25	22
Min.	3.50	0.90	1.70	ND
Max.	7.00	2.00	5.00	ND
Mean	5.50	1.60	3.69	ND
S.E.	0.091	0.063	0.171	ND
S.D.	0.700	0.361	0.855	ND

ND = not detected
Proteases activity ± standard deviations are reported as: Mean ration reflecting proteases activity = clearance zone diameter/ colony size

DISCUSSION

Many flora of spoilage bacteria have an effect on the shelf life of refrigerated food products. The main flora responsible for spoilage of fresh meat products during aerobic storage belong to genus *Pseudomonas* (Widders *et al.*, 1995). This genus include more than 140 species and represents the most psychrotrophic bacteria which are highly

proteolytic and/or strong lipolytic and lead to biological changes in the composition of meat and meat products particularly at low temperature (Gill and Newton 1982).

The results given in Table (1) revealed that the incidence of positive samples for total psychrotrophic and proteolytic *Pseudomonas* were 100% for each, while the mean values of the total psychrotrophic and proteolytic *Pseudomonas* counts were $7.2 \times 10^4 \pm 1.7 \times 10^4$ and $1.3 \times 10^4 \pm 1.8 \times 10^3$ CFU/g of ground beef respectively. These results were lower than the results recorded by Mayer, *et al.* (2003) and Ercolini, *et al.* (2007) but higher than the results recorded by Widders, *et al.* (1995) and Ercolini, *et al.* (2006). The high figures of our results may be attributed to the initial microbial loads of the samples (Emswiler, *et al.*, 1976 and Upmann *et al.*, 2000) and to the variation in the time/temperature storage condition (Ercolini, *et al.*, 2006), pH, O_2 availability and other bacterial flora (Gram, *et al.*, 2002).

The frequency distribution of the total psychrotrophic and proteolytic *Pseudomonas* counts of the examined ground beef samples presented in Table (2) showed that most of the total psychrotrophic and proteolytic *Pseudomonas* counts within the range of 10^3 - $<10^6$ and 10^3 - $<10^5$ CFU/g with an incidence 96% and 94 % respectively. Meanwhile 4% of total psychrotrophic counts and 6% of proteolytic *Pseudomonas* counts were less than $<10^3$ CFU/g in the examined samples. At these levels of no organoleptic changes and no sings of spoilage in the examined samples could be detected which agree with the results recorded by Jay (2000) and Eriksson *et al.* (1995) who reported that the signs of spoilage appear in counts more than 10^6 CFU/g food.

The obtained results in Table (3) revealed that 139 psychrotrophic and 99 proteolytic *Pseudomonas* isolates were recovered from the examined ground beef samples. The psychrotrophic *Pseudomonas* isolates were identified as *Pseudomonas fragi* (Cluster II), *Pseudomonas lundensis* (Cluster IV), *Pseudomonas fluorescens* (Cluster II) and *Pseudomonas putida* (Cluster II) with an incidence of 42.45%, 23.74%, 17.99% and 15.83% respectively. While the proteolytic *Pseudomonas* isolates were identified as *Pseudomonas fragi* (Cluster II), *Pseudomonas lundensis* (Cluster IV), *Pseudomonas fluorescens* (Cluster II) and *Pseudomonas putida* (Cluster II) with an incidence of 50.51%, 29%, 20.20% and 0% respectively.

The predominant of *Pseudomonas fragi* (Cluster II) over *Pseudomonas lundensis* (Cluster IV) and *Pseudomonas fluorescens* (Cluster II) is due to its ability to metabolize creatine and creatinine under aerobic conditions (Drosinos and Board, 1994) and the shorter of

its lag phase than the others strain (Gennari and Dragotto, 1992). Thus *Pseudomonas fragi* (Cluster II) accepted as the principle aerobic gram negative spoilage organism at chill temperatures (Banks and Board, 1983), while *Pseudomonas lundensis* (Cluster IV) and *Pseudomonas fluorescens* (Cluster II) constitute a significant part of the spoilage microflora on chill meat under aerobic conditions (Prieto *et al.*, 1992). On the other hand *Pseudomonas putida* (Cluster II) has ability to degrade toluene (Marqués and Ramos, 1993) needed for the activity of spoilage bacteria. The variation in the number of *Pseudomonas fragi* (Cluster II), *Pseudomonas lundensis* (Cluster IV) and *Pseudomonas fluorescens* (Cluster II) between psychrotrophic and proteolytic *Pseudomonas* was attributed to the difference in the media and temperature of incubation (Gram *et al.*, 2002).

The results reported in Table (4) showed that 22 (15.83%) of the isolated *Pseudomonas* strains showed no proteolytic activity and were identified as P. putida, while 33 (23.74%) and 14 (10.07%) of the examined isolates were of Grade I and were identified as *Pseudomonas lundensis* (Cluster IV) and *Pseudomonas fluorescens* respectively. On the other hand, *Pseudomonas fragi* (42.45%) and some strains of *Pseudomonas fluorescens* (7.91%) were of Grade II.

Regarding proteases activity of the proteolytic strains in Table (5), the mean values of the proteases activity of *Pseudomonas fragi* (Cluster II), *Pseudomonas lundensis* (Cluster IV) and *Pseudomonas fluorescens* (Cluster II) were 5.50 ± 0.700, 1.60 ± 0.361 and 3.69 ± 0.855 respectively. These results in addition to the count levels in Table (2) agree with the results reported by Cousin (1982) who found that proteases enzymes have been detected at psychrotrophic counts of 10^3 - $<10^6$ CFU/g food.

Pseudomonas fragi (Cluster II) has the highest proteolytic activity followed by *Pseudomonas fluorescens* (Cluster II) due to its ability to produce extracellular proteases in the late lag or early stationary phases of the bacterial growth (Kohlmann *et al.*, 1991 and Widders *et al.*, 1995).

Psychrotrophic *Pseudomonas* grows at refrigerated storage temperature with a maximum growth within 3-4 days and detection of heat resistant proteases at counts of 10^3-10^6 CFU/g, (Cousin, 1982). Besides its ability to make unacceptable changes in the meat and meat products (Gram *et al.*, 2002) and biofilms formation (Ryan and Ray, 2004). Therefore unsafe meat and meat products with lowering keeping quality will be produced. It is recommended that strict hygienic control measures should be applied before, during and after slaughtering, with a

rapid and accurate detection system for microbial spoilage, good time/temperature storage besides prevention the cross and secondary contamination. Also strictly hygienic measurement for prevention and removal the source of pollution and biofilms with the control of the intrinsic and extrinsic ecological factors of meat ecosystem such as pH, meat surface, O_2 availability and temperature will produce safe meat and meat products for the consumers with high keeping quality.

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