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Effect of various degrees of temperature on enzymes secreted by *Pseudomonas* species isolated from raw milk

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Abstract

The refrigerated storage of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for growth of psychrotolerant bacteria. These bacteria, mainly belonging to the genus *Pseudomonas*, they are capable of producing thermo resistant extracellular proteases and lipases, which can cause spoilage and structural defects in pasteurized and ultra-high-temperature-treated milk and milk products. Sixty raw cow's and buffalo's milk samples (30 of each) were collected from different shops at Assiut city, Egypt and examined using *pseudomonas* selective media. The obtained results of isolation and identification revealed that *pseudomonas* spp. was found in 40 % and 50 % of the examined cow's and buffalo's milk samples respectively. In positive samples of the examined milk; ***P. aeruginosa, P. fluorescens, P. putida, P. cepacia*** **and** ***P. stutzeri*** were detected in percentages of 25, 16.7, 41.6, 8.3 and 8.3% in cow's milk and in 13.3, 13.3, 40, 26.7 and 6.7 of buffalo's milk respectively. The growth of *pseudomonas* spp. in milk samples stored at 4 ºC increased continuously till the day 7 of storage, and at the 14th day *pseudomonas* spp. isolates were completely destroyed. The proteolysis and lipolysis enzymes activity were positive also till the 7th day of storage and disappear at the 14th day. However growth of *pseudomonas* spp. and the activity of proteolysis and lipolysis enzymes in milk samples stored at -18 ºC were continuously occur in an increasing proportion till the 14th day of storage.

Key words: *lipolytic, proteolytic enzymes and raw milk*

Introduction

Refrigeration in tropical countries has become essential to maintain the wholesomeness of milk. The refrigeration in farms and in processing plants has considerably improved the quality of raw milk and of dairy products. Unfortunately, the current practices for the collection and refrigerated storage of the raw milk favored the growth of psychrotrophic bacteria, regardless of their optimal growth temperature *Pseudomonas* spp. are the most common organisms in raw or pasteurized milk at the time of the spoilage **(Mc Phee and Griffiths, 2002)**. *Pseudomonas* species like *P. fluorescens*, *P. putida*, *P. fragi, P. putrefaciens*, and less frequently *P. aeruginosa* constitute the predominant microorganisms limiting the shelf life of processed fluid milk **(Gilmour and Rowe, 1990)**. Besides their rapid growth in refrigerated milk, *Pseudomonas* species produce heat stable extracellular proteases and lipases. Proteolytic and lipolytic activities of the psychrotrophs in general and Pseudomonas species in particular are valuable tools for the detection of spoilage of refrigerated foods and in assessing the shelf life of the foods. Lipases degrade the milk fat, causing rancid, soapy, and occasional bitter off-flavors through the formation of medium-chain fatty acids. Proteases that degrade casein cause a gray color, bitter off-flavors, and gelation of ultra high-temperature (UHT) products **(Datta and**

Deeth, 2001). Psychrotolerant bacteria have become more important for the shelf life of heat-treated dairy products because of the development of these bacteria during prolonged refrigerated storage of raw milk in the farm and at the dairy plant.

The combination of a longer storage time and a lower temperature creates a selective advantage for psychrotolerant bacteria, especially *Pseudomonas* members that enter raw milk via biofilms in the milk tanks, contaminated water, and soil **(Simo˜es *et al.*, 2009).** These *pseudomonads* are able to outgrow other bacteria, such as members of the *Aeromonas, Listeria, Staphylococcus*, and *Enterococcus* genera and the family *Enterobacteriaceae*, thus becoming the predominant microbiota in raw milk **(Sørhaug and Stepaniak, 1997)** and constituting up to 70 to 90% of the psychrotrophic raw milk microbiota **(Adams *et al.*, 1975).** Even though they are easily inactivated through pasteurization or UHT treatment, their heat-resistant enzymes persist upon processing of the milk **(Chen *et al.*, 2003).**

Consequent to the increasing economic constraints in the milk industry there is a demand for a method, which will allow longer storage of milk prior to pasteurization, without significant risk of subsequent detrimental effects **(Kumaresan *et al.*, 2007).** As *Pseudomonadales* play an important role in milk spoilage after long periods of cold incubation, more sensitive and efficient methods to evaluate the bacterial quality of raw milk are required. The present study firstly aimed to investigate the prevalence of, *Pseudomonas* spp. in raw cow's and buffalo's milk samples sailed in different shops in Assiut city, Assiut Governorate, Egypt and secondly to type the detected species and study the effect of different storage temperature on their growth and ability of producing proteolysis and lipolysis enzymes.

This study aimed to investigate the prevalence of pseudomonas spp. In raw milk samples, type the detected species and study the effect of different storage temperatures on their growth and ability of producing proteolysis and lipolysis enzymes.

Material and Method

A- Collection and Preparation of samples.

Cow's and buffalo's raw milk samples (30 samples of each) were collected from different localities at Assiut city, Assiut Governorate, Egypt. Each sample (100 ml) was mixed and prepared according to **A.P.H.A. (1992).**

B- Isolation and identification of *pseudomonas* spp.

1ml of the previously prepared samples was mixed with 9 ml of pseudomonas enriched broth. All enriched samples were incubated for 24-48 hrs. at 37°C. Loopfull from enriched broth was streaked *onto pseudomonas* selective which agar plates were incubated for 24-48 hrs. at 37°C and samples of no growth were incubated for another 48 hrs **(Collins, 1996).** The typical colonies were examined microscopically One colony from morphological typed samples was picked per plate and those showing bacteria morphologically similar to the genus *pseudomonas* underwent to diagnostic tests according to Meyer *et al.* (2002).

*** PH value: (A.P.H.A., 1992)**

It was detected by using electrical digital pH meter (an Orion Model).

Normal PH for milk 6.7

C- Proteolysis activity:

Using standard plate count agar with 10% added skim milk according to method recommended by **Harrigan and MacCance (1976).**

D- Lipolysis activity.

Using spirit blue agar according to method recommended by **Harrigan and MacCance (1976)**

E- Effect of different temp. storage on proteolytic and lipolytic activity of pseudomonas.

Pasteurized milk inoculated with a suspension of 24 hours incubation of *pseudomonas* strain secreted protease and lipase enzyme at a concentration of 10^5 and 10^7 cfu /ml. The samples were stored at 4°C and -18°C and analyzed and evaluated for growth and proteolysis and lipolysis activity on days 0, 3, 5, 7 and 14 according to **Kumaresan, *et al.* (2007).**

Results

The obtained results of isolation and identification revealed that *pseudomonas* spp. was found in 40 % and 50 % of the examined cow's and buffalo's milk samples, respectively (table 1).Findings of the prevalence of *psudomonas* spp. in the examined milk samples are illustrated in table (2). Five species of *pseudomonas* were recovered from the examined raw milk samples in this study which are: *P. aerugenosa*, *P. fluorescences*, *P. putida*, *P. cepacia* and *P. stutzeri* with incidence of 3 (25%), 2 (16.66%), 5 (41.67%), 1 (8.33%), 1 (8.33%) and 2 (13.33%), 2 (13.33%), 6 (40 %), 4 (26.67%), 1 (6.67%) in positive samples of cow's and buffaloes' milk respectively. Studying the effect of storage temperature on growth, proteolysis and lipolysis enzymes activities of *pseudomonas* spp. are shown in Table (3).

Table 1. Prevalence of *psudomonas* spp. in the examined raw milk samples.

Type of sample	Number of examined samples	*Positive samples*	
		No.	%
Cow milk	30	12	40
Buffalo milk	30	15	50

N=60

Table 2. Types and incidence of the isolated *psudomonas* spp. that recovered from positive milk samples**.**

Psudomonas Spp.	Cow's milk No.: 12/30		Buffalo's milk No.: 15/30	
	Number of isolates	%*	Number of isolates	%
P. aeruginosa	3	25	2	13.33
P. fluorescens	2	16.7	2	13.33
P. putida	5	41.7	6	40
P. cepacia	1	8.3	4	26.7
P. stutzeri	1	8.3	1	6.7

No.: Number of positive milk samples.
Percentages calculated according to the No. of positive samples

Table 3. Effect of storage temperature on growth, proteolytic and lipolytic activity of *pseudomonas* spp.

Temp. of storage	Counts of *pseudomonas* (cfu/ml)					Proteolysis activity					Lipolysis activity				
	Incubation period in day														
	0	3	5	7	14	0	3	5	7	14	0	3	5	7	14
4°C	10^5	$2x10^8$	$8x10^9$	$1x10^{10}$	0	+	+	+	+	-	+	+	+	+	-
	10^7	$8x10^{10}$	$8x10^{11}$	$1x10^{12}$	0	+	+	+	+	-	+	+	+	+	-
-18°C	10^5	$9x10^6$	$8x10^7$	$4x10^9$	$2x10^{10}$	+	+	+	+	+	+	+	+	+	+
	10^7	$9x10^7$	$1x10^9$	$5x10^{10}$	$1x10^{13}$	+	+	+	+	+	+	+	+	+	+

+ (positive): presence of enzymes, - (negative): deterioration of the samples

Discussion

Microbial contaminations of raw milk become critical problem especially in the time between milking and reaching to consumers. *Pseudomonadaceae* are among the most important spoilage bacteria in raw milk. It constitutes up to 78% of psychrotropic microflora **(Muir *et al.,* 1979 and Vela, 1997)**. In the current study microbiological investigations of raw milk samples cultured onto *pseudomonas* selected media revealed that *pseudomonas* spp. were detected in 12 out of 30 examined cow's milk samples (40%) and in 15 out of 30 examined buffalo's milk samples (50%) (Table 1). Contamination of raw milk by *pseudomonas* is mainly from soil and water **(Zaki *et al.,* 1996).** Milkers, udder surface and teats were reported to be of minor significance in contamination of raw milk with *pseudomonas* due to their direct contact with raw milk and the predominance of other skin micro flora. Many investigators in other studies recorded various results for the incidence and prevalence of *pseudomonas* in raw milk. **(Eman, 1992; zaki *et al.,* 1996; Dinsmore *et al.,* 2001 and Ahmed, 2008).**

Pseudomonas can gain access to milk via manure, polluted water, dairy equipments and dairy workers **(Khalil, 1992).** Such microbes as other psychrotrophes can multiply in milk stone deposits on equipment surfaces during shutdown periods and contaminated milk **(Shah, 1994).** The obtained results confirmed that there were no controlled conditions during milk production in our dairy farms confirm absolute absence of milk contamination.

Five species of *pseudomonas* (*P. aerugenosa*, *P. fluorescences*, *P. putida*, *P. cepacia* and *P. stutzeri*) were detected in the examined raw milk samples in this study (table 2). *Pseudomonas putida* was superior to other species and present in higher percentage (41.6%) in the examined cow's and buffaloe's milk samples, which may be attributed to the contamination of these samples with dust particles during milking transportation and other handing processed. **Anzai *et al.,* (2000)** reported that *P. putida* are saprophytic soil bacteria.

Pseudomonas aeruginosa present in 25% and 13.3 % of the positive samples of examined cow's and buffalo's milk respectively (Table 2). Higher incidence of *P. aeruginosa* was recorded in examined milk samples **(Otte et al., 1978; Katana, 1981; Grover and Strinivason, 1988 and Eman, 1992)**. Moreover, **Haiadova and Iacova (1982)** stated that 24 strains of *P. aerugenosa* were isolated from 60 samples of pasteurized milk. In several studies, the existence of *P. aeruginosa* in raw milk samples were found ranging between 4 % and 27% **(Mickova *et al.,* 1989).** Dilek and Sanver in 2007 isolated *P. aerugenosa* in 33.3% of examined milk samples. Different cases of food poisoning outbreaks due to consumption of milk contaminated with *P. aeruginosa* were

recorded **(Ahmed et al., 1989 and Eman, 1992; Todar 2002 and Mahrous and Mousa, 2012).**

Pseudomonas fluorescens was isolated in 16.7 and 13.3 % of the positive samples of examined cow's and buffalo's milk respectively (table 2). The existence of *P. fluorescens* in raw milk in various studies was differing from 7%-83% *(***King, 1978; Milliere and Veillet-Pance., 1979 and Keskin and Ekmekc, 2007).**

Pseudomonas cepacia detected in 8.3 and 26.7 % of the positive samples of examined cow's and buffalo's milk respectively (table 2). The existence of *P. cepacia* in raw milk samples was ranging between 1.1% and 12 % **(Stainer, 1966; King., 1978; Cheung, *et al;* 1983 and Jooste *et al.,* 1988)**. Higher incidence of *P. cepacia* was recorded by **King (1978) and Keskin and Ekmekc, (2007).**

Regarding *P. stutzeri*, it was detected in 8.33 and 6.67 % of the positive samples of examined cow's and buffalo's milk respectively (table 2). The presence of these strains in milk and its products was considered as a possible indicator of fecal contamination **(Todar, 2002 and Mahrous and Mousa, 2012)**. *Pseudomonas stutzori* was isolated in higher number in summer season **(Uraz and veCitak, 1995).** Results in the present study were recorded in summer season which come in agreement with that previously mentioned by **Uraz and veCitak (1995).**

Studying the relationship between storage temperature and the growth of *pseudomonas* spp. in milk samples (Table 3) revealed that:

- The growth of *pseudomonas* spp. in milk samples stored at 4 ºC increased continuously till the day 7 of storage, and at the 14th day, *pseudomonas* spp. isolates were completely destroyed. The proteolysis and lipolysis enzymes secreted by *pseudomonas* were positive till the 7th day of storage and disappear at the 14th day (table 3).
- The growth (multiplication) of *pseudomonas* spp. in milk samples stored at -18 ºC was continuously traced in an increasing proportion till the 14th day of storage. The proteolysis and lipolysis enzymes activities were found also positive till the 14th day of storage (Table 3). These findings indicated that *pseudomonas* spp. grow well at very low temperatures (-18C$^{\circ}$) and survive for long periods in such conditions. **Barbano et al. (2006) and Hantis-Zecharov and Halpern (2007)** recorded that rapid cooling and cold storage of raw milk favor growth of psychrotrophic bacteria in milk. They become dominant micro flora during cold storage in milk, and their extra cellular enzymes particularly proteases and lipases contribute to the spoilage of milk products.

Conclusion and Recommendation

It could be concluded that *pseudomonas* spp. can survive in milk at different very low temperature and produce proteolysis and lipolysis enzymes. Contamination of milk with *pseudomonas* spp. is a risk factor in limiting milk stability and reducing its quality. Raw milk deteriorates in only few days even when stored under refrigeration temperature. Enumeration of *pseudomonas* spp. in raw milk gives a good evaluation of the farm hygiene. This knowledge is increasing the attention toward the way by which the restriction of these microorganisms must be done. Such as, the cow is even milked; pathogens in the surrounding environment can get into the cow's feed or water. During milking, bacteria on the inside or outside of the cow's udder can get into the milk. If the milking device (human or mechanical) hasn't been properly sanitized it may contaminate the raw milk. As dairy equipment and utensils constitute the major source of many types of psychrotrophic bacteria in milk, so special attention should be considered in their cleaning and sanitation to produce milk of low bacterial count or even completely free of psychrotrophic bacteria **(Sun, 2006).**

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