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The Evaluation of Ascorbic Acid on Growth and Proteolysis Activity of *Pseudomonas fluoresencens* Isolates from Refrigerate Raw Milk

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Abstract: Psychrotrophic Gram-negative bacteria such as *Pseudomonas* species, pose a significant spoilage problem in refrigerated raw milk and dairy products due to secretion of hydrolytic enzymes, especially lipases and proteases. This study evaluated the effect of ascorbic acid against proteolysis activity produced by *Pseudomonas fluoresencens* in refrigerate raw milk. Strains of *Pseudomonas fluorescens* isolated from raw milk. Samples of pasteurized milk were inoculated with these strains and stored 10 days at 7°C. All the isolates showed proteolysis activity. Different concentrations of ascorbic acid were tested and the protein content was determined. The growth of each isolate tested was decreased and the content of protein was also decreased in accordance with the bacterial count.

Key words: *Pseudomonas fluorescens*, proteolytic activity, ascorbic acid, raw milk, enzymes

INTRODUCTION

The refrigerated storage of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for growth of psychrotolerant bacteria. Griffiths *et al.* (1981) reported that 53% of the psychrotrophic bacteria of dairy origin were *Pseudomonas* species. These bacteria, mainly belonging to the genus *Pseudomonas* 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 (De Jonghe *et al.*, 2011). Degradation of milk components through various enzymatic activities associated with the contamination of dairy products by *Pseudomonas* sp. can reduce the shelf life of processed milk (Dogan and Boor, 2003). The most common specie of psychrotrophic bacteria found in milk is *Pseudomonas fluorescens*. Proteases produced by this organism are resistant to both UHT and pasteurization treatments. Moreover, the production of extracellular protease in *P. fluorescens* also associated with the high cell density that is typically encountered towards the end of the exponential phase of growth (McKellar, 1989). Current practices for the control of psychrotrophic growth or the level or activity of extracellular enzymes may involve thermization, prompt refngeration or additives such as CO_2, the quality of milk can be adversely affected by these processes (Suhren, 1989) showed that heating milk samples to 95°C for 8.45 min resulted in a residual heat-resistant proteolytic activity of 73%. The enzyme, however was quite heat-stable requiring 15 h at 62.8°C, 8 h at 71.4°C and 9 min at 121°C for complete inactivation (Mayerhofer *et al.*, 1973). Several physical and chemical methods were suggested. The reduction of enzyme activity by metal chelating agents was suggested by Barach and Adams (1977) another Manothermosonication Method by Vercet *et al.* (1997). The use of peroxide hydrogen is recommended by FAO/WHO as a standard method for retarding bacterial growth in raw milk during collection and transportation to dairy processing plants in situations when refrigeration of the raw milk is not feasible (CAC, 1991). The use of peroxide hydrogen as preservative in raw milk is prohibits in several countries. Ascorbic acid as antioxidant were used in beverage and foods. Aim in the present study to evaluate the effect of ascorbic acid on the and the proteolysis of *Pseudomonas fluorencenc* strains isolated from refrigerate raw milk.

MATERIALS AND METHODS

Sampling, isolation and characterization of isolates: Ten samples of raw milk were taken from different farm in the region of Oran, Algeria. Samples were taken under sterile conditions. The milk was stored during 10 days at 7°C until it was analyzed in the laboratory. The 0.1 mL of each sample after storage was poured onto nutrient agar

medium and incubated at 25°C for 72 h. Colonies with different morphologies were picked and subcultured to obtain pure cultures. An average of 10 colonies per farm was collected. Isolated colonies were subcultured at least four times. Isolates were rod cell, gram positive, catalase positive and oxidase positive were retained to tested. It identified as *Pseudomonas fluorescens* all isolates respond to the following physiological and biochemical tests.

Grew at 4°C but not at 41°C, indole negative, methyl red positive, urease positive, no glucose fermentation, production of fluorescent pigment on king B, no production of pigment pyocianin on king A and nitrate reductase positive.

Extracellular proteolytic enzymes: The production of extracellular proteolytic enzymes was determined on PCA, containing 1% skim milk powder (Harper *et al*., 1978). The plates were incubated at 25°C for 72 h. The presence of clear zones around the colonies were indicative of proteolysis. The presence of transparent zones around the spots was recorded as positive strains referring to protease production and subsequently flooded with 10% v/v acetic acid solution. Clear zone around the colonies after 1 min exposure were regarded as positive (Harrigan and McCance, 1976). For each isolate, clearance zones around two to five colonies were measured and the ratio of the clearance zone diameter to the colony diameter was calculated.

Study of ascorbic acid effect on the proteolytic activitie: The effect of ascorbic acid on proteolytic activity was investigated on PCA containing 1% skim milk powder and supplemented by different concentration of ascorbic (0.01, 0.05, 0.08 and 0.1%). The effect of ascorbic acid on lipolytic activity was determined in same medium used for the determination of extracellar lipolytic enzymes production, supplemented with different concentration of ascorbic acid (0.1, 0.2, 0.3 and 0.4%).

Protein content determination: The Kjeldahl Method was used do determined the total content of proteins. This chemical method determines the nitrogen content of milk. Protein is precipitated from milk by Trichloroacetic Acid (TCA). Solution which contains non protein nitrogen components of a sample is separated from protein precipitate by filtration. Nitrogen content of protein precipitate is determined as described by AOAC Official Method 998.06. Samples were pasteurized and inoculated by selected isolates. Control samples and treated samples were stored 10 days at 7°C.

RESULTS

The results showed a high percentage of the psychrotrophs bacteria in refrigerated raw milk belonging to *Pseudomonas* genus. The highest count was found in the sample of Farm 5, the lowest in the Farm 1. *Pseudomonas fluorescence* species was dominant among the *Pseudomonas* genus. They exceed 50% of the pseudomonas species pre-identified in the eight samples (Table 1).

All the isolates identified previously as *Pseudomonas fluorencens* species were showed proteolysis activities (Table 2). Some isolates exert a strong activity, respectively 4.4, 4.2 and 4.1 mm and the lowest activity was 1.9 mm were expressed by PFF22, PFF42 and PFF81. The isolates were expressed different proteolysis activity in same culture medium and similar conditions and also same number of cell in their initial inoculum. All the isolates were affected by the different concentrations of ascorbic acid. Both bacterial count and protein content in samples treaite were decreased after storage (Table 3). The results confirm that the ascorbic acid is not anti-quorum sensing agent but act by inhibiting growth of *Pseudomonas fluorencens*.

Table 1: Percentage of *Pseudomonas* species and of *Pseudomonas fluoresencens* isolates

	Farm							
Isolates	1	2	3	4	5	6	7	8
A	60	90	80	65	85	55	80	55
B	30	45	40	75	65	40	65	35

Table 2: Proteolytic activity of *Pseudomonas fluoresens* isolates in PCA contained respectly 0.02, 0.04 and 0.06% of ascorbic acid. Activity was expressed by inhibition zone (mm)

		Treated		
Isolates	Control	0.02	0.04	0.06
PFF11	4.2	2.9	1.4	0.8
PFF12	3.6	3.1	2.0	0.6
PFF13	2.8	2.2	0.9	0.5
PFF21	3.9	3.1	2.3	0.9
PFF22	1.9	1.2	0.7	0.4
PFF23	3.2	2.5	1.8	0.9
PFF31	4.4	3.6	2.3	1.2
PFF32	3.5	2.4	1.3	0.8
PFF33	2.9	2.1	1.4	0.9
PFF41	3.0	2.6	1.8	1.1
PFF42	1.9	1.4	0.9	0.6
PFF43	2.8	2.1	1.4	0.7
PFF51	4.1	3.5	2.3	1.1
PFF52	2.8	2.1	1.0	0.6
PFF53	2.7	1.9	1.2	0.6
PFF61	2.5	1.3	0.9	0.5
PFF62	2.3	1.7	1.1	0.7
PFF63	2.8	2.1	1.7	0.9
PFF71	3.1	2.7	2.1	1.4
PFF72	3.0	2.9	2.3	1.2
PFF73	2.9	1.7	1.2	0.9
PFF81	1.9	1.1	0.7	0.4
PFF82	2.2	1.6	1.1	0.7
PFF83	2.6	1.8	1.3	0.8

Table 3: Protein content (gram per 100 mL) and bacterial count (log_{10} ufc. per mL) in refrigerate pasteurized milk and inoculated by *Pseudomonas fluorescens* isolates without ascorbic acid (control) and treated samples with 0.1% ascorbic acid after storage 10 days at 7°C

Origine	Isolates	Control	Bacterial count	Treated	Bacterial count
Farm 1	PF1	1.0	8.5	2.9	4.1
Farm 2	PF2	1.4	8.3	3.0	5.2
Farm 3	PF3	0.9	7.2	2.8	4.8
Farm 4	PF4	1.4	7.8	2.9	4.5
Farm 5	PF5	1.7	8.8	2.3	5.4
Farm 6	PF6	1.8	8.1	2.7	5.3
Farm 7	PF7	1.3	7.2	2.4	4.2
Farm 8	PF8	1.0	7.7	2.5	3.8

DISCUSSION

Typically, 65-70% of the psychrotrophs isolated from raw milk are *Pseudomonas* species (Garcia *et al*., 1989; Griffiths *et al*., 1987). Ternstrom *et al*. (1993) were found that refrigerate raw milk was exclusively spoilt by Gram-negative bacteria, the majority of which were *Pseudomonas fluorescens*. In similar and study based on the API 20 NE results, Dogan and Boor (2003) were found that 51% of the isolates were identified as *P. fluorescens*. In the present study the percentage of Pseudomonas isolates were between 55-90% among the psychotropic bacteria screened and among Pseudomonals isolates 30-75% belonging to *Pseudomonas fluorescens*. *Pseudomonas fluorescens* has safe growth among *Pseudomonas* species. *P. fluorescens* strains are able to replicate by binary fission in approximately 12 h in raw milk at 4-6°C (Cousin, 1982). The growth conditions permitting protease production are variable and do not depend on the genus of the producing strain (Nicodeme *et al*., 2005). According to Griffiths (1989), the differences in enzyme activity could be due to differences in the rate of release from the cells. Early, Rawal (1978) found that the bactericidal action of ascorbic acid on *Psuedomonas aeruginosa* is causing by the alteration of cell surface. Liu *et al*. (2007) were conclude that the protease gene in *P. fluorescens* is regulated by the AHL-based quorum sensing system at a transcriptional level during the late exponential growth phase. Contradictorily, Pinto *et al*. (2010) were found that the proteolytic activity of *Pseudomonas fluorescens* 07A strain isolated from milk is not regulated by quorum sensing signals. The results confirm that the ascorbic acid is not anti-quorum sensing agent but act by inhibiting growth of *Pseudomonas fluorencens*. The addition of CO_2 in raw milk to avoid spoilage refrigerate milk has been shown to increase the lag phase of growth and decrease the growth rate of microorganisms (Martin *et al*., 2003). *Lactobacillus* sp. are relatively CO_2 resistant and their growth may be enhanced by a CO_2-enriched environment (Hendricks and Hotchkiss, 1997). Excessive growth of *Lactobacillus* sp. in raw milk may lead to spoilage or development of off-flavors due to fermentation.

CONCLUSION

The use of ascorbic acid in refrigerate raw milk to minimize spoilage of raw milk by *Pseudomonas fluorencens* can present many advantages because the ascorbic acid is recognized a safety food additive and don not modifying the composition of milk.

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