Identification of *Pseudomonas jessenii* and *Pseudomonas gessardii* as the most proteolytic *Pseudomonas* isolates in raw milk and their impact on stability of sterilized milk during storage

Details of some methods used in this study

Detection of the aprX gene

Presumptive *Pseudomonas* colonies were individually grown overnight in 5 ml of TSB at 25°C. DNA was extracted using the usual boiling method for 5 min and the extracted DNA was analyzed using agarose gel electrophoresis. The presence of the *apr*X gene, encoding for a known alkaline heat-resistant metalloprotease, was investigated using an *apr*X-PCR test. PCR amplification of the *apr*X gene was performed with the set of primers SM2F/SM3R. PCR conditions were 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min and finally, an elongation step of 72 °C for 5 min. The nucleotide sequences of the primers used are listed in Table S1. The amplicon products of approximately 800 bp were detected on a 1 % agarose gel by electrophoresis (Duong *et al.*2001; Marchand *et al.*2009).

Molecular identification

In order to confirm the allocation of the isolates to the genus *Pseudomonas*, 16S rRNA gene was amplified, using universal primers (McCabe *et al.* 1999) to generate an amplicon of approximately 800 bp. PCR conditions were 94 °C for 5 min (initial denaturation), 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, and a final extension step of 72 °C for 5 min. The obtained PCR products were sequenced with 16S forward primer using a 310 automatic DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The partial sequences of the 16S rRNA gene were BLASTed against the 16S rRNA GeneBank (NCBI) database. Since the analysis of 16S rRNA

does not differentiate adequately to permit resolution of *Pseudomonas* intrageneric relationships, *rpoD* and *gyrB* genes were also amplified and sequenced. PCR amplification of *rpoD* was performed with the set of primers described by Mulet et al. (2009). The cycling conditions included a denaturation period at 94 °C for 5 min followed by 30 cycles of amplification (denaturation was performed at 94 °C for 1 min, primer annealing was performed at 55 °C for 1 min, and primer extension was performed at 72 °C for 1.5 min). Finally, an elongation step was performed at 72 °C for 5 min. *GyrB* was amplified by the set of primers described by Yamamoto and Harayama (1998) with the following conditions: 2 min at 94 °C; 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 7 min. The obtained PCR products were sequenced using a 310 automatic DNA Sequencer. Putative identifications of the *Pseudomonas* isolates were obtained through a combination of BLAST against the NCBI database for *rpoD* and *gyrB* genes. The nucleotide sequences of the primers used are presented in Table S1.

Casein zymography

After identification of the *Pseudomonas* isolates, in order to determine the production of the heat-resistant protease by *Pseudomonas* strains, casein zymography was performed based on Dufour et al. (2008) and Marchand et al. (2009), with some modification. Briefly, UHT semi-skimmed milk samples were inoculated with approximately 10^4 CFU/ml of each representative strains separately and incubated at 7 °C for 72 h. Subsequently, inoculated milk samples were heated at 95 °C for 8 min and 45 s as described by Marchand et al. (2009) to select for the heat-stable proteases. Milk samples were cooled on ice immediately after heating. After centrifugation (12,000 rpm for 15 min at 4 °C), 10 µl of cell free supernatant was diluted in 10 µl of zymogram loading buffer (50 mM Tris–HCl, 10% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue (pH 6.8) and

loaded for electrophoresis in a 12.5 % SDS-polyacrylamide running gel with 0.1% w/v of sodium caseinate, overlaid with a 4% stacking gel under non reducing conditions. Finally, proteolytic activity was appeared as clear bands on a blue background after distaining by solution containing 10% acetic acid and 40% methanol.

Physico-chemical characterization of sterilized milk during storage

The analysis of sterilized milk samples were performed in triplicate after 1, 20, 40 and 60 days of storage. In order to confirm the absence of bacterial contamination, viable counts of each milk sample were enumerated on PCA before each analysis. The pH range of all sterilized milk samples during the storage was of 6.62 to 6.84. The stability of milk samples was determined by measuring the proteolysis during storage time, as described below.

Non-protein nitrogen and non-casein nitrogen contents

Milk samples were precipitated at pH 4.6 and filtered to obtain the soluble fraction of non casein nitrogen (NCN); and precipitated with 12% trichloroacetic acid (TCA) and filtered for the soluble fraction of non protein nitrogen (NPN). The NCN and NPN contents were determined using the Kjeldhal method according to IDF standard 20B (IDF, 2001). The experimental error was ± 0.1 g of nitrogen expressed in protein kg^{-1}.

Evaluation of the progress in protein hydrolysis

O-phthaldialdehyde/N-acetyl-L-cysteine (OPA/NAC) fluorometric assay was performed to measure free amino groups of milk. OPA and NAC solutions were prepared separately in methanol and water, respectively. OPA/NAC reagent was prepared freshly by mixing OPA, NAC, and borate buffer 0.1 M (pH 9.5) solutions with a volumetric ratio of 1:1:8, respectively. This reagent was stored in the dark at 4 °C for at least 4 h to eliminate the background fluorescence (Devi *et al.*

2015; Lochmann *et al.* 2004). Milk samples were diluted 500 fold in deionized water and 100 µl was loaded into the wells of a black optiplate-96 flat-bottom well microplate (31496 SPL, Korea). Then, 200 µl of OPA/NAC reagent was added into each well and the microplate was shaken at medium speed for 5 min. Fluorescence reading was performed at an excitation wavelength of 340 nm and an emission wavelength of 434 nm. The fraction of free amino groups was the relative fluorescence unit (RFU) of milk sample after treatment divided by their initial RFU (F/F0) (Morales, Romero, Jiménez-Pérez , 1995).

SDS-PAGE

In order to observe the progress of proteins hydrolysis during storage, SDS-PAGE was also performed under reducing conditions with a 12.5% (w/v) separation polyacrylamide gel overlaid with 4% (w/v) stacking gel. Samples were diluted in SDS–PAGE sample buffer, containing b-mercaptoethanol, heated at 95 °C for 5 min and loaded into the gel. Peptide bands on gel were visualized after staining with Coomassie blue R250 and overnight destaining.

Table S1. The nucleotide sequences of the primers used in this study

Primer	Primer Sequence	Amplified region	References
SM2F	AAATCGATAGCTTCAGCCAT	*aprX* gene	Marchand et al., 2009
SM3R	TTGAGGTTGATCTTCTGGTT		
p8FPL	AGTTTGATCCTGGCTCAG	16S rRNA gene	Mc Cabe et al., 1995
p806R	GGACTACCAGGGTATCTAAT		
PsEG30F	ATYGAAATCGCCAARCG	*rpoD* gene	Mulet et al., 2009
PsEG790R	CGGTTGATKTCCTTGA		
UP1S	GAAGTCATCATGACCGTTCTGCA	*gyrB* gene	Yamamoto and Harayama, 1995
UP2R	AGCAGGGTACGGATGTGCGAGCC		

Table S2. GenBank accession numbers of the gene sequences of type strains used in this study

Type Strains	**16S rRNA**	***rpoD***	***gyrB***
P. lundensis	AB021395.1	FN554479.1	FN554197.1
P. corrugata	D84012.1	AB039566.1	AB039460.1
P. chlororaphis	FJ652610.1	D86036.1	FJ652718.1
P. baetica	FM201274.3	FN678357.1	FM201278.1
P. helmanticensis	HG940537.1	HG940516.1	HG940517.1
P. proteolytica	AJ537603.1	FN554220.1	FN554505.1
P. gessardii	AF074384.1	FN554468.1	FN554186.1
P. fluorescens	DQ207731.2	D86033.1	D86016.1
P. aeruginosa	HE978271.1	AJ633568.1	AJ633104.1
P. brenneri	AF268968.1	FN554457.1	FN554176.1
P. fragi	AF094733.1	FN554466.1	DQ887266.1
P. mucidolens	D84017.1	AB039409.1	AB039546.1
P. jessenii	AF068259.1	FN554473.1	AM293562.1
P. moraviensis	AY970952.1	FN554490.1	FN554206.1
P. putida	D84020.1	AB039581.1	AB039451.1
P. parafulva	AB060132.1	FN554500.1	FN554216.1
P. marginalis	AB021401.1	AB039575.1	AB039448.1
P. brassicacearum	AF100321.1	AM084334.1	AM084675.1
C. japonicus Ueda107	CP000934.1	CP000934.1	CP000934.1

Table S3. Characterization of presumptive *Pseudomonas* isolates

Sample Number	Isolate Code	*aprX* gene	Clear zone diameter (cm, mean of three replicates)	Pigment production on TSA at 7 °C
1	a	+	1.0	-
1	d	+	0.3	-
2	b	-	nd	-
2	c	+	1.1	-
2	d	+	1.0	-
2	e	+	1.0	-
3	c	+	0.8	-
3	d	+	0.1	-
3	e	+	0.2	--
4	a	-	nd	-
4	b	+	1.4	-
4	e	-	nd	-
5	c	-	nd	-
5	d	+	0.8	-
6	a	-	nd	Fluorescent green
6	c	+	0.4	Fluorescent green
7	a	+	1.2	Fluorescent green
7	b	-	nd	Fluorescent green
7	d	+	0.2	Fluorescent green
7	e	-	nd	Fluorescent green
8	a	+	1.4	Fluorescent green
8	d	-	nd	-
9	a	+	0.5	-
9	d	+	0.5	-
10	a	-	nd	-
10	c	+	1.0	Fluorescent green
10	d	-	nd	-
11	b	+	0.3	-
11	e	+	0.2	-
11	f	-	0.7	-
12	a	+	0.4	-
13	a	+	1.0	-
13	b	+	1.1	-
14	b	+	1.7	-
14	d	-	nd	-
15	e	+	0.7	-
15	f	-	nd	-
16	a	-	nd	-
16	b	+	0.2	-
16	e	-	nd	-
17	a	+	0.4	-
17	b	+	0.3	Fluorescent green
18	b	+	1.1	-
18	d	+	0.4	Fluorescent green
18	e	-	nd	-
19	a	-	nd	-
20	b	+	1.3	-
20	c	-	nd	-

20	d	-	nd	Fluorescent green
21	a	+	0.1	-
21	b	-	nd	-
21	c	+	1.0	Fluorescent green
22	d	-	nd	-
23	a	-	nd	-
23	b	-	nd	-
23	c	+	0.3	Fluorescent green
23	d	+	0.1	-
25	c	-	nd	-
25	f	-	nd	-
26	b	+	1.1	Fluorescent green
27	b	-	nd	-
27	d	+	0.1	Fluorescent green
27	e	+	0.1	-
28	a	-	nd	-
28	b	-	nd	-
29	e	+	0.6	-
29	f	+	0.6	-
29	b	+	1.2	-
30	c	+	0.7	-
30	d	+	1.2	-
30	e	+	1.3	-
31	a	-	nd	-
31	b	+	1.0	Fluorescent green
31	c	+	0.1	Fluorescent green
32	a	+	1.6	-
32	e	-	nd	-
33	b	+	1.3	-
33	c	+	1.0	-
33	d	+	1.0	-
33	e	-	nd	-
34	a	+	1.2	-
34	e	-	nd	-
35	a	+	0.7	Fluorescent green
35	d	+	0.9	Fluorescent green
36	b	-	nd	-
36	c	+	1.0	Fluorescent green
36	d	+	0.8	Fluorescent green
37	c	+	0.6	-
37	e	+	0.6	-
38	b	-	nd	-
38	d	+	1.0	Fluorescent green
39	a	+	0.3	-
40	a	-	nd	Orange
40	f	+	1.1	-
41	a	+	0.7	-
41	b	+	1.0	Fluorescent green
41	e	+	1.2	-
42	b	-	nd	-
42	a	+	0.6	-
43	b	-	nd	-
44	a	-	nd	-

44	b	+	1.7	-
45	c	+	1.2	-
45	d	+	1.2	-
45	e	-	nd	-
46	c	+	0.8	-
47	a	-	nd	-
47	c	+	0.8	-
47	e	-	nd	-
48	c	+	0.1	-
48	d	-	nd	-
49	c	-	nd	Fluorescent green
49	d	+	1.0	Fluorescent green
49	e	-	nd	-
50	b	+	0.7	-
51	a	+	0.7	-
51	b	+	1.0	Fluorescent green
51	f	-	nd	Fluorescent green
52	e	+	0.8	-
53	c	-	nd	Fluorescent green
54	c	+	1.3	Fluorescent green
54	d	+	0.7	Fluorescent green
55	f	+	0.7	Fluorescent green
56	a	+	1.0	Fluorescent green
56	e	+	1.1	-
57	a	-	nd	-
57	c	+	1.0	-
57	d	+	1.3	-
58	a	-	nd	-
59	c	+	1.7	-
59	d	+	0.9	Fluorescent green
59	f	+	1.3	Fluorescent green
60	a	+	0.1	-
60	b	+	1.4	-
60	d	-	nd	-
60	e	-	nd	-
61	b	+	1.1	-
61	e	-	nd	-
62	d	-	nd	-
63	a	-	nd	Fluorescent green
63	b	+	1.0	-
63	d	+	0.7	Fluorescent green
64	b	+	0.9	-
65	b	-	nd	-
66	a	+	1.0	Fluorescent green
66	e	-	nd	-
66	f	-	0.3	-
67	c	+	1.1	-
67	e	+	1.0	Fluorescent green
68	a	+	0.9	-
68	b	+	0.8	-
69	a	-	nd	-
69	b	-	nd	-
69	f	+	1.7	Fluorescent green

70	e	-	nd	-
71	b	+	1.4	-
71	c	+	1.4	-
71	d	-	nd	-
71	e	+	1.1	-
72	c	-	nd	-
73	a	+	1.0	-
73	d	-	nd	-
74	b	-	nd	-
74	f	+	1.0	-
75	a	+	0.8	Fluorescent green
75	b	+	0.8	Fluorescent green
75	d	+	1.0	Fluorescent green
75	e	+	1.0	-
76	d	-	nd	-
77	a	+	1.1	Fluorescent green
77	b	+	0.8	-
78	c	+	0.9	Fluorescent green
79	b	+	1.4	-
79	c	+	1.2	Fluorescent green
79	d	+	1.0	Fluorescent green
80	e	-	nd	Fluorescent green
81	a	+	0.8	-
81	d	+	1.3	Fluorescent green
82	e	-	nd	Fluorescent green
83	c	-	nd	Fluorescent green
84	f	+	1.1	Fluorescent green
84	a	-	nd	Orange
84	b	-	nd	Orange
85	a	+	0.9	-
85	c	+	1.0	-
85	d	-	nd	-
86	c	-	nd	-
87	d	+	0.5	-
88	e	+	0.5	-
89	a	-	nd	-
89	b	+	0.7	Fluorescent green
89	c	+	0.6	-
89	d	+	0.6	Fluorescent green
90	a	+	0.3	-
90	f	+	0.8	Fluorescent green
91	b	+	1.1	Fluorescent green
91	c	+	0.6	-
91	e	+	0.7	-
92	b	+	0.1	-
92	c	+	0.8	-
92	d	+	0.8	-
93	d	+	0.8	-
93	e	+	0.8	-
94	a	+	1.0	-
94	b	+	0.6	-
94	c	+	1.2	-
94	d	+	0.7	-

95	a	-	nd	Fluorescent green
95	b	+	0.9	Fluorescent green
96	e	+	1.0	-
97	d	+	0.2	-
97	e	+	0.6	-
98	a	-	nd	-
98	e	+	0.7	-
99	b	-	nd	-
99	c	+	0.7	-
99	d	-	nd	-
99	e	+	0.5	Fluorescent green
100	b	+	1.0	Fluorescent green
100	c	+	0.5	Fluorescent green

nd: Not determined

Table S4. GenBank accession numbers of the gene sequences obtained in this study

	***P. gessardii* 14b**	***P. gessardii* 32a**	***P. gessardii* 44b**	***P. jessenii* 59c**	***P. jessenii* 69f**
16S rRNA	MK715450	MK715466	MK715468	MK715470	MK715472
rpoD	MK773864	MK773865	MK773866	MK773867	MK773868
gyrB	MK764532	MK764533	MK764534	MK764535	MK764536

Figure S1. Destabilization and white sediments formation in sterilized milk samples after 60 days of storage.