Characterisation of the thermostable protease AprX in strains of *Pseudomonas fluorescens* and impact on the shelf-life of dairy products: preliminary results

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Abstract

Bacterial proteases are involved in food spoilage and shelf-life reduction. Among the bacterial proteases, a predominant role in spoilage of dairy products seems to be played by the thermostable metallo-protease AprX, which is produced by various strains of *Pseudomonas fluorescens*. Differences in AprX enzyme activity among different strains were highlighted, but the most proteolytic strains were not identified. In this study, the presence of the *aprX* gene was evaluated in 69 strains isolated from food matrices and 18 reference strains belonging to the *P. fluorescens* group, which had been previously typed by the multi locus sequence typing method. Subsequently, a subset of reference strains was inoculated in ultra-high temperature milk, and the expression of the *aprX* gene was evaluated at 22 and 6°C. On the same milk samples, the proteolytic activity was then evaluated through Azocasein and trinitrobenzenesulfonic acid solution assays. Finally, to assess the applicability of the former assay directly on dairy products the proteolityc activity was tested on industrial *ricotta* samples using the Azocasein assay. These results demonstrate the spread of *aprX* gene in most strains tested and the applicability of Azocasein assay to monitor the proteolytic activity in dairy products.

Introduction

Thermostable protease activity was indicated as one of the main factors responsible of dairy products spoilage, such as gelation of ultra-high temperature (UHT) milk, with consequences on the shelf-life and significant economic losses for the food industry. Proteases are predominantly active against the casein fraction, which causes gelation of UHT milk and/or the formation of bitter off-flavors. The gelation of UHT milk derives from the formation of complexes between the κ-casein and β-lactoglobulin that are denatured as a result of the heat treatment. Gelation is triggered by enzymatic processes of protein degradation (Rauh *et al.,* 2014; Datta and Deeth, 2001). The proteolysis during storage of UHT milk seems to be a consequence of the interaction between the plasminogen-plasmin system (endogenous protease of the milk) with proteases of bacterial origin. Among the bacterial proteases involved in spoilage processes a predominant role seems to be played by the thermostable metallo-protease AprX, which is produced by various strains of the species *Pseudomonas fluorescens*. The AprX protease, produced and released by bacteria in milk, is resistant to heat and is able to maintain unaltered its activity even after heat treatments that milk may undergo during its processing such as pasteurization, UHT treatment, and cheese-making (Ismail and Nielsen, 2010; Frohbieter *et al.*, 2005). Such enzyme activities could also have a role during cheese ripening for the formation of flavors in seasoned cheeses, and aromatic characteristics of milk cultures of the *P. fluorescens* group are strain-dependent (Morales *et al.,* 2005; Carraro *et al.,* 2011).

To evaluate proteolytic activity, several assays were developed and applied in *P. aeruginosa* (Kessler and Safrin, 2014) and some of them tested for AprX activity on *P. fluorescens* strains (Dufour *et al*., 2008; Marchand *et al.,* 2009a). The AprX protein and its encoding gene have been studied extensively (Liu *et al.,* 2007; Maunsell *et al.,* 2006; Nicodeme *et al*., 2005; Woods *et al*., 2001; Liao and McCallus, 1998). Differences in AprX enzyme activities were highlighted, but the identification of the most proteolytic strains was not completely elucidated (Dufour *et al*., 2008; Marchand *et al.,* 2009a, 2009b). This is due partially to the unreliability of methods currently used for the identification of *P. fluorescens* strains. Recently, *P. fluorescens* was recognized as a species group (Mulet *et al.,* 2010) and a molecular typing approach was developed and applied on several *P. fluorescens* reference and field strains (Andreani *et al.,* 2014). The determination of the genetic diversity among species and strains belonging to the *P. fluorescens* group provides an accurate method for strain identification.

In the present study the presence of the *aprX* gene was evaluated in 69 strains isolated from food matrices and 18 reference strains belonging to the *P. fluorescens* group. All strains were previously typed by multi locus sequence typing (MLST) method and allocated in a *subgroup* as genetically related to a specific reference type strain.

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Key words: *Pseudomonas fluorescens*; Spoilage; Dairy products; AprX.

Received for publication: 19 July 2016.
Revision received: 16 November 2016.
Accepted for publication: 19 November 2016.

Italian Journal of Food Safety 2016; 5:6175
doi:10.4081/ijfs.2016.6175

In a selected group of reference strains, the activity and gene expression of AprX were tested in milk. Finally, the applicability of Azocasein assay directly on dairy products was evaluated on industrial *ricotta* samples. The results demonstrated the spread of *aprX* gene in most of the strains tested and the applicability of trinitrobenzenesulfonic acid solution (TNBS) and Azocasein tests to monitor the proteolytic activity in dairy products.

Materials and Methods

Bacterial strains

P. fluorescens group strains are listed in Table 1. For each strain, the sequence type (ST) and the *subgroup* obtained by MLST analysis (Andreani *et al.,* 2014) are reported. Strains were conserved at -80°C in Tryptic Soy Broth [TSB; Oxoid, Basingstoke, UK; with 50% v/v glycerol (Sigma-Aldrich, Saint Louis, MO, USA)].

DNA and RNA extraction

For DNA extraction, a single colony from a fresh culture on CFC *Pseudomonas* Agar Base (CFC PAB; Oxoid) was re-suspended in 100 µL of nuclease-free water, vortexed at high speed for 5 seconds and incubated at 95°C for 10 minutes. The tube was vortexed again and centrifuged for 2 minutes at 14,000 rpm. The supernatant was transferred to a fresh tube and stored at -20°C (Martino *et al.,* 2011).

For RNA extraction, a single pure colony of each strain was inoculated in triplicate (giving 3 biological replicates for each strain) in 3 mL MBM Broth [0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% trisodium citrate, 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.2% glucose] and kept at 22°C for

Table 1. Species, subgroups, food origin, sequence type, presence of *AprX* gene and proteolytic activity in plate of *Pseudomonas fluorescens* strains.

Strain	Species	Subgroup	Source	ST	*AprX* gene	Proteolitic activity		
						6°C	22°C	31°C
DSM 17152T	*Pseudomonas gessardii*	*P. fluorescens subgroup*	Mineral water	7	+	+	++	-
DSM 15294T	*Pseudomonas brenneri*	*P. fluorescens subgroup*	Natural mineral water	8	+	+	++	-
DSM 17967T	*Pseudomonas mandelii*	*P. mandelii subgroup*	Mineral water	9	+	+	+	-
DSM 17150T	*Pseudomonas jessenii*	*P. jessenii subgroup*	Mineral water	10	+	-	+	-
DSM 16610T	*Pseudomonas koreensis*	*P. koreensis subgroup*	Agricultural soil	11	+	+++	+	++
DSM 17489T	*Pseudomonas orientalis*	*P. fluorescens subgroup*	Spring water	12	+	+++	++	-
DSM 18928T	*Pseudomonas synxantha*	*P. fluorescens subgroup*	Cream	13	+	++	+	-
DSM 18862T	Pseudomonas azotoformans	P. fluorescens subgroup	Paddies	14	+	+++	+++	-
DSM 6252T	*Pseudomonas lundensis*	*P. fragi subgroup*	Prepacked beef	15	+	-	-	-
DSM 14020T	*Pseudomonas rhodesiae*	*P. fluorescens subgroup*	Natural mineral water	16	+	++	+++	-
DSM 11331T	*Pseudomonas veronii*	*P. fluorescens subgroup*	Mineral water	17	+	++	++	-
DSM 17149T	*Pseudomonas libanensis*	*P. fluorescens subgroup*	Spring water	18	+	+++	++	-
DSM50415	*Pseudomonas fluorescens*	*P. fluorescens subgroup*	Soil	19	+	++	++	+
CECT124T	*Pseudomonas corrugata*	*P. corrugata subgroup*	Tomato: pith necrosis	20	+	-	++	++
CECT229T	*Pseudomonas marginalis*	*P. fluorescens subgroup*	*Cichorium intybus* (endive)	21	+	-	+++	-
CECT378T	*Pseudomonas fluorescens*	*P. fluorescens subgroup*	Pre-filter tanks, town water works	22	+	++	++	-
CECT446T	*Pseudomonas fragi*	*P. fragi subgroup*	Unknown	23	+	+++	+++	-
CECT4470T	*Pseudomonas chlororaphis*	*P. chlororaphis group*	Plate contaminant	24	+	++	++	++
ps_1		*P. fluorescens subgroup*	Mozzarella cheese	25	+	+	++	-
ps_2		*P. fluorescens subgroup*	Blue mozzarella cheese	26	+	++	++	-
ps_3		*P. fragi subgroup*	Mozzarella cheese	27	+	-	-	-
ps_4		*P. fluorescens subgroup*	Mozzarella cheese	28	+	-	+++	-
ps_5		*P. fluorescens subgroup*	Mozzarella cheese	29	+	-	++	-
ps_6		P. fluorescens subgroup	Blue mozzarella cheese	30	+	++	++	-
ps_7		*P. koreensis subgroup*	Mozzarella cheese	31	+	+++	+++	-
ps_8		*P. fluorescens subgroup*	Mozzarella cheese	32	+	+++	+++	-
ps_9		*P. fluorescens subgroup*	Mozzarella cheese	33	-	++	++	-
ps_10		*P. fluorescens subgroup*	Mozzarella cheese	34	+	++	++	-
ps_11		*P. fluorescens subgroup*	Mixed salad	35	+	+	++	-
ps_12		*P. fragi subgroup*	Butter	36	+	-	-	-
ps_13		*P. fluorescens subgroup*	Blue mozzarella cheese	37	+	++	++	-
ps_14		*P. koreensis subgroup*	Pork	38	+	-	+	+
ps_15		*P. fluorescens subgroup*	Salmo trutta marmoratus (trout)	39	+	++	++	-
ps_16		*P. fluorescens subgroup*	Salmo trutta fario (trout)	40	+	+++	+++	++
ps_17		*P. fragi subgroup*	Ricotta	41	+	++	+	-
ps_18		*P. fluorescens subgroup*	UHT milk	42	+	++	++	++
ps_19		*P. fragi subgroup*	UHT milk	43	-	-	+	++
ps_20		*P. fluorescens subgroup*	UHT milk	44	+	+++	++	+
ps_21		*P. koreensis subgroup*	Mozzarella cheese	45	+	+	+	-
ps_22		*P. fluorescens subgroup*	Blue mozzarella cheese	46	+	+	++	-
ps_23		*P. koreensis subgroup*	Mozzarella cheese	47	+	-	++	-
ps_24		*P. fluorescens subgroup*	Cheese	48	+	-	++	-
ps_25		*P. fluorescens subgroup*	UHT milk	49	+	-	+	-
ps_26		*P. koreensis subgroup*	Human	50	+	++	++	++
ps_27		*P. fluorescens subgroup*	Mixed salad	51	+	++	++	-
ps_28		*P. koreensis subgroup*	Mixed salad	52	+	++	+++	+++
ps_29		*P. fluorescens subgroup*	Mixed salad	53	+	+++	+++	-
ps_30		*P. fluorescens subgroup*	Mixed salad	54	+	+	+++	-
ps_31		*P. fluorescens subgroup*	Mixed salad	55	+	++	+++	-
ps_32		*P. chlororaphis group*	Mixed salad	56	+	-	-	-
ps_33		*P. fluorescens subgroup*	Mixed salad	57	+	++	++	-
ps_34		*P. fluorescens subgroup*	Mixed salad	58	+	+++	+++	-

Continued on next page.

24 h to reach 10^8 CFU/mL. Then, cultures were diluted five times in 3 mL of milk. After 24h of incubation at 22°C or five days at 5°C, 1 mL of culture was then extracted using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNase treatment was performed using the Qiagen RNase-Free DNase Set (Qiagen). RNA was eluted in 30 µL of RNAse-free H_2O. Purified DNA and RNA were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One microgram of total RNA for each sample was reverse transcribed to cDNA using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). To evaluate DNA contamination in RNA samples treated with DNAse, no-RT (reverse-transcriptase) samples were also checked in PCR amplification.

Polymerase chain reaction amplification

Primers for *aprX* gene amplification were designed using PriFi software (http://cgi-www.daimi.au.dk/cgi-chili/PriFi; Fredslund *et al.,* 2005) using as template the alignment of *aprX* sequences from complete genomes of *P. fluorescens* group already available in Genbank (http://www.ncbi.nlm.nih.gov/genome). The sequence of primers was Pse_AprXF1 CAGACCCTGACCCACGARATCGG and Pse_AprXR1 TGAGGTTGATCTTCTGGTTCTGGG. The *rpoD* housekeeping gene was used as positive control for DNA and RNA extraction using primers reported in Andreani *et al.* (2014).

PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 20 µL of amplification mix containing 1U of GoTaq polymerase (Promega, Madison, WI, USA), 1X GoTaq Buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 250 mM each primer and 5 ng of genomic DNA as template. The reaction mixture was subjected to the following thermal cycle: an initial step at 94°C for 2 min to activate the polymerase and 35 cycles each of denaturation at 94°C for 20 seconds, annealing of the primers at 60°C for 30 seconds and extension at 72°C for 1 minute and a final step of extension at 72°C for 7 min. Amplified products were analysed by electrophoresis on 1.8% agarose-Tris-acetate-EDTA (TAE) gels, stained with SYBR® Safe DNA Gel Stain (Invitrogen) and visualized on a UV transilluminator (Gel Doc XR™; Biorad, Hercules, CA, USA).

Table 1. Continued from previous page.

Strain	Species	Subgroup	Source	ST	*AprX* gene	Proteolitic activity		
						6°C	22°C	31°C
ps_35		*P. fluorescens subgroup*	Mixed salad	59	+	+++	++	-
ps_36		*P. fluorescens subgroup*	Mixed salad	60	+	+++	++	-
ps_37		*P. clororaphis subgroup*	Mixed salad	61	+	+	++	+++
ps_38		*P. clororaphis subgroup*	Mixed salad	62	+	+	++	+++
ps_39		*P. fluorescens subgroup*	Mixed salad	63	+	++	++	+
ps_40		*P. fluorescens subgroup*	*Ricotta*	64	+	+++	++	-
ps_48		*P. fragi subgroup*	Pork	72	+	++	+++	+
ps_50		*P. fragi subgroup*	Pork	74	+	++	+++	+++
ps_51		*P. clororaphis group*	Pork	75	+	++	+++	+++
ps_54		*P. fragi subgroup*	Pork	78	+	++	++	+
ps_55		*P. koreensis subgroup*	Pork	79	+	-	++	+++
ps_56		*P. koreensis subgroup*	Sashimi	80	+	+	++	+++
ps_57		*P. fluorescens subgroup*	Sashimi	81	+	++	+++	++
ps_58		*P. corrugata subgroup*	Sashimi	82	+	-	++	++
ps_59		*P. fluorescens subgroup*	Sashimi	83	+	+++	+++	++
ps_60		nd	Sashimi	nd	+	+++	+++	+++
ps_61		*P. fluorescens subgroup*	Sashimi	84	+	+	+++	++
ps_62		nd	Sashimi	nd	+	+	+++	+++
ps_63		nd	Sashimi	nd	+	++	+++	++
ps_64		*P. fluorescens subgroup*	Sashimi	85	+	-	+++	+
ps_65		*P. koreensis subgroup*	Rocket	86	+	++	+++	+++
ps_66		*P. koreensis subgroup*	Rocket	87	+	++	+++	+++
ps_67		*P. koreensis subgroup*	Valerian	88	+	++	+++	+++
ps_68		*P. corrugata subgroup*	Valerian	89	+	-	+++	+++
ps_69		*P. mandelii subgroup*	Dairy-product	90	+	-	+	+
ps_70		*P. fragi subgroup*	Dairy-product	91	+	+	++	-
ps_71		*P. fragi subgroup*	UHT milk	92	-	+	+	+
ps_72		*P. fragi subgroup*	Dairy-product	93	+	-	++	+
ps_73		*P. fluorescens subgroup*	Dairy-product	94	-	-	+	-
ps_74		*P. fragi subgroup*	Dairy-product	95	+	++	+	+
ps_75		*P. fluorescens subgroup*	Blue mozzarella cheese	29	+	-	++	-
ps_76		*P. fluorescens subgroup*	Mozzarella cheese	26	+	++	++	-
ps_77		*P. fluorescens subgroup*	Meat	96	+	-	++	-
ps_78		*P. fluorescens subgroup*	Mozzarella cheese	97	+	-	+	-
Ps_79		*P. fluorescens subgroup*	Meat	98	+	-	-	-

ST, sequence type; UHT, ultra-high temperature; nd, not determined.

Proteolytic activity in plate

The reference and field strains were tested for their proteolytic activity by agar diffusion assays at 6°C for 10 days, at 22 and 31°C for 7 days. All the strains were revitalized through a 72-hours-preinoculum in TSB at 22°C. A dilution of 10^5 CFU/mL was applied for the subsequent tests. The extracellular protease activity evaluation was conducted on Nutrient Agar (NA; Biokar diagnostics, Paris, France) with 2% UHT milk observing a clear zone around the colonies. The presence of a clear zone around the colonies after incubation was indicative for proteolysis.

Quantification of extracellular proteolytic activity (Azocasein assay)

Proteolytic activity of bacterial strains was quantified using Azocasein (Sigma-Aldrich) as substrate. One hundred μL of a 3% (w/v) Azocasein stock solution were added to 100 μL of cell free supernatant fluid and 300 μL of 50 mM Na_2HPO_4 pH 7.5. The negative control was set up with 100 μL of not inoculated milk. The mixture was incubated at 37°C for 1 h and the reaction was stopped by adding 500 μL of 20% (w/v) trichloroacetic acid (TCA). The sample was centrifuged at 12,000 g for 10 min and absorbance of the supernatant was measured at 366 nm using Multiskan GO UV/Vis spectrophotometer (Thermo Fisher Scientific). Absorbance of the blank (500 μL not incubated sample plus 500 μL of TCA 20%), and of the negative control were subtracted from sample absorbance. The results are reported as OD_{366}.

Quantification of proteolysis (trinitrobenzenesulfonic acid solution test)

The experimental set-up made it possible to calculate the net proteolytic activity produced during 2 weeks of storage at 37°C after a heat treatment simulating UHT process and storage (as described in Marchand *et al.*, 2008, 2009a). Hydrolysis of proteins was measured by the determination of the release of a-amino groups by the trinitrobenzenesulfonic acid (TNBS) method. The free amino groups react with the TNBS reagent (Sigma-Aldrich) at pH 9.2 in the dark. A yellow-orange colour develops and its intensity is determined by absorption measurements at 420 nm. The degree of proteolysis is calculated from the increase in absorption after 2 weeks of storage at 37°C and expressed as mmol of glycine equivalents mL-1 milk, using glycine (2.5, 2.25, 2, 1.75, 1.5, 1.25, 1, 0.75, 0.5, 0.25 e 0 mM; Sigma-Aldrich) to create a standard curve. The experiment was repeated twice, first in macro method (experiment A using a 7800 UV/VIS spectrophotometer; JASCO, Easton, MD, USA) and second time in micro method (experiment B using a Multiskan GO UV/Vis spectrophotometer (Thermo Fisher Scientific).

Proteolytic activity in industrial *ricotta* samples

Of *ricotta* samples, 10 g were inoculated with 1 mL of a 10^8 cell/mL of a fresh culture of *P. fluorescens group* strains growth in TSB in sterile 50 mL tubes and were maintained at refrigeration temperature for seven days. The Azocasein assay was carried out in order to evaluate the proteolytic activity of the inoculated ricotta in comparison to three industrial *ricotta* samples, belonging to the same lot, collected and analyzed 24 h from production. One gr of *ricotta* was sampled in duplicate from each of the four inoculated samples and the three fresh *ricotta* samples and diluted in 5 mL of Phosphate-buffered saline (PBS). After mixing, 2 mL were centrifuged at 12,000 rpm for 5 minutes, then 100 μL of the supernatant was used for the Azocasein assay as previously described.

Results

Distribution of the *aprX* gene in *Pseudomonas fluorescens* group

The distribution of the *aprX* gene was evaluated on 18 reference and 69 field strains and the result is reported in Table 1. Only for four strains (4.6%) the PCR amplification of *aprX* gene, repeated twice, gave a negative result. All these four strains resulted positive to the amplification of the *rpoD* gene. The four strains belonged to the *P. fluorescens* subgroup (ps_9 and ps_73) or to the *P. fragi* subgroup (ps_19 and ps_71).

Proteolytic activity of *Pseudomonas fluorescens* strains in plate

Proteolytic activity was observed at least at one temperature condition in 17 reference strains and 65 fields strains (for a total of 94.2%), indicating that proteolysis is a common spoilage mechanism for *P. fluorescens* group strains. The non-proteolytic strains are *P. lundensis* DSM6252T, ps_3, ps_12, ps_32, ps_79. The complete data are reported in Table 1.

Proteolytic activity and *aprX* gene expression in milk samples inoculated with *Pseudomonas fluorescens*

The experiment was set up to simulate the conditions during UHT milk production (as described in Marchand *et al.,* 2008, 2009a) and a schematic representation of the analyses is reported in Figure 1. Briefly, 100 μL (10^7 cells) of a fresh culture of each strain was inoculated in 10 mL of UHT milk and grown for 24 hours. An aliquot of 100 μL of the culture was then inoculated in 10 mL of UHT milk and grown for 24 hours. Double growth in milk until exponential phase was done to adapt bacterial strains to milk. The culture was then diluted to 10^3 cfu/mL in UHT milk and incubated at 6°C for five days. At the end of the incubation, the Azocasein test was performed as described in Materials and Methods to measure the global proteolytic activity (thermoresistent and non-

Table 2. Proteolityc activity and *AprX* gene expression in *Pseudomonas fluorescens* group references strains.

Type strains	Subgroups	Proteolitic activity in plate*			*AprX* gene expression°		Azocasein OD_{366}		TNBS# mM glicine		Azocasein§ *Ricotta* OD_{36}
		6°C	22°C	31°C	22°C	6°C	Pre-HT	Post-HT	Experiment A	Experiment B	
DSM 17489T	*P. orientalis*	+++	++	-	+++	+-+	0.28	0.22	3.69	11.85	1.02
DSM 6252T	*P. lundensis*	-	-	-	---	---	0.02	0.01	1.92	6.72	nd
DSM 14020T	*P. rhodesiae*	++	+++	-	+++	---	0.07	0,04	1.71	24.09	0.34
DSM 17149T	*P. libanensis*	+++	++	-	+++	---	0.37	0.17	-	-	1.38
CECT378T	*P. fluorescens*	++	++	-	++-	---	0.1	0.03	3.15	7.24	1.01
CECT446T	*P. fragi*	+++	+++	-	+++	---	0.28	0.18	-	-	nd
CECT229T	*P. marginalis*	-	+++	-	---	---	0.01	0.02	-	-	nd

TNBS, trinitrobenzenesulfonic acid solution; HT, high-temperature. *+, ++ or +++ depending on the size of the clear zone around the colonies; °+ or – is positive or negative result in the amplification of each triplicate; #experiment A was performed in macromethods, Experiment B in micromethods; §reported the average between the OD of the duplicates. nd, not determined.

thermoresistent proteases). The culture was then heat-treated (10 mL for 8 minutes and 45 seconds at 95°C to simulate UHT treatment) and a second Azocasein test was performed to measure the thermoresistent protease activity. The culture was then incubated for two weeks at 37°C. After incubation, the TNBS test was performed as described in Materials and Methods section.

This preliminary experiment was performed with six reference strains, including a negative control (not inoculated milk) and the results are summarized in Table 2. The gene expression study demonstrated that, in the condition tested, the *aprX* gene is not expressed in *P. lundensis* and *P. marginalis.* The Azocasein assay gave OD_{366} values ranging from 0.01 to 0.37. The TNBS assays gave values in mM Glycin from 0 (negative results for three strains) to 24.09 in micro method and from 0 to 3.69 in macro method. Proteolytic activity data is only partially in agreement with expression data.

Proteolytic activity in industrial *ricotta* samples

The results of proteolytic activity measured on *ricotta* samples are reported in Table 2 and Figure 2. The data evidenced variable activity in the freshly produced *ricotta* samples despite the three samples belonged to the same lot of production.

Discussion

The analysis of 87 *P. fluorescens* strains for the presence of the *aprX* gene demonstrated that the gene is widespread in this bacterial group. The *aprX* negative strains are not strongly genetically related (Andreani *et al.,* 2014) suggesting that the lack of *aprX* amplification, could be due to loss of the gene or mismatches in primer sites that occurred independently in each strain. However, the *aprX* gene expression study demonstrated strong variability across strains, which might explain the large variability in proteolytic activity reported in previous studies (Dufour *et al.,* 2008; Marchand *et al.,* 2009a, 2009b).

To evaluate the spoilage activity of AprX in dairy products, milk and *ricotta* cheese were used as template to inoculate strains positive to *aprX* gene. The experimental protocol for milk, reported in Figure 1, was designed to simulate UHT milk productive process, with milk samples incubated first at refrigeration temperature, and after heat treatment, incubated at 37°C. Similar experimental design to simulate UHT milk production was proposed and applied in previous studies (Marchand *et al.,* 2008, 2009a).

The proteolytic activity was measured with two different assays, the first, the Azocasein assay, measures directly the activity with a colorimetric reaction, the second, the TNBS assay, measures free amino acids (amino-groups), as products of the proteolysis. The two different assays are both easy and fast to be carried out, inexpensive and require a common spectrophotometer. In the present study, these assays were applied to compare their sensibility on dairy products inoculated with a bacterial culture. However, the results of the two assays are only partially comparable. The Azocasein assay results are in good agreement with *aprX* expression data, if considering value less to 0.05 OD_{366} as negative. On the contrary, TNBS assay results are discordant with *aprX* expression and Azocasein assay data for *P. lundensis* and *P. libanensis*. On the basis of these results the Azocasein assay might be more reliable as a direct measure of the enzyme activity, however some concerns remain. First, the Azocasein assay had not a standard curve to be used to compare the results. Secondly, protease activity immediately after the heat treatment might be slower as a consequence of the treatment itself and this effect might be different in the different strains. This effect could be due to differences in protein structure despite the nucleotidic sequence was reported to be very conserved among *P. fluorescens* strains (Marchand *et al.,* 2009b) or to different resistance of strains to heat treatment. The gelation of UHT milk, if occurs, usually takes place after weeks from production. This long time might depend on the time required by proteases to reactivate after heat treatment or to some chemical modification that might occur in milk and activate proteases.

Anyway, this preliminary study strongly confirms strain variability in protease activity. *P. lundensis* and *P. fragi* were indicated as the species mostly involved in spoilage of milk (Marchand *et al.,* 2009a). For these two strains, contrasting results were obtained in the present study. Regarding *P. lundensis* type strain, the *aprX* expression was negative in milk as it was proteolytic activity in plate and at Azocasein assay, even if the TNBS assay yielded a positive result. On the contrary, *P. fragi* type strain expressed *aprX* and was positive for proteolytic assay both in plate and with Azocasein assay, but was negative with TNBS assay. Thanks to MLST molecular typing, the *P. fluorescens* group fields strains were accurately identified and the application of the experiment in milk using fields strains belonging to *P. fragi* subgroup (that include *P. lundensis*) is interesting to confirm if the spoilage phenotype is related to this taxonomic group.

Finally, with the aim to test the applicability of these assays to analyze dairy products, in which spoilage activity might be due to thermoresistent proteases, industrial *ricotta* was chosen as a study case. In fact, industrial *ricotta* is produced at high temperature (90°C) and successively pasteurized (80°C) starting from milk whey that usually presents high bacterial loads (among these bacteria, *Pseudomonas* is

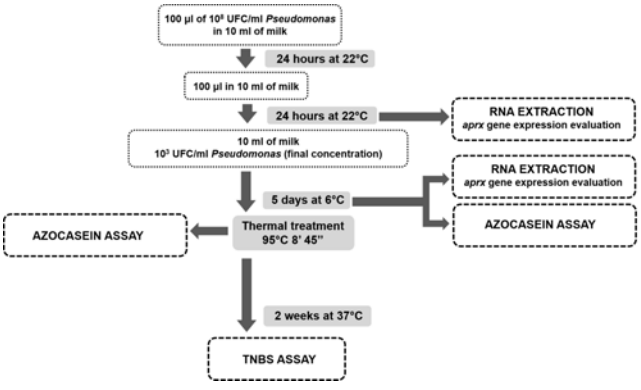

Figure 1. Schematic description of the experiment set-up simulating the conditions of ultra-high temperature milk production.

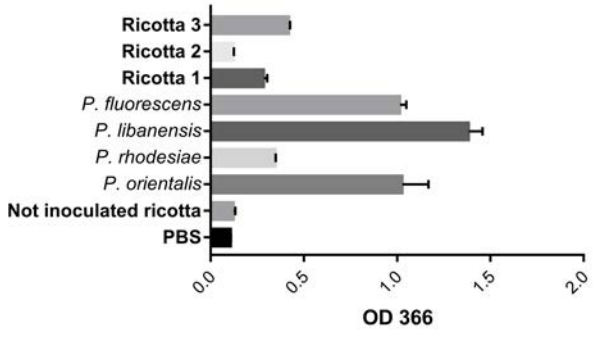

Figure 2. Proteolytic activity in *ricotta* samples.

often present). Industrial *ricotta* often shows premature spoilage that reduces product shelf-life. The application of Azocasein assay on freshly produced industrial *ricotta* samples showed protease activity that might be one of the responsible factors for premature spoilage. This result highlights the importance of good quality raw material to extend shelf-life also for heat-treated products.

Conclusions

These preliminary data highlight the interest, but also the complexity of studying the proteolytic activity of the *aprX* gene in *P. fluorescens*. The availability of MLST-typed strains might help to identify the major *aprX* producing strains and evaluate if such spoilage activity is a phenotypic trait linked to specific lineages in the *P. fluorescens* group. Azocasein and TNBS assay should be improved, but might be suitable (in particular the Azocasein assay) to evaluate proteolytic activity in dairy products such as industrial *ricotta* or UHT milk to assess in advance the spoilage potential during shelf-life.

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