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# Destabilization and off-flavors generated by *Pseudomonas* proteases during or after UHT-processing of milk

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## Abstract

**Background:** *Pseudomonads* play a major role in the spoilage of UHT processed dairy products, due to their growth-related protease production in raw milk.

**Results:** To assess the off-flavor generating capacity of these AprX proteases in milk after UHT-processing, six major milk spoiling *Pseudomonas* groups were investigated. Sensory evaluation of the different processed milk samples showed large differences in the degree of proteolysis related to onset of off-flavors. Nevertheless, it was illustrated that *P. fragi* has the greatest spoilage potential within the tested *Pseudomonas* groups, when it comes to generating off-flavors.

**Conclusions:** No clear correlation could be obtained between protein hydrolysis and the presence of off-flavors in UHT milk.

**Keywords:** *Pseudomonas*, Protease, Sensory analysis, Spoilage, Milk

## Background

Refrigerated storage of raw milk is universally accepted for prolonging shelf life and preventing spoilage by mesophilic bacteria. Due to evolutions in the dairy market in which dairies have become more and more centralized, milk is now stored longer at refrigerated temperatures (Gaafar and Ali 1995). To ensure good dairy products, the Belgian legislation foresees, that milk on farm should be collected within 72 h post-production (Anonymous 2007). Indeed, quality problems may arise if milk is stored too long at these refrigerated temperatures. This is mainly due to an outgrowth of pyschrotrophic microorganisms in the raw milk. Psychrotrophic *Pseudomonas* (especially *P. fragi, P. lundensis* and members of the *P. fluorescens*-like group) are the dominant microbiota of raw milk and are known to compromise heat-treated milk (e.g., UHT) due to the production of heat-stable enzymes during their growth in raw milk (Marchand et al. 2009a). While pseudomonads are readily eliminated by UHT heating conditions (minimal 135 °C for 1 s), their heat-stable proteases may remain active in the heat-treated products (Chen et al. 2003; Griffiths et al. 1981). The presence of heat-stable *Pseudomonas* protease, encoded by the AprX gene, may result in spoilage and destabilization of UHT milk during extended storage (Dufour et al. 2008). Although, this protease gene is widespread over numerous *Pseudomonas* spp. (Chessa et al. 2000), the production process of this protease is still not completely understood and appears to be very complex. Quorum sensing (Juhas et al. 2005), temperature (Nicodème et al. 2005), iron content (Woods et al. 2001) and phase variation (van den Broeck et al. 2005) regulate and influence the production process of proteases at different levels. Typically, within *Pseudomonas* spp. only one protease, AprX, an alkaline zinc metalloprotease with a pH optimum of 6.5–8, is produced (Woods et al. 2001). This AprX protease is solely responsible for casein hydrolysis as evidenced by casein zymography (Marchand et al. 2009b). The family of serralysin proteases, to which the AprX *Pseudomonas* protease belong, appears to be highly conserved in some domains. Typical similarities in amino acid sequence are observed: a zinc-binding motif (xxxQTLTHEIGHxxGLxxGLxHPx), a calcium binding domain characterized by the presence of four glycine rich

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repeats (GGxGxD), a high content of hydrophobic amino acids and no cysteine residues (Rawlings and Klostermeyer 1995; Kumeta et al. 1999). However, despite of this general interspecies conservation, genetic differences in the AprX sequence might be responsible for observed inter individual differences in proteolytic capacity and/or the specific activity of these sequence divergent AprX proteases.

Proteolysis of UHT milk causes the development of bitter off-flavors, through the generation of hydrophobic peptides by hydrolysis of casein (Chen et al. 2003; Datta and Deeth 2003). Next to *Pseudomonas* proteases, proteolysis in UHT milk may also be attributed to the native milk enzyme plasmin. Milk plasmin is associated with the casein micelle and the milk fat globular membrane and is also quite heat-resistant (Saint-Denis et al. 2001; Fox and Kelly 2006). It may even partially survive mild UHT-processing conditions. Plasmin exists in milk in both its active form as well as its inactive precursor plasminogen. Its activity in milk is controlled by a complex network of enzyme activators and inhibitors (Fox and Kelly 2006). In addition, *Pseudomonas* proteases may contribute to overall plasmin activity by acting as plasminogen activators to convert plasminogen to plasmin (Fajardo-Lira et al. 2000).

Former research of the authors of this paper identified six major *Pseudomonas* protease groups with great milk spoilage behavior (Marchand et al. 2009a, b). However, no data was gathered yet on the off-flavor generation in milk with relation to that proteolytic capacity. Therefore, this paper addresses the differences in off-flavor generation by six representatives of the major milk *Pseudomonas* protease groups and assesses the correlation between protein hydrolysis and of off-flavor perception in processed UHT milk.

## Methods

### Selection of milk *Pseudomonas* strains

The selection of the different *Pseudomonas* strains was based on the findings of Marchand et al. (2009a, b). They defined six major *Pseudomonas* AprX protease groups. From each of these groups (A-B-C1-C2-D and the *P. lundensis* group) a representative was chosen to use in this study: respectively, *Pseudomonas* sp. Z34a, *Pseudomonas* sp. W12b, *Pseudomonas* sp. Z34b, *Pseudomonas* sp. W2a, *P. fragi* W41b and *P. lundensis* W52b.

### Growth media

Cryopreserved *Pseudomonas* strains were first recovered in Brain Heart Infusion Broth (BHI) (Oxoid,

Basingstoke, Hampshire, England) before inoculation in UHT milk. The isolates were incubated in BHI at room temperature until growth was visually present. Next, 100 μL of incubated BHI broth was inoculated in 10 mL of UHT milk and incubated overnight at ambient temperature. The strains were checked for purity and bacterial counts showed that 24 h incubation at room temperature in UHT milk resulted in approximately $10^8$ cfu $mL^{-1}$. The six cultures were diluted in ringer solution (Oxoid) until $10^7$ cfu $mL^{-1}$.

### Raw milk collection, pasteurization, inoculation with *Pseudomonas* strains

575 L of raw milk was collected from a farm in East-Flanders, Belgium. This full fat milk was pasteurized (72 °C,15 s) and aseptically divided in seven batches of 60 L. One batch was used as the control milk for further follow up of the experiment. The other six pasteurized milk batches were each inoculated with 6 mL of the ringer solution containing approximately 7 log $mL^{-1}$ pseudomonads, in order to reach a final concentration of approximately 3 log pseudomonads in the 60 L batch.

### Total colony counts and *Pseudomonas* counts

Total colony counts of the raw and pasteurized milk were determined by pour plating serial dilutions on Nutrient agar (Oxoid) with incubation at 30 °C for 3 days. Immediately after inoculation, *Pseudomonas* counts of the six 60 L milk batches were determined on a selective medium for *Pseudomonas* that contains cetrimide (10 $mgL^{-1}$), fucidin (10 $mgL^{-1}$) and cephalosporin (50 $mgL^{-1}$) (CFC agar) (Oxoid) with incubation at 22 °C for 3 days.

### Cold milk storage

The inoculated milk batches were further stored for 3 (t3), 4 (t4) and 5 (t5) days at 6,5 °C until skimming and further UHT-processing.

### Skimming and UHT-processing

After cold storage, the seven different milk batches were further processed. Before UHT-processing the milk was skimmed using a Elecrem decreamer (Type 315 L/H,7800 tpm; Tomega, Marche-en-Famenne, Belgium). Indirect UHT-processing was performed on a Junior N326L apparatus, Process Pilot Plant, 200 Lh-1 (APV, Aartselaar, Belgium) under the following conditions: 2 steps homogenization: 200 bar, 65 °C; indirect UHT-processing: 5 s,140 °C; cooling to 20 °C. Milk was aseptically filled in high density poly ethylene (HDPE) bottles of 0.5 L and stored at 37 °C to accelerate possible proteolysis events. To ensure safe sensory evaluation, all produced milk samples were tested for sterility. Therefore 2 (HDPE) bottles of each milk batch were chosen randomly and incubated at 30 °C for 3 days. Total plate counts of the milk samples were determined by undiluted pour plating and incubation at 30 °C for 3 days.

### Sensory evaluation

Preliminary sensory evaluation and proteolysis measurements started after 3 days storage at 37 °C. The six *Pseudomonas* protease milk samples were compared with the reference control milk (by a taste panel of 5 persons) on a daily basis. If off-flavors were experienced, a larger sensory analysis took place. The panel consisted of 35 people (8 men and 27 women) who were staff of the Institute of Agriculture, Fisheries and Food Research. Their mean age was 37 years (range 24–55 years). All panelists had earlier experience in sensory evaluation of milk. Evaluations were conducted in a sensorial cabinet that was equipped with individually partitioned booths. Milk samples (30 mL) were served at 14 °C. The set-up was as follows: Milk with off-flavor was diluted in the following way: A) *Pseudomonas* protease milk undiluted, B) 2/3 *Pseudomonas* milk + 1/3 control milk, C) 1/3 *Pseudomonas* milk + 2/3 control milk, D) Control milk undiluted. Next, the taste panel was asked to rank the milk samples (A-B-C-D) according to preference. Statistical evaluation of the results was based on the Rank Test to Kramer (Kramer 1960) for $\alpha = 0.05$ but also compared with a newly developed sensory evaluation test. In short: The four milk samples (A-B-C-D) were presented at random to the tasting panel. The tasting panel was asked to arrange the milk samples on a line scale of 10 cm length according to preference. 10 cm was considered as an excellent taste, 5 cm satisfactory and 0 cm was considered as having a very bad taste. Two correction parameters were added to this test; (an example is given in Fig. 1). First, a correction within the tasting panel: the sensory analyst was enabled to choose between five statements to indicate the difference degree between the most extreme marks on the line scale. Each of these statements was correlated with a corrected difference in cm going from 0 cm (no noticeable difference) till 4 cm (very obvious difference) (Fig. 1). And second, a correction for the sensory analyst: the reference milk (the control undiluted milk, which is unknown to the tasting panel) and the other milk samples under evaluation can be placed by the sensorial analyst anywhere on the line scale. However, for the evaluation of the test, the control undiluted milk (D), is considered as satisfactory and is thus arbitrary associated with 5 cm on the line scale. Therefore, regardless of the place where the sensorial analyst has placed the reference, this milk gets de facto scaling 5. To obtain the corrected position for the other milk samples under evaluation, the distances between them and the reference mark need to be measured accurately. If the taste of the other milk samples was considered worse than the reference, the analyst would have placed its mark left from the reference on the line scale. If the taste was better, on the other hand, it would have been placed on the right hand side from the reference. Dependent if the mark is left or right from the reference, the measured distance between the two of them will be subtracted or added, respectively, from the arbitrary 5 cm scaling, resulting in the

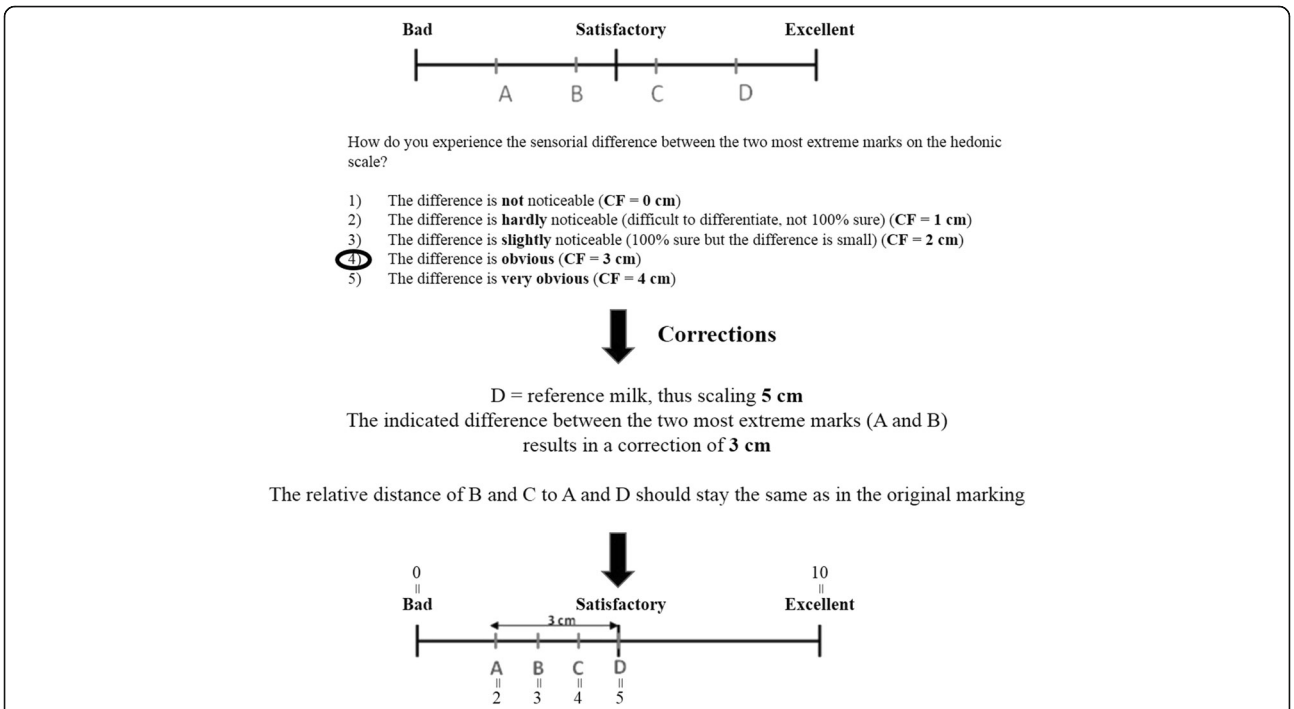


**Fig. 1** Example of the newly developed sensorial evaluation test

corrected values. All values derived from the different sensorial analysts were grouped and mean values and standard deviations (sd) for each milk sample were determined. Milk samples with mean values (±1 x standard deviation) were considered significant according to the Rank test to Kramer (Kramer 1960). Simultaneously, proteolysis was determined in each milk dilution (A-B-C-D).

### Measurement of proteolysis

Hydrolysis of proteins was measured by the determination of the release of a-amino groups directly in milk by the trinitrobenzenesulfonic acid (TNBS) method (Polychroniadou 1988), in which free amino groups react with the TNBS reagent (Sigma-Aldrich, Bornem, Belgium) at pH 9.2 in the absence of light. A yellow-orange color develops and its intensity is determined in duplicate by absorption measurements at 420 nm. The amount of proteolysis in the *Pseudomonas* milk samples and dilutions is calculated from the increase in absorption and expressed as μmol glycine equivalents $mL^{-1}$ milk using glycine (Sigma-Aldrich) as a standard curve.

## Results and discussion

### Milk processing and bacterial counts

575 L raw (full fat) milk was pasteurized and aseptically divided in seven batches of 60 L. Total bacterial count of the raw milk was 33.000 cfu/mL. After pasteurization total bacterial counts were reduced to 1900 cfu/mL in the pasteurized milk. Six 60 L batches were subsequently inoculated with *Pseudomonas* strains. *Pseudomonas* counts and total bacterial counts were determined at the moment of inoculation (t0) and before UHT-processing (t3, t4) for every milk batch. Bacterial counts can be retrieved in Table 1. All produced milk samples were sterile and were used for further sensory evaluation. In addition, the experiments showed that milk, which had been stored for 5 days or longer cannot be processed anymore under UHT conditions, because of destabilization of the milk, resulting in clogging of the heating exchanger.

### Sensory evaluation of the processed milk samples

Sensory evaluation of the different processed milk samples showed large differences in the onset of off-flavors. The majority of the *Pseudomonas* inoculated milk samples (strains Z34b, Z34a, W2a and W52b) were stored 4 days at 6,5 °C before sufficient proteases were produced. In *Pseudomonas* sp. Z34b milk, *Pseudomonas* sp. Z34a milk and *Pseudomonas lundensis* W52b milk, off-flavors occurred after 13 days of storage at 37 °C post UHT-processing, while in *Pseudomonas* sp. W2a, off-flavors were already present after 10 days. However, it was illustrated that not all pseudomonads contain equal spoilage threats for the dairy industry: *Pseudomonas fragi* Z41b and *Pseudomonas* sp. W12b produced already sufficient proteases after 3 days of storage at 6,5 °C. Off-flavors were detected in those milk samples, after 15 days and 10 days storage at 37 °C, post UHT production, respectively (Table 2). From these results, it can be deduced that refrigerated storage of milk should be limited in order to prevent or reduce *Pseudomonas* protease production. In addition, the sensorial analysis evaluation by the method of Kramer (Kramer 1960) and the method described in this paper showed identical results (Table 2).

### Correlation between off-flavors and protein hydrolysis in UHT milk by the six different *Pseudomonas* protease groups

In each sensory evaluated milk sample proteolysis was determined. TNBS-values of each milk sample can be retrieved in Table 2. Based on these grouped results the correlation between protein hydrolysis and off-flavors can be determined. First of all, control milk was checked for the occurrence of proteolysis events. During a period of 30 days, milk was monitored for possible proteolytic activity. The TNBS value remained constant over time and had a mean value of 1,01 ± 0,04 μmol glycine

**Table 1** Bacterial counts in milk before the different processing steps (In bold: Pseudomonas counts sufficient to induce off-flavors in the processed milk samples)

| MILK | After pasteurisation (t0) | | Before UHT processing 3 days storage at 6,5 °C (t3) | Before UHT processing 4 days storage at 6,5 °C (t4) |
|---|---|---|---|---|
| | TBC log (cfu/ml) | Added *Pseudomonas* count log (cfu/ml) | *Pseudomonas* count log (cfu/ml) | *Pseudomonas* count log (cfu/ml) |
| CONTROL | 3,28 | 0,00 | 0,00 | 0,00 |
| *Pseudomonas* sp. Z34a | 3,49 | 3,43 | 5,78 | **6,96** |
| *Pseudomonas* sp. W12b | 3,58 | 3,52 | **6,43** | 7,08 |
| *Pseudomonas* sp. Z34b | 3,57 | 3,45 | 6,23 | **6,60** |
| *Pseudomonas* sp. W2a | 3,61 | 3,41 | 6,63 | **6,75** |
| *P. fragi* Z41b | 3,18 | 3,04 | **6,48** | 6,93 |
| *P.* lundensis W52b | 3,38 | 3,20 | 6,48 | **6,79** |

**Table 2** Oversight of the sensory evaluation and proteolysis results ([a]Milk samples with off-flavors have values within the range indicated by Kramer and are thus significantly different from the milk samples with values outside that range)

| *Pseudomonas* culture | UHT production date | Time stored at 37 °C after UHT production / and time of sensory evaluation | Dilution with blanc milk | off-flavor | Sensorial evaluation method described in this paper | Rank test to Kramer | | | Proteolysis (μmolglycine equivalents / ml) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | value ± sd | milk sample rank sum | (lowest - highest) insignificant rank sum[a] | # sensorial evaluators | |
| Blanc | 02/12/2010 (t3) | 15 days | Undiluted | NO | 5 | 33 | (39–56) | 19 | 1,01 |
| Blanc | 02/12/2010 (t3) | 11 days | Undiluted | NO | 5 | 18 | (33–47) | 16 | 0,96 |
| Blanc | 03/12/2010 (t4) | 13 days | Undiluted | NO | 5 | $24^{Z34a}$ / $20^{Z34b}$ / $26^{W52b}$ | $(35–50)^{Z34a}$ / $(33–47)^{Z34b}$ / $(35–50)^{W52d}$ | $17^{Z34a}$ / $16^{Z34b}$ / $17^{W52d}$ | 1,04 |
| Blanc | 03/12/2010 (t4) | 10 days | Undiluted | NO | 5 | 17 | (33–47) | 16 | 0,92 |
| Z34a | 03/12/2010 (t4) | 13 days | 1:3 | NO | 4,69 ± 1,72 | 25 | (35–50) | 17 | 1,18 |
| | | | 2:3 | NO | 4.62 ± 1,68 | 26 | (35–50) | 17 | 1,39 |
| | | | Undiluted | YES | 3,43 ± 1,53 | 35 | (35–50) | 17 | 1,73 |
| W12b | 02/12/2010 (t3) | 11 days | 1:3 | NO | 4,37 ± 1,29 | 22 | (33–47) | 16 | 1,35 |
| | | | 2:3 | NO | 4,55 ± 1,40 | 22 | (33–47) | 16 | 1,58 |
| | | | Undiluted | YES | 3,00 ± 1,88 | 35 | (33–47) | 16 | 1,84 |
| Z34b | 03/12/2010 (t4) | 13 days | 1:3 | NO | 4,39 ± 1,13 | 29 | (33–47) | 16 | 1,42 |
| | | | 2:3 | YES | 3,89 ± 1,13 | 37 | (33–47) | 16 | 1,78 |
| | | | Undiluted | YES | 2,16 ± 1,24 | 53 | (33–47) | 16 | 2,24 |
| W2a | 03/12/2010 (t4) | 10 days | 1:3 | NO | 4,1 ± 1,38 | 23 | (33–47) | 16 | 1,58 |
| | | | 2:3 | YES | 3,12 ± 1,79 | 38 | (33–47) | 16 | 2,04 |
| | | | Undiluted | YES | 1,64 ± 0,75 | 47 | (33–47) | 16 | 2,48 |
| *fragi* Z41b | 02/12/2010 (t3) | 15 days | 1:3 | NO | 4,82 ± 0,82 | 32 | (39–56) | 15 | 1,06 |
| | | | 2:3 | YES | 3,61 ± 1,35 | 41 | (39–56) | 15 | 1,16 |
| | | | Undiluted | YES | 3,38 ± 1,35 | 53 | (39–56) | 15 | 1,2 |
| *lundensis* W52b | 03/12/2010 (t4) | 13 days | 1:3 | NO | 4,53 ± 1,54 | 29 | (35–50) | 17 | 1,41 |
| | | | 2:3 | YES | 3,02 ± 1,44 | 43 | (35–50) | 17 | 1,81 |
| | | | Undiluted | YES | 2,78 ± 1,45 | 54 | (35–50) | 17 | 2,2 |

[a]

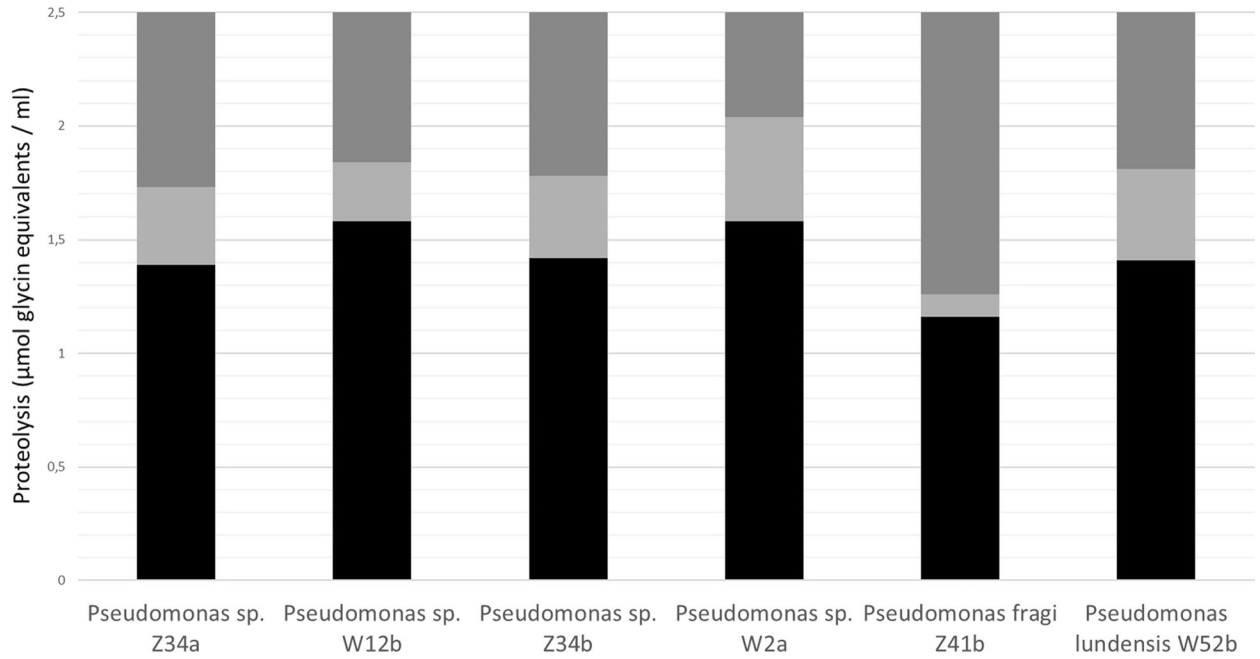


**Fig. 2** Correlation between off-flavors and protein hydrolysis in UHT milk by 6 different *Pseudomonas* protease groups. * No significant proteolysis off-flavors indicated by black bars, the uncertainty range by light gray bars (the panel did not reject the milk samples; the lower limit of the bar is determined by the TNBS-value of the most diluted sample that was not rejected by sensory analysis) and the significant proteolysis off-flavors by dark gray bars (panel rejected the milk samples and tasted off-flavors; the lower limit of the bar is determined by the TNBS-value of the least diluted sample)

equivalents $mL^{-1}$. It can be concluded that no plasmin activity was present and milk (raw or processed) of good quality should thus have a TNBS-value in that range. Next, all data concerning sensorial and proteolysis analyses were compiled in Fig. 2. This graph shows that no clear correlation can be obtained between the onset of off-flavors and the rate of protein hydrolysis in milk by the different *Pseudomonas* protease groups. The TNBS-values of the milk samples in which off-flavors were significantly tasted were different for each *Pseudomonas* protease under evaluation. For example, with *Pseudomonas* sp. W2a proteases, the TNBS-value was allowed to rise with 1,03 µmol glycin equivalents $mL^{-1}$ before any off-flavors were tasted. *P. fragi* proteases, on the other hand were capable in generating off-flavors after very limited proteolysis (a raise in TNBS-value of 0,15 glycin equivalents $mL^{-1}$). Therefore it can be speculated that not all *Pseudomonas* proteases have the same specificity for their casein substrates. The amino acid recognition sites within *Pseudomonas* proteases might thus be fundamentally different, resulting in peptide generation with a variable hydrophobic amino acid content. Further research, however is necessary to confirm this. Nevertheless, it is now clear that presence (of high numbers) of *P. fragi* strains prior to UHT-processing will severely compromise the shelf life of derived dairy products. To ensure good quality dairy products, milk should therefore be processed as quickly as possible or held refrigerated (≤2 °C) (Griffiths 1989; Haryani et al. 2005) awaiting further processing.

## Conclusions

High *Pseudomonas* counts and extended cold storage severely limits UHT-processing. Therefore, to ensure good quality dairy products, raw milk should be processed as quickly as possible or kept well refrigerated (≤2 °C) during the entire dairy chain (from farm to dairy). No clear correlation can be obtained between the degree of protein hydrolysis by the different *Pseudomonas* AprX proteases and the generation of off-flavors in UHT-milk. Nevertheless, *P. fragi* has the greatest spoilage potential within the tested *Pseudomonas* protease groups, when it comes to generating off-flavors.

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#### Authors' contributions

SM carried out the samplings, the sample analyses and drafted the manuscript. BD and JDB developed the new sensory evaluation test. MH, KC, JDB and BD participated in the design of the study. All authors read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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