

Dissemination and persistence of *Pseudomonas* spp. in small-scale dairy farms

Daniele Michele Nucera,¹

Sara Lomonaco,² Patrizia Morra,²

Marco Francesco Ortoffi,²

Daniele Giaccone,³ Maria Ausilia Grassi²

¹Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco (TO); ²Department of Veterinary Sciences, University of Turin, Grugliasco (TO); ³Regional Association of Farmers of Piedmont, Turin, Italy

Abstract

This study was aimed at collecting data on presence, dissemination and persistence of *Pseudomonas* in small-scale dairy farms. Six farms (located in Piedmont) were visited three times over 2014: 116 waters (wells and different faucets/pipes) and 117 environmental samples (milking equipments and drains) were collected. Enumeration of *Pseudomonadaceae* was performed, 3-5 colonies/samples were selected for identification via 16SrDNA/*oprI* polymerase chain reaction (PCR), and typed by enterobacterial-repetitive-intergenic-consensus (ERIC)-PCR. *Pseudomonadaceae* were detected in 77% of samples. No statistical differences were found among proportions of positives across farms, sample typologies and seasons. Most isolates were *Pseudomonas fluorescens* (45%), and ERIC-PCR showed 32 persistent types diffused across farms. All in all, *Pseudomonas* spp. represents a challenge, considering its presence over time in water as well as in teat cups, indicating a continuous source of contamination. Moreover, persistency of strains may indicate biofilm-formation and/or sanitisers resistance, therefore emphasising the role of primary production for preventing milk contamination by *Pseudomonas* spp.

Introduction

Psychrotolerant bacteria are mainly ubiquitous organisms able to grow at refrigeration temperatures regardless of their optimal growth temperature (Morita, 1975). *Pseudomonadaceae* represent a problem in primary production as well as in dairy-transforming plant: they may cause mastitis (Daly *et al.*, 1999) and milk/dairy products alterations even if these are kept under strict refrigeration temperatures (Ternström *et al.*, 1993). As contam-

inants of cheeses, *Pseudomonas* spp. can induce serious defects such as changes in colour of cheese surfaces (Leriche *et al.*, 2004; Martin *et al.*, 2011). In heat-treated milk, even in ultra high temperature milk, alterations due to *Pseudomonas* spp. enzymes are still possible, and these include gelation (Datta and Deeth, 2001). Some polymerase chain reaction (PCR) based methods have been applied for *Pseudomonas* spp. typing in order to detect genetic diversity and assess putative sources of contamination in dairy production, and methods were applied successfully (Eneroth *et al.*, 2000; Leriche *et al.*, 2004; Marchand *et al.*, 2009), identifying also persistent strains responsible for recurrent contamination. However, considering that *Pseudomonas* spp. may contaminate raw milk via biofilms in the milk tanks, contaminated water, or soil, few studies have been conducted focusing on primary production. For example, some authors (Leriche and Fayolle, 2012) revealed the presence and the level of *Pseudomonas* spp. and related species in milk just after milking and identified hygienic programmes and water quality as the most influential control measures. For this reason, the aim of this study was to collect new data on the presence of *Pseudomonadaceae* in milk producing farms, assess seasonal variation in frequency of contamination, and verify, through PCR-typing, the presence of persistent and largely diffused strains in a well defined food-production chain located entirely in Piedmont.

Materials and Methods

For this study it was selected a set of 6 farms producing milk for a large-scale production plant located in Piedmont. Farms were selected because of the presence and use of waters from underground wells: four of them were selected considering milk production and management procedures (representative of regional production: less than 100 milking cows, family owned and with few operators); two farms were selected because characterised by a similar production profile but also by having a small scale dairy production plant annexed.

Each farm was visited three times over 2014; during each visit water (from wells and different faucets/pipes) and environmental samples were collected. Samples of water were aseptically collected using 500 mL sterile bottles (intended for laboratory use) and placed at refrigeration temperature until analysis, few hours after collection. Environmental samples were collected from teat cups and from drains. The former, by scrubbing one sponge on the four cups of the same milking machine unit, whereas the latter by scrubbing an area of 100 cm² of drains located in the bulk-tank room

Correspondence: Daniele Michele Nucera, Department of Agricultural, Forest and Food Sciences, University of Turin, Largo Braccini 2, 10095 Grugliasco (TO), Italy.
Tel: +39.011.6708565 - Fax: +39.011.6708566.
E-mail:daniele.nucera@unito.it

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and in the two annexed dairy plants. Sponge bags were stored at refrigeration temperature and analyzed the same day of collection.

Enumeration of *Pseudomonadaceae* was performed using Cetrimide-Nalidixic Acid *Pseudomonas* agar, with previous membrane filtration (250 mL) for water samples. After incubation and enumeration, from 3 to 5 colonies/samples were selected for identification. Selection was applied to the highest dilution plated and when more than 10 colonies were present, 5 were used for further analyses. If less than ten colonies were present the number of selected ones was reduced to three. Selected colonies were identified via 16SrDNA/*oprI* PCR (De Vos *et al.*, 1997) and then sequenced. For identification, chromatograms were then retrieved, corrected (if needed) and compared through Blast-N similarity search (Genbank) with worldwide dataset.

After identification, each colony was cultured in Brian Hearth Infusion Broth and 1.8 mL used for DNA extraction with Ultra Clean Microbial DNA Extraction kit. The DNA was challenged with enterobacterial-repetitive-intergenic-consensus (ERIC) PCR, following the published protocol (Nucera *et al.*, 2013). After staining, profiles were visualised using a GelDoc UV transilluminator. These were first

checked by visual inspection, and then a software-assisted analysis was performed (Bionumerics®; Applied Maths NV, Sint-Martens-Latem, Belgium). Fingerprint comparison was carried out using Dice coefficient and unweighted pair group method with arithmetic mean algorithm. To set the threshold similarity level for indistinguishable patterns, a repeatability assay was performed by triplicate analyses (Corich *et al.*, 2005) on five strains. The lowest similarity level observed within each sample was used to define identical genotypes, minimising the intra-sample variation (Ravelo *et al.*, 2003).

Differences between proportion of positive samples of *Pseudomonas* spp. across time points was performed using chi-square tests, performed in SPSS® (v. 11.0). A proportion was calculated as the number of positive samples at each time point/number of total samples collected. The same test was also used to evidence differences across farms. In this latter case the proportions were calculated by dividing the number of positive samples in each farm by the total number of samples collected in each farm. Results were considered significant when $P < 0.05$.

Results and Discussion

Results showed that out of 233 samples retrieved (85 from teat cups, 32 from drains, and 116 from water pipes/faucets), 180 (77.3%) were positive for *Pseudomonadaceae*. They also showed that the proportion of positive samples was 73 and 75% for teat cups and drains, respectively; in samples of water the frequency of positive samples ranged from 86% in water collected from underground wells, to 73% in water used for milk processing (in annexed dairy plant); water collected from milking room and tank room showed both positive plates in 82% of the samples analyzed. Statistical analyses showed that there was no statistical difference among proportion of positive across sample types (Chi-square=0.15; $P=0.99$). Considering that sampling was performed in three different occasions, a comparison between frequency of positive samples per sampling visit for each sample type was also performed (Table 1).

Considering that there was no difference per visit nor per sample typology all sample were grouped together and the frequency of positive samples per farm was calculated. The

comparison between farms based on this variable was not significant (Table 2).

Polymerase chain reaction confirmed the presence of *Pseudomonas fluorescens* (45% of the isolates) as the predominant species in dairy environment, as previously reported by other authors (Wang and Jayarao, 2001; Dogan and Boor, 2004). However, typing results with ERIC PCR evidenced a genetic heterogeneity among the 250 tested strains (derived from 180 samples), all of which were similar at the 10% similarity level. A genetic heterogeneity was already observed within *Pseudomonas* species by other authors even if using different typing methods (Wang and Jayarao, 2001; Dogan and Boor, 2004). Considering the grouping of the PCR patterns, 18 clusters (group of strains with 80% or greater similarity) and 3 outliers were generated. There were 32 PCR profiles shared between strains collected in different time points from either the same or different farms in various sample types (Table 3).

These results show that 14 profiles were persistent over a period of 8 or more months and that 27 were shared between more than 2 farms, with three (P3, P10, and P14) being present in 4 of the 6 sampled dairies. Finally,

Table 1. Proportion of positive samples in different sample typologies considering the different sampling times.

Sample	Visit	Collected samples (n)	Positive samples (n)	f positive*	Significance
Sponge (teat cups)	I	28	18	0.64	Chi-square=3.09 P=0.21
	II	26	20	0.77	
	III	31	24	0.77	
	Total	85	62	0.73	
Sponge (drains)	I	11	7	0.64	Chi-square=0.28 P=0.87
	II	11	8	0.73	
	III	10	9	0.90	
	Total	32	24	0.75	
Well-water ^o	I	2	1	0.50	Chi-square=0.26 P=0.88
	II	2	2	1.00	
	III	3	3	1.00	
	Total	7	6	0.86	
Milking-water ^o	I	23	13	0.57	Chi-square=1.88 P=0.39
	II	19	19	1.00	
	III	19	18	0.95	
	Total	61	50	0.82	
Tank-water ^o	I	13	7	0.54	Chi-square = 1.21 P=0.55
	II	10	10	1.00	
	III	10	10	1.00	
	Total	33	27	0.82	
Processing-water ^o	I	4	0	0.00	Chi-square=3.47 P=0.18
	II	6	6	1.00	
	III	5	5	1.00	
	Total	15	11	0.73	
Total	-	233	180	0.77	-

I, winter/spring; II, summer; III, fall. *Number of positive samples per visit, regardless of the number of selected colonies; ^owater collected from different faucets/pipes located within the same room.

only 8 of the 32 persistent profiles were retrieved from only one sample typology, the remaining being present in two (N=17) or three (N=7) sample types.

All in all, these results highlight that the frequency of *Pseudomonadaceae* in dairy farm is high in all sample typologies, however no difference among proportions was observed among farms and seasons, therefore implying a constant environmental contamination. Considering that water is one of the most acknowledged sources of these family of Gram negative bacteria (Leriche *et al.*, 2004; Leriche and Fayolle, 2012) our results confirmed that *Pseudomonas* spp. is present in water used in primary production, both in well waters and in potable water, as already suggested (Leriche and Fayolle, 2012). The high frequency of positive in drains and in teat cups indicates that microbes are still present and alive after the sanitising procedures as well. In fact, specimens were all collected just after the washing cycle of the milking machine was concluded.

Our results seem to confirm what reported in the literature (Leriche and Fayolle, 2012). The presence of recurrent contamination suggests two possible scenarios: i) water may act as the primary vehicle of contamination in cleaned equipment, or ii) sanitising procedures are not effective in neutralising *Pseudomonas* spp. In our study no seasonal difference was observed in *Pseudomonadaceae* similarly to what observed by other researcher (Leriche and Fayolle, 2012). However, the lack of statistical significant differences across seasons for drains, well waters, processing waters and tank room waters may also be related to the low number of samples tested for each visit. This result may thus indicate that the contamination is diffused in primary production and no protective seasonal effect exists. However, in order to investigate this hypothesis a larger number of samples would need to be collected for each sample type and in each season.

Considering results of the typing PCR, even if the population examined showed great het-

erogeneity, 32 profiles were shared between strains collected in different farms or from different sample types (Table 3). These observations imply the presence of highly diffused genetic types, which may be spread within a well-defined geographic region, indicating that putative routes of transmission may exist in different environments. Among these, operators, equipments, milk transporting trucks, and water may represent the putative sources.

The presence of the same PCR type in different sample typologies within the same farm, indicates that, once the microbes are present, they can disseminate in all the dairy environments: from the barn to the milking room, from milking room, to tank room, and from this latter to the annexed dairy production plants. Profiles P14, P16, P18, P20, P23 e P32 were all present in primary production and in the annexed dairy transforming plants.

Another interesting result is the presence of PCR types that persist for periods lasting from 2 to 10 months. Persistent contamination was

Table 2. Proportion of positive samples in the six sampled farms.

Farm	Collected samples (n)	Positive samples (n)	f positive	Significance
A	35	23	0.66	Chi-square=1.11 P=0.71
B	43	32	0.74	
C	52	40	0.77	
D	34	27	0.79	
E	21	21	1.00	
F	48	37	0.77	
Total	233	180	0.77	-

Table 3. List of persistent profiles with relative persistence, sources and number of farms from which the strains were isolated.

PCR profile	Persistency (months)	Sources	Number of farms
P1, P2, P13	4	Water, teat cups	2
P3, P7	8	Teat cups, drains	4 (P3), 2 (P7)
P4, P5	4 (P4), 2 (P5)	Teat cups	2
P6	6	Drains	3
P8, P10, P31	10	Teat cups, drains	2 (P8, P31), 4 (P10)
P9, P16, P32	5 (P9, P16), 2 (P32)	Teat cups, drains	2
P11	9	Water, teat cups	2
P12	9	Drains	2
P14, P30	6	Teat cups, drains	4 (P14), 2 (P30)
P15	10	Water, teat cups, drains	1
P17, P21	9	Teat cups	1 (P17), 2 (P21)
P18	9	Teat cups, drains	3
P19, P20	2 (P19), 10 (P20)	Teat cups, water	2
P22, P23	8	Teat cups, water, drains	2
P24	3	Teat cups, water, drains	3
P25	10	Teat cups, water, drains	2
P26, P27	7	Teat cups, water, drains	3
P28, P29	4	Water	1

PCR, polymerase chain reaction.

also recorded previously (Eneroth *et al.*, 2000), showing a strain persisting in a dairy plant for 15 months. The persistence observed in the present research may be due to multiple reasons: i) the sanitation procedures are not effective (use of ineffective dose/contact time) (Leriche and Fayolle, 2012); ii) development of resistance to biocides (DeQueiroz and Day, 2007; Shen *et al.*, 2012); or iii) production of biofilms (Holm *et al.*, 2004; The *et al.*, 2014). Hence, future studies on typed strains are needed in order to investigate their susceptibility to sanitisers used in the selected farms, as well as *in vitro* studies for assessing the ability of persistence strains to form biofilm, using coupons made of different materials.

Conclusions

In conclusion, given that European Regulation set hygiene criteria for milk in primary production without including *Pseudomonas* spp. (which is not routinely detected, which is the reason why its presence is largely underestimated), this research highlighted that *Pseudomonadaceae* are extremely diffused in farm environments and water sources. Considering that this family of bacteria is well known for pigment production in dairies, it appears important the need of the elaboration of control programmes, by means of implementation of good hygienic practices manuals, particularly for small scale farms.

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