Pseudomonas fluorescens group bacteria as responsible for chromatic alteration on rabbit carcasses. Possible hygienic implications

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Abstract

Bacteria belonging to the genus Pseudomonas are ubiquitous and characterized by a high adaptation capability to different environmental conditions and wide range of temperatures. They may colonize food, sometimes causing alteration. Quite recently, a blue pigmentation due to Pseudomonas fluorescens has been widely reported in mozzarella cheese. In this report, we describe a blue coloration occurred on rabbit meat stored in the refrigeration cell of a slaughterhouse. The alteration was observed after about 72 hours of storage at 4-6°C. Bacteriological analyses were performed, and a microorganism included in the Pseudomonas fluorescens group was identified. The experimental contamination was planned, using a bacterial suspension with 1×10^8 UFC/ml load to spread on rabbit carcasses. The blue pigmentation appeared after 24 hours of storage in a cell with the same conditions of temperature. The bacterium was reisolated and identified as responsible for the alteration on meat. These findings highlight the importance of considering the members of the genus Pseudomonas and, more specifically, of the P. fluorescens group when the microbiological quality of food is to be ascertained. In fact, even if these bacteria are not considered a public health problem, their presence should be monitored by food industry operators in self-control plans because they may cause alteration in food. In fact, any altered product should be withdrawn from the market in agreement with Regulation (EC) No 178/2002 of the European Parliament and of the Council.

Introduction

The genus Pseudomonas includes rod cell, Gram-negative, aerobic, mesophilic and psychrotolerant bacteria with respiratory metabolism (Mailloux et al., 2011). Members of genus Pseudomonas appear as straight or slightly curved bacilli, from 0.5 to 1.0 μm in diameter and 1.5 to 5.0 μm in length, usually mobile for the presence of one or more flagella, unable to grow at a pH lower than 4.5 (De Jonghe et al., 2011). Their optimal growth temperature is equal to 25°C, but they can live in presence of lower temperatures (Decimo et al., 2014), increasing their survival capability.

The genus Pseudomonas includes several species, such as P. aeruginosa, P. fluorescens, and P. alcaligenes, which are all regarded as human opportunistic pathogens, chiefly in immune-deficient and/or nosocomial patients (Tümmeler et al., 2014; Peix et al., 2009), although some species are pathogenic for plants (P. pseudoalcaligenes, P. savastanoi, P. syringae) or for animals (P. anguilliseptica, P. chlororaphs, P. aeruginosa) (Caldera and Franzetti, 2014). These bacteria are commonly found in decaying organic material like rotting leaves and soil and have simple nutritional requirements (Anzai et al., 2000; Frapolli et al., 2007). In association with other bacteria such as Proteus spp, Escherichia coli, Citrobacter spp, Salmonella spp, Enterococci (Tassew et al., 2010; Soriano et al., 2001), Pseudomonas spp. constitute the microbial flora of a variety of foods, depending on several factors including the physical-chemical composition of the food, the storage conditions, the health status of animals and the nature of animal feed (Giraffa et al., 2014). However, some authors have identified Pseudomonas spp. as predominant bacteria species in the early stages of fermentation and storage of many foodstuffs such as refrigerated milk (De Jonghe et al., 2010), raw poultry (Dominguez et al., 2007) and fish (Tryfinopoulou et al., 2001; Zeng et al., 2015). Although most Pseudomonas species have an environmental origin, different species are often observed in foods, depending on the substrate: (i) in milk, P. lundensis, P. fragi, P. fluorescens, and P. germardi are commonly observed (Marchand et al., 2009); (ii) in meat, in processing facilities such as cutting and processing laboratories, it is common to observe P. fluorescens and P. fragi (Drosinos and Board, 1995); and (iii) in fish products, P. aeruginosa, P. putida, P. chlororaphs, and P. fluorescens are reported more frequently, all of which are considered opportunistic for fish species (Altinok et al., 2006; Angelini and Seigneur, 1988). The presence of P. fluorescens in food often triggers chromatic alterations, due to enzymatic reactions through which the bacterium uses quinoline as a source of carbon, nitrogen and energy, leading to the production of pigments (Andreani et al., 2014; Schwarz et al., 1989). Other pigment-producing strains are P. aeruginosa, P. lundensis, P. putida, P. chlororaphs subsp. Chlororaphis, and P. chlororaphis subsp. aureofaciens (Gennari and Dragotti, 1992). Chromatic alterations due to Pseudomonas fluorescens have been previously detected in dairy products (Martin et al., 2011; Nogarol et al., 2013). More recently, similar abnormalities caused by Pseudomonas azotoformans have been described in rabbit carcasses (Circella et al., 2020).

In this study, a superficial meat alteration, consisting of blue coloring, observed in rabbit carcasses during their preservation in the refrigeration cell of a slaughterhouse waiting for the sale, and the identification of the bacterium responsible for the alteration.

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are reported. The same contamination has been reproduced under experimental conditions using the identified bacterium.

**Materials and Methods**

**Farm and slaughterhouse layout**

The carcasses of rabbits were from an industrial rabbit farm provided with a regularly authorized slaughterhouse (Reg EU 853). The closeness between the sheds for breeding and the slaughterhouse allowed the slaughter *in situ* with consequent practical advantages and cost savings, since no transporting of live animals is needed. The produced carcasses were commercialized in local shops and markets in an area of 100 km radius.

The reared rabbits were commercial hybrids for meat production. At slaughterhouse, there was a refrigeration cell, where carcasses are usually stored before the sale at temperature of 4-6°C, for a time ranging from 24 hours to 3 days.

**Chromatic alteration occurrence**

The involved rabbits belonged to the same batch, and they did not show clinical sign at ante-mortem examination. Likewise, the carcasses did not have lesions or alterations at post-mortem inspection. A chromatic alteration consisting of a blue coloration in spots ranging from 5-6 to 9-10 cm in size appeared on the surface of the meat in some carcasses after about 72 hours spent in the refrigeration cell (Figure 1). Four carcasses were sent to Department of Veterinary Medicine of Bari, for laboratory investigations.

**Isolation**

Both direct and post-enrichment bacteriological tests were performed on the carcasses. Sterile swabs, humidified in sterile physiological solution, were rubbed over the blue spots of the carcasses for direct examination. The swabs were passed onto Trypticase Soy Agar (TSA-Oxoid, Milan, Italy) enriched media and selective media (Pseudomonas Agar Base-Oxoid, Milan, Italy). At the same time, portions of tissues with 1 cm² of extension were placed in pre-enrichment in peptone water (ratio of 1:10). After 24 h of incubation at 37°C, the broths were seeded onto solid media, TSA, and Pseudomonas Agar Base. Liquid and solid media were incubated under aerobic conditions at 37°C. The incubation time was 24 h for each step. The isolation of the colonies was performed on TSA and their identification was obtained by biochemical tests in micro-method (Api 20NE tunnels-Bio Merieux).

**Characterization**

In order to confirm the identification, a colony-PCR targeting the 16S rRNA gene was carried out. Briefly, a single, well-isolated colony from a pure culture was picked and resuspended in 10 μl of sterile distilled water. Two microliters of cell suspension were used as a template in the reaction, performed by using the Platinum II Got-Start Green PCR Mastermix (ThermoFisher Scientific, Milan, Italy) and adding 0.75 μM each of 27F (5’-AGAGTTTGATCMTG- GCTCAG-3’) and 1492R (5’-CTACGGGY- TACCTTGTTACGAC-3’) primers, modified from Garrido-Sanz et al. (2017). The gathered amplicon was purified by means of the PureLink Quick Gel Extraction and PCR Purification Combo Kit (ThermoFisher Scientific) and sequenced by the BigDye Terminator method at the facilities of Bio-Fab Research (Rome, Italy). Other than PCR primers, the 341f (5’-CCTACGGGAGGCAGCAG-3’) and 907r (5’-CCCCGTCAATTTCATTGGA GTTT-3’) primers (Lane, 1991) were used for sequencing. The reads were assembled by the online Cap3 Sequence Assembly Program (Huang and Madan, 1999) and the final nucleotide sequence, after removal of primers and low-quality regions, was compared by BLAST with those available in GenBank from type materials. The sequence was analyzed by the leBIBI IV 16S Automated Prokaryotes Phylogeny, available at https://umr5558-proka.univ-lyon1.fr/PKPhy/optimized_input.html.

**Experimental reproduction of the chromatic alteration**

To confirm the responsibility of the detected bacterium for the macroscopic lesions observed on the rabbit carcasses, an experimental reproduction of the alteration has been performed.

Three carcasses of commercial hybrid rabbits for meat production coming from a local market were used. The presence of *Pseudomonas* on the surface of carcasses was excluded by bacteriological analyses performed using the methods previously described. A bacterial suspension with 1×10⁸ UFC/ml concentration was prepared using the isolated bacterial colonies, based on the bacterial load used in a previous study on rabbit meat (Circella et al., 2020) and generally found in other matrixes with blue alteration due to *P. fluorescens* (>10⁷ cfu/g) (Bogdanova et al., 2010). The suspension was used to perform the experimental reproduction of the chromatic alteration. Therefore, 1 ml of the suspension was spread by brushing with sterile swabs approximately in the same spots onto the surface of the rabbit carcasses. The carcasses were immediately placed in a cold room at a temperature of about 4°C and were kept under observation for the following three days. Moreover, the microorganism was re-isolated out of the experimentally infected meat, and it was genetically analyzed.

![Figure 1. Chromatic alteration on the surface of the meat.](image-url)
Results

The bacteriological tests performed on the samples collected by the altered carcasses highlighted the growth of uniform bacterial colonies with the fluorescent pigmentation typical of *P. fluorescens* on the enriched media after 24 hours of incubation. The colonies were confirmed as *P. fluorescens* according to biochemical tests.

The BLAST analysis of the nucleotide sequence (QRY_356) of the 16S rRNA gene revealed it was 99.93% identical to the corresponding sequence of *Pseudomonas azotoformans* strain LGM 21611 (accession number LT629702) and 99.65% to the *Pseudomonas syxantha* strain NCTC10696 (LR590482). The phylogenetic analysis by the leBibi system included the sequence in close proximity, among the type strains, of a group of species belonging to the *P. fluorescens* group (Figure 2).

Experimental reproduction of the blue chromatic alteration was obtained on meat as soon as 24 h after refrigeration (Figure 3). The chromatic alteration was very similar to the one noticed on the carcasses preserved in the refrigerator cell of the slaughterhouse. *P. fluorescens* was re-isolated from the colored area of the carcasses in the experimentally contaminated meat and it was identified based on genetic analyses as the same bacterium involved in the chromatic alterations previously observed.
Discussion

The bacterium isolated in this study was responsible for the blue coloration observed on the rabbit carcasses. The strain responsible for the alteration was initially identified through biochemical tests as P. fluorescens, which is the representative species of the P. fluorescens group it belongs to (Palleroni and Genus, 2005).

P. fluorescens has been frequently found in food substrates, and it is well known for having been the cause of spoilage of dairy products, such as mozzarella, which exhibited a very typical blue coloration (Martin et al., 2011; Nogarol et al., 2013). Nevertheless, the BLAST analysis of the nucleotide sequence of the 16S rRNA gene of detected strain revealed an almost perfect match with the corresponding sequence of two species, Pseudomonas azotoformans and Pseudomonas synxantha respectively, due to the high identity among the 16S rRNA gene sequences of the species belonging to the P. fluorescens group, and, more generally, within the genus Pseudomonas.

Recently, the isolation of P. azotoformans from rabbit meat was reported (Circella et al., 2020). This specie has not frequently been found to be associated with food contamination, apart from a recent report of milk contamination (Evanowski et al., 2017). This may help to drive attention to an organism that is relatively unknown, and probably undervalued in its spoilage potential.

All the members of P. fluorescens group are microorganisms with poor nutritional requirements. Consequently, these bacteria have the ability to adapt even to hostile environments such as in cold rooms, where the conditions for their growth are not optimal (Anzai et al., 2000; Frapolli et al., 2007). Their ability to adapt to different environments may be likely related to the formation of biofilms, making the bacterial population more resistant (Rossi et al., 2018).

The primary source of meat contamination was not determined but the isolation of P. fluorescens-related microorganisms on rabbit carcasses suggests the importance of good sanitization procedures in the production chain, in agreement with other studies (Cenci-Goga et al., 2014). The meat represents an optimal substrate for replication of pseudomonads or other opportunistic microorganisms after contamination.

Furthermore, Pseudomonads live especially in seawater and fresh water (Mena and Gerba, 2009). Therefore, the water used to wash slaughter equipment and refrigeration rooms could represent a possible source of contamination (Asghari et al., 2013). Accordingly, P. azotoformans which was responsible for chromatic alterations in rabbit carcasses was detected in water used in the slaughter processes (Circella et al., 2020).

The refrigeration cell and each equipment used in the production chain should be disinfected frequently during slaughtering operations, to improve the environmental decontamination and avoid the alteration of carcasses. As previously suggested (Cenci-Goga et al., 2014; Circella et al., 2020), the alteration induced by P. fluorescens was directly correlated to storage time in addition to environmental temperature. In fact, the blue coloration appeared on carcasses after about 72 hours of storage in refrigeration cell, but it was not observed in the first two days. Moreover, the bacterial load seems to play a role in the appearance time of the alteration. After the experimental contamination performed on the carcasses, the blue coloration appeared within 48 hours. This was probably due to the high concentration of bacteria used for the experimental design, as previously observed under the same experimental conditions (Circella et al., 2020).

Although members of P. fluorescens are rarely, if not never, associated with human pathologies, there are several reports in which the presence of P. fluorescens in dairy products, fish, vegetables, and meat has led to marked deterioration of the products and withdrawal from the market (Garcia-Lopez et al., 2004; Nogarol et al., 2013). In addition, due to its environmental resistance, P. fluorescens is very difficult to eradicate once introduced into the production environment (Decimo et al., 2014). Therefore, although it is not considered therein, it could be regarded as a “process hygiene criterion” under Commission Regulation (EC) No. 2073/2005 as an environmental contaminant, just like Enterobacteriaceae. Consequently, although not mentioned in food regulations, a contamination due to species belonging to the P. fluorescens group should be considered unacceptable because it makes food unsuitable for human consumption. Therefore, any products altered by those microorganisms should be withdrawn from the market in agreement with Regulation (EC) No. 178/2002 of the European Parliament and of the Council. Accordingly, the presence of Pseudomonas spp. should be monitored by food industry operators in their self-control plans.

Conclusions

P. fluorescens was responsible for the chromatic alteration described in this report. Recently, a blue coloration on rabbit meat due to P. azotoformans belonging to the Pseudomonas group has been reported. Although these bacteria do not cause clinical sign in humans, those findings highlight the importance of their monitoring in the production chain because contaminated products are not available for consumption based on art. 14 comma 5 Reg. (CE) n. 178/2002.

References

Altinok I, Kayis S, Capkin E, 2006. Pseudomonas putida infection in rainbow trout. Aquaculture 261:850–5.

Andrei
e NATO, Martino ME, Fasolato L, Carraro L, Montemurro F, Mioni R, Bordin P, Cardazzo B, 2014. Tracking the blue: A MLST approach to characterise the Pseudomonas fluorescens group. Food Microbiol 39:116–26.

Angelini NM, Seigneur GN, 1988. Disease of the fins of Rhamdia sapo. Isolation of the etiological agents and experimental infection. Revista Argentina de Microbiologia 20:37–48.

Anzai Y, Kim H, Park JY, Wakabayashi H, Oyazu H, 2000. Phylogenetic affiliation of the pseudomonas based on 16S rRNA sequences. Int J Syst Evol Microbiol 50:1563–89.

Asghari FB, Niakeen M, Mirhendi H, 2013. Rapid monitoring of Pseudomonas aeruginosa in hospital water system: a key priority in prevention of nosocomial infection. FEMS Microbiology Letters 343:77-81.

Bogdanova T, Flores Rodas EM, Greco S, Torli R, Bilei S, 2010. Indagine microbiologica su campioni di mozzarella in occasione dell’allerta Mozzarella blu. In Proceedings of the XII Congresso Nazionale S.I.Di.L.V., 2010 ott 27-29, Genova, Italy, pp. 48–149.

Calderaz L, Franczaz L, 2014. Effect of storage temperature on the microbial composition of ready-to-use vegetables. Curr Microbiol 68:133–9.

Cenci-Goga BT, Karama M, Sechi P, Iulietto MF, Novelli S, Mattei S, 2014. Evolution under different storage conditions of anomalous blue coloration of Mozzarella cheese intentionally contaminated with a pigment-producing strain of Pseudomonas fluorescens. J Dairy Sci 97:6708–18.

Circella E, Schiavone A, Barrasso R,
Camarda A, Pugliese N, Bozzo G, 2020. Pseudomonas azotoformans belonging to Pseudomonas Fluorescens group as causative agent of blue coloration in carcasses of slaughterhouse rabbits. Animals 10:2-9

De Jonghe V, Coorevits A, Van Hoorde K, Messens W, Van Landschoot A, De Vos P, Heyndrickx M, 2011. Influence of storage conditions on the growth of Pseudomonas species in refrigerated raw milk. Appl Environ Microbiol 77:460–70.

Decimo M, Morandi S, Silvetti T, Brasca M, 2014. Characterization of Gram-negative psychrotrophic bacteria isolated from Italian bulk tank milk. J Food Sci 79:2081–90.

Domínguez SA, Schaffner DW, 2007. Development and validation of a mathematical model to describe the growth of Pseudomonas spp. in raw poultry stored under aerobic conditions. Int J Food Microbiol 3:287-95.

Drosinos EH, Board RG, 1995. Microbial and physicochemical attributes of minced lamb: Sources of contamination with pseudomonas. Food Microbiol 12:189–97.

European Commission, 2002. Regulation of the European Parliament and of the Council of 28 January 2002 Laying down the General Principles and Requirements of Food Law, Establishing the European Food Safety Authority and Laying down Procedures in Matters of Food Safety, 178/2002/CE. In: Official Journal, L 31, 01/02/2002.

European Commission, 2004. Regulation of the European Parliament and of the Council of 29 April 2004 Laying down Specific Hygiene Rules for Food of Animal Origin, 853/2004/CE. In: Official Journal, L 139, 30/04/2004.

European Commission, 2005. Regulation of the European Parliament on Microbiological Criteria for Foodstuffs,1/2005/CE. In: Official Journal, L 338, 22/12/2005.

Evanowski RL, Reichjler SJ, Kent DJ, Martin NH, Boor KJ, Wiedmann M, 2017. Pseudomonas azotoformans causes gray discoloration in HTST fluid milk. J Dairy Sci 100:7906–9.

Frapolli M, Défago G, Moënne-Loccoz Y, 2007. Multilocus sequence analysis of biocatalytic fluorescent Pseudomonas spp. producing the antifungal compound 2,4-diacylphloroglucinol. Environ Microbiol 9:1893–55.

García-Lopez I, Otero A, García-Lopez ML, Santos JA, 2004. Molecular and phenotypic characterization of non motile Gram-negative bacteria associated with spoilage of freshwater fish. J Appl Microbiol 96:878–86.

Garrido-Sanz D, Arrebolé E, Martinez-Granero F, García-Méndez S, Muriel C, Blanco-Romero E, Martin M, Rivilla R, Redondo-Nieto M, 2017. Classification of isolates from the Pseudomonas fluorescens complex into phylogenomic groups based in group-specific markers. Front Microbiol 8:413.

Gennari M, Dragotto F, 1992. A study of the incidence of different fluorescent Pseudomonas species and biovars in the microflora of fresh and spoiled meat and fish, raw milk, cheese, soil and water. J Appl Microbiol 72:281–8.

Huang X, Madan A, 1999. CAP3: A DNA sequence assembly program. Genome Res 9:868–77.

Lane DJ, 1991. 6S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M., Eds. Nucleic Acid Techniques in Bacterial Systematic. John Wiley and Sons, Chichester, UK, pp. 115–175.

Mailloux RJ, Lemire J, Appanna VS, 2011. Metabolic Networks to combat oxidative stress in Pseudomonas fluorescens. Antonie van Leeuwenhoek 99:433-42.

Marchand S, Heylen K, Messens W, Coudijzer K, De Vos P, Dewittink C, Herman L, De Block J, Heyndrickx M, 2009. Seasonal influence on heat-resistant proteolytic capacity of Pseudomonas lundensis and Pseudomonas fragi, predominant milk spoilers isolated from Belgian raw milk samples. Environ Microbiol 11:467–82.

Martin NH, Murphy SC, Ralyea RD, Mena KD, Gerba CP, 2009. Risk assessment of Pseudomonas aeruginosa in water. In: Review of Environmental contamination and Toxicology. Springer, New York, NY, pp 71-115.

Nogarol C, Acutis PL, Bianchi DM, Maurella C, Peletto S, Gallina S, Adriano D, Zuccon F, Borrello S, Caramelli M, Decastelli L, 2013. Molecular characterization of Pseudomonas fluorescens isolates involved in the Italian “blue mozzarella” event. J Food Prot 76:500–504.

Palleroni NJ, Genus I, 2005. Pseudomonas Migula 1894, 237AL (Nom. Cons., Opin. 5 of the Jud. Comm. 1952). In: Brenner DK, Krieg NR, Staley JT, Garrity GM, Eds. Bergey’s Manual of Systematic Bacteriology, 2nd ed. Springer: New York, NY, USA, Vol 2, Part B, pp 328–379.

Peix A, Ramirez-Bahena MH, Velazquez E, 2009. Historical evolution and current status of the taxonomy of genus Pseudomonas. Infect Genet Evol 9:1132–47.

Rossi C, Serio A, Chaves-López C, Annibali F, Auricchio B, Goffredo E, Cenci-Goga BT, Lista F, Fillo S, Paparella A, 2018. Biofilm formation, pigment production and motility in Pseudomonas spp. isolated from dairy industry. Food Control 86:241–8.

Schwarz G, Bauder R, Speer M, Rommel TO, Lingens F, 1989. Microbial metabolism of quinoline and related compounds. Degradation of quinoline by Pseudomonas fluorescens 3, Pseudomonas putida 86 and Rhodococcus spec B1. Biol Chem Hoppe-Seyler 370:1183-89.

Tassew H, Abdissa A, Beyene G, Gebreselassie S, 2011. Microbial flora and food borne pathogens on minced meat and their susceptibility to antimicrobial agents. Ethiop J Health Sci 20:137-43.

Tryptophonolou P, Drosinos EH, Nychas GJ, 2001. Performance of Pseudomonas CFC-selective medium in the fish storage ecosystem. J Microbiol Methods 47:243-7.

Tümmler B, Wiehlmann L, Klockgether J, Cramer N, 2014. Advances in understanding Pseudomonas. F1000 Prime Rep 6:9.

Zeng X, Chen X, Zhang W, 2015. Characterization of the microbial flora from suan yu, a chinese traditional low salt fermented fish. J Food Process Preserv 40:1093-3.