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 In fact, any altered product should be withdrawn from the market in agreement with Regulation (EC) No 178/2002 of the European Parliament and of the Council.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 (Tümmler *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. chloraphys, 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.,* 2004). 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. ludensis, P. fragi, P. fluorescens*, and *P. gessardi* 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. chloraphis*, 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. clororaphis* subsp. *Chlororaphis*, and *P. chlororaphis* subsp. *aureofaciens* (Gennari and Dragotto, 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 cm^2 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′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CTACGRVTACCTTGTTACGAC-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′-CCCCGTCAATTCATTTGAGTTT-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 1×10^8 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* (>10^7 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.

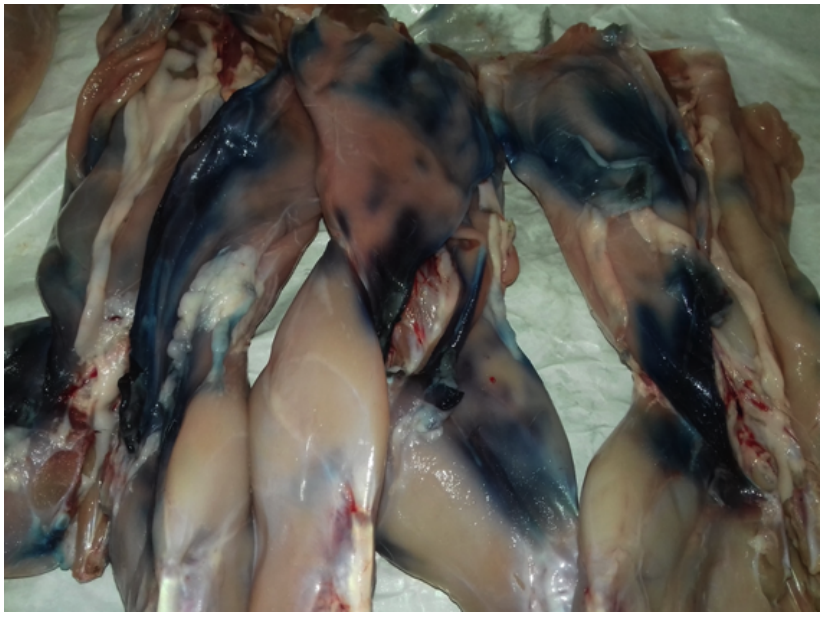

Figure 1. Chromatic alteration on the surface of the meat.

Figure 2. Phylogenetic analysis by the leBibi system.

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 synxantha* 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.

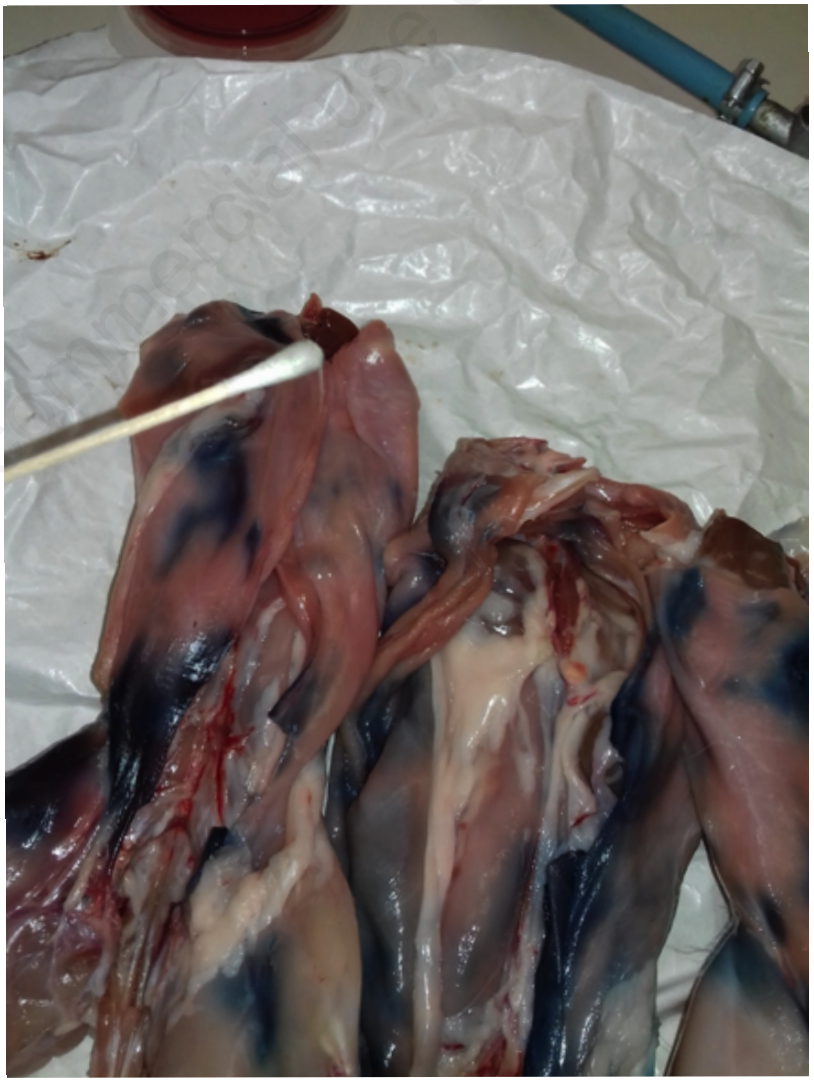

Figure 3. Experimental reproduction of the blue chromatic alteration.

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 pseudomonas 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.

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