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Evolution of Psychrotrophic Bacteria on Beef During Refrigeration

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Abstract Meat is a nutrient-dense food. This makes it a favorable environment for the proliferation of the most bacteria even if this food is refrigerated. The objective of this study was to follow the evolution of psychrotrophic bacteria mainly of the genus "*Pseudomonas*" on meat beef from the slaughterhouse of the municipality of Ouargla, during refrigeration. Samples were taken aseptically from the thighs of 15 bovine carcasses using the excision method. The psychrotrophic flora was counted on the nutrient agar and Plate Count Agar media, by mass inoculation of these culture media with the stock solution and its decimal dilutions. The phenotypic identification of the strains after isolation and purification was carried out by a morphological characterization of the colonies, microscopic of the bacterial cells and then the latter were subjected to a set of biochemical tests. The isolation of *Pseudomonas* was done on the two selective media: King A and King B (King A and King B media are used to guide the identification of *Pseudomonas* (the detection of the synthesis of pyocyanin and pyoverdin), the purification of the strains cultivated on these media was carried out on the nutrient broth then Nutrient Agar medium (GN) and the identification was accomplished by different biochemical tests. The count revealed the presence of psychrotrophic flora in all the meat samples analyzed. The means of contamination by this flora were 4.22±0.29 logufc/g on GN medium and 4.08±0.49 logufc/g on PCA medium. The isolated and purified strains belonged to ten strains, eight of which belonged to the genus: Pseudomonas, and two presented characteristics other than this genus. The bacterial diversity of this meat in the chilled state revealed six bacterial genera: *Pseudomonas*, *Micrococcus*, *Acinetobacter*, *Flavobacterium*, *Staphylococcus* and *Proteus*. This leads to the conclusion that the meat beef produced at the slaughterhouse of Ouargla and preserved by refrigeration contains a bacterial flora belonging to several genera of psychrotrophic bacteria.

Keywords: *contamination, conservation, meat, spoilage flora, pseudomonas*

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1. Introduction

Meat is a food of good nutritional quality, because it is rich in nutrients, especially in proteins of high biological value.

However, they constitute a favorable medium for the development of microorganisms. They are considered as a fragile raw material which must be strictly monitored because of the dangers following the possible presence of pathogenic germs [1,2].

Facultative psychrotrophic or psychrophilic bacteria are microorganisms that grow at low temperatures (from 25°C to 5°C) although this is not their optimum temperature [3,4,5]. These bacteria are refrigerated food spoilage agents [6].

The application of refrigeration increases the shelf life of meat, slowing down the rate of proliferation of microorganisms and intrinsic and extrinsic spoilage mechanisms and maintaining its organoleptic and nutritional properties. *Pseudomonas* is not very virulent, several strains are opportunistic pathogens for humans and spoilage agents of meat, fish and dairy products [7].

Meat is often implicated in food poisoning due to the presence of psychrotrophic flora, especially of the genus *Pseudomonas*. The latter is used as an indicator of deterioration of fresh meat.

This study aimed to assess the quality of meat beef produced at the Ouargla slaughterhouse, one of the largest slaughterhouses in Algeria. By following the evolution of the level of its contamination during refrigeration by the psychrotrophic flora: *Pseudomonas*. And the phenotypic identification of certain strains belonging to this flora.

2. Materials and Methods

2.1. Biological Material

The biological material used for this study was taken from beef produced at the slaughterhouse in the municipality of Ouargla in Algeria.

2.2. Experimental Protocol

2.2.1. Sampling

The samples were taken at the slaughterhouse of the municipality of Ouargla, just after slaughter and *post-mortem* inspection of slaughtered animals. Knowing that the animals were used without taking into account their race or their sex. The samples were taken from the same anatomical area of the carcasses: the thigh. The carcasses were chosen randomly, without taking into account either the age or the sex of the animal. Each sample of approximately 250 g was individually wrapped in a sterile bag. The transport was carried out under cold conditions in an isothermal cooler.

In the laboratory, the samples were immediately cut aseptically into pieces of about 10 g. Each sample was placed individually in a sterile bag of Stomacher, and the whole was placed in a refrigerator at a temperature of around +4°C.

The monitoring of the evolution of psychrotrophs during refrigeration was carried out by daily bacteriological analyses, from the first day of sampling for the duration of the study.

2.2.2. Preparation of Stock Suspension and Decimal Dilutions

The stock suspension was the first dilution prepared from meat. Each 10 g of beef was placed in a sachet of Stomacher in the presence of 90 ml of peptone water. Homogenization was carried out by grinding each test portion for 2-3 minutes in the stomacher. The ground material thus obtained constituted the stock solution 1/10 (10^{-1}). The preparation of the decimal dilutions was carried out in accordance with the French standard ISO 6887-2 [8]. Thus, one milliliter of this solution was transferred aseptically into a test tube containing 9 ml of peptone water sterile 0.1% (the 10^{-2} dilution). Proceed in the same way until dilution 10^{-4}.

2.2.3. Enumeration of Flora

The samples were subjected to the enumeration of the psychrotrophic bacterial flora, which represents the bacteria having the capacity to grow at refrigeration temperatures. This flora was counted on two culture media, according to the French standard ISO 17410 [9]. Plate Count Agar (PCA) and nutrient agar (GN), by mass inoculation. The Petri dishes thus inoculated were read after five to ten days of incubation at +4°C [10].

2.2.4. Characterization and Phenotypic Identification of Strains

A selection of the colonies according to their macroscopic characteristics was carried out, followed by a step of purification of the isolates, which consists of the seeding of each colony of different appearance separately in 9 ml of nutrient broth, then incubation for 18 hours at 24 hours, at +37°C. Each subculture thus obtained was inoculated by streaking on the two selective media for *Pseudomonas*: King A and King B and on the GN medium. The purification of the bacteria thus isolated was established by successive inoculation on nutrient broth, then on nutrient agar medium by the streaking method until clearly distinct and homogeneous colonies were obtained.

The latter were subjected to three characterizations, the first macroscopic based on the visual observation of the colonies and the culture medium, the second microscopic by the Gram staining of the bacterial cells and the third biochemical tests, by catalase and oxidase tests and the research of glucose and lactose fermentation and the production of gas on the TSI medium and of the respiratory type on meat-liver medium.

3. Results

3.1. Enumeration of the Psychrotrophic Flora of Chilled Beef

A variability of the levels of psychrotrophic bacteria of each sample was recorded according to the culture medium used (Table 1). The same contamination profile of this meat during refrigeration on both GN and PCA media was noted for these germs. They showed an increase in their number from the first day until the fifth day of refrigeration. A remarkable increase in the load of this flora was recorded on the second day of refrigeration on the GN medium, followed by a gradual increase on the two media, the levels becoming uncountable after five days of refrigeration (Table 1).

Table 1. Average levels of psychrotrophic bacteria according to the culture medium

	Psychrotrophic Flora	
Culture medium	**GN**	**PCA**
	Mean± standard deviation (logufc/g)	Mean± standard deviation (logufc/g)
T1 = 1d	3,76±0,54	3.24 ±0.28
T2 = 2d	4.21±0.46	3.84±0.34
T3 = 3d	4.31±0.07	4.25± 0.43
T4 = 4d	4,50±0,02	4.51±0.12
T5 = 5d	4.55±0.02	4,57±0,07
T6 = 6d	ind	ind
Mean± standard deviation (logufc/g)	4,27±0,29	4,08±0,49

Legend: T: time, d: day, ufc: colony-forming unit, g: gram, ind: uncountable, GN: nutrient agar, PCA: Plate Count Agar**.**

Table 2. Macroscopic appearance of colonies of psychrotrophic bacteria on nutrient agar

Isolates	Form	ElevationWith respect to the culture medium	Surface of the colonies and theirConsistency	Opacity of the colonies	Outline of the colonies	Size of the colonies	Color of the colonies
Sb1	Circular	Domed	Shiny and creamy smooth	Translucent	Regular	Big	Cream (beige)
Sb2	Circular	Domed	Shiny and creamy smooth	dark	Regular	Big	Yellow
Sb3	Circular	Domed	Shiny and creamy smooth	dark	Regular	Small	Brown
Sb4	Circular	Domed	Shiny and creamy smooth	Translucent	Regular	Medium	Beige
Sb5	Circular	Domed	Shiny and creamy smooth	dark	Regular	Small	Yellow
Sb6	Circular	Domed	Shiny and creamy smooth	dark	Regular	Medium	Brown
Sb7	Circular	Domed	Shiny and creamy smooth	dark	Regular	Small	Pink
Sb8	Circular	Domed	Shiny and creamy smooth	dark	Regular	Small	White
Sb9	Circular	Domed	Shiny and creamy smooth	dark	Regular	Small	Orange
Sb10	Elongated	Plate	Smooth and creamy	dark	Regular	Medium	Whitish
Sb11	Elongated	Flat	Smooth and creamy	dark	Regular	Medium	Beige to yellowish
Sb12	Ondulée	Flat	Rough and dry	dark	Irregular	Big	Whitish
Sb13	Circular	Domed	Shiny and creamy smooth	Translucent	Regular	Small	Beige
Sb14	Circular	Domed	Shiny and creamy smooth	Translucent	Regular	Big	Translucent with brown center

Legend: Sb: Strain grown on nutrient agar.

Table 3. Macroscopic appearance of colonies of psychrotrophic bacteria on King A and King B

Isolates	Culture medium/ Form	Elevation oppositeculture medium	Surface and consistency	Opacity of the colonies	Outline of the colonies	Size of the colonies	Color of the colonies
	King A Circular	Raised with domed center	Shiny and creamy smooth	Translucent	Regular	Medium	Whitish beige
	King A Circular	Domed	Smoothand creamy	Opaque	Regular	Big	Brown
	King A Elongated	Domed	Smoothand creamy	Translucent	Regular	Medium	Creamy
	King B Circular	Domed	Shiny smooth	Opaque	Regular	Big	Brown with orange center
	King B Circular	Flat high center	Shiny smooth	Opaque	Regular	Medium	Brown
	King A Elongated	Domed center	Shiny smooth	Opaque	Regular	Medium	White
	King B Circular	Domed	Smooth	Translucent	Regular	Big	Brown
	King B Elongated	Flat high center	Rough	Translucent	Regular	Medium	White
	King A Circular	Domed center	Shiny and creamy smooth	Opaque	Regular	Small	Beige - brown
	King B Circular	Domed	Shiny and creamy smooth	Translucent	Regular	Medium	White

Legend: S: Strain grown on King A or King B.

3.2. Isolation of Psychrotrophs and *Pseudomonas*

From the different colonies obtained on the PCA and GN media, after their simultaneous inoculation on the GN medium and the King A and King B selective media, it was noted that 14 different colonies (Sb1 - Sb14) developed on the GN medium, 5 strains (S1, S2, S3, S6 and S9) on King A medium and 5 strains (S4, S5, S7, S8 and S10) on King B medium (Table 2 and Table 3).

3.3. Macroscopic Characterization of Cultures

3.3.1. Macroscopic Characterization of Cultured Psychrotrophic Bacteria on Nutrient Agar

A wide range of appearances (smooth, creamy, dry) and sizes (small, medium and large), colonies with very varied colors (transparent white, creamy white, white, beige, yellow, orange, yellow orange…) was noted on the GN medium (Table 2). The smooth or viscous appearance of the colonies provides information on the synthesis of capsular exopolysaccharides by these bacteria.

3.3.2. Macroscopic Characterization of Cultured Psychrotrophic Bacteria on King A and King B Media

The observation of the cultures of the psychrotrophs after purification showed different types of colonies on the King A and King B media. The presence of circular or elongated, rounded or flat colonies, with a smooth or rough surface, opaque or translucent having a regular outline and of different colors (beige, white, cream and dark cream) and of different sizes (Small, medium and large; Table 3).

3.3. Microscopic Characterization of the Isolates Obtained

Microscopic characterization after Gram staining of bacteria cultured on King A and King B showed that all cells are Gram negative bacilli except for S4 and S5 strains which exhibited the Gram positive criteria. While those cultured on nutrient agar, the criterion of Gram negative bacilli and Gram positive or negative cocci was recorded (Figure 1).

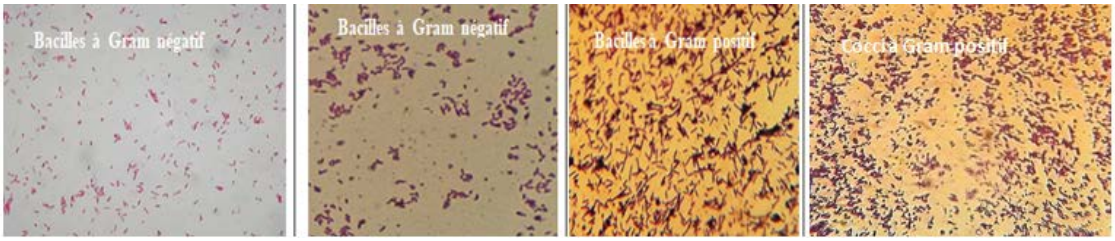

Figure 1. Microscopic characterization of strains at G 10×40

Table 4. Characterization of psychrotrophic bacteria cultured on nutrient agar

Strain	Cat	Ox	Form/ Gram	V.F	Mob	Glu	Lac/ Sac	H_2S	Gas (CO_2)	Bacterial genus
Sb1	+	+	Bacillus Gram-	Aérobic	+	-	-	-	-	*Pseudomonas*
Sb2	+	-	Bacillus Gram-	Aérobic	-	-	-	-	-	*Flavobacterium*
Sb3	+	+	Bacillus Gram-	Aérobic	+	-	-	-	-	*Pseudomonas*
Sb4	+	-	Bacillus Gram-	Aérobic	-	-	-	-	-	*Acinetobacter*
Sb5	+	+	Cocci Gram+	Aérobic	-	-	-	-	-	*Micrococcus*
Sb6	+	-	Bacillus Gram-	Aéro anaérobic	+	+	-	+	-	*Proteus*
Sb7	+	+	Cocci Gram+	Aérobic	-	-	-	-	-	*Micrococcus*
Sb8	+	+	Bacillus Gram-	Aérobic	+	-	-	-	-	*Pseudomonas*
Sb9	+	+	Cocci Gram+	Aérobic	-	-	-	-	-	*Micrococcus*
Sb10	+	+	*Bacillus* Gram-	Aérobic	+	-	-	-	-	*Pseudomonas*
Sb11	+	-	Bacillus Gram-	Aérobic	-	-	-	-	-	*Acinetobacter*
Sb12	+	-	Cocci Gram+	Aéro anaérobic	-	+	+	-	-	*Staphylococcus*
Sb13	+	+	Bacillus Gram-	Aérobic	+	-	-	-	-	*Pseudomonas*
Sb14	+	+	Bacille Gram-	Aérobic	+	+	-	-	-	*Pseudomonas*

Legend: Sb: Strain grown on nutrient agar, Cat: Catalase, Ox: Oxidase, V.f: breathing on liver meat, Mob: motility, Lac/Sac: Lactose/Sucrose, Glu: Glucose, H_2S: Hydrogen sulphide.

Table 5. Characterization of psychrotrophic bacteria cultured on King A or King B

Strain	Cat	Ox	Form/ Gram	V.F	Mob	Glu	Lac/ Sac	H_2S	Gas (CO_2)	Bacterial genus
S1	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S2	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S3	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S4	+	-	Bacillus Gram+	Aeroanaerobic	-	-	-	-	+	Genus other than *Pseudomonas*
S5	-	-	Bacillus Gram+	Strict aerobic	+	+	+	-	+	Genus other than *Pseudomonas*
S6	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S7	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S8	+	+	Bacille Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S9	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*
S10	+	+	Bacillus Gram-	Strict aerobic	+	-	-	-	-	*Pseudomonas*

Legend: S: Strain grown on King A or King B, Cat: Catalase, Ox: Oxidase, V .f: liver meat, Mob: motility, Lac/Sac: Lactose/Sucrose, Glu: Glucose, H_2S: Hydrogen sulphide.

3.4. Characterization of Isolated Strains

3.4.1. Bacteriological and Biochemical Characterization of Strains Isolated on Nutrient Agar

All purified strains possess the catalase enzyme. The cytochrome oxidase enzyme was produced by strains Sb1, Sb3, Sb5, Sb7. Sb8, Sb9, Sb10, Sb13 and S1b4. On the liver meat medium, all the strains showed an aerobic respiratory type except Sb6 and Sb12 which proved to be aeroanaerobic. The majority of strains are immobile bacteria. On TSI, all of these strains were unable to ferment all three sugars (lactose, sucrose, and glucose) and produce H_2S and gas, except strains Sb12 (fermented glucose and lactose) and Sb6 (fermented glucose with production of H_2S) (Table 4).

All the purified strains possess the catalase enzyme except S5. Also the enzyme cytochrome oxidase, was produced by all strains except S4 and S5. On the liver meat medium, these isolates showed a strict aerobic respiratory type with the exception of the S4 strain which proved to be optional aeroanaerobic. These strains are motile bacteria except S4. On TSI medium, all these strains did not ferment the three sugars (lactose, sucrose and glucose) and did not produce H2S and gas, except the S4 and S5 strains which presented gas in their tubing (Table 5).

4. Discussion

The bacterial load of refrigerated meats depends on their initial contamination at the slaughterhouse and the proliferation of certain flora during refrigeration following their adaptation to storage conditions. It is customary to qualify as psychrotrophic bacteria which retain significant activity at temperatures below or equal to +7°C.

The presence of psychrotrophic flora on beef was recorded from the first day of sampling (3.76±0.54 logufc/g and 3.24±0.28 logufc/g counted respectively on GN and PCA). A progressive evolution of the load in this flora during the refrigeration was noted. Mean contamination rates of the order of 4.27±0.29 logufc/g on GN and 4.08±0.49 logufc/g on PCA were recorded after five days of refrigeration. These rates are significantly higher than those reported by Benaissa et al. [11] (2.8 ± 0.27 logufc/cm^2), Benaissa et al. [12] (3.02 ± 0.71 logufc/g), Hamad, [13] (1.79 logufc/cm^2) and Hammoudi et al. [14] (3.17 log logufc/cm^2) on camel meat from the slaughterhouse of Ouargla and El Oued and beef from the Algiers slaughterhouse respectively. This can be explained by the poor hygienic conditions in which we proceed at the Ouargla slaughterhouse. Whereas, Nouichi et al. [15] and Okki et al. [16], announced higher rates than ours of the order of 4.48 logufc/cm^2 and 5.34 logufc/cm^2 on beef from the slaughter of El-Harrach and Constantine respectively. This difference can be explained by larger cattle slaughter, resulting in less compliance with hygiene standards. Bacteria of the genus *Pseudomonas* have the ability to grow at temperatures between +4°C and +43°C. Their presence at the level of the slaughter lines and in particular at low temperatures constitutes a permanent source of contamination and deterioration of meat [6]. This genus includes Gram negative, aerobic, motile, rarely immobile bacilli. They are bacteria with oxidative metabolism (oxidase positive) and catalase positive [17]. This agrees with our results noted on the strains studied. The isolation and purification of 14 strains on nutrient agar and 10 on King A and King B was carried out from the psychrotrophic flora. Microscopic examination after Gram staining of isolates on nutrient agar gave Gram negative bacilli and Gram positive cocci and on King A and King B both Gram negative and Gram positive bacilli. The fourteen isolates of psychrotrophic bacteria were assigned to the genera: *Pseudomonas*, *Micrococcus*, *Acinetobacter*, *Flavobacterium*, *Staphylococcus* and *Proteus*. Knowing that the genus *Pseudomonas* has the best capacity for development in the cold and shows significant activity up to a temperature of +2°C [18]. The genera, *Acinetobacter*, *Flavobacterium*, *Micrococcus* and *Staphylococcus*, are also frequently encountered in foodstuffs among psychrotrophic bacteria. Thus, at +4°C,

Pseudomonas grow slowly but show significant enzyme synthesis activity that hydrolyzes the food substrate [19]. Bacteria of the genera *Pseudomonas*, *Acinetobacter*, *Micrococcus* and *Flavobacterium* are characterized by proteolytic metabolism and significant lipolytic activity. Among the ten psychrotrophic isolates purified on King A and King B, 08 were attributed to the genus *Pseudomonas* and 02 did not show the criteria of this bacterial genus. The origin of bacteria of the genus *Pseudomonas* in this meat can be attributed to the washing water, the hands of the personnel, the equipment used or the ground. Our results agree with those reported by Arslan et al. [20].

5. Conclusion

The presence of psychrotrophic flora on beef was recorded from the first day of sampling, which means that contamination by this flora started just after slaughter.

The load of this flora increased during storage by refrigeration. So refrigeration does not inhibit the multiplication of this flora.

After the isolation and purification of the psychrotrophic strains, their phenotypic identification shows a diversity of bacterial genera: Pseudomonas, Micrococcus, Acinetobacter, Flavobacterium, Staphylococcus and Proteus.

Also, the presence of strains in bacilli, the majority of which are Gram-negative cultured on the selective culture media King A and King B, of the genus Pseudomonas, was highlighted.

So refrigeration does not contribute to the best preservation of beef against psychrotrophic bacteria.

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