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Consequences of packaging on bacterial growth. Meat is an ecological niche

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Abstract

Meat is a good support for bacterial growth and particularly for bacteria which are specific of meat and meat products. Little is known about the physiological and biochemical factors which could explain why some bacterial species are only isolated from meat. This review tentatively points out, from an ecological point of view, some of these factors in Gram negative and Gram positive micro-organisms influencing storage life. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Meat is a good support for bacterial growth as shown by the numerous reports dealing with the influence of micro-organisms on the storage life of meat products. The main property which explains rapid microbial growth on meats is its composition: 75% water and many different metabolites such as amino-acids, peptides, nucleotides, and sugars, (Lawrie, 1985). After slaughter, microbial contamination of carcasses is the consequence of the processing applied from skinning to conditioning. Processing influences not only the quantity of micro-organisms/cm^2 but also the type of micro-organisms present. Although some differences exist between animal species regarding the type of micro-organisms isolated from the carcasses, generally the same bacterial species can be isolated from beef, pork, sheep and even chicken carcasses. Further cutting, conditioning the muscles and final storage of meats will drastically modify the quantity and the type of micro-organisms which could grow and influence shelf life. Among the most important bacteria growing on meat in the dominant flora, several bacterial species are specific for meats, that is, they are only isolated from meats, slaughterhouses and the facilities necessary for the processing. Many papers have described the influence of bacteria on meat and meat products and many end products of microbial metabolism have been characterised. Although such information is important to assess the spoilage potentialities of micro-organisms, it does not allow an understanding of the peculiar relationship between meat, as an ecological niche, and these bacteria. In particular, it is difficult to explain why some bacteria grow more specifically on meat. The aim of this review is to show how conditioning and storage of meat could influence the microbial growth and to tentatively outline the factors which could explain why meat is the best support for several bacteria.

2. Meat as a selective agent for aerobic flora

Meat stored in air is rapidly spoiled by bacteria which are responsible for discoloration and off odours, causing its rejection. Generally, storage life depends largely on the quantities of bacteria on meat at the beginning of the storage and noticably the proportion of *Pseudomonas* spp within the flora (Dainty & Mackey, 1992). These bacteria are always the dominating bacteria after a few days at temperatures between 0 and 7°C (Molin & Ternström, 1982) whatever the type of meat. Temperature is one of the most important factors influencing bacterial growth in meat and meat products.

However, the proportion of bacteria present on meat surfaces is not only influenced by temperature. Oxygen availability and water activity (Jay, Kittaka, & Ordal, 1962) also determine the quantities and the type of bacteria

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growing on meats. In aerobically stored meats, *Pseudomonas* spp are, as already indicated, the micro-organisms which rapidly dominate the flora. In other food products stored in air, for instance, milk, fish and vegetables, *Pseudomonas* spp are also responsible for spoilage (Labadie, Dousset, & Hébraud, 1996). Such observations in many food products could indicate that the factors which select the flora are the same whatever the food product. In reality, even if *Pseudomonas* is a genus occupying many ecological niches, the species which grow on different food products are often different, although they share important common features. For example, *P. fragi* is the species most frequently isolated from meat (Molin & Ternström, 1982). *P. fragi*, which was characterized for a long time as nonpigmented *Pseudomonas* spp, grows more rapidly at low temperatures than fluorescent *Pseudomonas* spp (Gill, 1982; Gill & Newton, 1977). For instance, the generation time at 2°C (Gill & Newton, 1977; Lebert, Bégot, & Lebert, 1998) of these two groups of bacteria, are, respectively, 7.6 h and 8.2 h. What is the origin of such a difference? Could this difference be influenced by meat as a substrate for growth?

Dainty and Mackey (1992) indicated that *Pseudomonas* spp isolated from meat are certainly metabolizing its amino-acids, because the isolation, from spoiled meat, of many end products of their metabolism is probably correlated with their degradation. Although important, these results do not give clear evidence of a particular relationship between *Pseudomonas* spp and meat as a selecting agent.

It seems, however, that some feature of some *Pseudomonas* species isolated from meats, and which is not easily observed in vitro, could be correlated to their rapid growth on this substrate. For instance, the dominating species, that is the faster growing species when grown between 0 and 2°C (Lebert et al., 1998), are those which do not synthesize siderophore (a green fluorescent pigment). It is therefore possible that a relationship exists between this lack of synthesis and the more rapid growth at low temperatures. The green fluorescent pigment, pyoverdin, produced by many fluorescent species of *Pseudomonas*, is an iron chelator or a siderophore, overproduced in iron-starved media. Siderophore iron uptake systems are synthesized in order to provide the iron (Meyer & Abdallah, 1978) necessary for the aerobic metabolism of the bacterium. Champomier et al. (1996) have shown that most of the *P. fragi* isolated from meats do not synthesize pyoverdin and do not grow in the presence of an iron chelator (EDDHA, ethylene diamine di-hydroxyphényl acetic acid) in culture media. Although not synthesising pyoverdin, this bacterium is able to synthesize its receptors which are located in the outer-membrane. Such a feature indicates that *P. fragi* is able to compete for the siderophores produced by the growth of other *Pseudomonas* species in soil (Molin & Ternström, 1982) which is its ecological niche. As the growth of *P. fragi* is also strongly stimulated by siderophores of foreign origin, including aerobactin, *haemoglobin*, transferin and lactoferrin, it is likely that many sources of iron could be used by the bacterium, a property which suppresses the necessity for siderophore synthesis and which also probably saves energy for the bacterial cell. For full growth *P. fragi* exhibits a need for iron which is twice as much as the pathogen, *P. aeruginosa* (Champomier-Vergès, Stinzi, & Meyer, 1996). Meat represents an important source of iron, heminic or not (Lawrie, 1985). Thus, this bacterium could find in meat different sources of rapidly usable iron that could fullfil its important iron requirement. From all these properties it is tempting to speculate that the *Pseudomonas* spp, which would grow rapidly in meat, would be those which would save their energy at low temperature, while fullfilling their iron requirement from the different sources of iron available in the product. As *P. fragi* is able to use very diverse sources of iron, (Champomier et al., 1996), it seems adapted for dominating the flora in meats at low temperature.

Additional properties could also explain the growth of *P. fragi* in meat. In their taxonomical study of *Pseudomonas* spp isolated from meat of different origins, Molin and Ternström (1982) showed that 1/112 strains are not proteolytic in vitro, that is they do not excrete proteases in test tubes. Most of the authors working on *Pseudomonas* spp isolated from meats also indicated that *P. fragi* is not proteolytic in vitro. It seems, however, from some studies (Labadie, 1996; Tarrant, Pearson, Price, & Lechovich, 1971) that they indeed possess proteases located inside the cell but that these are not exported outside the bacterial cell in vitro. Most of the exoproteins of *Pseudomonas* sp are secreted via a two-step mechanism (Kadurugamuwa & Beveridge, 1995), the first one limiting the exportation into the periplasm of the cell and the second one depending on an energy source not identified. Thompson, Naidu, and Petska (1985) reported that meat extracts are inducers of the protease of *Pseudomonas fragi,* and it is tempting to propose that unknown meat proteins or peptides are necessary for secretion of proteases into the extracellular medium. Additionally, according to Thompson et al. (1985) proteases from *P. fragi* are secreted inside bleb-off structures, observed earlier in meats by Tarrant et al. (1971). Penetration of these blebs (Thompson et al., 1985) into the meat could allow the bacterium to penetrate the meat and finally help the organism to destroy the proteins. It is not known whether such bleb-off structures are specific for the strains isolated from meats, but the strains producing these blebs would have an evident advantage to colonize meat tissues. Interestingly, similar bleb-off membrane vesicles containing proteases are observed in culture media of the human pathogen *P. aeruginosa* (Kadurugamuwa & Beveridge,

1995). They seem also to be involved in the penetration of the bacterium into the infected tissues. Protease secretion involving bleb-off vesicles in several *Pseudomonas* spp could be a mechanism facilitating the invasion of the tissues to be degraded. In the case of *P. fragi* the capacity for using very diverse sources of iron, together with a particular secretion mechanism for exoproteases, could be important in explaining its particular importance in the spoilage of meat products.

For other bacteria, which could dominate in aerobically stored meats, their growth is influenced firstly by the temperature and their proportion in the initial flora. However, these are not the only factors since they do not explain why *Brochothrix thermosphacta* can only be isolated from slaughterhouses, cutting tables, chilling facilities, meats and meat products.

What are the properties which determine its presence in many meats products? Psychrotrophy is certainly important, but most foods are chilled before processing, and *B. thermosphacta* is not isolated from milk or vegetables. It is likely that meat (particularly pork and sheep) combine different chemical and biochemical parameters (Grau, 1980, 1981, 1983) which favour its growth. Although Gill and Newton (1977) showed that *B. thermosphacta* degrades glucose and glutamate *in beef*, it is unlikely that only two properties are sufficient to explain its growth in the dominant flora.

B. thermosphacta has numerous biochemical properties either shown in vitro or in meats. For instance, in aerobiosis, glucose is mainly degraded into lactic acid but small amounts of acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids were also produced (Gardner, 1982). In anaerobiosis, lactate and ethanol formed 85–90% of the end products of glucose metabolism (Gardner, 1982). *B. thermosphacta* possesses a glycerol ester hydrolase which is principally active on short chain fatty acids but proteins(gelatin or casein) are not degraded. Many of these reactions are used to identify the bacterium, or to characterise the spoilage but they cannot explain the specific growth of *B. thermosphacta* on meats. Interestingly Sutherland, Patterson, Gibbs, and Murray (1975) identified, in meat juice medium in which *B. thermosphacta* was grown, peptidolytic activity which is not observed in usual proteolysis media. Unfortunately, the specificity of the peptidase involved was not characterised. The determination of the type of peptides degraded could help to understand the particular aspects of *B. thermosphacta* metabolism when growing on meat. Talon (1984) tried to find out the ecological niche of *B. thermosphacta* in soil, hay, silages, lamb fleece, lamb faeces. *B. thermosphacta* was never isolated, or in so limited quantities (lamb fleece) that it was impossible to draw clear conclusions about the ecological niche of *B. thermosphacta*. Through these results, this work allowed the isolation from soil of a new species of *Brochothrix*, *B. campestris* (Talon, Grimont, Grimont-Gasser, & Boeufgras, 1988), which was further characterized as a bacterium producing a bacteriocin (Siragusa & Cutter, 1993) which was very efficient in inhibiting *B. thermosphacta* and *Listeria monocytogenes*. This result is important from two points of view, first soil could be the ecological niche of *B. thermosphacta*, and second, if this is true, isolating bacteria from the same ecological niche could help to find new molecules of microbial origin capable of inhibiting spoilage and/or pathogenic bacteria.

Comparisons of *B. campestris* and *B. thermosphacta* could also help to understand why only *B. thermosphacta* grows on meat. Interestingly, both strains are psychrotrophs (Talon et al., 1988), but only *B. thermosphacta* is able to grow in media containing 8 or 10% NaCl. This shows that *B. thermosphacta* resists harsher conditions than *B. campestris* and could explain its resistance and growth in cured products, but it does not explain why *B. campestris* is not isolated from meats.

For other bacteria which are not frequently involved in spoilage, such as *Acinetobacter* spp and *Psychrobacter* spp (Gill, 1982; Gennari, Parini, Volpon, & Serio, 1992) less is known, probably because of their low importance in meat spoilage. However, they behave differently in spoiled milk and meats. Some *Acinetobacter* spp produces a capsular polysaccharide, responsible for a phenomenon called ropy milk (Morton & Barrett, 1982; Wegemer & Gainor, 1954) while others produce a brown diffusible pigment in meat (Vanderzant, Savell, Harnby, Acuff, Cox, & Bailey, 1987). Such differences are undoubtedly due to the influence of specific relationship between the bacteria and their proteinaceous substrates.

3. Influence of modified atmospheres (MA) and vacuum packaging on the selection of a specific flora

Vacuum or MA packs exert an important effect on the micro-organisms. Vacuum packaged meat is generally very stable in the cold with the low temperature and limited quantity of oxygen inhibiting bacterial growth and lactic acid bacteria are the only bacteria producing important populations, that is at least 10^7 CFU/cm^2 (Gill & Newton, 1978). Other bacteria grow, but their growth is generally limited or very slow (Dainty & Mackey, 1992). As lactobacilli have little effect on the organoleptic properties of meats, vacuum packaged meats gives 3 to 4 weeks storage life close to 0°C. Apart from the MA containing only pure CO_2 which permits, with the aid of oxygen scavengers, conservation as long as 28 days at +2°C (Renerre, 1986), the MA containing two (O_2, CO_2) or three gases (O_2, CO_2, N_2) allow conservation which is considerably reduced. For instance, a mixture containing 66% O_2, 25% CO_2 and 9% N_2 gives a maximum of 2 weeks conservation at

+2°C (Christopher, Smith, Dill, Carpenter, & Vanderzant, 1980). This is due, to a small inhibitory influence of CO_2 on *Brochothrix thermosphacta* and to growth of other bacteria such as *Pseudomonas* spp or *Psychrobacter* spp which use the oxygen for growth and which is always present in the packs. Apart from CO_2, vacuum or residual oxygen, what are the other factors from the meat explaining the growth of the bacteria ?

For those which do not constitute an important part of the flora, but which are often or systematically present at low levels, psychrotrophy is certainly the main factor (Rosset, 1996). This is particularly evident for the *Enterobacteriaceae*, the species of which growing in MA conditioned and vacuum packaged meats, (*Serratia liquefaciens*, *Hafnia* spp) are always able to grow at temperatures between 0 and 10°C. The fact that they do not grow as the dominant flora is the result of poor metabolism in such conditions, which indicates they are not specifically adapted to meat. Moreover, as these species could be easily isolated in low quantities from other chilled food products (Blixt & Borch, 1996), meat is not a specific ecological niche for these bacteria.

Most of the bacteria growing on vacuum packaged or in MA conditioned meats, as the dominant flora, are species only isolated from meats. Apart from *B. thermosphacta* which grows in air (see above "meat as a selective agent of aerobic flora") and in MA, several lactic acid bacteria are only present in meat products. Two species, *L. sakei* and *L. curvatus*, largely dominate, although other species grow to constitute a minor part of the flora, for example *L. raffinolyticus*, *L. pentosus*, (Blixt & Borch, 1996). Similar conclusions could be made for the genera *Carnobacterium* (*C. piscicola, C. divergens*) (Hammes, Weiss, & Holzapfel, 1992) and *Leuconostoc* (*L. gelidum, L. carnosus*) (Collins, Samelis, Metaxopoulos, & Wallbanks, 1993). *L. sakei* uses arginine as a principal substrate to produce ATP when the glucose concentration is close to 0. 05% (w/w), which is the glucose concentration in meat (Montel, 1985); but the growth of *B. thermosphacta*, is not explained by only two biochemical characteristics. Meat as a medium for growth is the selecting factor. No clear explanation of such selection is available although growth at chill temperature (Reuter, 1982) is certainly one of the most important (but not the only) factors as already indicated for *B. thermosphacta*. As for this bacterial species, the real ecological niche of *L. sakei* is not clearly known, even if it has been reported present in sauerkraut and as a saké starter (Bergey's Manual of Determinative Bacteriology, 9th ed). The ecological niche of this species is undoubtedly plant material and probably some of those used for the production of silages (Reuter, 1982).

As *L. sakei* is an heterogeneous group (Montel, Talon, Fournaud, & Champomier, 1991), it would be interesting to determine precisely which plant species favour the growth of strains similar to those used as starters in meat products. If particular niches do exist in plants, identifying them could bring important information concerning the sugars degraded, the peptides which could be preferentially used and the differences between wild strains and those selected by the processing in meat technology. These natural sources of micro-organisms could be also used for the isolation of new starters with inhibiting or flavouring properties not yet identified.

Growth, and particularly growth rate as already outlined, is an important factor explaining the varying amounts of bacteria in foods. Although no particular study has been carried out to compare the amounts of the different species of Lactobacilli, *Leuconostoc* and *Carnobacterium* on meat, it is likely that at chill temperatures, in an atmosphere containing reduced O_2 and a varying amount of CO_2, the species which have already been cited are likely to grow faster than those which do not usually grow on meats, but which could be present at the onset of contamination. A combination of these factors certainly explains this ecological advantage but basically, the physiology of growth *on meat at a low temperature* is a key factor. Very little is known about the growth physiology of these bacteria at subzero temperatures. Most studies on microbial physiology in the cold are relatively recent. Most of them were carried out on four bacterial species, i.e. *Escherichia coli*, *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas fragi* (Hébraud, Dubois, Potier, & Labadie, 1994; Graumann, Schröder, Schmid, & Marahiel, 1996; Lee, Xie, Jiang, Etchegaray, Jones, & Inouye, 1994; Willimsky, Bang, Fisher, & Marahiel, 1992). These authors demonstrated that some transcription enhancers, the so-called Cold Shock Proteins and/or Cold Acclimation Proteins (Csps or Caps), are key biochemical factors explaining adaptation and/or growth at low temperatures by specific interactions with gene promoters activated at low temperatures. Recently, several studies have shown that these factors are also synthesised by lactic acid bacteria i.e. *L. plantarum* and *L. curvatus* (Derzelle, Hols, Ferain, Delplace, & Delcour, 1996; Wouters, Kuipers, de Vos, Rombouts, & Abee, 1996) and probably by all bacteria after a cold shock (Grauman et al., 1996). The role of such factors has not been clarified for true psychrotrophs such as *Pseudomonas fragi* (Hébraud et al., 1994) and is unknown for *Lactobacillus* spp. (Wouters et al., 1996). Up to now, studies on a few genes have been performed in the mesophilic bacterium *E. coli* to determine the metabolic pathways which are more specifically induced by these transcription factors (Lee et al., 1994). For instance, in *E. coli* and in *B. subtilis*, a nucleotidic sequence (CCAAT), identified in several promoter genes involved in the cold shock response, could be the target of these Csps (Willimsky et al., 1992). Considering the fact that Csps from lactic acid bacteria much resemble those from Gram negative

bacteria, (Wouters et al., 1996) they are likely to have a similar role in the cold.

For *L. sakei* which is not acid resistant (Reuter, 1982) the arginine degradation pathway is certainly involved at low temperatures as already shown by Gill and Newton (1978). Arginin degradation may lead to toxic amines, putrescine and spermine (Halasz, Barath, Simon-Sarkadi, & Holzapfel, 1994), which allow the bacterium to adapt its physiology to meat which has a relatively low pH (pH 5.4–5.8). Adaptation to mild acidic conditions, involving the production of amines from amino-acid decarboxylation, is also observed in *E. coli* and *Salmonella typhimurium* (Baik, Bearson, Dunbar, & Foster, 1996) and could represent a general mechanism of adaptation (Acidic Tolerance Response, or ATR) to media with acidic pH. We do not know the precise factors which could explain why meat, like other proteinaceous media containing arginine or other amino-acids, is well suited to *L. sakei*. Some key factors are the lactic acid content in a (semi) solid medium together with low free sugar and relatively small amounts of free amino-acids and peptides (Lawrie, 1985). These are certainly important. All bacteria possess sensors (Hoch & Silhavy, 1995) which give information to the cells concerning the availability of substrates in culture media surrounding them. Lactobacilli could detect culture conditions which resemble starvation (Hengge-Aronis, 1993) particularly in meats at low temperatures. Glucose concentrations in meat (< to 0.5g/l) (Rosset, 1996) are similar to those used for starving lactic acid bacteria of their source of carbon (Giard, Hartke, Flahaut, Benachour, Boutibonnes, & Auffray, 1996). A lag of seven days before growth of *L. sakei* in *chilled* vacuum packaged meats (Fournaud, 1987) could be related to an adaptation which takes into account that meat is a starvation medium. In many bacteria (Hengge-Aronis, 1993) and particularly the lactic acid bacteria (Giard et al., 1996) starved culture media are responsible for an increased resistance to stressful conditions. Specific experiments will have to be carried out to know whether this is also true for *L. sakei* or whether the combination of factors mentioned above are those which select the strains growing on meats. Apart from *L. carnosus* and *Carnobacterium* spp which could produce amines from arginine and other amino-acids in culture media, (Edwards, Dainty, Hibbard, & Ramantanis, 1987; Halasz et al., 1994; Masson, Compte, Talon, & Montel, 1996), very little is known about the other lactic acid bacteria. As for *L. sakei*, a combination of factors is likely to play a significant role on the strong growth of the *Leuconostoc* spp and *Carnobacterium* spp only isolated from meat.

Finally, for most lactic acid bacteria, our knowledge about their metabolism is insufficient to account for their specific growth in meat. Identification and studies of the regulation of some important metabolic pathways, and at least those involved in the production of amines, and/or the adaptation to meats as a particular medium, could be of major interest to understand the physiological relationship between the bacterial species only isolated from meat and meat as their main substrate.

4. Conclusion

This review of the microbial ecology of meat shows that the micro-organisms growing during storage result from the type of contamination introduced by the processing of meat and from the influence of the physicochemical factors applied during storage. These factors (temperature, pH, nutrients, A_w and composition of the atmospheres) constitute hurdles (Leistner, 1992) which play a crucial role in the activity and growth of micro-organisms. These hurdles (Leistner, 1992) are useful to explain the selective action of the different factors outlined above on a complex microflora, but they could not explain precisely why meat and meat products specifically select micro-organisms not isolated from other food products. This emphasises the need to study the particular relationship between bacterial growth and the availability of substrates in meat. It means that this availability is probably particular. Meat, as already indicated, is a semi solid medium and is relatively poor as a source of sugar for bacteria. As shown 20 years ago by Gill and Newton (1977, 1978), diffusion of the substrates from the inner part of the muscles to the contaminated surfaces could influence bacterial growth. Additionally, meat is not an important source of free amino acids (141 mg/100 g of *dry matter*) (Lawrie, 1985) but is an important source of proteins. As bacteria preferentialy use peptides for growth (Payne, 1976) these characteristics and those already outlined surely influence bacterial metabolism and, of course, growth and survival of some species. The recent identification of peptides (di or tripeptides) containing residues of glycine, proline and hydroxyproline imported intacellularly at high concentrations by cells of *Listeria monocytogenes* (Amezaga, Davidson, McLaggan, Verheul, Abee, & Booth, 1995) to protect them from damage due to culture media containing high salt concentrations, is the first experimental proof that peptides obtained from a protein only isolated from muscles, that is collagen or gelatin, could specifically influence the survival of a bacterium. As these peptides could easily be produced during the processing of meat, they could play a role in the growth and the survival of *L. monocytogenes* in cured meat products and on the processing lines. They could similarly play a role in the survival of *Lactobacillus* spp in meat products, particularly cured products. The recent finding (Glaasker, Konings, & Poolman, 1996) that *L. plantarum* increases its intracellular pool of the osmoprotectant amino-acids, proline, alanine, and glycine, when

the osmolarity of the media increases, is an element which could indicate that meat and meat products containing collagen and/or gelatin (glycine, proline, hydroxyproline, alanine represent 60% of the total amino-acids content of collagen) could select the bacteria which have the best system of importation of osmoprotecting peptides or amino-acids. Nothing is known about the osmoprotecting systems of *L. sakei* and those of the other lactic acid bacteria isolated from meats. These systems could play a major role in the selectivity of meat versus its specific microfora, particularly in cured products. Similar protection could exist with other micro-organisms, for instance *B. thermosphacta*, and specific utilisation of susbstrates such as peptides could play a role in the selectivity of meat versus other bacteria of the microflora in some circumstances. Limited knowledge is available about bacterial protein metabolism in meats. Studying this metabolism could bring precious information about the selectivity of meat on its *specific flora* and would indicate precisely how meat is an ecological niche for those bacterial species involved in its spoilage or in its stability in the cold.

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