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Identification of Low Molecular Weight Compounds Produced or Utilized by Psychrotrophic Meat Spoilage Organisms

by

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in partial fulfilment of the requirements for the degree of
Master of Science

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To the memory of my father,
Seyyed Khalil Moosavi-Nasab.

Abstract

Meat Juice Medium (MJM), an aqueous extract of meat, was inoculated with *Pseudomonas aeruginosa* and incubated for 7 d at 4°C under shaking conditions (100 rev.min^{-1}). Two predominant compounds produced during spoilage of MJM were detected using HPLC. These compounds with retention times (RT) of 21.48 and 32.04 min were tentatively identified as acetic and butyric acids, respectively. These compounds were also produced when MJM was replaced with Brain Heart Infusion Broth medium. In later experiments, the effect of glucose supplementation on the rate of MJM spoilage was examined. Glucose 0.5% (wt/vol) was added to the MJM, inoculated with *P. aeruginosa* and incubated at 30°C under shaking conditions (100 rev.min^{-1}). HPLC of samples after 1 d of incubation indicated the presence of 8 predominant compounds including acetic and butyric acids. Their concentrations were, in general, higher in control samples of MJM without added glucose. Using HPLC, TLC, Pyrolysis/GC/MS, FTIR and GC-MS methodologies, the compounds with RT of 8.91, 9.67, 11.96, 13.33, 17.74, 21.48, 26.07 and 32.04 min were tentatively identified as cadaverine, 2-keto gluconic acid, fructose, lactic acid, acetic acid, methanol and butyric acid. In contrast to the results of previous researchers, cadaverine was produced in large amounts while no putrescine was produced by *P. aeruginosa*. During the spoilage period, the levels of glucose, fructose and total carbohydrate were monitored. Addition of glucose to MJM delayed slime production by 4 days and increase to maximum pH of 8.3 by 7 days. Results suggest that addition of glucose to MJM delays spoilage by *P. aeruginosa*.

Résumé

Pseudomonas aeruginosa a été cultivé dans un extrait aqueux de viande (milieu au jus de viande, MJM) pour 7 j à 4°C sous des conditions d'agitation (100 rév.min^{-1}). Deux complexes prédominants, produits durant la détérioration de l'extrait MJM ont été détectés à l'aide de la chromatographie en phase liquide à haute performance (HPLC). Ces composantes, possédant des temps de rétention (RT) de 21.48 et 32.04 min, ont été identifiées, comme étant probablement de l'acide acétique et butyrique. Les mêmes complexes sont apparus quand l'extrait MJM a été remplacé par du "Brain Heart Infusion Broth" (BHIB). Durant des expériences ultérieures, l'effet de la supplémentation de glucose sur la vitesse de détérioration de l'extrait MJM a été examiné. Après l'addition de glucose 0.5% (wt/vol), l'extrait MJM inoculé de *Pseudomonas aeruginosa* a été cultivé à 30°C sous des conditions d'agitations (100 rév.min^{-1}). L'analyse des échantillons par HPLC, après 1 journée d'incubation, a relevé la présence de 8 complexes dont les acides acétique et butyrique. En général, les concentrations étaient plus élevées dans les échantillons de contrôle, composés de l'extrait MJM sans l'addition de glucose. En utilisant l'HPLC, la chromatographie en couche mince (TLC), la pyrolyse couplée à la chromatographie en phase gazeuse et la spectrométrie de masse (pyrolyse/CPG/SM), la spectroscopie infrarouge transformée de Fournier (IR-TF) et des méthodes de chromatographie en phase gazeuse (CPG), les composés à temps de rétention de 8.91, 9.67, 11.96, 13.33, 17.74, 21.48, 26.07 et 32.04 min ont été identifiés de façon non définitive comme étant respectivement de la cadavérine, de l'acide 2-céto gluconique, du fructose, de l'acide lactique, de l'acide acétique, du méthanol et de l'acide butyrique. Contrairement aux résultats présentés dans le passé, *Pseudomonas aeruginosa* a produit de la cadavérine en grande quantité mais n'a pas produit de putrescine. Durant la période d'altération, le niveau de glucose, de fructose ainsi que le niveau d'hydrate de carbone total ont été mesurés. L'addition de glucose à l'extrait MJM a eu l'effet de retarder la production de limon de 4 j, tout en augmentant le pH jusqu'à un maximum de 8.3 atteint après 7 j. Ces résultats viennent renforcer l'hypothèse que l'addition de glucose à la viande retarde sa détérioration.

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Introduction

In naturally spoiling foodstuffs, with which we are concerned here, it is highly unlikely that proteolytic microorganisms would always be absent, so the question whether proteolysis must always occur as a result of spoilage is therefore somewhat academic; but to insist that spoilage is the result of proteolysis is manifestly untrue.

(Shewan, 1974)

Meat, or the muscular tissue of red meat mammals, has been consumed by *homo sapiens* for nearly two million years. Its taste and nutritional value are recognized worldwide and have made meat a highly desirable food commodity. Meats are one of the most perishable of all important foods because of an abundance of all nutrients required for the growth of bacteria, yeasts, and molds (Jay, 1992). Table 1 lists the gross chemical composition of a typical adult mammalian muscle postmortem.

Meat spoilage is a complex event, in which a combination of microbial and (bio)chemical activities may interact. The detection levels for the metabolites formed during (bio)chemical spoilage are generally low making the detection of early signs of spoilage more possible, but a major problem is that the (bio)chemical processes related to meat spoilage appear to be poorly understood (Huis in't Veld, 1996). Even less is known about interactions between microbial and chemical spoilage reactions (Dainty, 1996; Dainty and Mackey, 1992). Therefore we are still far away from assuring the quality of a meat by predicting shelflife on the basis of specific spoilage indicators.

The microbiology of meat spoilage has received considerable attention over the years and the characterization of the typical microflora which develop on different types of meats during storage has been well documented. Therefore, the major problems are to find the relation between microbial composition and presence of microbial metabolites, related to the evaluation and possible prediction of microbial spoilage (Drosinons and Board, 1994). So, interest in defining the chemical changes associated with bacterial growth on meat arises from the possibility of using them as an index of microbial quality, shelf life, or consumer

Table1. Gross chemical composition of typical adult mammalian muscle after rigor mortis[a]

Component	% Wet weight
Water	75
Protein	20
Lipid	2.5-3
Carbohydrate	0.2-1.2
Miscellanous soluble nonprotein substances	
Nitrogenous	1.65
Inorganic	0.65
Vitamins	Trace amounts

[a] Derived from Lawrie, 1985; Dainty and Mackey, 1992.

acceptability (Dainty *et al.*, 1983).

The metabolic activities associated with the growth of psychrotrophic bacteria accelerate the deterioration of muscle foods during refrigerated storage. However, the precise nature of these deteriorative changes and their relevance to spoilage is only poorly understood. It is noteworthy that the role of proteolytic enzymes of psychrotrophs in the spoilage process is subject to considerable controversy. Although there is published evidence in support of protein degradation as a spoilage mechanism, an equal number of reports insist that low molecular weight nonprotein muscle constituents are precursors for meat spoilage (Greer, 1989).

A definite order of substrate utilization during growth of representative strains of many of the different types of bacteria commonly associated with the spoilage of meat stored in air or vaccum packs has been demonstrated and its relevance to shelf life established (Dainty *et al.*, 1983). The key role of glucose in meat and meat products is well documented. The glucose content of meat affects the composition of the microflora developing during storage, the metabolic products produced by the flora, and the cell density reached at the onset of spoilage (Lambropoulou *et al.*, 1996). According to Gill (1986), the addition of carbohydrates, particularly glucose, can be used to delay spoilage in dark, firm, dry (DFD) meat which is characterized by a high pH (>6.0) and depletion of carbohydrates.

Each year, in the USA, approximately thirty-four million pounds of meat and meat products are lost due to spoilage (Branen, 1978). Recently, it was estimated that the annual economic loss from spoilage of fresh meat and meat products in the United States is approximately 5 billion dollars (Ray *et al.*, 1992). So, an understanding of the bacterial processes involved in the spoilage of aerobically stored chilled meats should provide the knowledge to help prevent or reduce the amount of beef spoilage. It is of interest that comparative studies have shown that preferences for growth substances and the nature of spoilage catabolites are similar in the tissues of fish, poultry, and mammals (Greer, 1989). Thus, this information could be used to help preserve other refrigerated flesh foods such as chicken or fish.

This project thus consisted in measuring the disappearance or production of various

low molecular weight, water-soluble compounds from an aqueous meat extract (Meat Juice Medium, Gill, 1976) during aerobic incubation at 4 and 30°C with single spoilage organisms. The goal of this study is to identify some compounds which seem to be more predominant in the spoilage of meat, a continuation of Gauthier's work (1990). Also the effect of glucose supplementation on the production or utilization of these compounds and the rate of meat spoilage will be examined.

The following literature review will focus on the microbiological and chemical changes occurring during the spoilage of fresh, lean red meat (predominantly beef) stored at chill temperatures (1 to 10°C) or at 30°C in aerobic conditions (no wrapping at all, or gas-permeable wrapping). Only microbial species most often found in the spoilage microflora of meat in such conditions will be considered, namely *Pseudomonas* (fluorescent and non-fluorescent spp), *Enterobacter agglomerans* and *Brochothrix thermosphacta*.

1. Literature Review

1.1 Events Following Slaughter of Beef Animal

Upon the slaughter of a well-rested beef animal, a series of events take place that lead to the production of meat. Lawrie (1985) discussed these events in great detail, and they are here presented only in outline form. Following an animal's slaughter,

1. Blood circulation ceases; the ability to resynthesize ATP (adenosine triphosphate) is lost; lack of ATP causes actin and myosin to combine to form actomyosin, which leads to a stiffening of muscles.
2. The oxygen supply decreases, resulting in a reduction of the oxidation/reduction potential.
3. The supply of vitamins and antioxidants stops, resulting in a slow development of rancidity.
4. Nervous and hormonal regulations cease, causing the temperature of the animal to drop and fat to solidify.
5. Respiration and ATP synthesis cease.
6. Glycolysis starts, resulting in the conversion of most glycogen to lactic acid, which reduces pH from about 7.4 to its final level of about 5.6. This pH depression also initiates protein denaturation, releases and activates cathepsins, and completes rigor mortis. Protein denaturation is accompanied by an exchange of divalent and monovalent cations on the muscle proteins.
7. The reticuloendothelial system ceases to scavenge, thus allowing microorganisms to grow unchecked.
8. Various metabolites accumulate that also help protein denaturation.

These events occur between 24 and 36 h at the usual temperatures of holding freshly slaughtered beef (2° to 5°C). The flora associated with the carcass may have come from the animal's own lymph nodes (Lepovetsky *et al.*, 1953), the stick knife used for exsanguination, the hide of the animal, intestinal tract, dust, hands of handlers, cutting knives, storage bins, etc. High contamination was found at those sites associated with opening cuts and/or subject to hide contact during hide removal. In modern processing, the respiratory and intestinal tracts are not major sources of direct contamination (Bell, 1997). Upon prolonged storage at refrigerator temperatures, microbial spoilage begins.

1.2 Organisms Involved in the Microbial Spoilage of Chilled Fresh Meats

1.2.1 Definition of Spoilage and Psychrotrophs

Several authors have stated that the definition of spoilage is not an easy one, because it implies some personal analysis by the consumer (Gill, 1986). Spoilage should not be considered an individual condition, but the result of many changes in a food that make the product "unacceptable" to the consumer, e.g., oxidative rancidity. Spoilage can be considered as a condition resulting from microbial activity that is demonstrated by changes in odor, flavour, and appearance (including texture) or any other sensory impression that causes rejection of the food.

In 1892, Forster (Forster cited in Suhren, 1989) was the first to observe bacterial growth at 0°C, and since then the terminology used for microorganisms which are able to grow and multiply at refrigerating or freezing temperature has been confused. The use of the term "psychrophilic" was recommended by Eddy (1960) only when a low optimum temperature is implied, and "psychrotrophic" for bacteria able to grow at 5°C or less, whatever their optimum temperatures.

1.2.2 Selection of Spoilage Bacteria

Since the publication of the well-established review in which Mossel and Ingram (1955) defined food microbiology as a branch of microbial ecology, the selection of spoilage flora has been considered in the context of four interacting systems (Mossel, 1983; Hobbs, 1986): (i) the nutrient status of a food; (ii) the physiological attributes of spoilage organisms; (iii) extrinsic factors--temperature, gaseous composition of a storage environment, relative humidity, etc.; and (iv) processing factors.

Earlier literature (Ingram and Dainty, 1971) leaves the impression that proteolytic activity would be an early and essential action of meat spoilage bacteria. This controversy was supported also by the characteristic odors at the time of spoilage. It is now recognized that

bacterial proteolysis is a late, and relatively unimportant event in meat spoilage (Dainty *et al.* 1975). Also factors controlling the induction of proteolytic enzymes in bacteria should be considered. Harder's model (1979) assumed (i) that organisms produce very low basal levels of extracellular enzymes in the absence of an inducer, and (ii) that the regulation and extracellular production of proteinases are based on induction and end product and/or catabolite repression.

With a clinical isolate of *P. aeruginosa*, for example, proteinase was not produced in glucose basal medium unless an inorganic nitrogen source was replaced by an organic one, glutamic acid and glutamine being the most effective inducers (Jensen *et al.*, 1980). The proteinases are repressed by easily metabolizable C sources such as citrate; a proteinase was produced by *P. fluorescens* in protein containing medium under carbon but not under nitrogen limiting conditions in continuous culture (Fairbairn and Law, 1986) suggesting that the function of the proteinase is to ensure a supply of carbon (and energy) rather than amino acids for protein synthesis under conditions of carbon (and energy) limitation. Juffs (1976) showed that temperature and composition of the medium can influence markedly the production of proteinase by *P. fluorescens* and *P. aeruginosa*. He observed that glucose but not the nonutilizable lactose inhibited proteinase production by raw milk isolates of *P. fluorescens* and *P. aeruginosa* growing in peptone broth, the inhibition being most marked at 5°C. Extracellular proteinase production by these bacteria was found to be dependent on the presence of organic N in the growth medium. According to these observations, the possible involvement of microbial proteinases in the breakdown of polymers in meat can be considered not-significant which is in agreement with the conclusion of Dainty *et al.* (1983).

With modern slaughtering and butchering, we expect counts to be in the range of *ca.* 10^3 to 10^4 bacteria.cm^{-2} (Gill, 1986). Modern preservation methods associated with slaughter and butchering include physical, chemical and ionizing radiation methods (Varnam and Sutherland, 1995). The very small proportion of psychrotrophic bacteria among the initial contaminants (1-10%), and hence their spatial separation, obviously minimizes competition between psychrotrophic bacteria and the other organisms in the initial contaminants at least in the early stages of chill storage.

For finding the reasons why only a few types predominate in spoiled meats, it is helpful to return to the intrinsic and extrinsic parameters that affect the growth of spoilage microorganisms. Parameters such as pO_2, pCO_2, pH, and, in some instances, antibiotic production by other bacteria will select the relatively few organisms present in the initial contamination that become dominant during the storage of meat and certain meat products at chill temperatures (Nychas, 1988). The available evidence emphasizes a common characteristic of the spoilage organisms (Table 2) i.e., their capacity to grow rapidly and use glucose as the preferred energy source. Fresh meats such as beef, pork, and lamb, as well as fresh poultry, seafood, and processed meats, have pH values within the growth range of most of the organisms involved in the spoilage of meat. Nutrient and moisture contents are adequate to support the growth of all spoilage organisms. While the oxidation-reduction (O/R) potentials of whole meats are low (Table 3), O/R conditions at the surfaces tend to be higher so aerobic bacteria proliferate freely only on the surface of fresh meat, the interior favouring anaerobes (Jay, 1992). Of the extrinsic parameters, temperature of incubation is the most important factor in controlling the types of microorganisms that develop on meat because these products are normally held at refrigerator temperatures. Essentially all studies on the spoilage of meats, poultry, and seafood carried out over the past 45 years or so have dealt with low-temperature-stored products.

1.2.3 The Spoilage Flora of Chilled Meat

The generic distribution of psychrotrophic bacteria in red meats (Gill and Newton, 1977), poultry (Barnes and Thornley, 1966), and fish (Jay, 1992) has been documented in several, complete reviews. During refrigerated storage, *Pseudomonas* spp. appears as the dominant and most metabolically significant spoilage organism because of its ability to rapidly initiate growth at low temperatures, its comparatively rapid rate of growth, and its insensitivity to competition by other psychrotrophs. In addition, their growth is not restricted by pH within the range of 5.5 to 7.0. Metabolically, the spoilage pseudomonads have relatively extensive oxidative capabilities, and can readily utilize a variety of soluble, low molecular weight nitrogenous compounds as an energy source. This latter attribute offers a

Table 2. Important physiological attributes of principal meat spoilage bacteria

Attributes	Growth rates (h) at[a] 2°C Aerobic (anaerobic)	10°C Aerobic (anaerobic)
A. Simple nutritional requirements		
1. Obligate aerobes-*Pseudomonas*	7.6	2.8
Marked nutritional diversity. Glucose preferred substrate. Maximal ATP production from available substrate. High affinity uptake of substrate. External storage of energy source. Little if any secondary metabolism. Intolerance of CO_2 and sulfite[b,c].	(-)	(-)
2. Facultative anaerobes-*Enterobacteriaceae*.	11.1	3.5
Relatively simple nutrient requirement. ATP production determined by pO_2. Glucose preferred substrate; TCA cycle complete.	(55.7)	(8.5)
Incomplete TCA cycle-*Brochothrix thermosphacta*[d]. Glucose used. Tolerant of sulfite and CO_2.	12.0 (32.8)	3.4 (9.7)
B. Complex nutritional requirements.		
Facultative anaerobes *Lactobacillus* TCA cycle absent. Tolerant of acid conditions, sulfite and CO_2. Fermentative substrate (e,g., glucose) needed for growth. Antibiotics produced by some.	- (8.4)	- (4.6)

[a]Based on Gill, 1986; [b]Enfors *et al.*, 1979.
[c]Banks *et al.*, 1985; [d]Dainty *et al.*, 1983.

Table 3. Redox potentials in meats[a]

Foodstuff	Eh(mV)	pH	rH	References
Liver, raw minced	-200	7.0	8	Knight, 1930
Muscle				
raw, post-rigor	-150	5.7	7	Barnes & Ingram, 1955
raw, minced	+225	5.9	18	Rose & Petersen, 1953
minced, cooked	+300	7.5	24	Hirsch & Grinsted, 1954

[a]derived from Mossel and Ingram, 1955.

competitive advantage in muscle tissues where simple sugars are at a minimum. Besides, spoilage bacteria elaborate proteinases and lipases which may liberate growth substrates from muscle components and mediate tissue digestion (Farber, 1982; Greer, 1989). The frequent domination of *P. fragi* is suggested to be due to the ability of the bacterium to use creatine and creatinine in meat juice (Drosinos and Board, 1995; 1994).

Other frequently isolated but minor components of the psychrotrophic spoilage flora include *Moraxella, Acinetobacter, Brochothrix thermosphacta*, and *Alteromonas putrefaciens* (Varnam and Sutherland, 1995; Gill, 1986). Lactic acid bacteria and yeasts were also reported to be a part of the microbial composition of minced beef (Lambropoulou *et al.*, 1996). The spoilage potential of all the above-mentioned organisms is only manifested under conditions where the growth of pseudomonads is suppressed. Thus, major emphasis will be given to *Pseudomonas* spp. with only brief reference to the other organisms when their contribution to spoilage becomes relevant.

Most bacteria detected on refrigerated, aerobically stored meat are Gram negative rods (Dainty *et al.*, 1983). *Brochothrix thermosphacta*, formerly known as *Microbacterium thermosphacta* (Sneath and Jones, 1976), is the only Gram positive organism found in high numbers on refrigerated, aerobically stored meat. It was not described in detail when first isolated (Mcleans and Sulzbacher, 1953) and was not easy to recognize until Davidson *et al.* (1968) described its properties more fully. *B. thermosphacta* was not thought to be important in spoilage except possibly on lamb (Barlow and Kitchell, 1966). However, the common meat spoilage bacteria are recently reported as *B. thermosphacta*, *Carnobacterium piscicola* (non-aciduric strains of *Lactobacillus* formerly called *L. piscicola*), *L. curvatus*, *L. sake*, *P. fluorescens* and *Serratia liquefaciens* (Ouattara *et al.*, 1997).

Molds tend to predominate in the spoilage of beef cuts when the surface is too dry for bacterial growth or when the animal has been treated with antibiotics such as the tetracyclines. Molds virtually never develop on meats when bacteria are allowed to grow freely. The reason appears to be that bacteria grow faster than molds, thus consuming available surface oxygen, which molds require for their metabolic activities (Jay, 1992).

1.2.4 Interactions Between Microorganisms

The contribution that any group of organisms makes to spoilage will depend, in part, upon its relative numbers in a spoilage flora. In aerobic flora there appear to be no significant interactions between species until maximum numbers are approached (Table 4). At that time some species sequester the limited available oxygen at the expense of competitors (Gill and Newton, 1977). In anaerobic flora under anaerobic conditions, competition for glucose can produce similar effects when maximum numbers are approached (Newton and Gill, 1978). In addition, lactobacilli produce antimicrobial agents that inhibit competitors (Raccach and Baker, 1978), but even in this case numbers must be relatively high before such effects become obvious (Roth and Clark, 1975; Newton and Gill, 1978). For most of the time that the bacteria are growing in meat, the organisms are indifferent to the presence of competitors. Under this condition, dominance is primarily determined by the relative rates of growth of competing organisms, the fastest-growing species ultimately predominating. The relative growth rates of the various groups will, of course, be affected by the storage conditions. To prolong the shelf life of meat it is, therefore, desirable not only to delay the total growth of all bacteria, but to adjust conditions to produce a greater selective retardation of otherwise fast-growing organisms of high spoilage potential (Gill, 1986).

1.3 Spoilage Indicators

1.3.1 Criteria

Fields *et al.* (1968), in their excellent review of metabolic by-products of microorganisms as determinants of food quality, defined a chemical spoilage indicator as "a metabolic by-product which is produced by the dominant spoilage organisms as a result of their growth in the food". They further indicated that mixed cultures may sometimes be responsible for spoilage, so several compounds may need to be determined rather than a single metabolic by-product. In addition, the chemical composition of the particular food and the biochemical activity of the spoilage types are determinants of the choice of indicators that may be used in a given situation. Indicators have been described for foods high in protein, fat,

Table 4. Effect of a second species at maximum cell density on the aerobic growth of bacteria on meat at 10°C[a]

	Pure culture		Mixed culture		
Test species	Generation time (h)	Maximum cell density (CFU per cm^2)	Competing species at maximum cell density	Generation time	Maximum cell density
Enterobacter	3.5	1.8×10^9	*Pseudomonas*	10.4	6.2×10^7
			B. thermosphacta	6.0	7.6×10^8
B. thermosphacta	3.4	1.2×10^9	*Pseudomonas*	7.2	7.1×10^7
			Enterobacter	9.1	2.7×10^7
Pseudomonas	3.0	3.4×10^9	*Enterobacter*	3.2	1.1×10^9
			B. thermosphacta	3.2	1.6×10^9

[a]Data from Gill and Newton, 1977.

or carbohydrates (Fields *et al*, 1968). Criteria for a chemical indicator of food spoilage appear to be similar to specifications for microbial indicators of water or food quality. Jay (1986) reviewed previous works on indicators of spoilage of foods, including numbers and types of bacteria and various metabolites. Jay combined his criteria for a spoilage indicator with those of Fields *et al.* (1968) and other workers and listed these as:

1. The compound must be present at low levels or absent in healthy foods,
2. The indicator should increase in amount with increased spoilage,
3. The compound should make it possible to differentiate between low quality raw materials and poor processing conditions,
4. The indicator should be produced by the dominant spoilage flora . The indicator must be as reliable as organoleptic criteria, and should indicate stages of spoilage which cannot be established definitely by organoleptic testing,
6. To be useful as an index of quality for seafood and ground meats, the test for the compound must be rapid and the analysis simple,
7. The indicator should not give a false positive test, and a companion test is desirable,
8. If the indicator is a microbial metabolite, it should be produced by all strains and under all conditions that allow growth of the producer organisms,
9. The indicator should do more than indicate the obvious condition, it should aid in predicting shelf life (which to this author implies that the indicator would be useless if the food is at the end of its shelf life when the indicator is determined),
10. Ideally, the spoilage indicator should be responsive to certain pathogens so that a food is not considered sound (healthy) when it may be unsafe for consumption.

In considering all the criteria, Jay (1986) and recently Venkitanarayanan *et al.* (1997) believed that viable counts are more useful in detecting spoilage than is the detection of metabolic by-products. They stated that the total microbial count is the most commonly used method for assessing quality.

Recently, the polymerase chain reaction (PCR) was used as a powerful molecular technique for the rapid and sensitive detection of microorganisms (Hill, 1996; Candrian, 1995). When the results of estimation of spoilage bacterial load in aerobically stored meat by

a quantitative PCR and aerobic plate count were statistically analyzed, a correlation coefficient of 0.94 ($P < 0.001$) between aerobic plate count and QPCR luminosity units was found (Venkitanarayanan *et al.*, 1997).

1.3.2 Total Counts

According to Jay's (1986) "spoilage spectrum", foods with an aerobic plate count (APC) of $<10^4$ and most foods with 10^4 to 10^5 g^{-1} are free of spoilage, with the possible exception of raw or pasteurized milk, which may have detectable off flavours at the higher range of counts. As specified earlier, when the count reaches 10^7 to 10^8 bacteria.g^{-1}, definite spoilage has occurred (with the exception of fermented foods), and at a level of 10^9 or greater, changes in texture may be evident.

1.3.3 Bacterial Metabolites

Many attempts have been made to assess spoilage by measurement of microbial metabolites, based on the assumption that the metabolites are directly related to microbial growth and that their amount increases as spoilage proceeds (Jay, 1992).

Metabolites that may possibly be used as indicators with some accuracy for specific purposes include diacetyl for orange juice concentrate in which it gives a "buttermilk" odor; ethanol for apple juice; histamine for scombroid-type fish (e.g., tuna, mackerel); and lactic acid for certain canned vegetables subject to flat-sour spoilage (Jay, 1986). With anaerobically packaged and stored ground meats (Nassos *et al.*, 1984) and ground beef packaged in high-barrier materials, lactic acid was produced by the predominant lactic-acid bacteria after refrigerated storage. Other "promising" indicators include carbonyls (e.g., diacetyl), organic acids (iso-valeric, isobutyric, and acetic), and diamines (e.g., putrescine and cadaverine) produced by lactic-acid bacteria for vacuum- or gas-packaged meats. The diamines and ethanol may also serve as spoilage indicators for fish products.

For fresh aerobically stored meats, diamines are questionable as spoilage predictors, since some investigators showed that they may not be produced in sufficient amounts until spoilage was evident, with the APC in excess of 10^7. g^{-1}. Others indicated that putrescine

be a potential indicator of bacterial counts for ground beef (Edwards *et al.*, 1983; Sayem-el-daher *et al.*, 1985). "Tyrosine" value (mg tyrosine.g^{-1} meat) and decrease in color value were believed to be effective monitors of bacterial numbers on intact meat packaged in oxygen-permeable film and stored at -1 or 7°C for 20 d. However, the tyrosine value is subject to interference from intrinsic changes in meat (Strange *et al.*, 1977). Tyrosine value is also a measure of proteolysis, hence it could be an indicator of changes which might occur late, rather than early, in spoilage.

Strange *et al.* (1977) did not find that changes in pH, redox potential, hydration capacity, or thiobarbituric acid (TBA) number were useful as indicators of bacterial spoilage of intact meat. Indicators of spoilage of ground meat were not useful as indicators of spoilage of intact meat.

Decreases in substrates used by bacteria as they grow on meats has also been suggested as a means of detecting onset of spoilage. Reduction in nucleotides and amino acid levels are examples (Gill, 1983). Decrease in glutamine with subsequent increase in glutamic acid in meat has also been associated with bacterial growth and activity (Gardner and Stewart, 1966). However, as noted earlier, changes in amino acid content have generally been associated with high bacterial numbers, when spoilage is well advanced (Lea *et al.*, 1969 and Greer, 1989).

Analysis of the volatile profiles from meat inoculated with different spoilage organisms indicated that three requirements were necessary for evidence of a putrid odor:

(1) a minimum threshold value was necessary for total volatiles;

(2) except for hydrocarbons, sulfur compounds must be major components of the volatile compounds; and

(3) other classes of compounds must be present only in relatively minor amounts.

Considering these requirements and with meat stored at 5, 10, and 20°C, only four compounds qualified as potential indicators of microbial quality: acetone, methylethyl ketone, dimethyl sulfide, and dimethyl disulfide (Kraft, 1992). Diacetyl, acetoin, isovaleric, isobutyric, and acetic acids are of importance in detecting spoilage by *B. thermosphacta* (Jay, 1986). The actual concentrations of these products are markedly influenced by pH and the initial glucose

content of the medium (Table 5). Low glucose level and near neutral pH favour fatty acid formation, while high glucose level and lower pH values favour acetoin formation.

Volatiles that contain sulfur from the amino acid precursors cysteine and methionine are responsible for much of the off odor associated with spoilage of meats, poultry, and fish (Jay, 1986). Important sulfides that have been identified after growth of *Pseudomonas* spp. and that correlate well with sensory evaluation are: methanethiol, dimethyltrisulfide (in addition to the dimethylsulfides listed above), and methyl mercaptan (Greer, 1989). The bacterial production of hydrogen sulphide converts the muscle pigment to green sulphmyoglobin. Hydrogen sulphide is produced from cysteine and is triggered by glucose limitation (Borch *et al.*, 1996).

The sequence of odor development during spoilage of meats in relation to volatile compounds was summarized by Greer (1989). Initial odors were described as "dairy, butter, cheesy" from acetoin, diacetyl, 3-methyl-1-butanol, and 2-methyl propanol, as a result of glucose being metabolized by *B. thermosphacta*, when the organism was present in sufficiently high numbers. Following this, *Pseudomonas* spp. produced "fruity" odors from formation of esters of short chain fatty acids, and then putrid, "sulfide" odors were evidenced when populations reached $>10^9.g^{-1}$. In late spoilage, off odors arise from metabolism of amino acids by *Pseudomonas* spp, but Greer emphasized that the type of spoilage caused by *B. thermosphacta* specified above could occur first.

1.3.4 Hydration Capacity

One of the most important changes that occurs when fresh beef undergoes microbial spoilage at low temperatures is an increase in the hydration capacity of its proteins. Hydration of meat or water holding capacity (WHC) is the capacity of meat proteins to hold water under pressure or force, a phenomenon that was reviewed extensively by Hamm (1960). A method modified from that of Wierbicki and Deatherage (1958) which utilized pressure to measure free water on filter paper was used. The amount of water released was inversely related to WHC and degree of spoilage (Jay, 1986). Jay and Hollingshed (1990) have published quite extensively on the relation between WHC and meat quality and developed and used a simple

Table 5. The effect of pH and initial glucose concentration on end product formation by *Brochothrix thermosphacta*[a]

		μmol.mg^{-1} dry weight			
	Dry weight cells (mg.ml^{-1})	Acetoin	Acetic acid	Isobutyric acid	Isovaleric acid
Initial pH[b]					
5.5	1.18	3.9	3.5	0.1	0.7
6.0	1.52	2.9	4.0	0.1	1.1
6.5	1.62	1.8	4.9	0.3	1.9
7.0	1.76	1.3	5.5	0.6	3.1
7.5	1.76	1.0	6.0	0.9	4.1
Glucose (% wt/vol)[c]					
0	0.52	0	6.3	5.5	16.7
0.06	1.12	0.7	5.8	2.7	7.6
0.40	1.63	2.7	4.9	0.4	1.6
1.00	4.20	4.7	3.4	Trace	0.1

[a]Based on Dainty and Hofman, 1983.
[b]Growth substrate, glucose (0.2% wt/vol).
[c]Initial pH was 6.5 and cultures were grown for 24 h.

filtration method, termed the extract release volume (ERV) to measure hydration capacity. A decrease in ERV (increase in WHC) occurs as meat spoils and is generally linear, which Jay indicated would be useful for predicting shelf life. Those who are interested in more details can refer to an explanation of a possible mechanism for the ERV as an objective measure of spoilage by Dainty *et al.* (1983).

Increasing pH during spoilage results in a "loosening" of the previously tight protein matrix that exists at the normal pH of meat (near the isoelectric point of the major structural proteins). The increase in pH causes meat proteins to bind water to a greater extent and results in a lower ERV, but pH alone may not account for the results (Shelef, 1974). The amino sugars, particularly glucosamine, increase in meats undergoing spoilage as bacterial counts increase and these cause a raise in hydration capacity (Dainty *et al.*, 1983).

1.4 Bacterial Standards for Retail Meats

Oregon was the first state in the U.S. to set microbiological standards for retail meat products (Carl, 1975). Proposed Canadian microbiological standards were based on the data obtained from a 1974-75 survey. The proposed standards were: aerobic colony count (35°C), $<10^7$ CFU.g^{-1} for non-frozen and $<10^6$ CFU.g^{-1} for frozen products; *Escherichia coli* $<10^2$ CFU.g^{-1}; *Staphylococcus aureus*, $<10^2$ CFU.g^{-1}; and *Salmonella*, absent in 25 g portions from each of five subsamples. To accommodate the variable distribution of bacteria between packages in a single lot of product, a three-class plan based on a format suggested by the International Commission on Microbiological Specifications for Foods was used (Pivnick *et al.*, 1976). The recent standard for ground beef using three-class plan (except for *Salmonella*) in Canada (Health Canada, 1993) is as follows:

	n	c	m	M
Mesophilic aerobic colony count	5	3	1.0×10^7	1.0×10^8
E. coli	5	2	1.0×10^2	5.0×10^2
Staph. aureus	5	2	1.0×10^3	1.0×10^4
Salmonella	5	0	absent	--

where n is the number of sample units to be taken, c equals the number of samples within 'n' that may contain more than 'm' number of organisms but less than 'M' number of organisms and the 'lot' may still be acceptable. m is lower limit of organisms permissible in a 'lot' and M is the upper limit of organisms permissible in a 'lot'.

1.5 Growth Substrates in Muscle

Muscle is a biochemically and structurally a complex substrate comprising of proteins, lipids and numerous nonprotein, low molecular weight soluble constituents. Although there are some important intra- and interspecies differences, the gross chemical composition of postrigor muscle of poultry, fish, and mammalian species is similar.

1.5.1 Low Molecular Weight Components

The low molecular weight, nonprotein soluble constituents of muscle fluids account for about 1.2% of the wet weight of poultry muscle, 1.5% of fish muscle, and 3.5% of mammalian muscle (Greer, 1989). In addition to these quantitative interspecies differences, qualitative differences are evident from comparisons of the composition of the muscle "extractives" (Hobbs, 1983), from fish, Crustacea, poultry, and mammals. As noted by Newton and Gill (1981), however, there are many similarities when these substances are viewed as substrates for bacterial growth. Soluble components, common to the musculature of all three classes of animals, include nucleosides, nucleotides, free amino acids, lactic acid, glycogen, glucose, and glucose-6-phosphate (Dainty, 1982). From a bacterial growth perspective, the most important organic components include glucose, amino acids, and lactic acid (Gill, 1976; Jay, 1992). The main low molecular weight components of beef before and after rigor mortis are presented in Table 6.

The musculature of flesh foods is sufficiently rich in these low molecular weight constituents to support bacterial growth to considerable densities (*ca.* 10^9 bacteria.g^{-1}). This finding has been documented in fish (Lerke, 1967), poultry (Adamcic, 1970), and mammalian muscle (Gill and Newton, 1978). In fact, growth at meat surfaces becomes limited as a result of decreased oxygen tension as opposed to substrate depletion (Gill, 1982).

Table 6. The main low-molecular-weight components of beef pre- and post-rigor mortis[a]

Component	Concentration ($mg.g^{-1}$)	
	Pre	Post
Creatine phosphate	3.0	-
Creatine	4.5	6.5
Adenosine triphosphate	3.0	-
Inosine monophosphate	0.2	3.0
Glycogen	10	1.0
Glucose	0.5	0.1
Glucose 6-phosphate	1.0	0.2
Lactic acid	1.0	9.0
Amino acids	2.0	3.5
Carnosine, anserine	3.0	3.0
pH	7.2	5.5

[a]Based on Lawrie, 1985; Gill, 1986 and Tappel, 1966.

Substrate preferences by organisms growing on muscle were proposed as early as 1939 by Beatty and Collins. These investigators considered that the biodeterioration of fish muscle was characterized by two distinct phases. The first involved oxidation of lactic acid, sugars, and other carbohydrates while the second was an attack on amino acids and proteins. These concepts were supported in subsequent research where Jay and Kontou (1967) quantified changes in amino acids and nucleotides in naturally spoiling beef. It was concluded that primary muscle proteins were not attacked until the low molecular weight compounds were exhausted.

Major progress in the understanding of substrate utilization by meat spoilage bacteria comes from the conclusive studies of Gill and Newton (Gill, 1976; Gill and Newton, 1977). Their findings have been summarized in several excellent reviews (Gill, 1986; Gill, 1982). Using deproteinized, aqueous, beef muscle extracts and sterile meat slices, substrate preferences of spoilage bacteria were determined. The dominant spoilage organism *Pseudomonas* spp. showed a primary glycolytic phase of growth, where the metabolism of glucose supported bacterial growth to densities of $10^8.cm^{-2}$ of muscle surface. As the rate of diffusion of glucose from underlying tissues became limiting, the organisms entered a second metabolic phase where amino acids and then lactate were degraded. Farber and Idziak (1982), showed that the glucose oxidation products, gluconate, and 2-ketogluconate could also serve as growth substrates during this second metabolic phase of growth when glucose was depleted.

The role of glucose in *Pseudomonas* spp. growth on red meats and its potential to induce catabolite repression of amino acid metabolism has been discussed by Molin (1985). On the basis of results with mixed substrate carbon sources it was concluded that *P. fragi* used lactate simultaneously with amino acids, irrespective of the presence of glucose. The validity of the interpretation of these latter data, however, has been questioned (Greer, 1989).

Notwithstanding the importance of glucose, there is considerable evidence in support of a role for utilization of amino acids (Adamcic *et al.*, 1970). Lea and associates (1969) were among the first to demonstrate that mixed cultures of pseudomonads reduced the nonprotein nitrogen fraction and the amino acid content of chicken muscle. Subsequently, Adamcic *et al.*

(1970) observed that the growth of a nonpigmented *Pseudomonas* spp. decreased the amino acid content of chicken skin to below detectable levels during the most rapid phase of growth.

The most comprehensive studies of the role of amino acids in *Pseudomonas* spp. growth were conducted by Bannerjee and Chung (both cited in Greer, 1989). After a detailed study of bacterial growth in fish muscle extractives, it was concluded that the amino acids of the nonprotein nitrogen fraction could control proteinase synthesis until bacterial populations exceeded 10^8. ml^{-1} of fish protein medium. These researchers considered that pseudomonads dominated the spoilage flora as a consequence of their ability to more effectively compete for and oxidatively deaminate amino acids in fish muscle.

Although pseudomonads are metabolically and numerically the most significant species, some consideration should also be given to the minor components of the spoilage flora. Relative to this, Gill and Newton (1977) have compared the order of utilization of low molecular weight components by *Pseudomonas, Acinetobacter, Brochothrix thermosphacta,* and *Alteromonas (Shewanella) putrefaciens.* The *Acinetobacter/Moraxella* group cannot metabolize hexoses and are therefore initially dependent upon amino acids and lactate for growth. Some researchers have also determined that the metabolism of *B. thermosphacta* is primarily glucolytic and casamino acids (acid hydrolyzed casein), glutamate, or aspartate could not provide alternative energy or carbon sources (Grau, 1979). Contrarily, others have claimed that *B. thermosphacta* can degrade glutamate, valine, isoleucine, and leucine (Dainty and Hibbard, 1983). *Alteromonas putrefaciens* attacks glucose, serine, and cysteine simultaneously with the degradation of lactate being delayed (Gill, 1986).

Gill (1976) suggested that the presence of certain levels of glucose in meat inhibits the catabolism of alternative substrates (lactate, amino acids) by the spoilage flora. Other studies have confirmed and extended these findings (Drosinos and Board, 1994, 1995; Borch and Agerhem, 1992; Nychas and Arkoudelos, 1990). Several studies have been published that contradict this hypothesis. Lynch and Franklin (1978) found that the growth of *Pseudomonas* spp. in minimal media at 5°C was faster on organic acids than on glucose. However, Farber (1983) showed that in minimal media at 4°C, three *Pseudomonas* spp. and *E. agglomerans* reached higher cell densities when grown on glucose rather than organic acids and thus they

proposed that organic acids were used after glucose depletion.

Lynch and Franklin (1978) also found that, while using organic acids for growth, *P. fluorescens* converted glucose to gluconate and 2-ketogluconate (2-KG). Uptake activity for these two compounds was delayed until the end of the second growth phase, at which point glucose was exhausted. The same was noted by Nychas (1984), Farber (1983) and Whiting *et al.* (1976).

Glycerol was not among the carbon sources tested by Farber (1983) for the growth of meat spoilage organisms. *Pseudomonas* spp and *B. thermosphacta* utilize glycerol. *B. thermosphacta* reached similar cell densities when grown on 20 mmol.l^{-1} glucose or glycerol as a sole carbon source (Macaskie *et al.*, 1984). Gauthier (1990) found that glycerol can be utilized also by *Enterobacter* spp. In view of this, with a heterogenous mixture of spoilage psychrotrophs, several muscle substrates could be utilized concurrently. In summary, a proposed order of nutrient utilization by various spoilage species, based on Gauthier (1990) and supplemented with recent evidence, is given in Table 7.

Gauthier (1990) compared the order of utilization of low molecular weight components by single and mixed cultures of spoilage organisms (P. *fluorescens*, non-fluorescent *pseudomonas*, *E. agglomerans* and *B. thermosphacta)*. The HPLC chromatogram of the mixed culture agitated at 50 rev.min^{-1} after 7 d of incubation at 4°C showed two peaks of unknown nature. The first compound was originally present in MJM; whereas the second one appeared upon incubation in some cultures only.

In the mixed culture agitated at 50 rev.min^{-1}, the levels of the unknown no.1 first decreased slightly then increased until day 7. Levels of the second unknown compound increased continuously from day 3 to day 7. At 100 rev.min^{-1}, levels of both substances increased until *ca.* day 5, then rapidly declined, reaching non-measurable levels between days 6 and 7.

B. thermosphacta did not affect the levels of these two compounds when grown as a pure culture in MJM. In all other cultures, changes in concentration of the unknown peaks occurred between days 3 and 4. *P. fluorescens* and *E. agglomerans* first produced both compounds, then started to utilize the unknown no.2 between days 6 and 7. *E. agglomerans*

Table 7. Proposed order of utilization of various low-molecular weight substrates by meat spoilage organisms[a]

Pseudomonas spp	*Enterobacter* spp	*B. thermosphacta*	*Acinetobacter* spp
Organic acids[1,2]	Glucose[3,6,9,10]	Glucose[2,3,9]	Amino acids[2,3]
Glucose[3,6,9]	G-6-P[3,6]	Glycerol[6,7]	Lactate[2,3,8]
Gluconate[1,4,6]	Gluconate[6]	Glutamate[3]	
2-KG[1,4,6]	Glycerol[6]		
Amino acids[3]	Amino acids[2,3]		
Lactate[3]	Lactate[2,3]		
Glycerol[5,6]			

[a]derived from Gauthier, 1990.

1 Lynch & Franklin, 1978
2 Farber, 1982
3 Gill& Newton, 1977
4 Farber & Idziak, 1982
5 Lin, 1976
6 Gauthier, 1990
7 Macaskie *et al.*, 1984
8 Bøvre, 1984
9 Lambropoulou *et al.*, 1996
10 Drosinos and Board, 1995

produced higher amounts of the unknown no.1 than did *P. fluorescens*. The non-fluorescent *Pseudomonas* utilized some of the unknown no.1. Table 8 shows the order of utilization of some low molecular weight components (including unknowns 1 and 2) by meat spoilage organisms based on Gauthier's work.

The identity of these two compounds is still unknown. Since they appear as predominant compounds during spoilage of MJM, their identity seems to be important for a better understanding of the order of nutrient utilization of various spoilage species and consequently the mechanism of meat spoilage.

1.5.2 Protein

Protein constitutes from 12 to 22% of the wet weight of the lean muscle of fish, poultry, and mammals (Jay, 1992). Muscle proteins can be divided on the basis of solubility into the myofibrillar, sarcoplasmic, and stromal (connective tissue) components. Within each of these subdivisions there exists a complex, heterogeneous mixture of various proteins (Greer, 1989).

Since many spoilage bacteria showed proteolytic capabilities, earlier researchers assumed mistakenly that muscle proteins were an easily available source of nitrogen and carbon. It is apparent, however, from the previous discussion that there are sufficient nonprotein constituents in flesh foods to support relatively large bacterial populations. Thus, Jay (1966) found no changes in the total soluble proteins of beef muscle for up to 15 d at 7°C although the natural spoilage flora exceeded 10^{10} bacteria.g^{-1}. In a complementary study, Dainty *et al.* (1975) reported no changes in the proteins of slime-inoculated or naturally contaminated beef until bacterial numbers exceeded 3.2×10^{9}.cm^{-2}. Furthermore, others have shown that *P. fragi* proteinases were not detectable in growth media until the late exponential phase of growth and that they were degraded during the stationary phase (Tarrant *et al.*, 1973). However, Farber (1983) showed that in complex media *P. fluorescens* degraded both casein and gelatin, while *P. putida* degraded casein. With the aerobic atmosphere, Drosinos and Board (1995) showed that *P. fragi* catabolized creatinine in a meat (lamb) juice at 4°C.

Evidence negating a role for proteins as growth substrates includes the repeated

Table 8. Order of substrate utilization by spoilage organisms grown in MJM as pure cultures, at 4°C and 50 rev.min^{-1}.[a]

P. fluorescens	non-fluorescent *P.*	*E. agglomerans*	*B. thermosphacta*
Glucose	Glucose	Glucose	Glucose
Gluconate	Gluconate	Gluconate	Glycerol
Urea	Urea	Glycerol	
Lactate	**Unknown 1**	G-6-P	
Glycerol	Glycerol	**unknown 2**	
Unknown 2			

[a]Based on Gauthier, 1990.

observation that the population of nonproteolytic spoilage pseudomonads increases dramatically during the storage of muscle foods (Jay, 1967). It has been suggested that during the initial stages of growth the proteolytic strains grow in a nonproteolytic fashion both competing for low molecular weight nonprotein substrates (Greer, 1989). However, at the advanced stages of growth, when the low molecular weight substrates are depleted, the proteolytic strains would have a definite advantage. Indeed, the growth and proteinase secretion by the proteolytic psychrotrophs may synergistically support growth of nonproteolytic bacteria by providing the pool of amino acid substrates. There is now adequate evidence to conclude that although bacterial proteinases can attack muscle proteins, these activities are not manifest until after prolonged periods (weeks) of refrigerated storage. At this time, the concentration of the more readily digestable, nonprotein constituents have been reduced to below a critical level (Adamcic *et al.*, 1970; Dainty *et al.*, 1975).

Apart from the preferential utilization of soluble nonprotein substrates, there may be other reasons for a significant delay in proteolytic activities of spoilage bacteria. As *Pseudomonas* spp. grow on muscle substrates they liberate basic catabolites, such as ammonia, which significantly increase the pH. As a consequence of increased pH and an accumulation of amino sugars, the hydration capacity of the proteins is increased (Jay, 1972). Thus, at the advanced stages of bacterial growth the increased water-holding capacity may make insoluble refractory proteins more susceptible to bacterial proteinases which many psychrotrophic bacteria have. Increased pH would also be more favourable to proteinase function since the enzymes are optimally active within the neutral pH range (Tarrant *et al.*, 1973). Optimum pH for enzyme secretion is 7.0 to 8.5. This is of particular significance in postrigor, fresh mammalian muscle where the pH averages 5.6 but can approach 8.0 in spoiled meat.

The potential of psychrotrophic bacteria to synthesize and secrete extracellular proteinases has been documented in studies with whole muscle, aqueous muscle extracts, purified muscle proteins, and artificial substrates (Greer, 1989). Based upon research with *P. fragi* in pork, Tarrant *et al.* (1971, 1973) described the enzyme as a neutral extracellular proteinase with optimal activity between pH 6.5 and 8.0 and at a temperature of 35°C. The

regulation of proteinase synthesis and secretion in myosystems is quite complex and may be suppressed or induced by amino acids (Tarrant *et al.*, 1973). Catabolite repression by glucose has also been reported (Gill, 1982). More recent ultrastructural studies of extracellular proteinase production by a meat isolate of *P. fragi* have shown enzyme localization in "bleb"-like evaginations of the bacterial cell wall (Thompson *et al.*, 1985). It was hypothesized that the release of proteinase from these structures into the meat tissues could provide a source of amino acids following hydrolysis of muscle proteins.

Perhaps a typical example of research for the role of protein in bacterial growth and muscle spoilage is that of Lerke *et al.* (1967). By using Sephadex G-25 to fractionate muscle press juice from English sole, substances of molecular weight less than 5000 were excluded from the protein fraction. This muscle protein fraction, however, was as effective in supporting the growth of fish spoilage pseudomonads as was the unfractionated muscle juice. Subsequent research in other laboratories has shown that *Pseudomonas* spp. could grow in diazotized fish protein as the sole source of carbon and nitrogen, in filter sterilized beef actomyosin, and in beef sarcoplasmic and myofibrillar protein solutions (Greer, 1989). Thus, when the more readily digestable, nonprotein constituents are not available pseudomonads can utilize muscle protein as the sole source of carbon and nitrogen.

Some traditional methods for the detection of proteolytic bacteria used nutritionally complete media in which proteins were not the sole growth substrates. Sikes and Maxcy (1979), however, examined the ability of 122 bacterial isolates from chicken to hydrolyze water-soluble sarcoplasmic proteins from beef in the absence of additional carbon or organic nitrogen sources. A total of 51 of the 122 test strains could grow and secrete proteinases in this sarcoplasmic protein medium. These researchers concluded that the amino acids resulting from protein hydrolysis were essential to bacterial growth and enzyme production.

1.5.3 Lipids

Quantitatively, fat immediately follows moisture and protein as a major component of edible flesh. Adipose tissues are composed of neutral lipids, phospholipids, cholesterol, and some free fatty acids. Although the lipid content of muscle can be extremely low, it is subject

to considerable variation. In this regard, total lipids of raw, flesh-type foods can range from 0.7 to 15.7% for fish, 1.7 to 18.3% for chicken, and, depending upon the extent of intramuscular fat, 2.5 to 37.1 % for red meats. Indeed a layer of subcutaneous fat surrounds the dressed carcass of red meat animals (Greer, 1989).

Most researchers would agree that numerous spoilage psychrotrophs do elaborate extracellular lipases and can hydrolyze muscle lipids (Barnes and Melton, 1971). This has been confirmed with artificial substrates such as tributyrin and with the natural substrates lard and chicken fat. Relative to this, Barnes and Melton (1971) compared the lipolytic activities of *Pseudomonas* spp., *A. putrefaciens,* and *Acinetobacter* spp. using tributyrin, Difco lipase reagent, and chicken fat. The importance of using natural substrates was emphasized by the observation that although most strains were lipolytically active on tributyrin, lipolytic potential was markedly reduced when chicken fat was used as a test substrate (Greer, 1989).

Lipolytic activity is also demonstrable by determining increases in liberated free fatty acids and thiobarbituric acid values. Several researchers have used these chemical criteria in support of bacterial lipolysis in intact muscle and in natural muscle substrates (Mast and Stephens, 1973). It should be emphasized, however, that the lipids are susceptible to autooxidation and without appropriate control it is often insignificant to differentiate bacterial-associated lipolysis from autocatalytic oxidation (Gill, 1982). This is particularly evident with some fatty fish and pork where lipids are highly unsaturated and thereby more susceptible to autooxidation.

There is little published evidence on the mechanism of action, structure, or biosynthesis of extracellular lipase systems of psychrotrophic bacteria spoiling muscle foods. However, studies with a purified *P. fragi* lipase (active on lard) revealed that it hydrolyzed only glycerol esters of fatty acids and showed a 1,3-position specificity for triglycerides. Using tributyrin as substrate, the optimum pH of the purified enzyme was 8.6 to 8.7. Subsequently, Bala *et al.* (1979, 1977) reported a significant increase in free fatty acids in sterile beef muscle and in beef muscle extracts inoculated with a purified *P. fragi* lipase stored at 4°C. Lipase activity at low temperatures has also been determined with lard substrates where *P. fragi* lipases were active at subzero temperatures.

There are no known reports of muscle lipids (in their natural state in tissue) as growth substrates in the absence of other carbon sources. It is clear, however, that the adipose tissues of beef, pork, lamb, and chicken support bacterial growth to relatively large populations (Gill and Newton, 1980). Some researchers have also proposed that depending upon oxygen availability in fat tissue, distinct differences occur in the quantity and quality of the resident flora (Grau, 1983). These differences in part, can be attributed to the neutral pH of muscle lipids.

Vanderzant *et al.* (1986) determined that growth of *Hafnia alvei*, *Serratia liquefaciens*, and *Lactobacillus plantarum* was greater on refrigerated beef fat and pork fat when compared to lean meat probably because of the neutral pH of muscle fat. There are also conditions under which *Alteromonas putrefaciens* (Gill, 1986) and *B. thermosphacta* (Grau, 1983) are inhibited by the low pH of lean muscle but may multiply at the pH of fatty tissues.

It is obvious that psychrotrophic bacteria do grow on muscle surfaces and can attack lipids but significant lipolysis is only demonstrable when bacterial numbers are high. It has been reported that *Pseudomonas* spp. could grow to numbers exceeding $10^8.g^{-1}$ on chicken skin without increasing the levels of free fatty acids. Moreover, it was found that a source of readily metabolized carbohydrates would suppress lipase production by *P. fluorescens*.

Wooten *et al.* (1974) hypothesized that fat and lean beef muscles provided similar substrates for bacterial growth. This hypothesis was supported some years later by Gill and Newton (1980), who published the only definitive effort to assess substrate utilization in adipose tissue. These workers noted that unemulsified fat was resistant to bacterial attack and that growth rates reported for bacteria on fats were considerably too rapid for organisms using lipid substrates. It was proposed that the serum from cut blood vessels could provide organisms with easily assimilable low molecular weight substrates from adipose tissues. To provide evidence to support these contentions an original approach was taken by Gill (1986). Substrate preferences in lamb adipose tissue were assessed by examining the growth of a nonproteolytic and nonlipolytic *P. fluorescens* on a thin agar layer on freshly cut fat surfaces. Under these conditions, glucose, amino acids, and lactic acid provided by the serum from damaged blood vessels were found to be readily available substrates. As bacterial growth

started glucose was initially attacked, followed by amino acid utilization on day 2 and lactic acid on day 3. It was concluded that "triglyceride is not the substrate for microbial growth on adipose tissue." Furthermore, the above-mentioned researchers insisted that bacterial growth continues at a similar rate on lean and fatty tissues at the expense of the low molecular weight soluble components.

Although fats appear not to be used as growth substrates in muscle, there are instances where fatty substrates can support growth providing the physical characteristics of the substrate are suitable. Tan and Gill (1987) have found that both lard and mutton tallow could support the growth of *P. fluorescens* if these substrates were mechanically distributed i.e., emulsified and degraded and if the bacterial inoculum was induced for lipase. Bacterial growth occurred in the absence of added carbon sources and was attributed to the activities of both cell-associated and cell-free lipases.

As mentioned earlier, the potential of psychrotrophic bacteria to elaborate extracellular proteinases and lipases does not give any competitive advantages for growth in fat or lean muscle tissues. In fact, the synthesis and secretion of these extracellular enzymes is repressed by more readily utilized low molecular weight substances in the flesh. It should also be stressed that the proposed sequential use of muscle substrates may not be as well defined under natural conditions; that is, with an uneven distribution of a mixed spoilage flora the glucolytic and mixed substrate metabolic phases could occur simultaneously at distinct locations on muscle surfaces (Greer, 1989).

1.6 Glucose

1.6.1 Glucose Catabolism by Pseudomonads

Glucose metabolism by pseudomonads has been studied in considerable detail (Lessie and Phibbs, 1984). Two pathways are used in the production of 6-phosphogluconate (6PGA), a key intermediate of the Entner-Doudoroff pathway. The direct oxidation pathway acts on glucose extracellularly and the other pathway intracellularly to convert glucose to 6PGA by formation of different compounds e.g., formation of gluconate from glucose extracellularly and conversion of gluconate to 6PGA intracellularly. These extracellular and intracellular

pathways can be considered as truly alternative pathways in the production of the intermediate noted above in that one can compensate for the loss of the other. Midgley and Dawes (1973) proposed that glucose and gluconate dehydrogenase are located in the cytoplasmic membrane and that glucose is thereby catabolized in the periplasmic space. It has been believed that, in natural environments, the rapid oxidation of glucose may change this energy source in a form, gluconate, which is not so readily utilized by organisms other than pseudomonads. Consequently it can be considered to be an extracellular storage product (Varnam and Sutherland, 1995; Farber, 1983; Whiting *et al.*, 1976). Recent studies suggest that this strategy may enhance the competitiveness of pseudomonads growing on meat under chill conditions. It is evident that in addition to gross changes in substrates such as glucose, attention must be paid to the possible and probably temporary presence of intermediates such as gluconate (Nychas et al., 1988). This has been done in two excellent studies.

Farber and Idziak (1982) inoculated ϒ-irradiated (10 kGy) pieces of bovine *longissimus dorsi* muscle (3.0 x 1.5 -cm blocks) with a mixed population or pure cultures of pseudomonads, *B. thermosphacta*, or *Enterobacter agglomerans*. As was to be expected, the growth of the spoilage flora was associated with a decrease in the glucose and lactic acid content of meat wrapped in a gas-permeable film during storage at 4°C. There was also a notable increase in the gluconate concentration by the 6th day but a marked loss of this compound by the 12th day of storage. These workers demonstrated an increase in the concentration of glucose dehydrogenase activity throughout storage. Analysis of meat inoculated with pure cultures of bacteria revealed that gluconate occurred only with inocula of *P. fluorescens*, *P. putida*, or , nonpigmented pseudomonads.

Minced beef was used in the second study of Nychas and Board (1985). This effectively minimized problems with glucose diffusion, thereby ensuring that spoilage organisms had immediate access to this substrate from the beginning of an experiment. Selective conditions were used to promote spoilage associations of particular composition. Sulfite alone or in combination with CO_2 was used for this purpose. After 3 days of storage of minced beef in a normal atmosphere (without sulfite or CO_2), a continuous reduction in the glucose content of beef initially having a pH of 5.85 was demonstrated. The loss was

associated with an increase in the gluconate content in the 3- to 5-day period of storage, but thereafter the concentration of the latter declined. These trends were evident even if somewhat muted in minced beef having the characteristics of DFD meat, i.e., a relatively high pH (6.10) and a small content of glucose at the outset. Analysis of the spoilage flora showed that pseudomonads were dominant in these two experiments. When the growth of these organisms was suppressed by sulfite or sulfite and carbon dioxide, there was an increase in the glucose content of minced meat at 1 °C over the first 4 days of storage with, on three occasions, a slight reduction by the 10th day. Gluconate concentration increased to a significant extent only when there was an increase in the size of populations of pseudomonads (from $10^{5.1}$ to $10^{6.3}$). The meat containing preservatives supported a population of Gram-positive bacteria-*B. thermosphacta* and uncharacterized *lactobacilli*. In view of the known pathways of glucose catabolism in these organisms, the failure to demonstrate even a transient accumulation of gluconate in the experiment was not unexpected. The reduction in the glucose dehydrogenase activity of meat supporting the growth of Gram-positive facultative anaerobes provides further circumstantial evidence that pseudomonads were the major cause of the oxidation of glucose to gluconate. Indeed these two studies support a view expressed previously: pseudomonads out-grow would-be competitors on meat by sequestering glucose as gluconate (Farber and Idziak, 1982). This conclusion is of particular interest to those who seek chemical probes to assess meat freshness (Nychas *et al.*, 1988).

1.6.2 Spoilage Control by Addition of Glucose

As the glucose content of meat largely determines the cell density reached before spoilage onset, it would seem possible to extend the period of harmless bacterial growth by increasing the glucose available to the aerobic flora. In the case of DFD meat this approach is effective since adding glucose at a level of 100 $\mu g.g^{-1}$ to DFD meat will increase the shelf life to that of normal meat (Newton and Gill, 1978). It is, however, doubtful whether any actual extension of shelf life can be achieved by adding glucose to meat that already has a normal content of glucose in excess of 100 $\mu g.g^{-1}$.

Shelef (1975) observed liver spoilage by lactobacilli and concluded mistakenly that

these relatively harmless organisms had developed in response to the high carbohydrate content on the tissue. In fact, the storage conditions employed had produced an anaerobic environment, which accounts for the dominance of anaerobic and facultative anaerobic organisms (Gill and De Lacy, 1982). However, addition of glucose to meat at concentrations of 2% (wt/wt) or more under aerobic conditions did suppress odor and slime formation (Shelef 1977). Off-odors were not formed because amino acid utilization was suppressed by glucose.

Although the addition of large amounts of glucose delays the more obvious symptoms of spoilage, it is doubtful whether it could be of any practical value because of adverse flavor effects. Glucose at 2% (wt/wt) in meat would be unacceptable to many, while the presence of considerable concentrations of the acid derivatives might be unacceptable to most consumers. However, ordinary treatment of carcasses and cuts with much smaller amounts of glucose could be used to prevent the early spoilage of any undetected DFD muscle during aerobic chiller storage (Gill, 1986).

Addition of glucose to meat that will be vacuum-packaged is deleterious. The presence of glucose does not prevent early spoilage by greening of DFD meat as H_2S production by *A. putrefaciens*, the spoilage organisms responsible, is not inhibited by glucose. If anything, addition of glucose to vacuum-packaged DFD meat stimulates growth of this organism. The growth of *B. thermosphacta* in high pH meat will also be favoured by added glucose. Addition of glucose to normal pH meat will only increase the final cell density of the *Lactobacillus* flora and so accelerate the accumulation of volatile acids (Newton and Gill, 1980). However, it may be useful to add glucose on opening vacuum packages and subsequent storage to display the meat; as this would replace the glucose exhausted by the anaerobic flora and so delay the onset of aerobic spoilage by pseudomonads.

In summary, meat spoilage is a complex event whose mechanism is still poorly understood. The major problems are to find the relation between microbial composition and presence of microbial metabolites, related to the evaluation and possible prediction of microbial spoilage.

2. Material and Methods

2.1 Organisms

2.1.1 Source

The five organisms studied were isolated from the spoilage flora of beef stored at 4°C and at room temperature (Gauthier, 1990; this work, and D'Aoust, personal communication, 1996). They were identified as *Pseudomonas fluorescens*, two non-fluorescent *Pseudomonas* spp. (*Pseudomonas fragi* and *Pseudomonas aeruginosa*), *Enterobacter agglomerans* and *Brochothrix thermosphacta*.

The identities of the isolates were confirmed using the Biolog (Biolog, Inc., Hayward) and api 20E (bioMérieux Vitek, Inc., Hazelwood) test systems with additional biochemical tests where necessary.

2.1.2 Stock Cultures

Pure cultures of the five organisms were streaked on Brain Heart Infusion Agar (BHIA, Difco) slopes and incubated for 24 h at 23 ±1°C. The growth was resuspended in sterile Brain Heart Infusion Broth (BHIB), dispensed in Cryovial vials (Simport, Beloeil), and 90 μl Dimethyl Sulfoxide (DMSO) were added to each 1 ml of culture. Finally the cultures were kept at -80°C until required.

2.2 Meat Juice Medium (MJM)

The method of preparation of MJM was adapted from the method of Gill (1976) and Gauthier (1990) and is described below.

2.2.1 Extraction and Storage of the Crude Extract

Fresh, lean ground beef was purchased from a local butchery. The meat was mixed with an equal volume of 0.2 M phosphate buffer pH 6.0 (Costilow, 1981) and emulsified by mixing in a Hobart A-200 T bench model mixer for 1.5 min and then in a Braun food processor at low speed for 1 min. The emulsion was then heated to 70°C in an Escan steam jacketted kettle and maintained at that temperature for 5 min with slow agitation and scraping off the processor's walls to prevent overheating. The emulsion was then squeezed through a muslin bag. The liquid extracted was

dispensed in 1 liter portions in ziploc plastic bags and kept at -20°C until required. Eleven liters of crude extract and 6.6 kg of dry matter were obtained from 10 kg of meat and 10 l of phosphate buffer as starting material.

2.2.2 Clarification and Sterilization

The frozen crude extract was thawed in a 70°C water bath. It was then heated to 80°C and held at that temperature for 3 min. After cooling to 35°C in an iced-water bath, the extract was centrifuged (10,000 x g) at 25°C for 20 min in a Sorvall, model RC-5B refrigerated superspeed centrifuge.

The supernatant was collected, warmed to 35°C, and filtered under non-sterile conditions through whatman no.4 and no.1 filter papers followed by filteration through Millipore filters 12, 8, 3 and 0.45 μm and finally through 0.22 μm Gelman filter capsules, model no. 12140 (Gelman Sciences Inc., Ann Arbor), using a pressure chamber.

The clarified juice was finally filter sterilized by passing through a Gelman filter capsule (0.22 μ) (as described above) in a laminar flow hood under sterile conditions. The sterile MJM was kept at -20°C until required. One liter of crude extract yielded 750-800 ml of clarified, sterile MJM.

2.3 Growth of Single Spoilage Organisms in MJM

2.3.1 Preparation of the Working Cultures

Stock cultures were thawed at 23±1°C for 1 h and inoculated into BHIB. The organisms were incubated at 23±1°C and subcultured twice in fresh BHIB at 24 h intervals (1 loopful into 50 ml BHIB) under stationary condition. These cultures were subsequently used as working cultures.

2.3.2 MJM Conditioning and Inoculation

Sterile MJM was first defrosted in an 80°C water bath and then distributed into 250 ml sterile erlenmeyer flasks (100 ml per flask) and placed in a Gyrotory shaker (New Brunswick Scientific Company, New Brunswick; model G-25), set at 100 rev.min $^{-1}$. A working culture of *P. aeruginosa* was centrifuged (10,000 x g, 15 min at 4°C) and the supernatant was discarded. The cells were resuspended in sterile physiological saline. The suspension was diluted in sterile saline to obtain an

optical density of 0.025 units at 660 nm, followed by a further 10 fold dilution. One ml fractions of the last dilution of the culture were added to each of two MJM flasks. The flasks were incubated 7 days at 4°C and 50 rev.min^{-1}.

One uninoculated MJM flask was incubated as a control for each 2 inoculated flasks. The contents of the flasks were sampled on the 7th day of incubation, then centrifuged at 4°C and 10,000 x g for 20 min. The supernatants were filtered through 0.45μm filters and run through HPLC. Culture integrity was also verified at the end of the incubation period.

2.4 Effect of Glucose Supplementation on the Rate of MJM Spoilage

Five ml sterile distilled water containing 0.5 g D-glucose were added to 95 ml sterile MJM (0.5% wt/vol). As a control, 5 ml sterile distilled water without glucose were added to another flask containing 95 ml sterile MJM as a control. Both flasks were inoculated with *P. aeruginosa*, placed in a Gyrotory shaker (New Brunswick Scientific Company, N.B, model G-10) set at 100 rev.min^{-1}, and incubated at 30°C. The contents of the flasks were sampled on the day of inoculation and at 24 or 48 h intervals for the next 7 days. In the case of MJM containing glucose, sampling continued until day 21. A 3 ml sample was withdrawn from each flask and centrifuged at 10,000 x g for 20 min at 4°C. Sample supernatants were filtered (0.45 μm) and the filtrates kept at -20°C until required for pH, glucose, fructose, total carbohydrate, HPLC, GC-MS and LC-MS analyses.

2.4.1 Glucose, Fructose and Total Carbohydrate Measurement

Frozen samples from the glucose supplementation experiment stored at -20°C (section 2.4) were thawed at room temperature for 1 h. Total glucose and fructose were determined with the hexose kinase (HK)-glucose-6-phosphate dehydrogenase (G6PDH) test kit (Boehringer Mannheim, Cat.no. 139 106).

Fructose concentrations were determined with the same method, except that fructose was first phosphorylated to fructose-6-phosphate (F-6-P) and then was converted to G-6-P by phosphoglucose isomerase (PGI) (Boehringer Mannheim instruction, 1984). All absorbance measurements were done at 340 nm using a Beckman DU-70 spectrophotometer which was calibrated against air.

Reagent blanks were prepared and blank readings were subtracted from sample readings.

Concentrations were computed as follows:

$$C=\frac{V\times MW}{\varepsilon\times d\times v\times 1000}\times\Delta A$$

Where: C = concentration ($mg.ml^{-1}$)

V = final volume (3.02 ml)

v = sample volume (ml)

MW = molecular weight ; 180.16 $g.mol^{-1}$ for glucose

180.16 $g.mol^{-1}$ for fructose

d = light path (1 cm)

ϵ = absorption coefficient of NADPH at 340 nm (6.3 $l.mmol^{-1}.cm^{-1}$)

A = A_{glu} or A_{fru}

Total carbohydrate concentrations were determined using the phenol-sulfuric acid method (Dubois *et al.*, 1956).

2.4.2 pH Determination

The pH of the samples were measured with a Fisher Scientific accumet pH meter (model 50).

2.5 Identification of Unknown Compounds

2.5.1 Use of HPLC

A Waters model 510 HPLC , equipped with an automatic injector (Waters, model WISP 710 B) and a Shimadzu, HIC-6A column heater were used. The injection valve was fitted with a 15 μl loop. A Waters ultraviolet detector, model 441, set at a wavelength of 214 nm was used. The HPLC was linked to a Waters QA-1 data system.

The column, an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm i.d., Biorad, Hercules, CA) made specially for organic acid analysis was operated at a temperature of 35°C and

was preceded by a Biorad guard column containing a Cation H Micro-Guard cartridge, slurry-packed with material similar to that in the HPLC column they protect.

The mobile phase (0.008 N H_2SO_4 in distilled water) was prepared fresh and degassed by stirring under vaccum for 15 min before use. The flow rate of the eluant was set to 0.5 $ml.min^{-1}$. Samples were filtered (0.45 μm), and 15 μl aliquots were injected. The run time was 40 min.

2.5.2 Tentative Identification of Unknown Compounds

The retention times of two unknown compounds were compared to those of various organic acids. Standard solutions of various organic acids were prepared in distilled water, at a concentration of 0.5% (wt/vol) except fumaric acid, 0.05% (wt/vol). Solutions were filtered (0.45μ m) and analyzed with HPLC.

Acids whose retention times were close to those of the unknown peaks were selected to be used as internal standards. Solutions of these acids in distilled water were added into inoculated MJM containing the two unknowns and injected into HPLC to see if they co-elute with any of the unknown compounds. A fresh DL-lactate standard [0.5% (wt/vol) in distilled water] was included with each series of injections. Various amino acids and glucose were also examined.

Samples containing the two unknown compounds (RT 21 and 32 min) were injected into a second HPLC column (Ion 300), specific for sugars, in order to compare the two unknowns retention times. The second column was mounted on a Waters HPLC system (Millipore, Milford, MA) consisting of a 600E System Controller, a U6K Injector, a 486 Tunable Absorbance and Differential Refractometer R401 Detectors, a Waters Fraction Collector, and a Millennium 2010 Chromatography Manager. Portions (10 μ l) of water-soluble fractions were injected into the column and eluted with a mixture of acetonitrile : water : : 60 : 40 solution as a mobile phase.

Finally the unknowns were tentatively identified by comparing their relative retention times to those of the known library compounds. In this instance, the relative retention time of a compound is its retention time relative to the reference compound, lactic acid.

2.5.3 Preparation of Unknown Samples for Analyses

The contents of the two MJM flasks after growth of *P. aeruginosa* were centrifuged at 4°C and 10,000 x g for 20 min. The supernatants were filtered through 0.45 μm filters and were subjected to the following procedures. After each step the presence of the two unknowns was checked.

First, protein was removed from the sample by adding 1ml perchloric acid ($HCLO_4$ 70-72%) for each ml of sample. The mixture was homogenized and then centrifuged. The deproteinized supernatant was neutralized with NaOH (6M). Lipids were subsequently removed by adding chloroform-methanol solution (2:1 vol/vol) (Folch *et al.*, 1957). This proportion formed a miscible system with the water in the sample.

High molecular weight compounds were removed using amicon ultrafiltration membranes with 30,000 and 500 nominal cut-off levels respectively (10 PM30 76mm Lot 196 and 5 UM05 76mm Lot AB 01246A).

Waters Sep-Pak Cartridges (C18, Red and Silica, Black) were used to remove some interfering compounds. Finally the samples were lyophilized in the Thermovac freeze-dryer (FD-3, Island Park) and kept at -3°C for further analysis.

2.5.4 Thin Layer Chromatography (TLC)

Silica-gel thin layer chromatography (0.25 mm thickness) was used to separate and purify the two unknowns. The solvent system was ethanol : water : ammonia 25% : : 40 : 7 : 5 (vol / vol). Standard solutions of organic acids (acetic, butyric and formic acids) and lyophilized sample of MJM after 1 d growth of *P. aeruginosa* containing the two unknowns were prepared in distilled water at concentrations of 1 $mg.ml^{-1}$. For chromatography, the samples were spotted using capillary tubes, cold air fan dried, and the chromatograms developed in the above-mentioned solvent. The chromatograms were removed from the chambers and left overnight at room temperature to dry before spraying with bromocresol green to locate the spots (Krywawych, 1979). This prolonged drying period was necessary because failure to evaporate the solvent completely results in a background which contrasts poorly with the organic acid spots when using an acid-base indicator as a locating reagent.

2.5.5 Infrared (IR) Spectroscopy Analysis

IR spectroscopy was carried out with a Nicolet 8210 FTIR spectrometer (Nicolet. Instrument Corporation, Madison, WI) equipped with a deuterated triglycine sulfate (DTGS) detector. The spectrometer was interfaced to a 486/33 MHZ PC operating under Windows-based Nicolet Omnic 1.1 software. The lyophilized samples were dissolved in 16μ l distilled water and deposited on the CaF_2 window. The water was evaporated under a hot air dryer, leaving a uniform film. Sampling accessory was a demountable transmission cell with a CaF_2 window. The instrument and sample compartment were purged with dry air produced by a Balston dryer (Balston, Lexington, MA) to minimize water vapor and carbon dioxide interferences. Spectra were recorded by co-adding 256 scans at a resolution of 4 cm^{-1} and were ratioed against a 512-scan background spectrum recorded from the clean, empty cell.

2.5.6 Pyrolysis/GC/MS Analysis

A Hewlett-Packard GC/mass selective detector (5890 GC/5971B MSD) interfaced to a CDS Pyroprobe 2000 unit was used for the Py/GC/MS analysis. Two mg of solid samples were added to a quartz tube (0.3 mm thickness), which was then plugged with quartz wool, and inserted inside the coil probe with a total heating time of 20 s and pyrolyzed at 150, 200 or 250°C. The GC column flow rate was 0.8 $ml.min^{-1}$ for a split ratio of 92:1 and a septum purge of 3 $ml.min^{-1}$. The pyroprobe interface temperature was set at the temperature at which the sample was to be pyrolyzed (150, 200, or 250°C respectively; depending on the analysis), and the Pyroprobe was set at the desired temperature at a heating rate of 50°C.ms^{-1}. Capillary direct MS interface temperature was 180°C; ion source temperature was 280°C. The ionization voltage was 70 eV, and the electron multiplier voltage (EMV) was 1494 V. The mass range analyzed was 35-350 amu. The column was a fused silica DB-5 column (50 m length x 0.25 mm i.d. x 25 μm film thickness; Supelco, Inc.). The column initial temperature was -5°C for 3 min and was increased to 50°C at a rate of 30°C.min^{-1}; immediately the temperature was further increased to 250°C at a rate of 8°C.min^{-1} and kept at 250°C for 5 min.

2.5.7 GC-MS Analysis

Preliminary identification of 8 unknown compounds was performed on a Hewlett Packard HP 5890 gas chromatograph-mass selective detector equipped with a DB1 column (30 m x 0.32 mm). The oven was programmed from 80 to 280°C at an increment of 5°C.min^{-1}. The mode of operation was electron impact (EI) with injection port and detector temperature of 250°C and 200°C, respectively. Samples were silylated to form the trimethylsilyl (TMS) derivatives of unknown compounds.

2.6 Use of BHIB as a System Model for MJM

Five flasks containing 100 ml of BHIB were separately inoculated with *P. fluorescens*, *P. aeruginosa*, *P. fragi*, *E. agglomerans* and *B. thermosphacta* to see if the two unknown compounds which were produced and/or consumed in MJM at 4°C by *Pseudomonas* spp. were also produced /or consumed in BHIB. The inoculated media were incubated at 30°C under shaking conditions (100 rev.min^{-1}) for 1 d.

2.7 Replication of experiments

All growth experiments as well as analytical analyses were done in duplicate and the results presented as average values.

3. Results

3.1 Growth of *Pseudomonas aeruginosa* in MJM

The HPLC chromatogram of MJM inoculated with *P. aeruginosa* agitated at 100 rev.min^{-1} after 7 d of incubation at 4°C showed the two predominant unknown peaks previously observed by Gauthier (1990). The retention times of these two unknown peaks were *ca.* 21 and 32 min, respectively (Figure 1). The first compound was a component of MJM and *P. aeruginosa* increased its level in MJM, while the second one appeared only after the growth of the organism.

3.2 Effect of Glucose Supplementation on the Rate of MJM Spoilage

Addition of 0.5% (wt/vol) glucose to MJM led to a decrease in the production of all the HPLC detected MJM components by *P. aeruginosa* after 1 d of incubation at 30°C and 100 rev.min^{-1} (Figure 2). Similar component profiles were obtained after 7 d incubation at 4°C and 1 d incubation at 30°C (Figures 1 and 2). Other components with retention times (RT) of 8.91, 9.67, 11.96, 13.33, 17.74, 21.48, 26.07 and 32.04 min were produced during the growth of the organism in MJM. These compounds are also predominant during spoilage of meat. Since these compounds may play a role in the process of meat spoilage, efforts were made to identify them.

3.3 Carbohydrate Utilization by *P. aeruginosa* Grown in MJM

In MJM with added glucose (GMJM) *ca.* 80% of the initial glucose (6.42 mg.ml^{-1}) was consumed in the first day of incubation at 30°C. This was followed by a slight increase (day 2) and then a rapid decrease in the glucose concentration to 0.3 mg.ml^{-1}. After 5 d of incubation the level of glucose was the same in both MJM and GMJM (Figure 3).

There was 0.25 mg.ml^{-1} fructose in MJM on the day of inoculation, and this was consumed much faster in GMJM. There was a slight increase after the initial decrease in fructose levels in both MJM and GMJM (Figure 3).

Total carbohydrate increased after one day of incubation in both MJM and GMJM followed by a more rapid and extensive decrease in GMJM. In GMJM, the medium turned green with the production of slime on 10th day of incubation, while in MJM the medium remained light brown with

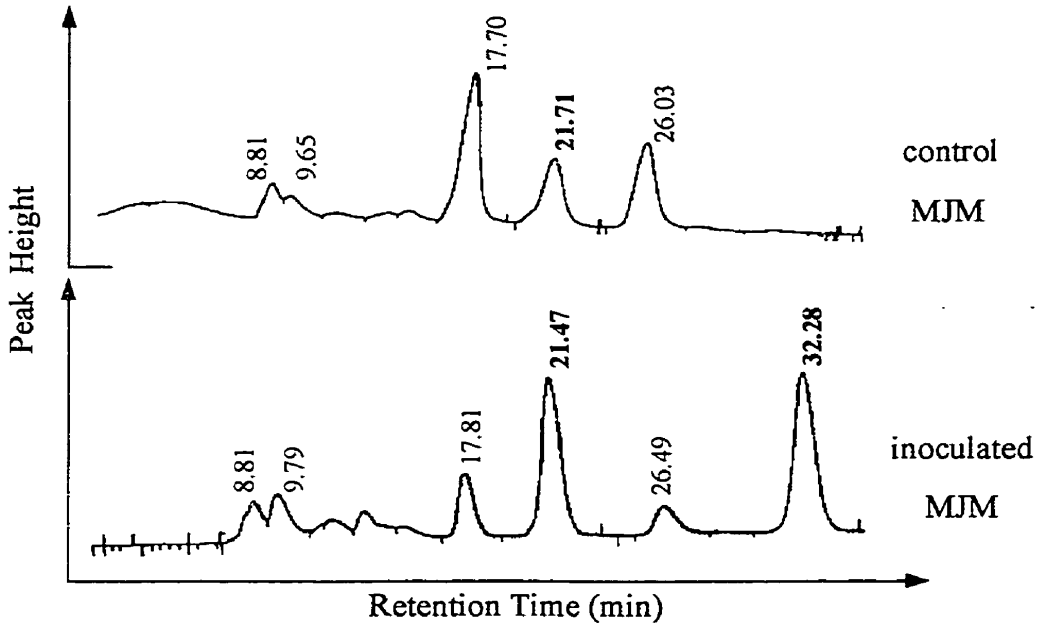


Figure 1. The HPLC chromatogram of control and inoculated MJM with *P. aeruginosa* after 7 d incubation at 4°C under shaking conditions (100 rev.min^{-1}).

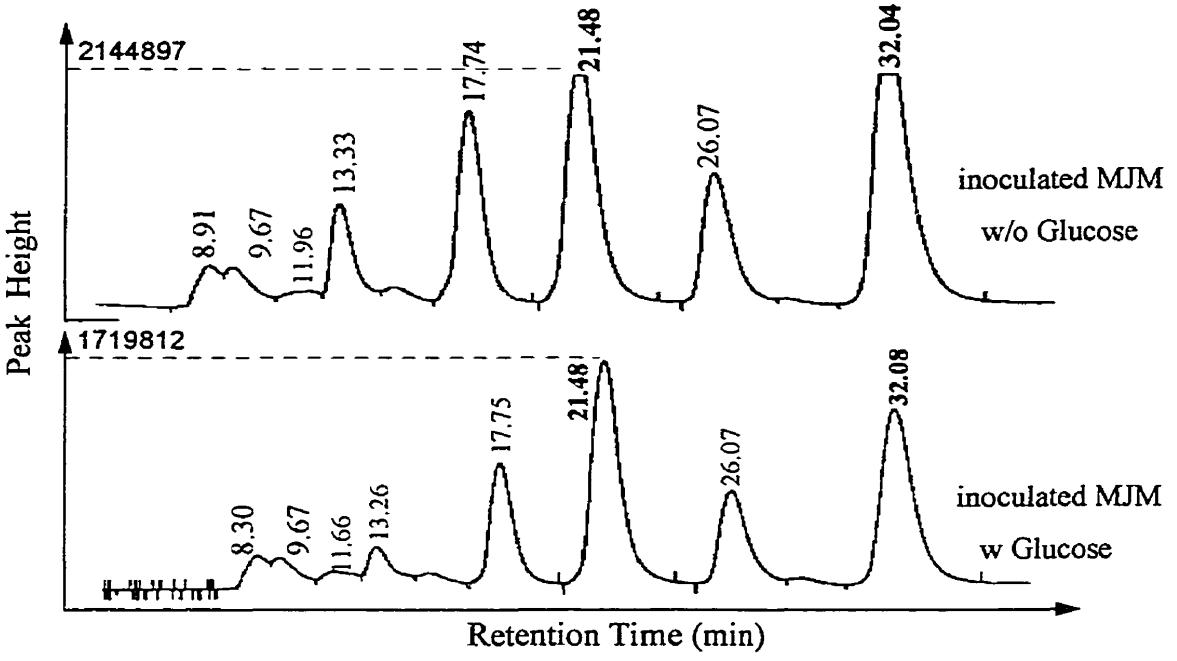
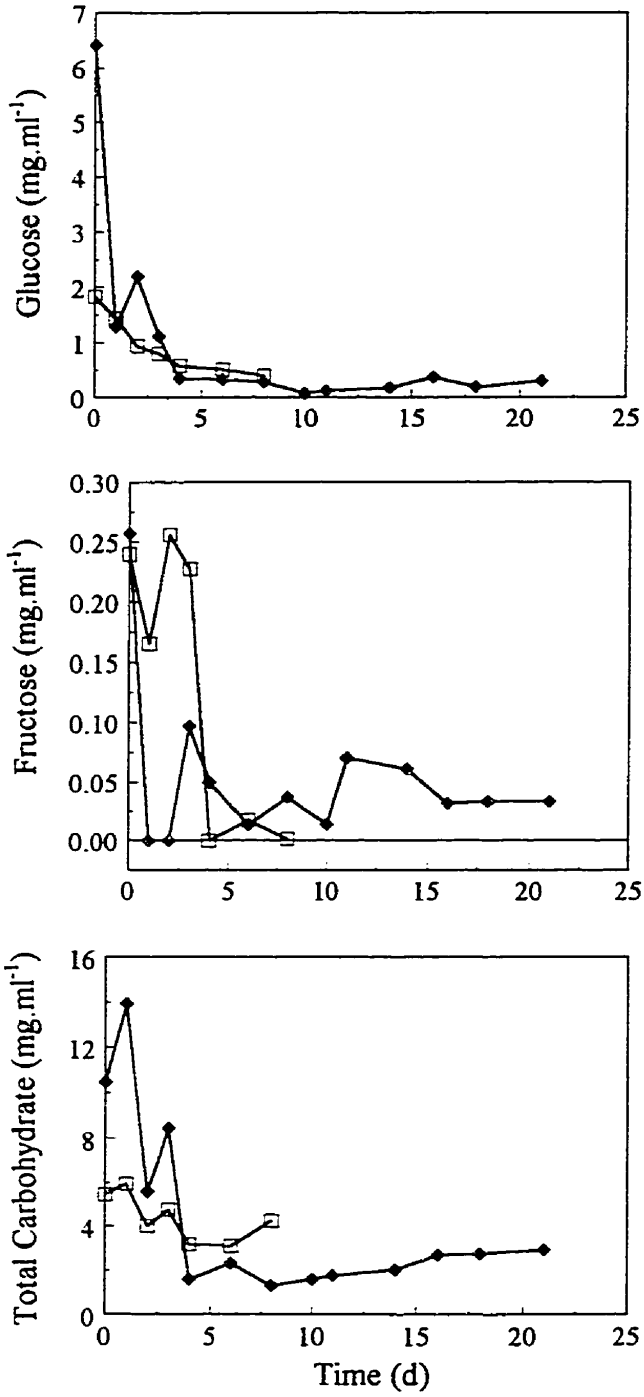


Figure 2. The HPLC chromatograms of inoculated MJM with *P. aeruginosa* with (w) and without (w/o) added glucose after one day incubation at 30°C under shaking conditions (100 rev.min^{-1}).

Figure 3. Utilization of glucose, fructose and total carbohydrate by *P. aeruginosa* in MJM incubated at 30°C under shaking conditions (100 rev.min^{-1}) with (♦) and without (□) added glucose.


Glucose (mg.ml-1)
Fructose (mg.ml-1)
Total Carbohydrate (mg.ml-1)
Time (d)

slime being formed on the 6th day of incubation. Thus, the slime production in GMJM was delayed by 4 days with the addition of glucose.

The pH of inoculated MJM increased more rapidly, but eventually a pH of 8.3 was attained in both media (Figure 4).

3.4 Concentration Changes in Unknown Compounds

The patterns of increasing / decreasing concentrations of unknowns 1, 2, 4, 5, 6, 7 and 8 (RT = 8.91, 9.67, 13.33, 17.74, 21.48, 26.07 and 32.04, respectively) in MJM and GMJM as a result of *P. aeruginosa* growth were similar (Figure 5). Differences in the levels of the unknowns in MJM and GMJM at time 0 are the consequence of using different batches of MJM. With one exception (unknown 3, RT=11.96), levels of the unknown were higher in GMJM than in MJM. Levels of unknowns 1, 2 and 7 decreased initially and then increased; whereas, concentrations of unknowns 4, 6 and 8 increased and then decreased as a result of *P. aeruginosa* growth. Unknown 5 was consumed by *P. aeruginosa* within the first 3 d with low levels recorded after that. Unknown 3 was the exception in that its concentration increased markedly in GMJM and then decreased whereas the level remained relatively unchanged in MJM. After one week just the unknowns 1, 2 and 7 remained in the growth medium in high amounts and their concentration continued to increase.

3.5 Identification of the unknown compounds

3.5.1 HPLC

The retention times of different acids and other suspected compounds were compared with the retention times of the two unknown compounds with RT of 21 and 32 min (Table 9). Acetic and butyric acid co-eluted with the first and the second unknowns, respectively at 35°C, but not at 60°C. The amino acids alanine, glycine and tyrosine as well as glucose were not detected by the system used.

The two unknowns (RT 21 and 32 min on the first HPLC system) were not detected in a second HPLC column, specific for sugars, connected to UV and Refractive Index detectors, showing that they cannot be a kind of sugar.

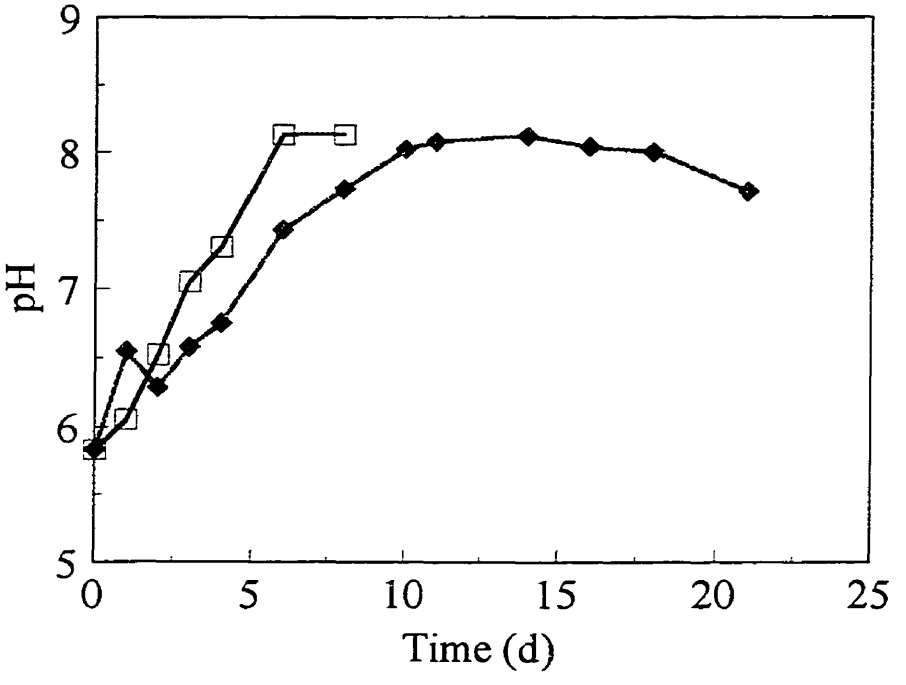


Figure 4. Effect of *P. aeruginosa* growth incubated at 30°C under shaking conditions (100 rev.min^{-1}) on pH of MJM with (♦) and without (□) added glucose.

Figure 5. Utilization or production of 8 unknown compounds in MJM inoculated with *P. aeruginosa* and incubated at 30°C under shaking conditions (100 rev.min^{-1}) with (♦) and without (□) added glucose,
RT = HPLC retention times.
..... , Peak recorded but peak height not registered.

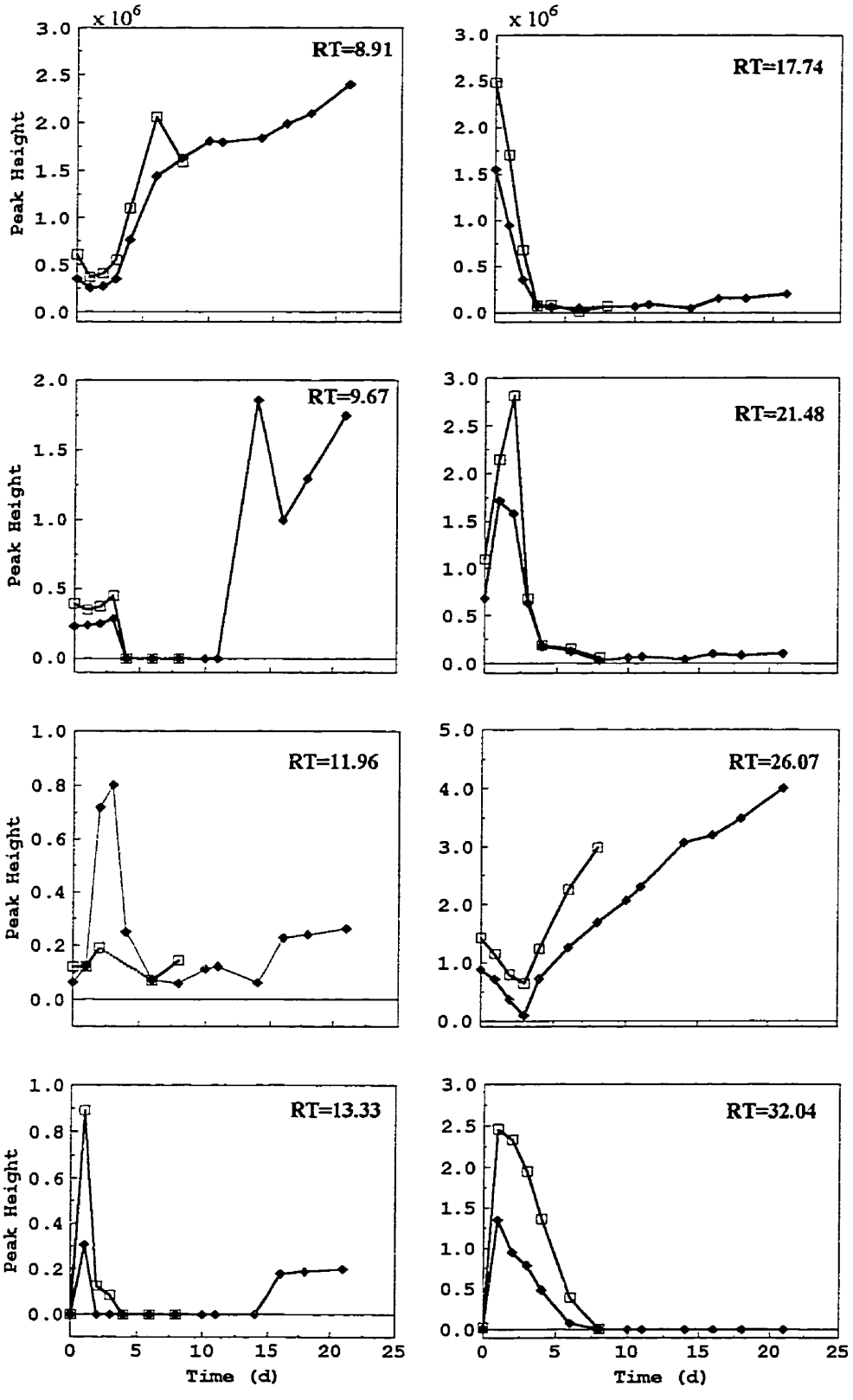

x 10^6
RT=8.91
RT=17.74
RT=9.67
RT=21.48
RT=11.96
RT=26.07
RT=13.33
RT=32.04
Peak Height
Time (d)

Table 9. Comparison of the HPLC[a] retention times of several known compounds and the two unknown compounds.

Compound	Retention time (min)
Unknown 1	21
Unknown 2	32
Acetic acid	21.77
Butyric acid	32.83
Fumaric acid	9.28
Formic acid	19.84
Citric acid	11.51
Tartaric acid	12.25
Malic acid	13.72
Succinic acid	17.04
Nicotinamide	8.62
Nicotinic acid	ND
Formamide	33.21

[a]HPLC system using Aminex HPX-87H ion exclusion column (see section 2.5.1)
ND, not detected.

3.5.2 Thin Layer Chromatography

Localization of the unknowns on the TLC chromatogram with bromocresol green was unsuccessful. An unstained developed chromatogram was sectioned into 5 fractions. The silicagel from each fraction was scraped off, suspended in distilled water, and filtered. The filtrates from each section were passed through the HPLC. The unknowns were present in fraction 4. This fraction was subsequently divided into 4 sub-fraction with each sub-fractions being prepared as above. The unknowns were located in the same sub-fraction 1 (Figure 6), indicating that the two unknowns may be very similar in composition.

3.5.3 Fourier Transform Infrared Spectroscopy

The IR spectra of the first and the second unknown and the comparison of the two spectra confirms the results of the thin layer chromatography, i.e., the two compounds are quite similar possibly an organic acid or a polyalcohol but not an aldehyde or a ketone (Figure 7).

3.5.4 Pyrolysis/GC/MS Analysis

The pyrograms after pyrolysis at 250, 200 and 150°C of MJM after the growth of *P. aeruginosa* are shown in Figure 8. Pyrolysis at 250°C showed the presence of butyric acid, 2,3-butanediol, formamide, glycerol, 5-methyl 2-ethyl pyrazine and trimethyl pyrazine (Figure 8, A). Pyrazine peaks were eliminated at a pyrolysis temperature of 200°C and acetic acid was observed after pyrolysis at 150°C (Figure 8, B and C). The second peak at 150°C could not be identified with the available library.

3.5.5 GC-MS Analysis

GC-MS chromatogram of MJM after one day of growth of *P. aeruginosa* at 30°C showed the presence of lactic acid, H_3PO_4, glycerine, pyroglutamic acid, creatinine, palmitic acid, and stearic acid (Figure 9). The last peak could not be identified with the available database. GC-MS and HPLC co-elution of above-mentioned compounds individually suggest that HPLC peak RT=17.74 min was lactic acid. Pyroglutamic acid (RT=24.54 min) eluted before the unknown with RT=26 min and did not co-elute with any of the unknown compounds. Phosphoric acid, glycerine, creatinine, palmitic and

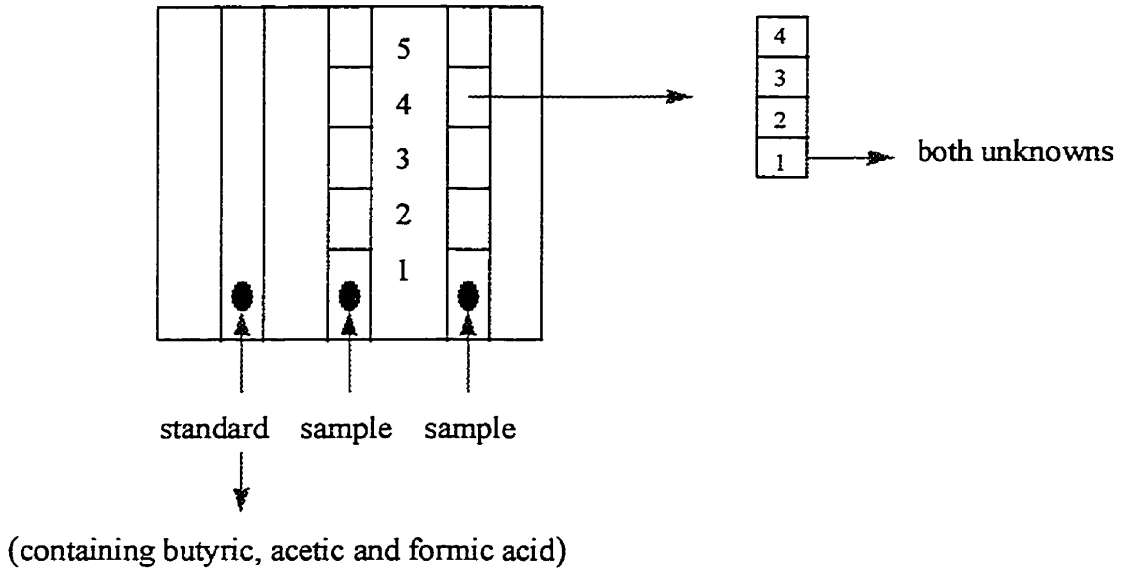


Figure 6. Schematic diagram of a TLC separation of the two unknowns (RT 21 and 32 min).

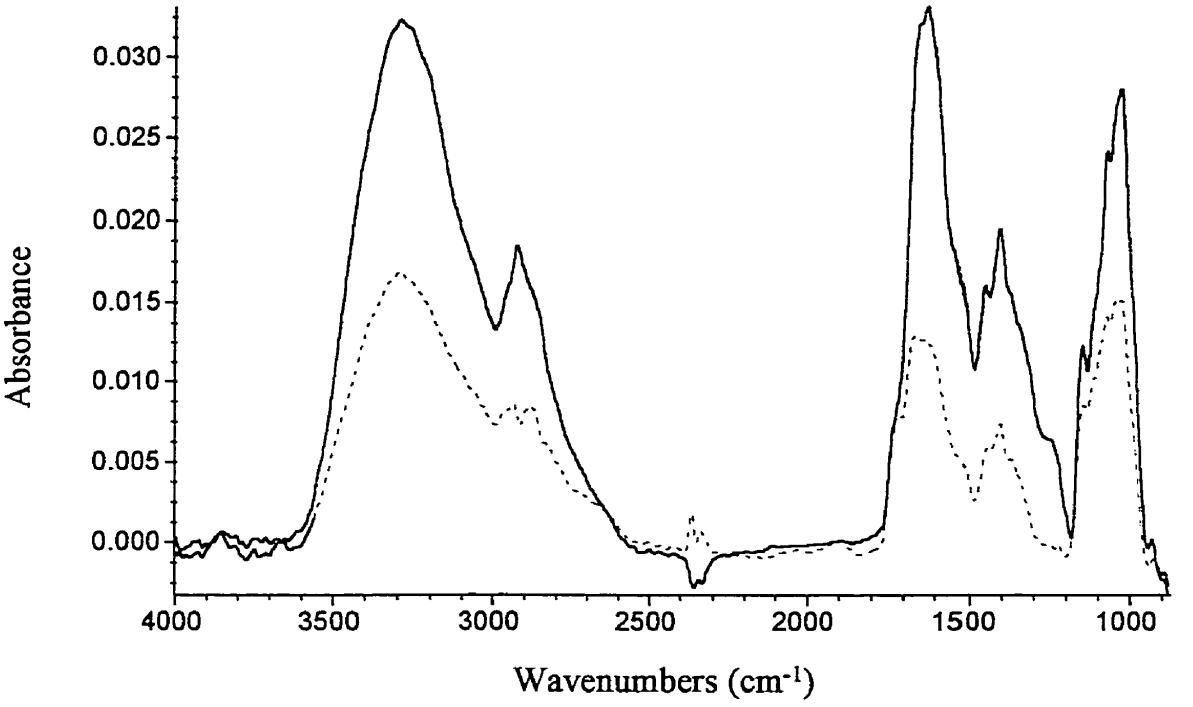
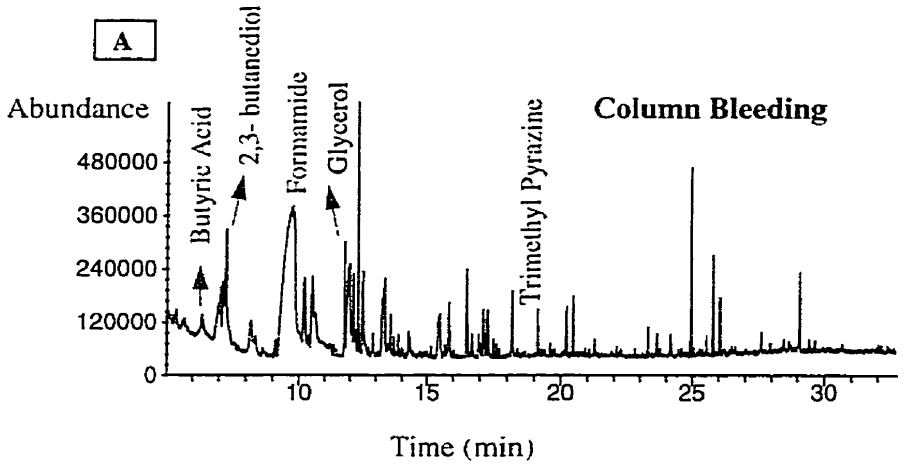
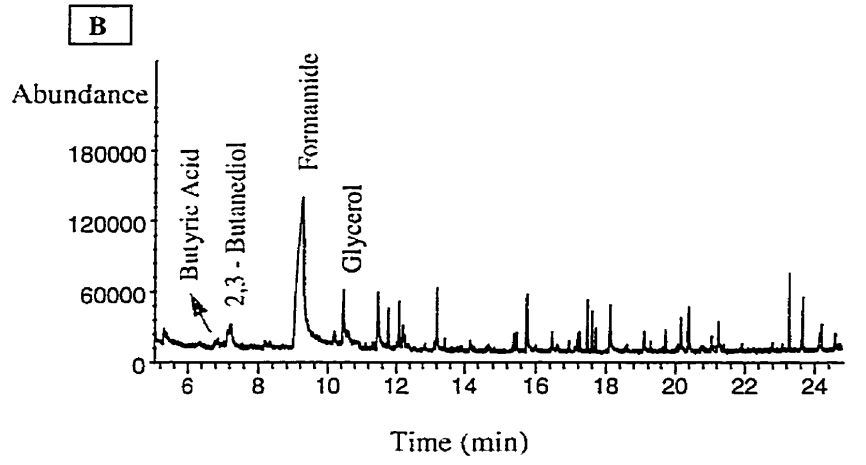


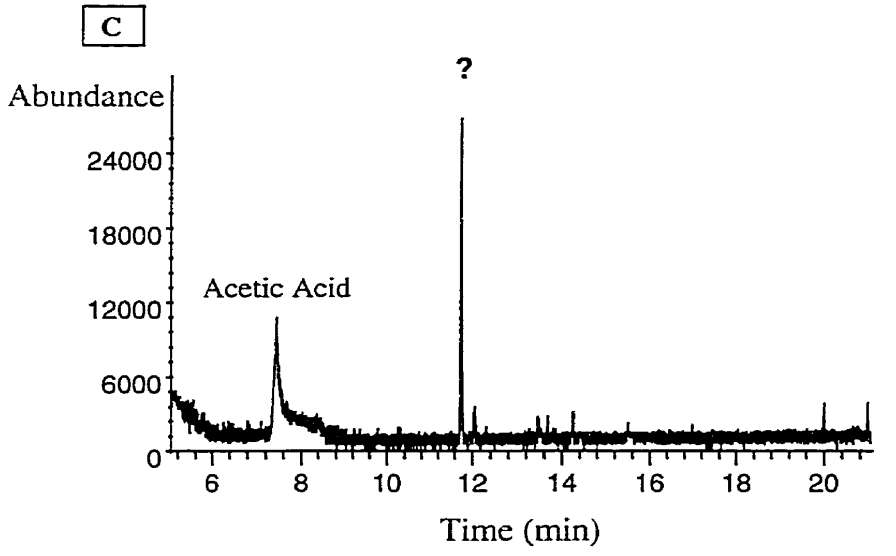
Figure 7. The FTIR spectra of the lyophilized samples of each unknown compound in distilled water after HPLC collection (RT 21 and 32 min).

(——), unknown 1; (·····), unknown 2

Figure 8. The pyrograms of the lyophilized sample of MJM after 1 d growth of *P. aeruginosa* at 30°C under shaking conditions (100 rev.min^{-1}) containing the two unknown compounds (RT 21 and 32 min) at A) 250°C, B) 200°C and C) 150°C.


A
Abundance
480000
360000
240000
120000
0
Butyric Acid
2,3- butanediol
Formamide
Glycerol
Trimethyl Pyrazine
Column Bleeding
10
15
20
25
30
Time (min)


B
Abundance
180000
120000
60000
0
Butyric Acid
2,3 - Butanediol
Formamide
Glycerol
6
8
10
12
14
16
18
20
22
24
Time (min)


C
Abundance
24000
18000
12000
6000
0
?
Acetic Acid
6
8
10
12
14
16
18
20
Time (min)

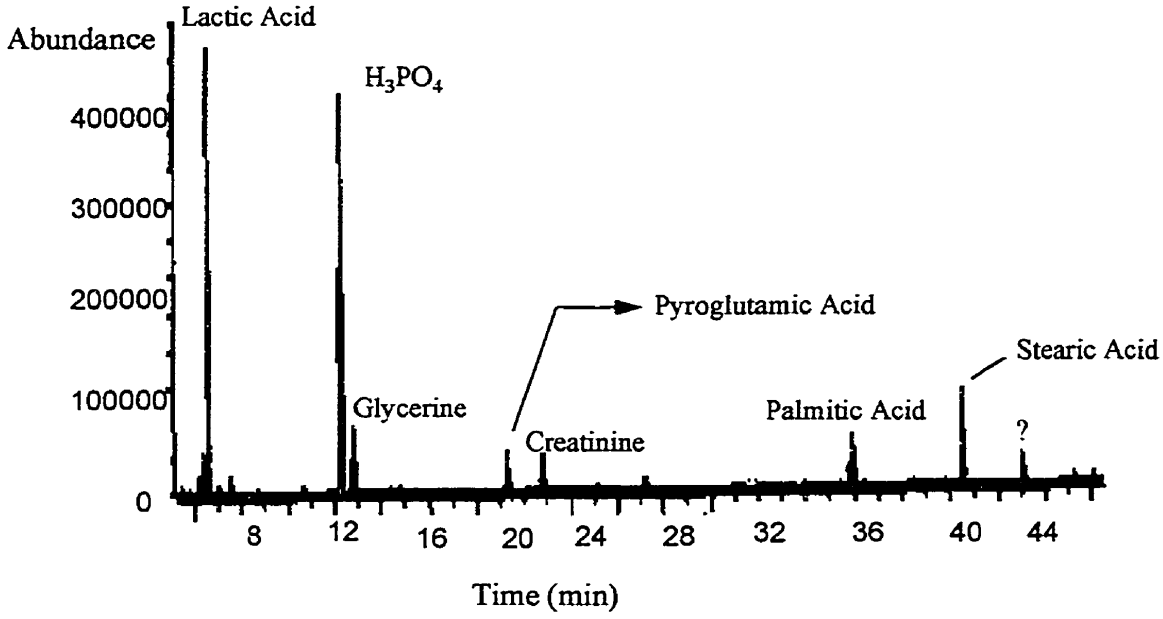


Figure 9. The GC/MS chromatogram of MJM after one day incubation with *P. aeruginosa* at 30°C under shaking conditions (100 rev.min^{-1}).

stearic acids were not detected by HPLC. Cadaverine and putrescine (the possible spoilage indicators of meats; Jay, 1992) were not among the compounds detected by GC-MS; cadaverine co-eluted with the unknown HPLC peaks 8.91 and 9.67 whereas putrescine eluted at 8.36 min before cadaverine and did not co-elute with any of the unknown compounds.

3.5.6 Tentative Identification of Unknowns

Combining the results from the different detection / identification methods used and finally comparing the relative retention times of the unknowns to those of known compounds, the compounds represented by the eight HPLC peaks were tentatively identified as cadaverine (as the first two peaks), 2-keto gluconic acid, fructose, lactic acid, acetic acid, methanol, and n-butyric acid, respectively (RT 8.91, 9.67, 11.96, 13.33, 17.74, 21.48, 26.07 and 32.04). Table 10 shows the relative retention times of the unknown compounds compared to the standard relative retention times of the known library compounds. HPLC supported the identity of the above-mentioned compounds except for fructose and methanol which were not detected. Fructose was , however, detected at 13.32 min RT in a solution containing a much higher concentration of fructose (0.25 $mg.ml^{-1}$) than that found in MJM and GMJM.

3.6 Use of BHIB as a System Model for MJM

The HPLC compound profiles of BHIB and MJM after growth of *P. aeruginosa* were somewhat different but in both media the unknowns 6 and 8 with RT of *ca.* 21 and 32 min were detected (Figure 10). In addition, *P. fluorescens*, *P. fragi*, *E. agglomerans* and *B. thermosphacta* also produced the unknown 6 and *P. fluorescens* and *E. agglomerans* produced unknown 8 in BHIB (results not shown). Since these two unknowns were produced in both media, BHIB could be used at least for the production and identification of the two unknown compounds.

Table 10. Relative retention times of the unknown compounds compared to the standard relative retention times of the known library compounds.

Compound	RT on our system[1]	T_R	ST_R
cadaverine	8.91	0.50	-
	9.67	0.54	-
2-keto gluconic acid	11.96	0.67	0.66
fructose	13.33	0.75	0.75
lactic acid	17.74	1.00	1.00
acetic acid	21.48	1.20	1.20
methanol	26.07	1.47	1.46
n-butyric acid	32.04	1.81	1.81

[1]HPLC system using Aminex HPX-87H ion exclusion column (see section 2.5.1)

T_R = RT (compound) / RT (lactic acid)

ST_R = Standard relative retention time using standard conditions in Biorad Bulletin (see section 2.5.2)

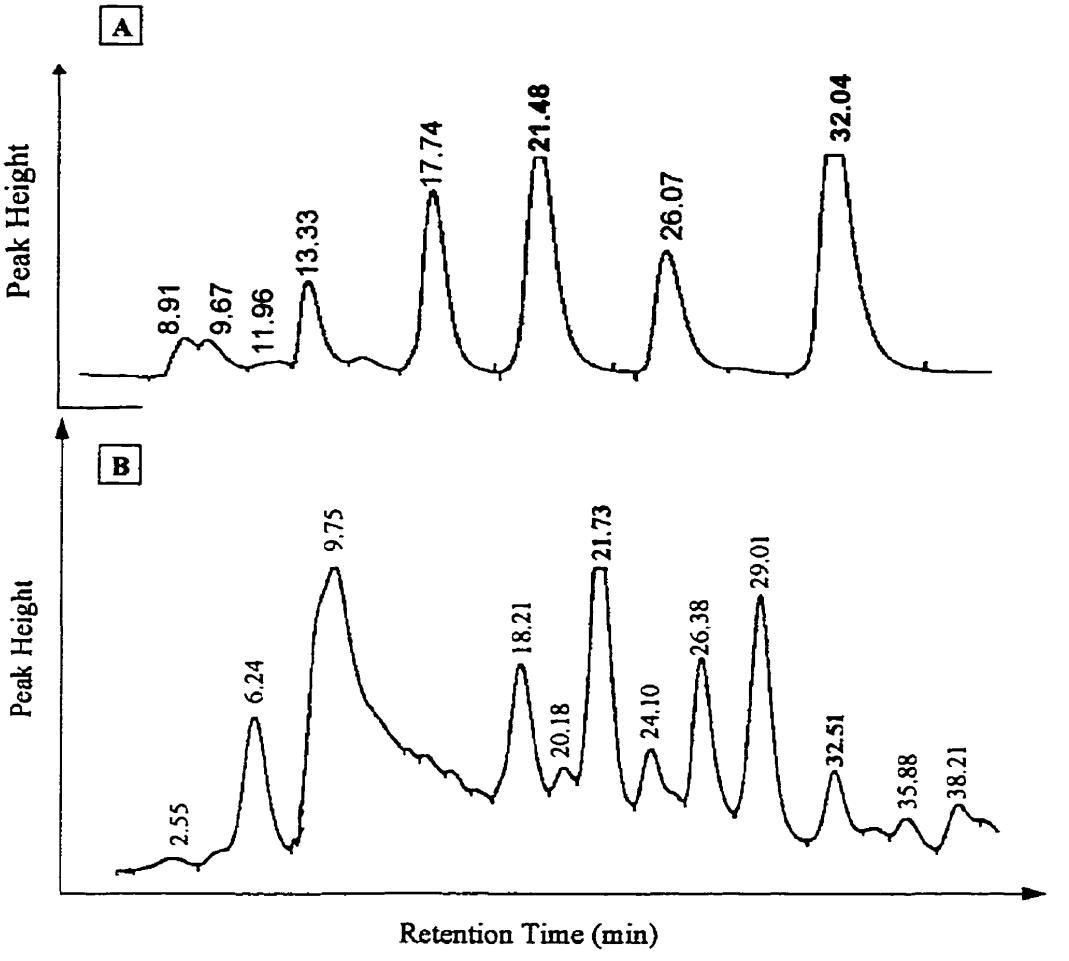


Figure 10. The HPLC chromatograms of inoculated A) MJM and B) BHIB with *P. aeruginosa* after 1 d incubation at 30°C under shaking conditions (100 rev.min^{-1}).

4. Discussion

To study the process of meat spoilage, MJM inoculated with *P. aeruginosa* was initially incubated at 4°C. To obtain results, the incubation period was of necessity, quite long. When it was observed that inoculated MJM incubated at 30°C contained the same compounds as those in MJM incubated at 4°C (Figures 1 and 2); therefore, 30°C storage became the norm. Other workers studying the utilization of low molecular weight soluble components of meat by various organisms have also used this temperature (Gill, 1976).

The glucose concentration in uninoculated MJM (1.62 $g.l^{-1}$) was greater than the levels measured in uninoculated MJM by Gauthier (1990, 0.67 $g.l^{-1}$) and in beef muscle by Nychas (1984, 0.077 $g.kg^{-1}$), Farber and Idziak (1982, 0.062 $g.kg^{-1}$) and Gill (1976, 0.4 $g.kg^{-1}$). One explanation can be the difference in the composition of meat used for MJM preparation.

P. aeruginosa used glucose at an early stage (Figure 3), as reported by Drosinos and Board (1995), Gauthier (1990), Gill and Newton (1977). Gauthier (1990) reported 0.11 $g.l^{-1}$ glucose (an 83.6% reduction) in MJM inoculated with a non-fluorescent *Pseudomonas* on day 4. The pseudomonads reduced glucose levels to 0.1 $g.l^{-1}$ before attacking secondary substrates (Gauthier, 1990 and Gill, 1982). This is in line with the fact that a minimum glucose concentration of 0.1 $g.kg^{-1}$ is required to maintain catabolic inhibition of amino acid metabolism.

The effect of glucose on the microbial development in meat is well documented. Shelef as early as 1977, stated that addition of *ca.* 2% glucose in meat delayed bacterial growth due to a decrease in pH. Working with DFD meat, stated that glucose in aerobically stored meat supports a high population of pseudomonads without meat spoilage (Newton and Gill, 1981; Newton and Gill, 1980; Newton and Gill, 1978).

Drosinos and Board (1994) found that addition of freshly chopped onion to minced lamb resulted in an increase in the carbohydrates concentration and a one log $CFU.g^{-1}$ higher pseudomonads population than that in the control. Lambropoulou *et al.* (1996) supplemented minced beef with 0.2% (wt/wt) glucose did not observe an increase in the pseudomonads population during storage. They suggested that difference in the amount of supplemented carbohydrate and not meat types were responsible for these differing results.

To study the effect of glucose supplementation we added 0.5% (wt/vol) glucose to the MJM.

After 1 d of incubation at 30°C the glucose concentrations were similar in MJM and GMJM. As a consequence one should expect either a substantial increase in the microbial population or an increase in some metabolites or both. In fact, we did observe an increase in the concentration of 2-keto gluconate (unknown 3) in GMJM. Indeed, gluconate production has been associated in meat with pseudomonads. This compound provides *Pseudomonas* spp. with a competitive advantage because gluconate is not utilized by the other typical meat spoilage bacteria (Nychas and Arkoudelos, 1990; Nychas *et al.*, 1988; Farber and Idziak, 1982). This study confirms that the addition of glucose resulted in a higher production of 2-keto gluconate by *P. aeruginosa* growing under aerobic conditions.

Carbohydrate utilization in meats has been centred on glucose metabolism. No data is available on the fate of fructose which was present at a concentration of 0.25 $g.l^{-1}$. Fructose was consumed in both MJM and GMJM but at a faster rate in GMJM. Why this should be so is not understood, but one reason may be the increased numbers of *P. aeruginosa* in the GMJM.

As spoilage of meats progresses, slime may develop on the meat surface. Ayres (1960) presented evidence that odors can be detected when the surface bacterial count is between log 7.0 and 7.5 $CFU.cm^{-2}$, followed by detectable slime with surface counts usually about log 7.5 to 8.0 $CFU.cm^{-2}$. So, the increase in total carbohydrate on the last days of storage of MJM might have been due to a high microbial population and/or slime formation. At the same time, the concentration of total carbohydrate was lower in GMJM (1.3 $mg.ml^{-1}$ compared to 4.25 $mg.ml^{-1}$ in MJM, Figure 3) possibly because of delay in slime formation.

Another parameter indicative of meat spoilage is a rise in pH. As observed by several authors (Gauthier, 1990; Farber, 1982; Gill and Newton, 1977; Gill, 1976), when grown in pure culture, *Pseudomonas* spp. caused the pH of MJM to rise. Ammonia, which is the major cause of the increase in pH at advanced stages of spoilage (Gill, 1976), is produced by many microbes, including pseudomonads, during amino acid metabolism. Farber (1982) reported that *P. fluorescens* and the non-fluorescent *Pseudomonas* produced ammonia when using urea as a source of nitrogen in minimal medium at 4°C. As shown by Gauthier (1990), the production of ammonia cannot be the only cause of increasing the pH because the recorded increases in pH were greater than could be expected from the quantities of ammonia produced. It is likely that the pseudomonads release other basic products,

e.g. amines (Dainty, 1982; Slemr, 1981) or increase the total amino acids (Drosinos and Board, 1995) that also contribute to the rise in pH. Lactate decreased in MJM during storage and this would also contribute to a rise in pH as suggested by Lambropoulou *et al.* (1996).

In ground beef, pH may rise as high as 8.5 in putrid meats, although at the time of incipient spoilage, mean pH values of about 6.5 have been found (Shelef and Jay, 1970). In our experiment the pH of MJM rose to 8.13. Causative factors may include the production of the diamine cadaverine, formation and accumulation of amino sugars (Shelef and Jay, 1969). The delay of one week for the pH increase to 8.13, in the case of GMJM, can be due to an increase in the lactic acid and 2-keto gluconic-acid from glucose and/or a delay in production of cadaverine and amino group containing compounds.

Although compounds responsible for the production of objectionable odors in the later stages of spoilage originate from the metabolism of amino acids by *Pseudomonas* it should be stressed that the onset of off odors can occur during glucose metabolism by *B. thermosphacta* (Dainty and Hibbard, 1983). In contrast to former beliefs (Gill, 1982; Gill, 1976), it is not inconceivable that shelf life failure could occur prior to amino acid degradation (Dainty and Hibbard, 1983; Dainty *et al.*, 1985).

In an effort to identify the two unknown compounds with RT of *ca.* 21 and 32 min, the TLC method used did not result in a separation of these compounds, suggesting that they are similar in structure.

The results of the FTIR also indicated that these two compounds have similar or the same functional groups (Figure 7). The positions of the major absorption bands for each of the two unknowns were compared with the infrared absorption spectra of reference functional groups (Robyt and White, 1987). Since there is so much overlap in the absorption range of some of the functional groups, it was not possible to identify the unknowns by using this method, which is not surprising as infrared spectroscopy is used more for the confirmation of a compound's identity. Nevertheless, we were able to conclude that these two unknowns are very similar in nature.

Pyrolysis/GC/MS was also used in an attempt to identify the two unknowns. The analytical pyrolysis approach is concerned with the characterization of the original molecule by the analysis of its pyrolysis products (pyrolyzates). The most popular detector has been the mass spectrometer, and

the major drawback is that fragments which are not volatilized will not be analyzed. In addition, depending on the conditions, this process may result in an increase in molecular weight through intermolecular events; generally this process leads to molecules of lower mass due to thermal fission (Keyhani, 1997).

In the analysis of MJM containing the two unknown compounds, pyrolysis at 250°C showed the presence of pyrazine products (Figure 8A). It has been reported that pyrazines can be produced biologically by species of *Bacillus*, *Corynebacterium* and *Pseudomonas* (Cook, 1994). Since pyrazines were not detected when lower temperatures of 200 and 150°C were used for pyrolysis (Figures 8B and 8C), it can be concluded that the pyrazine products detected after pyrolysis at 250°C were probably formed as a result of the Maillard reaction (interaction between amino acids and sugars) at high temperature (250°C) or column bleeding. Pyrolysis at 150°C, in fact, was desorption and the sample did not burn completely, thus acetic acid could be and was detected at this temperature (Figure 8C).

GC-MS analysis of MJM after one day's growth of *P. aeruginosa* at 30°C showed the presence of lactic acid, H_3PO_4, glycerine, pyroglutamic acid, creatinine, palmitic and stearic acids. The presence of glycerine, lactic acid and creatinine is in agreement with the observations of previous authors (Drosinos and Board, 1995; Gauthier, 1990). Gauthier showed that glycerol is present in substantial amounts in MJM (ca. 0.33 $g.l^{-1}$) and is likely to be used by the major meat spoilage organisms growing on meat.

The presence of H_3PO_4 is due to the use of phosphate buffer for the preparation of MJM. Presence of pyroglutamic acid (a cyclized internal amide of L-glutamic acid; Budavari, 1989) in MJM initially and/or after bacterial growth has not been reported. Pyroglutamic acid was detected by HPLC close to the unknown with RT=26 min, but did not co-elute with any of the unknown compounds. Since this compound with RT of 24.54 min was not detected in MJM by HPLC, it may be concluded that this compound was formed during the GC analysis. It is possible that pyroglutamic acid was formed from glutamic acid as a result of the high temperature of the GC.

Palmitic and stearic acids are initially present in MJM. Palmitic acid occurs as the glyceryl ester in many oils and fats. Stearic acid is also present as a glyceride in tallow and other animal fats and oils (Budavari, 1989). The presence of these acids as substrates for bacterial growth has not been

reported. Gauthier (1990) mentioned that it was not known if organic acids (other than lactate and amino acids were present in MJM at some time during the incubation period in sufficient levels to support bacterial growth. These acids could not be detected by HPLC even at high concentrations probably because of their insolubility in water. So, they cannot be any of the unknown compounds detected by HPLC.

Among the several methods used in this study, HPLC was the most often used one for detection and confirmation of the unknowns identities. Many of the reference compounds were run at Bio-Rad Laboratories to determine their retention times under standardized conditions using the Aminex HPX-87H column (Biorad Bulletin). Since 1978, Aminex HPX-87H columns have been recognized as the column of choice for small molecule analysis. A polystyrene based support matrix, the aminex HPX-87H resin possesses a sulfonic acid functional group in the hydrogen ionic form. Separation is achieved by the mechanism of ion moderated partitioning (IMP), an HPLC technique that employs multiple mechanisms, including ion exclusion, ion exchange, normal and reversed phase, size exclusion, and ligand exchange . This column can be used for separation of organic acids, carbohydrates, aldehydes, ketones, alcohols, cell metabolites and fermentation products (Biorad Bulletin). The column used in this study was specific for organic acids.

Cadaverine, 2-keto gluconate, fructose, lactic acid, acetic acid, methanol and butyric acid were tentatively identified, in this study, as some of the metabolites of *P. aeruginosa* growing in MJM. However, in the earlier studies (Dainty and Mackey, 1992; Dainty *et al.*, 1985), in which GC and GC/MS were used, complex mixtures of esters, sulfur containing compounds, branched chain alcohols, ketones such as acetoin and diacetyl, 3-methylbutanal and unsaturated hydrocarbons have been shown to constitute the headspace volatile compounds associated with naturally contaminated samples of meat stored chilled in air.

Off-odors such as sweet and fruity, putrid, sulphury and cheesy, characterize aerobically stored meat (Dainty and Mackey, 1992). *Pseudomonas* spp., specifically *P. fragi* produce ethyl esters coinciding with the early stages of spoilage. Sulphur-containing compounds contribute to the putrid and sulphur odors. The responsible compounds include dimethyl sulfide produced by *Pseudomonas* spp.

Among the metabolic by-products of meat spoilage, the diamines cadaverine and putrescine

have been studied as spoilage indicators of meats. The production of these diamines occurs in the following manner (Jay, 1996):

$$\text{Lysine} \xrightarrow{\text{decarboxylase}} H_2N(CH_2)_5NH_2 \text{ Cadaverine}$$

$$\text{Ornithine or Arginine} \xrightarrow{\text{decarboxylase}} H_2N(CH_2)_4NH_2 \text{ Putrescine}$$

Putrescine was not initially present in MJM while cadaverine was present in small amounts. During aerobic spoilage of MJM, cadaverine increased whereas putrescine was not produced at all; a result different from the findings of Slemr (1981) who reported that putrescine is the major diamine produced by pseudomonads; cadaverine, by *Enterobacteriaceae*. Our observations also differ from those of Edwards *et al.* (1985) who found that cadaverine increased less than putrescine in aerobically stored meats and Edwards *et al.* in 1983 found that significant changes in putrescine and cadaverine levels do not occur in beef until the plate count (APC) exceeds *ca.* 4×10^7 CFU.g^{-1}. This raises the question about their utility to predict meat spoilage. This is a common problem in using single metabolites to predict spoilage since their production and concentration tend to be related to specific organisms and/or numbers of organisms present. Nevertheless, Edwards et al. (1985) have suggested the use of cadaverine and putrescine as quality indicators for vacuum packaged meat stored at 1°C for up to 8 weeks. Here, cadaverine levels increased faster than putrescine.

With HPLC cadaverine produced two peaks suggesting the presence of two forms of cadaverine. It is possible that depending on the conditions, e.g. pH, one form can change to another form. Cadaverine and the unknown 7 (methanol?) were the predominant compounds in MJM after one week of incubation at 30°C.

The presence of fructose (at the level recorded in MJM and GMJM, 0.25 mg.ml^{-1}) and methanol were not confirmed by HPLC however their relative retention times were almost the same as unknowns 4 and 7, respectively . These compounds need a strong cation exchange stationary phase in order to be detected by HPLC (Biorad Bulletin) which can be the reason why fructose was detected when much higher concentrations were analyzed. It might be concluded that these two unknowns have some similarities with fructose and methanol.

When grown as pure cultures, *P. aeruginosa* produced gluconate while utilizing glucose, as noted by Gauthier (1990), Nychas (1984), Lee *et al.*, (1984) ; Farber and Idziak (1982). In this study, *P. aeruginosa* metabolized gluconate when grown in pure culture, which confirms the observations of Gauthier (1990) and Farber and Idziak (1982). Gluconate formation was less in samples without added glucose than that in MJM supplemented with glucose in agreement with the results of Lambropolou *et al.* (1996) from minced meat and also Lee *et al.* (1984) who found gluconate production by *Pseudomonas* spp. is substrate dependent, increasing with increasing amounts of available glucose. In agreement with the findings of Farber and Idziak (1982), the *Pseudomonas* used gluconate as a second substrate (after glucose).

Farber (1983) showed that lactate was utilized by *P. fluorescens* and non-fluorescent *Pseudomonas* in minimal medium at 4°C. However, Gauthier (1990) reported that lactate was metabolized only by *P. fluorescens* in MJM. In our study, *P. aeruginosa* was found to also metabolize lactate in MJM (Figure 5). The significant decrease in the concentration of lactate in minced meat stored at 4°C in air, as reported by Lambropoulou *et al.* (1996) was probably due to its metabolism by pseudomonads.

The changes in the concentrations of unknowns 5 (lactic acid) and 6 (acetic acid) (Figure 5) follows that observed with *P. fragi* growing in MJM at 4°C in air (Drosinos and Board, 1995). Acetic acid is probably formed from the oxidation of lactic acid. The possibility exists that a ratio of lactic:acetic acid could be used as a measure of the microbial quality of meat stored under aerobic conditions.

Our results on the utilization of unknown 6 (RT 21 min) and the production and subsequent consumption of unknown 8 (RT 32 min) are in agreement with those of Gauthier (1990). She found that of the four organisms grown in pure cultures (*P. fluorescens*, non-fluorescent *Pseudomonas*, *E. agglomerans* and *B. thermosphacta*) for 7 d at 4°C, only the non-fluorescent *Pseudomonas* consumed the unknown 6 and *P. fluorescens* synthesized and then consumed unknown 8 (these two unknowns in Gauthier's study are unknown no. 1 and 2). Acetic acid and butyric acid co-eluted with the unknowns 6 and 8, respectively, on HPLC analyses at 35°C but slightly preceeded the unknowns when the temperature was raised to 60°C. It is possible that the unknowns may be slightly modified forms of these acids, co-eluting with the acids at 35°C but not at 60°C.

Since making MJM is very time-consuming and there is a high risk of contamination while preparing the medium, it seems that the use of a possible substitute readily available medium to get the same or similar results would save both time and be more convenient. *P. aeruginosa* growing under aerobic conditions in BHIB produced the unknowns 6 and 8 (RT, 21 and 32 min), but the profile for the other metabolites were different (Figure 10). This variation can be due to the difference in the composition of these two media. BHIB is made from calf brain, beef heart, proteose peptone, dextrose, sodium-chloride and disodium phosphate (Difco manual, 1984). Thus, although BHIB may not be suitable for studying all the facets of meat spoilage, it can be used as a substitute in the production of unknowns 6 and 8 for further identification.

Conclusion

The results of this study have confirmed the fact that glucose is utilized preferentially by the major meat spoilage organisms and that glucose supplementation delays meat spoilage. The production of gluconate by pseudomonads and its utilization as a second substrate was also confirmed. In contrast to the results of previous researchers, cadaverine was produced in large amounts, while no putrescine was produced by *P. aeruginosa*. It is hypothesized that the increase in cadaverine level contributes to the increase in pH of stored meats. Also, in some cases, BHIB medium can be used as an alternative medium for MJM. Additional work is necessary to confirm the identity of the unknown compounds and to study the microbial interactions during the spoilage of meat. Although the data presented here add to our understanding of the process of meat spoilage, the elucidation of the complete process remains an enigma.

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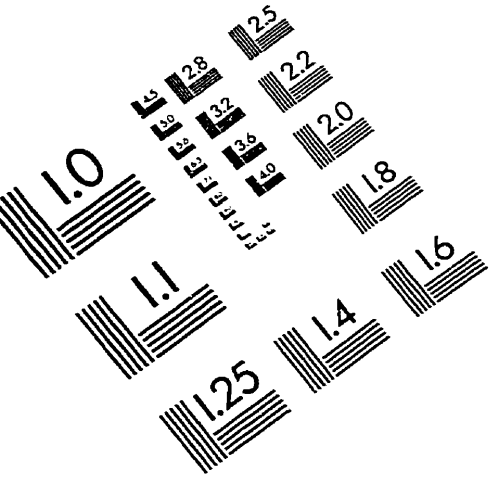

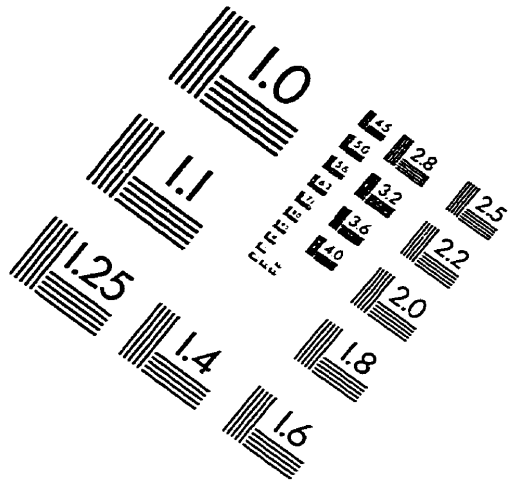

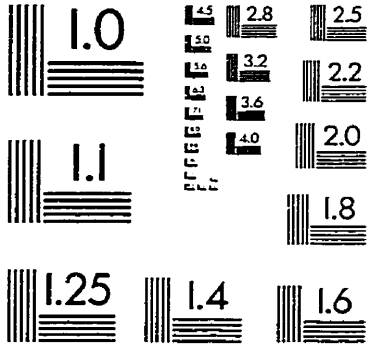

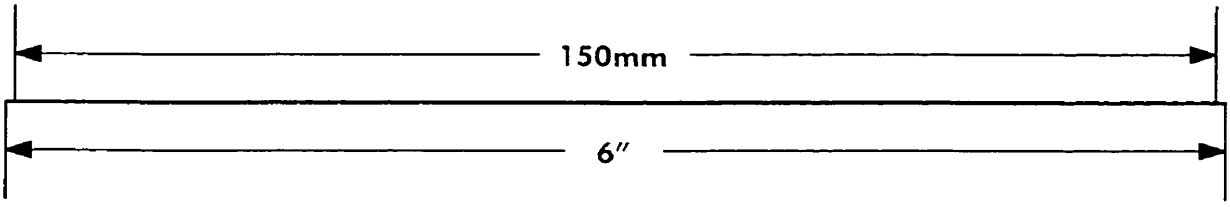

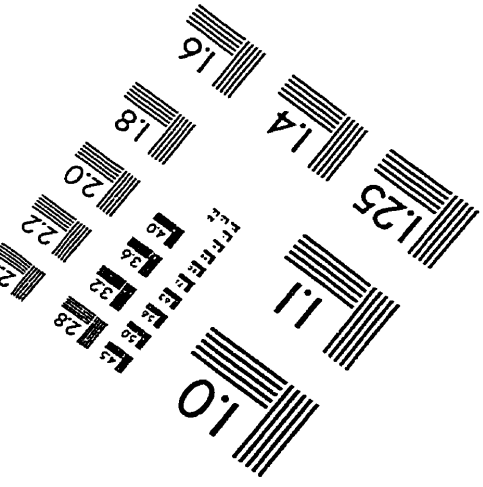

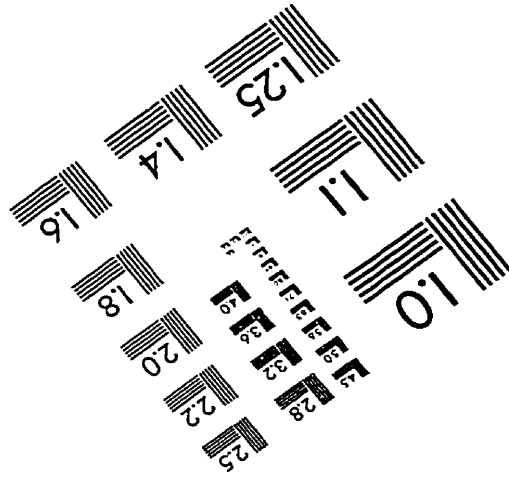

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