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Attributes of microbial associations of meat growing as xenic batch cultures in a meat juice at 4°C

E.H. Drosinos *, R.G. Board

University of Bath, School of Biology and Biochemistry, Claverton Down, Bath BA2 7AY, UK

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Abstract

Strains of Gram-positive (*Carnobacterium piscicola*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Brochothrix thermosphacta*) and Gram-negative (*Pseudomonas fragi* and *Hafnia alvei*) bacteria isolated from minced lamb packaged under a modified atmosphere were cultivated in a meat (lamb) juice at 4°C. Carbohydrates were catabolised in the order glucose > glucose 6-phosphate during the development of the population. Under an atmosphere enriched with carbon dioxide the Gram-negative portion of the population was suppressed during the exponential phase but *H. alvei* became the dominant organism towards the end of a protracted stationary phase of growth. With the aerobic atmosphere *P. fragi* catabolised creatine and became the dominant species in the stationary phase. The inability of *C. piscicola* to catabolise glucose 6-phosphate was reflected in its population being smaller than those of the other Gram-positive organisms (*C. piscicola* < *L. mesenteroides* subsp. *mesenteroides* < *B. thermosphacta*). During the stationary phase of growth, indigenous L-lactic acid and the D-isomer produced by leuconostocs were oxidised to acetic acid by the Gram-positive flora under an atmosphere enriched with carbon dioxide. These oxidations, which occurred after depletion of glucose, were supported by the oxygen in the system. D-Lactic and acetic acid appeared to be possible parameters for the estimation of the microbiological quality of packaged meat.

Keywords: Meat microbiology; Glucose; Glucose 6-phosphate; L,D-Lactic acid; Acetic acid

1. Introduction

Although the essentials of modified atmosphere packaging of red meat are known (reviewed by Gill and Molin, 1991), there are certain gaps in our knowl-

* Corresponding author. Present address: 23 Capaneus Street, Colonus, 104 44 Athens, Greece.

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edge. Not all commodities, for example minced lamb, have been studied in equal detail. Moreover certain determinants (Mossel and Ingram, 1955) – the partial pressure of carbon dioxide and oxygen, and temperature – change the patterns of development of a food ecosystem and hence the physico-chemical changes that accompany spoilage. These need to be identified and correlated with the development of microbial association, and hence with the freshness of products. Therefore, the metabolic attributes of microbial associations are important to understand these changes. Specific attributes could also reveal substrates and end products that may be useful for assessing meat quality and to design suitable biosensors for this purpose. For example, low concentration of glucose is associated with meat of interior quality (Gill, 1976; Nychas et al., 1988) and a biosensor to detect the concentration of glucose in meat was investigated by Kress-Rogers et al. (1988).

An approach to study the metabolic attributes of spoilage micro-organisms is to observe the pattern of chemical and microbiological changes in a deliberately contaminated food ecosystem (Borch and Agerhem, 1992) or in a model one (Jeppesen and Huss, 1993). In general the latter approach has been used mainly with axenic cultures in laboratory media (e.g. Borch and Molin, 1989). Although such an approach is valid in the sense that it may well identify major changes brought about by certain species, it may not identify emergent effects due to the interaction of members of a complex microbial association. In this study members of the microbial association isolated during a survey of minced lamb packaged under a modified atmosphere from local supermarkets were used as xenic cultures, in the juice obtained from minced lamb and incubated at 4°C.

2. Materials and methods

2.1. Bacterial strains and inocula

Strains were isolated from minced lamb packaged under a modified atmosphere. *Pseudomonas fragi* was identified according to Molin and Ternström (1986), *Carnobacterium piscicola*, *Brochothrix thermosphacta* and *Leuconostoc mesenteroides* subsp. *mesenteroides* according to McMullen and Stiles (1993) and Schillinger and Lücke (1987). *Hafnia alvei* was identified according to Holmes and Costas (1992).

Stock cultures of the Gram-negative and the Gram-positive organisms grown in Nutrient broth (Lab M, 14, Amersham, UK) and a modified MRS lactobacillus broth (De Man et al., 1960) with acetate and citrate omitted (Wilkinson and Jones, 1977) respectively were used as inocula. After overnight incubation at 20°C the cultures were harvested, washed and resuspended twice with saline (0.85% NaCl, w/v). Experimental cultures were inoculated with a portion from an appropriate dilution in 1/4 strength Ringer's solution (Lab M, 100 z) of the original suspension in saline to provide an initial inoculum of 10^2–10^3 cfu/ml.

2.2. Meat juice, culture conditions and apparatus

A meat juice was prepared as described by Gill (1976). It was used without further dilution as a culture medium under an aerobic or a modified gas atmosphere. The latter was established by sparging with carbon dioxide and oxygen (20:80%, v/v), for at least 5 min, at the time of inoculating the medium.

The culture apparatus, which minimised gaseous exchange with the ambient environment, was that described by Alterthum and Rose (1973). It consisted of a 500 ml round, flat-bottomed Pyrex flask fitted with a fermentation lock containing water and a sampling port which was covered by a latex-rubber 'Suba Seal' (Gallenkamp). Each flask contained a polythene-covered magnet rotated by a magnetic stirrer located beneath the flask. Meat juice (250 ml) was added to each flask and, after inoculation and modification of the gas atmosphere, the flasks were incubated in a cool room (ca. 4°C) for ca. 20–30 d.

2.3. Sampling and enumeration of the population

During the exponential phase of growth, each medium was sampled twice daily, but only daily during the early stationary phase. Thereafter the frequency of sampling decreased with the duration of the experiment. Growth and physico-chemical changes were followed by removing portions of the culture with a hypodermic syringe inserted through the sampling port with aseptic precautions.

Portion (0.1 ml) of an appropriate serial dilution in 1/4 strength Ringer's solution was spread on nitrite actidione polymyxin B agar (made according to Davidson and Cronin, 1973) and incubated at 30°C for 5 d, MRS lactobacillus agar without acetate and citrate (Wilkinson and Jones, 1977) supplemented with vancomycin (Sigma, V-2002) 400 mg/l and polymyxin B (Sigma, P-1004) 5 mg/l and incubated at 25°C for 4 d, STAA agar (Gardner, 1966, Oxoid CM 881, SR 151, UK) and incubated at 25°C for 3 d, and creatine agar (Molin and Ternström, 1986) and incubated at 25°C for 2 d, for enumeration of *C. piscicola*, *L. mesenteroides* subsp. *mesenteroides*, *B. thermosphacta* and *P. fragi* respectively. The utilisation of the modified MRS medium for enumeration of leuconostocs was based on the resistance of these bacteria to vancomycin (Orberg and Sandine, 1984). Creatine agar was based on the ability of *P. fragi* to use the substrate as sole carbon and energy source. Portions (1.0 ml) were used for pour plates with VRBG agar (Mossel et al., 1962, Lab M 88) for enumeration of *Hafnia alvei* incubated at 30°C for 2 d. Creatine and actidione (cycloheximide) were supplied by Sigma (Codes C-3630 and C-6255 respectively). 'Lab-Lemco' powder was supplied by Oxoid (Code L29). Other microbiological ingredients and chemicals were supplied by Lab M, UK and BDH, UK respectively.

2.4. Physico-chemical analysis

One ml of a culture was transferred to a 15 ml centrifuge tube and, 4.0 ml $HClO_4$ (1 M) were added. The contents mixed, then the tubes were centrifuged for

10 min at 3200 × *g* before being stored at −20°C. When a growth experiment was completed, the deproteinised samples were thawed and centrifuged as detailed above. The supernatant was transferred quantitatively into a universal vial and adjusted to a pH value 7.0–8.0 with 5 M KOH. Distilled water was then added to obtain a 10 ml final volume. The vial was shaken gently, then immersed for 20 min in an ice-bath to precipitate the potassium perchlorate. An appropriate portion of the clear supernatant used for the analyses.

The pH was determined with a glass electrode immersed in the sample taken from the flask. Glucose was assayed by glucose oxidase and peroxidase with [Trinder] reagent (Sigma, Diagnostic kit 315-100). Glucose 6-phosphate by an enzymatic procedure (Michal, 1984). L-lactic acid, D-lactic acid, acetic acid and creatine were assayed enzymatically by the methods of Noll (1984), Gawehn (1984), Beutler (1984), and Wahlefeld and Siedel (1985), respectively. Acetoin was determined by Westerfeld's method (Westerfeld, 1945). Ammonia was determined as described by Chaney and Marbach (1962) and total amino acids with a ninhydrin reagent (Rosen, 1957). Appropriate control assays were included in all determinations. Enzymes and coenzymes were supplied by Boehringer Mannheim, Germany.

3. Results

3.1. Xenic culture of C. piscicola, B. thermosphacta and P. fragi

The population under a CO_2-enriched atmosphere grew (Fig. 1A) during the first 100 h at the expense of glucose and glucose 6-phosphate (Fig. 1B). The pH changed from 6.1 to 5.9 (Fig. 1C). *Brochothrix thermosphacta* attained a larger biomass (9.0 log cfu/ml) than that of the other members of the association. *P. fragi* formed the smallest biomass (7.4 log cfu/ml). The eventual size of the population of *C. piscicola* was midway between those of the two other species (8.2 log cfu/ml). A steady population persisted after the growth phase. During the exponential and early stationary phase of growth the order of substrate utilisation was glucose > glucose 6-phosphate > L-lactate. D-lactic acid was not detected. Acetate (30 mg/dl) was formed mainly during exponential growth. A small amount of ammonia (12 mg/dl) was produced during the transition from the exponential to the stationary phase. This was linked with a slight decrease in the concentration of total amino acids which increased in concentration thereafter. Creatine was not catabolised. After the initial decrease during the development of the population, ammonia production and amino acid metabolism were associated with an increase of the medium pH from 5.9 to 6.2 (Fig. 1C).

The development of the population under aerobic conditions is summarised in Fig. 2A–C. Despite the equal growth rates of the members of the population during much of the exponential phase the distribution of biomasses at the end of the growth phase was again *B. thermosphacta* > *C. piscicola* > *P. fragi*. The catabolism of the available carbohydrates and the production of acetate were notable features of the exponential phase of growth. D-Lactic acid was not

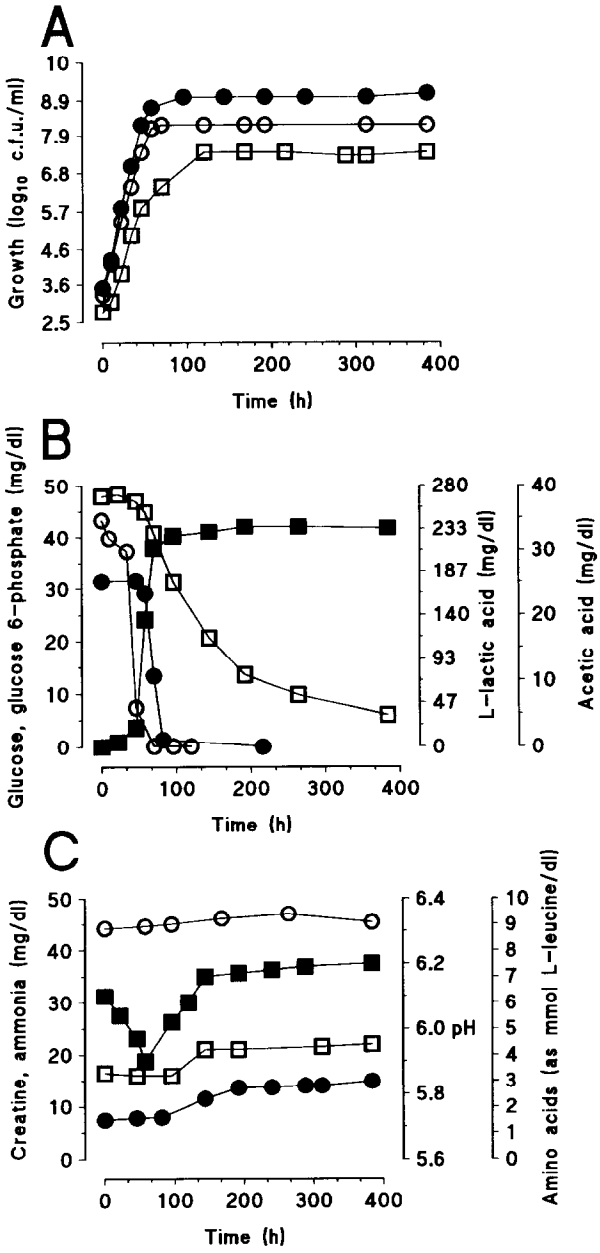

Fig. 1. Growth curves and metabolic attributes of members of a xenic culture growing in a meat juice at 4°C under an atmosphere enriched with carbon dioxide. (A) *Brochothrix thermosphacta* (●), *Carnobacterium piscicola* (○) and *Pseudomonas fragi*(□); (B) Catabolism of glucose (○), glucose 6-phosphate (●), L-lactic acid (□) and acetic acid (■); (C) creatine (○), ammonia (●), amino acids (□) and changes in pH value (■).

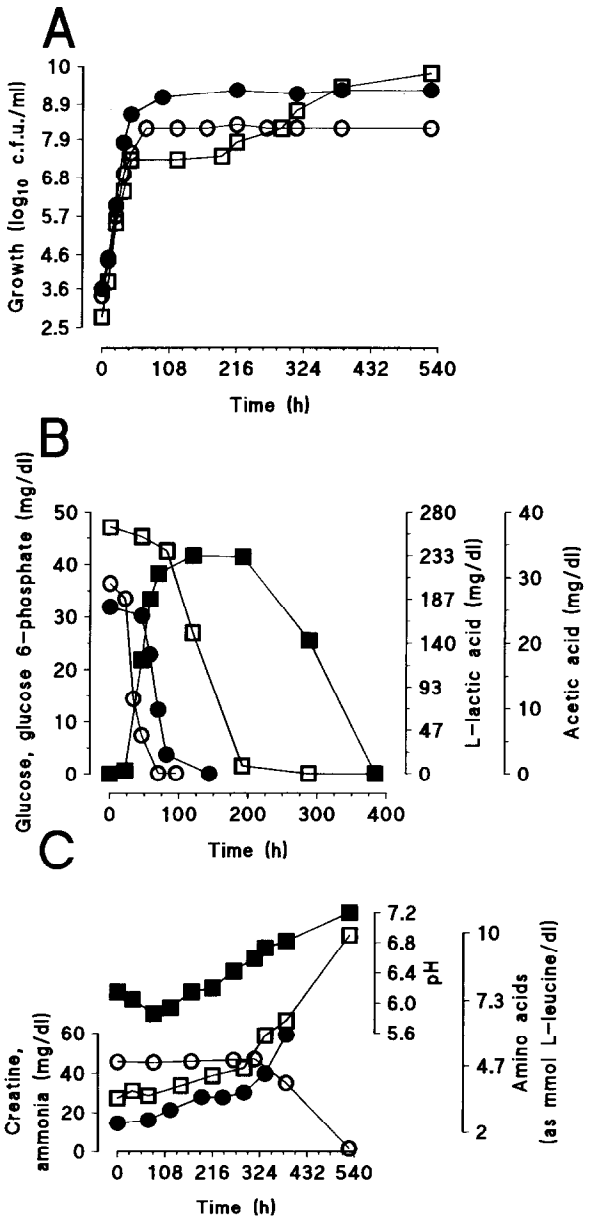

Fig. 2. Growth curves and metabolic attributes of members of a xenic culture growing in a meat juice at 4°C under an aerobic atmosphere. (A) *Brochothrix thermosphacta* (●), *Carnobacterium piscicola* (○) and *Pseudomonas fragi* (□); (B) Catabolism of glucose (○), glucose 6-phosphate (●), L-lactic acid (□) and acetic acid (■); (C) creatine (○), ammonia (●), amino acids (□) and changes in pH value (■).

detected. The order of substrate utilisation during the growth phase was similar to that observed with the modified atmosphere. During the relatively short steady state, L-lactate was catabolised at a rate higher than that in the modified atmosphere culture. *Pseudomonas fragi* became a climax species late in the stationary phase. At that time a multi-phase development was observed. There were small but successive increases in the pseudomonad population, these being associated with the catabolism of acetate (33 mg/dl) and creatine (45 mg/dl), the production of ammonia (60 mg/dl), increase of total amino acids (9.9 mmol/dl) and subsequent alkalinisation of the meat juice (pH 7.2).

3.2. Xenic culture of P. fragi, H. alvei, B. thermosphacta, C. piscicola and L. mesenteroides subsp. mesenteroides

Growth curves of these organisms and changes in the media compositions are shown in Fig. 3A–C. The Gram-positive organisms grew at equal rates and by the end of the growth phase, the growth of *P. fragi* had been inhibited attaining a population size of 6.8 log cfu/ml. *Hafnia alvei* grew initially at a rate slower than those of Gram-positive bacteria but its growth continued such that the size of its population (9.4 log cfu/ml) overshot those of the members of the Gram-positive population and eventually reached the total carrying capacity of the ecosystem. At the steady state of growth the distribution of biomasses was *H. alvei* > *B. thermosphacta* > *L. mesenteroides* subsp. *mesenteroides* > *C. piscicola* > *P. fragi*.

Development of the Gram-positive population was linked with sequential decreases in the concentrations of glucose and glucose 6-phosphate (Fig. 3B). In axenic cultures of the Gram-positive bacteria (data not shown) only *B. thermosphacta* and *L. mesenteroides* subsp. *mesenteroides* catabolised glucose 6-phosphate. In addition, none of these species was able to catabolise the 6-phosphogluconate present in the juice. At the end of the growth phase a slight decrease of the pH value from 6.0 to 5.8 coincided with the production of L-and D-lactate (Fig. 3C). Large amounts of acetic acid (74.4 mg/dl) were produced in the transitory phase. The formation of acetate was associated with declines in the concentration of both L-lactate and D-lactate.

3.3. Xenic culture of B. thermosphacta, C. piscicola and L. mesenteroides subsp. mesenteroides

The distribution of biomasses (Fig. 4A) the order of catabolism of the available carbohydrates (Fig. 4B) and the products (Fig. 4C) with these organisms were similar to those in the above experiment. However, the most distinguishing feature was the dramatic increase in the concentration of acetic acid (240 mg/dl) corresponding stoichiometrically to the summation of L-and D-lactate catabolised during that period.

3.4. Xenic culture of C. piscicola and L. mesenteroides subsp. mesenteroides

A meat juice exhausted, by growth of pseudomonads, of its content of L-lactate and glucose, and then, supplemented with D-glucose was used. The results are

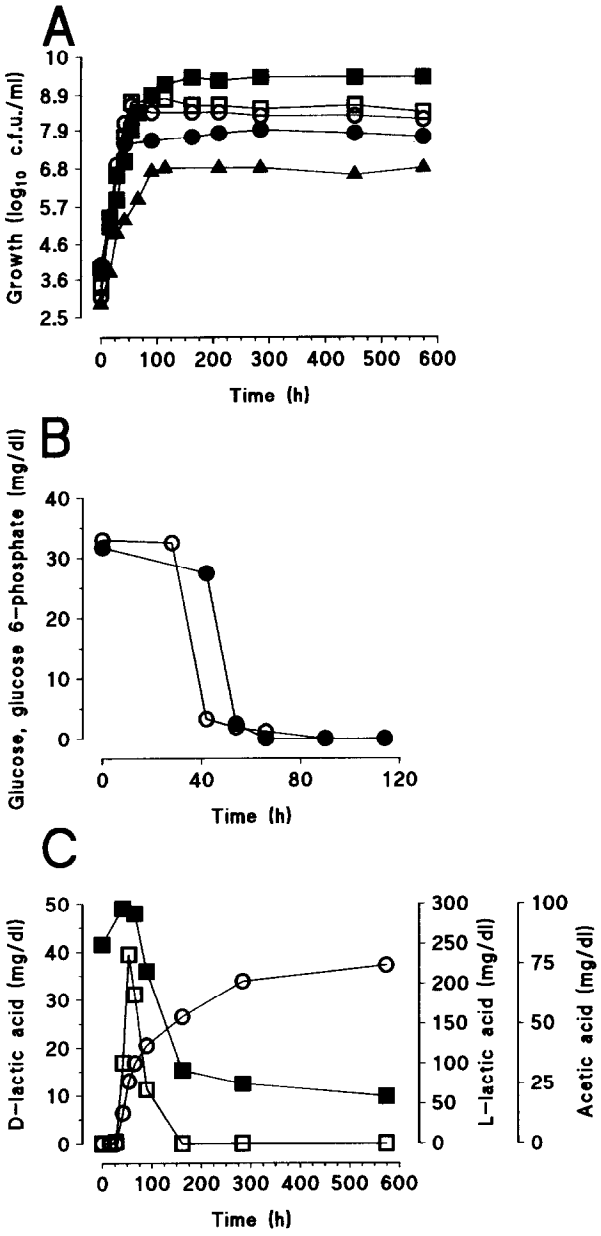

Fig. 3. Growth curves and metabolic attributes of Gram-positive and Gram-negative members of a xenic culture growing in a meat juice at 4°C under an atmosphere enriched with carbon dioxide. (A) *Leuconostoc mesenteroides* subsp. *mesenteroides* (○), *Carnobacterium piscicola* (●), *Brochothrix thermosphacta* (□), *Hafnia alvei* (■) and *Pseudomonas fragi* (▲); (B) Catabolism of glucose (○) and glucose 6-phosphate (●) and; (C) D-lactic acid (□), L-lactic acid (■) and acetic acid (○).

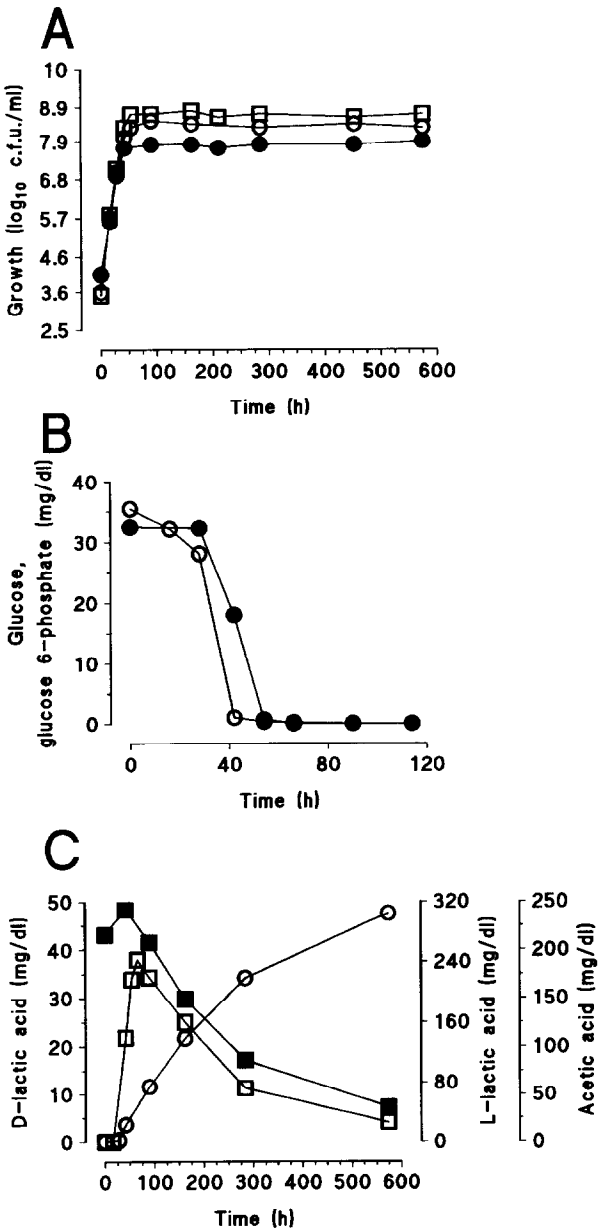

Fig. 4. Growth curves and synopsis of essential catabolism of Gram-positive members of a xenic culture growing in a meat juice at 4°C under an atmosphere enriched with carbon dioxide. (A) *Leuconostoc mesenteroides* subsp. *mesenteroides* (○), *Carnobacterium piscicola* (●) and *Brochothrix thermosphacta* (□); (B) Catabolism of glucose (○) and glucose 6-phosphate (●) and; (C) D-lactic acid (□), L-lactic acid (■) and acetic acid (○).

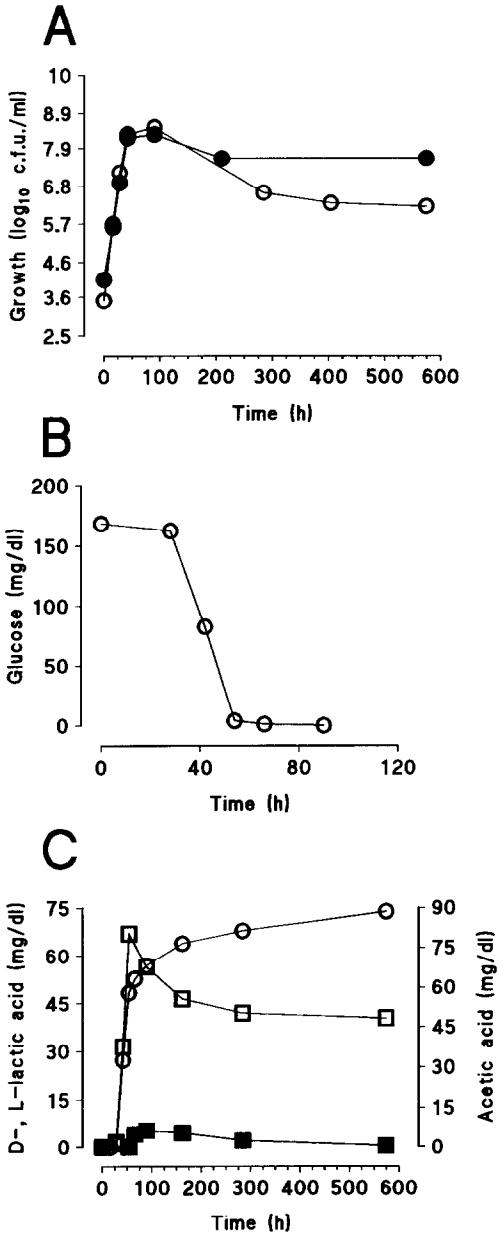

Fig. 5. Growth curves and synopsis of essential catabolism of lactic acid bacteria growing in a meat juice at 4°C under an atmosphere enriched with carbon dioxide. (A) *Leuconostoc mesenteroides* subsp. *mesenteroides* (○) and *Carnobacterium piscicola* (●); (B) Catabolism of glucose (○) and; (C) D-lactic acid (□), L-lactic acid (■) and acetic acid (○).

summarised in Fig. 5A–C. The percentage of D-lactate and L-lactate formed during the exponential phase of growth was 92.7% and 7.3% of the total lactic acid (72.2 mg/dl) respectively. L-Lactate was absent initially from the medium and a comparatively low concentration of acetate was formed (88.6 mg/dl). In this culture the smallest amount (2.0 mg/dl) of acetoin was detected comparatively to that observed with the others (results not shown).

4. Discussion

In a developing population the energy flow is in favour of growth with the accompanying depletion of carbohydrates and, in a climax population, the flow of energy is in favour of maintenance, with secondary biochemical activities being confined to further oxidation of possible substrates and harnessing of the remaining energy. Therefore, a first characteristic feature in a natural and/or model ecosystem of the type used in this study is the energy content, principally carbohydrate, that plays an important role in the evolution of the population. In addition, a selective pressure is presented by the inability of certain taxa to utilise available energy in the form of glucose 6-phosphate. Taxa (e.g. *C. piscicola*) unable to catabolise this substrate during the growth phase achieved a lower population size than those that did so.

During this period of utilisation of carbohydrates, one can observe different outcomes. On one hand, in an aerobic association, L-and D-lactate and creatine contribute to the energy budget such that they support and finally establish a climax population of pseudomonads. On the other hand in an ecosystem in which the determinants inhibit the development of an aerobic flora, at least during the development of the Gram-positive one, the outcome of this development was manifested by the major end products of the facultatively anaerobic Gram-positive flora.

After depletion of carbohydrates the populations entered a transitory period that led to a steady state. Maintenance energy during this phase was derived from secondary oxidations. Namely, the post-exponential catabolism of lactate, in the absence of glucose, was associated with the production of acetate. This observation is in accord with studies by Bryan-Jones and Whittenbury (1969). Moreover, Frey and Hubert (1993) noted that the oxidation of lactate to acetate by lactobacilli was induced by the presence of oxygen and glucose depletion. This phenomenon is referred to as a change from homo- to heterofermentative physiology (Kakouri and Nychas, 1994; Arkoudelos, 1992; Borch et al., 1991; Bobillo and Marshall, 1991).

It was at that period that the main body of acetate in the ecosystem was formed, as only minor production of acetate occurred during the catabolism of the carbohydrates. Obviously, the production in the first period is limited by interspecific antagonism for energy and this was clearly demonstrated when lactic acid bacteria alone were used as inocula. In this instance the concentration of end products, including acetate, were higher than those obtaining with the more diverse populations. In the second period, the role of a common resource for

energy was transferred from glucose and glucose 6-phosphate to L-, D-lactate. Despite major production during this phase, there was competition between the Gram-positive and Gram-negative population for lactate. When the Gram-positive population was isolated from that of the Gram-negative one, a threefold increase in the production of acetate was observed. At the other extreme, when lactate was initially absent, a limited amount of acetate formed. As the transitory phase reached the steady phase, it could be postulated that the system experienced anoxic conditions. Under these conditions the rate of lactate catabolism lessened when the population in the meat ecosystem entered into the steady state.

Susceptibility of *Pseudomonas* to acetic acid have been reported. Frey and Hubert (1993) observed inhibition of *P. fluorescens* by acetate produced from lactate in a xenic culture with lactobacilli. This supports the concept of the emergent property. Namely, that inhibition of the *Pseudomonas* caused by synergistic effect of an atmosphere enriched with carbon dioxide and the accumulation of acetate in the ecosystem. In addition to acetate, hydrogen peroxide produced by lactic acid bacteria has been reported by others (Price and Lee, 1970) as the agent accounting for the inhibition of *Pseudomonas*. Skytta and colleagues (Skytta et al., 1991) found also a broad spectrum antibacterial activity against *P. fragi* and *P. fluorescens* by bacteriocin-producing strains of *Pediococcus damnosus* and *P. pentosaceus* in minced meat.

The results of this study confirm and extend publications dealing with the detection of end products in meat and meat products (Kakouri and Nychas, 1994; Borch and Agerhem, 1992; Ordonez et al., 1991; De Pablo et al., 1989; Nassos et al., 1985). All these studies have demonstrated the production of D-lactic acid, acetic acid and acetoin and correlated these with a population of Gram-positive bacteria as judged by the results obtained with selective media.

Observations relating to the (a)xenic cultures of Gram-positive and lactic acid bacteria have been noted repeatedly in the literature. Arkoudelos (1992) found a progressive increase in acetic acid in (a)xenic cultures of *Lactobacillus plantarum* and *Staphylococcus carnosus* in a range of laboratory media under either static or agitated conditions at 37°C. The author advanced the view of lactate oxidation to acetic acid under conditions of glucose limitation. Borch and Molin (1989) found that under aerobic conditions with glucose as an energy and carbon source, leuconostocs produced only traces of L-lactic acid and no acetoin, the major products being D-lactic and acetic acid. With *B. thermosphacta* the major products were acetoin and acetic acid with no production of either stereo-isomers of lactate. *Carnobacterium piscicola* produced mainly acetic acid, L-lactate, acetoin but no D-lactic. In another study Blickstad and Molin (1984) obtained similar results with studies of *B. thermosphacta* under different gaseous atmospheres. Borch and Agerchem (1992) also noted the production of D-lactate in beef inoculated with leuconostoc and stored anaerobically. Similar findings were noted in this study, acetoin was mainly associated with *B. thermosphacta* and D-lactate with leuconostoc. Thus, it is confirmed that this stereo-isomer is of microbial origin and leuconostocs are the primary producers.

An analogous situation to the above development of meat ecosystems, particu-

larly those with determinants allowing the dominance of the Gram-positive population, has been noted repeatedly in dairy ecosystems (Murphy et al., 1985; Thomas et al., 1985). For example metabolism of lactate in cheese was induced after the exhaustion of D-glucose and was restricted by the limited amount of oxygen in the interior of the commodity. In conclusion, the metabolic attributes of the cultures studied, suggest that D-lactic and principally acetic acid appear to be important metabolites for assessment of freshness of a commodity packed under a modified atmosphere due to its accumulation in a food ecosystem. The key role of the latter as potential indicator was also noted by Kakouri and Nychas (1994) in poultry meat stored under modified atmosphere and vacuum packs.

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