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CHARACTERISATION OF PSYCHROTROPHIC LACTIC ACID BACTERIA ISOLATED FROM VACUUM PACKED BEEF.

Rita Mulala Sakala[1], Yukio Kato[1], Hideki Hayashidani[2], Choji Kaneuchi[1] and Masuo Ogawa[2].

[1]School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 229-8501. [2]School of Veterinary Medicine, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8538, Japan.

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BACKGROUND:

Lactic acid bacteria predominate the microflora of vacuum packed raw chill-stored meat and meat products where they serve a preservative or spoilage role (Schillinger & Lucke, 1989). Lactic acid bacteria that have been isolated from vacuum packed beef include *Lactobacillus*, *Carnobacterium*, *Leuconostoc* and *Brochothrix* species. Rapid growth or production of antibacterial substances early in the growth phase could be attributes facilitating the domination of these organisms in vacuum packed meat (Ahn & Stiles 1990a and b). In the past, some strains isolated from vacuum packed beef were not identified to species level and were therefore considered to be atypical streptobacteria or atypical betabacteria (Hitchener *et al.*, 1982).

OBJECTIVES:

The aims of this study were to identify some psychrotrophic lactic acid bacteria isolated from vacuum packed beef and to study the development of the microflora during the storage of such beef.

METHODS:

Samples. Five samples of beef (A, B, C, D and E) were obtained. Each sample was divided into 7 equal portions which were vacuum packed and stored at 2 °C up to 6 weeks. One portion from each sample was cultivated at weekly intervals by blending 1 g in 9ml of phosphate buffered saline (PBS). Ten fold serial dilutions of each sample (0.1 ml) were spread on plates of Trypticase Soy agar (TS agar; Nissui, Tokyo), MRS agar (Oxoid, UK) and BL agar (Nissui). TS agar plates were incubated aerobically at 7 °C for 7 days while the rest were incubated anaerobically at 7 °C for 14 days.

Strains. 15 colonies were picked from a dilution of each sample giving 30 to 300 colonies. A total of 1050 strains were isolated. The type strains of lactic acid bacteria were obtained from the Japan Collection of Microorganisms (JCM), Gifu University, School of Medicine (strains with NCFB numbers) and Deutsche Sammlung von Mikroorganismen (DSM). The following type strains were used in this study for reference purposes; *Brochothrix campestris* (DSM 4712^T), *Brochothrix thermosphacta* (DSM 20171^T), *Carnobacterium divergens* (JCM 5816^T), *Carnobacterium piscicola* (JCM 5348^T), *Lactobacillus agilis* (JCM 1187^T), *Lactobacillus mali* (JCM 1116^T), *Lactobacillus pentosus* (JCM 1558), *Lactobacillus plantarum* (JCM1149^T), *Lactobacillus sake* (JCM 1157^T), *Lactobacillus sharpeae* (JCM 1186^T), *Lactococcus garviae* (NCFB 2155^T), *Lactococcus lactis* subsp. *lactis* (JCM 5805^T), *Lactococcus piscium* (NCFB 2778^T), *Lactococcus plantarum* (NCFB 1869^T), *Lactococcus raffinolactis* (JCM 5706^T), *Leuconostoc gelidum* (JCM9697^T) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (JCM 6124^T). The strains were stored at -80 °C in TS broth with 10 % glycerol. Working cultures were grown on BL agar anaerobically at 20 °C for 48 hours and all tests performed at the same temperature unless otherwise stated.

Morphological, physiological and biochemical tests. Gram's reaction, cellular morphology, catalase test, oxidase test, gas production from glucose, ammonia production from arginine, motility, growth in broth with 6 % NaCl and arginine hydrolysis were determined according to the Cowan and Steel's Manual for the identification of Medical Bacteria (1993). The temperature range for growth was examined in Briggs Liver broth (BLB; Mistuoka, 1969) during incubation at 0, 5 and 10 °C for 14 days and 40 or 45 °C for 7 days. API 50CH systems for *Lactobacillus* (BioMeriuex,Tokyo) was used for acid production from carbohydrates. Presence of *meso*-diaminopimelic acid (*m*DAP) in the cell walls was performed as described by Becker *et al.* (1964).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (PAGE) of whole cell proteins. Preliminary grouping of the strains isolated was performed by whole cell PAGE. Cells harvested using a sterile wire loop were suspended in 1ml of 0.015M Tris buffer (pH 7.0). The preparation of cellular extracts and PAGE was performed as described by Pot *et al* (1994). The protein band patterns of the strains were compared and grouped according to similarities in protein profiles. Representative strains from each group were compared with related authentic reference strains.

16S rDNA sequencing and DNA-DNA hybridization. The PCR product was generated using universal primers for the 16S rDNA segment (Weisburg *et al.*, 1991) and purified using the Qiagen Kit (Qiagen, Germany). The sequencing reactions of gram positive unknown rods (M6 group) were run on an Applied Biosystems model 373A automated DNA sequencer while that of lactococci was carried out using Thermosequenase (Amersham Life Science Inc, USA) on a 1 dye-4 lane DNA sequencer (DSQ 2000S, Shimadzu Corp. Japan) as described by the manufacturer.

DNA-DNA hybridization was performed according to the colorimetric microplate method of Ezaki *et al.* (1989).

RESULTS AND DISCUSSIONS:

Characterisation of the strains. Most of the strains isolated during the whole period of study were gram positive rods or cocci. A few gram negative rods were isolated from all the five beef samples at 0 to 1 week only. They were identified as *Janthinobacterium*, *Aeromonas*, *Corynebacterium* and so forth at the genus level. Most strains isolated grew between 0 and 30 °C but not at 45 °C.

Some gram positive cocci produced gas from glucose and were identified as *Leuconostoc gelidum* on the basis of PAGE protein profile similarity and other biochemical characteristics. Non gas producing gram positive cocci were identified as *Latococcus* on the basis of growth at 10 °C, failure to grow at 40 °C or in broth with 6% NaCl and absence of motility. Since these strains did not hydrolyse arginine, they were compared by PAGE protein profile with some authentic reference strains of arginine dihydrolase negative lactococci. Their PAGE protein profile resembled that of *Lactococcus piscium* NCFB 2778^T with some minor band differences and sequencing of their 16SrDNA revealed a variation of 2 to 3 bases in the variable region. However, since the pattern of fermentation of sugars resembled that of *Lactococcus piscium*, these isolates were considered to be strains of this organism.

A few gram positive rod-shaped strains from 0 to 2 weeks' samples were catalase positive but oxidase negative. Some of these strains were well identified as *Brochothrix thermosphacta* on the basis of PAGE protein profile and biochemical characteristics although a few of these strains differed slightly in PAGE protein profile. These rods could be differentiated from *Brochothrix campestris* on the basis of PAGE protein profiles and failure to hydrolyse hippurate. The rest of the catalase positive strains were identified as *Bacillus* at the genus level.

Gram positive catalase negative rods with arginine dihydrolase and arginine deaminase were also isolated. A comparison of their

biochemical charcteristics and PAGE protein profiles showed that some strains resembled *Carnobacterium divergens* and others resembled *Carnobacterium piscicola*. All these strains possesed *m*DAP in their cell walls and were non motile.

Some *m*DAP positive, non motile, gram positive rods (M6 group) did not resemble biochemically and by PAGE protein profile any of the previously described authentic reference strains. Sequencing of the 16SrDNA of one of these strains and preliminary searches in the DDBJ data base using the program BLAST and alignment using GENETYX-MAC revealed some relation with *Lactobacillus* and *Pediococcus* but could not be identified as any one species from these genera. DNA-DNA hybridization with 13 representative strains from these genera failed to show any close relationship. These may represent a new species (*Lactobacillus algidus*) whose description is under way.

Development of microflora. Based on the numbers of strains isolated and identified from each sample at each respective week, the $\log_{10}$ numbers for each species per gram were calculated and the average for all the samples plotted (Fig. 1). *Lactococcus piscium*, *Leuconostoc gelidum* and the new strain (*Lactobacillus algidus*; M6 group) increased and persisted. They could be isolated from all the samples even up to 6 weeks. *Carnobacterium divergens* persisted at a slightly lower level but could not always be detected from all the samples. *Carnobacterium piscicola* was detected from 3 samples at week 0 but thereafter was inconsistently detected from only one sample (B). This species may show a tendency to be suppressed in vacuum packed beef. Further studies may be required to elucidate this trend. *Brochothrix thermosphacta* was detected in 1 to 3 samples for the first 2 weeks but could not be detected at and after 3 weeks from any one of the samples confirming its failure to compete with other lactic acid bacteria under anaerobic conditions (Newton & Gill, 1978).

CONCLUSIONS:

PAGE is useful in grouping and identifying strains isolated from vacuum packed beef. *Leuconostoc gelidum*, *Lactococcus piscium* and strains of a new species (M6 group) persist in vacuum packed beef stored at 2 °C. *Carnobacterium divergens* also persist but at a lower level than the former. Gram negative bacteria are rapidly eliminated from vacuum packed beef.

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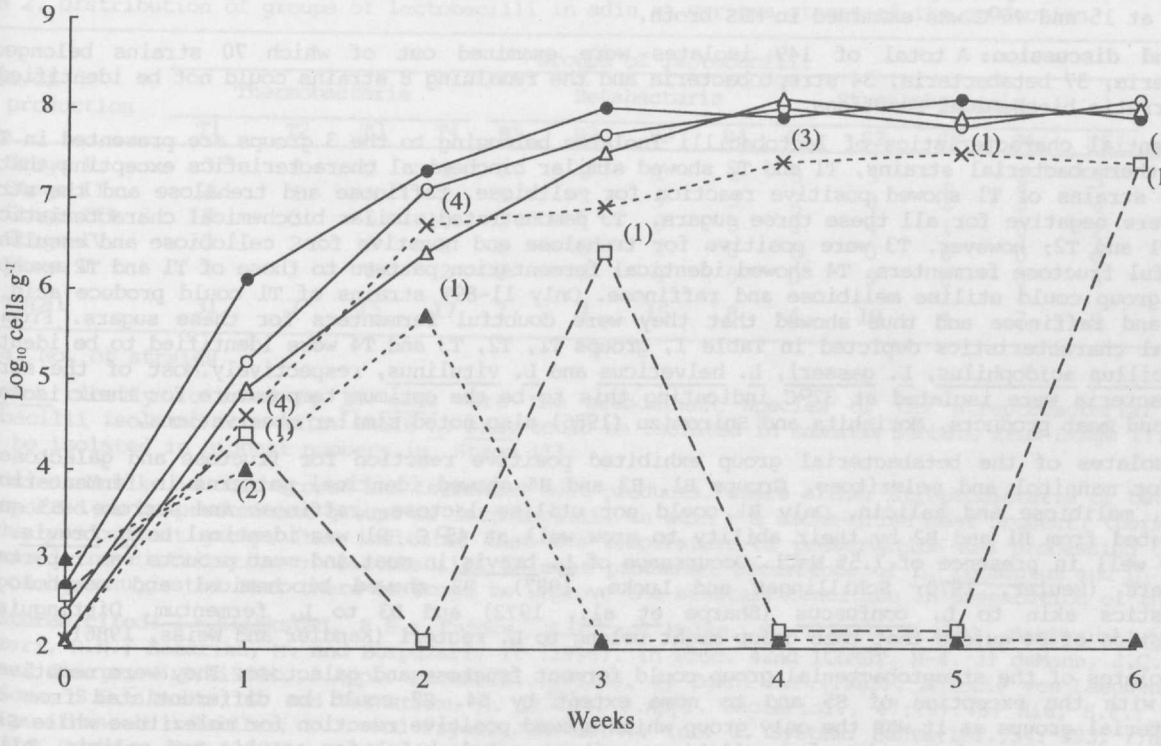

Fig. 1. $\log_{10}$ numbers/g of each species isolated at 7℃ from vacuum packed beef stored at 2℃ from 0 to 6 weeks. Numbers in parenthesis show the number of samples from which the species was detected

—●—, *M6 group* —○—, *Leuconostoc gelidum*
—△—, *Lactococcus piscium* ---×---, *Carnobacterium divergens*
---▲---, *Brochothrix thermosphacta* –□–, *Carnobacterium piscicola*