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ORIGINAL ARTICLE

Effects of vacuum, modified atmospheres and storage temperature on the microbial flora of packaged beef

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The flora growing on beef stored in vacuum and 100% CO_2 at 2 or 6°C (experiment 1) and in vacuum and different mixtures of CO_2 and N_2 at −1 or 2°C (experiment 2) was determined in two separate experiments. Both a high concentration of CO_2 and a low storage temperature inhibited bacterial growth, especially of the spoilage bacteria pseudomonads and Brochothrix thermosphacta*. No packaging conditions gave growth of coliforms. High numbers (log_{10} 5–6) of lactic acid bacteria after storage in vacuum or gas packs, inhibited growth of pseudomonads and* B. thermosphacta *during subsequent storage under retail conditions. Samples of the lactic acid bacteria obtained in experiment 2 were identified by genus-specific rRNA probes. Leuconostocs dominated in vacuum and CO_2 packs at both temperatures, whereas carnobacteria dominated in N_2 at −1°C.*

Introduction

The microbial flora and meat colour development are important determinants of the shelf life of fresh beef. By packaging the beef in a modified atmosphere and storage at low temperature, the shelf life can be prolonged considerably (Enfors et al. 1979, Young et al. 1983, Gill and Penney 1988). Many studies have shown that lactic acid bacteria are the predominating flora in vacuum and carbon dioxide (low oxygen) atmospheres (Erichsen and Molin 1981, Borch and Molin 1988), but little is known about the possible selective effects of different gas atmosphers for particular kinds of lactic acid bacteria.

Such knowledge could be important for several reasons. Although, as a group, lactic acid bacteria are generally regarded as having low spoilage potential, certain types can be more detrimental than others, e.g. heterofermentative gas and ethanol producers or producers of H_2S (Dainty and Mackey 1992). Furthermore, not all lactic acid bacteria are necessarily equally potent inhibitors of other organisms. Inhibition of the background flora by lactic acid bacteria, acting in conjunction with the inhibitory effects of the particular gas atmosphere in use, is frequently quoted as a factor in the rapid dominance of the flora of gas and vacuum packaged meats (Schillinger and Holzapfel 1990). Such inhibitory activity is also of special importance when the meat is subsequently exposed to air under retail display, when rapid growth of spoilage organisms such as *Brochothrix thermosphacta* and pseudomonads can occur.

Therefore, the aims of this study were (1) to determine to what extent different modified atmospheres (including vacuum) would influence the bacterial flora when the meat

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samples and background flora were essentially identical and conditions such as temperature and gas compositions were carefully controlled, (2) to determine the microbial flora of some of these meat samples after exposure to air for 1 week and (3) to use nucleic acid probes to identify which genera of lactic acid bacteria that were dominant on the meat.

Material and Methods

Meat sample preparation and storage plan

Experiment 1. A single loin (longissimus dorsi) was removed 3 days postslaughter from each of 12 different beef carcasses (pH< 5·9) and deboned. After removal of samples for initial microbial analysis, each loin was cut into eight sections of *c.* 0·5 kg each, giving a total of 96 samples. Equal numbers were packaged individually, under vacuum or in 100% CO_2 and stored at 2 or 6°C for 5 or 10 weeks in the dark. The 12 samples representing each of the different storage treatments came from different loins.

Experiment 2. Both loins were removed 3 days postslaughter from five different beef carcasses (pH<5·9) and deboned. After removal of samples for initial microbial analysis, each loin was cut into 10 sections of *c.* 0·4 kg each, giving a total of 100 samples. Equal numbers were packaged individually under vacuum or in 100% CO_2, 100% N_2, 20% CO_2/80% N_2 or 50% CO_2/50% N_2 and stored at –1 and 2°C for 5 and 10 weeks in the dark. The five samples representing each of the different storage treatments came from different loins.

Packaging

In both experiments, the loin sections were packaged on an Intevac IN30 chamber machine (Intevac Verpackungsmaschinen, Wallenhorst, Germany). The bags for experiment 1 were of polyamide/polyethylene (Sandvik, Bergen, Norway) with an O_2 transmission rate of 30 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23°C and 0% RH. The bags for experiment 2 were of polyamide/ethylene-vinyl alcohol/ polyethylene (Synclair Packaging BV, Almere, Netherlands) with an O_2 transmission rate of 8 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23°C at 60% RH. All packages were first vacuumized to 9 mbar. The gas packages were then flushed for 20 s with 1·25 bar pressure of the appropriate gas or gas mixture to give an initial gas to meat ratio of *c.* 2:1 by volume. One O_2 scavenger pack (Ageless R, Mitsubishi Gas Chem. Co. Inc., Tokyo) was included in every gas package, type FX-100 being used in experiment 1, types SPU-100 at 2°C and SS-100 at –1°C in experiment 2.

Display (experiment 2 only)

After 5 weeks storage, steaks of *c.* 2 cm thickness were cut from three loins, stored in vacuum, 100% N_2 or 100% CO_2. The steaks were placed on polystyrene trays and wrapped in polyvinyl chloride film, with an O_2 transmission rate of >10 000 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23°C and 0% RH. The steaks were displayed for 7 days at 2°C in a cold room under continuous 1000±100 lux warm white light (Warmton Lumilux L36W/31, Osram, Germany).

Gas analyses

Oxygen and CO_2 were analyzed with Toray LC 700-F and Toray PG-100 instruments respectively. Gas packages were tested immediately after packaging, after 3 days storage and at the end of storage. Headspace samples of 10 ml gas were taken with a syringe through a self-sealing, gas-tight rubber patch attached to the bag and injected into the instruments. (All the equipment was from Toray, Eng., Japan).

Microbial analyses

Analyses were done on 12 (experiment 1) or five (experiment 2) loins before packaging and after storage under each of the specified combinations of temperature, atmosphere and time, i.e. eight in experiment 1 and 20 in experiment 2, and on steaks subjected to

retail display (details given above). In each case 25 cm^2 of meat surface was delineated with a sterile aluminium template and a 2–3 mm thick layer removed with a scalpel, diluted in 100 ml physiological saline solution (PS, 0·87% NaCl, pH 7·0) and treated in a Colworth Stomacher (Model 400, Seward, London) for 1 min. A serial 10-fold dilution series was prepared in PS and 20 µl volumes spread in duplicate on five different media as follows: PCA (plate count agar; Difco, Detroit, MI, USA) for total viable counts; MRS agar (Difco) pH 5·7 for lactic acid bacteria (de Man et al. 1960); STAA (streptomycin thallous acetate actidione agar base, type CM 881, with selective supplement SR 151; Oxoid, Hampshire, England) for *B. thermosphacta*; Petrifilm Coliform Count Plates (3M, St. Paul, MN, USA) for coliforms; CFC (pseudomonads agar base type CM 559 with selective supplement SR 103; Oxoid) for pseudomonads. PCA, MRS, STAA and CFC were incubated at 20°C for 4 days and the Petrifilm at 30°C for 2 days, all aerobically. When no growth was observed on the MRS plates after 4 days, the plates were incubated for 4 days more. Counts were expressed as cfu cm^{-2}.

Identification of lactic acid bacteria by rRNA probes

Three replicate imprints were taken from MRS or PCA plates using colony lift membranes (Bio-Rad, Richmond, CA, USA). The colonies on the membranes were lyzed and baked before hybridizing with oligonucleotide probes. The genus-specific probes constructed for *Carnobacterium* spp. (CB1) and *Leuconostoc* spp. (LU2) (Nissen et al. 1994) were synthesized on a DNA synthesizer (Applied Bio-systems, model 381A, CA, USA) and end-labelled with ^{32}P. A universal probe (U), designed to hybridize all eubacterial 23S rRNA (Hertel et al. 1991) was synthesized and labelled in the same way. The hybridizations were done with overnight incubation at 40°C and a stringent wash with 2×SSC and 0·1% SDS at 45 and 50°C for the universal and genus-specific probes, respectively (Nissen et al. 1994, Nissen and Dainty 1995).

Statistical analysis

To test if the different treatments gave significantly different results, $\log_{10}$ bacterial counts were submitted to analysis of variance (ANOVA) using STATISTIX^R 4.1 (Analytical Software, Tallahassee, FL, USA). Although these counts were not strictly normally distributed—they were in fact left-censored—the ANOVA was considered robust enough to justify its use. The left-censored data were substituted by the midpoint between 0 and the value given, thus '<1' was replaced by 0·5. This change minimized the expected error on the assumption that a '<a' result is uniformly distributed over the interval (0,a).

Results and Discussion

To confirm that the packages contained the right gas mixture, the concentration of O_2 and CO_2 was measured initially, after 3 days and at the end of storage. In both experiments, the initial residual O_2 concentrations were below 0·8% in all gas packages and O_2 could not be detected later in the storage period. In the packages with CO_2 or CO_2 mixtures in experiment 2, concentrations of CO_2 constituted at least 90% of the nominal value. In packages with initially 100% N_2, the concentration was on average 5% CO_2 at end of storage (data not shown).

In experiment 1 in which bacterial growth on loins stored in vacuum and 100% CO_2 at 2 and 6°C for 5 and 10 weeks was compared, the initial bacterial counts were very low ($<\log_{10}$ 2·0), with pseudomonads being the largest group (Table 1). After 5 weeks the total bacterial counts were lower in 100% CO_2 than in vacuum and lower at 2 than 6°C. After 10 weeks the differences in counts were much less, indicating that CO_2 and low temperature gave longer lag periods and/or lower growth rates (after 10 weeks the fastest growing bacteria may already have reached the stationary phase). Lactic acid bacteria constituted most of the total counts under all conditions. After 5 weeks of storage, growth of *Pseudomonas* spp. and *Brochothrix thermosphacta* was significantly lower in beef packed in CO_2 than in vacuum. After 10

Table 1. Microbial counts (log_{10} cfu cm^{-2}) on beef stored in 100% CO_2 and vacuum at 2 and 6°C for 5 or 10 weeks

	Total count	Lactic acid bacteria	*Pseudomonas* spp.	*Brochothrix thermosphacta*	Coliforms
Before storage	1·9±0·3	0·5±0·2	1·3±0·3	0·4±0·2	0·1
After 5 weeks					
2°C					
100% CO_2	5·5^{a}	5·6^{a}	<1·0^{a}	<1·0^{a}	<0·6^{a}
Vacuum	6·4^{b}	6·2^{ab}	3·9^{b}	4·4^{c}	1·1^{a}
6°C					
100% CO_2	6·6^{bc}	6·4^{ab}	1·7^{a}	2·8^{b}	0·7^{a}
Vacuum	7·1^{c}	7·0^{b}	5·8^{b}	4·7^{c}	<0·6^{a}
After 10 weeks					
2°C					
100% CO_2	6·7^{a}	6·8^{a}	<2·0^{a}	<2·0^{a}	<0·6^{a}
Vacuum	6·8^{ab}	6·8^{a}	<2·0^{a}	3·5^{b}	1·1^{a}
6°C					
100% CO_2	7·0^{b}	6·9^{a}	2·0^{b}	3·6^{b}	n.a.
Vacuum	7·1^{b}	7·0^{a}	<2·0^{a}	3·2^{b}	<0·6^{a}

The initial counts are given as means±S.D. of 12 samples. Within counts (means of 12 samples) and a single storage period means having different superscripts differed at the 5% level (Tukey's test).
n.a.=not available.

weeks of storage, the number of *B. thermosphacta* had increased in 100% CO_2 at 6°C, whereas the number in the vacuum packages had decreased somewhat (Table 1). The number of *Pseudomonas* spp. after 10 weeks was low under all conditions, even in the vacuum packages which had exhibited growth of such organisms after 5 weeks storage at 2 or 6°C. The reason for this is not known.

In experiment 2, in which growth in different gas mixtures of CO_2/N_2 and vacuum was compared at –1 and 2°C, the initial bacterial counts (Table 2) were a little higher than in experiment 1. After 5 weeks at –1°C storage, the total count of bacteria in meat packaged in 100% CO_2 was significantly lower than the other treatments. The total counts from the other gas mixtures varied inversely with CO_2 concentrations. This effect was much less obvious at 2°C but counts in 100 and 50% CO_2 were somewhat lower than in the other atmospheres. This observation is in agreement with reports that the general bacteriostatic effect of CO_2 increases with lower temperatures (Gill 1988, Gill and Molin 1991). As in experiment 1, the lactic acid bacteria constituted most of the total counts. The low number compared with total counts in e.g. vacuum and N_2 at –1°C was probably owing to *Carnobacterium* spp. growing poorly on MRS.

As seen in experiment 1, the effects of the different storage conditions were much less evident after 10 weeks of storage. In experiment 2 most of the results obtained from storage at 2°C were in accordance with those in experiment 1. The higher number of pseudomonads in the vacuum packs in experiment 2 may have been caused by a higher number of pseudomonads at start.

The absence of pseudomonads, *B. thermosphacta* and coliforms is critical for obtaining a long shelf life (Dainty et al. 1983, Sørheim et al. 1995). In the present study, the numbers of pseudomonads and *B. thermosphacta* were low under all storage conditions compared with growth of lactic acid bacteria. However, faster growth and higher numbers were obtained under 100% N_2 and vacuum than in CO_2-containing atmospheres. The apparently better growth of pseudomonads than *B. thermosphacta* after 10 weeks in all atmospheres is difficult to explain in view of the generally accepted greater sensitivity of

Table 2. Microbial counts (log_{10} cfu cm^{-2}) on beef stored in different atmospheres at –1 and 2°C

	Total count	Lactic acid bacteria	*Pseudomonas* spp.	*Brochothrix thermosphacta*	Coliforms
Before storage	2·9±0·6	2·1±0·6	2·3±0·8	1·8±0·8	1·0±0·8
After 5 weeks					
–1°C					
1. 100% CO_2	$1·7^{a}$	$1·4^{a}$	$1·3^{a}$	$1·1^{a}$	$0·3^{ab}$
2. 50% CO_2/50% N_2	$3·5^{b}$	$2·0^{ab}$	$1·0^{a}$	$0·8^{a}$	$0·2^{ab}$
3. 20% CO_2/80% N_2	$4·6^{ab}$	$4·5^{bcde}$	$1·0^{a}$	$1·8^{ab}$	$0·2^{a}$
4. 100% N_2	$5·4^{cd}$	$2·4^{abc}$	$2·5^{ab}$	$2·0^{abc}$	$0·9^{ab}$
5. Vacuum	$4·6^{bc}$	$2·3^{abc}$	$2·6^{abc}$	$3·6^{c}$	$0·2^{a}$
+2°C					
1. 100% CO_2	$6·2^{de}$	$6·2^{e}$	$1·0^{a}$	$1·0^{a}$	$0·2^{a}$
2. 50% CO_2/50% N_2	$6·5^{de}$	$4·5^{cde}$	$1·8^{ab}$	$1·7^{ab}$	$0·8^{ab}$
3. 20% CO_2/80% N_2	$6·9^{e}$	$5·8^{de}$	$3·7^{cd}$	$2·9^{bc}$	$1·9^{b}$
4. 100% N_2	$6·8^{e}$	$3·2^{abcd}$	$4·3^{d}$	$3·2^{bc}$	$1·9^{ab}$
5. Vacuum	$7·0^{e}$	$6·1^{e}$	$4·2^{d}$	$4·1^{c}$	$1·6^{ab}$
After 10 weeks					
–1°C					
1. 100% CO_2	$4·9^{a}$	$4·8^{a}$	$1·8^{a}$	$1·6^{ab}$	$0·4^{ab}$
2. 50% CO_2/50% N_2	$6·3^{b}$	$6·2^{b}$	$1·2^{a}$	$1·4^{a}$	$0·1^{a}$
3. 20% CO_2/80% N_2	$6·4^{b}$	$6·2^{b}$	$2·6^{abc}$	$2·5^{abc}$	$0·1^{a}$
4. 100% N_2	$6·5^{b}$	$6·4^{b}$	$2·4^{ab}$	$3·2^{bc}$	$0·1^{a}$
5. Vacuum	$5·9^{b}$	$5·7^{ab}$	$3·3^{abcd}$	$3·9^{c}$	$0·1^{a}$
+2°C					
1. 100% CO_2	$6·7^{b}$	$6·6^{b}$	$1·4^{a}$	$1·2^{a}$	n.a.
2. 50% CO_2/50% N_2	$6·6^{b}$	$6·3^{b}$	$4·0^{bcd}$	$1·4^{a}$	$0·4^{ab}$
3. 20% CO_2/80% N_2	$6·4^{b}$	$6·0^{b}$	$4·5^{de}$	$2·8^{abc}$	$1·9^{b}$
4. 100% N_2	$6·7^{b}$	$6·4^{b}$	$4·7^{e}$	$3·3^{bc}$	$1·5^{ab}$
5. Vacuum	$6·7^{b}$	$6·4^{b}$	$4·1^{cde}$	$3·6^{c}$	$1·7^{ab}$

The initial counts are given as means±S.D. of five samples. Within counts (means of five samples) and a single storage period means having different superscripts differed at the 5% level (Tukey's test).
n.a.=not available.

Gram-negative bacteria to CO_2 (Dixon and Kell 1989, Lambert et al. 1991). One possible explanation could be that the CFC medium used was not entirely selective for pseudomonads, although no identifications were carried out to substantiate this observation.

Meat packaged in vacuum and N_2 acquired an unacceptable sour 'off' odour after 5 weeks of storage at 2°C and after 10 weeks in –1°C. A sour-aromatic smell produced by *B. thermosphacta* in vacuum-packaged meat has been described by Dainty et al. (1983) and Gill and Harrison (1989). However, in these reports the growth of *B. thermosphacta* reached 6 log_{10} cfu cm^{-2}. Whether the odour in our experiment was of microbial origin is not known.

Initial number of coliforms was very low and did not increase under any of the storage conditions. Gill and Penney (1988) found substantial fractions of *Enterobacteriacae* on vacuum treated beef and low concentrations of CO_2 even on normal pH beef. Gill and Harrison (1989) reported spoilage by higher numbers of enterobacteria or pork packaged in 100% CO_2 and stored at –1°C for 19 weeks. In both experiments, however, the initial number of enterobacteria was quite high.

Increases in bacterial counts after steak cut from loins, stored for 5 weeks in vacuum, 100% N_2 or 100% CO_2, re-packaged in oxygen-permeable film and exposed to light and air at 2°C for 7 days are shown in Fig. 1. The total count of bacteria before exposure was low at –1°C (log_{10} 1·7–5·4), but the exposure to air caused an increase in bacterial growth, especially for pseudomonads and *B. thermosphacta*. The beef that had been stored at 2°C

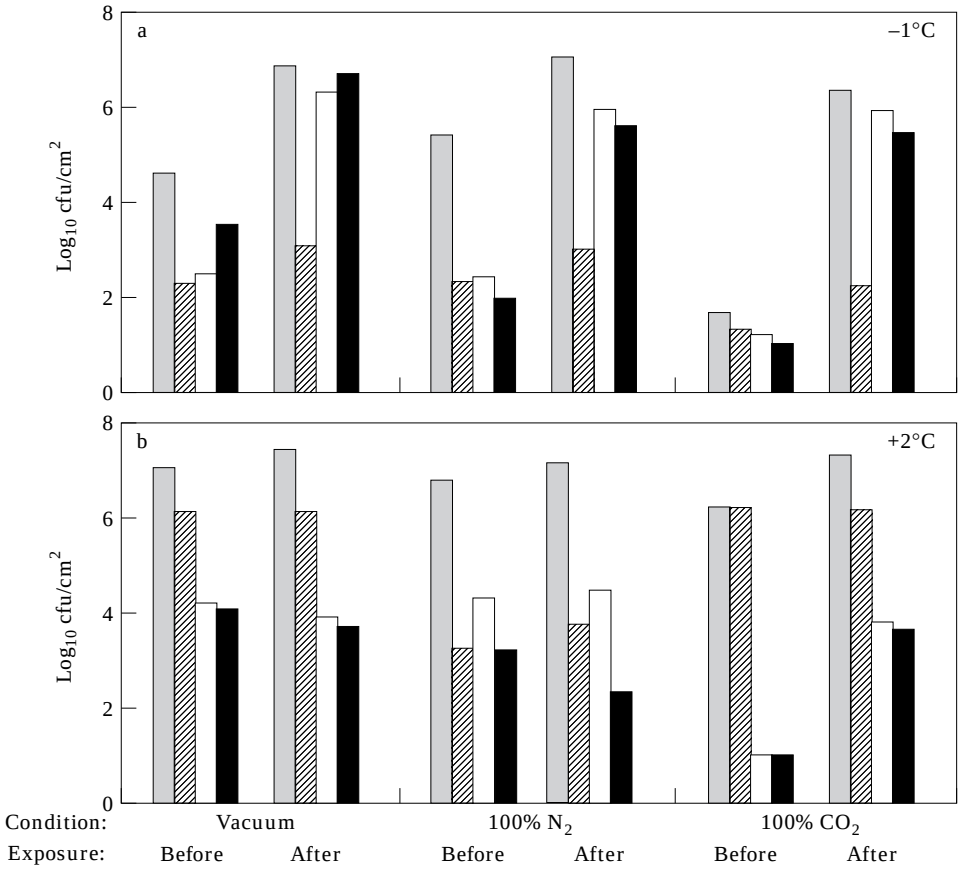

Figure 1. Number of colony forming units (cfu) (means of three samples) on beef stored in different atmospheres at –1°C (a) and 2°C (b) for 5 weeks and after subsequent exposure to air and light at 2°C for 7 days. (▒), total count; (▨), lactic acid bacteria; (□), *Pseudomonas* spp.; (■) *Brochothrix thermosphacta*.

in 100% N_2 and vacuum before exposure had initially higher counts of pseudomonads and *B. thermosphacta*, but the counts increased very little after exposure. This reduction in growth may have been caused by competition from lactic acid bacteria which were present in higher numbers on the 2°C stored samples than on the –1°C stored samples. This observation is in agreement with the finding that vacuum-packaged beef stored at 1·5°C had a short shelf life after they had been retail-packaged (Gill and Jones 1994). Such findings are clearly of relevance if meat is first stored in masterpacks with CO_2 and then exposed to air for retail display.

After 5 weeks storage, colony lift membranes were used to take replicas of the colonies growing on MRS or PCA plates from beef stored in 100% CO_2, 100% N_2 and vacuum packaged at both temperatures (Fig. 2 and Table 3). The membranes were hybridized with rRNA-directed probes for *Carnobacterium* sp. (CB1) and *Leuconostoc* sp. (LU2) (Nissen et al. 1994). To avoid false positives and to determine the percentage of total number of colonies giving hybridization, a probe for total eubacteria (U) (Hertel et al. 1991, Nissen and Dainty 1995) was also used. Fig. 2 shows that the colonies from beef stored in vacuum at –1°C and in CO_2 at 2°C for 5 weeks consisted almost entirely of *Leuconostoc* spp., as did colonies from beef packed in vacuum at 2°C (Table 3). This observation is in accordance with findings of Shaw and Harding (1984) for vacuum-packaged meat. For beef stored in 100% N_2 at –1°C, most of the colonies were *Carnobacterium* spp. (Fig. 2(c)). These colonies appeared

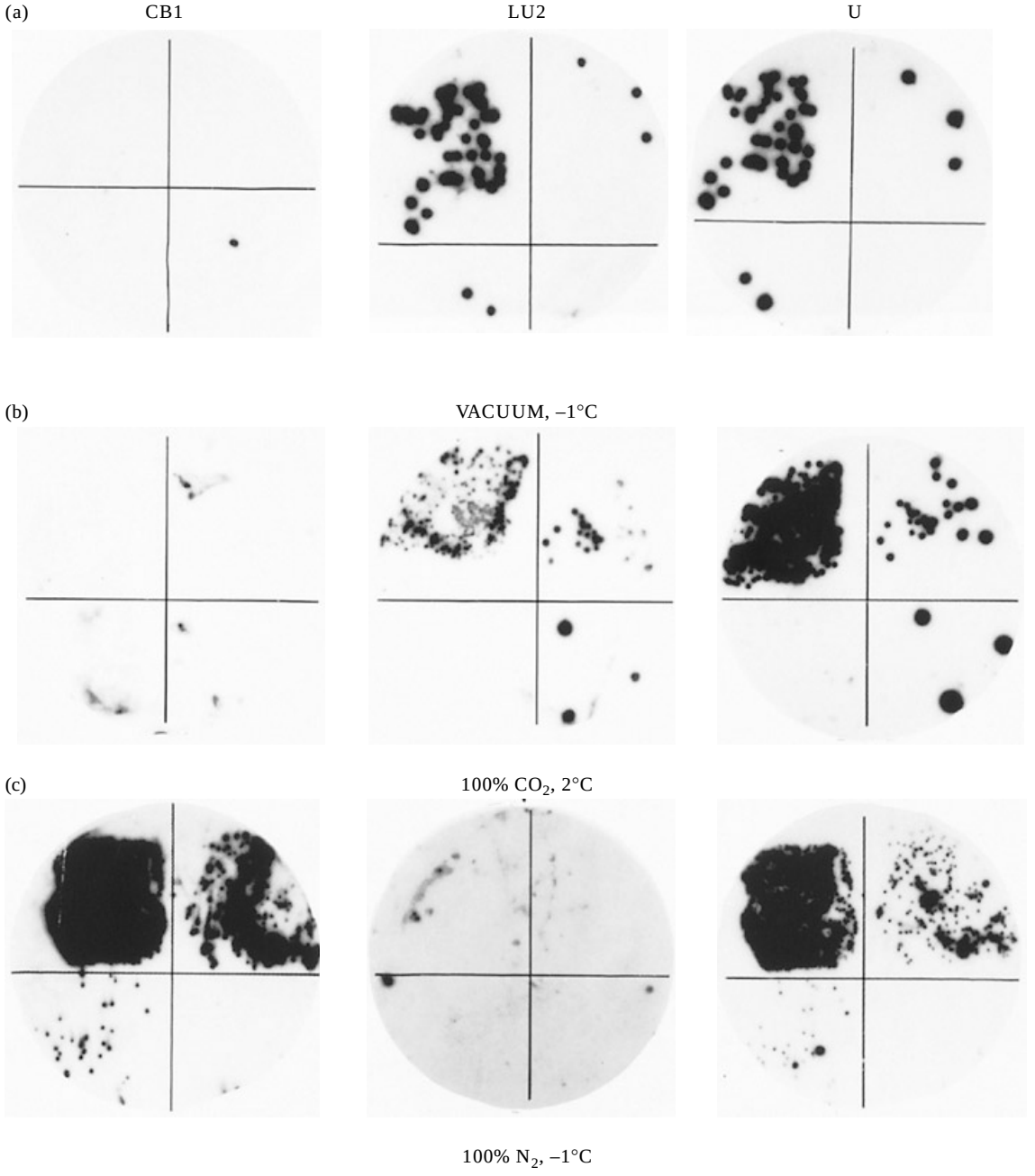

Figure 2. Bacterial colonies from beef stored under different conditions hybridized with the probes CB1, LU2 and U. The replicate imprints were made from MRS plates (a and b) or a PCA plate (c), inoculated with four different dilutions of the bacterial suspensions.

on the MRS plates only after prolonged incubation (8 days instead of 4 days at 20°C), but hybridization showed that they grew faster on the corresponding PCA plate (not shown), which is a probable explanation for the differences between total counts and counts of lactic acid bacteria in some samples. Poor growth of *Carnobacterium* on MRS plates has also been reported by others (Brooks et al. 1992, Millière et al. 1994) and is consistent

Table 3. Hybridization of bacterial colonies from beef stored under different conditions with rRNA directed probes for *Carnobacterium* spp. (CB1) and *Leuconostoc* spp. (LU2)

	% of total colonies			
	5 weeks		10 weeks	
	CB1	LU2	CB1	LU2
–1°C				
100% CO_2	n.a.	n.a.	<5%	>50%
Vacuum	<5%	>90%	<5%	>50%
100% N_2	>90%	<5%	>90%	<5%
2°C				
100% CO_2	<5%	>90%	<5%	>50%
Vacuum	<5%	>90%	<5%	<5%
100% N_2	n.a.	n.a.	<5%	5–20%

The percentages are estimated from the total number of colonies hybridized with the probe for Eubacterium (U).
n.a.=not available.

with their non-aciduric properties as a group (Shaw and Harding 1984).

After 10 weeks of storage, further hybridization showed that at –1°C, the dominating flora was still *Leuconostoc* spp. (Table 3). At 2°C fewer of the colonies hybridized with the CB1 and LU2 probe, probably because of increased growth of *Lactobacillus* spp. or *Weisella* spp. These results indicate that 100% N_2 atmosphere (at least at –1°C) will favour growth of *Carnobacterium* spp. whereas vacuum and 100% CO_2 will favour *Leuconostoc* spp. With time there is possibly also a succession to more *Lactobacillus* spp.

In our study we have shown that even when the starting material for each of the experiments was the same, different atmosphere and storage temperature had a significant influence on the flora, including different groups of lactic acid bacteria. Little information is known about which lactic acid bacteria are best at outcompeting typical spoilage bacteria, or whether they have significantly different spoilage potential themselves. The use of oligonucleotide probes for hybridizing membranes with replicates of colonies makes it possible to monitor the flora of lactic acid bacteria from different storage conditions faster and more accurately than by doing identification tests on individual purified colonies. Thus, information can be obtained on which groups of lactic acid bacteria are important for determining the shelf life of the meat.

In conclusion, this study of the growth of spoilage bacteria on beef packed in vacuum and different atmospheres indicates that both low storage temperature and high CO_2 concentration are necessary for obtaining maximum microbial shelf life. However, the display conditions and colour development of the meat will also affect the shelf life.

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