BACTERIOLOGICAL STUDIES ON THE EXTENSION OF THE

SHELF LIFE OF RAW MINCED BEEF

by

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I declare that the thesis herewith submitted by me for the degree
of M.Sc (Microbiology) at the University of Pretoria has not been
handed in for a degree to any other university.

ABSTRACT

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ABSTRACT
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Two hundred and thirty four samples of raw minced beef were subjected
to storage at 0° and 7°C over a period of 17 days. The samples were
subjected to four different treatments where the controls (TREAT 1)
were aerobically packed. The vacuum-packed samples (TREATS 2 and 3)
differed only by the addition of 0,5% L(+) ascorbic acid to the TREAT 3
samples. TREAT 4 represented aerobically packed samples to which a
commercial 'colour retainer' was added. The effects of these treatments
on the bacterial population in the samples was studied after storage
intervals of one, two, four, seven, 11 and 17 days.

The numbers of aerobic bacteria, Enterobacteriaceae, lactic acid bacteria,
Brochothrix thermosphacta, psychrotrophic bacteria and several indirect
determinations of pseudomonads were used to assess the microbiological
quality of the minced beef. Determinations of pH, redox potential and
lactic acid concentration were used in conjunction with microbial levels
and evaluations of physical and sensory characteristics to determine
shelf life of the raw minced beef under various conditions of storage
and treatment. One hundred and twenty eight representative psychrotrophic
spoilage isolates were identified in conjunction with seventy one
selectively isolated lactic acid bacteria.

Results indicated that lowered storage temperatures produced significant
increases in the shelf life of the minced beef as was evident by
significant reductions in the levels of most spoilage microorganisms.
In addition it was demonstrated that all forms of packaging and/or
additive treatment were greatly aided in their ability to prolong shelf
life by a lower storage temperature. This finding led to the conclusion
that temperature control around 0°C was the central element in achieving
shelf life extension of a highly perishable product such as raw minced
beef. Packaging and additive treatments enhanced the effect of low
storage temperatures. Compared to aerobic packaging (control), all
other treatments led to substantial increases in shelf life, especially
when samples were stored at 0°C.

Identification of psychrotrophic spoilage isolates revealed a predominance of Gram-negative organisms (63,3%), most of which were classified as pseudomonads (71,5%), the rest being Enterobacteriaceae. Among the Gram-positive isolates, lactobacilli predominated (44,7%), with the rest belonging to the coryneform group and the genera *Kurthia, Micrococcus* and *Streptococcus*.

Identification of lactic acid bacteria isolates revealed the presence of three predominant species, *Lactobacillus saké* (33,8%), *Lactobacillus curvatus* (22,5%) and *Lactobacillus bavaricus* (21,1%). Various other *Lactobacillus* and *Leuconostoc* species made up the remainder of isolates in small proportions.

Commonly observed oxygen relationships of typical meat spoilage bacteria were not found in this study. Pseudomonads proliferated even in vacuum packaged samples throughout the entire storage period, whilst lactic acid bacteria were also found in aerobically packed samples. These apparent anomalies could be attributed to the specific type of micro-environment prevailing in raw minced beef.

SAMEVATTING

BAKTERIOLOGIESE STUDIES OP DIE VERLENGING VAN DIE

RAKLEEFTYD VAN ROU GEMAALDE BEESVLEIS

deur

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SAMEVATTING

Tweehonderd-vier-en-dertig monsters rou, gemaalde beesvleis is by 0°C en 7°C vir 17 dae opgeberg. Die monsters is aan vier behandelings onderwerp, waarvan behandeling 1 (aerobe verpakking) die kontrole verteenwoordig het. Behandelings 2 en 3 is vakuum-verpak, met die verskil dat 0,5% L(+) askorbiensuur by behandeling 3 gevoeg is. Behandeling 4 is aëroob verpak na byvoeging van 'n kommersiele kleur-stabiliseerder. Die invloed van die behandelings op die bakteriese bevolking is na een, twee, drie, vier, sewe, 11 en 17 dae bestudeer.

Totale aërobiese plaattellings en die getalle van Enterobacteriaceae, melksuurbakterieë, *Brochothrix thermosphacta,* psigrotrofe asook verskeie indirekte bepalings vir pseudomonade is gebruik om die mikrobiologiese gehalte van die maalvleis te bepaal. Meting van pH, redokspotensiaal en melksuurkonsentrasie is in verbinding met mikrobiese tellings en evaluerings van fisiese en sensoriese eienskappe gebruik om rakleeftyd van die rou gemaalde beesvleis onder verskeie toestande van opberging en behandeling te bepaal. Eenhonderd-agt-en-twintig psigrotrofiese isolate en een-en-sewentig selektief-geïsoleerde melksuurbakterieë is geidentifiseer.

Resultate het getoon dat 'n lae opbergingstemperatuur beduidende verlengings in rakleeftyd van gemaalde beesvleis bewerkstellig het gepaardgaande met merkbare verminderings in die getalle van die belangrikste bederf-veroorsakende mikrobes. Daar is ook gevind dat alle vorms van verpakking en/of behandeling baie meer effektief was by 'n laer opbergingstemperatuur. Temperatuurbeheer rondom 0°C blyk dus van deurslaggewende belang in die verlenging van die rakleeftyd van 'n hoogs bederfbare produk soos rou maalvleis te wees. Die resultate het ook getoon dat verpakking en behandeling meer doeltreffend was in kombinasie met 'n lae opbergings-temperatuur. Daar is gevind dat alle verpakkings 'n beduidende verlenging van rakleeftyd kon teweegbring vergeleke met gewone aërobiese verpakking, veral by 'n opbergingstemperatuur van 0°C.

Identifikasie van psigrotrofiese isolate het 'n meerderheid van Gram-negatiewe organismes (63,3%) opgelewer, waarvan die meeste as pseudomonade (71,5%) en die res as Enterobacteriaceae geklassifiseer is. Die meeste van die Gram-positiewe isolate was lactobacilli (44,7%) en die oorblywende isolate is as coryneforme en lede van die genera *Kurthia, Micrococcus* en *Streptococcus* geïdentifiseer.

Identifikasie van die melksuurbakterieë het drie oorheersende spesies opgelewer, *Lactobacillus saké* (33,8%), *Lactobacillus curvatus* (22,5%) en *Lactobacillus bavaricus* (21,1%). Verskeie ander *Lactobacillus*-en *Leuconostoc*-spesies het die oorblywende persentasie verteenwoordig.

Tipiese vleisbederwende bakterieë het in hierdie ondersoek nie die verwagte verhoudings tot aërobiose en anaërobiose getoon nie. Pseudomonade het deurgaans 'n belangrike deel van die totale bakterie-populasie in vakuumverpakte monsters uitgemaak, terwyl melksuurbakterieë in groot getalle in aërobies-verpakte monsters voorgekom het. Hierdie oënskynlike teenstrydigheid met verwagte tendense kan moontlik aan die tipiese mikro-ekologie van rou maalvleis toegeskryf word.

ACKNOWLEDGEMENTS

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B.	*Brochothrix*
BTC	*Brochothrix thermophacta* count
ca.	circa (approximately)
CO	cytochrome oxidase
CV	coefficient of variation
D.F.	degrees of freedom
EC	Enterobacteriaceae count
EC-48	additional count to EC after incubation at room temperature for another 24 hours
EC-48-ox.	cytochrome oxidase positive fraction of EC-48
e.g.	for example
Eh	redox potential
et al.	and others
etc.	et cetera
Fig.	figure
g	gram
i.e.	that is
L.	*Lactobacillus*
LC	lactic acid bacteria count
LSD	least significant difference
P	probability
PC	psychrotrophic bacteria count
PC-ox.	cytochrome oxidase positive fraction of PC
p.p.m.	parts per million
r	coefficient of correlation
R-SQUARE	coefficient of determination
RUN	run (repetition of experiment)
spp.	species

STORE	storage time
TAPC	total aerobic plate count
TAPC-ox.	cytochrome oxidase positive fraction of TAPC
TEMP	storage temperature
TREAT	treatment
V	dependent variable (microbial count or analysis)
w/v	weight per volume
w/w	weight per weight
&	and
$\geq$	larger than or equal to
$<$	less than

CHAPTER 1

INTRODUCTION

Consumer selection of raw minced beef is based, primarily, upon appearance. Although the degree of leanness is an important factor, the bright, 'cherry-red' colour of fresh minced beef significantly affects its acceptability. The desirable characteristics of raw minced beef rapidly deteriorate, however, and various degrees of dis-colouration occur, sometimes within less than one day of retail display. Consequently, if the raw minced beef is not purchased and consumed within one or two days of retail display, the retailer can suffer losses.

Long shelf-life would mean continuous sales of products and profits to producers. A guaranteed extended shelf life would be a vital prerequisite for centralized production and country-wide distribution. However, microbes can ruin shelf life and, consequently, destroy profits. The question of how long a shelf life is necessary has always been debatable, but the reality of today's business world suggests that the longest possible shelf life should be sought.

Spoilage of refrigerated raw minced beef is primarily due to the proliferation and metabolic activities of psychrotrophic bacteria (Ayres, 1960; Greer & Jeremiah, 1980). Therefore efforts to extend the shelf life of this product should be directed at controlling its microbial load. The three best methods to achieve this are: (a) limiting contamination, (b) destroying contaminating microorganisms and (c) reducing the microbial growth rate.

The process of manufacturing minced beef involves grinding of cellular tissue. Bacteria normally present only on the surface of the meat are distributed by this process throughout the entire product. Ideal conditions for their multiplication are created by destructurization of the meat, resulting in increased availability of nutrients. Minced beef is not heated or otherwise processed to ensure the absence of pathogenic and spoilage microbes. Thus the microbiological quality depends firstly on the meat used for grinding, secondly the sanitary con-ditions and practices during preparation and thirdly, time and temperature

of processing and storage.   In fact, bacterial levels of minced beef are indicative of the product's history.

Establishment of centralized fabrication and distribution plants by many retail chains has resulted in mass production of fresh meat products in one location with little or no meat processing at the retail level. Therefore meat products such as minced beef are initially processed at locations some distance from the retail establishment where they are sold, making shelf life even more critical in view of long distribution pathways to warehouses or supermarkets.

Numerous studies (Seideman, Vanderzant, Smith, Hanna & Carpenter, 1976) have shown that vacuum-packaging of meat can reduce the growth and metabolic activities of aerobic meat spoilage bacteria and thus contribute to improved shelf life.   Under these conditions, lactic acid bacteria, particularly lactobacilli, became the predominant part of the microbial population, with a greatly reduced impact on appearance and palatability of the product.   Further shelf life extensions were attempted by packaging of meat products in modified gas atmospheres, but no clear-cut advantages over conventional vacuum-packaging were demonstrated (Christopher, Seideman, Carpenter, Smith & Vanderzant, 1979).

It was found, however, that in all studies of shelf life extension, temperature control was the most significant factor (Greer & Jeremiah, 1981).   In addition, and especially in the case of minced meats, satisfactory initial quality was rated as the prime determining factor of shelf life.   The combined effects of packaging, temperature control and any other treatment could only maintain, but not improve quality.

The largest proportion of research on shelf life extension of meat in general and raw minced beef in specific has been carried out in major producing countries such as the U.S.A. (Texas, Colorado and Maryland), Canada, Australia and New Zealand.   Despite South Africa's predominant role as a meat producer in Africa and an exporter to markets such as the European Economic Community (EEC), comparatively little local research on shelf life extension of refrigerated meats and especially raw minced beef has been undertaken.

Despite modern advances in sanitation and refrigeration there appears to be no evidence that South African consumers are protected against minced beef of inferior quality, especially when bacterial levels are taken as a criterion of quality. The aim of this study was to establish by bacteriological investigations the combined impacts of packaging methods, additive treatments and storage temperature on shelf life extension of raw minced beef in the South African context.

CHAPTER 2

LITERATURE REVIEW

2.1 Early research on minced meat spoilage

2.1.1 General

It was recognized in the early years of the twentieth century that ground, minced or comminuted meats ("hamburger") provided a highly favourable environment for the multiplication of bacteria. Fragmentation of tissues with the liberation of cell juices and the intimate mixing of the bacteria normally found on the meat surface with the macerated tissues resulted in a product subject to rapid modification by microbial action.

The bacterial population in minced meat is dependent on three main factors: bacteriological quality of the meats used for grinding; cleanliness of equipment contacting the meat before, during and after grinding; and time and temperature of storage. The number of microbes in market samples of minced meat were clearly indicative of the history of the product (Rogers & McCleskey, 1957).

The obvious relationship of bacterial numbers to quality led to several early microbiological investigations on minced meats. Weinzirl and Newton (1914) suggested that a maximum of 10 000 000 organisms per gram determined at 20°C be allowed in minced beef. They concluded, however, that there was no close agreement between the actual number of bacteria and the degree of spoilage. It was found that the determination of mere numbers of bacteria in meat had little significance by itself (Carey, 1916), but it was suggested by Lefevre (1917) that the bacterial count could be of value in detecting the use of improper raw materials and defects in handling of a product. Researchers in years to follow Lefevre's work derived numerous so-called spoilage levels for minced beef, most of which were scattered around the 1 000 000 to 10 000 000 bacteria per gram range. An example is shown in Fig. 2.1.

Fig. 2.1   Rates of growth of total bacteria on beef stored
at different temperatures (Ayres, 1960)
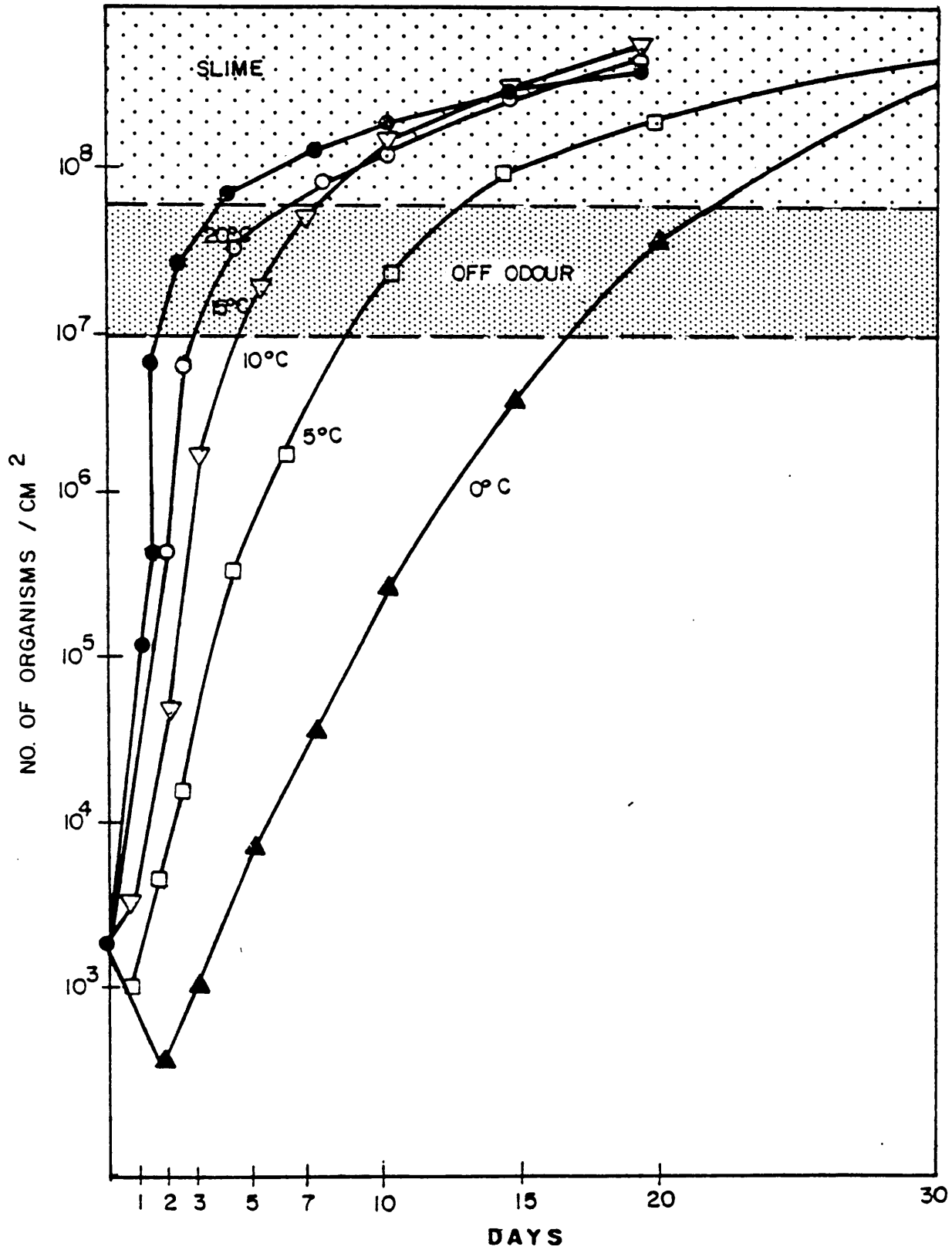
Kirsch, Berry, Baldwin and Foster (1952) determined the microbial population and shelf life at low storage temperatures of minced beef purchased from local American meat markets. They concluded that the hamburger products investigated were little, if any, better in quality than those investigated in the very early part of the twentieth century. Rogers and McCleskey (1957) were one of the earliest researchers to start placing emphasis on the presence and enumeration of coliform bacteria in meats, although no clear-cut evidence as to their role in minced beef spoilage was produced.

Halleck, Ball and Stier (1958) were the first researchers to conduct comprehensive studies on the effect of different packaging films on the shelf life of ground lamb at storage temperatures between 0 and 4°C. They also made use of differential media and special reagents to detect lipolytic, proteolytic and cytochrome oxidase-positive spoilage organisms, correlating their numbers with total bacterial populations. These researchers were the first to utilize the technique of artificial inoculation or 'spiking' of samples with known spoilage bacteria to simulate accelerated spoilage experiments. They found that storage temperature and time, packaging material and initial bacterial counts had definite effects on the shelf life of packaged meats. Furthermore, types of spoilage bacteria were found to differ, being of the souring type (lactic acid bacteria) for the oxygen impermeable films and the putrefying type (pseudomonads) for permeable films.

Murray, Kittaka and Ordal (1962) continued on evaluations of films with different permeabilities for packaging of minced beef stored at -1°C and 4°C. Bacteriological and organoleptic data indicated minced beef to retain acceptability for a longer period when packaged in gas-impermeable films ('Saran') and, likewise acceptability was maintained longer with storage at -1°C than at 4°C.

2.1.2 Taxonomy of spoilage bacteria

From the early years of the twentieth century, researchers have coupled bacteriological and shelf life studies on whole and minced meat with the systematics of predominant bacteria.

Glage (1901) reported that moist surfaces of meat stored at low temperature and high humidity became covered with bacterial colonies. These organisms, which he called 'Aromabakterien', were motile aerobes, growing well at 2°C but poorly at 37°C. Haines (1933) reported that with very few exceptions the bacteria growing on meat stored at 0 to 4°C all belonged to the *Achromobacter* group. At the same time, but independently, Empey and Vickery (1933) observed that 95% of the initial bacterial population of beef capable of growth at -1°C consisted of members of the genus *Achromobacter*; the remainder were species of *Pseudomonas* and *Micrococcus*. During storage, the relative numbers of *Achromobacter* and *Pseudomonas* increased, while those of *Micrococcus* decreased.

Empey and Scott (1939) considered the four principal genera of low temperature bacteria isolated from fresh meat to be: *Achromobacter*, 90%; *Micrococcus*, 7%; *Flavobacterium*, 3%; and *Pseudomonas*, less than 1%. In later years, Ayres, Ogilvy and Steward (1950), Kirsch *et al.* (1952) and Wolin, Evans and Niven (1957) reported that species of *Pseudomonas* were prominant. The last three groups of workers recognized that the discrepancy between their results and those of the previous workers resulted from changes in nomenclature used in the Sixth Edition of Bergey's Manual of Determinative Bacteriology (Breed, Murray & Smith, 1948) from that adopted in the Third Edition.

More recently, Brown and Weidemann (1958) reassessed the taxonomy of some 129 'psychrophilic' meat spoilage bacteria isolated by earlier workers and concluded that almost all of these were *Pseudomonas* species. Of 182 Gram-negative organisms studied, 170 (or 93%) were found to be species of *Pseudomonas*. Similar trends were discovered by Halleck *et al*. (1958), who reported that after 14 days storage of minced meat at 0 to 4°C, *Lactobacillus* and non-pigmented organisms of the *Pseudomonas/Achromobacter* type predominated, whereas *Pseudomonas fluorescens* types became dominant later.

Ayres (1960) reported that microorganisms isolated from refrigerated sliced beef held at 10°C or lower, and responsible for slime production, were almost without exception pseudomonads. When the meat was held at 15°C or above, there was an equal incidence of *Pseudomonas* and *Micrococcus*.

2.2 Food-borne disease and minced meat

The marketing of minced and whole meat with minimal probabilities of causing food-borne disease requires increased emphasis on the application of acceptable hygiene practices. These practices concern animal husbandry, sanitation and worker hygiene throughout all stages of shipping, killing, butchering, packaging and retail distribution.

In response to concern over rising production and distribution costs the meat industry has turned to centralized cutting, packaging and distribution of retail products. Because of lengthened storage times and increased opportunity for temperature abuse, however, centralized systems could promote deteriorative changes and development of health hazards in fresh, especially minced, meats.

Several studies have been conducted in conjunction with shelf life assessments on raw minced meat to determine the presence and behaviour of selected, ubiquitous food-borne pathogens.

Goepfert and Kim (1975) inoculated several strains of *Escherichia coli*, enterococci, salmonellae, staphylococci, *Bacillus cereus* and *Clostridium perfringens* into raw minced beef and observed changes in population levels at various storage temperatures, ranging from 1 to 12,5°C. It was demonstrated that food-borne pathogens were unable to compete effectively with natural spoilage bacteria of raw minced beef over a wide range of refrigeration temperatures.

Recent research demonstrated low-level incidence of salmonellas in minced meat (Surkiewicz, Harris, Elliott, Macaluso & Strand, 1975; Pivnick, Erdman, Collins-Thompson, Roberts, Johnson, Conley, Lachapelle, Purvis, Foster & Milling, 1976; Abbott & Robertson, 1980). Despite the low incidence and the fact that salmonellae in minced meat can be killed by adequate cooking, the occasional potential hazard of cross-contamination between raw and cooked foods is still to be emphasized.

Coagulase-positive staphylococci associated with food-borne intoxications were isolated from raw minced meat in numerous surveys (Duitschaever, Arnott & Bullock, 1973; Surciewicz *et al.*, 1975; Daly & Morrissey, 1976; Duitschaever *et al.*, 1977; Sumner, 1978; Sumner, Reay & Perry, 1979). The same researchers emphasized the presence of *C. perfringens* and *E. coli* in a large proportion of samples analysed by them. Controlled atmosphere packaging did not increase food-borne health hazards and should in fact reduce the risk of salmonellosis in moderately abused fresh meats (Silliker & Wolfe, 1980; Luiten, Marchello & Dryden, 1982).

The only dramatic observation relating to a possible food-borne health hazard recorded development of high numbers of *Yersinia enterocolytica*-like organisms on vacuum packaged beef and lamb at refrigeration temperatures (Hanna, Zink, Carpenter & Vanderzant, 1976). Whether this growth was selectively and specifically favoured by vacuum packaging was uncertain, making the public health significance of the observation unknown. The described finding was in keeping with reports of active growth of *Y. enterocolytica* on beef and pork at refrigeration temperatures (Lee, 1977). Isolated instances of growth and possible toxin production at low temperature (minimum 3,3°C) by *Clostridium botulinum* types E and F were also reported (Schmidt, Lechowich & Folinazzo, 1961; William-Walls, 1968).

The fact that, generally, food-borne disease related to raw minced meat is of little significance was supported by figures from the U.S. Centre of Disease Control (1975), attributing 3.6% (66) of 1827 food-borne disease outbreaks to minced meats. All of these could be traced to a specific post-purchase food handling error.

Despite reports that food-borne pathogens were found at often excessive levels in a variety of surveys on wholesale and retail raw minced meat (Holland, 1979), the report of an FAO/WHO working group on microbiological criteria for foods (1979) concluded that limits for pathogenic organisms should not be used for raw meats as they would not help to protect the consumer.

As emphasized at the beginning of the chapter, strictest temperature control (never exceeding 10°C) and adequate care in manufacturing, transportation and handling would go a long way to keep food-borne health hazards to a minimum.   This has to be followed by similar post-purchase care, excluding all suspect samples from consumption (Ali & van Dyne, 1981).

2.3 Microbiological standards and surveys of retail minced meat

The microbiological problems related to meats, whether raw or processed, may be resolved into two main areas: spoilage and public health hazards.   Therefore closely allied to assessments of food-borne health hazards on raw minced beef is the question of general bacterial standards, especially for retail purposes. Despite the benefits claimed for microbiological standards of raw minced beef (e.g. longer shelf life and reduced potential health hazards), establishment and legal enforcement of such standards gave rise to considerable controversy (Holland, 1979).

The centre of this controversy were the Oregon meat bacterial criteria, established as standards in May, 1973 and revised to criteria in October, 1977 (Sumner, Reay & Perry, 1979). These criteria of 1973 stated, that in Oregon the sale of ground beef with either an aerobic plate count in excess of 5 000 000/g or *Escherichia coli* in excess of 50/g became a criminal offence.   Numerous surveys on a worldwide basis were undertaken following the Oregon-criteria and their results were often used as guidelines for establishment of further criteria. Results of some of the more recent surveys are given in Table 2.1.

Generally speaking, suggested standards for total aerobic bacterial counts in minced beef varied from 100 000 to 10 000 000 per gram and suggested coliform standards ranged from 0 to 200 per gram.   Various limits set by different countries are summarized in Table 2.2.

Internationally, numerous authors indicated extreme difficulties with compliance to these guidelines by retail minced meat (Law, Yang & Mullins, 1971; Duitschaever, Arnott & Bullock, 1973 and 1977; Daly & Morrissey, 1976; Sumner, Reay & Perry, 1979; Holzapfel, 1980) and Duitschaever *et al.* (1973) concluded that, generally, minced meat quality was similar to that reported in investigations as far back as 1914!

Table 2.1   Survey summary of the hygienic status of ground beef based on results from different workers (Sumner, Reay & Perry, 1979)

Authors	Date	Location	Incubation Temp. °C	Percentage samples exceeding aerobic plate count of standard
Duitschaever *et al.*	1973	Ontario, Canada	32	$64\% - 1 \times 10^7$
Al-Delaimy & Stiles	1975	Alberta, Canada	32	$41\% - 1 \times 10^7$
Pivnick *et al.*	1976	Canada	21	$44\% - 1 \times 10^7$
		Canada	35	$12\% - 1 \times 10^7$
Duitschaever *et al.*	1977	Ontario, Canada	21	$91\% - 5 \times 10^6$
Chambers *et al.*	1976	Ohio, U.S.A.	32	$30\% - 1 \times 10^7$
Emswiler *et al.*	1976	Maryland, U.S.A.	35	$6\% - 1 \times 10^6$
Goepfert	1976	National, U.S.A.	20-22	$39\% - 5 \times 10^6$
Shoup & Oblinger	1976	Florida, U.S.A.	22	$50\% - 1 \times 10^7$
		Florida, U.S.A.	35	$35\% - 1 \times 10^7$
Westhoff & Feldstein	1976	Maryland, U.S.A.	28	$50\% - 1 \times 10^7$
		Maryland, U.S.A.	35	$18\% - 1 \times 10^7$
Field *et al.*	1977	Wyoming, U.S.A.	35	$10\% - 1 \times 10^7$
Foster *et al.*	1977	California, U.S.A.	32	$10\% - 1 \times 10^7$
Daly *et al.*	1976	Cork, Eire	30	$80\% - 1 \times 10^7$
Karim	1977	Tehran, Iran	37	$17\% - 1 \times 10^7$
Sumner	1978	Izmir, Turkey	25	$60\% - 1 \times 10^7$
Sumner *et al.*	1979	Christchurch, N.Z.	25	$32\% - 1 \times 10^7$

Table 2.2   Some microbiological limits for raw minced meat
(Sumner *et al*., 1979;   Holzapfel, 1980)

Country	Aerobic plate count per gram	Coliform count per gram	Status of limits
Australia	$1 - 10 \times 10^6$	-	guideline
Canada	$1 - 5 \times 10^7$	100(44°C)	guideline
Finland	1×10^8	1 000(44°C)	proposed guideline
France	5×10^5	100(faecal)	-
Massachusetts, U.S.A.	1×10^5	100	guideline
Oregon, U.S.A.	5×10^6	50(44°C)	guideline
Rhode Island U.S.A.	1×10^6	100	guideline
Sweden	1×10^8	10 000(44°C)	guideline

More recent research indicated that mandatory or legal micro-
biological standards on raw minced beef at retail level were
considered unnecessary and often undesirable (Holland, 1979;
Winslow, 1979), although the establishment of guidelines was
seen in a more favourable light.   Guidelines pertaining to
levels of *Escherichia coli* were generally regarded as superfluous,
because of the organism's doubtful usefulness as an indicator of
quality, faecal contamination and presence of other pathogens
in raw minced beef (Goepfert & Kim, 1975; FAO/WHO working
group report, 1979).   Even the use of the aerobic plate count
as an indicator of minced meat quality was questioned because
it did not show a consistent relationship to food safety (Daly
& Morrissey, 1976).   Also, no correlation was found between
microbiological quality and retail store sanitary conditions
(Wyatt & Guy, 1980; Greer & Jeremiah, 1980).

An important calculation emanated from a study by Holland (1979),
stating that a mandatory standard of an aerobic plate count of
10^7/g could remove in excess of 33% of minced beef sold at retail
level in Canada.   This placed a further question mark on the
applicability of mandatory standards in the currently experienced
era of protein shortage.   The same author concluded that temperature
control was probably one of the most significant quality standards
for minced beef and all raw meat in general, a view which was
supported by Holzapfel (1980).

Both previous authors felt that strict temperature control through
all facets of production, transportation and retail handling would
contribute to optimal quality of minced meat available to the
consumer.   This seemed a most realistic approach in view of the
fact that production of sterile minced beef is impossible, the
end product being an amalgam of the hygienic status and care of
handling from 'the hoof to the home' (Thatcher, 1974).

In the South African context, strict legislation has been in
force for many years to provide for maintenance of adequate
hygiene standards in the slaughtering of animals and the
handling of raw meats (Act No. 87, 1967; Act No. 54, 1972).

2.4   Review of topical research on psychrotrophic meat spoilage

Emphasis in research approaches to raw minced meat products
has shown considerable shifts in more recent years as spoilage
at low temperatures is probably one of the most important
facets.

The recognition of this fact led to a more fundamental approach
by many workers.   Investigations were predominantly on (a)
contamination patterns of raw minced meat from production to
sale, (b) temperature control and its relationship to quality,
(c) the development of the psychrotrophic concept and (d)
interrelationships and nutritional requirements of specific
spoilage groups.   Unfortunately, much of this research was
aimed at the study of intact meat in the form of wholesale
cuts, for example.   The direct applicability of such results
to minced meat could be questionable, although one would
expect the major patterns to apply also in the case of minced
meat.

The large-scale establishment of centralized fabrication and
distribution plants by many retail chains internationally
resulted in the processing of chopped or minced beef at
locations some distance from the retail establishment
(Berry & Chen, 1976).   It was long established that chopping
and grinding enabled bacteria present on the meat surface to
be distributed throughout the product (Rogers & McClesky, 1957).
In addition to this, Maxcy (1981) concluded that several factors
in the micro-environment of chilled minced meat determined
further penetration and spoilage patterns of initial surface
contamination.   To a certain extent, it appeared that such
selective effects already started at the abattoir (Newton,
Harrison & Wauters, 1978), the hide of the animal and the
carcass wash water being the principal sources of mesophilic
and psychrotrophic spoilage organisms.

Newton *et al.* (1978) re-emphasized earlier observations that
most psychrotrophic contaminants initially stemmed from soil,
water and vegetation as well as faecal material, but also
from walls and floors in chillers, cutting boards in boning
rooms and the like. Their results reinforced the belief
that the bacterial load on hides had an overwhelming influence
on that of the carcasses after dressing. It was thus
recognized as vitally important that abattoir hygiene
constituted the first determining factor of the microbiological
quality and shelf life prospect of chilled meats.

It was recognized by Grau and Macfarlane (1980) that the three
fundamental facets in extending shelf life of chilled meats
were the following: (a) limiting contamination (b) destroying
contaminating microorganisms, and (c) reducing the microbial
growth rate. Newton *et al.* (1978) stated that microbiological
quality of meat produced at the abattoir was relatively good,
and that further efforts in decreasing initial contamination
would not be commensurate with the minimal additional extension
of refrigerated storage life they achieved. Grau and
Macfarlane (1980) continued by recognizing that destruction of
microorganisms on the product was limited by the necessity to
maintain the meat in its 'fresh-like' condition. Reduction
of the microbial growth rate, therefore provided by far the
most promising method of increasing shelf life of refrigerated
meats. This they substantiated by an interesting calculation:
A 99% reduction of the initial bacterial load on mutton
carcasses was required to increase shelf life by about one
third. The same increase in shelf life was also achieved by
a one third reduction in growth rate of the spoilage bacteria.

This was already established by Scott (1937), who found that for the most significant psychrotrophic spoilage organisms under aerobic conditions, a lowering in storage temperature from 2°C to -1°C reduced growth rates by about 50%. These views received increasing support from quality assurance managers, sanitarians and executives involved in the meat industry in America, New Zealand, Australia and Ireland. Apart from factors such as Good Manufacturing Practices (GMP's), sanitation and good quality raw materials, temperature control and improvements in its strict implementation, received wide-spread publicity as major profit boosters (Del Giudice, 1982). Several investigations supporting this view were carried out in recent years (Greer & Jeremiah, 1980 and 1981; Greer, 1981). These studies emphasized the observation that even very small increments in temperature within the range of 0 to 7°C had a pronounced effect on psychrotrophic bacterial spoilage through exponential increases in growth rate.

In addition to temperature control, investigations on packaging methods and atmospheres and their contribution to low temperature shelf life increases of meats have dominated the research field. Packaging studies were first pre-empted by the observations that wrapping meat products with films of low water and high gas permeability did maintain a desirable bright red oxymyoglobin colour, but was also ideal for rapid growth of aerobic psychrotrophic spoilage organisms. Therefore, microbiologically speaking, shelf life was short. In most cases, shelf life was first limited to colour changes, the red oxymyoglobin being converted to the brown metmyoglobin by oxidation. This change was found to be accelerated by microbial growth lowering the oxygen tension at the meat surface (Grau, 1978).

In recent years, vacuum packaging started to play a prominent role in distribution of refrigerated fresh meats. Various studies showed that vacuum-packaging of meats could suppress the growth and metabolic activity of common psychrotrophic and mesophilic Gram-negative, aerobic meat spoilage bacteria and hence lead to extensions of low temperature storage life (Seideman, Carpenter, Smith & Hoke, 1976; Seideman, Vanderzant, Smith, Hanna & Carpenter, 1976). Additional advantages for vacuum packaging were reduction in weight loss due to dehydration, preservation of muscle colour and elimination of external contamination (Seideman *et al.*, 1976). The principle of the method involved use of a barrier film with low permeability to oxygen, which prevented re-entry of oxygen after evacuation of the air, and heat sealing (Christopher, Vanderzant, Carpenter & Smith, 1979). The residual oxygen was converted to carbon dioxide, possibly by meat tissue respiration and microbial activity. The gaseous environment in the package, primarily the presence of carbon dioxide which could comprise 20 to 30% of the final gas volume, was found to be responsible for suppression of growth of common spoilage bacteria such as *Pseudomonas*, and allowed development of facultative aerobes such as lactic acid bacteria. In some instances, Enterobacteriaceae also constituted a significant part of the spoilage organisms (Seideman, Vanderzant, Hanna, Carpenter & Smith, 1976). The rate of development of the spoilage bacteria depended on meat storage temperature. The inhibitory effect of carbon dioxide on growth and development of common aerobic, psychrotrophic Gram-negative bacteria was found to be due to suppression of decarboxylating enzymes, especially isocitric and malate dehydrogenases (King & Nagel, 1975). In addition, carbon dioxide was discovered to alter cell membrane fluidity and hence functional properties such as permeability and transport (Enfors & Molin, 1978).

However, some disadvantages to vacuum packaging were reported
like (a) economic losses associated with purge (meat fluid
exuding from cut muscle surfaces), (b) distortion of cuts,
and (c) the reduced myoglobin colour of vacuum packaged meat
which was generally considered to be unacceptable to the
consumer (Jeremiah, Carpenter & Smith, 1972). As physical
appearance and colour of retail meat were recognized to be
two of the most important attributes consumers used to select
and purchase, alternative methods of packaging had to be
looked for (Hall, Smith, Dill, Carpenter & Vanderzant, 1980).
Conventional packaging was compared with modified gas
atmosphere-packaging in numerous subsequent studies
(Christopher, Hall, Smith, Dill, Carpenter & Vanderzant, 1980;
Partmann, 1980; Seideman, Vanderzant, Smith, Dill & Carpenter,
1980). In these packaging methods the gas in the packages
was replaced with various porportional mixtures of carbon
dioxide, oxygen and nitrogen. In theory, carbon dioxide
was expected to inhibit aerobic spoilage bacteria, while the
maintenance of a supply of oxygen should keep the pigments
on the surface of the meat oxygenated. The effect of a
'filler' gas (nitrogen) on bacterial development was and is
still not certain (Christopher, Seideman, Carpenter, Smith
& Vanderzant, 1979). Based on microbiological criteria,
storage of refrigerated meats in modified gas atmospheres failed
to show up specific advantages over storage in conventional
vacuum packages (Christopher, Vanderzant, Carpenter & Smith,
1979). Specially differences in psychrotrophic and lactic
acid bacterial counts were rarely statistically significant,
although *Pseudomonas* species continued to persist to a
greater degree in vacuum packaged samples than in samples
stored in carbon dioxide/nitrogen atmospheres (Christopher,
Smith, Dill, Carpenter & Vanderzant, 1980).

The best success rating was awarded to packaging meat in an atmosphere of pure carbon dioxide, the inhibitory effect of which on spoilage bacteria persisted after opening of the package and storage of the meat at low temperature (Partmann, 1980).   Enfors, Molin and Ternström (1979) concluded that the clear predominance of lactic acid bacteria directly reinforced the shelf life prolonging effect of carbon dioxide.   Thus, carbon dioxide primarily inhibited the growth of carbon dioxide-sensitive organisms, i.e. *Pseudomonas* species (Brandt & Ledford, 1982).   This gave the more carbon dioxide-resistant lactic acid bacteria a chance to develop.   By virtue of their antagonistic properties, the lactic acid bacteria then inhibited development of other carbon dioxide-resistant organisms, such as *Brochothrix thermosphacta*, which, compared to the lactic acid bacteria, have the more deleterious effect on the meats (Roth & Clark, 1975; Collins-Thompson & Lopez, 1980).

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2.5 Pure culture investigations on predominant organisms and accelerated spoilage experiments

Research on fresh minced meat demonstrated the microbial population to be essentially heterogenuous and resulting from external contamination (Newton *et al.*, 1978). This population became more homogenuous as the meat underwent spoilage, its composition depending upon conditions of storage, chiefly atmosphere of packaging and storage temperature. It was found that several bacterial groups were predominant during low temperature spoilage of whole and minced meats, these being mainly species of the genus *Pseudomonas* under aerobic conditions, and lactic acid bacteria (species of *Lactobacillus* and *Leuconostoc*) under vacuum and modified gas atmosphere packaging conditions (Christopher *et al.*, 1979; Enfors *et al.*, 1979). Special relevance in spoilage of meats was attached to a relatively recently discovered organism, *Brochothrix* (B.) *thermosphacta* (earlier named *Microbacterium thermosphactum*), (McLean & Sulzbacher, 1953; Roth & Clark, 1972).

Taxonomic studies on psychrotrophic meat spoilage organisms led to speculations that the quantitatively predominant contaminants might not necessarily be the critical ones for spoilage. This was followed by several accelerated spoilage experiments, utilizing pure cultures of known meat spoilage bacteria which were often also psychrotrophic in nature (Ingram & Dainty, 1971; Enfors *et al.*, 1979). The role of *B. thermosphacta* in low temperature spoilage of meats was extensively investigated by several research teams. The organism was isolated and identified for the first time by McLean and Sulzbacher (1953) from pork sausages showing severe flavour deterioration. Its nutritional requirements were studied by Grau (1979) who found that they correspond to vitamin and amino acid requirements of the Lactobacillaceae into which the organism was re-classified (Sneath & Jones, 1976). Also in Australia, Stanley, Shaw and Egan (1981) studied the volatile compounds produced in the spoilage of vacuum-packed, sliced luncheon meats 'spiked' with *B. thermosphacta* at 5°C under aerobic conditions and found acetoin and diacetyl to be of major sensory significance. In similar experiments by

Dainty and Hibbard (1980), acetoin, acetic, isobutyric and isovaleric acid were identified as the major end-products of metabolism at 1°C and 20°C. Under anaerobic conditions, lactic acid appeared to be the major end-product of glucose fermentation. The relative spoilage potentials of B. *thermosphacta* and lactobacilli at 5°C on vacuum packaged sliced luncheon meats were studied by Egan, Ford and Shay (1980). They reported that B. *thermosphacta* caused rapid spoilage accompanied by 'off'-aromas at levels of 10^8/g. In contrast, the lactobacilli caused very slow spoilage and only some time after they had reached population levels of 10^8/g.

Further investigations on interrelationships between B. *thermosphacta* and other lactic acid bacteria at low temperatures and under anaerobic conditions were conducted by Collins-Thompson and Lopez (1980). Results showed that lactobacilli (chiefly L. *brevis* and L. *plantarum*) displayed antagonism towards B. *thermosphacta*, and actively contributed to limiting its growth. Results of a study undertaken by Grau (1980) indicated that L(+) lactic acid itself was an inhibitor to the anaerobic growth of B. *thermosphacta*, the concentration of undissociated lactic acid being the governing factor. This could mean that the lactate naturally present in meat could act as a pH-dependent inhibitor of anaerobic growth of B. *thermosphacta*. In a supplementary study along the above lines, Van Netten and Mossel (1980) described some ecological consequences of deliberately decontaminating raw meat surfaces with lactic acid. They found that treatment of the skin of freshly slaughtered pigs 'spiked' with Enterobacteriaceae (from pig colon) with a 2% solution of lactic acid (pH 2,5) achieved two to three log cycles reduction in Enterobacteriaceae counts with no apparent damage to the lactobacilli.

The influence of lactic culture-'spiking' on minced beef quality was investigated by Reddy, Hendrickson and Olson (1970). Their results indicated that a 10% inoculum of pure and mixed lactic cultures had a pronounced inhibitory effect on the Gram-negative bacteria in minced beef, and combined with 450 parts per million ascorbic acid even resulted in desirable organoleptic characteristics of the minced beef after seven days storage at 5°C. The addition of pure lactic acid was also found to inhibit microbial growth, but caused an undesirable colour and aroma. Follow-up studies (Hanna, Hall, Smith & Vanderzant, 1980; Smith, Hall & Vanderzant, 1980) indicated that inoculation of beef steaks with *Lactobacillus* species before vacuum packaging and storage at 1 and 3°C inhibited the growth of Gram-negative psychrotrophs during the first two weeks of storage. Parallel studies on other meat quality characteristics indicated, however, that the disadvantages of *Lactobacillus*-inoculation outweighed its advantages.

Several studies were performed on aerobic members of low temperature meat spoilage bacteria. Gill and Newton (1977) performed substrate utilization studies on two *Pseudomonas* species and one species each of *Acinetobacter, Enterobacter* and *B. thermosphacta* under aerobic conditions. The conclusion was reached that the species with the fastest growth rate would dominate the spoilage population, provided that all species had the same initial cell density. Also, it was noted that generation times of the pseudomonads were about 30% less than those of other species, giving these organisms a clear advantage during growth in mixed cultures. It was evident from the results that maximum cell density of an aerobic spoilage culture was in no small measure determined by the rate of oxygen availability to the cells.

Gill and Tan (1980) investigated the effect of carbon dioxide on growth of meat spoilage bacteria. The results reinforced earlier observations that *Enterobacter* and *B. thermosphacta* were unaffected by carbon dioxide. Inhibition on a fluorescent and non-fluorescent *Pseudomonas, Alteromonas putrefaciens* and *Yersinia enterocolytica* was incomplete, reaching maximum levels at low carbon dioxide concentrations.

For *Acinetobacter*, inhibition continued to increase with rising
carbon dioxide concentration.   In Ireland, Morrissey, Buckley
and Daly (1980) studied the effect of four species of spoilage
bacteria on minced beef stored at 7°C.   Results showed that
a non-pigmented *Pseudomonas* and *Pseudomonas fluorescens* produced
significant extracellular proteolytic activity when reaching
levels of 10^8/g.   *Enterobacter aerogenes* maintained an
intermediate and *Micrococcus* a low spoilage profile.

2.6   Rapid tests for evaluating meat quality during storage

A number of non-microbiological methods for evaluating microbial spoilage or predicting the remaining shelf life of refrigerated meats and meat products have been suggested.   These were aimed at obviating time-consuming procedures which often only gave results once the shelf life was already exceeded.   Also, for many meat products, including vacuum packaged fresh meat products, there appeared to be no clear correlation of total bacterial count with spoilage.

A promising dye reduction method utilizing methylene blue for estimating bacterial counts and spoilage in minced beef was investigated (Emswiler, Kotula, Chesnut & Young, 1976).   The data indicated that methylene blue reduction was an effective rapid method to make assessments of total bacterial and psychrotroph levels in minced beef.   However, further evaluations would be required to make the method commercially utilizable.

Release of aqueous extracts by beef homogenates (a phenomenon later called extract release volume or ERV) was studied by Jay (1964).   Results showed that ERV values of fresh beef were larger than those for spoiled beef at all temperatures and pH values between 5,0 and 5,8, but that the commercial applicability of the method was doubtful.

Seven rapid analytical tests for evaluating alterations in the quality of intact meat during refrigerated storage were investigated (Strange, Benedict, Smith & Swift, 1977).   It was found that only colour values and 'tyrosine' values provided effective monitoring of bacterial contamination.   Under the conditions of the study, thiobarbituric acid number, ERV, pH, pH_t and redox potential were rated as ineffective monitors of quality and shelf life.

Applicability of the *Limulus* amoebocyte lysate (LAL) test for
rapid estimation of microbial numbers in fresh ground beef was
investigated by Jay (1981). Results suggested that the LAL
test was workable to produce an estimate of aerobic plate
counts in one hour, although, for quality control purposes,
high results by the LAL test might need to be supplemented by
conventional methods.

The only rapid method of microbiological nature was produced by
Greer (1981), whose results showed a possible drastic time
reduction in the enumeration of psychrotrophs. It appeared
that incubation at 25°C for 24 hours (Plate Count Agar) resulted
in no loss of precision when compared to the conventional
incubation method of 10 days at 7°C. Moreover, the initial
bacterial load as determined by 24 hours of incubation at 25°C,
seemed to be directly related to retail case life of steaks.
Further investigation of this method could therefore be most
profitable to the meat industry, with the possible aim of
developing it into a routine quality assurance and shelf life
testing procedure.

To date, none of the rapid methods proposed were found suitable
for all types of refrigerated meats and meat products, since
they could at best only determine very limited aspects of the
total spoilage process. In addition to this, the analytical
chemical methods suffered from the handicap that considerable
variation in the measured parameter occurred between different
meats even before any microbial growth had occurred. Also,
significant changes in the measured parameter often occurred
only once spoilage was well advanced (Grau & Macfarlane, 1980)

2.7 <u>Miscellaneous treatments aimed at curtailing spoilage of</u>
<u>refrigerated meats</u>

In the last five years, several evaluations of other rather
unusual treatments aimed at increasing shelf life of
refrigerated meats were conducted.

Shelef (1977) investigated the effect of glucose addition to
ground beef stored at refrigeration temperatures.   Results
were most promising, indicating a possible shelf life increase
from five to eight to ten days by addition of 2% glucose.
The added glucose appeared to cause a shift in nutrient
utilization of the Gram-negative spoilage bacteria.   The
carbohydrate was used preferentially to other compounds (the
utilization of which normally led to alkaline by-products and
elevated the pH), and the oxidatively formed acids lowered the
meat pH, affected the growth rate of the spoilage flora and
hence retarded spoilage in the form of slime formation or
elevated pH values.

Conflicting results were obtained on bacterial growth in minced
beef prepared from electrically stimulated meat.   No effects
on *Lactobacillus, Acinetobacter, Pseudomonas, Moraxella* and
B. *thermosphacta* individually or in mixtures were observed by
Butler, Smith, Savell and Vanderzant (1981), whereas Mrigadat,
Smith, Dutson, Hall, Hanna and Vanderzant (1980) reported
consistently lower levels of *Pseudomonas putrefaciens* and a
Lactobacillus species in minced beef fabricated from electrically
stimulated carcasses.

No beneficial effects on shelf life increases were demonstrated
by refrigerated storage of meats in hypobaric environments
(Restaino & Hill, 1981), but radurization of prime beef cuts
by application of doses of 2 kilo - Gray (kGy) produced a
doubling of refrigerated-storage shelf life (Niemand, Van der
Linde & Holzapfel, 1981).

The increasing use of soy protein as an extender in minced
meat products destined for institutional feeding systems
prompted investigations into spoilage patterns at refrigeration
temperatures of some 'extended' products. It was found that
addition of textured soy protein (TSP) had no effect on the
total microbial load of regular minced beef, and that the
product did not present a public health hazard (Foster, Hunderfund,
Fowler, Fruin & Gutherz, 1978). Harrison, Melton and Draughton
(1981) reported, however that spoilage rates of TSP-extended
minced beef were faster than in regular minced beef.

CHAPTER 3

MATERIALS AND METHODS

3.1   Experimental design

Two blocks each of 120 samples of raw minced beef (200g) were subjected to various packaging and additive treatments.   These were kept for a total period of 17 days at storage temperatures of 0°C and 7°C.

Various bacteriological and chemical determinations were carried out on duplicate samples (referred to as internal replicates) at predetermined time intervals (see 3.2).

The entire experiment was carried out in duplicate for reasons of statistical viablility (referred to as Runs No. 1 and 2), and for each storage time and temperature two identical samples (internal replicates) were analysed by duplicate plating (single dilution series) for various bacterial groups.   Chemical determinations were not duplicated for each sample, but only carried out once on each replicate.

3.2   Diagrammatical layout of experiment

```
                  ┌─────────────────────────┐
                  │  30kg batch raw minced   │
                  │      beef per run        │
                  └─────────────────────────┘
          ┌───────────────┼───────────────┐
          ▼               │               ▼
 ┌──────────────────┐     │     ┌──────────────────┐
 │  56 small packs  │     │     │  56 small packs  │
 │   (200g each)    │     │     │   (200g each)    │
 └──────────────────┘     │     └──────────────────┘
          │     (various treatments,      │
          │      internal replicates)     │
          ▼                               ▼
 ┌──────────────────┐           ┌──────────────────┐
 │storage temperature│          │storage temperature│
 │       0°C        │           │       7°C        │
 └──────────────────┘           └──────────────────┘
                          │
                          ▼
                ┌─────────────────────┐
                │ Background analysis │
                │        DAY 0        │
              ┌─┴─────────────────────┴─┐
              │ Sample analysis on DAYS:│
              │ 1, 2, 3, 4, 7, 11 and 17│
              └─────────────────────────┘
                          │
                          ▼
```

┌───┐
│ Total aerobic plate count (TAPC)   │
├───┤
│ Psychrotrophic bacterial count (PC)   │
├───┤
│ Lactic acid bacteria count (LC)   │
├───┤
│ *Brochothrix thermosphacta* count (BTC)   │
├───┤
│ Enterobacteriaceae count (EC)   │
└───┘

Single dilution

Duplicate plating

- -

pH measurement	Eh measurement

Lactic acid determination

3.3   <u>Preparation of raw minced beef</u>

Two 30kg batches (one per run) of raw minced beef were prepared
(at different stages) from offcuts in the de-boning section of
an export abattoir during production.   A large commercial
mincer with an end-plate grid diameter of 0,5cm was used.
No special precautions to prevent normal microbiological con-
tamination during mincing, addition of additives, handling
and packaging of the meat were taken.   This was done to allow
the incorporation of a 'normal' bacterial population into the
minced beef, thus being able to simulate retail handling and
storage conditions during the course of the experiment.

3.4   <u>Treatment procedures</u>

Four distinct treatment processes consisting of different types
of packaging and/or additive addition were carried out on
samples of raw minced beef.   These are listed in Table 3.1
together with abbreviations assigned to them for statistical
analysis.

3.4.1 <u>Packaging</u>

Samples were vacuum-packaged in 200g portions by use of a
chamber-type, heat-seal vacuum-packaging machine (Multivac
AG-8) at the maximum capacity of the machine (747mm of Hg).
Barrier bags with the following characteristics were used
(Shorko SCX 26):

<u>Oxygen transmission rate (OTR)</u>   = 25cm^3/m^2/24 hours/atm
                                                 at 21°C, 44% RH.
<u>Carbon dioxide transmission rate (CTR)</u> = 65cm^3/m^2/24 hours/atm
                                                 at 21°C, 44% RH.

TABLE 3.1   DETAILS OF RAW MINCED BEEF TREATMENTS

TREATMENT DETAIL	STATISTICAL ANALYSIS ABBREVIATION
Aerobic packaging in freely gas-permeable wrapping (Resinite-RMF-S).	TREAT 1
Vacuum packaging in SCX-LDPE lamination (Shorko SCX 26).	TREAT 2
Vacuum packaging in SCX-LDPE lamination (Shorko SCX 26) with addition of 0,5%(w/w) L(+) ascorbic acid.	TREAT 3
Aerobic packaging in freely gas permeable wrapping (Resinite-RMF-S) with addition of 0,2%(w/w) commercial colour retainer (yielding 250 ppm SO_2).	TREAT 4

Water vapour transmission rate (WVTR) = 4,0g/m²/24 hours
                                           at 38°C, 90% RH.

The balance of samples were aerobically packed by wrapping
200g portions into freely gas permeable film with the
following characteristics (Resinite-RMF-S):

Oxygen transmission rate (OTR)   = 2700cm³/m²/24 hours/bar
                                           at 21°C, 44% RH.

Carbon dioxide transmission rate (CTR) = 1,5 to 2 times OTR

Water vapour transmission rate (WVTR) = 193g/m²/24 hours
                                           at 38°C, 90% RH.

3.4.2 Additives

Two types of additive were used for sample treatment.   For
TREAT 4, a commercial 'colour retainer' was employed.   This
compound was to be used at 3,6g per kg minced beef to yield
450 parts per million of SO_2, according to the manufacturer's
instructions.   Since addition of SO_2 to fresh meat is not
permitted by law, it was decided to use a reduced concentration
of the additive, namely 2g per kg of minced beef, yielding an
SO_2 concentration of 250 parts per million.   Samples treated
with the commercial 'colour retainer' were all wrapped in the
freely gas permeable Resinite-RMF-S film to test performance
of the additive under simulated retail-display conditions.

For TREAT 3, chemically pure, food-grade L(+) ascorbic acid
(Merck) was used at a concentration of 5g per kg minced
beef (final concentration 0,5%).   This level was somewhat
above the normal manufacturer's recommended level of 0,1 to
0,2% (for fruit, vegetable and mushroom preserves, sauerkraut
etc.).   Samples treated with L(+) ascorbic acid were all
vacuum packed in Shorko SCX 26 barrier bags to test for any
synergistic effects between the additive and conventional
vacuum packaging.

It was noted that L(+) ascorbic acid is chemically identical with naturally occurring vitamin C and could therefore be employed in almost all countries as a technical aid in the processing of foodstuffs without declaration of its presence and without its being subject to any quantitative restrictions. The relatively small quantities of ascorbic acid which were added, however, did not meet with the requirements normally placed on a vitamin-enriched foodstuff (Merck Information Brochure 21/II; H879/2.5/475). Minced meat processing with ascorbic acid as a technical aid as performed in this experiment, did not therefore, constitute a vitaminisation.

3.4.3 Sample assignment

Quantities of minced beef from the original 30kg batch (per run) were selected in such a way that 28 samples each were either aerobically wrapped only, vacuum packaged only, treated with L(+) ascorbic acid and then vacuum packaged, or treated with the commercial 'colour retainer' and subsequently wrapped aerobically.

These four blocks of 28 samples were each divided into two groups of 14, which were stored at 0°C and 7°C, respectively, until analysed. Labcon low temperature incubators (L.T.I.E. 20 and 40) were used for sample storage.

3.5 Chemical and microbiological composition of raw minced beef

The batches of minced beef used for Runs No. 1 and No. 2 showed considerable differences in chemical and microbiological composition. These are listed in Tables 4.2 and 4.1, respectively, under results and discussion (Chapter 4).

3.6   Sample preparation for analysis

Immediately after removal of each sample from the low
temperature incubator, a 20g aliquot was weighed into a
sterile polyethylene bag using aseptic precautions. After
addition of 180ml sterile quarter strength Ringer's solution
as diluent, the 20g sample was homogenized in a Colworth
Stomacher 400 for two minutes.

Several dilutions were subsequently made into sterile 9ml
dilution blanks of quarter strength Ringer's solution and
plated in duplicate by the spread-plate technique, using a
sterile glass rod bent into the shape of a 'hockey-stick'.
The plated liquid was allowed to settle into the culture
medium for 60 minutes before the plates were incubated at
the prescribed temperature in the inverted position.

3.7   Bacteriological studies

Five types of bacteriological counts were used to ascertain
the microbiological condition of minced beef samples after
various storage intervals at specific storage temperatures.
Growth media were in all cases prepared precisely to
manufacturer's specifications and sterilized at 121°C for
15 minutes unless otherwise specified.

All media were prepared and sterilized in bulk, after which
they were dispensed aseptically into presterilized disposable
plastic Petri dishes. After solidification of the media,
the Petri dishes were stored at 4°C until required. Petri
dishes were dried at 37°C for 30 minutes before being used
for sample plate-out.

Details of the specific growth media used for each count type
and parameters of incubation are listed in Table 3.2.

TABLE 3.2   DETAILS OF BACTERIOLOGICAL COUNT PROCEDURES

BACTERIOLOGICAL COUNT TYPE	ABBRE-VIATION	INCUBATION			GROWTH MEDIUM
		TIME	TEMPERATURE	ATMOSPHERE	
Total aerobic plate count	TAPC	3 Days	25°C	Air	Standard 1 Nutrient Agar (Merck)
Enterobacteriaceae count	EC	18-24 hrs	30°C	Air	Violet Red Bile Agar (Merck) + 1% (w/w) Glucose
Psychrotrophic bacteria count	PC	10 Days	7°C	Air	Standard 1 Nutrient Agar (Merck)
Lactic acid bacteria count	LC	5 Days	25°C	Air	Rogosa Agar (Lactobacillus Selective Agar, Merck) + 0,1% (w/w) L-cysteine chloride (monohydrate)
Brochothrix thermosphacta count	BTC	3 Days	25°C	Air	STAA - Selective Medium (Gardner, 1966)

After the required time of incubation, Petri dishes in the dilution series containing between 30 and 300 individual bacterial colonies were selected for counting on a Quebec darkfield colony counter.

3.7.1 Additional bacteriological studies

In order to obtain additional information on numbers of specific groups of spoilage microbes (chiefly the pseudomonads), use was made of a simple biochemical test for the presence of cytochrome oxidase (CO) (Kovacs, 1956; Steel, 1961). After counting, the following plates were subjected to the CO test: the total aerobic plate count (TAPC), the psychrotrophic bacteria count (PC) and the Enterobacteriaceae count after an additional incubation of 24 hours at room temperature (EC-48). Plates were flooded with a 1% (w/v) aqueous solution of tetramethyl-p-phenylenediamine hydrochloride (Merck). Colonies developing a pink colour which became successively dark red, purple and black in 10 to 30 minutes were scored as CO positive.

On several occasions, especially on plates from vacuum packed samples, no CO-positive colonies could be detected, and the count was scored as less than (<) the dilution factor applying to the specific plate. In the statistical analysis of results, data were analysed twice, once omitting all 'less than' values from the results and once including these values as representing the dilution factor. These data were referred to as incomplete and complete data, respectively. Cytochrome oxidase positive counts were denoted by addition of the suffix '-ox.' to the count type from which the plate originated (e.g. TAPC-ox., EC-48-ox. and PC-ox.). Details on the bacteriological count types and their abbreviations are listed in Table 3.3.

TABLE 3.3   MICROBIOLOGICAL AND CHEMICAL ANALYSES AND CORRESPONDING ABBREVIATIONS

MICROBIAL COUNT TYPE OR ANALYSIS	ABBREVIATION	VARIABLE NUMBER IN STATISTICAL ANALYSIS	UNIT OF MEASUREMENT
Total aerobic plate count	TAPC	V1	log number
Cytochrome oxidase positive fraction of total aerobic plate count	TAPC - ox.	V2	log number
Enterobacteriaceae count	EC	V3	log number
Additional count to EC after incubation at room temperature for another 24 hours	EC-48	V4	log number
Cytochrome oxidase positive fraction of 48 hour Enterobacteriaceae count	EC-48 - ox.	V5	log number
Lactic acid bacteria count	LC	V6	log number
Brochothrix thermosphacta count	BTC	V7	log number
Psychrotrophic bacteria count	PC	V8	log number
Cytochrome oxidase positive fraction of psychrotrophic bacteria count	PC - ox.	V9	log number
pH	pH	V10	pH - units
Redox potential	Eh	V11	mV
Total lactate percentage	L.A.	V12	mg lactic acid/ml sample

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3.8 Evaluation of physical and sensory characteristics

After removal of the 20g aliquots for bacteriological testing, samples were subjectively evaluated, taking into account physical appearance and morphology of the minced beef, as well as colour and odour attributes. No point scales for scoring were used in this evaluation. All vacuum packed samples were tested qualitatively for the production of H_2S immediately after opening of the barrier bags. For this test, Whatman No. 1 filter paper strips impregnated with a 5% solution (w/v) of lead acetate in distilled water were used. The strips were inserted into the bags between the bag and meat surfaces. Development of a black colour on the strip was taken as an indication for the presence of H_2S.

3.9 Determination of pH and redox potential (Eh)

Minced beef pH was measured by placing a combination glass-calomel electrode (connected to a T+C 1004 digital pH-meter) directly into the samples after removal of a 20g aliquot for bacteriological testing.

The redox potential was measured by placing a platinum electrode (coupled to a silver/silver chloride half cell connected to a Radiometer TYPE PHM 28 pH-meter) directly into the samples after removal of a 20g aliquot for bacteriological testing. The standard electrode potential was measured to have a value of 245,8 mV at 25°C. A figure of 246 mV was added to all readings taken to correct for the standard electrode potential.

3.10   Lactic acid determinations

Two-ml aliquots of each sample homogenate were withdrawn from
the polyethylene 'stomacher' bag after completion of the
bacteriological tests, stored in a small test tube covered
tightly with 'Parafilm' and immediately deep-frozen until
analysed.

Total lactate percentages as well as percentages of D- and
L- lactate were determined by the method of Hohorst (1970),
using 0,02 ml aliquots of homogenate.   Total lactate
percentages were multiplied by a factor of ten to obtain
figures in units of mg lactate per ml sample.   These figures
were subsequently corrected for background readings (taken
on DAY 0 samples).

3.11   Accelerated spoilage experiments on raw minced beef

Samples taken from the bulk of the raw minced beef (untreated)
used in each run were 'spiked' with identified, pure cultures
of known meat spoilage bacteria.   These included members of
the genera *Pseudomonas, Enterobacter* and *Lactobacillus*
(see Table 3.4).

'Spiked' samples were packaged aerobically in freely gas
permeable wrapping (Resinite-RMF-S) and stored at 0°C
and 7°C for four days.   Control samples were analysed
immediately after 'spiking', together with normal background
samples of raw minced beef.

TABLE 3.4   'SPIKING' CULTURES USED IN ACCELERATED
             SPOILAGE EXPERIMENTS ON RAW MINCED BEEF
         (All organisms listed were used for both runs)

CULTURE COLLECTION REFERENCE	ORGANISM IDENTIFICATION
3B/Pd 3	*Pseudomonas fluorescens*
2B/PV$_5$	*Pseudomonas fluorescens*
S$_{18}$U$_1$E$_2$	*Enterobacter agglomerans*
L$_{80}$	*Lactobacillus saké*
L$_{51}$	*Lactobacillus saké*

3.12 Isolation and identification of predominant psychrotrophic spoilage bacteria from raw minced beef

One hundred and twenty eight (128) isolated colonies were removed from the highest dilutions of psychrotrophic bacterial count (PC) plating ranges for all treatments and all storage intervals during Run No. 1. Each culture was subjected to further purification on Standard 1 Nutrient Agar. Once pure, it was stored on slants at 4°C until further identification testing. All incubations of psychrotrophic isolates were done at 25°C in an aerobic atmosphere.

As an initial step towards isolate identification, all cultures were allowed to grow on fresh Standard 1 Nutrient Agar slants for 18 to 24 hours at 25°C and subsequently Gram stained. Both conventional Gram staining (Harrigan and McCance, 1966) and the 4% KOH reaction method (personal communication W.H. Holzapfel) were used on all isolates. The outcome of the Gram reaction dictated the course of further identification testing.

3.12.1 Identification of Gram-negative psychrotrophic isolates

It was attempted to identify all Gram-negative psychrotrophic isolates by use of the API 20E minikit system (analytical profile index for Enterobacteriaceae and other Gram-negative bacteria). Considerable difficulties were encountered with this system (probably because it was chiefly intended for identification of medically important isolates), but after repeated attempts of testing and with the help of the computerized API data bank, all isolates could be identified, although not always to species level. This was especially true for many of the numerous *Pseudomonas* isolates, which could only be identified to the '*fluorescens* group'-level. A limited number of additional biochemical tests was carried out to aid in the identification of selected Gram-negative isolates (see Table 3.5).

TABLE 3.5   ADDITIONAL BIOCHEMICAL TESTS USED FOR THE IDENTIFICATION OF SELECTED GRAM-NEGATIVE PSYCHROTROPHIC ISOLATES FROM RAW MINCED BEEF

TEST	REFERENCE
1. Hydrolysis of gelatin	Harrigan & McCance, 1966.
2. Arginine dihydrolase test	Harrigan & McCance, 1966; Thornley, 1960.
3. Reduction of nitrate	Harrigan & McCance, 1966
4. Differentiation of oxidation and fermentation of carbohydrate	Hugh & Leifson, 1953; Harrigan & McCance, 1966.
5. Production of carbon dioxide from glucose	Harrigan & McCance, 1966.
6. Utilization of citrate as sole carbon source	Harrigan & McCance, 1966.
7. Cytochrome oxidase test	Kovacs, 1956; Steel,1961; Harrigan & McCance, 1966.
8. Lysine decarboxylase test	Stanier, Palleroni & Doudoroff, 1966.
9. Poly-β-hydroxybutyrate metabolism	Stanier, Palleroni & Doudoroff, 1966.
10. Motility in SIM-Medium (Merck)	Merck, Handbook of Microbiology .
11. Growth at 4° and 41°C	Stanier, Palleroni & Doudoroff, 1966.
12. Fluorescein production on King's media A and B	King, Ward & Raney, 1954; Stanier, Palleroni & Doudoroff, 1966; Harrigan & McCance, 1966.

13.12.2 Identification of Gram-positive psychrotrophic isolates

All Gram-positive isolates were re-purified on both Standard
1 Nutrient Agar and MRS Agar (Merck) before further
identification.   The isolates were then propagated on the
medium on which they showed optimal growth at 25°C, and
catalase reactions were confirmed on 18 to 24 hour cultures
of all isolates, using a fresh solution of 3% (v/v) hydrogen
peroxide (H_2O_2) in distilled water.   Depending on the result
of the catalase reaction, different identification schemes
were used for the isolates.

13.12.2.1 Identification of Gram-positive, catalase positive psychrotrophic isolates

The following identification schedule was used for all
Gram-positive, catalase positive isolates:

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┌─────────────────────────────────────────────┐
│ Gram-positive - catalase positive isolates   │
└─────────────────────────────────────────────┘
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obligately aerobic fat rods
⟶ *Bacillus*

positive sporulation (25°C)

⟶ cocci ── anaerobic fermentation ── + ⟶ *Staphylococcus*

of glucose (25°C)   - ⟶ *Micrococcus*

irregular cell shapes
⟶ Coryneform group
rods/coccobacilli
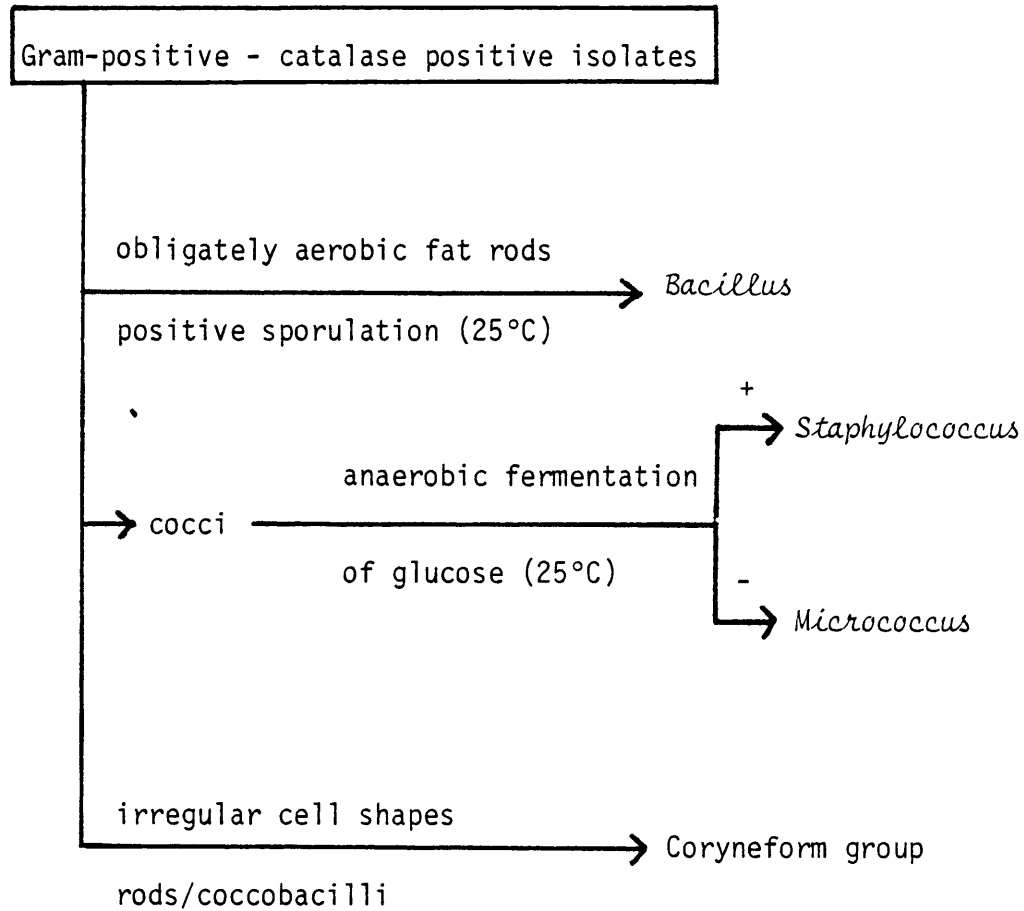
Considerable difficulties were experienced in accurately
identifying isolates falling into the Coryneform group
and extensive use had to be made of characteristics listed
in Bergey's Manual of Determinative Bacteriology
(Buchanan and Gibbons, 1974) to identify these isolates
to the genus level.

3.12.2.1.1 Anaerobic fermentation of glucose

Modified Hugh and Leifson's medium was used for this test
(Harrigan and McCance, 1966).

3.12.2.2 <u>Identification of Gram-positive, catalase negative</u>
<u>psychrotrophic isolates</u>

Details on the identification procedure for Gram-positive
catalase negative isolates can be found under section 3.13,
describing the identification of lactic acid bacteria
isolated from samples stored for 17 days during Runs No. 1
and No. 2.

3.13 <u>Isolation and identification of the predominant lactic</u>
<u>acid bacteria isolated from raw minced beef</u>

Seventy one (71) isolated colonies were removed from the
highest dilutions of the LC plating ranges for all treat-
ments after storage intervals of 17 days (Runs No. 1
and No. 2).   Each culture was subjected to further
purification on MRS-Agar before further identification
was carried out.   All incubations were carried out at
25°C in an aerobic atmosphere.

All isolates were subjected to a series of biochemical
tests for identification purposes.   For inoculation of
the test media, 24 hour (25°C) - MRS-Broth (Merck)
cultures were used.   With the exception of tests used to
determine lactic acid configurations, all other tests were
incubated for four to five days before reading results.

3.13.1 <u>Basal medium for carbohydrate fermentations (Sharpe, 1962)</u>

MRS-Broth without beef extract and glucose was used at a
pH of 6,5.   All carbohydrates, except maltose and arabinose,
were added at a concentration of 0,5% (w/v) to the basal
medium prior to sterilization at 121°C for five minutes,
followed by rapid cooling.   Solutions of maltose and
arabinose (5% w/v) were filter-sterilized and subsequently
added to the basal medium to a final concentration of 0,5%
using aseptic technique.

Chlorophenol red was used as indicator in all carbohydrate
fermentations (concentration 0,004% w/v), except aesculin.
For the aesculin fermentation, 0,05% (w/v) $FeCl_3$ was used.
Both indicators were added to the medium prior to ster-
ilization.

3.13.2   Carbohydrate fermentation tests

The fermentations of aesculin, amygdalin, arabinose,
cellobiose, galactose, gluconate, lactose, maltose,
mannose, melibiose, melezitose, raffinose, rhamnose,
ribose, sucrose, trehalose and xylose were investigated
in the basal medium described under section 3.13.1.
The production of gas was also checked for in the cases
of cellobiose and gluconate.

3.13.3   Growth at 15 and 45°C

All isolates were inoculated into test tubes containing
sterile MRS-Broth and incubated at the appropriate
temperatures in constant-temperature waterbaths.   Daily
checks were made on development of turbidity in the broth
as an indication of growth.

3.13.4   Determination of lactic acid configuration

The isolates were inoculated into MRS-Broth without beef
extract and sodium-acetate and incubated at 25°C for
48 hours.   Lactic acid configurations were determined
as described under Section 13.10 (Hohorst, 1970).

3.13.5   Arginine hydrolysis to ammonia

Isolates were inoculated into MRS-Broth containing 3g/ℓ
arginine monohydrochloride (Merck) instead of
di-ammoniumhydrogencitrate.   Nessler's reagent (Harrigan
and McCance, 1966) was used to detect formation of ammonia.

3.13.6 Slime formation from sucrose

Isolates were streaked onto pour plates of MRS-Agar containing 10% sucrose, added to the medium prior to sterilization. Plates were examined after five days for the presence of slimy growth.

3.13.7 Determination of the presence of diaminopimelic acid (DAP) in cell walls

The presence of DAP in the cell walls of the isolates was determined by one-dimensional thin-layer chromatography (Schleifer and Kandler, 1972; Harper and Davis, 1979).

The solvent used for DAP separation had the following composition:

Methanol	-	320ml
Pyridine	-	40ml
Distilled water	-	70ml
10 N HCl	-	10ml

3.13.8 Differentiation of homo- and heterofermentative isolates

Isolates were inoculated into test tubes of sterile MRS-Broth containing Durham tubes for the detection of gas-formation. Isolates showing gas production were classed as heterofermentative and isolates showing no gas production as homofermentative.

3.14 Statistical methods used for analysis of results

Before proceeding to the presentation and discussion of results, it is necessary to elaborate on statistical procedures employed in result analysis. Abbreviations used in the statistical analysis of results are listed in Table 3.6.

TABLE 3.6

ABBREVIATIONS USED IN STATISTICAL ANALYSIS OF RESULTS

FACTOR	ABBREVIATION	UNIT OF MEASUREMENT
REPETITION OF ENTIRE EXPERIMENT	RUN	-
MINCED BEEF TREATMENT (ADDITIVE/PACKAGING)	TREAT	-
STORAGE TEMPERATURE	TEMP	°C
STORAGE INTERVAL	STORE	DAYS
(DEPENDENT) VARIABLE (referring to chemical and microbiological analyses)	V	V1 to V9-log number V10 -pH units V11 -mV V12 -mg/ml sample

3.14.1   <u>Analysis of variance</u>

The following model was assumed:

$$Y_{ijk\ell v} = \alpha + \beta + \gamma + \delta + \varepsilon + \text{interaction terms} + E_{ijk\ell v}$$

Whereby:

$Y_{ijk\ell v}$ was the log count of a particular class of organism
(a dependent variable-V) recorded for the V-th internal
replicate in the ℓ-th run, at the k-th storage time, j-th
temperature and i-th treatment;

  α was the general mean log count
  β was the effect of the i-th treatment on the count
  γ was the effect of the j-th temperature on the count
  δ was the effect of the k-th storage time on the count
  ε was the effect of the ℓ-th run on the count

The interaction terms included all possible interactions
between the four factors treatment (TREAT), temperature
(TEMP), storage time (STORE) and run (RUN).

$E_{ijk\ell v}$ was the residual or error term of the $Y_{ijk\ell v}$-th
log count, unaccounted for by the model.

With the aid of analysis of variance tables, the
significance of the four factors and their interactions
were established for all dependent variables (V1 to V12),
using the SAS GLM - program (Barr, Goodnight, Sall &
Helwig, 1979). Counts for which only an upper limit
(see 3.7.1) was available were considered as missing
values and omitted from the initial analyses (referred
to as 'incomplete data'). The same analyses were
repeated for data which included the above-mentioned
upper limits (referred to as 'complete data'). Results
on both sets of data were similar to a high degree and
hence only results of the 'complete data'-set were used
for interpretation and discussion.

It was noted that interactions containing the factor RUN were highly significant in a large number of instances (see Table 3.7). Hence in order to simplify the discussion of results, it was decided to consider separate models for the two runs, i.e. identical to the one above but without the run-effect and interactions containing runs. These separate models were also subjected to analyses of variance to assess the significance of the factors treatment, temperature and storage time as well as their interactions - again considering both 'complete' and 'incomplete' sets of data and only using results of the 'complete data' set for interpretation and discussion.

It was noticeable that all data used for statistical analysis were of extremely high reproducibility with coefficients of determination (R-SQUARE) mostly higher than 0,9500 and coefficients of variation (CV) seldom exceeding 10%.

3.14.2 Graphical representation

Subsequent to the above analyses, mean log counts were calculated for the different treatment-temperature-storage time combinations, separately for each run. These means were used to construct the graphs presented in Chapter 4 (Results and Discussion). Included in the calculation of means were the logs of 'upper limit-counts', with the result that those true means were actually overestimated (biased upwards). This procedure inevitably led to an over-conservative view on the effectiveness of the treatments in question in controlling certain bacterial groups and in the graphical representations such values were all scored as 'open points'.

Table 3.7

Significance of RUN and RUN-interactions (Combined RUNS, 'complete' data)

FACTOR/SOURCE	D.F.	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
		P-VALUES ASSOCIATED WITH CALCULATED F-VALUES											
RUN	1	0,9573	0,0001	0,0001	0,2957	0,0001	0,0001	0,0001	0,0001	0,0939	0,0001	0,0001	0,0001
TREAT*RUN	3	0,0016	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0351	0,0269	0,0001	0,0047
TEMP*RUN	1	0,0664	0,0001	0,8543	0,4388	0,8740	0,4327	0,1115	0,0794	0,0001	0,0036	0,0001	0,7111
TREAT*TEMP*RUN	3	0,0060	0,0001	0,0021	0,0089	0,0001	0,0005	0,8361	0,0005	0,0001	0,1929	0,0364	0,2723
STORE*RUN	6	0,0001	0,0001	0,0187	0,0001	0,0015	0,0001	0,1325	0,0339	0,0001	0,0001	0,0001	0,0001
TREAT*STORE*RUN	18	0,0387	0,0001	0,1892	0,1830	0,0002	0,0001	0,0001	0,0001	0,0001	0,0575	0,0001	0,0252
TEMP*STORE*RUN	6	0,0742	0,0001	0,5457	0,0010	0,0005	0,0001	0,2015	0,0001	0,0001	0,1263	0,0001	0,0001
TREAT*TEMP*STORE*RUN	18	0,0004	0,0001	0,0709	0,0569	0,0017	0,0001	0,0001	0,0001	0,0001	0,0051	0,0001	0,9220
R-SQUARE	-	0,9601	0,9887	0,9671	0,9652	0,9710	0,9949	0,9711	0,9874	0,9935	0,9512	0,9642	0,9031
CV	-	7,1587	4,5957	7,1201	8,4301	7,5675	4,3486	11,0437	3,9596	3,7990	1,6463	(50,1231)	12,4920

Note: P-values $\geq$ 0,0500 are underlined.

3.14.3 Calculation of least significant difference (LSD)

Tukey's least significant difference values (Steel & Torrie, 1960) (both 5% and 1%) were calculated comparing the four treatments for each of variables V1 to V11. Calculations were carried out according to the formula:

$$LSD\ 5\% = 3{,}750 \times \sqrt{\frac{\text{Error mean square}}{r}}$$

$$LSD\ 1\% = 4{,}620 \times \sqrt{\frac{\text{Error mean square}}{r}}$$

$$r = 28 \left[7\ (\text{no. of storage intervals}) \times 2\ (\text{no. of internal replicates}) \times 2\ (\text{no. of temperatures}) \right]$$

Similar calculations were carried out for the comparison of differences between treatment means in the graphical re-presentations. A value of r = 2 (no. of internal replicates) was used in these calculations.

Differences between treatment means were rated as significant (S) if the difference was larger or equal to ($\geqslant$) LSD 5%. This was denoted by a single asterisk (*).

Differences between treatment means were rated was highly significant (HS) if the difference was larger or equal to ($\geqslant$) LSD 1%. This was denoted by a double asterisk (**).

3.14.4 Correlational analysis

Two sets of correlational analyses were carried out; firstly all variables (as defined under 3.14.1) were correlated with each other over all treatments, temperatures, storage times and both runs combined (N=221); and secondly the same variables were correlated within the different treatments (N=56), temperatures (N=111) and storage intervals (N=32) individually for both runs. Results of these analyses were used for determining high levels of association between variables (or specific bacteriological counts). Correlational analyses were carried out using the SAS PROC CORR-program (Barr *et al.*,1979).

CHAPTER 4

RESULTS AND DISCUSSION

Results are presented and discussed under five separate headings:

4.1 Background analyses on raw minced beef used for the study and accelerated spoilage experiments.

4.2 Analyses of variance and graphical representations of bacteriological studies, including assessments of physical and sensory characteristics.

4.3 Correlational analyses of bacteriological studies.

4.4 Significant differences between treatment means for the bacteriological studies.

4.5 Identification of representative spoilage isolates.

Tables too elaborate to be accommodated in the text of result presentation and discussion were collected in the Appendix. Due reference to this is made in the course of the presentation.

4.1   Background analyses and accelerated spoilage experiments

A complete set of bacteriological and chemical analyses was conducted on samples of raw minced beef used for both runs at day 0 (i.e. before treatment and storage) to assess initial bacteriological and chemical quality of the raw material used in the study.

Results of the bacteriological and chemical analyses are listed in Tables 4.1 and 4.2, respectively.

It was evident from both bacteriological and chemical results that the overall composition and quality of raw minced beef differed considerably for RUNS NO. 1 and NO. 2.

The most significant feature about the bacteriological results was the fact that all bacterial groups were present in higher numbers in the minced beef used for RUN NO. 2; in fact, levels were in all cases larger by at least one log number for the RUN NO. 2 raw material, except for the lactic acid bacteria count (LC or V6) which was practically identical for both runs. Noticeable features about the bacteriological results were the psychrotrophic bacterial count (PC or V8) and the cytochrome oxidase positive fraction of the total aerobic plate count (TAPC-ox. or V2) which were both nearly two log numbers higher for RUN NO. 2 than for RUN NO. 1.   It was also noted that the raw minced beef used for RUN NO. 2 had a rather elevated Enterobacteriaceae count (EC or V3).   Despite the fact that isolated higher counts were noted for some of the bacterial groups analysed, the microbiological quality of both batches of raw minced beef was rated as good when compared to results of numerous similar surveys of retail or market minced beef available for purchase in several countries (Sumner, Reay & Perry, 1979).

TABLE 4.1   MICROBIOLOGICAL CHARACTERISTICS OF RAW MINCED BEEF USED
FOR BACTERIOLOGICAL STUDIES

(Values are expressed as normal logarithms and represent averages of
internal replicate samples, each plated in duplicate)

MICROBIOLOGICAL CHARACTERISTIC	RUN NO. 1	RUN NO. 2
Total aerobic plate count (TAPC)	3,882	5,279
Cytochrome oxidase positive fraction of total aerobic plate count (TAPC-ox.)	2,000	4,202
Enterobacteriaceae count (EC)	3,330	4,838
Additional Enterobacteriaceae count after an extra 24 hours incubation at room temperature (EC-48)	2,362	3,777
Cytochrome oxidase positive fraction of EC-48 count (EC-48-ox.)	2,363	3,500
Lactic acid bacteria count (LC)	2,088	2,239
Brochothrix thermosphacta count (BTC)	1,847	2,500
Psychrotrophic bacterial count (PC)	3,378	5,089
Cytochrome oxidase positive fraction of psychrotrophic bacterial count (PC-ox.)	2,000	3,088

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TABLE 4.2   CHEMICAL CHARACTERISTICS OF RAW MINCED BEEF USED FOR
BACTERIOLOGICAL STUDIES

CHARACTERISTIC	RUN NO. 1	RUN NO. 2
MOISTURE	69,17%	54,67%
PROTEIN (NITROGEN)	17,33%	16,37%
FAT	11,18%	28,42%
ASH	0,92%	0,79%
TOTAL PERCENTAGE	98,60%	100,25%
DRY MATERIAL	30,83%	45,33%
pH	5,59	5,60
Eh (mV)	26	-144
LACTIC ACID (mg/mℓ sample) CONCENTRATION	0,6274	0,4352

The results of the chemical analyses clearly indicated that the raw minced beef used for RUN NO. 2 had a fat content of more than double that found in the raw material for RUN NO. 1. This was accompanied by an almost proportionately lower value for moisture in the RUN NO. 2 raw material. Protein, ash and pH values for both batches of minced beef were practically identical and slight differences were observed in dry material content and lactic acid concentrations.

Chemical characteristics of both batches of raw minced beef corresponded to values generally accepted as normal for retail material, with a relatively high value for fat content in the RUN NO. 2 raw material and low protein percentages for both batches (Law, Yang & Mullins, 1971). The pH-values of 5,59 and 5,60 for RUNS NO. 1 and NO. 2, respectively, were an indication that normal-pH meat was used for the fabrication of the minced beef. A pH of 6,0 to 6,1 is generally regarded as the cut-off point to high-pH meat (Morrissey, Buckley & Daly, 1980). The differences in composition of the raw materials used in RUNS NO. 1 and NO. 2 of the study were reflected in the statistical variance analysis of results. For this reason it was decided to carry out separate statistical analyses on data obtained from RUNS NO. 1 and NO. 2 (refer to Chapter 3, Section 3.14.1)

4.1.1 Accelerated spoilage experiments

Samples taken from the raw minced beef used in both runs were artificially inoculated or 'spiked' with pure broth cultures of known psychrotrophic meat spoilage bacteria (Chapter 3, Section 3.11). The inoculum level was chosen to fall between 5×10^5 and 1×10^6 organisms/g (6ml of culture were added for each kg of minced beef). Results of the experiments are presented in Table 4.3.

TABLE 4.3   RESULTS OF ACCELERATED SPOILAGE EXPERIMENTS ('SPIKED' SAMPLES), INOCULATED WITH TYPICAL MEAT SPOILAGE BACTERIA TO A LEVEL OF ca. 10^6/g

RUN	STORE (DAYS)	TEMP (°C)	VARIABLE (V)											
			V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
No. 1	0	-	5,715	3,000	5,227	4,477	5,134	3,000	2,253	5,470	3,678	5,68	-009	0,4284
No. 1	4	0	7,700	6,272	7,705	7,088	7,244	5,714	4,357	7,784	7,443	5,68	351	1,3210
No. 1	4	7	9,014	7,671	8,832	7,151	8,211	8,731	6,184	9,026	8,233	5,25	356	2,2886
No. 2	0	-	5,863	4,000	5,657	4,350	4,676	6,818	2,389	5,731	4,000	5,67	-139	0,0000
No. 2	4	0	8,242	6,000	8,042	6,088	7,426	8,218	4,213	8,126	6,477	5,56	-134	1,4668
No. 2	4	7	8,193	6,000	8,202	6,239	7,334	8,667	4,301	8,495	5,000	5,37	-224	1,7055

The results clearly indicated that considerable multiplication
and growth of all groups of bacteria used for 'spiking' occurred
over the four-day storage interval at both temperatures,
accompanied by rapid deterioration of physical and sensory
characteristics of the raw minced beef. Firstly, this finding
indicated that the bacteria employed in the artificial inocula-
tion were indeed capable of rapid growth in raw minced beef
stored at refrigeration temperatures.

Secondly, and most importantly, it was illustrated that the
microbiological techniques chosen allowed for satisfactory
recovery of organisms in the groups to be analysed. This
was evident by the fact that all bacterial groups covered by
the 'spiking' program showed between two and three log increases
after four days storage at 0°C and up to five log increases
after four days storage at 7°C. This pattern was in keeping
with results of similar studies reported in the literature
(Reddy, Hendrickson & Olson, 1970; Gill & Newton, 1977;
Morrissey, Buckley & Daly, 1980; Hanna, Hall, Smith & Vanderzant,
1980). In addition, lactic acid concentrations increased
noticeably compared to background values measured for both runs,
indicating considerable metabolic activity of the 'spiked'
Lactobacillus species.

Satisfactory performance of culture media and recovery
techniques for organisms was further apparent by excellent
correspondence of all results to the model used for the
statistical analysis of variance (high coefficients of
determination and low coefficients of variation).

4.2 Graphical representation and analysis of variance of bacteriological studies

Group responses over time are discussed separately for each bacterial group, also observing differences in patterns between the two storage temperatures. The two runs are discussed together whenever possible. Graphical representations, significance values established by analysis of variance and least significant differences (LSD) between treatment means were used extensively to aid in result interpretation.

Graphical representations were assigned to figure numbers 4.1 to 4.12, and LSD values (both 5% and 1%) were indicated to scale on each individual plot. Significance values of factors and interactions by analysis of variance are summarized in Tables 4.4 and 4.5 for RUNS No. 1 and No. 2, respectively.

On each graphical representation or plot microbiologically significant levels during spoilage were indicated and utilized for result interpretation. These arbitrarily chosen "guidelines" were 10^7 and 10^6 organisms per gram for V1 (TAPC), V2 (TAPC-ox.), V6 (LC), V8 (PC) and V9 (PC-ox.), 10^7 and 10^5 organisms per gram for V7 (BTC) and 10^7, 10^6 and 10^5 organisms per gram for V3(EC), V4 (EC-48) and V5 (EC-48-ox.). Periods of time taken for the bacterial counts to reach these levels were summarized and averaged for both runs (Table A.1, Appendix).

Levels of 10^5, 10^6 and 10^7 organisms per gram were chosen because of the widespread significance assigned to them by researchers as indicators of degree of spoilage and hygienic quality and hence shelf life of refrigerated minced beef. The 10^5 and/or 10^6 per gram levels were generally taken as signifying onset of spoilage, depending on bacterial type, whereas spoilage was taken to become prominent at 10^7 per gram and rising to an unacceptable level at numbers in excess of 10^7 per gram. In addition, physical and sensory characteristics of the minced beef were also assessed for each sample to add information to the extent of spoilage.

TABLE 4.4

Significance of factors and interactions in analysis of variance tables for RUN NO.1 (complete data)

(Values of $P \geqslant 0,05$ are printed in italics)

FACTOR/ SOURCE	D.F.	P-VALUES ASSOCIATED WITH CALCULATED F-VALUES											
		V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
TREAT	3	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	*0,1782*
TEMP	1	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
TR * TEMP	3	*0,1645*	0,0001	*0,7210*	0,0079	0,0008	0,0199	0,0003	0,0333	0,0001	0,0001	*0,1562*	0,0098
STORE	6	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
TR * STORE	18	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0011
TEMP * STORE	6	0,0001	0,0001	0,0001	0,0027	0,0001	0,0001	0,0001	0,0001	0,0001	*0,1846*	0,0001	0,0001
TR * TE * ST	18	0,0001	0,0001	0,0021	0,0015	0,0045	0,0001	0,0013	0,0001	0,0001	0,0306	0,0001	0,0018
R-SQUARE	-	0,9826	0,9878	0,9566	0,9525	0,9573	0,9942	0,9530	0,9859	0,9905	0,9204	0,9351	0,8329
CV	-	5,0411	5,4246	8,4871	10,4143	10,2302	3,8154	12,6246	4,2218	4,1916	2,1314	(30,1272)	10,0869

TABLE 4.5

Significance of factors and interactions in analysis of variance tables for RUN NO.2 (complete data)

(values of P ≥ 0,05 are printed in italics)

FACTOR / SOURCE	D.F.	P-VALUES ASSOCIATED WITH CALCULATED F-VALUES											
		V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
TREAT	3	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
TEMP	1	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	*0,1734*	0,0006
TR * TEMP	3	0,0231	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0009	*0,2881*
STORE	6	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
TR * STORE	18	*0,1604*	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0002
TEMP * STORE	6	0,0002	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0027
TR * TE * ST	18	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0059	0,0164
R-SQUARE	-	0,9293	0,9899	0,9773	0,9815	0,9895	0,9947	0,9867	0,9889	0,9957	0,9821	0,9701	0,8418
CV	-	8,8134	3,6783	5,6241	5,7783	3,9737	5,0211	8,1462	3,6851	3,3555	0,9536	(137,0269)	15,9894

The following discussions are based on a comparison of general
tendencies for the different treatments, storage temperatures,
times and runs. Smaller deviations from general patterns were
therefore not taken into consideration.

4.2.1 Effects of treatments, storage time and temperature on total
aerobic plate count (TAPC or V1), (Fig. 4.1)

In the analysis of variance for TAPC (V1) the factors temperature
(TEMP), treatment (TREAT) and storage time (STORE) as well as
the interactions of treatment and temperature (TREAT * TEMP),
treatment and storage time (TREAT * STORE), temperature and storage
time (TEMP * STORE) and treatment, temperature and storage time
(TREAT * TEMP * STORE) were found to be highly significant (P = 0,0001;
see Tables 4.4   and   4.5).   Only two exceptions were noted
with P-values in excess of 0,0500 (for TREAT * TEMP, RUN NO.1 and
TREAT * STORE, RUN NO.2, P = 0,1645 and 0,1604, respectively).

At a storage temperature of 0°C, the largest count increase over
the shortest time expectedly occurred in the aerobically packed
samples (TREAT 1) and the counts reached the 10^7/gram level after
five days (Table A.1 , Appendix) for both runs.   Counts for all
other samples took longer times to reach 10^7/gram, while the TREAT 4
samples did not reach this level at all in RUN NO. 1.   The same
pattern was observed in times taken for the counts to reach 10^6/gram,
only that correspondingly shorter times were required for all samples
(see Table A.1, Appendix).   It was noteworthy, however, that
for RUN NO. 2 at 0°C, counts for TREAT 2, 3 and 4 samples were
virtually constant up to seven days, whereafter normal increases
occurred.   For both runs at 0°C, terminal counts (after 17 days
of storage) were considerably lower for TREAT 2, 3 and 4 samples
than for TREAT 1 samples (10^7 to 10^8/gram as opposed to 10^9/gram).

At 7°C, differences in counts between treatments were not as
drastic as at 0°C (for TREAT * TEMP * STORE interaction, P = 0,0001).
For both runs, counts for all samples (except TREAT 4) crossed the
10^7/gram level before day 5 and the 10^6/gram level at or before
day 4 (including TREAT 4).   Count increases with time were more

rapid for RUN NO. 2 because of its higher initial bacterial load. Overall, both levels (10^6 and 10^7/gram) were reached first by TREAT 1 samples followed with increasing storage time by TREATS 2, 3 and 4, respectively (ignoring minor crossovers). Terminal counts were again highest for TREAT 1 samples (10^9/gram as opposed to 1 to 5 x 10^8/gram for the other treatments).

Comparing the effect of the two storage temperatures on total aerobic plate count, it was evident that TREATS 2, 3 and 4 had a more pronounced suppressive effect on counts at 0°C than at 7°C (for TEMP factor, P = 0,0001). This tendency was particularly obvious for RUN NO. 1 (0°C), where significant ($\geqslant 10^{1,53}$) and often highly significant ($\geqslant 10^{1,88}$) differences were noted between TREAT 1 and all remaining counts. This tendency was most noticeable in the day 5 to day 13 interval (for TREAT and STORE factors, P = 0,0001).

4.2.2 Effects of treatments, storage time and temperature on the cytochrome oxidase positive fraction of the total aerobic plate count (TAPC-ox. or V2),(Fig. 4.2)

In the analysis of variance for TAPC-ox. (V2), all factors as well as their interactions were rated as highly significant (P = 0,0001; see Tables 4.4 and 4.5).

At 0°C in both runs, counts remained virtually constant up to day 4 for all treatments. Subsequently, counts increased rapidly in all TREAT 1 samples, reaching 10^6 and 10^7/gram after six and seven to 10 days, respectively. Counts for TREAT 2, 3 and 4 samples were significantly lower than those for TREAT 1 samples over the entire storage interval ($\geqslant 10^{0,88}$ and $10^{0,63}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001). The TREAT 2, 3 and 4 sample counts also did not reach either the 10^6/gram or 10^7/gram levels over 17 storage days (except for TREAT 4, RUN NO. 2, exceeding 10^6/gram at 13 days). Internal differences between counts of TREAT 2, 3 and 4 samples were relatively small, constant and mostly not statistically significant ($< 10^{0,63}$ and $10^{0,88}$) for both runs. Terminal counts at day 17 generally showed a difference of 1,5 to two log numbers between TREAT 1 and TREATS 2, 3 and 4.

At 7°C, all counts were increased compared to 0°C (for TEMP factor, P = 0,0001) over the entire storage interval and differences between TREAT 1 and TREAT 2, 3 and 4 sample counts considerably smaller. Overall growth patterns were similar in samples of all four treatments, although again TREAT 2, 3 and 4 sample counts were lower than the corresponding TREAT 1 counts. These differences were often not statistically highly significant ($< 10^{0,88}$). The same tendency applied to the terminal counts for all treatments at 7°C (for times taken to reach 10^6 and 10^7/gram levels, see Table A.1 , Appendix). Despite several trend crossovers with increasing storage time at 7°C it appeared that TREATS 3 and 4 achieved several statistically significant count reductions to TREAT 1 ($\geqslant 10^{0,88}$ and $10^{0,63}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001).

As for total aerobic plate counts, the above tendency was more pronounced at 0°C storage (for TREAT * TEMP * STORE interaction, P = 0,0001) indicating a positive synergistic effect between storage temperature and treatments. As most, if not all organisms recovered under the V2 category were probable pseudomonads and hence typical spoilage organisms, this trend should be regarded as significant, especially with regard to possible practical applications.

It was also noted that overall growth patterns at both temperatures and for both runs were very similar for V1 and V2 (ignoring minor fluctuations), although absolute numbers were lower for V2.

4.2.3 Effects of treatments, storage time and temperature on the Enterobacteriaceae count (EC or V3), (Fig 4.3)

In the analysis of variance for EC (V3), all factors as well as their interactions with one exception, were rated as statiscally highly significant (P = 0,0001). For the TREAT * TEMP interaction in RUN NO. 1, P = 0,7210 (see Tables 4.4 and 4.5).

At 0°C in both runs the increase in counts was most rapid for the TREAT 1 samples.   The 10^5, 10^6 and 10^7/gram levels were reached after two, 3,5 and five days, respectively.   Terminal counts of TREAT 1 samples at 0°C reached 0,5 to 1 x 10^9/gram. Based on data in the literature (Oblinger, Kennedy, Rothenberg, Berry & Stern, 1982) this can be rated as extremely high for the low storage temperature.   Counts in TREAT 2, 3 and 4 samples were lower for both runs (statistically highly significant) compared to TREAT 1 samples from day 3 onwards ($\geqslant 10^{1,57}$ and $10^{1,11}$; for TREAT factor, P = 0,0001).   Counts for TREAT 3 and 4 samples were generally lower than those for TREAT 2 samples over the entire storage intervals, and in both runs, counts for TREATS 2, 3 and 4 did not reach the 10^7/gram level.   Terminal count differences between TREATS 2, 3 and 4 and TREAT 1 were, therefore, statistically highly significant in both runs ($\geqslant 10^{1,57}$ and $10^{1,11}$; for STORE * TREAT interaction, P = 0,0001).

At 7°C in both runs, counts for TREAT 1 samples showed rapid increases and the 10^5, 10^6 and 10^7/gram levels were already reached after one, 1,5 and two days, respectively.   Consequently, terminal (or stationary) levels of 10^9/gram were reached after only seven days of storage.   The tendency was also noted for TREAT 2 and 3 sample counts from day 7 onwards.

It was noticeable that counts for TREATS 2, 3 and 4 were numerically closer to TREAT 1 sample counts in RUN NO. 2 than in RUN NO. 1. Despite this fact, statistically highly significant differences between TREAT 2, 3 and 4, and TREAT 1 sample counts were recorded between days 3 and 13, the most noticeable exception being TREAT 2, RUN NO. 2 ($\geqslant 10^{1,57}$ and $10^{1,11}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001), (for times taken to reach levels of 10^5, 10^6 and 10^7/gram, refer Table A.1 , Appendix). At 7°C and for both runs, the initially excellent inhibitory effect of TREAT 4 ceased to persist after day 4, when steady increases set in, leading to high terminal counts of ca. 10^9/gram. Count differences between TREATS 1 and 4 were therefore not statistically highly significant from day 13 onwards.   This tendency was only slight in the runs at 0°C.

Comparing counts for the various treatments at 0 and 7°C,
it was once again noted that the lower storage temperature
increases the suppressive effects of TREATS 2, 3 and 4 on
growth of Enterobacteriaceae, although internal differences
existed between these three treatments (for TEMP factor and
TEMP * STORE interaction, P = 0,0001).

4.2.4 <u>Effects of treatments, storage time and temperature on
'Pseudomonadaceae' (additional Enterobacteriaceae after
another 24 hours incubation at room temperature) count
(EC-48 or V4),(Fig. 4.4).</u>

In the analysis of variance for EC-48 (V4) all factors as well
as their interactions were statistically highly significant.
For RUN NO. 2, P = 0,0001 for all factors and interactions,
whereas for RUN NO. 1 the TREAT * TEMP, TEMP * STORE and TREAT *
TEMP * STORE interactions had P - values of 0,0079, 0,0027 and
0,0015, respectively, the remaining values being equal to 0,0001
(see Tables 4.4 and 4.5).

At 0°C, counts for the TREAT 1 samples increased rapidly with
time in both runs, crossing the 10^5, 10^6 and 10^7/gram levels
at three, four and six to seven days, respectively, and reaching
high terminal counts around 5 x 10^8/gram.   Counts from TREAT 2,
3 and 4 samples were noticeably lower than those from TREAT 1
samples.   All differences were statistically highly significant
from day 3 ($\geq 10^{1,64}$ and $10^{0,92}$; for TREAT factor and TREAT *
STORE interaction, P = 0,0001).

In both runs at 0°C, TREAT 3 sample counts did not cross the
10^5/gram level but reached a low value of 10^4/gram after 17
days.   This led to statistically highly significant differences
between TREAT 3 and TREAT 2 and 4 terminal counts, the latter
reaching values of around 5 x 10^5/gram.

At 7°C, TREAT 1 sample counts showed rapid increases for both runs, crossing the 10^5, 10^6 and 10^7/gram levels after 1,5, two and three to four days, respectively. The TREAT 2 and 3 sample counts reached virtually stationary levels from day 4 in both runs, TREAT 2 values being higher than those for TREAT 3 in all cases. TREAT 3 values remained at or below 10^5/gram at all times with one exception (day 17, RUN NO. 2) For RUN NO. 1 TREAT 2 sample counts remained just above 10^5/gram. In both runs, TREAT 4 sample counts did not cross the 10^7/gram level with one exception (day 17, RUN NO. 2).

Overall, numerical values for TREAT 2, 3 and 4 counts were closer to those of TREAT 1 in RUN NO. 2 than in RUN NO. 1. Still, differences were all statistically highly significant between days 3 and 14 ($\geqslant 10^{1,64}$ and $10^{0,92}$). As for V3, TREAT 4 sample counts in both runs at 7°C increased noticeably from day 4, indicating less growth suppression of the 'Pseudomonadaceae' by this treatment.

As for other bacterial groups, EC-48 count differences between TREAT 1 and TREATS 2, 3 and 4 were larger at 0°C than at 7°C for both runs (for TEMP factor and TEMP * STORE and TREAT * TEMP * STORE interactions, P = 0,0001). Despite smaller internal differences between TREAT 2, 3 and 4 sample counts, TREAT 3 yielded the best inhibitions in both runs at both storage temperatures.

4.2.5 <u>Effects of treatments, storage time and temperature on the cytochrome oxidase positive fraction of the 'Pseudomonadaceae' count (EC-48-ox. or V5), (Fig. 4.5)</u>

In the analysis of variance of EC-48-ox. (V5), all factors and their interactions were statistically highly significant. All P-values with two exceptions, were equal to 0,0001 (in RUN NO.1, TREAT * STORE and TREAT * TEMP * STORE had P values of 0,0008 and 0,0045, respectively), (see Tables 4.4 and 4.5).

At 0°C in both runs, patterns for counts on samples from all treatments were strikingly similar to those obtained for EC-48 (V4). For both runs, counts on TREAT 2, 3 and 4 samples did not reach the 10^5/gram level over 17 days of storage; in fact, only a slight increase in numbers was recorded from day 0 to day 17. Differences between counts on TREAT 2, 3 and 4 samples were seldom statistically significant ($< 10^{1,23}$ and $10^{0,52}$). Differences between counts on TREAT 1 and TREAT 2, 3 and 4 samples were all statistically highly significant from day 3 onwards ($\geqslant 10^{1,54}$ and $10^{0,65}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001). Precise times taken for counts to reach the 10^5, 10^6 and 10^7/gram levels are listed in Table A.1 (Appendix).

At 7°C, both runs showed closely allied behaviour of counts from all treatment samples to that described for EC-48 (V4). In addition, TREAT 1 sample counts at 7°C showed an almost identical response to the one observed at 0°C for V5. As described for V4, counts on TREAT 3 samples levelled at or below the 10^5/gram mark from day 3 onwards. The steadily increasing trend in counts on TREAT 4 samples was again noticeable in both runs at 7°C after day 2. At 0°C, this pattern was not noticeable.

Results on V5 again reinforced the frequently made earlier observation of a positive synergism between low storage temperature and TREATS 2, 3 and 4. This synergistic action was strongest for TREAT 3 at 0°C. The fact that close pattern correspondences were noted between EC-48 (V4) and EC-48-ox. (V5) indicated that the two counts were microbiologically strongly related. This was in fact feasible, because it was expected to recover largely pseudomonads by the EC-48 procedure, most, if not all, of which should also show up as cytochrome oxidase positive by the EC-48-ox. procedure.

4.2.6 Effects of treatments, storage time and temperature on the lactic acid bacteria count (LC or V6), (Fig. 4.6)

In the analysis of variance for LC (V6), all factors and their interactions were highly significant (P = 0,0001) with one exception (for the TREAT * TEMP interaction, RUN NO. 1, P = 0,0199; see Tables 4.4 and 4.5).

In both runs at 0°C very similar trends were evident, although absolute counts were somewhat lower in RUN NO. 2 up to day 4. This finding is not readily explained in view of the fact that baseline counts were identical for both runs. However, it may be related to a difference in the type of dominating lactic acid bacteria between the samples. The similarity of counts in TREATS 1, 2 and 3 was remarkable for both runs. The 10^6 and 10^7/gram levels were crossed at days 8 and 11 in RUN NO. 1 and at days 11 to 13 and 14 to 17 in RUN NO. 2, respectively. Statistically highly significant lower counts were found on the TREAT 4 samples from day 2 in RUN NO. 1 and day 9 in RUN NO. 2. This finding also applied to terminal counts ($\geqslant 10^{0,70}$ and $10^{0,74}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001). For both runs, TREAT 4 sample counts did not reach the 10^6/gram level over the entire storage period.

At 7°C, similar patterns to 0°C were observed for both runs, although all individual counts were higher, as were the terminal counts on day 17 (for TEMP factor, P = 0,0001). Counts on TREAT 1, 2 and 3 samples increased in closely parallel fashion for both runs, crossing the 10^6 and 10^7/gram levels after two and three and after 2,5 and four days for RUNS NO. 1 and NO. 2, respectively, For both runs, the TREAT 4 sample counts were well below counts for the other treatments and differences were statistically highly significant over most of the storage period of 17 days ($10^{0,70}$ and $10^{0,74}$; for TREAT * TEMP * STORE interaction, P = 0,0001).

It was noticeable that growth patterns of the lactic acid bacteria
at 7°C bore a striking resemblance to the theoretical bacterial
growth curve, with a more noticeable lag phase for TREAT 4
samples.   Also, it was most surprising that lactic acid bacteria
levels were identical for aerobically and vacuum-packed samples.
One would expect these organisms to proliferate significantly
better in anaerobic or microaerophilic environments (Christopher,
Seideman, Carpenter, Smith & Vanderzant, 1979).   A possible
explanation could be the aerobic incubation of plates auto-
matically selecting against possible oxygen-sensitive strains
Also, lowering of the redox potential by vigorous metabolic
activities of aerobic bacteria and the natural poising
capacity of meat could provide an explanation.

It was once again noted that the lower storage temperature had
a strong reinforcing effect on TREAT 4.   Interestingly, this
effect was not observed for TREATS 2 and 3.   The high toxicity
of SO_2 for lactic acid bacteria in TREAT 4 compared to favourable
anaerobic conditions in TREATS 2 and 3 could provide an explanation
for this observation.

4.2.7   Effects of treatments, storage temperature and time on the _Brochothrix thermosphacta_ count (BTC or V7), (Fig. 4.7)

In the analysis of variance for BTC (V7), all factors as well
as their interactions were highly significant.   For RUN NO. 2,
all P-values were equal to 0,0001 and for RUN NO. 1 only two
P-values exceeded 0,0001, although both of these were below the
1% significance level (for TREAT * TEMP and TREAT * TEMP * STORE
interaction P = 0,0003 and 0,0013, respectively; see Tables
4.4 and 4.5).

At 0°C in both runs, counts in TREAT 1 samples increased rapidly,
crossing the 10^5 and 10^7/gram levels at 4,5 to seven and nine
to 11 days, respectively, reaching terminal levels of 5 x 10^7
to 10^8/gram.   The counts in TREAT 2, 3 and 4 samples stayed
virtually constant up to day 4, whereafter TREAT 3 sample counts
decreased in both runs to a terminal value of 2 to 2,5 x 10^2/gram.

This was very close to the baseline BTC on day 0. The counts for TREAT 2 and 4 samples increased in parallel to day 7, after which TREAT 2 counts levelled to below 10^5/gram in both runs. TREAT 4 sample counts showed a net increase, crossing the 10^5/gram level after 14 and 9,5 days, for RUNS NO. 1 and NO. 2, respectively. For RUN NO. 2, a parallel increase between TREAT 1 and 4 sample counts was noted from days 7 to 17.

Differences between counts for TREAT 1, and TREAT 2 and 3 samples were statistically highly significant from day 3 onwards in both runs ($\geqslant 10^{1,69}$ and $10^{0,92}$; for TREAT factor, P = 0,0001). This difference was not observed between TREAT 1 and TREAT 4 sample counts to the same extent, especially for RUN NO. 2.

At 7°C in both runs, similar tendencies to the 0°C runs were noted. Again, TREAT 1 sample counts increased rapidly to terminal values around 10^8/gram. In the case of RUN NO. 2, the TREAT 1 sample counts were closely followed by those of TREAT 4 up to day 17; for RUN NO. 1, this trend was less obvious but nevertheless present. A similar course of events was noted for TREAT 2 and 3 sample counts, although TREAT 2 counts were higher over the entire storage period. Both TREAT 2 and 3 sample counts did not reach the 10^5/gram barrier and levelled to a terminal value around 10^2/gram, which was again lower than the baseline value for day 0. Because of this fact, statistically highly significant differences were observed between sample counts for TREATS 1 and 4 and TREATS 2 and 3 from ca. day 5 onwards in both runs ($\geqslant 10^{1,69}$ and $10^{0,92}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001).

At both temperatures, significant differences in initial growth patterns were noted between the runs in that the lag times for samples from all treatment groups were visibly longer for RUN NO. 2, despite the fact that the RUN NO. 1 raw material actually contained lower initial numbers of B. *thermosphacta*.

In both runs and at both temperatures, excellent growth inhibition of B. *thermosphacta* in TREAT 3 samples (and TREAT 2 samples to a lesser extent) was evident. No net increase in numbers was recorded over 17 days of storage, a factor which could be of considerable practical significance in the light of the organism being regarded as a significant spoilage factor of raw minced beef stored at refrigeration temperatures (Collins-Thompson & Lopez, 1980). TREAT 2 and 4 samples generally showed similar behaviour up to day 7, whereafter TREAT 4 counts increased, indicating minimal inhibition by the commerical 'colour retainer'. For BTC, the previously found reinforcing effect of low temperature on treatment performance was not noted to any significant degree.

4.2.8 <u>Effects of treatments, storage time and temperature on</u>
<u>psychrotrophic bacterial count (PC or V8), (Fig. 4.8)</u>

In the analysis of variance for PC (V8), all factors and their interactions were highly significant (P = 0,0001), with one exception (for the TREAT * TEMP interaction in RUN NO. 1, P = 0,0003, falling between the 5% and 1% confidence limits), (see Tables 4.4 and 4.5).

For both runs at 0°C, striking similarities in patterns were observed in the corresponding TAPC (V1) plots (Fig. 4.1). This was especially true for the RUN NO. 2 plots which were practically identical regarding absolute numbers, differences between treatment sample counts, crossover points for the 10^6 and 10^7/gram levels and terminal counts reached after 17 days of storage (see Table A.1 , Appendix). As for V1, statistically highly significant differences were reached between TREAT 1 and TREATS 2, 3 and 4 from day 4 onwards ($\geqslant 10^{0,87}$ and $10^{0,78}$, for TREAT factor P = 0,0001); this pattern did not apply to TREAT 2 sample counts in RUN NO. 1, which were numerically fairly close to those of TREAT 1 samples.

At 7°C in both runs, the similarity to the corresponding V1 plots (Fig. 4.1) was again evident, especially in the case of RUN NO. 2. For RUN NO. 1, counts of samples from all treatments increased without a noticeable lag phase.

However, a lag phase of at least one day was evident for RUN NO. 2 (for STORE factor, P = 0,0001). Terminal counts once again closely corresponded to those observed for V1, as did the crossover points for the 10^6 and 10^7/gram levels.

Numerically, values for all sample counts were closer for all treatments over the entire storage, although differences between TREAT 1 and TREAT 3 and 4 sample counts were largely statistically highly significant ($\geqslant 10^{0,87}$ and $10^{0,78}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001) from day 3 onwards.

As recorded previously, the most noticeable difference between storage at the two temperatures was the improved suppressive effect on psychrotroph growth exerted by TREATS 2, 3 and 4 at the lower stage temperature (for TEMP factor and TREAT * TEMP * STORE interaction, P = 0,0001). In relative terms, TREAT 4 showed the best inhibition on PC although only in the earlier stages of storage (up to day 7), followed by TREATS 3 and 2, in that order.

The close correspondence between V8 and V1 indicated that a significant proportion of the total aerobic plate count consisted of psychrotrophic bacteria, and the microbes isolated by both procedures were governed by almost identical growth behaviour.

4.2.9 Effects of treatments, storage time and temperature on the cytochrome oxidase positive fraction of PC (PC-ox. or V9), Fig. 4.9)

In the analysis of variance for PC-ox. (V9), all factors as well as their interactions were highly significant. All P-values were equal to 0,0001 (see Tables 4.4 and 4.5).

At 0°C in both runs, similar patterns were observed to the corresponding TAPC-ox. (V2) plots (Fig. 4.2), except for a higher terminal count for TREAT 1 samples in RUN NO. 2. It was noticeable that for both runs, TREAT 2, 3 and 4 sample counts did not cross the 10^6/gram level until fairly late in the storage interval (day 11 onwards) and in no instance was the 10^7/gram level reached. In all cases, differences between TREAT 1 and TREAT 2, 3 and 4 sample counts were statistically highly significant from day 3 onwards ($\geqslant 10^{0,68}$ and $10^{0,54}$; for TREAT factor and STORE * TREAT interaction, P = 0,0001). A pronounced lag phase was also noticeable for TREAT 2, 3 and 4 sample counts in both runs at 0°C.

In both runs at 7°C counts in samples from all treatments increased in roughly parallel fashion, although a one-day lag was noticeable for RUN NO. 2 (for STORE factor, P = 0,0001). Numerous crossovers up to day 17 complicated an overall assessment of significant differences. However, differences between TREAT 1 and TREAT 3 samples were definitely statistically highly significant from day 2 onwards ($\geqslant 10^{0,68}$ and $10^{0,54}$). It was noticeable that even at 7°C, none of the TREAT 2, 3 and 4 sample counts crossed the 10^7/gram level (see Table A.1 , Appendix).

For both runs over both temperatures it was evident that TREATS 2, 3 and 4 achieved some count reduction compared to TREAT 1, although it was impossible to assign the best overall reduction to a specific treatment. As discussed for most of the other variables, the suppressive effect of TREATS 2, 3 and 4 on PC-ox. counts was more noticeable at the lower storage temperature (for TEMP factor and TEMP * STORE, TEMP * TREAT and TEMP * TREAT * STORE interactions, P = 0,0001).

A noticeable feature about all PC-ox. (V9) results was the fact that their response pattern over storage was very similar to that observed for TAPC-ox. (V2), although the degree of correspondence was not as high as for V1 and V8. It can be concluded that a similar spectrum of organisms was recorded by the V2 and V9 counting procedures. This agreed well with general observations (Ayres, 1960; Gill & Newton, 1977) that most meat spoilage pseudomonads (oxidase positive) have the ability to grow

under psychrotrophic conditions.

4.2.10 <u>Effects of treatments, storage time and temperature on sample pH (V10), (Fig 4.10)</u>

In this discussion, reduced emphasis is placed on the pH-data due to large potential for error in the technique of measurement and the possible influence of non-microbiological factors on readings.

In the analysis of variance of pH (V10) all factors and interactions were highly significant (P = 0,0001) with two exceptions (TEMP * STORE, RUN NO. 1, P = 0,1846; TREAT * TEMP * STORE, RUN NO. 1, P = 0,0306), (See Tables 4.4 and 4.5).

For both runs at 0°C, pH values for TREAT 1, 2 and 4 samples showed similar patterns. A marginal increase occurred from the initial value of 5,6, levelling back towards 5,6 around day 4. After another marginal increase terminal values scattered around the initial value. Differences between values for TREAT 1, 2 and 4 samples were not statistically significant ($< 10^{0,31}$ and and $10^{0,14}$). The pH values for TREAT 3 samples showed a sharp decline from day 0 to day 1 (i.e. after addition of 0,5% ascorbic acid) and levelled out to values around 5,2 for the entire storage period. The pH-value differences between TREAT 3 and TREAT 1, 2 and 4 samples were statistically highly significant over the entire storage period ($\geqslant 10^{0,38}$ and $10^{0,18}$; for TREAT factor and TREAT * STORE interaction, P = 0,0001).

At 7°C in both runs TREAT 3 samples behaved identically to 0°C, although further decreases occurred with increasing storage time to terminal values of around 5,0, indicating some additional acid-forming activity over and above the ascorbic acid influence. Considerable fluctuations in pH-values occurred for TREATS 1 and 4 at 7°C in both runs, generally around or above the initial value of 5,6. It was noticeable for both runs at 7°C that pH-values for TREAT 2 samples showed a definite decline from day 2 to day 3 onwards. Especially for RUN NO. 1 this decline was spectacular, leading to a terminal value of ca. 5,1.

Overall, in both runs at both temperatures, TREAT 3 sample
pH-values were significantly lower than those for the three
remaining treatments, although TREAT 2 values also showed
declining tendencies with increasing storage time at 7°C in
both runs (for TREAT * TEMP * STORE interaction, P = 0,0001).

4.2.11 Effects of treatments, storage time and temperature on Eh (Vll), (Fig. 4.11)

In the analysis of variance for Eh (Vll), most factors and
interactions were highly significant (P = 0,0001).   Four
exceptions were recorded (TREAT * TEMP, RUN NO. 1, P = 0,1562;
TEMP, RUN NO. 2, P = 0,1734;   TREAT * TEMP, RUN NO. 2, P = 0,0009;
TREAT * TEMP * STORE, RUN NO. 2, P = 0,0059), (see Tables 4.4
and 4.5).

Despite the significant values, comparatively little emphasis
is placed on the Eh-data because of a high degree of fluctuation
(especially in RUN NO. 1) making their reliability questionable.
This was in agreement with observations by several authors who
utilized the Eh-parameter in their research (Pierson, Collins-
Thompson & Ordal, 1970; Strange , Benedict, Smith & Swift, 1977).

The above authors reported tendencies of decreasing Eh patterns
to high negative values with increasing spoilage of raw minced beef.
This same tendency was observed after three days for TREAT 1 and
4 samples in RUNS NO. 1 at 0 and 7°C, but was not consistent up
to day 17.   A more definite decreasing tendency was observed in
Eh values for TREAT 2 and 3 samples in RUNS NO. 2 at 0 and 7°C,
compared to a fluctuating, although overall constant tendency for
TREAT 1 and 4 samples.

LSD values (5% and 1%) were calculated and inserted into the Eh
plots, but were not used for result interpretation because of
their dubious significance.   For the most parts of RUN NO. 2
at 0 and 7°C, Eh value differences between TREATS 2 and 3 and
TREATS 1 and 4 were very noticeable (and perhaps statistically
highly significant), as one would expect because of the effect of
vacuum packaging on redox potentials.   These differences became
less pronounced as storage time increased beyond 11 days.

4.2.12 <u>Effects of treatments, storage time and temperature on</u>
<u>(D+L) lactic acid concentrations (V12), (Fig. 4.12)</u>

For the analysis of variance on concentrations of lactic acid
(V12) samples were categorized into relative percentage ranges
only, allowing for superficial analysis of trends.   In a later
calculation (Table A.2, Appendix) absolute concentrations of
D+L lactic acid corrected for background levels were determined
for all samples.   These were not analysed statistically.   It
was evident from their graphical representation, however, that
no clear-cut differences existed between samples of the four
treatments (Fig. 4.12).

In RUNS NO. 1 at 0° and 7°C, no interpretation of trends was
possible at all, because of large fluctuations between storage
times in all samples, leading to no net increase in lactic
acid concentrations after day 2.   In RUNS NO. 2 at 0° and 7°C,
similar overall patterns were found for samples from all four
treatments.   Lactic acid concentrations increased steeply
from two to three days, stayed constant up to seven days and
then decreased to very low levels up to day 17.   This decrease
was more marked for RUN NO. 2 at 7°C, where virtually constant
low values were reached after 11 days.   No direct relationship
between lactic acid concentrations and lactic acid bacteria counts
was evident from an examination of the respective plots (Fig. 4.6).

Partial correspondence was shown by the V12 - RUN NO. 2 - 7°C plot,
where initial sharp increases in lactic acid concentration coincided
with similar increases in lactic acid bacteria for samples of
TREATS 1,2 and 3.   The stationary section was also corresponding
for both plots, but the decrease in lactic acid concentration after
seven days could not be explained by the lactic acid bacteria counts.
There could exist a possibility, however, that bacterial
groups other than the lactics utilized the accumulated lactic acid
preferentially, giving rise to the observed sharp decreases.   The
real significance of this observation is doubtful, however, as no
similar trends were discovered for RUNS NO. 1 at 0° and 7°C.

Fig. 4.1   Effects of treatments, storage time and temperature on total aerobic plate count (TAPC or V1)

(TREAT 1●—●;   TREAT 2▼—▼;   TREAT 3■—■;   TREAT 4◆—◆)
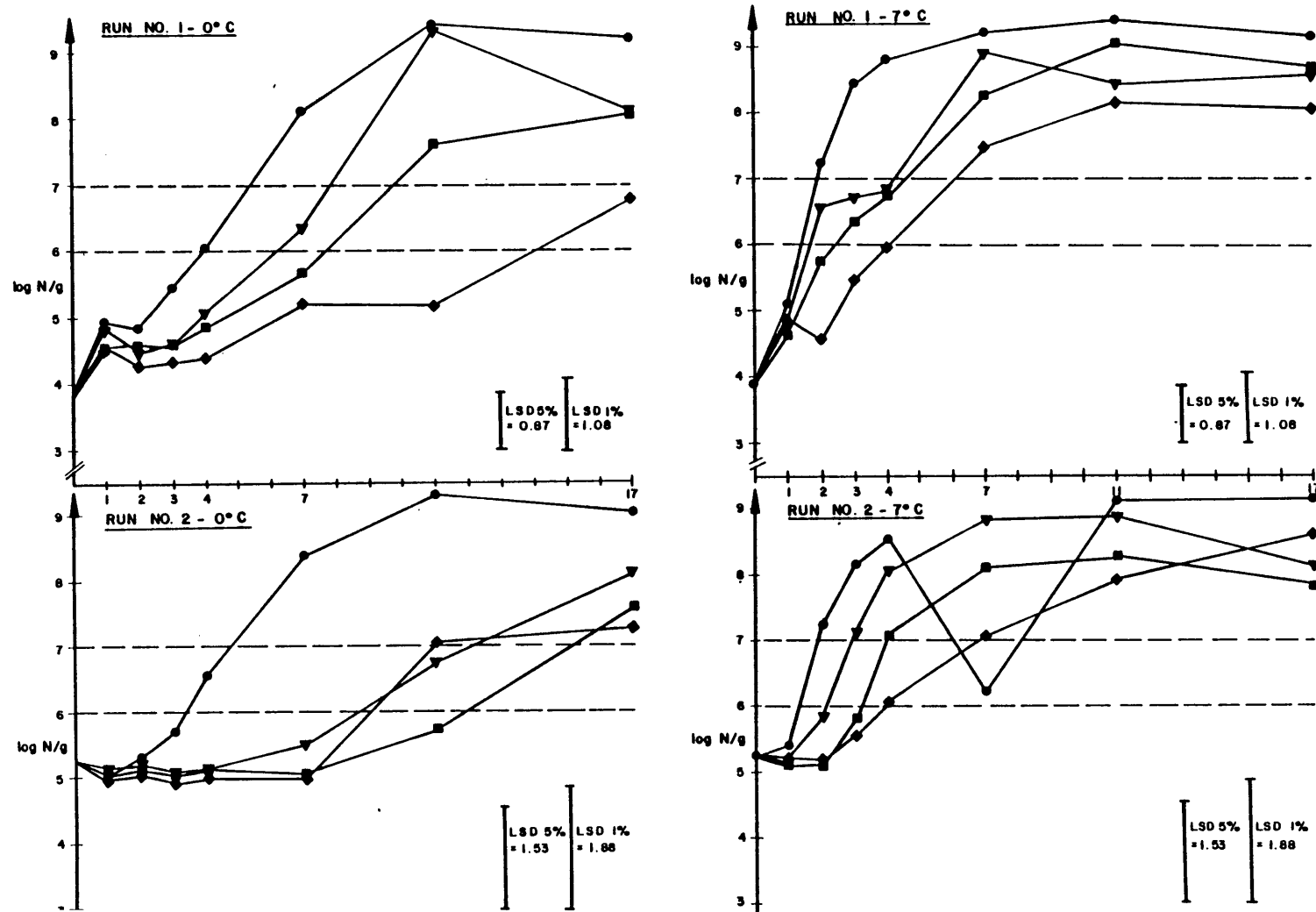
Fig. 4.2   Effects of treatments, storage time and temperature on the cytochrome oxidase
positive fraction of the total aerobic plate count (TAPC-ox. or V2)
(for legend see Fig. 4.1)
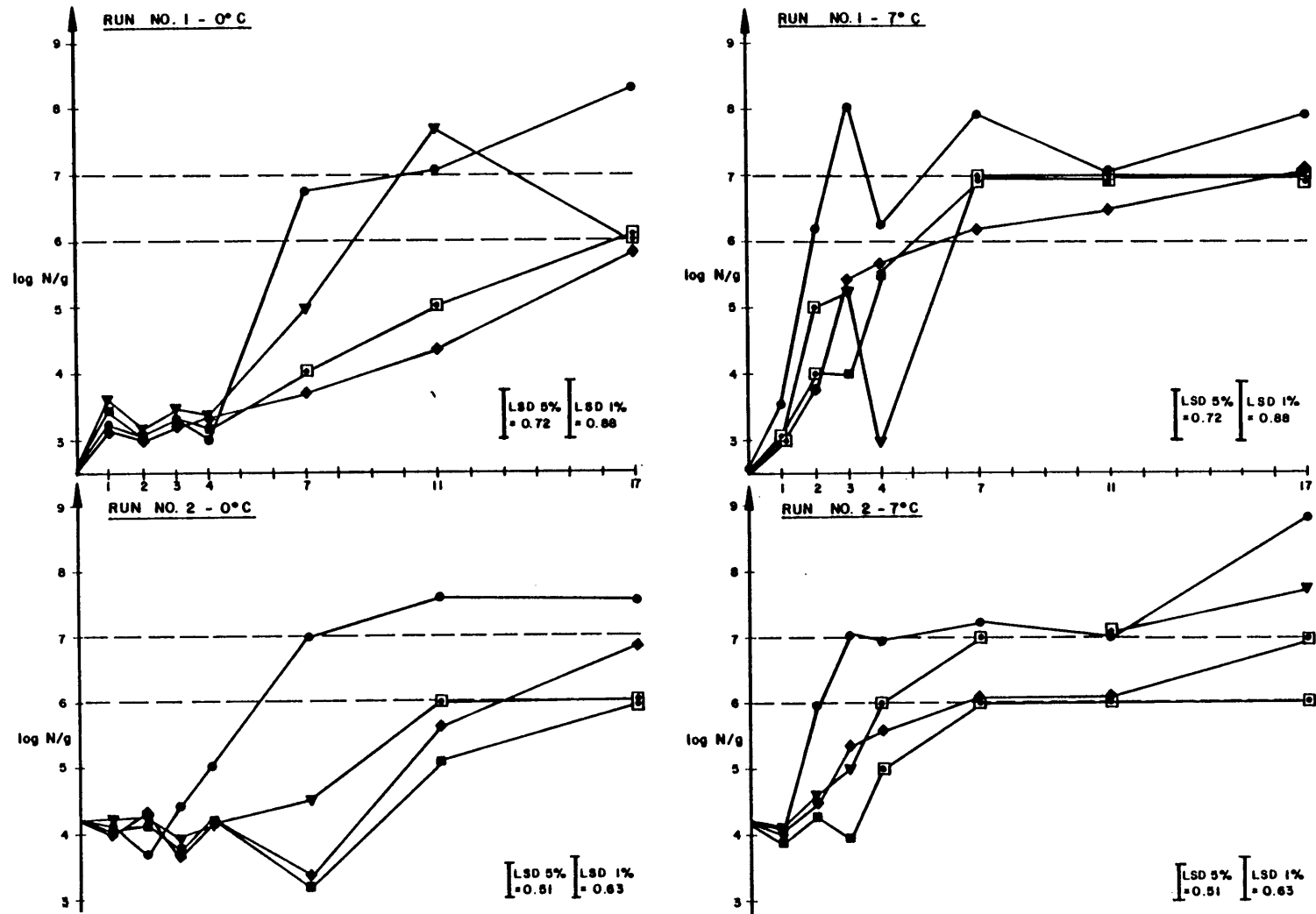
Fig. 4.3   Effects of treatments, storage time and temperature on the Enterobacteriaceae count (EC or V3)

(for legend see Fig. 4.1)
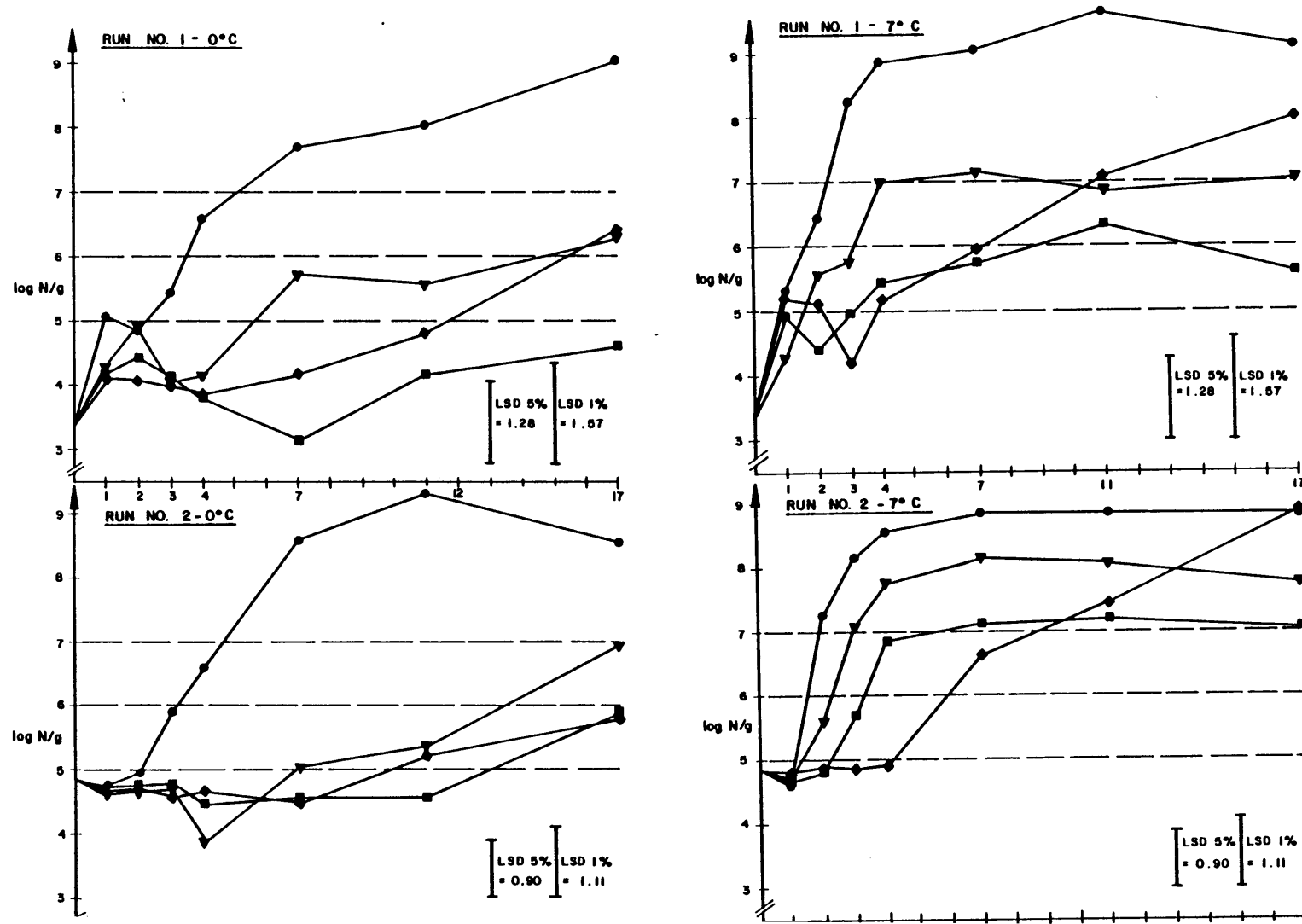
Fig. 4.4   Effects of treatments, storage time and temperature on 'Pseudomonadaceae'
(additional Enterobacteriaceae after another 24 hours incubation at room
temperature) count (EC-48 or V4)
(for legend see Fig. 4.1)
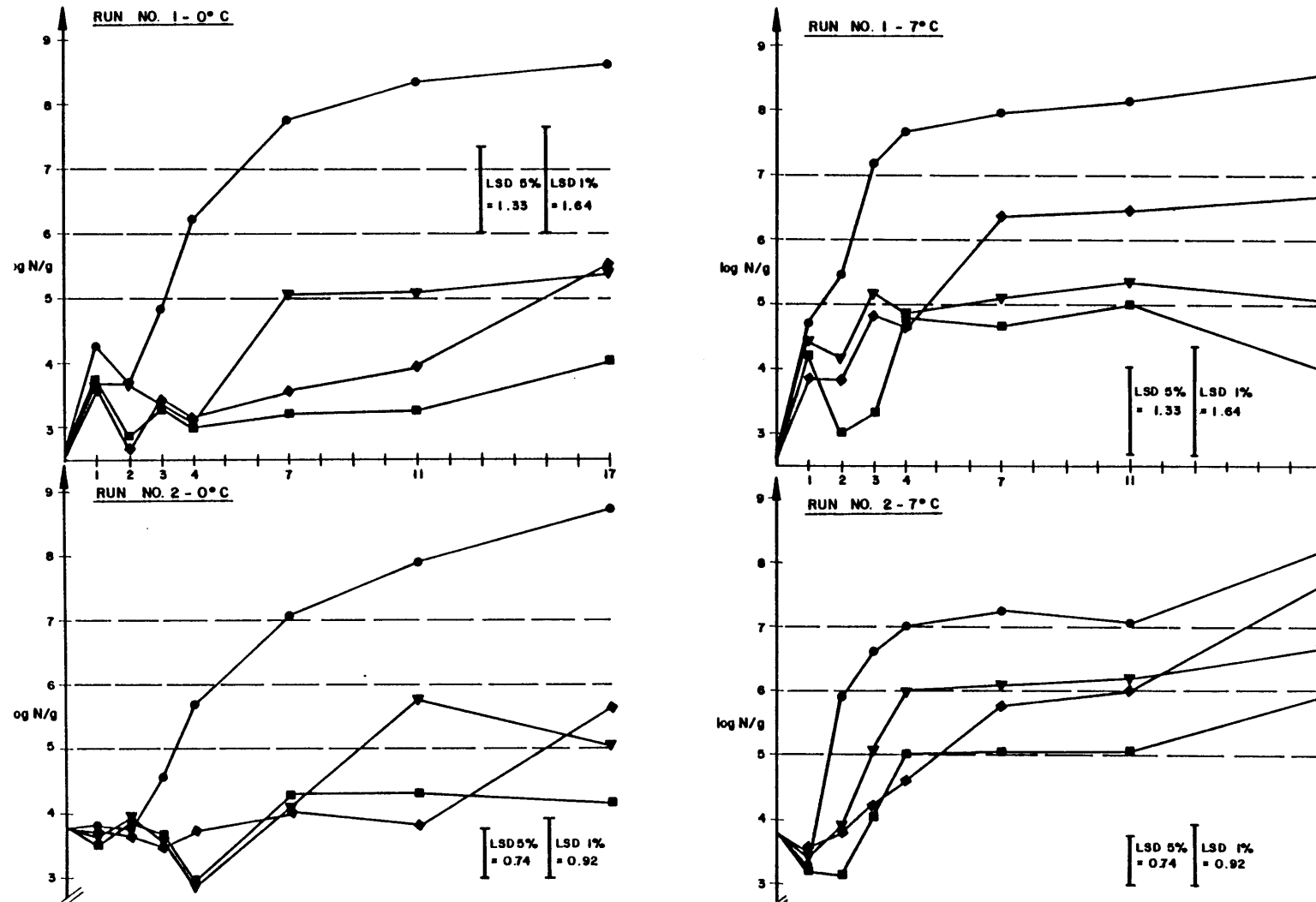
Fig. 4.5   Effects of treatments, storage time and temperature on the cytochrome oxidase
positive fraction of the 'Pseudomonadaceae' count (EC-48-ox. or V5)
(for legend see Fig. 4.1)
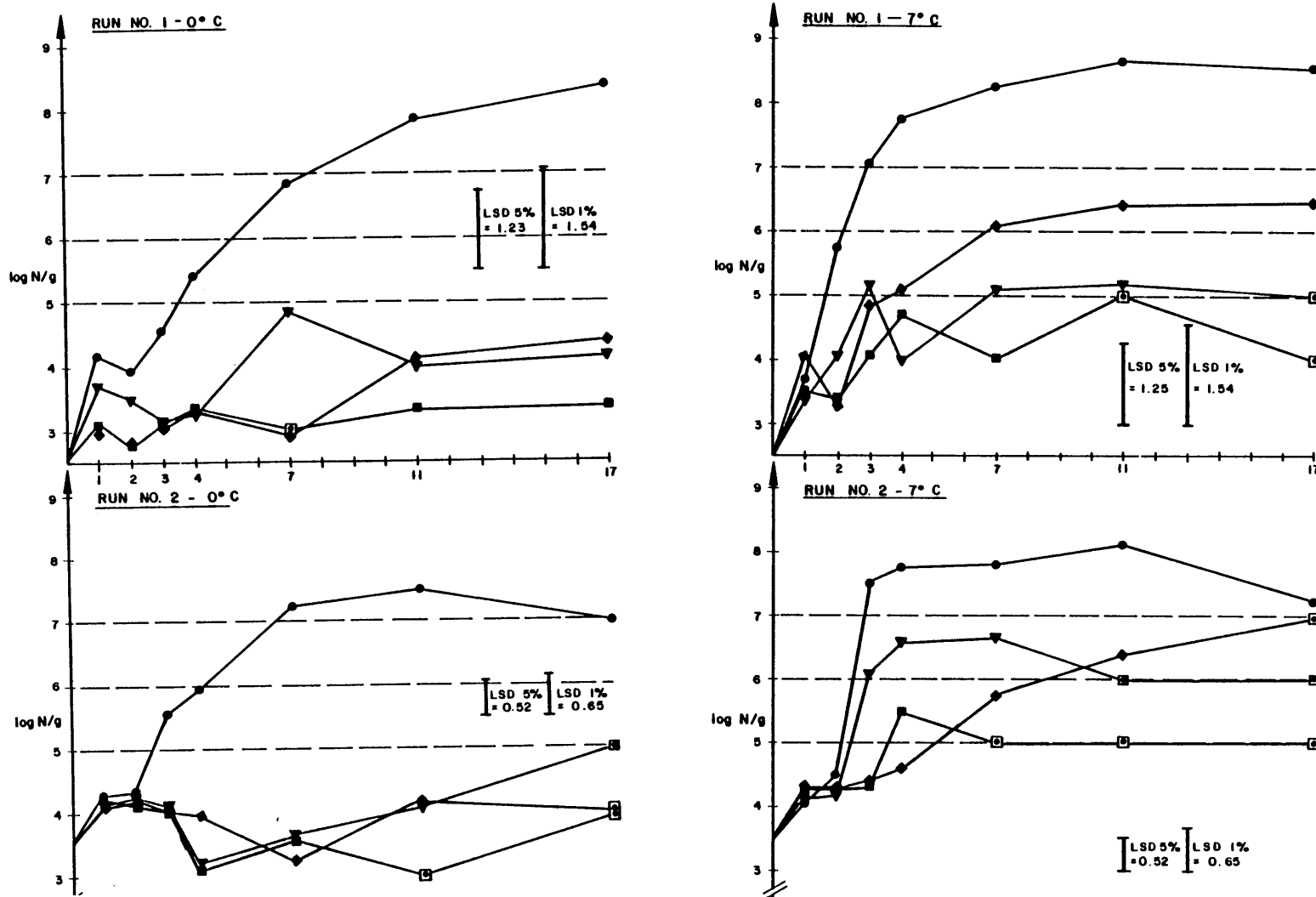
Fig. 4.6   Effects of treatments, storage time and temperature on the lactic acid bacteria count (LC or V6)
(for legend see Fig. 4.1)
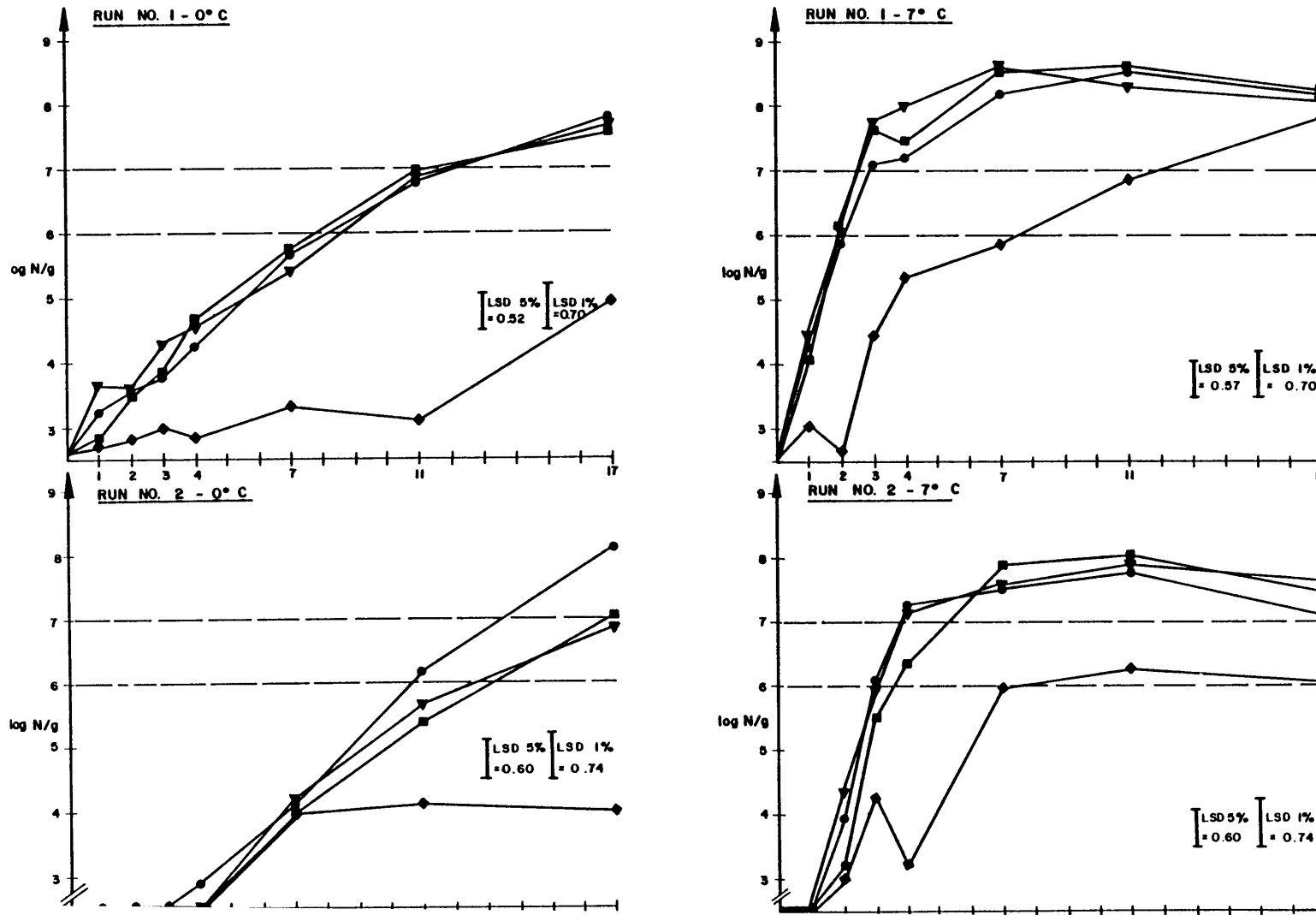
Fig. 4.7   Effects of treatments, storage time and temperature on the *Brochothrix*
thermosphacta count (BTC or V7)
(for legend see Fig. 4.1)
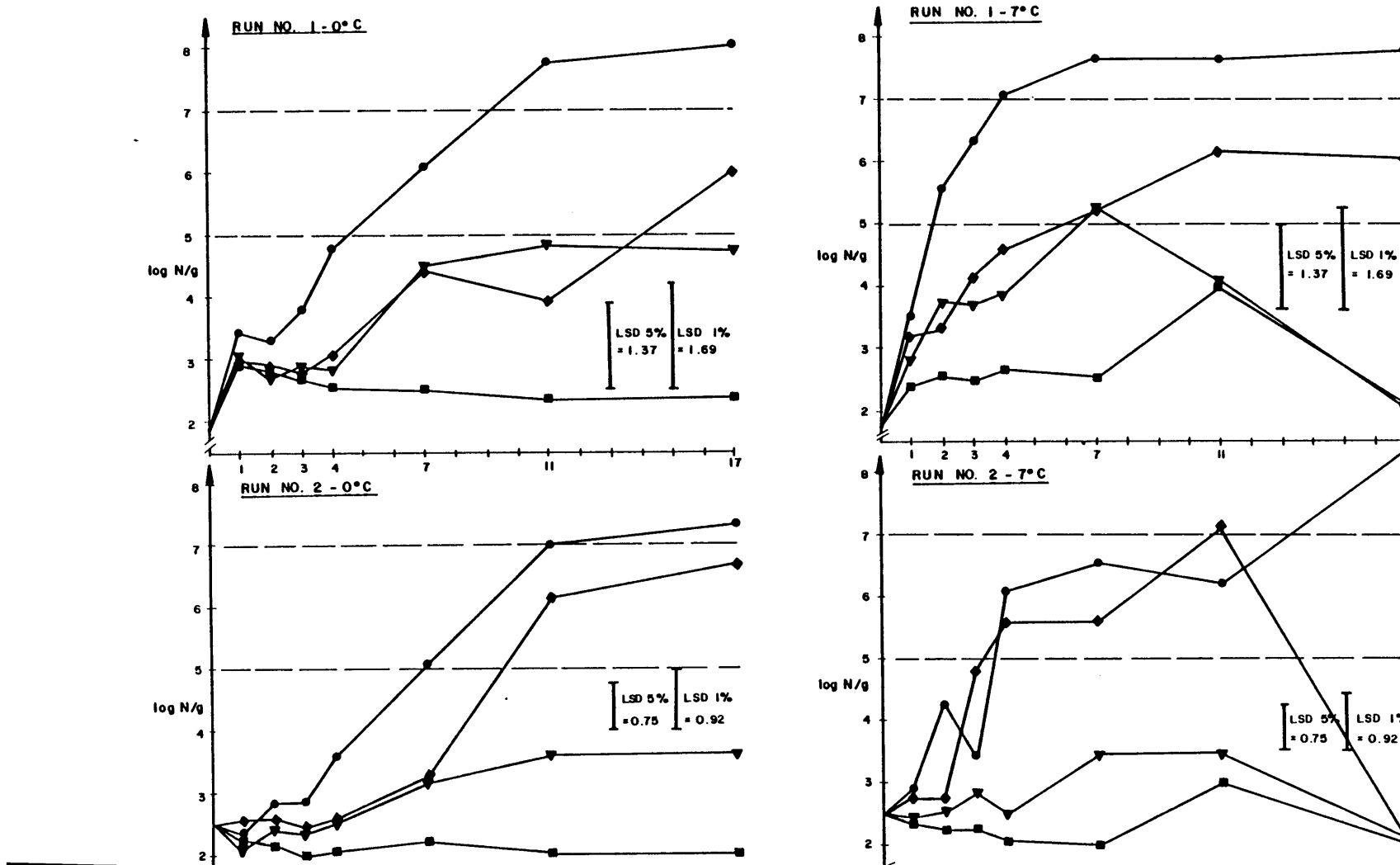
Fig. 4.8   Effects of treatments, storage time and temperature on the psychrotrophic
bacterial count (PC or V8)
(for legend see Fig. 4.1)
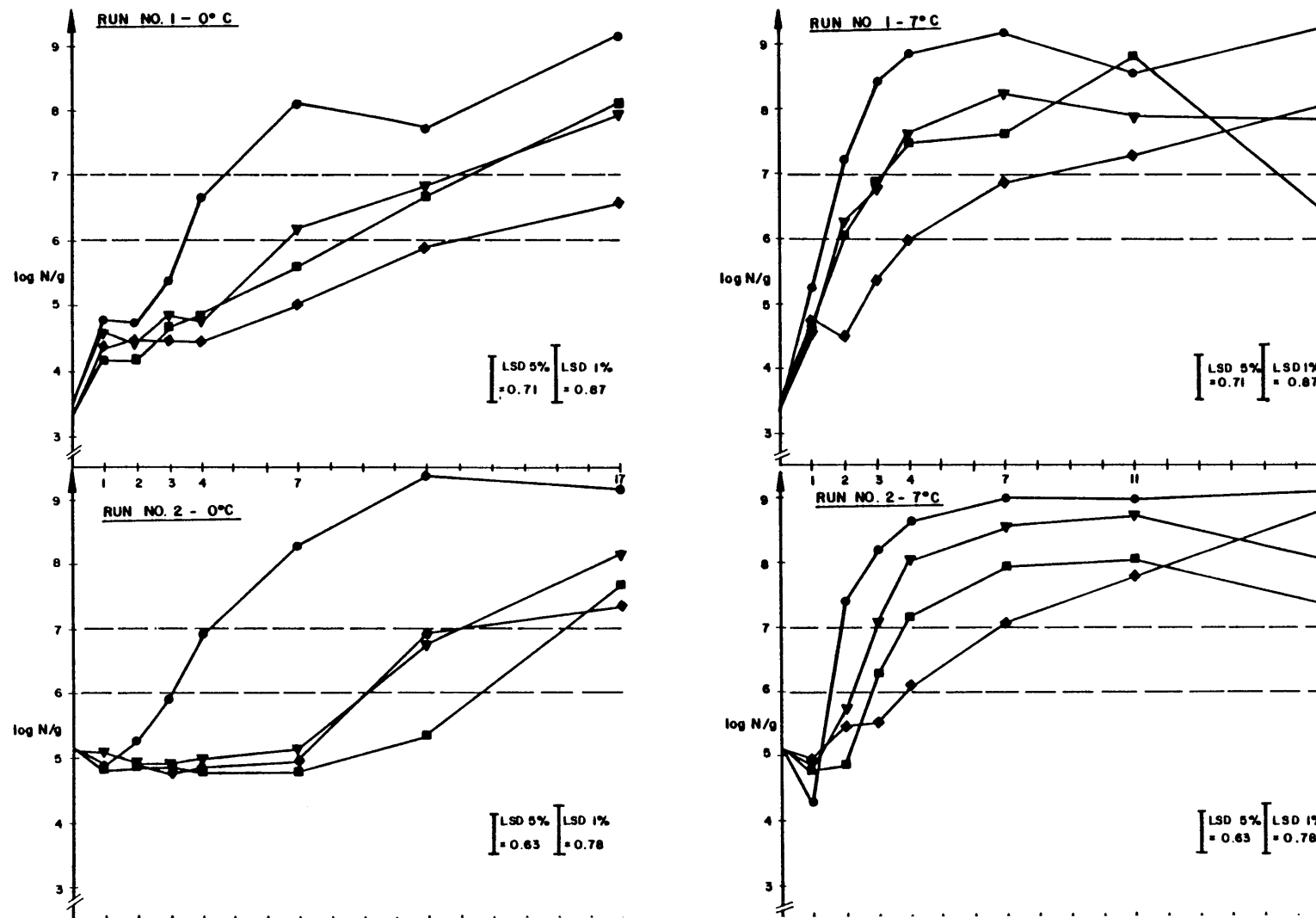
Fig. 4.9   Effects of treatments, storage time and temperature on the cytochrome oxidase positive fraction of PC (PC-ox. or V9)
(for legend see Fig. 4.1)
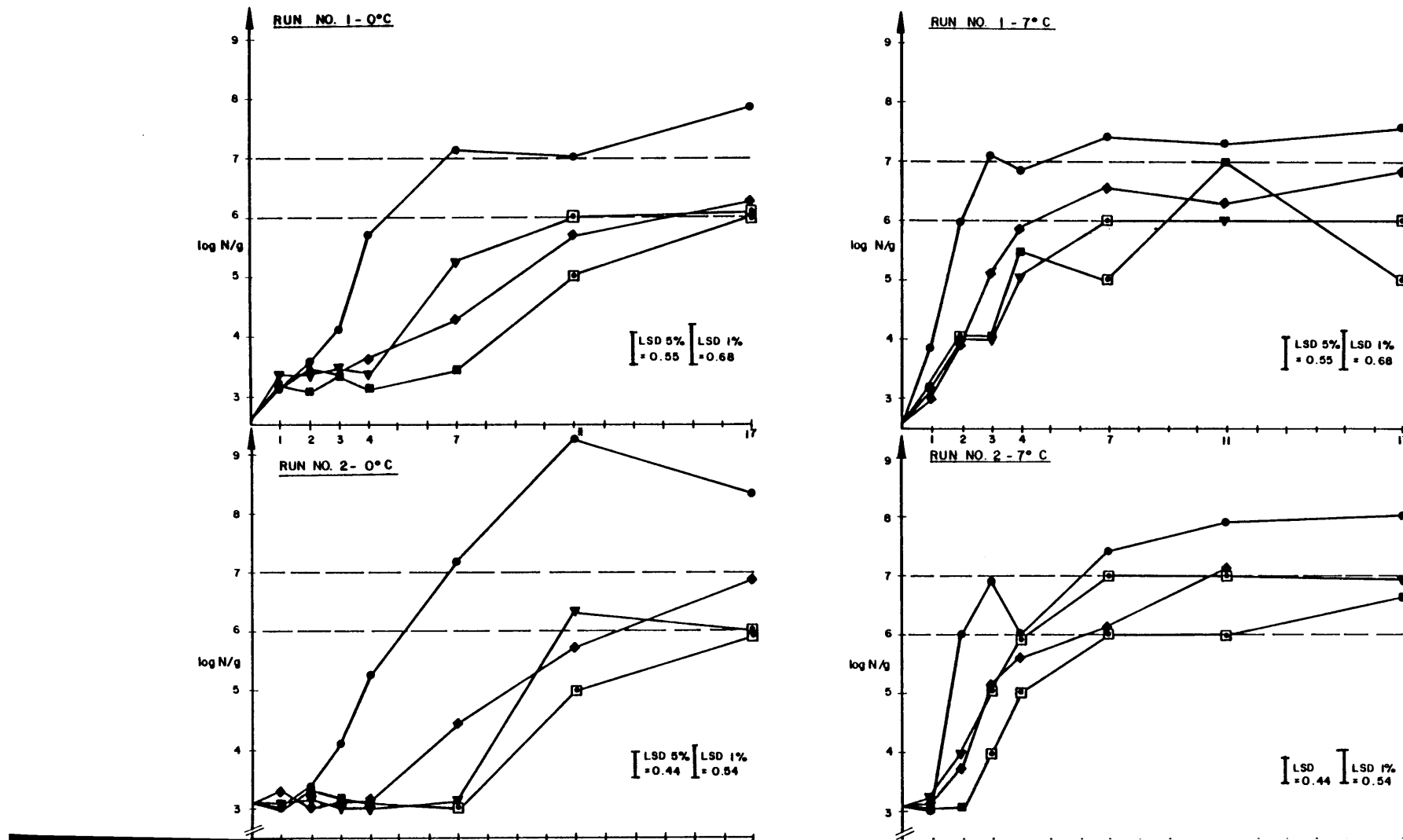
Fig. 4.10   Effects of treatments, storage time and temperature on sample pH (V10)
(for legend see Fig. 4.1)
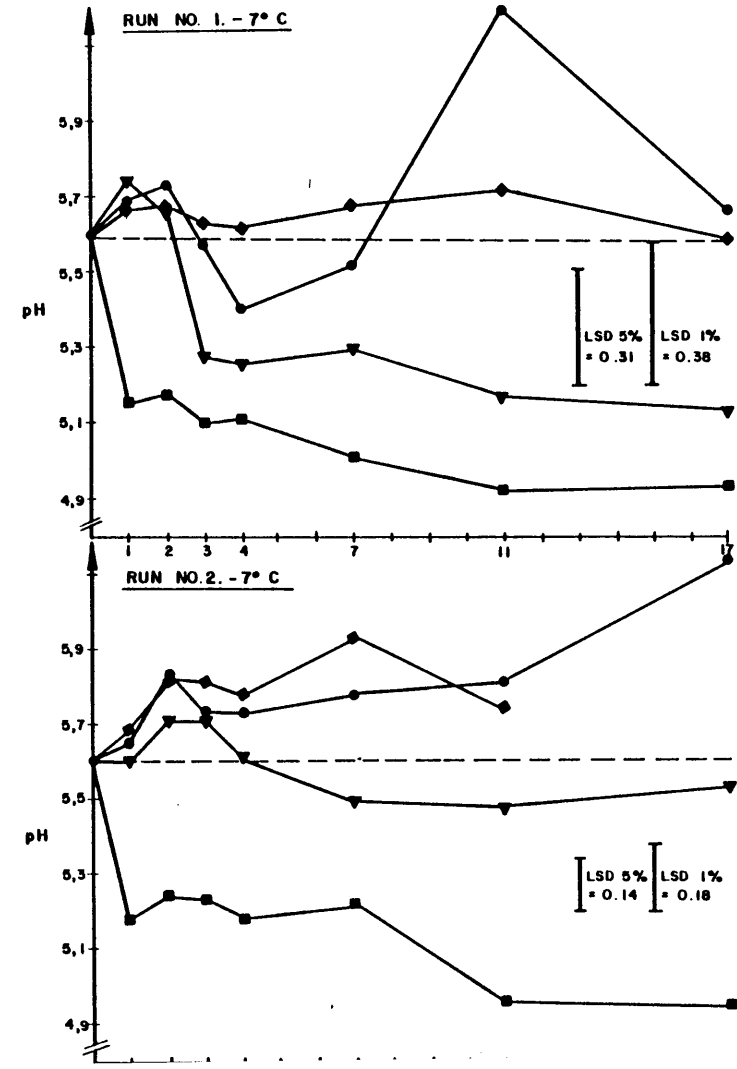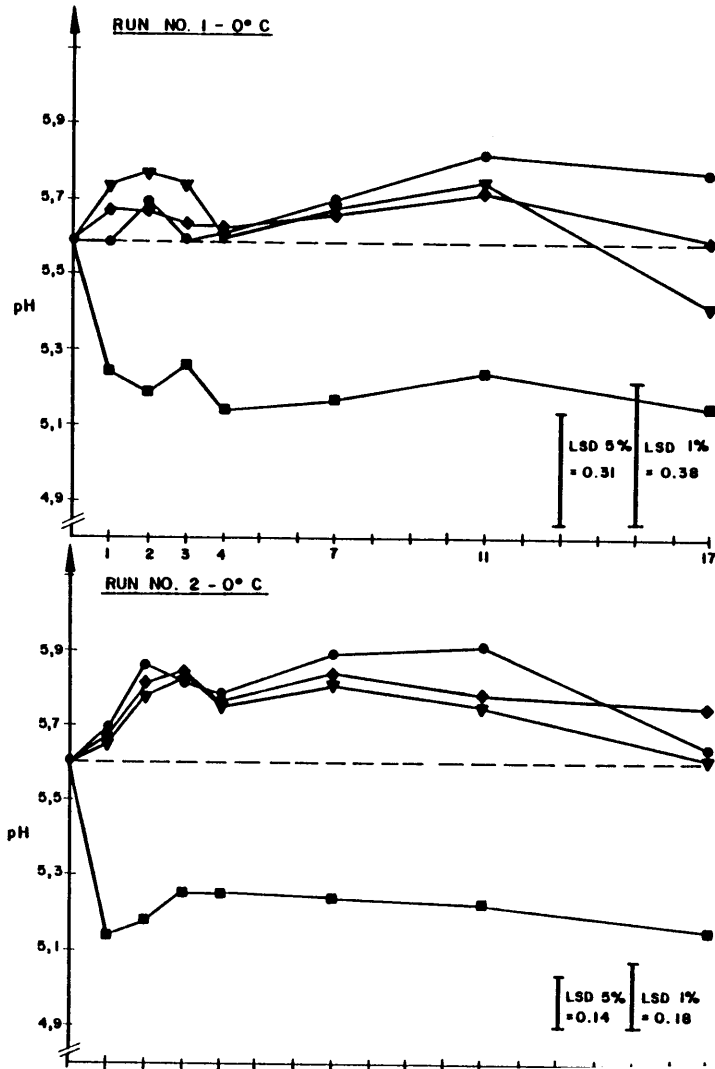
Fig. 4.11   Effects of treatments, storage time and temperature on Eh (V11)
(for legend see Fig. 4.1)
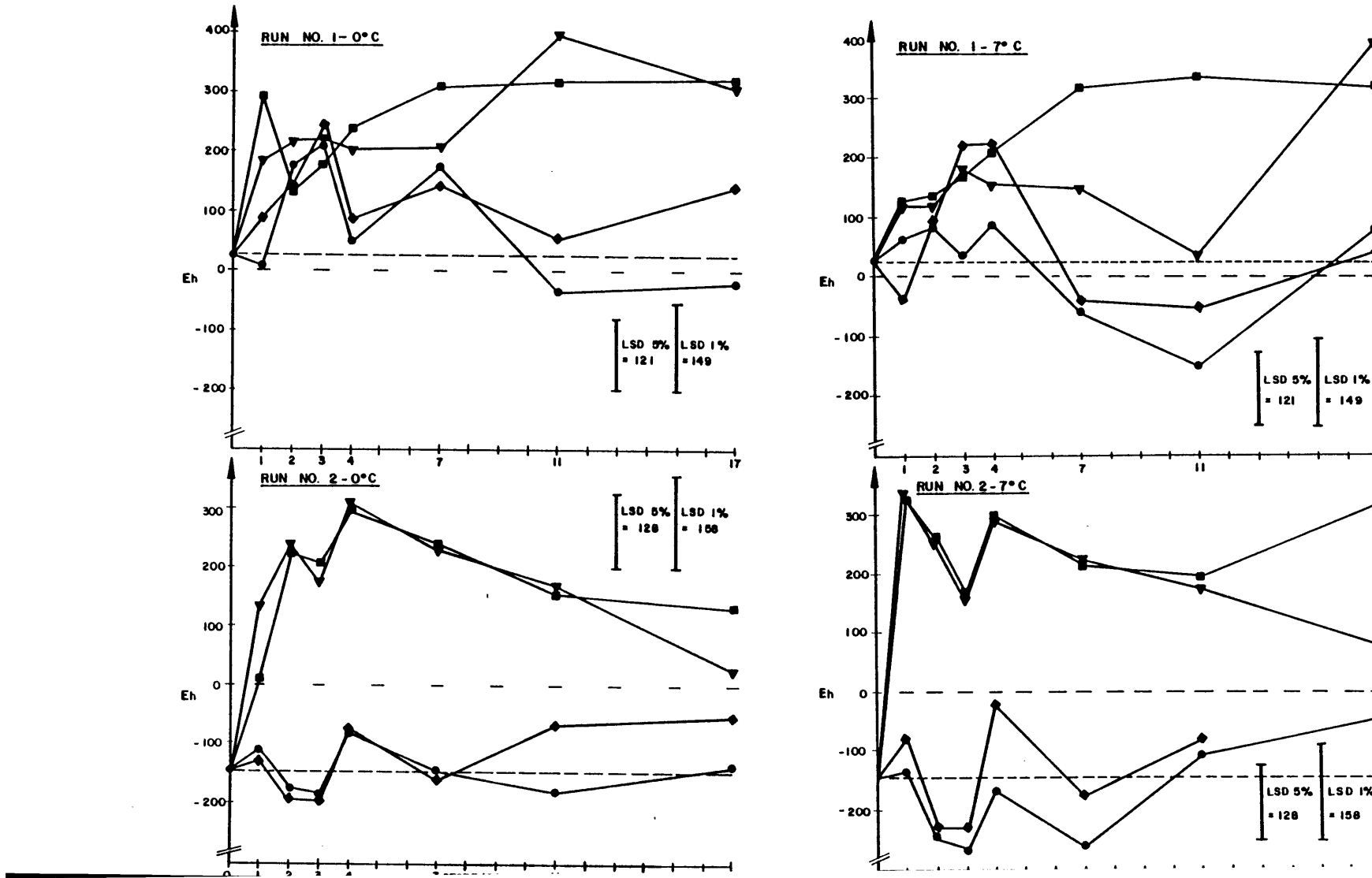
Fig. 4.12 **Effects of treatments, storage time and temperature on (D + L) lactic acid concentration (V12)**

(for legend see Fig. 4.1)
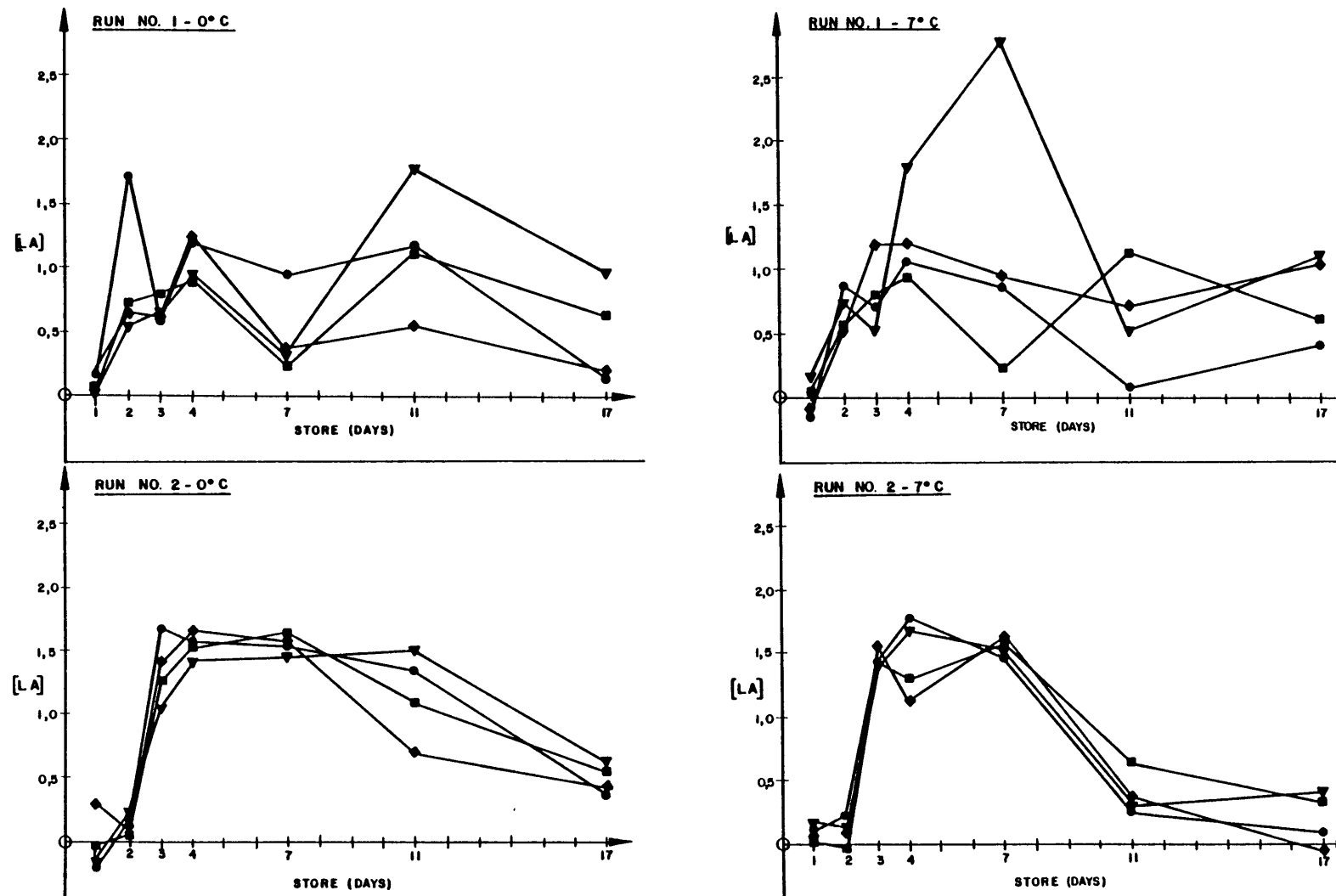
4.2.13 Effects of treatments, storage time and temperature on physical and sensory characteristics of raw minced beef

In the assessment of physical and sensory characteristics of the raw minced beef, several fundamental differences were observed between treatments and, as could be expected, between storage temperatures.

The packaging and/or additive treatments showed different effects on the raw minced beef on day 0. The TREAT 1 or 'aerobically' packed samples displayed a normal red fresh minced meat colour with a pleasant odour. A similar colour was retained by the vacuum packed samples (TREAT 2), although a certain amount of 'purge' was noted in each bag, which was not predominant enough at day 0 to be rated as objectionable. The addition of L(+) ascorbic acid to the raw minced beef (TREAT 3) caused a slight fade in red colour to an almost brown shade, which was retained after vacuum packaging. Limited amounts of purge were observed on day 0, but after addition of the L(+) ascorbic acid, the minced beef assumed a slightly sour odour. The addition of the commercial 'colour retainer' to the minced beef (TREAT 4) caused a lightening of the colour to an almost artificial bright red. No effect on the odour was observed on day 0 (Figs. 4.13 and 4.14).

At a storage temperature of 0°C, all samples from the four treatments showed excellent sensory and colour attributes for the first four days and no noticeable changes over the day 0 assessments were observed. After seven days, a definite colour deterioration was noted on the TREAT 1 samples, which assumed a uniform brown surface discolouration. After 11 days, this colour changed to brown/green and was accompanied by a strong off-sour odour. Complete spoilage and drying-out set in around 17 days. TREAT 2 and 3 samples showed no noticeable discolouration up to the end of the storage period, although the minced beef texture became increasingly 'soggy' from day 7 onwards and large amounts of purge were released from the meat, making the packages very unattractive (see Figs.4.15 and 4.16). TREAT 4 samples showed excellent colour retention over the entire storage period at 0°C, although a stale odour was noticeable on

all samples from day 11 onwards (Fig.4.17).

At a storage temperature of 7°C, changes in sensory and
physical characteristics were generally more pronounced and
set in earlier than at 0°C.   Samples of TREAT 1 already
started to show severe brown discolouration after four days,
accompanied by a slimy feel and a slight 'off' odour.   Complete
sensory spoilage set in after seven days.   Colour characteristics
on TREAT 2 and 3 samples were generally well-preserved over the
entire storage period, although, as at 0°C, purge increased with
storage time.   In the TREAT 2 samples a flat odour was noticeable
after 11 days accompanied by traces of H_2S.   After 17 days sub-
stantial amounts of H_2S were detected in the packages in addition
to a distinct off-sour odour.   Physical characteristics of TREAT
2 samples remained reasonably desirable up to day 11 in contrast
to TREAT 3 samples, which were all distinctly 'soggy' and brown.
A certain degree of break-up in texture was also noted for TREAT 3
samples, accompanied by increased purge volumes after day 11
(see Fig. 4.18). Organoleptic spoilage of TREAT 3 samples was
complete after 17 days, as was noticeable by a sour-off odour.
TREAT 4 samples remained desirable up to four days, after which a
noticeable surface browning, accompanied by a distinct flat
odour set in.   This tendency increased up to day 11, after which
the samples rapidly advanced to complete spoilage.

Observations on sensory and physical characteristics reinforced
the point discussed earlier, namely that low storage temperatures
were the key to increased shelf life of refrigerated raw minced
beef.   The lower temperatures tended to reinforce the suppressive
effect on microbial development by packaging and/or additive
treatments.   This comparison is pictured in Figs. 4.19 and 4.20, which
show samples from each treatment at both temperatures after 11
days of storage.

FIG. 4.13 Appearance of minced beef treated with commercial 'colour retainer' (TREAT 4) on day 0
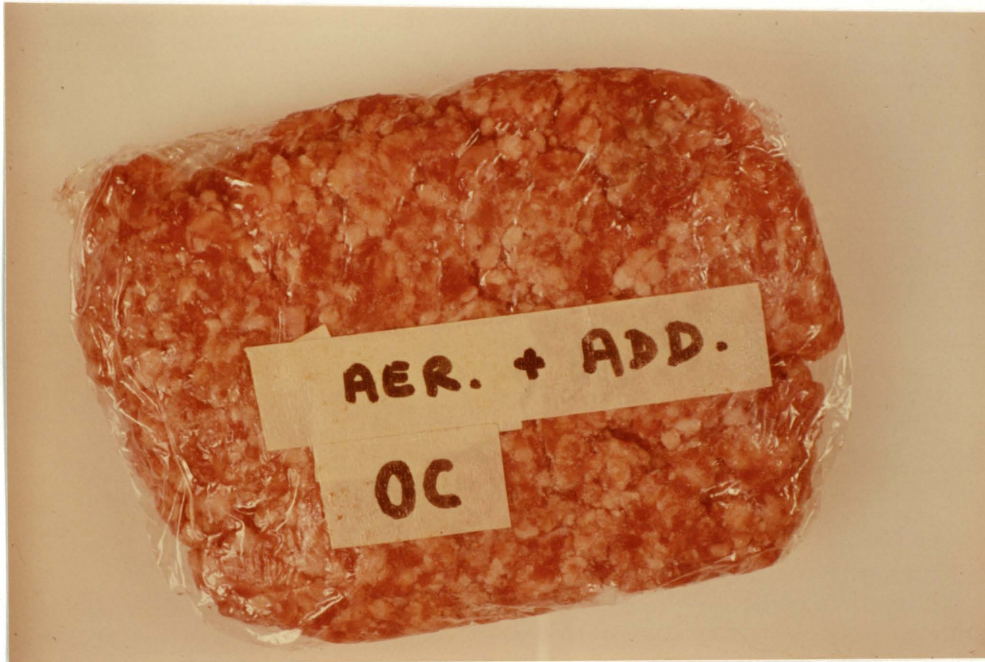
FIG. 4.14 Appearance of untreated minced beef (TREAT 1) on day 0
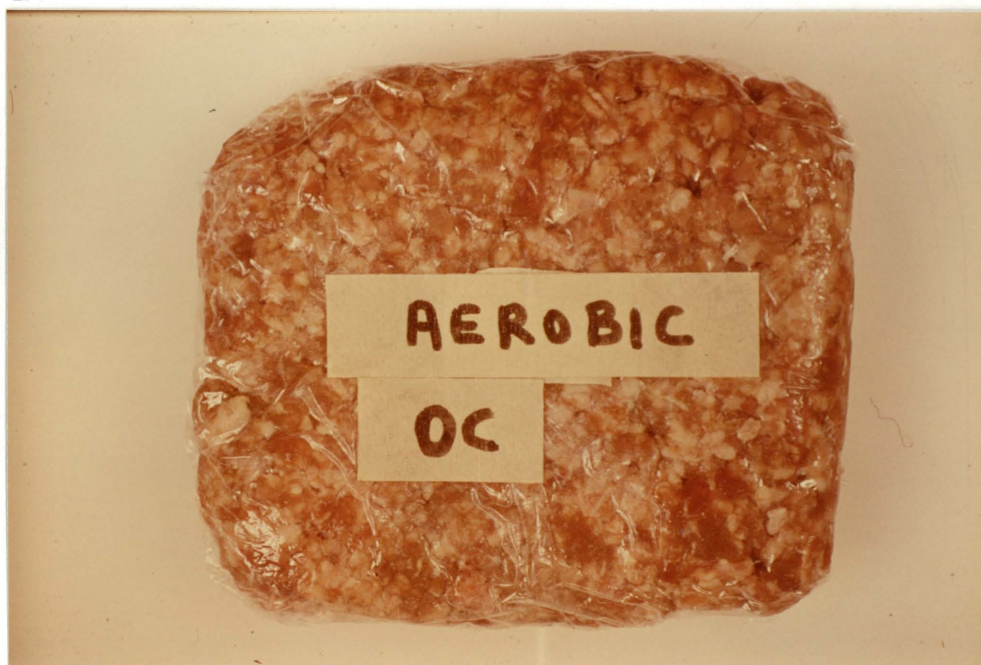
FIG. 4.15 Appearance of vacuum-packed minced beef (TREAT 2) stored at 0°C for seven days
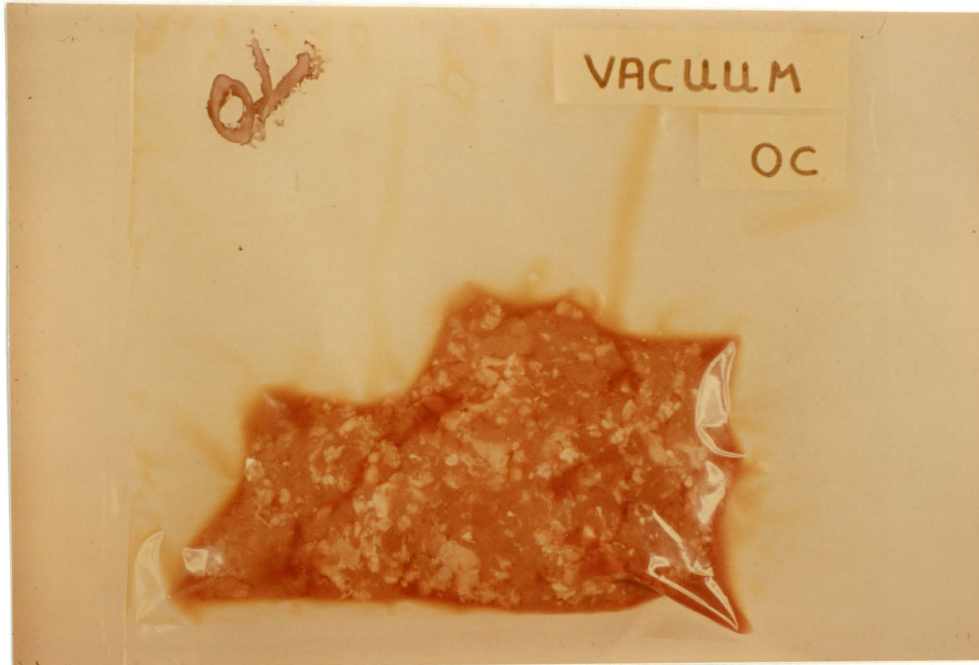
FIG. 4.16 Appearance of ascorbic acid treated, vacuum-packed minced beef (TREAT 3) stored at 0°C for seven days
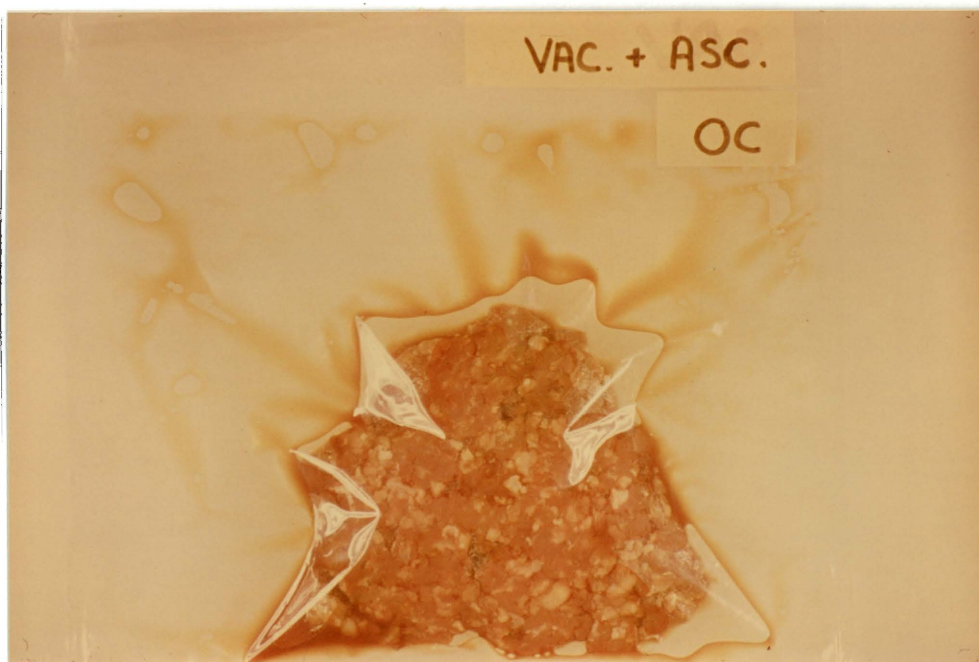
FIG. 4.17 Appearance of minced beef treated with the commercial 'colour retainer' (TREAT 4) after storage at 0°C for 11 days
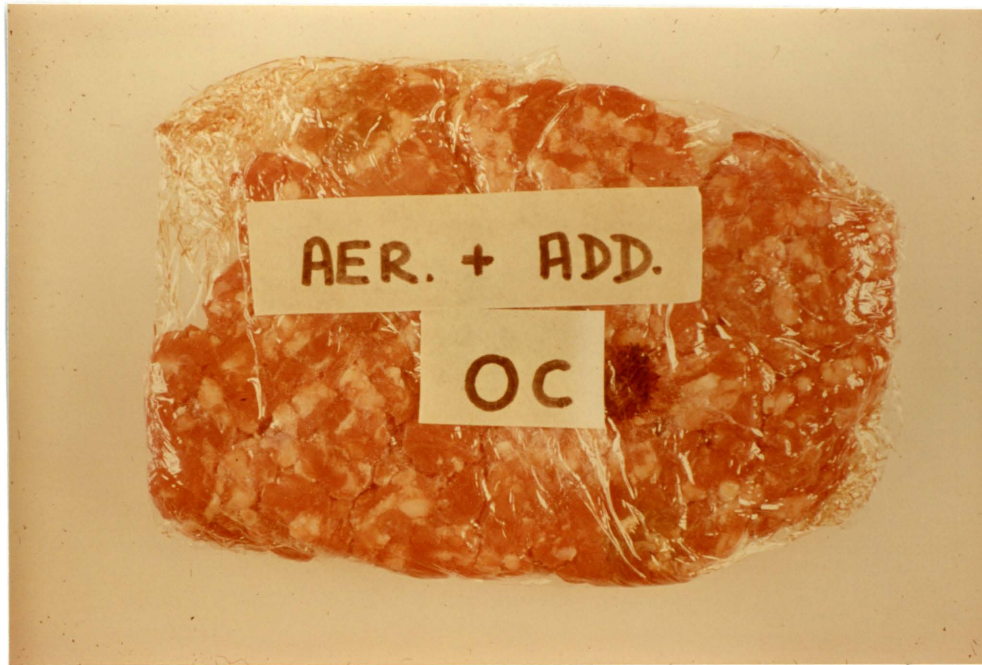
FIG. 4.18 Appearance of ascorbic acid treated, vacuum-packed minced beef (TREAT 3) stored at 7°C for 11 days
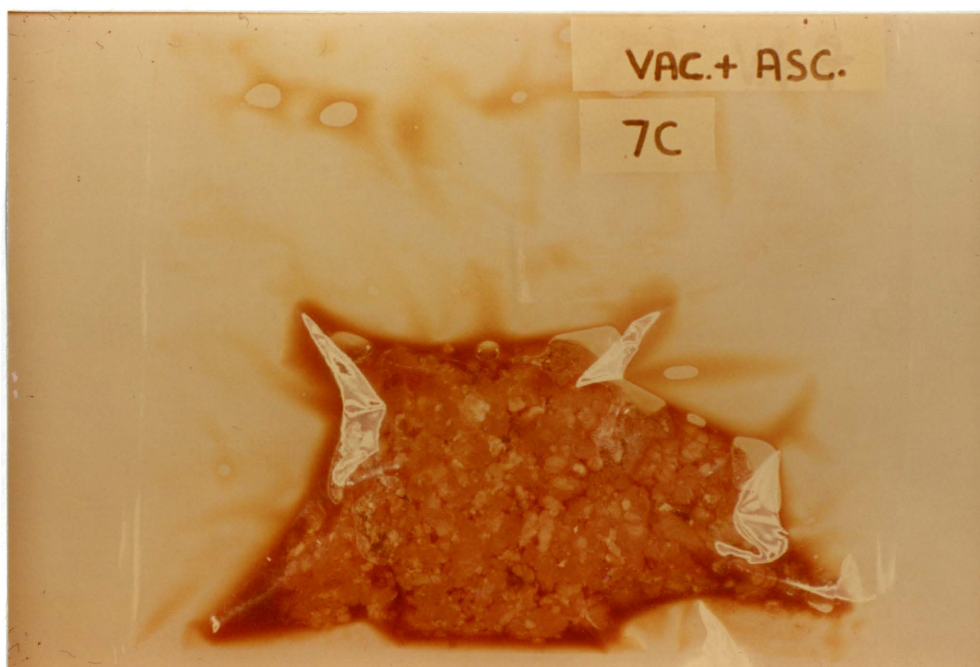
FIG. 4.19 Appearance of aerobically packed minced beef samples
(TREATS 1 and 4) stored at 0°C and 7°C for 11 days
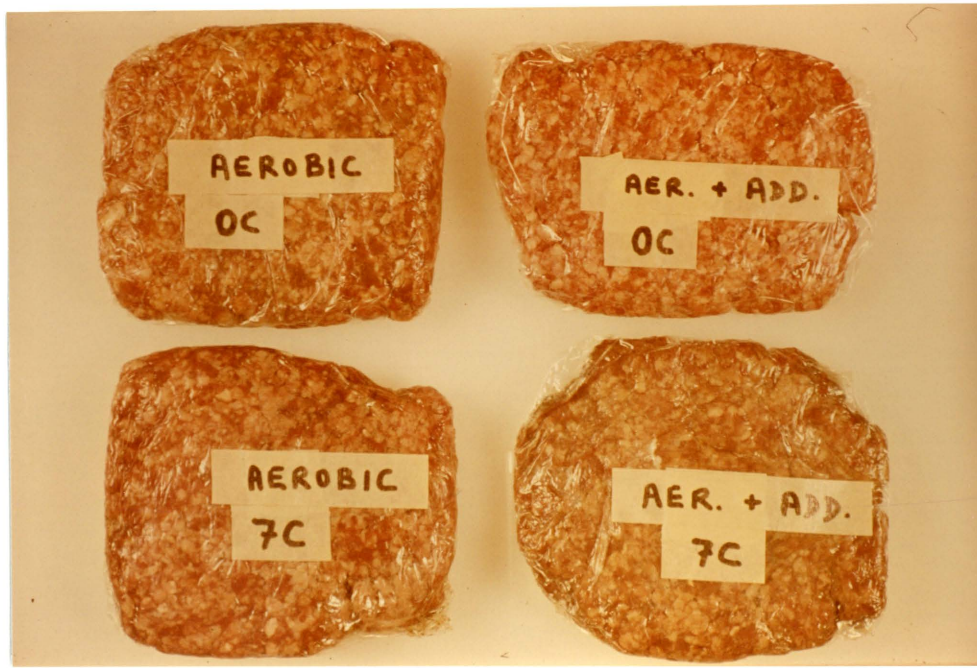
FIG. 4.20 Appearance of vacuum-packed minced beef samples
(TREATS 2 and 3) stored at 0°C and 7°C for 11 days
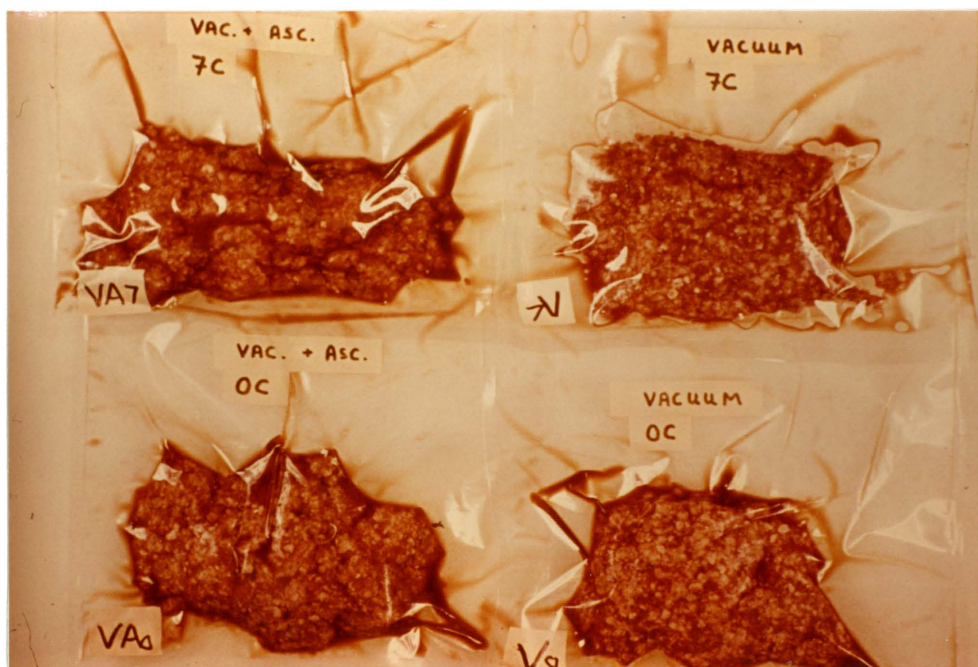
4.2.14 <u>Summarizing discussion - Effects of treatments, storage time</u>
        <u>and temperature on microbiological, physical and sensory</u>
        <u>characteristics of raw minced beef</u>

Several interesting observations on various bacterial groups were
made repeatedly in this study.

1. The lag phase for all bacterial groups (V1 to V9) was longer
    throughout in RUN NO. 2. This phenomenon was more pro-
    nounced at the low storage temperature of 0°C, but definitely
    also noticeable at 7°C. Surprisingly, the longer lag phase
    in RUN NO. 2 initially appeared to be quite independent of the
    treatments employed for different samples. Also, once
    growth had commenced, terminal levels were not noticeably
    different for both runs after a storage time of 17 days. The
    longer lag phase for all RUN NO. 2 samples displayed by all
    organisms was difficult to explain, especially in view of
    the fact that the raw material used for RUN NO. 2 showed
    significantly higher background levels of all bacterial
    groups, except lactic acid bacteria.

    The only plausible explanation for the observation could be
    the presence of a more extremely psychrotrophic population
    in the raw minced beef used for RUN NO. 1. This could
    account for the virtually immediate adaptation and growth with
    no noteworthy lag phase in RUN NO. 1 samples. This explan-
    ation is in agreement with the findings of several authors
    who indicated that the composition, initial level and
    especially the psychrotrophic nature of the spoilage bacteria
    depended vitally on conditions of production of the minced
    beef, and could vary substantially between batches (Newton,
    Harrison & Wauters, 1978; Greer, 1981).

2. For most of the bacterial groups investigated, the lower
    storage temperature showed a strongly positive synergism
    with other treatments with respect to reducing growth
    rates of spoilage organisms. This was very noticeable for
    the psychrotrophic bacterial and total aerobic plate counts,
    as well as the Enterobacteriaceae count. This tendency was

equally obvious for the cytochrome oxidase positive
fractions of the above counts. The most plausible
explanation for this observation was the fact that other-
wise near-ideal growth conditions are required by bacteria
to grow at low temperatures.

This was found to be absolutely critical especially with
regard to a plentiful supply of oxygen for strongly aerobic
spoilage organisms. The most obvious example for such
organisms are the pseudomonads which were isolated in the V2,
V4, V5 and V9 categories and displayed that exact response
(Brandt & Ledford, 1982). For these organisms both the
vacuum packaging and/or the additive treatments achieved
noticeable growth rate reduction at 0°C compared to 7°C,
also resulting in considerably lower terminal levels.

3. The lactic acid bacteria did not conform to the above des-
criptions, probably largely due to the fact that they are
mostly capable of rapid growth in anaerobic atmospheres and/
or in low-pH environments (TREAT 3). Additive treatment
combined with aerobic packaging (TREAT 4) appeared to pro-
duce a pronounced reduction in the growth rates of the lactic
acid bacteria. This phenomenon may be attributable to the
preserving effect of SO_2.

A noteworthy observation on lactic acid bacteria growth
was their rapid and seemingly uninhibited development in
the untreated, aerobically packed samples (TREAT 1). This
finding, which was observable in both runs at 0°C and 7°C,
can be rated as unusual, being not in agreement with the
commonly reported microaerophilic or anaerobic nature of these
organisms (Egan, Ford & Shay, 1980). To what extent the
development of lactic acid bacteria in the TREAT 1 samples
contributed to spoilage acceleration or inhibition was im-
possible to determine.

4. *B. thermosphacta* also showed little relation in growth
   patterns to any of the other groups investigated. Low
   temperature combined with vacuum packaging and low environ-
   mental pH (TREAT 3) appeared to cause virtual inability to
   grow, whereas vacuum packaging only and additive treatment
   in aerobic packaging led to partial inhibition of growth
   only. Growth rates of *B. thermosphacta* in the aerobic
   packagings appeared normal except for RUN NO. 2 at 7°C
   where somewhat erratic patterns were obtained.

   These findings agreed with literature reports (Dainty &
   Hibbard, 1980), and reinforced observations that *B. thermosphacta*
   was not normally regarded as a dominant spoilage organism in
   low temperature spoilage of raw minced beef (Pierson, Collins-
   Thompson & Ordal, 1970).

5. No positive evidence of antagonism between lactic acid bacteria
   and *B. thermosphacta* (Collins-Thompson & Lopez, 1980) could
   be produced from the results, although the pattern of low or
   no growth in vacuum package atmospheres at a pH below 5,5
   agreed with results of at least one previous report (Grau, 1980).

6. A point worth special discussion was the close correspondence
   found between total aerobic plate counts and psychrotrophic
   bacterial counts for both storage temperatures, especially for
   RUN NO.2. This reinforced literature reports (Greer, 1981) of
   a possible replacement of the lengthy psychrotrophic count by a
   25°C total aerobic count with minimal deviations.

7. Further excellent correspondence in patterns and numbers were
   found for the cytochrome oxidase positive fractions of the
   above counts, indicating similar applications of a revised
   method of detection of psychrotrophic pseudomonads. Although
   pseudomonad numbers counted on the Enterobacteriaceae count
   plates (V4 and V5) showed excellent correspondence to each
   other, this comparison was not shown to V2 and V9, both of
   which were essentially also pseudomonad counts.

The numerical levels obtained for V4 and V5 were noticeably
lower than for V2 and V9 (with the possible exception of
TREAT 1 samples), although an overall pattern correspondence
over 17 days did exist between the four counts (V2, V4, V5
and V9).   This indicated a possible, although limited,
application of the Enterobacteriaceae count plates in the
enumeration of pseudomonads, although it should be kept in
mind that the methods tended to underestimate numbers.

8. Two observations pointed to the fact that Enterobacteriaceae
    played a considerable part in low temperature spoilage of
    the raw minced beef.   Firstly, their multiplication to
    extremely high terminal levels (approximately 10^9/g) in both
    runs and for both temperatures indicated successful competition
    with the other bacterial groups involved in the spoilage process.

    Secondly, it was noted that the cytochrome oxidase positive
    fractions of the total aerobic plate count and the psychrotrophic
    bacterial count (V2 and V9, respectively) were numerically
    lower than the corresponding parent counts (V1 and V8), and
    thus did not account for all organisms isolated under V1 and V8.
    It was deemed as reasonable to assume that Enterobacteriaceae
    were amongst those organisms making up the observed differences
    in numbers.

    The proliferation of Enterobacteriaceae at 0°C is an unexpected
    phenomenon and stands in contrast to previous literature reports
    (Beebe, Vanderzant, Hanna, Carpenter & Smith, 1976).   It also
    reflects their ability to compete against other bacterial groups
    under these conditions.

9. An interesting observation that was consistent over several
    bacterial groups concerned the declining performance of TREAT
    4 (the commercial 'colour retainer') with increasing storage
    time at 7°C.   Although initial levels were mostly low,
    steady or other almost linear, growth ensued to terminal
    levels considerably higher than those for TREAT 2 and 3 samples.
    This tendency was observed chiefly for the total aerobic plate
    count, the Enterobacteriaceae count and the psychrotrophic
    bacterial count.   The practical importance of this observation

was questionable because of the satisfactory initial performance of TREAT 4 up to seven days of storage. This would probably represent the longest possible storage period under all treatments for a variety of other reasons such as physical and sensory attributes etc.

10. The relevance of the decreases in sample pH noted for TREATS 2 and 3 at 7°C could not be fully correlated to lactic acid bacteria development, as one would then have expected corresponding increases of lactic acid concentrations in the samples. Still, one would have to consider the possibility of the lactic acid being utilized preferentially by other spoilage organisms in the later stages of the storage period.

11. The comparatively poor reproducibility of redox potential (Eh) measurements was found to correspond to observations by other authors (Strange, Benedict, Smith & Swift, 1977). This led to the conclusion that the method in its current form was unsuitable for accurate quality assessments of refrigerated raw minced beef.

4.3   Correlational analysis of results on bacteriological studies

Before listing and discussing the correlational analysis of
results, it is of advantage to define the term 'correlation
coefficient between two variables'.   The correlation coefficient
between two variables describes the extent to which the variation
of the first variable is explained by that of the second variable
and vice versa.   With the scope of this definition in mind, it
was attempted to relate results of the bacteriological studies
to trends observed in the graphical representations that were
not covered by the analysis of variance.

The initial emphasis in the interpretation of correlational
analysis was placed on correlation coefficients (r) obtained
from Table 4.6, listing figures for correlations of the nine
variables (V1 to V9) over all treatments, temperatures, storage
days and both runs.   Although many of the variables correlated
reached the arbitrarily chosen minimum inclusion value of
r = 0,7746 (r^2 = 60%), it was decided to place most emphasis on
r-values $\geqslant$ 0,9000 ($r^2 \geqslant$ 81%), to single out the correlations most
important to the overall course of the study.   The decision was
taken on the basis of the high reproducibility and statistical
reliability of results.   By the use of strict criteria,
significant trends and patterns could be identified.   It was
thus found that VI (TAPC) and V2 (TAPC-ox.) correlated highly,
indicating that, overall, the total aerobic plate count was to
a large extent made up of cytochrome oxidase positive organisms,
probably *Pseudomonas* species, which had a deciding influence on
the change of the total aerobic plate count during storage.   The
same high correlation for these two variables was found in most
of the other correlation tables which were drawn up using fewer
observations (smaller N values), separating treatments and
storage temperatures (see Tables 4.7, 4.8, 4.9, 4.10, 4.11 and
4.12).

For the two storage temperatures, V1 and V2 were highly correlated at 0°C (r = 0,9341), but the correlation was lower at 7°C (r = 0,8369), although still above the minimum inclusion value. This fact reinforced the observation that many of the pseudomonads isolated on both V1 and V2 counts were indeed psychrotrophic in nature, being able to cause active spoilage even at very low storage temperatures.

Over the four treatments, V1 and V2 were again highly correlated (r ⩾ 0,9000) for TREATS 2, 3 and 4, with a lower value (r = 0,8490) for TREAT 1. This observation provided an indication that pseudomonads continued to contribute a significant part to total aerobic plate count and obviously played a part in the spoilage process in TREAT 2, 3 and 4. This observation was in contrast to trends found by other researchers for vacuum packed meats (Seideman, Vanderzant, Smith, Hanna & Carpenter, 1976; Christopher, Seideman, Carpenter, Smith & Vanderzant, 1979; Sison, De Mata, Baldonado, Olaguer, Gonzales, Pimentel & Beza, 1980.) These authors reported substantial reductions of pseudomonad percentages in the spoilage population on vacuum packaged meats with increasing storage time. It could of course be argued that vacuum packaged minced beef would contain substantially more residual oxygen than whole beef, because of the comminuted nature of the product, but the value of this observation is debatable.

Strong links between V1 and the psychrotrophic bacterial count (V8) were substantiated by the correlational analyses. V8, in turn, was found to be highly correlated to the oxidase positive fraction of the psychrotrophic bacterial count (V9). Both these interactions were highly correlated (r ⩾ 0,9000) in the table of overall correlations (Table 4.6).

Correlation coefficients in excess of 0,9000 were found for V1 and V8 at a temperature of 0°C (Table 4.11), and for TREATS 2, 3 and 4, indicating a close relationship between total aerobic plate count and psychrotrophic bacterial count. This observation supports suggestions in the literature for replacing the time-consuming psychrotrophic count (7°C, 10 days) with incubation at 25° for 24 hours (Greer, 1981). The high correlation of V8 and V9 (in addition to appearing in the table of overall

correlations) found for both temperatures and all four treatments, indicated that in almost all instances the pseudomonads were responsible for variations in the psychrotrophic bacterial count.

Correlations involving V1 and V9, V2 and V8, and V2 and V9 were mostly high in all analyses although values in excess of 0,9000 were not reached consistently. In addition, these three correlations did not achieve a value of 0,9000 or larger in the analysis of overall correlations (Table 4.6).

High correlations were also found between the Enterobacteriaceae count (V3), the 'Enterobacteriaceae' count after 48 hours ('pseudomonads' or V4) and the cytochrome oxidase positive fraction of the latter (V5). In the overall correlational analysis (Table 4.6) r-values in excess of 0,9000 were obtained for all of V3 and V4, V3 and V5 and V4 and V5. Similar values were obtained for the separate correlational analyses at 0°C, 7°C and TREAT 1. This observation indicated a relationship (maybe metabolic interdependence) between Enterobacteriaceae and pseudomonads involved in the low temperature spoilage process. The Enterobacteriaceae appeared after 24 hours at 30°C (under V3). The additional 24 hours' incubation of the V3 plates at room temperature and counting of newly-grown colonies (V4) followed by an oxidase test (V5) should theoretically have indicated pseudomonads only, with a close correspondence between V4 and V5. This interrelationship was operative at both temperatures (0° and 7°C) only for TREAT 1, i.e., in samples packed aerobically and containing no additive.

An interesting observation on the TREAT 1 correlational analysis (Table 4.7) was a series of high values ($r \geqslant 0,9000$) for the V3 and V8 or V9 interactions. The same was observed for V4 and V5 indicating a close link between Enterobacteriaceae count and psychrotrophic bacterial count, as well as a correspondence between the indirect pseudomonad enumeration procedures (V2, V4, V5 and V9). The fact that this series of high correlations was only observed for TREAT 1 samples indicated the overriding importance of pseudomonads in the aerobic spoilage of raw minced beef at refrigeration temperatures.

Looking at the overall correlational analysis (Table 4.6), it was noted that correlations between V7 (*B. thermosphacta*) and all other variables were below the minimum threshold value for inclusion. The same was observed for both temperatures and the TREAT 2, 3 and 4 correlational analyses. For the TREAT 1 correlations of V7 only one value exceeded 0,9000. It was also noted that the P-values associated with the V7 correlations were in many instances $< 0,05$, making the significance of the correlation coefficients doubtful. These results indicated that *B. thermosphacta* was not associated with any of the other bacterial groups to a significant degree, certainly not to the extent of its growth being regulated actively by such associations.

A similar trend was noted for correlations of V6 (lactic acid bacteria) with most of the other variables. The only correlational analysis yielding several high correlations of V6 was that for TREAT 1 (Table 4.7), none of which were above the value of 0,9000. The general absence of significant correlations indicated lack of association of the lactic acid bacteria with the other bacterial groups analysed except for V1. Several associations occurred in TREAT 1 samples, which also yielded some higher correlations for V7. This fact reinforced previous observations that high correlations were found between most variables in TREAT 1 samples, indicating considerable activity and association of the different bacterial groups in an additive-free environment with adequate supplies of oxygen.

4.4   Least significant difference values between treatments

Least significant differences (LSD) between treatments were calculated for variables V1 to V11 (for calculation details see Chapter 3, Section 14.3). Because of the excellent conformance of bacteriological results to the statistical model, LSD-values were very small in microbiological terms, leading to the establishment of a large number of statistically highly significant differences. This fact tended to obscure important trends and it was decided to judge differences according to microbiological rather than statistical criteria.

TABLE 4.6   CORRELATION COEFFICIENTS V1-9 X V1-9 OVER ALL TEMP , TREAT ,
STORE AND RUN (N = 221)
P-values for correlations are listed in bottom half of table
Arbitary threshold value chosen for significance: r = 0,7746
$(r^2 = 0,6000)$

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		*0,8994	0,8147	**0,7728	0,7286	0,8241	0,5580	0,9215	0,8694
V2	0,0001		0,7920	0,7920	0,7515	0,7261	0,6109	0,8816	0,8882
V3	0,0001	0,0001		0,9166	0,9148	0,6619	0,6556	0,8800	0,8436
V4	0,0001	0,0001	0,0001		0,9032	0,6110	0,7613	0,8252	0,8696
V5	0,0001	0,0001	0,0001	0,0001		0,5322	0,6964	0,7879	0,7790
V6	0,0001	0,0001	0,0001	0,0001	0,0001		0,4042	0,8338	0,7392
V7	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,6059	0,7120
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,9225
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	

Note: *   Values > 0,8900 are printed in italics
      **   Values ≤ 0,7746 are underlined

TABLE 4.7 CORRELATION COEFFICIENTS V1-9 X V1-9, TREAT 1 OVER ALL TEMP , STORE AND RUN (N = 54)

P-values for correlations are listed in bottom half of table

Arbitary threshold value chosen for significance: r = 0,8500

(r² = 0,7225)

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		0,8490	0,8683	0,8678	0,8425 **	0,8028	0,8104	0,8582	0,8563
V2	0,0001		0,9010 *	0,8703	0,8678	0,8174	0,8316	0,9199	0,8936
V3	0,0001	0,0001		0,9441	0,9394	0,8737	0,8645	0,9731	0,9453
V4	0,0001	0,0001	0,0001		0,9004	0,8685	0,9171	0,9386	0,9306
V5	0,0001	0,0001	0,0001	0,0001		0,8609	0,8272	0,9127	0,8729
V6	0,0001	0,0001	0,0001	0,0001	0,0001		0,8971	0,8673	0,8357
V7	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,8577	0,8566
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,9646
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	

Note: *   Values > 0,8900 are printed in italics

** Values ≤ 0,8500 are underlined

TABLE 4.8 CORRELATION COEFFICIENTS V1-9 X V1-9, TREAT 2 OVER ALL TEMP , STORE AND RUN (N = 56)

P-values for correlations are listed in bottom half of table

Arbitary threshold value chosen for significance: r = 0,8500

(r² = 0,7225)

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		0,9231 *	0,8373 **	0,7787	0,7102	0,8392	0,4249	0,9216	0,9093
V2	0,0001		0,7357	0,7553	0,6795	0,7041	0,2891	0,8211	0,8636
V3	0,0001	0,0001		0,8678	0,8785	0,8112	0,2591	0,9414	0,8875
V4	0,0001	0,0001	0,0001		0,7832	0,7703	0,3023	0,8441	0,8900
V5	0,0001	0,0001	0,0001	0,0001		0,5854	0,0469	0,7806	0,7265
V6	0,0001	0,0001	0,0001	0,0001	0,0001		0,5117	0,8894	0,8307
V7	0,0011	0,0307	0,0538	0,0236	0,7316	0,0001		0,4077	0,3826
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,9283
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	

Note: *   Values > 0,8900 are printed in italics

** Values ≤ 0,8500 are underlined

TABLE 4.9 CORRELATION COEFFICIENTS V1-9 X V1-9, TREAT 3 OVER ALL TEMP , STORE AND RUN (N = 56)

P-values for correlations are listed in bottom half of table
Arbitary threshold value chosen for significance: $r = 0,8500$
$(r^2 = 0,7225)$

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		0,9366*	0,6579**	0,6027	0,5187	0,8875	0,1650	0,9350	0,9129
V2	0,0001		0,6336	0,5998	0,4984	0,7764	0,1264	0,8334	0,8700
V3	0,0001	0,0001		0,7929	0,8102	0,5436	0,0405	0,6668	0,7019
V4	0,0001	0,0001	0,0001		0,7251	0,5445	0,2573	0,6144	0,7318
V5	0,0001	0,0001	0,0001	0,0001		0,3681	0,2042	0,5583	0,5385
V6	0,0001	0,0001	0,0001	0,0001	0,0052		0,2311	0,8872	0,8496
V7	0,2242	0,3522	0,7673	0,0556	0,1311	0,0865		0,2231	0,2292
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0982		0,9134
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0893	0,0001	

Note: * Values > 0,8900 are printed in italics
** Values ≤ 0,8500 are underlined

TABLE 4.10 CORRELATION COEFFICIENTS V1-V9 X V1-9, TREAT 4 OVER ALL TEMP , STORE AND RUN (N = 55)

P-values for correlations are listed in bottom half of table
Arbitary threshold value chosen for significance: $r = 0,8500$
$(r^2 = 0,7225)$

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		0,9204*	0,8706	0,8999	0,8340**	0,8531	0,7726	0,9665	0,8769
V2	0,0001		0,7555	0,8713	0,8094	0,8076	0,7636	0,9221	0,8978
V3	0,0001	0,0001		0,8703	0,8542	0,7885	0,5662	0,8453	0,6847
V4	0,0001	0,0001	0,0001		0,8631	0,8590	0,6326	0,8725	0,8323
V5	0,0001	0,0001	0,0001	0,0001		0,7963	0,5398	0,8054	0,6922
V6	0,0001	0,0001	0,0001	0,0001	0,0001		0,7117	0,8424	0,8503
V7	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,7601	0,8338
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,9169
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	

Note: * Values > 0,8900 are printed in italics
** Values ≤ 0,8500 are underlined

TABLE 4.11 CORRELATION COEFFICIENTS V1-9 X V1-9, TEMP = 0°C OVER ALL TREAT, STORE AND RUN (N = 111)

P-values for correlations are listed in bottom half of table

Arbitary threshold value chosen for significance: r = 0,8367

(r² = 0,7000)

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		0,9341	0,7963 * **	0,7949	0,6908	0,7824	0,7180	0,9485	0,8936
V2	0,0001		0,7614	0,7681	0,6660	0,6681	0,7000	0,8953	0,8721
V3	0,0001	0,0001		0,9357	0,9194	0,4976	0,7747	0,8335	0,8205
V4	0,0001	0,0001	0,0001		0,8947	0,5557	0,8325	0,8214	0,8514
V5	0,0001	0,0001	0,0001	0,0001		0,3408	0,7222	0,7248	0,6938
V6	0,0001	0,0001	0,0001	0,0001	0,0003		0,5107	0,7548	0,7278
V7	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,7207	0,8095
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,9397
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	

Note: * Values > 0,8900 are printed in italics

TABLE 4.12 CORRELATION COEFFICIENTS V1-9 X V1-9, TEMP = 7°C OVER ALL TREAT, STORE AND RUN (N = 110)

P-values for correlations are listed in bottom half of table

Arbitary threshold value chosen for significance: r = 0,8367

(r² = 0,7000)

	V1	V2	V3	V4	V5	V6	V7	V8	V9
V1		0,8369	0,7739 **	0,7003	0,6813	0,8098	0,4033	0,8721	0,8136
V2	0,0001		0,7495	0,7704	0,7486	0,6752	0,5281	0,8298	0,8828
V3	0,0001	0,0001		0,8942 *	0,8828	0,6471	0,5682	0,8775	0,8375
V4	0,0001	0,0001	0,0001		0,8974	0,5648	0,6998	0,7936	0,8628
V5	0,0001	0,0001	0,0001	0,0001		0,5012	0,6813	0,7725	0,8142
V6	0,0001	0,0001	0,0001	0,0001	0,0001		0,2853	0,8374	0,6944
V7	0,0001	0,0001	0,0001	0,0001	0,0002	0,0025		0,5051	0,6229
V8	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001		0,8942
V9	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	

Note: * Values > 0,8900 are printed in italics

** Values < 0,8367 are underlined

The following criteria were used for judgement:

(i) For V1 to V9, LSD values $\geqslant$ 1,000 were rated as highly
significant. This was based on a commonly employed
guideline stating that two microbiological values are
different only when they differ by one log number or
more.

(ii) For V10, LSD values $\geqslant$ 0,200 were rated as highly signifi-
cant. This rather small pH-difference was chosen
because of the fact that meat is a well-buffered system
and small pH changes tend to reflect considerable micro-
biological and physical changes.

(iii) For V11, LSD values $\geqslant$ 100 (mV) were rated as highly
significant, but because of the earlier-mentioned inherent
unreliability of the Eh measurements, results are only
presented but not discussed.

Data from the two runs of the study were separated for LSD
calculation but, with very few exceptions, correspondence between
the findings for both runs for each variable was excellent.
Results are presented in Tables 4.13 to 4.23. The results for
statistical calculations of LSD 5% and LSD 1% are given next to
each table. Differences judged as highly significant by the
criteria under (i) to (iii) above are printed in italics.

Many of the significant trends indicating differences between
treatments discovered in the graphical representations and
the correlational analyses were also shown up and reinforced
by the LSD calculations.

For V1 (TAPC) highly significant differences existed between
TREAT 1 and TREAT 3 as well as TREAT 4 for both runs (Table 4.13).
For RUN NO. 1 only, a highly significant difference was found
between TREATS 2 and 4. These results indicated that both TREATS
3 and 4 achieved microbiologically significant reductions in total
aerobic plate count as compared to the untreated, aerobically
packed control (TREAT 1). Less importance was assigned to the
TREAT 2-TREAT 4 result as it was not consistent over both runs.

For V2 (TAPC-ox.) the exact same trend to V1 was observed, this
time consistently over both runs (Table 4.14). It was noted that
also for the cytochrome oxidase fraction of the total aerobic
plate count TREATS 3 and 4 achieved significant reductions in
numbers. The fact that the same significance grouping of
treatments was found for V1 and V2 reinforced the fact that
these two variables were highly correlated and exhibited similar
responses to the different treatments.

A similar grouping effect to the one observed for V1 and V2 was
found for V3, V4 and V5, which differed from the V1-V2 grouping
by only one additional highly significant difference, namely
between TREATS 1 and 2 (Tables 4.15, 4.16 and 4.17). This trend
was consistent over both runs, indicating that for all of V3, V4
and V5, TREATS 2, 3 and 4 resulted in significant reductions of
bacterial numbers compared to TREAT 1. These results again
reinforced the already described high correlation between the
Enterobacteriaceae counts and counts of pseudomonads isolated
from the EC (V3) plates.

In addition to this, it was found that V8 and V9 displayed an
identical response to TREATS 2, 3 and 4 as did V3, V4 and V5
(Tables 4.20 and 4.21). Again this fact underlined the finding
that the psychrotrophic spoilage population, and especially the
psychrotrophic pseudomonads, were highly correlated to the
pseudomonads isolated under V4 and V5 as well as to the
Enterobacteriaceae. The latter observation pointed to the
fact that the Enterobacteriaceae played a significant part in
psychrotrophic spoilage of the raw minced beef.

For V6 (LC), a different set of least significant differences
was found (Table 4.18). It appeared that only TREAT 4 resulted
in significant reductions in numbers of lactic acid bacteria,
when compared to TREATS 1, 2 and 3. This finding was also clearly
evident from the graphical representations of storage time effect

on V6, where TREATS 1, 2 and 3 showed virtually identical (statistically not significantly different) growth patterns (Figure 4.6).   In addition, the above findings reiterated earlier observations that the responses of the lactic acid bacteria in the entire study were not linked to any of the other bacterial groups investigated.

For V7 (BTC) a large number of highly significant differences were observed, some of which were not consistent over both runs (Table 4.17). The differences consistent for both runs were between TREAT 1 and TREATS 2 and 3 as well as between TREATS 3 and 4.   In addition, significant differences were found between TREATS 2 and 3 and between TREATS 2 and 4 for RUNS NO. 1 and NO. 2, respectively.   Linking the above findings to the graphical representations of effect of storage time on V7, it was concluded that differences between TREAT 3 (and possibly TREAT 2) and the other treatments were the main underlying pattern.   This fact reinforced the pronounced inhibition on B. *thermosphacta* by the vacuum packaging/ascorbic combination evident from the graphs (Figure 4.7).   This trend was also displayed by TREAT 2 (vacuum packaging only), but to a lesser extent.   Again, the least significant difference calculations indicated that B. *thermosphacta* responses were not regulated to any significant extent by the other bacterial groups under study.

It was impossible to draw detailed conclusions as to the relative effectiveness of the treatments with regard to one another from the numerical values of the differences in the LSD tables.   This was because of inconsistent patterns between runs for each variable. It was decided that such assessments were best made by a detailed study of the graphical representations (Section 4.2).

For V10, the observation made from the graphical representations was reinforced by highly significant differences between TREAT 3 and the other treatments.   This effect was attributed to the addition of L(+) ascorbic acid to the raw minced beef under TREAT 3.   Also it was indicated by the LSD figures that differences observed between pH's of TREATS 1, 2 and 3 were not of major significance in a microbiological context.

Generally speaking, the calculation of least significant differences and the establishment of microbiologically significant differences for result discussion, further served to reinforce major trends that were observed in the other analyses.

TABLE 4.13 LEAST SIGNIFICANT DIFFERENCES IN TAPC (V1) BETWEEN TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF (microbiologically highly significant differences are printed in italics)

RUN NO. 1

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,8764	*1,0974*	*1,8658*
TREAT 2	★ ★		0,2210	*0,9895*
TREAT 3	★ ★			0,7684
TREAT 4	★ ★	★ ★	★ ★	

$LSD_{5\%} = 0,2337$

$LSD_{1\%} = 0,2879$

RUN NO. 2

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,7379	*1,2163*	*1,3025*
TREAT 2	★ ★		0,4784	0,5646
TREAT 3	★ ★	★		0,0862
TREAT 4	★ ★	★ ★		

$LSD_{5\%} = 0,4076$

$LSD_{1\%} = 0,5022$

Note: LSD values are given as natural logarithms

TABLE 4.14 LEAST SIGNIFICANT DIFFERENCES IN TAPC-ox.(V2) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

RUN NO. 1

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,8396	*1,1311*	*1,2219*
TREAT 2	* *		0,2915	0,3823
TREAT 3	* *	* *		0,0908
TREAT 4	* *	* *		

$$LSD_{5\%} = 0,1915$$

$$LSD_{1\%} = 0,2359$$

RUN NO. 2

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,8835	*1,4817*	*1,0926*
TREAT 2	* *		0,5982	0,2091
TREAT 3	* *	* *		-0,3891
TREAT 4	* *	* *		

$$LSD_{5\%} = 0,1372$$

$$LSD_{1\%} = 0,1691$$

Note: LSD values are given as natural logarithms

TABLE 4.15 LEAST SIGNIFICANT DIFFERENCES IN EC (V3) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,7914*	*2,7242*	*2,3198*
TREAT 2	* *		0,9328	0,5284
TREAT 3	* *	* *		-0,4044
TREAT 4	* *	* *	*	

RUN NO. 1

$LSD_{5\%}$ = 0,3409

$LSD_{1\%}$ = 0,4200

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,3534*	*1,9192*	*1,9526*
TREAT 2	* *		0,5658	0,5992
TREAT 3	* *	* *		0,0334
TREAT 4	* *	* *		

RUN NO. 2

$LSD_{5\%}$ = 0,2417

$LSD_{1\%}$ = 0,2976

Note: LSD values are given as natural logarithms

TABLE 4.16 LEAST SIGNIFICANT DIFFERENCES IN EC-48 (V4) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*2,1356*	*2,9122*	*2,2083*
TREAT 2	★ ★		0,7766	0,0727
TREAT 3	★ ★	★ ★		-0,7039
TREAT 4	★ ★		★ ★	

RUN NO. 1

$LSD_{5\%}$ = 0,3565

$LSD_{1\%}$ = 0,4392

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,4748*	*2,0494*	*1,6536*
TREAT 2	★ ★		0,5746	0,1788
TREAT 3	★ ★	★ ★		-0,3958
TREAT 4	★ ★		★ ★	

RUN NO. 2

$LSD_{5\%}$ = 0,1988

$LSD_{1\%}$ = 0,2449

Note: LSD values are given as natural logarithms

TABLE 4.17 LEAST SIGNIFICANT DIFFERENCES IN EC-48-ox.(V5) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*2,2672*	*2,3260*	*2,1623*
TREAT 2	* *		0,5588	-0,1049
TREAT 3	* *	* *		-0,6637
TREAT 4	* *		* *	

RUN NO. 1

$LSD_{5\%}$ = 0,3336

$LSD_{1\%}$ = 0,4110

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,4668*	*2,0829*	*1,7411*
TREAT 2	* *		0,6161	0,2742
TREAT 3	* *	* *		-0,3418
TREAT 4	* *	* *	* *	

RUN NO. 2

$LSD_{5\%}$ = 0,1400

$LSD_{1\%}$ = 0,1724

Note: LSD values are given as natural logarithms

TABLE 4.18 LEAST SIGNIFICANT DIFFERENCES IN LC (V6) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		-0,2433	-0,1216	*1,8433*
TREAT 2	* *		0,1217	*2,0866*
TREAT 3				*1,9649*
TREAT 4	* *	* *	* *	

RUN NO. 1

$LSD_{5\%}$ = 0,1525

$LSD_{1\%}$ = 0,1879

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,1103	0,2529	*1,2700*
TREAT 2			0,1426	*1,1597*
TREAT 3	* *			*1,0171*
TREAT 4	* *	* *	* *	

RUN NO. 2

$LSD_{5\%}$ = 0,1600

$LSD_{1\%}$ = 0,1972

Note: LSD values are given as natural logarithms

TABLE 4.19 LEAST SIGNIFICANT DIFFERENCES IN BTC (V7) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		2,2189	3,2118	1,7307
TREAT 2	* *		0,9929	-0,4882
TREAT 3	* *	* *		-1,4811
TREAT 4	* *	* *	* *	

RUN NO. 1

$LSD_{5\%}$ = 0,3666

$LSD_{1\%}$ = 0,4517

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		2,1919	2,7590	0,9012
TREAT 2	* *		0,5671	-1,2907
TREAT 3	* *	* *		-1,8578
TREAT 4	* *	* *	* *	

RUN NO. 2

$LSD_{5\%}$ = 0,1999

$LSD_{1\%}$ = 0,2463

Note: LSD values are given as natural logarithms

TABLE 4.20 LEAST SIGNIFICANT DIFFERENCES IN PC (V8) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,0363*	*1,2354*	*1,8196*
TREAT 2	* *		0,1991	0,7833
TREAT 3	* *	*		0,5842
TREAT 4	* *	* *	* *	

RUN NO. 1

$LSD_{5\%} = 0,1894$

$LSD_{1\%} = 0,2333$

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,0218*	*1,5526*	*1,5294*
TREAT 2	* *		0,5308	0,5076
TREAT 3	* *	* *		-0,0232
TREAT 4	* *	* *		

RUN NO. 2

$LSD_{5\%} = 0,1688$

$LSD_{1\%} = 0,2079$

Note: LSD values are given as natural logarithms

TABLE 4.21 <u>LEAST SIGNIFICANT DIFFERENCES IN PC-ox.(V9) BETWEEN</u>
<u>TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF</u>
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,3872*	*1,6934*	*1,2128*
TREAT 2	* *		0,3062	-0,1744
TREAT 3	* *	* *		-0,4806
TREAT 4	* *	*	* *	

RUN NO. 1

$LSD_{5\%}$ = 0,1466

$LSD_{1\%}$ = 0,1806

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		*1,3535*	*1,8259*	*1,3446*
TREAT 2	* *		0,4724	-0,0089
TREAT 3	* *	* *		-0,4813
TREAT 4	* *		* *	

RUN NO. 2

$LSD_{5\%}$ = 0,1177

$LSD_{1\%}$ = 0,1450

Note: LSD values are given as natural logarithms

TABLE 4.22   LEAST SIGNIFICANT DIFFERENCES IN pH (V10) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,1707	*0,5596*	-0,0011
TREAT 2	* *		*0,3889*	-0,1718
TREAT 3	* *	* *		*-0,5607*
TREAT 4		* *	* *	

RUN NO. 1

$LSD_{5\%} = 0,0829$

$LSD_{1\%} = 0,1022$

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		0,1414	*0,6328*	0,0264
TREAT 2	* *		*0,4914*	-0,1150
TREAT 3	* *	* *		*-0,6064*
TREAT 4		* *	* *	

RUN NO. 2

$LSD_{5\%} = 0,0382$

$LSD_{1\%} = 0,0470$

TABLE 4.23 LEAST SIGNIFICANT DIFFERENCES IN Eh (V11) BETWEEN
TREATMENTS FOR SHELF LIFE EXTENSION OF MINCED BEEF
(microbiologically highly significant differences are
printed in italics)

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		-157,3214	-195,8929	-46,9643
TREAT 2	* *		-38,5715	110,3571
TREAT 3	* *	*		148,9285
TREAT 4	* *	* *	* *	

RUN NO. 1

$LSD_{5\%}$ = 32,2491

$LSD_{1\%}$ = 39,7308

	TREAT 1	TREAT 2	TREAT 3	TREAT 4
TREAT 1		-42,7143	-377,1428	-23,5714
TREAT 2	* *		-17,8571	66,2857
TREAT 3	* *			353,5714
TREAT 4		* *	* *	

RUN NO. 2

$LSD_{5\%}$ = 34,3412

$LSD_{1\%}$ = 42,3083

4.5   Identification of representative spoilage isolates

Considerable difficulties were experienced with the
identification to species level of many spoilage isolates,
and in many cases only major characteristics were considered
in the identification process.

4.5.1   Psychrotrophic spoilage isolates

In view of the overwhelming role of psychrotrophic bacteria
in the spoilage of ground beef at refrigeration temperatures,
128 (one hundred and twenty eight) isolated colonies were
selected for identification from Standard 1 Nutrient Agar
plates representing the highest dilutions of psychrotrophic
counts (incubated at 7°C for 10 days).

All 128 isolates with once exception were identified, although
in some cases identification to species level was impossible
within the constraints of the biochemical and other tests used.
This was particularly evident in the case of the Gram-negative
isolates with special reference to the large number of
pseudomonads.   Similar problems using minikit identification
systems were reported in the literature (Cox & Mercuri, 1978).

In addition, considerable difficulties were experienced with
the accurate identification of many psychrotrophic lactic
acid bacteria.   Isolates number 60, 66, 81, 86, 87 and 115
were classified as *Lactobacillus* sp. (subgenus *Betabacterium*)
because of their short, rod-shaped morphology and their ability
to form carbon dioxide from glucose.   However, none of these
isolates (except 115) were typical *Betabacterium* spp. as all
produced only the L(+) isomer of lactic acid.   Isolate 115
closely resembled *L. vaccinostercus* although it was not
completely typical (Table 4.24),notably differing by several
carbohydrate fermentations (ribose, xylose, trehalose, cellobiose)
and the ability to grow at 15°C.   Isolates 60, 66, 81 and 87 were
identical in their test patterns, whereas isolate 86 differed from
the former four by fermentation of galactose and ribose.   This
group of four identical isolates was clearly different from both
isolate 115 and *L. vaccinostercus*.

TABLE 4.24 COMPARATIVE CHARACTERISTICS OF *LACTOBACILLUS VACCINOSTERCUS* AND ISOLATE 115

BIOCHEMICAL OR OTHER TEST	L. VACCINOSTERCUS	PSYCHROTROPHIC ISOLATE NO. 115
LACTIC ACID ISOMER	DL	L(+)
DAP IN CELL WALL	+	+
GROWTH AT 15°C	-	+
GROWTH AT 45°C	-	-
SLIME FROM SUCROSE	-	-
ARGININE HYDROLYSIS	+	+
FERMENTATION OF:		
ARABINOSE	+	+
RIBOSE	+	-
XYLOSE	+	-
MALTOSE	+	+
GALACTOSE	+	+
TREHALOSE	-	+
RAFFINOSE	-	-
MANNITOL	-	-
CELLOBIOSE	-	+
MELIBIOSE	-	±

Source and identification details for all psychrotrophic isolates are shown in Table A.3 (See Appendix). These results are summarized in Table 4.25, showing the distribution of psychrotrophic isolates over the four treatments. This summary shows that pseudomonads featured most prominently overall amongst the 128 isolates, followed by lactobacilli, yeasts and Enterobacteriaceae, in that order. Other Gram-positive isolates were only present in low numbers. When establishing the treatment type to isolate identification relationships (Table 4.26) it was found that pseudomonads featured most prominently in TREAT 1 samples, although significant numbers were also recovered from TREAT 2 and TREAT 3 samples. This was not in keeping with general literature reports indicating an almost total absence of pseudomonads on vacuum-packed, minced beef (Grau & Macfarlane, 1980). Few pseudomonads were isolated from TREAT 4 samples.

Lactobacillus isolates were expectedly most numerous on TREAT 2 and 3 samples (approximately equal proportions), with a surprisingly large incidence (11,4%) on TREAT 4 samples. No such isolates were found on TREAT 1 samples. All yeast isolates were made from TREAT 4 samples, indicating a significant occurrence of yeast in samples subjected to TREAT 4. This could be due to a selective effect of sulphur dioxide (SO_2), by which development of the yeast population was favoured. Earlier reports indicated that yeasts were not uncommon in raw minced beef, especially in conjunction with high total aerobic plate counts (Jay & Margitic, 1981).

Enterobacteriaceae isolates were mostly uniformly distributed over all treatments, except for a general absence in TREAT 3 samples, indicating a possible sensitivity to low pH values. Most of the remaining Gram-negative isolates were confined to TREAT 1 and 4 samples. This was evident from the Gram-positive and Gram-negative summary figures in Table 4.26, indicating almost total Gram-negative predominance in TREAT 1 samples, approximately equal proportions in TREAT 2 and 3 samples and approximately two thirds Gram-positive predominance in TREAT 4 samples.

TABLE 4.25

Distribution of psychrotrophic isolates according to treatments (% according to groups)

ISOLATE CLASSIFICATION GROUP	NO. OF ISOLATES	PERCENTAGE OF ISOLATES FROM:				PERCENTAGE OF TOTAL ISOLATES
		TREAT 1	TREAT 2	TREAT 3	TREAT 4	
Pseudomonas spp.	58	70,7	12,1	12,1	5,2	45,3
Lactobacillus spp.	21	0	47,6	33,3	19,0	16,4
Yeasts	13	0	0	0	100	10,2
Enterobacter spp.	9	22,2	55,6	0	22,2	7,0
Hafnia alvei	5	0	20	20	60	3,9
Coryneform group	4	0	25	25	50	3,1
Kurthia spp.	4	25	0	0	75	3,1
Achromobacter spp.	4	75	0	0	25	3,1
Acinetobacter spp.	3	0	33,3	0	66,7	2,3
Streptococcus spp.	2	0	0	100	0	1,6
Micrococcus spp.	2	0	50	50	0	1,6
Aeromonas sp.	1	100	0	0	0	0,8
Yersinia sp.	1	0	0	0	100	0,8
Unidentified	1	0	0	0	100	0,8
Gram-positive	47	2,1	25,5	23,4	48,9	36,7
Gram-negative	81	58,0	17,3	9,9	14,8	63,3
Total	128	37,5	20,3	14,8	27,3	100

TABLE 4.26

Distribution of psychrotrophic isolates over treatments (% according to groups)

Treatment / Isolate Group	*Pseudomonas* spp.	*Lactobacillus* spp.	Yeast	*Enterobacter* spp.	*Hafnia* spp.	Coryneform gp.	*Kurthia* spp.	*Achromobacter* spp.	*Acinetobacter* spp.	*Streptococcus* spp.	*Micrococcus* spp.	*Aeromonas* sp.	*Versinia* sp.	Unidentified	Gram-positive	Gram-negative	Total %	No. of isolates
TREAT 1	85,4	0	0	4,2	0	0	2,1	6,3	0	0	0	2,1	0	0	2,1	97,9	100	48
TREAT 2	26,9	38,5	0	19,2	3,8	3,8	0	0	3,8	0	3,8	0	0	0	46,2	53,8	100	26
TREAT 3	36,8	36,8	0	0	5,3	5,3	0	0	0	10,5	5,3	0	0	0	57,9	42,1	100	19
TREAT 4	8,6	11,4	37,1	5,7	8,6	5,7	8,6	2,9	5,7	0	0	0	2,9	2,9	65,7	34,3	100	35

The distribution of isolates over storage intervals is summarized in Table 4.27. Pseudomonads showed the highest incidence over the first four days, with a substantial presence up to day 17. *Lactobacillus* spp. only started to predominate after seven days but decreased from day 7 up to day 17. Yeasts showed a constant presence from day 3 to day 11, with a slight decrease to day 17. *Enterobacter* and *Hafnia* showed gradual increases from day 3 to day 17, whereas the other Enterobacteriaceae isolates predominated early in the storage interval (up to day 4). The remaining Gram-positive isolates predominated between days 7 and 11. Looking at the Gram-positive and Gram-negative summary figures, it was evident that Gram-negative isolates predominated up to day 4, followed by a marginal Gram-positive predominance on days 7 and 11. Day 17 revealed a definite Gram-negative predominance.

In addition, the distribution of psychrotrophic isolates over temperature is summarized in Table 4.28. Not unexpectedly, a higher percentage of pseudomonads were isolated at the lower incubation temperature of 0°C (75:25), whereas slightly more *Lactobacillus* spp. and yeasts were found at the higher incubation temperature (7°C) than at 0°C. Enterobacteriaceae were almost evenly distributed between the two temperatures, but *Hafnia* spp. were only isolated from samples incubated at 7°C. The Coryneform group and *Kurthia* isolates were chiefly found in samples stored at the lower temperature (0°C).

The summary figures show that Gram-positive isolates overall displayed a marginal predominance at 7°C (47:53), but that the Gram-negative isolates were most certainly more prevalent at 0°C (58:42). This finding agreed with numerous literature reports stressing the highly psychrotrophic nature of the meat spoilage pseudomonads, although it was generally found that Enterobacteriaceae were mostly marginally psychrotrophic (Beebe, Vanderzant, Hanna, Carpenter & Smith, 1976).

TABLE 4.27

Distribution of psychrotrophic isolates over storage interval (% according to groups) for all treatments

Storage Interval (days) \ Isolate Group	Pseudomonas spp.	Lactobacillus spp.	Yeast	Enterobacter spp.	Hafnia spp.	Coryneform gp	Kurthia spp.	Achromobacter spp.	Acinetobacter spp.	Streptococcus spp.	Micrococcus spp.	Aeromonas sp.	Yersinia sp.	Unidentified	Gram-positive	Gram-negative	Total %	No. of isolates
1 + 2	68,4	0	0	0	0	0	0	15,8	5,3	0	5,3	5,3	0	0	5,3	94,7	100	19
3 + 4	65,0	0	15,0	5	0	0	0	5,0	5,0	0	0	0	5,0	0	15,0	85,0	100	20
7	35,9	30,8	10,3	5,1	2,6	2,6	5,1	0	0	5,1	0	0	0	2,6	56,4	43,6	100	39
11	31,3	21,9	15,6	6,3	3,1	9,4	6,3	0	3,1	0	3,1	0	0	0	56,3	43,7	100	32
17	44,4	11,1	5,6	22,2	16,7	0	0	0	0	0	0	0	0	0	16,7	83,3	100	18

TABLE 4.28

Distribution of psychrotrophic isolates over temperature
(% according to groups)

Isolate Group	Isolation temperature		Total %	No. of isolates
	0°C	7°C		
Pseudomonas spp.	72,4	27,6	100	58
Lactobacillus spp.	42,9	57,1	100	21
Yeast	38,5	61,5	100	13
Enterobacter spp.	44,4	55,6	100	9
Hafnia spp.	0	100	100	5
Coryneform gp.	75,0	25,0	100	4
Kurthia spp.	100	0	100	4
Achromobacter spp.	50	50	100	4
Acinetobacter spp.	66,7	33,3	100	3
Streptococcus spp.	0	100	100	2
Micrococcus spp.	50	50	100	2
Aeromonas sp.	100	0	100	1
Yersinia sp.	100	0	100	1
Unidentified	0	100	100	1
Gram-positive	46,8	53,2	100	47
Gram-negative	64,2	35,8	100	81
Total	57,8	42,2	100	128

In summary, it appeared that the identification of psychrotrophic isolates reinforces results of many authors regarding the typical psychrotrophic nature of meat spoilage pseudomonads, although their occurrence in vacuum-packed samples was not in keeping with literature reports. Similarly, the general occurrence of *Lactobacillus* spp. on aerobically packed samples was an unusual observation (according to results for plate counts; see Fig. 4.6, V6 - graphs), although the predominance of the latter organisms on vacuum packed samples as well as their appearance in the later stages of the storage period was in keeping with results of several authors. In addition, it appeared that although TREAT 4 led to a marked reduction in pseudomonad levels, it selectively favoured development of rather exotic or uncommon microbial groups (including yeasts), many of which were Gram-positive.

4.5.2 Lactic acid bacterial isolates

Special attention was devoted to the lactic acid bacteria because of their reported antagonism against typical meat spoilage bacteria (Roth & Clark, 1975; Collins-Thompson & Lopez, 1980). Representative colonies were isolated from *Lactobacillus* Selective Medium and all 71 isolates were identified to species level. In most cases the identification was not clear-cut, and emphasis had to be placed on major characteristics such as lactic acid isomer and pentose fermentation. The identification procedure was made more difficult by the fact that very little taxonomic detail was available on lactic acid bacteria prevalent in the spoilage of raw minced beef and extensive use had to be made unpublished information (Holzapfel, personal communication). Source and identification details on the lactic acid bacterial isolates are listed in Table A.4, (see Appendix).

An assessment of the overall distribution of isolates (Table 4.29) revealed that three species were prevalent, namely *L. saké* (the largest single proportion by a considerable margin), *L. curvatus*

TABLE 4.29

Distribution of lactic acid bacteria isolates according to treatments (% according to groups)

ISOLATE CLASSIFICATION	NO. OF ISOLATES	PERCENTAGE OF ISOLATES FROM:				PERCENTAGE OF TOTAL ISOLATES
		TREAT 1	TREAT 2	TREAT 3	TREAT 4	
Lactobacillus saké	24	12,5	45,8	37,5	4,2	33,8
Lactobacillus curvatus	16	18,8	56,3	18,8	6,3	22,5
Lactobacillus bavaricus	15	33,3	40,0	6,7	20.	21,1
Lactobacillus alimentarius	7	0	14,3	71,4	14,3	9,9
Lactobacillus farciminis	3	0	0	66,7	33,3	4,2
Leuconostoc paramesenteroides	3	0	66,7	33,3	0	4,2
Lactobacillus sp. (subgenus *Betabacterium*)	1	0	0	0	100	1,4
Leuconostoc mesenteroides	1	0	100	0	0	1,4
Lactobacillus viridescens	1	0	100	0	0	1,4
Total	71	15,5	43,7	29,6	11,3	100

and *L. bavaricus*.   *L. alimentarius* occurred in much smaller
numbers and the remaining isolates were made up of miscellaneous
Lactobacillus and *Leuconostoc* spp. in small numbers.   The
distribution of lactic acid bacteria over the four treatments
is summarized in Table 4.30.   These results indicate that despite
numerous literature reports to the contrary, *Lactobacillus* spp.
reached high numbers ($> 10^6$/gram) in aerobically packed samples
(TREAT 1 and TREAT 4).   These results are supported by V6 counts
in Figure 4.6 .

This observation could possibly be explained by the fact that
all isolations were carried out on samples stored for 17 days,
but was nevertheless unusual in the light of the well-documented
microaerophilic nature of the lactobacilli.   It was noticeable
that *L. bavaricus* showed the highest occurrence on TREAT 1 samples
in addition to considerable numbers on TREAT 4 samples, with a
greatly reduced incidence on TREAT 2 and 3 samples.   This trend
was reversed for *L. saké* and *L. curvatus,* both of which were more
prominent on TREAT 2 and 3 samples.   Most of the other isolates
were found in TREAT 2 and 3 samples, although isolated occurrences
were recorded for TREAT 4 samples.   The reason for larger numbers
of isolates on TREAT 4 samples could be their relative resistance
to SO_2 (as compared with pseudomonads).

In summary, it was surprising to find considerable numbers of
lactic acid bacteria and especially *Lactobacillus* spp. in the
aerobically packed samples.   The fact that *Lactobacillus bavaricus,*
a relatively unknown organism, seemed to favour aerobic conditions
was most noteworthy.   Also, the association of this organism with
meat was not reported before (Holzapfel, personal communication).
A similar, although less pronounced aerobic growth habit was
recorded for the other two major species identified, namely *L. saké*
and *L. curvatus*.

TABLE 4.30

Distribution of lactic acid bacteria isolates over treatments (% according to groups)

Treatment (Isolate Group)	*L. saké*	*L. curvatus*	*L. bavaricus*	*L. alimentarius*	*L. farciminis*	*Leusonostoc paramesenteroides*	*L.* sp.(subgenus *Betabacterium*)	*Leusonostoc mesenteroides*	*L. viridescens*	Total %	No. of isolates
TREAT 1	27,3	27,3	45,5	0	0	0	0	0	0	100	11
TREAT 2	35,5	29,0	19,4	3,2	0	6,5	0	3,2	3,2	100	31
TREAT 3	42,9	14,3	4,8	23,8	9,5	4,8	0	0	0	100	21
TREAT 4	12,5	12,5	37,5	12,5	12,5	0	12,5	0	0	100	8

In addition, by overall proportions, *L. saké* was the most
prevalent spoilage isolate.   This finding is supported by
results from Kagermeier (doctoral thesis, University of Munich,
1981), where the preponderance of *L. saké*, especially in meat
and meat products, was clearly documented.

Unidentified isolate No. 11 (*Betabacterium* sp.) corresponded
to previously described psychrotrophic isolates No. 60, 66, 81,
86, 87 and 115.   This fact possibly reflects a major role of
this organism in psychrotrophic meat spoilage.   Since most of
the isolates were made from TREAT 4, a relatively high resistance
to SO_2 can be expected.

CHAPTER 5

CONCLUSIONS

Several general conclusions can be drawn from the results regarding effectiveness of treatments in prolonging the shelf life of raw minced beef. In addition, conclusions may be drawn on the prevalence and activity of various groups of spoilage bacteria.

(a) The results indicated that aerobic packaging and storage of even moderately contaminated raw minced beef did not allow for any inhibition of growth of spoilage microorganisms. Rapid micro-biological, physical and sensory spoilage set in even at a lowered storage temperature of 0°C, although shelf life at 0°C was much improved over storage life at 7°C.

(b) The two treatments employing vacuum packaging (TREATS 2 and 3) resulted in overall improvements of shelf life, and in most cases TREAT 3 produced superior results to TREAT 2. Much of this could have been related to the pH and E_h depression caused by addition of ascorbic acid. A most noteworthy point on TREAT 3 was its almost complete inhibition of *B. thermosphacta* growth, even at 7°C. This was a significant breakthrough in the light of the fact that *B. thermosphacta* was frequently in-criminated to be a major meat spoilage organism.

(c) The treatment with a commercial 'colour retainer' (TREAT 4) produced significant increases in shelf life of raw minced beef, even at a higher storage temperature and in conjunction with aerobic packaging. Microbiological results indicated, however, that the initially pronounced inhibitory effect of TREAT 4 decreased considerably from four days of storage onwards. The success of TREAT 4 was therefore limited to shorter storage intervals, although a more permanent inhibition of the pseudomonads over extended storage intervals was noted.

(d) Overall, TREAT 4 and TREAT 3 were rated as approximately equally
successful in increasing raw minced beef shelf life. Both
had disadvantages however: In TREAT 3, the vacuum packaging
combined with a lowered pH led to excessive destructurization
of the minced beef coupled to a considerable amount of purge,
both reducing the consumer appeal of the product. In TREAT 4,
the addition of colour stabilizing, antioxidant 'preservative'
compounds to raw minced beef is considered as undesirable from
a consumer point of view and illegal in terms of current
legislation on raw minced beef additives. Furthermore,
Enterobacteriaceae were suppressed more successfully by TREAT 3,
especially at 0°C.

(e) The microbiological results on all treatments indicated that a
strong positive synergism existed between lowered storage
temperature and effectiveness of treatment. This finding re-
emphasized the vital importance of strict temperature control
in the improvement of raw minced beef shelf life, especially
in the case of products with high initial contamination levels.

(f) The importance of strict temperature control at a level around
0°C was emphasized by the isolation and identification of a
large number of psychrotrophic pseudomonads obviously involved in
proteolytic spoilage. In contrast to reported observations,
many of these pseudomonads persisted in the oxygen-depleted
environments of TREAT 2 and 3 samples.

(g) Similarly, a significant proportion of the lactic acid bacteria
isolates (mostly *Lactobacillus* spp.) displayed distinct psychrotrophic
growth characteristics. In addition, the majority of these
isolates were apparently not significantly inhibited by aerobic
growth conditions. This finding contradicted numerous reports
describing microaerophilic conditions to favour these organisms.

(h)   Enterobacteriaceae were also found to be involved in psychrotrophic
      spoilage, although to a much lesser degree than the pseudomonads.
      However, most of the Enterobacteriaceae isolates displayed a
      somewhat marginal psychrotrophic habit when compared to the
      pseudomonads.   At 0°C, all treatments resulted in considerable
      growth inhibition of the Enterobacteriaceae compared to the
      controls (TREAT 1).

(i)   An almost perfect correlation between the total aerobic plate
      count and the psychrotrophic count was noted for all treatments
      at both storage temperatures.   This finding allowed the con-
      clusion that the time-consuming psychrotrophic count (10 days
      at 7°C) could be replaced by a more rapid count (72 hours at 25°C)
      with a minimal loss of precision in assessments of raw minced
      beef shelf life and general hygienic quality.

(j)   Similarly high correlations were found between the pseudomonad
      fractions of the total aerobic and the psychrotrophic counts.
      In addition, it was found that an extra 24 hour incubation of
      the Enterobacteriaceae count at room temperature followed by an
      oxidase test could be used as an approximate assessment of
      psychrotrophic pseudomonad numbers.   Although this procedure
      tended to underestimate numbers, the saving in time and materials
      is significant.

(k)   Chemical techniques of assessing shelf life and degree of micro-
      biological spoilage of raw minced beef yielded questionable
      results throughout.   It was therefore concluded that measurement
      of pH, redox potential (Eh) and lactic acid concentrations are of
      very limited use in shelf life assessments on raw minced beef.

(l)   *Lactobacillus saké* was found to be the most predominant re-
      presentative among lactic acid bacteria isolates (33,8%),
      followed by *L. curvatus* (22,5%), *L. bavaricus* (21,2%) and
      L. alimentarius (9,9%).   The association of *L. bavaricus* with
      meat was not reported before.

(m) *Lactobacillus* spp. also constituted the second largest group (16,4%) of the psychrotrophic isolates, although the pseudomonads predominated (45,3%).

CHAPTER 6

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APPENDIX
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TABLE A.1

SUMMARY OF TIMES TAKEN BY MICROBIAL COUNTS TO REACH CRITICAL LEVELS

(n.r. - not reached)

VARIABLE	TREAT	TEMP	STORAGE DAYS BEFORE REACHING LEVEL OF:					
			10^6/gram			10^7/gram		
			RUN 1	RUN 2	AVERAGE	RUN 1	RUN 2	AVERAGE
V1 TAPC	1	0	4	3	3,5	5	5	5
		7	1	1	1	2	2	2
	2	0	6	9	7,5	8	12	10
		7	1,5	2	1,8	4,5	3	3,5
	3	0	8	12	10	10	15	12,5
		7	2	3	2,5	4,5	4	4,5
	4	0	14	9	12,5	n.r.	11	11+
		7	4	4	4	6	7	6,5
V2 TAPC ox.	1	0	6	5,5	5,8	10	7	8,5
		7	2	2	2	2	3	2,5
	2	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	5	4	4,5	n.r.	11	11+
	3	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	5	n.r.	5+	n.r.	n.r.	n.r.
	4	0	n.r.	13	13+	n.r.	n.r.	n.r.
		7	6	7	6,5	16	n.r.	16+

TABLE A.1 (cont.)

SUMMARY OF TIMES TAKEN BY MICROBIAL COUNTS TO REACH CRITICAL LEVELS

(n.r. - not reached)

VARIABLE	TREAT	TEMP	STORAGE DAYS BEFORE REACHING LEVEL OF:					
			10^6/gram			10^7/gram		
			RUN 1	RUN 2	AVERAGE	RUN 1	RUN 2	AVERAGE
V8 PC	1	0	3,5	3	3,3	5	4	4,5
		7	1,5	1,5	1,5	2	2	2
	2	0	6,5	9	8	12	12	12
		7	2	2	2	3	3	3
	3	0	8,5	9	8,8	12,5	15	13,5
		7	2	3	2,5	3	4	3,5
	4	0	12	12	12	n.r.	12	12+
		7	4	4	4	9	7	8
V9 PC ox.	1	0	5	5	5	7	7	7
		7	2	2	2	3	6	4,5
	2	0	n.r.	11	11+	n.r.	n.r.	n.r.
		7	n.r.	11	11+	n.r.	17	17+
	3	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	9	11	10	11	n.r.	11+
	4	0	15	12	13,5	n.r.	n.r.	n.r.
		7	5	6	5,5	n.r.	11	11+

TABLE A.1 (cont.)

SUMMARY OF TIMES TAKEN BY MICROBIAL COUNTS TO REACH CRITICAL LEVELS

(n.r. - not reached)

VARIABLE	TREAT	TEMP	STORAGE DAYS BEFORE REACHING LEVEL OF:					
			10^5/gram			10^7/gram		
			RUN 1	RUN 2	AVERAGE	RUN 1	RUN 2	AVERAGE
V3 EC	1	0	2	2	2	5	5	5
		7	1	1	1	2	2	2
	2	0	6	7	6,5	n.r.	n.r.	n.r.
		7	3	1	2	4	3	3,5
	3	0	n.r.	13	13+	n.r.	n.r.	n.r.
		7	4	2	3	n.r.	6	6+
	4	0	12	10	11	n.r.	n.r.	n.r.
		7	1	4	2,5	11	9	10
V7 BTC	1	0	4,5	7	6	9	11	10
		7	2	3,5	2,8	4	13	8,5
	2	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	6,5	n.r.	6,5+	n.r.	n.r.	n.r.
	3	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
	4	0	14	9	11,5	n.r.	n.r.	n.r.
		7	6	3	4,5	n.r.	11	11+

TABLE A.1 (cont.)

SUMMARY OF TIMES TAKEN BY MICROBIAL COUNTS TO REACH CRITICAL LEVELS

(n.r. - not reached)

VARIABLE	TREAT	TEMP	STORAGE BEFORE REACHING LEVEL OF:					
			10^5/gram			10^7/gram		
			RUN 1	RUN 2	AVERAGE	RUN 1	RUN 2	AVERAGE
V4 EC-48	1	0	3	3	3	5,5	7	6,3
		7	1,5	1,5	1,5	3	4	3,5
	2	0	7	9	8	n.r.	n.r.	n.r.
		7	3	3	3	n.r.	n.r.	n.r.
	3	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	4	11	7,5	n.r.	n.r.	n.r.
	4	0	15	15	15	n.r.	n.r.	n.r.
		7	5	5	5	n.r.	15	15+
V5 EC-48 ox.	1	0	3,5	2,5	3	7.5	6,5	7
		7	1,5	2	1,8	3	3	3
	2	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	3	3	3	n.r.	n.r.	n.r.
	3	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	n.r.	3,5	3,5+	n.r.	n.r.	n.r.
	4	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	4	5	4,5	n.r.	n.r.	n.r.

TABLE A.1 (cont.)

SUMMARY OF TIMES TAKEN BY MICROBIAL COUNTS TO REACH CRITICAL LEVELS

(n.r. - not reached)

VARIABLE	TREAT	TEMP	STORAGE DAYS BEFORE REACHING LEVEL OF:					
			10^6/gram			10^7/gram		
			RUN 1	RUN 2	AVERAGE	RUN 1	RUN 2	AVERAGE
V6 LC	1	0	8,5	11	10	12	13,5	12,8
		7	2	3	2,5	3	4	3,5
	2	0	8,5	12	10,5	12	n.r.	12+
		7	2	3	2,5	2,5	4	3,3
	3	0	8	12	10	12	17	14,5
		7	2	3,5	2,8	2,5	5,5	4
	4	0	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
		7	7,5	7	7,3	12	n.r.	12+

TABLE A.2

(D+L) - LACTIC ACID CONCENTRATIONS

(CORRECTED FOR BACKGROUND)

TREAT	TEMP	STORE	D+L LACTIC ACID (mg/mℓ sample)
			RUN NO. 1
			(BACKGROUND 0,6274)
1	0	1	0,1988
1	0	2	1,5707
1	0	3	0,5832
1	0	4	0,1973
1	0	7	0,9455
1	0	11	1,2017
1	0	17	0,1326
1	7	1	-0,1414
1	7	2	0,8704
1	7	3	0,7025
1	7	4	1,0582
1	7	7	0,8461
1	7	11	0,0773
1	7	17	0,4043
2	0	1	-0,0066
2	0	2	0,5368
2	0	3	0,6296
2	0	4	0,9632
2	0	7	0,3027
2	0	11	1,7938
2	0	17	0,9808
2	7	1	0,1767
2	7	2	0,7422
2	7	3	0,5059
2	7	4	1,7982
2	7	7	2,8166
2	7	11	0,5125
2	7	17	1,0957
3	0	1	0,0287
3	0	2	0,7268

TABLE A.2 (cont.)

(D+L) - LACTIC ACID CONCENTRATIONS

(CORRECTED FOR BACKGROUND)

TREAT	TEMP	STORE	D+L LACTIC ACID (mg/ml sample)
			RUN NO. 1
3	0	3	0,7953
3	0	4	0,9433
3	0	7	0,2342
3	0	11	1,1355
3	0	17	0,6186
3	7	1	0,0354
3	7	2	0,5810
3	7	3	0,7953
3	7	4	0,9433
3	7	7	0,2342
3	7	11	1,1355
3	7	17	0,6186
4	0	1	0,1745
4	0	2	0,6340
4	0	3	0,6097
4	0	4	1,2105
4	0	7	0,2894
4	0	11	0,5324
4	0	17	0,1568
4	7	1	-0,1105
4	7	2	0,5412
4	7	3	1,1973
4	7	4	1,1885
4	7	7	0,9742
4	7	11	0,7202
4	7	17	1,0723
6	-	0	0,4284
6	0	4	1,3210
6	7	4	2,2886

TABLE A.2 (cont.)

(D+L) - LACTIC ACID CONCENTRATIONS

(CORRECTED FOR BACKGROUND)

TREAT	TEMP	STORE	D+L LACTIC ACID (mg/mℓ sample)
			RUN NO. 2
			(BACKGROUND 0,4352)
1	0	1	-0,1900
1	0	2	0,1723
1	0	3	1,6701
1	0	4	1,5574
1	0	7	1,5398
1	0	11	1,3498
1	0	17	0,3866
1	7	1	0,1348
1	7	2	0,2364
1	7	3	1,4381
1	7	4	1,7607
1	7	7	1,4580
1	7	11	0,2541
1	7	17	0,0994
2	0	1	-0,1878
2	0	2	0,1856
2	0	3	1,0560
2	0	4	1,4227
2	0	7	1,4470
2	0	11	1,5044
2	0	17	0,6384
2	7	1	0,1680
2	7	2	0,1458
2	7	3	1,4249
2	7	4	1,6767
2	7	7	1,5066
2	7	11	0,2784
2	7	17	0,3932

TABLE A.2 (cont.)

(D+L) - LACTIC ACID CONCENTRATIONS

(CORRECTED FOR BACKGROUND)

TREAT	TEMP	STORE	D+L LACTIC ACID (mg/mℓ sample)
			RUN NO. 2
3	0	1	-0,0244
3	0	2	0,0597
3	0	3	1,2747
3	0	4	1,5221
3	0	7	1,6038
3	0	11	1,1090
3	0	17	0,5324
3	7	1	0,0022
3	7	2	-0,0111
3	7	3	1,4337
3	7	4	1,2990
3	7	7	1,5177
3	7	11	0,6428
3	7	17	0,3468
4	0	1	0,3093
4	0	2	0,0619
4	0	3	1,3873
4	0	4	1,6590
4	0	7	1,5420
4	0	11	0,7025
4	0	17	0,3888
4	7	1	0,0265
4	7	2	0,1480
4	7	3	1,5486
4	7	4	1,1222
4	7	7	1,5309
4	7	11	0,3269
4	7	17	-0,1922
6	-	0	-0,0530
6	0	4	1,4668
6	7	4	1,705ɔ

TABLE A.3   SOURCE AND IDENTIFICATION DETAILS -
                  PSYCHROTROPHIC ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
1	1	0	1	3	Aeromonas hydrophila
2	1	0	1	4	Pseudomonas fluorescens gp.
3	1	0	1	4	Pseudomonas fluorescens gp.
4	2	0	1	4	Pseudomonas cepacia
5	3	0	1	4	Pseudomonas fluorescens gp.
6	4	0	1	4	Achromobacter sp.
7	1	7	1	4	Achromobacter sp.
8	1	7	1	4	Achromobacter xylosoxidans
9	3	7	1	4	Pseudomonas fluorescens gp.
10	4	7	1	4	Pseudomonas fluorescens gp.
11	4	7	1	4	Acinetobacter calcoaceticus var. anitratus
12	1	7	2	6	Pseudomonas cepacia
13	1	7	2	6	Pseudomonas cepacia
14	1	0	2	4	Pseudomonas fluorescens gp.
15	1	0	2	4	Pseudomonas fluorescens gp.
16	1	0	2	4	Pseudomonas fluorescens gp.
17	2	0	2	4	Micrococcus sp.
18	2	0	2	4	Pseudomonas cepacia
19	4	0	2	4	Pseudomonas fluorescens gp.
20	1	0	3	5	Pseudomonas fluorescens gp.
21	1	0	3	5	Pseudomonas aeruginosa
22	1	0	3	5	Pseudomonas fluorescens gp.
23	1	0	3	5	Achromobacter xylosoxidans
24	2	0	3	4	Pseudomonas aeruginoa
25	2	0	3	4	Pseudomonas fluorescens gp.
26	3	0	3	4	Pseudomonas fluorescens gp.
27	4	0	3	5	Yersinia sp.
28	4	7	3	4	Enterobacter cloacae
29	4	7	3	4	Yeast
30	4	7	3	4	Pseudomonas fluorescens gp.

TABLE A.3 (cont.) SOURCE AND IDENTIFICATION DETAILS - PSYCHROTROPHIC ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
31	1	7	3	6	*Pseudomonas fluorescens* gp.
32	1	7	3	6	*Pseudomonas fluorescens* gp.
33	1	7	3	6	*Pseudomonas fluorescens* gp.
34	1	7	3	6	*Pseudomonas* CDC VE-1
35	1	7	3	6	*Pseudomonas aeruginosa*
36	4	7	4	5	Yeast
37	4	7	4	5	Yeast
38	2	0	4	4	*Acinetobacter calcoaceticus var. lwoffi.*
39	3	0	4	4	*Pseudomonas fluorescens* gp.
40	1	0	7	5	*Pseudomonas fluorescens* gp.
41	1	0	7	5	*Pseudomonas fluorescens* gp.
42	1	0	7	5	*Pseudomonas aeruginosa*
43	1	0	7	5	*Pseudomonas aeruginosa*
44	1	0	7	5	*Pseudomonas fluorescens* gp.
45	1	0	7	5	*Pseudomonas fluorescens* gp.
46	1	0	7	5	*Pseudomonas fluorescens* gp.
47	3	0	7	4	*Pseudomonas fluorescens* gp.
48	3	0	7	4	Isolate lost
49	3	0	7	4	*Lactobacillus saké*
50	3	0	7	4	*Pseudomonas paucimobilis*
51	3	0	7	4	*Pseudomonas* CDC VE-1
52	1	7	7	6	*Pseudomonas* CDC VE-1
53	1	7	7	6	*Pseudomonas fluorescens* gp.
54	1	7	7	6	*Pseudomonas fluorescens* gp.
55	4	7	7	6	Yeast
56	4	7	7	6	Yeast
57	4	7	7	6	Yeast
58	4	7	7	6	Identification uncertain
59	2	7	7	7	Isolate lost
60	2	7	7	7	*Lactobacillus* sp. (subgenus *Betabacterium*)

TABLE A.3 (cont.) SOURCE AND IDENTIFICATION DETAILS -
PSYCHROTROPHIC ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
61	2	7	7	7	Isolate lost
62	2	7	7	7	Isolate lost
63	2	7	7	7	*Enterobacter* sp.
64	3	7	7	6	*Streptococcus lactis*
65	3	7	7	6	*Lactobacillus curvatus*
66	3	7	7	6	*Lactobacillus* sp. (subgenus *Betabacterium*)
67	3	7	7	6	Isolate lost
68	3	7	7	6	*Streptococcus lactis*
69	3	7	7	6	*Hafnia alvei*
70	2	0	7	5	Isolate lost
71	2	0	7	5	*Lactobacillus saké*
72	2	0	7	5	*Lactobacillus saké*
73	2	0	7	5	*Enterobacter agglomerans*
74	2	0	7	5	*Pseudomonas fluorescens* gp.
75	4	0	7	4	*Kurthia* sp.
76	4	0	7	4	*Kurthia* sp.
77	4	0	7	4	Coryneform gp.
78	4	0	7	4	Yeast
79	3	7	11	6	*Micrococcus* sp.
80	3	7	11	6	Coryneform/ *Propionibacterium* gp.
81	4	7	11	6	*Lactobacillus* sp. (subgenus *Betabacterium*)
82	4	7	11	6	Yeast
83	4	7	11	6	*Lactobacillus yamanashiensis*
84	2	7	11	7	*Hafnia alvei*
85	2	7	11	7	*Lactobacillus saké*
86	4	7	11	6	*Lactobacillus* sp. (subgenus *Betabacterium*)
87	4	7	11	6	*Lactobacillus* sp. (subgenus *Betabacterium*)

TABLE A.3 (cont.)   SOURCE AND IDENTIFICATION DETAILS -
PSYCHROTROPHIC ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
88	4	0	11	5	Yeast
89	4	0	11	5	Yeast
90	4	0	11	5	*Kurthia* sp.
91	4	0	11	5	Yeast
92	4	0	11	5	Coryneform gp.
93	4	0	11	5	Yeast
94	4	0	11	5	*Acinetobacter* sp.
95	1	7	11	6	*Pseudomonas aeruginosa*
96	1	7	11	6	*Enterobacter aerogenes*
97	1	7	11	6	*Pseudomonas fluorescens* gp.
98	2	0	11	8	*Enterobacter agglomerans*
99	2	0	11	8	Coryneform gp.
100	3	0	11	7	Isolate lost
101	3	0	11	7	Isolate lost
102	1	0	11	8	*Pseudomonas fluorescens* gp.
103	1	0	11	8	*Pseudomonas fluorescens* gp.
104	1	0	11	8	*Pseudomonas fluorescens* gp.
105	1	0	11	8	*Pseudomonas fluorescens* gp.
106	1	0	11	8	*Pseudomonas fluorescens* gp.
107	1	0	11	8	*Pseudomonas fluorescens* gp.
108	1	0	11	8	*Pseudomonas aeruginosa*
109	1	0	11	8	*Kurthia* sp.
110	1	0	11	8	*Pseudomonas fluorescens* gp.
111	2	7	17	7	*Enterobacter aerogenes*
112	2	7	17	7	*Pseudomonas fluorescens* gp.
113	1	0	17	8	*Pseudomonas fluorescens* gp.
114	1	0	17	8	*Pseudomonas fluorescens* gp.
115	2	0	17	7	*Lactobacillus* sp. (subgenus *Betabacterium*)
116	2	0	17	7	*Lactobacillus yamanashiensis*
117	2	0	17	7	*Pseudomonas fluorescens* gp.
118	2	0	17	7	*Enterobacter aerogenes*

TABLE A.3 (cont.)   SOURCE AND IDENTIFICATION DETAILS -
PSYCHROTROPHIC ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
119	1	0	17	8	*Pseudomonas fluorescens* gp.
120	1	0	17	8	*Pseudomonas fluorescens* gp.
121	1	0	17	8	*Pseudomonas fluorescens* gp.
122	1	0	17	8	*Enterobacter* sp.
123	1	0	17	8	*Pseudomonas fluorescens* gp.
124	4	7	17	7	*Hafnia alvei*
125	4	7	17	7	*Hafnia alvei*
126	4	7	17	7	Yeast
127	4	7	17	7	*Enterobacter cloacae*
128	4	7	17	7	*Hafnia alvei*

Note: All lost isolates were both Gram-positive and catalase negative.
Microscopic examination showed all of them to be short rods in
short chains and/or pairs.   This allowed the conclusion that all
eight lost isolates were lactic acid bacteria and probably
Lactobacillus species.

TABLE A.4   SOURCE AND IDENTIFICATION DETAILS -
LACTIC ACID BACTERIA ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
1	3	7	17	7	Lactobacillus alimentarius
2	3	7	17	7	Lactobacillus farciminis
3	3	7	17	7	Lactobacillus alimentarius
4	3	7	17	7	Lactobacillus alimentarius
5	3	7	17	7	Lactobacillus saké
6	3	7	17	7	Lactobacillus farciminis
7	3	7	17	7	Leuconostoc paramesenteroides
8	3	7	17	7	Lactobacillus alimentarius
9	3	7	17	7	Lactobacillus saké
10	3	7	17	7	Lactobacillus bavaricus
11	4	0	17	4	Lactobacillus sp. (subgenus Betabacterium)
12	2	0	17	6	Lactobacillus bavaricus
13	2	0	17	6	Lactobacillus bavaricus
14	2	0	17	6	Lactobacillus saké
15	2	0	17	6	Lactobacillus saké
16	2	0	17	6	Lactobacillus bavaricus
17	2	0	17	6	Lactobacillus curvatus
18	2	0	17	6	Lactobacillus curvatus
19	2	0	17	6	Lactobacillus bavaricus
20	1	0	17	6	Lactobacillus bavaricus
21	1	0	17	6	Lactobacillus bavaricus
22	1	0	17	6	Lactobacillus saké
23	1	0	17	6	Lactobacillus bavaricus
24	1	0	17	6	Lactobacillus bavaricus
25	1	0	17	6	Lactobacillus saké
26	1	0	17	6	Lactobacillus bavaricus
27	2	7	17	7	Lactobacillus saké
28	2	7	17	7	Leuconostoc mesenteroides
29	2	7	17	7	Lactobacillus alimentarius
30	2	7	17	7	Leuconostoc paramesenteroides

TABLE A.4 (cont.)   SOURCE AND IDENTIFICATION DETAILS - LACTIC ACID BACTERIA ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
31	2	7	17	7	Lactobacillus curvatus
32	2	7	17	7	Leuconostoc paramesenteroides
33	2	7	17	7	Lactobacillus saké
34	2	7	17	7	Lactobacillus bavaricus
35	2	7	17	7	Lactobacillus viridescens (subsp. minor)
36	4	7	17	7	Lactobacillus bavaricus
37	4	7	17	7	Lactobacillus farciminis
38	4	7	17	7	Lactobacillus bavaricus
39	4	7	17	7	Lactobacillus curvatus
40	4	7	17	7	Lactobacillus alimentarius
41	4	7	17	7	Lactobacillus bavaricus
42	4	7	17	7	Lactobacillus saké
43	3	0	17	6	Lactobacillus saké
44	3	0	17	6	Lactobacillus saké
45	3	0	17	6	Lactobacillus alimentarius
46	3	0	17	6	Lactobacillus saké
47	3	0	17	6	Lactobacillus curvatus
48	3	0	17	6	Lactobacillus saké
49	3	0	17	6	Lactobacillus saké
50	3	7	17	8	Lactobacillus curvatus
51	3	7	17	7	Lactobacillus saké
52	3	7	17	7	Lactobacillus curvatus
53	3	7	17	7	Lactobacillus saké
54	2	7	17	7	Lactobacillus curvatus
55	2	7	17	7	Lactobacillus curvatus
56	2	7	17	7	Lactobacillus saké
57	2	7	17	7	Lactobacillus curvatus
58	2	7	17	7	Lactobacillus curvatus
59	2	0	17	6	Lactobacillus saké
60	2	0	17	6	Lactobacillus saké
61	2	0	17	6	Lactobacillus curvatus
62	2	0	17	6	Lactobacillus saké

TABLE A.4 (cont.)   SOURCE AND IDENTIFICATION DETAILS -
                      LACTIC ACID BACTERIA ISOLATES

ISOLATE NO.	ISOLATED FROM			DILUTION FACTOR (POWER OF TEN)	IDENTIFICATION
	TREAT	TEMP	STORE		
63	2	0	17	6	*Lactobacillus saké*
64	2	0	17	6	*Lactobacillus saké*
65	2	0	17	6	*Lactobacillus saké*
66	2	0	17	6	*Lactobacillus curvatus*
67	1	7	17	7	*Lactobacillus curvatus*
68	1	0	17	7	*Lactobacillus curvatus*
69	1	0	17	7	*Lactobacillus saké*
70	1	0	17	7	*Lactobacillus curvatus*
71	2	0	17	6	*Lactobacillus bavaricus*