HEAT RESISTANCE AND INACTIVATION
OF MEAT SPOILAGE
LACTIC ACID BACTERIA

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DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Franz

CHARLES MARIE ANTOINE PAUL FRANZ

10th day of March, 1993.

This work is dedicated to two great women:
My mother Carla and my wife Ute.
You made it all possible.

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ABSTRACT

Heat resistance and inactivation of processed meat spoilage lactic acid bacteria was investigated in vitro and by in-package pasteurization of South African vacuum-packaged vienna sausages. In vitro heat resistance of four lactic acid bacteria strains was low, since reductions of at least one log cycle in bacterial numbers occurred upon heating at 57, 60 and 63°C in quarter-strength Ringers solution for one minute. In vitro heat resistance data were used to calculate three in-package pasteurization treatments of increasing severity for vacuum-packaged vienna sausages. Depending on treatment, pasteurization in a water cooker at 67°C increased microbiological shelf life of sausages to 10, 14 and 17 times that of control samples, during storage at 8°C. Although in-package pasteurization successfully decreased growth of spoilage lactic acid bacteria and increased product shelf life, it did not entirely prevent spoilage by pediococci. Since pasteurization also promoted growth of potentially pathogenic Bacillus and Clostridium, safety of pasteurized vacuum-packaged vienna sausages was compromised.

LIST OF ABBREVIATIONS

ANOVA	:	analysis of variance
°C	:	degrees Celsius
ca.	:	about
CFU	:	colony forming units
ch(s).	:	chapter(s)
cm	:	centimeter(s)
Ed(s).	:	edition, editor(s)
e.g.,	:	for example
et al.	:	and others
Fig(s).	:	figure(s)
g	:	gram(s)
i.e.	:	that is
LAB	:	lactic acid bacteria
LABC	:	lactic acid bacteria count
log	:	logarithm to base ten
min.	:	minute(s)
ml	:	milliliter
mm	:	millimeter
MMRS Agar	:	Modified MRS Agar
MRS Agar	:	De Man, Rogosa and Sharpe Agar
nm	:	nanometer

no.	:	number(s)
OD	:	optical density
PAST	:	pasteurization treatment
pp.	:	pages
QSRS	:	quarter-strength Ringers solution
sp.	:	species (singular)
spp.	:	species (plural)
STD1	:	Standard One Nutrient Agar
TAPC	:	total aerobic plate count
viz.	:	namely
VPVS	:	vacuum-packaged vienna sausages
vs.	:	versus
Vol.	:	volume
w/v	:	weight per volume
w/w	:	weight per weight

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CHAPTER ONE

INTRODUCTION

1.1 Microbial ecology of vacuum-packaged processed meats

1.1.1 Definition of processed meats

Processed meats are salted, or salted and cured. They may either be raw or cooked and are commonly subjected to some form of mechanical disruption during processing. They usually contain a variety of additives such as starch, spices and nitrite (Dodds and Collins-Thompson, 1984; Tompkin, 1986). Processed meats include products such as ham, bacon, corned beef, luncheon meats as well as raw, fermented and cooked emulsion-type sausages (Egan, 1983; Genigeorgis, 1984; Tompkin, 1986). Cured, emulsion-type sausages consist of meat, salt, sugar, sodium nitrite (or nitrate) and spices (Price and Schweigart, 1971). Many processed meat products are vacuum-packaged as a final processing step, in order to delay spoilage and increase shelf life (Sharpe, 1962; Mukherji and Qvist, 1981). Vienna sausages are smoke-cooked, emulsion-type sausages consisting of meat, spice and binding component emulsion filled into impermeable artificial casings. After the heat treatment, the casings are mechanically removed from the sausages and sausages are packed into gas impermeable bags and vacuum-sealed for refrigerated distribution and storage (Dykes et al., 1991). Vienna sausages and other processed meats provide an ideal environment for the growth of a variety of microorganisms, such as bacteria, yeasts and molds, since they are typically low acid (pH 6,0), have a high water activity (0,79 - 0,99) and contain a variety of freely available nutrients, such as fats, carbohydrates and vitamins (Allen and Foster, 1960; Nielsen and Zeuthen, 1985; Buchanan, 1986).

1.1.2 Microbiological contamination of vacuum-packaged emulsion-type sausages

Raw meat emulsion used in the manufacture of emulsion-type sausages is contaminated with a wide variety of microorganisms such as yeasts, molds, Enterobacteriaceae, Brochothrix (B.) thermosphacta and lactic acid bacteria

(Borch et al., 1988; Dykes et al., 1991). Lactic acid bacteria (LAB) and B. thermosphacta have been reported to occur at levels of ca. 10^3 - 10^5 colony forming units per gram ($CFUg^{-1}$) in raw meat emulsions (Kempton and Bobier, 1970; Borch et al., 1988; Dykes et al., 1991). Even though sausages are initially contaminated by a wide variety of microorganisms, the type, time and temperature of cooking processes employed during manufacture directly influence the resultant microbiological population of the finished product (Heizler et al., 1972; Carr and Marchello, 1986). Since cooking of processed meats raises the core temperature to at least 60°C and frequently above 70°C, vegetative microorganisms present in the product are destroyed (Carr and Marchello, 1986). Smoke-cooking of vienna sausages, for example, was capable of destroying the majority of microorganisms present in the raw emulsion (Mukherji and Qvist, 1981; Dykes et al., 1991). Thus vacuum-packaged emulsion-type sausages are usually not subject to internal contamination and consequently spoilage microorganisms seldom arise from survival of the cooking process. Most spoilage microorganisms are introduced by surface contamination from subsequent handling or slicing of product (Allen and Foster, 1960; Fruin et al., 1978; Nielsen and Zeuthen, 1984a,b; Korkeala and Lindroth, 1987; Mäkelä and Korkeala, 1987; Borch et al., 1988; Dykes et al., 1991).

Vacuum-packaged emulsion-type sausages and other processed meats have been reported to become recontaminated by diverse microbial populations including Gram-positive cocci, yeasts, Enterobacteriaceae and pathogens such as Bacillus (Ba.) cereus, Staphylococcus (S.) aureus, Escherichia coli, Salmonella spp., Clostridium (C.) perfringens, Listeria (L.) monocytogenes and Yersinia enterocolitica (Duitschaever, 1978; Fruin et al., 1978; Paradis and Stiles, 1978; Stiles and Ng, 1979; Nielsen and Zeuthen, 1985; Kokubo et al., 1986; Tompkin, 1986; Schwartz et al., 1988; Rorvik and Yndestad, 1991; von Holy et al., 1992). In addition, spoilage microorganisms such as B. thermosphacta and LAB also recontaminated sausages after heat processing (Allen and Foster, 1960; Egan et al., 1980; Borch et al., 1988; Dykes et al., 1991).

1.1.3 Factors influencing microbiological spoilage of vacuum-packaged emulsion-type sausages

The microbial spoilage ecology of vacuum-packaged emulsion-type sausages subsequent to initial contamination is influenced by a number of factors. These select which microbial groups will predominate in the spoilage population (Tompkin, 1986). Such parameters are either intrinsic, extrinsic or processing factors acting interdependently (Scott, 1989). Intrinsic factors are prime determinants in establishing the environment in which microorganisms compete for dominance. They include chemical, physical and biochemical characteristics such as nutrient composition, pH, water activity (a_w), antimicrobial factors (e.g., nitrate, nitrite, salt) and redox potential of the food product (Buchanan, 1986; Leistner, 1987; Scott, 1989). The pH and water activity of vacuum-packaged emulsion-type sausages and other processed meats are *ca*. 6,0 and 0,97 - 0,99, respectively, and therefore permit growth of most bacteria, yeasts and molds (Buchanan, 1986). Incorporation of antimicrobial compounds such as sodium nitrite or nitrate into emulsion-type sausages and other processed meats is effective in inhibiting growth of food-associated pathogens such as *C. botulinum*, *C. perfringens*, *S. aureus*, *Ba. cereus* and *L. monocytogenes* (Riemann *et al*., 1972; Sofos *et al*., 1979; Christiansen, 1980; Shahamat *et al*., 1980; Nielsen and Zeuthen, 1984b; Buchanan, 1986; Scott, 1989). While nitrite also inhibited the growth of spoilage microorganisms such as *B. thermosphacta*, Enterobacteriaceae and *Moraxella*, inhibition of Gram-positive cocci, yeasts and LAB was less pronounced (Nielsen, 1983; Dodds and Collins-Thompson, 1984; Botha and Holzapfel, 1987). Redox potential is also regarded as an intrinsic factor, but is influenced by extrinsic parameters such as packaging. The interior of meats was determined to have sufficiently low redox potential to prevent the growth of aerobic microorganisms such as *Pseudomonas* spp., while facultative anaerobic Enterobacteriaceae could grow (Silliker *et al*., 1980).

Extrinsic factors are mainly concerned with storage and processing conditions, for example storage times and temperatures, as well as the composition of the gaseous atmosphere surrounding meat products (Tompkin, 1986; Scott, 1989). Since most processed meats are only semi-preserved, refrigeration is generally required to retard the growth of contaminating microorganisms. Thus in properly refrigerated products, bacterial populations growing during storage of processed meats will predominantly consist of psychrotrophic species (species capable of growth at 5°C), while the growth of mesophilic pathogenic species such as C. botulinum (Types A and B), C. perfringens, Salmonella, S. aureus or Ba. cereus would not be expected (Paradis and Stiles, 1978; Stiles and Ng, 1979; Nielsen and Zeuthen, 1984a,1985; Buchanan, 1986; Scott, 1989).

Packaging is an important extrinsic factor since it directly influences the atmosphere and thus redox potential to which processed meats and associated microorganisms are exposed (Bell and Gill, 1982; Bell and de Lacy, 1983). The use of vacuum-packaging reduces the oxygen concentration in packs which retards the growth of aerobic or non-competitive facultatively anaerobic species (e.g., Pseudomonas, Brochothrix and Moraxella) while enhancing the growth of microaerophiles such as Lactobacillus and yeasts (Buchanan, 1986; Schillinger and Lücke, 1988).

Processing factors are procedures applied during food manufacture that affect microbial contamination, survival and growth (Scott, 1989). Heating of product was previously mentioned to be an important factor affecting microbial contamination of processed meat products, including vienna sausages. While product heating has been shown to eliminate most vegetative microorganisms (Carr and Marchello, 1986), thermotolerant sporeforming bacteria such as Bacillus or Clostridium are well-known to survive heat processing of processed meats (Houben, 1982; Nielsen and Zeuthen, 1984a,b; Buchanan, 1986; Kokubo et al., 1986).

1.1.4 Spoilage of vacuum-packaged emulsion-type sausages by lactic acid bacteria

Lactic acid bacteria constituted low initial numbers on processed meat products, when compared to other microbial groups such as Micrococcus and Pseudomonas (Borch et al., 1988; Dykes et al., 1991). Reuter (1969), however, found that regardless of initial numbers, LAB became the predominant spoilage populations of vacuum-packaged processed meats. Numerous studies also implicated the LAB as predominant spoilage populations of vacuum-packaged emulsion-type sausages, growing to high numbers (ca. $10^7 - 10^8$ CFUg^{-1}) during refrigerated storage (Sharpe, 1962; Nielsen, 1983; Korkeala et al., 1985; Holzapfel and Gerber, 1986; Korkeala et al., 1987; Borch et al., 1988; Zurera-Cosano et al., 1988; von Holy et al., 1991a,b; von Holy and Cloete, 1992). Recontamination of emulsion-type sausages by LAB from environmental sources occurred after heat processing as a result of manufacturing processes and handling (Borch et al., 1988; Dykes et al., 1991). This was reinforced by observations that spoilage populations were surface associated and did not originate from internal sources (Korkeala and Lindroth, 1987; von Holy et al., 1988). Contamination of sausages by heat resistant LAB surviving the cooking process, however, was reported on a few occasions (Niven et al., 1954; Milbourne, 1983; Borch et al., 1988).

When the growth of aerobic spoilage microorganisms such as Pseudomonas spp. is inhibited due to the use of low permeability packaging materials, the microaerophilic LAB can proliferate (Kitchell and Shaw, 1975; Egan, 1983; Buchanan, 1986; Schillinger and Lücke, 1988). In addition to vacuum-packaging, refrigeration and substrate composition also favor the growth and eventual domination of psychrotrophic LAB in emulsion-type sausages (Holzapfel and Gerber, 1986). The production of lactic acid during growth of LAB resulted in a pH decrease to levels sufficient to inhibit growth of other potential spoilage microorganisms such as B. thermosphacta and the Enterobacteriaceae (Schillinger and Lücke, 1988). The LAB themselves may, however, tolerate pH values as low as 4,0 (Silliker et al., 1980; Franz et al., 1991). The establishment of LAB as the predominant spoilage populations of vacuum-packaged emulsion-type sausages and other processed meats may also result from their antimicrobial activities. Lactic acid bacteria are known to produce antimicrobial compounds such as diacetyl and bacteriocins, which are inhibitory to the growth of a variety of microorganisms such as yeasts and molds, clostridia, *L. monocytogenes* and *S. aureus* (Tagg *et al.*, 1976; Jay, 1982; Nielsen and Zeuthen, 1985; Wang *et al.*, 1986; Klaenhammer, 1988; Harris *et al.*, 1989; Schillinger and Lücke, 1989; Spelhaug and Harlander, 1989; Schillinger and Holzapfel, 1990; Berry *et al.*, 1991; Hastings and Stiles, 1991; Okereke and Montville, 1991; Papathanasopoulos *et al.*, 1991; Schillinger *et al.*, 1991; Degnan *et al.*, 1992; Motlagh *et al.*, 1992; van Laack *et al.*, 1992).

In most spoilage associations of vacuum-packaged processed meats members of the genus *Lactobacillus* (atypical streptobacteria) predominated (Reuter, 1981; Holzapfel and Gerber, 1986; Morishita and Shiromizu, 1986), but were often accompanied by the enterococci, leuconostocs, pediococci and streptococci in varying proportions (Reuter, 1981; von Holy and Cloete, 1992). In spoiled, vacuum-packaged vienna sausages (VPVS) the homofermentative lactobacilli and leuconostocs dominated among spoilage LAB, jointly comprising 94,3% of total LAB populations (von Holy *et al.*, 1991b). Von Holy *et al.* (1992), therefore, argued that control measures against the spoilage of VPVS should be aimed specifically at homofermentative lactobacilli and leuconostocs.

1.1.5 Microbiological spoilage symptoms in vacuum-packaged emulsion-type sausages

Spoilage of vacuum-packaged emulsion-type sausages was characterized by milky, watery exudates, sour "off" flavors and gas which caused packages to blow (Borch *et al.*, 1988; Korkeala *et al.*, 1988; von Holy *et al.*, 1991b; von Holy and Cloete, 1992). All these defects result in products unacceptable to the consumer and thus represent an economic loss to the processor. Since vacuum-packaged vienna sausages account for approximately 23% of products manufactured by South African meat processors (Business and Marketing Intelligence, 1985), it is of considerable economic importance to achieve control over the microbiological spoilage of these products.

1.2 Control strategies for vacuum-packaged emulsion-type sausage spoilage

Although numerous preservation methods are used to make processed meats shelf-stable and safe, they are based on relatively few parameters (hurdles), i.e. F (high temperature), t (low temperature), a_w (less available water), pH (sufficient acidification), Eh (reduced oxygen potential), preservation (e.g., nitrite, smoke, CO_2) and radiation (e.g., gamma rays) (Leistner, 1987). The hurdle concept described by Leistner (1987) recommends the incorporation of combinations of these parameters at appropriate intensities into food products, so that undesirable microorganisms are unable to overcome them. Spoilage of the meat product would, therefore, be prevented or delayed. In addition, it was proposed that hurdles have an intensifying effect, and thus enhancements of individual hurdles would exert a synergistic effect on the microbiological stability of the product (Leistner, 1987; Scott, 1989).

Limited opportunities, however, exist for manipulating hurdles in VPVS without adversely affecting product characteristics. Vacuum-packaged vienna sausages, being typical processed meats, have pH's and water activities that allow for the growth of a wide variety of microorganisms, including LAB (Buchanan, 1986; Franz et al., 1991). Furthermore, meat spoilage LAB were shown to be psychrotrophic and capable of growth at 5°C, microaerophilic as well as resistant to inhibition by smoke, nitrite and salt (Egan, 1983; Dodds and Collins-Thompson, 1984; Buchanan, 1986; Franz et al., 1991). The use of high temperature (F) in addition to refrigerated product storage, however, was considered an attractive option to control the growth of spoilage LAB by lowering initial levels of these contaminants in a secondary or in-package pasteurization process. Spoilage control by using high temperature appeared viable since Bell (1983) found the size of microbial spoilage populations that survived a pasteurization process to be inversely related to the severity of the heat treatment applied. Furthermore, product recontamination would be prevented by the packaging material. Only microorganisms capable of surviving the in-package pasteurization process, therefore, would be capable of causing product spoilage (von Holy et al., 1991a).

1.3 Control of microbiological food spoilage by heat treatment

1.3.1 Pasteurization of foods

Heat treatment of foods is one of the most widely used methods for inactivating spoilage microorganisms (Moats, 1971). Although Pasteur's application of mild heat to wines to delay or prevent spoilage led to the designation of the process as 'pasteurization', the term has a broader meaning in modern food processing technology. In this sense, it refers to a heat treatment of food that is intended to destroy vegetative cells of pathogenic microorganisms, or that will destroy all or most vegetative cells of microorganisms that cause spoilage of a food product. For some foods, therefore, the principal objective of pasteurization is to reduce the level of specific spoilage microorganisms, so that the products may have adequate shelf life (Silliker et al., 1980). Different foods contain different target microorganisms that the pasteurization process is designed to inactivate (Witter, 1983). Since pasteurization processes do not inactivate all microorganisms present in foods, additional control parameters such as low temperature storage, acidity or reduced a_w may be necessary (Silliker et al., 1980). For pasteurization processes, temperatures below 100°C are generally used, in order to prevent overprocessing of foods which can lead to unacceptable losses in both food quality factors (color, flavor and texture) and nutritional value (Silliker et al., 1980; Witter, 1983).

In practice, in-package pasteurization was used successfully to delay spoilage and increase the shelf life of vacuum-packaged foods such as hot process (smoked) fish (Eklund et al., 1988), cooked pork chops (Prabhu et al., 1988) and South African vienna sausages (von Holy et al., 1991a). Von Holy et al. (1991a) pasteurized VPVS by immersing 500g packs into a water cooker held at 78 to 81°C for twenty minutes. Core temperatures measured in randomly chosen packs ranged from 52 to 57°C and peripheral temperatures from 58 to 59°C. While in-package pasteurization of VPVS increased shelf life of these products by ca. fourfold when stored at 7°C, it did not eliminate product spoilage by LAB (von Holy et al., 1991a). This documented ability of spoilage LAB to survive mild pasteurization of VPVS (von Holy et al., 1991a) was cause for concern, since growth of these bacteria still resulted in product spoilage after pasteurization. The need therefore arose to quantify heat resistance of predominant spoilage LAB in vitro to formulate appropriate in situ pasteurization regimes.

1.3.2 Heat resistance of microorganisms

When microorganisms are killed by moist heat at a constant temperature, death follows a logarithmic order. Numbers of viable microorganisms, therefore, decrease exponentially with time and when logarithms of survivors are plotted against time of exposure to the lethal temperature, a semilogarithmic straight-line survivor curve is obtained (Stumbo, 1965; Allwood and Russell, 1970; Cerf, 1977; Silliker et al., 1980; Witter, 1983; Jay, 1986; Pflug, 1987; Boyd, 1988; Müller, 1989). From this curve the decimal reduction time (D) value is determined as the time required at given temperature to destroy 90% (one log cycle) of microorganisms. The D-value, therefore, mathematically equals the slope of the survivor curve. By plotting logarithms of D-values against corresponding heating temperatures, a thermal death time (TDT) curve is obtained. The z-value is defined as the number of degrees Celsius required for the TDT curve to traverse one log cycle, and characterizes the relative heat resistance of microorganisms at different temperatures (Stumbo, 1965; Allwood and Russell, 1970; Cerf, 1977; King et al., 1979; Silliker et al., 1980; Witter, 1983; Jay, 1986; Pflug, 1987; Müller, 1989). The z-value, therefore, also specifies the number of degrees Celsius required to bring about a tenfold change in the death rate of a specific microorganism (King et al., 1979).

In general, the heat resistance (D-value) of microorganisms is related to their optimum growth temperatures.

Psychrophilic microorganisms are the most heat sensitive, followed by mesophilic and thermophilic microorganisms. Furthermore, sporeforming bacteria are more heat resistant than non-sporeformers, while thermophilic sporeformers are, in general, more heat resistant than mesophilic sporeformers (Jay, 1986). While heat treatments at 35°C would destroy true psychrophiles, psychrotrophs and heat sensitive mesophiles would only be eliminated at temperatures of 55°C or more. More heat resistant mesophilic non-sporeformers would require ca. 70°C for their destruction (Silliker et al., 1980).

Heat resistance data on meat spoilage LAB are largely lacking, but isolated reports showed that LAB surviving heat processing had high D-values and were therefore unusually heat tolerant (Houben, 1982; Milbourne, 1983; Table 1.1). Similarly, LAB surviving pasteurization of milk also had high D-values (Sanz Perez et al., 1982; Table 1.1). Lactic acid bacteria causing spoilage of VPVS, however, did not survive heat processing during product manufacture, but recontaminated sausages after heat processing and before packaging from environmental sources (Dykes et al., 1991). These LAB were, therefore, expected to have lower heat resistances than thermotolerant LAB (Houben, 1982; Sanz Perez et al., 1982; Milbourne, 1983). Heat resistances of spoilage LAB recontaminating processed meat products after heat processing are therefore expected to compare better to those of LAB involved in spoilage of beer and citrus juices (Adams et al., 1989; Parish, 1991; Table 1.1), since the latter bacteria stemmed from inadequately pasteurized beer and non-pasteurized citrus product, respectively. Furthermore, since LAB are closely related to Listeria monocytogenes (Jones, 1988), the reported heat resistance of the latter bacterium (Bradshaw et al., 1985; Donnelly and Briggs, 1986; Bradshaw et al., 1987; Table 1.2) might also compare favorably to those of meat spoilage LAB.

In general, z-values for most vegetative bacteria range from 4 to 6°C (Hansen and Riemann, 1963; Tomlins and Ordal, 1976). Experimentally determined D- and z-values allow the calculation of time/temperature combinations that will effectively inactivate spoilage microorganisms (Silliker et al., 1980). There are, however, a number of factors which affect the heat resistance of microorganisms and need to be taken into account in heat resistance determinations.

1.3.3 Factors affecting heat resistance of microorganisms

Factors that affect the heat resistance of microorganisms include inherent heat resistance, such as differences in heat resistance among different species or strains of the same species (Silliker et al., 1980; Jay, 1986). Furthermore, they include environmental influences prevailing during the growth of cells prior to heating. Cell age and temperature at which bacteria are grown before heat inactivation, for example, can affect their heat resistance (Stumbo, 1965; Silliker et al., 1980). In addition, environmental influences during the heating of cells, for example pH, water activity, type of suspension medium, presence of salts and other organic or inorganic compounds, can influence heat resistance (Stumbo, 1965; Silliker et al., 1980; Jay, 1986). The presence of carbohydrates, fats and proteins in the heating medium generally increase bacterial heat resistance (Moats et al., 1971b; Silliker et al., 1980; Jay, 1986). Thus, these factors need to be defined and kept constant when determining microbial heat resistance.

1.3.4 Heat process calculations for food products

Pasteurization or heat process calculations in the food industry are usually based on the semilogarithmic, straight-line model of bacterial death (Stumbo, 1965; Moats, 1971; Cerf, 1977; Pflug, 1987; Müller, 1989). This model relates the heat process (F_T) value to the initial microbial load of the food product (N_o), the decimal reduction time of microorganisms to be inactivated (D_T) and the resulting microbial population after heating (N_f) in the following way: $F_T = D_T (\log N_o - \log N_f)$. The heat process ($F_T$) value would thus be defined as the time of heating at a specific temperature to reduce the microbial load of a product to a predetermined level (Witter, 1983). The acceptability level for such heat processes, therefore, would be determined by the manufacturer and represents the desired reduction level in numbers of microorganisms in each unit (or pack), or the acceptable number of defective units (Witter, 1983).

1.4 RESEARCH MOTIVATION

Vacuum-packaged vienna sausages are a popular food in the South African food market and account for approximately 23% of products manufactured by South African meat processors (Business and Marketing Intelligence, 1985; von Holy and Cloete, 1992). Spoilage of these products prior to the expiry of the expected shelf life was attributed to the growth of LAB to high numbers during refrigerated storage. Premature spoilage of VPVS consequently has inflicted considerable economic losses on meat processors (Dykes _et_ _al_., 1991; von Holy _et_ _al_., 1991b; von Holy and Cloete, 1992). A practical method to control the growth of spoilage LAB and increase product shelf life is thus needed to decrease economic losses experienced by the manufacturers. Secondary, in-package heat treatment, in conjunction with low temperature storage has already been suggested as a strategy to control the growth of spoilage LAB in VPVS and increase product shelf life (Marshall, 1991; von Holy _et_ _al_., 1991a).

In previous trials involving in-package pasteurization of VPVS noticeable shelf life increases were achieved, but LAB still caused spoilage and predominated in spoilage populations of these products (von Holy _et_ _al_., 1991a). The above study, however, was performed on a "trial and error" basis and did not quantify the _in_ _vitro_ heat resistances of meat spoilage LAB. Since previously reported heat resistance data of meat spoilage LAB only pertained to unusually thermotolerant isolates (Niven _et_ _al_., 1954; Houben, 1982; Milbourne, 1983; Borch _et_ _al_., 1988; Table 1.1), this study undertook to quantify the heat resistance of predominant LAB causing spoilage of local VPVS in _in_ _vitro_ studies. Heat resistance of bacteria, however, is known to be greatly influenced by factors such as cell age and pH of the heating medium (Stumbo, 1965; Silliker _et_ _al_., 1980; Jay, 1986). Furthermore, clumping of bacterial cells during heat treatment and the media used for recovery of heat injured cells influence heat resistance determinations (Stumbo, 1965, Mackey and Derrick, 1982; Jay, 1986; Magnus et al., 1988). This study, therefore, also aimed to determine the effects of the above parameters on heat resistance and heat resistance determinations of meat spoilage LAB.

Since von Holy et al. (1991b, 1992) determined that homofermentative lactobacilli and leuconostocs predominated among the LAB spoilage populations in VPVS, they suggested that control measures against the spoilage of these products should be aimed specifically at these bacteria. This study therefore undertook to use in vitro heat resistance data of the most heat resistant homofermentative lactobacilli or leuconostocs to calculate pasteurization processes which would reduce numbers and growth of these bacteria in VPVS. Pasteurization of sausages thus aimed to delay spoilage of VPVS by the above bacteria, and consequently increase product shelf life.

In the previous study on the effect of pasteurization on spoilage and shelf life of VPVS, sausages were packed in double layers which resulted in poor heat penetration of packs (von Holy et al., 1991a). Furthermore, the use of double layer packs resulted in unequal heat distribution with temperatures varying from 52 to 57°C (center of packs) and 58 to 59°C (periphery of packs) (von Holy et al., 1991a). This study, therefore, also aimed to eliminate previous problems of unequal heat distribution, and to achieve higher pasteurization temperatures by packing sausages into single layers within vacuum bags. This study also undertook to determine the effect of higher pasteurization temperatures on the predominances of homofermentative lactobacilli and leuconostocs, as well as non-LAB in pasteurized VPVS.

In addition, the study of von Holy et al. (1991a) did not determine the effect of pasteurization on the predominance and growth of pathogenic bacteria which might be present in VPVS. This clearly required further study, especially since spores of potentially pathogenic Bacillus or clostridia have higher heat resistances than vegetative spoilage bacteria. Consequently, the effect of pasteurization on the predominance of potentially pathogenic bacteria such as L. monocytogenes and sporeforming Clostridium and Bacillus in VPVS was investigated.

Table 1.1 Summary of D- and z-values for spoilage lactic acid bacteria heated in various media.

Microorganism	D-values (min.) and z-values (°C)	Heating medium
Lactobacillus viridescens	D_{65} : 23,5 z = 38,5 (Milbourne, 1983)	MRS broth
Enterococcus faecium	D_{63} : 122,9 D_{66} : 84,8 z = 11,8 (Houben, 1982)	F [illegible] meat suspension
Streptococcus faecium Streptococcus durans Streptococcus faecalis subsp. faecalis Streptococcus faecalis subsp. liquifaciens	D_{62} : 12,6, D_{63} : 6,9, z = 3,7 D_{62} : 19,5 z = 3,8 D_{62} : 3,3, D_{63} : 1,6, z = 2,9 D_{62} : 2,8 z = 2,2 (Sanz Perez et al., 1982)	0,1M 3-3, Dimethyl-glutaric acid buffer (pH 6,0)
heterofermentative Lactobacillus	D_{60} : 2,6; D_{65} : 0,8 z = 12,1 (Adams et al., 1989)	Alcohol free (< 0,05% ethanol) lager beer
Lactobacillus plantarum Lactobacillus pseudoplantarum Leuconostoc mesenteroides	D_{53} : 1,9 D_{53} : 0,6 D_{53} : 1,5 (Parish, 1991)	Grapefruit serum adjusted to 17° Brix with glucose

Table 1.2 Summary of D- and z-values for Listeria (L.) monocytogenes heated in milk.

Microorganism	D-values (min.) and z-values (°C)	Heating medium
L. monocytogenes Scott A	$D_{63,3}$: 0,3; $D_{57,8}$: 4,8 z = 6,3 $D_{63,3}$: 0,6; $D_{57,8}$: 4,3 z = 6,5 $D_{63,3}$: 0,4; $D_{57,8}$: 4,1 z = 6,5 (Bradshaw et al., 1985; 1987)	Raw whole milk Sterile whole milk Sterile skim milk
L. monocytogenes 19113	$D_{62,7}$: 0,4, z = not determined	Sterile whole milk
L. monocytogenes 19115	$D_{62,7}$: 0,4, z = not determined	
L. monocytogenes F5069	$D_{62,7}$: 1,0, z = 4,3 (Donnelly and Briggs, 1986)	

CHAPTER TWO

IN VITRO HEAT RESISTANCE OF MEAT SPOILAGE LACTIC ACID BACTERIA

2.1 OPTIMIZATION OF VARIABLES INFLUENCING HEAT RESISTANCE DETERMINATION OF SPOILAGE LACTIC ACID BACTERIA FROM VACUUM-PACKAGED VIENNA SAUSAGES

ABSTRACT

Factors affecting in vitro heat resistance of a predominant Lactobacillus (Lb.) sake and a Leuconostoc (Lc.) mesenteroides strain from spoiled, South African vacuum-packaged vienna sausages were quantified. Heat resistances at 57 and 63°C in quarter-strength Ringers solution did not differ significantly between cells grown to either early or late logarithmic growth phase, or between heating media adjusted to pH 5,8 or 6,2. After heat resistance determinations, viable plate counts on MRS, Modified MRS and Standard One Nutrient Agar showed that bacteria were recovered in approximately equal numbers on all media. Survivor curves of bacteria after heat exposure were non-linear and of concave shape, suggesting that clumping of cells occurred during heating, or that bacteria were heterogeneous with respect to heat resistance. Prevention of cell clump formation during heating by adding 1,0% Tween 80 to the heating medium proved unsuccessful, since the detergent accelerated bacterial death rates. Consequently, the use of this detergent should be avoided during in vitro heat inactivation. It was concluded that the heat resistance of meat spoilage lactic acid bacteria could be assessed at either early or late logarithmic growth phase or in heating medium of pH 5,8 or 6,2, and cells could be recovered equally well on either MRS, Modified MRS or Standard One Nutrient Agar after heat treatment.

2.1.1 INTRODUCTION

Cured, emulsion-type sausages consist of meat, sugar, sodium nitrite (or nitrate) and spices (Price and Schweigart, 1971). The emulsion is filled and cooked in impermeable artificial casings (Hallerbach and Potter; 1981, Korkeala et al., 1989; von Holy et al., 1991b; von Holy and Cloete, 1992) and sausages are often vacuum-sealed into gas impermeable bags before refrigerated distribution and retail (Sharpe, 1962; Mukherji and Qvist, 1981; von Holy et al., 1991b).

Numerous studies have reported the lactic acid bacteria (LAB) as predominant spoilage populations of vacuum-packaged emulsion-type sausages (Allen and Foster, 1960; Sharpe, 1962, Mukherji and Qvist, 1981; Nielsen, 1983; Korkeala et al., 1987; Borch et al., 1988; Zurera-Cosano et al., 1988; von Holy et al., 1991b; von Holy and Cloete, 1992), reaching high numbers of ca. $10^7 - 10^8$ colony forming units per gram ($CFUg^{-1}$) during extended refrigerated storage (Kempton and Bobier, 1970; Hallerbach and Potter, 1981; Nielsen and Zeuthen, 1986; Korkeala and Lindroth, 1987; Zurera-Cosano et al., 1988; Korkeala et al., 1989; von Holy et al., 1991b, von Holy and Cloete, 1992).

Spoilage of vacuum-packaged vienna sausages (VPVS) by LAB is characterized by undesirable souring, gas formation and production of milky, slimy exudates in sausage packs. Such spoilage symptoms result in products unacceptable to the consumer and thus represent considerable economic losses to the producer (von Holy and Cloete, 1992). It was therefore considered necessary to control the growth of spoilage LAB in these products.

Preservation methods to make meats shelf-stable and safe are based on relatively few parameters or hurdles: F (high temperature), t (low temperature), a_w (less available water), pH (sufficient acidification), Eh (reduced oxygen potential), preservation (e.g., nitrite, smoke, CO_2) and radiation (e.g., gamma rays) (Leistner, 1987). Only limited opportunities, however, exist to manipulate such hurdles to control growth of spoilage LAB in vacuum-packaged emulsion-type sausages. These bacteria are psychrotrophic and hence capable of growth at refrigeration temperature (5°C), microaerophilic and resistant to inhibition by nitrite, salt and smoke (Enfors and Molin, 1980; Reuter, 1981; Egan, 1983; Dodds and Collins-Thompson, 1984; Buchanan, 1986; Holzapfel and Gerber, 1986; Franz et al., 1991). Consequently, high temperature (F) represents an attractive alternative for controlling the growth of spoilage LAB by lowering the initial numbers of these contaminants through a secondary or in-package pasteurization process. This control option appears viable since Bell (1983) noted that the size of spoilage populations surviving a pasteurization process was inversely related to the severity of the heat treatment applied and, furthermore, recontamination of product would be prevented by the packaging material. Thus, spoilage could only be caused by microorganisms surviving the pasteurization process.

Successful pasteurization processes for food products are best based on the semilogarithmic, straight-line model of bacterial destruction (Pflug, 1987), which to date provides the basis of most thermal process calculations in the food industry (Stumbo, 1965; Moats, 1971; Cerf, 1977). This method is based on the assumption that microorganisms exposed to moist heat at a constant temperature exhibit a death pattern of logarithmic order, and numbers of viable microorganisms decrease exponentially with time. Consequently, if logarithms of numbers of survivors are plotted against time of exposure to a constant lethal temperature, a straight-line survivor curve should be obtained. Two deviations from the logarithmic order of bacterial death have, however, been reported when bacteria are heated at a constant temperature. The first (described as convex survivor curves) is characterized by initial lags in death rates of bacteria, whereas the second (described as concave survivor curves) is characterized by an initial rapid death rate followed by a decrease in bacterial death rate (Stumbo, 1965; Dabbah et al., 1971a,b; Moats et al., 1971a; Cerf, 1977; Ababouch and Busta, 1987, Ababouch et al., 1987). Such deviations were explained on the basis of clumping of bacterial cells either before heating (convex curves) or during heating (concave curves)

(Stumbo, 1965; Cerf, 1977; Witter, 1983).

When bacterial death at constant, high temperature follows a logarithmic order, the decimal reduction time (D) value may be determined from straight-line survivor curves. The D-value is specific for the microorganism heated at the specified temperature. The D-value is also defined as the time required at a given temperature to destroy 90% (one log cycle) of microorganisms, and is mathematically equal to the slope of the survivor curve (Stumbo, 1965; Allwood and Russell, 1970; Cerf, 1977; Silliker et al., 1980; Witter, 1983; Jay, 1986; Pflug, 1987; Boyd, 1988; Müller, 1989).

Factors affecting heat resistance and hence D-values of microorganisms are of three general types: a) inherent resistance such as differences in heat resistance among species, or strains of the same species, as well as between spores and vegetative cells, b) environmental influences prevailing during growth of cells (e.g., age of cells and growth temperature) and c) environmental influences acting during the time of heating of cells (e.g., pH, a_w, type of suspension medium, salts and other organic and inorganic compounds) (Stumbo, 1965; Silliker et al., 1980).

Generally, bacteria are most heat resistant when grown at their maximum growth temperature and when heated in a medium at their optimum growth pH. Below and above these values, heat sensitivity increases (Hansen and Riemann, 1963; Stumbo, 1965; Dega et al., 1972; Silliker et al., 1980; Verrips et al., 1980; Ng, 1982; Sanz Perez et al., 1982; Witter, 1983; Jay, 1986; Humphrey, 1990; Linton et al., 1990; Bhaduri et al., 1991; Smith et al., 1991). Heat resistance of bacteria is also influenced by cell age, and bacteria are generally more heat resistant in the stationary than in the logarithmic phase of growth (Dega et al., 1972; Silliker et al., 1980; Houben, 1982; Jay, 1986; Palumbo et al., 1987; Kornacki and Marth, 1989; Linton et al., 1990; Boutibonnes et al., 1991; Condon et al., 1992). Furthermore, bacterial heat resistance increased with decreasing a_w of the heating medium. However, not only water activity, but also the type of solute used to decrease water activity affected bacterial heat resistance

(Goepfert et al., 1970; Corry, 1974; Silliker et al., 1980; Jay, 1986; Tuncan and Martin, 1989).

In addition, nutrient conditions of heating media and media used to grow bacteria prior to heating may also increase or decrease heat resistances (Stumbo, 1965; Moats et al., 1971b), but variable effects of different media components, singly or in combination, make generalization impossible (Silliker et al., 1980). The nutrient conditions of post-heating recovery media also influence heat resistance determinations, and recovery of sublethally injured cells is enhanced on nutritionally rich, non-selective media (Busta, 1978; Mackey and Derrick, 1982; Buchanan et al., 1988; Magnus et al., 1988; Crawford et al., 1989; Bailey et al., 1990; Harrison and Huang, 1990; Smith and Dell, 1990).

Thermal process calculations for canned foods or vacuum-packaged processed meats have been based on the semilogarithmic model of bacterial destruction, which takes into account the heat resistance or D-values of the particular microorganisms targeted for inactivation (Stumbo, 1965; Cerf, 1977; Pflug, 1987; Müller, 1989). Lactic acid bacteria, particularly homofermentative lactobacilli and leuconostocs are predominant spoilage populations of South African VPVS (von Holy et al., 1991b). Secondary or in-package heat process determinations for these products should, therefore, be based on thermal resistance data pertaining to the above genera. Since bacterial heat resistance determinations are influenced by factors such as age of cells, heating medium pH and type of recovery medium, this study determined the effect of these parameters on the in vitro heat resistance and recovery of spoilage LAB from processed meats. As cell clumping during heat inactivation of microbial cells results in non-logarithmic survivor curves (Stumbo, 1965; Cerf, 1977; Witter, 1983), detergent addition to counteract this phenomenon was evaluated.

2.1.2 MATERIALS AND METHODS

2.1.2.1 Culture selection and maintenance

Lactobacillus (Lb.) sake (1A73c) and Leuconostoc (Lc.) mesenteroides (1A15b) were identified as calculated mean organisms by numerical analysis of phenotypic characteristics of 61 predominant spoilage LAB from VPVS (Dykes, 1991). These strains were regarded as the most representative of previously described, predominant LAB spoilage populations of VPVS (von Holy et al., 1991b). Freeze dried stock cultures were grown in MRS Broth (BIOLAB) at 25°C for 48 hours and streaked onto MRS Agar (BIOLAB) to check for purity. Working cultures were grown up in litmus milk (100g litmus milk (BIOLAB); 50g calcium carbonate; 10g glucose; 2,5g liver digest; 2g yeast extract; water to one liter) at 25°C for 48 hours, stored at 4°C and subcultured in the same medium every three months.

2.1.2.2 Preparation of inoculum cultures

For heat resistance experimentation "seed" (inoculum) cultures were prepared to obtain inocula with sufficient cell densities to allow for fast growth. Inoculum cultures were prepared by transferring one loopful of culture from litmus milk to 50ml MRS Broth and growing the culture aerobically at 25°C for 48 hours.

2.1.2.3 Effect of growth phase and heating medium pH on bacterial heat resistance

To determine the effect of growth phase on LAB heat resistance, one ml of an inoculum culture (2.1.2.2) was used to inoculate 50ml MRS Broth. Cultures were then grown to either the early or late logarithmic growth phase (optical densities at 550nm of ca. 0,3 to 0,5 and 1,4 to 1,5, respectively) at 25°C. An incubation temperature of 25°C was chosen, since it approximated the optimum growth temperature of meat spoilage LAB studied here (Franz et al., 1991). At the desired growth phase, one ml was removed and diluted in quarter-strength Ringers solution (QSRS) pH 6,2, to standardize initial inoculum density to ca. log 7,00 CFU ml^{-1}. Standardization of bacterial inoculum density for heat resistance studies was necessary, since heat treatment parameters such as temperature, medium composition and concentration of cell numbers needed to be kept constant to obtain straight-line survivor curves (Cerf, 1977). Cells were then prepared for heat inactivation as shown in Fig. 2.1.1.

To determine the effect of heating medium pH on LAB heat resistance, one ml of an inoculum culture (2.1.2.2) was used to inoculate 50ml of MRS Broth, and the culture was grown to the early logarithmic growth phase (optical density at 550nm of ca. 0,3 to 0,5) at 25°C. One ml was removed and diluted in QSRS (pH 6,2) to standardize the inoculum density to ca. log 7,00 CFU ml^{-1}. To assess the effect of suspension medium pH on heat resistance, both the phosphate buffer used for washing cells, as well as the heating medium (QSRS) were adjusted to either pH 6,2 or 5,8 (Fig. 2.1.1). The pH value of 6,2 was chosen since unspoiled vienna sausages have typical pH values of ca. 6,2 (M.A. Jenkin, pers. comm.)*. Heat resistance of most microorganisms, however, was shown to decrease with increasing acidity of the heating medium (Silliker et al., 1980; Witter, 1983; Jay, 1986). A more rapid inactivation of spoilage LAB by pasteurization of VPVS was thus thought to be possible by decreasing product pH. Since the minimum value to which product pH could be lowered without adversely affecting organoleptic characteristics was pH 5,8 (M.A. Jenkin, pers. comm.)*, in vitro heat resistance of LAB was also determined at pH 5,8.

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2.1.2.4 Heat resistance determinations

Consequently, LAB heat resistance was determined at the early and late logarithmic growth phases in QSRS at pH 6,2 (Fig. 2.1.1), and in the early logarithmic growth phase in QSRS at pH 5,8 (Fig. 2.1.1). Quarter-strength Ringers solution was chosen as the heating medium since it would not permit LAB growth and since it did not contain compounds expected to protect cells against heat, for example fats or carbohydrates (Silliker et al., 1980; Jay, 1986).

After preparing cells for heat resistance determination (Fig. 2.1.1), 50 microliter aliquots of bacterial suspension (ca. $1x10^7$ $CFUml^{-1}$) were drawn into sterile glass capillary tubes of 125mm length and 1,4mm (± 0,007mm) internal diameter with a wall thickness 0,25mm (SOCOREX - Switzerland, Figs. 2.1.2 & 2.1.3), using a micropipette (SOCOREX - Switzerland, Fig. 2.1.4). Capillary tubes were heat sealed on both ends (Fig. 2.1.5), ensuring a ca. four to five cm distance of the bacterial suspension from the flame. After heat sealing each capillary tube end, it was immediately cooled in water (Fig. 2.1.6). Capillary tubes were placed in specially constructed perspex holders (Fig. 2.1.2) with spaces of at least one cm between tubes to allow for equal heating and unobstructed water flow. The construction of these holders also ensured that capillary tubes were fully submersed in water during heat exposure.

Five replicate capillary tubes were used at each of six time intervals to heat bacterial suspensions at temperatures of 63 or 57°C in a circulating, thermostat-controlled water bath (Fig. 2.1.7). Heating time intervals varied in length and depended on heating temperature (Table 2.1.1). The temperature of 57°C was chosen since meat spoilage LAB are psychrotrophic (Buchanan, 1986) and it was suggested that ca. 55°C would suffice to heat inactivate true psychrotrophs (Silliker et al., 1980). The temperature of 63°C was chosen to inactivate LAB more rapidly, and was also considered not to adversely affect product characteristics or packaging material during in-package pasteurization of VPVS (M.A. Jenkin, pers. comm.)*. Water bath temperature was measured using a conventional mercury thermometer and a Jenway 3100 microprocessor thermocouple (Fig. 2.1.8) and fluctuated by only 0,2°C during heat resistance determination. After each time interval one set of five capillary tubes was removed and rapidly cooled in ice at 0°C for five seconds (Fig. 2.1.9). Capillary tubes were then surface sterilized by immersing them in 70% ethanol for 20 minutes and air dried in a laminar flow cabinet (Fig. 2.1.3).

Each set of five capillary tubes was transferred to a sterile Down's-type homogenizer (Fig. 2.1.10), containing 2,25ml sterile QSRS, and crushed by grinding for one minute (10^{-1} dilution) (Fig. 2.1.11). This 10^{-1} dilution was vortexed and tenfold serial dilutions were prepared in QSRS. Volumes of 0,1 ml of appropriate dilutions were plated in triplicate onto MRS Agar using the spread plate technique. A control set of five capillary tubes was analyzed by identical procedures, but not subjected to heat treatment. Plates were incubated at 25°C for 48 hours and those showing between 30 to 300 CFU (or highest number if under 30) were counted. Counts from triplicate plates were meaned and converted to logarithms. In order to increase statistical significance of heat resistance data, all variables (growth phase, pH; Fig. 2.1.1) were tested three times on separate occasions.

2.1.2.5 Effect of detergent on cell clumping during heating

To determine the effect of Tween 80 on cell clumping during heating (Stumbo, 1965; Cerf, 1977; Witter, 1983), one ml inoculum culture (2.1.2.2) of _Lb_. _sake_ was used to

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inoculate 50ml MRS Broth. The culture was grown to the early logarithmic growth phase (optical density at 550nm ca. 0,3 to 0,5) at 25°C and prepared for heat inactivation as shown in Fig. 2.1.12. Cells were exposed to 63°C as described above (2.1.2.4), in heating medium (QSRS, pH 6,2) which contained 1,0% Tween 80 (Fig. 2.1.12). After heat exposure, survivors were enumerated as described above (2.1.2.4). The effect of Tween 80 on cell clumping during heating at 63°C was tested three times in separate experiments.

2.1.2.6 Effect of culture medium on recovery of heat treated bacteria

Cells of both strains were grown to the early logarithmic growth phase and prepared for heat inactivation as described above (2.1.2.5, Fig. 2.1.12), but were resuspended in heating medium (QSRS, pH 6,2) without adding Tween 80. Heat inactivation was performed as above (2.1.2.4) with the exception that bacteria were heated at 63°C for only two (short and extended) time intervals (Table 2.1.2). Moreover, for enumeration of survivors 0,1ml volumes of appropriate dilutions were plated in fivefold onto MRS Agar, Modified MRS Agar (MMRS) and Standard One Nutrient Agar (STD1) (Table 2.1.2). Plates showing 30-300 CFU were counted and counts were converted to logarithms. The effect of culture medium on recovery of heat treated bacteria was tested three times on separate occasions. Log survivor counts from different media of the three separate experiments were subjected to ANOVA with a comparison of means.

2.1.2.7 Computational methods

Log counts of survivors from triplicate heat resistance experiments assessing the effects of pH and growth phase were plotted against exposure time. A single, best fit regression line was calculated from points of triplicate data sets by the least square method. D-values were estimated by taking the absolute values of the inverse of slopes of linear regression lines (Figs. 2.1.13, 2.1.14 & 2.1.15) (Bradshaw et al., 1985; Donnelly and Briggs, 1986; Bradshaw et al., 1987). Linear regressions and ANOVA with comparison of means were computed using the STATGRAPHICS (version 5,0 - STSC Inc. and Statistical Graphics Corporation) statistical software program.

2.1.3 RESULTS AND DISCUSSION

2.1.3.1 Effect of culture medium on recovery of heat treated lactic acid bacteria

The effect of culture medium on recovery of Lc. mesenteroides and Lb. sake after heating at 63°C is shown in Table 2.1.3. Mean log numbers of Lb. sake grown on MRS, MMRS and STD 1 Agar media after fifteen seconds heating corresponded closely (Table 2.1.3). ANOVA of survivor counts with respect to recovery medium showed no significant differences in survivor recovery between the different media ($p > 0,05$; Table 2.1.3). Furthermore, ANOVA with a comparison of means also showed no significant differences in mean survivor counts between the different media. The ANOVA did, however, show a near-significant difference ($p = 0,061$) between log counts from the different media for Lb. sake after one minute's heating (Table 2.1.3). Using the ANOVA, a comparison of means of log survivor counts after one minute's heating showed that log survivor counts on MRS and MMRS Agar were significantly higher than on STD 1 Agar. This contradicted the finding that mean log counts and 95% confidence intervals for the means corresponded closely for all media (Table 2.1.3).

No statistically significant differences ($p > 0,05$) between log counts of Lc. mesenteroides on the different media were determined by the ANOVA, when bacteria were heated for either seven or 30 seconds (Table 2.1.3). Mean log survivor counts of Lc. mesenteroides from different media as well as their 95% confidence intervals also showed close correspondence (Table 2.1.3). ANOVA with a comparison of means did not show a statistically significant difference in log counts of Lc. mesenteroides on any of the media, for either seven or 30 seconds heating time.

The choice of recovery medium has been recognized as an important factor in bacterial heat resistance determinations. Since growth of sublethally injured bacteria is reportedly either delayed, or partially or completely inhibited in nutritionally defective or selective media (Busta, 1978; Mackey and Derrick, 1982; Buchanan _et al._, 1988; Bailey _et al._, 1990; Harrison and Huang, 1990; Smith and Dell, 1990), the choice of rich, non-selective recovery media is critical (Magnus _et al._, 1988; Crawford _et al._, 1989). Since heat injury affects bacterial cell membranes, substances which cannot pass through intact membranes may diffuse in or out of heat injured bacterial cells (Moats, 1971). Rich media, therefore, allow for improved recovery, since cell constituents lost as a result of membrane damage may be replenished from the medium. Selective media, on the other hand, could allow for toxic constituents to enter cells through damaged membranes and may thus lead to lower recovery compared to non-selective media (Moats, 1971).

MRS Agar, a nutritionally rich, non-selective medium suited to the complex nutritional requirements of LAB (de Man _et al._, 1960) was, therefore, evaluated in conjunction with the non-selective and nutritionally rich Standard One Nutrient Agar for recovering heat treated LAB. Since the oxidation-reduction potential of the environment reportedly influences the recovery of heat injured bacteria (Allwood and Russell, 1970; Linton _et al._, 1992) a Modified MRS medium with a lower oxidation-reduction potential was also used for recovery studies. The latter two media were also chosen since MMRS Agar and Standard One Nutrient Agar were previously used successfully in the isolation of LAB from in-package pasteurized and non-pasteurized VPVS (von Holy _et al._, 1991a,b; von Holy and Cloete, 1992).

Statistically significantly lower counts for _Lb. sake_ on Standard One Nutrient Agar after one minute's heating suggested that, in this case, MRS and MMRS Agar allowed for better cell recovery than STD1 Agar, especially after prolonged heat treatment. Differences between _Lb. sake_ mean log counts after one minute's heating on STD1 Agar and MRS and MMRS Agar, however, were small at 0,08 and 0,09 CFUml^{-1}, respectively (Table 2.1.3). Furthermore, since the 95% confidence intervals for _Lb. sake_ counts corresponded closely on all media after heating for one minute (Table 2.1.3), the differences in counts between STD1 and both MRS Agar media, was considered to be of low microbiological significance. Survivor counts of LAB heated for various times thus compared well for all media (Table 2.1.3), which suggested that these media were equally effective in recovering heat injured LAB. These results were supported by previous shelf life studies on pasteurized and non-pasteurized VPVS, where LAB counts on MMRS Agar and total aerobic plate counts on STD1 Agar showed close correspondence (von Holy et al., 1991a).

For further in vitro heat resistance studies on meat spoilage LAB, therefore, either of the culture media tested could be used to recover heat treated bacteria. As MRS Agar, however, is formulated to suit the specific nutritional requirements of LAB (de Man et al., 1960), this medium was preferred to STD1 Agar for further heat resistance studies. MRS Agar was also preferred to MMRS Agar for reasons of lower cost and simplicity of preparation.

2.1.3.2 Effects of growth phase and pH of heating medium on heat resistance of lactic acid bacteria

D_{63} and D_{57} values for Lb. sake in the early logarithmic growth phase were 32,5 and 52,9 seconds in QSRS (pH 6,2), respectively (Table 2.1.4). These were higher than D_{63} and D_{57} values of 28,9 and 42,1 seconds, respectively, for the late logarithmic growth phase (Table 2.1.4). For Lc. mesenteroides in the early logarithmic growth phase and in QSRS at pH 6,2, D_{63} and D_{57} values were 20,2 and 34,9 seconds, respectively (Table 2.1.4). In this case, cells in the late logarithmic growth phase had higher D-values than cells in the early logarithmic growth phase, and D_{63} and D_{57} values were 23,9 and 43,0 seconds, respectively (Table 2.1.4). This suggested that Lb. sake was more heat resistant in the early logarithmic growth phase, while Lc. mesenteroides was more heat resistant in the late logarithmic phase. Overlapping 95% confidence intervals for D-values at 57 and 63°C of both bacteria for both growth phases (Table 2.1.4), however, suggested that differences in D-values at the differe growth phases were not statistically significant (D.H. Meyer, pers. comm.)*.

Heat resistance of microorganisms is influenced by the growth phase (Silliker et al., 1980; Jay, 1986) and bacteria such as Staphylococcus aureus, Aeromonas hydrophila and Listeria monocytogenes were most heat resistant in the stationary phase of growth (Palumbo et al., 1987; Kornacki and Marth, 1989; Linton et al., 1990). Similarly, Boutibonnes et al. (1991), showed that Lactococcus lactis was more heat resistant in the stationary than logarithmic phase of growth. Moats et al. (1971a) argued that changes in bacterial heat resistance with cell age are determined by changes in cell physiology. Results of our study, therefore, suggested that the physiologies of cells grown to early and late logarithmic growth phases were similar and, therefore, no significant differences in cell heat resistance were observed.

Vienna sausages and other processed meats can be contaminated with low levels of spoilage LAB from environmental sources after heat processing (Paradis and Stiles, 1978; Steele and Stiles, 1981; Nielsen and Zeuthen, 1984a; Borch et al., 1988; Dykes et al., 1991). These bacteria subsequently grow to high numbers (ca. 10^7 - 10^8 CFUg^{-1}) during refrigerated product storage (Kempton and Bobier, 1970; Schneider et al., 1983; Korkeala et al., 1989; von Holy and Cloete, 1992). It can thus be deduced that spoilage LAB would enter the early logarithmic phase of growth in refrigerated VPVS shortly after processing, the time to reach this phase of growth being dependent on storage temperature. Since in-package pasteurization of VPVS is usually planned to occur immediately after packaging, such pasteurization processes

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should therefore be based on in vitro heat resistance data of LAB in the early logarithmic growth phase. In a "worst case" scenario, where pasteurization is delayed, LAB could theoretically enter the logarithmic or late logarithmic growth phases. In-package pasteurization treatments, however, could still be based on D-values of LAB in the early logarithmic growth phase, since our results clearly showed that D-values for early and late logarithmic growth phases did not differ statistically significantly. Further in vitro heat resistance determinations for meat spoilage LAB could, therefore, be performed on cells in the early logarithmic growth phase.

D_{63} and D_{57} values of Lb. sake heated in QSRS at pH 6,2 were 32,5 and 52,9 seconds, respectively, and therefore higher than corresponding D-values of 28,7 and 40,1 seconds for cells heated in QSRS at pH 5,8 (Table 2.1.4). D-values of Lc. mesenteroides heated in QSRS pH 6,2, were 20,2 and 34,9, respectively (Table 2.1.4). These values,however, were lower than corresponding D-values of 21,3 and 46,9 seconds for Lc. mesenteroides heated in QSRS at pH 5,8 (Table 2.1.4). This suggested that Lb. sake was more heat resistant at a pH of 6,2, whereas Lc. mesenteroides was more heat resistant at a pH of 5,8. Again, the 95% confidence intervals for D-values of both bacteria overlapped at the different pH's, and for both heating temperatures (Table 2.1.4). As for the growth phase effect on LAB heat resistance, this indicated that there were no statistically significant differences in D-values of meat spoilage LAB heated in QSRS at either pH 6,2 or 5,8.

Heat resistance of microorganisms was influenced by the pH of the heating medium in other studies, with microorganisms being most heat resistant at their optimum growth pH, and lowering of medium pH from this optimum value led to an increase in the heat sensitivity (Stumbo, 1965; Silliker et al., 1980; Witter, 1983; Jay, 1986). For example, Kornacki and Marth (1986) showed a decrease in D-value of Streptococcus faecium from 10,2 minutes to 4,8 minutes, when heated at 65°C in milk adjusted to pH 6,5 and 5,6, respectively. Sanz Perez et al. (1982) showed that D-values for enterococci decreased with decreasing buffer pH from maximum values at pH 6,0, at a constant heating temperature. These above authors did, however, not state whether the observed differences in D-values were statistically significant.

Although differences in D-values of LAB heated in QSRS at pH 6,2 and 5,8 in this study did not differ significantly, it was interesting to note that D-values for Lb. sake increased while those of Lc. mesenteroides decreased at the lower pH of 5,8. Reasons for this could possibly be that the Lb. sake strain had an optimum growth pH of above pH 5,8, while the optimum growth pH for the Lc. mesenteroides strain could have been lower than pH 6,2. The fact that no significant differences in D-values of LAB heated in QSRS adjusted to these pH values could be observed, suggested that the pH difference (0,4 units) was too small to increase the heat sensitivity of spoilage LAB. Lowering product pH to 5,8, therefore, was unlikely to affect the death rate of such spoilage bacteria during in-package pasteurization processes, and more rapid heat inactivation would not be achieved. Further in vitro heat resistance studies of LAB could, therefore, be performed in heating medium at pH 6,2 since this value corresponds to the established pH of most South African vienna sausage formulations.

2.1.3.3 Linear regressions and data fit validity

Regression analysis showed slopes of all lines to be highly significant (p = 0,00), validating the regression model. Correlation coefficients (r) for the linear regression of log survivor counts versus heating times ranged from -0,816 to -0,974, with most being higher than -0,910 (Table 2.1.4). This indicated a satisfactory linear relationship between log survivor counts and heating time for both LAB strains. On closer inspection of data points, however, survivor curves appeared concave rather than linear (Figs. 2.1.13, 2.1.14 & 2.1.15). Furthermore, when heat resistance data were analyzed for variance, plots of residuals versus predicted values (data not shown) showed trends in residuals with fan or parabolic shaped patterns (Neter et al., 1988). This indicated that the linear regression model did not fit the data, since residuals are estimates of the error terms of the linear regression model and are assumed to be randomly distributed (Neter et al., 1988).

Authors such as Rahn (1945) and Schmidt (1957), however, suggested that the assumption of the logarithmic order of bacterial death at constant temperature was convenient for comparisons of survival of bacteria at different temperatures, or survival of different bacteria under the same conditions. The logarithmic model of thermal death of bacteria was, therefore, considered appropriate to this study for comparisons of bacterial heat resistance at different growth phases and pH's.

Nonlinear, concave survivor curves have been reported previously when bacteria or bacterial spores were heated at constant temperature (Dabbah et al., 1971a,b; Moats et al., 1971a; Ababouch and Busta, 1987; Ababouch et al., 1987). Concave survivor curves have been explained on the basis of clumping of bacterial cells or spores during heating, and heterogeneity in heat treatments (Stumbo, 1965; Cerf, 1977; Witter, 1983; Beckers et al., 1987; Donnelly et al., 1987). Heterogeneity of heat treatment occurs when bacterial cells are heated in open test tubes. Cells covering test tube walls as a result of mixing and splashing would be positioned above the water level and thus escape the full effect of the heat treatment (Beckers et al., 1987; Donnelly et al., 1987). In our study, heterogeneity of heat treatment could not explain concave survivor curves of LAB, since bacterial suspensions were sealed in capillary tubes which were fully submersed in water during heating (Fig. 2.1.7). Concave survivor curves obtained upon heating meat spoilage LAB may, therefore, have resulted from clumping of bacterial cells during the heat treatment. To test whether this was actually the case, this study attempted to prevent clumping of cells during heating by addition of the detergent Tween 80 to the heating medium (QSRS).

2.1.3.4 Effect of Tween 80 on cell clumping during heating

Mean survivor counts for Lb. sake after heating at 63°C are shown in Table 2.1.5. While bacteria heated in QSRS (pH 6,2) without Tween 80 addition could be enumerated at all heating time intervals, survivors were reduced to below detectable levels (< log 2,0 $\mathrm{CFUml^{-1}}$) from 30 seconds onwards when the heating medium contained 1,0% Tween 80 (Table 2.1.5).

Attempts to prevent bacterial cell clumping during heating by incorporation of 1,0% Tween 80 into the heating medium resulted in more rapid kill in this study (Table 2.1.5). In addition, increases in bacterial death rates even occurred when cells were pre-treated with 1,0% Tween 80 during the first washing step (Fig. 2.1.12) and the detergent was not incorporated into the heating medium (results not shown). Clearly, in this case the detergent was not completely removed from bacterial cells when these were resuspended in the heating medium (Fig. 2.1.12). Increases in bacterial death rates during heating in QSRS containing detergent were speculated to result from detergent action on the lipid bilayer of bacterial cell membranes. This would make membranes more permeable and destroy the cells' osmotic balance. Injury to heat treated bacteria has previously been linked to damage of cell membranes (Iandolo and Ordal, 1966) and membrane damage was suggested to result from melting of the lipid component (Allwood and Russell, 1970). Since bacterial death was accelerated by the addition of 1,0% Tween 80 to QSRS during heating, it was decided not to use detergent to prevent cell clumping in further in vitro heat resistance studies.

Accelerated bacterial death by heating in QSRS with 1,0% Tween 80 precluded us from establishing whether cell clumping during heat treatment resulted in the concave survivor curves observed for the two meat spoilage LAB chosen for this pilot study. Moats et al. (1971a), however, dismissed the theory that concave survivor curves resulted from clumping of bacterial cells during heating, since clumps would be completely disrupted by normal dilution techniques employed for enumeration of survivors.

Thus, even though clumps would increase bacterial heat resistance by protecting bacteria from heat inactivation, dispersion of clumps by dilution techniques should nevertheless result in straight-line survivor curves. A more plausible interpretation of concave survivor curves such as the ones observed in this study, however, may be the fact that individual cells within a population of microorganisms differ from each other with respect to heat resistance (Licciardello and Nickerson, 1963; Allwood and Russell, 1970; Moats, 1971; Moats <u>et al</u>., 1971a, Witter, 1983). Since it was proposed that heterogeneity in populations of microorganisms with respect to heat resistance remained the only satisfactory explanation for concave survivor curves (Moats, 1971; Palumbo <u>et al</u>., 1987), the same explanation could be used with respect to the findings of this study.

2.1.4 CONCLUSION

Mean log counts of two heat treated meat spoilage LAB on MRS, MMRS and STD1 Agar media compared favorably in this study. We therefore concluded that any of these media could be used to recover heat treated LAB in further <u>in vitro</u> heat resistance studies. As MRS Agar is formulated to suit the specific nutritional requirements of LAB (de Man <u>et al</u>., 1960), this medium should, however, be used in preference to STD1 Agar for recovering heat treated LAB. Since MRS Agar is easier to prepare and less costly than MMRS Agar, it is recommended that MRS Agar should be used to recover heat treated meat spoilage LAB in further <u>in vitro</u> heat resistance studies.

Heat resistances of two meat spoilage LAB did not differ statistically significantly for cells in the early or late logarithmic growth phases or for cells heated in QSRS at pH 5,8 or 6,2. Since <u>in vitro</u> heat resistance data were to be used for calculating in-package pasteurization parameters for VPVS, a pH of 6,2, (which corresponds to the pH of South African vienna sausage formulations) is recommended for further <u>in vitro</u> heat resistance studies. Our results also implied that lowering vienna sausage pH to 5,8 would probably not increase the death rate of spoilage LAB during in-package pasteurization of VPVS.

As spoilage LAB contaminating VPVS would be in the early logarithmic growth phase after packaging (the time of which pasteurization is expected to occur), further in vitro heat resistance studies could be performed at this growth phase. Since the heat resistance of LAB did not differ significantly between the early and late logarithmic growth phases, pasteurization of sausages could still be based on heat resistance data for the early logarithmic growth phase, even if pasteurization is delayed and bacterial growth in sausages enters the logarithmic phase.

Survivor curves of two predominant spoilage LAB from VPVS in this study were non-logarithmic and of concave shape. Since concave survivor curves were previously argued to result from clumping of bacterial cells during heating (Stumbo, 1965; Witter, 1983), 1,0% Tween 80 was added to QSRS to prevent cell clumping. Addition of this detergent to QSRS, however, accelerated bacterial death rates, probably as a result of detergent damage to bacterial cell membranes. This led us to conclude that in vitro heat resistance of LAB should not be studied in media containing detergents, since this could lead to overestimation of actual heat sensitivity.

Accelerated bacterial death rates in QSRS containing detergent precluded an assessment on the causative factors for concave survivor curves. Clumping of cells during heating, however, was dismissed as a cause for concave survivor curves (Moats et al., 1971a), and the more plausible explanation for the occurrence of such curves in this study was that the two LAB strains were heterogeneous with respect to heat resistance. We therefore concluded that further in vitro heat resistance studies on meat spoilage LAB would result in similar curves. Although such curves could also be interpreted according to the linear model, the D-values obtained would be an underestimate of actual bacterial heat resistance. When D-values from concave survivor curves are used to calculate in situ in-package pasteurization parameters for VPVS, appropriate compensations have to be included to achieve the desired reduction in contaminating LAB.

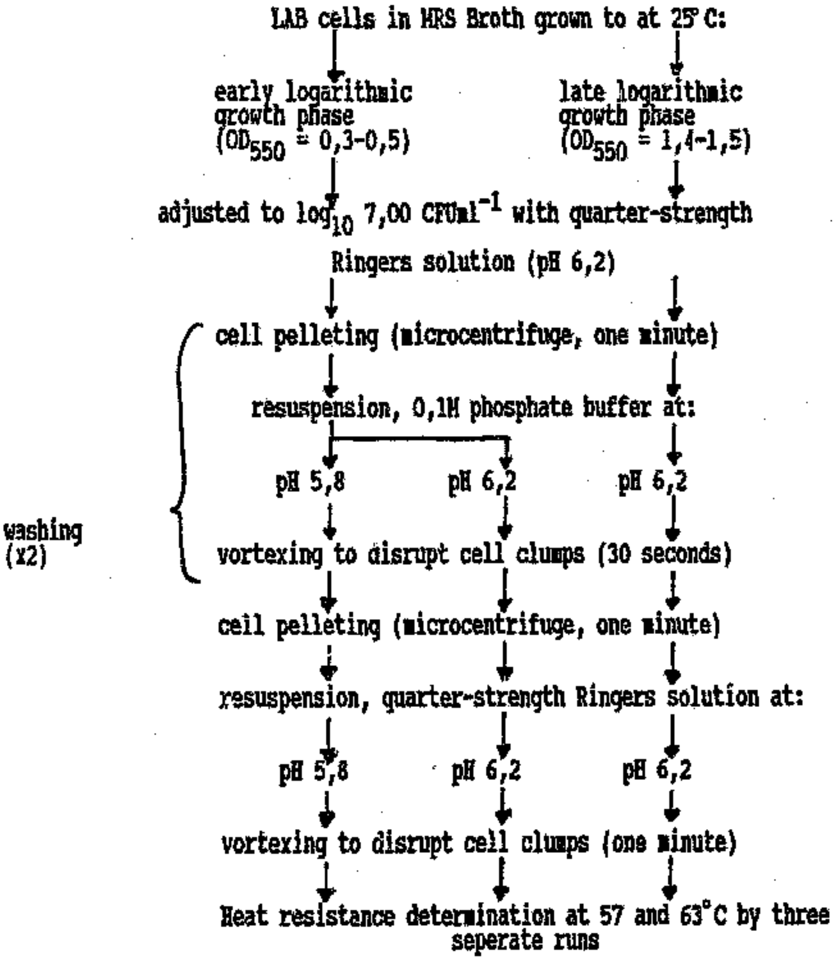


Fig. 2.1.1 Flow diagram illustrating procedure for cell preparation to assess the effects of growth phase and pH of heating medium on heat resistance of meat spoilage lactic acid bacteria (LAB).

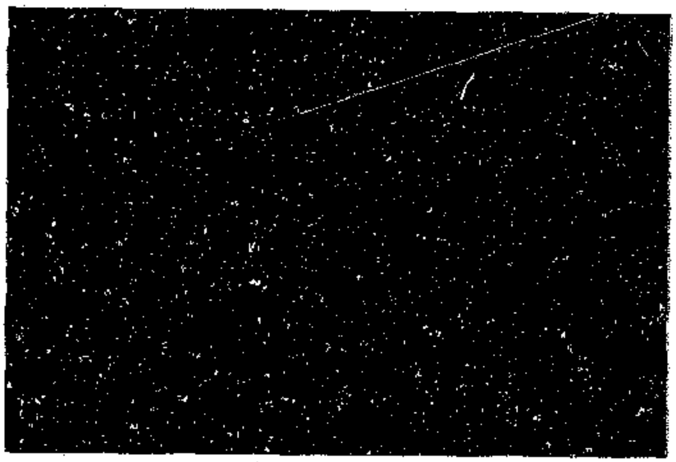


Fig. 2.1.2 Perspex holder containing a set of five 50 microliter capillary tubes, showing distances between tubes of ca. one cm.

Fig. 2.1.3 Sets of five capillary tubes in laminar flow cabinet to dry 70% ethanol used for surface sterilization.

Fig. 2.1.4 Bacterial suspension (50 microliter in quarter-strength Ringers solution is drawn into a capillary tube using the SOCOREX micropipette.

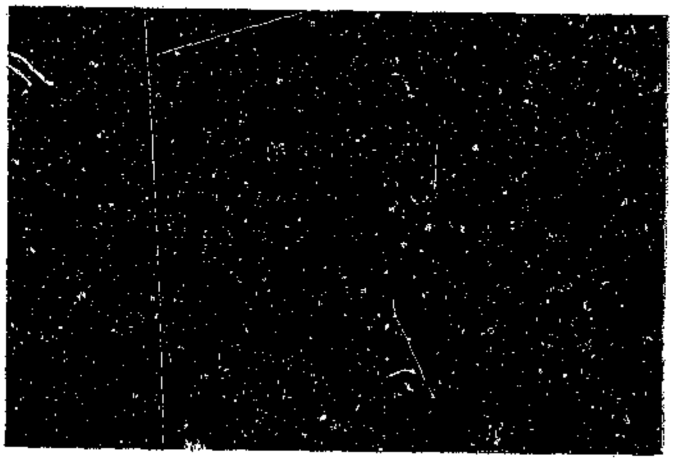


Fig. 2.1.5 Heat sealing of capillary tube containing 50 microliter bacterial suspension highlighting distance of *ca*. four to five cm of suspension from heated end.

Fig. 2.1.6 Cooling the end of a heat sealed capillary tube containing 50 microliter bacterial suspension in water.

Fig. 2.1.7 Heat inactivation of bacterial suspension in fully submerged 50 microliter capillary tubes in a thermostat-controlled circulating water bath.

Fig. 2.1.8 Thermostat-controlled circulating water bath with conventional mercury thermometer and Jenway 3100 microprocessor thermocouple used for heat inactivation studies.

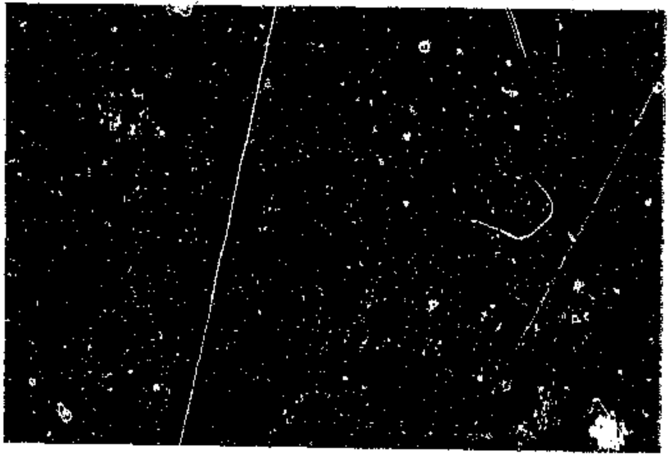


Fig. 2.1.9 Capillary tubes after heat treatment are cooled by fully submerging in melting ice.

Fig. 2.1.10 Down's-type homogenizers used to crush capillary tubes to dilute and enumerate bacteria after heat inactivation.

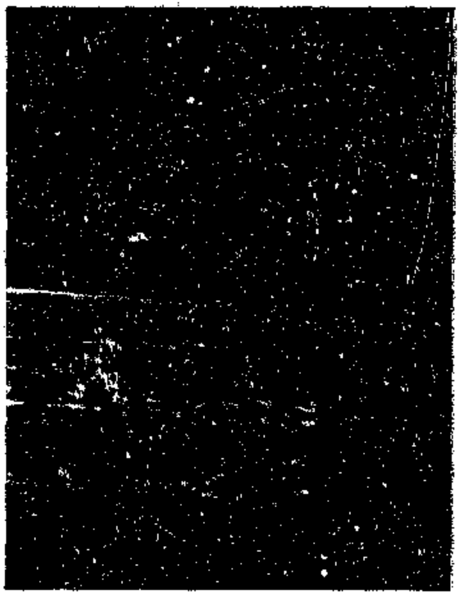


Fig. 2.1.11 Set of five 50 microliter capillary tubes crushed in a Down's-type homogenizer containing 2,25mℓ sterile quarter-strength Ringers solution (10^{-1} dilution).

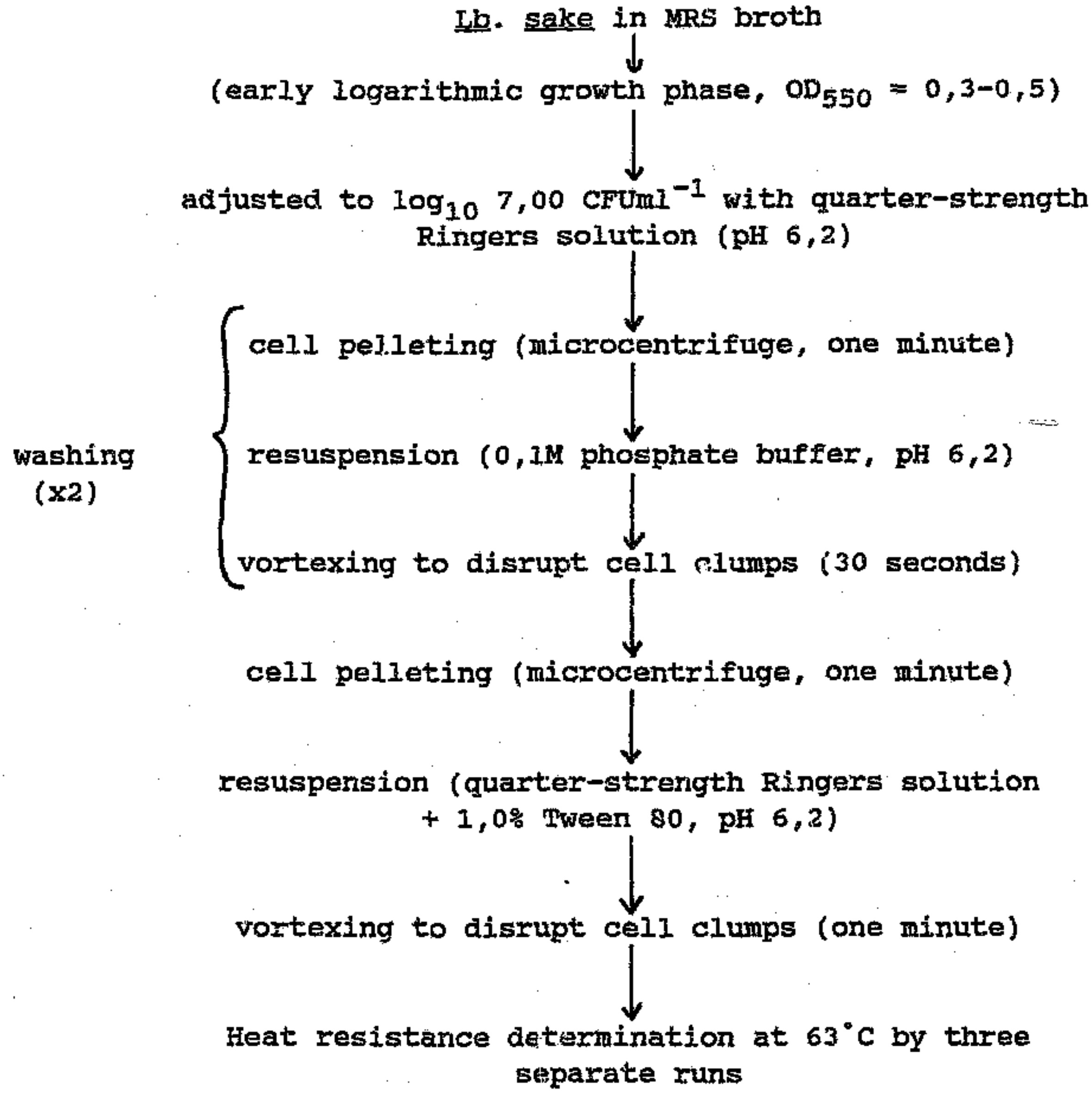


Fig. 2.1.12 Flow diagram illustrating procedure for cell preparation to assess the effect of 1,0% Tween 80 in heating medium on cell clumping during heat inactivation of _Lactobacillus_ (_Lb._) _sake_.

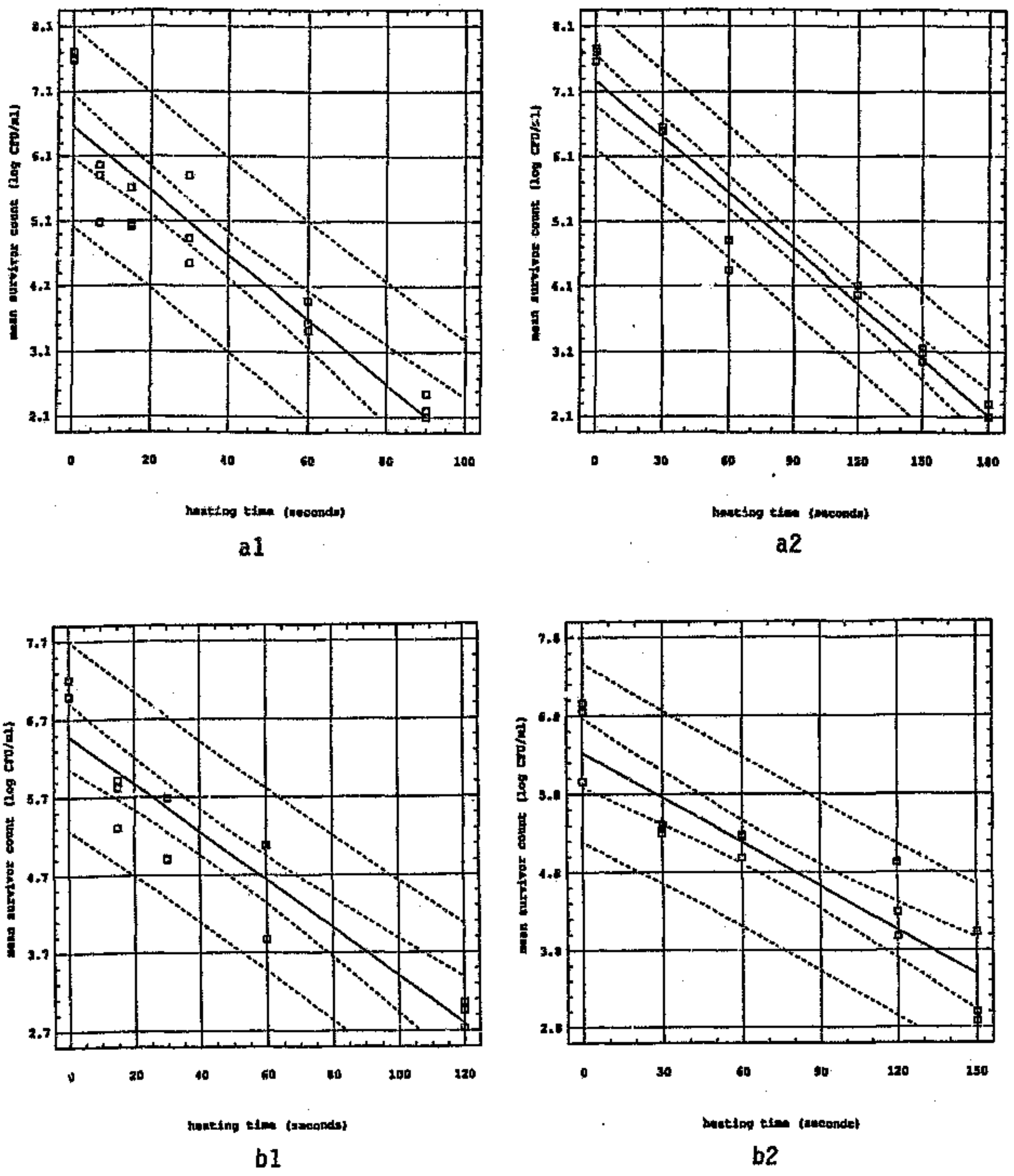
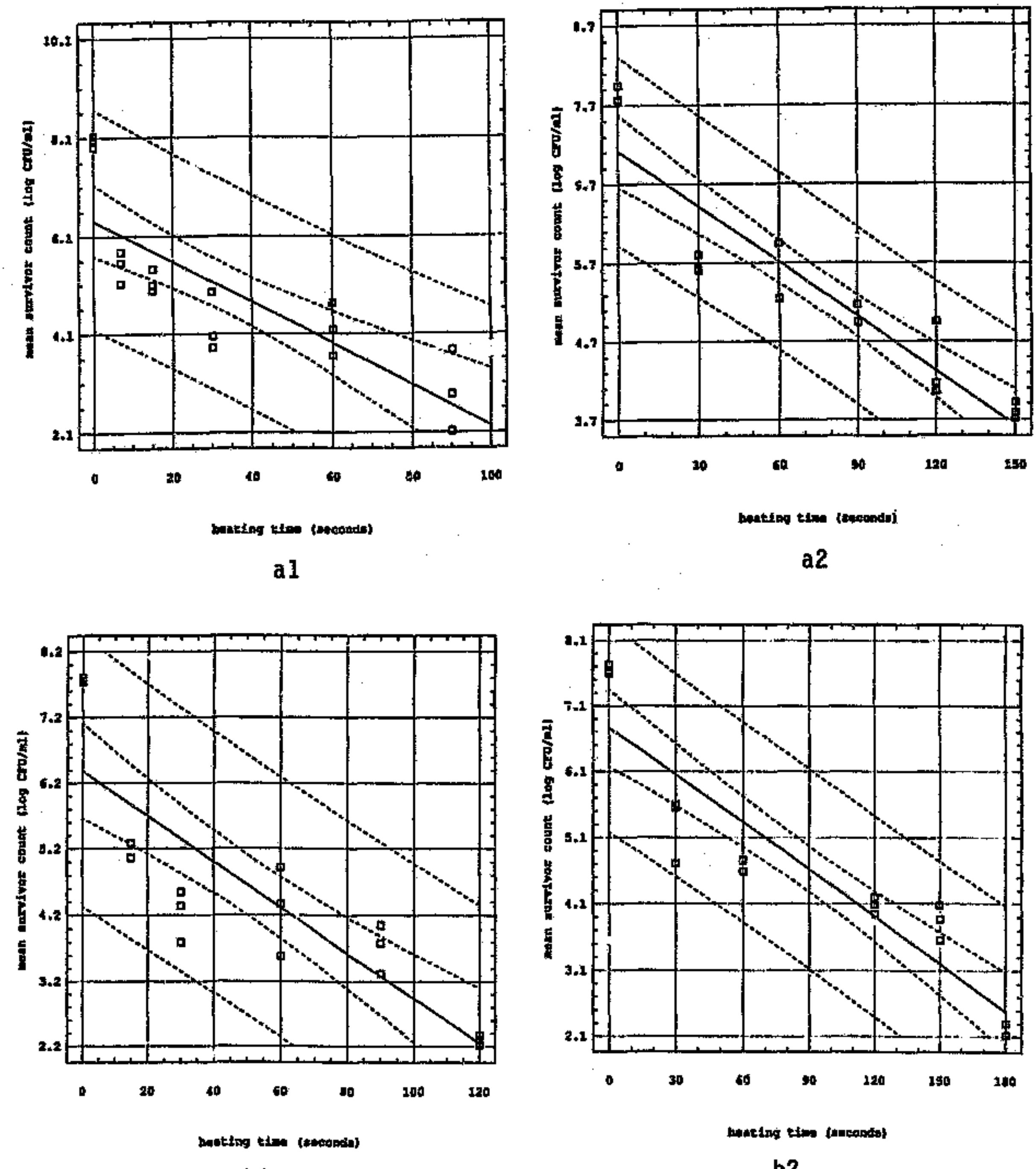


Fig. 2.1.13 Linear regressions of triplicate data sets of log $CFUml^{-1}$ vs. heating time for *Lc. mesenteroides* (a) and *Lb. sake* (b) heated in the early logarithmic growth phase (pH 6,2) at 63°C (1) and 57°C (2). Dotted lines represent 95 & 99% confidence intervals.



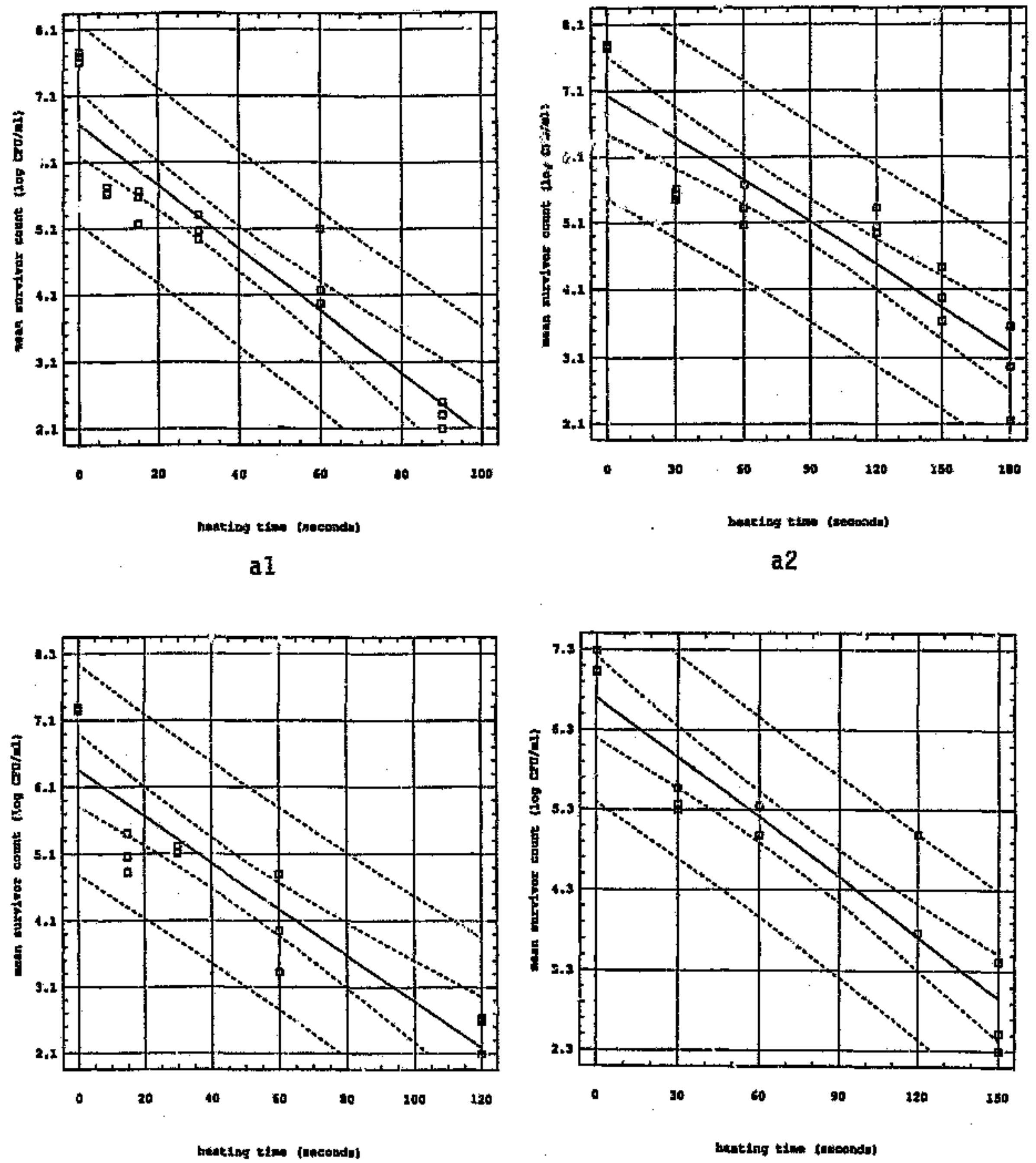
a1

a2

b1

b2

Fig. 2.1.14 Linear regressions of triplicate data sets of log CFUml^{-1} vs. heating time for Lc. mesenteroides (a) and Lb. sake (b) heated in the late logarithmic growth phase (pH 6,2) at 63°C (1) and 57°C (2). Dotted lines represent 95 & 99% confidence intervals.



a1

a2

b1

b2

Fig. 2.1.15 Linear regressions of triplicate data sets of log $CFUml^{-1}$ vs. heating time for Lc. mesenteroides (a) and Lb. sake (b) heated in the early logarithmic growth phase (pH 5,8) at 63°C (1) and 57°C (2). Dotted lines represent 95 & 99% confidence intervals.

Table 2.1.1 Heat treatment time intervals for *Lactobacillus sake* and *Leuconostoc mesenteroides* to determine effect of growth stage and heating medium[a] pH on heat resistance.

Growth stage and heating medium pH	Isolate	Temperature (°C)	Exposure time (seconds)
Early logarithmic, pH 6,2	*Lactobacillus sake*	57	0; 30; 60; 120; 150; 180
		63	0; 15; 30; 60; 120; 150
	Leuconostoc mesenteroides	57	0; 30; 60; 120; 150; 180
		63	0; 7; 15; 30; 60; 90
Early logarithmic, pH 5,8	*Lactobacillus sake*	57	0; 30; 60; 120; 150; 180
		63	0; 15; 30; 60; 120; 150
	Leuconostoc mesenteroides	57	0; 30; 60; 120; 150; 180
		63	0; 7; 15; 30; 60; 90
Late logarithmic, pH 6,2	*Lactobacillus sake*	57	0; 30; 60; 120; 150; 180
		63	0; 15; 30; 60; 90; 120
	Leuconostoc mesenteroides	57	0; 30; 60; 90; 120; 150
		63	0; 7; 15; 30; 60; 90

[a] Quarter-strength Ringers solution.

Table 2.1.2 Exposure times and recovery media used for heat inactivation of two meat spoilage lactic acid bacteria at 63 °C.

Mircoorganism	*Lactobacillus sake* (LA 73c)	*Leuconostoc mesenteroides* (LA 15b)
Heating time intervals	15 and 60 seconds	7 and 30 seconds
Plating media for cell recovery	MRS (de Man, Rogosa & Sharpe) Agar (BIOLAB); Modified MRS Agar (MRS Agar (BIOLAB) + 0,1% w/w cysteine monohydrochloride (MERCK) + 0,2% w/v potassium sorbate (UNILAB)); Standard One Nutrient Agar (STD 1) (BIOLAB)	

Table 2.1.3 Effect of culture medium[a] on recovery of *Lactobacillus sake* and *Leuconostoc mesenteroides* after heating in quarter-strength Ringers solution (pH 6,2) at 63°C.

Lactobacillus sake						
Heating time	15 seconds			60 seconds		
Medium	MRS	MMRS	STD1	MRS	MMRS	STD1
Mean[b] log$_{10}$ count (CFUml^{-1})[c]	4,96 (4,79-5,12)	4,97 (4,80-5,14)	4,90 (4,73-5,07)	4,62 (4,57-4,68)	4,63 (4,57-4,68)	4,54 (4,48-4,60)
ANOVA[d] significance level	p = 0,826			p = 0,061		
Leuconostoc mesenteroides						
Heating time	7 seconds			30 seconds		
Medium	MRS	MMRS	STD1	MRS	MMRS	STD1
Mean[b] log$_{10}$ count (CFUml^{-1})[c]	5,49 (5,32-5,65)	5,44 (5,27-5,61)	5,38 (5,22-5,56)	5,47 (5,30-5,64)	5,42 (5,25-5,59)	5,38 (5,21-5,55)
ANOVA[d] significance level	p = 0,718			p = 0,726		

[a] For abbreviations, see Table 2.1.2.

[b] Mean of independant triplicate experiments, 95% confidence intervals for means shown in brackets.

[c] Mean CFUml^{-1} calculated from five replicate plate counts.

[d] Analysis of variance for log counts (CFUml^{-1}).

Table 2.1.4 The effects of growth phase[a] and pH[b] of heating medium on the heat resistance (D-values)[c] of *Lactobacillus* (*Lb.*) *sake* and *Leuconostoc* (*Lc.*) *mesenteroides*.

D-values (seconds)			
Early logarithmic growth phase, pH 6,2			
Lb. sake		*Lc. mesenteroides*	
D_{63}	D_{57}	D_{63}	D_{57}
32,5	57,9	20,2	34,9
(26,6-41,8)	(42,0-71,3)	(16,6-25,8)	(31,1-39,9)
r = -0,937[d]	r = -0,918	r = -0,925	r = -0,974
Early logarithmic growth phase, pH 5,8			
Lb. sake		*Lc. mesenteroides*	
D_{63}	D_{57}	D_{63}	D_{57}
28,7	40,1	21,3	46,9
(22,9-38,6)	(32,7-51,7)	(17,4-27,6)	(37,6-62,4)
r = -0,919	r = -0,936	r = -0,919	r = -0,906
Late logarithmic growth phase, pH 6,2			
Lb. sake		*Lc. mesenteroides*	
D_{63}	D_{57}	D_{63}	D_{57}
28,9	42,1	23,9	43,0
(22,1-42,1)	(34,4-54,3)	(17,4-38,3)	(35,3-54,9)
r = -0,862	r = -0,921	r = -0,816	r = -0,925

[a] Early or late logarithmic phase.

[b] Quarter-strength Ringers solution, pH 5,8 or 6,2.

[c] D-values for bacteria heated at 63°C (D_{63}) or 57°C (D_{57}); corresponding 95% confidence intervals are shown in brackets.

[d] Correlation coefficients (r) of linear regression lines from which D-values were derived.

Table 2.1.5 Effect of Tween 80 treatment during heating[a] at 63°C on cell numbers of *Lactobacillus sake*.

% Tween 80	0			1,0			Heating time
Replicate no.	1	2	3	1	2	3	(seconds)
Microbiological	7,0	7,2	7,0	7,2	6,9	7,1	0
count	5,9	5,8	5,9	4,9	4,7	5,2	15
($\log_{10}$ CFUml^{-1})[b]	4,9	5,7	4,9	<2,0	<2,0	<2,0	30
	3,9	5,1	3,9	<2,0	<2,0	<2,0	60
	2,9	3,1	2,9	<2,0	<2,0	<2,0	120

[a] Heating in quarter-strength Ringers solution (pH 6,2).

[b] Mean of triplicate plate counts.

2.2 *IN VITRO* HEAT RESISTANCE OF SPOILAGE LACTIC ACID BACTERIA FROM VACUUM-PACKAGED VIENNA SAUSAGES

ABSTRACT

The *in vitro* heat resistance of four predominant lactic acid bacteria from spoiled, vacuum-packaged vienna sausages was determined in quarter-strength Ringers solution (pH 6,2). D-values at 57, 60 and 63°C were 52,9, 39,3 and 32,5 seconds for *Lactobacillus sake*, 34,9, 31,3 and 20,2 seconds for *Leuconostoc mesenteroides*, 22,5, 15,6 and 14,4 seconds for *Lactobacillus curvatus* and 49,9, 32,9 and 30,2 seconds for a *Pediococcus* strain, respectively. *Lactobacillus sake* was the most heat resistant, followed by *Pediococcus*, *Leuconostoc mesenteroides* and *Lactobacillus curvatus*, in decreasing order. All four lactic acid bacteria were considered heat sensitive, since one log cycle reductions in numbers could be achieved at 57°C in under one minute. Corresponding z-values ranged from a minimum of 25,2°C for *Leuconostoc mesenteroides* to a maximum of 30,9°C for *Lactobacillus curvatus*. Z-values were considered extraordinarily high, which could be explained by the fact that heat resistance did not change greatly over the 6°C temperature range in which heat resistances were determined. Survivor curves of lactic acid bacteria were not strictly linear, and D-values obtained were, therefore, regarded as underestimates of true heat resistance. In-package pasteurization processes for vacuum-packaged vienna sausages calculated on the basis of *in vitro* heat resistance data should, therefore, compensate for this deviation in order to achieve the desired reductions in levels of contaminating lactic acid bacteria.

2.2.1 INTRODUCTION

Spoilage of vacuum-packaged emulsion-type sausages is frequently due to the growth of lactic acid bacteria (LAB) to high numbers (Mukherji and Qvist, 1981; Nielsen, 1983; Korkeala et al., 1985,1987; Borch et al., 1988; Zurera-Cosano et al., 1988; von Holy et al., 1991b; von Holy and Cloete, 1992). Spoilage of South African vacuum-packaged vienna sausages (VPVS) by LAB is characterized by undesirable souring of product, gas formation and production of milky, slimy exudates in packages (von Holy et al., 1991b; von Holy and Cloete, 1992). Since such spoilage symptoms resulted in products unacceptable to consumers, they represented costly losses to producers (von Holy and Cloete, 1992). Homofermentative lactobacilli and leuconostocs predominated in LAB spoilage populations of VPVS (von Holy and Holzapfel, 1991; von Holy et al., 1991b; von Holy and Cloete, 1992) and it was, therefore, suggested that control measures aimed at reduced spoilage should address the inactivation of these specific bacteria (von Holy et al., 1992).

Lowering the numbers of spoilage bacteria in vacuum-packaged processed meats by in-package or secondary pasteurization was suggested as a control measure to decrease numbers and growth of contaminating bacteria, thus prolonging shelf life (Bell, 1983; von Holy et al., 1991a). In-package pasteurization was suggested as a feasible option to control spoilage of VPVS by LAB (von Holy et al., 1991a), since it was previously found that the size of the microbial population surviving a pasteurization process was inversely related to the heat treatment applied (Bell, 1983). In addition, recontamination of sausages would be prevented by the packaging material and only microorganisms surviving the heat treatment would be able to cause spoilage (von Holy et al., 1991a).

Pasteurization has previously been shown to improve the shelf life of semi-processed meats such as ham by destroying most of the spoilage microorganisms (Houben, 1982; Delaquis et al., 1986). Furthermore, the shelf life of vacuum-packaged products such as cooked pork chops (Prabhu et al., 1988), hot process (smoked) fish (Eklund et al., 1988) and vienna sausages (von Holy et al., 1991a; Marshall, 1991) also increased after a secondary, in-package heat treatment. Von Holy et al. (1991a) reported a fourfold increase in the shelf life of VPVS stored at 7°C, after in-package pasteurization for 20 minutes in a water cooker at 78-81°C. The double layer packs of sausages used in the above study were poorly heat penetrated and core temperatures ranged between 52°C and 57°C only. As the above study was performed on a "trial and error" basis, a more quantitative approach, based on heat resistances of predominant meat spoilage LAB as well as theoretically calculated heat process (F_T) values (Stumbo, 1965; Pflug, 1987; Müller, 1989) was considered essential to evaluate the effectiveness of any in-package pasteurization treatments.

When moist heat at a constant temperature is used to kill microorganisms, death follows a logarithmic order. Numbers of viable microorganisms, therefore, decrease exponentially with time and if logarithms of survivors are plotted against time of exposure to the lethal temperature, a straight-line survivor curve should be obtained (Stumbo, 1965; Allwood and Russell, 1970; Cerf, 1977; Silliker et al., 1980; Witter, 1983; Jay, 1986; Pflug, 1987; Boyd, 1988; Müller, 1989). The decimal reduction time (D) value may be determined from this curve which is a temperature specific value for the microorganisms heated. The D-value is defined as the time required at given temperature to destroy 90% (one log cycle) of microorganisms and mathematically equals the slope of survivor curves (Stumbo, 1965; Allwood and Russell, 1970; Cerf, 1977; Silliker et al., 1980; Witter, 1983; Pflug, 1987; Müller, 1989). Z-values are obtained when logarithms of the different D-values for a specific microorganism are plotted against the corresponding heating temperatures. Mathematically the z-value equals the slope of such a curve and is defined as the amount of temperature required to bring about a tenfold change in the death rate (Stumbo, 1965; King et al., 1979; Jay, 1986; Müller, 1989). Z-values for non-sporing bacteria ranged from 4 to 6°C (Hansen and Riemann, 1963; Tomlins and Ordal, 1976), and thus only small temperature increases would suffice to increase the death rates of most non-sporing bacteria tenfold. For instance, the z-value for Streptococcus faecium heated in buffer was 3,7°C (Sanz Perez et al., 1982; ch.1, Table 1.1) and since this bacterium had a D-value at 63°C of 6,9 minutes (ch.1, Table 1.1), raising the temperature by 3,7°C to 66,7°C would increase the death rate by a factor of ten to 41,1 seconds (0,69 minutes).

Heat process calculations are time/temperature combinations that take into account the D-values of microorganisms to be eliminated or reduced in food products. Such calculations are, therefore, based on the semilogarithmic, straight-line model of bacterial death. This model implies that log numbers of bacteria surviving a heat treatment plotted against time give rise to a straight-line survivor curve (Stumbo, 1965; Pflug, 1987; Müller, 1989). The model relates the microbial load of the product (N_o), the D-value of the microorganism to be inactivated (D_T) and the resulting microbial population after heating (N_f) to the heat process value in the following way: $F_T = D_T (\log N_o - \log N_f)$. Thus F_T would be defined as the time of heating required at a given temperature to reduce the microbial load of a product to a predetermined level (Witter, 1983). The acceptability level of such heat processes, therefore, would be determined by the manufacturer as the acceptable number of defective units, or the desired level of reduction of microorganisms in each unit (pack) (Witter, 1983).

Scientific data on the heat resistance of meat spoilage LAB are largely lacking. Isolated reports, however, addressed thermotolerant lactobacilli that survived heat processing of processed meat products and subsequently caused spoilage (Niven et al., 1954; Houben, 1982; Milbourne, 1983; Borch et al., 1988; Müller, 1989). Niven et al. (1954) showed that numbers of a heterofermentative Lactobacillus sp. that survived smoke-cooking of emulsion-type sausages and subsequently caused spoilage could be reduced only by three log cycles, from ca. log 6,0 to ca. 3,0 colony forming units per milliliter ($CFUml^{-1}$), when heated in tomato juice broth at 62,7°C for 40 minutes. Borch et al. (1988) described a heat resistant Lactobacillus viridescens that survived heat processing and caused spoilage of emulsion-type sausages. When this bacterium was heated in

ATP Broth at 60°C, numbers decreased by log 2,3 $CFUml^{-1}$ from an initial concentration of ca. log 6,7 $CFUml^{-1}$, during 40 minutes of heating (Borch et al., 1988). Although the above authors did not characterize heat resistances of these bacteria in terms of D-values, Milbourne (1983) and Houben (1982) showed that LAB surviving heat processing of processed meat products exhibited high D-values (ch.1, Table 1.1). In comparison, heat resistances of spoilage LAB from products such as non-pasteurized citrus juice (Parish, 1991) and inadequately pasteurized beer (Adams et al., 1989) were noticeably lower (ch.1, Table 1.1).

Although local vienna sausage emulsion was contaminated with LAB at ca. log 5,0 $CFUg^{-1}$, the smoke-cooking process was effective in reducing numbers of these bacteria to undetectable levels in plate count experiments. Lactic acid bacteria, however, recontaminated VPVS during subsequent handling and packaging (Dykes et al., 1991) and were, therefore, not expected to be as heat resistant as the LAB described by Niven et al. (1954), Milbourne (1983) and Borch et al. (1988). This study aimed to determine the in vitro heat resistance of LAB which predominated in spoilage populations of VPVS (von Holy et al., 1991b). D-values obtained for these bacteria would then be used to determine heat process (F_T) values for in-package pasteurization of VPVS.

2.2.2 MATERIALS AND METHODS

2.2.2.1 Culture selection and maintenance

Lactobacillus (Lb.) sake (1A73c), Lb. curvatus (1A8c), Leuconostoc (Lc.) mesenteroides (1A15a) and a Pediococcus strain (16P25Tb) were isolated from spoiled VPVS (von Holy et al., 1991b). Lb. sake, Lb. curvatus and Lc. mesenteroides were calculated mean organisms of a numerical analysis of phenotypic characteristics of 61 predominant spoilage LAB from VPVS (Dykes, 1991). These three strains were chosen for heat resistance studies since they were regarded as representative of previously described, predominant spoilage populations of VPVS (von Holy et al., 1991b). The Pediococcus strain was chosen since proportions of this genus were previously found to increase in pasteurized VPVS. Consequently it was proposed that these bacteria might be more heat resistant than homofermentative lactobacilli and leuconostocs (von Holy and Holzapfel, 1991). Freeze dried stock cultures were grown in MRS Broth (BIOLAB) at 25°C for 48 hours and streaked onto MRS Agar (BIOLAB) to check for purity. Working cultures were grown in litmus milk (100g litmus milk (BIOLAB); 50g calcium carbonate; 10g glucose; 2,5g liver digest; 2g yeast extract; water to one liter) at 25°C for 48 hours, stored at 4°C and subcultured in the same medium every three months.

2.2.2.2 Phase contrast microscopy

To illustrate morphologies of LAB used in this study (Fig. 2.1.1), cultures were prepared for phase contrast microscopy and photographed. Cultures from litmus milk were streaked onto MRS Agar and incubated at 25°C for 48 hours. Bacterial cells were transferred onto microscope slides, immobilized in 0,2% agar and examined using a LEITZ DIALUX 20EE phase contrast microscope at 1000x magnification using oil immersion. Bacteria were photographed using ILFORD PANF film.

2.2.2.3 Heat resistance determinations

For heat resistance studies, bacteria were grown in MRS Broth at 25°C to the early logarithmic growth stage, the inoculum density was adjusted to ca. log 7,00 $CFUml^{-1}$ and cells were suspended for heat inactivation in quarter-strength Ringers solution (QSRS) (pH 6,2) as described in ch. 2.1. Cells were heat inactivated in glass capillary tubes in a thermostat-controlled water bath (ch. 2.1) at 57, 60 and 63°C over six time intervals. Heating time intervals varied in length, depending on heating temperature (Table 2.2.1). The temperature of 57°C was chosen since VPVS spoilage LAB are psychrotrophic (Buchanan, 1986; Franz et al., 1991) and it was suggested that 55°C would suffice to heat inactivate true psychrotrophs (Silliker et al., 1980). Higher temperatures of 60 and 63°C were chosen for more rapid heat inactivation and 63°C was, furthermore, considered low enough not to destroy product quality or packaging material during in-package pasteurization of VPVS (M.A. Jenkin, pers. comm.)*.

After heating, LAB were recovered from capillary tubes and enumerated on MRS agar as previously described (ch. 2.1). Counts from triplicate plates were meaned and converted to logarithms (ch. 2.1). To increase statistical significance of heat resistance data, each heat resistance determination at a specific temperature (57, 60 or 63°C) was carried out three times and on separate occasions.

2.2.2.4 Replication and computational methods

Triplicate sets of log survivor counts for each heating temperature were plotted against heating time. A single best fit regression line was calculated for data sets at each temperature by the least square method (Bradshaw _et al_., 1985, Donnelly _et al_., 1986, Bradshaw _et al_., 1987). D-values were determined as the absolute value of the reciprocal of the slope of the regression line (Bradshaw _et al_., 1985, 1987). Z-values were determined as the absolute value of the reciprocal of the slope of the regression line obtained, when logarithms of D-values were regressed against the three heating temperatures (Bradshaw _et al_., 1985, 1987). All linear regressions were computed using the STATGRAPHICS (version 5,0 - STSC Inc. and Statistical Graphics Corporation) statistical software program.

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2.2.3 RESULTS AND DISCUSSION

2.2.3.1 Effect of non-logarithmic survivor curves on heat resistance determinations

Lactic acid bacteria survivor curves obtained by linear regression of log counts ml^{-1} vs. heating time with 95 and 99% confidence intervals are shown in Figs. 2.2.2 to 2.2.5. Regression analysis showed that slopes of all regression lines were highly significant ($p \leq 0,00005$), validating the linear regression model. Correlation coefficients for linear regressions of log survivor counts against heating times are shown in Table 2.2.2. Correlation coefficients ranged from -0,807 to -0,974, the majority being > -0.919 (Table 2.2.2). This indicated a satisfactory linear relationship between log survivor counts and heating times. On closer inspection of data points, however, survivor appeared concave, rather than linear (Figs. 2.2.2 to 2.2.5). When log survivor counts for the different heating times were subjected to analysis of variance and residuals were plotted against predicted values (data not shown), trends in residuals exhibited fan or parabolic patterns. This indicated that the linear regression model did not describe the heat resistance data accurately, since residuals are estimates of the error terms of linear regression models and should be randomly distributed (Neter _et al_., 1988).

When moist heat is used to kill microorganisms at a constant temperature, death follows a logarithmic order. If logarithms of numbers of survivors are thus plotted against time of exposure to a constant, lethal temperature, a straight-line survivor curve should be obtained (Stumbo, 1965; Allwood and Russell, 1970; Cerf, 1977; Silliker _et al_., 1980; Jay, 1986; Pflug, 1987; Boyd, 1988; Müller, 1989). Non-linear survivor curves of convex or concave shapes, however, were reported for various bacteria or bacterial spores when heated at constant temperature (Allwood and Russell, 1970; Dabbah _et al_., 1971a,b; Moats, 1971; Moats _et al_., 1971a; Dega _et al_., 1972; Sanz Perez _et al_., 1982; Ababouch and Busta, 1987; Ababouch _et al_., 1987; Palumbo _et al_., 1987; Humphrey, 1990; Bhaduri _et al_., 1991). Concave survivor curves such as the ones observed in this study reportedly result from clumping of bacterial cells during heat treatment (Stumbo, 1965; Cerf, 1977, Witter, 1983) or as a result of heterogeneity of heat treatment (Beckers et al., 1987; Donnelly et al., 1987; Donnelly, 1990). Moats et al. (1971a), however, argued that clumping of cells during heating could not account for concave survivor curves, since such clumps would be disrupted by dilution techniques employed for enumeration of survivors. Although bacteria in clumps might be protected from heat, which would lead to artificial increases in bacterial heat resistance, disruption of cells by appropriate dilution techniques would nevertheless result in linear survivor curves. Concave survivor curves for bacteria have also been explained on the basis of heterogeneity in heat treatment, where the use of open test tubes during heat inactivation allowed cells to coat test tube walls above the water bath level and escape the full effect of heat treatment (Beckers et al., 1987; Donnelly, et al., 1987, Donnelly, 1990). Since capillary tubes in our study were fully submersed in water during heat resistance determinations, heterogeneity of heat treatment could not explain the occurrence of concave LAB survivor curves in this study.

Heterogeneity of a population of microorganisms with respect to heat resistance was proposed to be the only satisfactory explanation for the occurrence of nonlinear, concave survivor curves (Moats, 1971, Palumbo et al., 1987). Concave survivor curves were previously reported for LAB heated at constant temperature (Sanz Perez et al., 1982) and were also attributed to heterogeneity with respect to heat resistance. Heterogeneity of LAB with respect to heat resistance could, therefore, explain concave survivor curves obtained for meat spoilage LAB also in this study.

When bacterial death follows a logarithmic order, D-values may be determined from plots of log numbers of survivors against heating times at constant temperature. Vas and Proszt (1957) and Moats et al. (1971a) cautioned against determinations of D-values from survivor curves of microorganisms heated at constant temperature, if these were not truly logarithmic. For convex survivor curves, therefore, Alderton and Snell (1970) and King et al. (1979) introduced a mathematical formula to linearize such survivor curves. This formula permitted the authors to calculate parameters analogous to those for the logarithmic death of microorganisms at constant temperature i.e. D- and z-values (Alderton and Snell, 1970; King et al., 1979). For concave survivor curves, however, no satisfactory mathematical procedure has yet been reported for accurate calculation of D-values or analogous parameters.

Published survivor curves which have been interpreted as logarithmic are, however, very seldom precisely straight-line and sometimes deviate considerably from linearity when individual data points are connected (Moats et al., 1971a). Furthermore, since Rahn (1945) and Schmidt (1957) suggested that the assumption of a logarithmic order of bacterial death at constant temperature was the most convenient for comparisons of survival of bacteria at different temperatures, the logarithmic model of bacterial death was considered appropriate to this study for determinations and comparisons of heat resistances of four LAB isolated from VPVS. The logarithmic model of bacterial death was applied by linearization of concave survivor curves using linear regression.

2.2.3.2 D-value determinations

Heat resistance characteristics at 57, 60 and 63°C of four LAB isolates from spoiled, VPVS are shown as D-values in Table 2.2.2. Lb. sake was the most heat resistant at all heating temperatures, followed by the Pediococcus strain, Lc. mesenteroides and Lb. curvatus isolates in decreasing order (Table 2.2.2). D_{57}, D_{60} and D_{63} values ranged from 22,5 to 52,9, 15,6 to 39,3 and 14,4 to 32,5 seconds, respectively (Table 2.2.2).

Heat inactivation of all LAB under the conditions of this study was deemed rapid, since at least one log cycle reductions could be achieved in a maximum of 71,3 seconds (upper limit of 95% confidence interval of D_{57} for Lb. sake, Table 2.2.2). These D-values were considerably lower than those of 1410 seconds at 65°C and 7374 seconds at 63°C reported by Milbourne (1983) and Houben (1982),respectively, for thermotolerant meat spoilage LAB (ch. 1, Table 1.1). Since smoke-cooking of VPVS reduced LAB contamination to below detectable levels (Dykes et al., 1991) and spoilage resulted from growth of LAB recontaminating sausages surfaces after heat processing these recontaminating LAB were expected, and indeed shown, not to be excessively thermotolerant. Only one report (Borch et al., 1988) on heat resistance of a LAB strain that did not survive heat processing of emulsion-type sausages, but recontaminated sausages after heat treatment, was found. Borch et al. (1988) showed that one log cycle reductions in numbers of this homofermentative Lactobacillus sp. occurred ca. every 90 seconds when heated at 63°C in ATP Broth. The thermal resistance of this strain therefore compared favorably to heat resistances of LAB documented in this study (Table 2.2.2).

Furthermore, D-values of a heterofermentative Lactobacillus sp. from inadequately pasteurized beer were 156 and 48 seconds, in low ethanol (<0,05%) beer at 60 and 65°C, respectively (Adams et al., 1989, ch.1, Table 1.1). The D_{60} value of this heterofermentative Lactobacillus spp., therefore, was ca. four times higher than corresponding D_{60} values of meat spoilage LAB in this study (Tables 2.2.2 & Table 1.1, ch.1). D-values of Lb. plantarum, Lb. pseudoplantarum and Lc. mesenteroides isolated from citrus products were 114, 36 and 90 seconds, respectively, at 53°C in grapefruit serum (pH 4,0) adjusted to 17° Brix with glucose (Parish, 1991). Heat resistance values of these citrus juice spoilage LAB (Parish, 1991) were lower than those determined for LAB in this study (Table 2.2.2), which could be explained by the lower heating temperature of 53°C. Even though the heat resistances of spoilage LAB from VPVS differed from those of a homofermentative Lactobacillus strain recontaminating processed meats after heat processing (Borch et al., 1988), and spoilage LAB from citrus juice and beer, they compared more favorably to the latter than to those of thermotolerant LAB surviving thermal processing of processed meats (Houben, 1982; Milbourne, 1983) and milk (Sanz Perez et al., 1982).

Differences in D-values of spoilage LAB from various products could be attributed to inherent differences in heat resistances of the various species or strains (Stumbo, 1965; Silliker et al., 1980; Jay, 1986). Furthermore, it is well-known that the physiochemical nature of the medium in which microorganisms are heated profoundly influences their heat resistance, with compounds such as fats, salts, carbohydrates and proteins protecting bacteria from heat inactivation (Stumbo, 1965; Moats et al., 1971b; Silliker et al., 1980; Jay, 1986). The comparatively low D-values determined for LAB in this study might therefore be attributable to heating cells in QSRS, a medium which does not contain carbohydrates, fats, proteins or other compounds that could exert heat protective effects. Media such as ATP Broth, citrus juices or beer previously used for heat resistance determinations of spoilage LAB, are likely to have contained such protective compounds (Borch et al., 1988; Adams et al., 1989; Parish, 1991). The higher D-values reported by the above authors could, therefore, be explained by the use of media which exerted heat protective effects.

Heat resistance characteristics of spoilage LAB from VPVS were also compared to those of Listeria (L.) monocytogenes, since these bacterial groups are closely related (Jones, 1988; van Laack et al., 1992). Heat resistance characteristics of L. monocytogenes were assessed extensively since major outbreaks of foodborne disease involving milk and milk products were linked to this pathogen. Concern was expressed that this bacterium could survive normal milk pasteurization (Bradshaw et al., 1985; Fleming et al., 1985; James et al., 1985; Donnelly and Briggs, 1986; Beckers et al., 1987; Bradshaw et al. 1987). Beckers et al. (1987), Donnelly et al. (1987) and Bradshaw et al. (1985; 1987) showed the inability of L. monocytogenes to survive milk pasteurization treatments (15 seconds at 71,7°C) and D-values ranged from ca. 18 seconds to 36 seconds at a heating temperature of 63,3°C, depending on the fat content of the milk used to heat cells (Bradshaw et al., 1985, 1987 - ch.1, Table 1.2). Donnelly and Briggs (1986) showed D-values of three L. monocytogenes strains to be less than or equal to 60 seconds, when heated in raw milk at 62,7°C (ch.1, Table 1.2).

D-values for *L. monocytogenes* heated in milk compared well to those of meat spoilage LAB heated at 63°C in QSRS (Table 2.2.2). $D_{57,8}$ values for *L. monocytogenes* in milk with different fat contents, however, ranged from *ca*. 246 to 288 seconds (Bradshaw *et al*., 1985, 1987, ch.1, Table 1.2), whereas D-values for LAB strains heated in QSRS at 57°C were considerably lower (Table 2.2.2). This indicated that *L. monocytogenes* might be more heat resistant than LAB at lower heating temperatures. This could be explained by the presence of compounds such as fats and proteins in milk which protected bacteria from heat inactivation, especially at low heating temperatures. These compounds are not present in QSRS and the LAB of this study were therefore comparatively more heat sensitive than *L. monocytogenes* heated in milk at 57,3°C.

Heat resistance of *L. monocytogenes*, moreover, increased when cells were heated in various processed meat products, as opposed to milk. Bhaduri *et al*. (1991) determined D_{57} and D_{60} values of 394 and 94 seconds, respectively, after heating the bacterium in a liver sausage slurry. Quintavalla and Campanini (1991) reported D_{60} and D_{63} values of 438 and 180 seconds, after heating *L. monocytogenes* in meat emulsion. Mackey and Bratchell (1989) reported D_{60} values of 498 and 318 seconds for *L. monocytogenes* heated in thick slurry of beef and chicken, respectively. The increased heat resistance of this bacterium in processed meats was argued to result from protective influences of compounds such as fats, salt and curing salt present in processed meats (Farber, 1989; Mackey and Bratchell, 1989; Mackey *et al*., 1990; Fain *et al*., 1991; Schoeni *et al*., 1991; Quintavalla and Campanini, 1991; Yen *et al*., 1991). Quintavalla and Campanini (1991), for example, reported D-values of *L. monocytogenes* heated in meat emulsion to be two to three times higher than those obtained when bacteria were heated in tryptic soy Broth. This increase in heat resistance was attributed to the presence of fats and curing salts in the meat emulsion. Curing salts in particular enhanced heat resistance of *L. monocytogenes* in cured meat based media (Farber, 1989; Yen *et al*., 1991) and Farber (1989) reported fivefold to eightfold increases in heat resistance when the bacterium was heated in ground meat to which cure (nitrite, dextrose, lactose, corn syrup and ca. 3% NaCl) was added.

Since vienna sausages also contain fats, salts and curing salts, the heat resistance of LAB in vienna sausage packs (in situ) is therefore likely to be greater than that determined in QSRS (in vitro). Since Quintavalla and Campanini (1991) determined the heat resistance of L. monocytogenes to be two to three times higher when heated in meat based media as opposed to broth, heat resistance of LAB heated in situ in vienna sausage packs could be estimated to at least double. Thermal processes for VPVS calculated on the basis of in vitro heat resistances of LAB, may, therefore, be expected to underestimate the required heating time by half.

2.2.3.3 Z-value determinations

Z-values for spoilage LAB from VPVS heated at 57, 60 and 63°C in QSRS (Table 2.2.2) ranged from a minimum of 25,2°C for Lc. mesenteroides to a maximum of 30,9°C for Lactobacillus curvatus. Although Lb. curvatus had the highest z-value, it was the most heat sensitive of the four LAB studied (Table 2.2.2). Z-values of the other three LAB, however, decreased with decreasing order of heat resistance (Table 2.2.2).

Z-values determined for the four meat spoilage LAB isolates in this study did not compare with those reported for other spoilage LAB from meat and beer products. The z-value of the Lb. viridescens strain reported by Milbourne (1983) at 38,5°C was ca. 8°C higher than the highest z-value of LAB (30,9°C for Lb. curvatus) in this study (Table 2.2.2). This, however, was expected since this Lb. viridescens isolate was also considerably more heat resistant than the LAB studied here (Table 2.2.2). The z-value is defined as the number of degrees Celsius required to bring about a tenfold change in D-value (Stumbo, 1965; King et al., 1979; Silliker et al., 1980; Jay, 1986). The Lb. viridescens isolate, being more heat resistant than LAB studied here, should thus also have a higher z-value. This is since a higher temperature would be required to bring about a tenfold change in bacterial death rates. The z-values for Enterococcus faecium and a heterofermentative Lactobacillus sp. determined by Houben (1982) and Adams et al. (1989) to be 11,8 and 2,2°C, respectively, were at least 13,4°C lower than those of the LAB of this study (Table 2.2.2). This, however, was unexpected since Enterococcus faecium and the heterofermentative Lactobacillus were noticeably more heat resistant than LAB in this study, respectively (ch.1, Table 1.1 & Table 2.2.2), and should therefore have higher z-values. Furthermore, heat resistances for LAB in this study compared favorably to those of L. monocytogenes in various milk media (Bradshaw et al., 1985; Donnelly and Briggs, 1986; Bradshaw et al., 1987), but corresponding z-values of L. monocytogenes did not compare well and were four to six times higher for meat spoilage LAB in this study (ch.1, Table 1.2 & Table 2.2.2).

Z-values for non-sporulating bacteria generally fall between 4 and 6°C (Hansen and Riemann, 1963; Tomlins and Ordal, 1976). Z-values of LAB determined in this study, however, compared neither to these values or to those reported for other LAB and L. monocytogenes. The high z-values for LAB in this study, therefore, were of questionable validity and could only be explained by the fact that D-values did not change by a factor of ten over the temperature range studied. D-values of all four LAB decreased by less than 50%, when the heating temperature was increased from 57 to 63°C (Table 2.2.2). Since the z-value measures tenfold changes in D-values and since such tenfold changes did not occur, unrealistically high z-values were obtained from regression of log D-values against heating times.

The fact that bacteria with higher D-values should also have higher z-values was true for LAB in this study, with the exception of the Lb. curvatus strain. Although this bacterium was the most heat sensitive of the four LAB strains, it had the highest z-value (Table 2.2.2). Again, this reinforced our earlier observation that z-values were extraordinarily high and may thus be of questionable validity. Moreover, z-values were obtained from regressions of three D-values against corresponding heating temperatures. Regressions through only three data points, therefore, might not have allowed for optimum accuracy. Consequently, heat resistance (D-value) determinations for these bacteria should be performed at more heating temperatures and over a greater temperature range, in order to obtain z-values which more accurately describe the effect of temperature on the relative heat resistances of meat spoilage LAB.

In this study, however, the temperature range used for in vitro heat resistance studies corresponded to temperatures that could be used in practise for in-package pasteurization of vienna sausages. The temperature range for heat resistance determinations was thus limited, since higher pasteurization temperatures were considered to detrimentally affect product characteristics, while lower pasteurization temperatures would not suffice to ensure rapid destruction of contaminating spoilage LAB in sausage packs. The unrealistically high z-values implied that extrapolation of heat resistance of meat spoilage LAB to temperatures higher than 63°C was impossible. Thus, even though product pasteurization at temperatures of ca. 70°C might be possible, inaccurate z-values would preclude extrapolation of bacterial heat resistance to these temperatures.

2.2.3.4 F_T-value determinations

Heat process (F_T) value determinations for thermal processing of foods have been based on the assumption that bacterial death follows a logarithmic order and straight-line survivor curves are obtained (Stumbo, 1965; Pflug, 1987; Müller, 1989). Pflug (1987), however, acknowledged that microbial destruction curves of almost all shapes were possible and that only 40% of reported data on bacterial heat resistance formed approximate straight-line survivor curves. However, the straight-line, semilogarithmic model of thermal destruction was the simplest to be used in engineering designs for thermal processing of foods in industry (Pflug, 1987). Therefore, thermal process calculations aimed at inactivating spoilage LAB in VPVS should be based on D-values for LAB determined in this study. Since survivor curves of bacteria from which D-values were derived were not strictly linear, true D-values were underestimated and consequently thermal process calculations should be expected to underestimate microbial destruction.

Control measures to inactivate spoilage LAB in VPVS should be aimed specifically at homofermentative lactobacilli and leuconostocs (von Holy _et_ _al_., 1992). Since high temperature was used as control parameter (hurdle) in in-package pasteurization, thermal process (F_T) value calculations should be based on the highest _in_ _vitro_ D-value determined in this study. Of the four isolates from spoiled VPVS, _Lb_. _sake_ showed the highest heat resistance (Table 2.2.2). The upper 95% confidence limit for the D_{63} value of this bacterium should thus be used in F_T-value calculations, since this was the maximum time needed for a heat process to reduce populations of this strain by one log cycle.

Two factors, however, were expected to influence F_T-value calculations. Firstly, since D-values were derived from non-logarithmic, concave survivor curves, they underestimated LAB heat resistance. F_T-values calculated on the basis of these D-values, therefore, would be lower than if calculated using D-values from logarithmic survivor curves. Secondly, D-values of LAB could be expected to increase when bacteria are heated in meat based media as opposed to QSRS. F_T-values, therefore, would underestimate LAB heat resistance in vienna sausage packs. The F_T-values used for pasteurization of VPVS will thus probably not suffice to reduce LAB contamination to the desired levels. Nevertheless, F_T-value determinations would be useful in estimating the time/temperature combinations required to heat packs of vienna sausages. The extent by which reductions in contaminating LAB after heat treatment of VPVS would be underestimated, however, would need to be determined by in-package pasteurization (_in_ _situ_) experiments.

2.2.4 CONCLUSIONS

Survivor curves of meat spoilage LAB heated at constant temperature were non-linear and concave. Although clumping of cells during heat treatment was proposed to cause concave survivor curves (Stumbo, 1965; Cerf, 1977; Witter, 1983), we concluded that concave survivor curves in this study more likely resulted from LAB being heterogeneous with respect to heat resistance. Calculation of heat resistance of meat spoilage LAB, however, was possible when such curves were linearized by linear regression.

D-values of LAB heated at 57, 60 and 63°C ranged from 22,5 to 52,9, 15,6 to 39,3 and 14,4 to 32,5 seconds, respectively. These D-values were considerably lower than those of 1410 seconds at 65°C and 7374 seconds at 63°C, respectively reported by Milbourne (1983) and Houben (1982) for thermotolerant meat spoilage LAB. However, they compared favorably to those of 156 seconds at 60°C, 36 to 114 seconds at 53°C and 246 to 288 seconds at 57,8°C reported for LAB heated in beer (Adams _et al_., 1989), citrus juice (Parish, 1991) and _Listeria monocytogenes_ heated in milk (Bradshaw _et al_., 1985; 1987), respectively. D-values for meat spoilage LAB in this study were expected to be lower than those of the thermotolerant bacteria studied by Houben (1982) and Milbourne (1983), since the latter survived heat processing of processed meats, whereas bacteria in this study did not and represented LAB populations that recontaminated sausage surfaces after heat processing (Dykes _et al_., 1991). D-values determined for LAB in this study compared favorably but were often lower than those of _L. monocytogenes_ heated in milk (Bradshaw _et al_., 1985, 1987), or those of LAB heated in beer (Adams _et al_., 1989) or citrus juice (Parish, 1991). This could have resulted from the presence of heat protective compounds such as fats or carbohydrates, which could be present in other heating media but were absent from the quarter-strength Ringers solution used in this study.

Z-values of meat spoilage LAB ranged from 25,2 to 30,9 and were considerably higher than those of 4 to 6°C reported for other non-sporing bacteria (Hansen and Riemann, 1963; Tomlins and Ordal, 1976). Such z-values were unrealistically high and could be explained by the fact that D-values did not change greatly over the 6°C temperature range in which heat resistance was determined. These z-values could thus not be used to extrapolate heat resistance to temperatures outside of those used for heat resistance determinations in this study. Consequently such extrapolations would over-estimate the increase in temperature required to decrease bacterial heat resistance by a factor of ten.

D-values of meat spoilage LAB at 57, 60 and 63°C were all lower than sixty seconds, and led us to conclude that spoilage LAB in VPVS could be eliminated by relatively mild heating. Furthermore, low heat resistance of meat spoilage LAB confirmed earlier findings (Dykes _et al_., 1991) that such LAB would not survive heat processing of vienna sausages (70 minutes to reach internal temperature of 72°C) during product manufacture. Since D-values of meat spoilage LAB were derived from non-logarithmic survivor curves and determined in a heating medium which did not offer heat protection, they were likely to underestimate bacterial heat resistance during in-package pasteurization of VPVS. Vienna sausages would, therefore, be underprocessed and desired reductions in initial numbers of LAB calculated by theoretical heat process (F_T) values would not be achieved. In-package pasteurization trials for VPVS based on _in vitro_ LAB heat resistance data, therefore, might have to be adjusted to achieve the desired reductions in contaminating LAB.

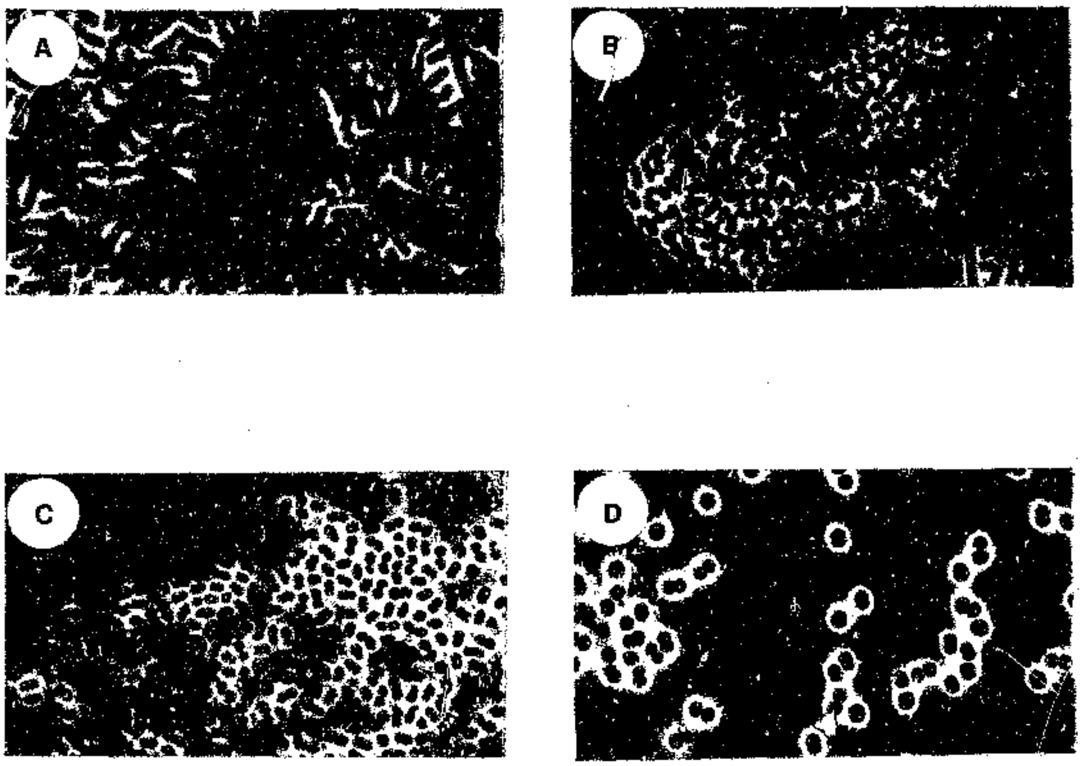
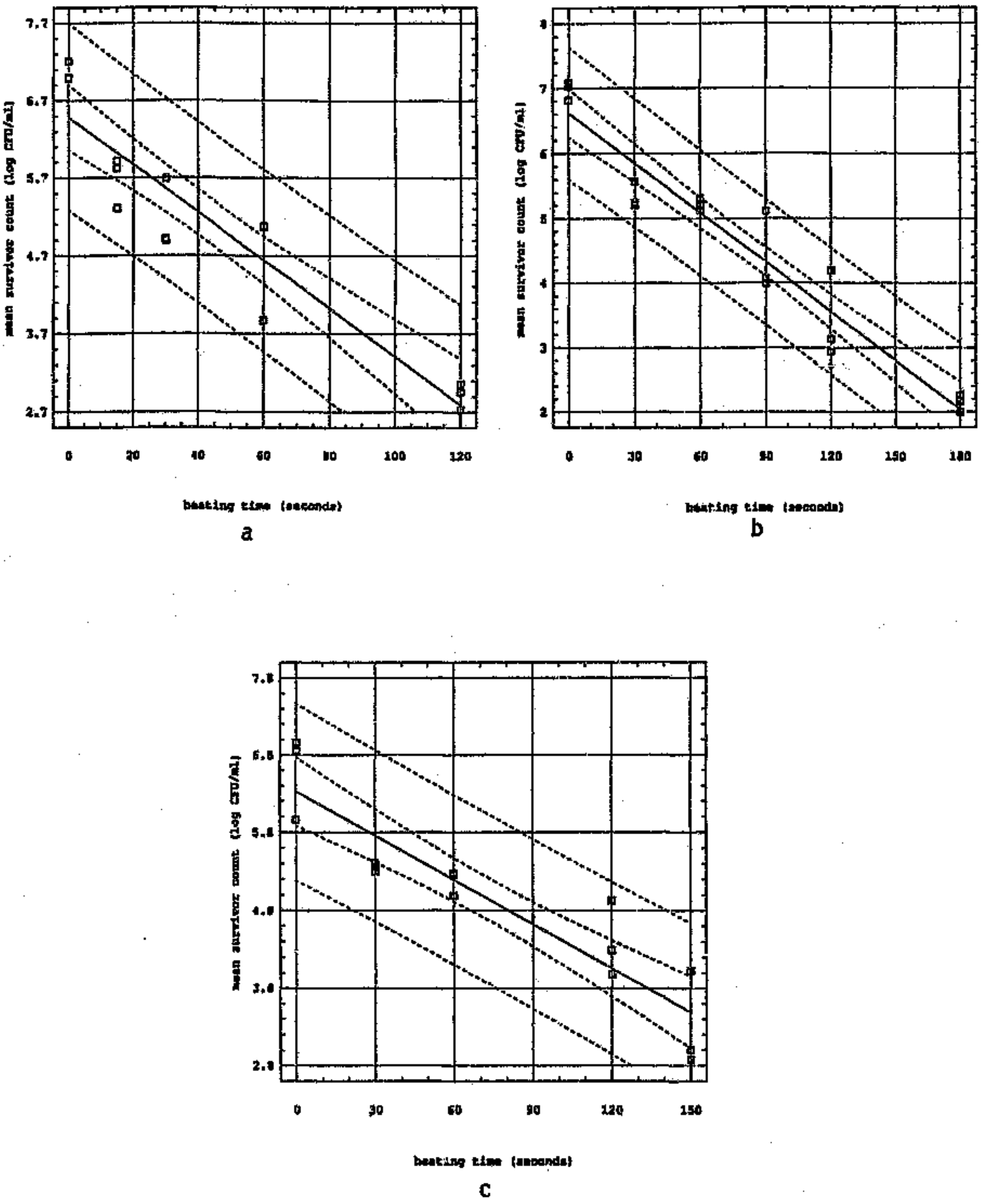


Fig. 2.2.1 Phase contrast micrographs (1000x magnification) of predominant *Lactobacillus sake* (a), *Lactobacillus curvatus* (b), *Leuconostoc mesenteroides* (c) and *Pediococcus* (d) isolates used for heat resistance determinations (├─┤ represents two micrometer).

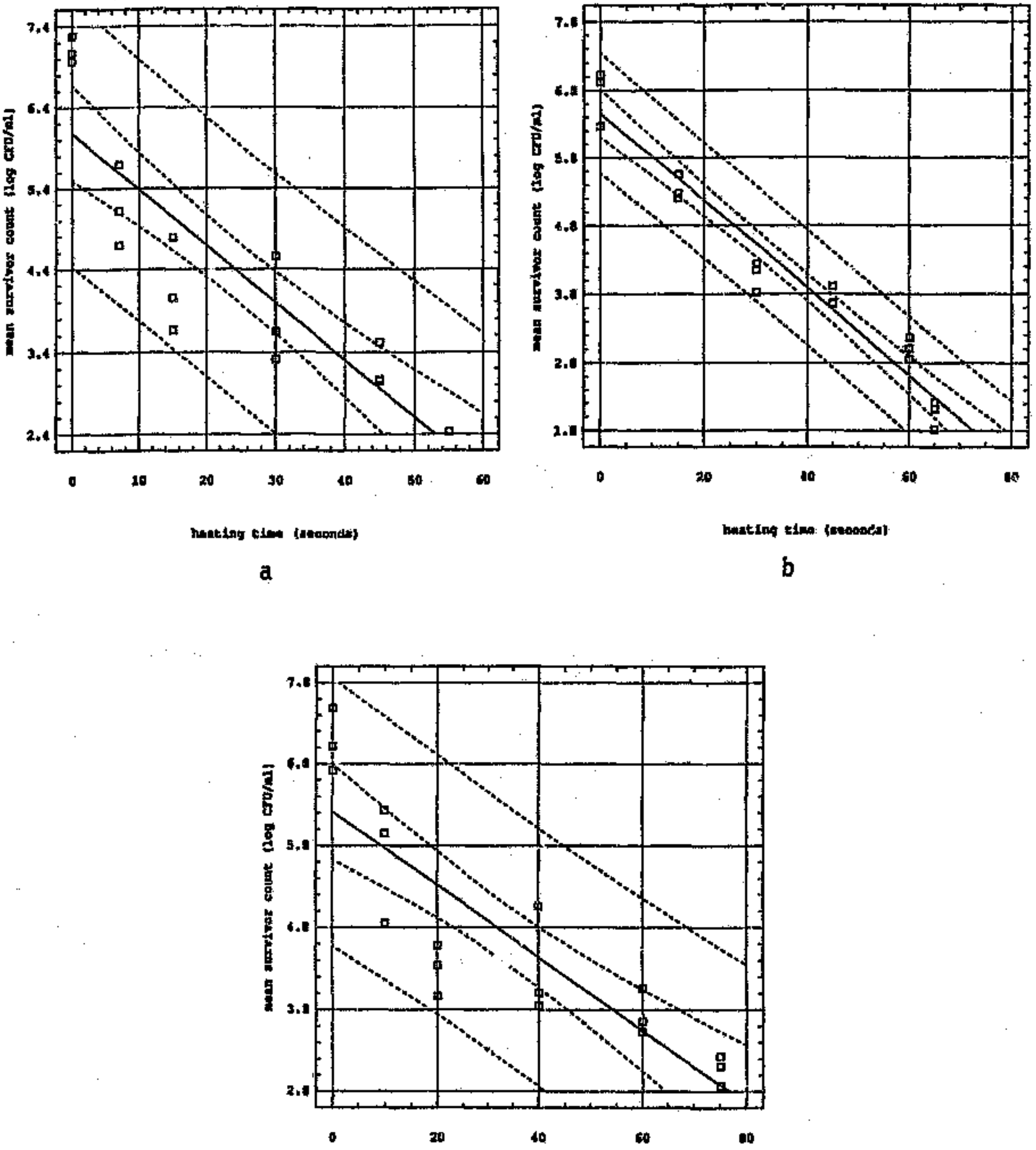


a

b

c

Fig. 2.2.2 Linear regression of triplicate counts of Lactobacillus sake heated at 63°C (a), 60°C (b) and 57°C (c) against heating time. Dotted lines represent 95 & 99% confidence intervals.



a

b

c

Fig. 2.2.3 Linear regression of triplicate counts of <u>Lactobacillus</u> <u>curvatus</u> heated at 63°C (a), 60°C (b) and 57°C (c) against heating time. Dotted lines represent 95 & 99% confidence intervals.

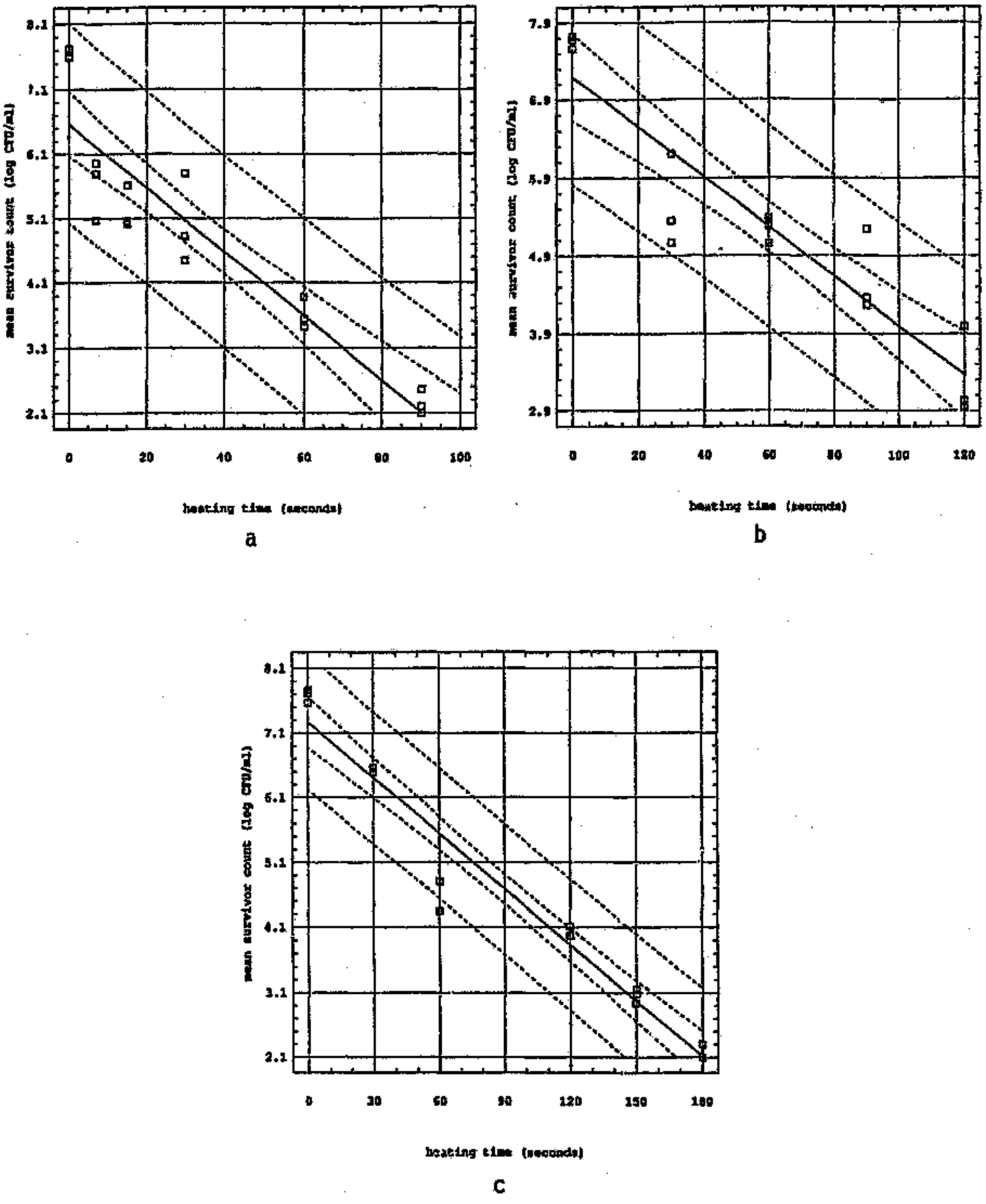


Fig. 2.2.4 Linear regression of triplicate counts of *Leuconostoc mesenteroides* heated at 63°C (a), 60°C (b) and 57°C (c) against heating time. Dotted lines represent 95 & 99% confidence intervals.

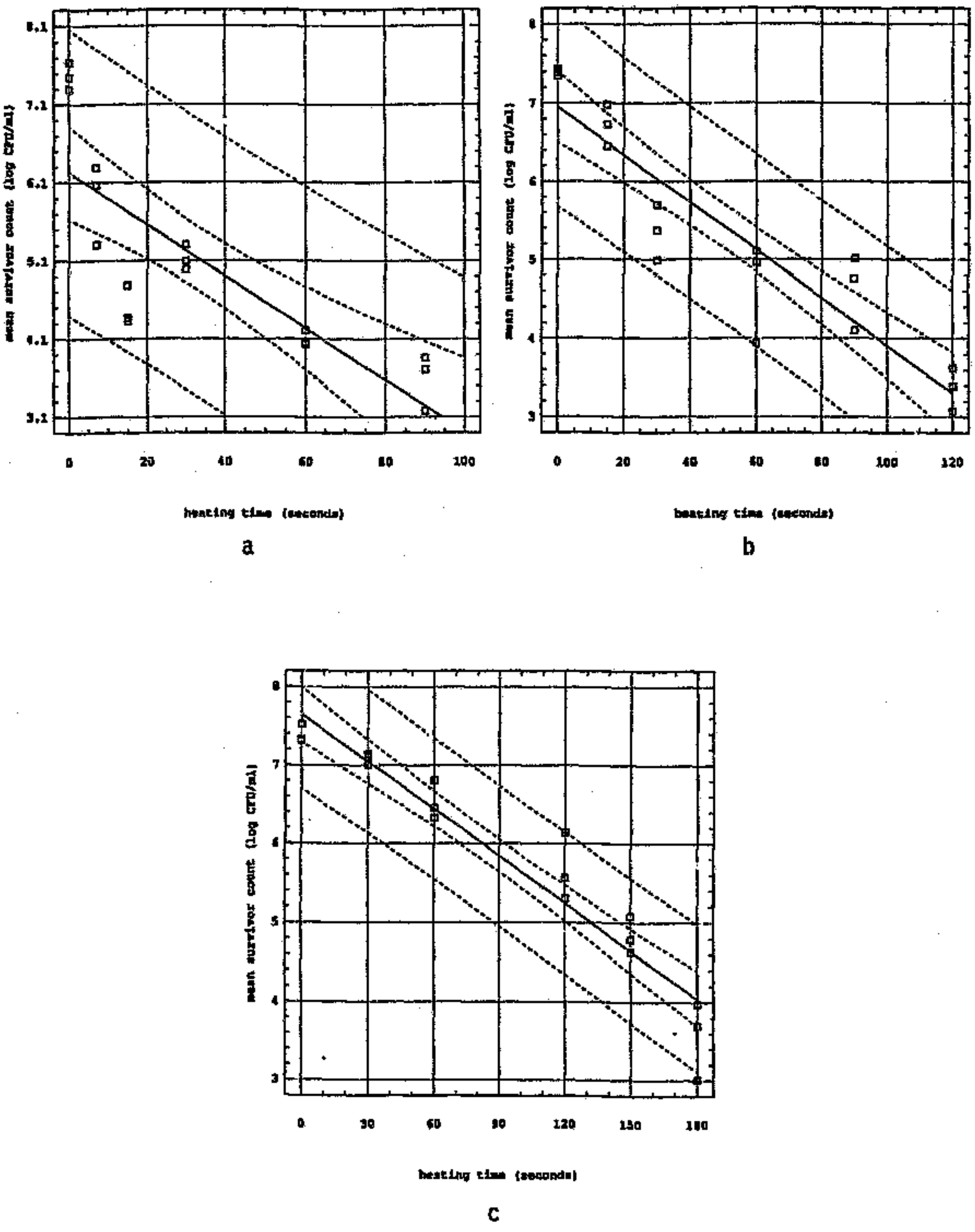


Fig. 2.2.5 Linear regression of triplicate counts of a <u>Pediococcus</u> strain heated at 63°C (a), 60°C (b) and 57°C (c) against heating time. Dotted lines represent 95 & 99% confidence intervals.

Table 2.2.1 Heat treatment time intervals for four lactic acid bacteria isolates from spoiled vacuum-packaged vienna sausages.

Isolate	Temperature (°C)	Exposure time (seconds)
Lactobacillus sake	57	0; 30; 60; 120; 150; 180
	60	0; 30; 60; 90; 120; 180
	63	0; 15; 30; 60; 120; 150
Lactobacillus curvatus	57	0; 10; 20; 40; 60; 75
	60	0; 15; 30; 45; 60; 65
	63	0; 7; 15; 30; 45; 55
Leuconostoc mesenteroides	57	0; 30; 60; 120; 150; 180
	60	0; 30; 60; 90; 120; 140
	63	0; 7; 15; 30; 60; 90
Pediococcus sp.	57	0; 30; 60; 120; 150; 180
	60	0; 15; 30; 60; 90; 120
	63	0; 7; 15; 30; 60; 90

Table 2.2.2 D[a]- and z-values for four meat spoilage lactic acid bacteria heated at 63, 60 and 57°C.

Heating temperature (°C)	*Lactobacillus sake*	*Pediococcus* sp.	*Leuconostoc mesenteroides*	*Lactobacillus curvatus*
63	32,5 (26,5-41,8) r = -0,937[b]	30,2 (21,8-49,3) r = -0,807	20,2 (16,6-25,8) r = -0,925	14,4 (11,4-19,6) r = -0,895
60	39,3 (34,2-46,1) r = -0,963	32,9 (26,9-42,1) r = -0,924	31,3 (25,3-41,2) r = -0,929	15,6 (13,8-17,9) r = -0,972
57	52,9 (42,0-71,3) r = -0,918	49,9 (43,1-59,2) r = -0,958	34,9 (31,1-39,9) r = -0,974	22,5 (17,2-32,2) r = -0,869
z-value (°C)	28,3	27,5	25,2	30,9

[a] D-values in seconds, 95% confidence intervals shown in brackets.

[b] Correlation coefficients (r) for linear regression lines from which D-values were derived are shown.

CHAPTER THREE

EFFECT OF IN-PACKAGE PASTEURIZATION ON THE MICROBIOLOGICAL SPOILAGE ECOLOGY OF VACUUM-PACKAGED VIENNA SAUSAGES

3.1 EFFECT OF IN-PACKAGE PASTEURIZATION ON SHELF LIFE AND SAFETY OF VACUUM-PACKAGED VIENNA SAUSAGES

ABSTRACT

The effect of three in-package pasteurization treatments of increasing severity on the microbial ecology of vacuum-packaged vienna sausages was assessed under constant temperature storage at 8°C. Treatment parameters were calculated from D-values of predominant lactic acid bacteria from spoiled vacuum-packaged vienna sausages. Microbiological shelf life values ($5x10^6$ $CFUg^{-1}$) were derived from total aerobic plate counts and lactic acid bacteria counts and compared to those of untreated control samples. Enterobacteriaceae and yeasts were also quantified and the incidence of <u>Listeria</u> and <u>Clostridium</u> was determined. Pasteurization lowered the initial contamination levels and significantly decreased growth of lactic acid bacteria. Depending on treatment, the microbiological shelf life of pasteurized samples increased to 10, 14 and 17 fold that of control samples. Enterobacteriaceae and yeasts failed to dominate the spoilage ecology of control samples and were almost completely eliminated from pasteurized samples. The incidence of <u>Listeria</u> and <u>Clostridium</u> was low in control samples. The absence of <u>Listeria</u> in pasteurized samples indicated inability to survive the heating treatments. Increasing incidences of clostridia and the presence of pathogenic <u>Clostridium</u> <u>perfringens</u> type A, however, were documented in pasteurized samples. Although the heat treatment effectively delayed spoilage by lactic acid bacteria and hence improved shelf life, results suggested that in-package pasteurization might compromise product safety.

3.1.1 INTRODUCTION

Processed meats have been salted, or salted and cured and may either be raw or cooked (Tompkin, 1986). Cooked processed meat products receive heat treatments to core temperatures of at least 60°C and frequently to above 70°C, which normally eliminate vegetative microorganisms, so that only bacterial spores of Bacillus and Clostridium (C.) spp. survive (Houben, 1982; Nielsen and Zeuthen, 1984a,b; Carr and Marchello, 1986; Delaquis et al., 1986; Kokubo et al., 1986). After heat processing, however, such products are usually recontaminated during handling, vacuum-packaging and possible slicing from environmental sources (Paradis and Stiles, 1978; Steele and Stiles, 1981; Nielsen and Zeuthen, 1984a,b; Borch et al., 1988; Dykes et al., 1991). Since cooked processed meats are typically low-acid (pH ca. 6,0), have a high water activity (0,97-0,99) and contain a wide variety of freely available nutrients, they provide a favorable environment for the growth and multiplication of a variety of microorganisms (Buchanan, 1986).

Numerous studies implicated the lactic acid bacteria (LAB) as the major spoilage populations of vacuum-packaged processed meats (Allen and Foster, 1960; Sharpe, 1962; Kempton and Bobier, 1970; Mol et al., 1971; Korkeala et al., 1987; Zurera-Cosano et al., 1988; von Holy et al., 1991a,b). The dominance of these bacteria in the spoilage association of processed meat products was attributed to their tolerance of microaerophilic conditions within vacuum packs, as well as their ability to grow at refrigeration temperatures (Kempton and Bobier, 1970; Enfors and Molin, 1980; Egan, 1983; Buchanan, 1986; Holzapfel and Gerber, 1986). In addition, the production of lactic acid during growth of LAB results in a decrease of pH to levels sufficient to inhibit growth of other potential spoilage and pathogenic microorganisms such as Brochothrix thermosphacta, Enterobacteriaceae and Clostridium spp. (Ritter, 1950; Riemann et al., 1972; Davidson and Webb, 1973; Christiansen et al., 1975; Shaw et al., 1978; Schillinger and Lücke, 1988; Hauschild, 1989). Furthermore, the production of antimicrobial compounds such as diacetyl and bacteriocins was recognized as an important selection factor for the predominance of LAB in processed meats and other foods (Daly et al., 1972; Jay, 1982; Nielsen and Zeuthen, 1985; Anderson, 1986; Wang et al., 1986; Klaenhammer, 1988; Schillinger and Lücke, 1989; Karunaratue et al., 1990). In addition, LAB may contribute to the safety of processed meats products, since they exhibit antimicrobial activity against foodborne pathogens such as Clostridium botulinum and Listeria (L.) monocytogenes (Spelhaug and Harlander, 1989; Schillinger and Holzapfel, 1990; Berry et al., 1991; Okereke and Montville, 1991; Schillinger et al., 1991; Degnan et al., 1992). Antimicrobial activity of LAB was recognized as an important factor in inhibition of clostridia in processed meats, since these products typically have pH's, water activity and salt concentrations that allow for growth of many clostridial strains (Riemann et al., 1972; Sperber, 1982; Hauschild, 1989; Labbe, 1989; Table 3.1.1).

Studies on vacuum-packaged processed meats under controlled temperature storage have shown that LAB dominated the spoilage populations irrespective of their initial numbers in individual packs (Reuter, 1969). The shelf life of processed meat products is also dependant on the type of product, as well as the storage temperature (Kempton and Bobier, 1970; Laleye et al., 1984; Zurera-Cosano et al., 1988; Korkeala et al., 1989). Korkeala et al. (1989) reported the shelf life of vacuum-packaged cooked ring sausages to be 55, 43, 29 and 17 days at 2, 4, 8 and 12°C storage, respectively, at which time the lactobacilli count reached 10^7 colony forming units per gram ($CFUg^{-1}$). Zurera-Cosano et al. (1988) found similar results for the shelf life of vacuum-packaged Frankfurters at 28 and 42 days during storage at 7 and 2°C, respectively and numbers of LAB reached 10^8 $CFUg^{-1}$. A variety of sliced, vacuum-packaged continental meats exhibited a shelf life of only 8 days at 7°C, after which bacterial numbers had reached 10^8 $CFUg^{-1}$ (Schneider et al., 1983). In this case, lowering the storage temperature to 2°C extended the shelf life to 25 days, but LAB were again the predominant spoilage populations. Shelf life in the above studies was taken as the time needed for products to become visibly spoiled, which occurred at LAB counts of 10^7 to 10^8 $CFUg^{-1}$. In a previous study, the shelf life of vacuum-packaged vienna sausages (VPVS) was 11 days at 7°C storage (von Holy et al., 1991a), based on maximum microbial numbers of $5x10^6$ $CFUg^{-1}$ and termed the microbiological shelf life. At this stage the product showed no visible spoilage symptoms (von Holy et al., 1991a) but the counts were taken to indicate a realistic criterion of imminent shelf life expiry (von Holy et al., 1991a).

The above studies demonstrated that shelf life of a variety of vacuum-packaged processed meats was relatively short, even at low temperature storage. The control of spoilage of such products by low temperatures alone, therefore, does not appear to be effective.

Numerous preservation methods (heating, freezing, chilling, freeze-drying, drying, curing, salting, sugar addition, acidification, fermentation, smoking, oxygen removal, carbon dioxide addition and irradiation) are used to make processed meats shelf-stable and safe (Leistner, 1987). These preservation methods, however, are based on relatively few parameters (or hurdles), i.e. F (high temperature), t (low temperature), a_w (less available water), pH (sufficient acidification), Eh (reduced oxygen potential), preservation (e.g., nitrite, smoke, CO_2) and radiation (e.g., gamma rays). The hurdle concept as described by Leistner (1987), recommends the incorporation of combinations of these parameters into food products at appropriate intensities, so that undesirable microorganisms are unable to overcome them. Spoilage would thus be prevented or delayed. Furthermore it was shown that hurdles have an intensifying effect and enhancements of individual hurdles exerted a synergistic effect on the microbiological stability of the product (Leistner, 1987; Scott, 1989).

Vacuum-packaged vienna sausages account for approx. 23% of products manufactured by South African meat processors (Business and Marketing Intelligence, 1985). These products are prone to spoilage by LAB during refrigerated storage, resulting in costly losses to producers (von Holy and Cloete, 1992). To control the microbiological spoilage of VPVS, only limited opportunities for manipulation of hurdles exist without adversely affecting product characteristics. Processed meats have water activities (a_w) and pH's which allow for the growth of spoilage LAB (Buchanan, 1986). Vacuum-packaged vienna sausages, for example the ones used in this study, have a typical a_w of 0,98 and pH of 6,2. Furthermore, many meat spoilage LAB are psychrotrophic and hence capable of growth at 5°C, microaerophilic as well as tolerant to high concentrations of nitrite (Dodds and Collins-Thompson, 1984; Buchanan, 1986, Franz et al., 1991). The use of high temperature (F) as an additional hurdle to low temperature product storage, however, was considered an attractive option to control the spoilage of VPVS by LAB, since the size of microbial populations surviving a pasteurization process was shown to be inversely related to the heat treatment applied (Bell, 1983). Furthermore, since recontamination of product would be prevented by the packaging material, only microorganisms surviving the heat treatment would be able to cause product spoilage (von Holy et al., 1991a).

In-package pasteurization of vienna sausages in double layers previously achieved noticeable shelf life increases (von Holy et al., 1991a) but was performed on a "trial and error" basis and did not take into account the heat resistance of spoilage LAB. Furthermore, von Holy et al. (1991a) found VPVS to be poorly heat penetrated during pasteurization, and maximum sausage core temperatures of only 57°C were achieved.

This study, therefore, aimed to use in vitro heat resistance data of meat spoilage LAB to calculate heat process (F_T) values (Stumbo, 1965; Pflug, 1987; Müller, 1989) that would better define heat process requirements for in-package pasteurization of VPVS. In-package pasteurization was undertaken to decrease LAB contamination and growth and increase product shelf life. This study was designed to achieve higher core temperatures during pasteurization, by packing sausages in single layers into vacuum bags. The study of von Holy et al. (1991a), furthermore, did not determine the effect of pasteurization on presence and growth of potentially pathogenic bacteria in VPVS. This clearly required further study and the effect of pasteurization on the incidences of potentially pathogenic bacteria such as clostridia and Listeria was quantified.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Determination of heat processing (F_T) values

Three different secondary or in-package heat treatments were applied to VPVS (Table 3.1.1). The first treatment (PAST1) was relatively mild and not based on a heat processing (F_T) value (Stumbo, 1965; Pflug, 1987; Müller, 1989). Instead, a core temperature of 60°C was arbitrarily selected. The second (PAST2) and third (PAST3) heat treatments of VPVS were, however, based on F_T values (Table 3.1.2).

3.1.2.2 Heat processing

Vienna-type emulsion sausages were manufactured according to a standard commercial recipe and method at a local meat processing plant (von Holy et al., 1991a,b). Sausages were vacuum-packaged in single layers (Fig. 3.1.1) into 400 gram packs. These were subsequently taken directly from the production line and split into four groups. Untreated packs served as a control, while the other three groups (PAST1, PAST2 and PAST3) each received a secondary, in-package pasteurization treatment. Pasteurization of each group occurred independently, immediately after one another, in a water cooker held at 67°C under continuous stirring (Fig. 3.1.2). Sausage core temperature and other pasteurization parameters are shown in Table 3.1.2. Water temperature was monitored before and during pasteurization using a Fluke $51^{K/J}$ thermocouple (John Fluke Mfg Co. Inc., Everett, Washington, U.S.A.) (Table 3.1.3). Sausage core temperatures (Table 3.1.3) were measured using two calibrated Jenway 3100 Microprocessor thermocouples. Core temperatures were measured on randomly chosen packs of sausages which were removed from the cooker every two minutes. One thermocouple each was inserted into the center of a sausage located in the middle of the pack (Fig. 3.1.3). After the required heat treatment, samples were removed from the cooker (Fig. 3.1.4) and cooled in ice (Fig. 3.1.5) to core temperatures below 4°C. Times needed to remove all sausage packs from the water cooker and core temperatures of the last pack removed are shown in Table 3.1.3. Samples were transported to the laboratory and stored at 8°C (±0,5°C) in a low temperature incubator (Fig. 3.1.6). A storage temperature of 8°C was used as it was representative of temperatures in retail display cabinets, which can reach 8 to 10°C or more in upper product layers (Mukherji and Qvist, 1981).

3.1.2.3 Microbiological analysis

Duplicate samples of each treatment were analyzed (Table 3.1.4) daily for the first week, then at two-daily intervals for the next 18 days, three-daily intervals for the next 12 days, ca. five-daily intervals for the next 45 days, followed by weekly intervals to the end of the storage period (128 days). In addition, duplicate packs of each treatment were also analyzed for the presence of Listeria and Clostridium after one, two, three, five, seven and eighteen weeks of storage at 8°C.

Packs were opened aseptically and samples taken by cutting across individual sausages and aseptically removing 20g from the middle of each vacuum bag. These were homogenized in 180ml of quarter-strength Ringers solution (OXOID) for two minutes using a Colworth 400 stomacher. Numbers of total aerobic bacteria (TAPC), lactic acid bacteria (LABC), Enterobacteriaceae (EC) and yeasts (YC) were determined by duplicate pour (10^{-1} dilution) and spread plating of a single serial tenfold dilution in quarter-strength Ringers solution (Table 3.1.4). Plates showing between 30 to 300 colony forming units (CFU) (or highest number if below 30) were counted and counts from duplicate plates were meaned and converted to logarithms. Corresponding log numbers from duplicate samples were meaned. Assuming one colony on one of duplicate plates from one sample only, the above procedure calculations would result in a lower detection limit of log 0,35 $CFUg^{-1}$ (1 (colony) ÷ 2 (duplicate plates) x 10 (dilution factor) log ÷ 2 (duplicate sample) = 0,35). To obtain a "worst case" scenario the log number of the sample showing the higher count was also used on its own.

For isolation of Listeria, 25 grams were aseptically removed and homogenized in Listeria Enrichment Broth (OXOID) using a Colworth 400 stomacher. For isolation of

Clostridium, a two-gram sample was weighed out into 10 ml Cooked Meat Broth (BURROUGHS WELLCOME) (Table 3.1.5). Conditions and procedures for enrichment, isolation, purification and identification are shown in Table 3.1.5. Clostridium perfringens isolates were typed according to the methods of Cruikshank et al. (1975).

Samples were assessed for organoleptic spoilage by recording development of milky exudates (Fig. 3.1.7), "blowing" (Fig. 3.1.8) or loosening of packaging material, and sour or "off" odors on opening of packs. Sample pH was determined from the 10^{-1} dilution using a Jenway 3100 microprocessor pH meter.

3.1.2.4 Shelf life determination

The microbiological shelf life (MSL) of VPVS was defined as the time needed for microbiological counts (LABC and TAPC) to reach $5x10^{6}$ $CFUg^{-1}$ (von Holy et al.,1991a).

3.1.2.5 Computational methods

To test for significant differences between the LABC and TAPC of control and pasteurized samples during storage, a paired students t test (Clarke and Cooke, 1983) was performed on the difference of these counts. A two way analysis of variance (ANOVA) with interaction was performed to test for significance of storage day effect and pasteurization treatment effects on microbial counts (LABC and TAPC), as well as for their interaction. All computations were carried out using the STATGRAPHICS (version 5,0 - STSC Inc. and Statistical Graphics Corporation) statistical software program.

3.1.3 RESULTS AND DISCUSSION

3.1.3.1 Effect of pasteurization on initial microbial numbers

Table 3.1.6 shows the mean LABC, TAPC, EC and YC of control and pasteurized samples on sampling day 0. Microbial numbers of control packs were noticeably higher than those of pasteurized packs, which was an expected trend, since control packs received no secondary (in-package) heat treatment. In the case of LABC and TAPC, initial bacterial numbers were reduced from ca. log 3,0 $CFUg^{-1}$ to ca. log 1,0 $CFUg^{-1}$ or less (Table 3.1.6). Pasteurization therefore effected a reduction of at least 2 log $CFUg^{-1}$. Enterobacteriaceae and yeasts were, however, reduced to below detectable levels by the pasteurization process (Table 3.1.6).

Surface contamination of emulsion-type sausages during processing has been reported after smoke-cooking from environmental sources (Korkeala and Lindroth, 1987; Borch et al., 1988; Dykes et al.,1991). Initial bacterial numbers (LABC and TAPC) of control samples (Table 3.1.6) corresponded to previously reported levels of log 2,52 (LABC) and 3,94 (TAPC) $CFUg^{-1}$, for the same type of product immediately after processing (Dykes et al., 1991). Initial numbers of Enterobacteriaceae and yeasts of this study, however, were noticeably lower than those reported at log 3,50 (EC) and log 3,00 (YC) $CFUg^{-1}$ in the previous study (Dykes et al., 1991). Differences in contamination levels by environmental microorganisms can be explained by varying levels of factory and/or personal hygiene at the different manufacturing times. Secondary (in-package) pasteurization, therefore, was effective in reducing numbers of initial bacteria and yeasts to below contamination levels in control packs.

For the PAST2 treatment, the heat processing (F_T) value was calculated to allow 10 $CFUg^{-1}$ LAB to survive the pasteurization treatment (Table 3.1.2) and initial numbers of LAB approximately decreased to this level (Table 3.1.6). For PAST3 treatments, however, the theoretical F_T-value was calculated to allow survival of LAB in only one out of a thousand packs. Table 3.1.6 clearly shows that this reduction was not achieved. This trend was not unexpected, since it has been reported that heat resistance of bacteria may vary considerably in response to environmental influences such as pH, a_w, type of heating medium and presence of salts and other organic or inorganic compounds (Silliker et al., 1980). Furthermore, it has been shown that decimal reduction time (D) values for

Listeria monocytogenes in meat emulsion were two to three times higher than those determined in tryptone soya broth (Quintavalla and Campanini, 1991). Since F_T-values for pasteurization in this study were calculated from in vitro heat resistance data (ch. 2.2), the in situ heat resistance of LAB was anticipated and confirmed to be greater (Tables 3.1.2 & 3.1.6) than in vitro heat resistance of the same bacteria in quarter-strength Ringers solution (ch. 2.2).

Lowering the initial numbers of bacteria in finished processed meat products to delay product spoilage has previously been suggested (Allen and Foster, 1960; Kempton and Bobier, 1970). Although improved plant sanitation was cited as a suitable method, our results indicated that pasteurization could be used to lower initial contamination levels of VPVS. Pasteurization, however, should not be regarded as a substitute for hygiene to achieve a reduction in initial numbers of spoilage bacteria. It has been documented, however, that there was no relationship between initial numbers and subsequent growth of LAB in processed meat products (Kempton and Bobier, 1970), and that LAB became the predominant spoilage populations regardless of initial contamination levels (Reuter, 1969). Microbiological analysis of VPVS was therefore regarded as necessary to determine whether a reduction of initial numbers of LAB by pasteurization would affect product shelf life, as well as composition of predominant spoilage populations.

3.1.3.2 Effect of in-package pasteurization on microbial numbers and shelf life

3.1.3.2.1 Effect of pasteurization on numbers of Enterobacteriaceae and yeasts

Counts of Enterobacteriaceae and yeasts for control samples fluctuated between < log 2,0 and 4,0 $CFUg^{-1}$ for the first 24 days (Fig 3.1.9a). Numbers of these microorganisms, however, decreased as storage time increased and after 68 days of storage at 8°C, no Enterobacteriaceae or yeasts were recovered from control samples (Fig. 3.1.9a).

The inability of Enterobacteriaceae and yeasts to dominate the spoilage of VPVS in this study confirmed the findings of numerous other studies on the spoilage ecology of vacuum-packed emulsion-type sausages (Fruin et al., 1978; Paradis and Stiles, 1978; Nielsen, 1983; Schneider et al., 1983; Zurera-Cosano et al., 1988; von Holy et al., 1991a). Antimicrobial activity of bacteriocinogenic LAB was documented as an important selection factor resulting in the reduced predominance of Enterobacteriaceae and yeasts in processed meats (Daly et al., 1972; Nielsen and Zeuthen, 1985; Andersson, 1986). In addition, diacetyl, a metabolic end product synthesized by LAB from pyruvate (Kandler, 1983), has been shown to inhibit yeasts (Jay, 1982). Furthermore, sensitivity of Enterobacteriaceae towards microbially produced lactic acid and associated pH decreases occurring during growth of LAB in foodstuffs, have been documented (Ritter, 1950; Davidson and Webb, 1973; Shay et al., 1978; Grau, 1981; Gill and Newton, 1982; Nielsen and Zeuthen, 1985).

The growth of LAB in control samples of this study resulted in concurrent pH decreases (Fig. 3.1.9a) and a noticeable decrease from initial product pH values of ca. 6,2 - 6,3 to below pH 6,0 occurred when the LAB count reached approx. log 7,0 $CFUg^{-1}$ (Fig. 3.1.9a). This correlated well to findings of Korkeala et al. (1990) who showed that pH values of cooked ring sausages were first at a constant level of ca. 6,3, but decreased sharply after the lactic acid bacteria count reached ca. log 7,7 $CFUg^{-1}$. Further pH decreases from pH 6,0 to 5,0 occurred as numbers of LAB increased to ca. log 8,0 $CFUg^{-1}$ (Fig. 3.1.9a). This corresponded to a decline in numbers of Enterobacteriaceae from ca. log 3,0 to 2,0 $CFUg^{-1}$ (Fig. 3.1.9a). Numbers of these bacteria declined further as the pH dropped to lower levels, until no more Enterobacteriaceae were recovered after 63 days (Fig. 3.1.9a). Microbially produced lactic acid and concurrent reductions of product pH may, therefore, explain the inability of these bacteria to dominate the spoilage association of VPVS in this study. Yeasts, however, are known to tolerate lactic acid and pH's of ca. pH 5,0 (Buchanan, 1986; Beuchat and Golden, 1989). The inability of yeasts to dominate in the spoilage ecology may therefore be a result of bacteriocin and/or diacetyl inhibition, as well as other factors such as slower growth rates and inefficient substrate utilization (von Holy et al., 1991a).

For pasteurized samples, numbers of Enterobacteriaceae and yeasts were generally lower than log 0,6 $CFUg^{-1}$ for the entire storage period (Fig. 3.1.9b). Moreover, these microbial groups were not recovered on all sampling occasions, which indicated almost complete elimination by pasteurization. Low heat resistance of most Enterobacteriaceae has been documented and is exploited commercially in, for example, milk pasteurization (Jay, 1986). This elimination of Enterobacteriaceae in VPVS was desirable from a practical point of view, since it could reduce the incidence of bacteria such as Yersinia enterocolitica and salmonellae in vacuum-packaged processed meats (Nielsen and Zeuthen, 1985) and, therefore, prevent the growth of these potential pathogens under conditions of product temperature abuse.

3.1.3.2.2 Effect of pasteurization on numbers of total aerobic and lactic acid bacteria

Microbial counts (LABC and TAPC) of non-pasteurized (control) and pasteurized samples over 128 days storage are shown in Fig. 3.1.10. For control samples, counts increased rapidly over the first 14 days to levels around log 8,0 $CFUg^{-1}$. Bacterial counts then stabilized at ca. log 8,0 $CFUg^{-1}$ (Fig. 3.1.10). For pasteurized samples, considerable fluctuations in microbial numbers were evident, but for PAST1 and PAST2 treatments, a gradual increase in numbers to ca. log 8,0 $CFUg^{-1}$ could be observed with increasing storage time (Fig. 3.1.10). Although counts of the PAST3 treatment also increased to ca. log 8,0 $CFUg^{-1}$, low counts at various sampling intervals indicated high variability between samples (Fig. 3.1.10). Figure 3.1.10 also shows noticeable differences in shelf life (Table 3.1.7) and bacterial numbers (LABC and TAPC) between control and pasteurized samples. Statistical analysis of the difference in numbers of these bacteria between control and pasteurized treatments was therefore not deemed necessary (D.H. Meyer, pers. comm.)*. Since bacterial numbers of all pasteurization treatments were comparable and ranged from log 1,0 to 4,0 $CFUg^{-1}$ for the first twenty days of storage (Fig. 3.1.10), statistical analysis to test for a significant difference between pasteurization treatments was only applied to counts after day twenty (D.H. Meyer, pers. comm.)*.

The two way ANOVA showed significant ($p = 0,00$) day and treatment effects for both LABC and TAPC. This implied that there was growth of microorganisms in sausage packs over time, and that pasteurization treatments differed significantly from each other in their ability to reduce numbers and growth of microorganisms. Moreover, there was no significant interaction between day and treatment effects for both the TAPC ($p = 0,28$) and LABC ($p = 0,18$). This implied that despite fluctuations in microbial numbers over time (Fig. 3.1.10), there were no significant differences in the shape of these curves for PAST1, PAST2 and PAST3 treatment.

Increases of LABC and TAPC to high numbers in a short time for control samples indicated that bacteria were ideally suited for growth in VPVS, which confirmed findings of previous studies on vacuum-packaged processed meats (Allen and Foster, 1960; Holzapfel and Gerber, 1986; Borch _et_ _al_., 1988; von Holy _et_ _al_., 1991a,b; von Holy and Cloete, 1992). In the case of pasteurized samples, however, counts fluctuated considerably and there was no consistent increase in bacterial numbers between all sampling intervals. Fluctuations in LABC and TAPC between pasteurized VPVS samples on successive sampling days have been reported previously, and were ascribed to inter-sample variation resulting from unequal heat penetration during pasteurization (von Holy _et_ _al_., 1991a). Unequal heat

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penetration of sausage packs thus might have occurred also in this study, despite attempts to minimize this effect by packaging sausages into single layers. Furthermore, inter-sample variations could have resulted from small differences in heating times, since not all packs could be removed from the water cooker at exactly the same time (Table 3.1.3). Counts (LABC and TAPC) of pasteurized samples in this study also took longer to reach the maximum of *ca*. log 8,0 $CFUg^{-1}$ compared to control samples (Fig. 3.1.10). It has been documented before that the size of the microflora surviving a pasteurization process is inversely related to the severity of the heat treatment applied (Bell, 1983). Furthermore, it has been reported that heat injured bacterial cells may remain viable but unable to reproduce until the damage is repaired (Silliker *et al*., 1980). Statistically significant differences between the pasteurization treatments observed in this study were, therefore, speculated to be the combined results of lower initial populations (Table 3.1.6) as well as slower growth of surviving bacteria (Fig. 3.1.10).

3.1.3.2.3 Effect of pasteurization on microbiological shelf life of vacuum-packaged vienna sausages

Microbiological shelf life (MSL) based on LABC and TAPC for the different treatments is shown in Table 3.1.7. Shelf life values derived from LABC and TAPC corresponded closely, as was the case in previous studies (von Holy *et al*., 1991a; Marshall, 1991). This implied that the LAB constituted the majority of the TAPC for all pasteurization treatments, which also was in agreement with an earlier report (von Holy *et al*., 1991a). The MSL of VPVS increased with increasing severity of the heat treatment applied (Table 3.1.7). For control samples, the "mean" (based on microbiological counts from duplicate samples) MSL was seven days. Microbiological shelf life of non-pasteurized VPVS has been previously reported to be eleven days at 7°C storage (von Holy *et al*., 1991a), which was marginally longer than observed in this study and may be explained by the slightly lower storage temperature. The "mean" MSL values of PAST1, 2 and 3 samples based on the LABC were 67, 99 and 119 days, respectively, whereas the "worst case" MSL for PAST1, 2 and 3 treatments were 28%, 8% and 5% shorter at 48, 91 and 113 days, respectively (Table 3.1.7). Thus shelf life increases of 10, 14 and 17 times (based on "mean" MSL) and 8, 15 and 19 times (based on "worst case" MSL) were achieved by pasteurization. Since the "worst case" scenario was based on the one sample of duplicate packs showing higher microbiological counts, "worst case" MSL may therefore express imminent expiry of the product with a margin of safety, i.e. product spoilage may be imminent even though the MSL suggests that product should still be unspoilt. Clearly, therefore, noticeable increases of VPVS shelf life were achieved by relatively mild in-package pasteurization processes.

Secondary or in-package pasteurization previously successfully increased shelf life of vacuum-packaged products such as ham (Houben, 1982; Delaquis et al., 1986), hot process (smoked) fish (Eklund et al., 1988) and cooked pork chops (Prabhu et al., 1988). Shelf life of VPVS packed in double layers (Marshall, 1991; von Holy et al., 1991a) also increased after a secondary, in-package heat treatment. Von Holy et al. (1991a) reported a fourfold increase in shelf life of VPVS stored at 7°C, when sausages were pasteurized for twenty minutes in a water cooker held at 78 to 81°C. Marshall (1991) also reported fourfold shelf life increases of vacuum-packaged vienna sausages stored at 8°C, after pasteurization in a water cooker for 30 minutes at 70°C. The double layer packs used in the above studies, however, showed poor heat penetration and von Holy et al. (1991a) reported temperatures at the center of packs to vary from a minimum of 52°C to a maximum of 57°C. Packaging of product in single layers (Fig. 3.1.1) clearly alleviated the occurrence of such "cold spots" and led to a quicker and more even heat distribution, which resulted in longer shelf life of VPVS pasteurized at lower heating temperatures. Product core temperatures in this study reached ca. 63°C (PAST2 and 3) in ca. ten minutes (Table 3.1.3). This, together with the fact that bacterial growth on emulsion-type sausages is essentially surface associated (Korkeala and Lindroth, 198?, Borch et al., 1988; Dykes et al., 1991; von Holy and Cloete, 1992) and therefore in direct proximity of the heat source, could explain the increased shelf life. Packaging of sausages into single, as opposed to double layers, for in-package pasteurization purposes is not only effective in increasing product shelf life, but also attractive to the meat industry in terms of saving energy and time during processing.

Figure 3.1.10 indicated that the LABC and TAPC of all treatments corresponded closely over most of the sampling period, and it was assumed that LAB dominated the TAPC even for pasteurized samples. To test this assumption a paired students t test was performed on the difference of LAB and total aerobic plate counts. This test showed no significant differences between LABC and TAPC of control ($p = 0,54$), PAST1 ($p = 0,68$) and PAST2 ($p = 0,09$) treatments. This, together with our earlier findings that the MSL of VPVS (based on LABC and TAPC) corresponded well (Table 3.1.7), confirmed that LAB constituted the majority of the TAPC for control, PAST1 and PAST2 treatments. The paired students t test, however, suggested a highly significant difference ($p = 1,61 \times 10^{-6}$) between LABC and TAPC of PAST3 counts, which indicated that in this case the TAPC was constituted of other bacterial populations in addition to LAB. Thus, our earlier assumption that LAB constituted the majority of the TAPC for PAST3 treatments since the MSL for these products (based on TAPC and LABC) corresponded well, no longer held true.

The fact that the TAPC for PAST3 treated samples on reaching the microbiological shelf life was constituted of bacterial populations other than LAB, contradicted the findings of Reuter (1969), that regardless of initial numbers LAB became the predominant spoilage populations of vacuum-packaged processed meats. Since PAST3 was the most severe heat treatment applied, the change in predominance might have been due to sporeforming bacteria such as <u>Bacillus</u> or <u>Clostridium</u>. Spores of these bacteria are known to be more heat resistant than vegetative cells (Stumbo, 1965; Silliker <u>et al</u>., 1980; Nielsen and Zeuthen, 1984a,b; Kokubo <u>et al</u>., 1986; Müller, 1989). Since von Holy (1989) reported an increased predominance of <u>Bacillus</u> spp. following pasteurization of VPVS, heating of product, therefore, appeared to select for more heat resistant sporeforming populations in this study. This could explain the significant differences between LABC and TAPC of PAST3 samples. The impact of this altered predominance pattern on safety of pasteurized VPVS would clearly require further study. A possibility of selecting for potentially pathogenic <u>Clostridium</u> spp. by pasteurization would, for example, imply a potential consumer hazard, and thus warrant further investigations on their incidence in pasteurized VPVS.

3.1.3.3 Effect of pasteurization on vacuum-packaged vienna sausage spoilage symptoms

Failure of bacterial counts to correlate with organoleptic data in studies on shelf life predictions has been reported previously (Egan and Shay, 1982; von Holy <u>et al</u>., 1991a). Although no professional organoleptic testing regimes were applied, most pasteurized and non-pasteurized samples were not organoleptically unacceptable when counts reached the MSL ($5x10^6$ $CFUg^{-1}$) (Figs. 3.1.11 to 3.1.16), apart from an occasional sour smell and slight loosening of packaging material. When total aerobic and lactic acid bacteria counts reached $1x10^8$ $CFUg^{-1}$, however, milky exudates occurred within packs and packaging material had loosened noticeably (Figs. 3.1.11 to 3.1.16). In addition, on opening of packs a distinct sour or "off" smell was noted. These observations were in accordance with other reports on a variety of vacuum-packaged processed meats, in which products were deemed spoiled when LAB or total aerobic plate counts reached $1x10^8$ $CFUg^{-1}$ (Allen and Foster, 1960; Mol <u>et al</u>., 1971; Egan <u>et al</u>., 1980; Korkeala <u>et al</u>., 1989). However, when counts reached $1x10^8$ $CFUg^{-1}$, spoilage symptoms of pasteurized samples were less severe than those of non-pasteurized samples (Figs. 3.1.11 to 3.1.16). For example, less exudate developed in vacuum bags and loosening of packaging material was not as pronounced as for non-pasteurized samples (Figs. 3.1.11 to 3.1.16). Speculatively, it was concluded that the less severe spoilage symptoms in pasteurized samples could be explained by a change in predominance of spoilage microorganisms brought about by the pasteurization process. Predominant microorganisms in pasteurized product were thus speculated to differ from those of control samples on account of less severe spoilage symptoms. Clearly, the composition of predominant spoilage populations in pasteurized and non-pasteurized VPVS would require further study.

3.1.3.4 Effect of pasteurization on predominance of Listeria and Clostridium

The incidence of Listeria and Clostridium in VPVS samples is shown in Table 3.1.8. One Listeria strain was isolated from a control pack in the first week of storage at 8°C (Table 3.1.8) and subsequently identified (Table 3.1.5) as Listeria innocua. No further Listeria were isolated from control packs during subsequent storage or from any packs of the three in-package pasteurization treatments (Table 3.1.8). Furthermore, only one control pack contained a Clostridium spp. (subsequently identified as C. butyricum) in the final week of storage. For pasteurized samples, however, clostridia were isolated more frequently (Table 3.1.8). Out of the 22 Clostridium-positive samples (Table 3.1.8), 25 isolates were recovered for identification to species level (Table 3.1.5). These 25 isolates included C. bifermentans, C. sporogenes, C. subterminale, C. litus-eburense, C. leptum, C. putrificum, C. sphenoides, C. butyricum and C. perfringens (type A).

Listeria monocytogenes, a bacterial foodborne pathogen, has been linked to foodborne illness outbreaks involving a variety of foods (Fleming et al., 1985, Linnan et al., 1988; Barnes et al., 1989). Although no outbreaks of listeriosis have to date been associated with meat products, risk factor analysis for sporadic listeriosis suggested an epidemiological association between eating non-reheated hot-dogs and human listeriosis (Schwartz et al., 1988, Glass and Doyle, 1989). Most infectious microorganisms are controlled in food by manipulating and controlling of one or more hurdles like pH, a_w, storage temperature and preservatives (Leistner, 1987; Scott, 1989). The control of L. monocytogenes in foods, however, has been regarded as difficult due to the microorganism's unique tolerance to control factors that would normally prevent, or severely limit growth of other common foodborne microbial pathogens (Leistner, 1987; Doyle, 1988; McClure et al., 1991). For example, Listeria has minimum growth temperatures and pH's of 0°C and pH 4,3 to 4,5, respectively (Farber et al., 1989; McClure et al., 1989; Grau and Vanderlinde, 1990). Furthermore, these bacteria have been reported to grow at decreased oxygen concentrations and to tolerate relatively high (ca. 10%) concentrations of NaCl as well as sodium nitrite (Seeliger, 1961; Shahamat et al., 1980; McClure et al., 1989). Shahamat et al. (1980) suggested that 100ppm nitrite inhibits growth of L. monocytogenes only when combined with more than 3,0% NaCl and a pH value at or below 5,5, at 5°C. The VPVS under study here had a pH of 6,2, contained 2,2% NaCl and a maximum of ca. 100ppm residual nitrite (M.A. Jenkin, pers. comm.)*, which might well allow for the growth of Listeria spp.

The isolation of one Listeria strain in this study, as well as reported incidences of Listeria at frequencies of 10 to 50% in other vacuum-packaged processed meats, showed that contamination by and growth of this pathogen could occur in these types of products (Buncic et al., 1991; Rorvik and Yndestad, 1991; Grau and Vanderlinde, 1992). Since only one Listeria strain was isolated from a control pack of sausages during the first week of storage in this study, and no Listeria could be isolated during subsequent product storage, it was speculated that these bacteria were successfully inhibited by competitive LAB populations. This was thought to be highly likely since numerous reports have shown that a diversity of LAB strains are antagonistic towards and inhibit growth of Listeria (Klaenhammer, 1988; Harris et al., 1989, Raccach et al., 1989; Spelhaug and Harlander, 1989; Ahn and Stiles, 1990; Schillinger and Holzapfel, 1990; Hastings and Stiles, 1991; Schillinger et al., 1991; Skyttä et al., 1991; van Laack et al., 1992)

Initial contamination levels of Listeria in vacuum-packaged processed meats varied from 0,3 g^{-1} for vacuum-packaged

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* M.A. Jenkin, Enterprise Foods, P.O. Box 570, 1400 Germiston, South Africa.

ham samples up to ca. 3×10^4 CFUg^{-1} in sliced vacuum-packaged processed meat products (Rorvik & Yndestad, 1991). As a potential for contamination of processed meats (including VPVS) by Listeria spp. does exist, and since these pathogens may have a low infective dose especially in susceptible persons (Varabioff, 1992), a need exists to control the presence and growth of these bacteria in such products. Post-processing pasteurization has been proposed for the control of L. monocytogenes in vacuum-packaged processed meat products (Glass and Doyle, 1989). Although there have been numerous detailed studies on the thermal resistance of L. monocytogenes in dairy products (Farber, 1989; Mackey and Bratchell, 1989; Knabel et al., 1990; Bradshaw et al., 1991), limited information is available on the fate of the pathogen during thermal processing of meat products (Johnson et al., 1990; Bhaduri et al., 1991). It has nevertheless been proposed that cooking processes normally applied to processed meat products should be adequate for the elimination of L. monocytogenes (Glass and Doyle, 1989; Zaika et al., 1990). Zaika et al. (1990) showed that the typical process for cooking Frankfurters (70 minutes to an internal temperature of 71,1°C) should kill L. monocytogenes at $<10^3$ CFUg^{-1} initial contamination. Similar heating processes employed during the manufacture of vienna sausages (70 minutes to reach an internal temperature of 72°C) (von Holy et al., 1991b) should, therefore, be adequate to destroy these pathogens also in this product, provided the initial contamination is $<10^3$ CFUg^{-1}. Contamination of vacuum-packaged processed meats by Listeria could, however, also occur as post-processing contamination (Varabioff, 1992). This contamination could then be eliminated by a secondary (in-package) pasteurization step.

Thermal resistance studies for L. monocytogenes have reported relatively low D-values of 0,65 minutes at 65°C and 1,1 minutes at 62,8°C, when these bacteria were heated in raw beef and liver sausage slurry, respectively (Mackey and Bratchell, 1989; Bhaduri et al., 1991). Farber (1989), however, found that addition of curing ingredients to ground meat increased the heat resistance of this pathogen between fivefold and eight fold. The reported D-value of L. monocytogenes in ground meat plus cure (cure included nitrite, dextrose, lactose, corn syrup and *ca*. 3% NaCl) was 1,28 minutes at 64°C (Farber, 1989). These data suggested that smoke-cooking of vienna sausages together with in-package pasteurization would effectively inhibit *Listeria* spp. when contamination levels are low. Failure to detect any *Listeria* spp. in any pasteurized VPVS samples (Table 3.1.8) suggested that this was indeed the case. It must be stressed, however, that effective sanitation programs should be maintained to keep initial contamination of vienna sausages by *Listeria* spp. to a minimum. Complete elimination of the pathogen by in-package pasteurization would then be assured.

The Gram-positive, anaerobic spore forming bacteria of the genus *Clostridium* include the well described foodborne pathogens *C*. *botulinum* and *C*. *perfringens* (Atlas, 1984). *C*. *botulinum* strains have been classified into seven types (A to G) on the basis of the serological specificity of characteristic neurotoxins they produce (Hauschild, 1989). *C*. *botulinum* types C and D are non-pathogenic to humans, and type G has not been implicated in human disease to date (Sperber, 1982). Most type E outbreaks of botulism were associated with consumption of fish or fish products (Sofos *et al*., 1979; Simunovic *et al*., 1985). Numerous reports indicated that *C.* *botulinum* is a rare contaminant of processed meats. Insalata *et al*. (1969) and Taclindo (1967) found only one out of 400 samples of vacuum-packed Frankfurters, and 73 samples of luncheon meats, respectively, contaminated with *C*. *botulinum* type B. While Abrahamson and Riemann (1971) found only six samples of 372 meats to be positive for *C*. *botulinum*, Kokubo *et al*. (1986) failed to detect the pathogen in any of 469 cooked meat products.

Enterotoxin producing *C*. *perfringens* strains have been classified into five types (A to E) according to the types of extracellular toxin (alpha, beta, epsilon and iota) they produce (Labbe, 1989). Virtually all food poisoning outbreaks of *C*. *perfringens*, however, were caused by type A strains (Labbe, 1989). Conflicting reports on the incidence of *C*. *perfringens* in processed meats are reported in the literature. Fruin *et al*. (1978) reported that less than 1% of 419 Bologna product samples contained these bacteria, and that counts in positive samples were <5 CFUg^{-1}. Duitschaever (1978) and Paradis and Stiles (1978) could not detect *C. perfringens* in 180 Frankfurter samples and 113 samples of vacuum-packaged sliced Bologna, respectively. By contrast, Kokubo *et al.* (1986) detected clostridia in 32,8% of 469 cooked meat product samples and 44,8% of isolates were *C. perfringens*. Contamination by clostridia in 90,9% of these positive samples, however, was at a level of <10 CFUg^{-1}. Although only a limited number of samples were evaluated for the presence of clostridia in our study, and no efforts were made to enumerate these bacteria, the presence of *Clostridium* spp. in only one control pack of vienna sausages (Table 3.1.8) indicated low contamination.

Even though clostridia were shown to contaminate processed meats at varying levels, these products have an exemplary safety record with respect to botulism (Sofos *et al.*, 1979; Sofos and Busta, 1980; Hauschild, 1982). Furthermore, *C. perfringens* has to date not been implicated in food poisoning outbreaks from vacuum-packaged cured meats (Steele and Stiles, 1981). This is probably due to the use of a combination of extrinsic and intrinsic control parameters (hurdles) in these types of products. Storage temperature, for example, was reported as a major factor contributing to the safety of processed meat products with respect to clostridial food poisoning (Riemann *et al.*, 1972; Sofos and Busta, 1980; Hauschild, 1982; Nielsen and Zeuthen, 1984a). Reported minimum growth temperatures of *C. perfringens*, proteolytic and nonproteolytic *C. botulinum* are shown in Table 3.1.1. These temperatures indicate that growth of proteolytic strains of *C. botulinum* types A and B and *C. perfringens* should not occur in adequately (*ca.* 5°C) refrigerated processed meat products. These products do, however, pose a potential risk to the consumer, since non-proteolytic types of *C. botulinum* can grow at refrigeration temperatures (Table 3.1.1). In addition, clostridia are known to survive long periods at low temperature and multiply once suitable conditions are established, for example during times of product temperature abuse (Christiansen, 1980; Sofos and Busta, 1980; Sperber, 1982; Nielsen and Zeuthen, 1984a).

Growth limiting salt concentration, a_w and pH values of C. botulinum and C. perfringens are shown in Table 3.1.1. Salt concentration, pH and a_w values reported by Buchanan (1986) for typical processed meats, would fall within the range that permit growth of these pathogens. As most cured meat products do not have sufficiently high levels of acid and or salt to completely inhibit the outgrowth of C. botulinum or C. perfringens spores, the addition of nitrite to meats has become an accepted practice (Cuppett et al., 1987). Nitrite is a well-known antibotulinal agent with increasing activity at increasing residual concentration in cured meats (Sofos et al., 1979; Sofos and Busta, 1980). To guarantee complete prevention of clostridial growth, Grever (1974) recommended that 100-200 micrograms nitrite per gram product should be added, depending on the heat treatment of the product, and the pH should be lower than 6,2. Nitrite effectiveness, however, was also positively synergistic when used in combination with salt or low pH. For example, Roberts and Ingram (1966), reported that the antibotulinal activity of nitrite increased ca. tenfold when the pH was reduced from 7,0 to 6,0. Thus, lower concentrations of nitrite had an inhibitory effect on clostridial growth in cured meats with increasing salt concentration and decreasing pH (Castellani and Niven, 1955; Sofos and Busta, 1980; Hauschild, 1982; Labbe, 1989). Furthermore, inhibitory activity of nitrite against clostridia in cured meats is reportedly enhanced when used in combination with other preservatives, such as nisin (Taylor et al., 1985), sorbate (Sofos et al., 1979) and ascorbic acid (Sofos and Busta, 1980).

Vienna sausages in our study had a pH of ca. 6,2, contained 2,2% sodium chloride, 150-200ppm ascorbic acid and <100ppm of residual nitrite (M.A. Jenkin, pers. comm.)*. The low incidence of Clostridium spp. in non-pasteurized packs of VPVS observed in this study (Table 3.1.8), may indicate

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that the combination and interaction of factors such as pH, refrigeration, nitrite and possibly ascorbic acid was indeed successful in inhibiting growth of these bacteria, as has been observed in other studies (Riemann et al., 1972; Sofos et al., 1979; Sofos and Busta, 1980; Sperber, 1982). One Clostridium butyricum isolate was detected during the last week of storage of non-pasteurized VPVS (Table 3.1.8), but this isolate was non-pathogenic. Although nitrite concentrations have been reported to decrease to sub-inhibitory levels during storage, growth of clostridia in processed meat products was still inhibited by product pH decreases resulting from the growth of concomitant spoilage LAB (Christiansen et al., 1975; Christiansen, 1980). Nitrite concentrations in VPVS could already have been depleted after 18 weeks of storage in our study, but the pH of control samples which at that time was ca. 4,4 to 4,6 (Figure 3.1.9a) was likely to have been inhibitory to the clostridia (Table 3.1.1). Furthermore, non-pasteurized VPVS were at that time spoiled (Fig. 3.1.16) and likely to be rejected by the consumer and/or removed from retail display. Non-pasteurized VPVS, like other processed meats, should therefore be safe from clostridial foodborne illness since growth of these bacteria would be inhibited not only by nitrite and refrigeration temperatures used for product storage, but also by competitive lactic acid bacteria populations.

For vacuum-packaged, cooked emulsion-style sausage, however, it has been suggested that growth of pathogenic clostridia may occur when levels of competing spoilage populations are low (Nielsen and Zeuthen, 1985). The in-package heat treatment used in our study was demonstrated to lower initial numbers of competitive LAB spoilage populations to $\leq$log 1,0 CFUg^{-1}, depending on the severity of the treatment (Table 3.1.6). In addition, Clostridium spores survive heat treatments normally employed for the processing of cooked meat products (Nielsen and Zeuthen, 1984a,b; Buchanan, 1986; Kokubo et al., 1986). These findings implied that a potential for growth of these pathogens existed, which was reflected by our results indicating a higher incidence (41,7% to 75,0%) of Clostridium in pasteurized samples, when compared to non-pasteurized samples (8,3%) (Table 3.1.8).

Furthermore, this incidence increased with increasing severity of heat treatment, from 41,7% in PAST1 treated packs to 58,3% and 75,0% in PAST2 and 3 treated packs, respectively (Table 3.1.8). The role of competitive LAB populations in the inhibition of clostridia has been attributed to their ability to produce lactic acid which results in pH decreases (Riemann et al., 1972; Christiansen et al., 1975; von Holy et al., 1991a), as well as production of bacteriocins (Spelhaug and Harlander, 1989; Okereke and Montville, 1991). Statistical analysis of numbers (TAPC and LABC) of spoilage bacteria in VPVS over the storage time (3.1.3.2.2) showed that there was a significant difference in the growth of spoilage LAB as a result of different pasteurization treatments. Growth of spoilage LAB decreased with increasing severity of heat treatment (3.1.3.2.2), and this appeared to result in the increasing incidences of clostridia (Table 3.1.8). This implied that spoilage LAB indeed played an important role in inhibiting the growth of clostridia in VPVS. Although pasteurization was effective in delaying spoilage of VPVS by LAB and eliminating Listeria, the heat treatment employed with the resultant reduction in spoilage LAB, was also responsible for increasing the incidence of potentially pathogenic clostridia.

Of the 25 Clostridium isolates from VPVS nine (36%) were identified as C. bifermentans, six (24%) as C. perfringens, two (8%) each as C. subterminale, C. butyricum and C. leptum and one (4%) each as C. sporogenes, C. litus-eburense, C. sphenoides and C. putrificum. Our results thus corresponded well to those of Kobayashi and Asami (1976, 1977) and Kokubo et al. (1986), indicating that among the clostridia from cooked meat products C. bifermentans was isolated with the highest frequency, followed by C. perfringens. Furthermore the above two species were acknowledged as predominant in processed meat raw materials (Baltzer and Wilson, 1965; Kokubo et al., 1986), suggesting that the clostridia in this study originated from similar sources.

Of the six C. perfringens isolates five were type A and one was undetermined, but produced alpha toxin. Interestingly, C. perfringens type A strains were only isolated from pasteurized products (Table 3.1.8). Increasing incidences of clostridia in pasteurized products and presence of pathogenic C. perfringens type A in these products, therefore, suggested that pasteurized VPVS might be unsafe for consumer use. Further work on numbers and identities of these clostridia in pasteurized VPVS samples would, however, be required to verify product safety.

3.1.4 CONCLUSIONS

Microbiological analysis of non-pasteurized VPVS showed that rapid growth of LAB to high numbers limited the shelf life to seven days at 8°C. Since LAB constituted the majority of the TAPC, these bacteria were recognized as the predominant spoilage populations of non-pasteurized VPVS. Enterobacteriaceae and yeasts, however, failed to establish themselves in the spoilage ecology, most probably as a result of inhibition by LAB.

Pasteurization effectively lowered initial levels of contaminating LAB in vacuum-packaged vienna sausages to ca. $\leq$log 1,0 CFUg^{-1} Furthermore, pasteurization successfully decreased growth of LAB significantly and corresponding to increasing severity of heat treatment. The above findings, therefore, were in contradiction to those of Kempton and Bobier (1970) indicating no relationship between initial numbers and subsequent growth of LAB in processed meat products. By decreasing initial numbers and growth of spoilage bacteria, in-package pasteurization of VPVS successfully achieved shelf life extensions of 10, 14 and 17 fold (based on the LABC) compared to non-pasteurized VPVS.

A report by Reuter (1969), that LAB become the predominant spoilage populations of vacuum-packaged processed meat products regardless of initial contamination levels held true for PAST1 and PAST2 pasteurization treatments in this study. For the most severe heat treatment (PAST3), however, statistical analysis on the difference of TAPC and LABC over the storage period suggested that other bacteria (e.g., Bacillus or Clostridium) reduced the predominance of LAB in VPVS. The impact of these bacteria on the spoilage and safety of VPVS clearly requires further study.

Incidences of both Listeria and Clostridium in non-pasteurized VPVS were low at 8,3% of samples analyzed, in each case. Low incidences of Listeria and clostridia in non-pasteurized sausages led us to conclude that growth of these bacteria was probably inhibited by a combination of control factors such as the relatively low storage temperature used (8°C), presence of nitrite and salt and competitive LAB populations. Isolation of one L. innocua strain during the first week of product storage, however, suggested that a potential for contamination by pathogenic Listeria existed after smoke-cooking of product. This emphasized the necessity for hygiene and good manufacturing processes during the production of VPVS, to keep product contamination by these bacteria to a minimum.

The observed inability of Listeria to grow in pasteurized VPVS indicated that these bacteria were unable to survive in-package pasteurization processes used in this study. Pasteurization of VPVS may therefore contribute to the safety of VPVS with respect to Listeria transmission, in addition to improving product shelf life. Incidences of Clostridium spp., however, increased to 42%, 58% and 75% in PAST1, 2 and 3 pasteurized samples, respectively. This, together with the fact that potential enterotoxin producing C. perfringens type A were isolated from samples of all pasteurization treatments, suggested that clostridia survived the pasteurization process and could render products unsafe for consumer use. It is not known whether clostridia in pasteurized products were present as vegetative cells or spores. Sublethal heat treatment, however, is known to be an effective germination activator (Stumbo, 1965). Since bacterial spores can survive heating for minutes at 120°C and hours at 100°C (Silliker et al., 1980), clostridial spores, if present in sausages studied here, could not only have survived but also been activated to germinate at the pasteurization temperatures used.

Further studies on control parameters for inhibition of growth of clostridia would therefore be necessary in order to assure the safety of pasteurized VPVS. In-package heat treatment, for example, may be used twice to control the growth of these bacteria. The first pasteurization thus would serve to activate bacterial spore germination, and after a suitable incubation period during which outgrowth of vegetative cells would occur, product could be re-pasteurized. In addition, investigations on the effects of preservatives such as nitrite, salt and sodium lactate might be of value to determine their efficacy of inhibiting growth of clostridia in pasteurized VPVS. Since residual nitrite concentrations in processed meats are well known to be affected by product heating as well as storage periods (Sofos *et al.*, 1979), the use of sodium lactate might be of special interest for inhibition of clostridia in processed meats that have been subjected to in-package pasteurization. Sodium lactate was previously shown to be successful in inhibiting growth and toxin production of *C. botulinum* during storage of cook-in-bag turkey products under conditions of temperature abuse (Maas *et al.*, 1989).

Consumer demands for decreased levels of salt, nitrite and other chemical preservatives, however, severely limit the prospects for control of clostridia in in-package pasteurized vacuum-packaged processed meat products by such methods. Thus, future work on controlling the spoilage of these products should focus on less severe pasteurization regimes i.e. lower heat processing values. This could lead to faster growth of spoilage LAB inhibitory to potential pathogens (e.g., clostridia) than observed in this study, while still allowing for shelf life increases.

Fig. 3.1.1 Vacuum-packaged vienna sausages packed in single layers to a mass of 400g.

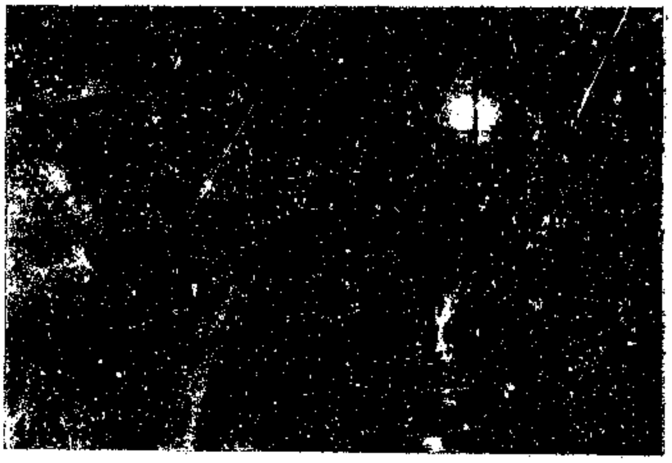

Fig. 3.1.2 Water cooker used to pasteurize vacuum-packaged vienna sausages in meat processing plant.

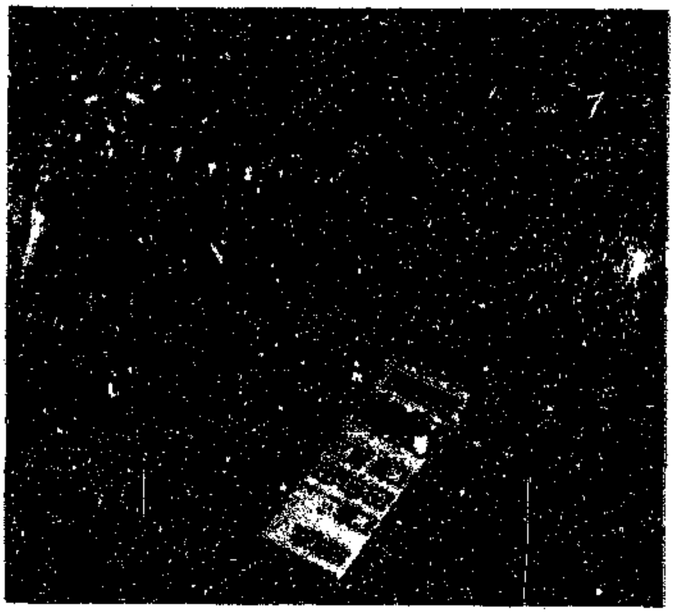

Fig. 3.1.3 Sausauge core temperature determination using a Jenway 3100 microprocessor thermocouple.

Fig. 3.1.4 Draining water cooker to remove packs of sausages after in-package pasteurization.

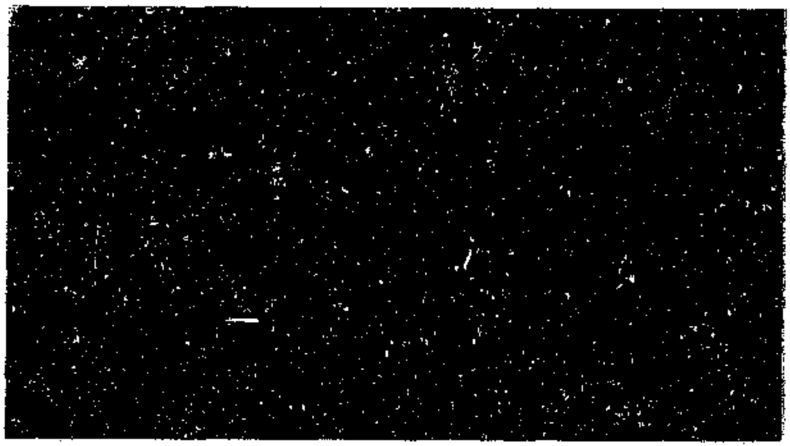

Fig. 3.1.5 Cooling of vienna sausage packs in melting ice after in-package pasteurization.

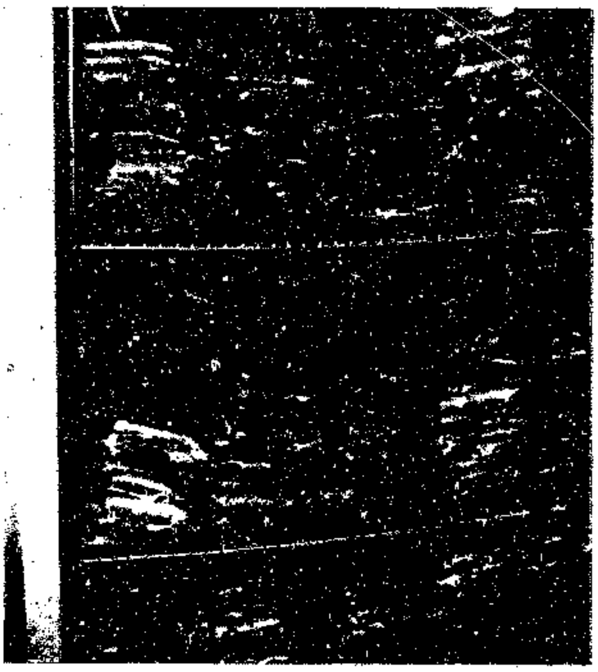

Fig. 3.1.6 Storage of non-pasteurized and pasteurized sausage packs in a low temperature incubator at 8°C for shelf life studies.

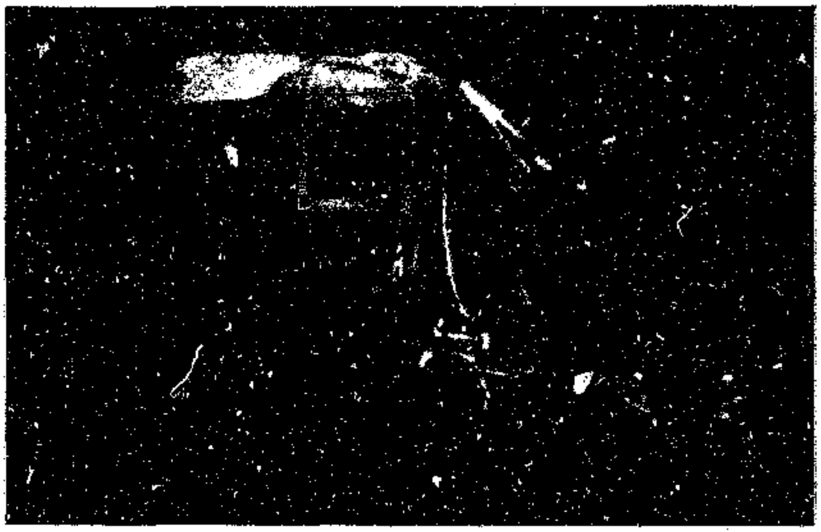

Fig. 3.1.7 Milky exudate drained from terminally spoiled vacuum-packaged vienna sausage pack.

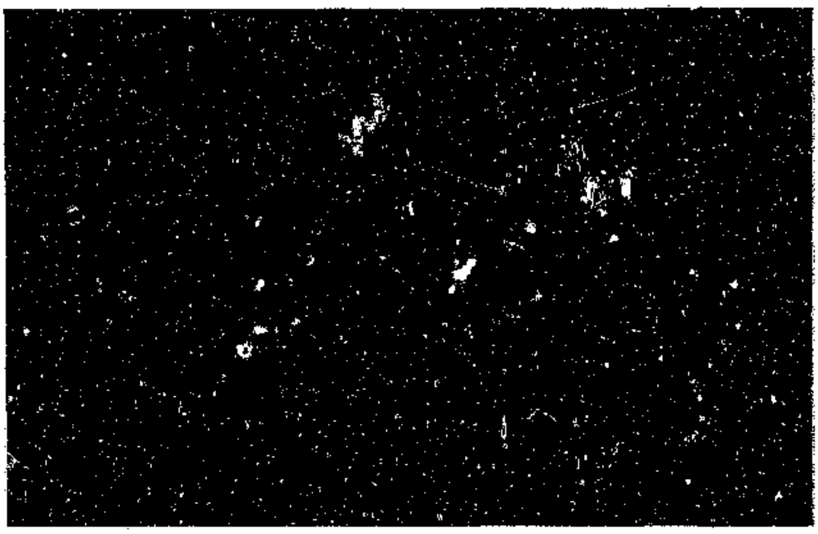

Fig. 3.1.8 Terminally spoiled vienna sausage pack showing severe gas development or "blowing".

a)

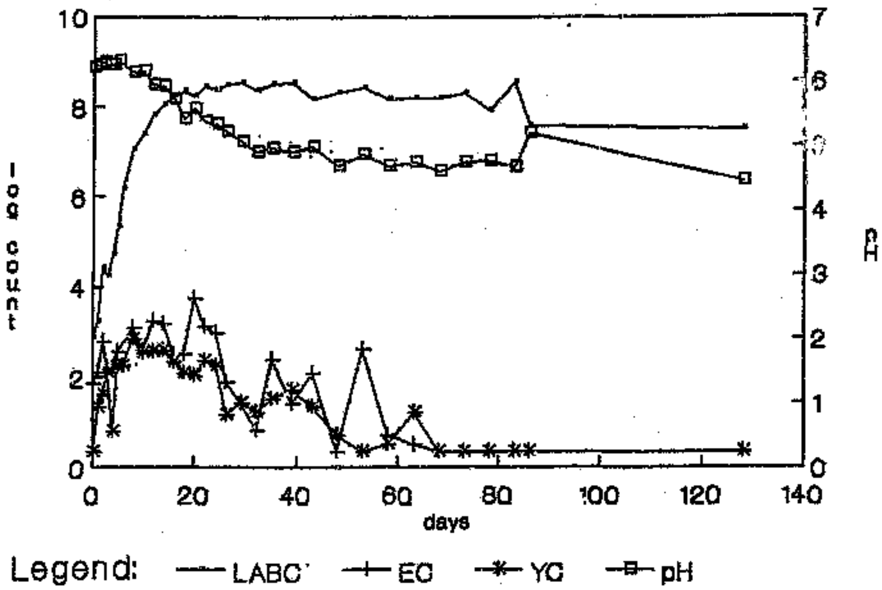

Fig. 3.1.9a Lactic acid bacteria (LABC), Enterobacteriaceae (EC), yeast and mold (YC) counts (log $CFUml^{-1}$) and pH of non-pasteurized vacuum-packaged vienna sausages over 128 days storage at 8°C.

b)

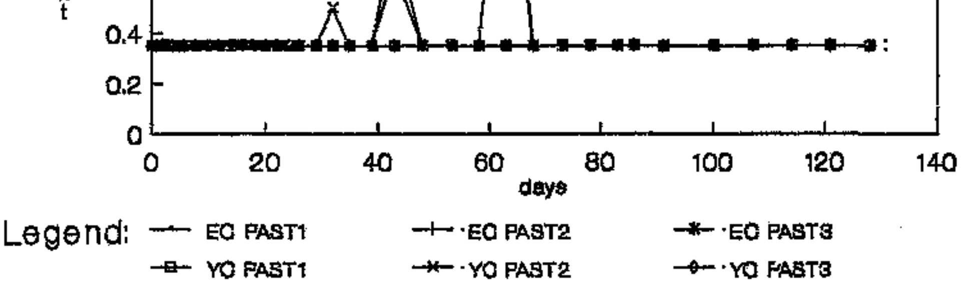

Fig. 3.1.9b Enterobacteriaceae (EC) and yeast (YC) counts (log $CFUml^{-1}$) of pasteurized (PAST1-3, see Table 3.1.2) vacuum-packaged vienna sausages over 128 days storage at 8°C.

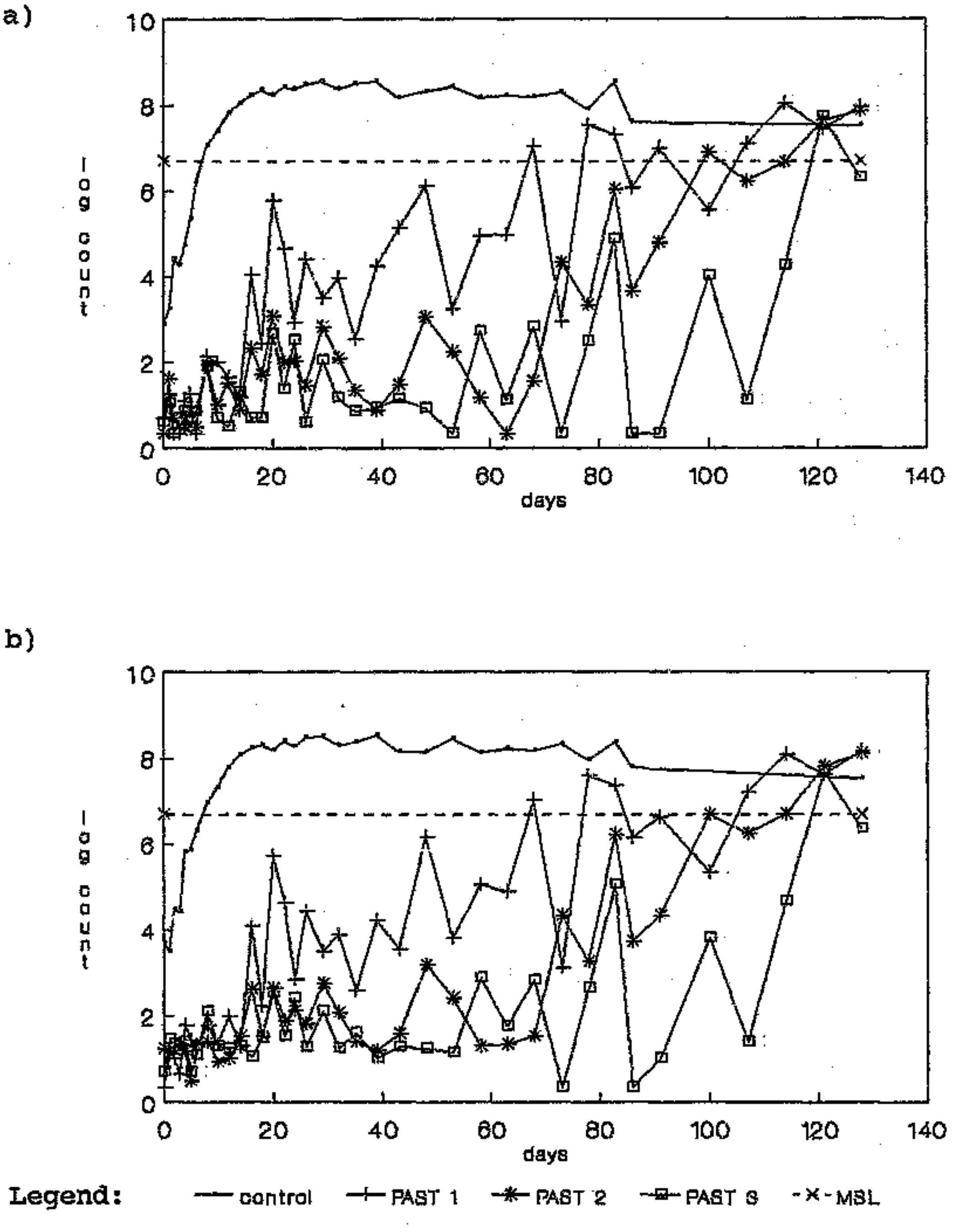

Fig. 3.1.10 Lactic acid bacteria counts (log CFUml^{-1}) (a) and total aerobic plate counts (log CFUml^{-1}) (b) of non-pasteurized (CONTROL) and pasteurized (PAST1-3, see Table 3.1.2) vacuum-packaged vienna sausages over 128 days storage at 8°C indicating microbiological shelf life (MSL) limit of $5x10^6$ CFUg^{-1}.

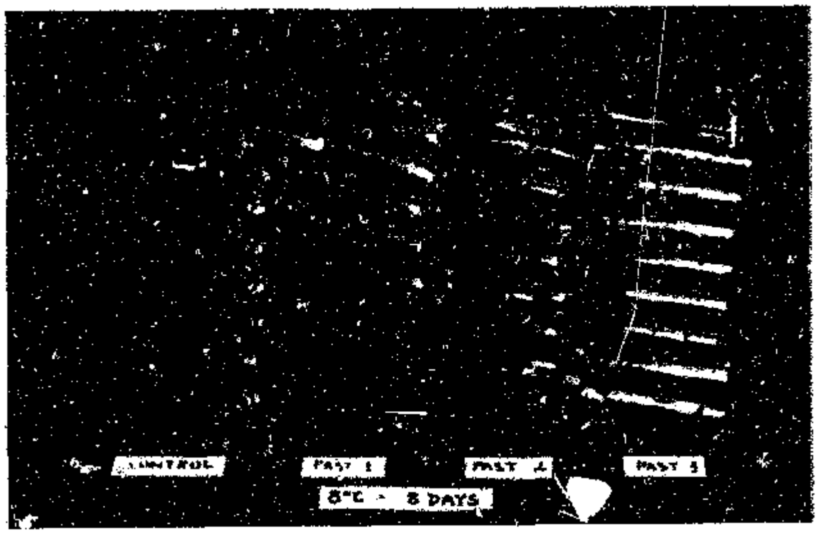

Fig. 3.1.11 Appearance of non-pasteurized (CONTROL) and pasteurized (PAST1-3) vacuum-packaged vienna sausages after 8 days storage at 8°C. Microbiological shelf life of CONTROL sausages was reached (Table 3.1.7)

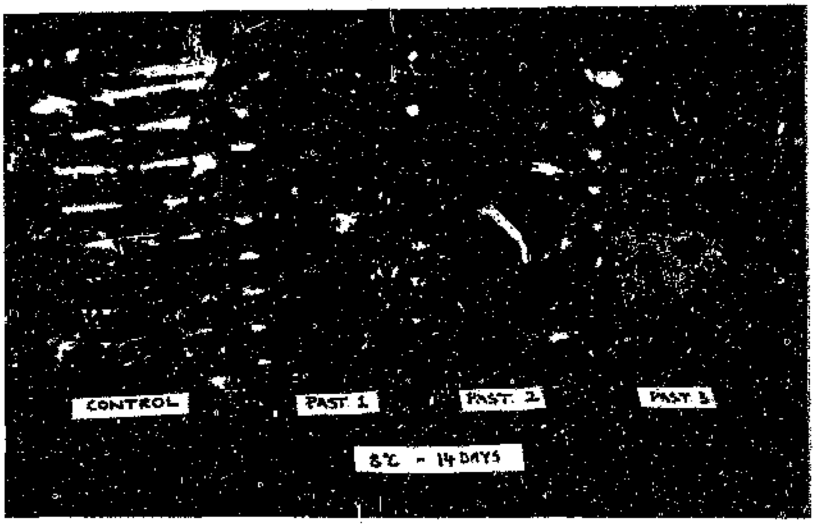

Fig. 3.1.12 Appearance of non-pasteurized (CONTROL) and pasteurized (PAST1-3) vacuum-packaged vienna sausages after 14 days storage at 8°C. Microbiological shelf life of pasteurized sausages was not yet reached (Table 3.1.7). Microbiological counts in CONTROL packs reached <u>ca</u>. log 8,0 CFUg^{-1}.

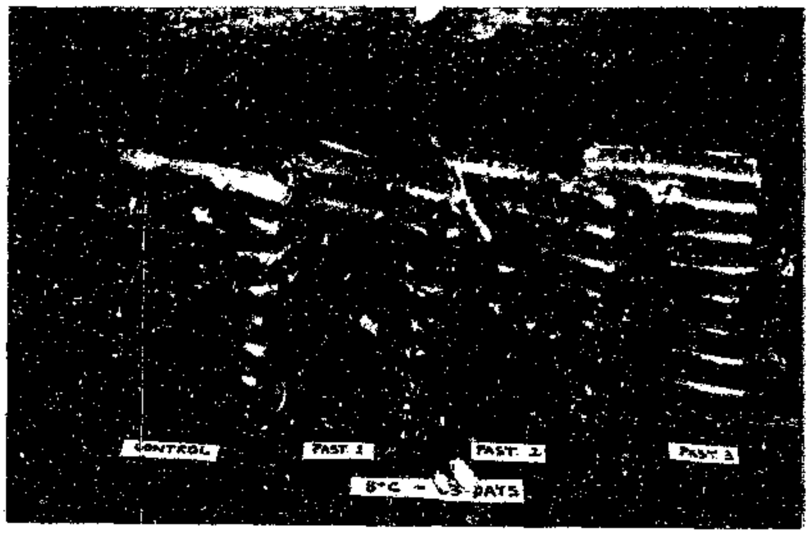

Fig. 3.1.13 Appearance of non-pasteurized (CONTROL) and pasteurized (PAST1-3) vacuum-packaged vienna sausages after 63 days storage at 8°C. Microbiological shelf life of PAST1 samples was almost reached (Table 3.1.7).

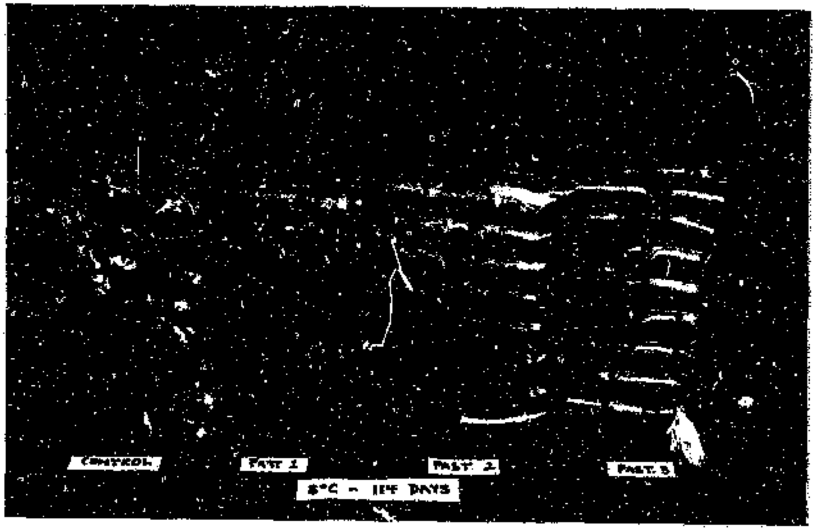

Fig. 3.1.14 Appearance of non-pasteurized (CONTROL) and pasteurized (PAST1-3) vacuum-packaged vienna sausages after 114 days storage at 8°C. Microbiological shelf life of PAST1 and PAST2 samples was surpassed (Table 3.1.7). Microbiological counts in PAST1 packs reached *ca.* log 8,0 $CFUg^{-1}$.

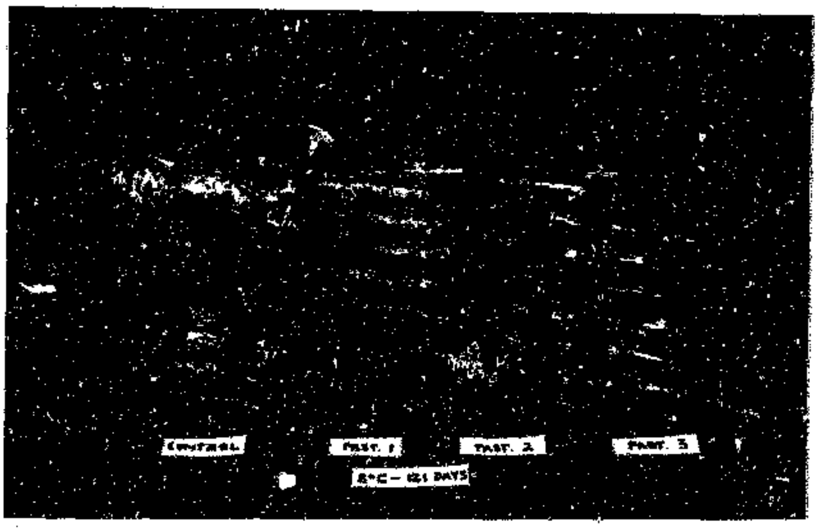

Fig. 3.1.15 Appearance of non-pasteurized (CONTROL) and pasteurized (PAST1-3) vacuum-packaged vienna sausages after 121 days storage at 8°C. Microbiological shelf life for all pasteurization treatments was surpassed. Microbiological counts in PAST1,2&3 packs reached <u>ca</u>. log 8,0 CFUg^{-1}.

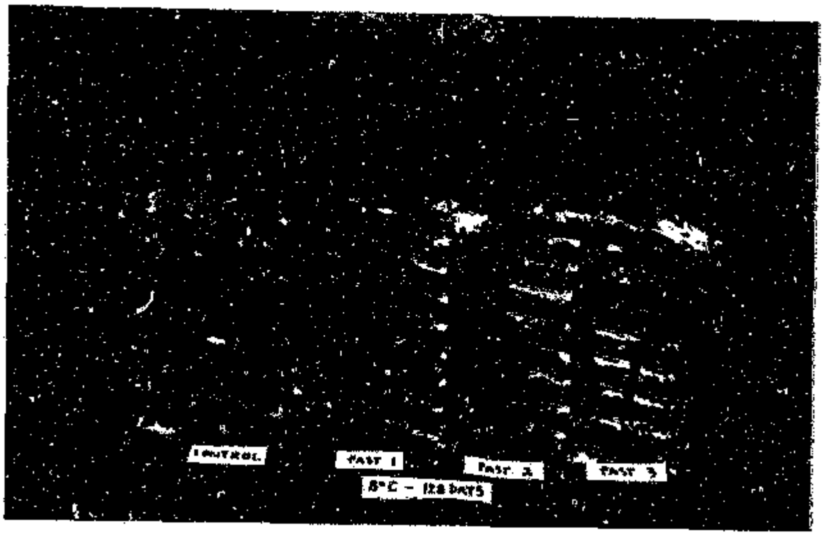

Fig. 3.1.16 Appearance of non-pasteurized (CONTROL) and pasteurized (PAST1-3) vacuum-packaged vienna sausages after 128 days storage at 8°C. Samples of all pasteurization treatments showed spoilage symptoms.

Table 3.1.1 Growth limiting a_w, pH and minimum temperature and salt concentration values for proteolytic and non-proteolytic *Clostridium* (*C.*) *botulinum* and *C. perfringens*[a].

Growth limiting factors	Proteolytic *C. botulinum*	Nonproteolytic *C. botulinum*	*C. perfringens*
a_w	0,94	0,97	0,95-0,97
NaCl (%)	10	5	7-8
pH	4,6	5,0	5,0
Temperature (°C)	10 Types A & B	3,5 Types B, E & F	15

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[a] Adapted from Riemann *et al.*(1972), Sperber (1982), Hauschild, (1989) and Labbe (1989).

Table 3.1.2 F_T-value parameters and determinations[a] for three in-package pasteurization processes at 60°C (PAST1) and 63°C (PAST2 and 3) applied to single layered 400g vacuum-packaged vienna sausage (VPVS) packs[b].

F_T-value parameters & determination	Pasteurization treatments		
	PAST1	PAST2	PAST3
Sausage layers per pack	1	1	1
Mass of a VPVS pack (g)	400	400	400
Initial no. ($CFUg^{-1}$) lactic acid bacteria per pack[c]	10^3	10^3	10^3
Lactic acid bacteria numbers per pack after pasteurization	1000>CFU>10	10 CFU	1 CFU in one of thousand packs
F_T-value calculation	ND[d]	42sec[e] (log 10^3 $CFUg^{-1}$ x 400g - log 10 CFU)	42sec[e] (log 10^3 $CFUg^{-1}$ x 400g - log 10^{-3} CFU)
F_T-value (minutes)	ND	3	6
Sausage core temperature (°C)	60	63	63

[a] After Mueller (1989).

[b] See Fig. 3.1.1 for 400g VPVS packs.

[c] Determined from replicate plate counts on MRS Agar (BIOLAB).

[d] Not determined.

[e] Upper limit of _in vitro_ D_{63} 95% confidence interval for meat spoilage _Lactobacillus_ _sake_ isolate (ch. 2.2).

Table 3.1.3 Mean sausage core and water cooker temperatures during three in-package pasteurization treatments (PAST1, 2 and 3)[a] of single layered, 400g packages of vacuum-packaged vienna sausages.

Time (minutes)	Pasteurization temperature (°C)					
	PAST1		PAST2		PAST3	
	Water	Sausage core[b]	Water	Sausage core[b]	Water	Sausage core[b]
0	66,7	11,0	68,5	12,4	67,8	11,1
2	65,9	35,5	66,8	34,8	66,0	39,3
4	66,9	49,4	67,0	48,1	67,5	51,3
6	68,3	55,6	66,9	55,9	67,7	57,2
8	68,7	60,7	67,0	59,9	67,8	60,9
10	ND[c]	ND	66,7	62,0	67,5	62,8
12	ND	ND	66,7	63,6	67,4	64,0
14	ND	ND	66,5	63,9	67,2	65,0
16	ND	ND	ND	ND	67,3	64,2
Water[d]	67,3	-	67,0	-	67,4	-
All on ice[e]	-	11,5 min	-	16 min	-	18,5 min

[a] For pasteurization parameters see Table 3.1.2.

[b] Mean core temperature of two individual sausages in the middle of an individual pack.

[c] Not determined.

[d] Mean water bath temperature (°C).

[e] Time (minutes) elapsed until last sausage pack was put on ice.

Table 3.1.4 Incubation conditions and culture media for microbiological counts of in-package pasteurized and non-pasteurized vacuum-packaged vienna sausages.

Microbiological count	Incubation			Growth medium
	Time (hours)	Temperature (°C)	Atmosphere	
Total aerobic plate count (TAPC)	72	25	Aerobic	Standard One Nutrient Agar (BIOLAB)
Lactic acid bacteria count (LABC)	72	25	Aerobic	MRS Agar (BIOLAB) + 0,1% (w/w) cysteine monohydrochloride (MERCK) + 0,2% (w/v) potassium sorbate (UNILAB) pH 5,7
Enterobacteriaceae count (EC)	18-24	30	Aerobic	Violet Red Bile Glucose Agar (OXOID)
Yeast count (YC)	96	25	Aerobic	Potato Dextrose Agar (OXOID) pH 3,7 (10% tartaric acid)

Table 3.1.5 Culture media, incubation conditions and characterization tests used for the enrichment, selective isolation, purification and identification of *Listeria* and *Clostridium* from vacuum-packaged vienna sausages.

Bacterium	*Listeria*	*Clostridium*
Enrichment	*Listeria* Enrichment Broth (OXOID) 30°C, aerobic, 48 hours	Cooked Meat Broth 37°C, anaerobic, 48 hours
Selective isolation	Oxford Formulation *Listeria* Selective Agar (OXOID) 30°C, aerobic, 48 hours	TSC Perfringens Agar (OXOID) + egg yolk free TSC Agar overlay 37°C, anaerobic, 24 hours
Presumptive identification	Colony morphology (OXOID Manual, 1990) esculin hydrolysis	Sulphite reduction, lecithinase production in basal agar Proteolytic isolates: gas and proteolysis of sausage sample
Purification	Oxford Formulation *Listeria* Selective Agar 30°C, aerobic, 24 hours	First on TSC Agar then Reinforced Clostridial Agar (RCA) (OXOID) 37°C, anaerobic, 24 hours
Identification tests	Gram stain, morphology, motility, catalase, esculin hydrolysis, nitrate reduction, growth in 40% esculin bile and at 4°C, Voges-Proskauer, Hemolysis of sheep red blood cells, CAMP-test with *S. aureus*, carbohydrate fermentations: glucose, lactose maltose, sucrose, xylose, rhamnose, mannitol	According to methods of Holdeman *et al.* (1977) omitting testing for motility, growth on bile agar, digestion of meat and utilization of amygdalin, arabinose, cellobiose, erythritol glycogen, inositol, melezitose, raffinose and rhamnose

Table 3.1.6 Initial microbial numbers of non-pasteurized (CONTROL) and in-package pasteurized (PAST1 - 3) vacuum-packaged vienna sausages.

Treatment	Microbiological count (log CFUg^{-1})			
	LABC[a]	TAPC	EC	YC
CONTROL	3,04	3,46	2,49	0,35
PAST1[b]	0,70	<0,35	<0,35	<0,35
PAST2	0,35	1,24	<0,35	<0,35
PAST3	0,59	0,70	<0,35	<0,35

[a] For abbreviations see Table 3.1.4.

[b] For pasteurization parameters see Table 3.1.2.

Table 3.1.7 Microbiological shelf life (MSL) comparisons of non-pasteurized (CONTROL) and in-package pasteurized (PAST1 - 3) vacuum-packaged vienna sausages.

Microbiological count type[a]	Treatment	"Mean" MSL[b] (days)	"Mean" MSL increase (fold)	"Worst case"[c] MSL (days)	"Worst case" MSL increase (fold)
LABC	CONTROL	7	-	6	-
	PAST1[d]	67	10	48	8
	PAST2	99	14	91	15
	PAST3	119	17	113	19
TAPC	CONTROL	7	-	6	-
	PAST1	67	10	48	8
	PAST2	100	14	97	16
	PAST3	119	17	113	19

[a] For abbreviations see Table 3.1.4.

[b] Time taken for bacterial counts (LABC and TAPC) to reach $5x10^6$ $CFUg^{-1}$.

[c] Value derived from duplicate sample showing the higher bacterial count.

[d] For pasteurization parameters see Table 3.1.2.

Table 3.1.8 Incidence[a] of *Listeria* and *Clostridium* in non-pasteurized (CONTROL) and in-package pasteurized (PAST1 - 3)[b] vacuum-packaged vienna sausages during storage at 8°C for 18 weeks.

Storage time	Listeria				Clostridium			
	CONTROL	PAST1	PAST2	PAST3	CONTROL	PAST1	PAST2	PAST3
Week 1	1/2	ND[c]	ND	ND	ND	1/2	2/2	2/2
Week 2	ND	ND	ND	ND	ND	*1/2	2/2	2/2
Week 3	ND	ND	ND	ND	ND	2/2	1/2	*2/2
Week 5	ND	ND	ND	ND	ND	ND	*2/2	*1/2
Week 7	ND	ND	ND	ND	ND	ND	ND	*1/2
Week 18	ND	ND	ND	ND	1/2	*1/2	ND	1/2
Total no. of packs	12	12	12	12	12	12	12	12
Total % positive packs	8,3%	-	-	-	8,3%	41,7%	58,3%	75,0%

[a] Number of positives from duplicate packs of VPVS, expressed as percentage of packs analyzed per treatment.

[b] For pasteurization parameters see Table 3.1.2.

[c] Not detected.

* Indicates presence of *C. perfringens* type A (isolate from PAST2 treated sausages from the 18th week of storage was not typable).

3.2 CHARACTERIZATION OF BACTERIAL POPULATIONS ASSOCIATED WITH PASTEURIZED AND NON-PASTEURIZED VACUUM-PACKAGED VIENNA SAUSAGES

ABSTRACT

Predominant bacterial populations associated with vacuum-packaged vienna sausages exposed to three different in-package pasteurization treatments of increasing severity (PAST1 2 and 3, respectively) and non-pasteurized controls were isolated during product storage at 8°C. The majority (> 52%) of isolates from pasteurized and non-pasteurized sausages were lactic acid bacteria, while the remainder were non-lactic acid bacteria and included Bacillus strains, Gram-positive, catalase positive cocci and Enterobacteriaceae. Non-lactic acid bacteria and pediococci were isolated in higher proportions from Standard One Nutrient Agar, while lactobacilli and leuconostocs were isolated in higher proportions from Modified MRS Agar plates. The type of medium from which isolates were recovered therefore affected the proportions of lactic and non-lactic acid bacteria in ecological studies. In-package pasteurization of sausages reduced proportions of LAB from 84,4% in non-pasteurized samples to 74,6%, 66,4% and 52,9% in PAST1, 2 and 3 treated samples, respectively. In non-pasteurized sausages leuconostocs and homofermentative lactobacilli jointly comprised the highest proportion (83,3%) of LAB isolates, while pediococci were isolated at a lower frequency of 16,7%. As a result of all pasteurization treatments proportions of leuconostocs and homofermentative lactobacilli decreased to ca. 40% of isolates, while proportions of pediococci increased to 50% or more. Although pasteurization led to diversification of the lactic spoilage populations, it did not prevent product spoilage by eliminating these bacteria. Since severe heating increased predominance of potentially pathogenic Bacillus spp. to 32,4% of total bacterial isolates, pasteurization of vacuum-packaged vienna sausages might compromise product safety.

3.2.1 INTRODUCTION

Despite storage of vacuum-packaged processed meats at refrigeration temperatures, these products are still prone to microbiological spoilage. Spoilage of such products was linked to the rapid growth of lactic acid bacteria (LAB) (Allen and Foster, 1960; Kempton and Bobier, 1970; Mol *et al.*, 1971; Fruin *et al.*, 1978; Paradis and Stiles, 1978; Egan *et al.*, 1980; Mukherji and Qvist, 1981; Egan, 1983; Nielsen, 1983; Korkeala *et al.*, 1987; Borch *et al.*, 1988). Lactic acid bacteria are ideally suited for growth in vacuum-packaged, refrigerated processed meats since they are both psychrotrophic and able to tolerate the microaerophilic conditions prevailing within vacuum-packs (Enfors and Molin, 1980; Reuter, 1981; Buchanan, 1986; Holzapfel and Gerber, 1986). Furthermore, LAB are tolerant to the concentrations of salt and nitrite commonly added to processed meats (Egan, 1983; Dodds and Collins-Thompson, 1984).

Other bacterial groups such as Enterobacteriaceae or yeasts are unable to dominate the spoilage ecology of vacuum-packaged processed meats (Fruin, *et al.*, 1978; Paradis and Stiles, 1978; Nielsen, 1983; Schneider *et al.*, 1983; Zurera-Cosano *et al.*, 1988; von Holy *et al.*, 1991a). Enterobacteriaceae are inhibited by microbially produced lactic acid and the consequent pH decreases which occur during the growth of LAB in these food products (Davidson and Webb, 1973; Shay *et al.*, 1978; Grau, 1981; Gill and Newton, 1982; Nielsen and Zeuthen, 1985). Since yeasts can tolerate lactic acid and pH's of *ca*. 5,0 (Buchanan, 1986; Beuchat and Golden, 1989), their inability to dominate in the spoilage ecology of vacuum-packaged processed meats results from other factors such as slower growth rates and inefficient substrate utilization (von Holy *et al.*, 1991a).

Although pathogenic bacteria such as clostridia, salmonellae, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphylococcus aureus* have been reported in vacuum-packaged processed meat products, their growth could be successfully inhibited by competitive LAB populations, incorporation of nitrite and storage at refrigeration temperatures (Weissman and Carpenter, 1969; Riemann et al., 1972; Duitschaever, 1978; Fruin et al., 1978; Paradis and Stiles, 1978; Christiansen, 1980; Shahamat et al., 1980; Sofos and Busta, 1980; Tiwari and Kadis, 1981; Nielsen, 1983; Nielsen and Zeuthen, 1984a,b; Nielsen and Zeuthen, 1985; Kokubo et al., 1986; Rorvik and Yndestad, 1991). Furthermore, LAB produce antimicrobial compounds termed bacteriocins, which inhibit the growth of pathogens such as Listeria monocytogenes, Staphylococcus aureus and clostridia (Klaenhammer, 1988; Harris et al., 1989; Schillinger and Lücke, 1989; Spelhaug and Harlander, 1989; Schillinger and Holzapfel, 1990; Berry et al., 1991; Hastings and Stiles, 1991; Okereke and Montville, 1991; Schillinger et al., 1991; Degnan et al., 1992). Bacteriocinicity of LAB may, therefore, further explain why pathogenic bacteria fail to establish themselves in the spoilage ecology of processed meat products.

Numerous studies on the identities of bacteria involved in spoilage of a variety of vacuum-packaged processed meat products have shown that the lactobacilli and leuconostocs predominate in the spoilage association to varying degrees (Allen and Foster, 1960; Reuter, 1969; Laleye et al., 1984; Holzapfel and Gerber, 1986; Morishita and Shiromizu, 1986; Schillinger and Lücke, 1987; Borch and Molin, 1988). These bacteria, however, were associated with closely related LAB such as pediococci, enterococci, streptococci and carnobacteria (Cavett, 1963; Reuter, 1981; Schillinger and Lücke, 1987; Borch and Molin, 1988).

Spoilage of South African vacuum-packaged vienna sausages (VPVS) was also attributed to the growth of LAB to high numbers during refrigerated storage (von Holy et al., 1991b; von Holy and Cloete, 1992). Since populations of LAB in spoiled VPVS samples were dominated by homofermentative lactobacilli and leuconostocs, which collectively comprised 94% of total LAB isolates, it was proposed that control measures to reduce VPVS spoilage should be aimed specifically at inactivating these bacteria (von Holy et al., 1991b; von Holy et al., 1992). In-package pasteurization of VPVS was suggested as a control measure, since the packaging material would prevent product recontamination and spoilage, therefore, could only be caused by microorganisms surviving the pasteurization process (von Holy et al., 1991a). In-package pasteurization of VPVS previously delayed product spoilage and increased the shelf life ca. fourfold (Marshall, 1991; von Holy et al., 1991a).

Even though pasteurization of VPVS successfully increased product shelf life, LAB still predominated among spoilage populations from pasteurized products (Marshall, 1991; von Holy et al., 1991a). Proportions of homofermentative lactobacilli and leuconostocs collectively decreased by 12,5% (von Holy and Holzapfel, 1991) or 30,8% (Marshall, 1991) following product pasteurization, but these bacteria still predominated and collectively comprised 71,5% (von Holy and Holzapfel, 1991) or 66,6% (Marshall, 1991) of LAB spoilage populations of pasteurized sausages stored at 7°C and 8°C, respectively. Pasteurization of VPVS, however, also led to a diversification in spoilage LAB populations (Marshall, 1991; von Holy and Holzapfel, 1991). Proportions of heterofermentative lactobacilli, enterococci, streptococci and pediococci increased in pasteurized products by ca. 3 to 5% (von Holy and Holzapfel, 1991). Marshall (1991), however, found proportions of only the pediococci to increase by 32,4% in pasteurized VPVS.

Enterobacteriaceae and yeasts were unable to establish themselves in the spoilage ecology of both non-pasteurized and pasteurized VPVS (Marshall, 1991; von Holy et al., 1991a). While Bacillus strains were also unable to establish themselves as predominant spoilage populations in either pasteurized or non-pasteurized VPVS, these bacteria could be isolated at higher frequencies from pasteurized products (von Holy, 1989). Marshall (1991) showed that proportions of Bacillus strains isolated from VPVS before reaching the microbiological shelf life (5×10^6 CFUg^{-1}), increased from 43% in non-pasteurized to 69% of bacterial isolates in pasteurized products. No Bacillus strains could, however, be isolated from either pasteurized or non-pasteurized products after reaching the microbiological shelf life, and it was concluded that these bacteria did not predominate in spoiled products (Marshall, 1991).

In this study, sausages were packed in single layers and higher sausage core temperatures were achieved when compared to those of Marshall (1991) and von Holy et al. (1991a) (ch. 3.1). This study, therefore, aimed to assess the role of LAB in predominant spoilage populations of pasteurized VPVS stored at 8°C and quantify inactivation of homofermentative lactobacilli and leuconostocs.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Isolation of predominant bacterial populations from vacuum-packaged vienna sausages

Colonies of predominant bacteria were isolated from Modified MRS Agar (MRS Agar (BIOLAB) + 0,1% w/w cysteine monohydrochloride (MERCK) + 0,2% w/v potassium sorbate (UNILAB)) and Standard One Nutrient Agar plates (STD1). Modified MRS and STD1 Agar plates were used for lactic acid bacteria counts (LABC) and total aerobic plate counts (TAPC), respectively, of VPVS that were non-pasteurized or pasteurized by three increasingly severe treatments (PAST1, 2 and 3; see ch. 3.1) and stored for 128 days at 8°C (ch. 3.1). Colonies were isolated from duplicate Modified MRS and STD1 plates used to determine LABC and TAPC of VPVS. For each VPVS sample, one colony was randomly picked from each of duplicate MRS and STD1 Agar plates of the highest dilution showing growth (von Holy, 1989; von Holy and Holzapfel, 1991). Colonies were purified on the medium from which they were isolated.

3.2.2.2 Characterization of predominant bacteria associated with pasteurized and non-pasteurized vacuum-packaged vienna sausages

The 467 predominant bacterial isolates (237 from Modified MRS Agar plates and 230 from STD1 Agar plates) were divided into Gram-positive and Gram-negative. Gram-negative bacteria were not identified further. Gram-positive isolates were further divided into lactic acid bacteria (LAB) and non-lactic acid bacteria (non-LAB) on the basis of catalase production. All aerobic, Gram-positive, catalase positive bacteria exhibiting rod shaped morphologies were assigned to the genus Bacillus (Fischer et al., 1986). Gram-positive, catalase positive cocci and Bacillus spp. were not identified further.

To assess the effect of pasteurization on relative proportions of lactic and non-lactic acid bacteria, populations from non-pasteurized (control) sausages were compared to those of PAST1, 2 and 3 treated sausages.

In order to determine changes in predominance of Enterobacteriaceae, Bacillus and Gram-positive, catalase positive cocci, proportions of these bacteria recovered during the first half of the storage interval (0 to 64 days) were compared to those obtained over the second half (65 to 128 days) of VPVS storage at 8°C. These intervals were chosen since PAST1, 2 and 3 sausages did not exceed microbiological shelf life (5×10^6 $CFUg^{-1}$, see ch. 3.1) during the first half of the storage interval. Bacterial populations isolated from this interval were thus not considered to comprise climax spoilage populations. Sausages of all pasteurization treatments, however, reached microbiological shelf life during the second half of the 128 days storage interval (see ch. 3.1), and corresponding populations were thus regarded as climax spoilage populations.

To determine the effect of culture medium on isolation of relative proportion of non-LAB, populations from Modified MRS Agar plates were compared to those from STD1 Agar plates.

3.2.2.3 Characterization of predominant lactic acid bacteria associated with pasteurized and non-pasteurized vacuum-packaged vienna sausages

Lactic acid bacteria were subdivided into six biogroups viz. heterofermentative lactobacilli (group I), leuconostocs (group II), homofermentative lactobacilli (group III), Pediococcus (group IVA), Streptococcus/Enterococcus (group IVB), Carnobacterium (group V) and thermobacteria (group VI) on the basis of six key characteristics i.e. morphology by phase contrast microscopy, growth at 15 and 45°C, gas production from glucose, hydrolysis of arginine and lactic acid enantiomers produced (Schillinger and Lücke, 1987; von Holy et al., 1991b; Fig. 3.2.1). Bacterial morphologies prompted division of isolates into biogroups. To illustrate different lactic acid bacteria morphologies, cultures were prepared for phase contrast microscopy and photographed as described in ch. 2.2. Isolates showed typical LAB morphologies ranging from cocci over coccobacilli to short and long rods (Sharpe, 1979; Dykes, 1991; Figs. 3.2.2 a to f & 3.2.3 g to j) and divided in one plane only, with the exception of pediococci which characteristically divided in two planes (Figs. 3.2.2 a,b) (Sharpe, 1979).

In order to asses the effect of pasteurization on relative proportions of LAB biogroups, LAB populations from non-pasteurized (control) sausages were compared to those of pasteurized sausages.

To determine changes in predominance of LAB biogroups from pasteurized sausages with storage time, proportions of LAB groups obtained over the first half of the storage interval (0 to 64 days) were compared to those obtained over the second half (65 to 128 days) of the period of storage of pasteurized VPVS at 8°C. These intervals were chosen for reasons mentioned previously (3.2.2.2). To determine the effect of the culture medium on composition of predominant LAB populations, LAB biogroup proportions from Modified MRS Agar plates were compared to those from STD1 Agar plates.

3.2.3 RESULTS AND DISCUSSION

3.2.3.1 Effect of pasteurization on predominance of lactic acid bacteria in vacuum-packaged vienna sausages

Proportions of predominant LAB isolated from pasteurized and non-pasteurized VPVS are shown in Fig. 3.2.4. Proportions of LAB decreased with increasing severity of the pasteurization treatment. Lactic acid bacteria were isolated from non-pasteurized (control) vienna sausages at a frequency of 84,4% (Fig. 3.2.4), but their proportions decreased to 74,6%, 66,4% and 52,9% in PAST1, PAST2 and

PAST3 treated sausages, respectively. Lactic acid bacteria, therefore, predominated both in pasteurized and non-pasteurized VPVS, since they comprised more than 50% of total isolates.

Previous reports of LAB predominating in spoilage populations of vacuum-packaged, non-pasteurized emulsion-type sausages correlated well with the findings in this study (Mukherji and Qvist, 1981; Egan, 1983, Nielsen, 1983; Korkeala et al., 1985; 1987; Borch et al., 1988; Zurera-Cosano et al., 1988; von Holy et al., 1991b; von Holy and Cloete, 1992). These results were, furthermore, in agreement with an earlier report that LAB predominated in spoilage populations of both non-pasteurized and pasteurized VPVS (von Holy et al., 1991a). The fact that LAB predominated in bacterial populations of non-pasteurized, PAST1 and PAST2 treated sausages also correlated well to our previous findings that these bacteria determined the shelf life of non-pasteurized, PAST1 and PAST2 treated VPVS (ch. 3.1).

For PAST3 treated sausages, however, only slightly more than half (52,9%) of the bacteria isolated over the storage period were LAB (Fig. 3.2.4). Clearly, therefore, the spoilage association of these products included a considerable (47,1%) non-lactic component. This finding corresponded to results of ch. 3.1., where statistically significant differences between total aerobic plate counts and lactic acid bacteria counts over the entire storage period were noted. This indicated that other bacteria significantly reduced the predominance of LAB in PAST3 treated VPVS. The statement of Reuter (1969) that LAB became the predominant spoilage populations of vacuum-packaged processed meats, regardless of initial contamination levels, was found true for non-pasteurized, PAST1 and PAST2 treated VPVS. For PAST3 treated sausages, however, this statement was arguable and although LAB still predominated at 52,9% of total isolates, this predominance was considerably reduced. If numbers of initial contaminating LAB are therefore reduced sufficiently by a heat process, they may fail to establish themselves as predominant spoilage populations.

3.2.3.2 Effect of pasteurization on predominance of non-lactic acid bacteria in vacuum-packaged vienna sausages

Proportions of non-LAB isolated from pasteurized and non-pasteurized VPVS are shown in Fig. 3.2.4. Proportions of non-LAB increased with increasingly severe pasteurization treatment. While non-LAB constituted 15,6% of isolates from non-pasteurized (control) VPVS, their frequencies of isolation increased to 25,4, 33,6 and 47,1% in PAST1, PAST2 and PAST3 treated sausages, respectively. Increasing predominance of non-LAB was expected to occur with increasing severity of pasteurization, since non-LAB would, for example, include spore forming Bacillus strains which can produce spores able to survive heat treatments lethal to vegetative cells (Silliker et al., 1980; Houben, 1982; Nielsen and Zeuthen, 1984a,b). The effect of pasteurization on proportions of different groups of non-LAB will now be discussed in detail.

3.2.3.2.1 Effect of pasteurization on predominance of Bacillus in vacuum-packaged vienna sausages

Proportions of Bacillus strains increased from 3,9% of total isolates from non-pasteurized sausages to 5,9%, 16,0% and 32,4% of total bacterial isolates of PAST1, 2 and 3 treated sausages, respectively (Fig. 3.2.4). Bacillus spp. have previously been shown to be unable to dominate the spoilage ecology of non-pasteurized VPVS (von Holy, 1989), which correlated well to the findings of this study which identified only 3,9% of total isolates as Bacillus strains. The inability of Bacillus spp. to dominate the spoilage of vacuum-packaged processed meats could be due to antimicrobial activity of LAB growing in such products (Spelhaug and Harlander, 1989; Marshall, 1991; Papathanasopoulos et al., 1991). Increasing proportions of Bacillus in pasteurized products, however, suggested these bacteria were present in the thermotolerant spore form upon pasteurization of VPVS. This bestowed a competitive advantage upon Bacillus strains to establish themselves in the spoilage association of pasteurized VPVS. The finding that Bacillus and their spores are frequently introduced to processed meats by ingredients such as flour, sugar and spices (Lynch and Potter, 1988) could, therefore, explain their predominance in heated products.

While increased proportions of Bacillus spp. in bacterial populations of VPVS upon pasteurization were previously noted, LAB still comprised predominant spoilage populations of pasteurized products (von Holy, 1989). The role of Bacillus in the spoilage ecology of pasteurized VPVS was, therefore, concluded to be of little significance (von Holy, 1989). Marshall (1991) found proportions of Bacillus spp. to increase from 43% of total bacterial isolates in non-pasteurized VPVS to 69% of total isolates in pasteurized VPVS. This predominance in Bacillus strains was, however, associated with the initial stages of storage and once pasteurized and non-pasteurized samples reached the microbiological shelf life limit ($5x10^6$ $CFUg^{-1}$), LAB populations predominated and no further Bacillus strains were isolated (Marshall, 1991). Similarly, Bacillus spp. in this study were isolated at frequencies of 23,3%, 47,5% and 60,4% of non-LAB isolates from PAST1, 2 and 3 samples, respectively, in the initial storage interval of 64 days (Table 3.2.1). No Bacillus could be isolated from PAST1 and PAST2 samples, and only 8,3% of total non-LAB isolates comprised Bacillus spp. in PAST3 samples, during the second half of the storage interval (65 to 128 days) (Table 3.2.1). Since pasteurized samples only reached the microbiological shelf life limit ($5x10^6$ $CFUg^{-1}$, ch. 3.1) and became organoleptically spoiled during the second half of the storage interval, our results complemented those of von Holy (1989) and Marshall (1991).

Increasing incidences in Bacillus spp. in pasteurized VPVS, on the other hand, raised concern since this genus contains Bacillus cereus which is a foodborne pathogen. This pathogen is widely distributed in nature and has been implicated in several outbreaks of food poisoning (Goepfert et al., 1972; Konuma et al., 1988). Furthermore, the pathogen has previously been isolated at frequencies of 18,3% from meat products such as sausages, hams, bacon, meat balls and hamburgers (Konuma et al., 1988). Since pasteurization of vienna sausages enhanced proportions of Bacillus isolates to up to ca. one third of the predominant bacterial populations (Fig. 3.2.4), a potential for growth of B. cereus in pasteurized VPVS existed and this might compromise product safety. This is especially so since pasteurization also decreased numbers and growth of spoilage LAB (ch. 3.1) which are inhibitory to the growth of Bacillus strains (Spelhaug and Harlander, 1989). Further studies on identities of Bacillus isolates from pasteurized VPVS would, therefore, be required to determine whether these products could pose a threat to consumer health.

3.2.3.2.2 Effect of pasteurization on predominance of Enterobacteriaceae and Gram-positive, catalase positive cocci

Gram-negative bacteria were consistently isolated from both non-pasteurized and pasteurized VPVS, but at relatively low proportions of ca. 5 to 8% of total isolates (Fig. 3.2.4). Failure of Enterobacteriaceae to dominate the spoilage ecology of non-pasteurized, vacuum-packaged processed meats was reported previously (Fruin et al., 1978; Paradis and Stiles, 1978; Nielsen, 1983; Zurera-Cosano et al., 1988; von Holy et al., 1991a) and correlated well to findings of this study. Our results furthermore correlated well with our earlier findings that numbers of Enterobacteriaceae remained low (< log 2,0 $CFUg^{-1}$) in non-pasteurized VPVS for most of the storage period (ch. 3.1), and that these bacteria, therefore, failed to establish themselves in the spoilage association.

The recovery of Enterobacteriaceae at low proportions from pasteurized samples did not compare to previous findings, where these bacteria were not recovered from pasteurized VPVS (von Holy, 1989). Although Gram-negative bacteria are known to be more heat sensitive than Gram-positive bacteria such as LAB (Jay, 1986), the consistent presence of Enterobacteriaceae suggested the presence of strains with increased heat resistance that could survive pasteurization of VPVS. Comparisons of Enterobacteriaceae proportions occurring during the two storage periods (0 to 64 and 65 to 128 days, respectively - Table 3.2.1), however, showed that these bacteria were predominantly isolated up to 64 days storage. This result compared well to our earlier findings that numbers of Enterobacteriaceae in pasteurized sausages had decreased to below detectable limits (< log 1,00 CFUg^{-1}) after 68 days storage at 8°C. These findings, therefore, supported the suggestion that Enterobacteriaceae of unusual heat resistance might have been present, since this would have bestowed an initial advantage to these bacteria to establish themselves in the spoilage ecology. Antimicrobial activity of LAB towards Enterobacteriaceae by way of pH inhibition has been well documented (Daly et al., 1972; Nielsen and Zeuthen, 1985) and could explain why these bacteria failed to establish themselves in the climax spoilage association of both non-pasteurized and pasteurized VPVS.

Proportions of Gram-positive, catalase positive cocci increased from 5,5 to ca. 10% of total isolates after pasteurization of VPVS (Fig. 3.2.4). Increasing proportions of these bacteria in pasteurized, vacuum-packaged processed meats have not been reported before. It was speculated that these bacteria were more heat resistant than LAB, and that pasteurization allowed their establishment in the spoilage association. Isolation frequencies with respect to storage period, however, showed that Gram-positive, catalase positive cocci were isolated at higher frequency (16,6 to 43,3% of total non-LAB isolates) during the first half, when compared to the second half of the storage period (0 to 4,2% of total non-LAB isolates) of pasteurized VPVS (Table 3.2.1). This implied that Gram-positive, catalase positive cocci failed to establish themselves in the climax spoilage association of both pasteurized and non-pasteurized VPVS, as did Bacillus spp. and Enterobacteriaceae. As before, this probably resulted from inhibition by LAB, which have been shown to have bacteriocinogenic activity against Gram-positive cocci such as Micrococcus and Staphylococcus aureus strains (Spelhaug and Harlander, 1989; Papathanasopoulos et al., 1991; Sobrino et al., 1991).

Although pathogenic strains of Enterobacteriaceae (e.g., Salmonella , Yersinia enterocolitica) and Gram-positive, catalase positive bacteria (e.g., Staphylococcus aureus) have previously been isolated from processed meats (Weissman and Carpenter, 1969; Duitschaever, 1978; Tiwari and Kadis, 1981; Nielsen and Zeuthen, 1985), their growth in such products was inhibited by control factors such as nitrite, low storage temperatures and competitive LAB populations (Paradis and Stiles, 1978; Tiwari and Kadis, 1981; Nielsen and Zeuthen, 1984a,b; 1985). Since proportions of Gram-negative and Gram-positive, catalase positive cocci were low and declined with storage time in pasteurized VPVS, growth of these bacteria seemed to be inhibited, possibly by a combination of factors named above. It was thus concluded that both the pasteurized and non-pasteurized VPVS studied here did not pose a health risk with respect to pathogenic Enterobacteriaceae and Gram-positive, catalase positive cocci.

3.2.3.3 Effect of culture media on composition of non-lactic acid bacteria populations from pasteurized and non-pasteurized vacuum-packaged vienna sausages

Proportions of non-LAB isolated from pasteurized and non-pasteurized VPVS on Modified MRS and STD1 Agar plates are shown in Table 3.2.2. Generally, non-LAB from both pasteurized and non-pasteurized sausages were isolated at higher proportions (> 67%) from STD1 Agar plates (Table 3.2.2). This was an expected trend since both media are non-selective, but MRS Agar was specially adapted to the specific growth requirements of LAB (de Man _et al._, 1960), whereas STD1 Agar is less complex and supports the growth of a wider variety of bacteria (MERCK Microbiology Manual, 1990). For quantification in ecological studies on non-LAB from pasteurized and non-pasteurized processed meats, therefore, use of a non-selective medium such as STD1 Agar was thought to be essential. Although proportions of non-LAB were isolated at a higher frequency from STD1 Agar plates, it was interesting to note that proportions of _Bacillus_ and Gram-negative bacteria marginally increased on MRS Agar plates after product pasteurization (Table 3.2.2). For proportions of Gram-positive, catalase positive cocci, however, no such consistent increases were observed. Proportions of _Bacillus_ isolates, for example, increased on Modified MRS Agar plates from 10,0% in control samples to 20,8% in PAST3 samples. Proportions of Gram-negative bacteria on Modified MRS Agar plates increased from 0% in control samples to 2,1-3,3% in PAST1, 2 and 3 samples. This could be explained by the fact that competition in mixed microbial populations on Modified MRS Agar plates by LAB was reduced as a result of the heat treatment.

3.2.3.4 Effect of pasteurization on composition of lactic acid bacteria populations in vacuum-packaged vienna sausages

3.2.3.4.1 Effect of pasteurization on predominance of heterofermentative lactobacilli (I), enterococci and streptococci (IVB), carnobacteria (V) and thermobacteria (VI)

The composition of LAB populations of non-pasteurized (control) and pasteurized VPVS stored at 8°C is shown in Table 3.2.3. No enterococci (group IVB), streptococci (group IVB), carnobacteria (group V) or thermobacteria (group VI) were isolated from either pasteurized or non-pasteurized VPVS (Table 3.2.3). Furthermore, no heterofermentative lactobacilli (group I) were isolated from non-pasteurized and PAST3 treated VPVS (Table 3.2.3). Heterofermentative lactobacilli were, however, isolated from PAST1 and PAST2 treated VPVS, at proportions of 13,6% and 5,1% of total LAB isolates, respectively (Table 3.2.3).

Carnobacteria, enterococci and streptococci have previously been isolated from vacuum-packaged processed and non-processed meats (Cavett, 1963; Mol <u>et al</u>., 1971; Schillinger and Lücke, 1987, 1988; Borch and Molin, 1988; von Holy and Holzapfel, 1991; von Holy <u>et al</u>. 1991b ; von Holy and Cloete, 1992). Although von Holy and Holzapfel (1991), von Holy <u>et al</u>. (1991b) and von Holy and Cloete (1992) reported isolation of streptococci and enterococci from VPVS at low frequencies, they failed to isolate <u>Carnobacterium</u> from these products. Failure to isolate the above biogroups in this study clearly demonstrated that they did not predominate in spoilage LAB populations of pasteurized or non-pasteurized VPVS. Similarly, thermobacteria, as well as heterofermentative lactobacilli (from non-pasteurized and PAST3 treated VPVS) did also not dominate the spoilage populations of these treatments. Thermobacteria, however, were expected not to grow in refrigerated, processed meats, since failure to grow at

15°C or lower is a diagnostic feature of this biogroup (Sharpe, 1962). Thermobacteria, enterococci, streptococci, heterofermentative lactobacilli and carnobacteria could, therefore, not have contributed significantly to spoilage of both pasteurized and non-pasteurized VPVS.

Observations that heterofermentative lactobacilli showed marked proportional increases from 0% of isolates (non-pasteurized sausages) to 13,6% and 5,1% (PAST1 and PAST2 treated sausages, respectively) (Table 3.2.3), were in agreement with previous findings where proportions of these bacteria increased from 4,9% of LAB isolates from non-pasteurized sausages to 10,0% of LAB isolates from pasteurized sausages stored at 7°C (von Holy and Holzapfel, 1991). These results suggested that heterofermentative LAB were more heat resistant than homofermentative lactobacilli and leuconostocs, since proportions of the latter biogroups decreased in PAST1 and PAST2 treated sausages (Table 3.2.3). This could have enabled the heterofermentative LAB to establish themselves in the spoilage associations of PAST1 and PAST2 pasteurized sausages. Several studies indeed showed that strains of heterofermentative lactobacilli such as Lactobacillus viridescens exhibited unusual thermotolerance (Milbourne, 1983; Borch et al., 1988) and were more heat resistant than homofermentative lactobacilli from similar environments (Borch et al., 1988). Failure to isolate heterofermentative lactobacilli from PAST3 sausages, however, suggested that members of this biogroup were unable to survive the prolonged heat treatment applied to PAST3 sausages (ch. 3.1.) and were therefore less heat resistant than pediococci. Pediococci were still at a high proportion of 57,4% of isolates from PAST3 treated sausages (Table 3.2.3).

Homofermentative lactobacilli and leuconostocs persisted in the spoilage ecology of PAST3 treated sausages, whereas heterofermentative lactobacilli did not. This could be explained by the former (although probably being more heat resistant than the latter) almost exclusively dominating (83,3% of isolates) the spoilage ecology of non-pasteurized VPVS (Table 3.2.3). Homofermentative lactobacilli and leuconostocs thus were expected to outnumber the heterofermentative lactobacilli even in PAST3 treated samples.

3.2.3.4.2 Effect of pasteurization on predominance of leuconostocs (II) and homofermentative lactobacilli (III)

Leuconostocs (group II) and homofermentative lactobacilli (group III) were isolated at the highest frequencies from non-pasteurized VPVS and jointly comprised 83,3% of LAB isolates (Table 3.2.3). Proportions of homofermentative lactobacilli decreased from 56,5% in non-pasteurized samples to <u>ca</u>. 30% of isolates from pasteurized samples. Similarly, proportions of leuconostocs decreased from 26,9% in non-pasteurized samples to 8,0-11,1% of LAB isolated from pasteurized samples (Table 3.2.3).

Homofermentative lactobacilli comprised the highest proportion of isolates from non-pasteurized VPVS and therefore dominated the spoilage ecology of these products. This finding was in agreement with other studies conducted on refrigerated processed and non-processed meats (Allen and Foster, 1960; Cavett, 1963; Mol <u>et</u> <u>al</u>., 1971; Laleye <u>et</u> <u>al</u>., 1984; Shaw and Harding, 1984; Holzapfel and Gerber, 1986; Morishita and Shiromizu, 1986; Borch and Molin, 1988; Korkeala <u>et</u> <u>al</u>., 1988; Korkeala and Mäkelä, 1989; Marshall, 1991; von Holy <u>et</u> <u>al</u>., 1991b; von Holy and Holzapfel, 1991). In this study, the leuconostocs comprised the second largest group of LAB isolates (26,9%) from non-pasteurized VPVS (Table 3.2.3). Leuconostocs were also reported to predominate in the spoilage ecology of processed and non-processed meats, although both higher and lower frequencies of isolation were reported (Egan, 1983; Borch and Molin, 1988; Korkeala and Mäkelä, 1989).

Von Holy and Holzapfel (1991) previously reported that leuconostocs and homofermentative lactobacilli jointly comprised 84% of 144 LAB isolated from non-pasteurized VPVS stored at 7°C. Similarly, Marshall (1991) found these bacteria to dominate in non-pasteurized VPVS stored at 8°C, collectively comprising 97,4% of 48 LAB isolates. Both these figures compared well to the 83,3% of this study. Our results thus confirmed previous findings that these biogroups dominated LAB spoilage populations of non-pasteurized VPVS and therefore were primarily responsible for spoilage of these products at refrigeration temperature.

Furthermore, von Holy and Holzapfel (1991) reported that homofermentative lactobacilli and leuconostocs still comprised predominant spoilage LAB populations from pasteurized VPVS, even though their proportions decreased to 71,5% of total isolates in these samples. Similarly, Marshall (1991) reported homofermentative lactobacilli and leuconostocs to decrease to a collective 66,6% of total LAB isolates upon pasteurization of VPVS. In this study, however, the homofermentative lactobacilli and leuconostocs from pasteurized samples jointly comprised only 36,4% (PAST1), 40,6% (PAST2) and 42,6% (PAST3) of total LAB isolates (Table 3.2.3). Greater reductions in proportions of these biogroups in this study were thought to result from packaging products into single layers within vacuum bags, thus achieving a higher sausage core temperature upon product pasteurization. By contrast sausages of the other studies (von Holy and Holzapfel, 1991; Marshall, 1991) were packed in double layers and poorly penetrated by heat. This is illustrated by reports that after pasteurization sausage core temperatures only reached 52 to 57°C (von Holy and Holzapfel, 1991; Marshall, 1991).

Even though joint proportions of homofermentative lactobacilli and leuconostocs decreased in pasteurized samples when compared to controls, their frequencies of isolation showed relative increases between pasteurized samples with increasingly severe pasteurization (PAST1, 2 and 3) (Table 3.2.3). Proportions of leuconostocs increased from 8,0% (PAST1) to 8,9% and 11,1% of total LAB isolates from PAST2 and PAST3 samples, respectively (Table 3.2.3). Similarly, proportions of homofermentative lactobacilli increased from 28,4% (PAST1) to 31,7% and 31,5% of total LAB isolates from PAST2 and PAST3 samples, respectively (Table 3.2.3). These relative increases could be explained by decreasing proportions of heterofermentative lactobacilli in PAST1, 2 and 3 treated samples (Table 3.2.3).

Since homofermentative lactobacilli and leuconostocs predominated in spoilage populations of non-pasteurized VPVS also in previous studies, control measures to prevent product spoilage were suggested to be aimed specifically at inactivating these biogroups (von Holy *et al.*, 1992). Consequently in-package pasteurization in this study was based on *in vitro* heat resistance data of a predominant *Lactobacillus sake* isolate from non-pasteurized VPVS (chs. 2.2 & 3.1). Our results clearly showed that reductions in proportions of these biogroups were successfully achieved by the pasteurization process, and that the predominance of these bacteria was lost in spoilage populations of pasteurized VPVS.

3.2.3.4.3 Effect of pasteurization on predominance of pediococci (IVA)

Pediococci (group IVA) comprised a frequency of 16,7% of isolates from non-pasteurized VPVS but showed marked proportional increases (>50% of isolates) in pasteurized samples (Table 3.2.3).

Although pediococci have often been reported to occur in conjunction with predominant spoilage lactobacilli and leuconostocs in processed or non-processed meat products (Reuter, 1981; von Holy and Holzapfel, 1991; von Holy *et al.*, 1991b; von Holy and Cloete, 1992), frequencies of isolation were usually lower than the 16,7% for non-pasteurized VPVS in this study. Low proportions of pediococci in non-pasteurized samples of this study suggested that these bacteria did not dominate spoilage populations of control samples. The marked proportional increase of pediococci in pasteurized samples correlated well to previous findings where proportions of pediococci increased from 4,9% to 9,2% (von Holy and Holzapfel, 1991) and from 0% to 32,4% (Marshall, 1991) of total LAB isolates after pasteurization of VPVS. Increases in proportions of pediococci and corresponding decreases in proportions of homofermentative lactobacilli and leuconostocs in pasteurized VPVS (Table 3.2.3) indicated that pediococci might be more heat resistant.

In our *in vitro* heat resistance studies (ch. 2.2), the

Pediococcus strain from spoiled, pasteurized VPVS was indeed more heat resistant than the *Leuconostoc mesenteroides* strain, but less heat resistant than the *Lactobacillus sake* strain. This *Pediococcus* strain was isolated from pasteurized double layer sausage packs (von Holy, 1989) in which lower sausage core temperatures were achieved than in this study. However, in a separate study (Morris, 1992) on *in vitro* heat resistance of predominant spoilage LAB isolated here from pasteurized VPVS (ch. 3.1), a representative *Pediococcus pentosaceus* spoilage isolate was *ca*. 8 to 9 times more heat resistant than representative homofermentative *Lactobacillus* and *Leuconostoc mesenteroides* isolates, respectively. This indicated that pediococci from pasteurized samples could indeed be more heat resistant than homofermentative lactobacilli and leuconostocs.

Pediococci were the dominant LAB biogroup associated with pasteurized VPVS in this study, since they were isolated at proportions of *ca*. 50 to 57% of total LAB from samples of all pasteurization treatments. This effect furthermore was more pronounced with increasing severity of the pasteurization treatment (Table 3.2.3). Pasteurization of sausages could, therefore, have afforded a competitive advantage to the heat resistant pediococci and enabled them to replace homofermentative lactobacilli and leuconostocs as predominant spoilage populations of VPVS.

3.2.3.5 Effect of storage interval on composition of predominant lactic acid bacteria populations in pasteurized, vacuum-packaged vienna sausages

Predominant lactic acid bacteria were isolated from pasteurized and non-pasteurized VPVS over 128 days at 8°C (3.2.2.1). It was, therefore, considered necessary to determine whether the predominance of pediococci in pasteurized VPVS occurred shortly after pasteurization and persisted throughout storage, or whether pediococci gradually replaced homofermentative lactobacilli and leuconostocs during storage. To this end, the frequencies of isolation of LAB from pasteurized and non-pasteurized VPVS samples from the first half of the storage interval (0 to 64 days) were compared with the second half of the storage interval (65 to 128 days) (Table 3.2.4).

In non-pasteurized VPVS homofermentative lactobacilli and leuconostocs jointly comprised 86,7% and 66,7% of LAB isolates from the first and second half of the storage interval, respectively (Table 3.2.4). This indicated that these biogroups not only dominated in non-pasteurized VPVS samples in the early (0 to 64 days) storage interval, but also dominated the climax LAB spoilage populations of these samples. These results again confirmed previous reports that homofermentative lactobacilli and leuconostocs dominated in spoilage populations of non-pasteurized, VPVS (Marshall, 1991; von Holy et al., 1991a,b; von Holy and Holzapfel, 1991; von Holy and Cloete, 1992).

Pediococci were isolated at a frequency of 29,6 to 30,2% in samples of all pasteurization treatments during the first half of the storage interval (Table 3.2.4). In the second half of the storage interval, however, pediococci comprised more than 83,3% of total LAB isolates (Table 3.2.4) and therefore clearly predominated. Conversely, the proportions of homofermentative lactobacilli and leuconostocs collectively decreased to ca. 9 to 14% of total LAB isolated from pasteurized products during the second half of the storage interval (Table 3.2.4). Since pasteurized sausages only became organoleptically spoiled during the second storage interval (65 to 128 days), and shelf life of PAST1, 2 and 3 sausages was 67, 99 and 119 days of storage at 8°C (ch. 3.1), respectively, the pediococci represented the climax populations of pasteurized sausages once they had spoiled.

The reason for the relatively late establishment (after ca. 64 days' storage) of pediococci as predominant LAB spoilage populations of pasteurized VPVS remains unclear. It was speculated, however, that these bacteria, as well as other LAB were heat injured and required time for recovery. Furthermore, pediococci could have slower growth rates than homofermentative lactobacilli and leuconostocs, which were previously reported to become the predominant LAB spoilage populations of pasteurized and non-pasteurized VPVS (von Holy et al., 1991b; von Holy and Holzapfel, 1991). As pediococci were more heat resistant than homofermentative lactobacilli and leuconostocs, this bestowed an advantage upon them to become predominant spoilage populations after pasteurization of VPVS. However, slow growth rates and slow recovery of heat injury might have delayed but not prevented their establishment as predominant spoilage populations in pasteurized VPVS.

The occurrence of pediococci as the most predominant LAB biogroup associated with the spoilage of in-package pasteurized vacuum-packaged processed meats has not been reported before. Conversely, the predominance of leuconostocs and homofermentative lactobacilli in pasteurized and non-pasteurized VPVS is well documented (von Holy et al., 1991b; von Holy and Cloete, 1992) and is associated with severe spoilage symptoms such as "blowing" of packs of sausages, "off" or sour smells and development of milky and slimy exudates. These spoilage symptoms are expected to result especially from the growth of leuconostocs, which are well-known for production of dextran slimes from sucrose, as well as gas by their heterofermentative metabolism (Sharpe, 1962; 1979). Since pediococci do not produce slimes or gas, their predominance in spoilage populations of pasteurized VPVS suggested reduced spoilage symptoms, such as "blowing" or loosening of packaging material, and the development of slimes in packages. Indeed these spoilage symptoms of VPVS in which LAB numbers reached ca. log 8,0 CFUg^{-1} were less severe for pasteurized samples compared to non-pasteurized samples (ch. 3.1, Fig. 3.1.16).

3.2.6 Effect of culture media on composition of lactic acid bacteria populations in pasteurized and non-pasteurized vacuum-packaged vienna sausages

Distribution of all LAB isolates from non-pasteurized and pasteurized VPVS with respect to culture medium (Modified MRS and STD1 Agar plates) is shown in Table 3.2.5. For non-pasteurized, PAST1 and PAST2 treated sausages, LAB isolates from Modified MRS Agar comprised proportions of 81,3%, 63,3% and 78,1%, respectively (Table 3.2.5). For PAST3 treated sausages, however, proportions of LAB isolates from Modified MRS Agar decreased to 39,7%.

Higher isolation frequencies for LAB were expected from MRS based Agar plates, since this medium is formulated specifically to satisfy the complex nutritional requirements of lactobacilli (de Man et al., 1960). In keeping with this expectation, higher proportions of homofermentative and heterofermentative lactobacilli were recovered from Modified MRS Agar (Table 3.2.5) which suggested that this medium allowed for improved recovery of these biogroups from pasteurized and non-pasteurized VPVS samples. Pediococci, however, were recovered at higher proportions from STD1 Agar plates for both pasteurized and non-pasteurized VPVS (Table 3.2.5). This suggested that this biogroup was better adapted to growth on STD1 Agar. For non-pasteurized, PAST1 and PAST2 treated sausages leuconostocs were recovered from Modified MRS Agar plates at higher frequencies than from STD1 Agar plates (Table 3.2.5). This suggested that the leuconostocs, like the lactobacilli were also better adapted to growth on Modified MRS Agar. Higher isolation frequencies for pediococci from STD1 Agar, and of lactobacilli and leuconostocs from Modified MRS Agar might reflect different nutritional requirements of these biogroups. Ecological studies on processed meat spoilage should, therefore, not only rely on the use of MRS-based Agars to isolate spoilage LAB, but should accommodate such phenomena by employing more than one suitable culture medium.

For PAST3 treated samples, however, leuconostocs were isolated at a proportion of 18,2% from STD1 Agar compared to 6,3% from Modified MRS Agar plates. (Table 3.2.5). Furthermore, the relative proportion of Leuconostocs isolation on Modified MRS Agar decreased with increasing severity of heat treatment (Table 3.2.5). This implied that the more severely heated bacteria recovered better on STD1 Agar than on Modified MRS Agar. Heat injury of bacteria to sub-lethal levels may be associated with changes in nutritional requirements (Buchanan et al., 1988; Magnus et al., 1988). The increased frequency of Leuconostoc isolation from STD1 Agar with increasing severity of pasteurization may thus be explained by changes in nutritional requirements.

Relative proportions of heterofermentative and homofermentative lactobacilli isolation increased on Modified MRS Agar with increasingly severe heat treatment applied to VPVS (Table 3.2.5). This implied that Modified MRS Agar allowed for better recovery of these heat treated biogroups than STD1 Agar. Relative proportions of pediococci decreased on STD1 Agar with increasing severity of heat treatment applied (Table 3.2.5). This implied that Modified MRS Agar also allowed for better recovery of heat treated pediococci than STD1 Agar. In the case of pediococci, a change in nutritional requirements of these bacteria as a result of sub-lethal heat injury (Buchanan, et al., 1988; Magnus et al., 1988) could again explain why relative proportions of these bacteria increased on Modified MRS Agar but decreased on STD1 Agar after heating. Since relative proportions of lactobacilli and pediococci increased, while those of leuconostocs decreased on Modified MRS Agar medium upon heating of product, Modified MRS Agar should be used in conjunction with STD1 Agar to recover the maximum diversity of LAB populations from in-package pasteurized products in ecological studies.

3.2.4 CONCLUSIONS

Lactic acid bacteria dominated (84,4% of 128 isolates) bacterial populations associated with non-pasteurized VPVS stored at 8°C. This was expected since both the microbiological shelf life and spoilage of this product was governed by the growth of these bacteria during refrigerated storage (ch. 3.1). Non-LAB such as Enterobacteriaceae, Bacillus strains and Gram-positive, catalase positive cocci were isolated from non-pasteurized VPVS at proportions of 6,3%, 3,9% and 5,5% of total isolates, respectively. We therefore concluded that the latter bacteria did not predominate in the spoilage ecology of VPVS, which was in agreement with other studies on the ecology of vacuum-packaged emulsion-type sausages (Fruin et al., 1978; Paradis and Stiles, 1978; Zurera-Cosano et al., 1988; von Holy et al., 1991a). Leuconostocs and homofermentative lactobacilli jointly predominated among predominant LAB populations (83,4%) and spoilage of non-pasteurized VPVS (ch. 3.1) was, therefore, a result of growth of these genera to high numbers. This was in agreement with earlier findings relating to the spoilage of these products at refrigeration temperatures (Marshall, 1991; von Holy and Holzapfel, 1991; von Holy et al., 1991b).

In-package pasteurization, proposed as a control measure to inactivate homofermentative lactobacilli and leuconostocs in VPVS, was successful in reducing proportions of these genera from a collective 83,4% in non-pasteurized, to 36-42% of LAB isolates in pasteurized VPVS. In all pasteurization treatments, however, LAB as a group still dominated (52% or more of total bacterial isolates) bacterial populations. Reductions in proportions of LAB, particularly homofermentative lactobacilli and leuconostocs, were correlated with increases in product shelf life (ch. 3.1). Pasteurization on the other hand, also increased proportions of Gram-positive, catalase positive cocci (ca. 8 to 10%) and Bacillus strains (ca. 6 to 32%). These increases were speculated to result from higher heat resistances of Bacillus and Gram-positive, catalase positive cocci, when compared to LAB. This could have bestowed a competitive advantage upon Bacillus and Gram-positive, catalase positive cocci to establish themselves with increasing predominance among spoilage populations associated with pasteurized VPVS. These non-LAB as well as Enterobacteriaceae, however, were recovered from pasteurized VPVS almost exclusively in the first storage interval (0 to 64 days). Since pasteurized sausages reached their microbiological shelf life (ch. 3.1) and became organoleptically spoiled only during extended storage (65 to 128 days) (ch. 3.1), spoilage of pasteurized product was not considered to be significantly influenced by the above bacteria.

Although Enterobacteriaceae and the Gram-positive, catalase positive cocci include pathogenic spp. such as salmonellae, Yersinia enterocolitica and Staphylococcus aureus which have previously been isolated from vacuum-packaged processed meats (Weissman and Carpenter, 1969; Duitschaever, 1978; Nielsen and Zeuthen, 1985), low isolation frequencies suggested that pasteurized and non-pasteurized VPVS did not pose a health risk to consumers. In addition, decreasing predominances of

Enterobacteriaceae and Gram-positive, catalase positive cocci with increasing storage time suggested that growth of these bacteria was inhibited by competitive LAB populations. Since pasteurization increased the proportions of Bacillus isolates, which may include the pathogenic species B. cereus, safety of VPVS might be compromised by pasteurization. Future work should, therefore, separately quantify and identify Bacillus strains from pasteurized VPVS, to assess potential safety risks.

Pasteurization of VPVS increased the predominance of pediococci from 16,7% of LAB isolates in non-pasteurized, to 50% or more of LAB isolates in pasteurized VPVS. Conversely, proportions of homofermentative lactobacilli and leuconostocs decreased from a collective 83,4% of LAB isolates in non-pasteurized to 36,4%, 40,6% and 42,6% of LAB isolates in PAST1, 2 and 3 treated sausages, respectively. The pediococci thus dominated the spoilage ecology of pasteurized VPVS. Speculatively, it was concluded that pediococci were more heat resistant than homofermentative lactobacilli and leuconostocs, which bestowed a competitive advantage on the pediococci to establish themselves as the dominant biogroup associated with pasteurized VPVS. Moreover, the pediococci were isolated at highest proportions (>83,3% of LAB isolates) from pasteurized sausages during the second storage interval (65 to 128 days). This clearly showed that pediococci represented the climax populations of pasteurized sausages once they had spoiled. Predominance of pediococci among spoilage LAB populations resulted in reduced severity of spoilage symptoms in pasteurized sausages. This was due to pediococci, unlike leuconostocs, not producing dextran slimes from sucrose and/or gas during growth.

All non-LAB from pasteurized and non-pasteurized sausages in this study were isolated in higher proportions on STD1 Agar, when compared to Modified MRS Agar. This was expected since MRS Agar is specially formulated to satisfy the complex nutritional requirements of LAB. Leuconostocs and lactobacilli were generally isolated in higher proportions on Modified MRS Agar, when compared to STD1 Agar. Conversely, pediococci were isolated in higher proportions on STD1 Agar than on Modified MRS Agar. These results highlighted the necessity of using more than one suitable medium to recover the maximum diversity of both non-LAB and LAB in ecological studies on vacuum-packaged processed meats.

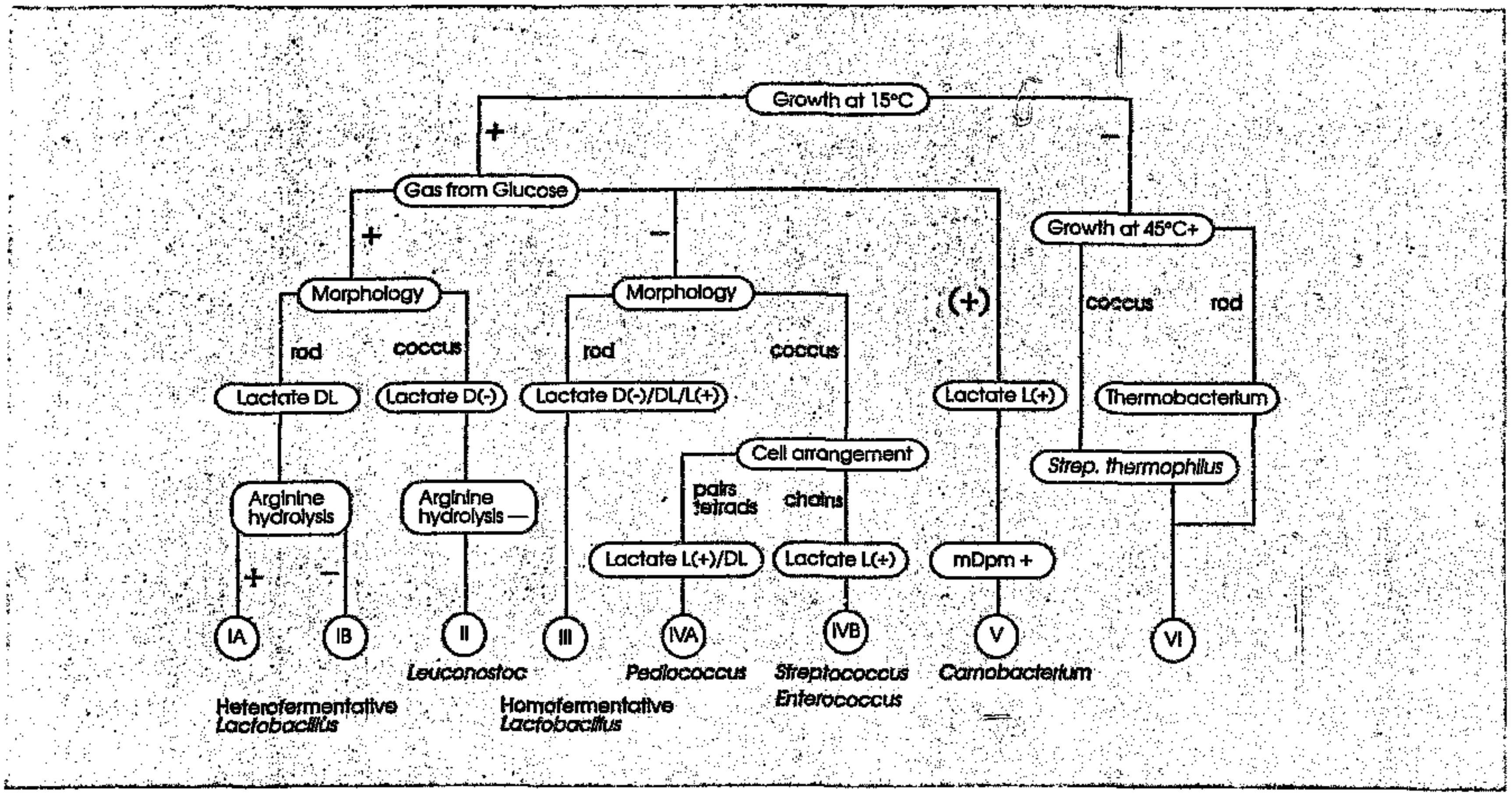


Fig. 3.2.1 Characterization key for the identification of predominant lactic acid bacteria from pasteurized and non-pasteurized vacuum-packaged vienna sausages (after von Holy *et al.*, 1991b).

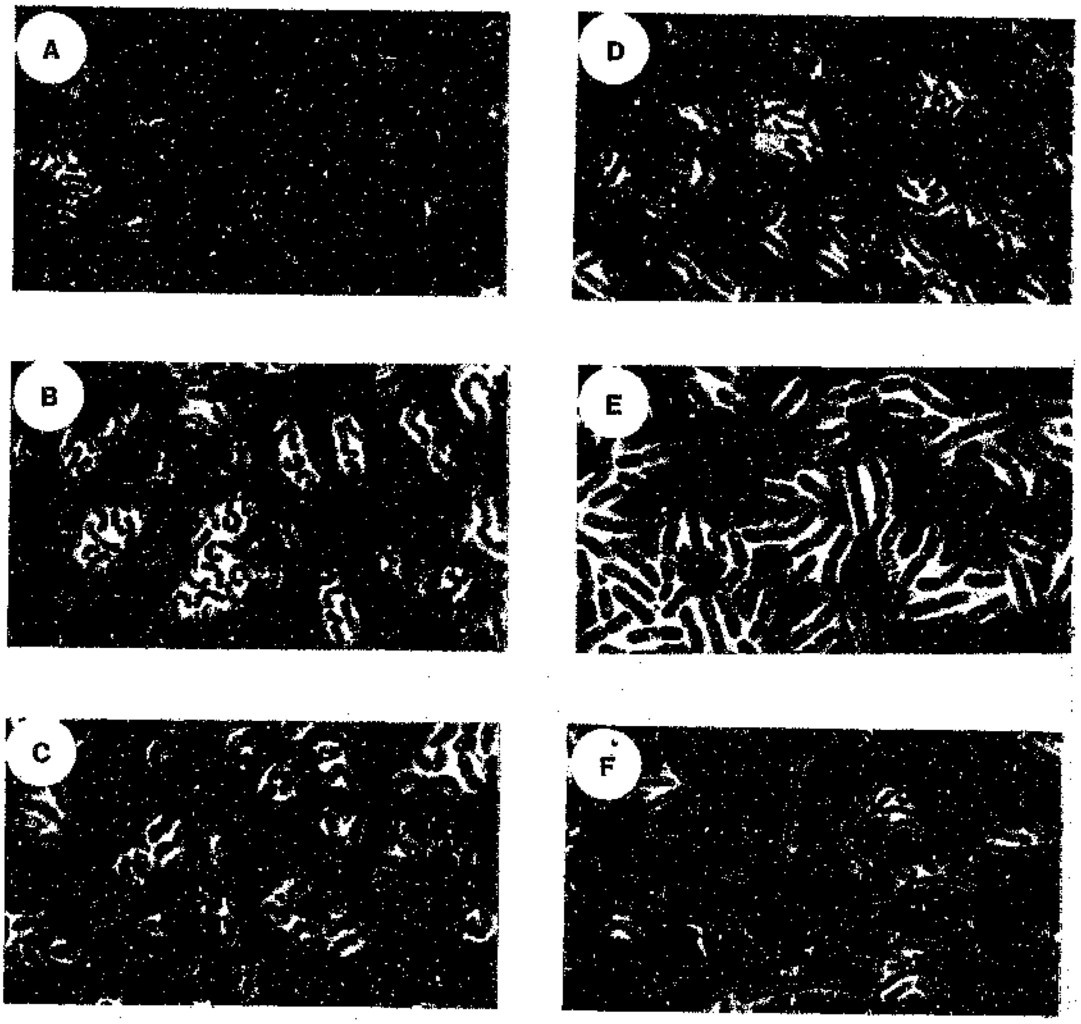


Fig. 3.2.2 Phase contrast micrographs illustrating morphological differences between lactic acid bacteria from pasteurized and non-pasteurized vacuum-packaged vienna sausages, showing rod shaped heterofermentative (a) and homofermentative (b - f) lactobacilli by increasing cell length. Note curved rods (b) and club shaped rods, typical for morphologies of Lactobacillus curvatus and Lactobacillus coryneformis, respectively (⊢—⊣ represents two micrometer).

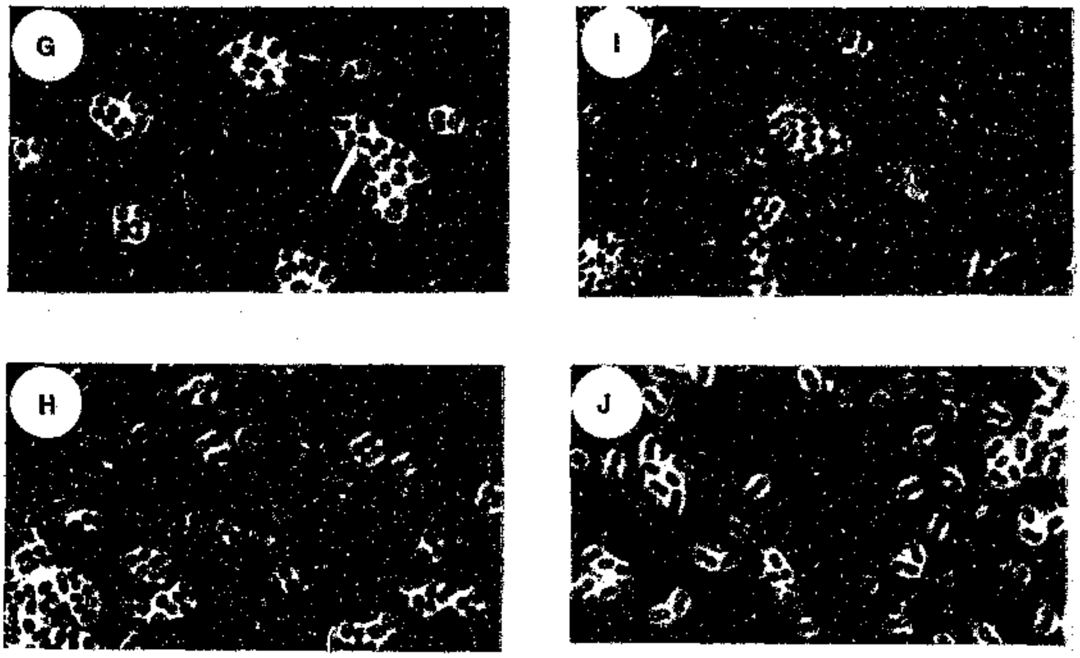
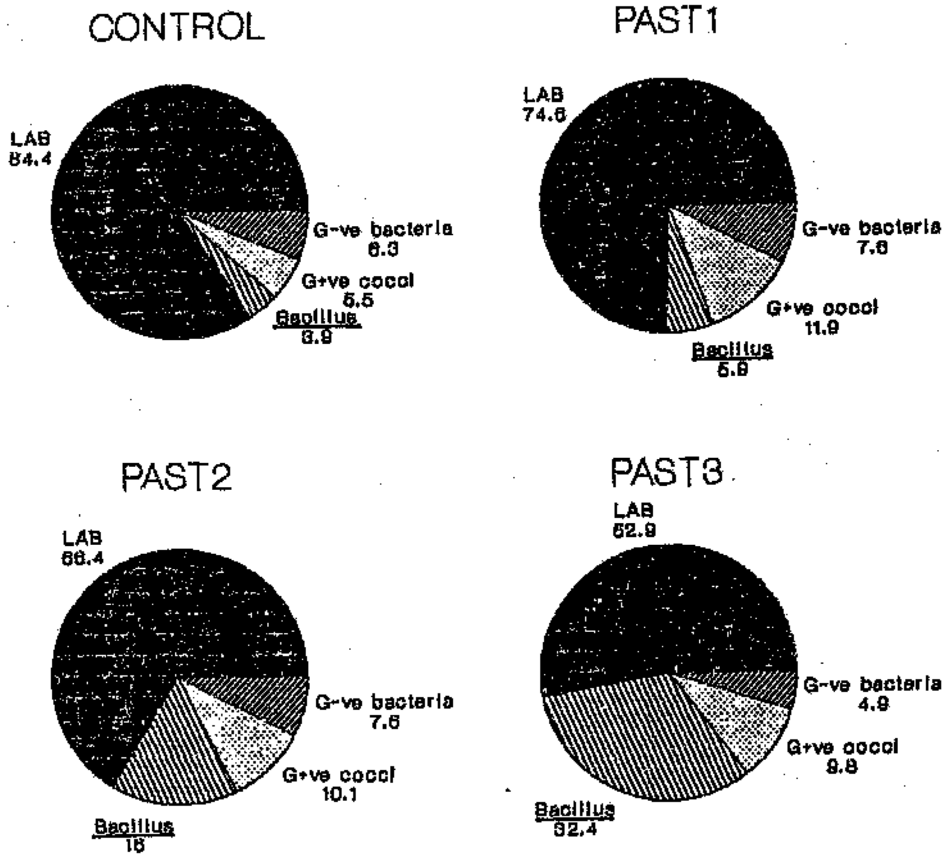


Fig. 3.2.3 Phase contrast micrographs of morphologically different lactic acid bacteria isolated from pasteurized and non-pasteurized vaciuum-packaged vienna sausages, showing coccoid and coccobacillus shaped pediococci (g & h, respectively) and coccobacillus shaped leuconostocs (i & j). The one *Leuconostoc* isolate (i) showed tendency towards chain formation, described as typical for *Lc. mesenteroides* (Dykes, 1991). Cell devision in two planes, characteristic for *Pediococcus* (Sharpe, 1979) could be observed to result in the formation of tetrads (g & h) (├──┤ represents two micrometer).



CONTROL

PAST1

PAST2

PAST3

Fig. 3.2.4 Distribution of microbial groups associated with non-pasteurized (CONTROL) and pasteurized (PAST1-3, ch. 3.1.) vacuum-packaged vienna sausages stored at 8°C for 128 days.

Table 3.2.1. Percentage distribution of 118 predominant non-lactic acid bacteria from pasteurized vacuum-packaged vienna sausages stored at 8°C for 128 days.

Treatment[a]	*Bacillus* strains	Gram-positive catalase positive cocci	Gram-negative bacteria	Total % of isolates	Total no. of isolates
PAST1	23,3[b] (-)[c]	43,3 (3,3)	30,1 (-)	100	30
PAST2	47,5 (-)	30,0 (-)	22,5 (-)	100	40
PAST3	60,4 (8,3)	16,6 (4,2)	10,5 (-)	100	48

[a] For pasteurization parameters see ch. 3.1, Table 3.1.2.

[b] Percentage for the first half of the storage interval (0 - 64 days).

[c] Percentage for the second half of the storage interval (65 - 128 days).

Table 3.2.2 Percentage distribution of 138 predominant non-lactic acid bacteria isolated from total aerobic plate counts on Standard One Nutrient Agar and lactic acid bacteria counts on Modified MRS Agar plates of vacuum-packaged vienna sausages stored at 8°C for 128 days.

Treatment	*Bacillus*	Gram-positive, catalase positive cocci	Gram-negative bacteria	Total non-lactic acid bacteria	Total % of isolates	Total no. of isolates
Control	15,0[a] (10,0)[b]	20,0 (15,0)	40,0 (-)	75,0 (25,0)	100 (100)	15 (5)
PAST1	16,7 (6,7)	33,3 (13,3)	26,7 (3,3)	76,7 (23,3)	100 (100)	23 (7)
PAST2	37,5 (10,0)	10,0 (20,0)	20,0 (2,5)	67,5 (32,5)	100 (100)	27 (13)
PAST3	47,9 (20,8)	12,5 (8,4)	8,3 (2,1)	68,7 (31,3)	100 (100)	33 (15)

[a] Percentage for Standard One Nutrient Agar plates.

[b] Percentage for Modified MRS Agar plates.

Table 3.2.3 Grouping of 329 lactic acid bacteria isolates from non-pasteurized (CONTROL) and pasteurized (PAST1 - 3, ch. 3.1) vacuum-packaged vienna sausages stored at 8°C for 128 days.

Treatment	Percentage isolates per biogroup[a]				Total no. isolates
	I	II	III	IVA	
CONTROL	-	26,9	56,5	16,7	108
PAST1	13,6	8,0	28,4	50,0	88
PAST2	5,1	8,9	31,7	54,4	79
PAST3	-	11,1	31,5	57,4	54

[a] For key to biogroups, see Fig. 3.2.1; no isolates belonging to biogroups IVB, V and VI were recovered.

Table 3.2.4 Percentage distribution of predominant lactic acid bacteria from pasteurized vacuum-packaged vienna sausages over first (0-64 days) and second (65 -128 days) storage intervals at 8°C.

Treatment	Percentage isolates per biogroup[a]				Total % of isolates	Total no. of isolates
	I	II	III	IVA		
CONTROL	-[c] (-)[d]	28,9 (16,7)	57,8 (50,0)	13,3 (33,3)	100 (100)	90 (18)
PAST1[b]	18,5 (5,9)	13,0 (-)	40,7 (8,8)	27,8 (85,3)	100 (100)	54 (34)
PAST2	7,0 (2,8)	16,3 (-)	46,5 (13,9)	30,2 (83,3)	100 (100)	43 (53)
PAST3	- (-)	14,8 (7,4)	55,6 (7,4)	29,6 (85,2)	100 (100)	27 (27)

[a] For definition of biogroups see Fig. 3.2.1; no isolates from biogroups IVB, V and VI were recovered.

[b] For pasteurization parameters see ch. 3.1, Table 3.1.2.

[c] Percentage for the first storage interval (0-64 days).

[d] Percentage for the second storage interval (65-128 days).

Table 3.2.5 Isolation frequencies of predominant lactic acid bacteria for pasteurized (PAST1-3) and non-pasteurized (CONTROL) vacuum-packaged Vienna sausages stored for 128 days from Modified MRS and Standard One Nutrient Agar.

Treatment	Percentage isolates per biogroup[a]								Total % of isolates	Total no. of isolates
	I	R.P.[b]	II	R.P.	III	R.P.	IVA	R.P.		
CONTROL	-[c] (-)[d]	-	28,6 (23,7)	1,2:1,0	62,8 (44,7)	1,5:1,0	8,6 (31,6)	1,0:3,7	81,3 (18,7;	70 (38)
PAST1[e]	18,9 (5,7)	2,5:1,0	9,4 (5,7)	1,6:1,0	37,7 (14,3)	2,6:1,0	34,0 (74,3)	1,0:2,2	63,3 (36,7)	53 (35)
PAST2	7,0 (2,8)	3,0:1,0	9,3 (8,3)	1,1:1,0	46,5 (13,9)	3,3:1,0	37,2 (75,0)	1,0:2,0	78,1 (21,9)	43 (36)
PAST3	- (-)	-	6,3 (18,2)	1,0:2,8	50,0 (4,6)	10,8:1,0	43,7 (77,2)	1,0:1,7	39,7 (60,3)	32 (22)

[a] For definition of biogroups see Fig. 3.2.1, no representatives of groups IVB, V and VI were found.

[b] Relative isolation proportions from Modified MRS Agar vs. Standard One Nutrient Agar.

[c] Percentage for Modified MRS Agar.

[d] Percentage for Standard One Nutrient Agar.

[e] For pasteurization parameters see ch.3.1, Table 3.1.2.

CHAPTER FOUR

SUMMARIZING DISCUSSION AND CONCLUSION

South African vacuum-packaged vienna sausages (VPVS) are spoiled by lactic acid bacteria (LAB), which grow to high numbers during refrigerated storage (von Holy et al., 1991a,b; von Holy and Cloete, 1992). Spoilage of VPVS prior to the expiry of the expected shelf life results in costly losses to the producers (von Holy et al., 1991b; von Holy and Cloete, 1992). Since final spoilage populations of VPVS were dominated by homofermentative lactobacilli and leuconostocs, it was suggested that control measures to curtail microbiological spoilage of VPVS be aimed specifically at these genera (von Holy et al., 1992). In-package pasteurization combined with low temperature storage was suggested as a viable control option, since the size of microbial populations surviving a heat treatment is inversely related to its severity (Bell, 1983), and recontamination after heat processing is prevented by the packaging material (Bell, 1983; von Holy et al., 1991a).

In-package pasteurization was used previously to increase microbiological shelf life of VPVS by ca. fourfold (von Holy et al., 1991a). Pasteurization, however, was performed on a "trial and error" basis and although VPVS shelf life was increased, LAB still caused product spoilage (von Holy et al., 1991a). To develop a quantitative and effective in-package pasteurization process for VPVS, knowledge on the heat resistance of meat spoilage LAB was required. This study quantified heat resistance and factors affecting heat resistance determinations of four predominant LAB from spoiled, VPVS by in vitro studies. Heat resistance data were used to calculate heat process (F_T) values for in-package pasteurization of VPVS aimed at reducing numbers and growth of homofermentative lactobacilli and leuconostocs. The effects of in-package pasteurization on shelf life and the composition of LAB and non-lactic acid bacteria (non-LAB) populations in VPVS were determined during product storage at 8°C.

Four predominant spoilage LAB from VPVS (Lactobacillus (Lb.) sake, Lb. curvatus, Leuconostoc (Lc.) mesenteroides and a Pediococcus strain, respectively) were heated in glass capillary tubes filled with quarter-strength Ringers solution (QSRS) for in vitro heat resistance determinations. Survivor curves deviated from the

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Four predominant spoilage LAB from VPVS (Lactobacillus (Lb.) sake, Lb. curvatus, Leuconostoc (Lc.) mesenteroides and a Pediococcus strain, respectively) were heated in glass capillary tubes filled with quarter-strength Ringers solution (QSRS) for in vitro heat resistance determinations. Survivor curves deviated from the logarithmic order of bacterial death (Stumbo, 1965; Pflug, 1987; Müller, 1989) and were of concave, instead of linear, shape. Since concave survivor curves reportedly result from clumping of bacterial cells during heating (Stumbo, 1965; Witter, 1983), detergent (Tween 80) was used during heating to prevent cell clumping. Addition of 1,0% Tween 80 to QSRS (pH 6,2), however, resulted in increased bacterial death rates, which appeared to be due to detergent damage to bacterial cell membranes. Use of detergent to prevent cell clumping in further <u>in vitro</u> heat resistance studies was thus avoided. Although concave LAB survivor curves might have resulted from cell clumping during heating, a more likely explanation was the heterogeneous heat resistance of these LAB. Heterogeneity of LAB with respect to heat resistance has previously been cited as the cause of concave survivor curves for these bacteria (Sanz Perez <u>et al</u>., 1982).

In a pilot study, <u>in vitro</u> heat resistance of a predominant <u>Lb</u>. <u>sake</u> and a <u>Lc</u>. <u>mesenteroides</u> isolate did not differ statistically significantly at 57 or 63°C in QSRS (pH 6,2) for cells in the early or late logarithmic growth phases, or for cells heated in QSRS at pH 5,8 or 6,2. Although bacterial heat sensitivity is known to increase with decreasing pH of the heating medium (Silliker <u>et al</u>., 1980), the pH decrease from 6,2 to 5,8 was not considered sufficient to increase heat sensitivity. Similarly, despite reports on bacterial heat resistance increases with cell age (Silliker <u>et al</u>., 1980), growing LAB to the late logarithmic phase did not result in increased heat resistance. Further <u>in vitro</u> heat resistance studies could thus be performed on LAB cells in either growth phase, or in heating medium at either pH.

Since South African VPVS have pH's of <u>ca</u>. 6,2, and in-package pasteurization of VPVS was envisaged to control low levels of LAB immediately after packaging, it was concluded that further <u>in vitro</u> heat resistance studies on meat spoilage LAB should be performed on cells in the early logarithmic growth phase and in QSRS at pH 6,2. Should pasteurization, however, be delayed and LAB reach the late logarithmic growth phase, <u>in vitro</u> heat resistance data of the early logarithmic growth phase would still be valid for

F_T-value calculations. Should in-package pasteurization be delayed and numbers of LAB increase, the F_T-value would need to compensate for the increased initial microbial load (N_o). Consequently it is suggested that in-package pasteurization on VPVS immediately follows product packaging, since bacterial growth would be minimal at that time. This is important since high microbial loads can detrimentally affect the flavor of the finished food product, even when contaminants are killed by heat processing (Warnecke _et_ _al_., 1966). Our _in vitro_ results further suggested that decreasing sausage pH to pH 5,8 would not result in faster heat inactivation of contaminating LAB.

In vitro heat resistance studies at 57, 60 and 63°C showed that the four predominant spoilage LAB from VPVS were heat sensitive, since at least one log cycle reductions in cell numbers were achieved by heating for _ca_. one minute at 57°C in QSRS. Since the four LAB selected for _in vitro_ heat treatment were originally isolated from spoiled VPVS, our data confirmed previous findings (Dykes _et al_., 1991) that LAB contaminating vienna sausage emulsion could not survive the heat processing (smoke-cooking) of product manufacture. Heat resistances of LAB in this study were thus expected and confirmed to be lower than those of previously described thermotolerant meat spoilage LAB (Niven _et_ _al_., 1954; Houben, 1982; Milbourne, 1983; Borch _et_ _al_., 1988). Heat resistances of LAB in this study, however, compared favourably to those of spoilage LAB from beer and citrus products (Adams _et al_., 1989; Parish, 1991) as well as _Listeria monocytogenes_ heated in milk (Bradshaw _et_ _al_., 1985; Donnelly and Briggs, 1986; Bradshaw _et al_., 1987). By implication, this indicated that the above pathogen should also not survive heat processing of emulsion-type sausage during manufacture, as was indeed found to be the case in a previous study (Zaika _et al_., 1990). Comparable heat resistances of LAB and _L_. _monocytogenes_ furthermore implied that _Listeria_ cells recontaminating sausages after heat processing were unlikely to survive in-package pasteurization.

Z-values determined from _in_ _vitro_ heat resistance determinations were _ca_. 4 to 5 times higher than the 4 to

6°C generally reported for non-sporulating bacteria (Hansen and Riemann, 1963; Tomlins and Ordal, 1976). Such high z-values were thought to arise since the temperature interval (6°C) over which LAB heat resistance was determined was too small to allow the thermal death time curve to traverse one log cycle. D-values used in heat process calculations could thus not be extrapolated to higher or lower temperatures than those used in this study. Should such extrapolations be required for pasteurization of VPVS at higher or lower temperatures, LAB heat resistance would need to be determined over a greater temperature interval to generate z-values which would allow for such extrapolations.

In situ VPVS pasteurization trials were based on the upper limit of the 95% confidence interval of the D_{63} value of a Lb. sake strain, which was the most heat resistant of the four predominant LAB isolates studied. Survivor curves of all four LAB strains heated in vitro, however, were non-linear and D-values derived from these curves, using linearization by linear regression, thus underestimated true heat resistance of LAB. In addition, D-values of LAB determined in QSRS in vitro were expected to be lower than those for bacteria heated in sausages, since carbohydrates, fats, salt and curing salts contained in sausages are known to protect bacteria from heat inactivation (Silliker, et al., 1980; Jay, 1986). Since the in vitro D-value for the Lb. sake strain was expected to underestimate its true heat resistance, three in-package pasteurization treatments of increasing severity (PAST1, 2 and 3, respectively) were applied to VPVS in single layers to determine their relative effectiveness in reducing numbers and growth of spoilage LAB.

In-package pasteurization of PAST1 treated sausages was performed to a sausage core temperatures of 60°C and not based on a heat process (F_T) value (Müller, 1989). PAST2 and PAST3 heat treatments were, however, performed to sausage core temperatures of 63°C and calculated by the F_T-values to reduce levels of spoilage LAB to log 1,0 CFU (PAST2) or leave one in a thousand packs of sausages contaminated with any LAB (PAST3). Although the calculated bacterial reduction was approximately achieved for PAST2 treated sausages, our results showed that the PAST3 pasteurization treatment only eliminated LAB from packages to approximately 0,6 $CFUg^{-1}$. This could be explained by the underestimate of the D-value for Lb. sake used in F_T value calculations, and the possible increased heat resistances of LAB in vienna sausages compared to QSRS.

Nevertheless, all in-package pasteurization treatments were successful in decreasing numbers of contaminating LAB from ca. log 3,0 $CFUg^{-1}$ in non-pasteurized to below log 1,0 $CFUg^{-1}$ in pasteurized sausages. In addition, in-package pasteurization significantly decreased the growth of LAB, a trend which increased with increasing severity of the heat treatment. Depending on the severity of the heat treatment, in-package pasteurization increased shelf life of VPVS to 10, 14 and 17 times that of non-pasteurized products and therefore effectively controlled the microbiological spoilage of VPVS stored at 8°C. In-package pasteurization of VPVS can, therefore, be recommended to producers of VPVS as a method to increase shelf life and decrease the losses experienced in form of returns of pre-maturely spoiled products from the marketplace. Approximately fourfold shelf life increases after pasteurization of double layer VPVS were previously reported for sausages stored at 8°C (Marshall, 1991) and 7°C (von Holy et al., 1991b). The noticeably larger increases in shelf life of VPVS achieved in this study were thought to result from packaging sausages into single layers. This not only resulted in higher sausage core temperatures when compared to those of 52 to 57°C achieved in the study of von Holy et al. (1991a), but also in quicker and more even heat transfer in sausage packs. Packing sausages into single, as opposed to double layers for in-package pasteurization of VPVS is therefore also recommended to the producers, since this practice would effectively increase shelf life, and also be attractive in terms of saving energy and processing time.

F_T-values calculated on the basis of in vitro heat resistances of LAB were thus useful for quantifying the time/temperature combinations required to reduce initial numbers and growth of spoilage LAB in VPVS. The time/temperature combinations for in-package pasteurizations utilized in this study were of sufficient accuracy to control spoilage and extend shelf life of VPVS from an industrial perspective. From a scientific perspective, however, it would be possible to quantify parameters for in-package pasteurization processes more accurately. For this purpose, LAB heat resistance could, for example, be determined in a vienna sausage slurry where bacteria are protected against heat damage by compounds such as fats, carbohydrates and salts. This would improve the accuracy of F_T-values and hence determinations of desired endpoint populations (N_f) after in-package pasteurization of VPVS. Furthermore, F_T-value calculations could be improved by compensating for non-linear survivor curves, which probably arose as a result of heterogeneity of LAB with respect to heat resistance. Since concave survivor curves are characterized by an initial rapid death rate stage followed by a slower bacterial death rate stage, a concave survivor curve may be subdivided into two linear phases, each representing one of the above stages in bacterial death rates. D-values may then be derived from the straight line with the greater slope in order to compensate for the fact that bacterial inactivation did not produce linear survivor curves. Speculatively, however, it is suggested that in-package VPVS pasteurization time should be increased by a factor of _ca_. one to two minutes during heating at sausage core temperatures of 63°C, to more accurately predict the endpoint LAB populations surviving the heat treatment.

Isolation of a _Listeria innocua_ strain from a non-pasteurized VPVS pack in the first week of storage at 8°C indicated a potential for post-heat processing (smoke-cooking during manufacture) contamination by _Listeria_. No _Listeria_ could, however, be isolated from any pasteurized VPVS stored at 8°C. This suggested that in-package pasteurization of VPVS not only effectively delayed spoilage by LAB, but also improved safety of these products with respect to _Listeria_ transmission. In-package pasteurization of VPVS, however, also increased the incidence of potentially pathogenic clostridia from 8,3% in non-pasteurized to 41,7% (PAST1), 58,3% (PAST2) and 75,0% (PAST3) in pasteurized samples.

Moreover, the isolation of potentially pathogenic *Clostridium perfringens* type A strains from sausages of all pasteurization treatments suggested that in-package pasteurization of VPVS might compromise product safety. In-package pasteurization of VPVS to control the spoilage of these products is, therefore, recommended to industry with caution. Clearly, a need for control factors in addition to high temperature exists to ensure the safety of pasteurized VPVS with respect to transmission of potentially pathogenic clostridia. Possible control factors will be discussed in more detail.

Even though the three in-package pasteurization treatments increased shelf life to 10, 14 and 17 times that of non-pasteurized VPVS, they did not prevent microbiological spoilage. Proportions of LAB decreased from 84,4% of predominant populations isolated from non-pasteurized samples to 74,6%, 66,4% and 52,9% for PAST1, 2 and 3 treated samples, respectively. The LAB thus dominated the spoilage ecology of all pasteurized products (> 52,9% of total bacterial isolates). This was in agreement with von Holy *et al.* (1991a) who also showed spoilage of in-package pasteurized VPVS to be associated with growth of LAB to high numbers (*ca.* log 8,0 CFUg^{-1}). However, in this study, proportions of non-LAB (*Bacillus*, Enterobacteriaceae and Gram-positive, catalase positive cocci) increased from 15,7% of predominant isolates in non-pasteurized to 25,4%, 33,7% and 41,7% of predominant isolates in PAST1, 2 and 3 treated samples, respectively. Increasing proportions of non-LAB with increasingly severe heat treatment suggested that these non-LAB were more heat resistant, which gave rise to a competitive advantage and led to establishment of non-LAB in the spoilage ecology of in-package pasteurized VPVS. Non-LAB, however, were isolated at highest proportions only during the first half (0 to 64 days) of the storage interval at 8°C and, therefore, did not predominate in climax populations of spoiled VPVS. The inability of non-LAB such as *Bacillus*, Enterobacteriaceae and Gram-positive, catalase positive cocci to dominate the climax spoilage ecology of both pasteurized and non-pasteurized VPVS was previously reported (von Holy, 1989; Marshall, 1991; von Holy *et al.*, 1991a) and supported the findings of this study.

In non-pasteurized VPVS, the homofermentative lactobacilli and leuconostocs dominated LAB populations and collectively comprised 83,3%. This was in agreement with results of Marshall (1991) and von Holy and Holzapfel (1991), who reported homofermentative lactobacilli and leuconostocs in non-pasteurized VPVS stored at 7°C to jointly comprise 97,4% and 84,0% of LAB isolates, respectively. In pasteurized products, however, joint proportions of leuconostocs and homofermentative lactobacilli decreased to 36,4%, 40,6% and 42,6% of predominant LAB isolates for PAST1, 2 and 3 treated samples, respectively. Conversely, proportions of pediococci increased from 16,7% of predominant isolates in non-pasteurized samples to 50,0%, 54,4% and 57,4% of predominant LAB isolates from PAST1, 2 and 3 samples, respectively. Increasing proportions of pediococci as a result of VPVS pasteurization were reported previously (Marshall, 1991; von Holy and Holzapfel, 1991), and it was suggested that this biogroup was more heat resistant than the homofermentative lactobacilli and leuconostocs. This could have bestowed a competitive advantage upon pediococci to establish themselves as dominant spoilage LAB in pasteurized VPVS. Moreover, pediococci were isolated from pasteurized VPVS at higher proportions (> 83,3% of predominant LAB isolates) during the second storage interval (65 to 128 days), suggesting that this biogroup dominated the climax LAB populations associated with spoiled, in-package pasteurized VPVS. This finding did not correlate to previous reports where the homofermentative lactobacilli and leuconostocs still predominated in LAB populations of pasteurized VPVS at proportions of 71,5% (von Holy and Holzapfel, 1991) and 66,6% (Marshall, 1991) of total LAB isolates.

The higher proportions of pediococci in predominant spoilage populations of pasteurized VPVS in this study are likely to have resulted from the arrangement of sausages into single layers within vacuum bags which produced higher sausage core temperatures. Since in-package pasteurization to higher sausage core temperatures than those used by Marshall (1991) and von Holy and Holzapfel (1991), was aimed at reducing the predominance of homofermentative lactobacilli and leuconostocs, this objective was achieved. Pediococci have not previously been implicated as predominant spoilage populations of vacuum-packaged processed meats. In this study, predominance of pediococci in pasteurized VPVS was associated with less severe spoilage symptoms such as loosening of packaging material and development of exudates in sausage packs. As a practical consequence, reduced spoilage symptoms would lead to a longer shelf life during retail display.

Although overall proportions of non-LAB increased with increasing severity of the heat treatment applied to VPVS, proportions of Gram-negative bacteria and Gram-positive, catalase positive cocci remained low (<11,9%) for all pasteurization treatments. Low proportions of Enterobacteriaceae and Gram-positive, catalase positive cocci in pasteurized VPVS implied that in-package pasteurization would not lead to a health risk with respect to transmission of pathogenic species of these groups (e.g., *Salmonella*, *Yersinia enterocolitica* and *Staphylococcus aureus*, respectively). These pathogens were previously reported to be associated with vacuum-packaged processed meat products (Weissman and Carpenter, 1969; Duitschaever, 1978; Tiwari and Kadis, 1981; Nielsen and Zeuthen, 1985). In-package pasteurization of VPVS did, however, increase the frequency of isolation of potentially pathogenic *Bacillus* strains in addition to that of of clostridia, including pathogenic *C. perfringens* type A. Proportions of *Bacillus* increased from 3,9% of predominant isolates from non-pasteurized VPVS to 32,4% of predominant isolates from pasteurized VPVS.

The increased isolation frequencies of clostridia (including pathogenic *C. perfringens* type A), as well as potentially pathogenic *Bacillus* strains as a result of VPVS pasteurization were concluded to result from spores surviving the pasteurization treatments. *Bacillus* and *Clostridium* spores are well known to survive heat treatments designed to inactivate vegetative cells (Nielsen and Zeuthen, 1985; Kokubo *et al*., 1986). Since growth of *Bacillus* and *Clostridium* spp. in processed meats is inhibited by competitive LAB as well as low temperature storage, decreased predominance of LAB in spoilage populations as a result of in-package pasteurization of

VPVS, and the potential for product temperature abuse during retail display could jointly compromise the safety of pasteurized VPVS.

In the practical situation, additional control parameters (or hurdles) may be effectively used to control the growth of pathogenic Clostridia and Bacillus strains in pasteurized VPVS. Pasteurization of VPVS could, for example, be performed at lower temperatures than those used in this study at, for example, 57°C core temperature for ca. 3 to 5 minutes. Our in vitro results showed that LAB could be heat inactivated at this temperature. Although this would imply a shorter shelf life, it would nevertheless allow for faster growth of spoilage LAB capable of inhibiting potentially pathogenic Clostridium and Bacillus strains.

Alternatively, in-package pasteurization of VPVS could be performed twice. The first pasteurization process would activate spores of Bacillus and Clostridium to germinate into vegetative cells during a suitable incubation period, after which sausage packs could be re-pasteurized. This would kill the more heat sensitive Bacillus and Clostridium vegetative cells. Further studies on the organoleptic implications and incidence of Bacillus and Clostridium in "double-pasteurized" VPVS would need to be performed to evaluate the feasibility of this approach. "Double-pasteurization", however, would probably not be practically feasible since increases in time and energy requirements would be of doubtful cost-benefit.

The high temperatures of in-package pasteurization could furthermore be used in combination with other hurdles to control the growth of potential foodborne pathogens. Low temperature product storage, for examples, is well known to inhibit growth of pathogenic mesophiles such as Bacillus and Clostridium. The cold chain, therefore, would need to be strictly adhered to, not only to inhibit the growth of spoilage populations, but also of pathogenic bacteria. Use of product pH as a hurdle to inhibit growth of pathogenic bacteria in VPVS is, however, difficult since lowering product pH to the "lower organoleptic limit" of pH 5,8 would still allow for growth of pathogenic B. cereus, C.

perfringens and C. botulinum (Buchanan, 1986; Hauschild, 1989; Labbe, 1989).

Nevertheless, chemical preservatives such as sodium nitrite or sodium lactate, in addition to in-package pasteurization, could be evaluated as hurdles to control the growth of pathogenic Bacillus strains and clostridia in vacuum-packaged processed meats. Nitrite is known to be effective in inhibiting these bacteria in processed meats, especially at low storage temperatures (Sofos et al., 1979; Nielsen and Zeuthen, 1984b; Buchanan, 1986) and is used in South African VPVS at levels of ca. 100ppm residual concentration. Since residual nitrite concentrations in processed meats are difficult to control as they are affected by product heating and storage time (Sofos et al., 1979), control of pathogenic bacteria in pasteurized VPVS by sodium lactate in conjunction with nitrite may be a viable option and should be investigated by further studies. Sodium lactate would be easier to control in pasteurized VPVS than sodium nitrite, as levels of the previous compound are expected not to be affected by heat treatments. Sodium lactate might thus successfully inhibit growth of pathogenic Bacillus and Clostridium spp. by virtue of its antimicrobial nature. Sodium lactate has previously been shown to inhibit growth and toxin production of C. botulinum in cook-in-bag turkey products under conditions of temperature abuse (Maas et al., 1989). Due to the ease of industrial application, sodium lactate combined with sodium nitrite and low temperature storage is recommended to manufacturers to enhance the safety of in-package pasteurized VPVS. Further studies on incidence of pathogenic Bacillus and Clostridium in pasteurized VPVS containing sodium lactate and sodium nitrite and stored at refrigeration temperatures should, however, be performed to validate the effectiveness of these control parameters.

To assure the safety of pasteurized VPVS, the effects of the above hurdles on numbers and identities of pathogenic Bacillus spp. and clostridia would require further study. It remains of utmost importance, however, that before control measures for the above pathogens are investigated or even instituted, efforts should be focused on prevention of contamination of vienna sausages with pathogenic bacteria in the meat processing environment. This would require that good manufacturing practices (GMP), effective sanitation and personal hygiene programs and raw material quality are maintained and even improved in South African meat processing plants. Thus GMP and the combined use of hurdles such as in-package pasteurization, preservation and low temperature storage may not only reduce spoilage and increase shelf life, but also assure the safety of VPVS.

In the final analysis, therefore, the statement that "Overall, the presence on meats of lactic acid bacteria is more desirable than that of the type of bacteria they have replaced" (Egan, 1983) was found to hold true in the case of in-package pasteurized, vacuum-packaged vienna sausages.

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