

BACTERIAL INTERACTION IN HIDE BIODETERIORATION  
WITH SPECIAL REFERENCE TO SELECTED *CLOSTRIDIUM* SPECIES

by

GILLIAN ANN THOMPSON

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## ABSTRACT

Animal hides are the basic raw material of the leather industry and they undergo rapid putrefaction unless "cured". This study investigated the role and interactive effects of three selected bacteria, *Pseudomonas aeruginosa*, *Clostridium histolyticum* and *Clostridium sporogenes* in in-situ cattle hide degradation using a model system set up for the purpose. The system consisted of 3cm diameter hide pieces contained in sealed jars and sterilised by ethylene oxide to remove resident microbes and inactivate autolytic tissue enzymes. The inocula were prepared either as individual cultures or as combinations of two inocula or all three inocula. Degradative changes during storage at 30°C were measured for up to 8 days using ten different parameters.

Initial trials confirmed that the selected inocula were readily isolated from raw hides and could outcompete resident populations to produce putrefactive decomposition. Growth rates and enzyme profiles of the organisms and the effects of nutrients and reductants on their relative denaturative effects were used to standardise the system. Trials on the effects of ethylene oxide indicated the suitability of the method for hide and collagen sterilisation.

The findings of in-situ trials with the selected inocula confirmed previous studies of protein putrefaction in that a bacterial succession was evident involving aerobic proteolytic bacteria, micro-aerophilic proteolytic bacteria and strictly anaerobic amino acid degrading bacteria. However, this study showed that the micro-aerophilic collagenase producing *C. histolyticum* degraded hides at a far greater rate when inoculated on its own than when in the presence of either or both of the other two inocula. It also demonstrated a bacterial antagonism between the two clostridia in which *C. sporogenes* prevented degradative changes occurring for up to 4-6 days possibly due to cysteine production by *C. sporogenes*. These findings have implications for hide preservation since maintenance of aerobic conditions and suppression of spore outgrowth could be used to delay growth of collagenase producing clostridia. The use of *C. sporogenes* as a biocontrol agent is also postulated.

The model system was also used to examine salted hides during storage and these studies indicated that *Halobacteriaceae* do not produce collagenase but that inadequately salted hides could possibly be subject to degradation by desulfobivibrios.

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GLOSSARY OF TERMS

$A_w$	Water activity
EO	Ethylene oxide
$T_s$	Hide shrinkage temperature (hydrothermal transition)
SAIMR	South African Institute for Medical Research
$T_m$	Transitional melting temperature
Double-mix	A 1:1 mix of two organisms
Triple-mix	A 1:1:1 mix of three organisms
Individual inocula	Pure culture of single organism
ATCC	American Type Culture Collection
NCTC	National Culture Type Collection
MIC	Minimum Inhibitory Concentration
<i>B.</i>	<i>Bacillus</i>
<i>C.</i>	<i>Clostridium</i>
<i>E.</i>	<i>Escherichia</i>
<i>S.</i>	<i>Staphylococcus</i>
<i>Ps.</i>	<i>Pseudomonas</i>
<i>V.</i>	<i>Vibrio</i>
<i>C.hist</i>	<i>Clostridium histolyticum</i>
<i>C.spor</i>	<i>Clostridium sporogenes</i>
<i>Ps.aer</i>	<i>Pseudomonas aeruginosa</i>

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## CHAPTER 1

### INTRODUCTION AND LITERATURE SURVEY

- 1.1. Summary.
- 1.2. *General Background to the Hide Industry*
- 1.3. Aspects of Hide Degradation
- 1.4. Historical Studies on Hide Degradation
- 1.5. Questions Arising from Literature Survey
- 1.6. Use of a Model System for Hide Degradation
- 1.7. Research Objectives
- 1.8. Thesis Rationale
- 1.9. Thesis Hypothesis

### 1.1 Summary:

Hides, the basic raw material of the leather industry, are subject to degradative changes which may be detrimental to final product quality. These changes are described and the fundamental factors affecting them are reviewed. These factors derive from both the nature of hide itself, in terms of structure, function and chemistry and the pre-tanning process treatments which they undergo. Other relevant aspects of hide degradation such as the role of bacteria and of general proteolytic and specific collagen degrading enzymes are discussed. Previous studies of bacterial degradation of protein substrates in general and of hides in particular are reviewed. Historical studies involving microbial aspects of hide degradation, and questions arising from the literature covered, indicate the need for more in-depth studies of microbial decomposition in hides. For this purpose the use of a model system in which bacteria could be inoculated to sterile hides was postulated. The choice of organisms for this system was based on the findings of previous studies involving the degradation of protein food, especially meats. The choice of inocula - *Pseudomonas aeruginosa*, *Clostridium histolyticum* and *Clostridium sporogenes* - is discussed and a thesis rationale outlining the steps taken to verify the thesis hypothesis is given. The thesis hypothesis is that hide degradation occurs as a result of a microbial succession involving the above three organisms.

### 1.2. General Background to the Hide Industry:

#### The raw material:

Research into the microbial degradation of hides and skins is important to the leather industry since these constitute the basic raw material of leather. Cattle hides are a valuable by-product of the meat industry and in South Africa, today, they sell at between R5 and R6 per Kg. so that a medium size (25 Kg) hide will cost between R100 and R150. This adds to the costs of producing leather goods, as do the numerous process treatments that raw hides need to undergo in order to convert them into leather. Leather, therefore, is an expensive product, the price and quality of which is directly related to the quality of the hide itself. Three main factors affect hide quality. The first is the condition of the hide before it is flayed (removed from the animal). The second is the degree of bacterial damage and post-mortem changes which occur after flay and the third factor is the degree of damage which may arise during the various chemical and mechanical treatments during the tanning process.

### Curing and tanning:

Hides removed at an abattoir are sold to a "curer" who preserves (cures) the hides prior to re-sale to a tanner who then processes them to leather. Hide quality is an important factor in these transactions but not always readily evaluated. The cause of poor quality hides and skins pre-mortem are both endogenous (poor nutritional status of the animal, Liebenberg *et al.* 1989) and exogenous (tick bites, insect bites, scarring from disease and brand marks, Halligan 1985). Where the latter defects are readily visualised they are used in the industry to grade raw hides on sale to curers and tanners. However, there is no practical means to detect either post-mortem tissue damage or bacterial spoilage and/or putrefaction in hides sold by the curer to the tanner except in extreme cases showing hairslip or malodour. In fact visible degradation due to these sources is frequently evident only at the semi or fully processed stage (Russell 1984)

Tanning procedures cannot reverse the effects of incorrect treatment of hides prior to tannage. The quality of the final leather product is, therefore, dependent on containment of bacterial action on raw hides. This is achieved by either putting freshly flayed hides straight into tanning treatments or, if hides have to be stored before tanning, subjecting the hides to some form of preservative treatment such as salting, chilling or biocide application (Elliott 1984).

Hides are preserved, as opposed to sterilised, and therefore remain potentially putrescible during the early stages of tannery processing. There are four preliminary wet-processes that are conducted in the early "beamhouse" stages prior to tanning, namely, soaking, liming, deliming and bating. Problems in these stages can be intrinsically linked with final leather quality (Russell 1984). Soaking returns hides to the normal hydrated state and removes the unwanted curing chemicals and some of the globular inter-fibrillary proteins. Soaking is followed by calcium hydroxide liming at pH 13, the purpose of which is to attack the outer proteoglycans of collagen fibres as well as the globular proteins and keratins of the epidermal layer. A sharpening agent such as sodium sulphide is added with the lime to accelerate rupture of cystine di-sulphide bonds present in hair follicle keratins in order to promote unhairing. Following liming, the hide pH is brought back to near neutrality by deliming with buffering salts such as ammonium chloride and ammonium sulphate. The final process, known as bating, involves the use of selected proteolytic enzymes at high pH (8-10) to remove unwanted residual inter-fibrillary proteins such as hair roots, elastins, reticular and muscle

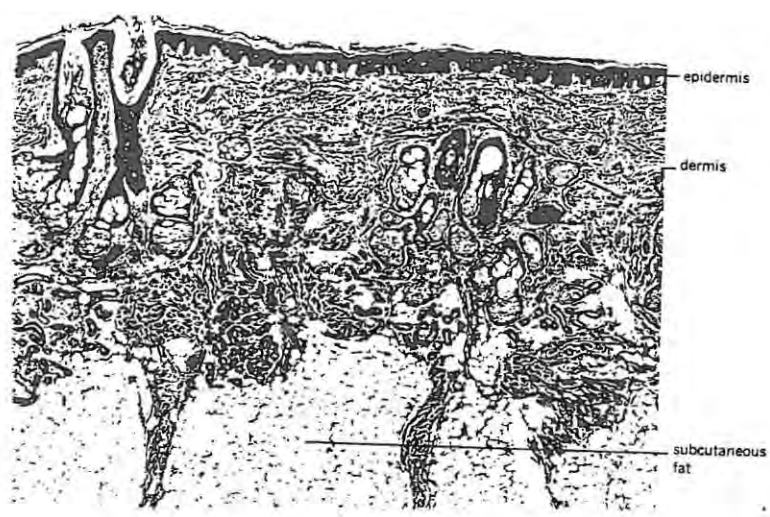
fibres. The tanning process itself which follows the beamhouse treatments first involves acid "pickling" followed by application of tanning agents. This results in the cross-linking of hide collagen fibres rendering them non-putrescible.

In life, the principal functions of skin and hides are to protect deeper tissues from injury, to act as a major barrier to microbes and chemicals and to control body heat. These functions cease in post-mortem skin and hides. Other intrinsic skin characteristics such as stress absorption and mechanical strength are retained and with these attributes are associated properties of elasticity, plasticity, shear resistance and viscosity which form the basis of good quality leather (Heidemann 1979; Glanville and Kuhn 1980). However, these properties may also be lost post-mortem and this can arise due to damage from two different sources. The first is autolytic from endogenous release of tissue enzymes as a result of cell death due to lack of oxygen. The second is exogenous and is related to bacterial proliferation and enzyme production (Malisoff 1943; Banwart 1987). Factors that will influence the microbial populations responsible for the bacterial attack include the physiology and chemistry of the hide, as well as prevailing conditions within the individual post-mortem tissues. Some of the pertinent facts concerning these aspects of hide degradation such as skin physiology, the chemistry of skin proteins and their individual susceptibility to specific and non-specific enzymes, are summarised below.

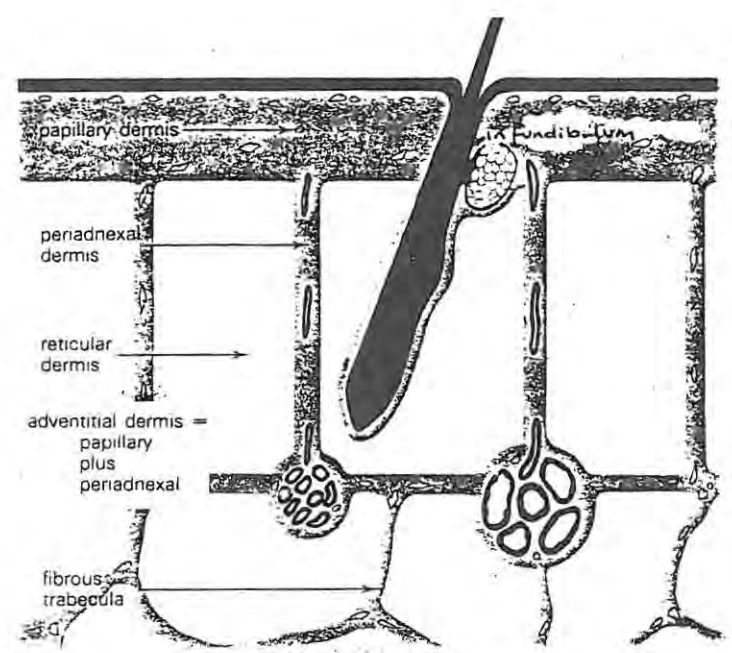
### **1.3. Aspects of Hide Degradation:**

#### **Skin structure and function:**

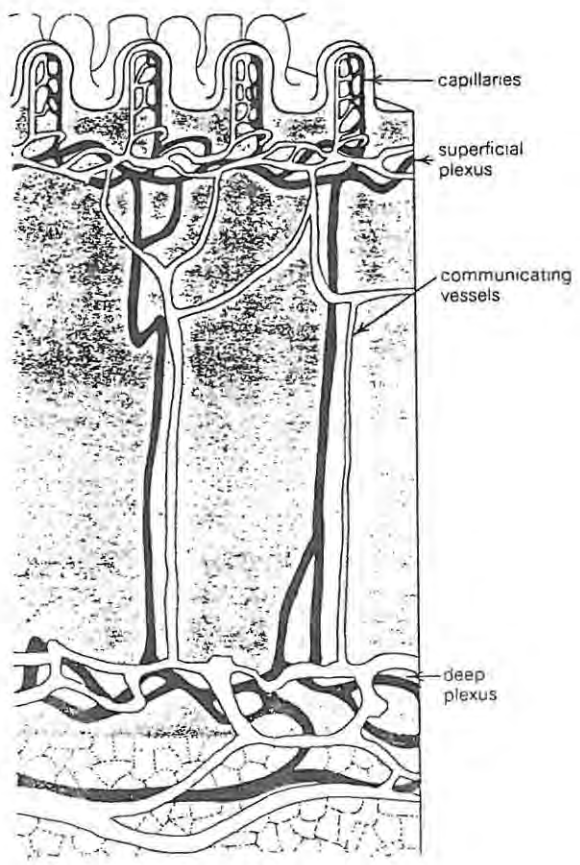
Skin/hide consists of three distinct skin layers, the epidermis, the dermis and the subcutaneous fatty layer, (Fig.1.1(a)). The epidermal layer consists mainly of keratinised epithelial cells. The dermis is between 15 and 40 times thicker than the epidermis and it can be divided into two distinct zones, the papillary dermis (known as the corium minor in the hide industry) and the reticular dermis (or corium major) (Fig.1.1(b)). The reticular dermis lies below the papillary dermis and extends to the flesh surface. It is composed of an orthogonal wickerwork of collagen fibres (Ackerman 1978). Twelve different types of collagen have been identified, based on primary amino acid chain configuration and in skin Collagen I and III predominate (Burgeson 1988; Stenn *et al.* 1992). Between the spaces are the ground substances composed primarily of mucopolysaccharides, of which heparin sulphate, dermatan sulphate,



(a)



(b)



(c)

Fig. 1.1 Skin Structure (a) Histological X-section showing three main areas (b) Diagrammatic representation of different dermal layers (c) Capillary network. (Reproduced from Ackerman 1978)

chondroitin-6 sulphate and hyaluronic acid predominate (Stenn 1992). These fluids cushion and lubricate the fibres. Water is also a major component comprising approximately two-thirds by mass of the tissue.

The papillary dermis lies directly under the epidermis, in conjunction with the periadnexal dermis (the dermis which surrounds hair follicles and blood vessels) and the two together form the advential dermis. (See Fig.1.1(b)). Papillary dermis is far richer in ground substance, fibroblasts and blood vessels than the reticular dermis and therefore more open to bacterial attack. This also applies to all cutaneous appendages such as hair follicles and glands. These extend to the dermis and penetrate the reticular layer and they are accompanied along their entire length by a thin layer of this peri-adnexal dermis (Montagna 1962).

The other main skin structure consists of the subcutaneous fat which is held in place by a fine network of collagen I fibrils. This layer is generally removed in the flaying of hides. Present throughout the hide/skin are elastin fibres and these form a delicately branched network between papillary dermal collagen fibres and cutaneous appendages and a thick wiry network with the Collagen I fibres of the reticular dermis (Ackerman 1978; Urmacher 1990). In the dermis also are hairs, hair follicles, sebaceous glands and sweat glands. Hair follicles are lined with a layer of collagen III fibres which form a basement membrane enclosing the outer root sheath of the follicle. The indigenous microflora of hides and skins, which include bacteria, yeasts and mites, are found in the infundibulum located at the upper end of the hair follicles adjacent to sebaceous glands and ducts (Ackerman 1978).

A capillary network of blood vessels lies throughout the whole dermal area and is connected to both hair follicle bases and the dermal-epidermal junction, but not to the epidermis. This whole vascular network of the skin is surrounded by a thin layer of fibrillar collagen (mostly collagen III). The capillaries and venules of this network form channels which cross both corium major and minor (Fig.1.1(c)) (Montagna 1962; Urmacher 1990).

#### **Skin chemistry:**

Skin consists mainly of fibrous protein, with smaller amounts of globular proteins, fats and carbohydrates. The two main proteins present are keratin and collagen. In both skins and

hides type I forms 80-90% of the collagen and type III forms between 10 and 20% (Kemp 1983; Burgeson 1988). Keratin is found in smaller amounts in the hair and epidermal tissue. Skin collagen constitutes up to 80% of the total protein present in hides and consists mostly of collagen I with smaller amounts of collagen III inter-linked to a carbohydrate/protein complex to form a macro-molecular glycoprotein (von der Mark 1981; Glanville 1987; Burgeson 1988). This results in a structured fibre bundle complex which underlies the epidermal layer.

Leather and gelatin chemists as well as medical and biochemical researchers have all contributed to our basic knowledge of collagen. In the early 1950's research focused on molecular studies in which physical, chemical and electron microscopic techniques were used to study collagenases and monomeric collagen in pure solution (Gallop *et al.* 1957; Seifter *et al.* 1959). More recently advances in monoclonal antibody, immunological and ultrastructural techniques have added additional information. Reddick (1974) used polyclonal fluorescent-labelled antibody to demonstrate collagen/collagenase interactions in the papillary dermis of human skins. Meigel *et al.* (1977) used the same basic techniques to show the uneven distribution of mature and precursor forms of Collagens I and III in the papillary and reticular dermis. Their findings indicated intertwining of the two collagens in the papillary dermis but predominance of collagen I in the reticular dermis. In 1981, von der Mark, in a review, concluded that both immuno-fluorescence and enzyme-linked immunohistochemical studies were a powerful supplement to the localisation of collagens I, II, III, IV and V in tissues. These techniques have also been applied to enzyme/inhibitor complex interactions in order to determine matrix turnover in femoral growth plates (Brown *et al.* 1989). Monoclonal antibodies, coupled to ELISA and immunoblotting techniques, have also been used to distinguish between collagens I, III and V in hides and skins in order to detail changes that occur during processing to leather (Werkmeister and Ramshaw 1988). From these studies, and other studies, twelve different collagen types are now recognised, based on both amino acid chain composition and the way in which individual chains are combined in the molecule (homopolymeric or heteropolymeric) as well as the steric configuration within the molecule (Fraser *et al.* 1979; Glanville & Kuhn 1980; Burgeson 1988). Of these twelve types seven are present in skin tissue but collagens I and III are predominant. Present in much smaller amounts, and found mainly in the basement membrane area, are the collagens IV, V, VI, VII

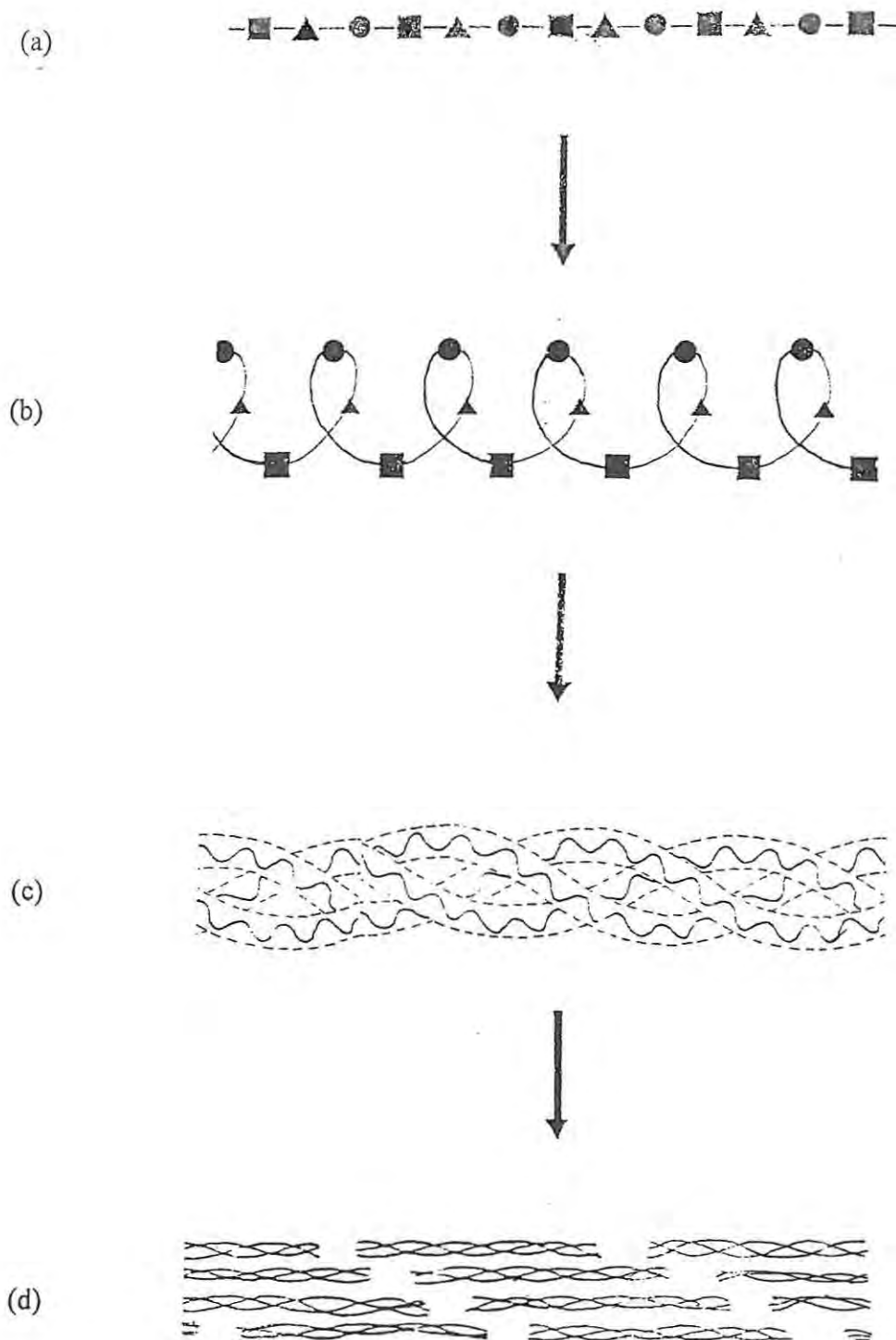


Fig. 1.2 Collagen Structure from Initial Amino Chain to Micro-Fibril Formation  
 (a) primary amino acid chain (b) secondary structure  
 (c) tertiary structure (d) micro-fibril

and VIII. (von der Mark 1981; Glanville 1987; Timpl and Engel 1987; Burgeson 1987; Kühn 1987, Burgeson 1988).

The collagen I molecule is a unique rod like triple helical structure containing over 1000 amino acids per individual chain in repeating triplets of X-Y-gly. Proline is generally present in the X position and hydroxyproline in the Y, so that the final molecule contains approximately 33% glycine and 20% hydroxyproline residues. The way in which the primary structure of collagen chains unite to form collagen is shown in Fig. 1.2. Each collagen I molecule is a heteropolymer consisting of three primary individual amino acid chains (two alpha 1 chains and one alpha 2 chain).

These chains hydrogen bond intra-molecularly to generate the secondary structure of collagen, a left-handed helix. The tertiary structure is formed when all three chains combine by intra-molecular hydrogen bonding which, together with water bridges, stabilises the helix (Glanville and Kühn 1980) to form a right-handed triple helical molecule. The helix is further stabilised by intra-molecular cross-links formed by aldol and condensation reaction between lysine and hydroxyproline residues of adjacent alpha chains in the non-helical region of the collagen molecule (Bornstein and Byers 1980; von der Mark 1981; Lawrie 1985) The quaternary structure occurs as these molecules aggregate to form a parallel staggered array which results in a micro-fibril (Bornstein and Byers 1980). Micro-fibrils so formed associate in bundles to form a fibre and the fibre bundles interlink to form a three dimensional pattern. This whole process results in a structural matrix that gives both strength and elasticity to skin.

Between this fibrous collagen structure are found other globular proteins, glycoproteins and proteoglycans which act as lubricants (Harkness; 1979; Pearse 1985). Amongst the proteoglycans are the ground substances of which chondroitin sulphate B (dermatan sulphate) is the principal, chondroitins A and C being found in lesser amounts. These substances are degraded by trypsin and chymotrypsin (Pearse 1985). Hyaluronic acid, another proteoglycan intrinsic to skin, is widely distributed in connective tissue and blood vessel walls. It is cleaved by hyaluronidases from bacteria and the invasiveness of *C. perfringens* is directly attributable to these enzymes which are able to decrease the viscosity of the extra-cellular matrix (Meyer and Rapport 1952).

### Hide degrading enzymes:

Since hides consist mainly of collagen the collagenase enzymes will be of prime importance in hide biodeterioration. True collagenases are defined as metallo-proteinases containing zinc in the active centre and being capable of causing the degradation of native collagen at physiological temperature and pH (Mandl 1972). Both tissue and bacterial collagenases can degrade collagen but only bacterial collagenases attack at multiple sites between the glycine and hydroxyproline/proline residues (Birkedal-Hansen 1987). Hydroxyproline and proline are imino acids and therefore no free hydrogen is available for bonding at the third amino acid down the helix as with other triplets. The effect is a bend or protrusion at this position disrupting the tight helical pattern (Boyd 1984). Mayer *et al.* (1980) postulated that regions of loose fluidity would represent protease sensitive sites and while non-specific protease attack on collagen can occur whether it causes degradation or not will depend upon the collagen type involved.

Collagen I is only degraded by collagenase and not by non-specific proteases, but collagen III is susceptible to trypsin and other non-specific proteases (Kemp 1983). Collagen IV, which co-distributes with collagen V, is sensitive to elastase, bone metallo-proteases and collagenase IV. Collagen V, which is found in muscle perimysium is susceptible to gelatinases. No reference was found concerning the susceptibility of collagen VI (which is found throughout the epidermis and especially in the peri-adnexal dermis) to non-specific proteases. Of significance is the susceptibility of collagen VII to non-specific proteases since this is the sole component of anchoring fibrils. These secure the lamina densa of epithelial tissues to underlying cytoplasmic membranes and are linked to anchoring plaques, and to each other, to form an enclosed network encompassing both collagenous and non-collagenous elements (Fig 1.3) (Burgeson 1988). It is thought that the collagen VII fibrils together with the plaques (which consist of type IV and VI collagen) may link the dermis and epidermis. (Timpl and Engel 1987; Glanville 1987). The susceptibility of collagen VII to both clostridial collagenases and pepsin is therefore of possible interest to the control of hide degradation.

Non-specific proteolytic degradation of collagen VIII occurs, but this collagen is only found in small amounts in the extracellular matrix of cells and so is not of major significance (Sage and Bornstein 1987).

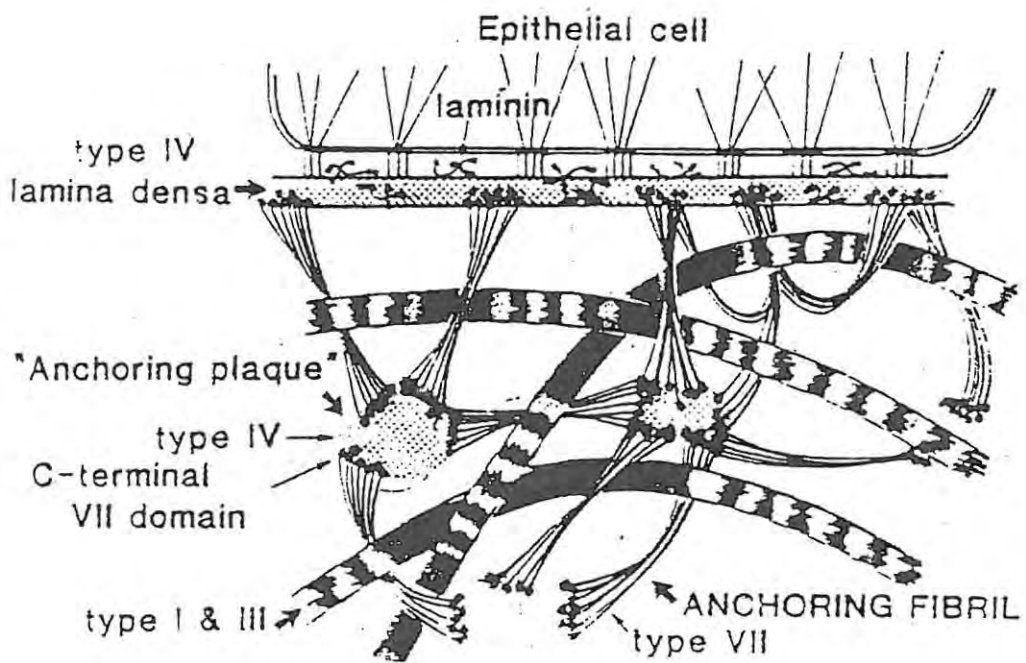


Fig. 1.3 Anchoring Fibrils and Plaques of the Dermal/Epidermal Junction  
(Reproduced from Burgeson, 1988)

### Bacterial invasion of hides

The bacterial degradation of hides results in two distinct forms of damage.

In the one type the hide is penetrated by bacteria with a progressive depletion of the collagen fibres and loss of hide substance (Dempsey 1948). This effect is highly visible and varies in degree of severity from structural looseness, to grain layer delamination, to individual holes of varying size and depth and ultimately multiple attack with its resultant "lace-curtain" appearance (Venkatesen 1979). Such gross biodeterioration results from an inadequate cure or no cure at all. As such it is not a common occurrence in the industry though when it does occur major financial losses can result.

The second biodeterioration effect is both more common and more subtle and is known in the industry as "sueded grain". This is best described as loss of the fine surface forming the skin (grain) layer of the papillary dermis, resulting in a scuffed and sueded appearance (Russell 1984). It is postulated, however, that chemical hydrolysis and mechanical treatments during pre-tannage and tannage can also give rise to sueded grain (Pfleiderer 1980; Heidemann 1983; Kemp 1983; Haines 1983(a)).

The role of different bacterial types in attacking either the surface papillary dermis grain layer or the general reticular dermis (corium major) has not yet been elucidated. This was investigated in 1949 by Anderson who found evidence for differential attack on hide protein layers by two strains of *Bacillus*, but did not identify either organism to species level.

During this period opinions differed as to whether the main bacterial access was via the epidermal and/or hair follicle route or via the flesh surface and along the adnexal dermis. Robertson and Haines (1947) showed evidence for the flesh route but Dempsey in 1948 showed that both routes provided access, although the initial attack occurred at the flesh surface. These findings were confirmed by Nandy *et al.* (1956).

The problem of which bacterial type and route of entry promotes sueded grain as opposed to general hide digestion, is compounded by the effects of post-cure processing. The beamhouse processes, described earlier, involve quite harsh chemical and mechanical treatments which on

their own could be a cause of sueded grain. Pfeleiderer (1980) attributed sueded grain to the improper use of process chemicals, while Heidemann (1983) ascribed it to both combined effects of prior bacterial attack and exposure to bating enzymes in processing. Haines (1983)(a) considered that liming at high temperature following initial bacterial attack and subsequent excess enzyme application was responsible. Tancous (1984) conceded that a chain of events was the most likely cause of this phenomenon. These interactive effects of hide degradation result in numerous changes within the hide tissue itself. Differential effects will obviously occur depending on the area under attack, the bacteria responsible, as well as the time and temperature at which degradation occurs.

#### **1.4. Historical Studies on Hide Degradation**

##### **General microbiological aspects**

Hides contain approximately 66% water and this, together with their high protein content will make them a good substrate for bacterial growth. The type of bacterial degradation that occurs will depend upon the bacterial populations present as well as the prevailing conditions on, and within, the hide itself. The rate and nature of the decomposition process will be governed by general microbial principles and these will include interaction between different bacterial types.

Bacteria in the environment co-exist in several ways which are based mainly on a series of interactions, either with other biota or with each other. Such interactions range from antagonism to synergism (Sinell 1980(a)). Beneficial effect of co-existence, such as proto-cooperation and potentiation, are well documented and symbiotic activity has been detected even in fossils (Meleney 1931; Boyd 1984).

Static or constant microbial composition only exists under defined conditions, these being due to chemical or physical extrinsic factors (e.g salt addition or freezing) or intrinsic factors, such as available nutrient depletion and/or toxic by-product accumulation (Ingram and Simonsen 1980). In the absence of these factors degradative processes can proceed pro-actively due to non-synchronous growth involving two or more species, often under conditions of bacterial succession involving mutualism or proto-cooperation ( Boyd 1984).

Evidence for bacterial interaction can be found in a broad spectrum of literature. Tissier and Martelly in 1902 showed that commensalism between aerobic bacteria and putrefactive anaerobes existed during the microbial digestion of meats. Much later, studies by Ishii et al. (1987 and 1988) confirmed that the same interactive aerobic/anaerobic microbial commensalism occurred during the dissolution of both raw hide and soluble collagen. In this case the bacteria involved an aerobic *Vibrionaceae* and *C. histolyticum*, were in log phase growth indicating that degradation occurred in a dynamic, as opposed to static, environment.

Forstner (1979), in a review, highlighted the essential role of many different bacteria in world geo-chemical recycling of metals and minerals. Bacterial succession in the degradation of a variety of substrates from many different environments has been well established. These include aquatic sediments, human and industrial wastes, oil, petroleum and paint products, dairy products, fish, meat, poultry, pharmaceutical products and cosmetics as well as animal, fish and avian tissues (Davis 1967; Sinell 1980(a); Hill 1981; Gottschalk et al. 1981; Brock et al. 1984; Boyd 1984; Marsh and Martin 1992).

In fact, in any degradative process where the substrate has not reached a state of microbial constancy there will be interactive microbial effects present often in the form of a succession. Succession has been defined as "the replacement of populations and community type that occurs with time" (Boyd 1984) and as a "microbial profile that changes continuously as one association (characteristic flora) succeeds another" (Sinell 1980)

Shifts of microbial population with time are of major interest to those concerned with the preservation of foodstuffs including meat products. Since meat is a high protein substrate containing collagen it could resemble hide in terms of substrate usage by microbes. Meat degradation is known to be caused as a result of bacterial succession involving preliminary colonisation with *Pseudomonas*, *Moraxella* and *Acinetobacter* followed by proteolytic microaerophilic *Clostridium* and then by strictly anaerobic, proteolytic and amino acid degrading *Clostridium* (Newton et al. 1977; Gill and Newton 1977; Banwart 1987). However, the role of specific bacteria in hide degradation remains to be fully elucidated. This includes both the process of the degradation as well as the effect of microbial succession on the degradation rate.

### Microbial degradation of hides

Studies to date concerning preservation and degradation of hides have focused on three areas. The majority of studies have concentrated on correlations between hide surface bacterial counts and subsequent leather quality (Nandy et al. 1956; Cooper et al. 1969; Cooper et al. 1970; Hendry et al. 1970; Bailey and Haas 1988). In some cases hide extrudates, as opposed to surface activity, were used to estimate total bacterial numbers together with bacterial collagenolytic activity in-situ (Woods et al. 1973; Hockey & Russell 1982). The findings of these studies are varied and often show a lack of correlation between total bacterial numbers, bacterial collagenolytic activity, and subsequent leather quality (Woods et al. 1970(b); Woods et al. 1971(a); Welton 1974). Most studies concentrated only on aerobic counts and bacterial species identification was omitted. The role of both specific bacterial types as well as anaerobic bacteria therefore remains undefined.

A second approach to bacterial hide degradation has been concerned with the role of specific collagenase producing bacteria e.g. *Vibrio alginolyticus* in the process. Welton et al. (1972) found no correlation between *V. alginolyticus* in salt cured hides and leather decay. Woods et al. (1973) found the opposite when examining natural populations, which would include the *Bacillus* group, from raw hides. *V. alginolyticus* is a bacterium of strictly marine origin which grows optimally at 3% salt concentration but only marginally at 20% (Thompson et al. 1986; Baumann and Schubert 1986). However, the bacteria of the *Bacillus* group vary in terms of salt tolerance, some strains growing up to 14% salt concentration (Christian 1980). They also vary in their ability to produce collagenases (Petushkova et al. 1985; Bruning 1991). The findings of Woods et al. (1973) and Welton (1972) therefore are in accordance with the individual characteristics of the bacteria involved and this also means that while *V. alginolyticus* has the most specifically active collagenase isolated from any bacterium (Keil 1979) it has no real place in raw hide degradation studies. Conversely, members of the *Bacillus* group, being both halotolerant and spore bearing (therefore capable of existing in high salt environments) may play a part in both raw and salted hide degradation.

The third approach used in hide degradation studies in the classical microbiological approach based on Koch's postulates in which bacteria are isolated from degraded hides and established in laboratory cultures prior to inoculation of hides or hide substitutes. The inoculated systems

are then subject to a number of microbiological, physical and chemical tests to establish evidence of specific microbial degradation. As early as 1922 McLoughlin and Rockwell, in an attempt to understand microbial succession in hides, isolated proteolytic and non-proteolytic bacteria from naturally decayed hides and used them to dose hide corium extracts. They used 41 parameters to classify their isolates but did not identify any to species level. The effects of pH, oxygenation and temperature on the rate of degradation were assessed using weight loss and general hide appearance. Their conclusion was that the two groups of bacteria acted synergistically in hide degradation but they stated that the anaerobic bacteria were not involved.

In 1949 Anderson used chemically sterilised (sodium metabisulphite and hydrogen peroxide) salted hide pieces and dosed them with strains of bacteria isolated from salted stock. He classified 80 isolates to generic level and found that only strains amongst those classified as *Bacillaceae* were capable of hide digestion. Parameters for assessment included weight loss, fluid extrusion and final leather quality. In 1955 further studies on salted stocks using chemically sterilised hide pieces were carried out by Everitt and Cordon. They grouped 67 hide isolates from salt brines and salted hides according to Gram reaction and morphology into four groups (Gram positive or negative; cocci or rods). A fifth group of Gram variable rods was included and all groups were dosed to hides either as individual or mixed cultures. Only strains from the fifth group mixed with those of the Gram negative rods showed evidence for definite collagen denaturation, as shown by the increase in hydroxyproline levels. They found no obligately anaerobic strains in their study (although Gram variability is a feature of both *Bacillus* and *Clostridium*).

Further classic work was done by Formisano in 1965. He isolated 97 halophilic bacteria from salted hides and after isolating them in pure culture and provisionally identifying them, he re-inoculated them to salted stock. From both visual and histological evidence of the stored hides he concluded that there was no real degradation of the hides despite some minor hypodermal effect. In 1986, Kallenberger and Lollar used hydrogen peroxide treated salted hides for inoculation with halotolerant bacteria isolated from tannery brines. They showed evidence of bacterial collagenolytic activity but their findings are suspect due to the long incubation times (50 days at 35-40°C) that were employed and the fact that the samples were

frozen prior to inoculation as well as not being completely sterile. Of interest, nevertheless, is the fact that their only active collagenolytic isolate was a halotolerant *Staphylococcus*. Waldvogel and Swartz (1969) also found collagenolytic activity in a *S. aureus* strain, albeit only under anaerobic conditions. As staphylococci tolerate high salt conditions (up to 15-20% salt) (Christian 1980) these findings suggest that bacterial succession involving halophilic halobacteria and halotolerant staphylococci could possibly exist in wet salted stock.

Perhaps the most relevant of the classic studies of hide degradation were those by Tancous who, in 1961, isolated a collagenase-producing organism from salted hides. Provisionally identified as *C. capitovale* and now reclassified as *C. cadaveris* (Koneman *et al.* 1983) this organism is not listed in the current literature as possessing a collagenase enzyme. In fact this property amongst the clostridia is ascribed only to *C. histolyticum*, *C. perfringens* and *C. difficile* (Cato *et al.* 1986; Seddon *et al.* 1990). However, with *C. capitovale*, Tancous demonstrated that when inoculated to calf skin corium (without epidermal layers) and held under moist incubation in a low salt solution, this organism caused degradation due to a proteolytic enzyme. This enzyme was shown to degrade soluble collagen and to cause an increase in total volatile nitrogen/ammonia values as well as histological changes of the inoculated corium. This is the first real evidence for the role of *Clostridium* species in hide degradation although their denaturative properties are well documented by food and medical scientists (MacFarlane and MacLennan 1945; Smith and George 1946; Hill 1981; Gottschalk *et al.* 1981). As stated and referenced earlier, the role of aerobic bacteria in enhancing micro-aerophilic/anaerobic clostridial decomposition of protein compounds is well established. This was further confirmed by Ishii *et al.* (1987) who inoculated two clostridial isolates (provisionally identified as *C. histolyticum*) and an aerobic "*Vibrionaceae*" from calf skins to skin specimens at 30°C. After 36 hours visual degradation was present on the skin inoculated with a combination of the aerobic and anaerobic bacteria. They did not follow Koch's postulates fully but instead inoculated their isolates to a broth culture containing casein, meat extract, gelatin and collagen. Their findings of increased digestion of soluble and insoluble collagen by the mixed cultures as compared to the mono cultures of the clostridia could be a reflection of their in vitro conditions rather than actual events in-situ but also represents a possible two-phase bacterial succession in hide degradation.

Few classical microbial studies involving hides have been done since 1961 other than that of Formisano (1965) and Kallenberger and Lollar (1986) as discussed above. However, of interest is the study by Jain and Zeikus (1989) involving the anaerobic digestion of gelatin. They found that an organism designated *C. collagenovans* (due to its collagenase production) synergistically caused degradation of gelatin in the presence of *Methanosarcina barkeri*.

### Microbial ecology of hides and other protein substrates

Venkatesen (1979) reviewed the literature on hide and skin degradation and mentioned the numerous bacterial types identified in different studies, of which *Bacillus* sp and *Proteus* sp were among the most common isolates. He concluded that both indigenous and environmental bacteria including human pathogens, would colonise hides. As such pure microbial ecological studies of hides would involve intensive surveys it is postulated that this may be the explanation for their almost total absence in the hide/skin literature.

In the absence of such studies it becomes necessary to select bacteria for raw hide deterioration investigations from another source. In this regard the literature on protein degradation in general becomes invaluable and this is further aided by the literature on bacteria capable of producing collagenases and amino acid degradative enzymes under post-mortem conditions of reduced redox potential.

High protein substrates, other than hides, which undergo bacterial degradation include a variety of foodstuffs (meat, fish, poultry and milk) as well as living human and animal tissues (muscle, cornea and gingival crevices). Degradation of proteins proceeds in a similar pattern for all these substrates viz:

protein > peptones > polypeptides > di-peptides > amino acids

The bacteria responsible for preliminary protein decomposition in foods have been identified as the Gram negative *Pseudomonaceae* (*Ps. fluorescens*, *Ps. aeruginosa* and *Ps. sp*) and *Neisseriaceae* (*Alcaligenes* sp. and *Acinetobacter* sp.) Their role as primary colonisers and decomposers of meat, fish and meat products has been studied in detail (Cloake 1923; Gill 1976; Gill and Newton 1977; Ingram and Simonsen 1980; Faber and Idziak 1982; Lawrie 1985; Hayes 1985; Nortje 1987). The organisms responsible for the secondary and tertiary

phases of protein degradation have been well documented in the food and meat science literature as the putrefactive micro-aerophilic and strictly anaerobic clostridia. These include *C. histolyticum*, *C. perfringens*, *C. bifermentans* and *C. sporogenes* (Rogers 1961; Eskin 1971; Mandelstam *et al.* 1975; Lawrie 1985; Banwart 1987). Of these *C. histolyticum* and *C. perfringens* are proven collagenase producing bacteria (Robb-Smith 1945; Kono 1968; Vencill *et al.* 1985; Tsiga *et al.* 1988). Studies in medicine concerning the causative agents of gas-gangrene, and its associated muscle myo-necrosis, have identified *C. histolyticum*, *C. perfringens* and *C. novii* as the major organisms responsible. Synergistic activity between these pathogenic clostridia and the non-pathogenic *C. sporogenes*, has been demonstrated and it has been shown that this results in increased morbidity and mortality in diseased patients (Smith and George 1946; Willis 1977; Freeman 1979; Joklik *et al.* 1984).

Studies by Nord *et al.* (1975) showed that *C. perfringens* and *C. sporogenes* possessed both casein and gelatin degrading alkaline proteases. Also oxidative and reductive deamination and transamination of coupled amino acids (including glycine, proline and hydroxyproline) via the Stickland reaction is brought about by both *C. histolyticum* and *C. sporogenes* (Doelle 1975; Costilow 1977; Costilow and Cooper 1978; Gottschalk 1979; Brock *et al.* 1984).

In the case of salted hides, the high salt environment (35% m/v) creates a unique ecosystem in which only the extreme halophiles, the Halobacteriaceae will be able to flourish (Larsen 1967) under normal circumstances. While these bacteria are known to produce proteases which are active in high salt concentration, no definite proof for collagenase production by these bacteria has been found (Lanyi 1974; Larsen 1986).

### 1.5. Questions arising from the literature survey

From this literature survey it can be seen that many aspects of hide degradation remain unresolved as to both cause and effect. In-depth pure microbiological investigations of the bacteria involved, their routes of entry and specific areas within hide tissue that are degraded still need to be established. In order to develop more exact and effective methods for preserving hides prior to tanning the following questions need to be answered:

- a. What are the major bacterial types that colonise hides immediately post-flay?

- b. What type of changes occur in hide populations post-flay and during transportation and storage?
- c. Which specific bacteria are involved in hide degradation involving surface degradation (sueded grain) and total hide digestion?
- d. Does hide degradation occur as result of interactive bacterial effects such as succession and, if so, which environmental parameters affect this?
- e. Which routes of entry are used by the specific bacteria involved in hide decomposition and which specific substrates within the hide do they colonise?
- f. Are there interactive effects between general proteolytic enzymes and collagenases during hide decomposition?

### 1.6. Investigation of a Model System for Hide Degradation

#### General considerations:

The examination of a model system, in which fresh hide samples subjected to sterilisation, and inoculated with selected organisms is a possible approach to answering some of the above questions. It has advantages over studies using naturally decomposing hides in that conditions in-situ can be well controlled. Also specific pure cultural isolates of bacteria can be used, either individually or mixed with other bacterial cultures, to study the interactive effects of bacteria in the degradative process. An added advantage is that autolytic tissue enzyme activity can be inactivated so that only bacterial effects can be studied. For the above reason the work of this thesis concentrated on developing such a system and using it to demonstrate possible bacterial succession in hide degradation.

#### The selected inocula

Due to the strong evidence in the literature that protein putrefaction occurs as a result of bacterial succession involving aerobic *Pseudomonas* sp, micro-aerophilic and collagenolytic *Clostridium* sp followed by anaerobic/proteolytic and amino acid degrading clostridia, representative type cultures of these organisms were chosen for the degradative studies in this thesis. While the initial criteria for the selection of these organisms was based on the literature survey, restrictions of both time and practical application limited the choice of bacteria to three, one for each of the postulated stages of raw hide degradation. In order to select these on a logical basis several factors, other than respiratory requirements and proteolytic activity, were taken into consideration.

the criteria used included the prevalence and distribution of the bacteria in both the natural environment as well as in specific niche habitats, such as soil, water and dung. Other specialised attributes considered included the ability of candidate organisms to outcompete natural and transient hide populations based on their ability to catabolise free amino acids and withstand "stress" situations. The utilisation of amino acids in putrefactive anaerobic energy yielding mechanism was also considered a pertinent criterion.

Using the above parameters suitable organisms were selected for the model study based on relevant literature (Nord *et al.* 1975; Adams 1959; Thacker 1969; Gryder and Adams 1969; Palleroni 1981; Cato *et al.* 1986; Hisano *et al.* 1989). The final choice of bacteria for the three stages of degradation were *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes* respectively. The reasons for the choice of these specific bacteria are given below:

#### *Ps. aeruginosa*

Pseudomonads in general are ubiquitous and are found as free living saprophytes of soil, water and a large number of other substrates (Stolp and Gadkari 1981; Palleroni 1981). They are mesophilic organisms which become secondary pathogens in disease processes but are able to survive for long periods in the environment (Palleroni 1981 and 1986). They are also implicated in the degradation of industrial products and in the spoilage of food and dairy products as well as meat, fish, poultry and eggs (Gould 1988). They act as primary degraders in sewage (Stolp and Gadkari 1981). *Ps. aeruginosa* in particular has been implicated in the degradation of meat (Banwart 1987) and many strains of this organism possess "stress"-resistance "R" factors which are plasmid mediated (Marques *et al.* 1979). *Ps. aeruginosa* also catabolises free amino acids and is a strictly aerobic organism. For these reasons it was considered a suitable choice to represent primary colonisation of raw hides.

#### *C. histolyticum*

The only known prolific collagenase producing clostridia are *C. histolyticum* and *C. perfringens*, and although *C. difficile* has been reported as a collagenase producer, it has only been found in one strain (Cato *et al.* 1986). Since both *C. histolyticum* and *C. perfringens* are proteolytic micro-aerophilic organisms that produce both hyaluronidase and elastase as well as collagenase, they would both fulfill the requirements for the secondary degradative organisms of a putrefactive succession (Sinell 1980). They are ubiquitous in all types of soil and are

natural residents in mammalian intestines from whence they are disseminated in faecal excretions (Smith 1975 (a) and (b); Hill 1981). Both organisms are spore bearers and this gives them competitive advantages over vegetative bacteria under "stress" conditions. Both organisms are mesophilic pathogens that degrade amino acids in oxidative-reductive Stickland reactions in which they reduce glycine, proline and hydroxyproline. *C. histolyticum* is used as a source for commercial collagenase production and therefore enzyme studies using in-situ samples and in-vitro enzyme controls could be compared using this organism. Therefore, *C. histolyticum* was selected as the choice for micro-aerophilic collagenase degrading organism representing secondary succession.

### *C. sporogenes*

This organism is a harmless saprophyte existing in nature under a wide range of conditions. It has been found in almost every specimen of soil which has been cultured and has been considered to be the primary cause of decomposition of protein material under natural conditions (Smith and Holdeman 1968). It is highly proteolytic, produces copious numbers of spores under both normal and "stress" conditions and will germinate within 1½ hours at 37°C (Smith and Holdeman 1968). These competitive characteristics together with its ability to transaminate glycine and alanine as well as deaminate other amino acids via the Stickland reactions (Eskin 1971; Costilow and Cooper 1978) make it an ideal choice for the strictly anaerobic tertiary organism involved in a bacterial succession resulting in the putrefaction of raw hides.

## 1.7. Main Research Objectives

- a. To utilise basic microbiological approaches to demonstrate whether bacterial succession, or any other form of bacterial interaction, is responsible for the degradation of raw hides.
- b. To establish a model system in which hide degradation studies in-situ could be undertaken without interference from either extraneous bacteria or tissue enzymes.
- c. To use the system to promote a better understanding of hide protein decomposition in general, and to elucidate the specific role of aerobic *Pseudomonas*, micro-aerophilic *Clostridium* and anaerobic *Clostridium* species in the degradative process.
- d. To use the findings of the study to formulate recommendations for more effective methods of raw hide preservation.

## 1.8. Thesis Rationale

This study concentrated on the role of selected *Clostridium species* in the degradation of raw hides. However, since a large number of hides undergo preservation (cure) by salting it was incumbent to study this aspect of hide degradation as well. Salted hides have their own unique populations, and evidence for bacterial succession was sought in this stock so as to promote a better understanding of hide degradation in general. In order to cover all of the various aspects of bacterial succession and interaction in hide tissues, a number of different investigations had to be performed. A thesis logic is included here to clarify the steps taken to prove the hypothesis of this thesis and at the same time to investigate hide degradation in a holistic manner. The research in this thesis was based on Koch's postulates i.e. isolating bacteria from infected material, maintaining them in pure culture, inoculating them to the same or similar material and studying their effects in-situ as confirmation of their role as causative agents in the primary infection. The thesis rationale for each chapter is given below.

### Chapter 1:

This covers a holistic literature survey to cover all aspects of hide structure and protein as well as bacterial degradation of hides.

### Chapter 2:

This describes the method development undertaken in order to optimise certain methods used in this thesis.

### Chapter 3:

This describes basic studies to:-

- a. Confirm the literature findings that hide degradation is due mainly to bacterial colonisation and proliferation and attack on various hide tissues.
- b. Confirm the choice of bacteria to be used as the inocula for the proposed model study by (i) isolating them from random hide samples and (ii) demonstrating their ability to outcompete resident hide populations and produce degradative changes to hide proteins.

### Chapter 4:

This describes the development of a system for in-situ hide degradation studies and does the following:

- a. Confirms ethylene oxide hide sterilisation does not affect hide integrity.
- b. Optimises the growth of selected bacterial inocula in the system so as to represent events observed in naturally decaying hides.
- c. Confirms that the general proteolytic and/or collagenolytic activity of the selected inocula accords with the expected hide tissue breakdown in a bacterial succession.

#### **Chapter 5:**

This chapter describes the use of the model system to determine events during hide degradation by dosing the selected bacteria to sterile hides either individually, or as double-mixes and as a mixture of all three organisms (triple-mix). It shows the changes that occur in the hides as a result of bacterial growth, proliferation and enzyme activity using a number of different parameters and confirms their recovery from the system in pure culture. The findings of this study are used to prove or disprove the main hypothesis of bacterial succession in hide degradation.

#### **Chapter 6:**

Investigates the cause of antagonistic effects found between the two selected clostridial strains.

#### **Chapter 7:**

This describes secondary studies to show evidence for bacterial, and possible halophilic collagenolytic activity, as a cause of degradation in salted hides as further proof of the role of bacterial interaction in hide biodeterioration.

#### **Chapter 8:**

Summarises the overall findings of this study and shows practical application of the results to hide preservation.

### 1.9. Thesis Hypothesis:

The hypothesis underlying this study is that bacterial degradation of raw hides is due to a succession of bacterial types in terms of respiratory requirements and enzyme production. The respiratory succession follows an aerobic, micro-aerophilic, anaerobic route, while the enzymatic succession involves proteolytic/saccharolytic, collagenolytic and proteolytic/amino acid degrading enzymes in that order. Secondary to this hypothesis is that degradation involves the interactive effects of ubiquitous aerobic respiratory colonisers (e.g *Pseudomonas species*), specific micro-aerophilic, collagenolytic *Clostridium species* and specific strictly anaerobic *Clostridium species*.

## CHAPTER 2

### METHODS DEVELOPED FOR USE IN THIS THESIS

- 2.1. Summary
- 2.2. Introduction
- 2.3. Materials and Methods
  - 2.3.1. Collagen gels and plugs
    - a. Collagen agarose gels for enzyme detection in fluids
    - b. Collagen agarose gels for enzyme detection in tissue
    - c. Collagen media for direct bacterial activity
    - d. Collagen plugs for enzyme inhibition studies.
  - 2.3.2. Hydroxyproline Assay
  - 2.3.3. Quantification and Visualisation of Bacteria
    - a. "Stamp Test"
    - b. Clostridia counts
    - c. Immunolocalisation
- 2.4. Results and discussion
  - 2.4.1. Collagen gels and plugs
    - a. Collagen agarose gels for enzyme detection in fluids
    - b. Collagen agarose gels for enzyme detection in tissue
    - c. Collagen media for direct bacterial activity
    - d. Collagen plugs for enzyme inhibition studies.
  - 2.4.2. Hydroxyproline Assay
  - 2.4.3. Quantification and Visualisation of Bacteria
    - a. "Stamp Test"
    - b. Clostridia counts
    - c. Immunolocalisation
- 2.5. Conclusion

### 2.1. Summary:

Ethylene oxide-treated soluble collagen gels, collagen media and collagen plugs were developed in order to detect collagenase activity in hide extrudates, inocula suspensions and tissue sections during in-situ hide degradation studies. The application of standard commercial collagenase and protease preparations and known collagenase positive and negative bacteria to the gels confirmed the specificity of the method. The gels were shown to be free from contaminating bacteria and did not cause over-solubilisation of the media in the presence of high collagenase concentrations. A good regression value was obtained when a range of collagenase concentrations was plotted against lysis zone size on the gels, indicating their suitability for use in semi-quantitative collagen assays. Collagen plugs were also shown to be specific for collagenase detection and suitable for use in testing of biocides for anti-collagenase activity.

Modifications to standard spectrophotometric hydroxyproline methods were made and these resulted in a sensitive assay for use in detecting this imino acid in hide extrudates.

A method to replace total counts of surface bacteria during hide degradation was developed. This method involved the transfer of surface bacteria from hides to laboratory media by use of a velveteen pad of a set area. A selective agar to detect early growth, and to semi-quantify the number of bacteria, as well as to detect proteolytic activity was also developed. This system called the "stamp" test was shown to be easy to use, non invasive of hide tissues, and generated individual assessments of bacterial colonisation on both hair and flesh surfaces.

To differentiate between the two individual *Clostridium* inocula used in this study attempts were made to find a suitable selective media. Dextrose fermentation was chosen as the identification factor but, due to the need to use a media overlay method because of the strictly anoxic characteristic of *C. sporogenes*, the method was unsuccessful. Similarly, attempts to raise antibodies in rabbits to the three selected inocula, for use in tissue immunolocalisation studies, were also unsuccessful. Therefore total clostridial counts only, not individual species counts, were chosen for use when the two selected *Clostridium* were in mixed culture.

## 2.2. Introduction:

### Collagenase assays:

Collagenase assay methods are numerous and they include viscometry (Gallop *et al.* 1957), titrimetry (Veiss 1964), photometric analysis of amino acid release from synthetic substrates (Welton *et al.* 1972; Barrett *et al.* 1989), hydroxyproline release (Morales 1978), radioactive labelling (Lefevre *et al.* 1979; van Wart and Bond 1982), fluorimetry (Steven and Lowther 1975), immunological labelling (Bauer *et al.* 1971 and 1972; Woolley *et al.* 1980), ELISA monoclonal assay (Birkedal-Hansen 1987), HPLC analysis of synthetic peptide degradation products (Biondi *et al.* 1988), collagen dye binding (Nethery *et al.* 1986), weight loss of acetone-dried collagen (Tint 1961), polarimetry (Swartland 1990, Brüning 1991) and haemagglutination of collagen coated erythrocytes (Kojer *et al.* 1978). Substrates used are generally soluble or fibrillated collagens and synthetic peptides but both human and animal tissues have also been used (Salthouse *et al.* 1970; Brown *et al.* 1989; Swartland 1990).

Most of the above techniques use specialised equipment or are time consuming. To avoid this, several studies have used semi-quantitative assays involving collagen films or gels utilising fibrillated or soluble collagen (Robbertse *et al.* 1977; Yankeelov *et al.* 1977; Giacomello *et al.* 1979; Hockey and Russell 1983). Soluble collagen is less reliable as a substrate than fibrillated collagen due to its greater susceptibility to heat denaturation, (41,0°C compared to 48,7°C respectively; Birkedal-Hansen 1987), and also soluble collagen is attacked inter-molecularly by a single enzyme whereas fibrillated collagen requires a succession of enzymes including inter-molecular depolymerases before denaturation occurs (Weiss 1984). Fibrillated collagen as a substrate therefore more nearly represents in-vivo conditions present in hides and as such have been shown to be sensitive to collagenase at 20 ng ml<sup>-1</sup> (Kasten *et al.* 1989) and used to detect 60 ng ml<sup>-1</sup> collagenase in 10 µl fluid samples (Giacomello *et al.* 1979). In a review of collagenase assay methods Harris and Vater (1980) confirmed the stability and linear response of fibrillated collagen gels. Fibrillated collagen gels were therefore investigated for use in the hide degradation studies of this thesis, both for collagenase detection in tissue and tissue extrudates as well as in actively growing cultures, and for future use in biocide mediated collagenase inhibition studies.

In order to optimise the system and prevent problems due to bacterial contamination the use of ethylene oxide-exposed collagen was investigated as preliminary trials had shown that unsterile collagen resulted in non-specific gel lysis. Also such gels were readily degraded at high collagenase concentrations making results interpretation difficult.

Initially both monomeric bovine corium collagen (prepared by the method of Cooper and Davidson [1965] and reconstituted as detailed by Brüning [1991] and polymeric collagen prepared according to the method of Merkel *et al.* [1975]) were used to form gels. Specificity of the gels was determined using both collagenase producing strains of bacteria and high purity commercial enzyme preparations. Details of these are given in Table 2.1.

**Table 2.1. Enzyme and Bacterial Controls for Collagen Gel Specificity Tests**

Sample	Source/ Code	Conc. mg ml <sup>-1</sup>	Origin	Active versus
Protease	*P5380	5	Microbial	Casein
Proteinase K	*P0390	5	Microbial	Casein, keratin
Pronase	*P5147	5	Microbial	Casein
Elastase	*E2058(IU)	5	Porcine pancreas	Denatured collagen, casein
Trypsin	*T0134	5	Porcine pancreas	Arginine, lysine, bovine albumin
Collagenase	*C9891(1A)	0,008- 0,50	Microbial ( <i>C.histolyticum</i> )	Collagen, proteins
<i>V. alginolyticus</i>	Strain 1	-	Estuarine	Collagen
<i>V. alginolyticus</i>	Strain 2	-	Estuarine	Collagen
<i>S. aureus</i>	10788	-	NCTC	#
<i>E.coli</i>	10148	-	NCTC	#
<i>B. cereus</i>	Strain 1	-	SAIMR	Collagen
<i>B. cereus</i>	Strain 2	-	SAIMR	#

\* Sigma Biochemicals

# Inactive against collagen (not tested against other proteins)

The monomeric collagen preparations were difficult to gel and proved unstable during incubation becoming susceptible to non-specific protease attack and had low sensitivity (levels of  $100 \mu\text{g ml}^{-1}$  not being detected until after 4-5 days incubation). Monomeric collagen was not included, therefore, in any further method development trials.

#### Hydroxyproline assay:

While assays of collagenase in hide tissues and hide extrudates are of prime importance to degradation studies, they are subject to inherent problems. This is because enzyme production may be potentiated or antagonised according to the test and environmental conditions (Gallop *et al.* 1957; Golub *et al.* 1987). Hydroxyproline release has been considered a good choice for the detection of collagen denaturation due to its virtual uniqueness to connective and dermal tissue and its relationship to hide collagen content (Heidemann 1974).

The method of choice of hydroxyproline assay has historically been a spectrophotometric assay based on hydrolysis of samples at high heat, followed by oxidation to form a pyrrole compound coupling to p-dimethylaminobenzaldehyde to form a red chromophore. The original method has undergone many modifications, the most notable being those of Neuman and Logan (1950) and Woessner (1961). Woessner's method in particular improved the stability and sensitivity of the chromogen as well as minimising the role of extraneous organics in depressing chromogen development. In order to overcome the effects of differing hydroxyproline/total protein ratios in various tissues, he developed an additional modification for quantification of the imino acid at concentrations of 2% or less. Similarly Heidemann (1974) adapted the assay for use for leather samples, (these containing approximately 7 mg hydroxyproline per 56 gm of milled sample) in which the action of dyes and tanning agents was also suppressed.

Jamall *et al.* (1981) modified Woessner's method in order to detect nanogram levels of hydroxyproline in  $100 \mu\text{l}$  extracts of rat tissue. The resulting sensitive and accurate assay was based on establishing correct concentrations for the standard controls and by determinations of the effect of tissue exudate on suppressing Chloramine T activity. Optimisation of pH, incubation time and temperature were also undertaken.

In order to achieve optimum results for this study a modified method was developed. This utilised standard concentrations of hydroxyproline of 4-16  $\mu\text{g ml}^{-1}$  compared to 0-5  $\mu\text{g ml}^{-1}$  (Woessner 1961); 0,5 -0,8  $\text{mg ml}^{-1}$  (Heideman 1974) and 0,2 - 0,16  $\mu\text{g ml}^{-1}$  (Jamall et al. 1981). In order to establish that no loss of activity occurred due to inactivation of Chloramine T by other proteins present in the tissues samples, extracts of aged EO hide plugs (1 month and 3 month respectively) were incorporated into the standard graph results. These control samples also served to confirm the integrity of EO collagen and to establish lack of autolytic degradation.

### **Quantification of Bacteria**

#### **The "stamp test":**

Studies of hide degradation have used total bacterial counts as indicators of deteriorative changes (Hendry et al. 1970; Woods et al. (1971(a)). As these are somewhat time consuming to perform a method was sought that would allow for a large number of samples to be examined with ease and at the same time give information concerning the degree of proteolytic activity of the bacteria present.

Bacterial counts on hides have generally been carried out on small hide plugs that have been extracted from larger (14,5 x 21,0 cm) pieces during storage (Hockey and Russell 1982). The plug is placed in a suitable diluent and shaken to extract any bacteria present and the diluent used to perform a bacterial count. This has three disadvantages, the first of which is that bacterial numbers on hair and on flesh surfaces cannot be differentiated. The second is that counts are not performed by direct transfer and therefore may not reflect actual numbers and the third disadvantage is that mutilation of the hide sample occurs so that possible differences may arise in niche areas during subsequent re-incubation and storage.

The "stamp" test was designed to overcome these problems by the use of pre-sterilised 3cm diameter velveteen pads that could be aseptically attached to a holder (designed for the purpose) with Prestik. The holder was designed with a swivel action so that the velveteen pad could be easily pressed over an area of hide and then transferred to the surface of an agar plate. To standardise the method, counts were initially performed on adjacent hide areas and bacterial numbers so obtained were correlated to the amount of growth on the agar (+ - +++). The agar media also incorporated a protein base in order to detect general proteolytic activity and

included an indicator of bacterial respiration as a means of easier visualisation of growing colonies.

**b. Clostridia Counts:**

In order to perform individual counts of *C. histolyticum* and *C. sporogenes* in fluid extrudates from hides (inoculated with mixed cultures of the two organisms), it was necessary to develop a selective medium. This medium was based on differences between the two species but unfortunately, due to their close phenotype relationships, only three choices out of some 37 characteristics were available (Smith and Holdeman 1968; Cato *et al.* 1986). The three biochemical reactions involved were lipolytic activity on egg yolk agar, aesculin hydrolysis and dextrose fermentation. Both lipolytic activity and aesculin hydrolysis were excluded as they resulted in large diffuse zones in the media causing cloudiness and blackening respectively and this prevented differential counts of individual colonies. Dextrose fermentation was therefore the final method of choice and this sugar was incorporated, along with Andrades indicator, into Reinforced Clostridia agar. It was then called Modified Reinforced Clostridia agar (MRCA). Two different modifications were tested called MRCA1 and MRCA2 dependent upon the agar and the starch content.

In order to obviate the problem of oxygen sensitivity of *C. sporogenes* the layer plate method for anaerobic counts was used (Appendix B).

**Immunolocalisation:**

The lack of a suitable media for the investigation of specific clostridial counts promoted the investigation of immunohistochemistry (Polak and van Noorden 1988) as a possible means of visualisation of the individual bacterial strains within the hide. It was also postulated that such techniques could help to determine whether the selected bacteria colonised specific areas within hide tissue as well as possibly locating initial entry routes of the different bacteria. By this means, it was hoped that the role of bacteria, in causing either sueded grain or full hide degradation, could be better understood.

## 2.3. Materials and Methods:

### 2.3.1. Collagen gels and plugs:

Neutral soluble polymeric collagen (at 5ml volumes in 160mm x 16mm glass tubes) was sterilised in a commercial EO steriliser (Appendix A). The result was a non-homogenous solution of high pH which proved immiscible in agar media. To overcome this problem pH adjustments were made using glacial acetic acid addition prior to EO-treatments. At pH values of 5,0 and 7,0, marked changes in fibrillation and homogeneity occurred rendering the collagen unsuitable for gel formation. At pH 6,0 pre-sterilisation, the result was a homogenous solution that gelled well and had a final pH value of 6,98.

#### 2.3.1.a. Collagen Agarose Gels for Enzyme Detection in Fluids:

High gel point agarose was prepared at a 2% m/v concentration in distilled water, heated to dissolve and cooled to 47°C. The pH was adjusted to 7,0 using 0,2 N NaOH. EO-sterilised bovine collagen calfskin (Merkel *et al.* 1975) was added in 4,5ml aliquots to 0,5ml of 10 x modified TRIS-buffer (Appendix A) at pH 7,6. This was brought to 37°C in a water bath and left for exactly 10 minutes to form fibrils after which 5ml of the cooled agarose was added, the mixture vortexed to homogenise, and poured to 90 mm diameter Petri dishes. All gels were dried by exposure to laminar flow (without UV) for 10 minutes.

Gel specificity was determined using Sigma proteases at 5mg ml<sup>-1</sup> (as detailed in Table 2.1) as well as Sigma *C. histolyticum* collagenase Type 1A [G9891(1A)] (with an activity of 380IU) prepared as a doubling dilution series ranging from 500 µg ml<sup>-1</sup> to 3,9 µg ml<sup>-1</sup>. 0,01ml of each solution was added to a sterile 6mm filter disc prior to transfer to the agar surface. Gels were incubated at 37°C for 24 hours and then the plates were flooded with 10% HgCl<sub>2</sub> to aid visualisation.

#### 2.3.1.b. Collagen Agarose Gels for Enzyme Detection in Tissue:

Hide plugs, 3cm diameter, were cut from small hide pieces and sterilised by ethylene oxide. *Ps. aeruginosa* NCTC 10662, *C. histolyticum* ATCC 1940 and *C. sporogenes* ATCC 3584 cultures were prepared at a concentration of ca 1 x 10<sup>5</sup> ml<sup>-1</sup> in Reinforced Clostridia media and 3 ml of each of these cultures, either individually, or as a 1:1, or a 1:1:1 mix, with each other, were added to the hide plugs in sealed jars. Incubation was at 37°C for 24 hour after which time the samples were blotted dry and

sequential 1-2cm x 0,5cm slices were cut aseptically from the middle area of each hide plug and placed on the surface of agarose collagen gels.

After incubation of the gels at 37°C for 24 hours, zones of lysis around the tissue were visualised using 10% HgCl<sub>2</sub> solution. Control plates consisting of 15% gelatin were prepared for each sample and visualisation of lysis after incubation of the tissue sections on these plates was achieved after application of 15% HgCl<sub>2</sub> in 20% m/v hydrochloric acid.

#### 2.3.1.c. Collagen Media for Direct Bacterial Activity:

The collagen media was prepared as for the collagen gels but with the substitution of Tryptone yeast extract agar base media (Appendix A : Collagen gels) in place of 2% agarose.

Specificity of the media was confirmed using the controls listed in Table 2.1 with the omission of the two *V. alginolyticus* strains and the inclusion of *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*. All control enzyme solutions were applied to agar surfaces in 0,1 ml volumes in 13mm antibiotic discs (Schleicher and Schull). Incubation was aerobic for 24 hours at 37°C except for the *C. histolyticum* and *C. sporogenes* cultures which were incubated anaerobically at 37°C for 48 hours.

#### 2.3.1.d. Collagen Plugs for Enzyme Inhibition Studies:

Collagen was prepared as above with the inclusion of 0,2% sirrius red in 10x TRIS-buffer. 0,2ml aliquots of the sterile collagen were transferred aseptically to 96 well (0,4 ml volume) micro-titre well plates and left to fibrillate at 37°C for 24 hours. The collagen plugs so formed were removed to sterile Petri dishes by means of washing with 0,002% sirrius red in 0,05M modified TRIS-buffer and they were stored in this buffer at 4°C for a maximum of 1 week.

0,5ml of Sigma Trypsin peptone and protease, at 1mg ml<sup>-1</sup> each, were added to collagen plugs in small plastic tubes and incubated at 37°C for 24 hours. The tubes were examined at 0; 0,25; 0,5; 1; 2; 3; 4; 5; and 24 hour intervals for degradative changes as evidenced by dissolution of the collagen plug and/or release of sirrius red into the surrounding buffer.

A second series of tests were performed using Sigma *C. histolyticum* collagenase (C 9891(1A)) in a concentration series of 0,5; 0,25; 0,05 and 0,025 mg ml<sup>-1</sup>.

### 2.3.2. Hydroxyproline Assay:

#### Standards:

Stock standard solution was prepared from 100 mg of hydroxyproline diluted in 500ml of distilled water and preserved with 0,1 gm of sodium azide. Working standards were prepared from the stock at 4, 8, 12 and 16  $\mu\text{g ml}^{-1}$  in distilled water.

Control standards were prepared from EO-treated hide pieces left to stand at 20°C for one month and 3 months respectively in 5ml volumes of nutrient broth. Extrudates were extracted manually using mechanical pressure (Appendix B) and these were used to generate background hydroxyproline levels. 0,5ml of each extrudate was hydrolysed at 105°C for 18 hours after the addition of 1,5ml of distilled water and 3,0ml of 10N HCl. 0,25ml of hydrolysate was added to each of four tubes and dried in vacuo over NaOH pellets. To each of these samples was added 1ml of standard solution and these resultant working standard samples were analysed as for the control standards.

#### Reagents:

Acetate-citrate buffer, Chloramine T reagent and Erhlich's reagent were prepared as detailed in Appendix A.

#### Assay procedure:

0,25 ml of standard or working standard sample was well mixed with 4,0 ml of Chloramine T and held for 20 minutes at 20°C. 1,5ml Erhlich's reagent was added and mixed well before incubation at 60°C in a water bath for 20 minutes. The samples were cooled rapidly in water and read within 20 minutes on a Beckman Varian DMS 100 at 558 nm using a reagent blank of 0,25ml distilled water treated as for the sample.

The working standards were treated as for the test samples. The results were plotted graphically as  $\mu\text{g ml}^{-1}$  of hydroxyproline (x axis) and absorbance at 558nm (y axis).

Results were calculated according to the following formula (see Appendix B):

$$\frac{\text{Sample absorbance} - \text{Blank}}{\text{Standard absorbance}} \times \mu\text{g ml}^{-1} \text{ hydroxyproline in standard} = \mu\text{g ml}^{-1} \text{ hydroxyproline}$$

### 2.3.3. Quantification and visualisation of Bacteria:

#### 2.3.3.a: "Stamp" Test:

**Media:** Modified Calcium Caseinate agar was used (see Appendix A).

**Velveteen pads:** Velveteen was cut into circles of 3 cm diameter and placed in glass Petri dishes for sterilising under moist heat at 121°C for 15 mins.

**Pad holder:** A solid stainless steel circular disc (3cm diameter x 1cm depth) is attached by hinges to a hollow cylindrical holder in which a wooden rod can be fitted to act as a handle. The hinge allows the disc to rotate through 90° and therefore gives flexibility of movement on hide surfaces. The surface of the disc is covered in Prestik so that the velveteen pads can be attached. Discs are then ready for pressing onto the selected hide surface with subsequent transfer to the media surface.

**Standardisation of bacterial growth:** 20 x 20cm raw hide pieces were taken from at least ten different animals and incubated at 30°C for 24 hours before removal of 3cm diameter plugs. These were placed in 10 ml of 0,1% buffered peptone. After vortexing for 2 minutes standard bacterial counts were performed using pour plate technique and Plate Count agar with incubation at 30°C for 24 hours. Adjacent samples were taken using the "stamp" agar media and the amount of bacterial growth recorded after incubation at 30°C for 24 hours. Growth was scored from +/- = scanty, + = visible, ++ = moderate, +++ = heavy to ++++ = very heavy growth. A control of biocide treated raw hide was included to represent no growth.

#### 2.3.3.b. Clostridia counts:

**Media:**

1. Reinforced Clostridia agar was modified by the addition of 1% extra agar (to prevent spreading individual colonies) and the omission of starch (to promote rapid dextrose fermentation) (Appendix A). To 1 litre of this media at 45°C was added 100 ml of 5% (0,45 µm filter sterilised) dextrose and 10ml of Andrades indicator (Appendix A). Final media pH was 7,0 = MRCA1.
2. As for media (1) but without the addition of 1% extra agar and with the inclusion of 0,1% starch pH adjustment to 6,8 = MRCA2.

**Bacterial inocula:** Individual cultures of *C. histolyticum* and *C. sporogenes* were incubated in Reinforced Clostridia broth for 24 hours at 37°C and then diluted to 10<sup>8</sup> bacteria ml<sup>-1</sup> in saline (as adjudged by 0,5 MacFarland Standard) (Appendix A). Each culture was further diluted to 10<sup>4</sup> bacteria ml<sup>-1</sup> and these were mixed together as a 1:1 ratio. This formed the test inocula.

**Hide samples:** EO-exposed raw hide samples were dosed with 5ml of the bacterial test inocula and kept at 30°C for up to 8 days.

Bacterial counts were performed on tissue extrudates taken from hides at intervals of 1, 2, 3, 4, 6 and 8 days. The layer plate method of counting was performed (Appendix B) and incubation was at 37°C for 24 and 48 hours in a Merck Anaerocult A gas generating kit system (Appendix A).

#### **2.3.3.c. Immunolocalisation:**

Bacterial antigen extracts of all three selected inocula were prepared according to the method of Chantler and McIlmurray (1987) and 1ml of each preparation, (mixed with 1ml of incomplete Freund's adjuvant) was injected intra-muscularly into the thigh of individual New Zealand white rabbits. A booster dose was given in the other thigh at 2 months and the animals were bled from the central ear vein two weeks later. The supernatant sera recovered after centrifugation of these samples were added to Ouchterlony plates containing specific antigen in the central well. Four fold dilutions of both sera and antigen (up to 1/4096) were used in a chequer board series of tests. Incubation was at 20°C for 24 and 48 hours.

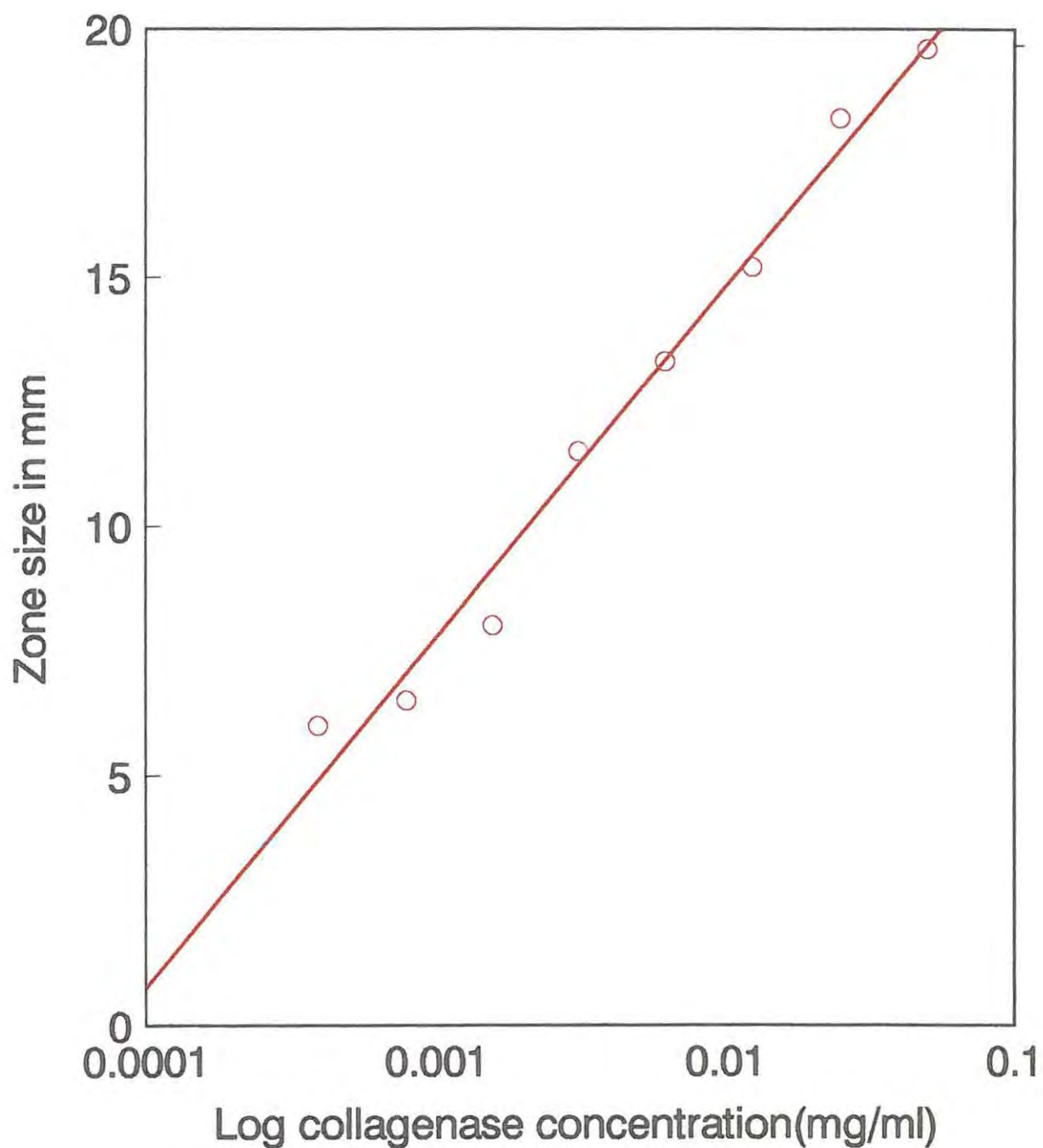
### **2.4.: Results and Discussion:**

#### **2.4.1: Collagen gels and plugs:**

##### **2.4.1.a. Collagen Agarose gels for enzyme detection in fluids**

None of the negative controls showed any lytic activity on the gels after incubation. The collagenase dilutions all gave clear, well defined symmetrical zones and none of the gels were contaminated by bacteria.

The zone size value for each of the collagenase concentrations is shown graphically in Fig. 2.1 as the log of the collagenase concentration versus the zone diameter in mm. The lowest



**Fig. 2.1. Zone Size Diameters on Collagen Gels as a Function of Collagenase Concentration.**

concentration showing lysis was  $7.9 \mu\text{g ml}^{-1}$ . This is in agreement with Brüning (1991) who used Sigma chromatographically pure collagen and *C. histolyticum* collagenase to assess polarimetry as a means of measuring collagenase activity. The agarose collagenase assay detailed here is both sensitive and specific for collagenase activity in the range  $7.9 \mu\text{g} - 500 \mu\text{g ml}^{-1}$  and is simple to prepare and easy to perform. It is also without interference from contaminating bacteria or excessive loss of gel viscosity at high collagenase concentrations. As such it is an ideal semi-quantitative assay for use in hide degradation studies.

#### **2.4.1.b. Collagen Agarose Gels for Enzyme Detection in Tissue:**

Tissue sections taken from hide plugs inoculated with individual and mixed cultures of the three bacteria gave variable results for both collagenase and gelatinase activity in gels (Table 2.2). The enzyme reactions were easily visualised as areas of lysis surrounding the tissues and visualisation was not impaired by contamination or solubilisation of the media. Only those samples containing *C. histolyticum* produced visible lysis on the collagen agarose gels which accords with the literature on known collagenase-producing bacteria. (Mandl *et al.* 1953; Yoshida and Noda 1965; Nord *et al.* 1975; Peterkofsky 1982; Bienkiewicz 1983; French *et al.* 1986). It also confirms the specificity of the gels. Conversely gelatinase activity was most pronounced in the *C. sporogenes*-inoculated hides which agrees with the proteolytic nature of this organism (Smith and Holdeman 1968). There was no evidence of tissue autolytic activity in non-inoculated controls confirming the alkylation effect of EO on tissue enzymes (Phillips and Kaye 1949; Gilbert *et al.* 1964).

Agarose gels containing EO collagen are therefore both sensitive and specific for the detection of bacterial collagenase activity in tissues during in-situ hide degradation studies.

Table 2.2. Detection of Collagenase and Gelatinase Activity in Tissue Sections of EO Hides Inoculated with *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*

Bacterial Inocula	Visible Lysis in Gels	
	Collagen	Gelatin
<i>Ps. aeruginosa</i>	-	-
<i>Ps. aeruginosa/C. histolyticum</i>	+++	+
<i>Ps. aeruginosa/C. sporogenes</i>	-	+++
<i>Ps. aeruginosa/C. histolyticum/C. sporogenes</i>	+++	+++

+ = slight; ++ = moderate; +++ = extensive

#### 2.4.1.c. Collagen Media for Direct Bacterial Activity:

The results, given in Table 2.3. below, indicate that good growth was achieved by all the cultures. Initial trials had indicated problems with regard to the anaerobic bacteria but the use of a 48 hour incubation time overcame this. Contamination of the media by extraneous isolates was not detected. Only collagenase enzyme, one strain of *B. cereus* and *C. histolyticum* were able to cause breakdown of the collagen incorporated into the gel with resultant lysis, indicating that the collagen-containing media was both sensitive and specific for the presence of collagenase. Variations between *Bacillus* strains and their ability to degrade collagen-based substrates was also found by Petushkova *et al.* (1985). Oversolubilisation of the media at high collagenase concentrations (0,25 mg ml<sup>-1</sup>) did not occur, either for the pure enzyme or the bacterial culture samples, demonstrating that the gels were suitable for use in screening programmes since several bacteria/enzyme solutions could be applied to a single plate without interference from positive readings.

Table 2.3. Bacterial Growth, Collagenolytic Activity and Enzyme Activity in Collagen Media Gels.

Sample	Conc. mg ml <sup>-1</sup>	Source	Activity on Collagen Media	
			Growth	Lysis
<i>Ps. aeruginosa</i>	-	NCTC 10662	+++	-
<i>C. histolyticum</i>	-	ATCC 1940	+++	+++
<i>C. sporogenes</i>	-	ATCC 3584	+++	-
<i>S. aureus</i>	-	NCTC 10788	+++	-
<i>E. coli</i>	-	NCTC 10148	+++	-
<i>B. cereus</i> (1)	-	ex S.A.I.M.R.	+++	+
<i>B. cereus</i> (2)	-	ex S.A.I.M.R.	+++	-
Protease	5,0	*P5380	-	-
Proteinase K	5,0	*P0390	-	-
Pronase	5,0	*P5147	-	-
Elastase	5,0	*E2058 (1)	-	-
Trypsin	5,0	*T0134	-	-
Collagenase	0,25	*C9891 (1A)	-	+

\* Sigma Chemical Co.; + = slight; ++ = moderate; +++ = extensive

#### 2.4.1.d. Collagen Plugs for Enzyme Inhibition Studies:

The collagen plugs underwent total degradation in the presence of commercial collagenase with release of sirrius red pigment into the buffer. The amount of degradation was graded from 0 (no degradation) to +++ (total degradation and diffuse red dye in buffer). Rates of degradation were varied according to collagenase concentration, but no degradation occurred with any of the other enzymes tested. (See Table 2.4. below).

Table 2.4.. Degradation Rate of Collagen Plugs by Collagenase

Enzyme	Conc. mg/ml <sup>-1</sup>	Time in Hours							
		0,25	0,5	1	2	3	4	5	24
Collagenase	0,500	+	++	++	+++	+++	+++	+++	+++
"	0,250	0	+	+	+++	+++	+++	+++	+++
"	0,050	0	+	+	++	+++	+++	+++	+++
"	0,025	0	0	+	+	++	+++	+++	+++
Trypsin	1,000	0	0	0	0	0	0	0	0
Pepsin	1,000	0	0	0	0	0	0	0	0
Protease	1,000	0	0	0		0	0	0	0

0 = plug intact, media colourless; + = plug slightly degraded, media pink  
 ++ = plug 50% degraded, media red; +++ = plug totally degraded, media red.

There was a definite positive correlation between time to total degradation and enzyme concentration although all four enzyme concentrations caused total degradation within 4 hours. These results confirm that collagen plugs can be used for semi-quantitative estimates of collagenase in hide extrudates and other fluids. This test also has particular use for inhibition studies of biocides and other anti-microbials that form coloured or turbid solutions which renders them unsuitable for standard optical or spectrophotometric assays. Similarly this test could supplement the standard MIC (minimum inhibitory concentration) test for biocides with low diffusability and/or for those that bind to media ingredients.

#### **2.4.2. Hydroxyproline Assay:**

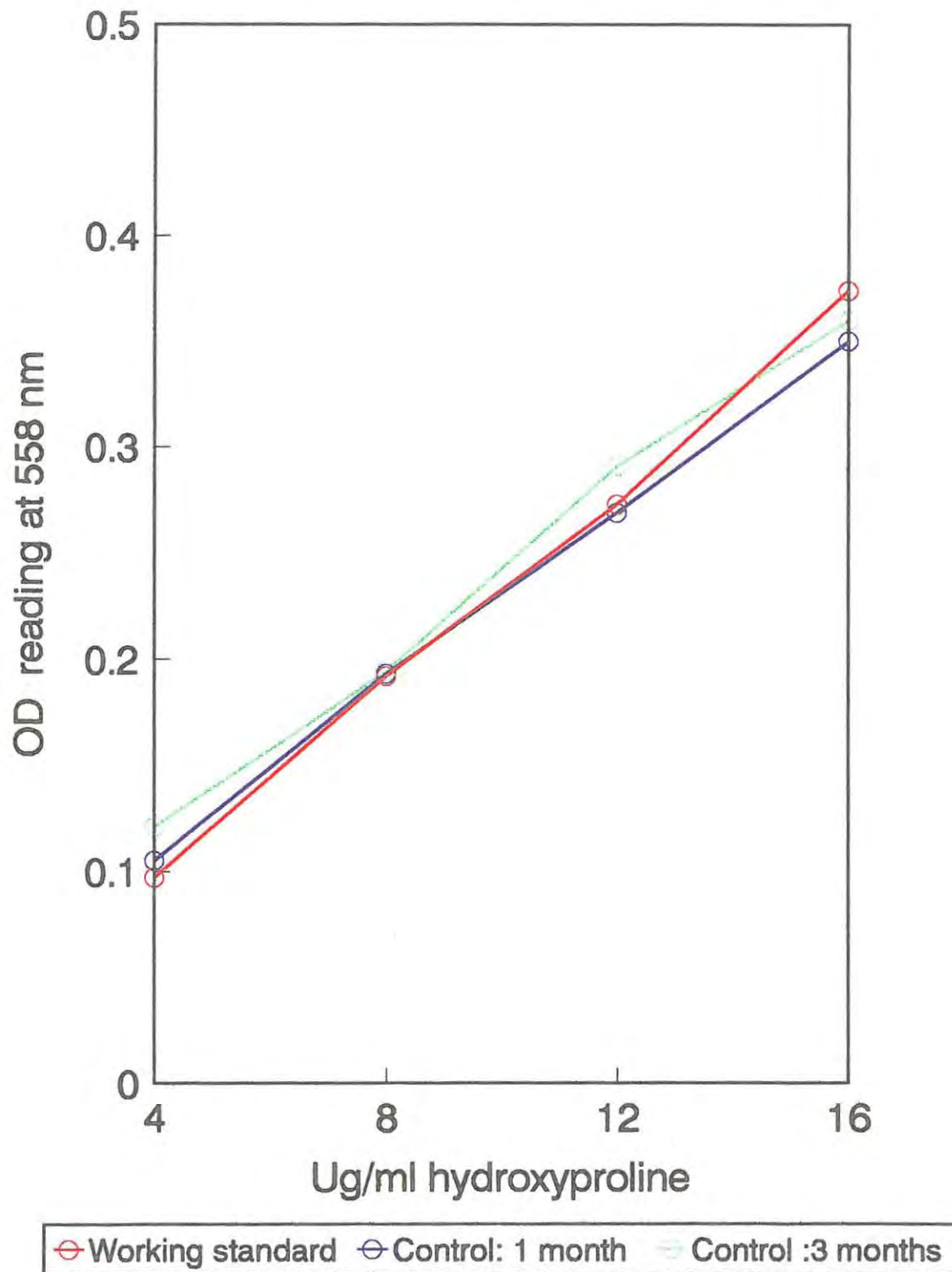
The results of the studies are shown graphically in Fig. 2.2, and these results give a regression value of 0,999. The results of the control standards demonstrate that, even when exposed to nutrient broth for 3 months, EO-exposed hide plugs showed no major breakdown of collagen and this confirms the validity of EO treatments for hide sterilisation. Values of greater than 1,0  $\mu\text{g hydroxyproline ml}^{-1}$  were not found indicating that hide tissue enzymes are inactivated during EO treatment.

The modified hydroxyproline method detailed here was shown to be suitable for use in hide degradation studies as it could accurately detect a range of hydroxyproline levels from 4  $\mu\text{g ml}^{-1}$  to 16  $\mu\text{g ml}^{-1}$  in hide extrudates and this could be extended up to 1600  $\mu\text{ ml}^{-1}$  by preparing dilutions of hide extrudates and inocula suspensions at 1/100 and 1/1000 prior to testing. This means that in undiluted, 1/10 and 1/100 diluted samples the method can cover a wide range which would include the maximum hydroxyproline levels of 788  $\mu\text{g ml}^{-1}$  found by Hockey and Russell (1982) in limed/delimed hides left to "stale" naturally at 35°C.

#### **2.4.3. Quantification and Visualisation of Bacteria:**

##### **2.4.3.a. "Stamp" test:**

Visualisation of growth was clear and easy to detect with heavy to very heavy growth evident in most of the hides tested. Counts of below  $2,9 \times 10^4$  per  $\text{cm}^{-1}$  hide were not found. Counts from  $10^6$  up to  $10^{10}$  per  $\text{sq.cm}$  of hide correlated to "stamp" test readings of +++ or ++++ for both hair and flesh surfaces. The control hide, that had been treated with industrial biocide, showed complete absence of growth. From these results a table for the semi-quantitative estimation of bacterial growth by the stamp test method was generated (see Table 2.5.).



**Fig. 2.2 Hydroxyproline Standard Graph.**

Table 2.5. Semi-quantitative Estimation of Surface Bacteria on Hides Using the Stamp Test

No. Bacteria per cm <sup>2</sup>	Amount of Bacterial Growth	
	Flesh	Hair
2,9 x 10 <sup>4</sup>	+	+/-
1,6 x 10 <sup>5</sup>	++	++
6,5 x 10 <sup>5</sup>	+++	+++
1,7 x 10 <sup>6</sup>	++++	++++
5,8 x 10 <sup>6</sup>	++++	++++
Control *	0	0

\* Control = biocide treated hides

Proteolytic activity varied amongst the different hides tested and results indicated that bacterial growth and proteolytic activity did not always correspond. This finding was to be expected as bacteria differ in their ability to digest casein. The amount of proteolytic activity was easily visualised after the addition of 10% mercuric chloride to the plates.

The "stamp" test as developed here therefore appears satisfactory for use as a measure of bacterial activity in hide degradation studies as well as for biocide application studies. It can also be used to detect bacterial recolonisation of hides in both laboratory trials and in industrial stock after treatment with biocides.

#### 2.4.3.b. Clostridia Counts:

The use of MRCA1 resulted in difficulties in visualisation of colonies and their reaction with dextrose. The colonies appeared very small and it was postulated that this was due to the higher agar content and/or the lack of starch in the media. Starch was therefore reintroduced in MRCA2 and the media pH made more acid in an attempt to enhance visualisation of glucose fermentation and the agar content was also reduced. The results of the MRCA2 trials indicated that although glucose fermentation was slightly more enhanced it was still difficult to determine the difference between the *C. sporogenes* (glucose fermenter) and the *C. histolyticum* (non-fermenters). The colonies of both organisms were larger, and therefore more visible, after 48 hours incubation than at 24 hours but differentiation was still unclear.

It was concluded that no satisfactory method had been found for differentiation between the two *Clostridium* when in mixed culture in hide extrudates during degradation. It would only be possible in mixed cultures, therefore, to record the clostridia as total clostridia present and not as individual species counts.

#### 2.4.3.c. Immunolocalisation:

Despite numerous test runs none of the Ouchterlony plates showed any evidence of antibody production by any of the three rabbits for the three inocula used.

It is hoped that future work will provide a logical explanation for this seeming lack of reactivity and a immunohistochemical method for tissue localisation of individual clostridia be developed. For this study it was decided to use a general Gram stain reaction coupled to morphological appearance in order to determine the presence of the two chosen clostridia in histological tissue sections.

#### 2.5. Conclusion:

Satisfactory methods were developed for semi-quantitative assay of collagenase in fluids and detection of the enzyme in tissues as well as in actively growing bacterial cultures. A method was also developed, using collagen in plug form, which could be used to screen potential hide preservatives for collagenase inhibition activity. Standard hydroxyproline methods were modified to form a suitable method for use in hide studies. This was shown to be sensitive to microgram quantities of this imino acid in both fresh and naturally decayed hide pieces. A method for quantification of bacteria in conjunction with assessing relative proteolytic activity was developed for use in determination of bacterial loads on hide surfaces both in laboratory and production trials. Method development for differentiation of *C. histolyticum* and *C. sporogenes* either in total counts or in tissue sections was not successful.

## CHAPTER 3

### BACTERIAL DEGRADATION OF RAW HIDES

- 3.1. Summary
- 3.2. Introduction
- 3.3. Research Objectives
- 3.4. Results and Discussion
  - 3.4.1. Surface Colonisation and Activity during Storage..
  - 3.4.2. Identification of Aerobic and Anaerobic Bacteria from Fresh and Degraded Hides
  - 3.4.3. Competitive Inhibition between Selected and Resident Bacteria
- 3.5. Conclusion.

### 3.1 Summary:

Raw and biocide-treated hides were used to study degradative changes during storage in order to confirm the general role of bacteria in hide decomposition. Bacterial involvement in the degradation process was monitored using surface bacterial growth estimates, organoleptic changes, pH variation and proteolytic activity in agar media. These initial trials indicated that there were limitations to the study of naturally decaying hides for confirmation of the major role of bacteria in hide degradation and this limitation was compounded when assessment parameters were non-specific.

To overcome these problems a model system was proposed in which sterile hides could be degraded by inoculating with selected bacterial types. The selection of the bacteria was based on current literature on protein degradation in general. The selected organisms were *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*. Evidence that these organisms routinely colonised hides was obtained using raw hide pieces and laboratory media. To confirm the suitability of the selected bacteria as representative hide degrading organisms, their ability to outcompete normal hide populations was investigated. The results showed that all three selected inocula could be readily isolated from hides and that the organisms, when in mixed culture, were able to both outcompete resident and transient populations and to produce grain surface and/or gross collagen damage.

### 3.1. Introduction:

#### Bacteria and protein degradation:

The hypothesis of this thesis, that hide degradation occurs as a result of bacterial succession, was based on literature evidence which showed that general protein degradation involves bacteria with different catabolic activities and redox requirements working in succession (Meleny 1931; Lawrie 1985; Ishii *et al.* 1987; Marsh and Martin 1992).

The changes that occur as a result of the bacterial activity in protein substrates are well documented and this has led to a series of definitions covering the three main stages of protein degradation (Tissier and Martelly 1902; Eskin 1971; Sinell 1980(a); Boyd 1984; Hayes 1985; Banwart 1987). They can be summarised thus:

- a. Spoilage - surface aerobic bacterial proliferation involving decomposition at the expense of available glucose, amino acids and other low molecular weight compounds without significant protein breakdown. Slime, off odours and foul smelling breakdown products of amino acid degradation occur.
- b. Decay - aerobic or facultatively anaerobic bacterial attack on protein with oxidation of metabolic products to yield a large number of volatile compounds such as ketones and dimethyl sulphides with resultant off-odours.
- c. Putrefaction - micro-aerophilic or anaerobic bacterial degradation of oligopeptides, di-peptides and amino acids with release of ammonia, hydrogen sulphide and foul smelling amino acid degradation products.

Although these definitions are somewhat broad they do have pertinent application in hide studies, both for preservation and degradation studies.

Hides are post-mortem protein material and deteriorative changes are governed by two prime factors affecting all mammalian post-mortem tissue. These are autolytic enzyme degradation and bacterial proliferation with concomitant enzyme production. Immediate post-mortem autolytic changes include cell death, rapid reduction in redox potential (Eh) as a result of oxygen removal by tissue respiration, elevated temperature and endogenous enzyme release from cells. To some extent all these changes are self-limiting although both tissue respiration and enzyme activity can continue to act for several weeks (Lawrie 1985; Banwart 1987; Gould 1988). However this autolytic activity is minimal in comparison to post-mortem bacterial growth and enzyme production, since both of these increase with time post-mortem.

Previous studies in the hide industry have not focused so much on specific bacterial action but have sought to establish general cause and effect relationship between bacteria and hide damage. Woods *et al.* (1971 (a) and (b)) investigated salted hides and found variable correlation between bacterial numbers (surface activity), proteolytic activity and final leather quality. Ishii *et al.* (1987) found increased bacterial loads ( $10^4$  -  $10^9$ ) on calfskins held at 30°C for 2 days. Nandy *et al.* (1956) detailed levels of  $10^8$  bacteria  $\text{gm}^{-1}$  initially on raw hides rising to  $10^{11}$  after 3 days at 30°C and in raw hide storage trials at 25°C, Hockey and Russell (1982) recorded rates of collagenolytic and proteolytic degradation up to 5 days at final bacterial levels

of  $10^8 \text{ gm}^{-1}$ . Bacterial counts have also traditionally been used to detect bacterial proliferation on general protein surfaces (Dainty *et al.* 1975; Nortje 1987).

Bacterial counts on hides are comparable to the initial bacterial loads of  $10^3 - 10^7 \text{ gm}^{-1}$  that have been reported for meat and meat products (Gill 1976; Lawrie 1985; Nortje 1987). While spoilage of meat occurs at levels as low as  $10^3 - 10^5 \text{ gm}^{-1}$  (Ingram and Simonsen 1980) and  $10^6 \text{ gm}^{-1}$  (Banwart 1987), Arganosa *et al.* (1987), in a study on pork products, found that bacterial numbers per se were not as important as type species in causing degradation.

In order to confirm the role of bacteria in hide decomposition both bacterial numbers and organoleptic parameters have been used to assess "spoilage and decay". The three most traditionally used organoleptic parameters in South Africa are:

- a. Hairslip - indicators of proteolytic activity in-situ due to digestion of hair follicles (Welton 1974).
- b. Malodour - ammoniacal and other odours due to protein and amino acid digestion (Gerbi 1986).
- c. Appearance - surface slime due to bacterial build up and collagen gelatinisation causing discoloration of hide flesh surfaces. (Russell and Galloway 1982).

Increase in hide pH has also been used as a measure of proteolytic hide degradation (Hockey and Russell 1982).

Initial studies of this thesis focused on determinations of bacterial growth and activity, together with non-specific (organoleptic) assessments, as indicators of hide damage during storage of raw and biocide-treated hides.

#### **Bacterial colonisation of hides:**

The microbial ecology of human skin is well documented (Holland and Kearney 1985) as is that of meat and meat products (Hayes 1985; Lawrie 1985) but few studies have focused on the microbial ecology of raw hides. It is possible, from the general microbiological literature, to list some 150 genera of bacteria that could colonise hides (Frobisher *et al.* 1974; Ross 1986; Sneath 1986). This is shown diagrammatically in Fig 3.1. which indicates both some possible

sources of transient micro-organisms as well as organisms which may be introduced as a result of specific hide processing treatments. While salted hides present a unique environment which is selective for halotolerant and halophilic bacteria (Schlegel and Jannash 1981), raw hides present a much more general substrate for colonisation. Venkatesen (1979) reported on a variety of bacterial types isolated from both hides and skins. While these included both Gram positive and Gram negative organisms none of these were specifically identified as being primary colonisers, or specific to secondary spoilage/degradative ability.

Literature references identifying bacteria associated with each level of hide degradation were not found other than that of Ishii *et al.* (1987). They isolated two collagenase producing strains of *C. histolyticum* from hides which, when mixed with a member of the *Vibrionaceae*, caused major visual degradative changes within 36 hours at 30°C. In contrast, Tancous (1961) isolated a single collagenase producing strain of *C. capitovale* (now *C. cadaveris* [Koneman *et al.* 1983]) capable of degrading salted hides, while Welton (1974) isolated collagenase producing *Achromatobacter iophagus* (now *V. alginolyticus* [Baumann and Schubert 1986]) from degraded salted stock.

Bacterial succession is well documented with regard to deterioration of meat and meat products and it is established that the primary colonisers are the aerobic bacteria of the genus *Pseudomonas*, *Moraxella* and *Acinetobacter*, followed by the micro-aerophilic putrefactive organisms, *C. histolyticum* and *C. perfringens* (Eskin 1971; Lawrie 1985; Banwart 1987). While the majority of studies have indicated that the *Clostridium spp.* were zymogenous in origin, evidence for endogenous invasion came from Gill (1979) as well as Lawrie (1985) who found  $10^3$  *Clostridium spp.* gm<sup>-1</sup> as sole colonisers of horse muscle 8 hours post mortem.

The role of specific clostridia in raw hide putrefaction has not been fully proven as studies have been focused more on general proteolytic activity of bacteria in salted hides (Robertson and Haines 1947; Anderson 1949) or on collagenase producing strains of bacteria in finished leather products (Petushkova *et al.* 1985). These facts, together with the lack of microbial ecology studies, made it necessary to select organisms for use in the proposed hide degradation study of this thesis. The choice of organisms was discussed extensively in Chapter 1 and was based on those found on other high protein substrates and/or found to be in some way responsible for putrefactive degradation. It was necessary to confirm the ubiquity and the

presence in raw hides of the selected organisms, *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*, and this was achieved by culturing randomly selected hide cuttings aerobically and anaerobically in media. Any bacteria present were subcultured to appropriate solid nutrient media prior to identification by morphological or biochemical techniques.

**Competitive inhibition between inoculated selected bacteria and resident hide populations:**

Evidence that the selected inocula can be readily isolated from hides does not itself constitute proof of their degradative capacity in-situ, nor that they will be able to outcompete resident bacteria for available nutrients. Both indigenous and transient hide populations will be very variable depending upon existing environmental conditions. Pre-flay conditions such as pasture type, feed lots and holding pens will also exert an influence and this will affect the initial bacterial populations of individual hides. Post-flay hides are subject to further environmental exposures including transportation from abattoir to curer and from curer to tanner as well as application of preservative chemicals (including salt) and storage under tannery conditions. Each will contribute to the bacterial load, increasing its diversity (Fig. 3.1).

It is established that protein substrates undergo several changes in microbial flora during decomposition and these are directly related to both external environmental influences and endogenous conditions during storage. These endogenous conditions include bacterial exhaustion of available nutrients and production of toxic metabolites from catabolic activities (Eskin 1971; Hayes 1985; Nortje 1987). These events affect the four main factors which influence microbial growth viz. pH, redox potential ( $E_h$ ), temperature and availability of free water as measured by water activity ( $A_w$ ) (Sinell 1980(a)).

Hides possess a good buffering capacity and since *Ps. aeruginosa* grows at a pH range of 4.4 - 9.0, *C. sporogenes* at 5.0 - 9.0 and *C. histolyticum* from 6.0 - 8.5 (Cato et al. 1986; Banwart 1987) these organisms should thrive under the near neutral pH conditions of raw hides.

Redox levels for optimum growth of aerobic bacteria range from +350 to +500mV. For facultatively anaerobic bacteria the levels are +100 to +350 mV, for aerotolerant/microaerophilic bacteria +287 to -125 mV, and +85 to -150mV for anaerobic bacteria. In post-mortem muscle tissue, tissue respiration accounts for 80% of the oxygen

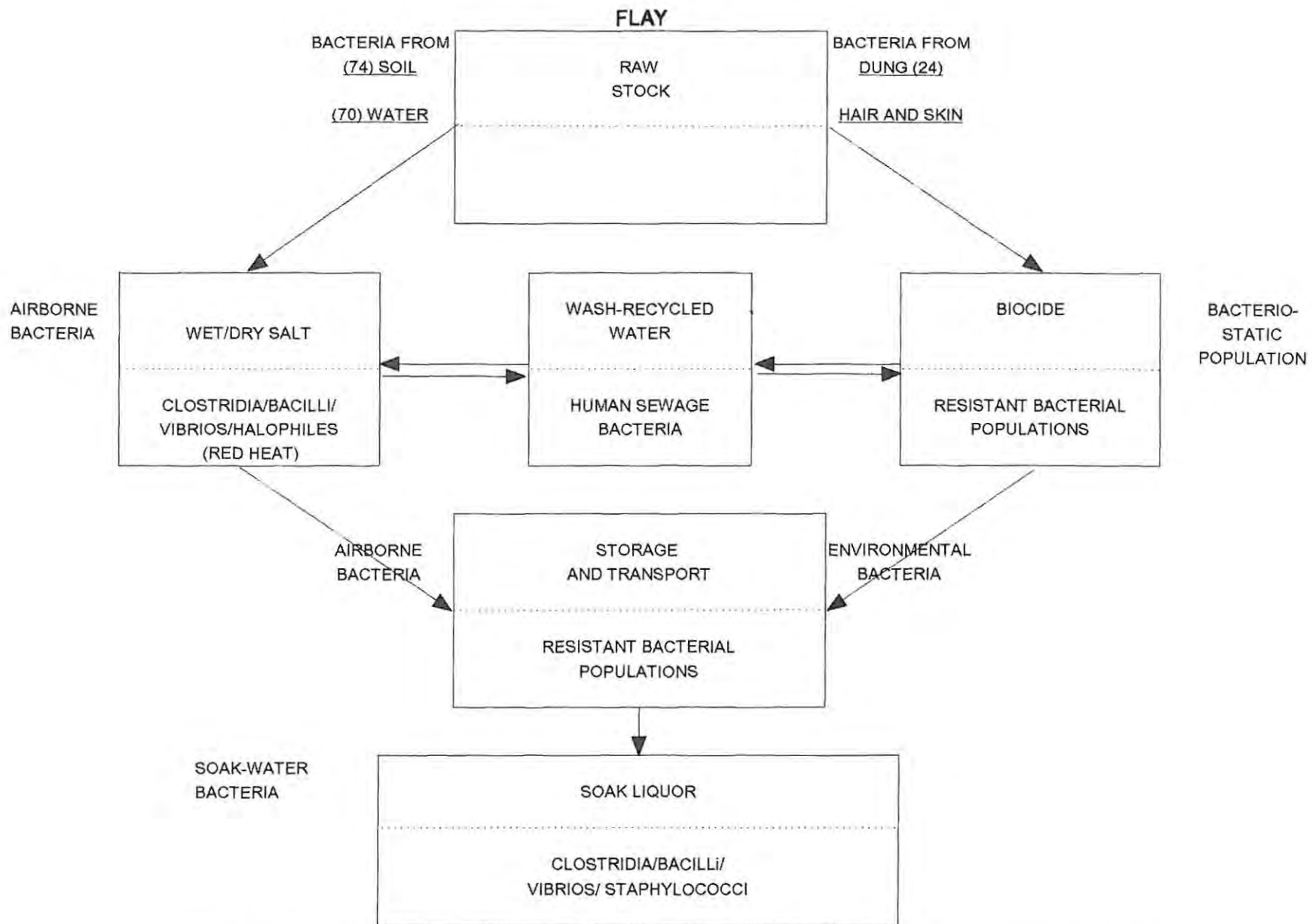


Figure 3.1. Bacteria from flay to soaking; Those outside the boxes are sources for bacterial contamination. [( ) = No. of genera in group]. Those inside the boxes are introduced or selected out by the nature of the process.

uptake and this is increased in deep seated tissue (Banwart 1987). In post-mortem hides similar changes would take place and in stored hides a redox gradient from hide surface to deeper tissue would exist. This would provide ideal conditions for the growth of micro-aerophilic *C. histolyticum* followed by the anaerobic *C. sporogenes*, and these should therefore be able to outcompete the strictly aerobic resident flora.

Temperature tolerances of the three inocula are similar, 37°C being optimal for *Ps. aeruginosa* and *C. histolyticum*, and 30-40°C for *C. sporogenes*. Temperatures in hides will vary according to whether they are measured directly post flay or during storage, as well as to the way in which the hides are stored. Generally hides are stored in piles or stacks and this will result in the initial increased temperatures that occur in post-mortem tissue (Banwart 1987) being maintained for longer in the middle of the stack. Summer conditions also promote increased hide temperature levels. In South Africa a mean summer temperature of 25°C was found for five major cities over a ten year period, with individual temperatures reaching 33°C and above (Thompson 1987(a)). While these temperatures are ideal for many hide colonisers they would tend to promote the growth of the mesophilic organisms.

Water activity levels ( $A_w$ ) of raw hides are adequate for bacterial growth as they contain approximately 66% water, although some of this is bound. The industry practice of washing hides to remove dirt and dung will leave a film of moisture on hide surfaces which will tend to promote rapid bacterial growth.

Since the pH and  $A_w$  of hides is near optimal for the selected inocula, temperature and redox will be the two parameters most likely to affect their competitive inhibition on hides. Generally, hide conditions of warm, moist storage, coupled with post-mortem tissue reduction of  $E_h$  levels, will create ideal conditions for proliferation of mesophilic, putrefactive micro-aerophilic and anaerobic bacteria and as such it would be expected that collagenase producing and amino acid degrading *Clostridium spp.* would have an advantage over psychrophilic and non-proteolytic/collagenolytic resident bacteria.

### 3.3. Research Objectives:

The general purpose of the initial studies for this thesis was to confirm the role of bacteria in hide degradation and to establish the ability of these selected bacteria to initiate and maintain degradation in the presence of existing raw hide populations.

The overall research objectives of this chapter were as follows:

1. To confirm that hide degradation is primarily a result of bacterial action.
2. To assess some existing parameters used in degradation studies of naturally decaying hides for their effectiveness in reflecting in-situ conditions.
3. To establish some of the major bacterial groups colonising raw hides.
4. To isolate and identify from raw hides the bacterial inocula selected for the proposed model system.
5. To demonstrate the ability of the selected bacterial inocula to outcompete hide populations and cause degradation during storage of raw hides.

### 3.4. Results and Discussion:

#### 3.4.1. Surface Colonisation and Activity during Storage:

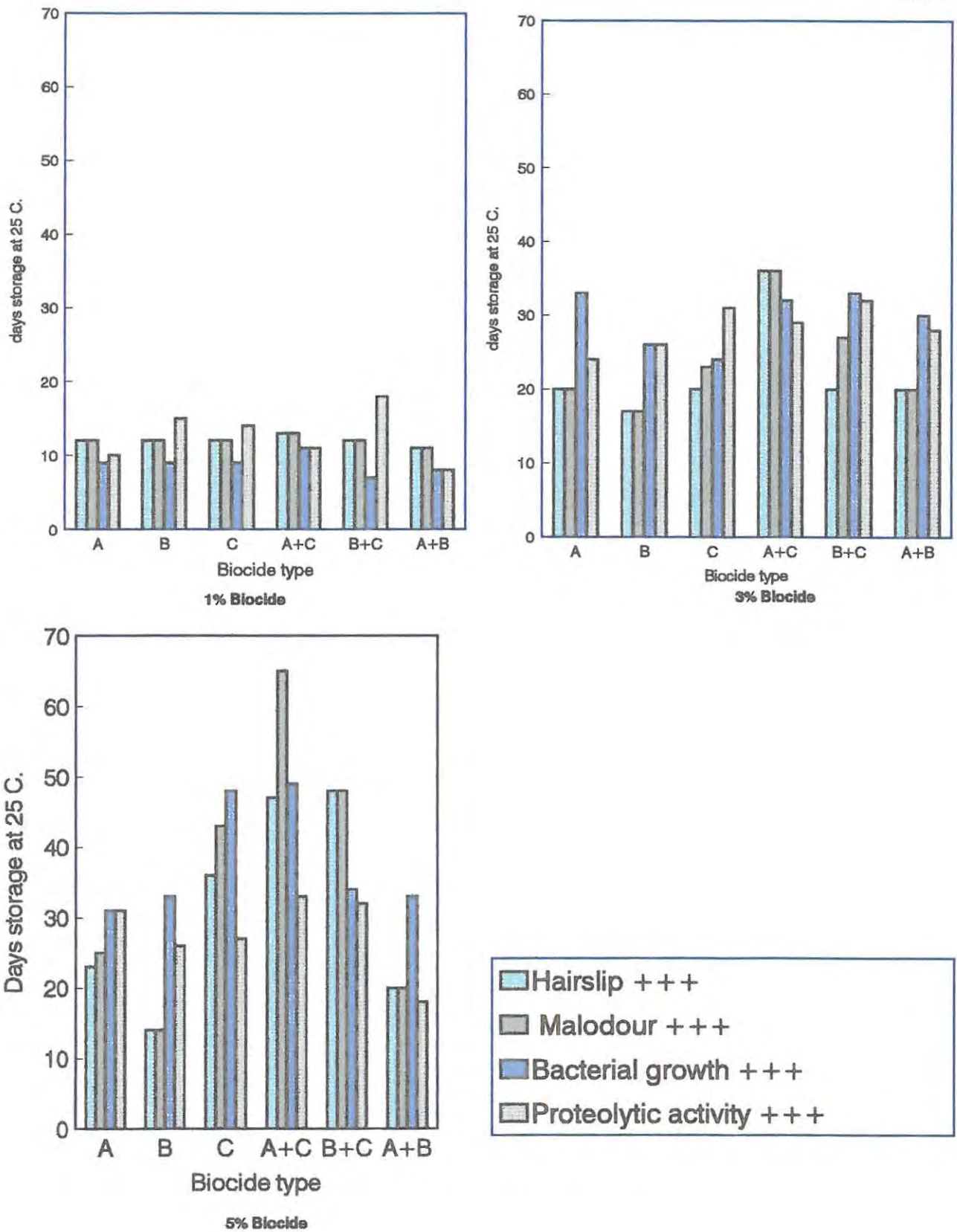
Two storage trials were performed using 14,5 cm x 21,0 cm size raw hide cuttings some of which were pre-exposed to biocide treatments. The biocides used were (A) SAPCO STA , (B) BUCKMAN Bulab LV and (C) boric acid together with 1:1 combinations of A:C, B:C and A:B. In the first trial the cuttings were exposed by 6-h drumming in Wacker glass tumbrils with 100% float (Appendix A) to three individual biocides and three 1:1 mixes of the biocides (at individual and 1:1 final concentrations of 1, 3 and 5% v/v). Control non-treated hides were included and storage of all samples was at 25°C. Parameters assessed were bacterial growth and proteolytic activity (by the "stamp" test), surface pH, hairslip, malodour and appearance. (Appendix B). In the second trial, pH values of both raw and biocide-treated hides were taken during storage in order to establish the value of changes in this parameter in detecting degradation in hides.

#### Trial 1 - Bacterial Action:

All control (raw) hide pieces without biocide treatment deteriorated very rapidly (scores of +++ bacterial growth at day 1 and +++ for all organoleptic parameters at day 3). Slime and malodour were pronounced at day 3 indicating decay/putrefaction (Table 3.1). This confirmed the findings of Hockey and Russell (1982) who found severe surface damage of

hides at elevated temperatures (35°C) within 2-3 days and visible leather damage (grain surface) in less than 24 hours.

Biocide-treated cuttings gave prolonged storage allowing for longer monitoring. The individual results of these trials are given in Table 3.1. and the overall means are depicted graphically in Fig. 3.2. These confirm the findings of Russell (1988) that differential storage times are achieved according to biocide type and concentration. Degradation was considered present when all four storage parameters reached the +++ level. Ranking of the parameters showed that hairslip and malodour were closely correlated but that bacterial growth and proteolytic activity were not as well correlated to these parameters (Thompson *et al.* 1988(b)). On the 1% biocide-treated hides +++ bacterial growth and to some extent +++ proteolytic activity appeared before hairslip and malodour (+++). However, this order was largely reversed for most of the 3% and 5% biocide-treated hide pieces and these also gave variable results in terms of proteolytic activity. Malodour, attributed to general protein degradation as well as amino acid deamination and transamination with resultant formation of metabolites such as cadaverine and putresine (Eskin 1971; Lawrie 1985), has been considered a useful means of assessing degradative changes in hides, and was used as long ago as 1939 by Shuttleworth and Sebba in their hide preservation studies. The results of this study indicated that while such organoleptic assessments can give some indication of combined deteriorative changes, they cannot differentiate between the different stages of protein degradation such as spoilage, decay and putrefaction. It also showed that while the stamp test depicts spoilage, the organoleptic assessments (at +++) are perhaps better indicators of decay. Putrefaction could not be confirmed using organoleptic assessments and aerobic bacterial activity alone since there was no proof of increased anaerobic bacterial activity.



**Fig. 3.2 Organoleptic and Bacteriological Assessments of Biocide Treated Hides During Storage at 25 C.**

Biocide A = SAPCO STA, biocide B = BULAB LV, biocide C = Boric acid

Table 3.1. Days Storage at 25°C of Raw and Biocide Treated Hide Cuttings based on Organoleptic and Bacterial Assessments

Biocide Type	Final % Conc.	Days storage to score (+++)					
		Hairslip	Malodour	Bacterial growth		Proteolytic Activity	
				H	F	H	F
A	1	12	12	2	7	3	7
	3	20	20	16	17	13	11
	5	25	25	16	15	13	18
B	1	12	12	2	7	6	9
	3	17	17	13	13	13	13
	5	14	14	11	12	10	13
C	1	12	12	2	7	7	7
	3	20	23	13	11	20	11
	5	36	43	18	30	18	9
A + C*	1	13	13	6	5	6	5
	3	36	36	16	16	13	16
	5	47	65	23	26	11	12
B + C*	1	12	12	2	5	9	9
	3	20	27	16	17	15	17
	5	48	48	16	18	17	15
A + B*	1	11	11	2	6	2	6
	3	20	20	15	15	13	15
	5	20	20	17	16	9	9
Control raw hide	-	3	3	1	2	2	3

Key: \* At 1:1 ratio; H = hair side; F = flesh side

### Trial 2 - pH values of raw and treated hides:

The pH values (Table 3.2) obtained from raw hides at day 0 and day 8 of storage at 25°C indicated definite differences between pH levels of hair ( $\bar{x} = 8,3$ ) and flesh ( $\bar{x} = 7,3$ ) surfaces. Despite the fact that organoleptic and bacterial parameters reached +++ within 3 days of storage, there was no real significant change in pH levels during the whole 8 day storage period. This finding was similar to that of Gerbi (1986) who examined raw hide storage at 25°C and found hide degradation occurred at 2½ days, based on total volatile nitrogen release, as well as scanning electron and light microscopy of the resultant leather damage; the pH level at this time was 7.1. Hockey and Russell (1982) found definite progressive increase in pH

levels of hide extrudates, according to time and temperature of storage, indicating that in-situ changes in pH are more representative of degradative changes than surface pH values.

**Table 3.2. pH of Control Hide Pieces during Storage at 25°C**

Days Storage	Flesh		Hair	
	pH*	Range	pH*	Range
0	8.2	7.6 - 8.3	7.4	6.7 - 7.7
2	8.4	8.1 - 8.5	7.1	6.4 - 7.7
4	8.4	8.3 - 8.5	7.0	6.4 - 7.7
6	8.2	7.6 - 8.3	7.5	6.8 - 8.2
8	8.4	8.2 - 8.5	7.5	6.5 - 7.8

\*  $\bar{x}$  of five samples

The pH levels of the biocide treated hide cuttings during storage are shown in Table 3.3 together with the biocide float pH values taken during biocide application. These results indicate that hides have a good buffering capacity and bring pH levels to near neutrality fairly rapidly. pH changes to onset of +++ bacterial growth range from 6.8 - 8.6 and for +++ proteolytic activity the range is 6.6 - 8.6. This indicates the limitations of shift in surface pH values in monitoring of hide degradation.

Table 3.3. pH of Biocide Treated Hide Pieces during Storage at 25°C

Biocide	Final % Conc.	pH								
		Float	Hairslip (+)		Odour (+)		Bacterial growth +++		Proteolytic Activity +++	
			h	f	h	f	h	f	h	f
A	1	10.4	7.7	7.8	7.7	7.7	7.7	7.7	7.5	7.8
	3	10.6	7.9	8.1	7.9	8.0	7.9	8.0	7.9	8.0
	5	11.2	8.3	8.6	7.8	8.5	8.2	8.5	8.3	8.6
B	1	9.4	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
	3	9.6	8.0	7.6	8.0	7.6	7.7	7.7	7.8	7.6
	5	9.7	8.2	8.1	8.2	8.1	8.0	8.0	8.0	8.1
C	1	5.8	7.9	7.8	7.9	7.8	7.9	7.8	7.9	7.8
	3	4.6	7.3	7.5	7.4	7.5	7.7	7.4	7.8	6.8
	5	4.2	6.8	6.8	7.5	7.2	6.8	7.2	6.8	6.6
A+C*	1	7.4	7.5	7.7	7.6	7.7	7.5	7.6	7.5	7.7
	3	7.0	7.7	8.0	7.7	8.0	7.7	7.7	7.7	7.8
	5	6.6	8.2	8.2	8.3	8.1	8.0	8.3	8.0	8.0
B+C*	1	7.2	7.6	7.6	7.6	7.6	7.6	7.7	7.7	7.7
	3	6.8	7.6	7.5	7.6	7.6	7.8	7.6	7.8	7.6
	5	6.4	7.6	7.7	7.9	7.8	7.9	7.7	7.9	7.8
A+B*	1	9.7	7.7	8.0	7.7	8.0	7.7	8.0	7.7	7.7
	3	9.9	7.7	8.3	7.7	8.2	7.8	8.2	7.8	8.3
	5	10.2	8.3	8.6	8.3	8.3	8.3	8.3	8.3	8.6

\* at a 1:1 ratio    h = hair side    f = flesh side

### 3.4.2. Identification of Aerobic and Anaerobic Bacteria from Fresh and Degraded Hides:

This identification was carried out in two trials. In the first trial, the major bacterial groups colonising degraded biocide-treated hides were studied and their general cell morphology examined. This was achieved by the use of bacterial growth from 50 "stamp" agar plates taken during hide preservation trials involving four different biocide applications. In the second trial, in order to determine if the selected bacteria were present as part of the resident/transient

population of raw hides, aerobic and anaerobic bacterial isolates from 40 raw hides were obtained. Small cuttings from these individual hides were used to isolate the bacteria which were then identified by standard methods (Appendix B).

#### Trial 1 - General morphology of bacteria from degraded hides:

Of the 50 hide samples tested 25 yielded a mixture of bacterial types; 47 had Gram negative rod-shaped organisms present; 35 had Gram positive organisms present of which 17 were of the micrococci/staphylococci/streptococci groups and 12 resembled diphtheroids. The remainder consisted of large bacillus-like rods (see Table 3.4).

**Table 3.4. Variations in Bacterial Types Recolonising Biocide Treated Hide Cuttings**

Biocide Trial No.	Bacterial Morphology and Gram Reaction						
	Gram Negative			Gram Positive			Mixed *
	Rods	Cocci	Total	Rods	Cocci	Total	
1	19	0	19	0	10	10	10
2	4	0	4	6	2	8	3
3	19	0	19	6	4	10	7
4	5	0	5	6	1	7	5
Total	47	0	47	18	17	35	25

\* Includes Gram positive and Gram negative organisms.

The morphology of the Gram negative organisms was less variable but different reactions and colonial appearance on MacConkey agar indicated that several different coliforms as well as *Pseudomonas* were present. The overall results are shown in Table 3.4. and they indicate that variations occur between recolonised hides treated with different preservatives (biocides). The predominance of Gram negative rods is in accordance with Sinell (1980) who stated Gram negative flora predominate in high  $A_w$ , high protein, low carbohydrate substrates. It also confirms the findings that pseudomonads are primary colonisers of proteinaceous foods prior to spoilage and decay (Gill 1976; Lawrie 1985; Hayes 1985).

#### Trial 2 - Aerobic and anaerobic bacteria from fresh hides

All 40 samples yielded a mixture of Gram positive and Gram negative bacteria of varying morphological types. The aerobic Gram positives consist mainly of *Bacillus sp.* and

*Staphylococcus* while the aerobic Gram negatives were mainly Enterobacteriaceae (*E. coli*, *Klebsiella sp* and *Proteus mirabilis*) and Pseudomonaceae (including *Ps. fluorescens*, *Ps. aeruginosa* and *Ps. sp.*). There were also a few unidentifiable bacteria present.

Provisional identification of the bacteria is given in Table 3.5 and more specific (API) identification recorded in Table 3.6. These results indicate that a variety of bacterial types exist on raw hides which is in accordance with McLaughlin and Rockwell (1922) who isolated 24 strains of bacteria from hides which they placed into 10 different groups.

**Table 3.5 Provisional Identification of some of the Major Aerobic Bacterial Groups isolated from Raw Hides**

Growth at 30°C		Gram Reaction	General Morphology	Spores	Provisional Identification
BA	MA				
+	-	+	Large rods	CO	<i>Bacillus sp.</i>
+	-	+	Large rods	ST/T R/O	<i>Bacillus sp.</i>
+	-	+	Small rods	-	Diphtheroids
+	-	+	Clusters of cocci	-	Staphylococci
+	-	+	Irregular clusters of cocci	-	Micrococci
+	-	+	Chain and diplococci	-	Streptococci
+	-	+	Clusters of cocci	-	Pigmented staphylococci
+	+NLF/G	-	Thin rods	-	Pseudomonads
+	+LF	-	Rounded end rods	-	Coliforms
+	+LF/M	-	"	-	<i>Klebsiella sp.</i>
+	+NLF/S	-	"	-	<i>Proteus sp.</i>
+	+NLF/F	-	"	-	Pseudomonads
+	+LF	-	Cocco bacilli	-	Coliforms

Key: NLF = non lactose fermentor      C = central  
 LF = lactose fermentor                ST = subterminal  
 M = mucoid                                T = terminal  
 G = green pigment                      O - oval  
 F = fluorescent                          R = round  
 + = growth or positive                - = no growth or negative  
 BA = blood agar                         MA = MacConkey agar

All 40 samples yielded a variety of anaerobic bacteria and in many cases more than one species was present per sample. *Clostridium sp.* were the dominant anaerobes present and out of 24 pure colony isolates *C. perfringens*, *C. histolyticum*, *C. sordelli* and *C. sporogenes* were

identified to species level. *Clostridium sp.* and two strains of *Actinobacillus* were also identified but several of the anaerobic cultures proved unidentifiable. The API 32A results of the anaerobic isolates are given in Table 3.6 together with the API 10S results of the aerobic isolates.

**Table 3.6. API (10S and 32A) Identification of Aerobic and Anaerobic Bacteria from Raw Hides**

Incubation Conditions	Bacteria Identification
<b>Aerobic</b>	<i>E. coli</i> <i>Klebs. aerogenes</i> <i>Prot. mirabilis</i> <i>Ps. aeruginosa</i> <i>Ps. fluorescens</i> <i>Ps. sp.</i>
<b>Anaerobic</b>	<i>C. perfringens</i> <i>C. histolyticum</i> <i>C. sordelli</i> <i>C. sporogenes</i> <i>A. acetocomitans</i>

These results indicate that a large variety of bacterial types colonise both raw hides and re-colonise biocide-treated hides. All three organisms selected for the inocula in this thesis study were readily isolated from raw hides and therefore are natural colonisers of raw hides.

#### 3.4.3. Competitive Inhibition between Selected and Resident Bacteria:

This aspect was examined also in two separate trials. In Trial 1, 3 cm diameter raw hide plugs were inoculated with a mixture of the three selected inocula prepared initially as individual saline suspensions at  $10^8$  organisms  $\text{ml}^{-1}$  (as detailed in Appendix B). 1 ml of each of these suspensions was added to 97 ml of fresh saline to form a 1:1:1 triple-mix of ca  $3 \times 10^6$  organisms  $\text{ml}^{-1}$ . 10 ml of this triple-mix inoculum was added to each of the following: (a) 90 ml of saline; (b) 90 ml of nutrient broth; (c) 90 ml of Fluid thioglycollate broth. This resulted in three separate inocula suspensions (saline, Nutrient broth or Fluid thioglycollate broth) each containing a final concentration of ca  $3 \times 10^5$  bacteria  $\text{ml}^{-1}$ . After incubation at  $37^\circ\text{C}$  for 24h followed by  $25^\circ\text{C}$  for 48h, heat shrinkage, organoleptic assessment, hydroxyproline levels, collagenase activity tests and post liming/delimiting stereo-microscopy examinations were carried

out. In Trial 2, the selected inocula were prepared as above but were also used individually and as 1:1 (double-mixes) at a final concentration of ca  $1$  and  $2 \times 10^5$  bacteria  $\text{ml}^{-1}$  respectively as well as the 1:1:1 (triple mix) of all three organisms. Incubation was as for Trial 1 as were the assessment parameters except that histological examination was substituted for heat shrinkage. Non-inoculated controls were included in both trials.

#### **Trial 1 - Mixed inocula of all three bacteria:**

There were marked differences between the unsterile control hides without inocula and the inoculated (triple-mix) hides in terms of heat shrinkage and collagenase production. The inoculated hides showed decreased hide shrinkage temperatures ( $T_s$ ,  $\bar{x} = 35^\circ\text{C}$  compared with  $T_s$ ,  $\bar{x} = 67^\circ\text{C}$ ) which is in accordance with the decreased thermal denaturation temperatures of degraded native and soluble collagens (Birkedal-Hansen 1987; Swartland 1990). Similarly collagenase activity was greatest in the hides containing the additional triple-mix inocula but absent or only marginal in the non-dosed controls, indicating lower collagenolytic activity associated with the resident/transient bacteria. (The mean zone size measurements (Appendix B) were 4,7 mm for the triple-mix dosed hides compared to 2,0 mm for the non triple-mix dosed controls). See Table 3.7. Collagenolytic activity by *Bacillus spp.* has been found previously (Petushkova et al. 1985; Mackinen and Mackinen 1987), and it is possible that this could be the cause of activity in the control hides. The increased degradation shown in the reduced media (FTB) is in accordance with previous findings of the predominance of micro-aerophilic *Clostridium sp.* in protein degradation (Gill 1979; Banwart 1987). The low hydroxyproline levels for all the hide samples, despite the stereo microscopic findings of marked degradative changes in the hides suspended in the reduced media, could be attributed to the short incubation time in which only spoilage and decay, as opposed to putrefaction, would be present. The overall conclusion for this initial trial was that the triple-mix of the selected inocula outcompeted resident and/or transient bacterial populations to produce degradative changes in-situ, and this was most pronounced under reduced conditions.

Table 3.7. Degradation of Raw Hides inoculated with a 1:1:1 Mix of *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes* compared with Non-Inoculated Hides

Inocula Type	Heat Shrinkage (°C)	Organoleptic			Collagenase zone (mm)	Hydroxyproline (mg/ml <sup>1</sup> )	Post lime/delime		
		H	M	A			EP	PD	RD
Triple mix in saline	31	+++	+++	+++	5.6	<20.0	+	-	-
Triple mix in FTB	36	+++	+++	+++	3.4	"	++	+++	++
Triple mix in NB	37	+++	+++	++	5.2	"	+	-	-
No inocula/saline	65	++	++	+	Nil	"	-	-	-
No inocula/no fluid	69	+++	+++	+	2.0	"	+	-	+

Key: H= hairslip; M - malodour; A = appearance (slime and/or gelatinisation)  
 - = absent; + = slight; ++ = moderate; +++ = extensive  
 ED = epidermis; PD = papillary dermis; RD = reticular dermis  
 FTB = fluid thioglycollate broth; NB = nutrient broth

### Trial 2 - Individual and mixed inocula effects:

The second trial examined the ability of individual strains of *C. histolyticum* and *C. sporogenes*, either alone or as a 1:1 double-mix with *Ps. aeruginosa*, or as a 1:1:1 triple-mix of all three organisms to outcompete hide populations. The effects of added nutrients and of reductants on degradative changes was also examined. Hide shrinkage tests on very degraded hides had proved too difficult to carry out in the first trial. Therefore in this trial these were replaced by histological examinations of limed/delimed hide pieces. The fact that the raw hides in both trials were untreated, and so would contain numerous bacteria (some of which could be the same as the selected inocula types) must be considered when interpreting the results. This was borne out by the organoleptic assessments which showed little difference between test and control hides (Table 3.8.) except with respect to black deposits and foul putrefactive odours detected only in the inoculated hides. *C. sporogenes* produces copious hydrogen sulphide and blackening in meat (Cato *et al.* 1986) and it is postulated that this caused the black deposits in the anaerobic systems containing this organism. In the aerobic systems the black deposits could be due to either hydrogen sulphide producing micro-aerophilic clostridia or to *Proteus sp.* The latter organisms are aerobic and they also produce H<sub>2</sub>S and attack hair follicle proteins causing hairslip (Venkatesen 1979).

**Table 3.8. Effect of Dosing Raw Hides with Individual and Mixed Strains of Selected Bacteria : I. Organoleptic Changes during Storage in Different Media**

Bacterial Inocula	MEDIA								
	Saline			FTB			NB		
	H	M	A	H	M	A	H	M	A
<i>Ps. aer</i> + <i>C. hist</i> + <i>C. spor</i>	+++	+++ (a)	+++	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)
<i>Ps. aer</i>	+++	+++	+++ (b)	+++	+++	+++	+++	+++	+++ (b)
<i>C. hist</i>	+++	+++ (a)	+++	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++
<i>C. spor</i>	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)
<i>Ps. aer</i> + <i>C. hist</i>	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)
<i>Ps. aer</i> + <i>C. spor</i>	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)	+++	+++ (a)	+++ (b)
Control - no inocula	+++	+++	++	+++	+++	++	+++	+++	++

Key: H = hairslip; M = malodour; A = appearance  
 + = slight; ++ = moderate; +++ = extensive  
 (a) = foul odour; (b) = black discolouration  
 FTB = fluid thioglycollate broth; NB = nutrient broth

The pH values, collagenase activity and hydroxyproline levels of the dosed raw hides of Trial 2 are shown in Table 3.9. pH values were very stable and did not reflect the degradative changes shown by other parameters. This confirms the findings of 3.4.1 above that internal pH changes values would be more useful for assessing denaturation in hides.

Hydroxyproline levels were below 20 µg ml<sup>-1</sup> of inoculating fluid throughout, confirming the findings of the initial trial and indicating the need to examine hide extrudates, as opposed to inocula suspensions, for increased levels of this imino acid. Collagenase activity appeared relatively high for both test and controls. These trials were carried out before sterile collagen plates were perfected and a standard graph developed, therefore zone size differences were used as an indication of relative activity only. (Appendix B). As in Trial 1, lower activity in reduced media was evident and attributed to hide extrudate (internal) activity not being detected.

Table 3.9. Effect of Dosing Raw Hides with Individual and Mixed Strains of Selected Bacterial Inocula : II. pH, Collagenase and Hydroxyproline Levels during Storage in Different Media.

Bacterial Inocula	MEDIA								
	Saline			FTB			NB		
	pH	C	H	pH	C	H	pH	C	H
<i>Ps. aer</i> + <i>C. hist</i> + <i>C. spor</i>	6.9	4.6	<20.0	6.8	4.5	<20.0	6.6	4.4	<20.0
<i>Ps. aer</i>	6.8	5.4	"	6.8	4.0	"	6.6	5.0	"
<i>C. hist</i>	6.9	4.4	"	6.5	4.2	"	6.6	5.2	"
<i>C. spor</i>	7.0	3.4	"	6.5	0.9	"	6.3	1.1	"
<i>Ps. aer</i> + <i>C. hist</i>	6.9	4.4	"	6.8	4.3	"	6.7	3.7	"
<i>Ps. aer</i> + <i>C. spor</i>	6.6	5.8	"	6.6	4.5	"	6.2	4.5	"
Control - no inocula	7.0	4.9	"	6.5	3.0	"	6.6	3.0	"
Mean	6.9	4.7	"	6.6	3.8	"	6.5	4.0	"

Key: C = Collagenase zone size in mm (disc edge to zone edge)

H = Hydroxyproline µg/ml media

FTB - Fluid thioglycollate broth; NB = nutrient broth

The histological findings (see Table 3.10) showed that the selected inocula degraded hides to a greater degree than normal hide populations. This finding held true for both the triple-mix and 1:1 double-mix of the inocula. This confirms the findings of Trial 1 in that the selected bacteria outcompete natural populations to cause degradative changes in hide tissue. However, there are differences between individual bacterial types and their action on specific hide tissues. In terms of increased degradation of the epidermal area compared with the dermal area, the inocula rank as follows:

*Ps. aer.* > *Ps. aer./C. hist./C. spor* > *Ps. aer./C. hist.* > *C. spor./C. hist.*>

*Ps. aer/C. spor.* > control

These findings confirm both the greater overall hide degradation by *C. histolyticum* and the specific association of *Ps. aeruginosa* with surface epidermal area degradation. *C. sporogenes* appears to have a unique micro-niche in hides localised directly under the epidermis at the epidermal/papillary dermal junction.

Table 3.10: Effect of Dosing Raw Hide with Individual and Mixed Strains of Selected Bacterial Inocula : III. Histological Changes during Storage in Different Media.

Bacterial Inocula	MEDIA											
	Saline				FTB				NB			
	ED	HG	PD	RD	ED	HG	PD	RD	ED	HG	PD	RD
<i>Ps. aer</i> + <i>C. hist</i> + <i>C. spor</i>	++++	+++	+	++	++	++	-	+	++++	+++	-	+/- (b)
<i>Ps. aer</i>	+++	++	-	+(b)	++++	++	-	+/-	++++	++	+	++
<i>C. hist</i>	+++	++	-	++	+++	++	+	+	+++	++	+	++
<i>C. spor</i>	++++	+++	+(a)	++	++++	++++	++++	+/-	++++	++++	++	+
<i>Ps. aer</i> + <i>C. hist</i>	+++	+++	+/- (a)	+	++++	++	+	++	++++	-	-	+
<i>Ps. aer</i> + <i>C. spor</i>	++	-	+	+	+++	+++	+	++	++++	++	-	++
Control - no inocula	++++	++++	+++ +	-	+	-	-	-	-	-	-	-

Key: FTB = fluid thioglycollate broth; NB = nutrient broth  
 +/- = slight; + = trace; ++ = moderate; +++ = extensive;  
 ++++ = abundant degradation  
 E = epidermis; HG = hair follicles and sebaceous glands;  
 PD = papillary dermis; RD = reticular dermis  
 (a) = gas in tissues; (b) = degradation mainly in centre of hide at upper papillary dermal/epidermal junction

Stereo microscopy of limed/delimed hide plugs confirmed that the triple-mix inocula outcompeted raw hide population to produce degradative changes in-situ (Table 3.11). Similar findings occurred for the 1:1 double-mix containing *C. histolyticum* indicating that this organism is a primary cause of hide degradation. Neither control hides nor those dosed with *Ps. aeruginosa* on its own showed any evidence of damaged grain. The overall ranking of different inocula in effecting sueded and degraded grain decreased in the following order:

*Ps. aer./C. hist./C. spor.* > *C. hist.* > *Ps. aer./C. hist.*;

*Ps. aer./C. spor* > *C. spor* > *Ps. aer.* > Control

Of interest is the link between *C. histolyticum* and grain damage.

The influence of nutrient additions and reduced conditions was demonstrated by the histological appearances (Table 3.10) and stereo-microscopy findings (Table 3.11) in that the

reduced medium (FTB) promoted the growth of *C. sporogenes* both individually and when mixed with *Ps. aeruginosa*. To a lesser extent similar findings were found for *C. histolyticum*. The addition of nutrients to the system generally resulted in a slight increase in degradative changes.

**Table 3.11: Effect of Dosing Raw Hide with Individual and Mixed Strains of Selected Bacterial Inocula : IV. Stereo-microscopy of Limed/Delimed Hides**

Bacterial Inocula	Stereo-microscopy Appearance of Hide Pieces					
	Material			Longitudinal X-section		
	Epidermis	Grain		Epidermis	Papillary Dermis - D	Reticular Dermis - D
	D	S	D	- D		
<i>Ps. aer</i> + <i>C. hist.</i> (1)	-	+	+	+	++(b)	++(b)
	+++	-	+++	+++(*)	++(b)	+++ (b)
	++	++	++	+++(*)	+++	+++
<i>Ps. aer</i> (1)	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
<i>C. hist</i> (1)	+	+	-	+(*)	+	+
	+	+	-	+	++	++
	+	+	-	+	++	+
<i>C. spor</i> (1)	+	+++	-	+	+	+
	-	-	-	+(*)	-	-
	-	-	-	+	-	-
<i>Ps. aer</i> + <i>C. hist</i> (1)	+	++	-	++(*)	++	++
	+	+	-	+(*)	-	-
	+	++	-	+/-	-	-
<i>Ps. aer</i> + <i>C. spor</i> (1)	+	++	-	+	+	+
	+	++	-	+++(*) (a)	+	-
	NT	NT	NT	NT	NT	NT
Control no inocula	+/-	-	-	-	-	-
	-	+/-	-	+	+/-	+/-
	-	-	-	-	-	-

Key: (1) Saline media; (2) Fluid thioglycollate broth; (3) Nutrient broth.

D = Degradation of tissue; S = surface sueding

+/- = Slight; + = trace; ++ = moderate; +++ = extensive

(\*) = degradation at ED/dermal junction

(a) = Black discoloration

(b) = Tissue very soft and pulpy

### 3.5. Conclusion:

Bacterial growth and proteolytic activity were found to be useful parameters for detecting spoilage in hides and to confirm the involvement of bacteria in hide degradation. Hairslip and malodour acted as secondary indicators of general protein degradation but they did not differentiate between the various stages of onset of degradation such as spoilage and early decay, nor did they differentiate between bacterial and tissue (autolytic) activity, so limiting their value as degradation indicators.

Surface pH shift was also found to have a limited application in monitoring protein breakdown in both hide degradation and preservation studies due to buffering effects. The absence of definite evidence of putrefactive changes in these studies indicates the need for more specific parameters to be applied to hide degradation studies. The role of bacteria as the major cause of hide degradation was evident but a contribution from autolytic breakdown could not be discounted in these studies since autolytic enzyme activity was not suppressed. It is postulated that the use of sterile hide pieces inoculated with specific bacterial types would prove to be a better method for in-situ studies.

Microbial colonisation of raw hides was found to be effected by a number of different bacterial types which included environmental, enteric and pathogenic isolates. Hides preserved with biocides became recolonised during subsequent storage and although no specific pioneer species were identified, Gram negative bacilli predominated. In raw hides Gram negative bacilli, mainly pseudomonads and enteric bacteria, also predominated indicating their possible role in primary spoilage. The organisms selected as inocula for the model system (*Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*) were readily isolated from raw hides selected at random. This confirms that hides are a natural reservoir for these organisms.

The three selected inocula for hide degradation studies in the model system (*Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*) successfully outcompeted resident and/or transient hide populations when dosed to raw hide pieces as a 1:1:1 triple-mix, or as a 1:1 double-mix of either *C. histolyticum* or *C. sporogenes* or *Ps. aeruginosa*. Degradative changes occurring due to the inocula included sueded grain, surface grain damage and reticular dermal degradation. These changes varied according to the bacterial type in the inocula as well as the presence of nutrient and/or reducing conditions.

## CHAPTER 4

### THE DEVELOPMENT OF A MODEL SYSTEM FOR IN-SITU HIDE DEGRADATION STUDIES

- 4.1. Summary
- 4.2. Introduction
- 4.3. Research Objectives
- 4.4. Results and Discussion
  - 4.4.1. Sterilisation of Hides
    - a. Sterility Testing
    - b. Hide Integrity after EO Treatment
  - 4.4.2. Studies on the Selected Inocula
    - a. Growth Curves
    - b. Factors affecting Bacterial Growth
- 4.5. Conclusion

#### 4.1. Summary:

Ethylene oxide (EO) was applied to hide cuttings and to soluble collagen in order to generate bacteria-free systems for use in biodegradation studies. To ensure that the EO treatment did not significantly modify the hide/collagen, either chemically or structurally, the following tests were performed:

1. Polarimetric analysis of the collagen
2. Heat shrinkage of hides
3. Enzyme dosing of hides with subsequent histological studies

Sterility testing of the hide cuttings was also carried out. The results indicate that EO treatment can successfully sterilise hide, and autolytic enzyme systems be inactivated, without sacrificing basic collagen integrity. This makes EO-treated hides an acceptable substrate for degradation studies.

To optimise the model system for biodegradation studies with the selected three inocula it was necessary to establish a storage period which would cover both early and late degradative changes in inoculated hides in-situ. One way this was achieved was by the calculation of individual growth rate constants for each of the three inocula. These constants were determined in laboratory broth cultures and the results extrapolated to generate the time in hours to early stationary phase growth for each culture (assuming an initial bacterial level of either  $10^2$  organisms  $\text{ml}^{-1}$  or of  $10^5$  organisms  $\text{ml}^{-1}$ ). These times were found to be 5 days at  $37^\circ\text{C}$  at  $10^2$  bacteria  $\text{ml}^{-1}$  which was reduced to 3 days if the inoculum size was increased to  $10^5$   $\text{ml}^{-1}$ . Physical factors affecting the degradation rate were also investigated in order to generate standard parameters for the system. This resulted in a storage (incubation) time of 8 days at a temperature of  $30^\circ\text{C}$  being established as optimal.

During these studies bacterial growth in-situ was found to be delayed in EO-sterilised hides compared to raw hides. To overcome this the effects of adding small amounts of nutrients (in the form of liquid media from the overnight broth cultures of the bacteria) was investigated. Similarly, the effects of reductants on bacterial growth were studied. The use of inocula suspensions consisting of  $10^5$  bacteria  $\text{ml}^{-1}$  (in 5ml saline containing 1:10 volume of nutrient broth) was found to be effective in promoting bacterial growth and catabolic activity but reductants were found to have no beneficial effect in the system. Bacterial enzyme activity was also investigated. All three selected inocula were shown to produce both caseinolytic and

gelatinolytic enzymes to a varying degree but only *C. histolyticum* produced collagenase. These findings were compatible with the enzyme requirements for putrefactive degradation of protein substrates containing collagen.

From the results of these studies it was possible to establish a model system for hide degradation studies using 3 cm diameter hide pieces placed in 40ml plastic jars with screw on lids. The hide pieces were sterilised by ethylene oxide, inoculated with the selected bacteria and incubated for 8 days at 30° C.

#### 4.2. Introduction:

##### Hide sterilisation:

As discussed in Chapter 3, conclusive evidence for the action of bacteria in hide degradation cannot be obtained solely by studies of naturally decaying hides. Such studies cannot differentiate between autolytic tissue enzymes and bacterial enzymes in any quantitative way. Another disadvantage of using naturally decaying hides is the inherent variations that will occur in the microbial populations, both spatial and temporal. Added to this are the multiplicity of post-flay environmental conditions to which various hides are subject.

Prior to initiating a major degradation study there is a need to establish a standard system in which individual bacterial strains can be added, either in pure or mixed cultures. Ideally the hides used should be devoid of autolytic tissue enzymes and all viable resident and transient bacteria should be eradicated. In establishing such a model certain practical criteria must be met and these would include considerations such as the size and consistency of the hide pieces as well as the ease of manipulation of both hides and bacteria within the system.

One of the most important requirements to meet is that of eradication of all viable bacteria in the hides prior to inoculation with the selected strains. Concomitant to this is the maintenance of basic hide integrity and evidence for non-denaturation of proteins and other organic components as a result of the chosen sterilisation process. In general there are many chemical and physical methods available for bacterial eradication and the method chosen will depend upon the sample/product being treated as well as the number of bacteria present. Previous hide studies have indicated levels of bacteria on wet salted hides to be between  $10^3$  -  $10^6$  g<sup>-1</sup> and that these can be reduced to lower levels by application of various liquid biocides, (Cooper and

Galloway 1965; Woods *et al.* 1969(a); Hendry *et al.* 1970; Woods *et al.* 1971(b)). However, biocides, like other means of controlling bacteria in organic substances, tend to cause only temporary effects as bacterial activity is generally merely inhibited or reduced. Alternatives to biocides such as exposure to UV radiation, sonication, heavy metals, chilling, freezing, dehydration, antibiotics as well as alterations in pH and/or osmotic pressure, have all been used to inhibit bacteria under a wide range of conditions (Hugo 1971; Hurst 1980; Christensen 1982; Kostenbauder 1983; Happel 1983; Gardner and Peel 1986). In the case of antibiotics, heavy metals and biocides, total bactericidal activity can be achieved but problems can arise due to residual chemical activity. This would eliminate these methods as a choice for sterilisation of hides for the model since any extraneous chemical activity in the system could prove detrimental to the selected inocula.

Methods which can achieve sterility without residual activity are few and are as follows: heat treatments, ionising radiation and gaseous application. Heating cannot be used for hide sterilisation since hide proteins will be denatured at temperatures well below those required to sterilise them (Glanville & Kühn 1980). Therefore the choice of methods for use in hide sterilisation is limited to those that do not cause denaturation of the major skin proteins nor leave any residues that could be toxic to the selected inocula. Those limitations restrict the choice of method to gaseous application or radiation.

#### **Irradiation:**

Ultra-violet radiation cannot be used for hide sterilisation due to its poor penetrative power and low activity in the presence of high microbial loads (Block 1983). Gamma and electron beam irradiation have both been successfully applied to a range of proteinaceous materials. Radiation affects microbes non-specifically, being targeted mainly at RNA and DNA, although both cell membranes and enzymes are attacked. Ionising radiation selectively affects different microbial types in the order fungi > vegetative bacteria > spores > viruses with a minimum dosage level of 2.5M rad (25 kGy) being required to achieve sterility (Christensen *et al.* 1982).

Radiation does have some disadvantages as a sterilising agent in that, while its effects are mainly due to the formation of free radicals, polymers and peroxides, indirect breakdown of some organics as a result of ionised water radicals can occur (Christensen *et al.* 1982). Such denaturation occurs in ionised meats due to their high aqueous phase and since hides contain

approximately 66% water (Bienkiewicz 1983), denaturation of hides in this way is possible. Lawrie (1985) showed that at 5M rad (50 kGy) doses radiation, loss of water holding capacity occurred in meats and that dosage levels sufficient to kill  $10^2$  bacteria results in alteration of 0,2% of the proteins, 0,3% of the carbohydrates and 0,4% of the fats although that, even at 70M rad many enzymes remained unaltered. Since for the model system of this thesis residual enzyme activity is undesirable, this could be a limiting factor. Lakritz & Maerker (1988) discussed the effect of radiation on raw meat and concluded that proteolytic enzyme levels were mostly unaffected by low dosage (4 kGy [0,4M rad]) but that a drop of 42% in enzyme activity occurred after exposure to 10 kGy, with 50% loss occurring at 13 kGy and 75% loss at 45 kGy.

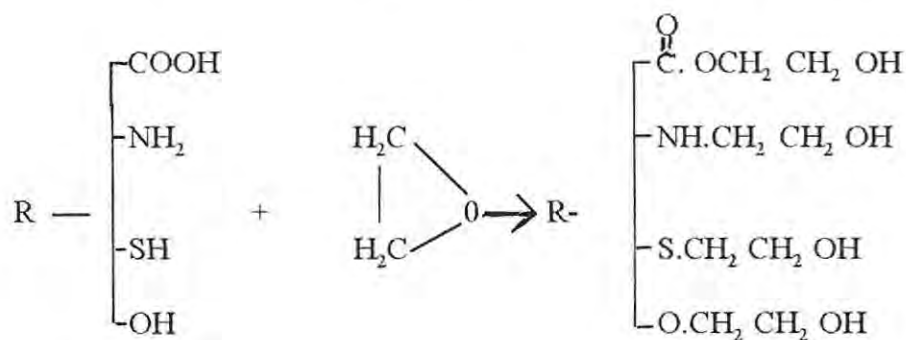
Nevertheless, despite residual autolytic enzyme activity many foods are preserved by irradiation. These range from astronaut provisions (Sonsino 1987) to frozen proteinaceous foods radiated at 5-10 kGy (Farkas 1987) to spices irradiated at 10 kGy (Munasiri *et al.* 1987). Preservation of hides by irradiation was investigated and Russell *et al.* (1982) who found that levels of 25 kGy were required for sterilisation but that this could be reduced to 5-8 kGy if a combined biocide/radiation treatment was applied. Six weeks storage at ambient was achieved for hides thus treated and tests on the finished leather indicated no evidence of deterioration. However appreciable damage did occur after radiation dosage at 10 kGy followed by storage for four weeks at ambient temperature. Bailey and Haas (1988) and Bailey and Wang (1989) investigated the use of both electron beam and gamma irradiation on raw and salted hides. Results indicated loss of tensile strength (grain and fibre break) and general fibre splitting, even at low dose levels of 5M rad. Lawrie (1985) reported changes in shrinkage temperature of irradiated dry collagen from 61°C to 47°C at 4M rad falling to 27°C at 40M rad. Liu *et al.* (1989) working with injectable human amnion collagen irradiated by gamma rays at levels of 0.25 to 2-5M rad demonstrated significant changes in physical characteristics. These included changes in neutral solubility at 45°C, increase in fibril diameter, greater resistance to collagenase and increased sensitivity to trypsin. Therefore due to loss of water holding capacity, changes in organoleptic properties, possible residual enzyme activity and the need for specialised treatment facilities, irradiation was not considered a suitable means for sterilisation of hides for the proposed model system of this study.

### Gaseous Sterilisation:

Gases that have been used for sterilisation purposes include methyl bromide,  $\beta$ -propiolactone, propylene oxide, glutaraldehyde, formaldehyde and ethylene oxide. The first three are rarely used due to their high toxicity (Christensen and Kristensen 1982). Glutaraldehyde has high sporicidal activity but has a variable activity (Hugo 1971). McPherson *et al.* (1986) studying bovine corium collagen, found that increased collagen X-linking occurred after exposure to 0.1% and 1% glutaraldehyde, as determined by increased transition temperature and decreased sensitivity to trypsin indicative of tanning effects.

Formaldehyde has long been used as a sterilant for inanimate articles in medicine (Cruickshank *et al.* 1975) but, while rapidly sporicidal to some bacteria, it fails to kill *C. sporogenes* even after 2 hours exposure (Hugo 1971). Its unpleasant odour, tendency to polymerise at room temperature and creation of allergic reactions in humans reduces its value as a sterilising agent. (Christensen and Kristensen 1982).

Ethylene oxide (EO) like formaldehyde, acts by alkylation of carboxyl, amino, sulphhydryl and hydroxyl groups of proteins, including enzymes to form alkylethyl groups, e.g.:



The effect of EO on bacteria therefore results in the blocking of many reactive groups needed for essential metabolic activities and is possibly also due to alkylation of DNA and nucleic acids (Ingram & Roberts 1980).

In order for EO to be effective, conditions governing concentration, exposure time, relative humidity and temperature must be rigorously controlled. For this reason commercial EO sterilisers are available in which an optimum relative humidity of 28-33% is obtainable. Levels below 30% allow for survival of resistant bacteria and even at 33% some resistant cells have

been found to survive (Gilbert et al. 1964). The carrier state of the material plays an important role as water is essential to allow permeation through polar type films and for the rehydration of spores, these being 2-10 times more resistant than vegetative cells (Gilbert et al. 1964). For example Hugo (1971) showed that  $10^5$  spores of *C. sporogenes* in 0.15 ml of infusion media on a porous surface were sterilisable but that  $10^6 - 10^7$  spores of the same organism from media dried onto a solid surface were resistant to sterilisation by ethylene oxide.

It would appear that ethylene oxide as a means of sterilising hides for the proposed model study is appropriate as it does not cause denaturation of the major proteins, does inactivate enzymes and sterilising conditions are readily controlled due to the availability of commercial EO sterilisers. However, in order to ensure complete sterility, factors involving the hide itself need to be considered since bacterial numbers present need to be minimal and any spores present must be in a moist state. This is easily achieved by thorough washing of the hides without subsequent drying.

In 1957, Thonard and Scherp used bovine achilles tendon for dental caries studies after exposing the collagen to EO vapour for 18 hours followed by freezing and thawing. They found collagen denaturation after this rather prolonged process and showed that this was not present in non-EO exposed frozen collagen.

Similar findings emerged when Mergenhagen et al. (1960), working with dental bacteria and acid-soluble rabbit skin collagen gels, found marked changes in collagen exposed to 11.0% EO for 16 hours. Loss of fibrillogenesis and defined cross striation pattern (as shown by electron microscopy) correlated with greater susceptibility of the gels in broth cultures of gingival accumulations compared with non-EO exposed gels. However, gels exposed to 0.1% EO, showed much less change and neither gels had decreased hydroxyproline content indicating that the collagen molecules were, in fact, still intact. Similarly Chakraborty and Chandra (1984) treated a series of soluble collagens including rat tail, calf skin, carp-swim bladder and achilles tendon using a 12 hour EO exposure at  $1\text{ml L}^{-1}$  dessicator area and then used them to detect collagenase production by a streptomycete.

Soluble collagen is far more susceptible to denaturation generally than hide tissue ( $T_m$  25°C and 65°C respectively). It is therefore logical to expect that if collagen is not denatured by EO

exposure then hide tissue would not be denatured either (providing no further treatments such as freezing ensued). In fact, effects on tissue components, being those of alkylation, would be expected to result in greater fibre cross-linking and therefore increased resistance to denaturation. EO was therefore selected as the means of sterilisation for hide pieces to be used in the model system as well as for collagen gels for use in enzyme detection and quantification.

#### **Studies of the selected inocula:**

In the raw hide dosing studies of Chapter 3 the selected inocula were shown to give different results in a number of test parameters according to whether saline, fluid thioglycollate broth or nutrient broth was added to the system. The findings of increased degradation of EO hides in the presence of small amounts of nutrient addition indicated a lack of freely available micro-nutrients after EO treatment, probably due to their alkylation. Simple carbon sources such as glucose are often the primary target of bacteria as opposed to macro-molecular proteins. Hides contain only small amounts of sugars, glucose and galactose being present in collagen at approximately 4% and mannose at lower concentration. These sugars are attached to collagen via glycosidic links at hydroxyproline residues, mainly in the non-helical regions in the form of glycoproteins (Harkness 1979; Glanville & Kühn 1980).

Other sugars present in skin are in the form of the proteoglycans such as dermatan and chondroitin sulphate, both of which contain galactose and galactosamine bound to hyaluronates (which themselves contain small amounts of glucose). Proteoglycans coat the collagen molecules and also line blood vessel walls (Pearse 1985) and therefore present a readily available source of carbon for initial hide colonisation post-mortem. In the EO hides however, due to alkylation, these carbohydrates would be less prone to catabolic activity.

A precedent for nutrient addition in hide studies was set by Tancous (1961) investigating degradation by *C. capitovale* of calf skin coriums. Inoculating these coriums after 14 days storage in 4-6% salt solution resulted in no growth unless egg white and yolk was added to the system. In order to obviate growth suppression in the model system of this thesis, initial investigations were made of the growth-promoting effect of low nutrients as well as chemical reductants (Chapter 3). In this Chapter generation times of each of the three individual bacteria were calculated in order to obtain an indication of the optimum bacterial inoculum size, incubation (storage) time, and the temperature to be used for this study. The results of these

trials were used to predict the probable rate of denaturation in the model system. These rates were used to set the model parameters at a time scale that would encompass both onset and cessation of bacterial degradation with all three inocula. Finally the selected strains were screened for their proteolytic activity against casein (general protein), collagen (specific hide protein) and gelatin (collagen denaturation product) to ensure their suitability to the study.

#### **4.3. Research Objectives:**

The research covered in this chapter was aimed at investigating both the sterilisation of hide pieces and the parameters affecting growth and activity of the selected inocula in order to establish the final model dosing system.

The research objectives were as follows:

- a. To sterilise hides without causing destruction of the native collagens and other major proteins present or any alteration to the general skin structure.
- b. To establish base line levels for incubation times, growth rates and proteolytic enzyme activity of the three selected bacteria types for use in the main degradation study.

#### **4.4. Results and Discussion:**

##### **4.4.1. Sterilisation of Hides:**

3 cm diameter hide pieces were washed and sonicated, placed in 40 ml plastic jars and then sterilised using an ethylene oxide sterilant as detailed in Appendix B. In order to ensure that sterility was achieved hide pieces were subjected to 14 day aerobic and anaerobic sterility tests post EO treatment. In order to ensure the integrity of the hide collagen after sterilisation, tests for heat shrinkage and in-situ enzyme susceptibility tests, were performed. For confirmation of autolytic enzyme denaturation, hide pieces were stored at 20°C and 30°C for 3 months prior to histological examination of the tissues and hide extrudates were screened for proteolytic activity. Acid-soluble bovine collagen was also tested for denaturation changes after EO exposure by means of hydrothermal transition ( $T_g$ ) and polarimetry.

#### 4.4.1.a. Sterility Testing:

Raw hide cuttings were taken directly post-flay using a minimum of 30 different animals. One third of these samples were left "as is", one third were washed in tap water for twenty minutes and the remaining third were washed and then sonicated for twenty minutes. All samples underwent EO treatment and twenty samples from each of these three pre-treatment series underwent standard sterility testing for 48 hours (Appendix B).

The comparative effectiveness of these hide treatments was assessed by scoring the number of samples with visible growth in sterility test broth medium after 48h incubation. Samples receiving no pre-wash had a 15% positive culture rate, those which were pre-washed had 5%, while those that had received both sonication and washing had 0%. Therefore sonication/wash was the most efficient of the three methods of pre-treatment. To confirm this finding 50 randomly selected raw hides pieces were pre-treated in this way and after EO sterilisation underwent a 14 day sterility test at 37°C. Only 2 pieces were positive on sub-culture (one aerobically and one anaerobically) and growth was present after 24 hours in each case. Therefore EO was found to be a satisfactory method for hide sterilisation, provided hides are well washed and sonicated for a minimum of 20 minutes prior to treatment and, as an additional precaution, some form of sterility test was carried out to ensure that no contaminated samples were used in the model system. It was found that this could be best achieved by swabbing each hide piece with a sterile swab prior to inoculation with the selected bacteria. The swabs were then incubated in individual 5ml volumes of fluid thioglycollate broth at 37°C for 24 hours. Any hide samples that gave growth in the media were discarded from the test series under investigation.

#### 4.4.1b. Hide integrity after EO treatment

In order to confirm that EO treatment did not cause either hide or soluble collagen denaturation the following determinations were carried out:

##### Heat shrinkage

The standard hydrothermal transition ( $T_g$ ) test (Appendix B) was applied to both soluble collagen and raw hide pieces. The results of these tests show that there was no real difference between the mean shrinkage temperature ( $T_g$ ) of EO-exposed and non-EO exposed collagen plugs (53.0 and 55.0 respectively). The results are shown in Table 4.1. below:

**Table 4.1. Heat Shrinkage Temperature ( $T_s$ ) of EO Exposed and Non-EO Exposed Collagen Plugs**

Sample No.	Heat Shrinkage Temperature °C	
	EO Exposed	Non-EO Exposed
1	53	53
2	53	56
3	53	55
4	53	55
5	53	55
Means	53	55

Similar results were found for heat shrinkage temperatures of EO and non-EO exposed raw hides. These were compared to raw hides exposed to 5 kGy of cobalt -60 gamma radiation in a MCI JS-6500 industrial irradiator (Isother, Kempton Park) and the results are tabulated in Table 4.2.

**Table 4.2. Heat Shrinkage Temperatures ( $T_s$ ) of EO Exposed, Non-EO Exposed and Gamma Irradiated Raw Hides.**

Sample No.	Raw Hide Heat Shrinkage Temperature (°C)		
	EO Exposed	Non-EO Exposed	Gamma Irradiated
1	61.0	65.5	56.5
2	61.5	64.5	56.0
3	62.0	65.5	55.0
4	61.5	64.0	55.5
5	61.0	66.5	56.0
6	59.0	65.5	56.0
Means	61.0	65.3	55.9

The slight difference in mean  $T_s$  between EO exposed (61.0°C) and non-EO exposed hides (65.3°C) could be due to the masking (alkylation) of charged basic groups, which while resulting in a slight shift in iso-electric balance, will not affect the molecular structure of collagen where the enzyme reaction takes place. The greater difference in  $T_s$  of the irradiated hide (55.9°C) compared to raw hide (65.3°C) could be due to interfibrillary bond breaking (A.E.Russell 1990 personal communication). This greater degree of change in  $T_s$  of irradiated hides compared to EO hides reinforces the choice of EO as opposed to irradiation as a method of hide sterilisation.

### Enzyme Hydrolysis

These tests were carried out using EO-treated neutral-salt soluble polymeric collagen which was exposed to a range of commercial proteolytic enzyme solutions at 20°C including collagenase (See Table 2.1. Chapter 2). Optical activity measurements of any changes in specific levorotation at 436nm were recorded over a six hour period (see Appendix B). These results are tabulated in Table 4.3. below:

**Table 4.3. Effect of Proteolytic Enzymes on EO-Exposed Soluble Collagen: Changes in Optical Rotation**

Enzyme	Conc. mg ml <sup>-1</sup>	Specific levorotation at 436nm			
		0h	2h	4h	6h
Collagenase	0,25	567	258	160	138
Trypsin	0,25	623	611	600	588
Protease	0,25	594	559	541	535
Control 1	-	582	578	575	571
Control 2	0,25	582	278	171	138

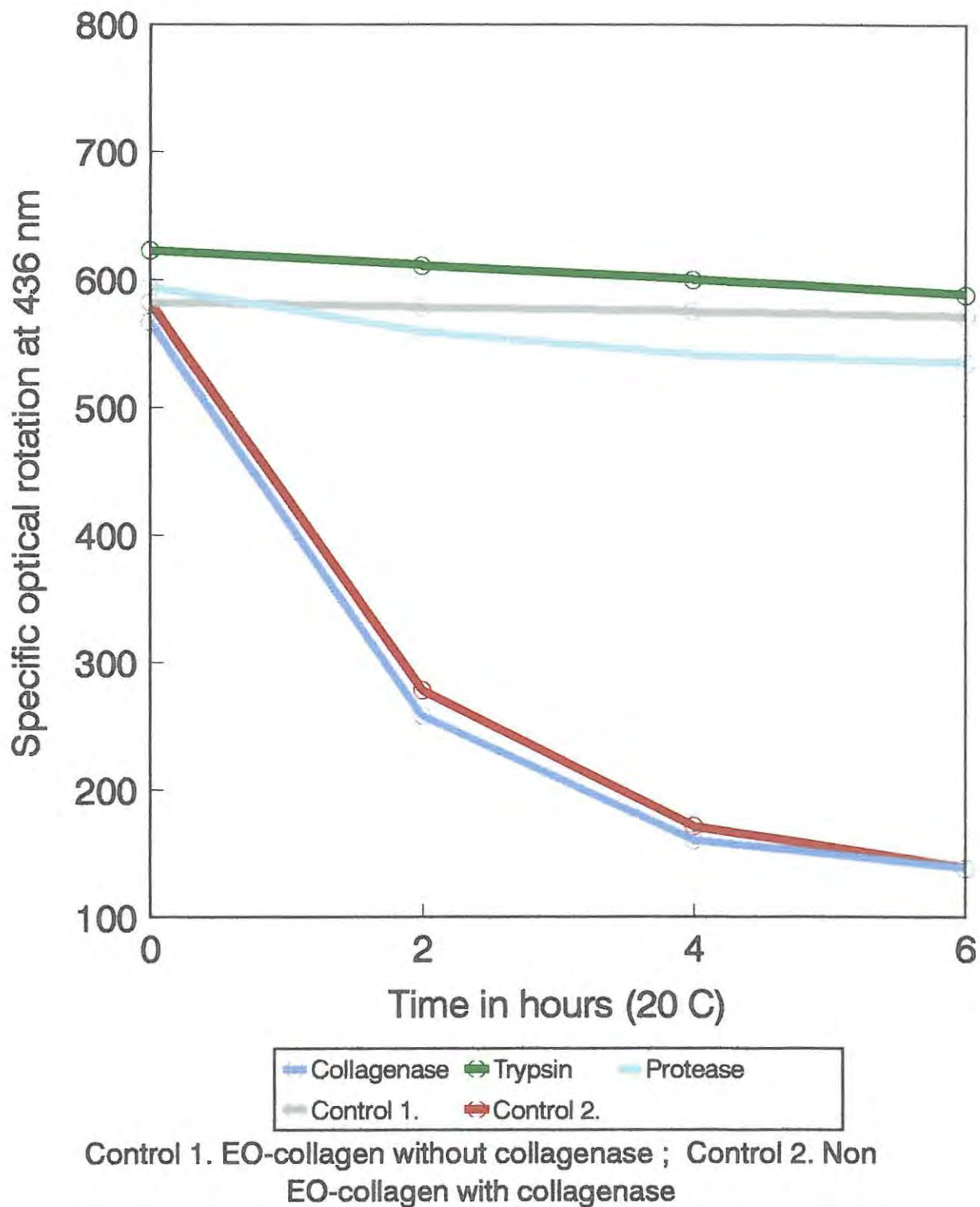
Control 1 = EO exposed soluble collagen (no collagenase)

Control 2 = Non EO exposed soluble collagen (plus collagenase)

Ethylene oxide-treated soluble collagen was susceptible to Sigma collagenase at 0.25mg ml<sup>-1</sup> and showed a similar response to the control non-EO exposed collagen plus collagenase. Comparatively small changes in optical rotation occurred in EO exposed soluble collagen subject to 0.25mg ml<sup>-1</sup> of either trypsin or proteinase. (Fig.4.1) These findings confirm that EO treatment does not affect the molecular integrity of collagen or render it more susceptible to non-specific proteinase cleavage.

### Enzyme Specificity on EO, Raw and Delimed Hides:

To confirm the findings of the heat shrinkage and polarimetry assessments of hide integrity post EO treatment, a range of commercial proteases (at 2.5mg ml<sup>-1</sup> in normal TRIS-buffer [Appendix A]) were applied to hide surfaces. Specific enzyme attack on hide collagens was detected using *C. histolyticum* collagenase at 0,5 and 2,5mg ml<sup>-1</sup> in modified TRIS-buffer (Appendix A). All enzymes were left to react for 24 hours at 37°C.



**Fig.4.1 Effect of Proteolytic Enzymes on EO-Exposed Soluble Collagen : Changes in Optical Rotation**

Initial trials involved enzyme application directly to both epidermal (or grain in delimed hide) and flesh surfaces of EO-treated hides (either as 30mm frozen sections or small hide cuttings). Evidence of any degradation was visualised using light microscopy of Masson stained tissue sections either directly for the frozen sections or after histological processing for the hide cuttings. (See Appendices A and B). A second series of trials utilised 3cm x 3cm raw and delimed hides and exposed these to trypsin, pronase and collagenase at 2.5mg ml<sup>-1</sup>. The enzymes were contained in antibiotic discs placed on the grain surface of the hides. Visualisation of the samples after liming and deliming was achieved using stereo-microscopy .

### EO Hides:

Of the enzyme series used, only collagenase caused any degradation of the collagen fibres of the test samples as visualised by normal histological procedure using light microscopy. The effects were more clearly defined in the processed tissue pieces from the hide cuttings than in the frozen sections (Table 4.4.)

**Table.4.4. Effect of Proteolytic Enzymes on the Collagen Structure of EO Hides.**

Sample Type	Amount of Degradation after Enzyme Exposure (24h at 37°C)					
	Collagenase (1)	Collagenase (2)	Trypsin (2)	Elastin (2)	Proteinase k (2)	Pronase (2)
Frozen Sections	++	+++	-	-	-	-
Tissue Pieces	++	+++	-	-	-	-

++ = moderate damage

+++ = heavy damage

- = no damage

(1) 0,5mg ml<sup>-1</sup>

(2) 2,5mg ml<sup>-1</sup>

### Raw and Delimed Hides:

Collagenase was the only enzyme to cause visual degradation as seen by stereo-microscopy of the hide surfaces. The effects were present in both raw and delimed samples as sueded grain and slight "pitting". The effects were enhanced on the samples delimed before enzyme application possibly due to the synergistic effect of bacterial enzymes with chemical processing in sueded grain (Heidemann 1983; Tancous 1984) (Table 4.5.) Another explanation is that absence of epidermal layer in delimed hides would allow more enzyme access but this would be counterbalanced by the high enzyme concentration and long exposure time.

**Table 4.5. Effect of Proteolytic Enzymes on the Grain Surface of Raw and Delimed Hides**

Sample Type	Amount of Degradation after Enzyme Exposure (24h 37°C)		
	Collagenase (*)	Trypsin (*)	Pronase(*)
Raw	+	-	-
Limed & Delimed	+++	-	-

\* Enzyme concentration = 2,5mg ml<sup>-1</sup>

- = No action

+ = Slight

+++ = heavy grain damage

The overall results of the enzymes studies confirmed that the EO sterilisation of hides used in this study did not cause any collagen denaturation nor render the hides susceptible to non-specific enzyme attack.

#### Autolytic enzyme activity

Five replicas each of EO-exposed 3 cm diameter hide pieces were stored at 20°C and 30°C respectively for three months. Controls of non-EO treated hide pieces were included. Hide degradation was visualised histologically using Masson stain as for the enzyme treated EO hides above. The results of these studies showed that no changes occurred to any of the EO-treated hide pieces throughout the storage period at either temperature. The control samples had to be discarded within the first few weeks due to tissue degradation and offensive odours.

#### **4.4.2 Studies on the Selected Inocula:**

After establishing the efficacy and appropriateness of the chosen sterilization method, the next most important factor to be established concerned the parameters governing the inocula. The choice of inocula to be used in the degradation study, as well as the ability of the chosen organisms to outcompete natural hide populations, have been discussed in detail in Chapter 1 and Chapter 3 respectively.

Further considerations relating to the inocula, which will influence the outcome of trials using the system, are the relative generation times of the bacteria involved and their ability to produce appropriate specific proteolytic enzymes. Similarly, incubation (storage) time, temperature and relative humidity needed have to be optimised to allow for overall optimal activity that could reflect in situ hide degradation. Results of these investigations are given below.

#### 4.4.2.a. Growth Curves:

Bacterial growth curves were carried out at 37°C on all three bacteria selected as inocula for this study (Chapter 1) using a Bausch & Lomb colorimeter at 600nm for optical density measurements. Cultures were prepared in Nutrient broth for *Ps. aeruginosa* and Fluid thioglycollate broth for *C. histolyticum* and for *C. sporogenes* (as detailed in Appendix B). To confirm these results total bacterial counts were also performed at the same time intervals (Appendix B).

Fig.4.2 below shows the graphical plots of the log phase growth for all three organisms individually. The generation times of the bacteria (g) were obtained directly from these plots using a minimum of two doubling times. The times for one generation i.e. the doubling times, were:

<i>Ps. aeruginosa</i>	g = 1,0 h	=	(60 mins.)
<i>C. histolyticum</i>	g = 1,2 h	=	(73 mins.)
<i>C. sporogenes</i>	g = 0,57 h	=	(34 mins.)

Growth rates and generation times were also calculated from the total count data (not shown) according to Frobisher *et al.* 1974 as follows:

$$1. \quad \text{Growth rate (gr)} = \text{number of doublings per time unit } gr = \frac{f}{tu}$$

Where f = total number of generations (doublings) and tu = time unit between initial and final total counts.

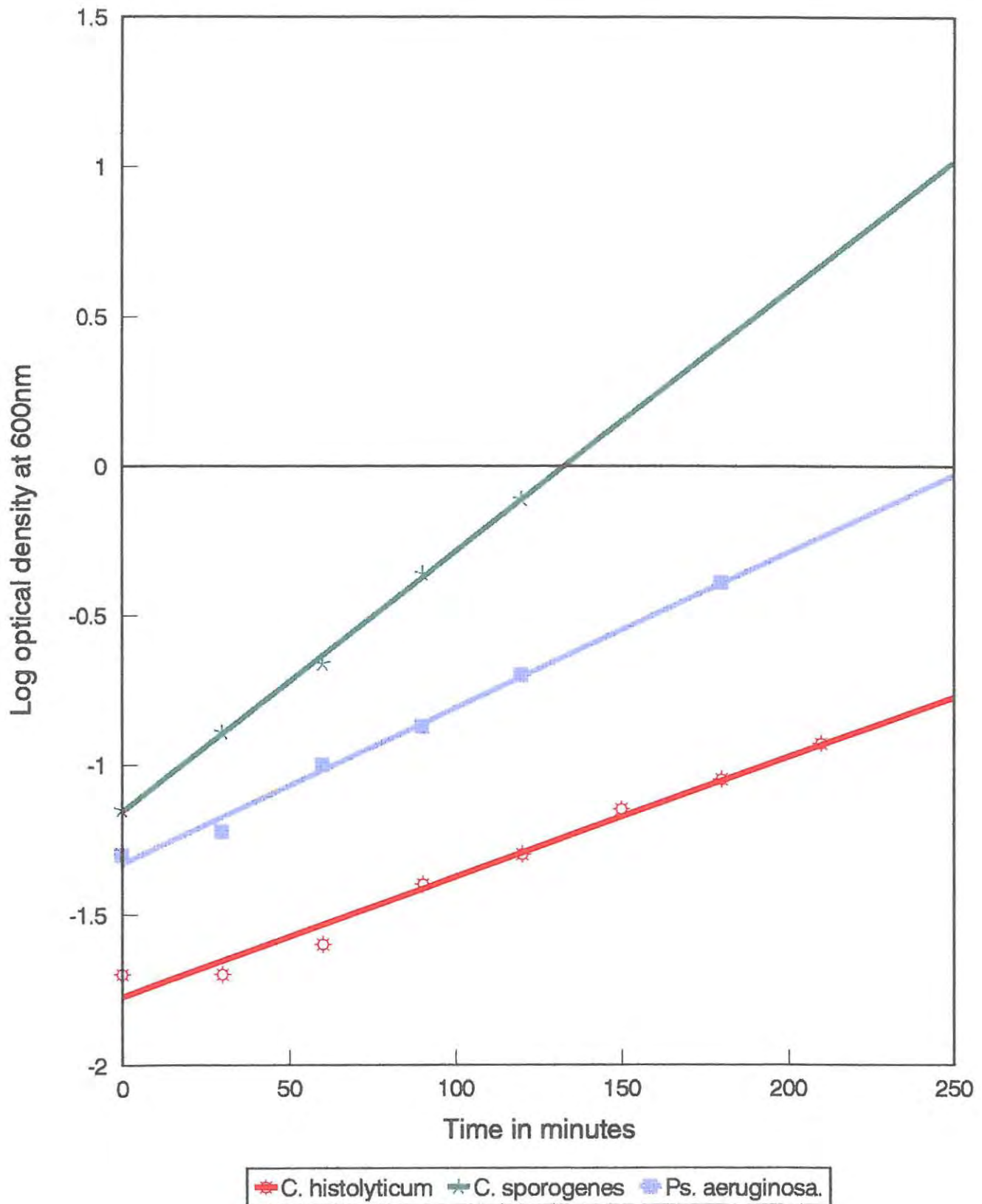
$$f \text{ is calculated as } \frac{\log_{10} C_1 - \log_{10} C_2}{0,301}$$

Where  $C_1$  = final count and  $C_2$  = initial count (if Tu = 1h then  $gr \text{ h}^{-1} = f/tu$  (in hours))

$$2. \quad \text{Generation time (g)}$$

$$g = \frac{1}{gr}$$

Where g = generation time and gr = growth rate.



**Fig. 4.2 Log Phase Growth of *Ps. aeruginosa* NCTC 10662 (in Nutrient Broth) and *C. histolyticum* ATCC 1940 and *C. sporogenes* ATCC 3584 (in Reinforced Clostridia broth) at 37 C.**

The total count readings were more variable than OD readings but nevertheless gave generation times of 0,86 h (52 mins), 0,95 h (57 mins) and 0,45 h (27 mins) for *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes* respectively which were comparable to the OD findings. Using the OD readings the specific grow rate constants (K) for each organism were calculated according to the formula (Boyd 1984).

$$K = \frac{\ln 2}{g} \text{ where } g = \text{generation time in h ( = doubling time)}$$

These gave values for K of 0,693, 0,568 and 1,215 for *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes* respectively.

Ka *et al.* (1994) used generation times of individual strains of 2, 4-dichlorophenoxyacetic acid-degrading bacteria growing as axenic and mixed culture in broths to predict the outcome of competition between the species when inoculated into soil microcosms. For the study of this thesis, growth rate constants (K) were used to predict a possible time period involved to putrefactive degradation of hides in a model system. To achieve this a hypothetical inoculum of  $10^2$  organisms  $\text{ml}^{-1}$  was substituted for  $N_1$  in the formula:

$$x = \frac{2,303 \times \ln (N_2 - N_1)}{K}$$

where K = growth rate constant, x = the time (in hours) to stationary phase growth and  $N_1$  = initial bacterial count and  $N_2$  = total count of the inocula at the stationary phase growth (as determined from the growth curve of each organism). This calculation represents conditions of minimal added inocula. To predict events at a higher inoculum size the values were recalculated based on an initial inoculum ( $N_1$ ) of  $10^5$  organisms  $\text{ml}^{-1}$ . Since broth and microcosm conditions can vary due to the differences between homogenous and isolated niche environments (Ka *et al.* 1994), the figures were re-calculated based on a doubling of the actual generation time to allow for these variables. The results of all these calculations are given in Table 4.6. below:

hide tissues due to an aerobic/anaerobic bacterial succession. These predictions, based on the generation times, are therefore consistent with actual events in naturally degraded hides.

The above findings are also relevant in determining optimum temperature for the model. The optimum growth temperature for both *Ps. aeruginosa* and *C. histolyticum* is 37°C but *C. sporogenes* has an optimum temperature between 30°C and 40°C. The predictions of degradation times from the growth curves would therefore be expected to be close to the degradation times of hides stored in high summer temperatures. However, since the pH of EO-hides was found to be approximately 8,6 ( $\bar{x}$  of 20 hides) and some strains of *C. sporogenes* are inhibited at this pH (Sneath 1986) this organism could exhibit prolonged generation times in the model system. To overcome these variables affecting degradation rates, and to achieve degradation in the model system within a set time scale, the inoculum concentration for the system was set at the higher level of  $10^5$  organisms ml<sup>-1</sup>. Similarly, the experimental time scale was set at 8 days in order to ensure that both early and advanced degradative changes would occur during the study period. Sampling was set to occur daily for four days and subsequently at 48 hour intervals to ensure that sufficient samples were obtained without being unmanageable.

#### 4.4.2.b. Factors affecting bacterial growth:

##### Effect of temperature:

EO treated hide pieces (3cm in diameter) were incubated at three different temperatures (25, 30 and 37°C) after the addition of 2ml of a triple-mix inoculum (1:1:1 of *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes*). The inoculum was prepared in saline at ca  $3 \times 10^8$  organisms ml<sup>-1</sup> (as detailed in Appendix B) and further diluted 1/100 in saline to give a final concentration of ca  $3 \times 10^6$  organisms ml<sup>-1</sup>. Incubation was for 24 hours. Hide shrinkage (T<sub>s</sub>) values as well as mass gain/loss of EO-hides after incubation were used as degradation criteria and the results are shown in Table 4.7. below:

Table 4.7. Effects of Temperature on Bacterial Growth in EO Exposed Hides after 24h Incubation

Temperature °C	Heat Shrinkage °C	Mass gain/loss (gms)
25	63	+0.50
30	61	+0.60
37	63	+0.60
Control *	66	0.00

\* Non-inoculated at 20°C

These results indicate that minimal bacterial activity, in terms of degradation took place in any of the three systems. The results also indicated that variations in incubation temperature between 25°C and 37°C did not significantly affect the test results. This could be attributed to the lack of additional nutrients to the system as well as the short incubation time.

#### Effects of nutrients, reductants and temperature:

Since little reactivity, and therefore bacterial growth, was present in the initial temperature trials these were repeated with a longer incubation time, as well as with the addition of either nutrients or nutrients plus reductants to the original saline suspension of the inocula. This was achieved by preparing the triple-mix 1:1:1 inoculum of all three bacteria at ca  $3 \times 10^6$  organisms  $\text{ml}^{-1}$  in saline as detailed above. To determine the effects of nutrients on degradative rates, 10 ml of Nutrient broth was added to 90 ml of this inoculum to give a final concentration of ca  $3 \times 10^5$  organisms  $\text{ml}^{-1}$  in 10% Nutrient broth. To assess the effect of reductants and nutrients the inoculum was prepared exactly as for the 10% Nutrient broth inoculum but with the addition of 0,5g of L-cystine and 0,5g of sodium thioglycollate per 100 ml inoculum suspension. 5 ml of one or the other of these solutions was added to individual 3cm diameter hide pieces followed by incubation at 37°C. These trials involved a six day incubation period with organoleptic and hide shrinkage tests being performed on days 1 and 6 for comparative purposes.

The results (Table 4.8.) clearly demonstrate the positive effects of nutrient addition to the system. Although these nutrients were minimal (a 1/10 dilution of nutrient broth in the inocula saline suspensions) they actively promoted bacterial growth as indicated by the changes in hide shrinkage temperature and organoleptic values. This is in agreement with both Anderson

(1949) and Tancous (1961) both of whom found it necessary to add some form of extraneous protein to chemically sterilised hides in order to promote bacterial growth.

**Table 4.8. Effect of Nutrients, Reductants and Temperature on Bacterial Growth in EO Exposed Hides Incubated for 6 days at (1) 25°C, (2) 30°C and (3) 37°C.**

Temp. °C	Saline Additions	Days Incubation							
		1				6			
		HS	H	M	A	HS	H	M	A
25	N	59.0	-	+	+/-	56.0	++	++	-
	N + R	57.0	-	+/-	+/-	55.0	+/-	+	+++*
30	N	56.0	-	+	+/-	54.5	+++	++	+++*
	N + R	62.0	-	+	+/-	55.0	+++	++	+*
37	N	57.0	-	+/-	+/-	56	++	+++	+++*
	N + R	60.0	-	+	+/-	56.0	++	+++	+++*
Control (No bacteria)	N	61.0	-	-	-	60.0	-	-	-
	N + R	60.5	-	-	-	60.0	-	-	-

HS = heat shrinkage temperature in °C

N = 10% nutrient broth

H = hair slip

R = 0.5% L-cystine + 0.5% Na.thioglycollate

M = malodour

A = appearance

+/- = slight; + = some; ++ = moderate; +++ = heavy; \* = black discoloration

The effects of reductants in the system were negligible and less degradation occurred as judged by hide shrinkage  $T_m$  in this series at both 30 and 37°C than in the nutrient addition series. The organoleptic results showed that malodour and gelatinisation were more pronounced at 37°C than at either 25° or 30°C. This was attributed to optimal activity of both *Ps. aeruginosa* and *C. histolyticum* at this temperature. Blackening of the hide samples was ascribed to hydrogen sulphide production from *C. sporogenes*.

Degradative effects, based on heat shrinkage value at day 6, were marginally greater at 30°C therefore 30°C was set as the incubation temperature for the model system. This choice was confirmed by the fact that after 6 days the triple-mix inoculated hide pieces, while degraded, were still suitable as test

samples. This indicated that the selection of an 8-day incubation period for the model system would be appropriate to the elucidation of progressive deteriorative changes.

#### Proteolytic activity:

EO-treated 3 cm diameter hide plugs inoculated with individual pure cultures of *Ps. aeruginosa*, *C. histolyticum* or *C. sporogenes* respectively were held in the model system at 30°C for 8 days. Hide extrudates were taken daily and examined for lysis on collagen, casein and gelatin gels. (Chapter 2 and Appendix B). *C. histolyticum* was the only organism to show proteolytic activity against all three substrates. Although casein and gelatin degrading activity was evident from the first day of incubation, collagenase was not evident until day 2 (Table 4.9.). *Ps. aeruginosa* was much less active, producing small amounts of caseinase and gelatinase, these only appearing after 8 days of incubation. Collagenase was absent throughout. Similarly *C. sporogenes* did not produce any collagenase although caseinolytic and gelatinolytic activity was detected after 4 days of incubation.

These enzyme profiles are consistent with the literature on the individual bacteria as well as the projected role of each species in protein degradation (Hill 1981; Palleroni 1981, 1986; Cato et al. 1986; Banwart 1987; Mandelstam et al. 1975). Both collagenolytic and gelatinolytic activity has been found in extrudates from raw hides allowed to decay at 35°C (Hockey and Russell 1982). These enzymes were detected after only one day's incubation indicating that the findings in this model degradation system therefore compare closely with a natural system in reflecting in-situ hide degradation events.

Table 4.9. Proteolytic Activity of Exudates from Hides after Inoculation with *Ps. aeruginosa*, *C. histolyticum* and *C. sporogenes* and Incubation for 8 days.

Organism	Days	Lysis in Gels		
		Casein	Collagen	Gelatin
<i>Ps. aeruginosa</i>	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	6	-	-	-
	8	+	-	+
<i>C. histolyticum</i>	1	+++	-	++
	2	++	+++	+++
	3	+++	+++	+++
	4	+++	+++	+++
	6	+++	+++	+++
	8	+++	+++	+++
<i>C. sporogenes</i>	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	++	-	++
	6	+++	-	+++
	8	+++	-	+++

+ = weak; ++ = moderate; +++ = heavy lysis

#### 4.5. Conclusion:

It was demonstrated that ethylene oxide exposure was satisfactory as a means of sterilising small hide pieces under the conditions described, providing that the samples received a pre-wash/sonication treatment prior to EO exposure. Degradative effects were not detected in the basic hide collagen fibres after EO exposure as determined by heat shrinkage, optical activity and enzyme exposure tests. Also, autolytic enzymes were shown to be absent up to 8 days post treatment and histological analysis of samples stored for 3 months at 20°C and 30°C showed no evidence of degradation. Therefore ethylene oxide treated hide pieces (3 cm in diameter) contained in small plastic capped jars were shown to be a good base for a model dosing system in selected microbial hide degradation studies.

Putrefaction of EO-exposed hides by the selected inocula occurred in the model system at varying times of incubation (storage), this being related to both the inoculum type and concentration as well as the individual generation times of the bacteria. Nutrient addition to the system was shown to be essential for initiation of early growth but the addition of reductants was not necessary to accelerate the process. Incubation temperatures of 30-37°C were near optimal for the growth of mixed inocula with degradative changes in hide pieces clearly evident after 6 days incubation with a 1:1:1 mix of all three bacteria. The suitability of the selected inocula was confirmed by the detection of weak caseinolytic and gelatinolytic activity in *Ps. aeruginosa* strong caseinolytic, gelatinolytic and collagenolytic activity in *C. histolyticum* and moderate caseinolytic and gelatinolytic activity in *C. sporogenes*.

It was concluded that the model system, as described here, was appropriate for in-situ hide degradation studies and would to a high degree represent the main degradative effects occurring in naturally putrefying hides.

## CHAPTER 5

### THE ROLE OF BACTERIAL SUCCESSION IN HIDE DEGRADATION AS DETERMINED IN A MODEL SYSTEM

- 5.1. Summary
- 5.2. Introduction
- 5.3. Research Objectives
- 5.4. Results and Discussion
  - 5.4.1. Organoleptic Assessment
  - 5.4.2. pH Change
  - 5.4.3. Mass Loss/gain and Fluid Volume Increase/Decrease
  - 5.4.4. Bacterial Counts
  - 5.4.5. Ammonia Release
  - 5.4.6. Hydroxyproline Release
  - 5.4.7. Proteolytic Activity
  - 5.4.8. Stereo-microscopy of the Grain Surface
  - 5.4.9. Histological Appearance.
- 5.5. General Discussion
- 5.6. Conclusion

### 5.1. Summary:

The model system developed in the previous chapter was used to study the effects of the selected inocula on hide degradation in-situ under controlled conditions. A number of parameters were used to assess the degradative changes that took place during an 8 day storage period at 30°C. The findings of this study were that, although in mixed cultures of the organisms bacterial succession was evident, the greatest degradation that took place was in the hides exposed to *C. histolyticum* as an individual culture. This suggests that bacterial succession is incidental to, and not essential to degradation in hides and that redox conditions and substrate composition play an essential role in the degradative process. Antagonistic reactions were found in the mixture of *C. histolyticum* with *C. sporogenes* and this could possibly be exploited as a means of hide bio-preservation.

The individual parameters used to assess the degradative changes occurring in the hides during storage were shown to be variable in their predictive value of actual events. Organoleptic, pH and ammonia values appeared to be the least representative of hide putrefaction, while hydroxyproline and enzyme activity were among the more reliable parameters. Histological analyses coupled with mass loss and fluid volume increase, together with aerobic and anaerobic bacterial counts of the individual organisms, also gave a comprehensive picture of events in-situ.

The model system developed in this thesis proved to be a reliable means of studying hide degradation in-situ and the results supported the thesis hypothesis of bacterial interaction in hide degradation. Bacterial penetration and proliferation in hides were shown to be dependent upon the type and mixture of organisms present in the system.

### 5.2. Introduction:

The role of specific bacteria as the causative agents of specific infectious diseases was the basis of the formulation of Koch's postulates (Isenberg and Balows 1981). The postulates are not confined to a medical context only since their basic premise holds good for degradative processes in general i.e. to find bacteria in the diseased/degraded material; to isolate and maintain them in pure laboratory culture; to reproduce and monitor the disease/degradative process in a secondary (similar) substrate and to recover the organisms from the diseased/degraded sites within that substrate.

Previous studies in hide degradation have occasionally involved these postulates as their basis but these studies are few in number (McLaughlin and Rockwell 1922; Anderson 1949; Everitt and Cordon 1955; Tancous 1961; Formisano 1965; Kallenburger and Lollar 1986). Of these only McLaughlin and Rockwell worked with raw hides, the other studies used salted hides as their source material.

This deficiency in classic microbiological studies of raw hide degradation amplifies the need to establish a system in which the role of specific proteolytic and putrefactive aerobic and anaerobic bacteria can be studied in-situ. Such studies are also required to establish both the bacterial types involved as well as the areas within hide which they preferentially attack. A greater understanding of these factors will benefit studies on hide preservation since these fundamental aspects of degradation are essential to the generation of better preservation techniques targeted at specific bacterial types. To achieve this the model system of this thesis was generated and the previous chapters have dealt with the establishment of the system and confirmation of its validity for use in raw hide studies. Probably the most important factor in establishing the model was the chosen method for sterilisation of hide pieces without causing denaturation and this was dealt with extensively in Chapter 4. Parameters governing storage incubation temperature and time were established and the second most important parameter, choice of inocula, was investigated in detail (Chapters 1, 3 and 4). The inocula used in this study were represented by type cultures from established culture collections in order that the results produced here could be reproduced by other interested persons elsewhere. The inocula were as follows:

<i>Ps. aeruginosa</i>	NCTC	10662
<i>C. histolyticum</i>	ATCC	1940
<i>C. sporogenes</i>	ATCC	3584

Ten different parameters were chosen to determine in-situ degradative effects and these, together with an outline of the model, are shown schematically in Fig. 5.1 below:

<b>BACTERIA</b>	<ol style="list-style-type: none"> <li>1. Aerobic <i>Ps. aeruginosa</i></li> <li>2. Micro-aerophilic <i>C. histolyticum</i></li> <li>3. Anaerobic <i>C. perfringens</i></li> </ol>
<b>HIDE</b>	Green (raw) hide pieces (3 cm diameter) sterilised by ethylene oxide
<b>SYSTEM</b>	Plastic container (40 ml volume) enclosing a hide piece with either the individual, double-mix or triple-mix of the bacteria prepared as inocula at ca $1 \times 10^5 \text{ ml}^{-1}$ in 5 ml saline containing 1/10 volume of nutrient broth.
<b>TEST</b>	1. Organoleptic (hairslip, malodour and appearance).
<b>PARAMETERS</b>	<ol style="list-style-type: none"> <li>2. pH.</li> <li>3. Mass gain/loss and fluid increase/decrease..</li> <li>4. Total bacterial count.</li> <li>5. Ammonia release.</li> <li>6. Hydroxyproline release.</li> <li>7. Proteolytic activity.</li> <li>8. Grain surface appearance after lime depilation.</li> <li>9. Histological examination of tissues (pre and post lime depilation (Masson and Gram stains).</li> </ol>

Fig. 5.1 Schematic Representation of the Model System

The tests chosen to assess denaturation were based on previous studies involving protein breakdown. Organoleptic assessments were included to confirm the findings of Chapter 3, that they did not necessarily reflect putrefactive changes in-situ. As discussed previously, bacterial counts of decaying hides have shown that numbers per se do not necessarily reflect degradation. For this study specific media to count the bacteria were used and both external and internal surface counts performed. To further differentiate sites of colonisation during storage the hides were divided into hair and flesh surfaces by sectioning horizontally and counting extrudates from each of the two pieces so produced.

Mass loss and extrudate fluid volume increase have been used as a simple, but effective, means of assessing protein degradation (Jennison 1947; Anderson 1949). Shifts towards alkaline pH indicate ammonia due to amide hydrolysis, which together with increase nitrogen levels are said to indicate non-specific protein loss (Heidemann 1979; Lawrie 1985). In 1942 Somer used nitrogen release as a measure of deterioration and gave 10% volatile nitrogen as a definite indicator of hide decay.

In contrast to ammonia and nitrogen, hydroxyproline is almost totally specific for collagen so that this parameter is considered a reliable indicator of collagen hydrolysis, especially for bacterially damaged collagen (Venkatesen 1979). Everitt and Cordon (1955) showed that liming caused an increase in hydroxyproline release of up to 14 times that of the unlimed sample, making this a useful parameter for degradation in both raw and limed hide studies.

Detection of proteolytic enzymes, both in-situ and in laboratory experiments, has been used to study prokaryotic and eukaryotic hide deterioration (Welton *et al.* 1972; Keil 1979; Lecroissey and Keil 1985; Bowles *et al.* 1988; Brüning 1991). The detection of general proteolytic activity, especially in the papillary dermis, is essential to elucidating the causes of sueded grain, while collagenolytic attack of the underlying reticular dermis needs to be correlated to other degradative parameters (Pfleiderer 1980; Tancous 1984).

The value of histological evidence in conjunction with microbiological and biochemical assessments of degradative processes as was clearly demonstrated by Robb-Smith in 1945. He demonstrated that toxic bacterial proteases (isolated by MacFarlane and MacLennon in 1945 from bacteria from gangrenous muscle tissue) caused specific attack on collagen fibres surrounding muscle tissue, but not on the muscle itself, when injected into rabbits. This was the first complete proof that a specific protease, collagenase, was produced by certain bacteria and was responsible for pathological changes in-vivo.

Histological examination of poly-microbial infected tissue was advocated by Roberts in 1969 as a means of determining the main infectious agent. Flint and Lyons (1975) used Masson stained sections to detect changes in human skin due to burns. They found that changes in

collagen at the molecular level were visualised as changes in staining reaction and these correlated with more sophisticated physio-chemical techniques. Evidence of bacterial degradation of hides has been used by Haines (1983)(b) in conjunction with biocide studies.

Great advances in histological techniques have improved the effective use of histology as a scientific tool (James and Tas 1984; Pearse 1985). New methods for collagen visualisation and location, as well as the use of polarising microscopy for protein degradation studies of a variety of tissues and fibres, have enhanced the value of histological diagnosis (Constantine and Mowry 1968; Wolman 1975; Fatou 1978; Von der Mark 1981; Kalsmark *et al.* 1988; Whittaker *et al.* 1989; Pickering and Boughner 1990). Histological appearance was used in this present study, both for detection of tissue damage during storage, and for comparison of general hide integrity in both raw and limed/delimed samples.

### 5.3. Research Objectives:

- a. To determine if hide putrefaction is a direct result of bacterial succession involving strictly aerobic primary colonisers, micro-aerophilic collagenase producing *Clostridium spp.* and strictly anaerobic, proteolytic but non-collagenase producing *Clostridium spp.*
- b. To investigate specific and non-specific attack on hide proteins with respect to bacterial type and to visualise the main routes by which denaturation occurs.
- c. To establish whether degradation of the upper papillary hide surfaces is the result of collagenolytic bacterial action solely or whether general proteolytic attack by bacteria is also involved. Similarly, to establish whether a combination of bacterial action (pre-beamhouse processing) and chemical application (during liming and de-liming) is a secondary cause of sueded or damaged grain.
- d. To establish the most effective methods of assessing deteriorative changes of significance during in-situ hide degradation studies.

### 5.4. Results and Discussion:

The model system was set up as shown in Fig. 5.1 using sixty 3 cm diameter EO-treated hide plugs per inoculum type. The individual inocula were prepared from stock cultures at  $10^8$  organisms  $\text{ml}^{-1}$  in saline as detailed in Appendix B. 10 ml of these solutions were further

diluted to 90 ml of saline to give a bacterial concentration of  $10^7$  ml<sup>-1</sup>. These suspensions were well mixed and 1 ml added to 99 ml of saline containing 10% Nutrient broth (prepared as 90 ml saline to 10 ml Nutrient broth). The final bacterial concentration for each individual bacterial inoculum was therefore ca  $1 \times 10^5$  organisms ml<sup>-1</sup>. Double-mix inocula were prepared in the same way except 1 ml of each of two inocula was added to 98 ml of saline containing 10% Nutrient broth to give a final concentration of ca  $2 \times 10^5$  organisms ml<sup>-1</sup>. The triple-mix inoculum was prepared as above from all three organisms using 1 ml of each to 97 ml of saline containing 10% Nutrient broth to give a final inoculum concentration of ca  $3 \times 10^5$  organisms ml<sup>-1</sup>.

5 ml of each inoculum so prepared was added to individual 3 cm diameter EO-treated hide pieces. Total counts were prepared from each inocula to generate actual bacterial concentrations at time of inoculation. These results were as follows:

<i>Ps. aeruginosa</i>	$1,1 \times 10^5$ ml <sup>-1</sup>
<i>C. histolyticum</i>	$1,0 \times 10^5$ ml <sup>-1</sup>
<i>C. sporogenes</i>	$7,1 \times 10^4$ ml <sup>-1</sup>
<i>Ps. aeruginosa/C. histolyticum</i>	$1,7 \times 10^5$ ml <sup>-1</sup> (as <i>Ps. aeruginosa</i> ) and $1,2 \times 10^5$ ml <sup>-1</sup> (as <i>C. histolyticum</i> )
<i>Ps. aeruginosa/C. sporogenes</i>	$2,0 \times 10^5$ ml <sup>-1</sup> (as <i>Ps. aeruginosa</i> ) and $7,2 \times 10^4$ ml <sup>-1</sup> (as <i>C. sporogenes</i> )
<i>C. histolyticum/C. sporogenes</i>	$1,2 \times 10^5$ ml <sup>-1</sup> (as <i>C. histolyticum</i> / <i>C. sporogenes</i> mixed count)
<i>Ps. aeruginosa/C. histolyticum/C. sporogenes</i>	$1,1 \times 10^5$ ml <sup>-1</sup> (as <i>Ps. aeruginosa</i> ) and $2,1 \times 10^5$ ml <sup>-1</sup> (as <i>C. histolyticum</i> / <i>C. sporogenes</i> mixed count)

One set of hides remained uninoculated as a control series. Thus eight different inoculum systems were set up as follows:

- i. *Ps. aeruginosa*
- ii. *C. histolyticum*
- iii. *C. sporogenes*

- iv. *Ps. aeruginosa* + *C. histolyticum*
- v. *Ps. aeruginosa* + *C. sporogenes*
- vi. *C. histolyticum* + *C. sporogenes*
- vii. *Ps. aeruginosa* + *C. histolyticum* + *C. sporogenes*
- viii. Uninoculated control.

The inoculated hides were stored at 30°C for 8 days and ten parameters were used for the detection of degradative changes in-situ (see Fig 5.1 for test descriptions and Appendix B for details of test methods) Tests were performed on days 1, 2, 3, 4, 6, and 8 and the results of the organoleptic, physical, microbiological and biochemical parameters are depicted graphically. To make logical interpretation easier, the graphs have been divided into two sets, the first comprising the results of the aerobic and aerobic/anaerobic mixed inocula systems. The second set consists of anaerobic inocula only and includes the control (uninoculated) system. The findings for each series of graphs are summarised after the graphs and an overall summary of the results for each inoculum type is included at the end of all the results.

The data for the histological studies was fairly extensive as each sample was stained by Masson and Gram stain (Appendices A and B) and was examined with respect to the following aspects:

1. Epidermis (cornified layer; granular layer);
2. Hair (fibre and follicle);
3. Sebaceous glands and other non-fibrous structures;
4. Dermis (papillary fibres and reticular fibres (at upper, mid and lower corium) and cellular nuclei).

The results of the findings for both the raw and limed/delimed samples were summarised and these combined summaries are given covering the eight day storage period for each inocula type.

The combined graphical and histological data were used to generate overall conclusions concerning bacterial action in hide degradation and these are given at the end of this chapter.

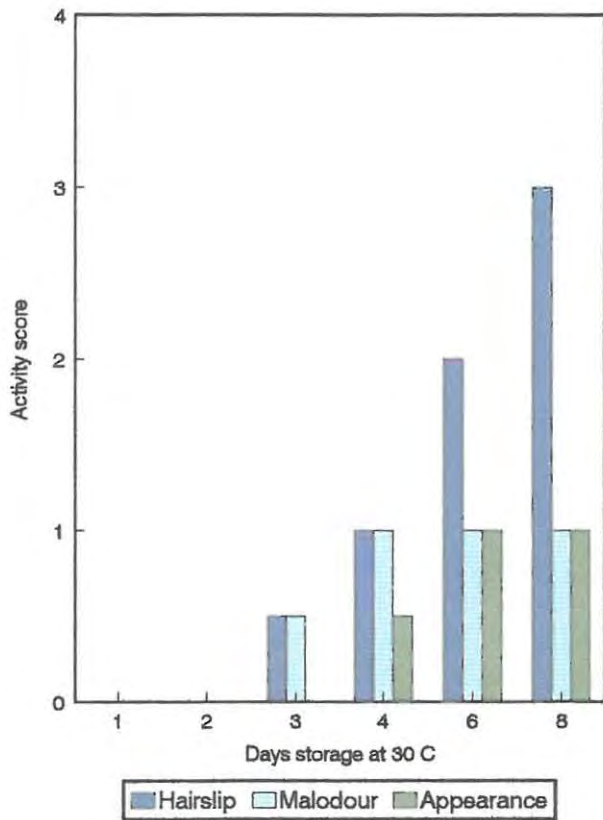
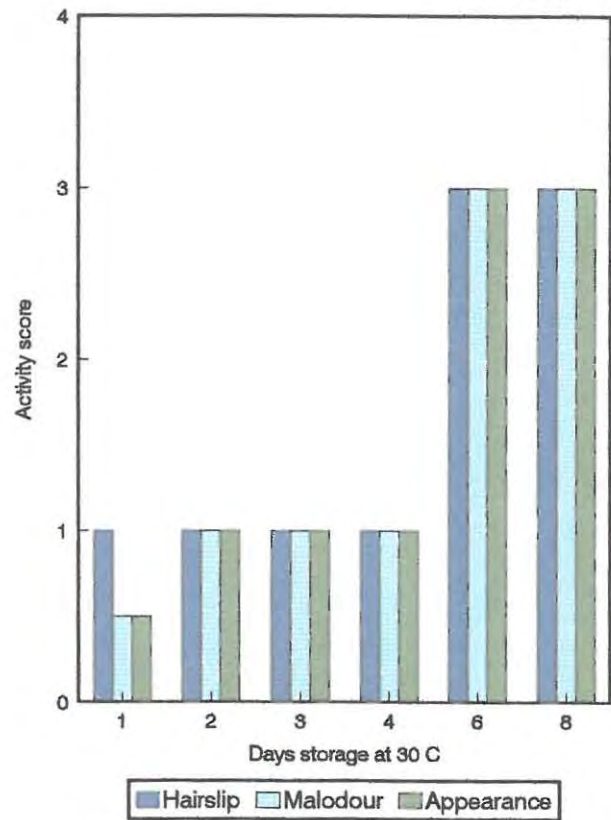
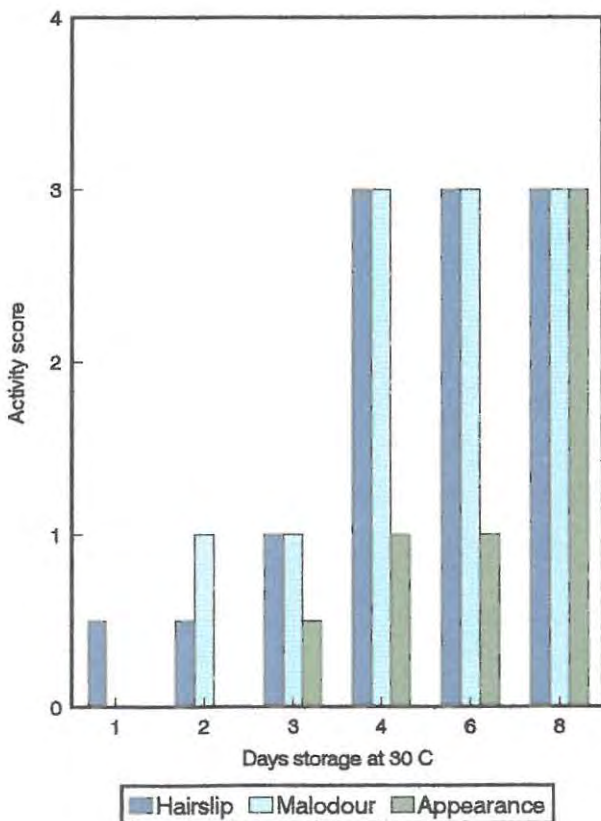
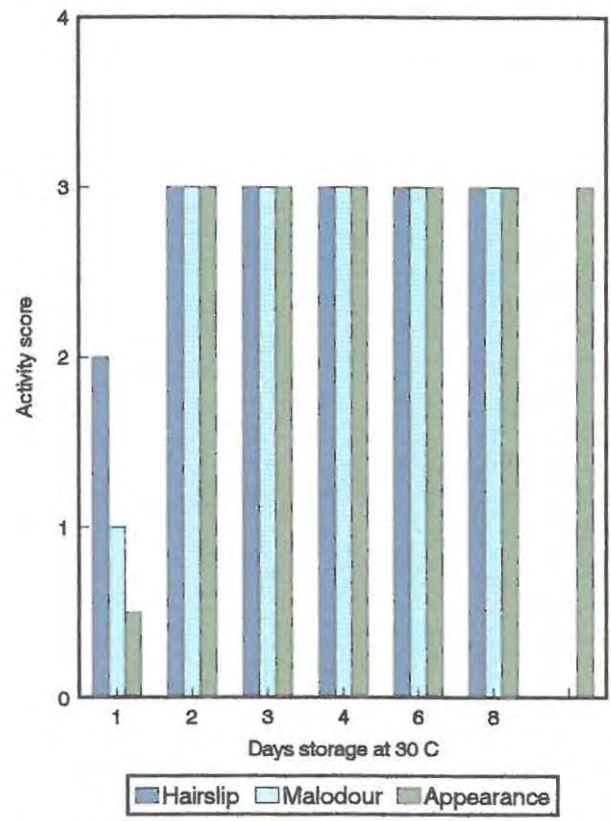
Details of all the methods used and the calculation of results (where applicable) are given in Appendix B.

The individual test results are as follows:

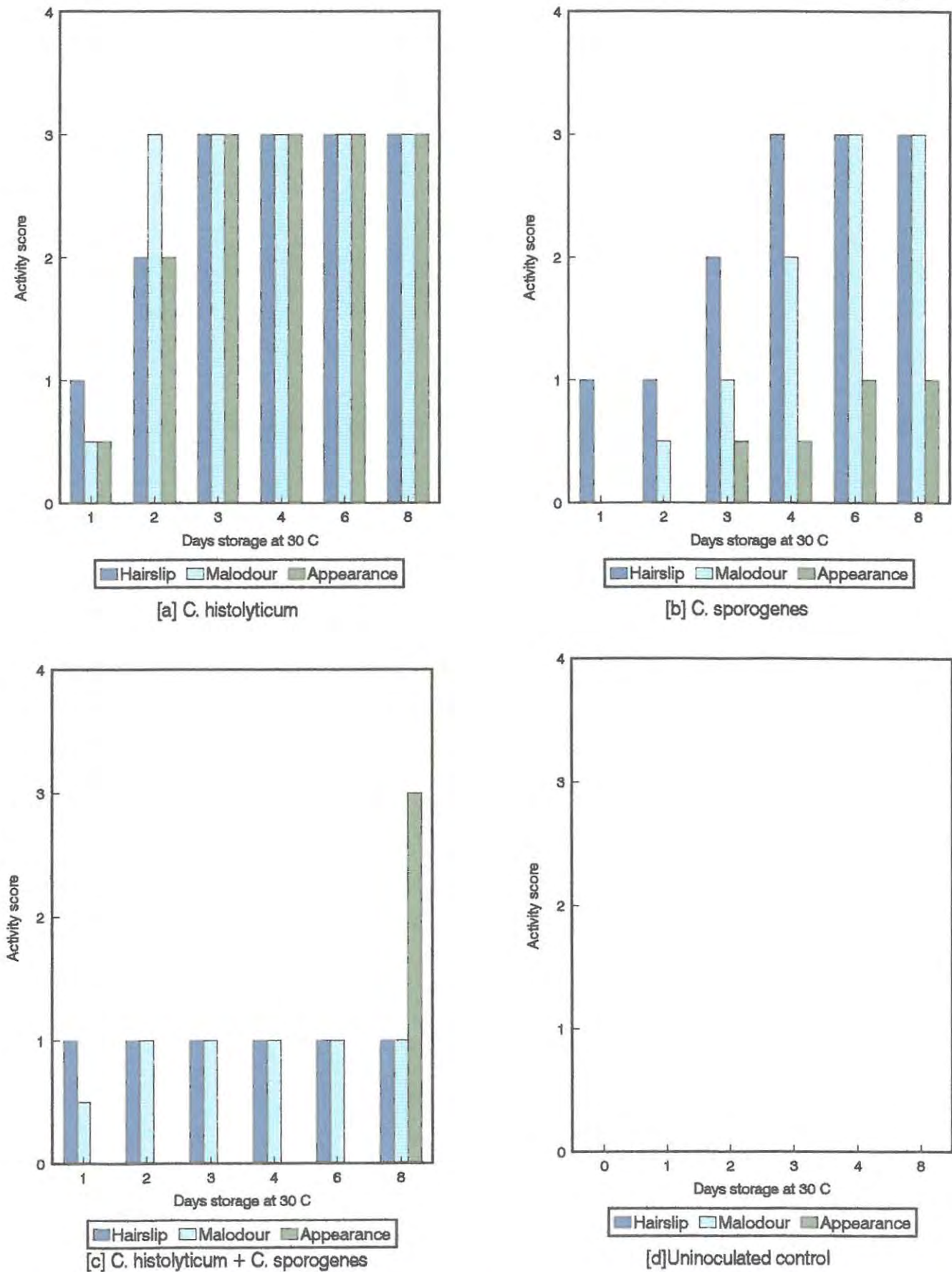
#### 5.4.1. Organoleptic assessment:

Assessments of the degree of hairslip, malodour and appearance are depicted graphically in Figs. 5.2 and 5.3. In the first data set, hides dosed with *Ps. aeruginosa* showed little change in any parameter until day 8 when hairslip was well advanced, but the other two parameters remained near normal. Hides dosed with a double-mix of *Ps. aeruginosa/C. sporogenes* had advanced hairslip and malodour by day 4 as also occurred with hides dosed only with *C. sporogenes*. Hides dosed with the double-mix of *Ps. aeruginosa/C. histolyticum* only showed advanced levels of all three parameters at day 6. This was in contrast to the *C. histolyticum*-dosed hides, in which advanced levels of all three parameters were evident by day 3, indicating some suppression of the *C. histolyticum* activity in the presence of *Ps. aeruginosa*. In the second data set (Fig. 5.3) hide pieces inoculated with the double-mix of *C. sporogenes/C. histolyticum* showed strong evidence for bacterial suppression. This was shown by the lack of changes that occurred in all three parameters during the first four days storage, compared to the changes that took place in the individual cultures of each organism, as well as their activity in the triple-mix inoculum containing all three organisms. These latter hides showed the greatest rate of change in all three parameters, indicating possible bacterial succession or synergism.

Detectable odour may be a late indicator of protein putrefaction since Banwart (1987) found levels of  $10^8$  *C. perfringens*  $g^{-1}$  in meat in which proteolysis was present but detectable odour was absent. When the present data was compared to other, more specific, test results (mass loss, hydroxyproline etc. Figs. 5.6, 5.7, 5.12 and 5.13) there did not appear to be a correlation. In fact in many instances changes in either one or more of the organoleptic parameters occurred well in advance of a more specific test parameter and no specific pattern of results emerged with the organisms tested.

[a] *Ps. aeruginosa*[b] *Ps. aeruginosa* + *C. histolyticum*[c] *Ps. aeruginosa* + *C. sporogenes*[d] *Ps. aeruginosa* + *C. histolyticum* + *C. sporogenes*

**Fig. 5.2 Organoleptic Assessments of Hide Pieces During Storage at 30 C :  
[1] After Inoculation with Individual Aerobic bacteria and Mixed  
Aerobic/Anaerobic bacteria**

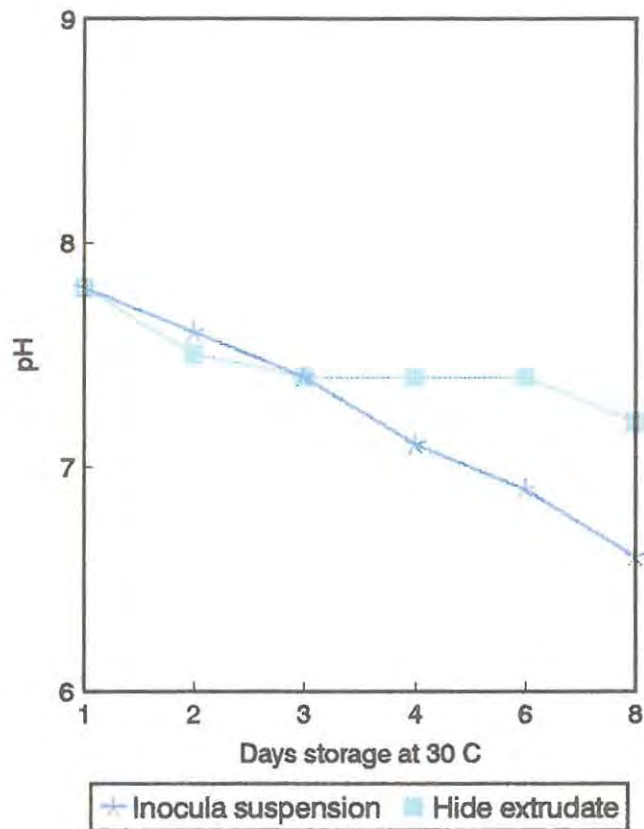
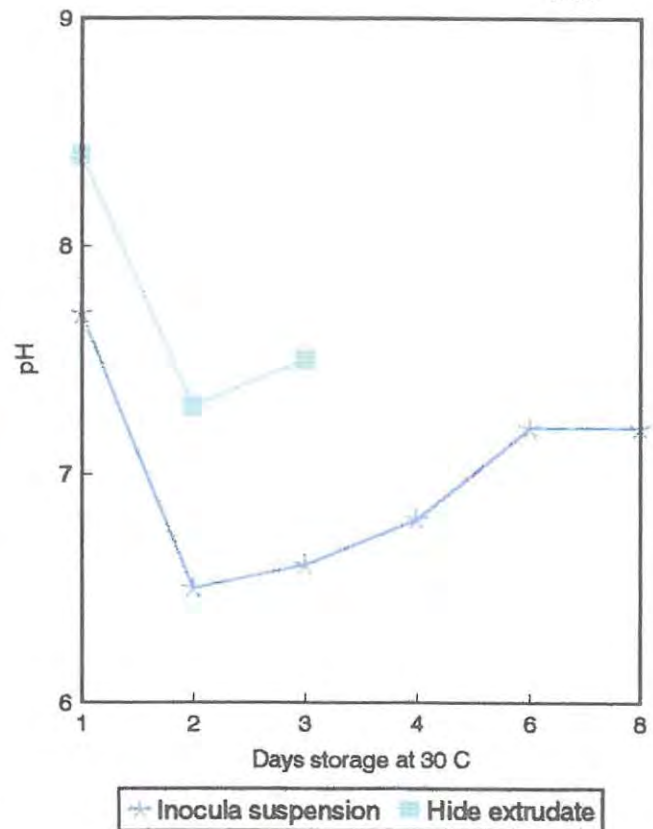
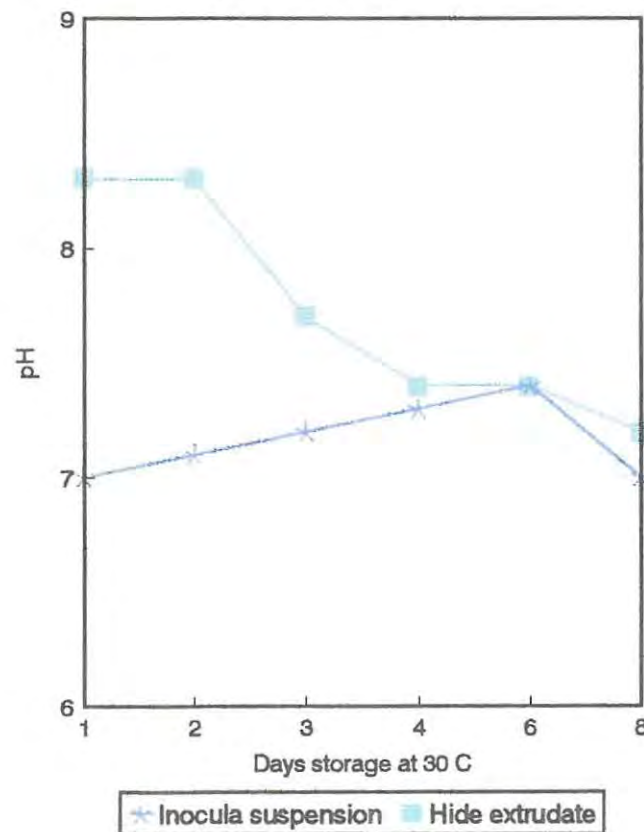
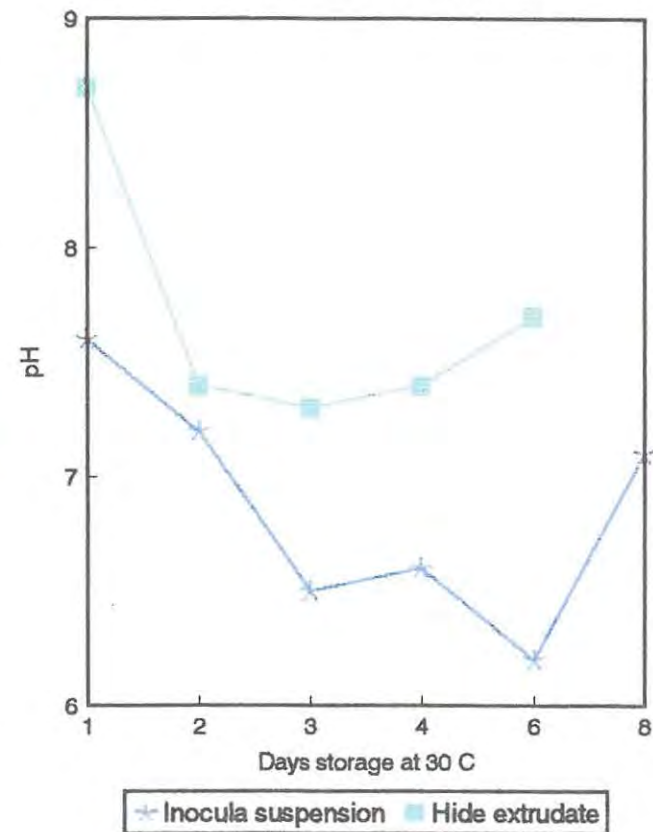


**Fig. 5. 3 Organoleptic Assessments of Hide Pieces During Storage at 30 C : [2] After Inoculation with Anaerobic Bacteria and Uninoculated Control**

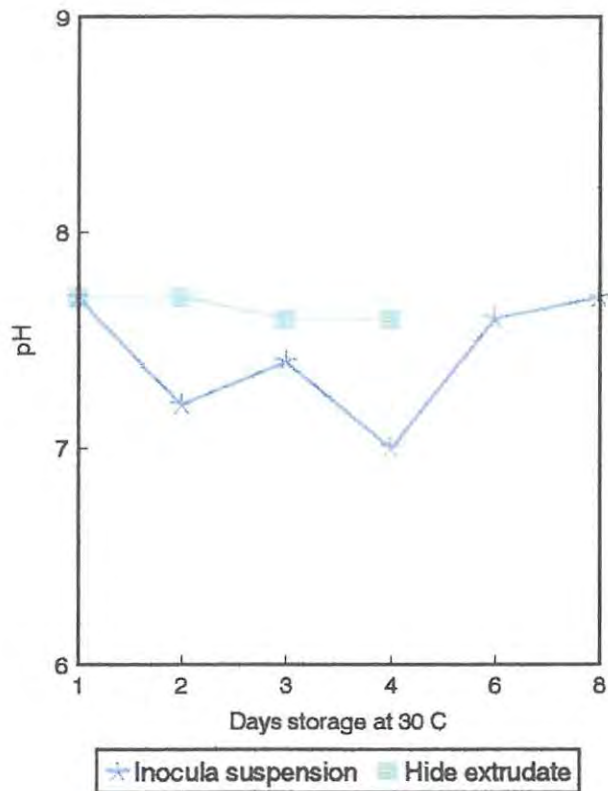
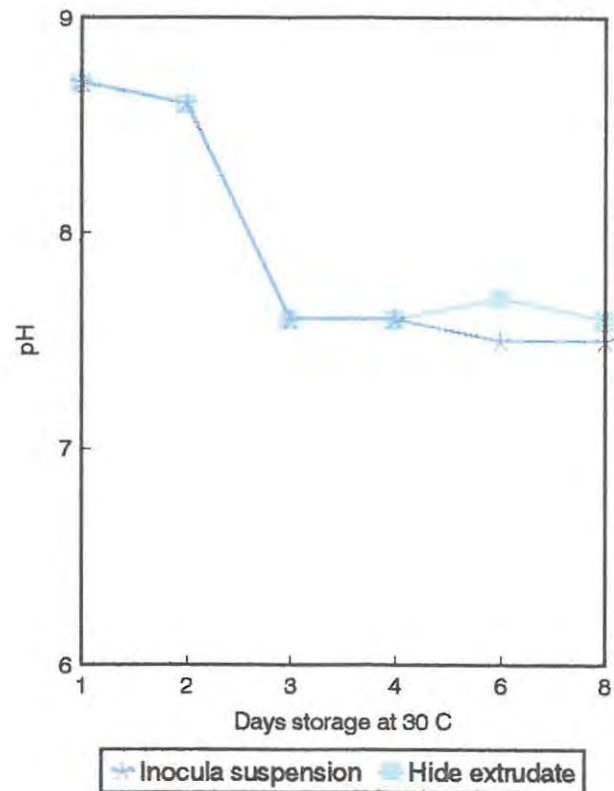
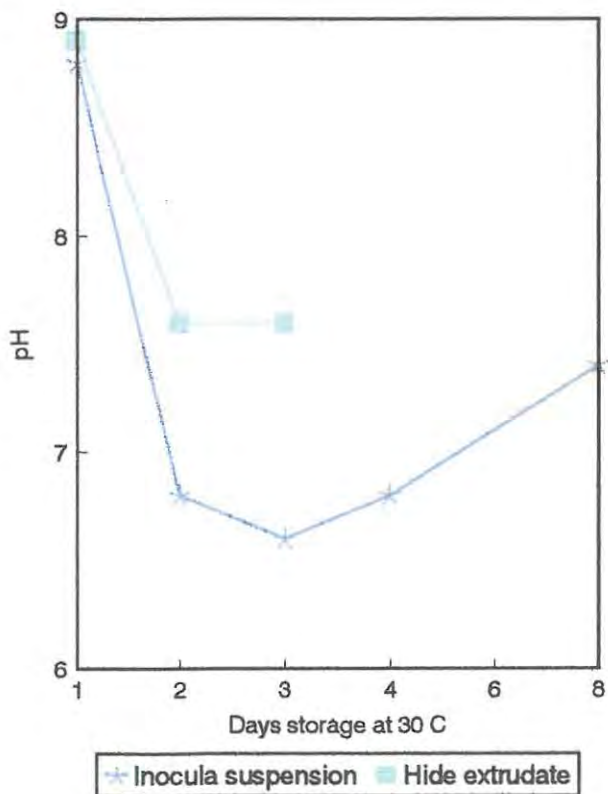
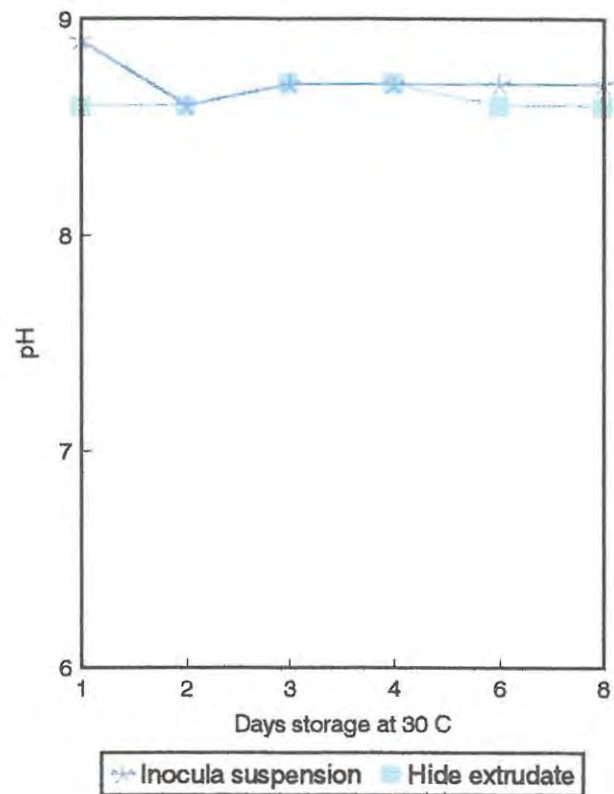
#### 5.4.2. pH:

The pH of post mortem animal tissue normally drops rapidly from that of the initial living tissue pH of 6,8 - 7,6 (Malisoff 1943). In the present study the pH values were variable as shown graphically in Figs. 5.4, 5.5, the high pH values found in the control hides (Fig.5.4(d)) are attributed to alkylation of protein material as a result of EO sterilisation of the hide pieces (Christensen and Kristensen 1982). Despite this effect definite changes occurred in pH values within the tissues. While these levels were higher than, or similar to, those of the inocula suspensions, they generally showed a progressive drop during the first three days of storage to reach levels of pH 7,4 - 7,6 by day 4.

The pH levels of inoculum suspension fluids were more variable. This was especially so in the inocula containing *C. histolyticum*, either individually or as part of a mixed inocula, with the lowest pH level (6,6) being recorded at day 4 in the triple-mix dosed samples. All four *C. histolyticum* containing inocula gave upward shifts at days 6 and 8 with individual *C. histolyticum* inocula giving a higher value (7,7) than the triple-mix (7,1) at day 8. These increased pH levels correlated to peak bacterial counts (Fig. 5.9a) of this organism indicating that early growth was at the expense of carbohydrates and later growth was dependent upon protein catabolism. In contrast, hides inoculated with *Ps. aeruginosa* showed little initial pH change which, in the inoculum suspension, decreased steadily to a final pH of 6,6 at day 8. Tissue pH levels reached only 7,2 at 8 days and these results, in conjunction with increased total counts with time without increased proteolytic activity (Fig. 5.8a, Fig 5.14a), indicate that this organism was probably utilising carbohydrates and free amino acids as energy sources. In contrast the *C. sporogenes* internal and external pH values showed similar trends to each other, with no change up to day 3, and only a slight drop thereafter. This correlates with findings of lack of growth until day 3 (Fig 5.9b) and lack of proteolytic activity until day 4 (Fig. 5.15b). Although, in general, all pH levels at day 2 were in the range between optimum and maximum for all three inocula they were too variable to clearly indicate in-situ events. This was especially apparent in the mixed inocula and it is concluded, therefore, that neither surface, nor internal pH values are a good method for detecting the various stages of protein degradation.

[a] *Ps. aeruginosa*[b] *Ps. aeruginosa + C. histolyticum*[c] *Ps. aeruginosa + C. sporogenes*[d] *Ps. aeruginosa + C. histolyticum + C. sporogenes*

**Fig. 5.4 pH Changes in Inocula Suspensions and Hide Extrudates During Storage at 30 C : [1] After Inoculation with Individual Aerobic Bacteria and Mixed Aerobic/Anaerobic Bacteria**

[a] *C. histolyticum*[b] *C. sporogenes*[c] *C. histolyticum* + *C. sporogenes*

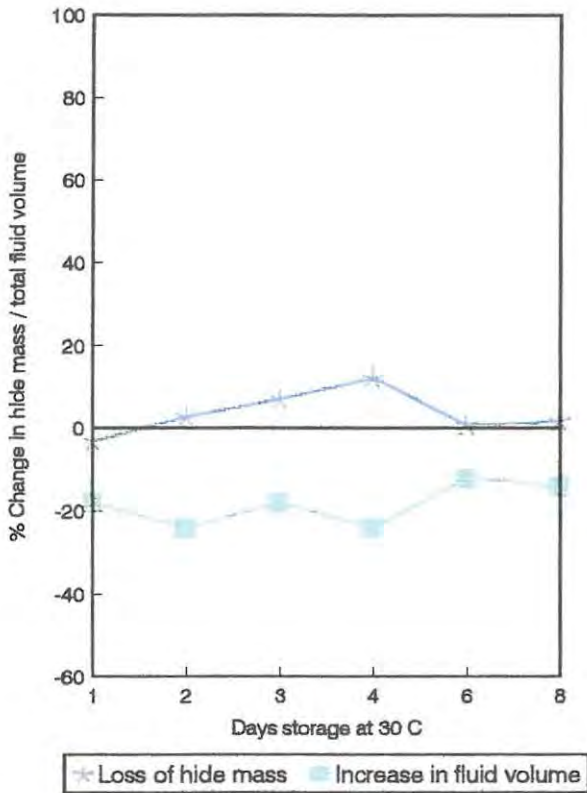
[d] Uninoculated control

**Fig. 5.5 pH Changes in Inocula Suspensions and Hide Extrudates During Storage at 30 C : [2] After Inoculation with Anaerobic Bacteria and Uninoculated control**

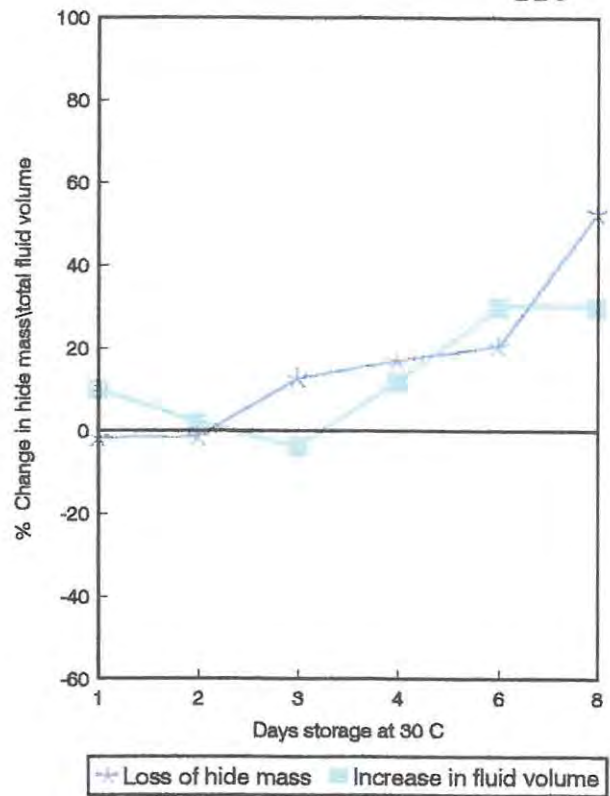
#### 5.4.3. Mass loss/gain and total fluid increase/decrease

These parameters were found to be interlinked and are therefore discussed together. The results from both test series are depicted graphically in Figs. 5.6 and 5.7. The results showed definite changes according to inoculum type. The greatest consistent mass loss and fluid volume increase occurred with the samples dosed with *C. histolyticum*. These findings were reflected in the mixed inoculum dosed hides containing this organism except in the case of the *C. histolyticum/C. sporogenes* double-mix. In this series mass loss and fluid volume increase did not escalate continuously, as for other inocula containing *C. histolyticum*, but remained almost static until day 4. At day 6 both parameters increased somewhat, to reach a maximum at day 8. In fact, mass gain, as opposed to loss, was apparent until day 4 indicating that *C. sporogenes* was successfully either inhibiting or out-competing *C. histolyticum*. Mass gain also occurred with the individual inocula of *C. sporogenes*, and, to a lesser extent, with the *Ps. aeruginosa/C. sporogenes* mix. Mass gain was not evidenced in *Ps. aeruginosa/C. histolyticum* inoculated samples but increase in fluid volumes was higher than in the other mixed cultures containing *Pseudomonas*. Increased mass with decreased volumes, as shown in the *C. sporogenes* inoculated hides was shown to correlate to the differential attack of this organism on hide proteins (as indicated by the histological findings - see 5.4.9) which showed that epidermis, sebaceous glands, hair follicles and interstitial proteins were degraded by these organisms. It is postulated that this loss of epidermal barrier would allow passage of fluids into the reticular dermis resulting in mass gain and fluid volume loss. While mass loss or gain could also be due to tissue shrinkage or swelling the uninoculated control remained at fairly constant mass levels throughout the storage period (Fig.5.7(d)) indicating that the decrease in mass and increase in fluid volumes of the other samples can be attributed to bacterial degradation of hide tissues.

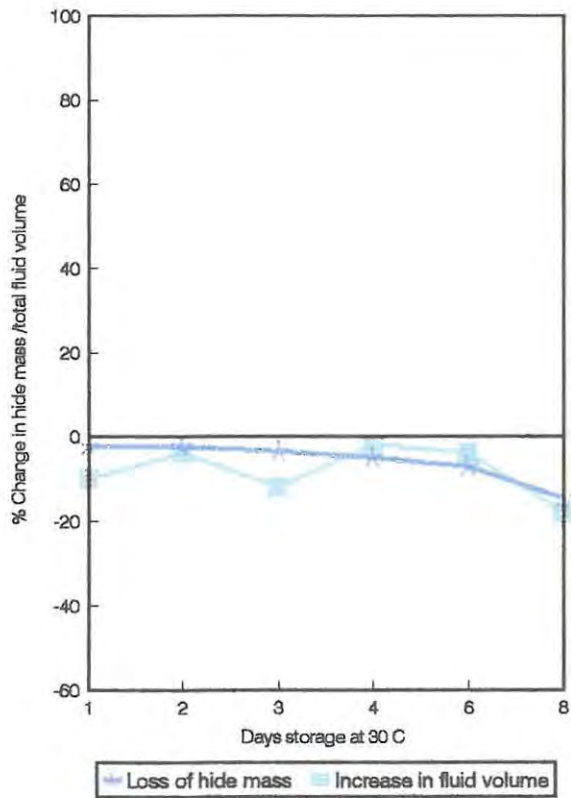
In general the mass gain/loss and fluid volume increase data showed good correlation with levels of ammonia and hydroxyproline release and the appearance of proteolytic enzymes (Figs. 5.10, 5.11, 5.12, 5.13, 5.14 and 5.15) as well as with surface grain damage after liming/delimiting and the general histological findings.



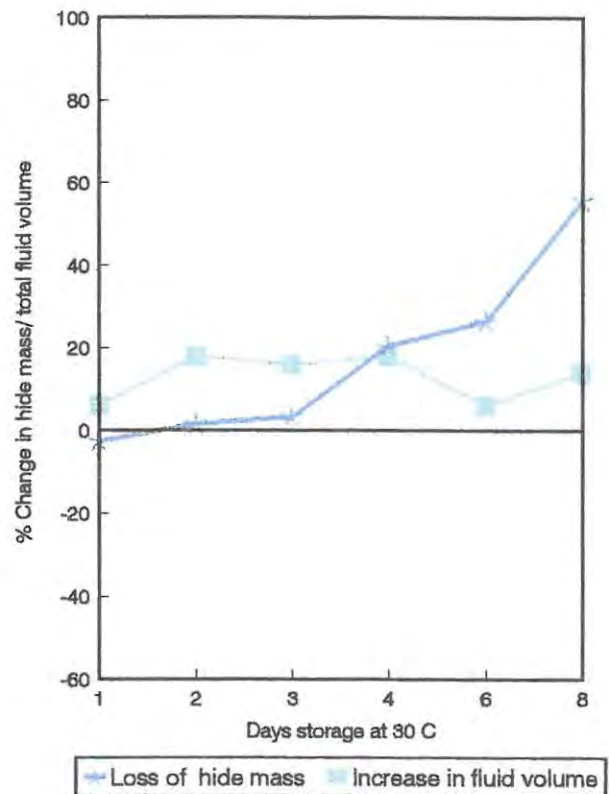
[a] *Ps. aeruginosa*



[b] *Ps. aeruginosa + C. histolyticum*

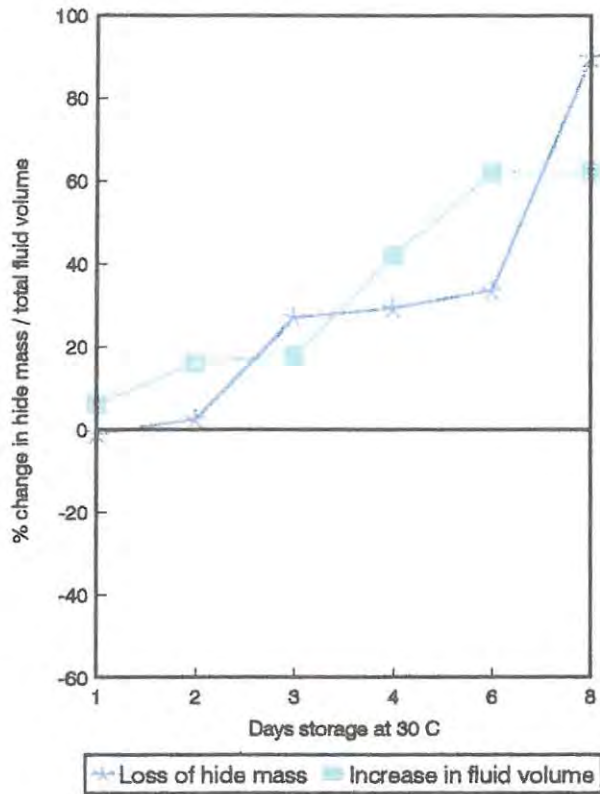
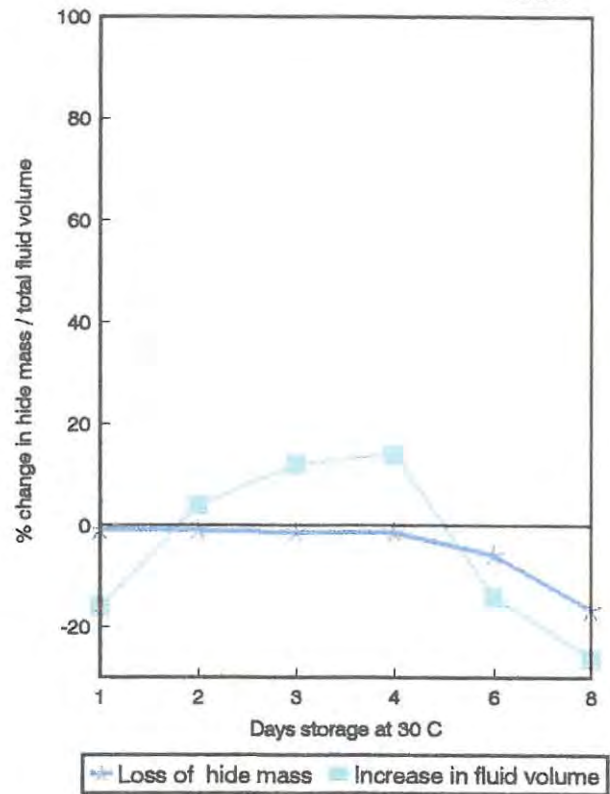
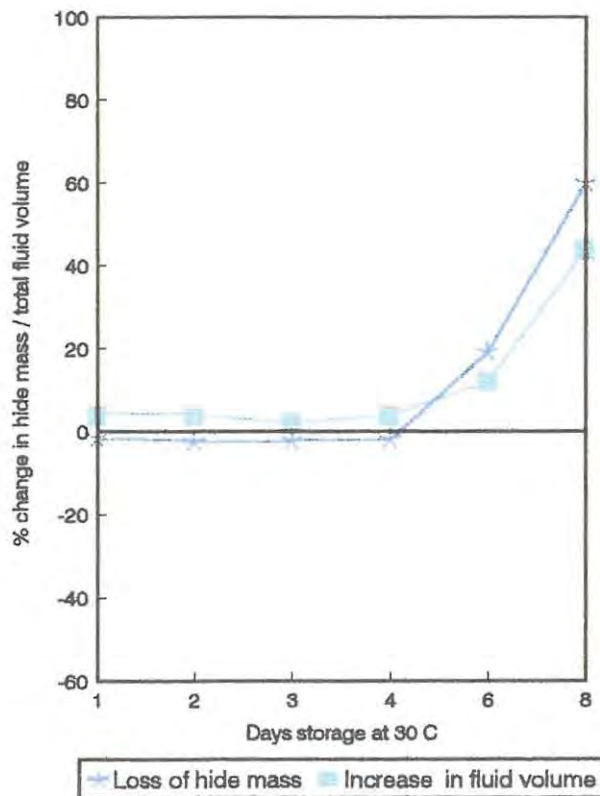
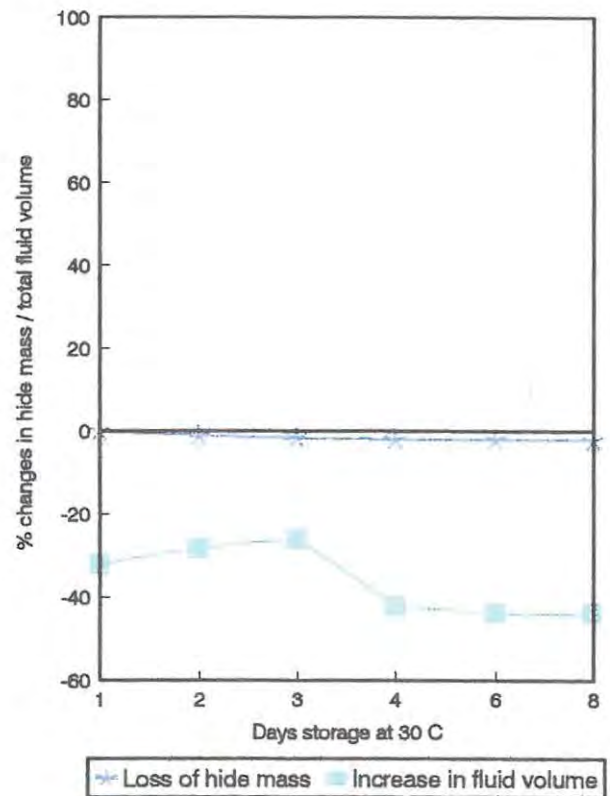


[c] *Ps.aeruginosa + C. sporogenes*



[d] *Ps. aeruginosa + C. histolyticum + C. sporogenes*

**Fig. 5. 6 Changes in Hide Mass and Total Fluid Volumes During Storage at 30 C : [1]After Inoculation with Aerobic and Mixed Aerobic/Anaerobic Bacteria**

[a] *C. histolyticum*[b] *C. sporogenes*[c] *C. histolyticum + C. sporogenes*

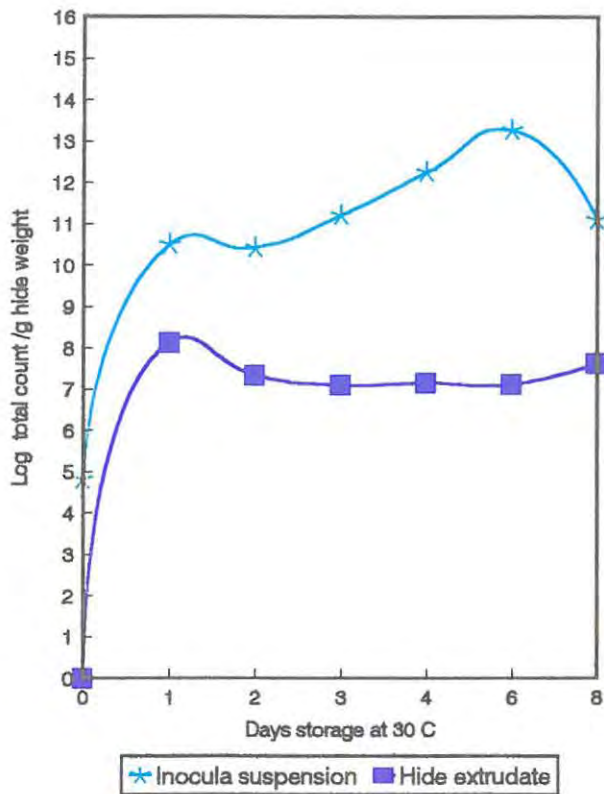
[d] Uninoculated control

**Fig. 5. 7 Changes in Hide Mass and Total Fluid Volumes During Storage at 30 C : [2] After inoculation with Anaerobic Bacteria and Uninoculated control**

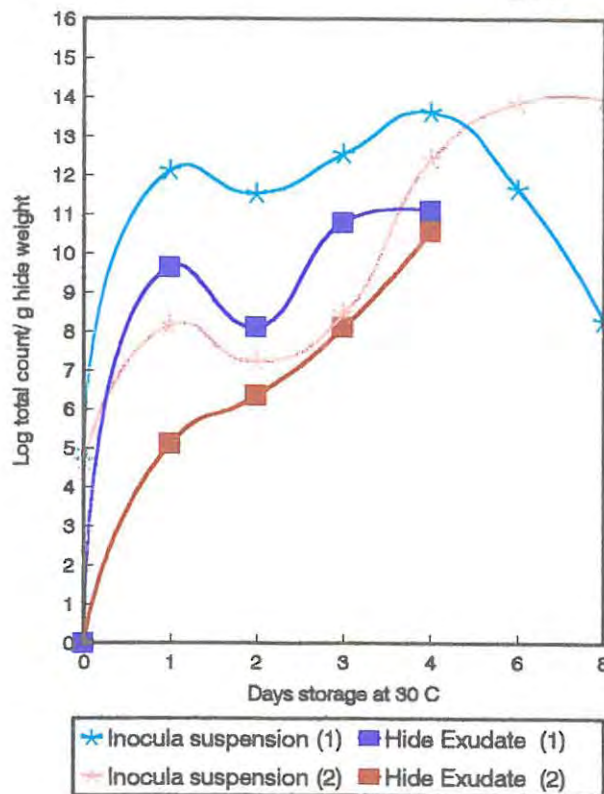
#### 5.4.4. Bacterial counts:

The results of the bacterial counts are shown graphically in Figs 5.8 and 5.9. Where mixed inocula were added to the hide samples both the aerobic and anaerobic counts were performed in order to differentiate between the individual inocula. Unfortunately it was not possible to differentiate the individual organisms in the mixed cultures of *C. histolyticum* and *C. sporogenes* and where these were present together only one total value was obtained (Chapter 2). The counts were performed on both the overlying inocula suspension as well as extrudates extracted from the hide tissues. In view of the controversy regarding the modes of entry of hide degrading bacteria (Robertson and Haines 1947; Anderson 1949; Formisano 1965; Halligan 1985), the hide pieces in this study were sliced horizontally at the epidermal/dermal junction. Extrudates were obtained from both flesh and hair pieces so formed and bacterial counts done on each extrudate. The results of comparing "flesh" bacterial counts to "hair" bacterial counts for each inocula are however not given here. The reason for this is that there were no major differences found between the counts of the two areas in any of the seven inocula sets tests and for clarification of the total count graphs, only the total hide extrudate counts (i.e. the sum of "hair" and "flesh" values) are given.

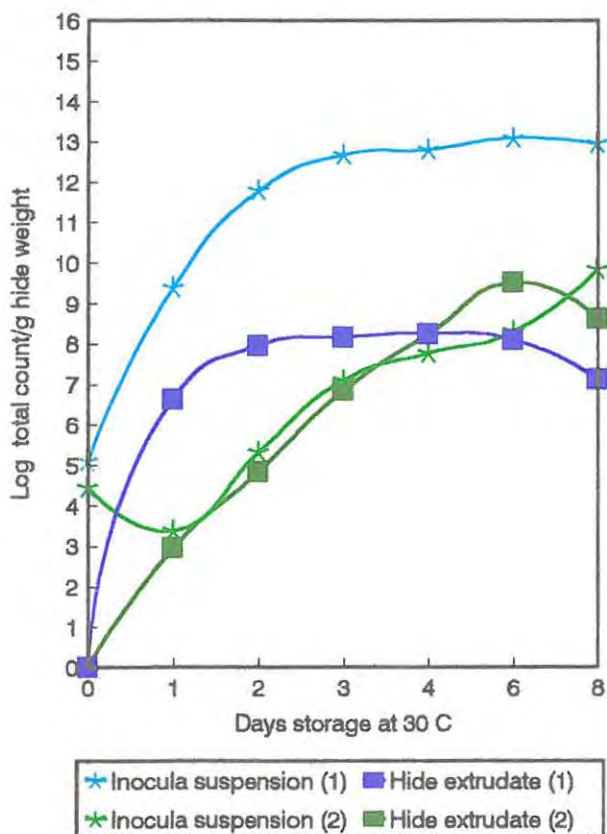
Bacterial counts of inoculated hides ranged from  $10^3 \text{ g}^{-1}$  (*C. sporogenes* extrudate) to  $9,5 \times 10^{13} \text{ g}^{-1}$  (*C. histolyticum* in the inocula suspension of the double-mix of *Ps. aeruginosa*/*C. histolyticum* where the *Ps. aeruginosa* count was  $10^{11} \text{ g}^{-1}$ ). The total counts found in this study clearly demonstrated that different effects occurred according to both inoculum type and inoculum combination. This resulted in a number of different interactions which included bacterial succession, synergism and antagonism. These various reactions are reflected in the total counts of the individual organisms within each system. Studies on meat degradation have shown that pseudomonads, at levels of  $10^9$  and  $10^{10} \text{ g}^{-1}$ , cause spoilage due to proteolysis (Dainty et al. 1975; Faber and Idziak 1982; Hayes 1985). Nandy (1956) reported bacterial levels of  $10^{10} \text{ g}^{-1}$  in 3 day old naturally decaying salted hides. Hockey and Russell recorded surface counts of  $10^9 \text{ g}^{-1}$  hide stored for 24 hours at  $37^\circ\text{C}$ . Ishii et al. (1987) recorded bacterial levels of  $10^9 \text{ ml}^{-1}$  in liquid cultures of calfskin showing putrefactive grain damage after 48 hours at  $30^\circ\text{C}$ . In this study inocula suspension counts of *Ps. aeruginosa* (from both the individual and double-mix dosed samples containing these organisms) exceeded these levels within 2-3 days of hide storage. However, lower counts of these organisms generally occurred in the hide extrudates



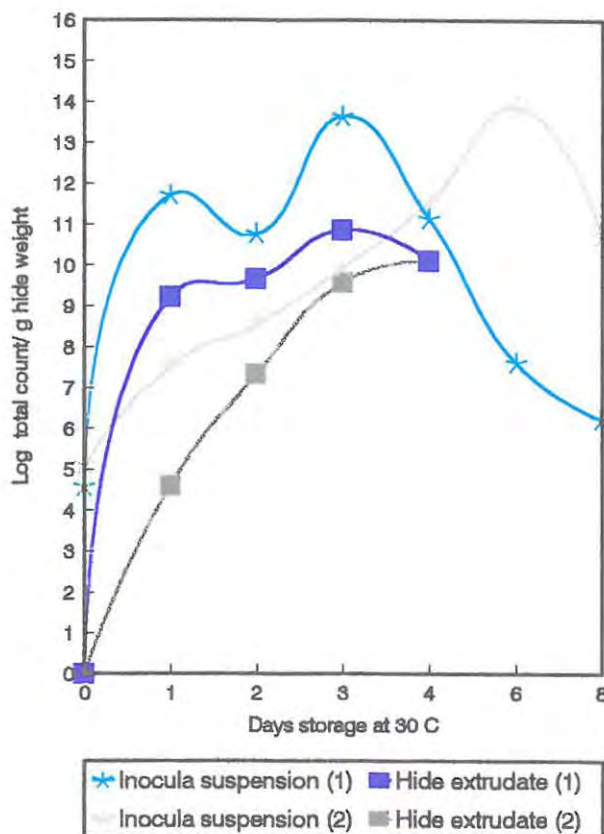
[a] *Ps. aeruginosa*



[b] *Ps. aeruginosa* (1) + *C. histolyticum* (2)

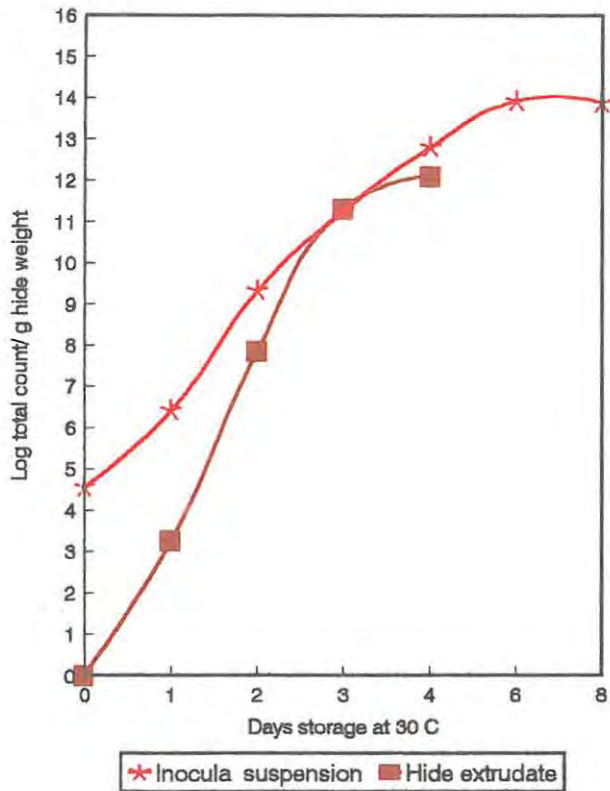
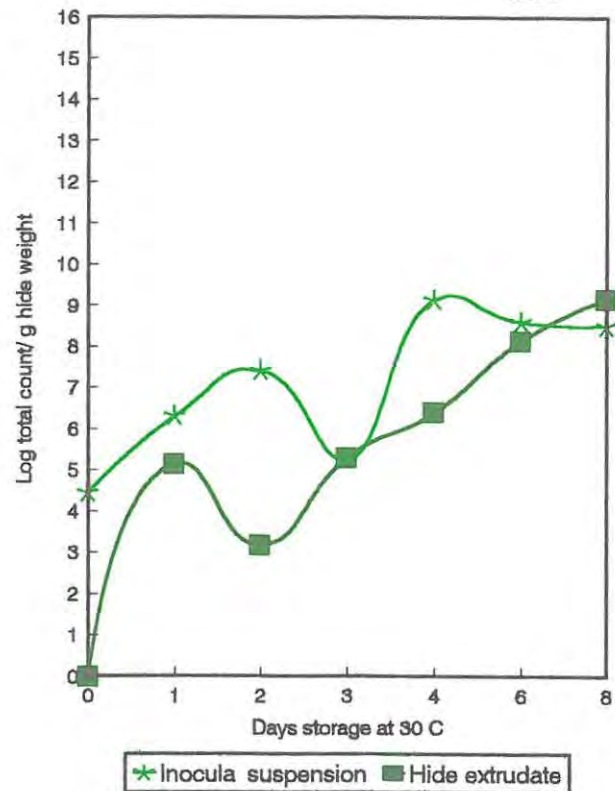
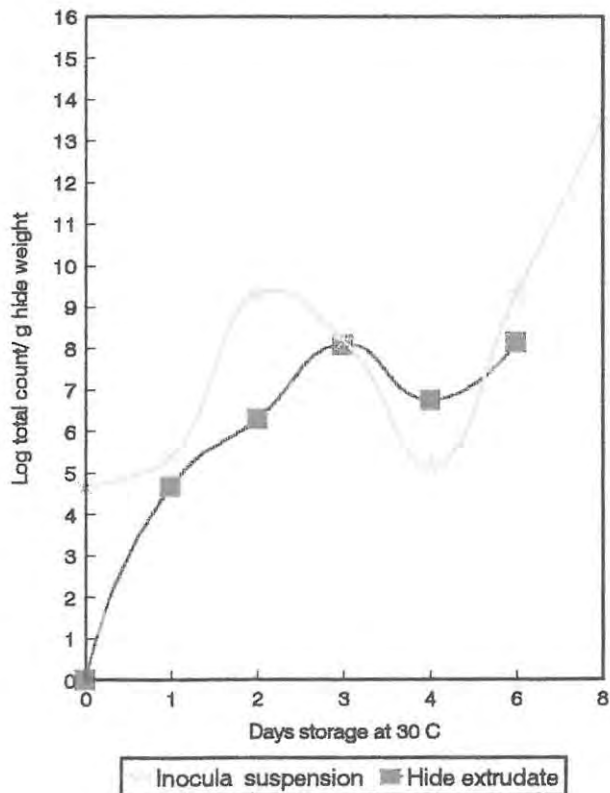
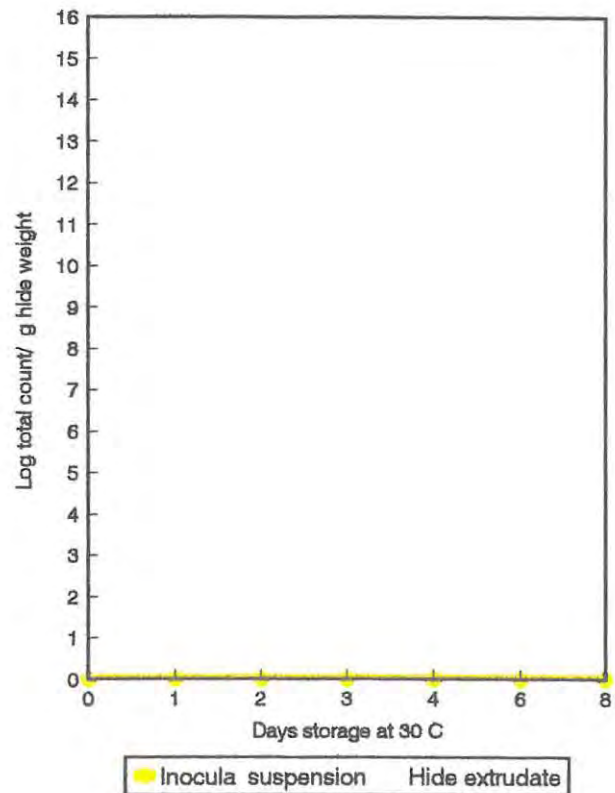


[c] *Ps. aeruginosa* (1) + *C. sporogenes* (2)



[d] *Ps. aeruginosa* (1) + *C. histolyticum/C. sporogenes* (2)

**Fig. 5.8 Total Bacterial Counts in Inocula Suspensions and Hide Extrudates During Storage at 30 C : [1] After Inoculation with Individual Aerobic Bacteria and Mixed Aerobic/Anaerobic Bacteria**

[a] *C. histolyticum*[b] *C. sporogenes*[c] *C. histolyticum / C. sporogenes*

[d] Uninoculated control

**Fig. 5. 9 Total Bacterial Counts in Inocula suspensions and Hide Extrudates During Storage at 30 C : [2] After Inoculation with Anaerobic Bacteria and Uninoculated Control**

throughout the incubation time, a finding compatible with the strictly aerobic nature of the bacterium. Hide extrudate counts were slightly lower than those of the inocula suspensions in the individual and mixed *Clostridium* inoculum systems. This is attributed to spore formation by these bacteria in the liquid suspension denoting potential, if not actual, viable bacterial activity. It is not always possible to compare in-vitro growth rates to in-vivo rates due to total viable counts being inclusive of both growth and death rates (Linton 1982). These findings emphasise the need to determine both external and internal bacterial counts, and to relate them to specific parameters of collagen breakdown, in order to accurately reflect in-situ changes in hide degradation.

The findings of this study are also interesting in that, the prediction from the growth rates of the selected inocula that putrefaction would occur at 2-6 days depending upon initial inocula concentration (see Chapter 4) was confirmed by the maximum growth levels at days 4-6 found in both the individual *C. histolyticum* and the other *C. histolyticum* containing inocula (except for the *C. histolyticum/C. sporogenes* double-mix).

These results are of significance to the hide industry, since hides themselves are too expensive to be used freely in preservation studies and so reliance must be placed on initial small scale laboratory studies. If bacterial growth in nutrient broths can fairly accurately reflect in-situ conditions then this represents a powerful, but inexpensive, way of screening biocides for hide preservation. Changes in growth rate arising from biocide inhibition would significantly alter the outcome of biocide preservation, especially if slower growing but more potentially damaging species emerged.

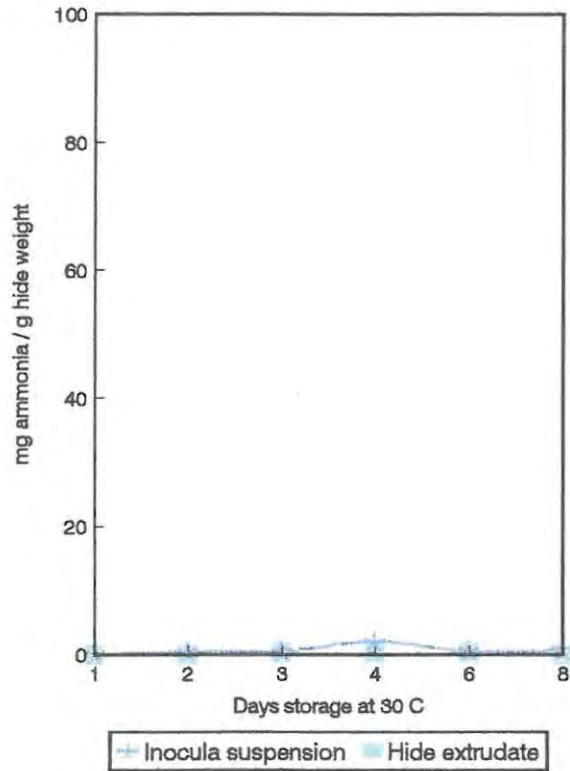
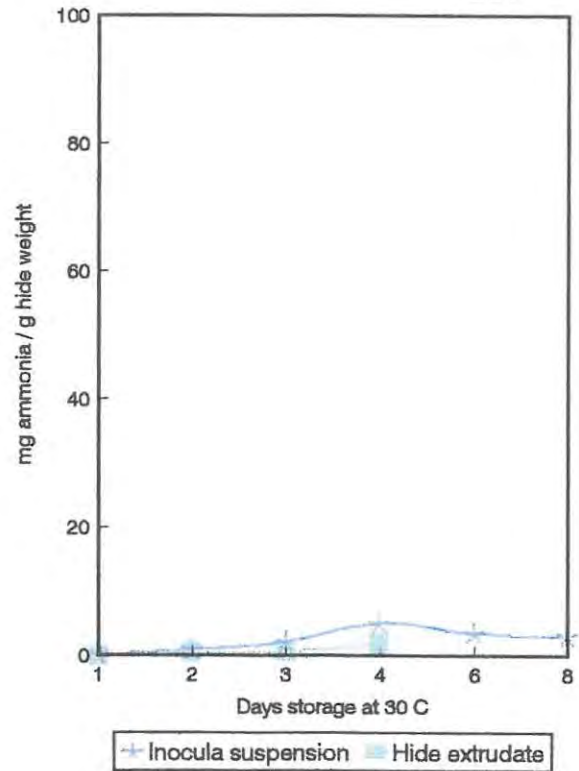
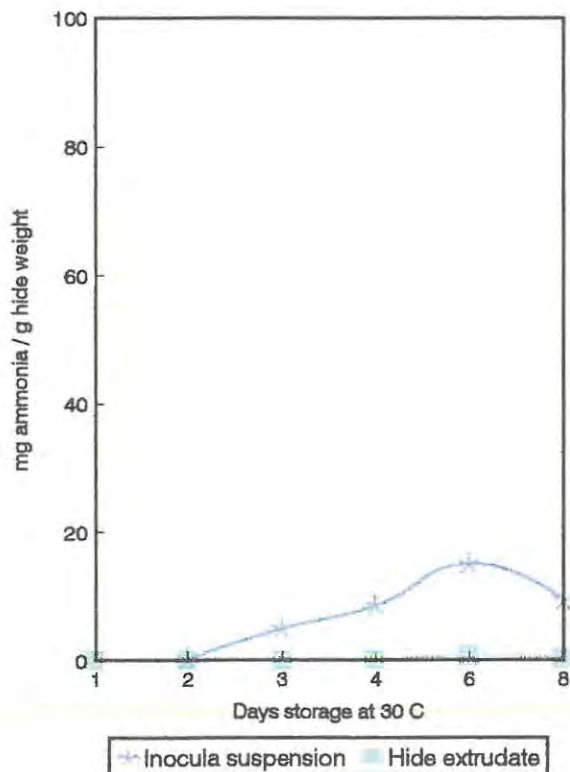
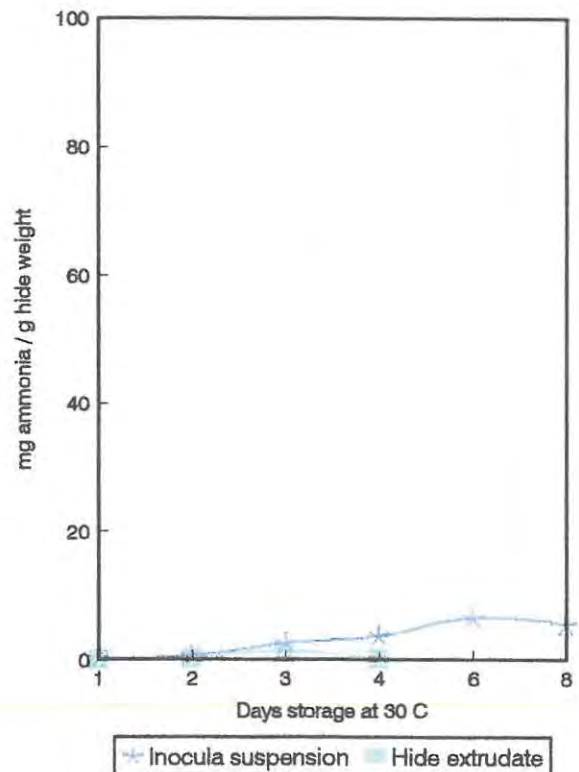
It has been shown that, in mixed cultures, organisms with the faster growth rate generally become dominant (Veldkamp and Jannasch 1972) and that substrate limitation does not play a role in competitive inhibition leading to bacterial succession (Gill and Newton 1977). Conversely, bacterial respiration, although insignificant when compared to tissue respiration (Banwart 1987), has been shown to play an important role in bacterial succession. Therefore it would be expected that the collagenase producing micro-aerophilic *C. histolyticum* should contribute greatly to any bacterial succession in hides. Figs 5.8 (b) and (c) show that, as in Fig. 8(d) (the triple-mix bacterial system), double-mixes of the strictly aerobic *Pseudomonas*

and the two *Clostridium spp.* also exhibit evidence of various degrees of bacterial succession, with *Clostridium* being the secondary colonising organism.

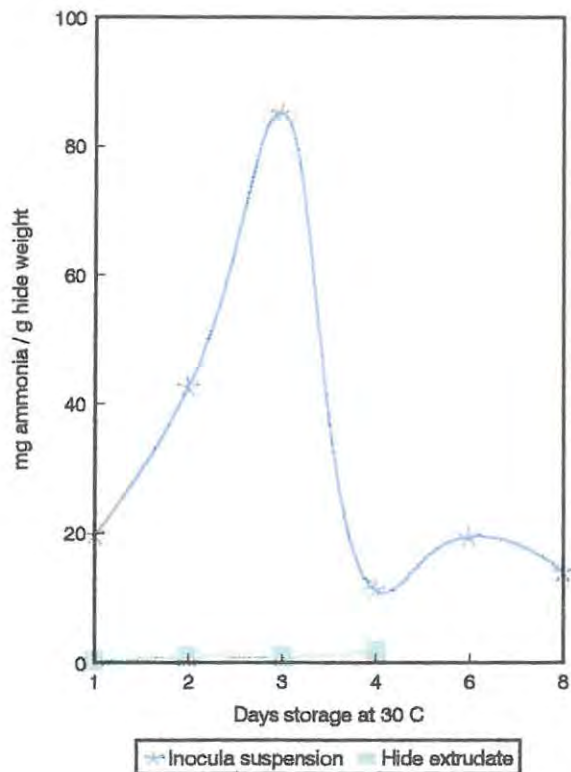
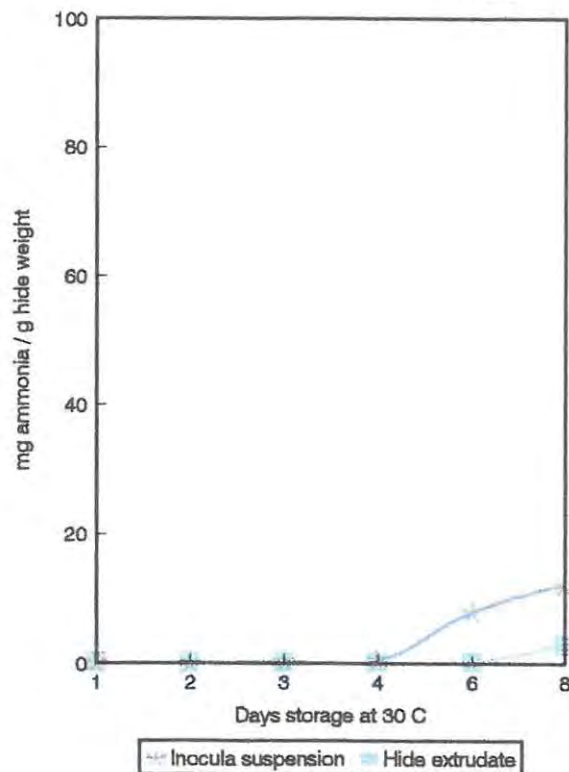
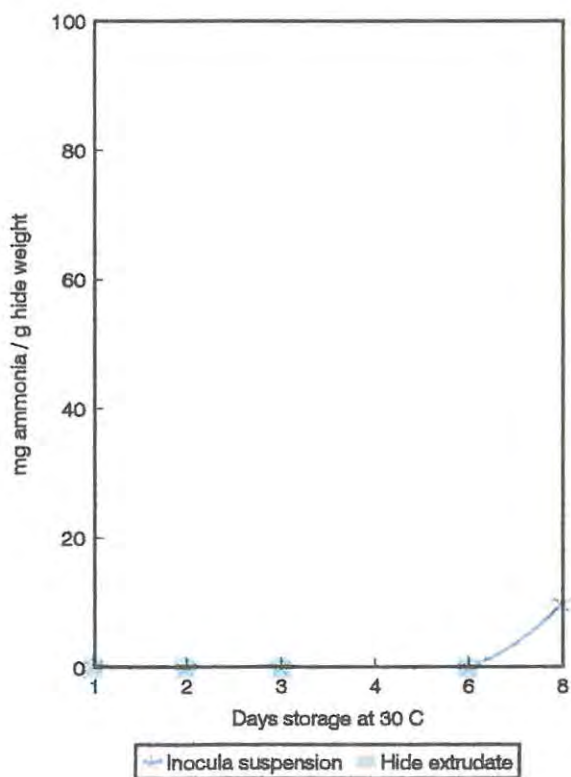
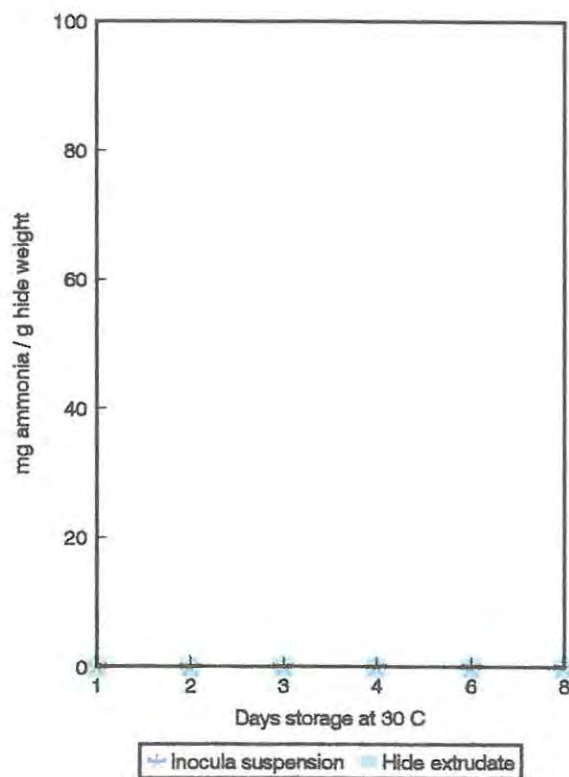
What is far more significant is the finding of the individual *C. histolyticum* culture which not only showed the steepest rise in growth rate but exhibited also greater degradative changes than when mixed with either of the other two inocula, or in the triple-mix [Fig 5.9(a)]. This increased degradation is also reflected in greater mass loss and fluid volume increase (Figs 5.6 and 5.7) ammonia levels (Figs 5.10 and 5.11) and hydroxyproline release values (Figs. 5.12 and 5.13). This clearly demonstrates that, while bacterial succession does occur in hide degradation, it has less significance to the phenomenon of hide substance loss than the predominance of collagenase producing micro-aerophilic clostridia (which would include *C. histolyticum* and *C. perfringens*). These bacteria possess a double advantage in post-mortem hide tissue due to both their respiratory preferences and enzyme specificity for the substrate.

*C. sporogenes* growth curves were also of interest in that the known oxygen sensitivity of this organism was evident during early phase growth in the form of decreased total counts from extrudate samples. The inoculum suspension counts were somewhat higher than those of the extrudates but the sporulation of this organism under stress (oxygenation) conditions could account for this. Studies using a counting method which differentiated between viable and dormant bacteria would, if available, eliminate this anomaly. However, the above findings are confirmed by the lack of decreased *C. sporogenes* counts in the double-mix of *Ps.aeruginosa/C.sporogenes*. In this culture *C. sporogenes* would be favoured due to *Ps.aeruginosa* being a rapid oxygen consumer (Smith and George 1946; Cato et al. 1986) and it can be seen from the growth curves of the double-mix of these organisms that synergism occurs resulting in an increase in growth rate and higher total biomass for *C. sporogenes*.

Besides succession and synergism the results of the total counts indicated antagonism between *C. histolyticum* and *C. sporogenes* in the double-mix systems containing these organisms. Although it was not possible to count these organisms separately it was possible to predict which organism was dominant, based on the data from the other test parameters. A bi-modal curve was present indicating some initial growth of either *C. sporogenes* and/or *C. histolyticum*. This was followed by a phase in which *C. sporogenes* dominated the system (as

[a] *Ps. aeruginosa*[b] *Ps. aeruginosa* + *C. histolyticum*[c] *Ps. aeruginosa* + *C. sporogenes*[d] *Ps. aeruginosa* + *C. histolyticum* + *C. sporogenes*

**Fig. 5.10 Soluble Ammonia in Inocula Suspensions and Hide Extrudates During Storage at 30 C: (1) After Inoculation with Individual Aerobic Bacteria and Mixed Aerobic/Anaerobic Bacteria**

[a] *C. histolyticum*[b] *C. sporogenes*[c] *C. histolyticum* + *C. sporogenes*

[d] Uninoculated control

**Fig. 5.11 Soluble Ammonia in Inocula Suspensions and Hide Extrudates During Storage at 30 C: (2) After Inoculation with Anaerobic Bacteria and Uninoculated Control**

shown by the decrease in bacterial numbers). After day six the order was reversed and the predominance of *C. histolyticum* was seen in the sudden increase in mass loss, fluid increase and ammonia [Fig 5.7(c), Fig. 5.11(c)]. The lack of ammonia and decreased hydroxyproline levels [Fig 5.13(c)] as well as the total suppression of collagenase until day 8 [Fig 5.15(c)] confirmed the predominance of *C. sporogenes* in this system. The initial increase of bacterial growth at days 1 and 2 could be due to *C. histolyticum* utilising free amino acids and sugars and this is reflected in the lower pH values of the inocula suspensions during this time [Fig. 5.5(c)].

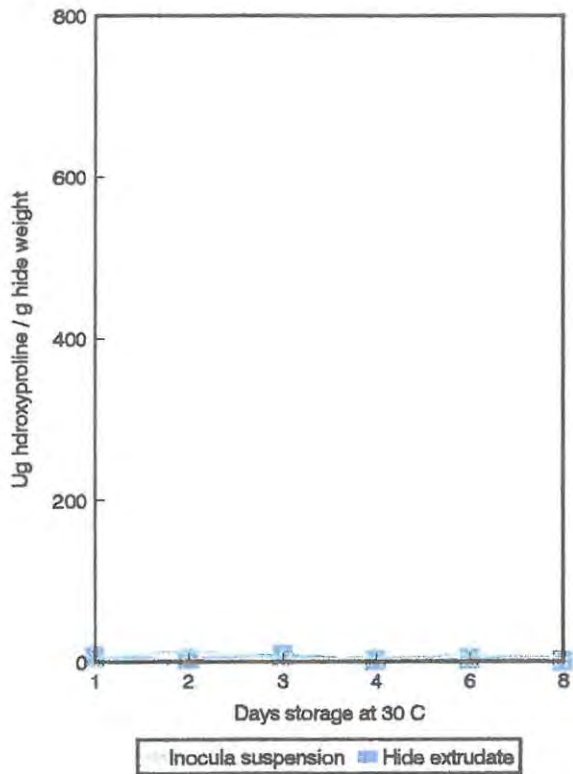
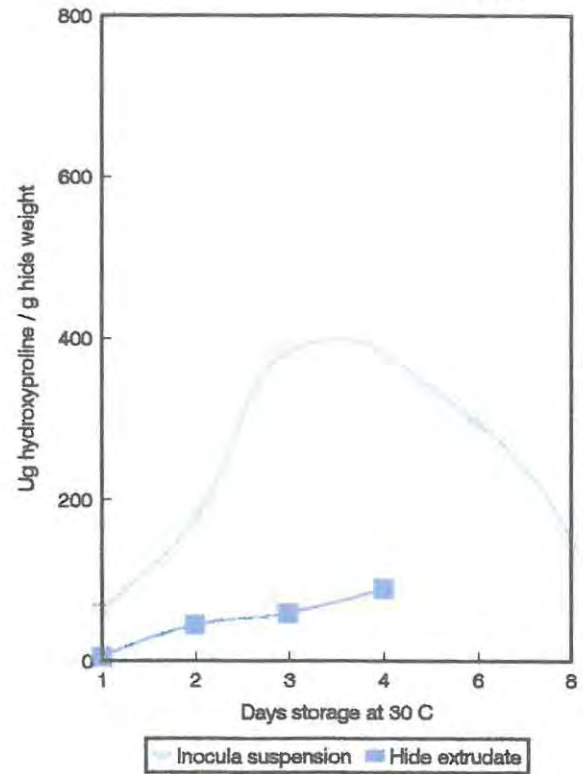
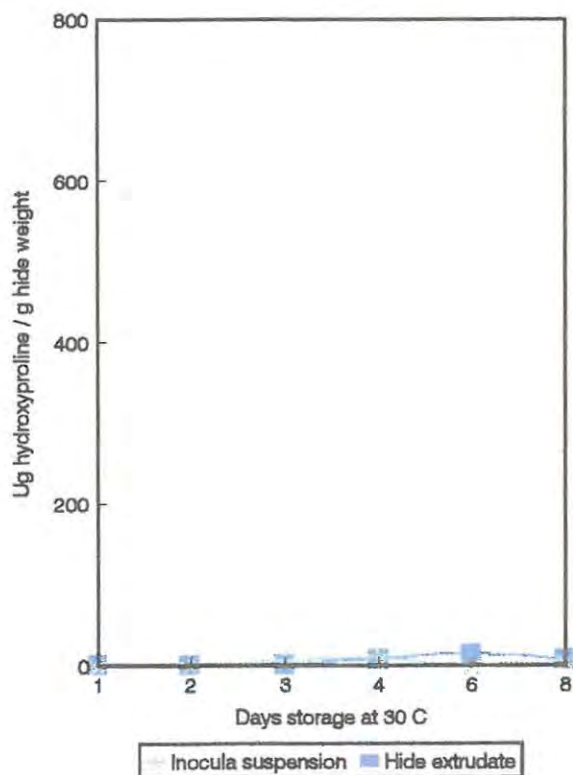
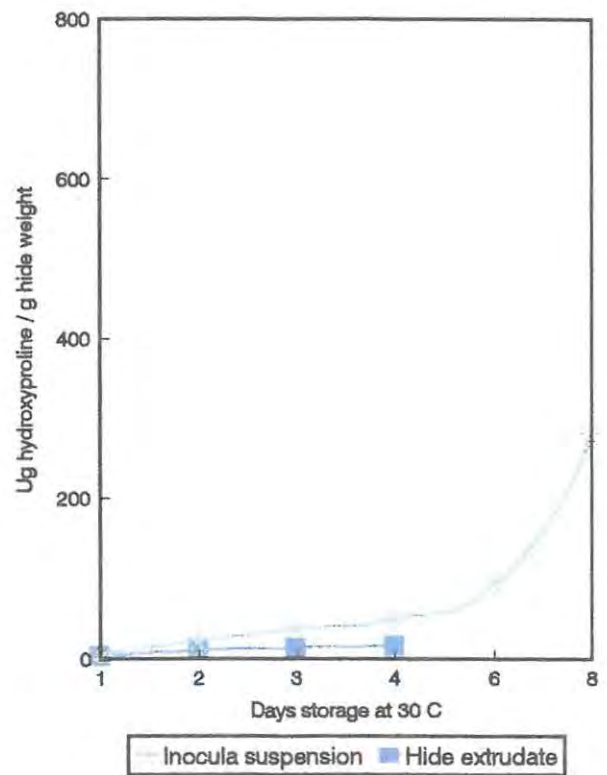
#### 5.4.5. Ammonia release:

The only really significant ammonia levels found were those of the individual *C. histolyticum* system (Fig.5.11a) which achieved a maximum ammonia level of 85,1mg g<sup>-1</sup> hide mass at day 3 in the inoculum suspension. This fell rapidly to 11,2 mg g<sup>-1</sup> hide mass at day 4. Gerbi (1986) studying the effects of antibiotics on hide storage, found volatile ammonia levels of 6,8mg g<sup>-1</sup> after six days storage at 25°C. Volatile ammonia has been attributable to bacterial breakdown of proteins and amino acids (Mellon *et al.* 1954) and Somer (1942) stated that volatile ammonia levels greater than 3,0% were indicative of a poor cure.

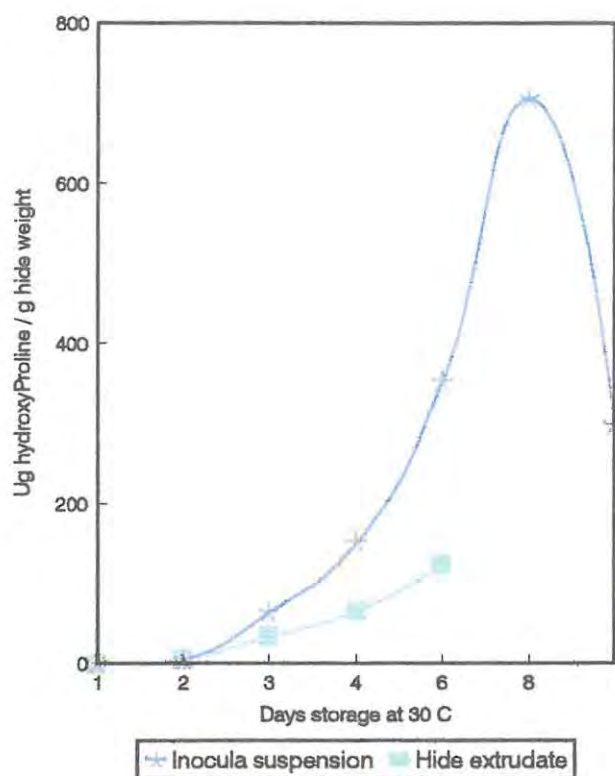
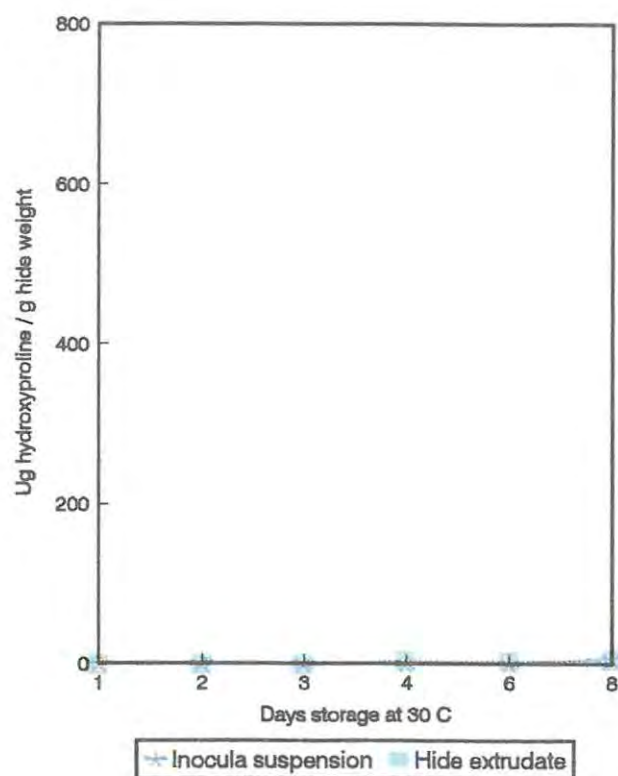
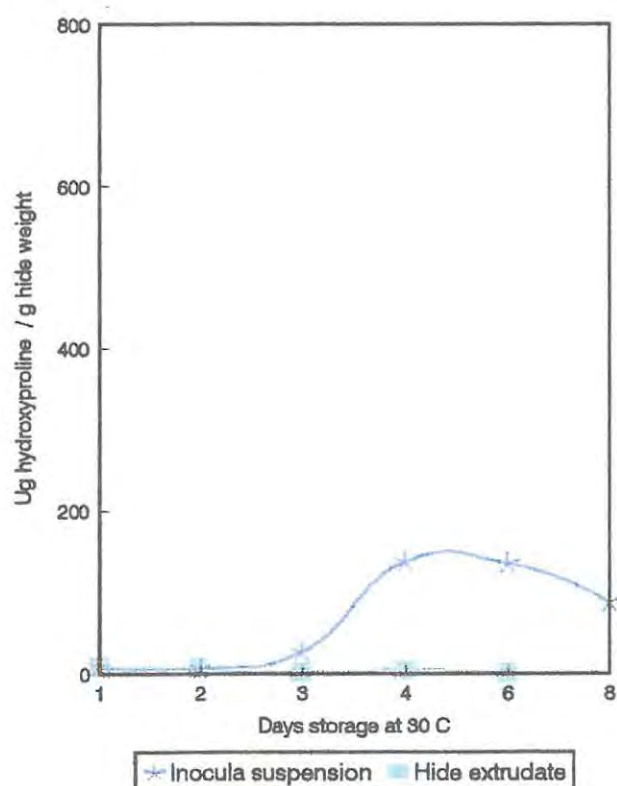
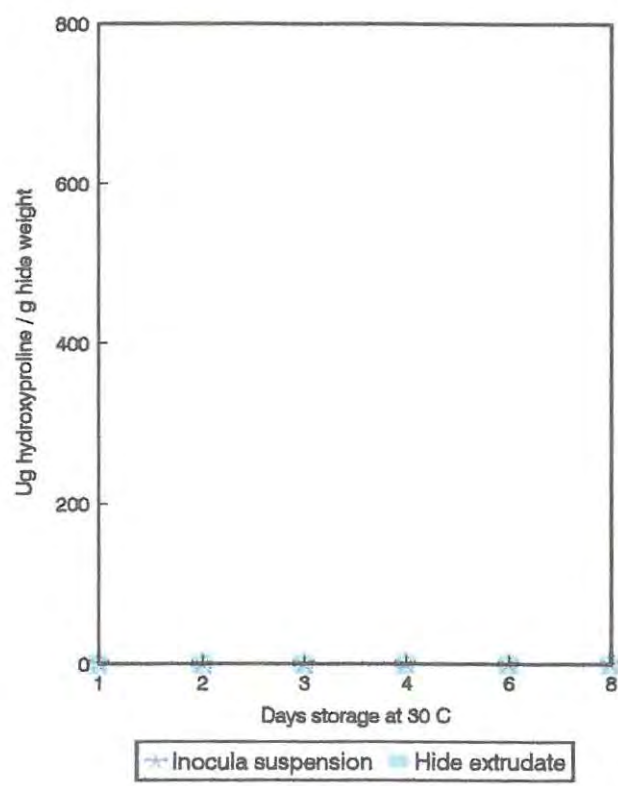
In this study only *C. histolyticum* showed levels of >3,0% and these were present on days 2 and 3 only (Fig. 5.11), although almost 2,0% ammonia was detected at day 1. *C. sporogenes*, *Ps. aeruginosa*/*C. sporogenes* and *C.histolyticum*/*C.sporogenes* all had slightly elevated ammonia levels (approximately 1%) by day 8 indicating that free amino acid breakdown had occurred. No other significant levels were detected in any of the other systems. Ammonia was virtually absent from all of the hide extrudates and this is attributed to the volatile nature of this compound which would readily solubilise in inoculum suspension (Figs. 5.10 and 5.11).

#### 5.4.6. Hydroxyproline release:

These results are shown in Figs 5.12 and 5.13. Volatile nitrogen (ammonia) is a non-specific assessment of hide protein breakdown since it is produced as a result of general bacterial action on globular proteins, free amino acids and denatured fibrillar proteins. Hydroxyproline is therefore considered to be a more reliable method of measuring collagen hydrolysis (Nandy

[a] *Ps. aeruginosa*[b] *Ps. aeruginosa* + *C. histolyticum*[c] *Ps. aeruginosa* + *C. sporogenes*[d] *Ps. aeruginosa* + *C. histolyticum* + *C. sporogenes*

**Fig. 5. 12 Hydroxyproline Release in Inocula Suspensions and Hide Extrudates During Storage at 30 C :(1) After Inoculation with Individual Aerobic Bacteria and Mixed Aerobic/Anaerobic Bacteria**

[a] *C. histolyticum*[b] *C. sporogenes*[c] *C. histolyticum* + *C. sporogenes*

[d] Uninoculated control

**Fig. 5.13 Hydroxyproline Release in Inocula Suspensions and Hide Extrudates During Storage at 30 C : (2) After Inoculation with Anaerobic Bacteria and Uninoculated Control**

et al. 1956; Chakraborty and Chandra 1984). The results of this study showed that, while ammonia levels in the *C. histolyticum*-inoculated series showed a peak at day 3 (Fig 5.11) the corresponding hydroxyproline peaks appeared only after six days incubation [Fig.5.13a]. This corresponded to onset of stationary growth phase for this organism. However, the ammonia upswing occurred during early log phase growth [Figs 5.9a]. This confirmed that volatile ammonia represents early catabolic attack on interstitial amino acids and oligopeptides while hydroxyproline release is more directly related to breakdown of fibrillar components by specific enzyme action. Hydroxyproline is almost unique to collagen-based tissues, (Glanville and Kuhn 1980; Weiss 1984; Buckley et al. 1988) and so the detection of this imino acid is very relevant to collagen hydrolysis studies.

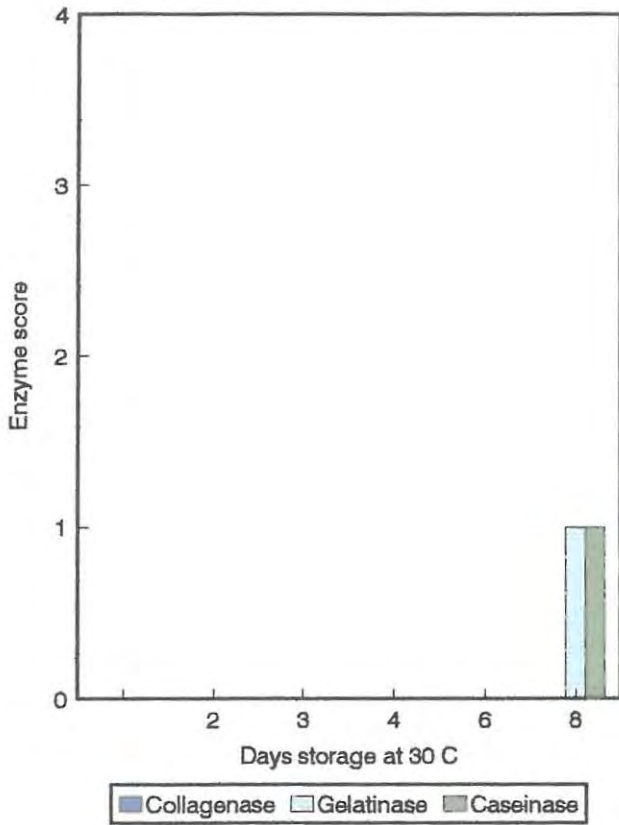
The results indicated that only the cultures containing *C. histolyticum*, either alone or in mixed cultures, showed the presence of hydroxyproline. The greatest amount was found in the *C. histolyticum* individual system which gave values ranging from  $4.6 \mu\text{g g}^{-1}$  hide mass at day 1 to a maximum of  $705 \mu\text{g g}^{-1}$  hide mass at day 6, which corresponded to the time for maximum total count values of this organism. In the *Ps. aeruginosa* + *C. histolyticum* mix levels of  $294 \mu\text{g}$  were present at day 6, again corresponding to late log/early stationary phase growth of the *C. histolyticum*. In the triple-mix inoculum system a maximum level of  $279 \mu\text{g}$  hydroxyproline was found at day 6, (also during clostridia stationary phase growth) but levels before day 8 were below those for the *Ps. aeruginosa* + *C. histolyticum* mix. In the *C. histolyticum* + *C. sporogenes* mix low levels were found at days 1,2 and 3 but at day 4 and 6 hydroxyproline at  $138$  and  $136 \mu\text{g g}^{-1}$  hide mass was detected respectively. These values fell to  $88 \mu\text{g g}^{-1}$  by day 8. Lower amounts of hydroxyproline were found in the hide extrudates, compared with the inocula suspension, for all the test systems.

Hockey & Russell (1982) found levels of  $788 \mu\text{g ml}^{-1}$  of hydroxyproline in the hide extrudate of hides "staled" at  $35^\circ\text{C}$  for three days and this level dropped to  $165 \mu\text{g ml}^{-1}$  at 5 days. The surface aerobic count data of their study showed that maximum bacterial numbers occurred at day 1. This indicated a lag between maximum bacterial numbers and hydroxyproline release. However, anaerobic counts were not performed, therefore, it is not possible to determine whether increased anaerobic bacterial levels correlated with the hydroxyproline peak levels.

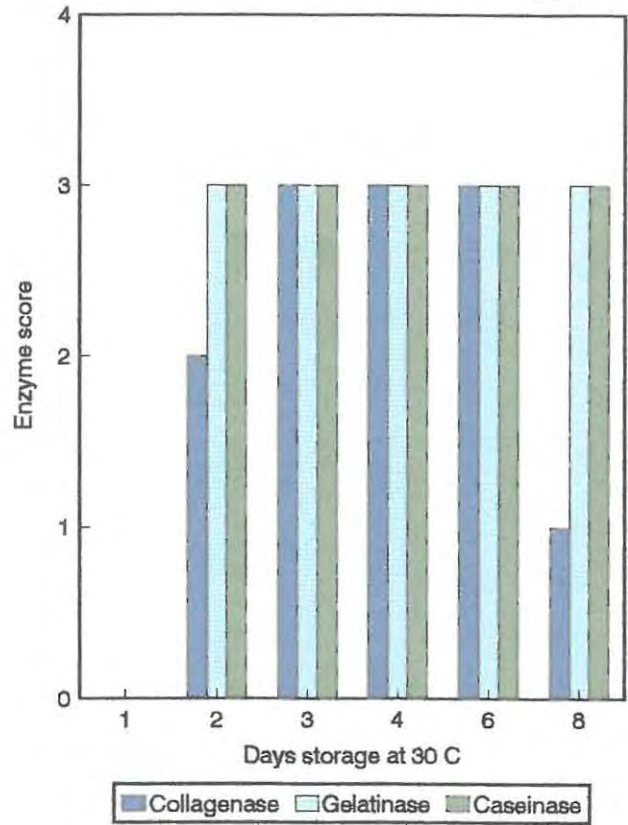
In this present study there was a very strong correlation between maximum total count values and maximum hydroxyproline levels. The rapid drop in hydroxyproline levels in the *C. histolyticum* and *C. histolyticum* mixed series is probably due to either its oxidation to an  $\alpha$ -keto acid by *Ps. aeruginosa* or to its trans-amination via the Stickland reaction by *C. sporogenes*. Hydroxyproline degradation has been studied in several pseudomonads including *Ps. putida* and *Ps. fluorescens* while the fermentation of paired amino acids by *C. sporogenes* and *C. histolyticum* is well documented (Eskin 1971; Doelle 1975; Gottschalk 1979).

The hydroxyproline results of this study confirm the specificity of this parameter for collagen breakdown and indicates that ammonia levels are non-specific and tend to indicate decay, not putrefaction (see Chapter 3). Ammonia remains, however, a useful parameter for detecting early bacterial build up and attack on globular proteins of hide tissue, but it does not necessarily indicate any putrefactive degradation of hide collagen.

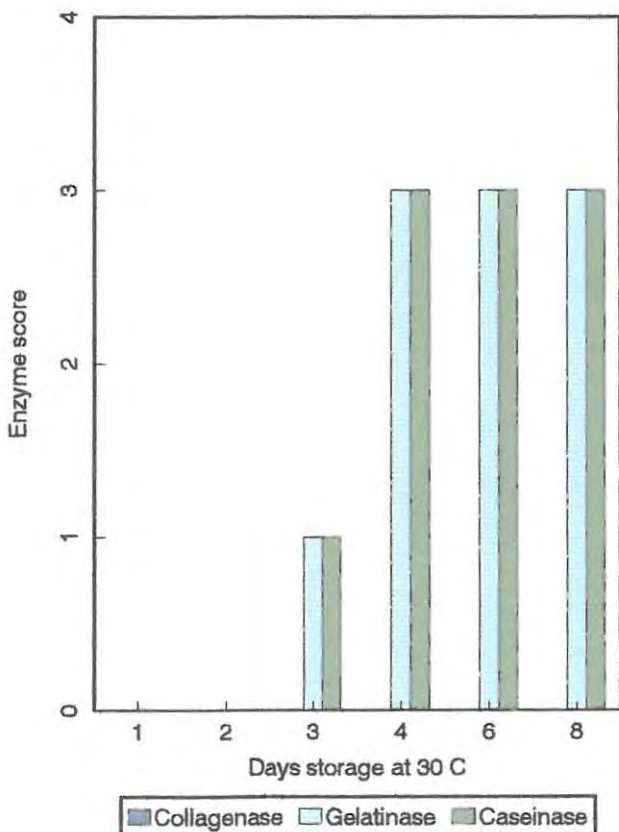
From these results the greater degradation occurring in the individual *C. histolyticum* system as opposed to when combined with the other bacteria, again emphasises that this collagenolytic clostridium is more active in causing hide loss when alone than in the presence of other organisms, either interactively, or in the form of a succession. This is of significance to hide degradation since individual niche areas of lowered redox potential will be caused by the industry practice of dropping hides haphazardly to form piles. Hair-to-flesh contact of individual areas within these hides will allow for the transfer of bacteria from dung and soil on the outer hair area onto the previously clean flesh surfaces, where exposed capillary networks will act as channels for invasion. Since both *C. histolyticum* and *C. perfringens* possess powerful hyaluronidases, rapid proliferation will occur through any exposed blood capillaries. Since the capillary network extends up to and just below the dermal/epidermal junction both general substance losses and sueded grain will occur. Folded and piled hides therefore will be more vulnerable to collagenolytic degradation than those left fully open and exposed to the air, since in the latter case the aerobic colonisers such as *Ps. aeruginosa* will commence growth first. As shown in the results of this study, this will generate a bacterial succession which will delay the onset of denaturation. Confirmation of the inhibitory effect of *C. sporogenes*, on *C. histolyticum* is evident in these hydroxyproline results, where, in the double-mix only by day 4 and in the triple-mix only by day 7, is there evidence of moderate



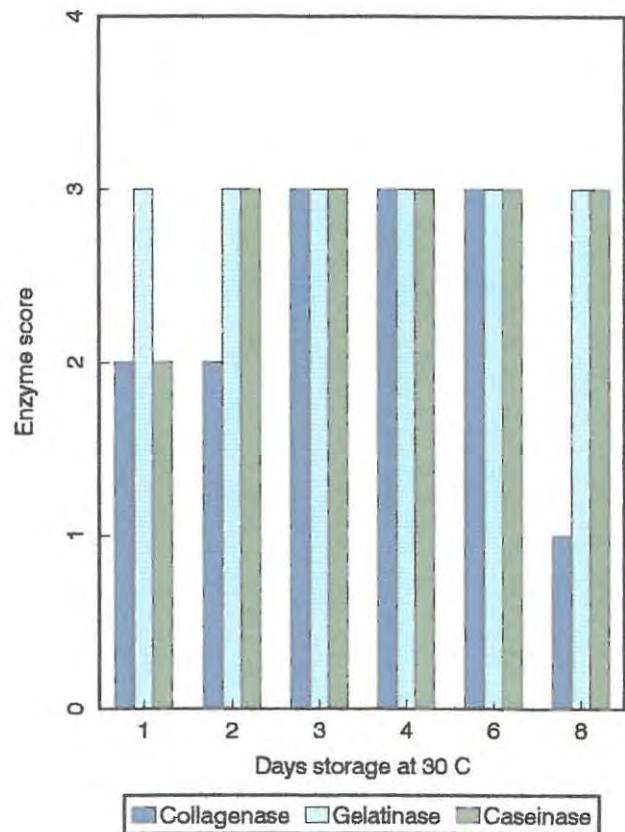
[a] *Ps. aeruginosa*



[b] *Ps. aeruginosa* + *C. histolyticum*

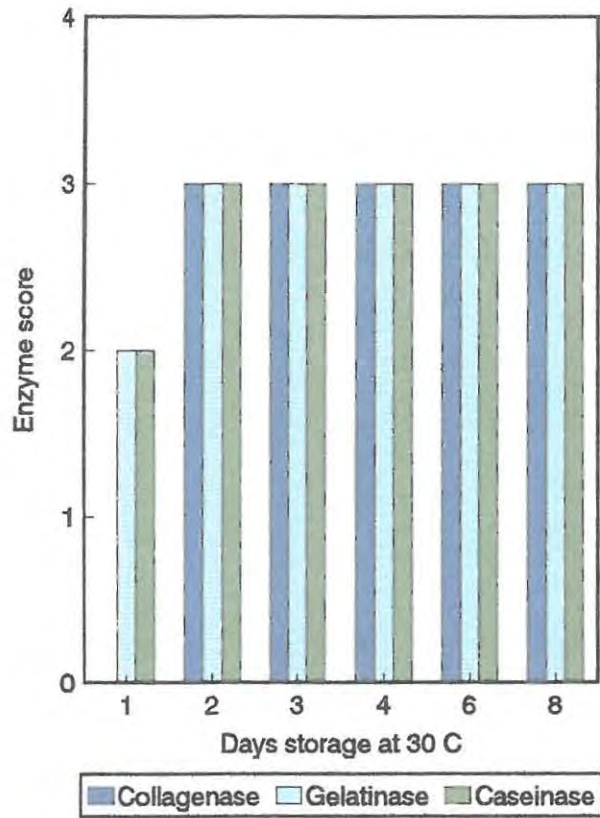
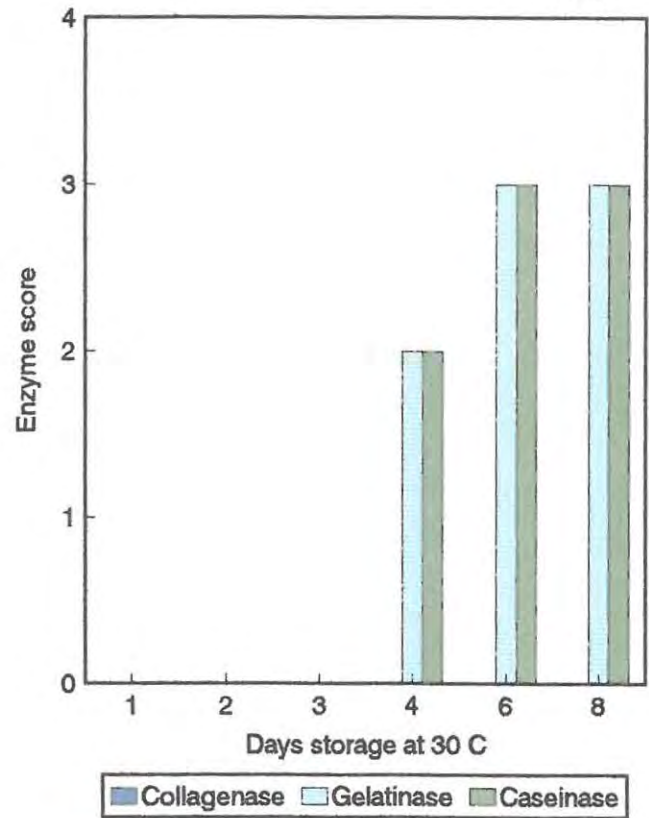
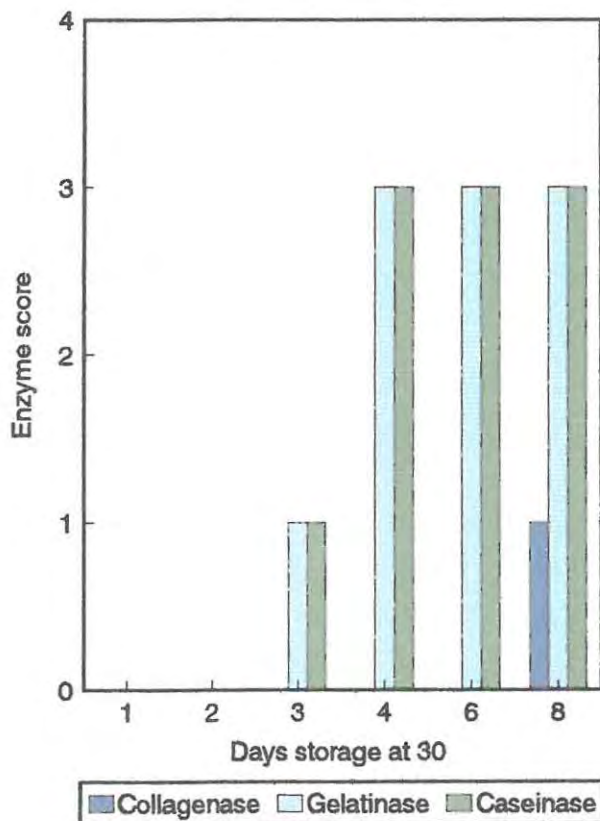
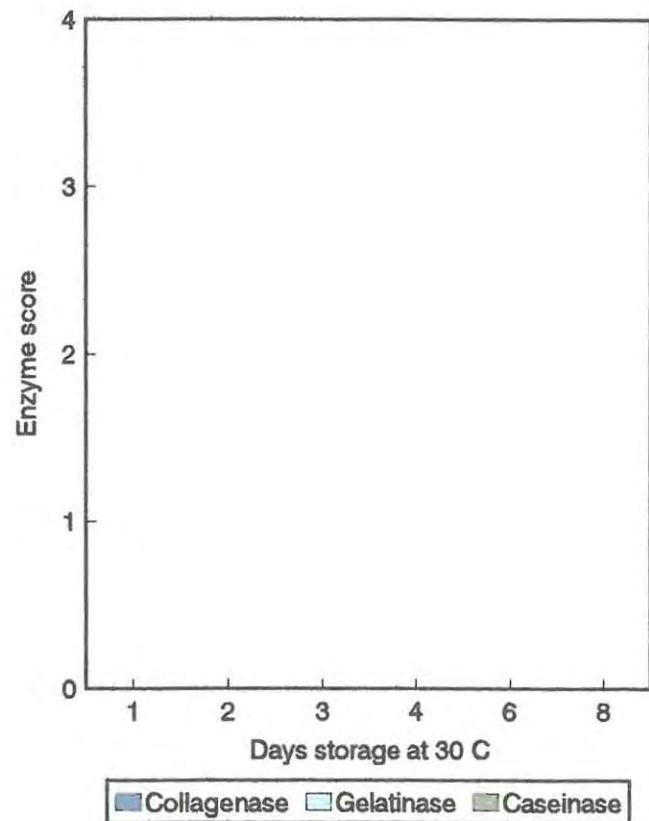


[c] *Ps. aeruginosa* + *C. sporogenes*



[d] *Ps. aeruginosa* + *C. histolyticum* + *C. sporogenes*

**Fig. 5. 14 Proteolytic Activity in Hide Extrudates During Storage at 30 C : (1) After Inoculation with Aerobic Bacteria and Mixed Aerobic/Anaerobic Bacteria**

[a] *C. histolyticum*[b] *C. sporogenes*[c] *C. histolyticum* + *C. sporogenes*

[d] Uninoculated control

**Fig. 5. 15 Proteolytic Activity in Hide Extrudates During Storage at 30 C : (2) After Inoculation with Anaerobic Bacteria and Uninoculated Control**













activity and then only at a minimal level compared to that occurring in the individual *C. histolyticum*-inoculated hides.

#### 5.4.7. Proteolytic activity:

The results of activity testing of hide extrudates on collagen, gelatin and casein gels is shown in and Figs. 5.14 and 5.15. The findings are compatible with the enzyme profiles of each organism as demonstrated in Chapter 4. These results also parallel those of the other test parameters such as mass and fluid changes, bacterial counts, ammonia and hydroxyproline release as well as those of the histological findings (see 5.4.9.). Most evident is the suppression of *C. histolyticum* collagenase during the first 6 days incubation in the presence of *C. sporogenes*. The weak proteolytic activity against the selected substrates by *Ps. aeruginosa* indicates that this organism is fairly inactive in hide tissue. The triple-mix system appears more degradative based on these tests, than it does by other measures such as hydroxyproline release.













#### 5.4.8. Stereomicroscopy of the Grain Layer:

Changes in the grain layer (papillary dermis) are described as sueded grain when they are restricted to superficial attack on the upper papillary dermis (also called grain "enamel") (Kemp 1983). There remains some controversy as to whether all, or part, of the basement membrane of the dermal-epidermal junction is removed during beamhouse processing (V. Stirtz and Schroder 1981 and 1982; Loutis *et al.* 1984; Werkmeister and Ramshaw 1988). There is also some controversy over the distribution of collagens I and III in the papillary and reticular dermis (Epstein and Munderloh 1978; Weber and Meigel 1976) and therefore an exact definition of sueded grain is not available. For the purpose of this study therefore, sueded grain (s) was defined as loss of well - defined surface structure (as seen under the stereomicroscope at x120 magnification). General grain damage (g) was defined as visible degradation of the deeper grain tissues. These two aspects were scored from 0 = Nil to +++ = extensive. Photographs of the stereomicroscopic appearance of the samples (x120 magnification) are shown in Figs. 5.16 and 5.17. These demonstrate that both types of grain damage were present in all samples dosed with either *C. histolyticum* or *C. histolyticum/Ps. aeruginosa* or *Ps. aeruginosa/C. histolyticum/C. sporogenes* and these showed progressive increase in the amount of damage with duration of storage. However, in the *C. histolyticum/C. sporogenes* dosed samples, general grain damage was absent until day 8, confirming the

Inoculum Type	Stereomicroscopy of Surface Grain (x120)		
	Day 1	Day 4	Day 8
<i>Ps. aeruginosa</i> s g	 0 0	 0 0	 0 0
<i>Ps. aeruginosa</i> + <i>C. histolyticum</i> s g	 0 0	 +++ ++	 ns +++
<i>Ps. aeruginosa</i> + <i>C. sporogenes</i> s g	 0 0	 0 0	 0 0
<i>Ps. aeruginosa</i> + <i>C. histolyticum</i> + <i>C. sporogenes</i> s g	 0 0	 +++ ++	 ns +++

**Fig. 5.16. Stereo-microscopy of the Grain Surface of Limed/Delimed Hides:  
(1) After Prior Inoculation and Storage at 30°C with Aerobic Bacteria  
and Mixed Aerobic/Anaerobic Bacteria**

**Key:** s = sueded grain; g = general grain damage  
ns = extensive loss of surface enamel  
+ = slight; ++ = moderate; +++ = extensive

Inoculum Type	Stereomicroscopy of Surface Grain (x120)		
	Day 1	Day 4	Day 8
<i>C. histolyticum</i>	 s + g +	 s +++ g ++	 s ns g +++
<i>C. sporogenes</i>	 s 0 g 0	 s 0 g 0	 s 0 g 0
<i>C. histolyticum</i> + <i>C. sporogenes</i>	 s 0 g 0	 s 0 g 0	 s +++ g ++
Uninoculated Control	 s 0 g 0	 s 0 g 0	 s 0 g 0

**Fig. 5.17. Stereo-microscopy of the Grain Surface of Limed/Delimed Hides:  
(2) After Prior Inoculation and Storage at 30°C with Anaerobic Bacteria  
and Uninoculated Control**

**Key: s = sueded grain; g = general grain damage  
ns = extensive loss of surface enamel  
+ = slight; ++ = moderate; +++ = extensive**

observation of inhibition of *C. histolyticum* collagenase activity in this system. Hides inoculated with either *Ps. aeruginosa* or *C. sporogenes* or *Ps. aeruginosa/C. sporogenes* double-mix showed no general degradative changes throughout the storage period.

#### 5.4.9. Histological Examinations:

Sections from the processed raw tissue and the limed/delimed samples were examined under 100 and 400 x magnification using a light microscope. The hide samples were stained by Masson and divided into areas and the amount of degradative change in each area has recorded on a scale of + (little change) to +++ (gross damage). Some representative samples are showed in Fig. 5.18. Control inoculated samples, not shown in the photographs of Fig. 5.18, showed no evidence of degradation throughout the 8 day storage period. They therefore appeared very similar to those shown in Fig. 5.18 - 3(a) with the exception that the cornified layer of the control samples was completely intact. Since there were no real differences between the processed and the fresh samples these results are not recorded separately here.

Gram stains were performed on all sections and some representative samples showing different areas of hide colonisation according to bacterial type are shown in Fig 5.19.

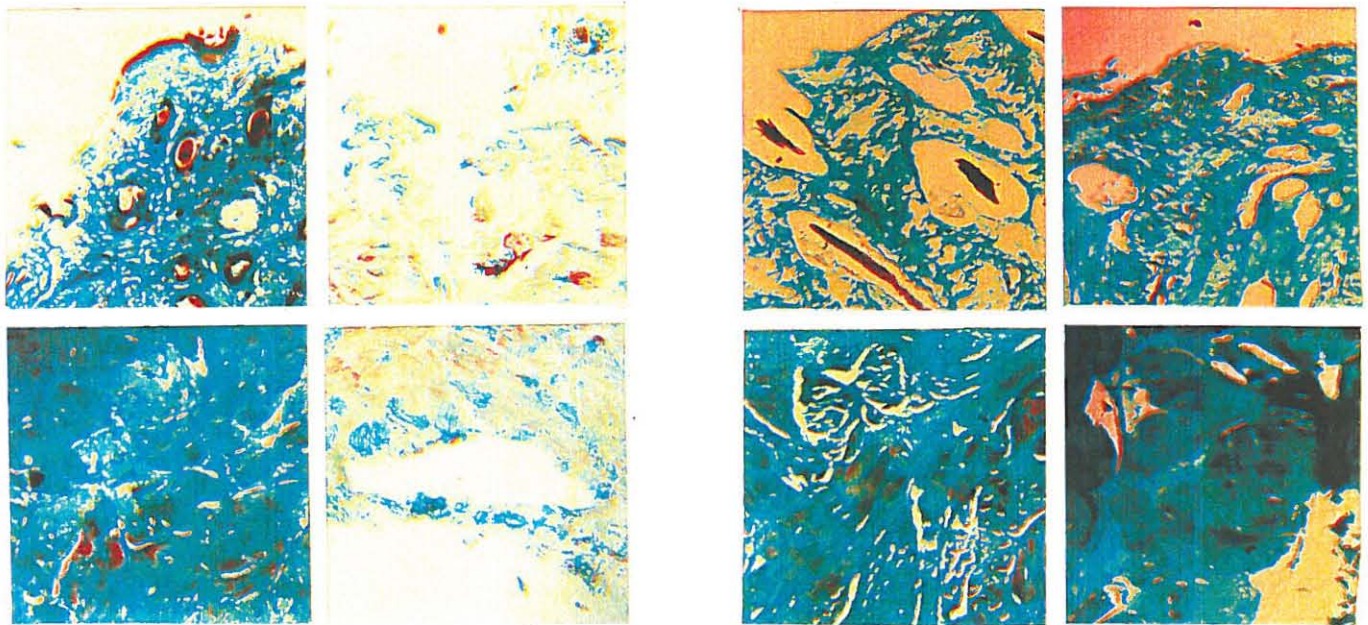
#### *Ps. aeruginosa:*

**Masson Stain:** No marked changes occurred throughout the tissue for up to 4 days storage. At day 6 there was some epidermal/dermal separation and by day 8 both the cornified and granular epidermal layers were absent. From day 2 there was some evidence of attack on the hair follicles, mostly at the root bulb and hair shaft-epidermal junction.

**Gram Stain:** Bacteria were not evident either in the epidermal area, papillary dermis or reticular dermis until day 3 when both epidermal and sub-epidermal colonisation was apparent. No infiltration of the reticular dermis occurred either at the upper or lower (flesh edge) At day 8 the epidermal layer was absent and extensive infiltration of the papillary dermis had occurred.

#### *C. histolyticum:*

**Masson Stain:** Epidermal granular layers, hair follicles and papillary collagen all showed slight degradative change by day 1 but cell nuclei and reticular collagen remained intact, although slight fibrolysis of the reticular collagen was present. Progressive degradation of these areas as

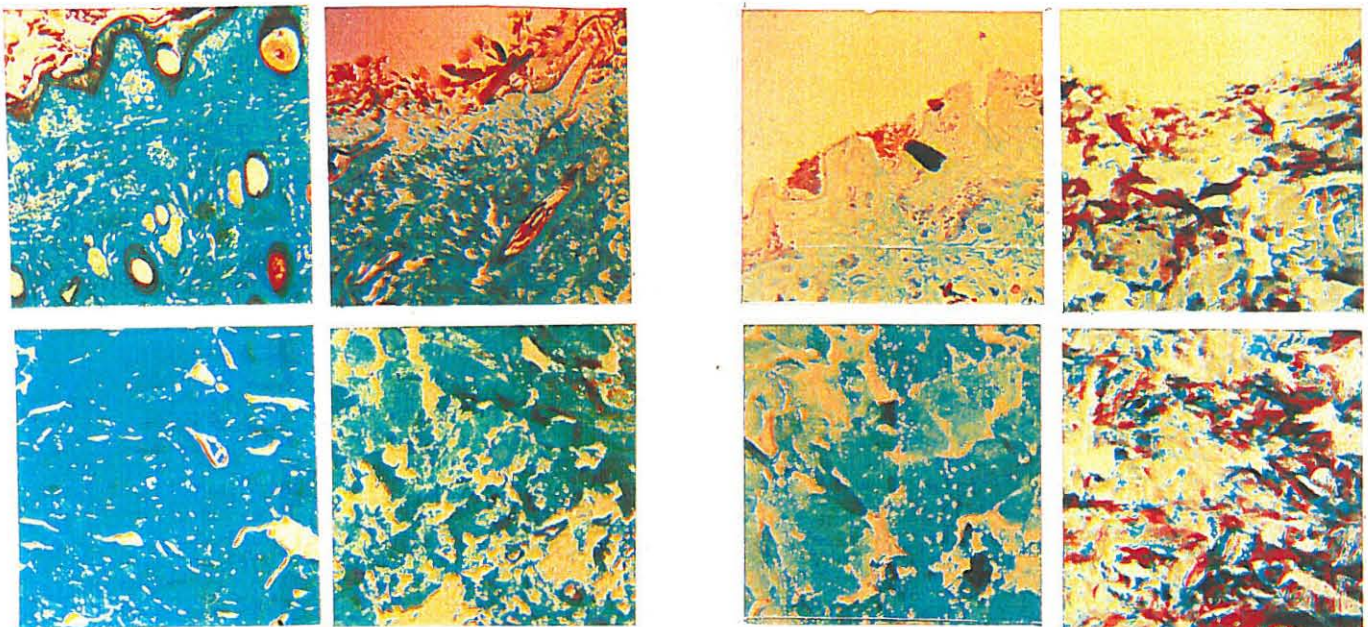


1(a)

1(b)

2(a)

2(b)



3(a)

3(b)

4(a)

4(b)

**Fig. 5.18. Hide Cross Section (x100) taken after (a) 2 Days and (b) 8 Days Post Inoculation with (1) *C. histolyticum*; (2) *C. sporogenes* (3) *C. histolyticum*/*C. sporogenes*; (4) *Ps. aeruginosa*/*C. histolyticum*/*C. sporogenes*. Upper photo = epidermis + papillary dermis; Lower photo = reticular dermis**

**Note:** Uninoculated controls showed no degradative changes throughout and resembled the samples shown in 3(a) above.

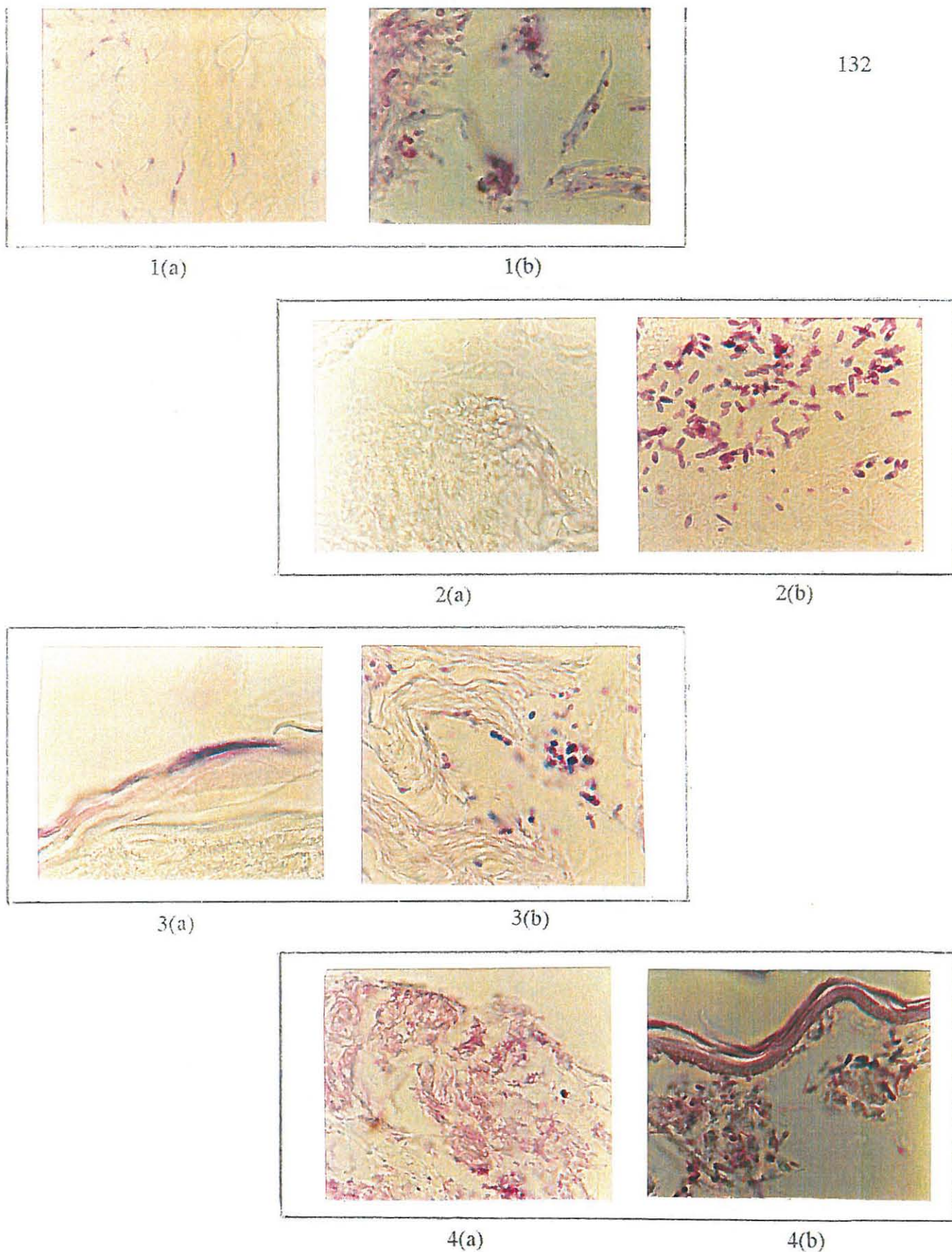
well as initial loss of cornified epidermis, cellular nuclei and reticular collagen occurred at day 2. Total absence of epidermal layers and increasing loss of hair and hair follicles occurred from days 3 to 8 but sebaceous gland degradation was only evidence from day 4 onwards. Marked reticular and papillary collagen loss occurred by day 3 and increasing fibrolysis of the remaining collagen occurred with increased incubation time resulting in marked hide mass loss. (See Fig.5.18[1a & 1b]).

**Gram Stain:** Scanty spores were present in the epidermal area, and to a lesser extent in the papillary dermis, at day 1 and these had not increased in number by day 2 in these areas but there were numerous Gram positive bacteria both in the central area of the lower corium and a moderate number on the flesh edge of the hides. At day 3 moderate numbers were present in the epidermal area and papillary dermis approximately half of which were in the vegetative state. By day 3 the bacteria were numerous at the lower hide edge and had proliferated throughout the reticular dermis invading the tissue fibres as well as the interstitial areas. (See Fig. 5.19 [1a & 1b]).

**C. sporogenes:**

**Masson Stain:** The cornified layer of the epidermis showed loss from day 1. There was evidence of attack on hair follicle epidermal layers, causing separation from the hair itself, and also degradative changes in some sebaceous glands at days 1 and 2. Papillary dermis showed basic fibre weave integrity but there appeared to be a loss in general fibre bulk, indicating possible non-specific proteolytic attack on collagen III present in this area. Similar slight loss of finer fibres occurred in the reticular dermis but otherwise this area remained intact. At day 3 marked evidence of degradation was present in the epidermal layers with almost complete loss of the whole area. There was also absence of all but a few hairs and loss of cellular nuclei throughout the tissue. From day 4 to 8 hair the epidermal loss continued to be almost total and fine fibre loss in the papillary and reticular dermis was more evident. At day 8 there was some loss and fibrolysis of the papillary collagen but the reticular dermis strong fibre weave structure remained intact throughout the incubation period. (See Fig.5.18 [2a & 2b]).

**Gram Stain:** Bacteria were absent from the inter-epidermal strata throughout but were present as spore forms in the lower epidermis and hair follicles at day 2 and showed a few early development forms at the papillary/reticular dermal junction at this time. By day 3 they were present in profuse numbers in individual niches especially in the area of the papillary



**Fig. 5.19. Hide Cross Section (x100) Stained by Gram**

(1) *C. histolyticum*: Reticular dermis (a) day 2, (b) day 8; (2) *C. sporogenes*: lower papillary dermis (a) day 2, (b) day 8; (3) *C. histolyticum*/*C. sporogenes*: Epidermal/dermal junction (a) day 2, (b) day 4; (4) *Ps. aeruginosa*/*C. histolyticum*/*C. sporogenes*: Papillary dermis (a) day 2, (b) day 8.

**Note:** Uninoculated controls showed no bacteria present at all and resembled the sample shown in 3(a) above.

dermis, epidermal-dermal junction and at the centre of the hide in both vegetative and spore-bearing form. They were also present in the upper and lower reticular dermis in profuse numbers but only in moderate numbers in the mid-dermis, until day 8 when they became numerous. (See Fig. 5.19 [2a & 2b]).

**C. histolyticum/C. sporogenes:**

**Masson Stain:** Apart from a slight loss of cornified epidermis there was no evidence of degradative changes occurring in the hides during the first three days of incubation. By day six little change had occurred except for some loss of reticular collagen. By day 8 marked changes had occurred throughout the tissue including increased reticular collagen loss with fibrolysis, some loss and fibrolysis of papillary collagen, absence of nuclei and almost total absence of the epidermal region and associated hair follicles and sebaceous glands. Fine fibre loss was present but not marked. (See Fig. 5.18 [3a & 3b]).

**Gram Stain:** Bacteria were not detected within the tissue at day 1 and were present only in small numbers in the papillary dermis on day 2 but were absent at the dermal/epidermal junction. By day 3 scanty to moderate bacteria were found in the whole of the reticular dermis and scanty bacteria were present in the epidermal area. From day 4 to day 6 progressively increasing numbers were found with most being located in the lower reticular dermis. While these were mostly vegetative forms at day 4, by day 8 spores predominated and the area under the epidermis showed the heaviest load while there was a slight reduction in load at the lower reticular dermis. (See Fig. 5.19 [3.b]).

**Ps. aeruginosa/C.sporogenes:**

**Masson Stain:** All areas of the hide remained intact during the first two days of incubation but by day 3 slight loss of general cell nuclei had occurred. There was moderate degradation in the epidermal layer with some epidermal delamination at the dermal/epidermal junction. Hair, hair follicles and sebaceous glands were slightly depleted. By day 4 there was total absence of epidermis and its adjacent structures including follicles and sebaceous glands with both the papillary and reticular fine fibres showing signs of degradation, and there was some slight loss in reticular collagen. On days 6 and 8 both reticular and papillary collagen showed slight loss with some fibrolysis in the reticular area. Cell nuclei were absent from day 4 onwards.

**Gram Stain:** Bacteria were not detected at days 1-3 in the internal tissues but by day 4 there were moderate numbers of Gram positive bacteria in the papillary dermis, many bearing spores and many free spores being present. In the upper and mid reticular dermis there were moderate to profuse spore-bearing Gram positive bacteria and a few also in the lower reticular dermis. This pattern of distribution of bacteria was evident at day 6 and also 8, except that the lower reticular dermis bacterial content had increased from slight to moderate.

**Ps. aeruginosa/C. histolyticum:**

**Masson Stain:** Slight losses of cornified epidermis and reticular fine fibres were evident at day 1 but all other areas remained intact. At day 2 the epidermis was absent and hair, hair follicles depleted in number but sebaceous glands were not degraded. Both papillary and reticular collagen were slightly depleted. At day 3 this depletion was increased and all collagen areas showed slight fibrolysis. Sebaceous glands were also moderately degraded. These, together with hair follicles and cell nuclei, were totally absent from day 4 onwards. By day 6 the papillary dermis was moderately degraded with some areas showing total degradation and, while the reticular dermis was less damaged, there were areas (close to the centre of the hide and papillary dermis) that showed total degradation. A similar picture occurred at day 8 but by this time the papillary dermis was completely absent.

**Gram Stain:** At day 1 bacteria were detected only in the lower reticular dermis and only in small amounts. By day 2 numerous Gram negative, and a few Gram positive bacteria, were seen in this area, while scanty Gram positive bacteria were also seen in the epidermis near the sebaceous glands. At this time also moderate numbers of these bacteria were present in the sporing state in the upper and mid-reticular collagen. Some Gram negative bacilli were detected in the epidermis at day 3 and these were also still dominant in the lower reticular dermis while the Gram positive bacteria in this area had progressed inward toward the hide centre in moderate numbers. By day 6, in the absence of the epidermis, a few Gram negative bacilli had invaded the papillary dermis along with moderate numbers of Gram positive spore bearers which, in the lower reticular collagen had become pleomorphic in shape. By day 8 there were both scanty Gram negative and numerous Gram positive bacilli throughout the remaining tissue.

*Ps. aeruginosa/C. histolyticum/C. sporogenes:*

**Masson Stain:** Evidence for some epidermal degradation, but not reticular collagen attack, occurred from day 1 onwards and this increased from moderate degradation at day 2 to absence of epidermis with almost total loss of hair follicles by day 3. This trend continued to day 6, by which time hair follicles, sebaceous glands and cell nuclei were totally absent. Collagen degradation was also progressive and by day 3 moderate attack of both papillary and reticular collagen was seen. Reticular collagen showed heavy fibrolysis from day 6 to 8, while papillary collagen underwent extensive fibrolysis by day 6 and was totally absent by day 8. (See Fig 5.18 [4a & 4b]).

**Gram Stain:** Bacteria were absent from the epidermal, papillary collagen, upper-reticular and mid-reticular collagen areas at day 1 but numerous Gram negative bacilli were present at the lower-reticular collagen edge. These bacilli remained in moderate numbers to day 4 by which time they had invaded both the sides and bottom edges of the hides. They were not found subsequently (day 6 to 8). The Gram positive spore bearing bacilli were not present in the inter epidermal area throughout but were found in moderate amounts in the papillary dermis and in moderate to numerous amounts in the upper and mid-reticular collagen. They were present in numerous numbers in the lower reticular collagen, showing only a slight fall off in numbers by day 8. (See Fig. 5.19 [4a & 4b]).

**Control - No bacteria:**

**Masson Stain:** The tissue remained intact throughout the incubation period and no loss of any layer or cell nuclei occurred.

**Gram Stain:** Bacteria were absent in all the sections examined.

**Bacterial colonisation of hides:**

The above Gram stain results of the seven different inocula types showed that *Ps. aeruginosa* colonised outer flesh surfaces only, *C. sporogenes* colonised areas between epidermal and papillary dermal junctions, and *C. histolyticum* colonised both flesh and internal areas including reticular and papillary dermis. Some examples of the Gram staining reactions by the different inocula are shown in Fig.5.19 where the individual inocula are shown colonising selected hide tissues. Note also the low numbers of *C. sporogenes* at day 2 in the individual

*C. sporogenes* inocula (Fig. 5.19[2a]) and the absence of *Ps. aeruginosa* at day 8 in the triple-mix inocula (Fig. 5.19 4[b]).

#### Limed and delimed samples:

None of these samples showed any evidence of accentuation in degradative changes for any of the inocula tested. Epidermal, hair fibre, hair follicle losses present were compatible with the process treatments that the hide samples had undergone. It therefore appears that the liming/deliming procedure as carried out in this thesis did not result in synergistic degradative effects.

#### 5.5. General Discussion:

Hides inoculated with *Ps. aeruginosa* showed no real evidence of degradation by any of the test assessments, although high total bacterial counts were evident in both inocula fluids and hide extrudates. The indication is that this organism successfully utilises free, interstitial amino acids and carbohydrates for growth without causing either decay or denaturation within the hide tissues.

This is the exact opposite of the effects produced in hides inoculated with *C. histolyticum* in which over 25% loss of hide mass occurred within 3 days of storage and after 8 days some 87% of the original hide piece was degraded. Ammonia and hydroxyproline levels were higher in this series than in any other with maximum values for each being recorded at days 3 and 6 respectively. This shows that collagen is not the initial protein catabolised by this organism and this is born out in the enzyme studies where moderate amounts of gelatinase and caseinase are produced at least 24 hours prior to collagenase. Organoleptic assessments up to +++ for all three parameters at day 3 corresponded to maximum ammonia levels and these preceded advanced putrefactive changes, as evidenced by maximum hydroxyproline levels, by at least 3 days. General histological findings were compatible with the other degradative parameters and both fibrolysis and dissolution of collagen fibres in-situ was visualised. Numerous bacteria were present in the mid and lower reticular dermis by day 2 and moderate numbers were also present at the lower epidermal/dermal junction, mainly in the form of spores.

*C. sporogenes* had a completely different distribution pattern within the hide to that of *C. histolyticum*. Bacteria were not present on either hair or flesh surfaces at all during storage but appeared in large numbers at the papillary/epidermal junction by day 2, many as free spores. From there they spread gradually throughout the hide. Bacterial numbers dropped to below pre-inoculation levels initially, probably due to the oxygen sensitivity of the organism. *C. sporogenes* attack appeared limited to hair follicles, interstitial proteins and epidermal layers probably due to a lack of collagenase as well as a requirement for absolute anaerobiosis. The strong proteolytic activity of *C. sporogenes* could be the cause of the papillary dermal fibre loss found in this series, since the increased collagen III content of this area would be open to attack by non-specific proteases.

Several different interactions occurred when the individual cultures were mixed as a 1:1 basis. With regard to *Ps. aeruginosa* and *C. histolyticum* in the double-mix of these organisms there was a drop in both collagenase and hydroxyproline levels, compared to *C. histolyticum* on its own but this was in proportion to the dilution factor involved. Histological evidence showed moderate progressive damage to papillary and reticular dermis and moderate grain damage from day 2 onwards.

Possible synergistic effects of reduced oxygen tension due to the aerobic respiratory activity of *Ps. aeruginosa* could have been the cause of the findings that *C. sporogenes/Ps. aeruginosa* double-mix resulted in greater activity of *C. sporogenes* compared to the individual *C. sporogenes* series. Bacterial succession was evident in this mix as *Ps. aeruginosa* gave counts that maximised at day 3 followed by maximal *C. sporogenes* counts at day 6. This was confirmed by histology which indicated absence of internal bacteria until day 4 when many free spores were present in the papillary dermis and moderate Gram positive bacteria in the reticular dermis. Ammonia levels were low throughout and neither hydroxyproline or sueded grain were seen in any of the samples.

Perhaps the most interesting of the interactive effects encountered in this study were those occurring between *C. histolyticum* and *C. sporogenes* as definite antagonism of *C. histolyticum* by *C. sporogenes* was shown for up to 4-6 days of storage, after which conditions reversed. All parameters, including organoleptic changes, reflected this antagonism and its sudden

disappearance at day 8 at which stage histological studies showed both fibrolysis of collagen tissues and general denaturation. The bi-modal growth curve of these two organisms combined, in conjunction with pH and mass loss values, indicates initial dominance of *C.histolyticum* followed by *C.sporogenes* followed by *C. histolyticum*. Suppression or loss of the collagenase activity of *C. histolyticum* occurred throughout storage except for the last day when slight amounts were present. This correlated to the appearance of moderate sueded grain after eight days storage. Bacteria were present in both the reticular and papillary dermal areas from day 3 onwards.

Bacterial succession was evident in the triple-mix inoculum system in which *P. aeruginosa* was the primary coloniser followed by the *C.histolyticum/C.sporogenes* mix. Collagenase was not suppressed in this series, being present from day 1, and attack on reticular dermis and sueded grain appeared between days 3 and 4. This correlated negatively with decreased total counts of *Ps. aeruginosa* and positively with increased total counts of *C. histolyticum* between days 3 and 4 indicating that *C. histolyticum* had become the dominant organism. This was confirmed by the increased hydroxyproline and collagenase levels at this time as well as the presence of Gram positive bacteria in both papillary and reticular dermis.

The finding of this study, that greater activity occurred in the *C. histolyticum* inoculated hides than in hides containing mixed inocula with this organism, is opposite to the established medical and food industry literature. As discussed in Chapter 1, *C. sporogenes* promotes invasiveness of *C. histolyticum* and *C. perfringens* in muscle myelonecrosis and is also involved in a microbial succession leading to the degradation of meat and meat products. In the case of myelonecrosis, the possible explanation for these different findings could be the differences in redox potential between living and post-mortem tissue. This could selectively enhance the growth of the micro-aerophilic clostridia. Another, perhaps more pertinent factor, common to both the medical and food findings is that of substrate composition, in that meat and muscle tissue are composed mainly of myosin with low collagen content and minimal keratin present, whereas skin (hide) contains high collagen content with small amounts of myosin but moderate amounts of keratin (Marieb 1992).

Post-mortem skin therefore provides both suitable redox potential conditions and abundant substrate availability for the proliferation of micro-aerophilic, collagenolytic clostridia.

Similarly the antagonistic effects of *C. sporogenes*, when dosed in mixed culture with *C. histolyticum* to hide samples, could be due to specific bacterial enzyme action on the different proteins present in skin (as compared to muscle and other internal tissues) resulting in metabolic by-products toxic to *C. histolyticum*.

### 5.5 Conclusion:

The overall conclusion of this in-situ hide study using *Ps. aeruginosa* and two selected clostridia, *C. histolyticum* and *C. sporogenes*, is that interactive effects take place in mixed cultures of these organisms and these have variable effects on hide degradation. These effects were most clearly demonstrated using hide mass and fluid changes, hydroxyproline levels, total bacterial counts and histological parameters. Bacterial succession was found in both the *Ps. aeruginosa* + *C. histolyticum* mix and the *Ps. aeruginosa* + *C. sporogenes* mix as well as in the triple-mix (*Ps. aeruginosa* + *C. histolyticum* + *C. sporogenes*) An important finding was that this succession was incidental to hide degradation in that pure cultures of *C. histolyticum* showed greater degradation rates in-situ than when present in any of the mixed cultures.

Another important finding was that of antagonism between *C. histolyticum* and *C. sporogenes* in the mixed inocula of these two organisms. This effect was present during the initial few days of storage but a reversal occurred between 4-6 days and thereafter degradative effects occurred due to the action of *C. histolyticum*. The initial suppression of activity was attributed to competitive inhibition by the *C. sporogenes*.

These findings have implication for use application to bio-control of hide preservation. Studies would need to be undertaken to determine the exact causes of the inhibitory effects. The bio-control method employed could either be indirect i.e. involve extraction, purification and application of the inhibitory substance to production hides (where applicable) or could involve direct applications of bacterial cultures under controlled conditions.

In terms of the routes and modes of bacterial tissue invasion the findings were that hide tissue was colonised differently according to the organisms inoculated. *Ps. aeruginosa* did not penetrate into internal tissues but stayed as a surface coloniser at the expense of interstitial soluble nutrients and as it was only weakly proteolytic, it caused no major damage to hide tissues nor any sueded grain. Conversely, *C. histolyticum* penetrated the hide from the lower (flesh) areas and spread rapidly throughout both the reticular and papillary dermis. Collagenase production by this organism resulted in severe hide damage including major substance loss and sueded grain. *C. sporogenes* occupied a unique niche within the hide at the dermal/epidermal junction and the base of hair follicles from where it spread through the hide tissue but, being non-collagenolytic, it produced little hide denaturation and no sueded grain. Mixtures of the cultures produced variations of the above findings depending upon the organisms involved. Sueded grain was only present if *C. histolyticum* formed part of the inocula except when it was in combination with *C. sporogenes* in which case no sueded grain was detected until the eighth day of storage.

## CHAPTER 6

### INVESTIGATION OF ANTAGONISTIC REACTIONS BETWEEN *CLOSTRIDIUM HISTOLYTICUM* AND *CLOSTRIDIUM SPOROGENES*

- 6.1. Summary
- 6.2. Introduction
- 6.3. Research Objectives
- 6.4. Results and Discussion
- 6.5. Conclusion

### 6.1. Summary:

The antagonism between *C. sporogenes* and *C. histolyticum* used in the model system was investigated. Crude extracts of collagenase and protease were prepared from the organisms, individually as well as the double-mix, using ammonium sulphate precipitation of cell free broth cultures (Appendix B). The broth cultures were prepared using normal anaerobic broth media with, and without, the addition of either 0,05% or 0,5% cystine respectively. Collagenase was applied to collagen gels either singly or with proteases.

The results indicated that two mechanisms could be responsible for antagonism: Either the production of cysteine due to cystine reduction and hydrogen sulphide deposition by *C. sporogenes*, or proteolytic digestion of *C. histolyticum* collagenase by *C. sporogenes* protease.

### 6.2. Introduction:

One of the major findings of the main experimental degradation study of this thesis was that of antagonism between the two strains of *Clostridium*. This result was unexpected as accounts of interactions between *C. sporogenes* and pathogenic proteolytic clostridia, responsible for myelonecrosis of muscle tissue in gas gangrene infections, indicated a synergistic role for this organism in the disease process (Smith and Holdeman 1968; Cruickshank et al. 1975; Willis 1977). In fact, for many years *C. sporogenes* was considered a pathogen due to its presence in gas gangrene (Katitch et al. 1964; Ross 1986) but it is now well established as a harmless saprophyte that acts as a contaminant in many infected wound cultures (Gillies and Dodds 1973; Willis 1977; Freeman 1979).

Unlike the collagenolytic clostridia (*C. perfringens* and *C. histolyticum*), *C. sporogenes* is virtually non-pathogenic for laboratory animals despite its highly proteolytic nature and its role in general putrefactive decomposition of proteins (Smith and Holdeman 1968). Its use of both glycine and cystine in amino acid energy-yielding fermentations of the Stickland reaction (Eskin 1971; Gottschalk 1979; Banwart 1987) would indicate that part of its synergistic effect could be due to the removal of collagen breakdown products within the decomposing system. There are however, reports in the literature of inhibition of various strains of *Clostridium* by *C. sporogenes* and this could arise from four different sources as discussed below.

It is well established that clostridia produce biocins against both their own and different species. Betz and Anderson (1964) showed that antagonistic reactions occurred between two strains of *C. sporogenes* due to biocin production. Smith (1975a) demonstrated the ability of both *C. sporogenes* and *C. perfringens* to inhibit *C. botulinum*, while evidence for biocin production between strains of *C. botulinum* (Lau *et al.* 1974) and between *C. botulinum* and bacteria from mud (Graham 1978) are recorded. Similarly there are reports of bacteriophages that cause inhibition between different clostridial strains (Betz and Anderson 1964; Nieves *et al.* 1981).

A third cause of inhibition that could account for the lack of activity in the double-mix *C. histolyticum*/*C. sporogenes* system could be the digestion of *C. histolyticum* collagenase by *C. sporogenes* protease. Woolley in 1980 demonstrated digestion of Collagenase III by the tissue enzyme trypsin, while Brüning in 1991 demonstrated that pre-exposure of *C. histolyticum* collagenase to protease, trypsin, thermolysin and chymotrypsin had detrimental effects on its activity. Mandl (1961 and 1972) showed that collagenase inhibition occurred due to microbial proteolytic enzymes other than non-specific clostridial proteases.

Another, less direct, cause of collagenase inhibition could be due to the release of inhibitory substances in the hide tissue arising directly, or indirectly, from *C. sporogenes* metabolic activities. Inhibitors of both microbial and tissue collagenases, have been studied fairly extensively. Collagenase has an absolute requirement for calcium to maintain activity while zinc is present at the active site of the enzyme so these metals play an important role in enzyme dynamics (Mandl 1961). Since collagenase is a metallo-proteinase metal chelators will inactivate the enzyme and in this regard, EDTA, cysteine and hydroxamic acids have all been shown to reduce activity. (Bienkiewicz 1983; Brown *et al.* 1989). Vencill *et al.* (1985), showed that zinc ligating groups in hydroxamic acids, carboxymethyl and thiols were responsible for enzyme inactivation. Tsigas *et al.* (1988) demonstrated inhibitory action of synthetic tri-peptides on collagenase activity while Birkedal-Hansen *et al.* (1988) demonstrated the inhibition of *Bacteroides gingivalis* collagenase by thiols. Both *Bacteroides gingivalis* and *B. cereus* collagenase were shown to be inactivated by Tetracycline and its chemically modified analogues, and this was attributed to their metal binding ability (Golub *et al.* 1987).

Many studies report on the role of cysteine in collagenase inhibition (Seifter *et al.* 1959; Lecroisey *et al.* 1975; Keil 1979) while Eisen *et al.* (1971) lists serum also as an inhibitor. Brüning 1991) cited glucose, sucrose and glycerol in laboratory media as a cause of collagenase suppression in actively growing bacterial cultures.

All the above aspects needed to be considered as the possible causes of the antagonistic effects of this study. The antagonistic effects in the double-mix *C. histolyticum/C. sporogenes* inoculated hides resulted in both decreased degradation rate and collagenase inhibition during the first six days of storage. (See Chapter 5). Evidence for some form of biocin, or bacteriophage, was not fully investigated here, as no noticeable antagonistic reactions between individual colonies of these organisms grown on solid media, nor any suppression of growth in broth culture, was evident throughout the study period (other than that recorded for the *C. histolyticum/C. sporogenes* double-mix).

Peptide fragments, carbohydrates and serum are all present in hides and if these substances were inhibitory they would be expected to react in both the individual and double-mix cultures containing *C. histolyticum*, which was not the case. Therefore it was postulated that the main cause of inhibition was due to an additional factor present in the double-mix culture, as opposed to the pure *C. histolyticum* culture.

In all the *C. sporogenes* containing cultures of this study, copious amounts of hydrogen sulphide were produced, as is characteristic of this organism. This organism was also shown in this study to localise in the sub-epidermal/upper papillary dermal area of inoculated raw hides. This area of the dermis contains large amounts of keratin and more collagen III than is found in other dermal areas (Stenn *et al.* 1992). Collagen III is distinguished from collagen I partly by the presence of inter-chain di-sulphide bonds at the carboxyl terminus of the triple helical sequences (Gerrard *et al.* 1983; Weiss 1984) The ability of *C. sporogenes* to attack hair follicle keratins with their associated cystine residues, and its use of cystine via the Stickland reaction, indicates that when inoculated to hides this organism could be capable of conversion of this amino acid to cysteine with resultant inhibition of any collagenase present.

The depression of *C. histolyticum* collagenase production in cultures mixed with *C. sporogenes* could also arise as a result of direct attack on collagenase itself, as this enzyme possesses two and a half cystine residues per mol and these play a role in its molecular stability (Keil 1979).

### 6.3. Research Objectives:

This chapter details the experimental tests which were carried out in order to show that antagonistic reactions between *C. sporogenes* and *C. histolyticum* were probably the result of cysteine production by *C. sporogenes* in the presence of available cystine. At the same time evidence was sought for proteolytic digestion of collagenase in the double-mix system. The research objectives are listed below:

1. To demonstrate that collagenase production in the double-mix of *C. histolyticum*/*C. sporogenes* broth cultures is reduced in the presence of increased cystine levels in the media.
2. To determine whether *C. sporogenes* proteases could significantly digest or denature *C. histolyticum* collagenases in mixed cultures of these organisms.

In order to demonstrate the possible role of cysteine production, as a result of *C. sporogenes* catabolism of keratins and other high cystine proteins in hides, an experimental trial was undertaken in which *C. histolyticum* and *C. sporogenes* were inoculated at  $10^6$  organisms  $\text{ml}^{-1}$  to a series of three broth types. The three broth series used were Robertson's Artificial Cooked Meat broth (RCB), RCB broth containing 0,05% cystine and RCB broth containing 0,5% m/v cystine.

Preliminary crude extracts of both proteases and collagenases were obtained by ammonium sulphate precipitation (Appendix B) and these were used to demonstrate the effect of both the low and high concentrations of cystine on collagenase production by the different inocula. The same enzymes and broth cultures were used to determine if digestion of *C. histolyticum* collagenase occurred in the presence of *C. sporogenes* protease.

#### 6.4. Results and Discussion:

##### Collagenase inhibition by cysteine

The crude ammonium sulphate preparations of collagenase, obtained from cell free extracts of broth cultures, of either *C. histolyticum*, *C. sporogenes* or their double-mix, incubated at 37°C for 24 h, were applied to collagen gels. Zone size measurements were taken (after overnight incubation of the gels at 37°C) and converted to mg ml<sup>-1</sup> collagenase by reference to a prepared standard graph (Chapter 2 and Appendix B). The results, given below in Table 6.1, indicate that in the broth with no additional cysteine there was a greater concentration of collagenase in the mixed inocula compared to the pure *C. histolyticum* culture.

**Table 6.1. Inhibition of *C. histolyticum* Collagenase Activity in the Presence of *C. sporogenes* during Incubation in Broth Cultures containing Cysteine.**

Organism	mg ml <sup>-1</sup> Collagenase in Crude Extracts of Broth Culture		
	(1) Normal Broth	(2) Low cystine broth	(3) High cystine broth
<i>C. histolyticum</i>	0,100	0,017	0,013
<i>C. sporogenes</i>	0	0	0
<i>C. histolyticum</i> / <i>C. sporogenes</i> 1:1 mix	0,150	0,007	0

(1) Robertson's Artificial Cooked Meat Broth - no additive.

(2) Robertson's Artificial Cooked Meat Broth + 0,05% cystine as cystine hydrochloride

(3) Robertson's Artificial Cooked Meat Broth + 0,5% cystine as cystine hydrochloride

Collagenase was not detected in any of the *C. sporogenes* precipitates. In the cystine broths there was suppression of activity in both the individual *C. histolyticum* inocula and in the double-mix. In the case of the double-mix there was complete absence of collagenase in the high cystine media. The decrease in collagenase in the individual *C. histolyticum* cultures with increasing cystine content, compared to that produced in the normal RCB broth, is attributed to the lowered Redox potential that would occur in the presence of cysteine. *C. histolyticum* is a micro-aerophile and as such its preferred Redox range will be between +85mV to +160mV (compared to levels of -150mV favoured by the strictly anaerobic bacteria) (Banwart 1987). It is therefore possible that reduced growth, with concomitant reduced enzyme activity, would occur in the lowered Redox levels of the cystine broths compared to those of the non-cystine broths.

The complete absence of collagenase in the double-mix inocula containing high cystine levels was in contrast to the synergistic collagenase response in normal RCB broth media, indicating that an inhibitory factor was present. In both the double-mix and the individual *C. sporogenes* media there was evidence of hydrogen sulphide production (as shown by blackening of the media). This was absent in the *C. histolyticum*-inoculated media. These findings indicated that cystine in the media was reduced in the *C. sporogenes*-containing cultures with the formation of cysteine and this was eventually metabolised with the release of hydrogen sulphide. Cysteine is a known inhibitor of *C. histolyticum* collagenase so that this finding is in accordance with Werb and Burleigh (1974), who showed that tissue collagenase activity was reduced by 64% in the presence of cysteine. Seifter *et al.* (1959 and 1970), showed that this reaction could be reversed in the presence of high zinc concentrations.

It is postulated by this author *C. sporogenes* cystine reductase activity in the double-mix culture resulted in cystine reduction to cysteine and this amino acid chelated zinc from the active site of *C. histolyticum* collagenase. Direct reduction of cystine residues in the enzyme itself by *C. sporogenes* could also have contributed to the loss of collagenase activity (Keil 1979).

#### **Collagenase denaturation by *C. sporogenes* proteases:**

To determine if the loss of activity was also directly, or partially, caused by proteolytic digestion of collagenase, the same collagenase extracts from the individual inocula of *C. histolyticum* and the *C. histolyticum/C. sporogenes* double-mix were added to crude proteolytic enzyme extracts of *C. sporogenes* (prepared by concentration with 20% saturated ammonium sulphate as detailed in Appendix B). Although the double-mix of *C. histolyticum* and *C. sporogenes* originally contained both *C. histolyticum* collagenase and *C. sporogenes* protease, previous trials by this author (unpublished data) had shown that variations in activity of the enzyme mixes occurred according to whether they were mixed directly in actively growing cultures or were first extracted and then mixed. These preparations were made at a 1:1 ratio (i.e. a 1/2 dilution of collagenase to protease) and 10 $\mu$ l volumes were then applied to 6 mm discs which were transferred to the surface of collagen agar gels. The preparations were then allowed to react at 30°C for 2 hours after which a further 10  $\mu$ l volume of each was added to 6 mm discs prior to application to collagen gels as above.

The results indicated that similar trends occurred as for the individual collagenase extracts since synergistic reactions occurred in both samples prepared from the normal RCB broth cultures (see Table 6.2.)

**Table 6.2. Interaction between *C. histolyticum* Collagenase and *C. sporogenes* Protease with and without Pre-exposure\***

Organism	Exposure Period at 30°C	mg ml <sup>-1</sup> Collagenase in extracts of broth cultures		
		Normal broth (1)	Low cystine broth (2)	High cystine broth (3)
<i>C. histolyticum</i>	Nil	0,074	0,016	0,011
<i>C. histolyticum</i> / <i>C. sporogenes</i>		0,089	0	0
<i>C. histolyticum</i>	120 min.	0,046	0,009	0,009
<i>C. histolyticum</i> / <i>C. sporogenes</i>		0,067	0	0

(1) Robertson's Artificial Cooked Meat Broth - no additive.

(2) Robertson's Artificial Cooked Meat Broth + 0,05% cystine as cystine hydrochloride

(3) Robertson's Artificial Cooked Meat Broth + 0,5% cystine as cystine hydrochloride

\* Prepared as a 1/2 dilution of *C. histolyticum* collagenase in *C. sporogenes* protease

This synergism is quite marked since the original collagenase concentration of the individual sample is diluted half in each of the test samples of this series ( 1:1 *C. histolyticum* collagenase: *C. sporogenes* protease) Therefore 0,074 mg ml<sup>-1</sup> collagenase as found in the *C. histolyticum* collagenase/*C. sporogenes* protease sample is equivalent to 0,148 mg ml<sup>-1</sup> collagenase in the undiluted sample. This is 0,048 mg ml<sup>-1</sup> more than that found for pure *C. histolyticum* collagenase alone (Table 6.1)

Similarly in the double-mix sample the collagenase content is equivalent to 0,178 mg ml<sup>-1</sup> collagenase compared to 0,150 mg ml<sup>-1</sup> in the individual *C. histolyticum* collagenase only sample (Table 6.1.) A similar synergistic response was also recorded by this author in previous trials (unpublished data) in which the two organisms were inoculated to hide pieces followed by ammonium sulphate enzyme precipitation with subsequent transfer to gels, either individually or as 1:1 mixtures of proteases and collagenases. The *C. sporogenes* samples (both protease and collagenase extracts) consistently gave no reactivity on collagen gels which indicates that proteolytic/collagenolytic synergism is the cause of the increased collagenase activity occurring

in these samples. Similar synergistic effects of collagenase with non-specific proteases have been reported in the literature (van Wart Bond 1982; Nethery *et al.* 1986).

Decreased enzyme activity was again evident in the cystine-containing broths of the *C. histolyticum* inoculated cultures and there was no activity at all in the *C. histolyticum/C. sporogenes* double-mix low cystine broth, probably due to the level being diluted below the detection limit of  $7,8 \mu\text{g ml}^{-1}$ .

The pre-exposed collagenase/protease samples showed loss of activity for both inocula in the normal broth samples ( $0,046 \text{ mg ml}^{-1}$  for *C. histolyticum* compared to the non-pre exposed sample level of  $0,074$ ) and a drop from  $0,089 \text{ mg ml}^{-1}$  to  $0,067 \text{ mg ml}^{-1}$  in the double-mix. Levels of collagenase in the *C. histolyticum* inoculated cystine broths showed a loss from  $0,016 \text{ mg ml}^{-1}$  to  $0,09 \text{ mg ml}^{-1}$  in the low cystine broth and a marginal loss from  $0,011 \text{ mg ml}^{-1}$  to  $0,009 \text{ mg ml}^{-1}$  for the high cystine broths. These results indicate that, as found by Brüning (1991), some digestion of collagenase does occur when collagenase is pre-exposed to non-specific proteases. In this study this was minimal especially in view of the long pre-exposure time (2h) at elevated temperature ( $30^{\circ}\text{C}$ ).

These results indicate that loss of collagenase activity in the model study of this thesis (as found for the double-mix of *C. histolyticum/C. sporogenes* samples) was probably not due primarily to digestion by non-specific proteases. However the above two experiments do not constitute proof, nor disprove, either of the two main theories concerning the cause of the antagonistic effects observed when *C. sporogenes* was mixed with *C. histolyticum*. It would be necessary to examine the levels of cystine and cysteine in both broth cultures and hide fluids during storage in order to confirm these initial findings.

It was beyond the scope of this thesis to take these investigations further. Nevertheless the antagonistic effects found during this study could play a major role in future hide preservation studies. If the effects are mainly due to the action of *C. sporogenes* on cystine then this could form the basis of a bio-control method of preservation to replace the more costly and time consuming industrial treatments presently in common use e.g. salting, biocide application and chilling. Of particular importance to a successful outcome of such studies would be the control

of the treatment temperature and redox levels to ensure growth promotion of *C. sporogenes* and growth suppression of micro-aerophilic/anaerobic collagenolytic clostridia. An added incentive to investigate this phenomenon further is the possibility of combining preservation methods with preliminary beamhouse processing as *C. sporogenes* cultures in the model system produced marked hair and epidermal loss. Neither the hair nor papillary dermis was degraded, nor was there any evidence of sueded grain, during the initial six days of incubation at 30°C (in either the fresh or limed samples). This is despite the general highly proteolytic nature of the organism. Most importantly there was no evidence of collagenase activity by this organism throughout the studies of this thesis and this further motivates for the choice of *C. sporogenes* in any future bio-technological studies involving combined bio-control/unhairing processes.

#### 6.5. Conclusion:

The antagonistic effect of *C. sporogenes* on the growth and collagenase activity of *C. histolyticum* was investigated. This was achieved by the use of crude collagenase extracts prepared from *C. histolyticum*, *C. sporogenes* and their double-mix (*C. histolyticum/C. sporogenes*) cultures after growth in normal anaerobic broth (Robertsons Artificial Cooked Meat Broth) with and without either 0,05% or 0,5% cystine addition. In the *C. histolyticum/C. sporogenes* double-mix inoculated cultures some synergistic activity was present in both the mixed collagenase, and mixed collagenase/ protease extracts. In the cultures containing cystine there was a notable antagonistic response with loss of collagenolytic activity and this was greater in the double-mix samples than in the individual *C. histolyticum* samples. The results indicated that inhibition of collagenolytic activity could possibly be attributable to catabolic cystine reduction to cysteine by *C. sporogenes*. Samples containing a 1:1 mix of *C. sporogenes* proteolytic enzymes with *C. histolyticum* collagenolytic enzymes (with and without prior exposure) also demonstrated a loss of enzyme activity in cystine media. Some slight loss of collagenase activity due to digestion by *C. sporogenes* proteases was noted in the pre-exposed samples. The possible use of some of these findings for bio-control and bio-technological processing of hides prior to tannage is postulated.

## CHAPTER 7

### BIODETERIORATION OF SALTED HIDES

- 7.1 Summary
- 7.2. Introduction
- 7.3. Research Objectives
- 7.4. Results and Discussion:
  - 7.4.1. Studies to determine if extreme halophiles produce collagenase.
  - 7.4.2. Investigation of a possible halophilic/halotolerant bacterial interaction in biodeteriorated salted hides.
- 7.5. Conclusion.

### 7.1. Summary:

Investigations into two aspects of hide degradation in salted stock were undertaken in order to extend the scope of bacterial hide degradation studies in general. The first was to establish whether members of the family *Halobacteriaceae* could be intrinsically involved in the degradation of salted hides by determining if they could produce collagenases active in high salt concentrations. This was achieved using individual strains of halobacteria isolated from salt used in the curing and tanning industry. The strains were inoculated, either as pure cultures, or mixtures of pure cultures, to solid halophilic media containing soluble collagen or collagen plugs. After incubation at 35°C for 30 days, subsequent transfer of syneresis fluid from each of these cultures was made to small test tubes containing collagen plugs, followed by incubation at 35°C for a further 30 days. Visual collagen loss was the criterium used to determine the presence of collagenase activity in these trials.

Two sets of liquid cultures containing collagen were also inoculated with the halophilic organisms and the first set was used for direct visualisation of the breakdown of collagen. The second set was used for ammonium sulphate precipitation of culture supernatants in order to concentrate any collagenase present prior to detection on collagen gels. No evidence of collagenolytic activity was detected in any of the 20 pure or 4 mixed cultures tested.

The second aspect of bacterial degradation in wet-salted hides concerned the investigation of a case of production stored stock showing signs of deterioration in the form of black stains on the flesh surface. Halophilic and halotolerant bacteria were isolated from the hides using nutrient broth cultures at different salt concentrations. Studies of hydrogen sulphide production, general cell morphology as well as histological effects on hide tissue (both deteriorated and EO hide pieces) were carried out to determine the cause of the stains. Bacterial isolates from a 15% salt broth culture were grouped into four groups based on their salt and respiratory requirements. These isolates, either individually or mixed, were inoculated to sterilised salted hides and incubated for 28 days at 30°C to determine the effects of these organisms on hide tissue as well as their ability to produce proteolytic and collagenolytic enzymes. The results indicated that the stains were probably a result of a bacterial succession or interaction involving aerobic *Halobacteriaceae* and anaerobic dissimilatory sulphate reducing bacteria of the genus *Desulfovibrio* or *Desulfotomaculum*. This appears to be the

first reported evidence for the possible role of these organisms in hide biodeterioration and further studies are needed to confirm these findings.

## 7.2. Introduction:

The first stage of hide processing post-flay involves curing or preserving the hides against bacterial invasion. While a number of different methods are available, the two most preferred methods of preservation in industrial use are salting (for extended storage) and biocide application (for short-term preservation). Sometimes a combination of these methods is used. During the course of the main study of this thesis two industrial enquiries arose which were pertinent to hide degradation studies, even though both involved salted stock as opposed to raw stock.

The first enquiry required an investigation of the colonisation of hides by a unique group of bacteria, the halobacteria. These bacteria are known to be implicated in the degradation of salted protein products including fish and hides (Hess 1942; Halligan 1985). The second enquiry concerned salted production hides which exhibited black discolouration of the flesh surfaces from causes unknown.

Collagen gels and plugs were used in the investigations of the hides colonised by halobacteria and an adaptation of the model system was used to help solve the problem of the stained production stock. It was considered pertinent to record the findings of these studies here as both have relevance to hide degradation and preservation. Also, in both cases, the methods developed for the main study of this thesis were shown to be adaptable for use in studies of both raw stock and salted stock, indicating their usefulness to degradation studies in general.

The traditional method of salt curing by saturation of the hide with sodium chloride is based on the historical use of salt which, since before 2700 BC, has been employed for both food and hide preservation (Baas-Becking 1931; Thompson 1988; Russell and Kohl 1989). Biocide application is a good alternative to salt as, when applied as spray or dip treatments, it ensures prompt inhibition of surface bacteria and rapid penetration into hide tissues (Russell and Galloway 1982). Salt, however is slower acting and takes much longer to penetrate hide tissues. Salt itself is not actively bactericidal but acts due to osmotic effect, ionic toxicity and

oxygen limitation within the system (Formisano 1965). Cooper (1971) showed that salt penetration of raw hides six hours after application was at approximately 50% of the initial load and that internal salt levels were only 8% (calculated on an "as is" basis). After 10 hours this value increased to 10% but it required 48 hours before saturation levels of 35% m/v salt were reached.

These figures are fundamental to prediction of the bacterial population shifts that will occur in salted stock during the initial stages of salt curing and since salting is the method of choice for long-term hide preservation, an understanding of these microbial interactions is essential to both hide degradation studies and hide preservation as a whole (Cooper and Galloway 1965; Russell 1984).

The addition of saturation levels of salt to hides will result in a unique ecosystem arising from the extreme abiotic environment so generated. Under normal raw hide conditions such as prior to salting, bacterial species diversity will be high but under the adverse conditions of high salt concentration, low species diversity, few physiological flora and obligate populations will prevail (Schlegel and Jannasch 1981). In fact only the extremely halophilic bacteria, the *Halobacteriaceae*, will proliferate on hides that are adequately salted (Larsen 1986). In the initial stages of salting only halotolerant and halophilic bacteria will survive and of these the halotolerant bacteria will only flourish if the salt cure is inadequate. Such conditions may arise due to the use of low salt brines or where salted stock is washed ("wetted back") to remove visible halophilic growth.

The halobacteria have a natural red pigment which consists of bacteriorubin (Larsen 1986). This highly visual pigment is responsible for the phenomenon of "red heat" which in the hide industry is regarded as evidence for poor or deficient cure. None of the halotolerant bacteria present would be detected by simple visualisation but any detrimental effects caused by them would be visualised as loss of quality in the finished leather product. Halotolerant bacteria could in fact be responsible for down-graded leather since collagenase activity has been recorded for the following halotolerant species, *C. histolyticum*, *C. perfringens*, *B. cereus* and *V. alginolyticus* (Kono 1968; Lecroisey *et al.* 1975; Makinen and Makinen 1987).

Conversely, although Kallenberger and Lollar (1986) postulated the production of collagenases by *Halobacteriaceae*, no definite proof of this has been established.

Additional factors in the degradation of salted hides include the effect of allochthonous organisms from salt, in creating a novel microbial community. These will consist of not only *Halobacteriaceae*, as discussed above, but also halotolerant species from marine and brackish environments. These will include *V. alginolyticus* and members of the genus *Desulfovibrio* and *Desulfotomaculum* (Pfennig *et al.* 1981; Larsen and Willenberg 1984). The presence of these organisms poses a potential threat to salted hides, to which raw or biocide treated hides are not exposed. This is especially so in the case of *V. alginolyticus* since many strains of this organism have been shown to produce highly active collagenases (Welton 1974; Thomson *et al.* 1971; Merkel 1972; Merkel *et al.* 1975).

Studies by Woods *et al.* (1971 a & b) indicated some correlation between aerobic and anaerobic bacterial counts in salted stock and leather decay but the species responsible were not identified. Optimum growth of *V. alginolyticus* will not occur at levels of greater than 30% m/v salt as is present in wet-salted material, and no growth will occur at levels of 35% m/v salt as occurs in dry salted stock. Since optimal enzyme activity usually occurs at optimal growth activity (which for *V. alginolyticus* is 3.0% m/v salt [Blake *et al.* 1980]), it would appear unlikely that this organism would be active in hide degradation, except marginally, and then only during the initial stages of salt curing.

In contrast to *V. alginolyticus* the halobacteria, which form the major flora of salted stock, could be responsible for hide damage, especially if they possessed collagenolytic enzymes capable of acting in high salt environments. These organisms are unique in that their proteins are both highly acidic and hydrophobic (Brown 1963; Lanyi 1974) and salt is responsible both for activation of their proteolytic enzymes as well as the stabilisation of their hydrophobic protein bonds. Salt is also required for the neutralisation of the ionic forces and this affects the creation of stable internal structures (Lanyi 1974). Unlike in other organisms, decreased salt levels result in reduced enzyme activity in halobacteria (Larsen 1967; Larsen 1986).

The proteolytic activity of halobacteria and their possible role in the degradation of salted protein products, including fish and hides, is well documented (Cloake 1923; Hess 1942; Venkataraman and Soreenivason 1954). The role of the extreme halophiles in hide degradation has been extensively studied by Formisano (1965) who isolated seven *Halobacter* strains from salted hides and used them to re-infect green hides. He found that all were capable of utilising amino acids including glycine, proline and valine and some also showed proteolytic activity. He did not test for collagenolytic activity in his isolates. Kallenberg and Lollar (1986) investigated halophilic collagen degradation using bacterial isolates from salted hides and hide brines. They used calfskins as a substrate to demonstrate collagenolytic and gelatinolytic activity from these organisms. The calfskins were pre-treated with 5% hydrogen peroxide, frozen, thawed and reconstituted in clean brines at 4°C for 24 hours. The mechanical stress of freezing together with the use of unsterile brines and incubation conditions of 35-40°C for up to 50 days, precludes definite confirmation of their findings of collagenolytic activity in some of the strains. Therefore, collagenolytic activity in halophiles, as a possible source of degradation in salted hides, remains an unresolved issue.

The biodeterioration of salted stock is not fully determined. While "red heat" provides visible evidence of red bacterial growth, other stains occur on the hides, some of which may be due to chemical application and others may be bacterial in origin. Cases of black-stained salt-cured hides have been reported in South Africa (A.E. Russell 1992 personal communication) but the cause of this was not fully identified. No reports of black deposits due to growth of *Halobacteriaceae* were found in the literature survey of this thesis. Therefore the possibility that this reaction occurred as a result of the growth of halotolerant organisms must be considered.

The process of "wetting back" salted stock to remove pigmented halophilic growth, as had occurred in the production salted stock of this study, could reduce salt levels in hide stocks to around 3,4M (20% m/v). Several halotolerant bacteria will grow at 2,5M (15% m/v) and a few will grow up to 3,5M. Changes which result in a reduction in the salt concentration of the stock will result in a shift in bacterial populations away from the aerobic *Halobacteriaceae* towards halotolerant aerobic, micro-aerophilic or anaerobic mesophiles (Schlegel and

Jannasch 1981). Since many of these possess collagenolytic enzymes this could prove detrimental to the hides both in terms of biodeterioration and degradation (Larsen 1967).

Bacteria capable of surviving high salt environments to become secondary colonisers in moderate salt-level conditions would require specialised means (such as spore production) to overcome salt stress. Also, both the strictly aerobic nature of the halobacteria together with the action of the salt would create anoxic conditions within hide tissues and this would limit the types of bacteria able to proliferate in such conditions to those possessing micro-aerophilic or anaerobic respiratory metabolisms. Other factors influencing the growth and survival of secondary colonisers would be relative humidity within the system and the water activity ranges ( $A_w$ ) of the individual organisms. Since  $A_w$  and solution osmotic pressure are inter-related it is possible to predict possible secondary colonisers of inadequately salted hides based on  $A_w$  values prevailing at selected sodium chloride concentrations. Another pre-requisite for secondary colonisation would be the possession of proteolytic enzymes, especially collagenases.

Using the above parameters, it is possible to select from the literature those bacteria that would be potential candidates as secondary colonisers in salted stock under conditions of reduced salt concentration (Waldvogel and Swartz 1969; Welton 1974; Blake *et al.* 1980; Christian 1980; Gottschalk *et al.* 1981; Hill 1981; Larsen and Willenberg 1984; Halligan 1985; Baumann and Schubert 1986; Cato *et al.* 1986; Kloos and Schleifer 1986; Mäkinen and Mäkinen 1987). A list of these bacteria and their salient properties is tabulated below in Table 7.1.

**Table 7.1. Halotolerant Bacteria Meeting the Criteria for Secondary Colonisers of Salted Hides under Conditions of Lowered Salt Content**

Organism	Water Activity		Spores	Preferred Respiration	Proteolytic enzymes	
	A <sub>w</sub>	NaCl (m/v %)			General	Collagenase
<i>V. alginolyticus</i>	0.86	18.18	Absent	Aerobic	+	+
<i>S. aureus</i>	0.86	18.18	Absent	Aerobic	+	D
<i>B. cereus</i>	0.95	8.0	Present	Aerobic	+	+
<i>B. megaterium</i>	0.95	8.0	Present	Aerobic	+	+
<i>B. subtilis</i>	0.90	14.1	Present	Aerobic	+	+
<i>C. perfringens</i>	0.95	8.0	Present	Anaerobic	+	+
<i>C. histolyticum</i>	ND	ND	Present	Anaerobic	+	+
<i>Desulfotomaculum</i>	ND	ND	Present	Strictly anaerobic	+	ND
<i>Desulfovibrio</i>	ND	ND	Absent	Strictly anaerobic	+	ND

Key: ND = No data D = Doubtful

Only the spore-bearing organisms from this list would be able to remain viable during storage of salted hides at saturation levels. If the hides underwent "wetting back" the anaerobic bacteria would have a competitive advantage in terms of regrowth due to anoxic conditions that would prevail post-halophilic bacterial colonisation. Of the bacteria listed in Table 7.1. the *Desulfovibrio* and *Desulfotomaculum* have the best competitive advantage in a salt environment since they use sulphides in salt as a terminal electron acceptor in dissimilatory energy-conserving reactions. Of the two genera, *Desulfotomaculum* are the most likely to survive the initial saturation salt levels of salted stock because they are spore producing organisms. Dissimilatory sulphate utilising bacteria are widespread in the environment, more particularly in estuaries and marine sediments, salt lakes and evaporation ponds. The copious amounts of hydrogen sulphide they produce from sulphates present in salt give rise to distinctive odours and also characteristic black deposits as a result of metal sulphide formation (Pfennig *et al.* 1981; Postgate 1986; Campbell and Singleton 1986). Schlegel and Jannasch (1981) emphasised the role of environmental changes (in the form of redox reduction and selective pressure of sulphate) in promoting growth of the anaerobic *Desulfovibrio* and *Desulfotomaculum*. For the above reasons these organisms were considered before the others named in Table 7.1. as prime candidates for the cause of blackening in salted hides.

### 7.3. Research Objectives:

The overall objectives of the studies in this chapter were to develop a greater understanding of hide degradation in general by examining salted hides for (a) collagenolytic activity at high salt concentrations and (b) deteriorative and/or degradative changes occurring as a result of bacterial interaction arising due to inadequate salting. The main research objectives were as follows:

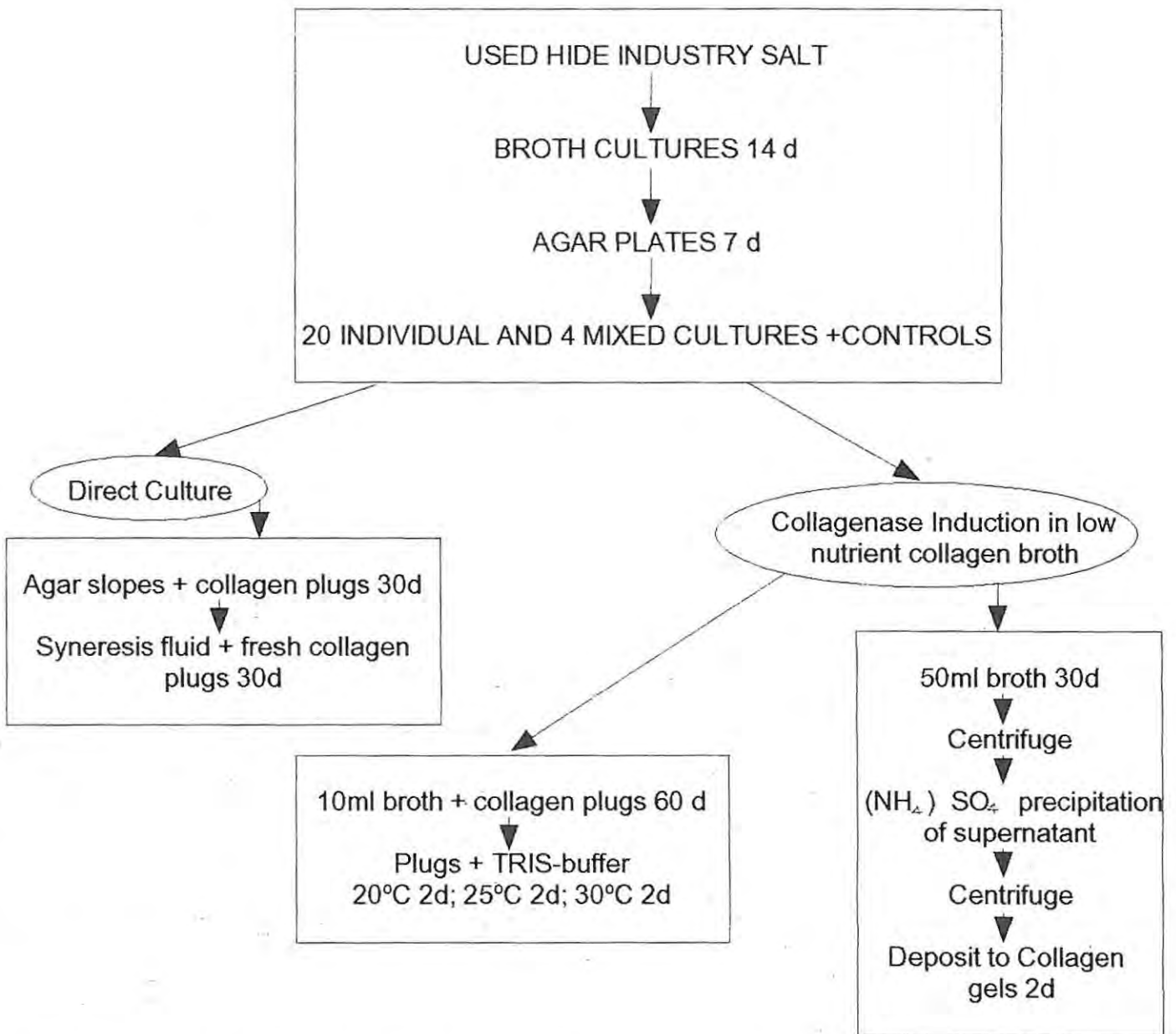
1. To show whether *Halobacteriaceae* produce collagenases that are active at a salt concentration of 4.3M (25% m/v).
2. To determine if black stains on salted hides are of bacterial origin and, if so, whether specific bacterial types are implicated.
3. To determine whether the process of "wetting back" salted hides results in a bacterial interaction which results in hide degradation.

To achieve these objectives the study was divided into two parts. In the first part pure strains of halobacteria were used to determine if *Halobacteriaceae* could produce collagenase. In the second part an industrial case of black staining of salted hides was investigated to determine the role of specific bacteria in this type of biodeterioration.

### 7.4. Results and Discussion:

#### 7.4.1 Studies to Determine if Extreme Halophiles produce Collagenase:

Pure strains from halobacteria isolated from industrial salts were inoculated to solid agars and broths, either individually or as a mixture of individual strains, and incubated at 35°C for 30-60 days. Detection of collagenolytic activity by the bacteria was achieved either directly by detection of collagen lysis in both agar and broth cultures, or indirectly by enzyme precipitation from broth culture supernatants. (See Chapter 2 and Appendix B for method details) Denaturation of fibrillated collagen plugs or lysis of collagen gels respectively was used as the criteria for evidence of collagenase production (Chapter 2). This approach involved the use of three different test series and these are shown in more detail in the experimental scheme depicted in Fig 7.1.



**Fig 7.1.** Experimental Scheme for the Investigation of Collagenolytic Activity during Growth of Halophilic Bacteria

**Note:** All agars and broths = 25% salt base  
All incubation at 35°C

A list of the cultural isolates and controls used in both the direct and collagenase induced trials is given in Table 7.2.

**Table 7.2. List of Bacterial Isolates used for the Investigation of the Action of Halophilic Bacteria on Fibrillated Collagen Plugs**

Code	Sample Nos.	Type of Culture	Source	Name
A	1-10	Pure	Clean unused sea salt	"Red Heat" extreme halophile
B	11-20	Pure	Dirty used sea salt ex hide curer	"
C	21	Mixed	Dirty used sea salt ex tannery stock	"
D	22	Mixed	Streak from cultures A1-10	"
E	23	Mixed	Streak from cultures B11-20	"
F	24	Mixed	Mixture C, D and E	"
G	25	Pure	Ex CSIR/environmental	<i>V. alginolyticus</i> *
H	26	Pure	Ex SAIMR	<i>B. cereus</i> *
I	27	Pure	Ex Rhodes University	<i>B. subtilis</i>
J	28	Pure on 3.TSA	Ex CSIR/environmental	<i>V. alginolyticus</i> *

Key: 3 TSA = 3% salt tryptone soya agar

\* = collagenase producing strains on agarose collagen gels

Controls (1) = non-inoculated halophile agar or broth

Controls (2) = 0,1 mgm ml<sup>-1</sup> sigma C. histolyticum collagenase in TRIS buffer pH 7.6

#### Direct cultures:

None of the halophilic isolates produced noticeable degradation of the collagen plugs contained in the halophilic agar slopes throughout the 30 day incubation period. Although growth of the *V. alginolyticus* was limited on the halophile agar, deteriorative effects were apparent after 7 days incubation, as visualised by slight degradation of the plug, but no increase in degradation was observed thereafter. Good growth of the *V. alginolyticus* occurred in the 3% tryptone-soya agar and moderate degradation of the plug occurred by the first day of incubation followed by strong degradation at 3 days and total plug dissolution by 10 days. The collagenolytic strain of *B. cereus* grew poorly on the media and failed to degrade the plug.

None of the plugs in the tube tests utilising the syneresis fluid from the agar cultures were degraded. This confirms the lack of activity found in the agar slopes with added plugs. Only the *V. alginolyticus* growing on 3% salt media was active amongst the positive control organisms. The *C. histolyticum* control enzyme solution diluted in the syneresis fluid of the non-inoculated halophilic agar slope proved negative. The syneresis fluid salt content of the slopes were analysed and found to be 23% m/v and this could account for the inactivity of this control enzyme solution. Russell and Pinchuck (1988), investigating the action of collagenase and collagenase inhibitors on limed hides, found that salt levels above 15% m/v inhibited *C. histolyticum* collagenase at 5mg ml<sup>-1</sup> concentration. Using a collagen film test Hockey and Russell (1983) demonstrated that collagenase at 0,15mg ml<sup>-1</sup> was inhibited by 20% m/v salt but not by 15% m/v salt. The findings of this study therefore confirm the inhibitory action of salt on collagenase produce by non-halophilic bacteria. The findings also indicate that the halophilic bacteria tested did not produce collagenases capable of activity at high (4.3M) salt concentrations.

#### **Collagenase induction:**

None of the plugs added to the test samples in the 10ml low nutrient collagen halophile broths were degraded during the 60 day incubation period. The *C. histolyticum* control enzyme solution was also inactive, and it is again postulated that this is a function of the high salt levels in the system. Once the plugs were removed from the broths to 0,05M TRIS buffer and incubated at sequential temperatures for 2 days each (20°, 25° and 30°C) degradation of some of the plugs occurred. This ranged from very slight to slight and was present in six cultures. These cultures consisted of one of the halophilic pure strains from the unused salt ex supplier, two of the halophilic pure strains from the salt ex curer, one of the mixed streak cultures from the unused salts, the mixed streak culture form the salt ex tanner and the mixture of all three streaks from the three salt types. Degradation was also present in the control *C. histolyticum* enzyme set. These results are shown in Table 7.3 below for the positive cultures only, as all other cultures gave negative results throughout.

Table 7.3. Halophilic Bacterial Degradation of Collagen Plugs after Pre-incubation for 60 Days at 30°C followed by 2 Days Sequential Incubation each at 20°, 25° and 30°C.

Sample Code	Degradation after 2 days at different temperatures					
	20°C		25°C		30°C	
	1	2	1	2	1	2
A2	0	0	+/-	0	+/-	0
B11	+	0	+	0	+	0
B19	0	0	+	0	+	0
C	0	0	+/-	0	+/-	0
D	0	0	+/-	0	+/-	0
F	+/-	0	+/-	0	+/-	0
Collagenase 0.1mg ml <sup>-1</sup>	+/-	+	+/-	+	+/-	+

Key: A-2 = pure culture ex supplier  
 B11,B19 = pure cultures ex curer  
 C = mixed culture ex tanner  
 1 = plug of monomeric collagen  
 2 = plug of polymeric collagen  
 D = mixed streaks culture ex supplier  
 F = mixed streak culture ex supplier and curer and tanner  
 +/- = very slight; + = slight degradation

The activity of the commercial collagenase in these tests as opposed to the previous direct tests is probably due to the TRIS-buffer maintaining the pH level around neutral. pH levels below 6.5 and above 9.0 are known to be inhibiting to collagenase activity (Mandl 1972; Bienkiewicz 1983) and this factor could have affected the outcome of the previous trials. The low reactivity of the commercial collagenase is, as before, attributed to the inhibition of these enzymes in the presence of high salt concentrations. The overall finding of this trial is one of minimal collagenase reactivity of the halophiles at salt concentrations ideal for structural stability of their proteins. This minimal activity is not conclusive proof of collagenase production since the long pre-incubation time of the plugs could have rendered them susceptible to non-specific proteolytic activity. This is indicated by the greater susceptibility generally shown by the plugs (1) which were formed from monomeric collagen as opposed to the polymeric form of plugs (2) [see Chapter 2]. None of the ammonium sulphate precipitated cultural supernatants from the 30 day cultures of the organisms in 50 ml volumes of low nutrient halophile broth caused lysis in the collagen agarose gels, again confirming the lack of collagenase activity in halophilic bacteria. This could, however, be ascribed to the collagenases of halophiles possibly possessing different molecular weights to those of

mesophilic bacteria. If so, then these would require extraction at different ammonium sulphate concentrations to those used in this study. This aspect of *Halobacteriaceae* proteolytic enzymes needs further investigation.

No conclusive evidence of collagenolytic activity from 20 pure and 4 mixed strains of extreme halophilic bacteria was detected by direct culture techniques in the presence of collagen plugs. Similarly, the results of the collagenase induction techniques (using collagen broth media and direct incubation with collagen plugs as well as indirect detection of enzyme activity by ammonium sulphate precipitation) indicated that extreme halophilic bacteria do not produce collagenases active at high salt concentrations.

#### **7.4.2. Investigation of a Possible Halophilic/Halotolerant Bacterial Inter-action in Biodeteriorated Salted Hides:**

The role of halotolerant bacteria as the cause of black stains in industrially salted hide stock (which had undergone "wetting back" to remove "red heat") was examined.

##### **Bacterial growth in the selective salt media**

Samples of the salt stained hides were cultured in fluid media at 15%, 20% or 30% m/v salt concentration (Appendix A) in which were suspended strips of blotting paper that had been dipped in 10% lead acetate in order to detect hydrogen sulphide production. Visual and histological examination of these cultures took place after incubation for up to 28 days at 37°C. The cultural broths were also examined after 8 days incubation for bacterial growth, cell morphology, cell motility and hydrogen sulphide production.

##### **Interaction between different bacterial groups**

To determine if bacterial interaction occurred during the biodeterioration evidenced in the form of black deposits on the salted hides, bacteria present in the 15% salt broth cultures were isolated by subculture to four media as follows:

- (1) Tryptone soya agar
- (2) Halophile agar
- (3) Postgate lactate enrichment media

(4) Robertson's cooked meat media followed by anaerobic blood agar.

The resultant isolates were grouped according to their growth on these media into four categories respectively:

- (1) aerobic halotolerant bacteria (AH)
- (2) aerobic halophilic bacteria (H)
- (3) anaerobic dissimilatory sulphate reducing bacteria (ANS)
- (4) anaerobic non-dissimilatory sulphate reducing bacteria (ANH)

Cultures from these organisms (at  $10^5 \text{ ml}^{-1}$  in 2ml volumes of 25% m/v salt solution) were added to EO sterilised 3 cm diameter hide cuttings and incubated for 28 days at 30°C. Fluid extrudates were used to determine fluid volume increase, hydroxyproline levels and enzyme activity on collagen, gelatin and casein gels. (Appendix B). Organoleptic assessments of the hide pieces were also effected. (Appendix B).

The results of these trials show that bacterial growth and hydrogen sulphide production was present in both 15% and 20% salt broths by the fourth day of incubation but visible growth in the 30% salt broth was present only after 8 days incubation. Hydrogen sulphide was not detected in the 30% salt broth culture even after 28 days incubation. All three cultures showed marked variation in the colour of the media after incubation (Fig 7.2).

**Fig 7.2. Hydrogen Sulphide Production and Media Discoloration due to Halotolerant and Halophilic Bacteria from Salted Hides after 28 Days Incubation**



Left = 15% m/v salt broth; Middle = 20% m/v salt broth;  
Right = 30% m/v salt broth.

The 30% salt broth changed to a reddish brown colour but the other two broths both turned black. Although the 15% salt broth was blacker than the 20% media both of these media showed hydrogen sulphide production as evidenced by the blackening of the lead acetate strip, and this was greater in the 15% salt broth culture. The media colour change from neutral brown to black only took place as bacterial growth occurred and this indicated that the cause of the black strains in the original hide sample was caused by bacteria and not by direct chemical reaction. This was further confirmed when wet preparations of the broth cultures, made with a standard 30 $\mu$ l loop, were examined for motility. It was seen that the amount of bacterial growth in these preparations correlated with the degree of media blackening and hydrogen sulphide production as detected by the lead acetate strips. The morphology of the bacterial cells was indistinct for the 30% salt broth cultures but the 15% and 20% broth cultures consisted of numerous rod shaped bacteria many of which were curved and resembled desulfovibrios bacteria. This tendency was more marked in the 15% salt broth preparation. These results are tabulated in Table 7.4 below.

**Table 7.4. Incubation of Black Stained Hide Pieces in 15%, 20% and 30% m/v Nutrient Broths : I. Bacterial Growth, Motility, Cell Morphology and Hydrogen Sulphide Production after 8 days at 37°C**

Broth Sample Type	Bacterial Growth and Appearance			H <sub>2</sub> S Production
	Growth	Motility	Morphology	
15% salt	+++	Very active	Mainly curved rods	+++
20% salt	++	Active	Mainly rods, curved and straight	++
30% salt	+/-	Nil	Indistinct	-

Similar findings concerning differences in cell morphology between the two lower salt concentration cultures and the 30% salt broth cultures were also shown in the Gram staining results of the histological section (Table 7.5 below).

Table 7.5. Incubation of Black Stained Hide Pieces in 15%, 20% and 30% m/v Nutrient Broths : II. Histological Changes in Hide Tissue after 8 days at 37°C

Broth Sample Type	Gram Stain		Collagen Stain *		
	Reaction	Shape	Hair	Epidermis	Corium
15% salt	Positive	Cocci	+++	++	+
	Negative	Rods			
20% salt	Positive	Cocci	+	+	-
	Negative	Rods			
30% salt	Positive	Cocci			
	Negative	Rods	+	++	-

\* Amount of degradation/loss + = slight; ++ = moderate; +++ = heavy  
- = no degradation/loss

Gram negative rod shaped bacteria were absent from the 20% salt broth exposed tissues but present in both the 15% and 30% salt samples. However the morphology of these rods varied between the two samples, with the 15% broth bacteria being much longer and thinner than the 30% broth bacteria. Curved forms present in 15% salt broth cultures were not found in the 30% salt broth culture. Anomalous results from Gram stains of *Halobacteriaceae* have been reported (Formisano 1965) and therefore the 30% broth Gram stain results should be viewed accordingly. The collagen stain reaction of this series indicated that hair loss together with epidermal and corium degradation occurred in the 15% salt broth incubated hide pieces but that while both hair follicle and epidermal attack was present in both the 20% and 30% salt broth tissues, there was no evidence for any collagen (corium) degradation in either of these two samples.

The overall findings from both the broth culture and tissue results indicate increased degradative effects in hides incubated in 15% salt broth compared to hides in the 20% or 30% salt broths. Since Gram positive cocci were found throughout the series it is concluded that the Gram negative curved and straight rods were most likely to be responsible for both the black stains and the degradative effects within the hide tissues. The cellular morphology of these organisms together with their ability to produce hydrogen sulphide and to degrade salted organic material suggests that they may be *Desulfovibrio* or *Desulfotomaculum spp.* derived from the original salt used to cure the hides. It is postulated that the Gram positive cocci in the 15% and 20% salt broth samples are most probably staphylococci, since these are halotolerant and grow actively in up to 20% salt concentrations (Christian 1980).

The anomalous results that occur in staining halophiles, as discussed above, precludes predictions based on Gram reaction as to the cause of growth in the 30% salt broths. However, the red pigment present in the media indicates the presence of *Halobacteriaceae* and the moderate epidermal loss that occurred in these samples in accordance with the proteolytic, but not collagenolytic, nature of this organism (Formisano 1965). Studies by Russell and Kohl (1989) concerning biocide application to control "red heat" on salted hides showed that grain damaged occurred in untreated samples after a few days storage at 35°C. In view of the active proteolytic enzymes possessed by this organism (Larsen 1986), these findings indicate a possible role for *Halobacteriaceae* in surface hide degradation.

#### Interaction between different bacterial species:

Isolates from the bacterial subculture of the 15% salt broths on to selected agars were divided into four groups (as described earlier):

- (1) AH = aerobic halotolerant bacteria
- (2) H = aerobic halophilic bacteria
- (3) ANS = anaerobic dissimilatory sulphate reducing bacteria
- (4) ANH = anaerobic non-dissimilatory sulphate reducing bacteria

Individual colonies from these groups were preliminarily identified using Gram stain reaction and cell morphology as well as colonial morphology. The ANH group were additionally identified using the API 32A system. Three strains of *Bacillus* (AH); three strains of *Clostridium*, (two of *C. difficile* and one of *C. perfringens*), (ANH); some presumptive *Desulfovibrio* (ANS) and several halobacteria strains (H) were isolated. These bacteria were maintained as separate mixed cultures prior to dosing at a final concentration of ca  $10^5$  ml<sup>-1</sup> to EO sterilised 3 cm diameter raw hide plugs contained in 5ml volumes of 25% sterile salt solution. This was followed by incubation at 30°C for 28 days. Unfortunately, the anaerobic dissimilatory sulphate reducing bacteria (ANS) cultures did not remain viable in stock culture and could therefore not be included in this study. The results of inoculating the other strains to hide pieces either as individual groups (H; ANH or AH) or as 1:1 mixtures of groups (H/ANH; H/AH or H/ANH/AN) are given in Table 7.6 below:

Table 7.6. Effects of Halophilic (H), Anaerobic Halotolerant (ANH) and Aerobic Halotolerant (AH) Bacteria on EO Hides Stored at 30°C for 28 Days

Bacterial Inocula	Hide Extrudate		Enzyme Action on Gels			Organoleptic	
	Vol.*	Hydro. <sup>x</sup>	Collagen	Gelatin	Casein	Hairslip	Malodour
H	0.14	2.4	-	-	-	+	+
ANH	0.25	2.5	-	-	-	++	+
AH	0.49	3.8	-	+++	++	+++	+
H/ANH	0.45	6.2	-	++	+	++	++
H/AH	0.33	1.1	-	+++	+++	+	+
A/ANH/ AH	0.59	4.3	-	-	-	+	+
Nil	0.28	0.7	-	-	-	+	-

Key: H = halophilic; ANH = anaerobic halotolerant; AH = aerobic halotolerant  
 \* = ml cm<sup>-3</sup> hide; <sup>x</sup> = µg hydroxyproline per hide piece.

Evidence of significant degradation, as determined by elevated tissue extrudate and/or hydroxyproline levels (see *C. histolyticum* Chapter 5 for comparison) were not found. Although, interestingly the halophilic/anaerobic halotolerant combination (H/ANH) gave marginally greater values for these parameters than the other organisms in this test series. The ANH group consisted of two strains of *C. difficile* and one of *C. perfringens* and while collagenase activity has been reported in both of these clostridia (Cato *et al.* 1986), proteolytic activity in non-halophilic bacteria has been considered inactive at levels of 10% m/v salt and above (Halligan 1985). It would appear, therefore that these findings could be due to some synergy between the two groups of organisms. Collagenase activity was not detected in any of the fluid extrudates but gelatinase and caseinase activity was present in the aerobic halotolerant group (AH) as well as the halophilic (H) combinations with both the aerobic halotolerants (AH) and the anaerobic halotolerants (ANH). All hides showed some degree of hairslip and malodour during the 28 day incubation period indicating the presence of mild proteolytic activity.

There was no evidence of hydrogen sulphide production or black stains on the hides in any of the samples tested but red discoloration was present on hides inoculated with the halophilic bacteria (H). The ANS strains growing in the Postgate media as black colonies were cut from the media and exposed to air for 24 hours. During this time the colonies changed colour from black to white, a finding which correlates with the disappearance of the black stains on the actual industrial stock of hides when these hides were taken and exposed to the air overnight.

The black stains were only present in hides that were stored at the bottom of hide stacks. It was concluded that lack of oxygen played a causal role since conditions arising at the bottom hides, from the pressure of the stacks, would promote anaerobic conditions.

It was concluded therefore that the most likely cause of salted hide discolouration, in the form of black stains, was due to a bacterial succession or interaction in which the combination of aerobic halophilic growth, reduced oxygen tension at the bottom of the piles of stacked hides and moderately high salt levels created conditions favourable to growth of the dissimilatory sulphate reducing bacteria. The proliferation of the anaerobic sulphate reducing bacteria in stock initially salted at  $>5,0M$  salt could occur as a result of lowered salt concentration occurring due to the process of "wetting back".

Survival of vegetative *Desulfovibrio*, particularly if surrounded by pockets of dung on the hide, is possible even in highly salted stock. The presence of Gram negative curved rods in the 15% salt broth cultures containing stained hide samples is indicative of this. However, the spore bearing *Desulfotomaculum* would have a competitive advantage of survival in salted hides and it is suggested that these would be the most likely secondary colonisers in any aerobic/anaerobic succession of salted stock. While there is established evidence for petroleum degradation as a result of an aerobic/anaerobic bacterial succession involving *Desulfovibrio* (Davis 1967), the role of these bacteria in hide degradation has not been previously considered.

It could prove of great interest to carry out further studies in which hides were dosed with these bacteria and *Halobacteriaceae* under varying conditions of redox potential.

The lack of collagenolytic activity in halobacteria, as shown in the first part of this study, indicates that they do not play a prime role in hide degradation except possibly in the formation of surface sueded grain due to general proteolytic attack. In contrast, possible interactive effects of *Halobacteriaceae* and either *Desulfovibrio* or *Desulfotomaculum*, under conditions favourable to organic degradation by the dissimilatory sulphate reducing bacteria, could prove to be a major factor in the degradation of inadequately controlled salted hide stocks. The cause of the black stains of the hides under investigation could also be attributable to hydrogen sulphide production by these bacteria.

### 7.5. Conclusion:

No evidence was found that *Halobacteriaceae*, the cause of "red heat" in salted hides, produced collagenolytic enzymes active at salt concentrations of 4.3M. None of the 20 pure or 4 mixed cultures of these bacteria, isolated from three different sources, were able to denature fibrillated collagen under conditions of 35°C and up to 60 days of incubation. No collagenolytic enzyme activity was detected in primary precipitated protein extracts of supernatants from broth cultures of these organisms as determined by lysis of collagen gels.

Black stains on salted hides were found to be of bacterial origin and were attributed to the process of "wetting back" of the hides. Both spore-bearing aerobic and anaerobic halotolerant bacteria, as well as dissimilatory sulphate reducing bacteria, were recovered from the hides at 15% and 20% salt levels. Studies on the aerobic and anaerobic halophilic strains as well as halophilic isolates showed that these were not responsible for the stains. Studies on the morphology of bacteria that generated black stains in 15% and 20% salt media as well as producing hydrogen sulphide, indicated that these were possibly *Desulfovibrio* or *Desulfotomaculum*. Histological studies on hide tissues indicated that degradative changes could occur as a result of their presence. Further in-depth studies are required to confirm the above findings and the possible interactive role of *Halobacteriaceae* with these organisms.

CHAPTER 8

**CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH**

### Degradation of hides:

Biodegradation studies in any industry are intrinsically linked to some form of microbial control such as preservation, sterilisation or disinfection (Block 1983; Busta and Foegeding 1983; Lawrie 1985; Gardner and Peel 1986;). In the hide and skin industry preservation of the raw material also involves an integrated approach using both microbiology and chemistry. From the early studies on hide preservation (Shuttleworth and Sebba 1939) to the later ones of the mid-1950's and mid 1960's (Nandy *et al.* 1956; Cooper and Galloway 1965) the microbiology of preservation was centred mainly on the estimation of surface bacterial numbers. At this time very few in-depth microbial studies were recorded in the literature and, somewhat surprisingly, this situation remains today.

In fact, surface bacterial counts have traditionally been used as a measure of hide degradation (Nandy *et al.* 1956; Cooper *et al.* 1969; Bailey and Haas 1988). Total surface bacterial numbers are important to certain industries as they represent possible spoilage and decay. This is of concern for products such as food, meat and pharmaceuticals where organoleptic factors play a role in product quality and acceptance by the purchaser. Conversely, for hides, initial spoilage or decay, providing it were well controlled, could contribute to preliminary removal of unwanted interstitial elements with possible reduction in subsequent early beamhouse treatments. Therefore total surface bacterial counts are probably more relevant to this aspect than they are to studies on putrefactive degradation.

Surface bacterial counts give some idea of total numbers per se but fail to differentiate between viable and non-viable organisms. They also do not reflect bacterial numbers within deeper hide tissues where putrefaction, as opposed to spoilage and decay, takes place. Bacterial count levels on meats (which should be microbiologically "clean" as they do not contain intrinsic resident populations) have been recorded at  $10^4$ - $10^7$   $\text{gm}^{-1}$  (Nortje 1987) and  $3 \times 10^7$  to  $4 \times 10^8$  Gill (1976). Bacterial numbers colonising hides post flay (which would be expected to be microbiologically "loaded") have, in contrast, been reported at  $2,5 \times 10^3$   $\text{gm}^{-1}$  (Bailey and Haas),  $6,8 \times 10^3$  -  $1,9 \times 10^6$   $\text{gm}^{-1}$  (Woods *et al.* 1971(b)) and  $5,6 \times 10^5$   $\text{ml}^{-1}$  (Nandy *et al.* 1956). It is postulated by this author that the use of various non-vigorous extraction techniques, as well as low hide mass to

extractant fluid ratios, may have resulted in non-recovery of more deep-seated resident bacterial populations.

Studies using either fluid extracts from hides or a combination of aerobic and anaerobic counts have also been used to estimate bacterial numbers (Woods *et al.* 1971(b); Woods *et al.* 1973; Woods *et al.* 1970(a)). Similarly, in order to correlate total bacterial counts with final leather quality, a number of assessment methods have been employed. These include collagenolytic activity, hydroxyproline release, histological examination and soluble and volatile nitrogen values. The number of tests, as well as the number of hides used varies according to individual author.

From the late 1960's through to the 1980's the scope of preservation and degradation studies in South Africa was increased. At this time some, or all, of the above methods were incorporated while the role of collagenolytic bacteria was specifically investigated (Woods *et al.* 1970(a); Woods *et al.* 1971(b); Woods *et al.* 1973). With the discovery of a collagenase-producing bacteria from salted hides (Thomson *et al.* 1972; Welton *et al.* 1972 and Welton 1974), the microbiological aspects of preservation changed focus, from non-specific total bacterial counts to collagenase producing bacteria. In particular a specific collagenase producing bacterial strain, isolated from salted hides and provisionally identified as *Achromatobacter iophagus* (later reclassified as *Vibrio alginolyticus*), was thoroughly investigated (Woods *et al.* 1973; Reid *et al.* 1978; Keil 1979).

As discussed in Chapter 1, *Vibrio alginolyticus* has no real place in raw hide degradation studies since it is of marine origin and has an optimum salt requirement of 3% m/v. Although it can exist in high salt environments (up to 20% m/v) (Thompson *et al.* 1986) it does not possess any special mechanisms, such as sporulation, to overcome the levels of salt above 25 % m/v which are found in adequately salted stock. Therefore, except for the use of this bacteria as a source of extracellular collagenase for biocide inhibition trials, studies on this bacterium appear to be of limited value to the hide and skin and leather industry.

Venkatesen (1979) reviewed the literature pertinent to the microbiology of leather manufacture from 1920 -1979 and he included a total of 117 references of which the first 74 were applicable

to raw hides and skins. Of these only seven references referred to the isolation of one or more specific bacterial types from hides and skins. Of these, three reported on the isolation of several different bacteria from salted skins, one reported the isolation of a staphylococcus from an unspecified skin and the other two reported the isolation of several different bacteria from salted goatskins. This indicates that there is a deficiency of pure microbiological studies in the hide industry and this was confirmed by the literature survey undertaken for this thesis.

Studies carried out in South Africa in the 1970's and 1980's investigated the preservation of hides and skins with "antiseptics" (Russell 1977; Russell and Galloway 1982; Russell and Kohl 1989) but did not involve extensive pure microbiological investigations. Similarly, reports of previous international hide degradation studies in which specific bacterial types or groups have been investigated, are extremely few in number (McLaughlin and Rockwell 1922; Anderson 1949; Everitt and Cordon 1955; Tancous 1961; Formisano 1965; Kallenberger and Lollar 1986; Ishii *et al.* 1987) compared to similar literature in the food and meat sciences. The majority of hide studies have been carried out on salted hides, which are mainly colonised by a small group of specific bacteria, the halophiles that have their own unique environmental niche. Raw hides, in comparison, will be colonised by a much wider range of different bacterial types, many of which will derive from the immediate environment in which the hide is placed.

#### **Hide degradation studies based on Koch's postulates:**

Hide studies based on Koch's postulates, in which bacteria recovered from hides are re-inoculated to fresh hides, are almost totally absent from the literature concerning hide decomposition. This author found only six pure microbiological studies, based on these postulates, in the literature (McLaughlin and Rockwell 1922; Anderson 1949; Everitt and Cordon 1955; Tancous 1961; Formisano 1965; Kallenberger and Lollar 1986). Formisano (1965) working with halophiles and Tancous (1961) working with an anaerobic *Clostridium* from salted hides did some classic work in this regard, however, neither worked with raw hides or included the role of bacterial succession in their studies.

Evidence of an aerobic/anaerobic bacterial succession involving clostridia in meat degradation and putrefaction process was established in 1902 by Tissier and Martelly. These findings were reflected

in the work of Ishii *et al.* (1987) who used broth cultures of aerobic bacteria and clostridia, isolated from raw hides, to demonstrate an aerobic/anaerobic interaction. They did not study micro-aerophilic /anaerobic inter-actions nor did they apply Koch's postulates in their experimental work. The importance of these postulates is that visualisation of, and specific evidence for, bacterial associated degradative changes within the tissue under study, is essential to the establishment of specific bacteria as causative agents of a degradative (disease) process.

The main purpose of this study, therefore, was to use basic microbiological principles (based on Koch's postulates) in order to develop a better understanding of in-situ hide events leading to putrefaction of hide tissues. This was to be achieved with special reference to the role of selected clostridia, *C. histolyticum* and *C. sporogenes*. It was also the aim to demonstrate whether hide degradation, like other forms of protein degradation, was just a "decay" process (as reported in a much of the hide and skin literature), or whether it involved the successive steps of spoilage, decay and putrefaction.

#### **Bacterial interaction in hides:**

The food industry literature, especially in the field of meat science, has established the role of bacterial succession in the degradation of meat proteins (Gill 1979; Banwart 1987). Meat as a substrate is similar to hide, in that it is a high protein, high moisture, post-mortem tissue containing collagen. The bacteria involved are documented as the *Moraxella/Acinetobacter/Pseudomonas* group for primary aerobic colonisation, followed by the micro-aerophilic clostridia (mainly *C. histolyticum* and *C. perfringens*) and then finally by the strictly anaerobic clostridia, including *C. sporogenes* and *C. novyi*. (Eskin 1971; Lawrie 1985). In the absence of any comprehensive microbial ecology studies of hides, it seemed logical to select representative bacterial inocula for hide bio-degradation studies from these bacteria found to be responsible for the putrefaction of raw meats. One representative bacterial species, *Ps aeruginosa*, *C. histolyticum* and *C. sporogenes* respectively, was chosen for each of the three stages of putrefaction as determined by respiratory and proteolytic characteristics (aerobic, micro-aerophilic and anaerobic; proteolytic, collagenolytic and amino acid degrading).

In order to confirm the suitability of the selected inocula for hide degradation, studies were carried out to show that these organisms were hide colonisers and that they had the ability to outcompete resident and/or transient hide populations. In all cases the findings confirmed these events.

#### **Sterilisation of hides:**

One of the main basic hurdles to achieving a suitable system for use in implicating specific bacteria as being responsible for differential degradative attack on hide proteins was the elimination of all extraneous bacterial activity. While previous studies had attempted to sterilise hides by use of chemical application (Everitt and Cordon 1955; Kallenberger and Lollar 1986) there was evidence that sterility of their samples was not achieved. This study undertook to investigate the use of ethylene oxide to achieve both sterile hides for a model system, and sterile collagen for enzyme studies, without resultant hide collagen denaturation. The results indicated that the sterilisation method was successful in that sterility was achieved without resultant tissue/collagen degradation.

The sterilisation of hides without denaturation of the proteins was a major achievement because most sterilisation techniques result in either protein denaturation or residual chemical activity (Gardner and Peel 1986) which could prove detrimental to any subsequent bacterial study. The sterilisation of hide pieces, as developed for this study, should prove of great value for other studies as both pure cultures and inter-actions between actively growing bacteria and enzyme preparations can be studied in-situ. The use of EO-sterilised hide pieces in the model system was shown to be adaptable to salted hide studies. With regard to the current interest in hide preservation by chilling (Gavend and Rabbia 1986) the system should prove to be invaluable for the detection of degradative changes in raw stock after inoculation with psychrophilic bacteria. It could be used to select out and study the recently reported strains of proteolytic *Bacillus cereus* (Nagarajan et al. 1990) as these could possibly play a role in chilled hide decomposition since some strains of *B. cereus* also possess collagenases (Petushkova et al. 1985).

#### **Use of a model system for hide degradation studies:**

The use of a system in which in-situ changes could be followed by the visualisation of bacterial invasion and tissue degradation and the simultaneous monitoring of other parameters, such as

bacterial numbers and enzyme activity, was essential to understanding bacterial dynamics in hide breakdown. A system was designed in which the simultaneous use of a large number of inoculated hide pieces enabled several different assessment parameters to be applied to individual samples during storage under controlled conditions. The use of a large number of different hides is also essential for statistically valid studies to be effected. As hides are expensive, it was important that any system set up was based on the use of small hide cuttings taken from many different animals.

#### **Results of the raw hide degradation studies using the model system:**

Using the model system of this thesis it was shown that the use of a large number of test parameters in degradation studies for hides was unnecessary. Only certain test parameters gave reliable indications of actual in-situ putrefactive changes, as opposed to spoilage and decay, events. These parameters were changes in hide weight, hydroxyproline content, aerobic and anaerobic counts (from hide surfaces and hide extrudates) and histological analyses of Gram and Masson stained 3  $\mu\text{m}$  tissue sections.

The most important findings that have arisen from the use of the selected inocula and model system of this thesis are those directly related to the elucidation of bacterial interactions during hide degradation as these have significance to both hide quality and preservation techniques.

The evidence of correlation between anaerobic bacterial counts in hides with putrefactive changes, as opposed to increased aerobic counts without putrefactive changes, was of significance to future hide studies as well as to the understanding of hide degradation dynamics. It emphasises the necessity to establish meaningful correlations between bacterial counts and in-situ degradative changes, as measured by other parameters such as ammonia and hydroxyproline release.

Another very important finding of this thesis which is very relevant to hide preservation is that bacterial succession in hide degradation was shown to occur as an aerobic (*Ps. aeruginosa*) /micro-aerophilic (*C. histolyticum*) sequence of events with the aerobic phase being dependent on interstitial nutrient availability without any attack on basic collagen molecules. The micro-aerophilic phase involved catabolic use of collagen and other polymeric proteins resulting in

the degradation of hide tissues. The study also showed very clearly that putrefactive degradation of hide collagen was effected only by the collagenolytic activity of *C. histolyticum*. However, contrary to a bacterial succession being a requirement for this degradative process, the results clearly indicated that *C. histolyticum* by itself can produce more rapid and more pronounced degradative changes within the tissue than when combined with either one or both of the other selected inocula. This is of great significance to the hide industry since this will affect all three aspects of hide preservation viz. laboratory trials of biocides, field trials involving individual hides and general industrial practices in current use.

### Hide preservation:

In terms of laboratory trials of new biocides and preservatives, one of these concerns the correlation of data from hide preservation studies with laboratory-based minimum inhibitory concentration (MIC) studies. Studies to date have often used mixed hide populations as the challenge inoculum (Chan-Henry and Russell 1987; Russell *et al.* 1990) and therefore these will be less likely to reflect in-situ events than those using selected bacteria based on the findings of this and future studies in which specific bacterial types causing degradation are identified. This study, therefore, has highlighted the importance of including a range of individual bacterial challenges in laboratory MIC studies which should encompass both Gram negative aerobic spoilage bacteria (*Ps. aeruginosa*, [Hayes 1985]), the facultative Gram positive anaerobic decay producing bacteria (*Bacillus sp* [Krieg 1981; Gordon 1981]) and especially the putrefactive anaerobic Gram positive collagenase producing bacteria (*C. perfringens*/*C. histolyticum* [Cato *et al.* 1986]).

These findings are also of prime importance in terms of general preservation of hides, because if certain bacteria are established as the prime degraders of hides then preservation techniques can be targeted specifically against these organisms. This would prove much more effective than the present non-specific use of biocides and biostatic agents used to kill or inhibit bacterial populations in general. (Elliott 1984). Since all chemical preservatives have varying degrees of activity against both Gram negative and Gram positive vegetative bacteria, as opposed to spore-bearers, selection of products based on sporicidal activity could eliminate unnecessary (and sometimes costly) field trials on new products. Clostridia, being spore bearing organisms, will be more resistant to

anti-microbials in general and will also be able to remain dormant on hides until circumstances allow for the outgrowth and germination of their spores. Since these organisms are also implicated in the putrefaction of foods, especially meat and meat products, much of the wealth of literature available on meat preservation will be relevant to hide preservation. (Robach 1980; Busta and Froegeding 1983; Lawrie 1985; Oloyede and Abalaka 1989). Preservation in the meat industry has often concentrated on the inhibition of spore germination and/or spore outgrowth ((Hugo 1971; Robach 1980; Blocher and Busta 1985). Other methods of preservation such as gaseous application (Newton et al. 1977; Clark and Takacs 1980; Davies and Gibbs 1994) as well as combinations of different preservative techniques, could be applicable to hides.

With regard to current industrial practices concerning hide preservation and storage, the findings of this thesis that collagenolytic micro-aerophilic clostridia can act on their own as primary degraders, emphasises the importance of generating good housekeeping practices that could prevent the proliferation of this and the other micro-aerophilic collagenolytic clostridia. A similiar approach was promulgated by Duerden (1994 ) who, in a review of anaerobic infections in man from 1890 to 1994, found that that most were a result of synergistic mixed infections with aerobic bacteria. His approach to the control of degradative tissue changes was twofold, namely to remove necrotic tissue and establish well perfused tissue and to apply appropriate anti-microbial treatment. To keep hides "well-perfused" requires the avoidance of anoxic situations during handling and storage.

Collagenolytic micro-aerophilic clostridia exist in niche environments within individual hides, and these bacteria will undergo selective enrichment in raw hides as post-mortem tissue respiration creates lowered redox potential. Similar micro-aerophilic environments can be created due to extraneous factors governed by the hide industry itself. These will include any folding/stacking or pile formation of hides or individual areas within a given hide. Hair to flesh configurations will promote the transference of clostridia from their natural habitat on hide surfaces to the more readily degradable flesh surfaces. The presence of dung on the hide surfaces will serve as an extra rich reservoir of clostridia as it contains all three of the officially recognised collagenase-producing strains of clostridia (*C. perfringens*, *C. histolyticum* and *C. difficile*) in relatively large amounts (Cato et al. 1986). Folding or creasing of the hides during handling post-flay, in which

hair-to-flesh or flesh-to-flesh contact occurs, will create micro-aerophilic niche environments. Redox reduction will be more pronounced where there are flesh to flesh contacts since collagen surfaces, being very gelatinous, form almost glue-like adhesions between one surface and the other.

The industrial practice of forming high piles or stacks will result in increased pressure on the bottom samples which could result in sufficient oxygen depletion to initiate spore germination and outgrowth in otherwise dormant clostridia. Similarly if hides have undergone a wash and not received adequate draining, the excess moisture on the hide surfaces will promote spore re-growth. Gavend and Rabbia (1986) in a study of preservation of skins by chilling and freezing found that skins washed before freezing had increased bacterial loads of forty times the original within four months of storage, compared to only twice the original for non-washed hides. These findings were also reflected in increased grain damage on the processed leather.

Other industrial practices, such as the use of tarpaulin and other light-excluding covers to protect hides in transport, will also promote the growth of bacteria in the enclosed hides. This would also promote clostridial outgrowth, more especially if the covers were pulled tightly over stacked hides so creating reduced oxygen tension. (In this regard the model system used in this study obviated problems that can be encountered in laboratory trials when storing hide cuttings in plastic bags since areas of moisture on the hides will result in clinging of the plastic to the hide surfaces, creating areas of reduced oxygen potential and promoting micro-aerophilic growth (Sinell 1980(b)). This will be particularly pronounced where the plastic is impermeable to oxygen although, conversely, this will have the effect of retarding psychrophilic pseudomonads in chilled products (Lawrie 1985), resulting in possible increased storage margins).

Gill (1976) studying bacterial succession in meat degradation showed that, where two organisms are utilising the same fermentable substrate, the organism with the fastest growth rate will dominate initially and subsequently the organism with the greatest affinity for the substrate will be dominant. In this study *C. histolyticum* not only had a similar growth rate to that of *Pseudomonas*, as determined by its generation time but it also possessed the greatest affinity for hide tissue of all the

three selected inocula due the possession of highly active collagenases, gelatinase, hyaluronidase, and elastase (Cato *et al.* 1986). It is postulated that *C. perfringens*, which has the same growth characteristics and the same enzyme profile, will also degrade hides in the same manner. Therefore preservation methods applied to raw hides must take cognisance of this and aim to achieve well-aerated storage conditions in which the prevention of germination and outgrowth of spores is a priority. In this regard the practice of dropping hides in piles at abattoirs to await transportation to the curers must be avoided. All transportation of hides prior to preservation should be carried out in open, well ventilated containers and/or vehicles. A system of individual storage using palletisation of hides on open mesh pallets would prevent hair to flesh contact, retain aerobic conditions and delay bacterial putrefaction.

When hides are not preserved they will biodeteriorate which will promote bacterial proliferation as there are numerous niche environments that can exist on hides and the findings of this study of the competitive degradation of hides by *C. histolyticum* explains why hide degradation is rarely seen as a total destruction of the hide but appears as patches in different areas, some of which are in a far greater state of decomposition than others (Woods *et al.* 1973) It is also notable that only *C. histolyticum*, out of all three of the organisms tested, caused sueded grain, despite the fact that *C. sporogenes* produced strong proteolytic activity both in hide extrudates and directly in hide tissues.

#### **Bio-preservation of hides:**

Another very important finding of this study was the antagonistic effects between *C. histolyticum* and *C. sporogenes*. The preliminary investigation into the cause of this indicated that cysteine, formed as a result of the action of cystine reductase by *C. sporogenes* in the epidermal regions, was responsible. This, together with the proven non-collagenolytic status of *C. sporogenes*, as well as its ability to unhair rapidly and attack papillary interstitial proteoglycans without denaturation of the basement membrane indicates that it has high potential for bio-preservation. Unlike the collagenolytic clostridia it has a broad optimum temperature range, retaining its activity at temperatures below that of maximum collagenase production by the collagenolytic clostridia. It is also active at much lower redox potentials than the collagenase producing strains. These two facts could be used to create conditions for its competitive growth in raw hides. Such conditions

should be relatively inexpensive to provide in a modern tannery or curing operation and since partial beamhouse process could be achieved simultaneously no extra capital equipment need be entailed.

New methods for hide and skin preservation are being sought to replace some of the existing ones, since they all have some drawbacks. Chilling requires capital expenditure, while the salt from salted stock is a problem as it is environmentally a pollutant, and biocide treatments are expensive but not necessarily active against the degradative bacteria. Some new form of preservative action which could effectively reduce both the time and costs of treatment would be invaluable. To generate such a preservative method from the findings of this study is feasible and the use of the model system would facilitate this. Variations in the time of application, the temperature of the treatment and the inoculum concentration could be effectively studied in-situ without recourse to costly chemicals and a large number of hides as samples.

#### **Shortcomings of this thesis:**

As long ago as 1922 McLaughlin and Rockwell, who did some excellent classic microbiological studies on the effects of proteolytic bacteria on hide decay stated that "while previous contributions had proved the important role of bacteria and their enzymes in the curing and tanning of hides, no systematic studies on the bacteria as a whole have been carried out". They also commented that "the subject will not become clear to us until we understand the action of bacteria in each successive step of the process".

It is felt that this study has generated a considerable amount of further information that will contribute not only to the basic knowledge of hide degradation but will also form the base for more specific and possibly innovative methods for hide preservation. In order to maximise the findings of this study the shortcomings in this work must be rectified. While the model system proved to be useful for both raw and salted hide studies, it nevertheless remains an artificial system. It could have been more representative of in-situ storage conditions if the bacterial inocula had not been added as 5 ml volumes of bacterial suspension as this created a somewhat artificial environment. It would be better for future studies if they were sprayed onto the hide surface. Alternatively the

hides pieces could undergo a short dip treatment into pre-prepared inocula and then be drained before placing in the jars. This would more nearly represent industrial treatments of raw hides.

Another improvement to the system would be the inclusion of a small perforated stainless steel or plastic platform at the base of the jar holding the sample. This would ensure that no micro-niche environments that could promote anaerobic growth above that in the rest of the jar, were present.

#### Future research areas:

Very many more bacterial types need to be investigated for their action on hides and these need to be studied particularly in relation to sueded grain. While this study demonstrated that different bacteria could and did enter the hide via different routes, the role of proteolytic bacteria in sueded grain was not elucidated. In this regard the role played by *Proteus* sp. in de-hairing (Venkatesen 1979) and therefore by implication its possible attack on membrane proteins needs investigation. In particular investigations into the collagenolytic strains of *Bacillus* are long overdue as these proteolytic, aerobic and facultatively anaerobic bacteria could be candidates for the cause of sueded grain. The discovery of psychrophilic *B. cereus* strains (Nagarajan et al. 1990) has implications for possible degradation of chilled hides, as have the highly proteolytic psychrophilic *Pseudomonas* sp.

Hide degradation by anaerobic bacteria, purported to produce collagenase enzymes, other than *C. histolyticum* and *C. perfringens* has not yet been investigated. These would include *Bacteroides gingivalis* (Marsh and Martin 1992), *C. collagenovarans* (Jain and Zeikus 1989) and *C. difficile* (Cato et al. 1986) as well as other *Bacteroides* species and *Actinobacillus actinomycetemcomitans* (Robertson et al. 1982). Of great interest too, is the finding of this study that *Desulfovibrio* sp. could be implicated in the degradation of salted hides and this is the first reported reference in the literature to this organism as a possible cause of hide degradation. Since it is ubiquitous in salt environments it is essential that this organism be considered as a potential cause of deteriorative changes in salted hides and skins. Studies on salted stock should therefore include this as a challenge organism for MIC studies of biocides for use in salted stock. Both the raw and salted hide studies of this thesis have resulted in highlighting the necessity of using specific bacterial types

in laboratory MIC studies of biocides for use in the hide and skin industry. This is in accordance with the findings of Thompson (1987(a),(b),(c) and 1988) and Thompson *et al.* (1988 (a) and (b)) concerning method development for comparative testing of biocides for green hide application.

Perhaps the biggest oversight of this, and previous, studies has been the omission of investigations into the action of bacterial elastases and their effect on final leather quality. Similarly the effect of specific bacterial proteases, other than collagenase I, on the laminin, collagen IV and collagen VII of the basement membrane needs to be assessed in order to define events leading to sueded grain.

The model system set up for this study proved to be a very useful tool for studying bacterial and enzyme effects in hides and could be adapted to many other research studies. The use of immuno-histochemistry, both to detect enzyme reaction in denatured tissues within hides, as well as to identify specific bacterial types in histological sections would greatly aid the elucidation of bacterial/ enzyme dynamics in hide degradation.

In summary this study established that interactive effects occurred between aerobic *Ps. aeruginosa*, micro-aerophilic *C. histolyticum* and anaerobic *C. sporogenes*. These effects included both bacterial succession as well as bacterial antagonism. Succession was incidental to hide degradation in that pure cultures of *C. histolyticum* achieved greater degradation than mixed cultures containing either, or both, of the other two organisms. Antagonism between the two clostridia species delayed hide degradation by six days and this was attributed to cysteine release as a result of the metabolic activity by *C. sporogenes*. It was postulated that this finding could be used as a basis for better preservation, including possible bio-preservation of hides.

## APPENDIX A

### MATERIALS

- A.1. Bacterial Media
- A.2. Solutions and Buffers
- A.3. Histological Fixatives and Stain
- A.4. Equipment

### A.1. Bacterial Media and Stains:

#### General:

1. All sterilisation was done by autoclaving at 121°C for 15 mins. at 15 lb inch<sup>-2</sup> unless otherwise stated.
2. All techniques for bacterial manipulation, subculture and maintenance were carried out under aseptic conditions either by use of laminar flow or heat sterilisation by gas burner.
3. Unless otherwise stated all solutions were made up in de-ionised water.

#### BLOOD AGAR:

	(g/l)
Tryptose	10,0
Lab-lemco Powder	3,5
Agar	12,0
Sterile Horse Blood	70,0ml
pH 7,4	

#### CASEIN GELS:

	(g/l)
Hammerstein casein	2,5
Sodium chloride	5,0
CaOH	0,15
CaCl <sub>2</sub>	0,05
Agar No. 1. (Oxoid)	20,00
Skim Milk	5,0
pH 7,0	

#### COLLAGEN GELS (for enzyme detection in fluids and tissues):

1. Agarose (high gel point) is prepared at a 2% concentration in water, melted and cooled to 47°C and the pH adjusted to 7,0 using 0,2N NaOH and distributed in 5ml volumes.
2. 4,5ml neutral EO collagen solution (prepared according to Merkel *et al.* 1975) plus 0,5ml of modified 10 x TRIS-buffer is incubated for exactly 10 mins. in a 37°C water bath.
3. 5ml 2% agarose gel at 47°C is added to the 5ml neutralised collagen at 37°C, vortexed lightly, and poured into 90 mm pre-warmed Petri dishes.
4. After setting, the gels are exposed to 10-15 mins. laminar flow (without UV light) to dry.

5. After incubation 20%  $\text{HgCl}_2$  is added to aid lytic area visualisation.

#### COLLAGEN NUTRIENT MEDIA:

This is made as above with the substitution of Tryptone Yeast Extract Agar media for 2% agarose.

<u>TRYPTONE YEAST EXTRACT AGAR:</u>	(g/l)
Tryptone	2,0
Sodium chloride	2,0
Zinc acetate (0,002M)	0,044
Yeast Extract	1,0
Noble agar	3,0

Heat to dissolve and cool to 47°C\*, adjust pH to 7,4 - 7,6. Sterilise 121°C 15 minutes.

\*(It is very important to get this temperature exact, higher temperatures will cause collagen denaturation and lower temperatures will result in a non-homogenous collagen/media mixture).

#### COLLAGEN PLUGS:

Collagen prepared as for collagen gels (but with addition of 0,2% sirrius red in the modified 10 x TRIS-buffer) is added in 0,2 ml aliquots to 96 well (0,4 ml volume) micro-litre wells and left to fibrillate at 37°C for 24 hours. The plugs are removed to sterile Petri dishes (for storage at 4°C prior to use) by means of washing with 0,002% sirrius red in 0,05M TRIS-buffer.

<u>FLUID THIOGLYCOLLATE BROTH:</u>	(g/l)
L - Cystine	0,50
Agar	0,75
D - Glucose (monohydrate)	5,50
Yeast Extract	5,00
Pancreatic Digest of Casein	15,00
Na thioglycollate	0,50
Resazurin solution 1/1000	1,00 ml
pH 7,3	

GELATIN GELS: (g/l)

Agar No. 1 (Oxoid)	10,0
Gelatin	4,0

After incubation flood plates with 15% HgCl<sub>2</sub> in 20% m/v HCl to visualise lytic areas

HALOPHILE BROTH: (g/l)

Sodium chloride	250,00
MgSO <sub>4</sub> 7H <sub>2</sub> O	20,00
KCL	2,00
Tri-sodium citrate	3,00
FeCl <sub>2</sub> (0,46%)	0,5 ml
Yeast extract	10,00
Casamino acids(Difco)	7,5

pH 7,5 - 7,8, heat 121°C 5min. cool, filter, adjust pH to 7,4. Autoclave 121°C 15 min.

HALOPHILE AGAR:

As far Halophile broth with the addition of 20g of agar per litre.

LOW NUTRIENT HALOPHILE COLLAGEN BROTH:

	(g/l)
Sodium chloride	250,00
MgSO <sub>4</sub> 7H <sub>2</sub> O	20,00
KCL	2,00
Tri-sodium citrate	3,00
FeCl <sub>2</sub> (0,46%)	0,5 ml
Yeast extract	1,00
Casamino acids(Difco)	0,75
Acid collagen solution	400 ml

Add dry ingredients to 600 ml of distilled water and add in acid collagen solution. pH 7,6. Acid collagen solution = preliminary glacial acetic acid extract of collagen (prepared according to Merkel et al 1975) in 300 ml modified TRIS-buffer at pH 7,6.

<u>McCONKEY AGAR:</u>	(g/l)
Peptone	20,0
Lactose	10,0
Bile Salts No. 3	5,0
Sodium chloride	5,0
Neutral Red	5,0
Agar No. 3	0,075
pH 7,4	

<u>MODIFIED CALCIUM CASEINATE AGAR:</u>	(g/l)
Peptone (Oxoid)	5,0
Lab Lemco (Oxoid)	3,0
Casein (Hammerstein)	2,5
CaOH.6H <sub>2</sub> O	0,15
CaCl <sub>2</sub>	0,05
Agar	30,00
Skim milk powder (Oxord)	10,0
pH 7,0	

After melting and cooling add 1 ml of 1% 2, 3, 5 triphenyltetrazolium chloride per litre of media.

#### MODIFIED REINFORCED CLOSTRIDIA MEDIA:

For counting *C. histolyticum* and *C. sporogenes* in mixed cultures - see Appendix B.

<b>1. Modified Reinforced Clostridia Agar:</b>	(g/l)
Yeast Extract	3,0
Lam-Lemco Powder	10,0
Peptone	10,0
*Soluble starch	1,0
Sodium chloride	5,0
Sodium acetate	3,0
Cysteine HCl	0,5
Agar	20,0

Note: Increased agar content to prevent spreading of colonies.

\* Omitted from Modified Reinforced Clostridia Agar 1 (MRCA1) and included in MRCA2

Andrades indicator 10,00ml

pH 6,8

Sterilise and cool to 45°C. Add 100ml 5% (0,45 µm filter sterilised) Dextrose

#### ANDRADES INDICATOR

0,5% acid fucshin adjusted with 1N NaOH till just pale yellow.

## **2. Reinforced Clostridia Broth**

As for Modified Reinforced Clostridia Agar but with omission of the agar.

<u>NUTRIENT AGAR:</u>	(g/l)
Lab-lemco Powder	1
Yeast Extract	2
Peptone	5
Sodium chloride	5
Agar No. 3	15
pH 7,4	

<u>NUTRIENT BROTH:</u>	(g/l)
Lab-lemco Powder	1
Yeast Extract Powder	2
Peptone	5
Sodium chloride	5
pH 7,4	

<u>PLATE COUNT AGAR:</u>	(g/l)
Tryptone	5,0
Yeast Extract	2,5
Dextrose	1,0
Agar	12,0
pH 7,4	

POSTGATE LACTATE ENRICHMENT AGAR:

Enrichment and Isolation of Sulfate-reducing Bacteria with Lactate

**Solution 1:**

Distilled water	980 ml
K <sub>2</sub> HPO <sub>4</sub>	0,5 g
NH <sub>4</sub> SO <sub>4</sub>	1,0 g
CaCl <sub>2</sub> 2H <sub>2</sub> O	0,1 g
MgSO <sub>4</sub> 7H <sub>2</sub> O	2,0 g
Na-lactate	3,5 g
Yeast extract	1,0 g
Agar	

**Solution 2:**

Distilled water	10 ml
FeSO <sub>4</sub> 7H <sub>2</sub> O	0,5 g

**Solution 3:**

Distilled water	10 ml
Ascorbic acid	0.1 g
Na-thioglycolate	0.1 g

The pH of solutions 1 and 3 is adjusted to pH 7,4 and the three solutions are sterilised separately by autoclaving for 20 min. at 121°C. The three solutions are combined and mixed and the mixture is distributed aseptically into sterile culture vessels. For the isolation of pure cultures the ready-mixed, autoclaved agar medium in test tubes is held at 40-44°C and inoculated from serial dilutions of the sample to be tested. After mixing, the tubes are placed in cold water and sealed with a 1,5 to 2 cm plug of agar to prevent access of air to the inoculated portion. The tubes are incubated in air for 3-16 days until the number of black colonies shows no further increase. Incubation temperature for mesophilic strains is 28-30°C. For enumeration the number of black coloneis is counted. Pure cultures are obtained by using sterile Pasteur pipettes to ioslate black colonies, suspending them in 0,5 ml sterile medium (without agar) and examining microscopically. The agar shake method is repeated until the liquid culture grown from a single colony fulfils the requirements of a pure culture.

ROBERTSON'S ARTIFICIAL COOKED MEAT BROTH: (g/l)

Peptone	10
Lam-lemco powder	10
Textured soya protein	30
Sodium chloride	5
pH 7,0	

"STAMP" AGAR: (g/l)

Peptone	5,0
Lab-lemco	3,0
Sodium chloride	5,0
Casein (Hammerstein)	2,5
Calcium hydroxide	0,15
Calcium chloride	0,05
Agar	30,0
Oxoid (L31) Skim milk powder	10,0

Sterilise 121°C 15 minutes, cool to 45°C and add 1 ml<sup>-1</sup> 2, 3, 5 triphenyltetrazolium chloride 1000 ml<sup>-1</sup> media.

SOYBEAN CASEIN DIGEST AGAR: (g/l)

Pancreatic Digest of Casein	17,0
Papaic Digest of Soybean Meal	3,0
K <sub>2</sub> HPO <sub>4</sub>	2,5
Dextrose	2,5
pH 7,3 ± 0,2	

TRYPTONE SOYA AGAR: (g/l)

Tryptone	15
Soya Peptone	5
Sodium chloride	5
Agar	15
pH 7,3	

**A.2. Solutions and Buffers:**BUFFERED PEPTONE WATER: (g/l)

Peptone	1,0
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	9,0
KH <sub>2</sub> PO <sub>4</sub>	1,5
pH 7,0	

MACFARLAND STANDARD (0.5)

BaCl <sub>2</sub> (0,048M)	0,5 ml
H <sub>2</sub> SO <sub>4</sub> (0,350M)	99,5 ml

MODIFIED TRIS-BUFFER: (g.l)

TRIS	6,0570	(0,05M)
Sodium chloride	8,5000	0,85%
CaCl <sub>2</sub>	0,5495	(0,005M)
Zn acetate	0,0133	(0,0002M)
In de-ionised water		
pH 7,6		

MODIFIED 10 x TRIS-BUFFER:

As above but in 100 ml of water not 1L.

(0,5M TRIS; 8,5% NaCl; 0,05M CaCl<sub>2</sub>; 0,002M zinc acetate)

NORMAL TRIS-BUFFER: (0,05M) (g/l)

TRIS	6,0570
pH 7,6	

PHOSPHATE BUFFERED SALINE: (g/l)

Sodium chloride	8,00
K Cl	0,20
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	2,90
KH <sub>2</sub> PO <sub>4</sub> monohydrate	0,20
pH 7,2	

<u>SALINE (Physiological)</u>	(g/l)
Sodium chloride	8,5

HYDROXYPROLINE SOLUTIONS:

**1. Acetate - citrate buffer**

Sodium acetate trihydrate	57,0 g
Tri-sodium citrate	37,5 g
Citric acid monohydrate	5,5 g
Isopropanol	395 ml

Make up to 1000 ml with de-ionised water.

**2. Chloramine T Reagent**

Chloramine T	2,82 g
Acetate - citrate buffer	180 ml

Made up to 400 ml with de-ionised water.

**3. Ehrlich's Reagent**

p-aminodimethylbenzaldehyde	60 g
Isopropanol	250 ml
60% perchloric acid	104 ml

Solution kept in dark and prepared fresh daily.

VOLATILE AMMONIA SOLUTIONS:

Note: De-ionised double distilled ammonia-free distilled water was used for all solutions.

**1. Borate buffer**

Sodium tetraborate $10\text{H}_2\text{O}$	9,5 g
0,1N NaOH	88 ml

Make to 500 ml with distilled water.

**2. 6N Sodium hydroxide solution**

NaOH	240 g
------	-------

Distilled water to 1 litre.

**3. Mixed indicator solution**

Methyl red	200 mg
95% ethyl alcohol	100 ml
Methylene blue	100 mg
95% ethyl alcohol	50 ml

Combine both solutions. Storage life = 1 month.

**4. Indicating boric acid solution**

Boric acid	20 g
Mixed indicator solution	10 ml
Distilled water	1 L

**5. 0,05N sulphuric acid**

0,1N standard H <sub>2</sub> SO <sub>4</sub>	500ml
Distilled water	1 L

Standardise by titrating against 15,0 ml of 0,05N sodium carbonate solution which has been incorporated into the indicating boric acid solution.

**6. Distillation apparatus cleaning solution**

Borate buffer	20 ml
Distilled water	500 ml

pH 9,5 with 6N NaOH.

**A.3. Histological Fixatives, Section Treatments and Stains:****BOUINS FLUID FIXATIVE:** (ml)

Formaldehyde 40%	25,0
Picric acid (sat)	75,0
Glacial acetic acid	5,0

**POST FLXATIVE 70% ALCOHOL:**

Absolute ethanol	70 ml
Distilled water	30 ml

SECTION TREATMENTS:**1. Dehydration**

Absolute alcohol (3 changes)	3-5 min. each
Abs. alc: Xylene 1:1 (2 changes)	3-5 min. each
Xylene (3 changes)	3-5 mins.

**2. Rehydration**

Xylene x 2	2 min.
Abs. alcohol x 2	30 sec.
90% alcohol	30 sec.
70%	30 sec.
Tap water	

MOUNTING MEDIA:

Entellen

STAINS:MASSON TRICHROME STAIN FOR COLLAGEN:**Solutions:****1. Weigerts Solution A:**

Haematoxylin	2 g
95% ethanol	200 ml

**2. Weigerts Solution B:**

29% Ferric chloride	8 ml
HCl	2 ml
Distilled water	190 ml
Mix A:B 1:1, discard after 12 hours.	

**3. 2/3 Saturated Picric acid solution:**

Picric acid	6 g
Absolute ethanol	100 ml

4. Xylidene-Ponceau:

1% Xylidene-ponceau in 1% acetic acid	2 parts
1% acid fuchsin in 1% acetic acid	1 part

5. Phosphomolybdic acid:

Phosphomolybdic acid	3 gm
Distilled water	100 ml

6. Light Green Stain:

Light green stain SF	6 gm
1% acetic acid	300 ml

Method:

1. Section.
2. Stain Weigerts iron haematoxylin 5 min.
3. Differentiate to a pure nuclear stain in 2/3 sat. picric acid 1 min.
4. Wash under running tap water. 15 min.
5. Stain Xylidine ponceau/acid fuchsin 5 min.
6. Rinse in distilled water.
7. Mordant with 1% phosphomolybdic acid 5 min.
8. Drain stain 1% light green solution 5 min.
9. Differentiate in 1% acetic acid. 2 min.
10. Dehydrate section and mount.

GRAM STAIN:

Kopeloff-Beerman (Conn and Darrow Modification):

Crystal Violet Solution:

5% Sodium bicarbonate (Freshly made)	4 ml
1% aqueous crystal violet	15 ml

Sodium Hypo-iodate:

Iodine	2 gm
N-NaOH	10 mls
Distilled Water	90 mls

Decolourant:

Acetone or 30% acetone/ether

Counter stain:

Basic Fuchsin	0,1 g
Distilled water	100 ml

**Method:**

1. Rehydrate section.
2. Crystal violet solution 5 min.
3. Wash with sodium hypo-iodate
4. Immerse in sodium hypo-iodate 2 min.
5. Wash in tap water, blot lightly once.
6. Decolorise acetone/ether approx. 10 secs.
7. Counter-stain 0,1% basic fuchsin 5-10 secs.
8. Wash in tap water.
9. Dehydrate section and mount.

**A.4. Equipment:**INCUBATOR:

Incubation of cultures throughout was in a Memmert Series B- incubator (operating - sensitivity 0,5%. Maximum temperature (120°C). Operating - uniformity 1,5% of the maximum temperature) and relative humidity 25%.

WATER BATH:

Memmert W 350 0-100°C  
 Model DIN 40050 - 1P20  
 Operating sensitivity 0,25°C

AUTOCLAVE:

HMC speedy vertical SF 721.

ETHYLENE OXIDE STERILISER:

Steri-vac automated/vacuum model 202

PHOTOMETER:

Varian DMS 100

BALANCE:

Sartorius 1201 MP2 4 place.

VORTEX:

Vortex genie 2, model G 560E  
Scientific Industries, Bohemia NY 117116.

MICROSCOPES:

Zeiss standard and Zeiss stereomicroscope.

pH METER:

Orion Research pH meter, model 221, with surface electrode.

ANAEROBIC SYSTEM:

Merck Anaerocult A and Merck Anaerobic jar from E. Merk, D-6100, Darmstadt, Germany.

BIOCHEMICAL IDENTIFICATION:

API 10S (for Gram negative bacteria) and API 32A (for anaerobic bacteria) from Bio Mérieux, 69280, Marcy-l'Etoile, France.

WACKER DRUM APPARATUS:

Glass tumbril system giving continuous rotation of hide pieces, with or without fluid addition (float); built by Leather Industries Research Insitutute, Grahamstown, South Africa

## APPENDIX B

### METHODS USED IN THIS STUDY

- a. Organoleptic Assessments:
  - i. Hairslip
  - ii. Malodour
  - iii. Appearance
- b. Ethylene Oxide Sterilisation.
- c. Spectrophotometric Measurements.
- d. Heat Shrinkage (thermal transition temperature).
- e. pH Measurements.
- f. Hair/flesh Separation of Hides.
- g. Histological Processing.
- h. Optical Activity of Soluble Collagen.
- i. Liming and Deliming of Hide Samples.
- j. Sterility Testing.
- k. Preparation of Hide Extrudates.
- l. Preparation of the Selected Inocula.
- m. Bacterial Counts:
  - i. Growth curves of the selected inocula.
  - ii. Total aerobic bacterial counts.
  - iii. Total anaerobic bacterial counts.
  - iv. Surface aerobic bacterial counts combined with proteolytic activity.
- n. Bacterial Identification:
  - i. Gram stain.
  - ii. Biochemical identification.
  - iii. Sulphate reducing bacteria.

- o. Detection of Proteolytic Activity in Fluids and Cultures.
- p. Soluble Ammonia Release.
- q. Hydroxyproline Assay.
- r. Mass Loss/Gain
- s. Fluid Volume Increase/Decrease.
- t. Ammonium Sulphate Precipitation of Enzymes from Bacterial Cultures.
  - i. General proteases.
  - ii. Collagenases

### Methods Used in this Study:

#### a. Organoleptic assessments:

- i. Hairslip was determined by use of gentle pulling on several hairs in different areas of the hide with the aid of forceps. Release of hair was scored from + (slight hair loss) to +++ (marked hair loss).
- ii. Malodour was scored from + (slight off odour) to +++ (strong odour either ammoniacal or foul/putrefactive)
- iii. Appearance was scored from + (surface slime) to +++ (excess slime and discolouration of the flesh surface).

#### b. Ethylene oxide sterilisation:

Hide samples from at least 40 different animals were taken at the Port Elizabeth and Grahamstown abattoirs. The samples for the studies in Chapters 4 and 5 were taken within 20 minutes of animal kill and during the initial flay process, transported at 4°C to the laboratory and washed in running tap water for 20 minutes. They were then cut into 3cm diameter hide plugs and sonicated for 20 minutes in a Sorval sonicator at 230-250V. After blotting to remove excess moisture the plugs were placed in individual 40ml plastic jars with screw-on lids and subject to ethylene oxide sterilisation for 210" at 29°C in a STERI-VAC automated steriliser (model 202/54 litres) at 5lbs atmosphere pressure with 100% ISO = 1240mg/L. After sterilisation the jars and contents were stood at 20°C for 48 hours to allow dissipation of any residual EO.

#### c. Spectrophotometric measurements:

Turbidity measurements for bacterial growth curve studies were carried out in a standard Bausch and Lomb colorimeter using 500 ml side arm flasks and a wave length of 600 nm.

#### d. Heat shrinkage (thermal transition temperature):

These tests were performed on a LIRI heat shrinkage apparatus in which a hide plug is held between two clamps and immersed in a beaker of water placed on a heater/stirrer apparatus. One clamp is fixed, the other is attached via string to a weighted pulley. As hide fibres reach transitional temperatures ( $T_t$ ) they begin to melt and the resultant tension change results in release of the string which in turn causes movement of an arm across a temperature dial.

e. **pH measurements:**

An Orion Research pH meter model 221 with surface electrode, was used for pH measurements of hide surfaces and for solutions.

f. **Hair/flesh separation of hides:**

This was achieved using an electric Bosch domestic meat slicer in which the hide plug was cut horizontally at a setting of 0,5 cm giving a hair and a flesh sample.

g. **Histological processing:**

Processing was carried out in a Miles Scientific Tissue Tek VIP Vacuum processor and embedding was achieved using a Tissue Tek Embedding Console system. Section cutting at 3  $\mu\text{m}$  was achieved using a Bright Instrument Co. (ENG. ) microtome. Masson and Gram stains were performed as detailed in Appendix A.

h. **Optical activity of soluble collagen:**

This was tested on a Perkin-Elmo 141 digital polarimeter at 436 nm at 25°C under standard conditions as per Brüning (1991). These tests were carried out by personnel of the Leather Industries Research Institute, Grahamstown, South Africa.

i. **Liming and deliming of hide samples:**

Samples were limed and delimed individually in the 40 ml plastic containers used for their storage. The samples (in their containers) were treated throughout in a mini-Wacker drum system built at Leather Industries Research Institute, Grahamstown. After an initial soak in 150% float of water at 28°C for two hours, the hides were subject to warm liming at 100% float and 28°C using 3% lime and 3% sodium sulphide (with 0,1% Perdol M) for 5 hours. Drumming was every hour for 5 min. After an additional 100% water float the samples were left to stand overnight, washed for 20 minutes and delimed at 50% float at 25°C using 3% ammonium sulphate (with 0,2% Fluidol W) for two hours or until the pH value reached pH 8-9. The samples were then rinsed in water and stored in Boiuns fluid for 24 hours followed by 70% ethanol prior to examination by stereomicroscopy and histological sectioning.

**j. Sterility testing:**

Sterility test media (Soya Bean Casein Digest broth and Fluid Thioglycollate Broth) were prepared according to the US Pharmacopoeia (1984) and small hide samples were placed in 20 ml volumes of this media and incubated at 37°C for up to 14 days. All turbid cultures were plated to Soya Bean Casein agar and Reinforced Clostridia Media and incubated at 37°C for 24 hours.

**k. Preparation of hide extrudates:**

Hide pieces and plugs were blotted to remove surface moisture, placed in small sterile bags, between two pieces of Velcro (hook side only) and subjected to 4 tons of mechanical pressure using an industrial press. The fluids so obtained were transferred to sterile 5 ml screw cap tubes using sterile Pasteur pipettes.

**l. Preparation of the selected inocula:**

Stock cultures of all three organisms were maintained at 41°C on either Typtone Soya agar (*Ps. aeruginosa*) or in Robertsons Cooked Meat broth (*C. histolyticum* and *C. sporogenes*). Subcultures were made from these stock cultures to blood agar and incubation was either aerobic (*Ps. aeruginosa*) or anaerobic (*C. histolyticum* and *C. sporogenes*) for three successive days at 37°C. Pure, isolated colonies from each of the final subculture plates were inoculated to Nutrient broth (*Ps. aeruginosa*) and Reinforced Clostridial broth (*C. histolyticum* and *C. sporogenes*) and incubated at 37°C for 24 h. Bacterial cells were recovered by centrifugation at 3,500 g for 20 minutes. These cells were diluted in saline to give ca  $1 \times 10^8$  organisms  $\text{ml}^{-1}$  (as judged by MacFarland Standard [Appendix A] and confirmed by total bacterial count after incubation at 37°C for 24 h). These saline preparations (at  $10^8$  organisms  $\text{ml}^{-1}$ ) formed the basis of the inoculum suspensions used throughout the thesis for raw hide degradation studies. Individual variations of these inocula included further dilution, addition of nutrients (1/10 volume nutrient broth), addition of reductants (cystine) and changes in volume of inocula added to hide pieces. These individual variations are detailed where appropriate in each Chapter.

m. **Bacterial counts:**

i. **Growth curves of the selected inocula:**

*Ps. aeruginosa* was incubated in Nutrient broth and *C. histolyticum* and *C. sporogenes* in Fluid Thioglycollate broth for 24 hours at 37°C. Each inocula was adjusted to  $10^8 \text{ ml}^{-1}$  using 0,5 MacFarland standard before further diluting in a Klett side arm flask in 150 ml of the appropriate broths to achieve Ca  $5 \times 10^7$  organisms  $\text{ml}^{-1}$ . Readings were taken on a Klett Colorimeter at 600 nm at times 0, and at 0,5 hours intervals up to 4 hours and thereafter at 1 hour intervals up to 10 hours and again at 18 hours. Total bacterial counts were performed at these times using the standard pour plate method and Plate Count agar (aerobic) and Reinforced Clostridia Agar (anaerobic) for media. The results were plotted graphically (time v abs) and generation times calculated both from the graphs and the total count data. The MacFarland standard was prepared using 0,5 ml of 0,048 M  $\text{BaCl}_2$  and 99,5 ml of 0,350 M  $\text{H}_2\text{SO}_4$ .

ii. **Total aerobic bacterial counts:**

These were performed using standard pour plate techniques with 0,1% buffered peptone as the diluent and Plate Count agar as the nutrient media. Unless otherwise stated incubation was at 37°C for 24 hours. Results were calculated as the number of organisms  $\text{ml}^{-1}$  in each fluid sample. The final total count per extrudate or inocula suspension was used to calculate the total count per gram of hide weight according to the formula:

$$\text{Final total count g}^{-1} \text{ hide weight} = \frac{N \times F}{g}$$

Where:

N = number bacteria  $\text{ml}^{-1}$  fluid under test

F = ml of fluid (either as extrudate or as inocula suspension) per sample

g = weight in grams of sample before storage.

iii. **Total anaerobic bacterial counts:**

These were performed using a media overlay pour plate method and Modified Reinforced Clostridia agar for *Clostridium*. 10 ml of agar was used per 0,5 ml dilution of sample per 90 mm Petri dish. When this media had set 3 ml of extra media was added as a thin film on top of the previous agar surface (overlay). Incubation was anaerobic in a Merck Gas-Pak Anaerobic system (Appendix A) at

either 30°C or 37°C as stated. Results were calculated as the number of organisms ml<sup>-1</sup> in each fluid sample and as final total count per gram of hide weight as in (ii) above.

iv. **Surface aerobic bacterial counts and general proteolytic activity:**

These were determined by use of the "Stamp" test detailed in Chapter 2 and using "Stamp" agar (Appendix A).

n. **Bacterial identification:**

i. **Gram Stain:**

Films of bacterial colonies were prepared in saline on glass slides and stained by standard Gram stain technique (Cruickshank *et al.* 1975).

ii. **Biochemical identification:**

Bacterial identification was established using API 10S (for Gram negative enteric bacteria) and API 32A (for anaerobic bacteria) Appendix A.4. Hydrogen sulphide detection in broth cultures was performed using strips of filter paper impregnated with 10% lead acetate which were hung above, but not in touch with, the broth surface.

iii. **Sulphate reducing bacteria:**

Sulphate reducing bacteria counts were performed using Modified Postgate Agar and sealed with agar plugs, as detailed in this Appendix. Incubation was at 30°C for 8 days.

o. **Detection of proteolytic activity in fluids and cultures:**

Details of the methods for proteolytic enzyme detection are given in Chapter 2. The methods for media and/or gel preparation for collagen, gelatin and casein are given in Appendix A. Unless otherwise stated 0,1 ml of hide extrudates/inocula suspensions were added to 6mm filter paper discs and these were transferred to the gel surfaces. Incubation was at 37°C for 24h after which the zones of lysis were measured (mm diameter including 6mm disc). These results were used to quantify collagenase concentration, where necessary, by reference to the standard graph (Chapter 2). All other results were scored according to an arbitrary range which was + = 6,5 - 10,0mm; ++ = 10,0 - 15,0 mm; +++ = >15,0 mm. For cultures such as the "stamp" test (see chapter 3) the degree of activity was scored in a similar manner except the measurement (in mm) was taken from colony edge to zone edge.

p. **Soluble ammonia release:**

Soluble ammonia was measured in both hide extrudates and inoculum suspensions using a steam distillation Micro-Kjeldahl nitrogen determination apparatus. (All solutions used in the assay are listed in Appendix A).

**Distillation:**

The apparatus was cleaned by distillation of a solution containing 20ml of borate buffer in 500 ml water to which a few glass beads were added.

1. 0,1 ml of sample was added to 500 ml of distilled water and 25 ml of borate buffer in a distillation flask and the pH adjusted to 9,5 with 6N NaOH.
2. After steam generation from the steam generator was completed, the flask containing the sample was connected to the distillation apparatus.
3. Distillation was for 6 mins. at a rate of 6-10 ml min<sup>-1</sup> and at least 200 ml of distillate was collected.
4. A blank solution (no sample) was treated in the same way.

**Titration:**

The distillate was diluted to 500 ml with distilled water and titrated with 0,05N H<sub>2</sub>SO<sub>4</sub> as titrant. (This solution had been standardised against 0,05N sodium carbonate).

**Calculation:**

$$1. \quad \text{mg NH}_3 \text{ ml}^{-1} = t - b \times c \times \text{nf} \times 0,5 \times 10$$

Where:

t = titre (ml) of back titration using 0,05N H<sub>2</sub>SO<sub>4</sub>

b = blank solution titre

c = 1,7 (1,7 mg NH<sub>3</sub> = 1 ml of 0,1N H<sub>2</sub>SO<sub>4</sub>)

nf = 0,05N H<sub>2</sub>SO<sub>4</sub> normality factor (1,272).

0,5 = correction factor using 0,05N H<sub>2</sub>SO<sub>4</sub> as opposed to 0,1N H<sub>2</sub>SO<sub>4</sub>

10 = correction factor using 0,1 ml sample as opposed to 1,0 ml.

$$2. \quad \text{mg NH}_3 \text{ -N g}^{-1} \text{ hide weight} = \frac{\text{mg NH}_3 \text{ ml}^{-1} \times V}{g}$$

Where:

$V$  = total volume of extrudate or inoculum suspension from hide piece

$g$  = gram weight of hide sample before inocula addition

q. **Hydroxyproline assay:**

Hydroxyproline assay was carried out according to the method developed for this thesis (detailed in Chapter 2), 0,5 ml g sample (either hide extrudate or inoculum suspension) was hydrolysed at 105°C for 18h after the addition of 1,5 ml of distilled water and 3,0 ml of 10N - HCl. The assay was performed on the cooled hydrolysates and on standard control samples (see Chapter 2). The results were read at 558 nm on a Varian DMS 100 Spectrophotometer and the hydroxyproline concentration per gram of hide weight was calculated as follows:

$$1. \quad \text{Hydroxyproline } \mu\text{g ml}^{-1} = \frac{t}{s} \times cs$$

Where:

$t$  = absorbance reading of test sample

$s$  = absorbance reading of standard sample

$cs$  = concentration ( $\mu\text{g ml}^{-1}$ ) of standard

$$2. \quad \mu\text{g hydroxyproline g}^{-1} \text{ hide weight} = \frac{\mu\text{g hypro ml}^{-1} \times V}{g}$$

Where:

hypro = hydroxyproline

$V$  = Volume of extrudate or inoculum suspension from hide piece.

$g$  = gram weight of hide sample before inocula addition.

r. **Mass loss / gain:**

Hide samples were weighed on a four place balance (using a minimum of four samples per test) after blotting off excess moisture with thick paper towels. The mean mass of the sample (corrected to 1 decimal place) of day 1 was used to calculate % gain or loss of subsequent sample.

s. **Fluid volume increase / decrease:**

Inocula fluids and hide extrudate volumes were measured against a series of graded volumes prepared using a Gilson Pipetman 1000  $\mu\text{l}$  pipette) in standardised 5 ml tubes. Fluid volumes at day 1 were used as a base to calculate increase or decrease in subsequent volumes. A minimum of four samples per test were used to calculate the values.

t. **Ammonium sulphate precipitation of enzymes from bacterial cultures:**

i. **General proteases:**

The cultures were centrifuged at 4500 g for 20 minutes to remove bacterial cells.  $(\text{NH}_4)_2 \text{SO}_4$  was added to 20% saturation ( $1,06 \text{ g} / 10 \text{ ml}^{-1}$  supernatant) and left to react for 24 hours at  $4^\circ\text{C}$ . The samples were re-centrifuged at 4,500 g for 20 minutes and the supernatants were removed and 0,5 ml modified N-TRIS buffer (Appendix A) added to the deposit.

ii **Collagenases:**

$(\text{NH}_4)_2 \text{SO}_4$  was added to the supernatants from the general protease preparations above at 60% saturation ( $2,6 \text{ g} 10 \text{ ml}^{-1}$ ) and left to react at  $4^\circ\text{C}$  for 24 hours. Recovery of deposits was achieved by centrifuging at 4,500 g for 20 minutes and these were re-suspended in 0,5 ml of modified N-TRIS buffer (Appendix A) for raw hide degradation studies and in 2 ml g modified N-TRIS with added 0,05% Brij for the halophilic studies.

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