CHANGES IN THE AEROBIC SAPROPHYTIC MICROBIAL FLORA DURING BILTONG PRODUCTION WITH SPECIAL REFERENCE TO THE MICROCOCCACEAE

by

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SUMMARY

Ninety-four presumptive Micrococcus and Staphylococcus strains isolated from both commercial beef biltong and game biltong, were identified using a scheme based on the system used by Baird-Parker.

The changes occurring in both the aerobic, saprophytic microbial flora and the environmental factors, during conversion of beef to biltong, were examined. The predominantly Gram-negative, halo-sensitive flora initially present on the meat, was replaced by Gram-positive, halo-tolerant staphylococci and micrococci, which form the dominant component of the microflora of the final product. This replacement was attributed to changing environmental factors, principally to the increasing sodium chloride concentration and associated decline in water activity.

The presence of the antifungal antibiotic, pimaricin, during processing did not influence the bacterial flora of the product. However, the addition of potassium sorbate altered the microbial profile of the product significantly. The presence of these two preservatives, at the concentrations used, could not be detected organoleptically.

The importance of the saprophytic microflora of the product in relation to the environmental factors during processing, is also discussed.

SAMEVATTING

Vier en negentig vermoede Micrococcus- en Staphylococcusstamme, geïsoleer uit monsters van kommersiële bees- en wildsbiltong, is met behulp van 'n skema wat op die sisteem van Baird-Parker berus, geïdentifiseer.

Die veranderinge in die aërobe, saprofitiese mikroflora met betrekking tot omgewingsfaktore tydens die verwerking van beesvleis na biltong, is ondersoek. Die oorheersende, Gram-negatiewe, soutgevoelige flora wat oorspronklik op die vleis teenwoordig is, word deur Gram-positiewe, souttolerante stafilokokke en mikrokokke, wat die hoofkomponent van die mikroflora van die finale produk uitmaak, verplaas. Hierdie verplasing word aan veranderinge in die milieu, hoofsaaklik die toename in die natriumchloriedkonsentrasie en die gepaardgaande afname in wateraktiwiteit, toegeskryf.

Die teenwoordigheid van die skimmelwerende antibiotikum, pimarisien, tydens verwerking het nie die samestelling van die bakterieflora van die produk noemenswaardig beïnvloed nie. Die toevoeging van kalium-sorbaat daarenteendeel, het wel tot veranderinge in die samestelling van die mikroflora gelei. Die aanwesigheid van hierdie twee preserveer-middels kon, in die gebruikte konsentrasies, nie organolepties waargeneem word nie.

Die belangrikheid van die saprofitiese mikroflora van die produk met betrekking tot omgewingsfaktore tydens verwerking word ook bespreek.

CONTENTS

I. GENERAL INTRODUCTION

1. THE NATURE OF BILTONG AND RELATED PRODUCTS

Biltong, a traditional salted, air-dried, raw-meat product is a delicacy well known to South Africans.

1.1 The derivation of the word 'biltong' is uncertain although many suggestions as to it's origin have been made. One such suggestion is that it was derived from the Malay word 'dendang', the name used for an Indonesian dried meat product. Another is that it was derived from 'trepang', dried smoked sea-cucumber, also consumed in the Orient. However, the more generally accepted origin is a combination of 'bil' referring to buttock or the hindquarter (*de billen* in Dutch) and 'tong' referring to tongue or fillet thereby implying 'tongue of meat' or 'portion of the hindquarter in the shape of a tongue'.

Its use dates back to the early settlers at the Cape and to the Boer commandos who during the South African War could remain independent of fresh supplies with a diet of biltong and dried fruit. Lewis et al. (1957) determined the food value of biltong and found that it was a useful addition to the diets used on Greenland expeditions. He also found that if necessary, it could be rehydrated and cooked to provide a solid meat-like meal.

Biltong is an important commercial commodity, but as its production is not controlled, the exact quantities produced in South Africa and their retail value is unknown.

1.2 Biltong is usually prepared from beef and antelope meat but with the animal culling operations, the use of elephant, buffalo and ostrich meat is becoming increasingly popular. The portions used are

usually the dorsal muscles on either side of the spinal column and the haunches i.e. the topsides and silverside or muscles thereof. In Afrikaans, biltong prepared from a specific muscle is given a specific name viz. 'binnebiltong' from the *M. psoas major*; 'regte biltong' from the *M. semimembranosus* and 'rugstringbiltong or garingbiltong' from the *M. longissimus dorsi* (van den Heever, 1970a).

Strips of meat are cut along the muscle fibres and then salted, either in brine or by dry-salting, and allowed to pickle for 16-24 hours at low temperatures. Sodium chloride is the principal curing ingredient used, although other ingredients such as coriander, pepper, commercial spices, brown sugar and saltpetre are also known to be used in some pickling mixtures. The strips of pickled meat are then hung to dry. Originally drying was done in the cooler winter months in a shady, protected area exposed only to the wind and after 10-14 days the product was ready for consumption. However, with the present demand for biltong, the manufacturers sought ways of drying meat in as short a time as possible. Large rooms equipped with hot and cold air fans are used as drying chambers where the meat is dried and ready for marketing within a period of 4-6 days.

1.3 The curing and drying of meat as a form of preservation is by no means unique to South Africa and dates back to time immemorial. The ancient Egyptians used the theory of curing and dehydration in the preparation of mummies, while the salting of fish was practised by the ancient Greeks. The Romans were known to use salt in the curing of pork and fish and the Iron Age people from the North are reported to have practised salt preservation and dessication of meat products (Jensen, 1954).

This form of preservation of fish and meat is usually associated with a hot climate and low humidity where it is easily prepared and kept. In North America, 'pemmican' is prepared from beef or venison by smoking the meat before dessication. After the meat is dried it is flaked and an equal amount of fat is added to it before it is stored. 'Charqui' or 'Xarqui' sundried, salted fore-quarter beef is an important local and export product of Uruguay and Brazil. In Peru, 'charqui' is prepared

for local consumption from Llama and Alpaca flesh. A similar product 'chalone' is also prepared from sheep flesh (Thornton, 1962). In both North and South America 'jerky' or 'jerked beef' is prepared by curing and drying strips of beef and venison. Dried, cured meats are also encountered in Europe with the 'Graubündnerfleisch' of Switzerland resembling a very moist biltong.

2. INVESTIGATIONS CONCERNING THE PATHOGENIC MICROFLORA OF BILTONG

2.1 Meat and meat products if handled incorrectly create an ideal environment for the proliferation of micro-organisms. In South Africa, the laws concerning meat and meat products do not include biltong in their definitions or specifications (van den Heever, 1970a). The South African Bureau of Standards has a set of specifications for ready-to-eat processed meats, but biltong is not mentioned specifically. There is therefore no direct control over the production of biltong commercially and consequently public health hazards such as microbial infection and chemical contamination pass unchecked.

Biltong, compared to other meat products, is not subjected to any form of heating or smoking during processing or before consumption. It thus creates a potential health hazard if any pathogens, either from the original meat or environmental contamination survive the curing and dehydration procedures. Heating or smoking will not necessarily render the product free of pathogens as *Clostridium botulinum* type F was isolated from venison jerky, a smoked product, implicated in an outbreak of food poisoning (Midura et al., 1972).

Food poisoning is not notifiable in South Africa (except Durban). One can only assume that cases of food poisoning due to biltong consumption were attributed to other gastric disorders without any further investigation.

2.2 The first report of biltong-induced food poisoning was by Jansen (1949), who isolated *Salmonella lomita* from game biltong

responsible for an outbreak of gastro-enteritis. Subsequently, a number of other cases of biltong-induced food poisoning have been reported. Neser et al. (1957), investigating an outbreak of food poisoning in a school hostel resulting in 21 children ill and one fatality, isolated *S. newport* from samples of the remaining biltong. Viable salmonellae were isolated from samples of the same biltong stored for two years at room temperature. This indicated that the presence of sodium chloride and dehydration did not eliminate the organisms completely.

A short outbreak of gastro-enteritis due to biltong infected with *S. anatum* was mentioned by Bokkenheuser (1963). In his investigations concerning the hygienic quality of biltong, Bokkenheuser (1963) examined 121 samples of sliced biltong obtained from various sources in Johannesburg. Only one sample (0,8%) was found to be infected with salmonella (*S. poona*). Thirty-four samples (28,1%) were infected with *Escherichia coli* type I (considered to be indicative of faecal contamination) and no coagulase-positive staphylococci were isolated. From these results he concluded that biltong was hygienically unsatisfactory and that quality was not related to the retail outlet. He suggested that the isolation of group D streptococci may be more significant than *E. coli* type I as an indication of faecal contamination, as *E. coli* type I was thought to survive poorly out of the gut.

Botes (1966) reported a further case of biltong-induced food poisoning where *S. enteritidis* var. *typhimurium* was incriminated. In this case and those reported by Bokkenheuser (1963) and Neser et al. (1957), the available data suggested that the animal itself was the source of infection and not external contamination during processing.

In these investigations the microbial infections are discussed without reference to the physico-chemical nature of the environment. An understanding of the environment is essential as the conditions encountered in biltong i.e. high sodium chloride concentration, low moisture content and low water activity, would selectively enhance the proliferation of halo-tolerant micro-organisms able to withstand certain levels of dessication and adversely affect the proliferation and viability of halo-sensitive micro-organisms.

2.3 Van den Heever (1965, 1970a, b) examined various aspects concerning the hygienic quality of biltong in relation to the environmental factors and in particular, investigated the incidence of pathogens and their viability both *in vitro* and during biltong production.

In a survey of 60 samples of commercial biltong van den Heever (1970a, b) found biltong to have an average moisture content of 25,2% (with minimum 9,6% and maximum 51,5%), NaCl content of 6,6% (with minimum 3,4% and maximum 12,0%), pH 5,88 (with minimum pH 5,6 and maximum pH 6,6) and water activity of 0,742 (with maxima and minima of a_w 0,924 and a_w 0,296 respectively). Thus commercial biltong exhibits a wide range of physico-chemical properties.

Microbial analysis of these 60 samples showed the total aerobic plate count to range from $1,2 \times 10^3$/g to $5,0 \times 10^8$/g. Salmonella (types *S. blutwa* and *S. woodstock*) were present in 3,3% of the samples while 45% contained *E. coli* type I and 98,3% yielded faecal streptococci. From these results van den Heever (1970a, b) concluded that commercial biltong was microbiologically unhygienic thus confirming Bokkenheuser's conclusions. Both van den Heever (1970a, b) and Bokkenheuser (1963) based their conclusions on the high frequency of *E. coli* type I and group D streptococci infection. However, the presence of group D streptococci in biltong need not necessarily be indicative of faecal contamination as Deibel (1964), reported that group D streptococci could also be recovered from sources other than from faecal origin. On the other hand, the presence of salmonella immediately incriminates the product as being a potential health hazard.

Chemical analyses of the 60 commercial biltong samples indicated that the level of contaminating chemicals such as boron and zinc present posed no immediate danger to consumers, but that precautions were necessary to prevent these compounds present in low concentrations reaching toxic levels.

2.4 Van den Heever (1965) compared the viability of salmonellae in biltong prepared from meat from an animal infected with *S. dublin* with that of biltong prepared from sound meat which had been artificially

infected with a culture of *S. typhimurium* prior to salting. In the former instance, salmonellae were still recoverable from the biltong after six months while in the latter, no viable salmonellae were detected after 22 days.

The environmental conditions encountered during biltong processing therefore appear to be inadequate to control salmonellae in biltong prepared from meat obtained from salmonella-infected animals or meat infected during slaughter and pre-salting procedures. Van den Heever (1965) therefore concluded that "the use of meat of healthy animals held at low temperatures is essential in biltong production, and care in slaughter and pre-salting procedures is necessary to avoid salmonella contaminations".

2.5 Van den Heever's investigations appear not to have found application as Prior and Badenhorst (1974), in a survey of salmonellae in some meat products found seven biltong samples out of 45 to harbour salmonella i.e. 16% of the samples tested yielded salmonella (types *S. typhimurium*, *S. thompson*, *S. london* and *S. johannesburg*), compared to the frequency of 3,3% reported by van den Heever (1970a, b) just four years previously. This increase of salmonella infection in biltong could be indicative of gross mishandling and contamination of the meat prior to processing or of the product before marketing, or of improved methods for the detection of these organisms.

2.6 The processing of meat to biltong also appeared not to influence the growth of *Staphylococcus aureus* and *Streptococcus faecalis* as these organisms were found to survive extremely well throughout the various stages of biltong processing (van den Heever, 1970a, b). Although the commercial biltong samples examined by both Bokkenheuser (1963) and van den Heever (1970a, b) yielded no coagulase-positive *Staphylococcus aureus*, van den Heever (1970b) emphasized the need for steps to avoid staphylococcal contamination.

E. coli type I, however, was found to be susceptible to the environmental changes, but was not completely eliminated (van den Heever, 1970a, b).

2.7 From van den Heever's investigations (van den Heever, 1965; 1970a, b) it can be concluded that the salting and dehydration of meat is inadequate to control or eliminate pathogenic organisms satisfactorily and that extreme care should be exercised during biltong production to prevent contamination of the meat and final product.

3. INVESTIGATIONS CONCERNING THE AEROBIC, SAPROPHYTIC MICROBIAL FLORA OF BILTONG

3.1 Meat is a substrate which favours the growth of a wide range of organisms and the microflora of fresh beef is reported to comprise principally organisms from the genera *Achromobacter* (90%), *Micrococcus* (7%), *Flavobacterium* (3%±) and *Pseudomonas* (1%±). With the salting and dehydration of the meat during biltong production, a change in the microflora can be anticipated. However, very little was known of this saprophytic microflora which was expected to be characteristic of biltong.

Bokkenheuser (1963), who did not undertake a quantitative investigation of biltong, reported *Staphylococcus epidermidis* to be present in more than half the samples of biltong he analyzed.

In his investigations, van den Heever (1970a, b) reported commercial biltong to have a total number of organisms ranging from $1,2 \times 10^3$/g to $5,0 \times 10^8$/g with a mean value of $6,8 \times 10^7$/g. Organisms tolerant to 6,5% and 10% sodium chloride (determined by plate counts on agar containing 6,5% or 10% sodium chloride) constituted a large proportion of the total number of organisms present. Van den Heever (1970b) considered these high numbers of micro-organisms present to be indicative of unhindered microbial proliferation or of heavy contamination during processing and accepted total counts as an indication of the sanitary quality of the product. However, Mossel (1971) pointed out that microbial proliferation was not always deleterious to both food or consumer as many fermented meat and fish products containing millions of microbes/g are consumed without harmful effects. As van den Heever (1970b) omitted to characterize these micro-organisms, his use of total

counts as an index of sanitary quality of biltong is unacceptable.

The first attempt at characterizing the aerobic saprophytic microflora of biltong was made in a short investigation by Reiche (1972). In both beef and game biltong she found salt-tolerant micrococci to be the predominant flora with lactobacilli and bacilli occurring to a lesser extent.

It therefore became necessary to establish what these organisms proliferating during biltong production were and what their significance was.

4. THE AIMS OF THIS INVESTIGATION

The purpose of this investigation was therefore two-fold.

The first section deals with the problems associated with the taxonomy and identification of the salt-tolerant, Gram-positive, catalase-positive cocci found on biltong.

The second section is concerned with the changes occurring in the saprophytic microbial flora during the conversion of meat to biltong and the factors causing these changes. The possible contribution of the saprophytic bacterial flora to flavour production was also investigated.

II. IDENTIFICATION OF THE MICROCOCCACEAE

1. INTRODUCTION AND REVIEW OF THE TAXONOMY OF THE MICROCOCCACEAE

1.1 INTRODUCTION

During the course of her introductory survey of the saprophytic bacterial flora of biltong, Reiche (1972) isolated a number of bacteria from dilution plates of different selective media. As she reported salt-tolerant cocci to constitute the dominant component of the microflora of biltong, it became necessary to identify these strains to the species level for future investigations.

When the present study was commenced, no standard system for the classification of this group of bacteria appeared to be in use.

Patterson (1966) used the scheme of Shaw, Stitt and Cowan (1951) while Baird-Parker (1962), in addition to the scheme of Shaw, Stitt and Cowan (1951), also used the 7th edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957) and then later developed his own scheme (Baird-Parker, 1963). Davis and Hoyling (1974) used the abridged Baird-Parker scheme (Baird-Parker, 1966) while Pohja and Gyllenberg (1962) also went as far as to develop their own classification scheme. It was evident that before any method of classification could be applied without restraint, the trends in the taxonomy of the Micrococcaceae had to be reviewed.

1.2 REVIEW OF THE TAXONOMY OF THE MICROCOCCACEAE

"The genus *Micrococcus* provides what is probably the worst example in bacteriology of uncritical systematic work" (Abd-el-Malek and Gibson, 1948).

The problems associated with the taxonomy of the Micrococcaceae are not recent developments, but the culmination of numerous disputes arising from the chaotic state which has characterized this family ever since the description of the first species, *Micrococcus prodigiosus*, by Cohn (1872). However, a detailed account of the taxonomic disputes centred around the Micrococcaceae falls beyond the scope of this study. For further information reference may be made to the extensive reviews by Cowan (1962) and Baird-Parker (1972), and the comments on the Gram-positive cocci in the 8th edition of *Bergey's Manual* (Buchanan and Gibbons, 1974).

1.3 PROBLEMS ASSOCIATED WITH THE CLASSIFICATION OF THE MICROCOCCACEAE

For the classification of the Gram-positive, catalase-positive cocci isolated from biltong, only an efficient method of reference and communication was sought. Therefore, the problem did not require the development of a new classification scheme, but more an adaptation of the methods applicable to the present situation. The scheme proposed by Baird-Parker (1963) and its subsequent modifications (Baird-Parker, 1965; 1966) was chosen as the basis for the classification of the isolates from biltong. This scheme circumvented the immediate use of binomials to describe the isolates and provided an orderly, acceptable system of subgroups which, it was presumed, could later be related to accepted, named species. This however, proved more difficult to realize than originally hoped for, as the relationships between the Baird-Parker subgroups and named species were not clear (Baird-Parker, 1962; 1965). The situation was further confused by the subsequent changes in the number of named species and genera accepted by the different taxonomic laboratories involved with the study of the Micrococcaceae.

In their re-organization of the taxonomy of the Micrococcaceae, the I.C.S.B. Subcommittee on the Taxonomy of Staphylococci and Micrococci (1971) proposed that four genera of Gram-positive, catalase-positive and cluster-forming cocci be recognized, viz. *Staphylococcus* Rosenbach, *Micrococcus* Cohn, *Planococcus* Migula and *Aerococcus* Williams, Hirch and

Cowan. All the species[1] which the I.C.S.B. Subcommittee (1971) recognized within the genera *Staphylococcus* and *Micrococcus* [except *Micrococcus morrhuae* (Farlow) Klebahn 1919] could be related to the subgroups demarcated by Baird-Parker (1965, 1966), whereas those of the genera *Aerococcus* and *Planococcus* could not be accommodated by the Baird-Parker scheme.

Within the genus *Staphylococcus* Rosenbach 1884 [type species *Staphylococcus aureus* Rosenbach 1884], the two recognized species, i.e. *Staphylococcus aureus* Rosenbach 1884 and *Staphylococcus epidermidis* (Winslow and Winslow) Evans 1916, corresponded to Baird-Parker's *Staphylococcus* subgroup I and Baird-Parker's *Staphylococcus* subgroups II, III, IV and VI respectively. Of the five recognized species within the genus *Micrococcus* Cohn 1872 [type species *Micrococcus luteus* (Schroeter) Cohn 1872], four could be related to the subgroups defined by Baird-Parker (1965, 1966). These were *Micrococcus luteus* (Schroeter) Cohn 1872 (≡ Baird-Parker's *Micrococcus* subgroup 7); *Micrococcus roseus* Flügge 1886 (≡ Baird-Parker's *Micrococcus* subgroup 8); *Micrococcus lactis* Shaw, Stitt and Cowan 1951 (≡ Baird-Parker's *Micrococcus* subgroups 5 and 6); and *Micrococcus saprophyticus* Fairbrother *emend.* Shaw, Stitt and Cowan 1951 (≡ Baird-Parker's *Micrococcus* subgroups 1-4).

The recently isolated halophilic species, *Micrococcus halobius* Onishi and Kamekura 1972 and *Micrococcus mucilaginosus* Migula 1900, which was reisolated by Bergan et al. (1970), were not included in the discussions of the I.C.S.B. Subcommittee (1971).

Subsequently, on the basis of available information, a number of amendments were made to the proposals of the I.C.S.B. Subcommittee (A.C. Baird-Parker and M. Kocur; pers. comm.). The species *Micrococcus saprophyticus* (≡ Baird-Parker's *Micrococcus* subgroups 1-4) was transferred to the genus *Staphylococcus* and cited *Staphylococcus saprophyticus*. *Micrococcus morrhuae* was transferred to the genus *Halococcus*, which included only the extremely halophilic cocci, and *Micrococcus lactis* (≡ Baird-Parker's *Micrococcus* subgroups 5 and 6) was cited as *Micrococcus varians*

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[1] The citation of species and authors follows the system adopted by the 8th edition of *Bergey's Manual* (1974).

Migula 1900. The taxonomic status of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus* and *Micrococcus roseus* and their relationships to Baird-Parker's subgroups remained unchanged. No ruling was taken on the taxonomic positions of *Micrococcus freudenreichii* Guillebeau 1891, *Micrococcus halobius* and *Micrococcus mucilaginosus*.

1.4 RECENT DEVELOPMENTS IN THE CLASSIFICATION OF THE MICROCOCCACEAE

The classification of the Micrococcaceae was revised for the 8th edition of *Bergey's Manual* (1974) by Baird-Parker and Kocur. It was hoped that the proposed scheme would resolve the taxonomic disputes within the family and assist in relating the Gram-positive, catalase-positive cocci isolated from biltong to internationally recognized species. However, the proposed scheme only served to confuse the situation further.

This scheme, compared to that of the 7th edition of *Bergey's Manual* (1957) not only reduced the number of genera within the family from six to three but also the number of fully recognized species within each genus. Although this reduction in the number of recognized genera and species resolved many of the disagreements in the taxonomy, it is not the ultimate solution to the problem. Of the 21 species mentioned in the scheme, only seven fully recognized species are described while 14 species are cited as *species incertae sedis*.

Within the genus *Micrococcus* Cohn 1872, three fully recognized species were defined. These were *Micrococcus luteus* (Schroeter, Cohn 1872 (≡ Baird-Parker's *Micrococcus* subgroup 7); *Micrococcus roseus* Flügge 1886 (≡ Baird-Parker's *Micrococcus* subgroup 8); and *Micrococcus varians* Migula 1900, previously cited as *Staphylococcus lactis* Shaw, Stitt and Cowan 1951 or *Micrococcus pulcher* Müller 1961 (≡ Baird-Parker's *Micrococcus* subgroups 5 and 6).

Six *Micrococcus* species of uncertain taxonomic position were also mentioned viz. *Micrococcus colpogenes* Campbell and Williams 1951;

Micrococcus cryophilus McLean, Sulzbacher and Mudd 1951; *Micrococcus radiodurans* Raj, Duryee, Deeney, Wang, Anderson and Elliker 1960; *Micrococcus freudenreichii* Guillebeau 1891; *Micrococcus mucilaginosus* Migula 1900; and *Micrococcus halobius* Onishi and Kamekura 1972. No mention was made as to the relationship between these *species incertae sedis* and the Baird-Parker subgroups, nor could this information be obtained from the current literature.

Three species were recognized within the genus *Staphylococcus* Rosenbach 1884 viz. *Staphylococcus aureus* Rosenbach 1884 (≡ Baird-Parker's *Staphylococcus* subgroup I); *Staphylococcus epidermidis* (Winslow and Winslow) Evans 1916, comprising 4 biotypes (≡ Baird-Parker's *Staphylococcus* subgroups II, III, IV and VI); and *Staphylococcus saprophyticus* (Fairbrother) *emend. mut. char.* Shaw, Stitt and Cowan 1951 (≡ Baird-Parker's *Micrococcus* subgroups 1-4).

Staphylococcus salivarius Andrewes and Gordon 1907 is the only *species incerta sedis* mentioned in the genus *Staphylococcus* even though there is evidence for its transfer to the genus *Micrococcus* (Baird-Parker, 1972; Bergan et al. 1970).

The third genus, *Planococcus* Migula 1894 [type species *Planococcus citreus* Migula 1894] encompasses all the Gram-positive, motile cocci with *Planococcus citreus* Migula 1894 the only fully recognized species. Seven species viz. *Planococcus roseus* (Winogradsky) Migula 1895; *Planococcus casei* Migula 1900; *Planococcus ochroleucus* (Prove) Migula 1900; *Planococcus löffleri* Migula 1900; *Planococcus luteus* (Adametz) Migula 1900; *Planococcus agilis* (Ali-Cohen) Chester 1901[1)]; and *Planococcus europaeus* (Winogradsky) Vuillemin 1913 were mentioned as *species incertae sedis*. None of these species could be related to Baird-Parker subgroups.

From this discussion, it is evident that the scheme for the classification of the Gram-positive, catalase-positive and cluster-forming cocci as outlined in the 8th edition of *Bergey's Manual* (1974) does not measure up to expectations. Although the Baird-Parker subgroups could

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[1)] Recently Kocur and Schleifer (1975), against the ruling of the I.C.S.B. Subcommittee (1971), transferred this motile species to the genus *Micrococcus*!

all be related to specific recognized species of *Micrococcus* and *Staphylococcus*, the presence of so many species of uncertain taxonomic positions and which had not been related to Baird-Parker subgroups, added to the uncertainties associated with the taxonomy of the Micrococcaceae.

The situation was not improved upon by the recent isolation and description of a number of new species. Kloos et al. (1974) described two new *Micrococcus* species viz. *Micrococcus lylae* and *Micrococcus kristinae* and reisolated species similar to *Micrococcus sedentarius* and *Micrococcus nishinomiyaensis*. Kloos and Schleifer (1975) and Schleifer and Kloos (1975) described seven new *Staphylococcus* species from human skin viz. *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus hominis*, *Staphylococcus simulans*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus* and *Staphylococcus xylosus*. They also amended the descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. The taxonomic status of these newly described species and their relationship to Baird-Parker subgroups must be considered as uncertain till further evidence justifying their recognition is presented.

1.5 PROPOSED SCHEME FOR THE CLASSIFICATION OF THE GRAM-POSITIVE, CATALASE-POSITIVE COCCI ISOLATED FROM BILTONG

It was therefore evident that a more comprehensive, but not more complicated scheme was needed to assist in the classification of the cocci isolated from biltong.

A system, based on Baird-Parker's scheme (Baird-Parker, 1965; 1966) which avoided the problems encountered in determining the relationships between the Baird-Parker subgroups and recognized species of Gram-positive, catalase-positive cocci was devised. This system enabled the isolates to be related directly to named species of *Staphylococcus*, *Micrococcus*, *Planococcus* and *Aerococcus*. The named species selected, included the type strains of the fully recognized species described in the 8th edition of *Bergey's Manual* (1974), strains corresponding to Baird-Parker's subgroups and a number of recently isolated species, and strains of *species incertae sedis*.

2. METHODS AND MATERIALS

2.1 CULTURES USED

2.1.1 Ninety-four presumptive *Staphylococcus* and *Micrococcus* strains isolated by Reiche (1972) from biltong and maintained were used in this investigation. All these isolates were screened on the basis of Gram-reaction, morphology, oxidase and catalase reactions to verify their placement within the Micrococcaceae. Pure cultures were initially maintained on Nutrient Agar (Difco; BBL) and later on Stock Culture Agar (Difco) at 4 °C.

2.1.2 Thirty-two lyophilized strains of named species and subgroups of *Staphylococcus*, *Micrococcus*, *Planococcus* and *Aerococcus* were obtained from the American Type Culture Collection (ATCC), Czechoslovak Collection of Microorganisms (CCM) and the Colworth Bacterial Culture Collection (CBCC). The names and culture numbers of these strains are presented in Tables 1 and 2.

These lyophilized cultures were reconstituted in Brain Heart Infusion broth (Difco) at 30 °C, screened to check their purity and then maintained on Stock Culture Agar (Difco) at 4 °C.

2.1.3 All cultures were grown in Brain Heart Infusion broth (Difco) or on Brain Heart Infusion agar (Difco) or I.S.O. agar (Barraud et al., 1967) at 30 °C for 18-24 hours before they were utilized as inocula.

TABLE 1

The names and culture numbers of strains obtained from the American Type Culture Collection (ATCC) and the Czechoslovak Collection of Microorganisms (CCM)

CULTURE NUMBER	SPECIES
ATCC 418	*Micrococcus roseus*
ATCC 15306	*Staphylococcus lactis*
ATCC 4698	*Micrococcus luteus*
ATCC 15305	*Staphylococcus saprophyticus*
ATCC 14404	*Planococcus citreus*
ATCC 12600	*Staphylococcus aureus*
ATCC 14990	*Staphylococcus epidermidis*
ATCC 27566	*Micrococcus lylae*
ATCC 27570	*Micrococcus kristinae*
ATCC 14392	*Micrococcus sedentarius*
CCM 169	*Micrococcus luteus*
CCM 884	*Micrococcus varians*
CCM 679	*Micrococcus roseus*
CCM 764	*Micrococcus freudenreichii*
CCM 885	*Staphylococcus aureus*
CCM 2124	*Staphylococcus epidermidis*

TABLE 2

The subgroups and culture numbers of strains obtained from the Colworth Bacterial Culture Collection (CBCC) and their relationship to named species of *Staphylococcus* and *Micrococcus*

CULTURE NUMBER	SUBGROUP	ORIGINAL SOURCE	RELATIONSHIP TO NAMED SPECIES (BAIRD-PARKER, 1973; PERS. COMM.)
CBCC 272	Baird-Parker *Staphylococcus* subgroup I		*Staphylococcus aureus*
CBCC 1512	Baird-Parker *Staphylococcus* subgroup II	Dust	
CBCC 1462	Baird-Parker *Staphylococcus* subgroup III	Pig skin	
CBCC 1466	Baird-Parker *Staphylococcus* subgroup IV	Human	*Staphylococcus epidermidis*
CBCC 1477	Baird-Parker *Staphylococcus* subgroup V	Nasal swab	
CBCC 1464	Baird-Parker *Staphylococcus* subgroup VI	Human	
CBCC 1557	Baird-Parker *Micrococcus* subgroup 1	Human urinary tract	
CBCC 1588	Baird-Parker *Micrococcus* subgroup 2	Bacon	*Staphylococcus saprophyticus*
CBCC 1587	Baird-Parker *Micrococcus* subgroup 3	Dust	
CBCC 1839	Baird-Parker *Micrococcus* subgroup 4	Cheese	
CBCC 1469	Baird-Parker *Micrococcus* subgroup 5	Bacon	*Micrococcus varians*
CBCC 1463	Baird-Parker *Micrococcus* subgroup 6	Pig skin	
CBCC 1465	Baird-Parker *Micrococcus* subgroup 7	Pig skin	*Micrococcus luteus*
CBCC 1489	Baird-Parker *Micrococcus* subgroup 8	Pig skin	*Micrococcus roseus*
CBCC 1649	*Sarcina lutea*	Not given	
CBCC 1522	*Aerococcus* NCTC 7597	Not given	

2.2 BIOCHEMICAL TEST METHODS

2.2.1 Catalase test

Catalase production was determined by either one of the following methods:

(i) A loopful of growth (from a plate or broth culture) was emulsified with a droplet of 3% ($^{v}/_{v}$) hydrogen peroxide on a glass surface;

(ii) One ml of culture from a broth culture was added to 1 ml hydrogen peroxide in a small test tube.

Effervescence indicated the presence of the enzyme in the culture.

2.2.2 Oxidase test

The method of Kovacs (1956) as outlined in Harrigan and McCance (1966, p.65) was used.

2.2.3 Oxidative or fermentative utilization of glucose

The modified Hugh and Leifson medium recommended by the I.C.S.B. Subcommittee (1965) and Baird-Parker (1966) was used. Incubation was at 37 ^{o}C for 7 days and the tubes were examined daily. If acid was produced throughout both tubes, the organisms were assigned to the genus *Staphylococcus* whereas those which did not produce acid or produced acid in the aerobic tube were relegated to the genus *Micrococcus*.

2.2.4 Growth in Evans and Kloos' medium (Anaerobic utilization of glucose)

The modified Brewer's Thioglycollate medium (Oxoid) and methods recommended by Evans and Kloos (1972) were used. Incubation was at 37 ^{o}C for 3 days and cultures were examined daily for anaerobic growth.

2.2.5 Acid production from sugars

The plate method recommended by Baird-Parker (1966) proved unsatisfactory. Therefore the modified Hugh and Leifson medium in tubes (Subcommittee, 1965) with the relevant 10% ($^{w}/_{v}$) filter sterile, aqueous sugar solution added to a final concentration of 1% ($^{w}/_{v}$) was used. Incubation was at 30 ^{o}C for 7 days and tubes were examined daily for acid production.

2.2.6 Acetoin production from glucose

Acetoin production was detected using the method recommended by Baird-Parker (1966).

2.2.7 Phosphatase production

Phosphatase production was determined using the method described by Baird-Parker (1966).

2.2.8 Reduction of nitrate

Nitrate reduction to nitrite was determined by the method described by Harrigan and McCance (1966, p.56) and results were recorded after incubation at 30 ^{o}C for 5 days.

2.2.9 Production of ammonia from urea

Urease production was determined using Christensen's Urea Agar (Oxoid) and the method described by Harrigan and McCance (1966, p.56). Tubes were incubated at 30 ^{o}C for 5 days and examined daily.

2.2.10 Hydrolysis of Tween 80

The method of Sierra (1957) as described by Harrigan and McCance (1966, p.64) was used. The plates were incubated at 30 ^{o}C and examined daily for 5 days. Opaque zones surrounding the microbial growth were taken as being indicative of positive lipolytic activity.

2.2.11 Hydrolysis of suet

The modified Eijkman-plate method (Tuynenburg Muys and Willemse, 1965) with an I.S.O. agar (Barraud et al., 1967) overlay was used. Plates were incubated at 30 °C and examined daily for 5 days.

2.2.12 Hydrolysis of gelatin

Hydrolysis of gelatin was tested using Frazier's gelatin agar and the method outlined in Harrigan and McCance (1966, p.51). Plates were incubated at 30 °C for 3 days before recording results.

2.2.13 Sodium chloride tolerance and growth response at 10 °C and 45 °C

The presence or absence of visible growth in broth cultures was considered to be indicative of a positive or negative growth response under specific growth conditions.

(i) Sodium chloride tolerance was determined in a peptone-yeast extract broth [0,5% ($^{w}/_{v}$) peptone (Difco); 0,3% ($^{w}/_{v}$) yeast extract (Difco)] to which 10% ($^{w}/_{v}$) NaCl (Merck) had been added. Tubes were incubated at 30 °C and the results recorded after 3 days.

(ii) Growth response at 10 °C and 45 °C was determined in yeast extract-glucose broth as outlined in Collins and Lyne (1970, p.118). Tubes were incubated in either a waterbath set at 45 °C or in a cooled waterbath at 10 °C for 3 days.

2.2.14 Egg-yolk clearing

The method outlined in Harrigan and McCance (1966, p.66) using Nutrient Agar (Difco; BBL) and Egg Yolk Emulsion (Oxoid), was followed. Plates were incubated at 30 °C and examined daily for 3 days.

2.2.15 Production of ammonia from arginine

The method of Abd-el-Malek and Gibson (1948) as described by Harrigan and McCance (1966, p.54) was used. Results were recorded after incubation at 30 °C for 5 days.

2.2.16 Coagulase production

Using Bacto-Coagulase Plasma (Difco) and the method recommended in the *Difco Manual* (1953, p.330), presumptive *Staphylococcus aureus* strains were tested for coagulase production.

2.2.17 Pigmentation

Pigment characteristics were recorded from the phenolpthalein diphosphate plates (see 2.3.7) after 3 days incubation (Baird-Parker, 1966).

2.3 IDENTIFICATION OF THE ISOLATES

For the identification of the Gram-positive, catalase-positive cocci isolated from biltong by Reiche (1972), a scheme based on the Baird-Parker scheme (Baird-Parker, 1965; 1966), was used to group the isolates initially with the idea of eventually relating the Baird-Parker subgroups to named species of staphylococci and micrococci.

2.3.1 In an experimental system, 20 randomly selected isolates were subjected to a series of 42 biochemical tests and grouped according to the Baird-Parker scheme (Baird-Parker, 1965; 1966). Some emphasis was placed on the biochemical characters described in Baird-Parker's abridged scheme (Baird-Parker, 1966) and consequently the grouping of certain isolates was biased. This scheme was tedious and time-consuming and the results proved unsatisfactory, as although nearly all the isolates could be assigned to Baird-Parker subgroups, difficulties were encountered in trying to relate the subgroups to named species or groups of micrococci or staphylococci.

2.3.2 Initial grouping

A second scheme, also based on Baird-Parker's scheme (Baird-Parker, 1965; 1966) but using only 25 of the biochemical tests used previously (see 2.3.1) was devised. Isolates were not grouped according to Baird-Parker's scheme but by direct comparison with the results obtained for type material, i.e. 32 strains of named species and subgroups, under the same conditions, and then related to Baird-Parker subgroups using available information. Adansonian principles of each character carrying the same weight were applied in most cases, but weighting of some biochemical characters e.g. anaerobic or aerobic acid production from glucose and acetoin production was often used to group controversial isolates.

2.4 COMPUTATION OF IDENTIFICATION OF ISOLATES BY BIOCHEMICAL METHODS

To try and exclude personal bias from the identification system based on biochemical methods, two computer programs were prepared whereby the biochemical characters of each isolate were compared to those obtained for the standards (32 named species and subgroups of *Staphylococcus*, *Micrococcus*, *Planococcus*, *Aerococcus* and *Sarcina*) as given in Tables 1 and 2.

2.4.1 First program

Using this program the biochemical characters of each isolate were compared with those obtained for all 32 standards and then related to a specific named species or subgroup by the following two methods:

(i) Strict Adansonian principles of each character carrying the same weight were applied and corresponding positives (++) and negatives (--) were recorded as hits and counted to give a score with a maximum of 25 hits; and

(ii) Eight biochemical characters viz. aerobic or anaerobic acid production from glucose; acid production from L(+)-arabinose, mannitol, maltose and lactose; acetoin and phosphatase production; were weighted (simulating personal bias) and again corresponding positives (++) and negatives (--) were recorded as hits with a maximum of 40 hits.

2.4.2 Second program

This program enabled the *Staphylococcus* and *Micrococcus* isolates to be differentiated separately. Personal bias and weighting could not be excluded from this system of grouping as prior to being processed by the computer, the isolates were separated into the genera *Staphylococcus* and *Micrococcus* on the basis of their anaerobic or aerobic acid production from glucose, acetoin production and growth in the medium recommended by Evans and Kloos (1972). Subsequently ammonia production from arginine was also used to differentiate between these two genera. The biochemical characters of those isolates grouped initially as *Staphylococcus* were then compared with those obtained for the 15 named species and subgroups of *Staphylococcus* while the biochemical characters of those isolates relegated to the genus *Micrococcus* were compared to those obtained for the 16 named species and subgroups of *Micrococcus*, *Sarcina* and *Aerococcus*. Using strict Adansonian principles of each character carrying the same weight, corresponding positives (++) and negatives (--) were recorded as hits and counted to give a score with a maximum of 25 hits after which the isolate could be related to a specific species or subgroup in the corresponding genus.

3. RESULTS AND DISCUSSIONS

3.1 INITIAL IDENTIFICATION OF THE GRAM-POSITIVE COCCI ISOLATED FROM BILTONG

The initial identification of the Gram-positive, catalase-positive cocci isolated from biltong is presented in Table 3. Detailed information concerning the biochemical characters of each isolate and of the named species and subgroups of *Staphylococcus*, *Micrococcus*, *Planococcus*, *Aerococcus* and *Sarcina* and their possible relationships, appear in Appendix A.

The initial method (see 2.3.2) used to identify the isolates proved most satisfactory as all the isolates could be related to a named species or subgroups of *Staphylococcus* or *Micrococcus*. *Staphylococcus saprophyticus* was the predominant species, comprising 65% of the isolates identified, while *Micrococcus luteus*, *Micrococcus varians* and *Staphylococcus epidermidis* occurred in lower numbers comprising only 8%, 19% and 7% of the isolates identified respectively.

3.2 THE INTERRELATIONSHIPS BETWEEN THE COMPUTER GROUPING AND THE INITIAL GROUPING OF THE GRAM-POSITIVE COCCI ISOLATED FROM BILTONG

The results obtained from the computer analyses of the same data used for the initial grouping, were not in complete agreement with those obtained from the initial grouping (see Table 4, the details of which appear in Appendix B).

From Table 4 it is evident that only 46% complete correlation between the results obtained for the initial grouping and those obtained from computer analysis using the first program (see 2.4.1) was found, compared to the 51% complete correlation observed if the second program (see 2.4.2) was used.

TABLE 3

Initial identification of the Gram-positive, catalase-positive cocci isolated from biltong

	SOURCE OF ISOLATE														TOTAL	
	BILTONG												GRAUBUNDNER-FLEISCH (GB)			
	BEEF (B)*		BEEF (C)		BEEF (D)		ELEPHANT (E)		BUFFALO (F)		OSTRICH (G)					
Species	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
S. saprophyticus	10	91	10	67	14	82	13	100	9	82	5	20	1	33	62	66
M. varians	1	9	4	27	1	6			1	9	9	36	2	67	18	19
M. luteus			1	6	2	12					5	20			8	8
S. epidermidis									1	9	6	24			7	7

(B)* - (GB) denote the source of the isolate in Appendices A and B

TABLE 4

Comparison between initial grouping and computer grouping (1st program); comparison between initial grouping and computer grouping (2nd program); and comparison between computer grouping (1st program) and computer grouping (2nd program)

		Number	%
Total number of strains processed		94	100
(i) Initial grouping vs. computer grouping (1st program)	Complete correlation (++)*	43	46
	Partial correlation (+-)	2	2
	Partial correlation (-+)	8	8
	No correlation (--)	41	44
(ii) Initial grouping vs. computer grouping (2nd program)	Complete correlation	48	51
	No correlation	46	49
(iii) Computer grouping (1st program) vs. computer grouping (2nd program)	Complete correlation (++)*	65	69
	Partial correlation (+-)	8	9
	Partial correlation (-+)	5	5
	No correlation (--)	16	17

(++)* The computer grouping (1st program), using both weighted and unweighted characters, agrees with the initial grouping and the computer grouping (2nd program).

(+-) The computer grouping (1st program), when no characters are weighted, agrees with the initial grouping and the computer grouping (2nd program).

(-+) The computer grouping (1st program), using weighted characters, is in agreement with the initial grouping and the computer grouping (2nd program).

(--) The computer grouping (1st program), using both weighted and unweighted characters, differs from both the initial grouping and the computer grouping (2nd program).

However, when the results obtained from the two computer groupings are compared [see Table 4 (iii)], 69% complete correlation is observed with only 17% of the isolates showing no correlation at all.

It therefore appears that the results obtained from computer analyses are fairly uniform and independent of the program used.

When the results of the initial grouping are compared to the computer grouping using the first program [see Table 4 (i)] the following is observed. If those isolates exhibiting partial correlation (-+), i.e. where the results obtained using weighted characters are in agreement with the initial grouping, are considered in conjunction with those exhibiting complete correlation with the initial grouping, 54% correlation with the initial grouping is obtained. This increase in correlation, based on results obtained when certain characters were weighted, corresponds closely to the 51% correlation found between the initial grouping and computer grouping using the second program [cf. Table 4 (ii)], where the weighting of characters and personal bias influences the differentiation of the isolates prior to computer analysis (see 2.4.2).

This increased correlation between the initial grouping and the computer grouping derived from results obtained when the weighting of characters was employed, is considered indicative of how the weighting of certain characters and personal bias may have affected the initial grouping of the isolates.

3.3 FINAL IDENTIFICATION OF THE GRAM-POSITIVE, CATALASE-POSITIVE COCCI ISOLATED FROM BILTONG

In view of the discrepancies encountered between the different methods of grouping, it became necessary to review the initial identification of the isolates. The biochemical characters of each isolate had to be re-evaluated, and where discrepancies occurred, the computer grouping was also evaluated and was either accepted or rejected before the isolate was finally identified.

The final identification of the isolates is presented in Table 5, the details of which appear in Appendix B.

TABLE 5

Final identification of the Gram-positive, catalase-positive cocci isolated from biltong

	SOURCE OF ISOLATE														TOTAL	
	BILTONG												GRAUBUNDNER-FLEISCH (GB)			
	BEEF (B)*		BEEF (C)		BEEF (D)		ELEPHANT (E)		BUFFALO (F)		OSTRICH (G)					
Species	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
S. saprophyticus	10	91	10	67	14	82	11	85	9	82	5	20	1	33	60	63
S. epidermidis							2	15	1	9	6	24			9	9
M. luteus			3	20	1	6					2	8			6	6
M. varians	1	9	2	13					1	9	8	32	2	67	14	15
M. kristinae											2	8			2	2
M. lylae					1	6									1	1
Sarcina lutea (≡ *M. luteus*)					1	6					1	4			2	2
M. freudenreichii											1	4			1	1

(B)* - (GB) denote the source of the isolate in Appendices A and B

From Table 5, it is evident that *Staphylococcus saprophyticus* remains the dominant component of the Gram-positive cocci. This species only comprises 63% of the isolates identified compared to the 65% obtained after initial identification (see Table 3). The number of isolates cited as *Staphylococcus epidermidis* increased from 7% to 9% of the isolates identified whereas the number of isolates identified as *Micrococcus varians* decreased from 19% to 14% of the isolates identified. Four of the isolates previously cited as *Micrococcus varians* or *Micrococcus luteus* were transferred to the species *Micrococcus kristinae*, *Micrococcus lylae* and *Micrococcus freudenreichii* forming 2%, 1% and 1% of the isolates identified respectively. Two of the isolates previously identified as *Micrococcus varians* i.e. GL_{3y} and DH_3 were re-classified as *Sarcina lutea*, which is now considered to be synonymous to *Micrococcus luteus* (Baird-Parker, 1965; *Bergey's Manual*, 1974).

Of the isolates showing no correlation when the results of the initial grouping was compared to those of the computer grouping using the first program [see Table 4 (i)], the initial grouping was retained for 10 of the isolates while the computer assisted in the final identification of the remaining 31 isolates.

Therefore, although the computer is a useful aid for the efficient identification of the Gram-positive, catalase-positive cocci, the computer results cannot be accepted without a personal evaluation of each isolate being made before the isolate is finally grouped.

3.4 RELATIONSHIPS BETWEEN THE SOURCE AND THE FINAL IDENTIFICATION OF THE GRAM-POSITIVE, CATALASE-POSITIVE COCCI ISOLATED FROM BILTONG

From Table 5 it appears that from beef biltong, buffalo biltong and elephant biltong, *Staphylococcus saprophyticus* was the predominant species isolated, comprising 67-91% of the isolates identified. *Micrococcus varians*, *Micrococcus luteus*, *Micrococcus lylae*, *Micrococcus luteus* (≡ *Sarcina lutea*) and *Staphylococcus epidermidis* occurred in lower numbers, constituting 6-20% of the isolates identified. The ostrich biltong, however, exhibited a completely different profile of cocci; *Micrococcus varians* predominated, comprising 36% of the isolates identified with

Staphylococcus epidermidis and *Staphylococcus saprophyticus* present at similar, lower frequencies i.e. 24% and 20% of the isolates identified respectively. *Micrococcus luteus, Micrococcus kristinae, Micrococcus luteus (≡ Sarcina lutea)* and *Micrococcus freudenreichii* comprised only 4-8% of the isolates identified.

All these species are common to soil, dust, water and warm-blooded sources. Their presence on biltong could therefore be attributed to one or more of the following:

(i) contamination during slaughter;

(ii) the subsequent handling of the carcass and meat cuts;

(iii) contamination during the handling and processing of the product;

(iv) chance aerial contamination;

(v) the curing salt; as well as

(vi) spices and other curing additives when used.

III. CHANGES IN THE AEROBIC SAPROPHYTIC MICROBIAL FLORA DURING BILTONG PRODUCTION

1. INTRODUCTION AND REVIEW OF THE STAPHYLOCOCCI AND MICROCOCCI ASSOCIATED WITH MEAT AND CURED MEAT PRODUCTS

1.1 INTRODUCTION

In the past, the microbiological investigations concerning biltong have centred mainly around the public health aspects of this commodity (see I.2), with relatively little notice being taken of the saprophytic microbial flora and their relationship with the environment (see I.3). As salt-tolerant micrococci and staphylococci were reported to be the dominant component of the microflora of commercial biltong (Reiche, 1972), compared to the Gram-negative rods characteristic of fresh meat (Kitchell, 1962), an investigation into the changes occurring in the microbial flora during biltong production in relation to the environment could lead to a greater understanding of the microbial associations within the product and thereby assist in improving methods of production.

1.2 THE STAPHYLOCOCCI AND MICROCOCCI ASSOCIATED WITH MEAT AND CURED MEAT PRODUCTS

Cured meat products are reported to have a completely different bacterial flora to that of fresh meat (Lechowich et al., 1956) and lipolytic micrococci and coagulase-negative staphylococci were found to be the major component of the microflora of products such as charque (charqui) (Gutheil, 1958), Italian type raw hams (Giolitti et al., 1971), bacon and bacon curing brines (Patterson, 1966; Kitchell, 1958; Gardner and Patton, 1969; Dempster, 1973) and fermented meat products (Pohja and

Gyllenberg, 1962; Smith and Palumbo, 1973), with lactobacilli and Gram-negative rods occurring to a lesser extent. This replacement of the *Pseudomonas/Achromobacter* group by a flora comprising predominantly micrococci and coagulase-negative staphylococci, has been ascribed to the selective activity of meat curing salts (Lechowich et al., 1956; Dainty, 1971) of which sodium chloride has been implicated as the major selective agent. The addition of sodium chloride and other curing salts e.g. sodium nitrite and potassium nitrate is used to prevent the growth of putrefactive organisms while permitting the proliferation of ostensibly harmless saprophytic organisms.

The role of sodium chloride as an inhibitor has been reviewed by Ingram and Kitchell (1967), who pointed out that the selective, preservative activity of sodium chloride is dependent on a number of factors. These include the sensitivity of different organisms to this salt e.g. organisms less tolerant to sodium chloride will be excluded while halophilic and halo-tolerant organisms will be selected for, and the environmental conditions such as pH, temperature and availability of oxygen. For example, micrococci and staphylococci were found to be the predominant species present in lean Wiltshire bacon of low pH stored under aerobic conditions at 10 ^{o}C (Gardner, 1971), whereas the incidence of lactic acid bacteria, organisms not readily recovered from fresh bacon, increased during the storage of vacuum packed, 'sweet-cured' bacon at 10 ^{o}C with a corresponding decrease in the staphylococci and micrococci population (Dempster, 1973). However, the micrococci, coagulase-negative staphylococci and lactobacilli have been implicated in the spoilage of vacuum packed bacon (Kitchell, 1962).

The role of other curing agents such as nitrate and nitrite in meat preservation is not clear, although nitrite at a low pH forms nitrous acid which is known to possess antibacterial properties, especially against the micrococci (Dainty, 1971). However, their use in food products is not entirely desirable, as Ayanaba and Alexander (1973) have demonstrated a number of microbiologically catalazed steps leading to the appearance of nitrosamines from amines and nitrite.

Micrococci have also been found to be present in canned cured meat products which have either been pasteurized or processed to 'commercial

sterility'. Their presence is ascribed to the increased heat resistance of micrococci in the presence of fat or to contamination after processing (Kitchell, 1962).

The activities and the presence of these micrococci and coagulase-negative staphylococci in cured meat products can be both deleterious, by causing meat spoilage (Kitchell, 1962), or advantageous, by reducing nitrate to nitrite to give the product a good cured meat colour (Smith and Palumbo, 1973).

For further information concerning these aspects, reference may be made to the review article by Kitchell (1962).

The presence of micrococci and staphylococci in meat products has been related to flavour production within the product. Pohja (1960) proposed that the presence of micrococci in dry sausages improved its colour, aroma, flavour and keeping quality whereas Lerche, according to Kitchell (1962), found that the best flavour and aroma production in dry sausage was produced by a combination of yeasts and micrococci. It has also been suggested that diacetyl production by organisms such as coagulase-negative staphylococci may be involved in bacon flavour production (Kitchell, 1962). Gutheil (1958) believed that moderate growth of micrococci developed the flavour of charque by the mild rancidification of the fats present in the meat.

1.3 THE USE OF PRESERVATIVES DURING BILTONG PRODUCTION

Biltong, both sticks and packeted slices, is reported to be susceptible to yeast and mould spoilage (van den Heever, 1972). In addition, certain species of fungi encountered in biltong e.g. *Aspergillus flavus* have been known to product aflatoxin, a powerful carcinogen, in country-cured hams at 30 ^{o}C (Bullerman et al., 1969). Therefore, a need to control fungal growth in biltong exists for both economic and hygienic reasons. At present, only one preservative is permitted in biltong. In terms of the amended Food, Drugs and Disinfectants Act, 1929 (1972), sorbic acid at a concentration of 2 000 ppm, calculated on a wet weight basis (as is in the sample), is permissible.

Sorbic acid, a six carbon α, β-unsaturated fatty acid, and its salts are mainly used as fungistatic agents in foods (Nury and Bolin, 1962; Frazier, 1967). Sorbic acid is known to inhibit some aerobic bacteria (Frazier, 1967, p.138) but has little effect against lactic acid bacteria and anaerobes at pH values greater than pH 5 and pH 7 respectively (Bell et al., 1959). Van den Heever (1972) found that immersion of salted strips of meat in a solution containing 20% sodium chloride and 2% potassium sorbate prior to drying resulted in the final product containing 2 000 ppm sorbic acid, which he considered sufficient for good control of fungal and yeast growth on biltong during commercial processing and handling procedures. However, no mention was made regarding the bacterial flora of biltong in the presence of potassium sorbate.

Delvocid S, a fungicide used to control mould spoilage in the meat and cheese industry in Europe (Gist-Brocades N.V.; technical brochure) may also have its applications in the biltong industry. The activity of Delvocid S, compared to that of organic acids such as sorbic acid, is relatively independent of pH although pH values lower than pH 3 and higher than pH 9 may result in a decrease in the activity of the active ingredient. As the pH of biltong ranges from pH 5,6 to pH 6,6 (van den Heever, 1970a, b) it falls within the pH range in which Delvocid S is stable.

Pimaricin, an antibiotic produced by *Streptomyces natalensis*, is the active ingredient of Delvocid S, and is a white, odourless and tasteless powder which, in low concentration, inhibits the growth of a large number of yeasts and moulds. It is not known to affect bacterial growth and the manufacturers claim that it does not interfere with the bacterial growth required during the manufacture of certain cheeses.

Although its use is not permitted in the South African meat industry at present, the use of pimaricin for control of mould spoilage in biltong is under consideration. It was therefore necessary to ascertain whether the presence of pimaricin in biltong would affect the saprophytic bacterial flora of this product.

Biltong was therefore prepared and dried in the laboratory under controlled conditions to ascertain:

(i) What changes occurred in both bacterial numbers and species during the process;

(ii) What the possible factors causing these changes were;

(iii) How these changes were affected by the presence of preservatives; and

(iv) What the possible contribution of the saprophytic bacterial flora to biltong flavour production was.

2. METHODS AND MATERIALS

2.1 PREPARATION OF BILTONG UNDER CONTROLLED CONDITIONS

2.1.1 Standard method for the preparation of biltong

Throughout this investigation topside beef, i.e. the semimembranosus muscle and surrounding meat, was purchased for the preparation of biltong.

The method of biltong production used during this investigation was based on the method adopted by the Food Technology Division of the National Food Research Institute of the C.S.I.R.

Using hygienic conditions and sterile utensils, the semimembranosus muscle was separated from the rest of the meat and trimmed free of visible extraneous fat, tendon and connective tissue. The muscle was cut along the line of the fibres into strips measuring approximately 2,5 cm x 4 cm x 20-30 cm. The strips of meat were placed in a sterile stainless steel container and dry-salted with heat sterilized (160 ^{o}C for 2 h) sodium chloride (Merck) to a final concentration of 2,5% ($^{w}/_{w}$) and left to pickle for 22 hours at 4 ^{o}C. To ensure the uniform salting of each strip of meat, the strips were turned regularly. After pickling,

the strips of meat were hung to dry in an especially designed drier set to the constant conditions of 35 ^{o}C, 30% relative humidity and an airspeed of 3 m/sec.

After drying for 144 hours, the biltong was removed from the drier and stored, in unsealed polythene bags, at ambient room temperature for a further 144 hours.

To monitor changes, samples were removed at regular time intervals throughout the process for both microbial and physico-chemical analyses.

2.1.2 Preparation of biltong from commercially available beef

In order to simulate the commercial process, six samples of meat (see Table 6) were purchased from four different retail sources and prepared following the standard method described previously (see 2.1.1).

TABLE 6

Summary of the nature of and subsequent treatment of the meat

MEAT SOURCE	NATURE OF AND TREATMENT OF THE MEAT
Butcher A	One topside, 5 kg, was purchased. Meat was lean with healthy pink colour. Gloves were not worn during laboratory preparative procedures.
Butcher A	One topside, 5,5 kg, was purchased. Meat was lean with healthy pink colour. Gloves were worn throughout laboratory preparative procedures.
Butcher A	One topside, 9,2 kg, was used. Meat was dark red with much blood present; very fatty; once it was minced it became sticky and dough-like in texture. Gloves were worn during laboratory preparative procedures.

TABLE 6 (Cont.)

MEAT SOURCE	NATURE OF AND TREATMENT OF THE MEAT
Butcher B	Two semi-membranosus muscles, totalling 9,8 kg, were bought. Both muscles were lean with healthy pink colour. The strips cut from each muscle were mixed during preparative procedures and two strips were chosen at random for each sample. Gloves were worn during laboratory preparative procedures.
Butcher C	Two topsides, totalling 7,4 kg, were used. Meat lean with healthy pink colour. Gloves were worn during laboratory preparative procedures.
Butcher D	Two topsides, totalling 9,5 kg, were bought. Meat was lean with healthy colour. Gloves were worn during laboratory preparative procedures.

2.1.3 Preparation of biltong using aseptically dissected beef

As a control for the biltong prepared from commercially available beef, three samples of aseptically dissected beef were obtained from the Veterinary Research Institute, Onderstepoort and processed using the technique described previously (see 2.1.1).

The term 'aseptically dissected beef' is used to described topside beef removed, using sterile hooks and knives, from a freshly slaughtered animal. The muscle was transported from the abattoir to the laboratory in a sterile container and stored at 4 ^{o}C for approximately 22 hours before processing. This storage period was necessary to facilitate some of the biochemical and biophysical changes necessary for the conversion of the muscle to meat e.g. post-mortem glycolysis (Lawrie, 1974, p.127).

Gloves were worn throughout preparative procedures and thus organisms present on aseptically dissected beef and the resultant biltong

could be considered to be derived from sources other than from human origin.

2.1.4 Preparation of biltong from commercially available beef treated with potassium sorbate during salting

In an investigation to ascertain how the presence of 1 500-2 000 ppm sorbate in biltong would affect the saprophytic bacterial flora of biltong, biltong was prepared from topside beef obtained from a retail source (Butcher A), using the standard method described previously (see 2.1.1).

Potassium sorbate (Merck) was added to the meat during dry-salting at a concentration of a 1 000 ppm (wet weight basis). The use of this concentration was based on the assumption that as biltong loses about 50% moisture during processing, the potassium sorbate would be concentrated to between 1 500 ppm and 2 000 ppm in the final product. Potassium sorbate is used in preference to sorbic acid as sorbic acid is highly insoluble.

Each sample of meat removed for microbial and physico-chemical analysis was also analyzed for sorbate using the method of Nury and Bolin (1962).

2.1.5 Preparation of biltong from commercially available beef treated with pimaricin during salting

In a controlled experiment similar to those described previously (see 2.1.1) biltong was prepared from commercially available beef (purchased from Butcher A). Pimaricin (sample from Gist-Brocades N.V.) was added during dry-salting at a final concentration of 50 ppm (wet weight basis). This concentration was used as it is reported to inhibit the growth of a wide range of yeasts and moulds (Gist-Brocades N.V.; technical brochure).

2.2 STANDARD METHOD FOR THE PREPARATION OF SAMPLES FOR ANALYSIS

Using hygienic conditions, the sample strips of meat, pickled meat and biltong, weighing between 100 g and 200 g were cut into smaller manageable pieces and then, depending on their degree of dehydration, were treated using one of two methods. The samples were either ground in a sterile meat grinder or pulverized in a sterile 'Braun' coffee grinder for 30-60 seconds at slow speed (setting 1), to ensure thorough mixing of the sample. The resultant ground or pulverized sample was homogenized in the M.S.E. Atomix blender.

2.3 MICROBIOLOGICAL ANALYSIS OF MEAT AND BILTONG

2.3.1 Enumeration of bacteria

The method recommended by the International Organization of Standardization (I.S.O.) (Barraud et al., 1967) for the determination of the total aerobic count of bacteria in meat and meat products was used as the basis for the bacteriological analysis of samples (see Appendix C). In addition, a number of different selective media were used to differentiate between groups of organisms in order to establish what changes occurred in both number and species of bacteria during processing.

2.3.2 Media used

The media used, along with the incubation temperatures and times are presented in Table 7.

Initially the number of coliforms, group D streptococci, aerobic and anaerobic spore-formers, anaerobic total count, lipolytic organisms and proteolytic organisms present were also enumerated. However, as the results obtained were not significant, the enumeration of these organisms was omitted from later investigations.

TABLE 7

The media used for evaluating the flora of meat and biltong

GROUP	INCUBATION		MEDIUM
	Temp. (°C)	Time (h)	
Total aerobic count	30	72	I.S.O. medium (Barraud et al., 1967) at pH 7,2
Organisms tolerant to 10% NaCl	30	72	I.S.O. medium (Barraud et al., 1967) plus 10% ($^{W}/_{V}$) NaCl (Merck)
Micrococcaceae	30	48	Mannitol Salt Agar (Oxoid; Gibco)
Pseudomonas/ Achromobacter spp.	25	72	Masurovsky et al. (1963)
Lactobacilli	25	120	Rogosa et al. (1951)
Yeasts and moulds	25	120	Potato Dextrose Agar (Oxoid; Gibco) at pH 3,8

For the investigations concerning the effect of the presence of pimaricin or sorbate on the saprophytic microbial flora of biltong, a number of media were modified to select for organisms tolerant to 50 ppm pimaricin and 2 000 ppm sorbate at both pH 5,8 and pH 7,2 (see Appendix D).

As the anti-microbial action of preservatives is often pH dependent, media at both pH 5,8 and pH 7,2 were used. Thus, the effectiveness of the preservative as detected using media at pH 5,8, can be directly related to its effectiveness in biltong, which is reported to have an average pH 5,8.

2.3.3 Isolation and identification of bacteria

The plates used to enumerate the total aerobic counts were divided into sectors to give approximately 30 colonies per sector. All the

colonies from a single sector were picked into Nutrient Broth (Difco; BBL) or onto I.S.O. agar (Barraud et al., 1967) plates and incubated at 30 °C. Thus 20-30 isolates per sample were obtained.

The isolates were purified by the successive streaking of a suspension (broth or distilled water) of the isolate across Nutrient Agar (Difco; BBL) or I.S.O. agar (Barraud et al., 1967) plates. Using the scheme described by Gardner (1968) (Table 8) the isolates were differentiated into presumptive groups.

TABLE 8

Scheme used for the initial differentiation of isolates

GRAM REACTION	MORPHOLOGY	CATALASE PRODUCTION	PRESUMPTIVE GROUP
+	Rods or cocci	-	Lactic acid bacteria
-	Rods or coccobacilli	+	Pseudomonadaceae and Enterobacteriaceae
+	Rods and spores	+	*Bacillus* spp.
+	Rods	+	*M. thermosphactum*
+	Pleiomorphic rods	+	*Corynebacterium* spp.
+	Cocci	+	Micrococcaceae

The organisms tentatively grouped as Micrococcaceae were further identified using the scheme described previously (cf. II.).

2.4 PHYSICO-CHEMICAL ANALYSIS OF MEAT AND BILTONG

2.4.1 Water activity (a_w)

The water activity of the ground or pulverized samples was measured in Durotherm "a_w-Wert-Messers" using the method of Rödel and Leistner (1971).

Enough sample to form a 5-10 mm layer in the base of the meter was placed in a meter, pre-calibrated to an a_w-value similar to that expected for the sample. The meter was held at 25 °C for 3½ hours to stabilize before the a_w-value was recorded.

2.4.2 pH

The pH of the fresh and pickled meat was determined by inserting the electrode of a Polymetron type 55 or Beckman Zeromatic SS-3 pH meter into the ground sample. The pH of the drier biltong samples was determined by suspending 5 g pulverized biltong in 7,5 ml distilled water for 15-30 minutes and then measuring the pH of the resultant slurry.

2.4.3 Sodium chloride determinations

The sodium chloride content of the samples was determined using either the method recommended by I.S.O. (1970) for meat and meat products or the method recognized by the Association of the Official Agricultural Chemists (A.O.A.C.) (1965, p.347), for meat and meat products.

2.4.4 Moisture content

The moisture content of the ground or pulverized samples was determined using the methods recommended by the A.O.A.C. (1965, p.347), for meat and meat products.

(i) Air drying

The moisture content of the meat and pickled meat and meat dried for up to 72 hours was determined by mixing 10 g ground sample with 2-5 g pre-weighed sea sand (Merck) in a glass weighing bottle and then air-dried in an air circulation oven for 5 hours at 125 °C. Loss in weight was reported as percentage moisture.

(ii) Drying *in vacuo*

Five to ten grams of the drier, pulverized samples were dried in a glass weighing bottle in a vacuum oven for 16-18 hours at 80 °C. Loss in weight was reported as percentage moisture.

The moisture content of the drier samples was also determined by air drying [see 2.4.4 (i)] of 5-10 g of the sample.

2.4.5 Sorbate analysis

The method developed by Nury and Bolin (1962) for the determination of sorbate in dried fruits was used.

2.5 ORGANOLEPTIC EVALUATION OF BILTONG SAMPLES

The organoleptic evaluation of biltong samples prepared by means of different methods (see 2.1) was carried out to ascertain:

(i) Whether the presence of preservatives could be detected organoleptically; and

(ii) Whether the saprophytic bacterial flora associated with biltong contributed to the biltong flavour production.

Two separate tests were used to investigate the following aspects:

(i) Whether or not there were any significant organoleptic differences between treated samples and untreated controls;

(ii) Whether there was a significant preference for either of these samples; and

(iii) If a significant difference was observed, what the nature of this difference was i.e. a difference detected in flavour, texture, colour or taste.

2.5.1 Triangular test

Panelists were asked to detect which sample out of three was different and also to state their preference for a particular sample or

two samples depending on the combination of the samples evaluated. The results were interpreted using the statistical tables of Larmond (1967).

2.5.2 Independent evaluation of each sample

Panelists were given both samples and asked to evaluate each sample independently using taste, colour, flavour and texture as criteria. The results obtained for each criterion in the first sample were then compared to those obtained for the same criterion in the second sample. Using Freidman two way analysis of variance (Miller, 1966) any significant difference between samples for a single criterion could be detected at any critical value.

3. RESULTS AND DISCUSSION

3.1 THE CHANGES OCCURRING IN THE PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTRATE DURING BILTONG PRODUCTION

The physico-chemical characteristics of biltong prepared from both untreated commercially available beef and commercially available beef treated with preservatives as well as biltong prepared from aseptically dissected beef corresponded well with those reported for commercial biltong (cf. I.2.3). In addition, the results illustrating the changes occurring in the physico-chemical properties of the substrate of all four preparative treatments used during biltong production likewise showed close agreement (see Appendix E). These results are represented graphically (see Figures 1 and 2) using as an example, the results obtained from biltong prepared from commercially available beef.

3.1.1 Changes in pH values

The pH values obtained from fresh meat and biltong were pH 5,72 and pH 5,80 respectively and from Figure 1 it was evident that no significant changes occurred during the processing of meat to biltong. This stability of pH was ascribed to the buffering effect of the myofibrillar proteins in the meat and to a lesser extent to the non-protein components of the meat (Honikel and Hamm, 1974).

The pH values observed for one of the samples of commercially available beef (purchased from Butcher A) ranged between pH 6,36 and pH 6,7 during processing, compared to the range of pH 5,21 to pH 6,0 found during the processing of the other samples from retail sources. This difference in pH corresponded to a marked difference in the quality of the meat (see Table 6), and could be indicative of:

(i) post-mortem bacterial infection of the carcass or subsequent meat cuts (Ingram and Dainty, 1971; Dainty, 1971); or

(ii) the animal was exposed to stress conditions prior to slaughter e.g. excitement or fatigue with the resultant depletion of glycogen reserves causing a high ultimate post-mortem pH (Lawrie, 1974, p.127).

3.1.2 Changes occurring in moisture content

The percentage moisture represents the total moisture present in the sample.

From Figure 2, it appears that after salting of the meat (-22 h) there is a small decrease in the moisture content from 75% to 73% which then remains constant during the pickling process (-22 to 0 h). Upon drying (0 h), there is an initial steady decrease in moisture content till 72 hours of drying after which it decreases more slowly to a final level of 25% and then remains relatively constant during storage.

3.1.3 Changes in sodium chloride content during biltong production

From Figure 2 it is evident that the sodium chloride content, calculated on a wet weight basis (as is), increases rapidly on the addition of salt and remains constant during the pickling process. During drying, there is a steady increase in the sodium chloride content of the pickled meat and after 144 hours of drying reaches a plateau, corresponding to 6,6% sodium chloride.

3.1.4 Changes observed in water activity

Fresh meat was found to have a water activity of $a_{w_{25}}$ 0,986, which allows the growth of a wide range of organisms (Scott, 1957; Mossel, 1971).

From Figure 2, it appears that the changes observed in water activity during the processing of meat to biltong exhibit similar trends to those observed for the changes occurring in moisture content. However, during the first 72 hours of drying, a linear decrease in water activity is found compared to the non-linear decrease observed in moisture content.

On removal of the biltong from the drier and during its subsequent storage at ambient atmospheric conditions, the water activity of the product remained relatively constant.

3.1.5 Changes in sorbate concentration

The changes in the sorbate concentration (calculated on a wet weight basis) during biltong production from meat treated with potassium sorbate (see Figure 3), show similar trends to those observed for sodium chloride (cf. Figure 2). The addition of potassium sorbate to meat at a concentration of 1 000 ppm appeared to maintain the sorbate concentration within the legal limit of 2 000 ppm.

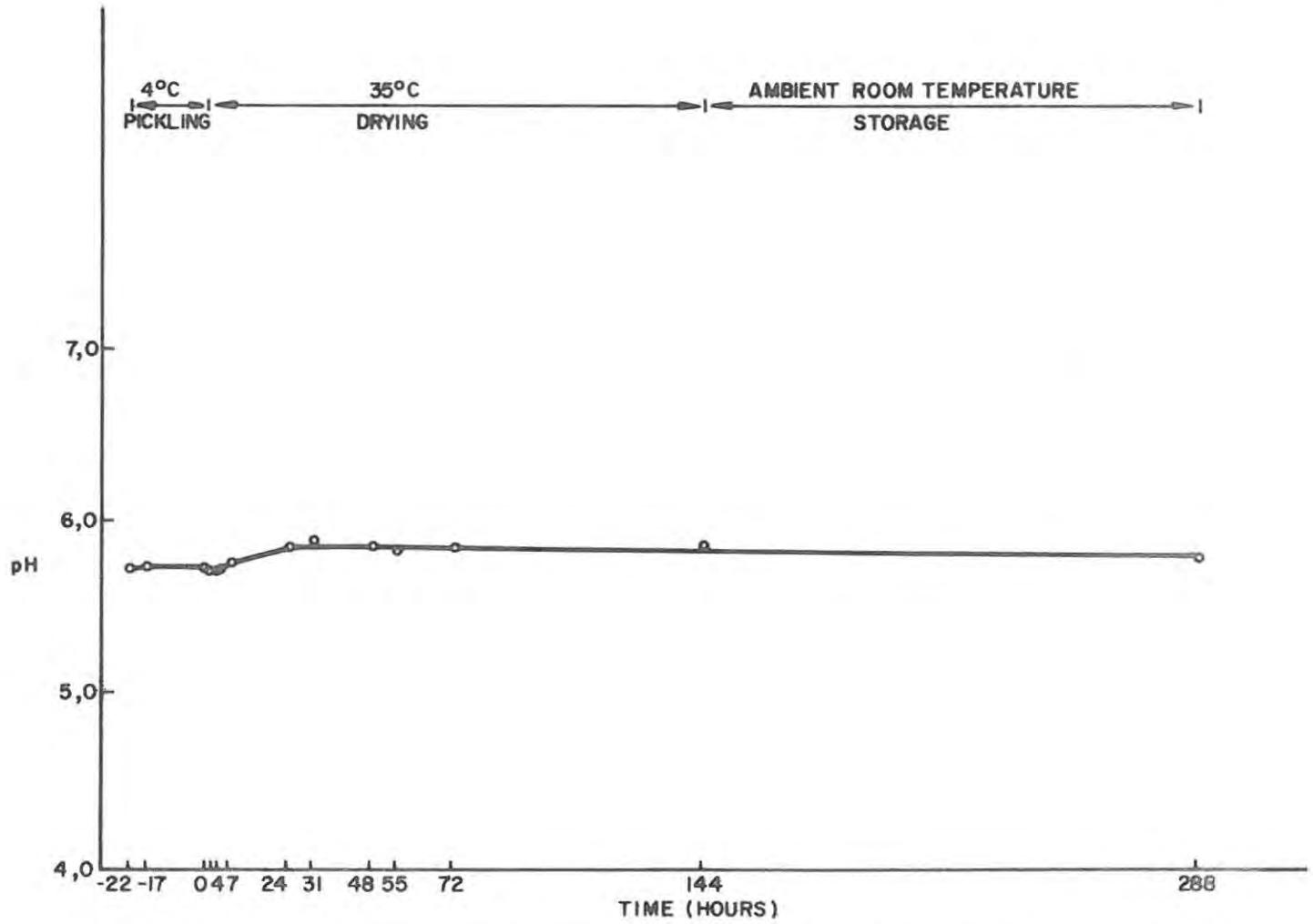

FIGURE 1 pH CHANGES DURING BILTONG PRODUCTION

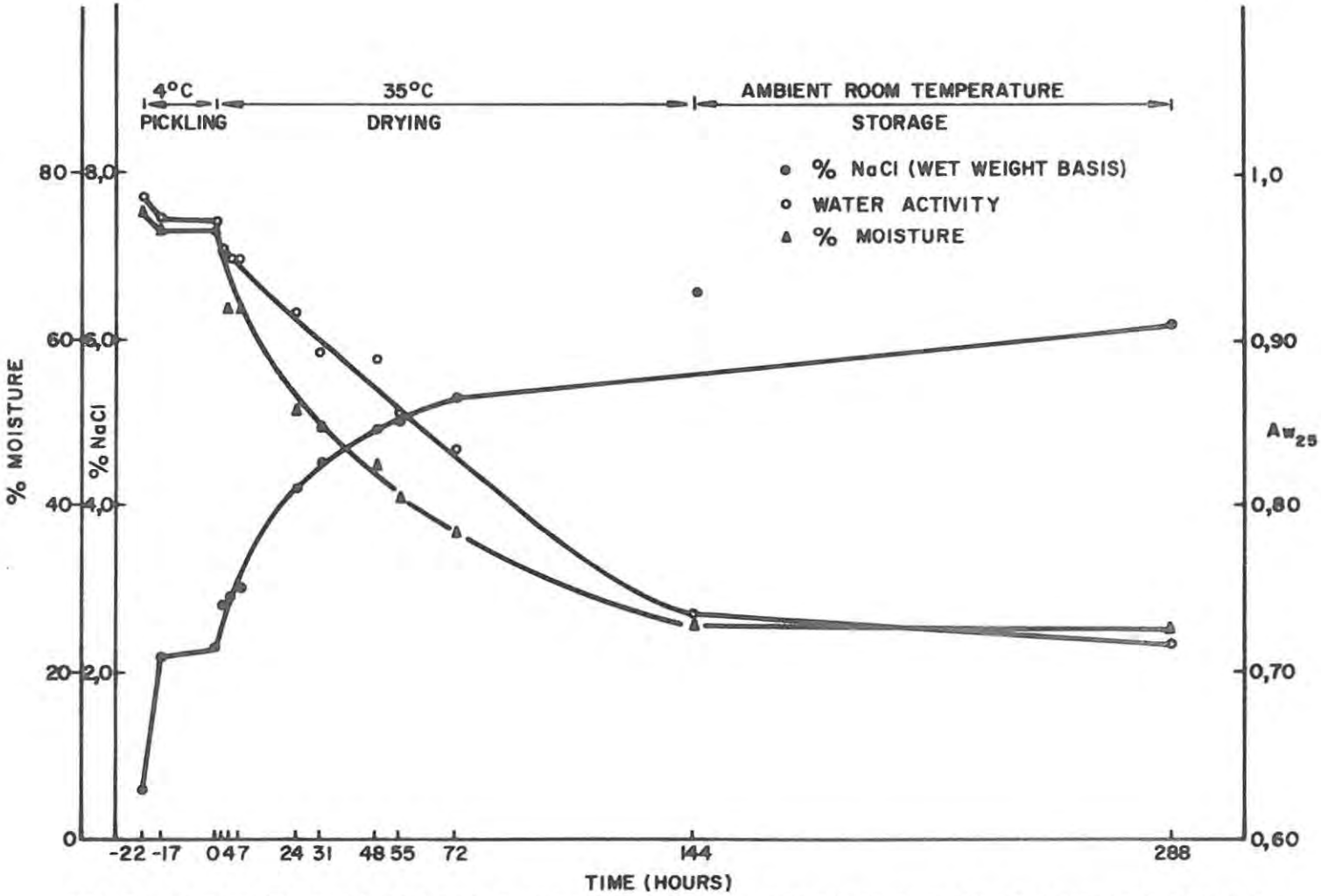

FIGURE 2 CHANGES IN MOISTURE CONTENT, WATER ACTIVITY AND NaCl CONTENT DURING BILTONG PRODUCTION

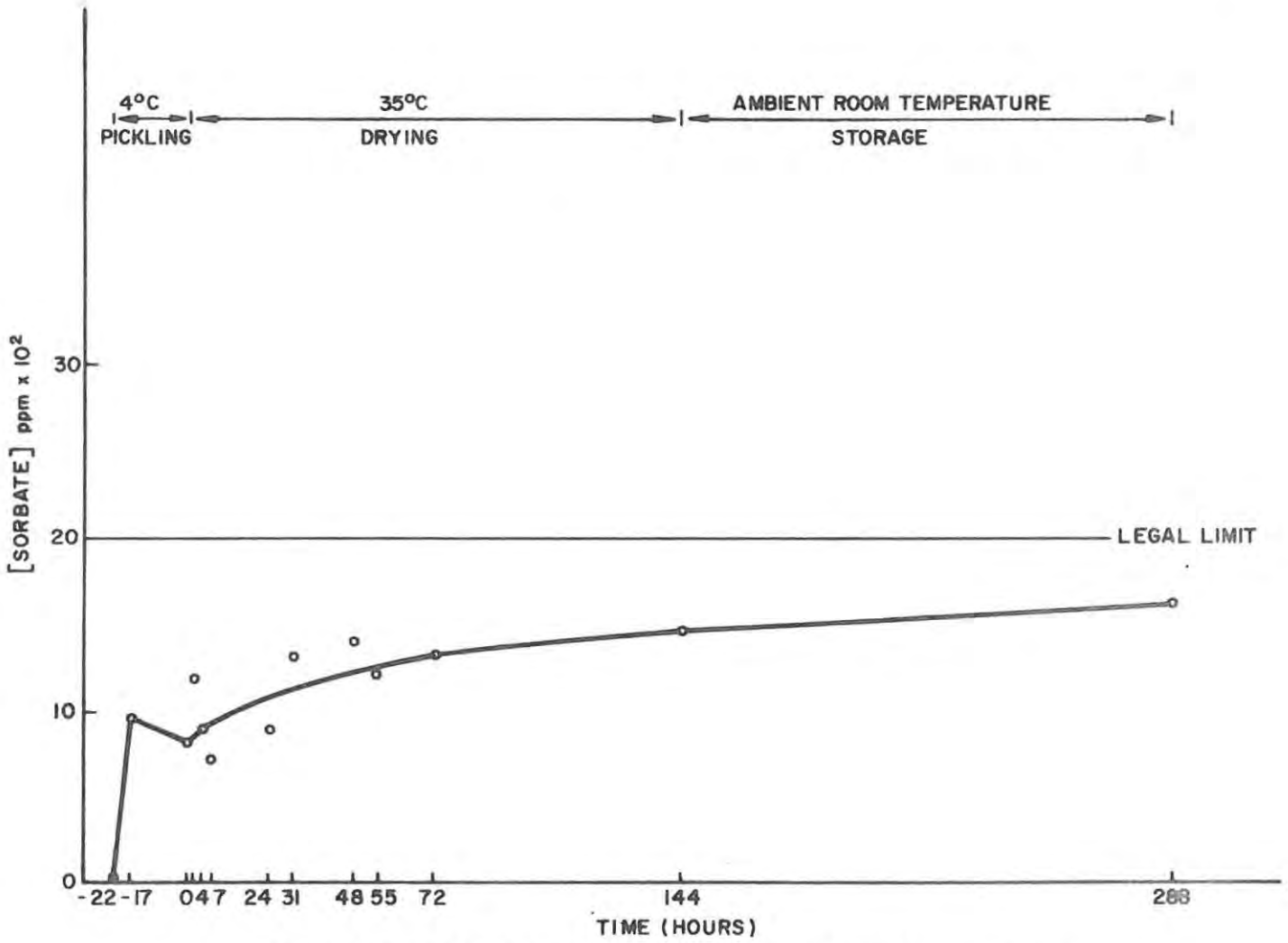

FIGURE 3 CHANGES IN SORBATE CONCENTRATION DURING BILTONG PRODUCTION

3.2 THE CHANGES OCCURRING IN THE SAPROPHYTIC MICROBIAL FLORA DURING BILTONG PRODUCTION

In both the untreated biltong prepared from commercial beef (see 2.1.2) and that prepared from aseptically dissected beef (see 2.1.3), two main groups of bacteria appeared to feature during processing i.e. the Micrococcaceae and the *Pseudomonas/Achromobacter* group.

3.2.1 Changes occurring in the saprophytic microbial flora during biltong production from commercially available beef

The results representing the changes in both number and groups of organisms during biltong production appear in Appendix F and are represented graphically in Figures 4 and 5.

From Figure 4, where the changes in the total aerobic count are compared to the changes in the counts of the Micrococcaceae and the *Pseudomonas/Achromobacter* group, the following is observed.

During the pickling process (-22 h to 0 h), the total number of organisms of the *Pseudomonas/Achromobacter* group present in the product decreases, and accounts for 40-55% of the investigated flora (see Appendix F). Their numbers decrease further during the first seven hours of drying, that is from 40% to 3% of the investigated flora. After seven hours of drying, there is a 10-fold increase in their numbers, but these organisms do not constitute a significant proportion of the investigated flora. After 48 hours of processing, the number of these organisms present in the product decrease rapidly.

The initial number of Micrococcaceae present in fresh meat was found to be 10-fold less than that recorded for the *Pseudomonas/Achromobacter* group. During the pickling period, the Micrococcaceae numbers decreased and accounted for 25-36% of the investigated flora. In the first seven hours of drying, their numbers, like those of the *Pseudomonas/Achromobacter* group decreased rapidly. During this period, when the proportion of the

Pseudomonas/Achromobacter group decreased, that of the Micrococcaceae increased from 36% to 83% of the investigated flora. After seven hours of drying, there was a 100-fold increase in the number of Micrococcaceae present which, after 55 hours of processing, declined again. During the period from 7 to 288 hours of processing, the Micrococcaceae comprised 82-100% of the investigated flora.

Identification of the Micrococcaceae isolated at different time intervals during processing (see Appendix G) indicated that *S. saprophyticus* was the predominant species present throughout the drying process, with *S. epidermidis*, *M. luteus*, *M. varians*, *M. freudenreichii*, *M. sedentarius* and *M. kristinae* encountered to a lesser extent. These results corresponded well with those reported for commercial biltong (cf. II.3.4).

Figure 5 illustrates the changes in the number of organisms tolerant to 10% sodium chloride, the lactic acid bacteria as well as the yeasts and moulds. It is evident that the trend in the changes in the number of organisms tolerant to 10% sodium chloride closely follows those observed for the Micrococcaceae (cf. Figure 4). This observation implies that the staphylococci and micrococci are salt-tolerant and that the changing environment i.e. increasing sodium chloride content of the product (see 3.1.3) favoured their selection and subsequent proliferation.

The trends displayed in the changing yeast and mould and the lactic acid bacteria populations were similar to those observed for the other groups of organisms, but at a much lower level. Instead of their numbers declining steadily after 55 hours, as found with the other organisms, their numbers declined rapidly after 48 hours of processing.

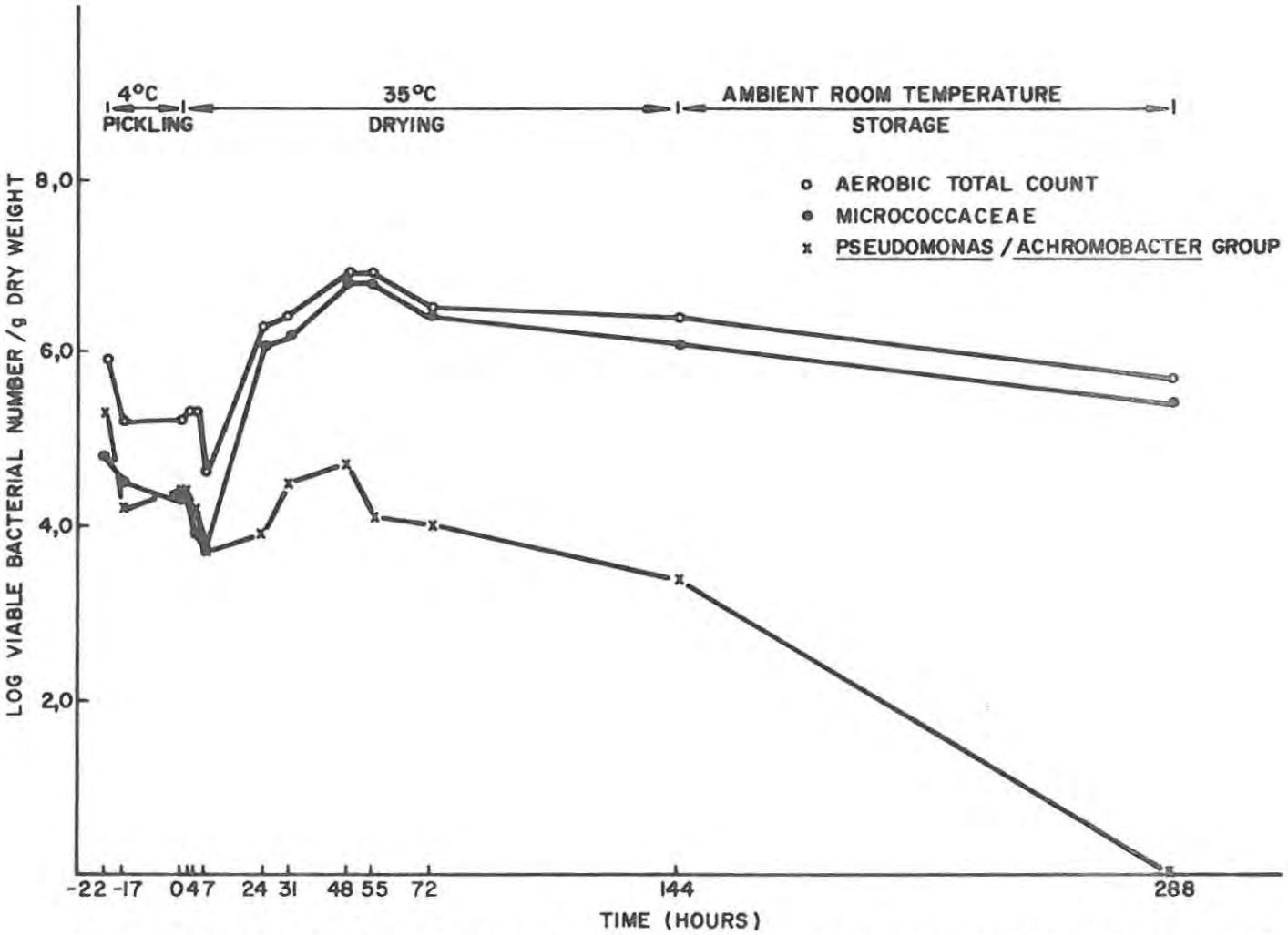

FIGURE 4 CHANGES IN BACTERIAL NUMBERS DURING BILTONG PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF

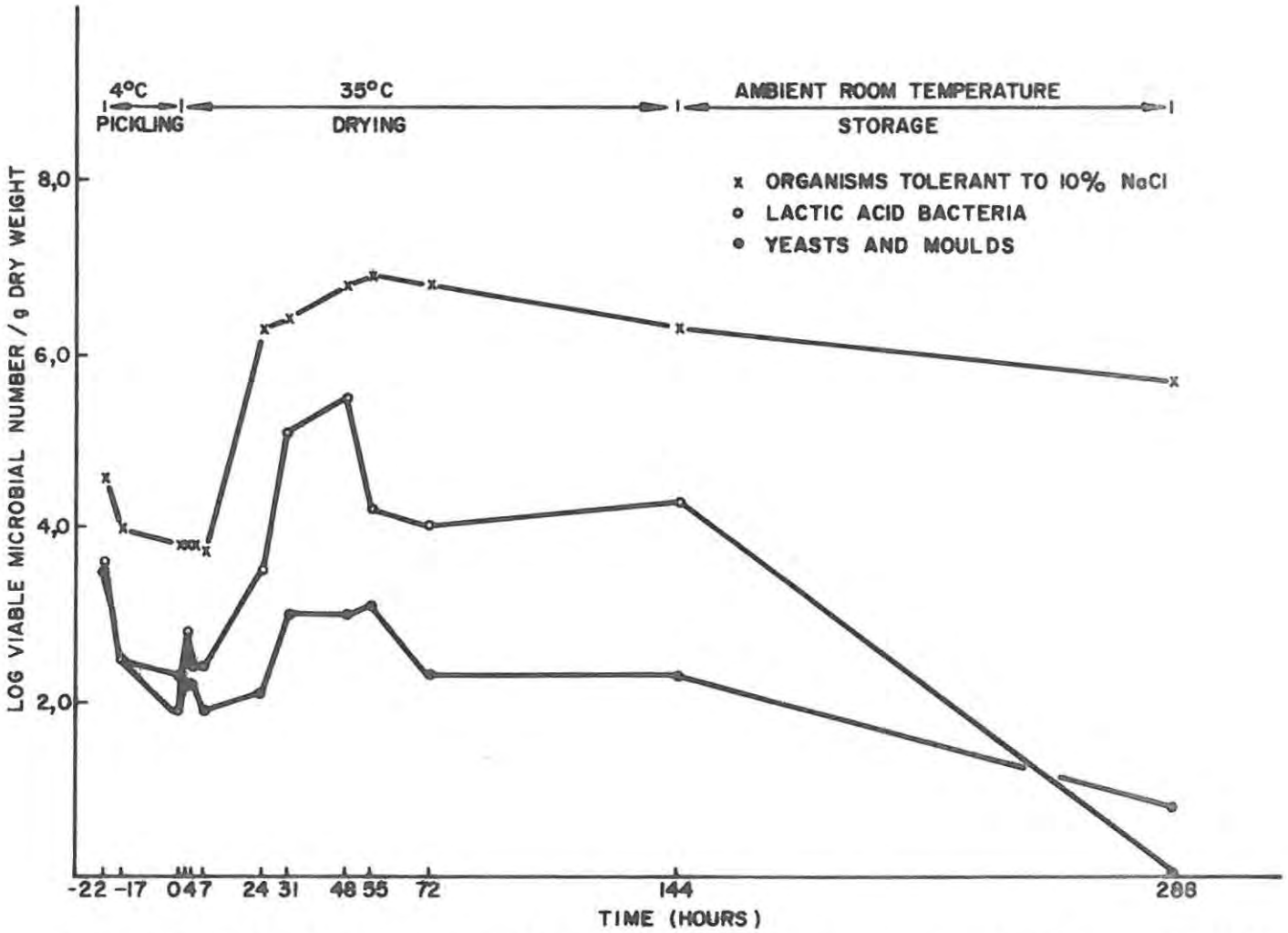

FIGURE 5 CHANGES IN MICROBIAL NUMBERS DURING PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF

3.2.2 Changes occurring in the saprophytic microbial flora during biltong production from aseptically dissected beef

The microbial counts obtained during biltong production from aseptically dissected beef (see Appendix F) were found to be substantially lower than those observed for biltong prepared from commercial beef although their graphic representation showed similar trends (see Figures 6 and 7).

As was observed with biltong prepared from commercial beef, the *Pseudomonas/Achromobacter* group predominated in the fresh meat and pickled meat (see Figure 6) and accounted for 37-55% of the flora investigated while the Micrococcaceae comprised only 12-21% of the investigated flora. During the first seven hours of drying, the proportion of the *Pseudomonas/Achromobacter* group decreased to 11% of the total flora while that of the Micrococcaceae increased to 88%. During the period from 7 to 288 hours of processing, the Micrococcaceae formed 83-100% of the investigated flora.

Identification of these Gram-positive, catalase-positive cocci indicated that *S. epidermidis* was the predominant species with *M. varians*, *M. roseus* and *S. saprophyticus* present only infrequently (see Appendix H).

No lactic acid bacteria were detected during the processing of aseptically dissected beef and yeasts and moulds were encountered only in isolated samples (see Figure 7).

3.2.3 Comparison between the microflora present on biltong prepared from commercially available beef and the microflora present on biltong prepared from aseptically dissected beef

The observed differences in the total aerobic counts and the numbers of the Micrococcaceae and the *Pseudomonas/Achromobacter* group, between biltong prepared from commercial beef and biltong prepared from aseptically

dissected beef, were thought to be significant. However, statistical analysis of variance proved these differences to be not significant, but a highly significant feature was that the species of Micrococcaceae isolated were different, i.e. isolates from biltong prepared from commercial beef comprised predominantly *S. saprophyticus*, while isolates from biltong prepared from aseptically dissected beef were predominantly *S. epidermidis*.

The absence of lactic acid bacteria and infrequent occurrence of yeasts and moulds in biltong prepared from aseptically dissected beef compared to their presence in biltong prepared from commercially available beef and commercial biltong (Reiche, 1972) was considered to be extremely significant.

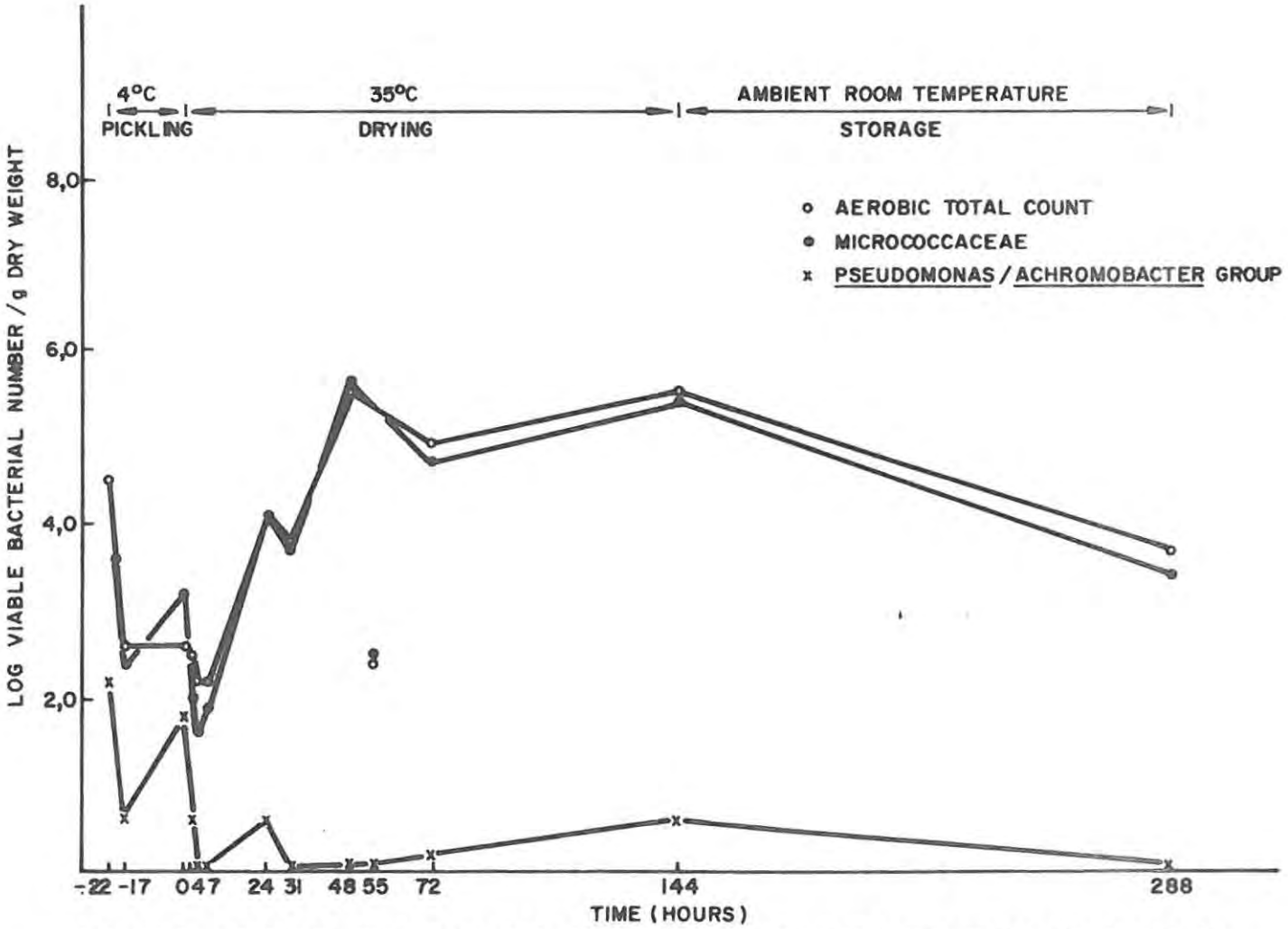

FIGURE 6 CHANGES IN BACTERIAL NUMBERS DURING BILTONG PRODUCTION FROM ASEPTICALLY DISSECTED BEEF

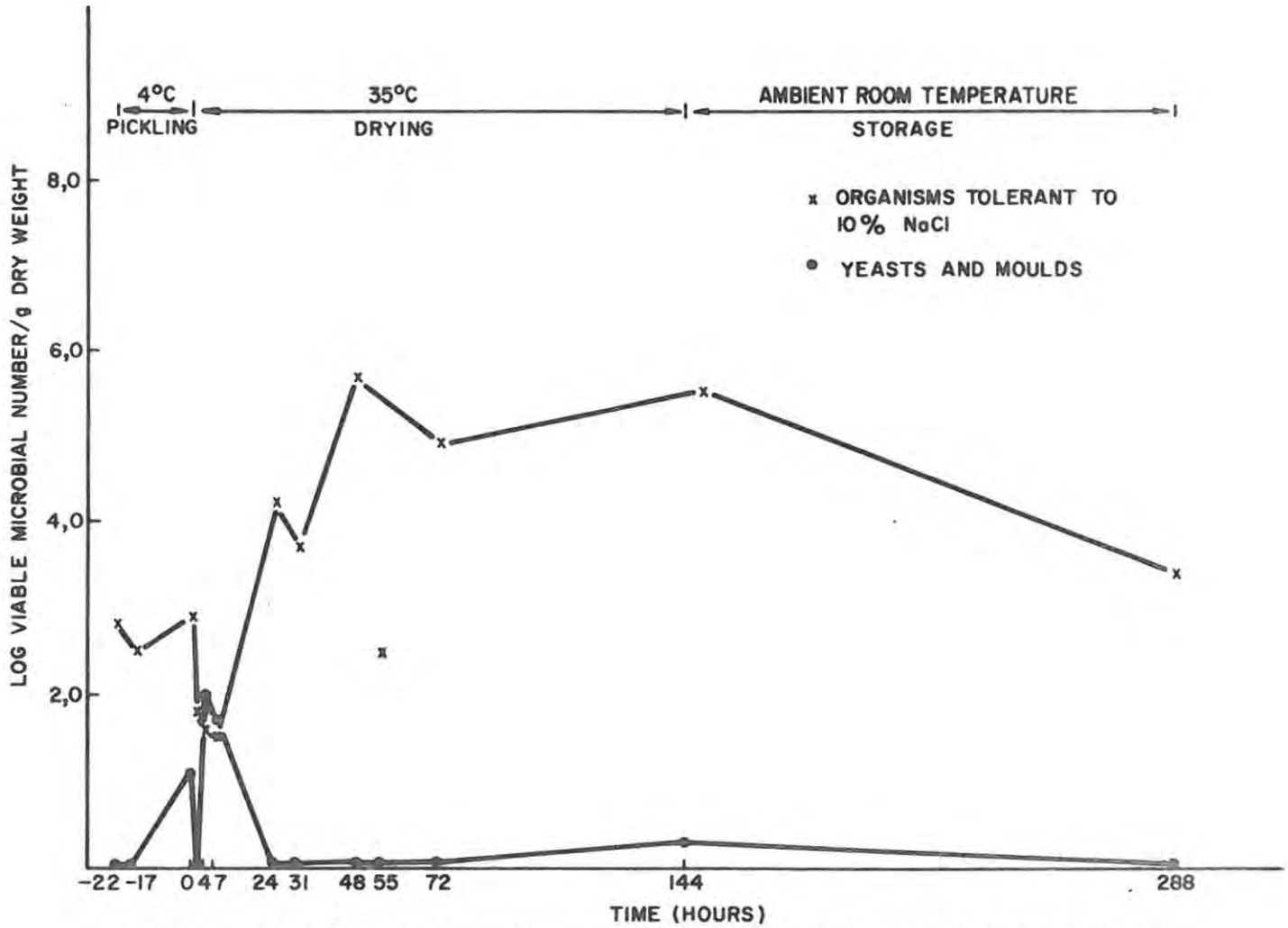

FIGURE 7 CHANGES IN MICROBIAL NUMBERS DURING BILTONG PRODUCTION FROM ASEPTICALLY DISSECTED BEEF

3.2.4 Changes in the saprophytic microbial flora during biltong production from commercially available beef treated with potassium sorbate during salting

The presence of potassium sorbate initially reduced the number of organisms present in biltong (see Figure 8 and Appendix F), but after 48 hours of drying, a group of organisms that were either resistant to or able to utilize potassium sorbate emerged and proliferated. Isolation and partial characterization of these organisms revealed that they were not typical of those usually associated with biltong prepared from commercial beef and comprised mainly Gram-positive, catalase-positive, spore-forming rods. It appears that the presence of potassium sorbate creates an elective environment selecting for organisms previously suppressed by the sorbate-sensitive organisms.

The observation that the presence of sorbate only reduces the bacterial numbers initially is not unique to biltong and reportedly occurs in hake treated with potassium sorbate (A. Atkinson, Fishing Industry Research Institute, Cape Town; pers. comm.).

Although fungi and yeasts were initially present on the meat, these organisms were not detected during the latter stages of processing.

Acids usually exert their preservative effect by increasing the hydrogen-ion concentration in the foodstuff (Frazier, 1967, p.138). However, the antimicrobial activity of sorbic acid, as is the case with benzoic acid, is dependent on the concentration of the undissociated acid present (Bell et al., 1959; Frazier, 1967, p.138). This dissociation is dependent on pH (Figure 9) and at pH 5,8, the average pH of biltong, only about 9% undissociated sorbic acid is present. Therefore of the 2 000 ppm sorbic acid permitted by law, only 180 ppm or less would be effective.

Even though the effective sorbic acid concentration is very low, it does alter the microbial profile of the final biltong product. Its

use could, however, be hazardous, as it selects for aerobic, spore-forming bacteria which might cause intestinal disorders.

Whereas the addition of potassium sorbate during biltong production arrests the fungi and yeasts and reduces the initial aerobic count, it cannot be relied on to serve as a bactericidal or bacteriostatic agent.

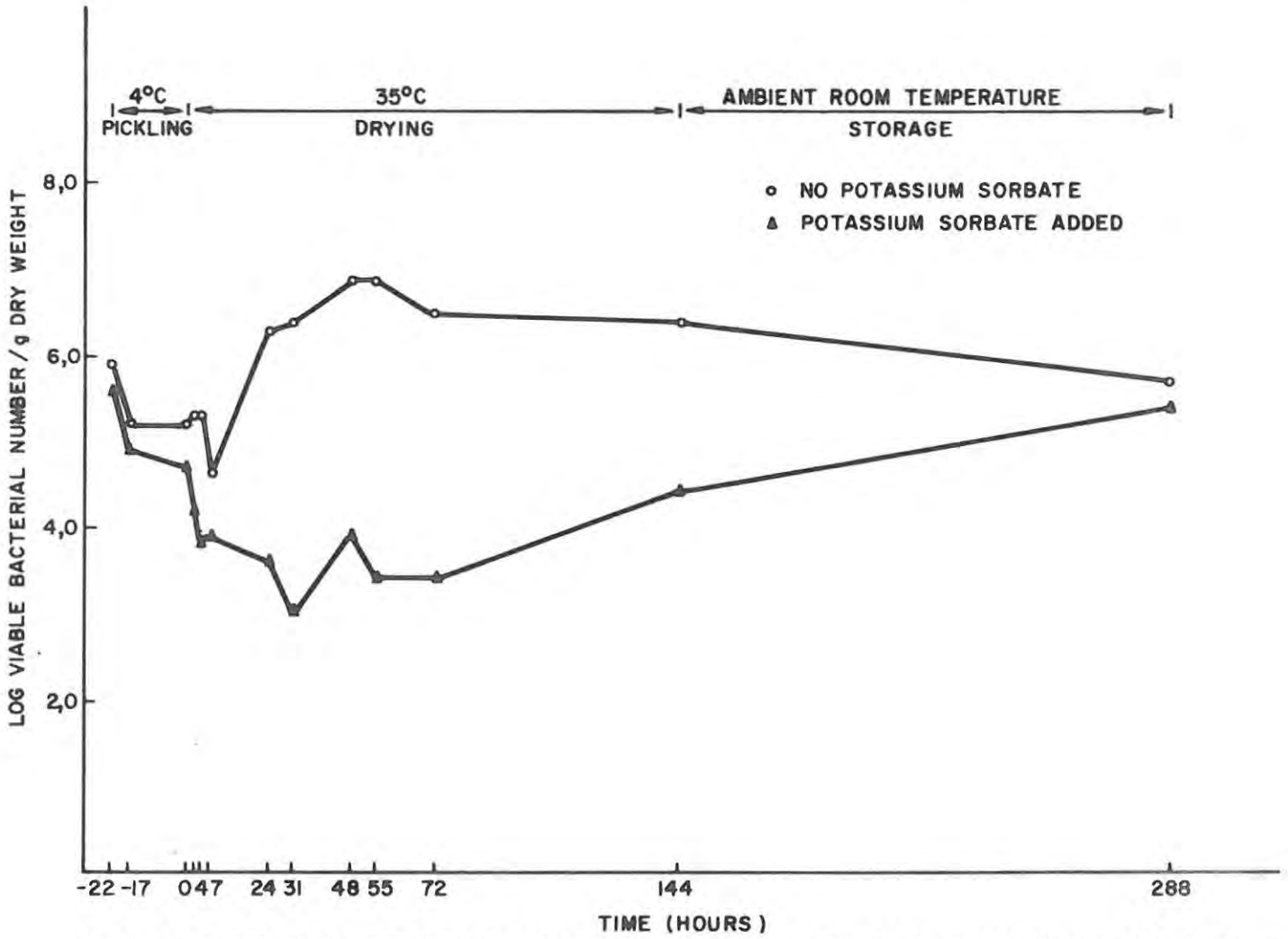

FIGURE 8 CHANGES IN THE TOTAL NUMBER OF AEROBIC BACTERIA DURING :(i) BILTONG PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF AND (ii) BILTONG PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF TREATED WITH POTASSIUM SORBATE DURING SALTING

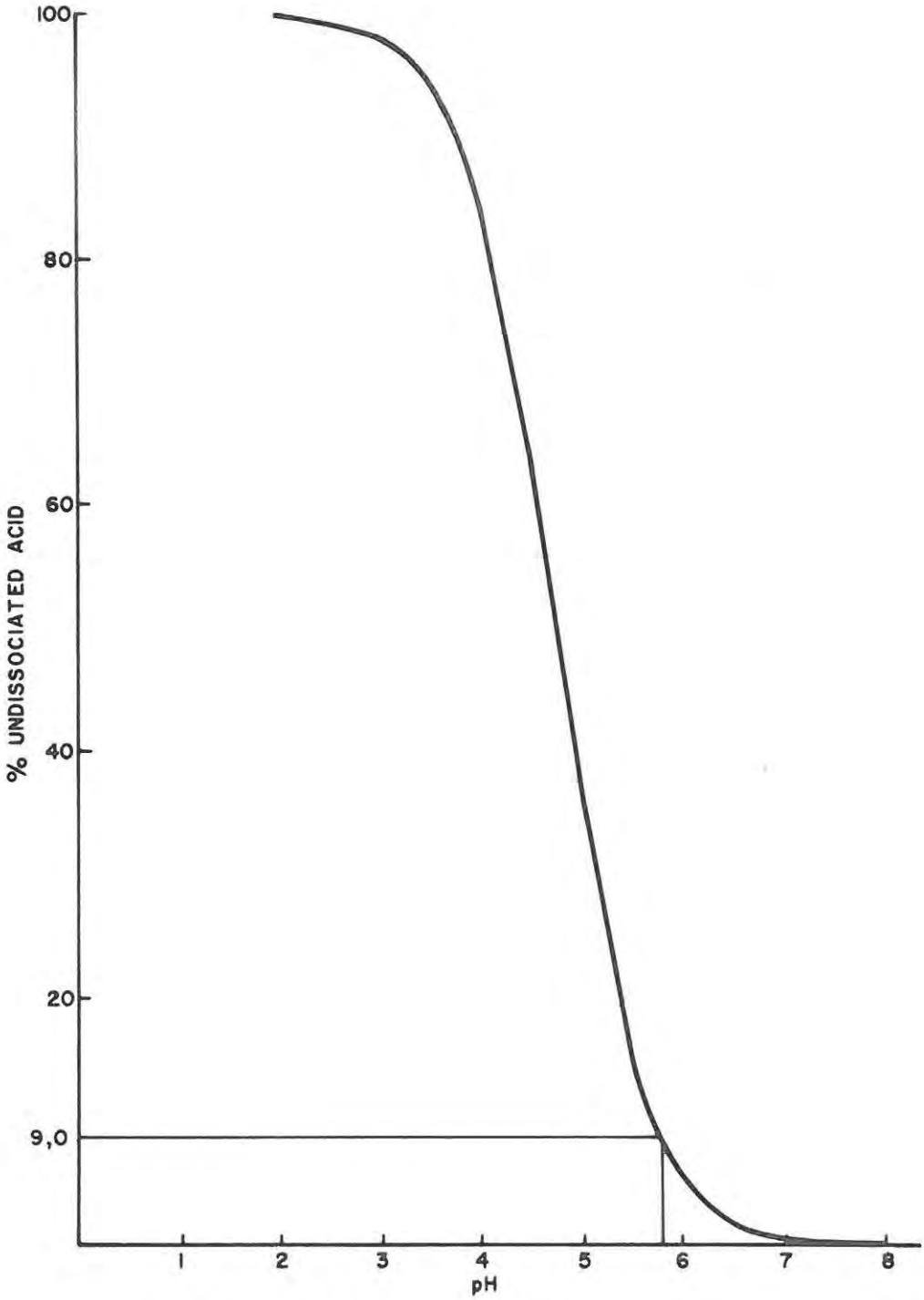

FIGURE 9 DISSOCIATION OF SORBIC ACID vs pH

3.2.5 Changes occurring in the saprophytic microbial flora during biltong production from commercially available beef treated with pimaricin during salting

Bacteriological analysis of samples indicated that the presence of pimaricin had no effect on the total aerobic population of organisms present during biltong production. Their counts (see Appendix F) and representative curves (see Figure 10) were similar to those observed when no preservative was added (cf. Figure 4). Isolation and partial characterization of bacteria present in the final product indicated that Gram-positive, catalase-positive cocci formed the predominant component of the microflora, indicating that pimaricin had no observed effect on the type of aerobic bacteria flora present either.

These results confirm the claims (Gist-Brocades N.V.; technical brochure) that pimaricin is by and large inactive against bacteria.

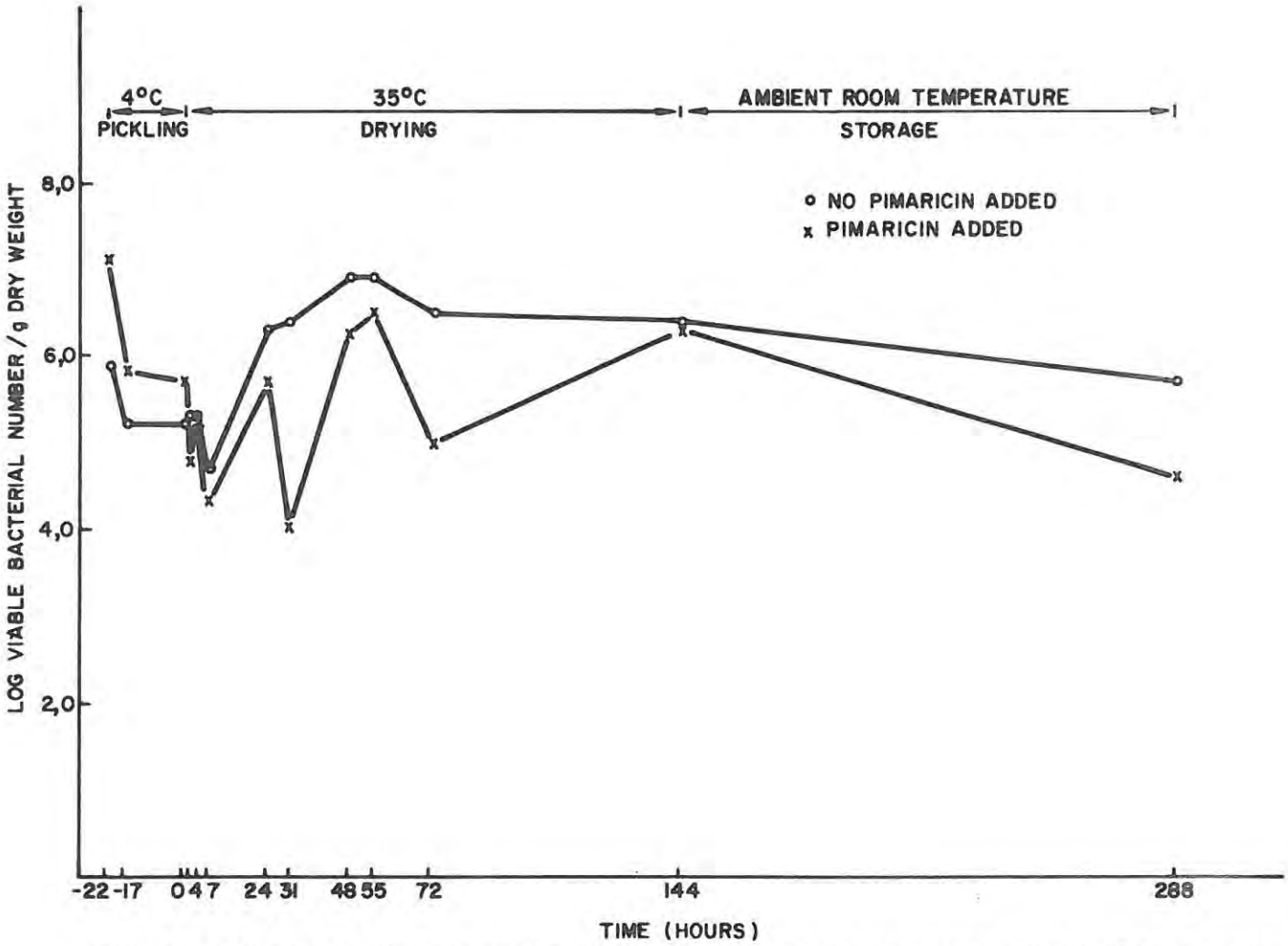

FIGURE 10 CHANGES IN THE TOTAL NUMBER OF AEROBIC BACTERIA DURING:(i) BILTONG PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF AND (ii) BILTONG PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF TREATED WITH PIMARICIN DURING SALTING

3.3 ORGANOLEPTIC EVALUATION OF BILTONG AND THE ROLE OF THE SAPROPHYTIC BACTERIAL FLORA IN BILTONG FLAVOUR PRODUCTION

Organoleptic evaluation of biltong prepared from meat treated with either potassium sorbate or pimaricin during salting indicated that there were no organoleptically detectable differences between the treated biltong and untreated biltong, nor was there any preference for either the biltong with or the biltong without added preservative. It was therefore apparent that the presence of either potassium sorbate or pimaricin, at the concentrations used, had no effect on the organoleptic properties of the biltong. If these findings were considered together with the observation that the presence of potassium sorbate alters the microbial profile of the product (see 3.2.4), the staphylococci and micrococci normally associated with biltong prepared from commercial beef, therefore appear not to contribute to biltong flavour production.

However, a significant organoleptic difference could be detected between biltong prepared from aseptically dissected beef and biltong prepared from commercial beef although there was no preference for either sample. The exact nature of this organoleptic difference remains undetermined.

Although there is an observed difference in the number and species of bacteria present during biltong production from commercially available beef and biltong production from aseptically dissected beef, there was also a difference in the ages of the meat used. Therefore no final conclusions as to the contribution of the saprophytic bacterial flora to biltong flavour production can be drawn, as factors such as the age of the beast at slaughter and the period of storage of the carcass prior to processing may also affect the organoleptic qualities of the final biltong product.

4. GENERAL DISCUSSION

The changes in the bacterial flora during conversion of meat to biltong appear to relate to the competitive growth of micro-organisms and their interrelationships with the environmental changes taking place during processing.

Evidence for competition in natural ecosystems and investigations concerning the interrelationship of staphylococci and micrococci other than *S. aureus* with bacteria commonly associated with food and the environment, is difficult to obtain. Consequently the previously established interrelationships between *S. aureus*, food-associated bacteria and the environment as determined *in vitro*, will be related to the present investigation, assuming also that the tolerance ranges of *S. saprophyticus* and *S. epidermidis* to the physico-chemical properties investigated, are of the same order as *S. aureus*.

During the pickling process and early stages of drying, the dominant *Pseudomonas/Achromobacter* group encountered on meat and possibly other food-associated bacteria, may prevent the proliferation of the staphylococci and micrococci present on meat by competing for available nutrients (Troller and Frazier, 1963a), by exuding a staphylolytic enzyme affecting the salt tolerance of the staphylococci, thus limiting their growth (Collins-Thompson et al., 1973), or by the production of antibiotic-like substances active against staphylococci and micrococci (Troller and Frazier, 1963a).

Environmental conditions were reported to affect the influence of food-associated micro-organisms on staphylococcal growth.

Troller and Frazier (1963b) reported that maximal inhibition occurred at a temperature range of 20-25 ^{o}C while McCoy and Faber (1966) found 27% of the food micro-organisms they examined using a plate test inhibited staphylococcal growth at 35 ^{o}C. However, in meat slurries,

inhibition of staphylococcal growth was found to be greater at 25 °C than at 35 °C. Maximal inhibition also occurred within the range of pH 6,2 to pH 7,4 while oxygen availability had no influence on inhibition (Troller and Frazier, 1963b).

On the other hand, the presence of sodium chloride concentrations above 3,5% or the addition of sucrose, thereby implying a decrease in water activity, favoured the proliferation of staphylococci (Peterson et al., 1964). However, the effect of sodium chloride was temperature-dependent as at 0 °C and 5 °C, the presence of 9,5% sodium chloride prevented the growth of psychrophilic saprophytes, whereas lower concentrations of sodium chloride were unable to do this. Staphylococci failed to grow at these two temperatures. A temperature of 10 °C enabled the saprophytes to flourish and inhibited staphylococcal growth no matter what concentration of sodium chloride was used. Above 3,5% sodium chloride and at temperatures of 20 °C and above, staphylococcal inhibition was markedly decreased, while the saprophytes were increasingly inhibited, with their lag phases materially lengthened as the salt concentration was increased. Peterson et al. (1964) therefore intimated that the salt acted directly on the staphylococcal population and also, by repressing saprophytic growth, decreased competition which allowed the staphylococci to proliferate. This ability of *Staphylococcus aureus* to tolerate and proliferate in the presence of high levels of sodium chloride was further demonstrated by Troller (1971). The addition of 10% sodium chloride to Brain Heart Infusion broth, resulting in a water activity of a_w 0,92, was found not to affect staphylococcal growth although enterotoxin production was inhibited.

Therefore, the physico-chemical conditions encountered during biltong production i.e.

(i) the pH 5,80 and pH 5,66 found for biltong prepared from commercially available beef and aseptically dissected beef respectively,

(ii) the processing temperature of 35 °C, and

(iii) the ever increasing sodium chloride concentration and resultant decrease in water activity,

would appear to depress the growth of the saprophytic organisms, other than the staphylococci and micrococci, thus reducing competition and thereby favouring the proliferation of the staphylococcal population (Ingram and Kitchell, 1967).

This depression of the *Pseudomonas/Achromobacter* group's growth is probably due to the physico-chemical conditions being unfavourable for their proliferation i.e.

(i) the pH is 1,2 units lower than the optimum reported for pseudomonads (*Bergey's Manual*, 1974, p.218),

(ii) the temperature is 5 °C higher than the optimum for pseudomonads (*Bergey's Manual*, 1974, p.218),

(iii) the *Pseudomonas/Achromobacter* group are very sensitive to decreases in water activity, having limiting water activities of a_w 0,97 and a_w 0,96 respectively (Frazier, 1967, p.40).

As the water activity was ever decreasing during processing with $a_{w_{25}}$ 0,955 reached only 2 hours after the onset of drying, the combination of these factors increase the lag phase of these organisms and reduce their subsequent growth rate. This resulted in a decline in their number with the resultant reduction in competition.

The staphylococci and micrococci appear not to be as sensitive to the changing environmental factors and as they are no longer inhibited by the *Pseudomonas/Achromobacter* group, are able to proliferate till 55 hours of drying where $a_{w_{25}}$ 0,856 is reached (cf. Figures 2 and 4). As a_w 0,86 was the limiting water activity reported for *S. aureus* under aerobic conditions (Scott, 1953) and a_w 0,89 for both *S. albus* (≡ *S. aureus*) and *S. citreus* (≡ *S. aureus*) (Christian and Waltho, 1961), it appears that the limiting water activities of these organisms were reached between 48 and 55 hours of drying, resulting in a reduction in

their growth rates and the consequent decline in the staphylococcal population of the biltong after 55 hours of processing (see Figure 4).

It is not certain whether it is an ionic effect or decreasing water activity which plays the major role in the changes in the microflora (Ingram and Kitchell, 1967).

This preferential increase in the numbers of the staphylococci compared to those of the micrococci, which predominate the Micrococcaceae present in fermented meat products (Pohja and Gyllenberg, 1962) and bacon (Baird-Parker, 1962; Kitchell, 1958), could be attributed to the elevated temperature used during processing. In the spoilage of vacuum packed bacon at 30 °C, the coagulase-negative staphylococci were reported to be the dominant organisms present, while at 20 °C, the oxidative micrococci were most common (Cavett, 1962). This ability of the staphylococci to withstand higher temperatures than the micrococci is characteristic of the genus *Staphylococcus* (*Bergey's Manual*, 1974, p.483) and has been exploited by the I.C.S.B. Subcommittee (1965) in their method for differentiating between the genera *Staphylococcus* and *Micrococcus*.

A possible explanation as to why *S. epidermidis* predominated in biltong prepared from aseptically dissected beef is that *S. epidermidis* is usually associated with the skin and mucous membranes of the live animal [Baird-Parker (*Bergey's Manual*, 1974, p.488)] and may spread to the carcass during slaughtering and subsequent removal of the topside. As the meat was handled aseptically and used within 24 hours of slaughter, the predominant staphylococci normally associated with the surface of carcasses viz. *S. saprophyticus* [Baird-Parker (*Bergey's Manual*, 1974, p.489)] may not have been able to establish themselves, hence the original flora remained dominant.

Although van den Heever (1970a, b) and Bokkenheuser (1963) failed to demonstrate the presence of coagulase-positive *S. aureus* in commercial

biltong and Badenhorst (1972) only detected *S. aureus* in 2 out of 20 commercial biltong samples investigated, Peterson et al. (1962) mentioned that staphylococcal food poisoning occurs in foods which selectively favour the growth of staphylococci while inhibiting the growth of other genera. From the preceding discussion it is evident that the environmental changes during the processing of meat to biltong would favour the proliferation of *S. aureus*, thereby creating a potential health hazard if meat contaminated with *S. aureus* was used. However, Troller (1971) found the presence of sodium chloride and consequent decrease in water activity to reduce *S. aureus* enterotoxin B production even though the actual numbers of organisms were not affected. He was therefore of the opinion that the presence of rapid growth and high numbers of potentially enterotoxigenic strains of *S. aureus*, although undesirable, would not necessarily indicate the presence of enterotoxin.

However, a potentially dangerous situation still exists if biltong manufacturers, for both economic and aesthetic reasons, abuse the use of potassium sorbate and pimaricin and market biltong with a relatively high moisture content. The micro-environment within the product would therefore no longer influence enterotoxin B production, or affect the growth of organisms such as *Salmonella* spp., *Escherichia coli* and *Bacillus* spp. with limiting water activities of a_w 0,945; a_w 0,960; and a_w 0,90 respectively (Scott, 1957; Frazier, 1967, p.40) thereby creating a potential health hazard.

Furthermore, Mossel (1971) reported that the growth of aerobic spore-forming bacteria in food of water activity near the limiting water activity of *Clostridium botulinum* (a_w 0,95) raises the moisture content significantly and so increases the possibility that the latter should grow.

As the use of sorbate alters the microbial profile of biltong and selects for aerobic spore-forming bacteria, it would therefore appear that the use of pimaricin to control mould spoilage of biltong may be more advantageous than sorbic acid and its salts.

Van den Heever (1970b) stated that "Although high microbial counts in foods are not necessarily harmful as such, and food poisoning may be induced by foods containing small numbers, total counts are generally accepted as indication of the sanitary quality, organoleptic acceptability, safety and usefulness of food". In the light of the present investigation, this statement concerning the interpretation of total counts cannot be condoned as it is the type of organisms present rather than their number, and their relationship with the environment which determines whether or not the product is acceptable both aesthetically and with regard to the sanitary quality.

In the production of biltong under controlled conditions, where the meat and biltong were handled hygienically, no lactic acid bacteria or yeasts and moulds were isolated or if they were it was at a very low level. These groups of organisms are present in commercial biltong in numbers of the order of 10^6-10^7 orgs/g (Reiche, 1972). The presence of indicator organisms such as *Escherichia coli* type I and group D streptococci is therefore not essential to indicate poor handling procedures, as the saprophytic lactic acid bacteria and the yeasts and moulds could possibly be used as indicators of poor handling procedures as well.

5. SCOPE FOR FUTURE MICROBIOLOGICAL INVESTIGATIONS

Although the present investigation into the aerobic, saprophytic microbial flora of biltong enabled an interrelationship to be established between the aerobic, saprophytic microflora and the environmental factors, a number of aspects still need to be investigated to substantiate the observed relationship.

Such aspects should include:

(i) The individual and combined effect of water activity, pH and temperature on the relative growth rates of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and of the Gram-positive, spore-forming rods isolated from biltong prepared from meat treated with potassium sorbate during dry-salting;

(ii) Identification of the Gram-positive, spore-forming rods isolated from biltong treated with potassium sorbate during dry-salting and their significance;

(iii) As the present investigation dealt specifically with the aerobic, saprophytic, microbial flora of biltong, a similar investigation concerning the anaerobic microbial flora of biltong is necessary to establish whether or not these organisms are influenced by the same intrinsic factors which affect the aerobic microflora of the product;

(iv) In addition, the microflora of the commercial salts and other curing additives used in biltong manufacture should be investigated to establish whether or not they contribute to the total microbial load of the final product.

IV. GENERAL DISCUSSION

From this investigation into the saprophytic microbial flora of biltong in relation to the environment, a number of salient features have emerged which may have their application in the manufacture of biltong.

As biltong is a raw-meat product which is consumed as such, the use of sound meat, obtained from healthy animals and preferably of pH 6 or below, is essential, as pH values greater than pH 6 could be indicative of spoiled meat.

Hygienic procedures both during slaughter and processing must be practised to prevent contamination and proliferation of pathogens and potential pathogens.

Rapid drying is essential, especially in warm, humid conditions to prevent the growth of mesophiles e.g. *Clostridium perfringens* implicated in the spoilage of meat under warm conditions, 25-40 ^{o}C, (Ingram and Dainty, 1971) and in food poisoning.

The indiscriminate use of preservatives such as pimaricin and potassium sorbate must be prevented as these are antifungal agents and cannot be relied on to serve as bactericidal or bacteriostatic agents.

However, no matter what precautions may be taken, biltong will always carry a certain microbial load determined by the intrinsic, extrinsic and implicit properties of this commodity (Mossel, 1971).

APPENDIX A

THE BIOCHEMICAL CHARACTERS OF EACH ISOLATE AND OF THE NAMED SPECIES AND SUBGROUPS OF *STAPHYLOCOCCUS*, *MICROCOCCUS*, *PLANOCOCCUS*, *AEROCOCCUS* AND *SARCINA* AND THEIR POSSIBLE RELATIONSHIPS

APPENDIX A

BIOCHEMICAL CHARACTERS OF NAMED SPECIES AND SUBGROUPS OF MICROCOCCACEAE

BIOCHEMICAL TEST / CULTURE NUMBER	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM								Acetoin	Phosphatase	Reduction of nitrate to nitrite	Urease production	Hydrolysis of Tween 80	Hydrolysis of suet	Hydrolysis of gelatin	GROWTH				Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol								10 °C	45 °C	0% NaCl	15% NaCl						
272	+	+	Anaer	–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	–	+	–	+	+	–	+	Pale yellow	I
12600	+	+	Anaer	–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	Yellow/ orange	I
885	+	+	Anaer	–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	Yellow/ orange	I
2124	+	+	Anaer Aer	–	–	+	+	–	+	–	–	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	White	II
14990	+	+	Anaer Aer	–	–	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	White	II
1512	+	+	Anaer Aer	–	–	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+	+	+	–	+	–	+	White	II
1462	+	+	Anaer Aer	–	–	–	–	–	–	–	–	–	+	+	+	–	–	+	–	+	+	–	–	+	–	+	Bright yellow	III
1466	+	+	Anaer Aer	–	–	+	–	+	–	–	–	+	–	–	+	+	+	–	–	+	+	+	+	+	–	+	White	IV
1477	+	+	Anaer Aer	–	–	+	+	+	+	–	–	+	–	+	+	+	+	+	–	–	+	+	–	+	–	+	White	V
1464	+	+	Anaer Aer	–	+	+	–	+	–	–	–	+	–	+	+	–	–	–	–	+	+	–	–	–	–	+	White	VI
15306	+	–	Aer	–	–	–	+	–	+	–	–	+	–	+	+	–	–	–	–	+	+	–	–	–	–	+	Yellow	5 or 6
15305	+	–	Aer	–	+	+	+	+	–	–	–	+	–	–	+	–	–	–	+	–	+	+	–	–	–	+	White	1 – 4
1649	+	+	Anaer Aer	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	+	+	+	–	–	+	+	Bright yellow	*S. lutea*
14404	–	–	Aer	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	+	+	+	+	–	–	–	+	Yellow	*Planococcus*
1522	+	–		–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	White	*Aerococcus*
418	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	+	–	+	–	–	+	Pink	8
1489	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–		–	–	–	–	–	–	–	+	Pink/ red	8
679	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–	+	–	–	–	–	+	Pink	8

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BIOCHEMICAL CHARACTERS OF NAMED SPECIES AND SUBGROUPS OF MICROCOCCACEAE

BIOCHEMICAL TEST / CULTURE NUMBER	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM									Acetoin	Phosphatase	Reduction of nitrate to nitrite	Urease production	Hydrolysis of Tween 80	Hydrolysis of suet	Hydrolysis of gelatin	GROWTH				Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol									10 °C	45 °C	0% NaCl	15% NaCl						
4698	−	−	Aer	−	−	−	−	−	−	−	−		−	−	−	+	−	−	+	−	−	+	−	−	−	−	+	Pale yellow	7
1465	+	−		−	+	+	−	+	−	−	−		−	−	+	−	−	−	+	+	+	+	−	+	−	−	+	White	7
169	−	−	Aer	−	−	−	−	−	−	−	−		−	+	−	+	+	−	+	−	−	+	−	−	−	−	+	Pale yellow	7
884	+	−	Aer	−	−	−	+	−	+	−	−		−	−	+	+	−	−	+	−	+	+	−	−	−	−	+	Bright yellow	5 or 6
764	−	−	−	−	−	−	−	−	−	−	−		−	−	−	−	−	−	+	−	−	+	−	+	−	−	+	White	5 or 6
1469	+	−	Aer	+	+	+	+	+	+	+	−		−	−	+	+	−	−	−	+	−	+	+	+	−	−	+	White	5
1463	+	−	−	+	+	+	+	+	−	+	−		−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	Pale yellow	6
1557	+	−	Aer	−	−	+	−	−	−	−	−		+	−	−	−	+	+	−	+	+	+	+	+	−	−	+	White	1
1588	+	−	Aer + Anaer	−	−	+	+	+	−	−	−		+	−	−	+	−	+	−	+	+	+	+	+	−	−	+	White	2
1587	+	−	Aer + Anaer	−	+	+	+	+	−	−	−		+	−	−	+	−	+	+	+	+	+	+	+	−	−	+	White	3
1839	−	−	Aer + Anaer	−	+	−	+	+	−	+	−		−	−	+	+	−	−	+	+	−	+	+	+	−	−	+	White	4
14392	−	−	−	−	−	−	−	−	−	−	−		−	+	−	−	−	−	+	+	+	+	−	+	−		+	Off white	
27566	−	−	Aer	−	−	−	−	−	−	−	−		−	−	−	−	+	−	+	+	+	+	+	+	−		+	Off white	
27570	+	−	Aer + Anaer	−	−	+	−	+	−	−	−		−	−	−	+	−	−	+	+	+	+	+	+	−		+	Off white	

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BIOCHEMICAL CHARACTERS OF ISOLATES

BIOCHEMICAL TEST / ISOLATE	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM										Hydrolysis of gelatin	Hydrolysis of suet	Hydrolysis of Tween 80	Urease production	Reduction of nitrate to nitrite	Phosphatase	Acetoin	GROWTH			Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol										10 °C	45 °C	0% NaCl	15% NaCl						
BL_1	+	–	Aer + Anaer	–	–	+	+	+	+	–	–				+	+	–	+	–	+	+	+	+	+			+	+		2
BL_2	+	–	Aer + Anaer	–	–	+	+	+	–	–	–				+	+	–	+	–	+	+	–	+	+			–	+		2
BTc_1	+	–	Aer + Anaer	–	+	+	+	+	+	–	–				–	+	–	–	–	–	–	–	+	+			–	+		5
BTc_2	+	–	Aer	–	–	+	+	+	–	–	–				+	+	–	+	–	+	+	+	+	+			–	+		2
BH_2	+	–	Aer	–	+	+	+	+	–	–	–				+	+	–	+	–	+	+	+	+	+			–	+		3
BH_4	+	–	Aer + Anaer	–	–	+	+	+	–	–	–				+	+	–	+	–	+	–	+	+	+			–	+		2
BH_5	+	–	Aer + Anaer	+	–	+	+	+	–	–	–				+	+	–	+	–	+	+	+	+	+			–	+		2
BH_6	+	–	Aer	–	+	–	–	+	–	–	–				+	+	+	+	–	+	+	+	+	–			–	+		1
BM_1	+	–	Aer + Anaer	–	+	+	–	+	+	–	–				+	+	–	–	–	+	+	–	+	–			–	+		3
BM_2	+	–	Aer + Anaer	–	+	+	–	+	–	–	–				+	+	–	+	+	+	+	–	+	–			–	+		3
BM_3	+	–	Aer	–	+	+	–	+	+	–	–				–	+	–	+	+	+	+	–	+	–			–	+		3
CT_3	+	–	Aer + Anaer	–	–	+	+	+	–	–	–				+	+	–	+	–	+	+	+	+	–			–	+		2
CH_1	+	–	Aer	–	+	–	–	+	–	–	–				–	+	–	+	+	+	+	–	+	–			+	+		3
CH_2	+	–	Aer + Anaer	–	+	+	+	+	–	–	–				–	+	–	+	+	+	+	–	+	–			–	+		3
CH_3	+	–	Aer + Anaer	–	–	+	–	+	+	–	–				+	+	–	+	–	+	–	–	+	–			–	+		1
CH_4	+	–	Aer	–	–	+	+	+	+	–	–				+	+	–	+	+	+	+	+	+	–			–	+		2
CH_5	+	–	Aer	–	–	+	+	+	+	–	–				+	+	–	+	–	+	+	+	+	–			–	+		2
CH_6	+	–	Aer + Anaer	+	–	+	+	+	+	–	–				+	+	–	+	+	+	+	–	+	–			–	+		6
CH_7	+	–	Aer + Anaer	–	+	+	+	+	–	–	–				+	+	–	+	–	+	+	–	+	–			–	+		5

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BIOCHEMICAL TEST / ISOLATE	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM										Hydrolysis of gelatin	Hydrolysis of suet	Hydrolysis of Tween 80	Urease production	Reduction of nitrate to nitrite	Phosphatase	Acetoin	GROWTH				Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol										10 °C	45 °C	0% NaCl	15% NaCl						
CM_1	+	-	Aer	+	-	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-			-	+	Yellow	6
CM_2	-	-	Aer	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	+	+	-			-	+		1
CM_3	+	-	Aer + Anaer	-	-	+	+	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-			-	+		3
CL_1	+	-	Aer	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	-			+	+		5
CL_3	+	-	Aer	-	-	+	+	+	+	-	-	-	+	-	-	+	+	-	-	+	+	+	+	-			-	+		2
CL_4	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	-	+	-	-	+	+	+	-	+	-	+	-	-			-	+	Yellow	3
CL_5	-	-	Aer	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	+	-	+	-	-			-	+		7
DH_1	+	-	Aer + Anaer	-	-	+	+	+	-	-	-	-	+	-	+	-	+	+	-	+	+	+	+	-			-	+		2
DH_2	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	-	+	-	+	-	+	+	-	+	-	+	+	-			-	+	Yellow	3
DH_3	+	-	Aer + Anaer	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	-	+	+	-			-	+		5
DH_4	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	-	+	-	+	-	+	+	-	+	-	+	+	-			-	+	Pale yellow	3
DH_5	+	-	Aer	-	+	+	+	+	-	-	-	-	+	-	+	+	+	+	-	+	-	+	+	-			-	+	Yellow	3
DH_6	+	-	Aer + Anaer	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	-	+	+	-			-	+		7
DH_7	+	-	Anaer	-	-	+	+	+	-	-	-	-	+	-	+	+	+	+	-	+	-	+	+	-			-	+		2
DH_8	+	-	Aer + Anaer	+	+	+	+	+	-	+	-	-	+	-	+	+	+	+	-	+	-	+	+	-			-	+		3
DTc_3	-	-	Aer	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	-			-	+		7
DTc_4	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	+	-			-	+		3
DLa_2	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	-	+	-	+	+	+	+	-	+	-	+	+	-			-	+		3

APPENDIX A

BIOCHEMICAL CHARACTERS OF ISOLATES

BIOCHEMICAL TEST / ISOLATE	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM										Phosphatase	Reduction of nitrate to nitrite	Urease production	Hydrolysis of Tween 80	Hydrolysis of suet	Hydrolysis of gelatin	GROWTH				Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol	Acetoin							10 °C	45 °C	0% NaCl	15% NaCl						
DM_2	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	+	–	+	+	–	–	+	–	–		–	+		3
DM_3	+	–	Aer	–	+	+	+	+	–	–	–	+	–	+	+	–	+	+	+	–	+	–	–		–	+	Pale yellow	3
DL_1	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	+	–	+	+	+	–	+	+	–		–	+		3
DL_2	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	+	–	+	+	+	–	+	+	–		–	+	Yellow	3
DL_3	+	–	Aer + Anaer	–	+	+	+	+	+	–	–	+	–	+	+	–	+	+	–	+	+	+	–		–	+		3
DL_5	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	–	–	+	+	+	+	+	+	–		–	+	Yellow	3
EM_1	+	+		–	+	+	+	+	+	–	–	+	–	+	–	–	+	+		+	+	+	–		–	+	Pale yellow	3
EM_2	+	–		–	+	+	+	+	+	–	–	+	–	+	+	–	+	+	+	+	+	+	+		–	+	Pale yellow	3
EM_3	+	+	Aer + Anaer	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–	+	+	+		–	+		1
EH_4	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	–	–	+	–	+	+	+	+	+		–	+		3
EH_5	+	–		–	+	+	+	+	+	–	–	+	–	+	+	–	+	+	+	+	+	+	+		–	+	Pale yellow	3
EH_6	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	–	–	+	+	–	+	+	–	+		–	+	Yellow	3
EH_7	+	–		–	+	+	+	+	+	–	–	+	–	+	–	–	+	+		+	+	+	–		–	+	Pale yellow	3
ETc_1	+	–		–	+	+	+	+	+	–	–	+	–	+	+	–	+	+		+	+	+	–	+	+	+	Pale yellow	3
ETc_3	+	+		–	+	+	+	+	+	–	–	+	–	+	–	–	+	+		+	+	+	+		–	+	White	3
EL_1	+	+		–	+	+	+	+	+	–	–	+	–	+	–	–	+	+		+	+	+	+		–	+	Pale yellow	3

APPENDIX A

BIOCHEMICAL CHARACTERS OF ISOLATES

BIOCHEMICAL TEST / ISOLATE	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM										Acetoin	Phosphatase	Reduction of nitrate to nitrite	Urease production	Hydrolysis of Tween 80	Hydrolysis of suet	Hydrolysis of gelatin	GROWTH				Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol										10 °C	45 °C	0% NaCl	15% NaCl						
EL_2	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	–	+	+	–	–	+	+	+	+	+	–	+	–	+	Pale yellow	3
EL_3	+	+		–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	+		+	+	+	–	+	–	+	White	3
ETc_4	+	+	Aer + Anaer	+	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–			–	+		IV
FH_2	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	–	–	+	–	+		+	–	+	+			–	+	Yellow	3
FH_4	+	–	Aer + Anaer	–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	+		+	+	+	–		–	+	Pale yellow	3
FH_6	+	–		–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	+		+	+	+	+		–	+	Pale yellow	3
FL_1	+	–		–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	+		+	+	+	–		–	+	White	3
FL_2	+	–		–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	–		+	+	+	–		–	+	Pale yellow	3
FL_3	+	+		–	–	–	–	+	+	–	–	–	–	–	–	+	–	+	–	–		+	+	+	+	+	–	+	Pale yellow	V
FL_4	+	–		–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	+		+	+	+	–		–	+	Pale yellow	3
FM_3	+	–		–	+	+	–	+	+	–	–	+	–	+	–	–	+	–	+	+		+	+	+	–		–	+	Pale yellow	3
FTc_2	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	+	–	+	–	–	+	–	+	+	+	–	+	+	–		–	+	Pale yellow	3
FTc_3	+	–	Aer + Anaer	–	+	+	+	+	–	–	–	–	–	–	–	–	+	–	+	+		–	+	+			+	+		5
FM_1	+	–		–	+	+	+	+	+	–	–	+	–	+	–	–	+	–	+	+	+	+	+	+	–		–	+	Pale yellow	3
GH_1	+	+	Aer + Anaer	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	+	–			–	+		VI
GH_2	+	–	Aer + Anaer	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	+	+	+			–	+	Yellow	7

APPENDIX A

BIOCHEMICAL CHARACTERS OF ISOLATES

BIOCHEMICAL TEST / ISOLATE	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM									Acetoin	Phosphatase	Reduction of nitrate to nitrite	Urease production	Hydrolysis of Tween 80	Hydrolysis of suet	Hydrolysis of gelatin	GROWTH			Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol									10 °C	45 °C	0% NaCl	15% NaCl						
GH_3	+	-	Aer + Anaer	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	+	-	-		-	+	Bright yellow	7
GH_5	+	-	Aer + Anaer	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-		+	+	Yellow	5
GH_7	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-		-	+		VI
GH_8	+	-	Aer + Anaer	-	+	+	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	+	+		+	+		6
GH_9	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+		-	+		3
GH_{10}	+	-	Aer	-	+	+	+	+	-	-	-	-	+	+		-	-	-	+	-	+	-	+		+	+	Yellow	6
GH_{11}	+	-	Aer + Anaer	-	+	+	+	+	+	-	-	+	-	-		+	-	+	+	-	+	-	+		-	+		3
GM_2	+	-	Aer	-	-	-	+	-	+	-	-	-	-	-		+	-	-	+	-	+	-	+		-	+		7
GM_3	+	-	Aer + Anaer	-	+	+	+	+	+	-	-	-	+	-		-	-	-	+	+	+	-	+		-	+		6
GM_4	+	+	Aer + Anaer	-	+	-	-	-	-	-	-	-	-	-		-	-	-	+	-	+	-	+		+	+		VI
GC_1	+	-	Aer + Anaer	-	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	+	+	-		-	+		5
GC_2	-	-	Aer + Anaer	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-		-	+	Yellow	7
GC_3	+	-	Aer + Anaer	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	-	-	+	-	-		-	+		4
GL_2	+	-	Aer + Anaer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-		-	+	Yellow	7
GL_{3w}	+	+	Aer + Anaer	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	+	-	-			+		4
GL_4	+	+	Aer + Anaer	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-		-	+		VI
GL_5	+	-	Aer + Anaer	-	-	-	+	-	+	-	-	-	-	-		+	+	-	-	-	+	-	-		-	+	Pale yellow	5

APPENDIX A

BIOCHEMICAL CHARACTERS OF ISOLATES

BIOCHEMICAL TEST / ISOLATE	ACID FROM GLUCOSE		Growth in Evans and Kloos' Medium	ACID PRODUCTION FROM											GROWTH							BAIRD-PARKER SUBGROUP
	Aerobic	Anaerobic		L(+)arabinose	Mannitol	Maltose	Lactose	Sucrose	D(+)galactose	D(+)xylose	Meso-inositol	Acetoin	Phosphatase	Reduction of nitrate to nitrite	Urease production	Hydrolysis of Tween 80	Hydrolysis of suet	Hydrolysis of gelatin	10 °C	45 °C	0% NaCl	15% NaCl	Egg-yolk clearing	Ammonia from arginine	Oxidase	Catalase	Pigment	
GL_7	+	-	Aer + Anaer	-	-	-	+	+	+	-	-	-	-	+	+	-	-		-	+	+	+			-	+	Yellow	5
GL_{3y}	+	-	Aer	-	-	-	-	-	-	-	-	-	-		+	-	+		+	-	+	+			+	+	Yellow	5
GTc_1	+	+	Aer + Anaer	-	-	-	-	-	-	+	-	-	-		+	-	-		+	+	+	+			-	+	Yellow	VI
GTc_2	+	-	Aer	-	-	-	-	-	-	+	-	-	-		+	-	-		+	+	+	-				+	Yellow	5
GTc_3	+	+	Aer + Anaer	-	+	-	-	-	-	-	-	-	-	+	+	-	-		+	+	+	-			-	+		VI
GTc_4	+	-	Aer	-	-	-	-	-	-	-	-	-	-	+	+	-	-		+	-	+	+			-	+	Pale yellow	7
$GBTc_3$	+	-	Aer + Anaer	+	+	-	+	+	-	+	-	-	-		+	-	+		+	+	+	+			+	+		4
$GBTc_4$	+	-	Aer	-	+	+	+	+	-	-	-	-	+		+	-	+		+	-	+	-			-	+		6
$GBTc_5$	+	-	Aer	-	+	+	+	+	-	-	-	-	+		+	-	-		+	-	+	-			-	+	Yellow	6

APPENDIX B

THE INTERRELATIONSHIPS BETWEEN THE COMPUTER GROUPINGS AND THE INITIAL GROUPING OF THE GRAM-POSITIVE COCCI ISOLATED FROM COMMERCIAL BILTONG AND THEIR FINAL CLASSIFICATION

APPENDIX B

SAMPLE NO.	INITIAL GROUPING	COMPUTER GROUPING					FINAL GROUPING	SPECIES
		Agreement with initial grouping			Agreement between 1st and 2nd Program			
		1st Program		2nd Program				
		Unweighted	Weighted		Unweighted	Weighted		
BL_1	1588	+	+	+	+	+	1588	*S. saprophyticus*
BL_2	1588	+	+	+	+	+	1588	*S. saprophyticus*
BTc_2	1588	+	+	+	+	+	1588	*S. saprophyticus*
BH_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
BH_4	1588	+	+	+	+	+	1588	*S. saprophyticus*
BH_5	1588	+	+	+	+	+	1588	*S. saprophyticus*
BH_6	1557	+	+	+	+	+	1557	*S. saprophyticus*
BM_1	1587	+	+	+	+	+	1587	*S. saprophyticus*
BM_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
BM_3	1587	-	-	-	+	+	15305	*S. saprophyticus*
CTc_3	1588	+	+	+	+	+	1588	*S. saprophyticus*
CH_1	1587	-	-	-	-	-	1465	*M. luteus*
CH_2	1587	-	-	-	+	+	15305	*S. saprophyticus*
CH_3	1557	-	-	-	+	-	1588	*S. saprophyticus*
CH_4	1558	+	+	+	+	+	1588	*S. saprophyticus*
CH_5	1588	+	+	+	+	+	1588	*S. saprophyticus*
CM_2	1557	-	-	-	-	-	1557	*S. saprophyticus*
CM_3	1587	-	-	-	+	+	1588	*S. saprophyticus*
CL_3	1588	+	+	-	-	-	1588	*S. saprophyticus*
CL_4	1587	-	-				15305	*S. saprophyticus*
DH_1	1588	+	+	+	+	+	1588	*S. saprophyticus*
DH_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
DH_4	1587	+	+	+	+	+	1587	*S. saprophyticus*
DH_5	1587	-	-	-	+	+	15305	*S. saprophyticus*

APPENDIX B

SAMPLE NO.	INITIAL GROUPING	COMPUTER GROUPING					FINAL GROUPING	SPECIES
		Agreement with initial grouping			Agreement between 1st and 2nd Program			
		1st Program		2nd Program				
		Unweighted	Weighted		Unweighted	Weighted		
DH_7	1588	+	+	+	+	+	1588	*S. saprophyticus*
DH_8	1587	+	+	+	+	+	1587	*S. saprophyticus*
DTc_4	1587	+	+	+	+	+	1587	*S. saprophyticus*
DLa_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
DM_2	1587	-	+	+	-	+	1587	*S. saprophyticus*
DM_3	1587	-	-	-	+	+	15305	*S. saprophyticus*
DL_1	1587	+	+	+	+	+	1587	*S. saprophyticus*
DL_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
DL_3	1587	+	+	+	+	+	1587	*S. saprophyticus*
DL_5	1587	+	+	+	+	+	1587	*S. saprophyticus*
EM_1	1587	-	-	+	-	-	1587	*S. saprophyticus*
EM_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
EM_3	1557	-	-	-	-	-	1462	*S. epidermidis*
EH_4	1587	+	+	+	+	+	1587	*S. saprophyticus*
EH_5	1587	+	+	+	+	+	1587	*S. saprophyticus*
EH_6	1587	+	+	+	+	+	1587	*S. saprophyticus*
EH_7	1587	+	+	+	+	+	1587	*S. saprophyticus*
ETc_1	1587	-	+	-	+	-	1587	*S. saprophyticus*
ETc_3	1587	+	+	+	+	+	1587	*S. saprophyticus*
EL_1	1587	+	+	+	+	+	1587	*S. saprophyticus*
EL_2	1587	-	-	-	+	+	15305	*S. saprophyticus*
EL_3	1587	+	-	+	+	-	1587	*S. saprophyticus*
ETc_4	1466	-	-	-	-	-	1466	*S. epidermidis*

APPENDIX B

SAMPLE NO.	INITIAL GROUPING	COMPUTER GROUPING					FINAL GROUPING	SPECIES
		Agreement with initial grouping			Agreement between 1st and 2nd Program			
		1st Program		2nd Program				
		Unweighted	Weighted		Unweighted	Weighted		
FH_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
FH_4	1587	+	+	+	+	+	1587	*S. saprophyticus*
FH_6	1587	+	+	+	+	+	1587	*S. saprophyticus*
FL_1	1587	+	+	+	+	+	1587	*S. saprophyticus*
FL_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
FL_3	1477	-	-	-	+	-	1466	*S. epidermidis*
FL_4	1587	+	+	+	+	+	1587	*S. saprophyticus*
FM_3	1587	+	+	+	+	+	1587	*S. saprophyticus*
FTc_2	1587	+	+	+	+	+	1587	*S. saprophyticus*
FM_1	1587	+	+	+	+	+	1587	*S. saprophyticus*
GH_1	1466	-	-	-	+	+	1462	*S. epidermidis*
GH_7	1466	-	-	-	+	+	1464	*S. epidermidis*
GH_9	1587	+	+	+	+	+	1587	*S. saprophyticus*
GH_{11}	1587	+	+	+	+	+	1587	*S. saprophyticus*
GM_4	1466	-	-	-	-	-	1462	*S. epidermidis*
GC_3	1839	-	-	-	+	+	1839	*S. saprophyticus*
GL_{3w}	1839	-	-	-	-	-	1839	*S. saprophyticus*
GL_4	1466	-	-	-	-	-	1462	*S. epidermidis*
GTc_1	1466	-	-	-	-	-	1462	*S. epidermidis*
$GBTc_3$	1839	+	-	+	+	-	1839	*S. saprophyticus*
GTc_3	1466	-	-	-	+	-	1462	*S. epidermidis*
$GBTc_4$	1463	-	+	+	-	+	1463	*M. varians*
$GBTc_5$	1463	-	+	-	-	-	1469	*M. varians*

APPENDIX B

SAMPLE NO.	INITIAL GROUPING	COMPUTER GROUPING					FINAL GROUPING	SPECIES
		Agreement with initial grouping			Agreement between 1st and 2nd Program			
		1st Program		2nd Program				
		Unweighted	Weighted		Unweighted	Weighted		
BTc_1	1469	-	+	+	-	+	1469	*M. varians*
CH_6	1463	+	+	+	+	+	1463	*M. varians*
CH_7	1469	-	-	-	-	-	1587	*S. saprophyticus*
CM_1	1463	-	+	-	+	+	1463	*M. varians*
CL_1	1469	-	-	-	+	+	1465	*M. luteus*
CL_5	4698	-	-	-	+	+	4698	*M. luteus*
DH_3	1469	-	-	-	-	-	1649	*Sarcina lutea*
DH_6	4698	-	-	-	+	+	4698	*M. luteus*
DTc_3	4698	-	-	-	+	+	27566	*M. lylae*
FTc_3	1469	-	-	-	-	-	1469	*M. varians*
GH_2	4698	-	-	-	+	+	27570	*M. kristinae*
GH_3	4698	-	-	-	-	+	884	*M. varians*
GH_5	1469	-	-	-	+	+	27570	*M. kristinae*
GH_8	1463	-	+	-	+	+	1463	*M. varians*
GH_{10}	1463	-	+	-	-	+	1463	*M. varians*
GM_2	4698	-	-	-	+	+	15306	*M. varians*
GM_3	1463	-	-	-	+	+	1463	*M. varians*
GC_1	1469	-	-	-	-	-	1587	*S. saprophyticus*
GC_2	4698	-	-	-	+	+	764	*M. freudenreichii*
GL_2	4698	-	-	-	+	+	1649	*M. luteus*
GL_5	1469	-	-	-	+	+	884	*M. varians*
GL_7	1469	-	-	-	+	-		*M. varians*
GL_{3y}	1469			-			1649	*Sarcina lutea*
GTc_2	1469	-	-	-	+	-	884	*M. varians*
GTc_4	4698	-	-	-	+	+	4698	*M. luteus*

APPENDIX C

STANDARD METHOD USED FOR THE MICROBIOLOGICAL ANALYSIS OF SAMPLES

The method specified by Barraud et al. (1967) for meat and meat products was used for the microbiological analysis of samples.

Eleven grams of the ground or pulverized sample were weighed out aseptically and diluted with 99 ml saline-peptone diluent [0,85% ($^{w}/_{v}$) NaCl (Merck); 0,1% ($^{w}/_{v}$) peptone (Difco)] in a sterile M.S.E. Atomix blender to give a $^{1}/_{10}$ dilution of the original sample. The sample was macerated in the blender for 30 seconds at 6 000 rpm (half speed) plus 60 seconds at 12 000 rpm (full speed of the blender).

Serial 10-fold dilutions of the homogenate were prepared in 90 ml saline-peptone diluents. Aliquots of 0,1 ml of the appropriate dilutions were spread onto the surface of dry agar plates using a sterile glass 'hockey stick' or 1 ml aliquots were introduced into sterile plastic petri-dishes and over-poured with 15 ml agar previously cooled to 45 ^{o}C.

APPENDIX D

SELECTIVE MEDIA FOR THE ISOLATION OF BACTERIA TOLERANT TO SORBATE OR PIMARICIN

1. TOTAL NUMBER OF BACTERIA TOLERANT TO SORBATE

I.S.O. agar was prepared as specified by Barraud et al. (1967) and prior to sterilization, the pH of the medium was adjusted to either pH 5,8 or pH 7,2 using 1(N)HCl and 10(N)NaOH respectively. Aliquots of a 10% ($^{w}/_{v}$) filter-sterilized, aqueous potassium sorbate (Merck) solution were aseptically introduced into the medium, precooled to 50 ^{o}C, to the final concentrations of 1 500 ppm and 2 000 ppm potassium sorbate. The medium was mixed thoroughly prior to pouring plates.

2. MICROCOCCACEAE TOLERANT TO SORBATE

Mannitol Salt Agar (Oxoid; Gibco) was adjusted to pH 5,8 with 1(N)HCl prior to sterilization. Aliquots of a 10% ($^{w}/_{v}$) filter-sterilized, aqueous potassium sorbate solution were aseptically introduced into both adjusted and unadjusted media, precooled to 50 ^{o}C, to a final concentration of 2 000 ppm potassium sorbate and mixed thoroughly prior to pouring plates.

3. TOTAL NUMBER OF BACTERIA TOLERANT TO PIMARICIN

I.S.O. agar was prepared and the pH adjusted as described above (cf. 1.). As pimaricin is highly insoluble in a number of solvents and weakly soluble in glycerol (Gist-Brocades N.V.; technical brochure), a 0,5% ($^{w}/_{v}$) pasteurized suspension of pimaricin (sample from

Gist-Brocades N.V.) in glycerol (Merck) was used. Aliquots of the suspension were aseptically introduced into the media, precooled to 50 °C, to a final concentration of 50 ppm pimaricin and mixed thoroughly prior to pouring plates.

4. MICROCOCCACEAE TOLERANT TO PIMARICIN

Aliquots of a 0,5% ($^{w}/_{v}$) pasteurized suspension of pimaricin in glycerol were aseptically introduced into both Mannitol Salt Agar (Oxoid; Gibco) adjusted to pH 5,8 with 1(N)HCl and unadjusted Mannitol Salt Agar, precooled to 50 °C, to a final concentration of 50 ppm pimaricin. Thorough mixing of the media was necessary prior to pouring plates.

APPENDIX E

SUMMARY OF THE CHANGES OCCURRING IN THE PHYSICO-CHEMICAL PROPERTIES OF THE SUBSTRATE DURING BILTONG PRODUCTION UNDER CONTROLLED CONDITIONS

APPENDIX E

PHYSICO-CHEMICAL CHANGES DURING BILTONG PRODUCTION

PHYSICO-CHEMICAL PARAMETERS	SAMPLE / MEAT TREATMENT	1 -22 h	2 -17 h	3 0 h	4 2 h	5 4 h	6 7 h	7 24 h	8 31 h	9 48 h	10 55 h	11 72 h	12 144 h	13 288 h
pH	Commercially available beef (mean of 6 samples)	5,72	5,74	5,73	5,72	5,72	5,76	5,85	5,89	5,86	5,84	5,85	5,86	5,80
	Aseptically dissected beef (mean of 3 samples)	5,87	5,73	5,62	5,58	5,58	5,63	5,65	5,58	5,66	5,64	5,66	5,69	5,66
	Potassium sorbate treatment (single sample)	5,46	5,40	5,46	5,43	5,41	5,48	5,56	5,55	5,56	5,56	5,58	5,65	5,71
	Pimaricin treatment (single sample)	5,63	5,51	5,50	5,53	5,48	5,50	5,65	5,56	5,65	5,65	5,75	5,65	5,65
WATER ACTIVITY	Commercially available beef (mean of 6 samples)	0,985	0,974	0,971	0,955	0,949	0,948	0,917	0,893	0,888	0,856	0,833	0,733	0,718
	Aseptically dissected beef (mean of 3 samples)	0,989	0,982	0,979	0,972	0,968	0,966	0,937	0,928	0,893	0,879	0,828	0,741	0,717
	Potassium sorbate treatment (single sample)	0,999	0,997	0,981	0,980	0,972	0,984	0,957	0,956	0,915	0,904	0,900	0,840	0,815
	Pimaricin treatment (single sample)	0,990	0,984	0,981	0,980	0,980	0,969	0,964	0,926	0,960	0,957	0,876	0,786	0,747
% MOISTURE	Commercially available beef (mean of 6 samples)	75,52	73,48	73,14	70,47	63,92	63,96	51,30	49,27	44,45	40,72	36,32	25,84	25,09
	Aseptically dissected beef (mean of 3 samples)	76,05	74,01	74,26	70,10	66,26	64,14	50,60	49,05	39,94	36,18	31,30	22,52	21,44
	Potassium sorbate treatment (single sample)	75,15	71,82	71,69	69,51	67,09	64,63	52,42	48,69	44,58	40,18	39,82	28,46	28,31
	Pimaricin treatment (single sample)	75,64	73,34	73,14	70,95	67,98	66,54	56,53	46,60	46,25	43,89	41,43	27,96	22,84

APPENDIX E

PHYSICO-CHEMICAL CHANGES DURING BILTONG PRODUCTION

PHYSICO-CHEMICAL PARAMETERS	SAMPLE / MEAT TREATMENT	1 -22 h	2 -17 h	3 0 h	4 2 h	5 4 h	6 7 h	7 24 h	8 31 h	9 48 h	10 55 h	11 72 h	12 144 h	13 288 h
% NaCl (WET WEIGHT BASIS)	Commercially available beef (mean of 6 samples)	0,602	2,219	2,271	2,829	2,929	2,990	4,233	4,485	4,902	5,044	5,297	6,586	6,227
	Aseptically dissected beef (mean of 3 samples)	0,124	2,125	2,123	2,721	2,999	3,397	4,645	4,438	5,303	5,432	6,279	6,296	6,413
	Potassium sorbate treatment (single sample)	0,055	2,910	2,553	3,210	3,375	2,989	4,370	4,795	4,883	4,932	5,788	4,683	4,783
	Pimaricin treatment (single sample)	0,088	2,762	2,849	2,635	2,913	3,482	3,384	5,260	4,499	4,616	4,587	5,879	5,775
POTASSIUM SORBATE ppm (WET WEIGHT BASIS)	Potassium sorbate treatment (single sample)	35	965	825	1175	895	720	895	1312	1387	1200	1325	1455	1610
POTASSIUM SORBATE ppm (DRY WEIGHT BASIS)	Potassium sorbate treatment (single sample)	140	3424	2914	3853	2719	2035	1881	2557	2503	2006	2201	2033	2245

APPENDIX F

SUMMARY OF THE CHANGES OCCURRING IN BOTH THE NUMBER AND TYPES OF SAPROPHYTIC MICRO-ORGANISMS FOUND DURING BILTONG PRODUCTION UNDER CONTROLLED CONDITIONS

APPENDIX F

CHANGES IN MICROBIAL NUMBERS DURING BILTONG PRODUCTION

		LOG OF VIABLE BACTERIAL NUMBER/g DRY WEIGHT OF SAMPLE												
	SAMPLE / MEAT TREATMENT	1 -22 h	2 -17 h	3 0 h	4 2 h	5 4 h	6 7 h	7 24 h	8 31 h	9 48 h	10 55 h	11 72 h	12 144 h	13 288 h
AEROBIC TOTAL COUNT	Commercially available beef (mean of 6 samples)	5,9	5,2	5,2	5,3	5,3	4,7	6,3	6,4	6,9	6,9	6,5	6,4	5,7
	Aseptically dissected beef (mean of 3 samples)	4,5	2,6	2,6	2,5	2,2	2,2	4,1	3,8	5,5	2,4	4,9	5,5	3,7
	Potassium sorbate treatment (single sample)	5,6	4,9	4,7	4,2	3,8	3,9	3,6	3,0	3,9	3,4	3,4	4,4	5,4
	Pimaricin treatment (single sample)	7,1	5,8	5,7	4,8	5,3	4,3	5,7	4,0	6,3	6,5	5,0	6,3	4,6
MICROCOCCACEAE	Commercially available beef (mean of 6 samples)	4,8	4,5	4,3	4,3	3,9	3,7	6,1	6,2	6,8	6,8	6,4	6,1	5,4
	Aseptically dissected beef (mean of 3 samples)	3,6	2,4	3,2	2,0	1,6	1,9	4,1	3,7	5,6	2,5	4,7	5,4	3,4
	Potassium sorbate treatment (single sample)	4,2	3,5	3,3	2,8	2,8	3,0	3,1	2,8	3,5	3,5	3,1	4,3	5,3
	Pimaricin treatment (single sample)	3,7	3,3	2,7	2,0	2,3	*	5,3	3,6	5,5	6,3	4,8	6,3	4,4
Pseudomonas/Achromobacter GROUP	Commercially available beef (mean of 6 samples)	5,3	4,2	4,4	4,4	4,2	3,7	4,0	4,5	4,7	4,1	4,0	3,4	*
	Aseptically dissected beef (mean of 3 samples)	2,2	0,6	1,8	0,6	*	*	0,6	*	*	*	0,2	0,6	*
	Potassium sorbate treatment (single sample)	4,6	3,7	3,6	3,1	2,7	2,5	1,8	*	*	*	*	*	*
	Pimaricin treatment (single sample)	6,8	5,1	4,4	3,7	4,1	2,5	*	*	*	2,1	2,8	1,6	*

* ≡ < 10^2 orgs/g dry weight of sample

APPENDIX F

CHANGES IN MICROBIAL NUMBERS DURING BILTONG PRODUCTION

		LOG OF VIABLE MICROBIAL NUMBER/g DRY WEIGHT OF SAMPLE												
	SAMPLE / MEAT TREATMENT	1 -22 h	2 -17 h	3 0 h	4 2 h	5 4 h	6 7 h	7 24 h	8 31 h	9 48 h	10 55 h	11 72 h	12 144 h	13 288 h
ORGANISMS TOLERANT TO 10% NaCl	Commercially available beef (mean of 6 samples)	4,6	4,0	3,8	3,8	3,8	3,7	6,2	6,4	6,8	6,9	6,8	6,3	5,7
	Aseptically dissected beef (mean of 3 samples)	2,8	2,5	2,9	1,8	1,6	1,5	4,2	3,7	5,7	2,5	4,9	5,5	3,4
	Potassium sorbate treatment (single sample)	4,2	3,9	3,5	3,1	2,7	3,2	3,0	2,7	3,3	3,3	3,3	4,4	5,3
	Pimaricin treatment (single sample)	3,7	3,3	2,6	*	2,5	2,3	5,4	3,7	5,4	6,3	4,7	6,3	4,4
LACTIC ACID BACTERIA	Commercially available beef (mean of 6 samples)	3,6	2,5	1,9	2,9	2,4	2,4	3,5	5,1	5,5	4,2	4,0	4,3	*
	Aseptically dissected beef (mean of 3 samples)	*	*	*	*	*	*	*	*	*	*	*	*	*
	Potassium sorbate treatment (single sample)	-†	-	-	-	-	-	-	-	-	-	-	-	-
	Pimaricin treatment (single sample)	-	-	-	-	-	-	-	-	-	-	-	-	-
YEASTS AND MOULDS	Commercially available beef (mean of 6 samples)	3,5	2,5	2,3	2,2	2,2	1,9	2,1	3,0	3,0	3,1	2,3	2,3	0,8
	Aseptically dissected beef (mean of 3 samples)	*	*	1,1	*	2,0	1,7	*	*	*	*	*	0,3	*
	Potassium sorbate treatment (single sample)	2,6	2,0	*	*	2,0	1,9	*	*	*	*	*	*	*
	Pimaricin treatment (single sample)	-	-	-	-	-	-	-	-	-	-	-	-	-

* ≡ < 10^2 orgs/g dry weight of sample

-† ≡ not determined

APPENDIX F

CHANGES IN MICROBIAL NUMBERS DURING BILTONG PRODUCTION

		LOG OF VIABLE BACTERIAL NUMBER/g DRY WEIGHT OF SAMPLE												
	SAMPLE / MEAT TREATMENT	1 -22 h	2 -17 h	3 0 h	4 2 h	5 4 h	6 7 h	7 24 h	8 31 h	9 48 h	10 55 h	11 72 h	12 144 h	13 288 h
AEROBIC VIABLE COUNT TOLERANT TO pH 5,8	Potassium sorbate treatment (single sample)	5,5	4,6	4,6	3,4	3,7	3,8	3,1	2,9	3,8	3,5	3,4	4,4	5,4
	Pimaricin treatment (single sample)	7,1	5,7	5,5	4,7	5,4	4,2	5,6	4,0	6,1	6,5	4,8	6,3	4,5
TOTAL NUMBER OF ORGANISMS TOLERANT TO pH 5,8 AND 1500 ppm K-SORBATE	Potassium sorbate treatment (single sample)	4,6	3,5	3,5	2,6	2,3	2,5	3,0	2,9	3,9	3,5	3,4	4,4	5,3
TOTAL NUMBER OF ORGANISMS TOLERANT TO pH 5,8 AND 2000 ppm K-SORBATE	Potassium sorbate treatment (single sample)	4,4	3,2	3,3	2,7	2,0	2,7	3,1	2,7	3,9	3,5	3,4	4.3	5.3
MICROCOCCACEAE TOLERANT TO pH 5,8 AND 2000 ppm K-SORBATE	Potassium sorbate treatment (single sample)	3,7	3,2	2,7	2,6	2,5	2,2	3,2	2,4	3,8	3,3	3,3	4,4	5,2
TOTAL NUMBER OF ORGANISMS TOLERANT TO pH 5,8 AND 50 ppm PIMARICIN	Pimaricin treatment (single sample)	7,1	5,7	5,6	4,8	5,1	4,1	5,5	4,0	6,1	6,5	4,8	6,3	4,8
MICROCOCCACEAE TOLERANT TO pH 5,8 AND 50 ppm PIMARICIN	Pimaricin treatment (single sample)	3,7	3,1	2,6	2,5	2,5	*	5,4	3,6	5,5	6,3	4,7	6,3	4,4

* $\equiv < 10^2$ orgs/g dry weight of sample

APPENDIX F

TYPES OF BACTERIA ISOLATED DURING BILTONG PRODUCTION

PRESUMPTIVE BACTERIAL GROUP	SAMPLE / MEAT TREATMENT	PERCENTAGE OF INVESTIGATED FLORA												
		1	2	3	4	5	6	7	8	9	10	11	12	13
		-22 h	-17 h	0 h	2 h	4 h	7 h	24 h	31 h	48 h	55 h	72 h	144 h	288 h
MICROCOCCACEAE	Commercially available beef	27	25	37	34	23	83	100	92	96	100	100	100	
	Aseptically dissected beef	21	13		14	100		89	83	100	100	94	100	84
LACTIC ACID BACTERIA	Commercially available beef			3	7	19	7		4	4				
	Aseptically dissected beef													
GRAM-POSITIVE CATALASE-POSITIVE RODS	Commercially available beef	27	20	13	24	35	7		4					
	Aseptically dissected beef	14	38		29				17					11
PSEUDOMONADACEAE OR ENTEROBACTERIACEAE	Commercially available beef	46	55	40	34	23	3							
	Aseptically dissected beef	57	38		57			11				6		5

APPENDIX G

THE NUMBER AND SPECIES OF STAPHYLOCOCCI AND MICROCOCCI ISOLATED AT DIFFERENT TIME INTERVALS DURING BILTONG PRODUCTION FROM COMMERCIALLY AVAILABLE BEEF

SAMPLE NUMBER	TIME INTERVAL (h)	SPECIES
1	- 22	*M. luteus* (2)*; *M. freudenreichii* (1); *M. varians* (1).
2	- 17	*M. luteus* (1); *S. saprophyticus* (2).
3	0	*M. luteus* (2); *M. varians* (1); *S. saprophyticus* (4).
4	2	*M. luteus* (2); *M. varians* (1); *M. sedentarius* (1); *S. saprophyticus* (3).
5	4	*M. kristinae* (1); *S. saprophyticus* (4).
6	7	*M. luteus* (1); *M. kristinae* (1); *M. freudenreichii* (1); *S. epidermidis* (1); *S. saprophyticus* (12).
7	24	*M. varians* (1); *M. kristinae* (1); *M. luteus* (1); *S. saprophyticus* (21).
8	31	*M. luteus* (1); *M. varians* (1); *M. kristinae* (1); *S. saprophyticus* (19).
9	48	*M. varians* (6); *M. kristinae* (2); *S. epidermidis* (2); *S. saprophyticus* (15).
10	55	*M. kristinae* (2); *S. epidermidis* (2); *S. saprophyticus* (19).
11	72	*M. kristinae* (1); *S. epidermidis* (3); *S. saprophyticus* (15).
12	144	*M. varians* (1); *S. epidermidis* (1); *S. saprophyticus* (19).

(2)* = number of strains identified

APPENDIX H

THE NUMBER AND SPECIES OF STAPHYLOCOCCI AND MICROCOCCI ISOLATED AT DIFFERENT TIME INTERVALS DURING BILTONG PRODUCTION FROM ASEPTICALLY DISSECTED BEEF

SAMPLE NUMBER	TIME INTERVAL (h)	SPECIES
A	- 22	*M. varians* (1)*; *S. epidermidis* (1); *S. saprophyticus* (1).
B	- 17	*M. roseus* (1).
C	0	
D	2	
E	4	*M. varians* (1); *S. epidermidis* (1).
F	7	
G	24	*S. saprophyticus* (4); *S. epidermidis* (3).
H	31	*S. epidermidis* (9).
I	48	*S. epidermidis* (19).
J	55	*S. saprophyticus* (2).
K	72	*S. saprophyticus* (5); *S. epidermidis* (12).
L	144	*S. epidermidis* (7).
M	288	*S. epidermidis* (13); *S. saprophyticus* (2).

(1)* = number of strains identified.

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