The effect of honeybush (*Cyclopia subternata*) extract in dry-cured and fermented warthog salami with reduced added nitrate

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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GENERAL ABSTRACT

The aim of this study was to investigate the effect of incorporating honeybush (*Cyclopia subternata*) extract as a natural antioxidant in dry-cured and fermented salami. This is the first study attempting the addition of honeybush extract to a processed red meat product and is therefore regarded as baseline data for future research. The data forms part of a relevant research focus, being botanical extracts in meat exhibiting antimicrobial and/or antioxidant activity and could expand the knowledge surrounding the potential of a native South African extract intended for the food industry.

The first research trial was regarded as a pilot study to familiarise with the skill of salami making and to better comprehend the technical challenges when incorporating honeybush extract to salami. For this purpose, a typical pork Italian salami was chosen as the model. A total of 68 pork salami were produced, which were subdivided into three treatments: Control= no added honeybush or nitrate; Nitrate= with added sodium nitrate (100 mg/kg) and ascorbic acid; and Honeybush= with 0.5% (w/w) added honeybush extract. After 35 days of controlled ripening, the honeybush treated salami had a higher water activity (a_w= 0.928, p ≤ 0.01) compared with the control (a_w= 0.923) and nitrate salami (a_w= 0.924). This was also reflected in the higher moisture content of the honeybush treated salami (35.3%), compared to the control (33.5%; p ≤ 0.01). Final product salami pH (5.35-5.24) was not affected by treatment, however, salami with honeybush extract had less spontaneous outer surface mould growth or coverage at the end of ripening.

The subsequent research trial entailed the use of warthog meat and pork fat to create salami with reduced added nitrate and increasing amounts of honeybush, where: Control= 0%; Treatment 1= 0.125%; Treatment 2= 0.25%; Treatment 3= 0.375%; and Treatment 4= 0.5% (w/w) of added honeybush extract. A total of 75 mg/kg of sodium nitrate (in the absence of nitrite) was added to all salami treatments. This addition of nitrate equalled a 70% reduction compared to the maximum permitted amount of added nitrate by the European Union (250 mg/kg) for non heat treated cured meat products (in the absence of added nitrite). After a 40-day ripening period, all salami sufficiently decreased in moisture (30.1%) and were well within the recommended a_w for microbial safety (a_w= 0.8333- 0.873), with T4 resulting in a significantly higher a_w (0.873, p < 0.001). Although the highest honeybush addition (T4= 0.5% w/w of added honeybush) significantly lowered the salami pH (p= 0.004), the salami pH overall failed to decrease sufficiently after stuffing (with the typical low acidity salami pH reduction being between 5.0-5.3), resulting in a strangely high final product salami pH (6.71-7.46). It was hypothesised that this strangely high pH was ascribable to surface mould (*Penicillium nalgiovense*) growth on the small diameter (~ 33 mm) salami with the ability of utilising lactic acid, counteracting the acidifying effect of the added starter culture. Furthermore, the possible outgrowth of the starter culture lactic acid bacteria strains by background competing microflora (including *Enterococcus faecium*) presumably hindered a successful pH reduction. Positively, the retention of the red salami colour was best in salami with the highest honeybush inclusion levels

(T3, CIE a*= 10.07 and T4, CIE a*= 10.18) after five and a half months of frozen storage (-20°C). The addition of honeybush (in a maximum concentration of 0.5%) did not significantly affect the proximate composition (moisture, protein and total lipid content) and texture attributes (hardness, chewiness and cohesiveness) of final product salami.

Final product salami microbial analyses were conducted to gain insight regarding the food safety when incorporating such an extract to dry-cured and fermented game salami with reduced added sodium nitrate. All salami treatments (C, T1-T4) were free from major pathogens: *Escherichia coli; Listeria monocytogenes; Salmonella* spp.; and *Staphylococcus aureus*. Lactic acid bacteria were lowest in the control salami (6.57 log CFU/g) and increased with increasing honeybush concentration, with T3 and T4 resulting in the highest counts (> 7.0 log CFU/g). This finding corresponded with the lower pH of T4 salami. This study proved the feasibility of producing game salami with a high pH (6.71-7.46) free from major pathogens with a 70% reduction in added sodium nitrate (75 mg/kg) with the addition of a natural extract, honeybush. The honeybush addition presumably promoted the growth of wanted acidifying bacteria, with no effect with regard to bacterial proliferation inhibition.

The aim of the final research chapter was to determine the effect of the honeybush extract on the fatty acid methyl ester (FAME) composition and volatile organic compound (VOC) profile of the warthog salami. Honeybush significantly affected the fatty acid composition (C12:0, C14:1, C20:1, C18:3n6, C18:3n3, C20:3n3, C20:4n6, C20:5n3 and subsequently also total *n*-3 polyunsaturated fatty acids (PUFA) and the *n*-6/*n*-3 PUFA ratio). Nonetheless, the overall FAME composition of the final product salami were comparable with European and American dry-cured and fermented sausages. The VOC results indicated that a honeybush addition of 0.5% to warthog salami may contribute to floral, sweet and spicy aromas due to the presence of (*R*/*S*)-linalool, terpinene-4-ol and *α*-terpineol in the final product salami. Positively, honeybush treated salami resulted in lower amounts of compounds related to rancidity compared to the control after a 40-day ripening period, including: hexanal; nonanal; 1-pentanol; 1-octen-3-ol; 2-pentylfyran; and undecane. Of these, hexanal and 1-octen-3-ol reduced the most significantly in T2, T3 and T4 salami (0.25%, 0.375% and 0.5% honeybush addition, respectively) compared to the control salami (p < 0.01). These results were indicative of the suppression of rancid volatile compounds originating from oleic (C18:1n9*c*) and linoleic (C18:2n6*c*) fatty acid oxidation suggesting that honeybush suppressed the formation of the above-mentioned VOCs linked to unwanted aromas in meat products.

ALGEMENE UITTREKSEL

Die doel van hierdie studie was om die effek van 'n natuurlike anti-oksidant, naamlik heuningbosekstrak (*Cyclopia subternata*), te toets in gedroogde, gekuurde en gefermenteerde wors (wat bekend staan as salami). Dit is die eerste studie wat poog om heuningbosekstrak in 'n verwerkte rooisvleisproduk te inkorporeer en word daarom beskou as 'n basis datastel vir toekomstige navorsers. Die data wat gegenereer is in die tesis vorm deel van 'n relevante navorsingsonderwerp wat die gebruik van botaniese ekstrakte met antimikrobiese en/of anti-oksidant eienskappe in vleis behels. Hierdie data sal die begrip rondom die toepassingspotensiaal van so 'n inheemse ekstrak vir die voedselbedryf verbreed.

Die eerste navorsingsproef was beskou as 'n loodstudie om vertroud te raak met die vaardighede wat benodig word vir salami produksie en om die tegniese uitdagings wat gepaard gaan met die byvoeging van heuningbosekstrak tot salami beter te verstaan. Vir hierdie doel was 'n tipiese Italiaanse vark salami gekies as die studiemodel. 'n Totaal van 68 salami's was vervaardig wat bestaan het uit behandelinge: Kontrole= geen bygevoegde heuningbos of nitrate; Nitraat= met bygevoegde natriumnitraat (100 mg/kg) en askorbiensuur; en Heuningbos= met 0.5% (w/w) bygevoegde heuningbosekstrak. Na 35 gekontroleerde rypwordingsdae, het die heuningbos salami 'n hoër wateraktiwiteit gehad (a_w= 0.928, p ≤ 0.01) in vergelyking met die kontrole (a_w= 0.923) en nitraat salami (a_w= 0.924). Hierdie bevinding was ook gereflekteer in die hoër voginhoud van die heuningbos salami (35.3%) in vergelyking met die kontrole (33.5%, p ≤ 0.01). Die pH van die finale salami produk (pH= 5.24-5.35) was nie beïnvloed deur die heuningbosbehandeling nie. Die heuningbosbehandelde salami het wel minder suksesvolle spontane groei van muf op die buite-oppervlak gehad na die rypwordingstydperk.

Die daaropvolgende navorsingsproef het die gebruik van vlakvarkvleis en varkvet ingesluit om salami te vervaardig met verminderde bygevoegde nitraat en toenemende hoeveelheid heuningbosekstrak, waar: Kontrole= 0%, Behandeling 1= 0.125%; Behandeling 2= 0.25%; Behandeling 3= 0.375%; en Behandeling 4= 0.5% (w/w) bygevoegde heuningbosekstrak behels het. Al die behandelinge het 0.75 mg/kg bygevoegde natriumnitraat bevat, sonder die byvoeging van nitriet. Hierdie byvoeging van nitraatsout was gelykstaande aan 'n 70% vermindering in vergelyking met die maksimum toelaatbare hoeveelheid soos deur die Europese Unie gestipuleer (250 mg/kg) word vir gedroogde, bereide en gefermenteerde vleis wat nie onderhewig gemaak is aan 'n hittebehandeling nie (in die afwesigheid van nitriet). Na 'n 40-dag rypwordingsperiode het die voginhoud (30.1%) in al die salami genoegsaam afgeneem en was alle behandelinge binne die aanbevole minimum wateraktiwiteit (a_w) vir mikrobiese veiligheid (a_w= 0.8333-0.873). Behandeling 4 het 'n betekenisvolle hoër a_w-waarde gehad (0.873, p < 0.001). Alhoewel die hoogste heuningbos byvoeging (behandeling 4= 0.5% bygevoegde heuningbosekstrak) die salami pH aansienlik verlaag het (p = 0.004), het die salami pH in totaal nie voldoende gedaal tydens die fermentasietydperk nie.

Dit het gelei tot 'n eindproduk salami met 'n onverwagte hoë pH (6.71-7.46). Die laasgenoemde verskynsel was toegeskryf aan die groei en metabolisme van die buite-oppervlak muf (*Penisillium nalgiovense*) op hierdie relatiewe klein deursnee salami (~ 33 mm) wat die bekende vermoë het om melksuur te metaboliseer en sodoende die versuring van die melksuurbakterieë teen te werk. Dit, te same met die kompeterende groei van bakterieë in die vleis (insluitend *Enterococcos faecium*), kon die bygevoegde melksuurbakterieë onderdruk het. 'n Belowende resultaat was die behoud van salami rooiheid (CIE a*) gepaardgaande met die byvoeging van heuningbosekstrak. Na vyf-en-'n-half-maande se vriestydperk (-20ºC), het salami met die hoogste heuningbos toevoeging (T3, CIE a*= 10.07 en T4, CIE a*= 10.18) hul rooiheid die beste behou in vergelyking met die ander behandelinge met laer heuningbosekstrak inhoud. Die byvoeging van heuningbos (tot 'n maksimum konsentrasie van 0.5% w/w) het nie die onmiddellike samestelling (vog, proteïen en lipied-inhoud) en tekstuureienskappe (hardheid, koubaarheid en sameklewing) van die finale vlakvark salami verander nie.

Mikrobiese analise op die finale vlakvark salami was uitgevoer om die voedselveiligheidsaspek in ag te neem wanneer so 'n tipe ekstrak in wildsalami gevoeg word met 'n 70% vermindering in bygevoegde nitraat. Alle salami Behandeling C, T1-T4 was vry van patogene: *Escherichia coli; Listeria monocytogenes; Salmonella* spp.; en *Staphylococcus aureus*. Melksuurbakterieë was die laagste in die kontrole salami (6.57 log CFU/g) en het toegeneem met toenemende heuningbosekstrak met Behandeling T3 en T4-salami wat gevolglik die hoogste hoeveelheid gehad het (> 7.0 log CFU/g). Laasgenoemde bevinding stem ooreen met die laer pH van Behandeling-T4-salami. Hierdie studie het die moontlikheid bewys om wildsvleis salami (spesifiek vlakvarkvleis) met 'n hoë pH (6.71-7.46), 70% verminderde bygevoegde nitraat (75 mg/kg) en met toenemende heuningbosekstrak konsentrasies te produseer wat vry is van getoetsde patogene. Die heuningbosekstrak byvoeging het ook die groei van verlangde melksuurbakterieë bevorder, met geen effek ten opsigte van bakteriese groei inhibisie nie.

Die finale navorsingshoofstuk se doel was om die effek van heuningbosekstrak op die vetsuurmetielester samestelling en vlugtige organiese verbindings van die vlakvark salami te bepaal. Heuningbosekstrak het geselekteerde vetsure betekenisvol beïnvloed (C12:0, C14:1, C20:1, C18:3n6, C18:3n3, C20:3n3, C20:4n6, C20:5n3 en gevolglik ook die totale *n*-3 en *n*-6/*n*-3 verhouding). Die algemene vetsuursamestelling van al die finale produk vlakvark salami was egter steeds vergelykbaar met Europese en Amerikaanse gedroogde en gefermenteerde worse. Die resultate rakende die vlugtige komponente het aangedui dat heuningbos kan bydra tot 'n soet, blomme en kruie aroma as gevolg van die teenwoordigheid van (*R*/*S*)-linalool, terpien-4-ol en *α*-terpineol in die finale salami produk. Verder het die heuningbosekstrak 'n afname in ongewensde, galsterige aromas getoon in vergelyking met die kontrole salami na 40 rypwordingsdae, insluitend: heksanaal; nonanaal; 1-pentanol; 1-okten-3-ol; 2-pentielfuran; en undekaan. Van die laasgenoemde

komponente, het al die heuningbosekstrak behandelde salami (T1-T4) betekenisvol minder heksanaal en 1-okten-3-ol inhoud gehad in vergelyking met die kontrole ($p < 0.01$). Hierdie resultate was 'n aanduiding van die onderdrukking van galsterige komponente met 'n oorsprong van oleïensuur (C18:1n9*c*) en linoleïensuur (C18:2n6*c*) oksidasie wat gevolglik daarop dui dat heuningbos die potensiaal toon om die rakleeftyd van salami met verminderde bygevoegde nitraat kan verleng.

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NOTES

The language (UK English) and style used in this thesis are in accordance with the requirements of the International Journal of Food Science and Technology (IJFST). This thesis represents a compilation of manuscripts where each chapter is an individual entity, thus some repetition between chapters (Materials and Methods) was unavoidable.

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CHAPTER 1

General Introduction

Salami, classified as dry-cured and fermented sausages, are part of a rich culinary history and the production thereof evolved along with technology and science (Leroy *et al.*, 2013; Franciosa *et al.*, 2018). Salami are worth appreciating considering the intricate, non-mutual biochemical reactions occurring during the fermentation and ripening periods to create the final product. Its popularity and importance is reflected in the many designation of origin certified salami in Europe and the economical contribution per year due to the trade thereof (Van der Lans & Van Ittersum, 2001; Marcoz *et al.*, 2016). These certifications reflect the sense of pride and cultural heritage connected to dry-cured and fermented sausages. The action of curing is defined as the mutual action of salting with the addition of nitrate and/or nitrite salt (Honikel, 2007; Hammes, 2012). Salting, drying, fermenting, curing and smoking meat in modern-day are not acknowledged as necessities to ensure meat supply, as the benefit of the cold chain can ensure meat supply all year round. These meat preserving techniques to produce salami are rather still applied today due to consumer preference for a fermented and cured taste, together with sense of culinary heritage (Bianchi *et al.*, 2007; Guerrero *et al.*, 2009).

Despite the popularity of salami, these sausages have been scrutinised over time for a number of health-related reasons (Bolger *et al.*, 2017; Holck *et al.*, 2017). Salami are classified as high in salt (~ 4-6%) and fat (~ 20-50%) (Meynier *et al.*, 1999; Zanardi *et al.*, 2004; Di Cagno *et al.*, 2008; Dos Santos *et al.*, 2015). A diet high in sodium and saturated fat diet is linked with increase risks of coronary heart diseases (Ha, 2014; Zong *et al.*, 2016). Many studies have attempted at reducing or substituting the amount of sodium in salami or altering the fatty acid profile to be more favourable for human consumption (e.g. increase the unsaturated fatty acids and lower the saturated fatty acids) (Muguerza *et al.*, 2003; Del Nobile *et al.*, 2009; Chen *et al.*, 2019; da Silva *et al.*, 2020).

Salami commonly contain preservatives known as nitrate (NO_3) and/or nitrite (NO_2) either as sodium or potassium salts. The revolutionary discovery of nitrite dates back centuries and although not documented, it is hypothesised that the use of nitrite as we know it, originated from the accidental contamination of sodium chloride with saltpetre (Sindelar & Milkowski, 2011; Taormina, 2014). Little did the scientists foresee then how complex the nitrate/nitrite chemistry in cured meat actually is, or which challenges the meat industry would face in the future due to the application of these preservatives. In order for any of the activities of nitrate to manifest in meat, it must first be reduced to its active counterpart, nitrite (Hammes, 2012; Bedale *et al.*, 2016). These activities include a highly effective antioxidant, antimicrobial and colour fixating ability (Skibsted, 2011; Majou & Christieans, 2018). A unique and desired cured flavour free from excessive rancidity (i.e. warmed-

over flavours) and an attractive pink-red colour are achieved through nitrite addition in small amounts (Møller & Skibsted, 2002; Sebranek & Bacus, 2007; Sindelar & Milkowski, 2011). The European Union allows a maximum permitted limit of 150 mg/kg nitrate and nitrite, respectively, or 250 mg/kg nitrate when nitrite is omitted to dry-cured and fermented, non heat treated sausages (Directive 2008/1333/EC). Nitrite is said to be an irreplaceable compound ascribable to its multifunctionality, high effectiveness in small dosages and rather mysterious contribution to create cured meats with superior sensory characteristics (Sindelar & Milkowski, 2011). Unfortunately, the addition of nitrate/nitrite in meat has proven to lead to the formation of carcinogenic *N*-nitrosamines when exposed to high temperatures (e.g. such as frying, > 130 ºC) and an acidic environment (IARC, 1978; Hammes, 2012; Sindelar & Milkowski, 2012; Butler, 2015; Bedale *et al.*, 2016). The lowering of Denmark's maximum permitted limit of nitrite in cured meats to 60 mg/kg (as opposed to the maximum EU limit of 150 mg/kg), together with the vast amount of research dedicated at attempting to reduce or partially replace nitrate/nitrite in cured meats portray the importance of the current research topic (Christiansen *et al.*, 1974; Cenci-Goga *et al.*, 2012; Herrmann *et al.*, 2015; Hospital *et al.*, 2015; Mainar *et al.*, 2016). The meat industry is faced with a great challenge as the consumer demand to lower the amount of nitrate/nitrite contradicts the fear of food pathogens which are successfully inhibited by these preservatives (Bedale *et al.*, 2016).

With regards to exploring nitrate/nitrite substitutes, botanical extracts exhibiting antimicrobial and/or antioxidant effects in processed meats have received much attention (Shah *et al.*, 2014; Aziz & Karboune, 2018; Jin *et al.*, 2018; Alirezalu *et al.*, 2019). Honeybush (*Cyclopia subternata* Vogel, Family: Fabaceae; Tribe: Podalyrieae) is a native South African shrub enjoyed as hot brewed tea after fermentation of the stems and leaves (De Beer *et al.*, 2012). This tea has proven to contain a vast number of polyphenolic compounds and exhibits antioxidant capacity in both the fermented and unfermented (green) state (Joubert *et al.*, 2008). The latter has received attention as a potential food extract due to its higher polyphenolic content compared to the former (Joubert *et al.*, 2008, 2019). Honeybush is rich in hesperidin and mangiferin, although an abundant number of polyphenolics contribute to the overall antioxidant capacity (Kamara *et al.*, 2004; De Beer *et al.*, 2009). Recently, Joubert *et al* (2019) published an article summarising a 20-year research milestone in the South African tea industry. This ongoing research partially portrays the potential of honeybush as a pharmaceutical and food extract. In fact, the growing demand in health promoting, antioxidant rich foods gained honeybush its international recognition and economic importance for Southern Africa (Kokotkiewicz *et al.*, 2012). Previous researchers have patented the extraction process of green honeybush extract (De Beer & Joubert, 2002), developed a rapid method for the quantification of polyphenolic content (De Beer & Joubert, 2010), developed a sensory wheel for different *Cyclopia* species (Theron *et al.*, 2014; Alexander *et al.*, 2019) and detected a vast amount of volatiles in honeybush (Le Roux *et al.*, 2012; Ntlhokwe *et al.*, 2018). Apart from the antioxidant

capacity, honeybush, mangiferin and hesperidin have shown antimicrobial, -fungal and -viral properties, although to a variable extend (Coetzee *et al.*, 2008; Singh *et al.*, 2009, 2012; Iranshahi *et al.*, 2015; Dube *et al.*, 2017).

Warthog meat is comparable to other South African game meat and have been proven to be low in total fat (< 2.2%) and high in protein (> 20%) with a percentage carcass yield resembling that of domestic pig carcasses if the head, skin and trotters are taken into consideration (Hoffman & Sales, 2007; Swanepoel *et al.*, 2014, 2016b,a). These free-roaming creatures are considered as agricultural pests in some regions in South Africa, causing crop, fence and endemic field plant destruction (Swanepoel *et al.*, 2016b). The culling of warthogs to mitigate damages may lead to surplus game meat fit for human consumption. In contrast, warthog meat is considered underrepresented in the South African game meat market (Swanepoel *et al.*, 2016c). The production of game salami poses a unique solution to utilise surplus game meat, including less valuable cuts (e.g. the warthog forequarter muscles, *Infraspinatus*, IS, and *Supraspinatus*, SS) to create a value added meat product with an extended shelf life (Swanepoel *et al.*, 2016c). Salami also creates the possibility of all year availability of game meat, as restricted seasonal availability was identified as one market gap in the South African game meat industry (Hoffman & Wiklund, 2006). Furthermore, salting, drying, fermenting and curing can mask potential negatively perceived gamey flavours of fresh warthog meat. The production of salami with warthog meat with a free roaming (i.e. organic) status can be further developed to suit the increasing demand for cleaner labelled foodstuffs, by lowering the amount of nitrate and/or nitrite (Di Vita *et al.*, 2019).

The potential of honeybush extract as a natural antioxidant in processed meat products, together with the potential of warthog meat as primary meat source for salami production remain unexplored. The aim of the present study was to determine the effects of honeybush (*Cyclopia subternata*) extract in warthog salami with reduced added nitrate.

References

Alexander, L., De Beer, D., Muller, M., Van der Rijst, M. & Joubert, E. (2019). Bitter profiling of phenolic fractions of green *Cyclopia genistoides* herbal tea. *Food Chemistry*, **276**, 626–635.

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

Aziz, M. & Karboune, S. (2018). Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, **58**, 486–511.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and

evolving perceptions. *Meat Science*, **120**, 85–92.

Bianchi, F., Cantoni C., Careri, M., Chiesa, L., Musci, M. & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, **72**, 1552-1563.

Bolger, Z., Brunton, N.P., Lyng, J.G. & Monahan, F.J. (2017). Comminuted meat products - Consumption, composition, and approaches to healthier formulations. *Food Reviews International*, **33**, 143–166.

Butler, A. (2015). Nitrites and nitrates in the human diet: Carcinogens or beneficial hypotensive agents? *Journal of Ethnopharmacology*, **167**, 105–107.

Cenci-Goga, B.T., Rossitto, P. V., Sechi, P., Parmegiani, S., Cambiotti, V. & Cullor, J.S. (2012). Effect of selected dairy starter cultures on microbiological, chemical and sensory characteristics of swine and venison (*Dama dama*) nitrite-free dry-cured sausages. *Meat Science*, **90**, 599–606.

Chen, J., Hu, Y., Wen, R., Liu, Q., Chen, Q. & Kong, B. (2019). Effect of NaCl substitutes on the physical, microbial and sensory characteristics of Harbin dry sausage. *Meat Science*, **156**, 205–213.

Christiansen, L.N., Tompkin, R.B., Sharapis, A.B., Kueper, T. V, Johnston, R.W., Kautter, D.A. & Kolari, O.J. (1974). Effect of sodium nitrite on toxin production by *Clostridium botulinum* in bacon. *Applied Microbiology*, **27**, 733–737.

Coetzee, G., Marx, I.J., Pengilly, M., Bushula, V.S., Joubert, E. & Bloom, M. (2008). Effect of rooibos and honeybush tea extracts against *Botrytis cinerea. South African Journal for Enology and Viticulture*, **29**, 33–38.

da Silva, S.L., Lorenzo, J.M., Machado, J.M., Manfio, M., Cichoski, A.J., Fries, L.L.M., Morgano, M.A. & Campagnol, P.C.B. (2020). Application of arginine and histidine to improve the technological and sensory properties of low-fat and low-sodium bologna-type sausages produced with high levels of KCl. *Meat Science*, **159**, 1–5.

De Beer, D., Jerz, G., Joubert, E., Wray, V. & Winterhalter, P. (2009). Isolation of isomangiferin from honeybush (*Cyclopia subternata*) using high-speed counter-current chromatography and high-performance liquid chromatography. *Journal of Chromatography A*, **1216**, 4282–4289.

De Beer, S.W. & Joubert, E. (2002). Preparation of tea-like beverages S.A. paptent number 2002/2802.

De Beer, D. & Joubert, E. (2010). Development of HPLC method for *Cyclopia subternata* phenolic compound analysis and application to other *Cyclopia* spp. *Journal of Food Composition and*

Analysis, **23**, 289–297.

De Beer, D., Schulze, A.E., Joubert, E., De Villiers, A., Malherbe, C.J. & Stander, M.A. (2012). Food ingredient extracts of *Cyclopia subternata* (honeybush): Variation in phenolic composition and antioxidant capacity. *Molecules*, **17**, 14602–14624.

Del Nobile, M.A., Conte, A., Incoronato, A.I., Panza, O., Sevi, A. & Marino, R. (2009). New strategies for reducing the pork back-fat content in typical Italian salami. *Meat Science*, **81**, 263–269.

Di Cagno, R., Lòpez, C.C., Tofalo, R., Gallo, G., De Angelis, M., Paparella, A., Hammes, W.P. & Gobbetti, M. (2008). Comparison of the compositional, microbiological, biochemical and volatile profile characteristics of three Italian PDO fermented sausages. *Meat Science*, **79**, 224–235.

Di Vita, G, Blanc, S., Mancuso, T., Massaglia, S., La Via, G. & D'Amico, M. (2019). Harmful compounds and willingness to buy for reduced-additives salami. An outlook on Italian consumers. *International Journal of Environmental Research and Public Health*, **16**, 1-9.

Dos Santos, B.A., Campagnol, P.C.B., Da Cruz, A.G., Galvão, M.T.E.L., Monteiro, R.A., Wagner, R. & Pollonio, M.A.R. (2015). Check all that apply and free listing to describe the sensory characteristics of low sodium dry fermented sausages: Comparison with trained panel. *Food Research International*, **76**, 725–734.

Dube, P., Meyer, S. & Marnewick, J.L. (2017). Antimicrobial and antioxidant activities of different solvent extracts from fermented and green honeybush (*Cyclopia intermedia*) plant material. *South African Journal of Botany*, **110**, 184–193.

Franciosa, I., Alessandria, V., Dolci, P., Rantsiou, K. & Cocolin, L. (2018). Sausage fermentation and starter cultures in the era of molecular biology methods. *International Journal of Food Microbiology*, **279**, 26–32.

Guerrero, L., Dolors Guà Rdia, M., Xicola, J., Verbeke, W., Vanhonacker, F., Zakowska-Biemans, S., Sajdakowska, M., Sulmont-Rossé, C., Issanchou, S., Contel, M., Scalvedi, M.L., Granli, B.S. & Hersleth, M. (2009). Consumer-driven definition of traditional food products and innovation in traditional foods. A qualitative cross-cultural study. *Appetite*, **52**, 345–354.

Ha, S.K. (2014). Dietary salt intake and hypertension. *Electrolytes and Blood Pressure*, **12**, 7–18.

Hammes, W.P. (2012). Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. *Food Microbiology*, **29**, 151–156.

Herrmann, S.S., Duedahl-Olesen, L., Christensen, T., Olesen, P.T. & Granby, K. (2015). Dietary exposure to volatile and non-volatile *N*-nitrosamines from processed meat products in Denmark. *Food and Chemical Toxicology*, **80**, 137–143.

Hoffman, L.C. & Sales, J. (2007). Physical and chemical quality characteristics of warthog (*Phacochoerus africanus*) meat. *Livestock Research for Rural Development*, **19**.

Hoffman, L.C. & Wiklund, E. (2006). Game and venison - Meat for the modern consumer. *Meat Science*, **74**, 197–208.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M. & Heir, E. (2017). Health and safety considerations of fermented sausages. *Journal of Food Quality*, **2017**, 1–25.

Honikel, K.-O. (2007). Principles of Curing. In: *Handbook of Fermented Meat and Poultry*. Pp. 17–30. Iowa: USA: Blackwell Publishing Professional.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Iranshahi, M., Rezaee, R., Parhiz, H., Roohbakhsh, A. & Soltani, F. (2015). Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin. *Life Sciences*, **137**, 125–132.

Jin, S., Choi, J.S., Yang, H., Park, T. & Yim, D. (2018). Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science*, **146**, 34–40.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. & Gelderblom, W.C.A. (2019). Formal honeybush tea industry reaches 20-year milestone – Progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, **127**, 58–79.

Joubert, E., Richards, E.S., Van der Merwe, J.D, De Beer, D., Manley, M. & Gelderblom, W.C.A. (2008). Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush Tea). *Journal of Agricultural and Food Chemistry*, **56**, 954–963.

Kamara, B.I., Brand, J.D., Brandt, Vincent, E. & Joubert, E. (2004). Phenolic metabolites from honeybush Tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391–5395.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Le Roux, M., Cronje, J.C., Burger, B. V & Joubert, E. (2012). Characterization of volatiles and aroma-active compounds in honeybush (*Cyclopia subternata*) by GC-MS and GC-O Analysis. *Journal of Agricultural and Food Chemistry*, **60**, 2657–2664.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L. & Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: A historical outlook. *Trends in Food Science and Technology*, **31**, 130–137.

Mainar, M.S., Xhaferi, R., Samapundo, S., Devlieghere, F. & Leroy, F. (2016). Opportunities and limitations for the production of safe fermented meats without nitrate and nitrite using an antibacterial *Staphylococcus sciuri* starter culture. *Food Control*, **69**, 267–274.

Majou, D. & Christieans, S. (2018). Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. *Meat Science*, **145**, 273–284.

Marcoz, E.M., Melewar, T.C. & Dennis, C. (2016). The value of region of origin, producer and protected designation of origin label for visitors and locals: The case of Fontina cheese in Italy. *International Journal of Tourism Research*, **18**, 236–250.

Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E. & Gandemer, G. (1999). Volatile compounds of commercial Milano salami. *Meat Science*, **51**, 175–183.

Møller, J.K.S. & Skibsted, L.H. (2002). Nitric oxide and myoglobins. *Chemical Reviews*, **102**, 1167–1178.

Muguerza, E., Ansorena, D., Bloukas, J.G. & Astiasarán, I. (2003). Effect of fat level and partial replacement of pork backfat with olive oil on the lipid oxidation and volatile compounds of Greek dry fermented sausages. *Journal of Food Science*, **68**, 1531–1536.

Ntlhokwe, G., Muller, M., Joubert, E., Tredoux, A.G.J. & De Villiers, A. (2018). Detailed qualitative analysis of honeybush tea (*Cyclopia* spp.) volatiles by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry and relation with sensory data. *Journal of Chromatography A*, **1536**, 137–150.

Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. URL: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02008R1333-20170320&from=ES.

Sebranek, J.G. & Bacus, J.N. (2007). Cured meat products without direct addition of nitrate or nitrite: What are the issues? *Meat Science*, **77**, 136–147.

Shah, M.A., Don Bosco, S.J. & Mir, S.A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, **98**, 21–33.

Sindelar, J.J. & Milkowski, A.L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. In: *American Meat Science Association*. Pp. 1–14.

Singh, S., Dhyani, S.K., Kumar, S.S. & Sharma, S. (2009). Antimicrobial evaluation of mangiferin

analogues. *Indian Journal of Pharmaceutical Sciences*, **71**, 328–331.

Singh, S.K., Tiwari, R.M., Sinha, S.K., Danta, C.C. & Prasad, S.K. (2012). Antimicrobial evaluation of mangiferin and its synthesized analogues. *Asian Pacific Journal of Tropical Biomedicine*, **2**, S884–S887.

Skibsted, L.H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, **24**, 176–183.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2014). The influence of season and sex on the carcass yield of common warthog (*Phacochoerus africanus*). *South African Journal of Wildlife Research*, **44**, 179–188.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2016a). Comparative analyses of the chemical and sensory parameters and consumer preference of a semi-dried smoked meat product (cabanossi) produced with warthog (*Phacochoerus africanus*) and domestic pork meat. *Meat Science*, **114**, 103–113.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2016b). Farmers' perceptions of the extra-limital common warthog in the Northern Cape and free state provinces, South Africa. *Wildlife Society Bulletin*, **40**, 112–121.

Swanepoel, M., Leslie, A.J., Van der Rijst, M. & Hoffman, L.C. (2016c). Physical and chemical characteristics of warthog (*Phacochoerus africanus*) meat. *African Journal of Wildlife Research*, **46**, 103–120.

Taormina, P.J. (2014). Meat and Poultry, Curing of Meat. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 501-507.

Theron, K.A., Muller, M., Van der Rijst, M., Cronje, J.C., Le Roux, M. & Joubert, E. (2014). Sensory profiling of honeybush tea (*Cyclopia* species) and the development of a honeybush sensory wheel. *Food Research International*, **66**, 12–22.

Van der Lans, I.A. & Van Ittersum, K. (2001). The role of the region of origin and EU certificates of origin in consumer evaluation of food products. *European Review of Agricultural Economics*, **28**, 451-477.

Zanardi, E., Ghidini, S., Battaglia, A. & Chizzolini, R. (2004). Lipolysis and lipid oxidation in fermented sausages depending on different processing conditions and different antioxidants. *Meat Science*, **66**, 415–423.

Zong, G., Li, Y., Wanders, A.J., Alssema, M., Zock, P.L., Willett, W.C., Hu, F.B. & Sun, Q. (2016). Intake of individual saturated fatty acids and risk of coronary heart disease in US men and women: Two prospective longitudinal cohort studies. *British Medical Journal*, **355**, 1–11.

CHAPTER 2

Literature review: Salami production, essential ingredients and the potential of warthog (*Phacochoerus africanus*) as primary meat source

Abstract

Dry-cured and fermented sausages (in particular, salami) are part of a fascinating gustatory history. Early production techniques used to prepare traditional salami lead to standardised processing techniques and ingredients and a better comprehension of the intricate processes involved during fermentation and drying. The basic food and meat science, microbiology and biochemistry theory involved in salami production needs to be comprehended before recipe or processing formulations can commence. This literature review focusses on the history of salami production and subsequent modern-day processing, of which the use of functional starter cultures comprises a big part of the latter. The raw ingredients used directly influences salami end product safety and quality. In this regard, the essential ingredients required for salami production are discussed and the potential of warthog meat for game salami is considered based on the literature surrounding the characteristics of the meat derived from this game species.

***Keywords*:** salami; warthog; functional starter culture

2.1 The origin of salami to modern-day delicacies

The evolution of salami production is part of a rich cultural history truly worth appreciating. The word "salami" (salame: singular, salami: plural) is postulated to originate from the Medieval Latin word, "*salumen*", meaning salted stuffs (Leroy *et al.*, 2013), which is closely related to the Italian word, "*sale*", meaning salt. Arguably, the word originated from the ancient town, Salamis, were these sausages were created from early times (Pederson, 1979; Leroy *et al.*, 2013). Similar to the Italian *la cucina povera* philosophy, meaning cooking of the poor or poor kitchen, fermented sausages created the possibility for optimal use of leftover meat and entrails. Fresh meat with a high water activity (a_w), protein and mineral content, fermentable glycogen, and a favourable pH is an optimum growth medium for many microorganisms (Garriga & Aymerich, 2007; Woraprayote *et al.*, 2016; Holck *et al.*, 2017). Ancient, though highly effective preserving techniques, including: salting; drying; fermenting; and smoking (Zeuthen, 2007), originated from the need to simply preserve highly perishable meat, to modern-day delicacies (Holck *et al.*, 2017). Proud and nostalgic European settlers practiced the techniques to produce their traditional foods and familiar tastes, spreading fermented meats to America, Australia and South Africa (Campbell-platt, 1994; Leroy *et al.*, 2013). Modern-day processing techniques with the maintenance of the meat product cold chain is sufficient to supply meat with acceptable microbiological safety and shelf life, essentially eliminating the necessity to ferment raw meat (Ordóñez & De la Hoz, 2007). The current demand for cured, fermented meats is probably largely due to emotional nostalgia associated with ancient family

recipes and techniques, as well as a sense of identity and preference for this acquired taste forming part of a gustatory experience (Bianchi *et al.*, 2007; Leroy *et al.*, 2013; Todorov *et al.*, 2017).

The extremely large variety of fermented sausages makes product classification challenging. Salami can be classified as dry (long ripened), semi-dry (sliceable), or moist (spreadable), (Ockerman & Basu, 2007; Talon & Leroy, 2014). While this classification seems simple, when solely considering semi-dry sausages in Europe one can distinguish between many types within this category, not to mention the vast majority found in other countries classified under the same product category (Incze, 2007). When comparing northern to southern Europe, an extremely large variety of fermented sausages exist as a result of ingredients used and processing steps applied according to region and climatic conditions (Flores, 1997; Demeyer *et al.*, 2000; Polka *et al.*, 2015; Romano *et al.*, 2018). In northern European countries such as Hungary and Germany, a typical fermented sausage is produced with a short ripening time (~3 weeks) and subsequent rapid acidification to reach a final pH < 5 (Demeyer *et al.*, 2000; Lebert *et al.*, 2007a; Holck *et al.*, 2017). These products may contain both pork and beef meat and are microbiologically safe due to a rapid pH decrease, the addition of nitrite (NO_2) salt to the meat batter and the occasional smoking of the outer surface (Demeyer *et al.*, 2000; Leroy *et al.*, 2013; Talon & Leroy, 2014; Holck *et al.*, 2017). The smoking procedure alone can create variety in sausage flavour and colour, depending on the type of wood and smoking parameters used (Flores, 1997). In contrast, southern Mediterranean fermented sausages, including: Italian salami; Spanish chorizo; and French saucisson are produced with mainly pork meat and undergo a long ripening period (~40 days, or up to several months) (Demeyer *et al.*, 2000; Talon & Leroy, 2014; Holck *et al.*, 2017). Furthermore, the acidification occurs gradually, creating a mild acidic product with a final pH > 5 and these products are generally not smoked (Blaiotta *et al.*, 2004; Lebert *et al.*, 2007b; Holck *et al.*, 2017). Shelf life and safety of Mediterranean salami are established mainly by lowering of the a_w (~0.85-0.90) over a longer drying period, as compared to the northern European salami with a higher a_w (~0.92-0.94) (Talon & Leroy, 2014). The extended ripening period in Mediterranean salami is associated with a range of biochemical reactions contributing greatly to the typical appreciated flavour of these salami (Flores, 1997). The outer surfaces are typically coated with desired moulds (i.e. *Penicillium nalgiovense*) and yeasts (i.e. *Debaryomyces hansenii*) rather than smoking, which aids in microbiological protection and creating a unique sensory profile (Leroy *et al.*, 2006; Holck *et al.*, 2017). The much appreciated Mediterranean dry-cured flavour is in fact said to be dependent on the surface inoculum (Flores, 1997). Moreover, the use of nitrate (NO_3) as opposed to its reduced form, nitrite, is preferred in Mediterranean cured products with longer ageing periods (Flores, 1997; Blaiotta *et al.*, 2004). Other than the processing and curing techniques, the difference in pig breeds and rearing conditions across Europe may also influence final product quality traits (Dirinck *et al.*, 1997).

Today, these ancient family recipes and techniques are acknowledged as part of a valuable gastronomic heritage (Guerrero *et al.*, 2009). The European Union (EU) promotes product differentiation on regional level to distinguish, what they refer to as typical products (Marcoz *et al.*, 2016). Regulation EEC No. 2081 from 1992, (No. 1151/2012 currently in force) focusses on the protection of geographical indications and designations of origin for agricultural products and foodstuffs and Regulation EEC No. 2082/92 focusses on product certification (Marcoz *et al.*, 2016). Subsequently, the Directorate-General for Agriculture and Rural Development (DG AGRI) established official product trademarks which are linked with the above-mentioned regulations (Marcoz *et al.*, 2016). Protective Title Ⅱ in the regulation represents the *Protected Designation of Origin* (PDO) and *Protected Geographical Indication* (PGI) or simply, *Geographical Indication*. Title Ⅲ represents *Traditional Specialty Guaranteed* (TSG) and Title Ⅳ, miscellaneous product descriptions i.e. *Mountain Product.* Protected Designation of Origin certified products are the most common (Marcoz *et al.*, 2016) and is associated with specific quality characteristics due to human and environmental interventions linked within a given geographical area. To receive this certification, the entire product must be processed and produced within a defined region. Protected Geographical Indication in contrast, assures a geographical connotation with at least one of the production, processing or preparation steps and differing from PDO and PGI. Although TSG does not link the food product to a specific geographical area, it however, guarantees the application of traditionally used raw ingredients and processing methods to create a specialty product unique to those in the same category (EU, Regulation No. 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs). The large amount of certified products in Europe, especially in Italy (Montanari *et al.*, 2016; Roccato *et al.*, 2017) and the positive correlation between quality association and purchase intent by consumers (Fandos & Flavián, 2006), emphasize the significance of regional products. Many cured, fermented meats in European countries are certified according to one of the above-mentioned trademarks (Blaiotta *et al.*, 2004; Casaburi *et al.*, 2007), as illustrated in Fig. 2.1.

Figure 2.1 From left to right: Protected Designation of Origin; Protected Geographical Indication; and Traditional Speciality Guaranteed trademarks commonly certified to fermented meats in Europe (Adapted from the European Quality Schemes URL: https://ec-europa-eu.ez.sun.ac.za/info/food-farming-fisheries/food-safety-and-quality/certification/qualitylabels/quality-schemes-explained#latest).

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2.2 Salami production

Salami production simply entails the comminution of meat and fat (in a ratio of ~7:3/6:4 meat:fat) with added salt and/or other ingredients (including: sugars; nitrate and/or nitrite salt; starter cultures; herbs; and spices) which are stuffed into natural or artificial casings, fermented and dried (Lücke, 1994; Talon & Leroy, 2014; Holck *et al.*, 2017; Franciosa *et al.*, 2018). The combination of a high sodium chloride concentration, the lowering of the pH during fermentation, the preserving action of nitrate and nitrite salts, the anaerobic environment created through stuffing, and the drying step all contribute to a multiple microbial hurdle effect creating a shelf stable salami (Leroy *et al.*, 2013; Holck *et al.*, 2017). The ingredients used and processing steps applied greatly influence the final product quality (Lücke, 1994). Natural or artificial casings varying in diameter can be used (Djordjevic *et al.*, 2015), which influences the drying rate and final appearance of the product. Natural casings are often preferred, especially in Mediterranean countries, contributing to artisanal sausage aesthetics. In contrast, artificial casings either derived from plant cellulose or animal collagen were developed to better suit industrial stuffing machines and for improved salami uniformity (Djordjevic *et al.*, 2015). Impermeable synthetic polymers cannot be used for the purpose of salami making as it will not allow water migration (Djordjevic *et al.*, 2015). Whether animal intestines or artificial casings are used, the material should have tensile strength and be water, gas and smoke permeable (Djordjevic *et al.*, 2015).

The end product salami is cylindrical in shape, varying based on processing methodology used in weight, length and diameter. Typical Italian salami weighs between 200-2500 g, with a length of 10-50 cm and diameter of 2-10 cm (Demeyer *et al.*, 2000; Romano *et al.*, 2018). When sliced, salami have a characteristic reddish or pinkish colour (Sebranek & Bacus, 2007) with visible fat particles uniformly distributed throughout the product (Romano *et al.*, 2018). Traditional salami based on family recipes and processing techniques have been produced for centuries without following a scientific approach and paved the way for industrial, standardised processing methods (Fernández *et al.*, 2000). An overview of the major differences in traditional and commercially produced fermented sausages are summarized in Table 2.1.

Table 2.1 Major differences between traditional and modern salami production[1]

Traditional salami	Modern-day salami
Spontaneous fermentation by means of a variety of naturally occurring bacteria in variable, unknown quantities	The deliberate addition of selected, predetermined amounts of bacterial strains as functional starter cultures
Nitrate addition in the form of saltpetre or contaminated sodium chloride in unknown quantities	Nitrate or nitrite salts are directly added as preservatives in regulated amounts
Intestinal animal casings used	Synthetic casings used
Open-air drying	Drying in controlled atmosphere chambers
A non-homogenous, artisanal product appearance and dimensions	A more homogenous, standardised appearance and dimensions
Small scale production linked to regional areas	Large scale, commercial productions, not limited to regional areas
At greater risk for microbial contamination	Reduced risk for microbial contamination
More complex or unique sensory profile	A more easily defined and almost uniform sensory profile

[1]Adapted from Leroy *et al.* (2013) and Franciosa *et al.* (2018).

The introduction of modern-day processing aided tremendously in product safety and quality control, like the use of selected starter cultures to ensure sufficient acidification and ripening chambers to decrease the a_w of salami, as demonstrated in Greek fermented sausages (Blaiotta *et al.*, 2004). Traditional processing and spontaneous fermentation are still used, however, the application of modern-day processing increased parallel with the need for product standardization and a shorter production time (Lücke, 1994; Franciosa *et al.*, 2018).

2.2.1 Fermentation and ripening

Based on Adams's simple classification of fermented foods, salami is a true fermented product wherein the growth of microorganisms and the subsequent metabolic products are vital (Adams, 1986). In biochemical terminology, fermentation is defined as an anaerobic microbial process involving oxidation-reduction reactions to yield adenine triphosphate (ATP) as energy source, mainly through substrate level phosphorylation, as opposed to the electron transport chain (Müller, 2001). Fermentation and ripening (the latter is also referred to as maturing, ageing or drying) are often mentioned together as they are closely associated, however, they can be separated according to desired biochemical and microbial changes occurring in each period, respectively (Demeyer *et*

al., 2000). In summary, the fermentation period can be mainly defined by a pH decline and nitrate reduction to nitrite, as opposed to the ripening period which entails salami texture and flavour development to a great extend (Ordóñez *et al.*, 1999; Fernández *et al.*, 2000). After an initial decrease in the pH of salami during fermentation (which commences just after stuffing), a slight increase in pH is expected during the ripening period, which can last weeks to months (Comi *et al.*, 2005; Petäjä-Kanninen & Puolanne, 2007; Tabanelli *et al.*, 2012). In some countries, the duration of the ripening period determines whether nitrate or nitrite salt is preferred and is often used as point of difference between Mediterranean and northern European fermented sausages, where an extended ripening period and the use of nitrate salt at lower temperatures are associated with the former (Flores, 1997; Blaiotta *et al.*, 2004). Ripening is often tedious, contributing to high costs of fermented foodstuffs, however, accelerating this process with the use of higher environmental temperatures may result in sub-optimal quality or microbiological safety issues (Fernández *et al.*, 2000).

The first days after salami are stuffed into casings are crucial from a food safety point of view (Hospital *et al.*, 2015). The a_w is still fairly high, the pH is suitable for pathogen growth, the salt concentration has not yet increased and the favourable starter culture bacteria may not yet be dominant. After stuffing, salami visually loose water through drip loss, thereafter water must effectively move from the salami centre to the surface to evaporate. This evaporation process should occur gradually to prevent the unwanted phenomenon known as case hardening (Olivares *et al.*, 2010). The latter results in an outer crust formation which ultimately delays water diffusion from the salami centre to the surface, with a possible pathogen risk as the inner a_w remains high (Andrés *et al.*, 2007). This diffusion process is driven by appropriate air conditioning (temperature, relative humidity (RH) and airspeed) within ripening chambers (Rizzi, 2003). For instance, high speed dry air (low RH) will accelerate salami water loss (Rizzi, 2003). Higher drying temperatures may prevent case hardening by promoting diffusion of tightly bound water molecules from the salami centre, but in turn could also promote unwanted microbial growth (Andrés *et al.*, 2007). The extent of drying (and lowering of the a_w) not only plays a pivotal food safety role, but also influences the textural properties of the final product salami such as hardness, chewiness and cutting resistance (Tabanelli *et al.*, 2012). The environmental conditions during fermentation and ripening may vary greatly depending on the product type (Rantsiou *et al.*, 2005). Numerous factors influence the choice of environmental parameters during fermentation and ripening, including (but not limited to): the quality of the raw meat; the type of salami (slow, medium or fast ripening); the dimensions of the product; the type of casings used; the meat:fat ratio; particle size of the meat and fat; and the salt concentration (E Novelli 2018, personal communication, 18 June). As it is difficult to completely control all of the above-mentioned factors, the salami producer should ideally be involved during the ripening process to inspect the salami visually and by touch for an indication of the product's

internal environment. Physical-chemical changes during ripening can alternatively be measured to guide in decision making regarding adjustment of the environmental parameters, including the pH, a_w, weight loss (%) and firmness (Lücke, 1994).

Salami fermentation is a rather complex phenomenon dependent on intrinsic product and extrinsic environmental parameters during which a range of interlinked biochemical and physical changes occur (Leroy *et al.*, 2013). To elaborate on the previously mentioned summary of salami fermentation, the process entails the following: lactic acid bacteria becomes the dominating microflora causing the matrix pH to decline due to organic acid (mainly lactic acid) production; subsequently, dehydration is promoted as the pH decreases and lowering of the matrix pH promotes solubilization of actin-myosin and sarcoplasmic proteins that contribute to the typical salami texture. Simultaneously, nitrate is enzymatically reduced to nitrite and nitric oxide is formed in the aforementioned acidic environment. Subsequently, nitric oxide causes the formation of the nitrosomyoglobin colour pigment responsible for the typical salami colour (Fernández *et al.*, 2000; Casaburi *et al.*, 2007). Although lowering of the pH is probably the most commonly associated result of salami fermentation, it involves far more than just that.

Irrespective of their fundamental acidification role in salami production, lactic acid bacteria merely outcompete other microorganisms in the meat environment. When acknowledging this, one cannot solely focus on lactic acid production as a result of meat fermentation, but should rather evaluate the process holistically to understand how the proliferation of lactic acid bacteria and their metabolic reactions greatly influences the sensory and texture characteristics of salami. The antagonistic effects of lactic acid bacteria, including lactate and bacteriocin production to outcompete acid sensitive and closely related competitors, are not only to their advantage, but also to the advantage of the salami producer, as pathogenic growth is inhibited while the a_w of salami is still high (Petäjä-Kanninen & Puolanne, 2007). The pH reduction additionally promotes water loss in salami in two ways: firstly, the denaturation of protein leads to water secretion; and secondly, actin-myosin proteins reach their iso-electric point (pH of ~5.3-5.5) resulting in an overall zero net charge decreasing their ability to bind water (Miralles *et al.*, 1996; Flores, 1997; Warriss, 2010; Stajić *et al.*, 2014). Protein coagulation subsequently contributes to a characteristic salami texture (Flores, 1997; Ravyts *et al.*, 2012). Furthermore, the secretion of extracellular lactic acid bacteria enzymes as an additional energy source promotes protein and lipid breakdown (Petäjä-Kanninen & Puolanne, 2007), creating a vast number of volatile compounds contributing to the sensory quality of the final product, salami.

The fermentation process is crucial to decrease the initial pH of raw meat of ~5.5-5.7, depending on the meat species used (Warriss, 2010), down to ~4.2-5.5, depending on the type of salami (Petäjä-Kanninen & Puolanne, 2007), but generally close to the iso-electric point of the myofibril meat proteins (Hierro *et al.*, 1997). During fermentation a higher RH (90-95%) is

maintained followed by a decrease during ripening (70-80%), to create a a_w gradient between the wet salami and the environment to promote drying as seen in the methodology followed by numerous researchers (Flores, 1997; Samelis *et al.*, 1998; Meynier *et al.*, 1999; Andrés *et al.*, 2007; Ravyts *et al.*, 2012; Coloretti *et al.*, 2014; Hospital *et al.*, 2015). Relative humidity of 75% can be used as a benchmark to distinguish dry from wet air, where anything above is considered a wet environment which will slow down salami water loss (E Novelli 2018, personal communication, 18 June). Salami fermentation can last for up to one week at lower temperatures (10-11°C) or can be accelerated (1-2 days) at higher temperatures (18-22°C, Lebert *et al.*, 2007a), indicating that the acidifying rate is dependent on the environmental temperature. Although higher temperatures may reduce the fermentation time, it may also favour the growth of unwanted pathogens (Lücke, 1994). The fermentation temperature is often controlled to balance the growth of wanted bacteria, with the inhibition of unwanted bacteria. In contrast, the amount of acid production (as opposed to the rate of acidification) primarily depends on the initial quantity of available fermentable nutrients (predominantly sugars) in the meat batter (Lücke, 1994; Holck *et al.*, 2017). The rate of drying (and thus reduction in a_w) and surface colonisation of moulds are also important factors which influence the extent of acidification. A low a_w may eventually also inhibit acid production by lactic acid bacteria and moulds are able to utilise lactic acid, counteracting the acidification process (Lücke, 1994). When the a_w decreases to ≤ 0.92 during ripening, the acid production may become insignificant. By this point (a_w ≤ 0.92) in the ripening period, the internal salami pH should have decreased to ≤ 5.3 to ensure microbial safety (Lücke, 1994). Apart from the environmental conditions inside the drying chamber, even the salami dimensions (especially the diameter) also influence drying and subsequently, acidification. Salami with a greater diameter naturally loses water more gradually than a smaller diameter salami under the same environmental conditions. Finely ground meat and fat particles stuffed in larger diameter casings may reach a lower final pH than coarsely ground salami with a smaller casing diameter, ascribed to the rate of water loss and oxygen availability influencing the growth of lactic acid bacteria (Lücke, 1994).

During ripening, intricate lipolysis and proteolysis reactions from endogenous meat enzymes and arguably to a lesser extent, from microbial exoenzymes, also known as extracellular enzymes (such as lipase, proteinase, peptidase, aminopeptidase and esterase), contribute greatly to salami texture and flavour (Montel *et al.*, 1996, 1998; Hierro *et al.*, 1997; Casaburi *et al.*, 2007; Pasini *et al.*, 2018). The fermentation of carbohydrates and hydrolysation of proteins and lipids (yielding free amino acids and free fatty acids, respectively) are both prerequisites for salami flavour development in the form of volatile (i.e. alcohols, ketones and aldehydes) and non-volatile (i.e. amino acids, nucleotides and sugars) compounds (Montel *et al.*, 1998; Champomier-Vergès *et al.*, 2001; Rantsiou & Cocolin, 2006; López *et al.*, 2015). It is generally believed that endogenous meat proteases (i.e. calpains and cathepsins) and lipase are predominantly responsible for the

hydrolysation of sarcoplasmic proteins, actin-myosin (myofibril) proteins and lipids during ripening (Montel *et al.*, 1996; Hierro *et al.*, 1997; Toldrá, 1998; Sanz *et al.*, 1999; Vestergaard *et al.*, 2000a; Zanardi *et al.*, 2004). In contrast, previous researchers reported a weak endogenous meat protease activity during salami ripening (Casaburi *et al.*, 2007). More recent research prompted the re-evaluation of the importance of microbial contribution to proteolysis and lipolysis in fermented meats and highlighted not to neglect the effect of starter cultures on fermented meat flavour (Chen *et al.*, 2017; Gallego *et al.*, 2018), nonetheless, there are still discrepancies surrounding this argument in literature (López *et al.*, 2015; Xu *et al.*, 2019). Manipulating fermentation and drying according to the desired final salami product is an extremely challenging task, often only mastered through experience. Apart from the raw materials, the producer has a great role in controlling the environmental conditions over time, ultimately determining the safety and quality of the final salami product.

2.3 Functional starter cultures

2.3.1 Spontaneous fermentation

Early traditional salami were spontaneously fermented with the producer dependent on wild-type acidifying bacteria originating from the raw meat, processing environment and equipment (Leroy & De Vuyst, 2004; Talon *et al.*, 2007; Baka *et al.*, 2011). This process was gradually refined by inoculating fresh salami batter with previously successfully fermented meat resulting in proliferation of well adapted lactic acid bacteria strains, a technique referred to as backslopping (Holley *et al.*, 1998; Leroy & De Vuyst, 2004; Petäjä-Kanninen & Puolanne, 2007). Spontaneous fermentation with naturally selected, highly competitive microflora can create a market advantage for the artisanal salami producer, as it is able to create salami with unique flavours not easily reproduced with commercial starter cultures often lacking in genetic diversity (Samelis *et al.*, 1994; Comi *et al.*, 2005; Leroy *et al.*, 2006; Ravyts *et al.*, 2012; Franciosa *et al.*, 2018). The quality contribution of these so called, "wild-type" strains substantiates the importance of geographical indication to distinguish niche, small scale produced salami directly related to the salami microflora (Leroy & De Vuyst, 2004).

Unfortunately, spontaneous fermentation is less controlled, posing the risk of inconsistent salami safety and quality (Ockerman & Basu, 2007; Wang *et al.*, 2015), making this method less practical for large-scale, commercial salami production. Subsequently, salami starter cultures were developed to aid in product standardisation, homogeneity, safety and quality (Rantsiou *et al.*, 2005; Wang *et al.*, 2015). The use of salami starter cultures furthermore reduce production costs, shortens the fermentation time and ensures that an adequate amount of added nitrate is reduced to nitrite though nitrate reducing bacteria (Lücke, 2000; Cocolin *et al.*, 2001; Hammes, 2012). When applying salami starter cultures, a predetermined sufficient concentration of colony forming units (CFU) per

kg of meat can be introduced as opposed to relying on fluctuating concentrations of inherit meat and/or environmental lactic acid producing and nitrate reducing bacteria (Ockerman & Basu, 2007).

The microflora of spontaneously fermented sausages (also referred to as autochthonous starters) are often studied to better understand the characteristics of naturally high competitive bacterial strains to apply them commercially (Lücke, 1994; Samelis *et al.*, 1998; Casaburi *et al.*, 2007; Baka *et al.*, 2011*;* Casquete *et al.*, 2011; Franciosa *et al.*, 2018). In this regard, traditional (culture dependent) or molecular (culture independent) methodologies are used, where the latter provides a more in depth understanding of the genetic makeup of the naturally occurring microbial ecosystem in less time (i.e. DNA amplification of the RNA 16S gene with the polymerase chain reaction and denaturing gradient gel electrophoreses, PCR-DGGE) (Cocolin *et al.*, 2001; Rantsiou & Cocolin, 2006; Talon & Leroy, 2014; Polka *et al.*, 2015; Stavropoulou *et al.*, 2018).

Different strains from the same bacterial species may portray different metabolic activities, as seen from two *Staphylococcus xylosus* strains isolated from spontaneously fermented salami (Casaburi *et al.*, 2007). This clarifies why artisanal sausages, rich in a variety of bacterial strains, are often of superior quality (Blaiotta *et al.*, 2004). On the contrary, strain dependent genetic variety of autochthonous starters may also cause unsatisfactory fermentation results, including the presence of harmful biogenic amines (ascribed to decarboxylase activity) or hydrogen peroxide (H_2O_2) production (Tabanelli *et al.*, 2012; Talon & Leroy, 2014). Literature emphasises to predetermine the robustness (or competitiveness) and absence of unwanted genetic traits (i.e. decarboxylase activity, toxin production or antibiotic resistance) of autochthonous bacteria strains before applying as a starter culture strain. The adaptability and proliferation of these strains are not guaranteed, especially when applied in various meat sources or extrinsic environmental conditions (e.g. change in temperature) (De Vuyst, 2000; Leroy *et al.*, 2006; Talon & Leroy, 2014; Todorov *et al.*, 2017).

Lactic acid bacteria have repeatedly been reported as the dominant group during fermentation and ripening of both spontaneously fermented and starter culture added salami, with *Lactobacillus sakei* (previously known as *L. sake*), *L. curvatus* and *L. plantarum* as the dominant species ascribed to a natural high competitiveness, exceptional adaptability and robustness (Samelis *et al.*, 1994, 1998; Coppola *et al.*, 2000; Papamanoli *et al.*, 2003; Comi *et al.*, 2005; Rantsiou *et al.*, 2005; Talon & Leroy, 2014; Aquilanti *et al.*, 2016; Castellano *et al.*, 2017; Cardinali *et al.*, 2018). Other lactic acid bacteria (although to a lesser extent) are also isolated from salami, including *Pediococcus* spp. and *Enterococcus* spp. (Samelis *et al.*, 1998; Papamanoli *et al.*, 2003; Aquilanti *et al.*, 2016). *Pediococci* are not as commonly isolated from naturally fermented European sausages, insinuating that these strains may be poor adapted competitors (Samelis *et al.*, 1998). They are more often associated with commercial starter cultures applied for accelerated acidification at higher fermentation temperatures (up to ~40°C), especially in American type fermented sausages (Leory *et al.*, 2006;

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Talon & Leroy, 2014). Forming part of the lactic acid bacteria group, *Enterococci* are occasionally detected in fermented sausages (more often in Mediterranean products; Leroy *et al.*, 2006) and have been reported throughout the fermentation and ripening period of spontaneously fermented sausages, reaching counts of >10^5 CFU/g (Samelis *et al.*, 1998).

Following the lactic acid bacteria group, the Gram-positive catalase-positive cocci (GCC+), specifically coagulase negative *staphylococci* (CNS), are the second most abundant bacterial group isolated from a variety of salami, followed by yeasts and moulds (Aymerich *et al.*, 2003; Comi *et al.*, 2005; Talon & Leroy, 2014).

2.3.2 Lactic acid bacteria

Functional starter cultures refer to selected microbial strains with inherent properties contributing to food safety and quality, including: organoleptic; nutritional; technical; health promoting traits; without secreting harmful substances; or promoting unwanted biochemical processes (Lücke, 2000; Leroy & De Vuyst, 2004; Leroy *et al.*, 2006). Regulating bodies only allow the addition of microorganisms as part of starter cultures if they are generally recognised as safe (GRAS) or in other terms, certified as qualified presumption of safety (QPS) (Franciosa *et al.*, 2018). Lactic acid bacteria have been granted a GRAS status ascribed to their natural presence in fermented foods and application in foodstuffs over centuries (Castellano *et al.*, 2017). The first functional starter cultures were applied in the early 1920-1940s in the United States (Zeuthen, 2007). The need to industrially standardise fermented sausage characteristics and shorten fermentation to achieve desired sensory traits in less time, promoted the application of these microorganisms in salami (Lücke, 2000; Zeuthen, 2007). Functional salami starter cultures should effectively limit the growth of unwanted bacteria (i.e. *Enterobacteriaceae*), which are inevitably present to some extent in the raw materials and should out compete potentially dangerous moulds (Casaburi *et al.*, 2007; Talon & Leroy, 2014). Reflecting the microflora of spontaneously fermented sausages, the two most important bacterial groups in modern-day functional salami starter cultures are a variety of lactic acid bacteria and coagulase negative *Staphylococci,* where the former predominantly consists of *Lactobacillus spp.* (i.e. *L. sakei, L. curvatus* and *L. plantarum*) due to their naturally high competitiveness (Ammor & Mayo, 2007; Bedia *et al.*, 2011; Talon & Leroy, 2014; Aquilanti *et al.*, 2016; Castellano *et al.*, 2017; Franciosa *et al*., 2018). This competitive trait is pivotal, as possible ill adapted (low acidifying) naturally occurring lactic acid bacteria strains may not always be superior to ensure a sufficient pH reduction and should be outcompeted together with other unwanted bacteria (Casaburi *et al.*, 2007). Lactic acid bacteria are naturally present in fresh meat in the range of 10^2-10^4 CFU/g under good hygienic practices and will multiply under favourable conditions typically associated with intrinsic salami parameters (anaerobic, high salt, nitrate/nitrite added environment) (Rantsiou & Cocolin, 2006; Ravyts *et al.*, 2012). Lactic acid bacteria are commonly added in the order of 10^6 CFU/g to increase

up to 10^8 CFU/g if not injured during harvesting or freeze drying (Lücke, 1994; Champomier-Vergès *et al.*, 2001; Talon & Leroy, 2014).

Lactic acid bacteria are grouped according to their sugar metabolism as strict homofermentative, strict heterofermentative, or facultative heterofermentative (Ravyts *et al.*, 2012). The first mentioned involves the metabolization of simple six carbon sugars (hexoses) through the glycolytic or Embden-Meyerhof-Parnas (EMP) pathway, yielding predominantly lactic acid. Strict heterofermentation involves the phosphoketolase pathway by metabolising both hexoses and five carbon sugars (pentoses), yielding lactate, ethanol and/or acetate. Facultative heterofermentation is a combination of hexoses and pentoses metabolism through the glycolysis and phosphoketolase pathway respectively, yielding lactic acid, carbon dioxide (CO_2), ethanol, acetic acid and acetaldehyde (Ravyts *et al.*, 2012). In this regard, sugar metabolism is an important factor to consider before applying lactic acid bacteria strains as salami starter cultures, as certain metabolic products (i.e. acetic acid associated with a sharp acidity or CO_2 disrupting gelling of the salami matrix) may not be desired (De Vuyst, 2000). In the case of applying a homofermentative lactic acid bacteria starter culture, pyruvate may still lead to the formation of many other compounds, including: acetate; ethanol; diacetyl; and acetaldehyde, all contributing to characteristic flavours (Leroy & De Vuyst, 2004).

Organic acid production and subsequent lowering of the matrix pH is the main, but not the only preserving technique achieved by utilising lactic acid bacteria (Ammor & Mayo, 2007; Castellano *et al.*, 2017). These bacteria also have the ability to produce a variety of antimicrobial metabolites, including: a number of bacteriocins; H_2O_2; reuterin; reutericyclin; diacetyl; 3-hydroxy fatty acids; propionate; phenyl-lactate; hydroxyphenyl-lactate; and 3-hydroxy fatty acids (Castellano *et al.*, 2017). Bacteriocins are proteins (or smaller peptides) synthesised in the ribosomes of bacteria, which kill or inhibit the growth of other closely related bacterial strains (Woraprayote *et al.*, 2016). The functionality and solubility of secreted bacteriocins are pH dependent, therefore, the acidity of the salami matrix will influence the activity of these compounds (Leroy & De Vuyst, 2000). Binding of bacteriocins to meat and fat particles and protease could furthermore demolish their activity (Talon & Leroy, 2014). Nonetheless, literature reports the inhibitory action of lactic acid bacteria bacteriocins against *L. monocytogenes* (Lücke, 2000), including those secreted by *L. sakei* CTC 494 (Ravyts *et al.*, 2008), *P. pentosaceus* BCC 3772 (Kingcha *et al.*, 2012) and *E. faecium S-32-81* (Ananou *et al.*, 2005). The potential of lactic acid bacteria bacteriocins to inhibit *Listeria monocytogenes* is especially advantageous, as this ubiquitous pathogen can proliferate at refrigerated temperatures and survive in acidic (pH < 5.0) and high salt (10%) environments (Papamanoli *et al.*, 2003), typically associated with salami. However, the reproducibility of the anti-listerial bacteriocins are questionable when salami formulation, processing steps and endogenous microflora are adapted (Hugas *et al.*, 2003). Although H_2O_2 has a strong antimicrobial activity,

excessive production may lead to sensory defects, including: high amounts of aldehydes linked to rancidity due to accelerated lipid and protein oxidation; or discolouration due to H_2O_2 reaction with nitrosomyoglobin (Ammor & Mayo, 2007; Talon & Leroy, 2014). Deteriorative effects of H_2O_2 can be counteracted by introducing catalase positive strains able to facilitate the breakdown of H_2O_2, or by avoiding the use of lactic acid bacteria strains known for excessive H_2O_2 production (Ammor & Mayo, 2007).

As reviewed by other researchers, lactic acid bacteria seem to exhibit a weaker proteolytic activity towards myofibril proteins as compared to sarcoplasmic proteins (Rantsiou & Cocolin, 2006; Ammor & Mayo, 2007). This bacterial group most likely contribute to salami flavour mainly through carbohydrate fermentation and subsequent acid production, although some strains (such as *L. sakei, L. curvatus* and *L. plantarum*) have shown proteolytic and lipolytic activity (Papamanoli *et al.*, 2003; Leroy *et al.*, 2006; Todorov *et al.*, 2017).

2.3.3 Enterococci

Enterococci are related to lactic acid bacteria and classified as Gram-positive, catalase-negative diplococci (grouped in pairs) (Foulquié Moreno *et al.*, 2006; Lorenzo *et al.*, 2010; Jahan *et al.*, 2015). Although ubiquitous, they are primarily associated with the human and animal gastrointestinal tract (GIT), (Giraffa, 2002). They are quite robust, with the majority of strains growing over a wide temperature range (10-45°C), in a high bile (40%), high salt (6.5% NaCl) and high pH (~9.6) environment with the ability to survive heat exposure (60°C for 30 min), (Sherman, 1937; Franz *et al.*, 2003; Foulquié Moreno *et al.*, 2006). Their competitiveness and robustness lead to the application of enterococci as part of probiotic strains and starter cultures, able to survive and compete in the human GIT and fermented products known for harsh intrinsic environments (high in salt, but low in a_w and pH), (Franz *et al.*, 1999; Lund *et al.*, 2002; Foulquié Moreno *et al.*, 2006). Their food application seems primarily oriented towards the dairy industry, playing a role in flavour and aroma development of ripened cheeses most likely through lipolysis, proteolysis and citrate breakdown (Sarantinopoulos *et al.*, 2001; Giraffa, 2003; Foulquié Moreno *et al.*, 2006). High numbers of enterococci (predominantly *E. faecium* and *E. faecalis*) have been isolated from ripened cheese, reaching counts up to 10^7 CFU/g (Franz *et al.*, 1999; Foulquié Moreno *et al.*, 2006).

Bacteriocins produced by enterococci, known as enterocins, have proven to be inhibitory against *Listeria* spp. and *Clostridium* spp., luring attention towards the application of these strains, especially in fermented meat products (Tarelli *et al.*, 1994; Giraffa, 1995; Franz *et al.*, 1999; Aymerich *et al.*, 2000a,b; Callewaert *et al.*, 2000; Poeta *et al.*, 2007). The effects of different stress exposures on *E. faecium* (RZS C5) growth and bacteriocin production were investigated and furthermore promoted the robustness of this microorganism exhibiting continuous growth and bacteriocin production in adverse environmental conditions, such as a high pH (6.0-7.5) (Giraffa, 1995; Aymerich *et al.*, 2000a; Callewaert *et al.*, 2000; Leroy & De Vuyst, 2002; Leroy *et al.*, 2003).

On the contrary, the presence of enterococci in processed meats, especially when heat treated, is acknowledged as a spoilage problem (Hugas *et al.*, 2003; Foulquié Moreno *et al.*, 2006; Franz *et al.*, 2011) and their ability to form biofilms in food processing areas are worrisome (Fernandes *et al.*, 2015). The deliberate use of enterococci as part of food starter cultures became a controversial topic due to their arising importance in clinical microbiology, potential antibiotic resistance and inheritance of virulence genes (Franz *et al.*, 1999; Giraffa, 2002; Jahan & Holley, 2014). Although some strains are safer than others (Franz *et al.*, 2011), many studies have demonstrated the multidrug resistance of the enterococci of husbandry and food related origin, in particular of *E. faecium* and *E. faecalis* strains (Jahan *et al.*, 2013; Pesavento *et al.*, 2014; Soares-Santos *et al.*, 2015; Guerrero-Ramos *et al.*, 2016). Furthermore, the ability to transfer these resistant genes to clinical strains have been proven (Jahan *et al.*, 2015). Of these previous studies, the evidence of vancomycin-resistant *E. faecium* from husbandry, meat and clinical origin (Klare *et al.*, 1995; López *et al.*, 2009) were the greatest warning signs, as this antibiotic is used as a last reserve in treating infections in some cases (Wegener *et al.*, 1999). The cumbersome ability of enterococci to exchange genes could impose higher antibiotic resistance in the future, including manifestation in the human intestinal pathogens when exposed to antibiotic resistant genes via enterococci from the food chain (Giraffa, 2002). Another risk factor to consider when applying enterococci strains as part of a starter culture mixture, is the presence of decarboxylase activity, as certain strains could lead to biogenic amine accumulation in salami (Bover-Cid *et al.*, 2001; Sarantinopoulos *et al.*, 2001; Lebert *et al.*, 2007a; Komprda *et al.*, 2010).

The application of robust enterococci in starter cultures and probiotics appeared useful in previous studies, however, their usefulness is now questionable (Franz *et al.*, 1999, 2003, 2011). Enterococci can easily be introduced in salami through the food processing environment or raw materials, especially animal carcasses (Franz *et al.*, 1999; Talon *et al.*, 2007). Their ability to multiply during fermentation and to survive the adverse environment created in dry-cured and fermented sausages explain the frequent isolation of enterococci in final product hams and salami (Samelis *et al.*, 1998; Giraffa, 2002, 2003; Hugas *et al.*, 2003; Martin *et al.*, 2005; Lebert *et al.*, 2007a; Lorenzo *et al.*, 2010; Jahan *et al.*, 2013). Although they are highly competitive and most likely capable of outcompeting other pathogenic strains, they are not necessarily wanted in salami due to reasons elaborated above. Due to their association with animal and human intestines and resistance to adverse environmental conditions such as a high temperature exposure, these microorganisms are often used as sanitary indicators (Franz *et al.*, 1999; Giraffa, 2002). On the other hand, if carefully selected enterococci strains free from unwanted genetic traits are applied in fermented sausages, they could contribute uniquely to the product quality (Martin *et al.*, 2005) and food safety, especially through antilisterial bacteriocin production as demonstrated in a Spanish-style dry fermented sausage (Callewaert *et al.*, 2000).

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2.3.4 Coagulase negative staphylococci (CNS) and micrococci

Starter cultures can consist purely out of lactic acid bacteria, however, the final product salami might be predominantly acidic with little flavour depth not favoured by many consumers and have a limited shelf life due to rancidity (Lücke, 1994). Non-pathogenic CNS, recently classified as part of the *Staphylococcacea* family, (Gherardi & Di Bonaventura, 2018) and *micrococci* spp. (part of the *Micrococcaceae* family) are acknowledged for their contribution to salami flavour via protein and lipid breakdown. These bacteria also supress the onset of rancidity through enzymatic reduction of nitrate to nitrite, a process elaborated on later in this literature review study (Ordóñez *et al.*, 1999; Casaburi *et al.*, 2005; Ravyts *et al.*, 2012; Montanari *et al.*, 2018). Perhaps their most important function is their ability to impart the stable, pink-red nitrosylmyoglobin/nitrosomyoglobin ($MbFe^{2+}NO$) colour complex in processed meats containing nitrate as opposed to nitrite (Blaiotta *et al.*, 2004; Mauriello *et al.*, 2004; Gøtterup *et al.*, 2008). However, the nitrate reduction ability is strain dependant and should be predetermined before applying strains as part of cultures (Mainar & Leroy, 2015).

The staphylococcus genus is categorised according to strains which have the ability to coagulate rabbit plasma (coagulase-positive) and those which are unable to do so (coagulase-negative) (Rupp & Fey, 2015). In this regard, coagulase positive *Staphylococcus aureus* is associated with food poisoning as a result of enterotoxin formation (Talon & Leroy, 2011). To identify both species and strain in the CNS group is challenging, but worth studying, as genetic differences amongst strains may reveal the key to distinguishable, highly favoured salami sensory profiles linked to PDO and PGI certification (Blaiotta *et al.*, 2004). This is evidently seen in different salami volatile profiles associated with different staphylococcal strains, for instance *S. xylosus* produced more 2-butanone, acetoin and diacetyl, as a result of amino acid degradation, compared to *S. carnosus* (Søndergaard & Stahnke, 2002). Different strains may also impart a different reddening effect due to their inherit ability to reduce nitrate (Gøtterup *et al.*, 2008). Furthermore, the absence of staphylococcal enterotoxin gene variants need to be confirmed before applying these strains in salami starter cultures (Talon & Leroy, 2011).

The most prevalent CNS species in spontaneously fermented sausages are *S. xylosus*, *S. equorum*, *S. saprophyticus* and *S. carnosus* (Papamanoli *et al.*, 2002; Mauriello *et al.*, 2004; Pisacane *et al.*, 2015). Although, a number of other species (i.e. *S. epidermidis*, *S. haemolyticus* and *S. warneri*) have been isolated depending on the environmental conditions (temperature and pH fluctuation) during fermentation and ripening (Stavropoulou *et al.*, 2018). Of the most prevalent species, the first mentioned (*S. xylosus*) is commonly associated with mildly acidic Mediterranean salami with a more rounded aroma (Samelis *et al.*, 1998), including: Italian sausages (Coppola *et al.*, 1997; Cocolin *et al.*, 2001; Rossi *et al.*, 2001; Blaiotta *et al.*, 2004; Mauriello *et al.*, 2004); Spanish chorizos (García-Varona *et al.*, 2000); and Iberian dry-cured sausages (Martín *et al.*, 2007).

In addition to the nitrate reducing activity of micrococcus and staphylococcus strains, their catalase (breaks down H$_2$O$_2$) and superoxide dismutase (breaks down peroxide radicals) activities inhibit oxidation and prevent the formation of unwanted grey-brown appearances and off flavours in salami (Hammes & Knauf, 1994; Miralles *et al.*, 1996; Barrière *et al.*, 2001). Apart from suppressing rancid flavours, different CNS strains contribute uniquely to salami volatile profiles according to their amino acid metabolism and lipid ß-oxidation properties (Montel *et al.*, 1996; Søndergaard & Stahnke, 2002; Marco *et al.*, 2008).

Rapid and extensive acidification by means of lactic acid bacteria may inhibit *Micrococcaceae* and/or *Staphylococcacea* proliferation, compromising on the successful reduction of nitrate to its active counterpart, nitrite (Samelis *et al.*, 1998; Ordóñez *et al.*, 1999; Marco *et al.*, 2008). An environmental pH of 5.4-6.0 is favourable for nitrate reductase activity (Flores, 1997).

2.3.5 Moulds and yeasts

Salami surface inoculations (reflecting spontaneously fermented salami) predominantly consist of *Penicillium nalgiovense* mould and *Debaryomyces hansenii* yeast, although numerous other species have been isolated and the dominant yeast strains may vary during the ripening period (Lücke, 2000; Cocolin *et al.*, 2006; Flores *et al.*, 2015; Delgado *et al.*, 2019; Vila *et al.*, 2019). Moulds and yeasts can be applied by spraying or the product can be submerged into a suspension thereof (Flores, 1997).

Mould ripened sausages are particularly popular in Mediterranean countries (Leroy *et al.*, 2006). Mould growth on salami is encouraged to impart wanted characteristics, including: sensory traits (taste, smell and appearance); protection against oxygen and light (Lücke, 2000; Moore, 2004; Leroy *et al.*, 2006); the prevention of harmful mycotoxins produced by unwanted moulds (Delgado *et al.*, 2019); to aid in gradual water release during ripening creating a final product salami with an easy-peel casing (Grazia *et al.*, 1986; Leroy *et al.*, 2006); and to retard lipid oxidation (Bruna *et al.*, 2001a,b).

As with starter culture bacteria, caution should be taken before applying moulds and yeasts. White-grey to yellow moulds free from mycotoxin and antibiotic production are desirable (Lücke, 2000; López-Díaz *et al.*, 2001; Canel *et al.*, 2013). In contrast, black mould growth (i.e. *Cladosporium oxysporum*) is unwanted (Lozano-Ojalvo *et al.*, 2015) and can be inhibited by the smoking of salami (Asefa *et al.*, 2009).

The proteolytic and lipolytic action of moulds and yeasts greatly contribute to the final product salami flavour (Bruna *et al.*, 2001b,a; Sunesen & Stahnke, 2003; Flores *et al.*, 2015). The proteolytic action is especially what often gives ripening chambers a strong ammoniac smell as a result of oxidative deamination of amino acids to ammonia (Flores, 1997). In this regard, mould growth may bring about a rise in the pH of salami towards the end of ripening (sometimes leading to a final pH

higher compared to the initial raw meat pH) due to ammonia production and lactic acid metabolization (Grazia *et al.*, 1986; Sunesen & Stahnke, 2003). Furthermore, yeasts may exhibit an acid neutralising effect through enzymatic expression (glutaminase) responsible for ammonia generation (Durá *et al.*, 2002). These metabolic reactions should be considered when applying moulds and yeast in salami starter cultures, as a drastic rise in pH can create pathogen and spoilage problems.

2.4 Meat and fat

Salami are predominantly produced with pork meat and fat in Mediterranean countries (Demeyer *et al.*, 2000), however, innovative researches have studied the use of alternative, exotic meat species such as: ostrich (Cullere *et al.*, 2013); seal; whale (Koep, 2005); and various South African ungulates (Todorov *et al.*, 2007; Van Schalkwyk *et al.*, 2010; Chakanya *et al.*, 2018a). The meat source, varying in glycogen and intramuscular fat content, may greatly influence fermentation and drying and thus final organoleptic properties of salami (Feiner, 2006). Raw meat that is utilised for salami production should ideally have a pH of 5.3-5.8 (Chakanya *et al.*, 2018b). Meat with an initial high pH (> 5.8) is not ideal for salami production, posing a risk for an insufficient pH decrease (Petäjä-Kanninen & Puolanne, 2007; Lücke 1994). Chakanya *et al.* (2018b) reviewed the challenges when attempting to utilise suboptimal PSE (Pale, Soft and Exudative, pHu < 5.2-5.4) and DFD (Dark, Firm and Dry, pHu > 6.2) meat for salami production, including binding properties, water holding capacity and susceptibility to unwanted bacterial growth.

Salami is classified as a high fat (~30-40%) product with firm (highly saturated) fat tissue preferable for the production of high quality salami (Lücke, 1994; Herranz *et al.*, 2008). Reducing the fat content of salami may create quality and sensory defects as the fat contributes to mouth feel, texture and flavour (Olivares *et al.*, 2010; Holck *et al.*, 2017). Furthermore, many aroma compounds are fat soluble and the lipid oxidation process creates a vast amount of volatiles contributing to the typical salami flavour (e.g. 2-heptanone, 2-butanone) or rancidity (e.g. hexanal) (Montel *et al.*, 1996; Gilles, 2009; Olivares *et al.*, 2011; Tabanelli *et al.*, 2012). The fat particle size and distribution also affects the overall appearance of salami and may influence consumer purchase choice (Romano *et al.*, 2018). Furthermore, a high fat content aids in moisture retention to prevent salami from becoming extensively dry and hard, especially when ripened for longer periods (Olivares *et al.*, 2010). Altering the fatty acid profile of comminuted meat from predominately saturated to less saturated by replacing animal fat with fish or plant oils poses many challenges (Bolger *et al.*, 2017). Nonetheless, previous studies showed promising results after partially replacing pork backfat in fermented sausages with soya oil (Muguerza *et al.*, 2003), olive oil (Muguerza *et al.*, 2001; Del Nobile *et al.*, 2009) and grapeseed oil (Stajić *et al.*, 2014). A more saturated fat source, however, is less susceptible to oxidation and may prolong the shelf life of salami delaying the onset of rancidity due to lipid oxidation (Yildiz-Turp & Serdaroglu, 2012; Fuentes *et al.*, 2014).

During salami processing, meat and fat can either be minced or reduced in particle size by means of a bowl cutter, in turn influencing the texture and rate of water diffusion. During mixing of meat and fat particles, two variables are important to consider: the extent of mixing; and the meat to fat ratio. Over-mixing of the meat batter may cause smearing of the fat particles, creating a dense structure hindering water diffusion from the centre of the product and consequently causing crust formation (Andrés *et al.*, 2007). In contrast, under-mixing may result in a crumbly salami texture due to a lack of salt soluble protein extraction and emulsion formation. Salt soluble actin and myosin myofibril proteins allow for the formation of a gel-like structure or emulsion of meat and fat upon salting and mixing (Munasinghe & Sakai, 2004; Sun & Holley, 2011).

2.4.1 The potential of warthog (Phacochoerus africanus) meat for salami production

The common warthog (*Phacochoerus africanus*) is hunted in South Africa for meat production purposes, as recreational activity, as trophies and for damage control (Swanepoel *et al.*, 2016a). The physical-chemical traits of warthog meat has been studied to optimise meat quality for the purpose of commercialisation as consumer criteria used to judge the quality of domestic meat products is also applicable to game meat products (Hoffman & Wiklund, 2006; Swanepoel *et al.*, 2014). Warthog meat, similarly to other South African game meat species, has an attractive nutritional composition being low in total lipid content (< 2.2%) and high in protein (> 20%) (Swanepoel *et al.*, 2016c). The carcass yield competes well with domestic pigs ready for slaughter if the head, skin and trotters (which are commonly removed during warthog dressing procedures) are taken into account (Hoffman & Sales, 2007; Swanepoel *et al.*, 2014, 2016b). Furthermore, comparing warthog to domestic South African pork meat, the former showed more favourable fatty acid profiles (PUFA:SFA= 1.33) than the latter (PUFA:SFA= 0.46-0.64) with the physical-chemical traits (i.e. pH, drip loss %, cooking loss % and CIEL*a*b* surface colour) comparing well between the two species (Hoffman & Sales, 2007).

The redness of different warthog muscles vary (CIE a*= 7.87-14.84) depending on the physiological location (Swanepoel *et al.*, 2016c). The forequarter muscles (*Infraspinatus*, (IS) and *Supraspinatus*, (SS)) of adult warthogs were more intense red with higher CIE a* (14.80-13.70) and CIE Chroma (16.32-17.39) values, respectively (Swanepoel *et al.*, 2016c). This phenomenon was ascribed due to possible higher oxidative myofibers, resulting in a higher ultimate pH (pHu) and subsequently darker and redder meat (Swanepoel *et al.*, 2016c). Higher physical activity has shown to greatly impact the colour of fresh red meat, due to a shift in muscle fibre type towards a higher quantity of slow contracting, oxidative fibres with a greater myoglobin content for oxygen carrying capacity, resulting in darker meat (Vestergaard *et al.*, 2000b; Díaz *et al.*, 2002; Hoffman *et al.*, 2005). The high level of physical activity together with a low intramuscular fat content contribute to the naturally darker appearance of warthog meat (Swanepoel *et al.*, 2016c; Neethling *et al.*, 2017).

Warthogs have a high fertility rate, with an average of four to five piglets per litter after a 167-175 day gestation period (Hoffman & Sales, 2007) and average annual population growth of 75% (Furstenburg, 2009). The ideal population density is 7 ha per individual warthog or 15 ha for a sow and piglets in optimum habitat conditions (Furstenburg, 2009). If these populations are not managed, the proliferation and natural activities of warthogs could pose a threat to both crop and game farmers (Swanepoel *et al.*, 2016a). Different from other angulates, these creatures are considered to be completely free-roaming not easily kept within fenced properties (Swanepoel *et al.*, 2016a). Negative impacts due to warthog activities were identified as damaging towards: boundary fences; field plants and trees; the soil system; crops; and small livestock (Swanepoel *et al.*, 2016a).

Processing of surplus warthog meat (from trophy hunting or culling for the prevention of overpopulation and/or crop devastation) has been suggested for commercial consumption (Swanepoel *et al.*, 2016a,b) to promote sustainable wildlife utilisation and contribute to food security (Swanepoel *et al.*, 2014, 2016c). Nonetheless, warthog meat is still said to be underrepresented in the game meat market (Swanepoel *et al.*, 2016c). Whole warthog cuts can be sold at a premium price based on physical-chemical and nutritional properties discussed earlier. The majority of the commercially viable cuts will originate from the *Longissimus lumborum* (LL) muscle and hind quarter muscles, *Biceps femoris* (BF) and *Semimembranosus* (SM). The less valuable forequarter muscles (*Infraspinatus*, IS, and Supraspinatus, SS) can be optimally used in processed game meat products, such as salami (Swanepoel *et al.*, 2016c). Salami, requiring no or minimal processing and having an extended stable shelf life, can combat the restricted seasonal availability of warthog meat (Hoffman *et al.*, 2004; Hoffman & Wiklund, 2006). Furhermore, the typical cured flavour achieved through salalmi production can mask potential negatively portrayed flavours related to game meat (Wauters *et al.*, 2017; Chakanya *et al.*, 2018b).

2.5 Salt

Salt (NaCl) plays a pivotal role in salami production, contributing to the shelf life and microbial safety, taste, texture, water binding capacity, emulsion and colour formation (Ruusunen & Puolanne, 2005; Desmond, 2006; Skibsted, 2011; Holck *et al.*, 2017).

Sodium chloride effectively binds free water molecules and subsequently limits the engagement in chemical reactions and microbial growth, a process referred to as water activity reduction (Forsythe, 2010; Henney *et al.*, 2010; Delgado-Pando *et al.*, 2018). The unwanted natural occurring Gram-negative bacteria in raw meat (i.e. *Pseudomonas*) are especially high salt sensitive, in contrast to beneficial lactobacilli, micrococci and staphylococci, emphasising the importance of a high salt environment selecting the growth of salt and nitrite tolerant bacteria (Holley *et al.*, 1988; Petäjä-Kanninen & Puolanne, 2007). In this sense, adjusting the salt concentration in salami may alter the microbial population responsible for volatile compounds formation and flavour development (lactobacilli and staphylococci), (Olesen *et al.*, 2004; Corral *et al.*, 2013). Salt is typically added in

the range of 2-4% to the raw sausage mince (Ockerman & Basu, 2007; Stahnke & Tjener, 2007) and increases in final product salami depending on the extent of product drying.

Apart from microbial safety reasons, the addition of salt to salami allows for the extraction of salt soluble proteins (mainly the actin and myosin proteins), crucial for the formation of a gel-like structure or emulsion (Ordóñez *et al.*, 1999; Sun & Holley, 2011; Leroy *et al.*, 2013). A salt concentration of 2-3% is adequate to solubilise the myofibril proteins (Sun & Holley, 2011) and in this sense, NaCl has superior protein extraction ability compared to potassium chloride (KCl) and lithium chloride (LiCl) (Munasinghe & Sakai, 2004). Another interesting functionality of salt is the partaking in the typical pink-red salami colour, known as nitrosomyoglobin. Salt (a strong oxidizing agent) can react with nitrite to form nitrosyl chloride (NOCl), which is an even stronger nitrosalyting agent compared to nitrite, accelerating the formation of the wanted nitrosomyoglobin colour complex (Møller & Skibsted, 2002; Skibsted, 2011). This beneficial reaction, however, also entails that salt (an oxidizing agent) may promote unwanted oxidation reactions, yielding brown discolouration and rancid flavours in salami (Flores, 1997; Min *et al*., 2010; Mariutti & Bragagnolo, 2017).

Unfortunately, a high sodium intake is known to contribute to cardiovascular diseases and the intake thereof should be restricted (Kotchen & McCarron, 1998; Ha, 2014). In this sense, the South African Department of Health (DoH) recently amended salt reduction guidelines for specific food groups, including cured meat products (from a maximum permitted amount of 1300 to 1150 mg sodium/100 g) (DoH, 2019). Salt reduction in salami is particularly challenging as it may lead to impaired sensory qualities (aroma, taste and juiciness) and starter culture growth (Corral *et al.*, 2013; Aaslyng *et al.*, 2014). Nonetheless, researchers have partially replaced sodium chloride with potassium or calcium chloride in fermented sausages (Holck *et al*., 2017).

2.6 Sugar

Fermentable sugars may either be inherently present in the meat matrix from the glycogen stores (glucose) or ATP hydrolysis (ribose) or may be added in the salami formulation (Champomier-Vergès *et al*., 2001; Ockerman & Basu, 2007). Milk powder may form part of salami recipes as an additional fermentable sugar source (Mauriello *et al.*, 2004). The initial pH of the raw meat and the available glycogen will determine whether acidification during fermentation is successful (Lücke, 1994). Raw meat with a pH > 5.9 is said to have insufficient lactate and available sugar to ensure adequate fermentation (Lücke, 1994). In this sense, the fibre type and species of meat used will influence the acidity of the product when considering that some meat species and muscle fibre types have a naturally higher glycogen content than others (Hiscock *et al*., 2004; Feiner, 2006; Choe *et al*., 2008).

Although meat contains glycogen, this energy source is not easily metabolised by meat bacteria (Petäjä-Kanninen & Puolanne, 2007). Monosaccharides such as glucose (also known as

dextrose) are the primary energy source for lactic acid bacteria and are commonly added as part of salami ingredients. A concentration of 0.2-2.0% fermentable sugars is said to be sufficient to achieve a desired pH decline, taking into consideration that too much added sugar may lead to less controlled fermentation (Lücke, 1994; Talon & Leroy, 2014). Fermentable substrates are not limited to sugars: amino acids; purines and pyrimidines; organic acids; and polyols can also be metabolised by bacteria (Müller, 2001).

2.7 Nitrate and nitrite

Cured meats can be distinguished from the vast majority of processed meat products by the addition of sodium chloride, nitrite and nitrate, or the combination thereof (Flores, 1997; Honikel, 2007; Hammes, 2012). The curing process dates back to ancient times and although not documented, it is generally accepted that the accidental contamination of sodium chloride with potassium nitrate (saltpetre) led to the commercial use thereof today (Honikel, 2008; Sindelar & Milkowski, 2011; Taormina, 2014). Although the curing process is ancient, nitrite chemistry was only established at the start of the 20th century (Hospital *et al.*, 2012). The ability of the nitrogen atom to exist in one of many oxidation-reduction states (Honikel, 2008), together with the complex nature of processed meat products make nitrite chemistry in cured meats anything but easy to comprehend.

Nitrate must be reduced to its reactive counterpart, nitrite, to initiate this complex phenomenon known as curing (Sebranek & Bacus, 2007; Hammes, 2012; Bedale *et al.*, 2016). This process can be accelerated by adding reducing agents (ascorbate or ascorbic acid and erythorbate) to salami containing nitrate (Cassens, 1997; Gøtterup *et al.*, 2008). The multifunctionality of nitrite in cured meat is repeatedly emphasised as an antimicrobial, antioxidant and a partaker in the development of the characteristic cured meat colour and flavour (Sofos *et al.*, 1979; Sebranek & Bacus, 2007; Alahakoon *et al.*, 2015), which are probably brought about as a result of nitric oxide (Møller & Skibsted, 2002a; Skibsted, 2011). The effectiveness of nitrite is, however, dependent on numerous factors, including: the pH; temperature; added concentration of nitrate and/or nitrite; sodium chloride; ascorbate and erythorbate reducing agents; and the initial microbial load (Majou & Christieans, 2018).

Polenske (1891) first observed and proved the microbial action involved in generating nitrite from a nitrate source. The role of nitrite (as opposed to nitrate) in the formation of the heat stable, pink-red meat colour complex was later confirmed by Lehmann (1899) and Kisskalt (1899). Haldane (1901) gave insight surrounding the reaction between nitrite and myoglobin to create nitrosomyoglobin, while Hoagland (1910, 1914) demonstrated the acidic environment required for this reaction to occur and contributed to the understanding of the importance of nitric oxide and nitrous acid in meat curing (Sofos *et al.*, 1979; Honikel, 2008).

Today, sodium nitrate (E252), potassium nitrate (E251), sodium nitrite (E250) and potassium nitrite (E249) are controlled as food additives with maximum and minimum permitted limits as determined by regulating bodies including: Food and Drug Administration (FDA); US Department of Agriculture (USDA) (Hord *et al.*, 2009); and the European Union (Müller-Herbst *et al.*, 2016). The European Union (Directive 2008/1333/EC) allows a maximum of 150 mg/kg (ppm) of nitrate and nitrite each (NaNO$_2$/KNO$_3$; E252, E251) to non heat treated cured meat products or 250 mg/kg of nitrate when no nitrite is added to long ripened (minimum of 30 days) cured products such as chorizo (Perea-sanz *et al.*, 2018). The South African Department of Health (DoH) allows a maximum ingoing amount of 160 mg/kg of sodium nitrite and 200 mg/kg of sodium nitrate (DoH, Act of 54 of 1972).

2.5 Conclusion

While salami production in itself may be a simple technique, the processes involved are intricate and not mutually exclusive. Salami are extensively studied throughout literature due to a vast amount of relevant research topics, including, but not limited to: functional starter culture strains to improve salami quality and safety; inhibition of unwanted microbial growth; utilising exotic meat species; reducing salt and fat content; and the curing process. From this literature review it is clear that the altering of salami recipe or processing steps may pose a number of challenges as the ingredients, microflora balance and extrinsic parameters determine the product quality and safety.

2.6 References

Aaslyng, M.D., Vestergaard, C. & Koch, A.G. (2014). The effect of salt reduction on sensory quality and microbial growth in hotdog sausages, bacon, ham and salami. *Meat Science*, **96**, 47–55.

Adams, M.R. (1986). Fermented flesh foods. *Progress in Industrial Microbiology*, **23**, 159–198.

Alahakoon, A.U., Jayasena, D.D. & Jo, C. (2015). Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science and Technology*, **45**, 37–49.

Ammor, M.S. & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, **76**, 138–146.

Ananou, S., Garriga, M., Hugas, M., Maqueda, M., Martínez-Bueno, M., Gálvez, A. & Valdivia, E. (2005). Control of *Listeria monocytogenes* in model sausages by enterocin AS-48. *International Journal of Food Microbiology*, **103**, 179–190.

Andrés, A., Barat, J.M., Grau, R. & Fito, P. (2007). Principles of Drying and Smoking. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 37–48. Iowa: USA.

Aquilanti, L., Garofalo, C., Osimani, A. & Clementi, F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other

mediterranean countries: An overview. *International Food Research Journal*, **23**, 429–445.

Asefa, D.T., Gjerde, R.O., Sidhu, M.S., Langsrud, S., Kure, C.F., Nesbakken, T. & Skaar, I. (2009). Moulds contaminants on Norwegian dry-cured meat products. *International Journal of Food Microbiology*, **128**, 435–439.

Aymerich, T., Artigas, M.G., Garriga, M., Monfort, J.M. & Hugas, M. (2000a). Effect of sausage ingredients and additives on the production of enterocin A and B by *Enterococcus faecium* CTC492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. *Journal of Applied Microbiology*, **88**, 686–694.

Aymerich, T., Garriga, M., Ylla, J., Vallier, J., Monfort, J.M. & Hugas, M. (2000b). Application of enterocins as biopreservatives against *Listeria innocua* in meat products. *Journal of Food Protection*, **63**, 721–726.

Aymerich, T., Martín, B., Garriga, M. & Hugas, M. (2003). Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. *Applied and Environmental Microbiology*, **69**, 4583–4594.

Baka, A.M., Papavergou, E.J., Pragalaki, T., Bloukas, J.G. & Kotzekidou, P.(2011). Effect of selected autochthonous starter cultures on processing and quality characteristics of Greek fermented sausages. *LWT - Food Science and Technology*, **44**, 54–61.

Barrière, C., Centeno, D., Lebert, A., Leroy-Sétrin, S., Berdagué, J.L. & Talon, R. (2001). Roles of superoxide dismutase and catalase of *Staphylococcus xylosus* in the inhibition of linoleic acid oxidation. *FEMS Microbiology Letters*, **201**, 181–185.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, **120**, 85–92.

Bedia, M., Méndez, L. & Bañón, S. (2011). Evaluation of different starter cultures (*Staphylococci* plus lactic acid bacteria) in semi-ripened salami stuffed in swine gut. *Meat Science*, **87**, 381–386.

Bianchi, F., Cantoni, C., Careri, M., Chiesa, L., Musci, M. & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, **72**, 1552–1563.

Blaiotta, G., Pennacchia, C., Villani, F., Ricciardi, A., Tofalo, R. & Parente, E. (2004). Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented sausages. *Journal of Applied Microbiology*, **97**, 271–284.

Bolger, Z., Brunton, N.P., Lyng, J.G. & Monahan, F.J. (2017). Comminuted meat products - Consumption, composition, and approaches to healthier formulations. *Food Reviews*

International, **33**, 143–166.

Bover-Cid, S., Izquierdo-Pulido, M. & Vidal-Carou, M. (2001). Changes in biogenic amine and polyamine contents in slightly fermented sausages manufactured with and without sugar. *Meat Science*, **57**, 215–221.

Bruna, J.M., Hierro, E.M., De la Hoz, L., Mottram, D.S., Fernández, M. & Ordóñez, J.A. (2001a). The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. *Meat Science*, **59**, 97–107.

Bruna, J.M., Ordóñez, J.A., Fernández, M., Herranz, B. & De la Hoz, L. (2001b). Microbial and physico-chemical changes during the ripening of dry fermented sausages superficially inoculated with or having added an intracellular cell-free extract of *Penicillium aurantiogriseum*. *Meat Science*, **59**, 87–96.

Callewaert, R., Hugas, M. & De Vuyst, L. (2000). Competitiveness and bacteriocin production of *Enterococci* in the production of Spanish-style dry fermented sausages. *International Journal of Food Microbiology*, **57**, 33–42.

Campbell-platt, G. (1994). Fermented foods – A world perspective. *Food Research International*, **27**, 253–257.

Canel, R.S., Wagner, J.R., Stenglein, S.A. & Ludemann, V. (2013). Indigenous filamentous fungi on the surface of Argentinean dry fermented sausages produced in Colonia Caroya (Córdoba). *International Journal of Food Microbiology*, **164**, 81–86.

Cardinali, F., Milanović, V., Osimani, A., Aquilanti, L., Taccari, M., Garofalo, C., Polverigiani, S., Clementi, F., Franciosi, E., Tuohy, K., Mercuri, M.L., Altissimi, M.S. & Haouet, M.N. (2018). Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites. *International Journal of Food Microbiology*, **278**, 61–72.

Casaburi, A., Aristoy, M., Cavella, S., Di Monaco, R., Ercolini, D., Toldra, F. & Villani, F. (2007). Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (southern Italy) as affected by the use of starter cultures. *Meat Science*, **76**, 295–307.

Casaburi, A., Blaiotta, G., Mauriello, G., Pepe, O. & Villani, F. (2005). Technological activities of *Staphylococcus carnosus* and *Staphylococcus simulans* strains isolated from fermented sausages. *Meat Science*, **71**, 643–650.

Casquete, R., Benito, M.J., Martín, A., Ruiz-Moyano, S., Hernández, A. & Córdoba, M.G. (2011). Effect of autochthonous starter cultures in the production of "salchichón", a traditional Iberian dry-fermented sausage, with different ripening processes. *LWT - Food Science and Technology*, **44**, 1562–1571.

Cassens, R.G. (1997). Composition and safety of cured meats in the USA. *Food Chemistry*, **59**, 561–566.

Castellano, P., Mariana, P., Massani, M.B. & Vignolo, G.M. (2017). Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: A focus on meat ecosystems and industrial environments. *Microorganisms*, **5**, 1–25.

Chakanya, C., Arnaud, E., Muchenje, V. & Hoffman, L.C. (2018a). Changes in the physico-chemical attributes through processing of salami made from blesbok (*Damaliscus pygargus phillipsi*), eland (*Taurotragus oryx*), fallow deer (*Dama dama*), springbok (*Antidorcas marsupialis*) and black wildebeest (*Connochaetes gnou*) in comparison to pork. *Meat Science*, **146**, 87–92.

Chakanya, C., Arnaud, E., Muchenje, V. & Hoffman, L.C. (2018b). Fermented meat sausages from game and venison: What are the opportunities and limitations? *Journal of the Science of Food and Agriculture,* doi: 10.1002/jsfa.9053.

Champomier-Vergès, M.-C., Chaillou, S., Cornet, M. & Zagorec, M. (2001). *Lactobacillus sakei*: Recent developments and future prospects. *Research in Microbiology*, **152**, 839–848.

Chen, Q., Kong, B., Han, Q., Xia, X. & Xu, L. (2017). The role of bacterial fermentation in lipolysis and lipid oxidation in Harbin dry sausages and its flavour development. *LWT - Food Science and Technology*, **77**, 389–396.

Choe, J.H., Choi, Y.M., Lee, S.H., Shin, H.G., Ryu, Y.C., Hong, K.C. & Kim, B.C. (2008). The relation between glycogen, lactate content and muscle fibre type composition, and their influence on post mortem glycolytic rate and pork quality. *Meat Science*, **80**, 355-362.

Cocolin, L., Manzano, M., Aggio, D., Cantoni, C. & Comi, G. (2001). A novel polymerase chain reaction (PCR) - Denaturing gradient gel electrophoresis (DGGE) for the identificacation of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages. *Meat Science*, **57**, 59–64.

Cocolin, L., Urso, R., Rantsiou, K., Cantoni, C. & Comi, G. (2006). Dynamics and characterization of yeasts during natural fermentation of Italian sausages. *FEMS Yeast Research*, **6**, 692–701.

Coloretti, F., Tabanelli, G., Chiavari, C., Lanciotti, R., Grazia, L., Gardini, F. & Montanari, C. (2014). Effect of wine addition on microbiological characteristics, volatile molecule profiles and biogenic amine contents in fermented sausages. *Meat Science*, **96**, 1395–1402.

Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C. & Cocolin, L. (2005). Characterisation of naturally fermented sausages produced in the North East of Italy. *Meat Science*, **69**, 381–392.

Coppola, R., Iorizzo, M., Saotta, R., Sorrentino, E. & Grazia, L. (1997). Characterization of

micrococci and staphylococci isolated from soppressata molisana, a southern Italy fermented sausage. *Food Microbiology*, **14**, 47–53.

Coppola, S., Mauriello, G., Aponte, M., Moschetti, G. & Villani, F. (2000). Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage. *Meat Science*, **56**, 321–329.

Corral, S., Salvador, A. & Flores, M. (2013). Salt reduction in slow fermented sausages affects the generation of aroma active compounds. *Meat Science*, **93**, 776–785.

Cullere, M., Hoffman, L.C. & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products. *Meat Science*, **95**, 72–77.

Delgado-Pando, G., Fischer, E., Allen, P., Kerry, J.P., O'Sullivan, M.G. & Hamill, R.M. (2018). Salt content and minimum acceptable levels in whole-muscle cured meat products. *Meat Science*, **139**, 179–186.

Delgado, J., Peromingo, B., Rodríguez, A. & Rodríguez, M. (2019). Biocontrol of *Penicillium griseofulvum* to reduce cyclopiazonic acid contamination in dry-fermented sausages. *International Journal of Food Microbiology*, **293**, 1–6.

Del Nobile, M.A., Conte, A., Incoronato, A.I., Panza, O., Sevi, A. & Marino, R. (2009). New strategies for reducing the pork back-fat content in typical Italian salami. *Meat Science*, **81**, 263–269.

Demeyer, D., Raemaekers, M., Rizzo, A., Holck, A., De Smedt, A., Hagen, B., Montel, C., Zanardi, E., Murbrekk, E., Leroy, F., Vandendriessche, F., Lorentsen, K., Venema, K., Sunesen, L., Stahnke, L., De Vuyst, L., Talon, R., Chizzolini, R. & Eerola, S. (2000). Control of bioflavour and safety in fermented sausages: First results of a European project. *Food Research International*, **33**, 171–180.

Department of Health, Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972, amended by government notice 812, 2019. URL: https://www.foodfocus.co.za/assets/documents/UPDATED_Notice%20214%20-Regulations%20relating%20reduction%20of%20sodium.pdf.

Department of Health, Foodstuffs Cosmetics and Disinfectants Act 54 of 1972, Regulation on Preservatives and Additives. URL: http://www.health.gov.za/index.php/shortcodes/2015-03-29-10-42-47/2015-04-30-09-10-23/2015-04-30-09-11-35/category/181-act.

De Vuyst, L. (2000). Technology aspects related to the application of functional starter cultures. *Food Technology and Biotechnology*, **38**, 105–112.

Díaz, M.T., Velasco, S., Cañeque, V., Lauzurica, S., Ruiz De Huidobro, F., Pérez, C., González, J. & Manzanares, C. (2002). Use of concentrate or pasture for fattening lambs and its effect on carcass and meat quality. *Small Ruminant Research*, **43**, 257–268.

Dirinck, P., Van Opstaele, F. & Vandendriessche, F. (1997). Flavour differences between northern and southern European cured hams. *Food Chemistry,* **59**, 511-521.

Djordjevic, J., Pecanac, B., Todorovic, M., Dokmanovic, M., Glamoclija, N., Tadic, V. & Baltic, Milan, Z. (2015). Fermented sausage casings. *Procedia Food Science*, **5**, 69–72.

Durá, M.A., Flores, M. & Toldrá, F. (2002). Purification and characterisation of a glutaminase from *Debaryomyces* spp. *International Food Microbiology*, **76**, 117–126.

European Union (EU) Regulation No. 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs. URL: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:343:0001:0029:en:PDF.

Fandos, C. & Flavia, C. (2006). Intrinsic and extrinsic quality attributes, loyalty and buying intention: An analysis for a PDO product. *British Food Journal*, **108**, 646–663.

Feiner, G. (2006). Raw Fermented Salami. In: *Meat Products Handbook: Practical Science and Technology*. Pp. 314–375. New York: Woodhead Publishing Limited Cambridge: England.

Fernandes, M.S., Kabuki, D.Y. & Kuaye, A.Y. (2015). Biofilms of *Enterococcus faecalis* and *Enterococcus faecium* isolated from the processing of ricotta and the control of these pathogens through cleaning and sanitization procedures. *International Journal of Food Microbiology*, **200**, 97–103.

Fernández, M., Ordóñez, J.A., Bruna, J.M., Herranz, B. & De la Hoz, L. (2000). Accelerated ripening of dry fermented sausages. *Trends in Food Science and Technology*, **11**, 201–209.

Flores, J. (1997). Mediterranean vs northern European meat products. Processing technologies and main differences. *Food Chemistry*, **59**, 505–510.

Flores, M., Corral, S., Cano-garcía, L., Salvador, A. & Belloch, C. (2015). Yeast strains as potential aroma enhancers in dry fermented sausages. *International Journal of Food Microbiology*, **212**, 16–24.

Forsythe, S.J. (2010). Basic aspects. In: *The Microbiology of Safe Food*. Pp. 52–100. West Sussex, United Kingdom: Blackwell Publishing Ltd.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E. & De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology*, **106**, 1–24.

Franciosa, I., Alessandria, V., Dolci, P., Rantsiou, K. & Cocolin, L. (2018). Sausage fermentation

and starter cultures in the era of molecular biology methods. *International Journal of Food Microbiology*, **279**, 26–32.

Franz, C.M.A.P., Holzapfel, W.H. & Stiles, M.E. (1999). Enterococci at the crossroads of food safety? *International Journal of Food Microbiology*, **47**, 1–24.

Franz, C.M.A.P., Huch, M., Abriouel, H., Holzapfel, W. & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, **151**, 125–140.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H. & Holzapfel, W.H. (2003). Enterococci in foods - A conundrum for food safety. *International Journal of Food Microbiology*, **88**, 105–122.

Fuentes, V., Estévez, M., Ventanas, J. & Ventanas, S. (2014). Impact of lipid content and composition on lipid oxidation and protein carbonylation in experimental fermented sausages. *Food Chemistry*, **147**, 70-77.

Furstenburg, D. (2009). Focus on the Warthog (*Phacochoerus africanus*). *Game Species Biology*, 1–15.
URL:https://www.researchgate.net/publication/316166219_Focus_on_the_Warthog_Phacoch oerus_africanus.

Gallego, M., Mora, L., Escudero, El. & Toldrá, F. (2018). Bioactive peptides and free amino acids profiles in different types of European dry-fermented sausages. *International Joural of Food Microbiology*, **276**, 71–78.

García-Varona, M., Santos, E.M., Jaime, I. & Rovira, J. (2000). Characterisation of *Micrococcaceae* isolated from different varieties of chorizo. *International Journal of Food Microbiology*, **54**, 189–195.

Garriga, M. & Aymerich, T. (2007). The Microbiology of Fermentation and Ripening. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 125–135. Iowa: USA: Blackwell Publishing Professional.

Gherardi, G. & Di Bonaventura, G. (2018). Staphylococcal Taxonomy. In: *Pet-To-Man Travelling Staphylococci A World in Progress* (edited by V. Savini). Pp. 1–10. Academic Press.

Gilles, G. (2009). Dry cured ham quality as related to lipid quality of raw material and lipid changes during processing: A review. *Grasas Y Aceites*, **60**, 297–307.

Giraffa, G. (1995). Enterococcal bacteriocins: Their potential as anti-*Listeria* factors in dairy technology. *Food Microbiology*, **12**, 291–299.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiology Reviews*, **26**, 163–171.

Giraffa, G. (2003). Functionality of enterococci in dairy products. *International Journal of Food*

Microbiology, **88**, 215–222.

Gøtterup, J., Olsen, K., Knøchel, S., Tjener, K., Stahnke, L.H. & Møller, J.K.S. (2008). Colour formation in fermented sausages by meat-associated staphylococci with different nitrite- and nitrate-reductase activities. *Meat Science*, **78**, 492–501.

Grazia, L., Romano, P., Bagni, A., Roggiani, D. & Guglielmi, G. (1986). The role of moulds in the ripening process of salami. *Food Microbiology*, **3**, 19–25.

Guerrero-Ramos, E., Cordero, J., Molina-González, D., Poeta, P., Igrejas, G., Alonso-Calleja, C. & Capita, R. (2016). Antimicrobial resistance and virulence genes in enterococci from wild game meat in Spain. *Food Microbiology*, **53**, 156–164.

Guerrero, L., Guàrdia, M.D., Xicola, J., Verbeke, W., Vanhonacker, F., Zakowska-Biemans, S., Sajdakowska, M., Sulmont-Rossé, C., Issanchou, S., Contel, M., Scalvedi, M.L., Granli, B.S. & Hersleth, M. (2009). Consumer-driven definition of traditional food products and innovation in traditional foods. A qualitative cross-cultural study. *Appetite*, **52**, 345–354.

Ha, S.K. (2014). Dietary salt intake and hypertension. *Electrolytes and Blood Pressure*, **12**, 7–18.

Haldane, J. (1901). The red color of salted meat. *Journal of Hygiene*, **1**, 115–122.

Hammes, W.P. & Knauf, H.J. (1994). Starters in the processing of meat products. *Meat Science*, **36**, 155–168.

Hammes, W.P. (2012). Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. *Food Microbiology*, **29**, 151–156.

Henney, J.E., Taylor, C.L. & Boon, C.S. (2010). Preservation and Physical Property Roles of Sodium in Foods. In: *Strategies to Reduce Sodium Intake on the United States*. Pp. 91–118. Washington, D.C.: National Academic Press.

Herranz, B., Ordóñez, J.A., De la Hoz, L., Hierro, E., Soto, E. & Cambero, M.I. (2008). Fatty acid composition of salami from different countries and their nutritional implications. *International Journal of Food Sciences and Nutrition*, **59**, 607–618.

Hierro, E., De la Hoz, L. & Ordóñez, J.A. (1997). Contribution of microbial and meat endogenous enzymes to the lipolysis of dry fermented sausages. *Journal of Agriculture and Food Chemistry*, **45**, 2989–2995.

Hiscock, N, Chan, M.H.S., Bisucci, T., Darby, I.A. & Febbraio, M.A. (2004). Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: Evidence of fiber type specificity. *The Federation of American Societies for Experimental Biology*, **9**, 992-994.

Hoffman, L.C., Kritzinger, B. & Ferreira, A.V. (2005). The effects of region and gender on the fatty

acid, amino acid, mineral, myoglobin and collagen contents of impala (*Aepyceros melampus*) meat. *Meat Science*, **69**, 551–558.

Hoffman, L.C., Muller, M., Schutte, D.W. & Crafford, K. (2004). The retail of South African game meat: Current trade and marketing trends. *South African Journal of Wildlife Research*, **34**, 123–134.

Hoffman, L.C. & Sales, J. (2007). Physical and chemical quality characteristics of warthog (*Phacochoerus africanus*) meat. *Livestock Research for Rural Development*, **19.** URL: http://www.lrrd.org/lrrd19/10/hoff19153.htm.

Hoffman, L.C. & Wiklund, E. (2006). Game and venison - Meat for the modern consumer. *Meat Science*, **74**, 197–208.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M. & Heir, E. (2017). Health and safety considerations of fermented sausages. *Journal of Food Quality*, **2017**, 1–25.

Holley, R.A., Lammerding, A.M. & Tittiger, F. (1988). Occurrence and significance of streptococci in fermented Italian type dry sausage. *International Journal of Food Microbiology*, **7**, 63–72.

Honikel, K.-O. (2007). Principles of Curing. In: *Handbook of Fermented Meat and Poultry*. Pp. 17–30. Iowa: USA: Blackwell Publishing Professional.

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, **78**, 68–76.

Hord, N.G., Tang, Y. & Bryan, N.S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *The American Journal of Clinical Nutrition*, **90**, 1–10.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Hospital, X.F., Hierro, E. & Fernández, M. (2012). Survival of *Listeria innocua* in dry fermented sausages and changes in the typical microbiota and volatile profile as affected by the concentration of nitrate and nitrite. *International Journal of Food Microbiology*, **153**, 395–401.

Hugas, M., Garriga, M. & Aymerich, M.T. (2003). Functionalty of enterococci in meat products. *International Journal of Food Microbiology*, **88**, 223–233.

Incze, K. (2007). European Products. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 307–318. Iowa: USA: Blackwell Publishing Professional.

Jahan, M. & Holley, R.A. (2014). Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25°C and 37°C. *International Journal of Food Microbiology*, **170**, 65–69.

Jahan, M., Krause, D.O. & Holley, R.A. (2013). Antimicrobial resistance of *Enterococcus* species from meat and fermented meat products isolated by a PCR-based rapid screening method. *International Journal of Food Microbiology*, **163**, 89–95.

Jahan, M., Zhanel, G.G., Sparling, R. & Holley, R.A. (2015). Horizontal transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to clinical isolates of *E. faecium* and *Enterococcus faecalis. International Journal of Food Microbiology*, **199**, 78–85.

Kingcha, Y., Tosukhowong, A., Zendo, T., Roytrakul, S., Luxananil, P., Chareonpornsook, K., Valyasevi, R., Sonomoto, K. & Visessanguan, W. (2012). Anti-listeria activity of *Pediococcus pentosaceus* BCC 3772 and application as starter culture for Nham, a traditional fermented pork sausage. *Food Control*, **25**, 190–196.

Klare, I., Heier, H., Claus, H., Reissbrodt, R. & Witte, W. (1995). *vanA*-mediated high-level glycopeptide resistance in *Enterococcus faecium* from animal husbandry. *FEMS Microbiology Letters*, **125**, 165–171.

Koep, K.S.C. (2005). *Production of salami from meat of aquatic and terrestrial mammals*. MSc Thesis, Stellenbosch University. URL: https://core.ac.uk/download/pdf/37320667.pdf.

Komprda, T., Sládková, P., Petirová, E., Dohnal, V. & Burdychová, R. (2010). Tyrosine- and histidine-decarboxylase positive lactic acid bacteria and enterococci in dry fermented sausages. *Meat Science*, **86**, 870–877.

Kotchen, T.A. & McCarron, D.A. (1998). Dietary electrolytes and blood pressure: A statement for healthcare professionals from the American Heart Association Nutrition Committee. *Circulation*, **98**, 613–617.

Lebert, I., Leroy, S., Giammarinaro, P., Lebert, A., Chacornac, J.P., Bover-Cid, S., Vidal-carou, M.C. & Talon, R. (2007a). Diversity of microorganisms in the environment and dry fermented sausages of small traditional French processing units. *Meat Science*, **76**, 112–122.

Lebert, I., Leroy, S. & Talon, R. (2007b). Microorganisms in Traditional Fermented Meats. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 113–124. Iowa: USA: Blackwell Publishing Professional.

Leroy, F. & De Vuyst, L. (2002). Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. *International Journal of Food Microbiology*, **72**, 155–164.

Leroy, F. & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology*, **15**, 67–78.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L. & Scholliers, P. (2013). Meat fermentation at the

crossroads of innovation and tradition: A historical outlook. *Trends in Food Science and Technology*, **31**, 130–137.

Leroy, F., Vankrunkelsven, S., De Greef, J. & De Vuyst, L. (2003). The stimulating effect of a harsh environment on the bacteriocin activity by *Enterococcus faecium* RZS C5 and dependency on the environmental stress factor used. *International Journal of Food Microbiology*, **83**, 27–38.

Leroy, F., Verluyten, J. & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology*, **106**, 270–285.

López-Díaz, T., Santos, J., García-López, M. & Otero, A. (2001). Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of *Penicillium* isolates. *International Journal of Food Microbiology*, **68**, 69–74.

López, C.M., Bru, E., Vignolo, G.M. & Fadda, S.G. (2015). Identification of small peptides arising from hydrolysis of meat proteins in dry fermented sausages. *Meat Science*, **104**, 20–29.

López, M., Sáenz, Y., Rojo-Bezares, B., Martínez, S., Del Campo, R., Ruiz-Larrea, F., Zarazaga, M. & Torres, C. (2009). Detection of *vanA* and *vanB2*-containing enterococci from food samples in Spain, including *Enterococcus faecium* strains of CC17 and the new singleton ST425. *International Journal of Food Microbiology*, **133**, 172–178.

Lorenzo, J.M., García, M.C., Cachaldora, A., Franco, I. & Carballo, J. (2010). Study of the lactic acid bacteria throughout the manufacture of dry-cured lacón (a Spanish traditional meat product). Effect of some additives. *Food Microbiology*, **27**, 229–235.

Lozano-Ojalvo, D., Rodríguez, A., Cordero, M., Bernáldez, W., Reyes-Prieto, M. & Córdoba, J.J. (2015). Characterisation and detection of spoilage mould responsible for black spot in dry-cured fermented sausages. *Meat Science*, **100**, 283–290.

Lücke, F.-K. (1994). Fermented meat products. *Food Research International*, **27**, 299–307.

Lücke, F.-K. (2000). Utilization of microbes to process and preserve meat. *Meat Science*, **56**, 105–115.

Lund, B., Adamsson, I. & Edlund, C. (2002). Gastrointestinal transit survival of an *Enterococcus faecium* probiotic strain administered with or without vancomycin. *International Journal of Food Microbiology*, **77**, 109–115.

Mainar, M.S. & Leroy, F. (2015). Process-driven bacterial community dynamics are key to cured meat colour formation by coagulase-negative staphylococci via nitrate reductase or nitric oxide synthase activities. *International Journal of Food Microbiology*, **212**, 60–66.

Majou, D. & Christieans, S. (2018). Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. *Meat Science*, **145**, 273–284.

Marco, A., Navarro, J.L. & Flores, M. (2008). The sensory quality of dry fermented sausages as affected by fermentation stage and curing agents. *European Food Research and Technology*, **226**, 449–458.

Marcoz, E.M., Melewar, T.C. & Dennis, C. (2016). The value of region of origin, producer and protected designation of origin label for visitors and locals: The case of Fontina cheese in Italy. *International Journal of Tourism Research*, **18**, 236–250.

Mariutti, L.R.B. & Bragagnolo, N. (2017). Influence of salt on lipid oxidation in meat and seafood porducts: A review. *Food Research International*, **94**, 90-100.

Martín, A., Colín, B., Aranda, E., Benito, M.J. & Córdoba, M.G. (2007). Characterization of *Micrococcaceae* isolated from Iberian dry-cured sausages. *Meat Science*, **75**, 696–708.

Martin, B., Garriga, M., Hugas, M. & Aymerich, T. (2005). Genetic diversity and safety aspects of enterococci from slightly fermented sausages. *Journal of Applied Microbiology*, **98**, 1177–1190.

Mauriello, G., Casaburi, A., Blaiotta, G. & Villani, F. (2004). Isolation and technological properties of coagulase negative staphylococci from fermented sausages of Southern Italy. *Meat Science*, **67**, 149–158.

Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E. & Gandemer, G. (1999). Volatile compounds of commercial Milano salami. *Meat Science*, **51**, 175–183.

Min, B., Cordray, J.C. & Ahn, D.U. (2010). Effect of NaCl, Myoglobin, Fe (II), and Fe (III) on lipid oxidation of raw and cooked chicken breast and beef loin. *Journal of Agricultural and Food Chemistry*, **58**, 600-605.

Miralles, M.C., Flores, J. & Perez-Martinez, G. (1996). Biochemical tests for the selection of *Staphylococcus* strains as potential meat starter cultures. *Food Microbiology*, **13**, 227–236.

Møller, J.K.. & Skibsted, L.H. (2002). Nitric oxide and myoglobins. *Chemical Reviews*, **102**, 1167–1178.

Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. & Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry*, **192**, 736–744.

Montanari, C., Gatto, V., Torriani, S., Barbieri, F., Bargossi, E., Lanciotti, R., Grazia, L., Magnani, R., Tabanelli, G. & Gardini, F. (2018). Effects of the diameter on physico-chemical, microbiological and volatile profile in dry fermented sausages produced with two different starter cultures. *Food Bioscience*, **22**, 9–18.

Montel, M.-C., Reitz, J., Talon, R., Berdagué, J.-L. & Rousset-Akrim, S. (1996). Biochemical activities of *Micrococcaceae* and their effects on the aromatic profiles and odours of a dry

sausage model. *Food Microbiology*, **13**, 489–499.

Montel, M.C., Masson, F. & Talon, R. (1998). Bacterial role in flavour development. *Meat Science*, **49**, S111–S123.

Moore, J.E. (2004). Gastrointestinal outbreaks associated with fermented meats. *Meat Science*, **67**, 565–568.

Muguerza, E., Ansorena, D. & Astiasarán, I. (2003). Improvement of nutritional properties of Chorizo de Pamplona by replacement of pork backfat with soy oil. *Meat Science*, **65**, 1361–1367.

Muguerza, E., Gimeno, O., Ansorena, D., Bloukas, J.G. & Astiasarán, I. (2001). Effect of replacing pork backfat with pre-emulsified olive oil on lipid fraction and sensory quality of Chorizo de Pamplona - A traditional Spanish fermented sausage. *Meat Science*, **59**, 251–258.

Müller-Herbst, S., Wüstner, S., Kabisch, J., Pichner, R. & Scherer, S. (2016). Acidified nitrite inhibits proliferation of *Listeria monocytogenes* - Transcriptional analysis of a preservation method. *International Journal of Food Microbiology*, **226**, 33–41.

Munasinghe, D.M.S. & Sakai, T. (2004). Sodium chloride as a preferred protein extractant for pork lean meat. *Meat Science*, **67**, 697–703.

Neethling, N.E., Suman, S.P., Sigge, G.O., Hoffman, L.C. & Hunt, M.C. (2017). Exogenous and endogenous factors influencing color of fresh meat from ungulates. *Meat and Muscle Biology*, **1**, 253.

Ockerman, H.W. & Basu, L. (2007). Production and consumption of fermented meat products. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldra). Pp. 9–15. Iowa: USA: Blackwell Publishing Professional.

Olesen, P.T., Meyer, A.S. & Stahnke, L.H. (2004). Generation of flavour compounds in fermented sausages - The influence of curing ingredients, *Staphylococcus* starter culture and ripening time. *Meat Science*, **66**, 675–687.

Olivares, A., Navarro, J.L., Salvador, A. & Flores, M. (2010). Sensory acceptability of slow fermented sausages based on fat content and ripening time. *Meat Science*, **86**, 251–257.

Ordóñez, J.A., Hierro, E.M., Bruna, J.M. & De la Hoz, L. (1999). Changes in the components of dry-fermented sausages during ripening. *Critical Reviews in Food Science and Nutrition*, **39**, 329–367.

Ordóñez, J.A. & De la Hoz, L. (2007). Mediterranean Products. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 333–347. Iowa: USA: Blackwell Publishing Professional.

Papamanoli, E., Kotzekidou, P., Tzanetakis, N. & Litopoulou-Tzanetaki, E. (2002). Characterization of *Micrococcaceae* isolated from dry fermented sausage. *Food Microbiology*, **19**, 441–449.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E. & Kotzekidou, P. (2003). Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science*, **65**, 859–867.

Pasini, F., Soglia, F., Petracci, M., Caboni, M.F., Marziali, S., Montanari, C., Gardini, F., Grazia, L. & Tabanelli, G. (2018). Effect of fermentation with different lactic acid bacteria starter cultures on biogenic amine content and ripening patterns in dry fermented sausages. *Nutrients*, **10**, 1–16.

Pederson, C.S. (1979). Fermented Sausage. In: *Microbiology of Food Fermentation*. Pp. 210–234. Westport: USA: AVI Publishing Inc.

Pesavento, G., Calonico, C., Ducci, B., Magnanini, A. & Nostro, A. Lo. (2014). Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. *Food Microbiology*, **41**, 1–7.

Petäjä-Kanninen, E. & Puolanne, E. (2007). Principles of Meat Fermentation. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 31–36. Iowa: USA: Blackwell Publishing Professional.

Pisacane, V., Callegari, M.L., Puglisi, E., Dallolio, G. & Rebecchi, A. (2015). Microbial analyses of traditional Italian salami reveal microorganisms transfer from the natural casing to the meat matrix. *International Journal of Food Microbiology*, **207**, 57–65.

Poeta, P., Costa, D., Rojo-Bezares, B., Zarazaga, M., Klibi, N., Rodriguez, J. & Torres, C. (2007). Detection of antimicrobial activities and bacteriocin structural genes in faecal enterococci of wild animals. *Microbiological Research*, **162**, 257–263.

Polka, J., Rebecchi, A., Pisacane, V., Morelli, L. & Puglisi, E. (2015). Bacterial diversity in typical Italian salami at different ripening stages as revealed by high-throughput sequencing of 16S rRNA amplicons. *Food Microbiology*, **46**, 342–356.

Rantsiou, K. & Cocolin, L. (2006). New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: A review. *International Journal of Food Microbiology*, **108**, 255–267.

Rantsiou, K., Drosinos, E.H., Gialitaki, M., Urso, R., Krommer, J., Gasparik-Reichardt, J., Tóth, S., Metaxopoulos, I., Comi, G. & Cocolin, L. (2005). Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. *Food Microbiology*, **22**, 19–28.

Ravyts, F., Barbuti, S., Frustoli, M.A., Parolari, G., Saccani, G., De Vuyst, L. & Leroy, F. (2008). Competitiveness and antibacterial potential of bacteriocin-producing starter cultures in different types of fermented sausages. *Journal of Food Protection*, **71**, 1817–1827.

Ravyts, F., De Vuyst, L. & Leroy, F. (2012). Bacterial diversity and functionalities in food fermentations. *Engineering in Life Sciences*, **12**, 356–367.

Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives URL: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02008R1333-20170320&from=ES.

Rizzi, A. (2003). Development of a numerical model for the fluid dynamic simulation of an ascending flow ripening chamber. *Journal of Food Engineering* , **58**, 151–171.

Roccato, A., Uyttendaele, M., Barrucci, F., Cibin, V., Favretti, M., Cereser, A., Dal, M., Pezzuto, A., Piovesana, A., Longo, A., Ramon, E., De Rui, S. & Ricci, A. (2017). Artisanal Italian salami and soppresse: Identification of control strategies to manage microbiological hazards. *Food Microbiology*, **61**, 5–13.

Romano, A., Masi, P. & Cavella, S. (2018). Visual evaluation of sliced Italian salami by image analysis. *Food Science and Nutrition*, **6**, 153–159.

Rossi, F., Tofalo, R., Torriani, S. & Suzzi, G. (2001). Identification by 16S-23S rDNA intergenic region amplification, genotypic and phenotypic clustering of *Staphylococcus xylosus* strains from dry sausages. *Journal of Applied Microbiology*, **90**, 365–371.

Rupp, M.E. & Fey, P.D. (2015). *Staphylococcus epidermidis* and other Coagulase-Negative Staphylococci. In: *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. Pp. 2272-2282. New York: Sauders.

Ruusunen, M. & Puolanne, E. (2005). Reducing sodium intake from meat products. *Meat Science*, **70**, 531–541.

Samelis, J., Maurogenakis, F. & Metaxopoulos, J. (1994). Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology,* **23,** 179-196.

Samelis, J., Metaxopoulos, J., Vlassi, M. & Pappa, A. (1998). Stability and safety of traditional Greek salami - A microbiological ecology study. *International Journal of Food Microbiology*, **44**, 69–82.

Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.-C., Oliver, G. & Toldrá, F. (1999). Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. *International Journal of Food Microbiology*, **53**, 115–125.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M.D., Rea, M.C., Lombardi, A., Cogan, T.M., Kalantzopoulos, G. & Tsakalidou, E. (2001). Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal*, **11**, 621–647.

Sebranek, J.G. & Bacus, J.N. (2007). Cured meat products without direct addition of nitrate or nitrite: What are the issues? *Meat Science*, **77**, 136–147.

Sherman, J.M. (1937). The streptococci. *Bacteriological Reviews*,**1**, 3–97.

Sindelar, J.J. & Milkowski, A.L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. In: *American Meat Science Association*. Pp. 1–14.

Skibsted, L.H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, **24**, 176–183.

Soares-Santos, V., Barreto, A.S. & Semedo-Lemsaddek, T. (2015). Characterization of enterococci from food and food-related settings. *Journal of Food Protection*, **78**, 1320–1326.

Sofos, J.N., Busta, F.F. & Allen, C.E. (1979). Botulism control by nitrite and sorbate in cured meats: A review. *Journal of Food Protection*, **42**, 739–770.

Søndergaard, A.K. & Stahnke, L.H. (2002). Growth and aroma production by *Staphylococcus xylosus*, *S. carnosus* and *S. equorum* - A comparative study in model systems. *International Journal of Food Microbiology*, **75**, 99–109.

Stajić, S., Živković, D., Tomović, V., Nedović, V., Perunović, M., Kovjanić, N., Lević, S. & Stanišić, N. (2014). The utilisation of grapeseed oil in improving the quality of dry fermented sausages. *International Journal of Food Sciences and Technology*, **49**, 2356–2363.

Stavropoulou, D.A., Filippou, P., De Smet, S. De Vuyst, L. & Leroy, F. (2018). Effect of temperature and pH on the community dynamics of coagulase-negative staphylococci during spontaneous meat fermentation in a model system. *Food Microbiology*, **76**, 180–188.

Sun, X.D. & Holley, R.A. (2011). Factors influencing gel formation by myofibrillar proteins in muscle foods. *Comprehensive Reviews in Food Science and Food Safety*, **10**, 33–51.

Sunesen, L.O. & Stahnke, L.H. (2003). Mould starter cultures for dry sausages - Selection, application and effects. *Meat Science*, **65**, 935–948.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2014). The influence of season and sex on the carcass yield of common warthog (*Phacochoerus africanus*). *South African Journal of Wildlife Research*, **44**, 179–188.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2016a). Farmers' perceptions of the extra-limital common warthog in the Northern Cape and free state provinces, South Africa. *Wildlife Society Bulletin*, **40**, 112–121.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2016b). Comparative analyses of the chemical and sensory parameters and consumer preference of a semi-dried smoked meat product

(cabanossi) produced with warthog (*Phacochoerus africanus*) and domestic pork meat. *Meat Science*, **114**, 103–113.

Swanepoel, M., Leslie, A.J., Van der Rijst, M. & Hoffman, L.C. (2016c). Physical and chemical characteristics of warthog (*Phacochoerus africanus*) meat. *African Journal of Wildlife Research*, **46**, 103–120.

Tabanelli, G., Coloretti, F., Chiavari, C., Grazia, L., Lanciotti, R. & Gardini, F. (2012). Effects of starter cultures and fermentation climate on the properties of two types of typical Italian dry fermented sausages produced under industrial conditions. *Food Control*, **26**, 416–426.

Talon, R., Lebert, I., Lebert, A., Leroy, S., Garriga, M., Aymerich, T., Drosinos, E.H., Zanardi, E., Ianieri, A., Fraqueza, M.J., Patarata, L. & Laukova, A. (2007). Traditional dry fermented sausages produced in small-scale processing units in Mediterranean countries and Slovakia. 1: Microbial ecosystems of processing environments. *Meat Science*, **77**, 570–579.

Talon, R. & Leroy, S. (2011). Diversity and safety hazards of bacteria involved in meat fermentations. *Meat Science*, **89**, 303–309.

Talon, R. & Leroy, S. (2014). Fermented Foods: Fermented Meat Products and the Role of Starter Cultures. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 870-874.

Taormina, P.J. (2014). Meat and Poultry: Curing of Meat. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 501-507.

Tarelli, G.T., Carminati, D. & Giraffa, G. (1994). Production of bacteriocins active and against *Listeria monocytogenes* and *Listeria innocua* from dairy enterococci. *Food Microbiology*, **11**, 243–252.

Todorov, S.D., Koep, K.S.C., Van Reenen, C.A., Hoffman, L.C., Slinde, E. & Dicks, L.M.T. (2007). Production of salami from beef, horse, mutton, blesbok (*Damaliscus dorcas phillipsi*) and springbok (*Antidorcas marsupialis*) with bacteriocinogenic strains of *Lactobacillus plantarum* and *Lactobacillus curvatus. Meat Science*, **77**, 405–412.

Todorov, S.D., Stojanovski, S., Iliev, I., Moncheva, P., Nero, L.A. & Ivanova, I.V. (2017). Technology and safety assessment for lactic acid bacteria isolated from traditional Bulgarian fermented meat product "lukanka." *Brazilian Journal of Microbiology*, **48**, 576–586.

Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Science*, **49**, S101–S110.

Van Schalkwyk, D.L., Mc Millin, K.W., Booyse, M., Witthuhn, R.C. & Hoffman, L.C. (2010). Physico-chemical, microbiological, textural and sensory attributes of matured game salami produced from springbok (*Antidorcas marsupialis*), gemsbok (*Oryx gazella*), kudu (*Tragelaphus*

strepsiceros) and zebra (*Equus burchelli*) harvested in Namibia. *Meat Science*, **88**, 36–44.

Vestergaard, C.S., Schivazappa, C. & Virgili, R. (2000a). Lipolysis in dry-cured ham maturation. *Meat Science*, **55**, 1–5.

Vestergaard, M., Oksbjerg, N. & Henckel, P. (2000b). Infuence of feeding intensity, grazing and finishing feeding on muscle fibre characteristics and meat colour of *semitendinosus*, *longissimus dorsi* and *supraspinatus* muscles of young bulls. *Meat Science*, **54**, 177–185.

Vila, G., Segura, J.A., Ludemann, V. & Pose, G.N. (2019). Surface mycobiota of home-made dry cured sausages from the main producing regions of Argentina and morphological and biochemical characterization of *Penicillium nalgiovense* populations. *International Journal of Food Microbiology*, **309**, 1–10.

Wang, X., Ren, H., Wang, W., Zhang, Y., Bai, T., Li, J. & Zhu, W. (2015). Effects of inoculation of commercial starter cultures on the quality and histamine accumulation in fermented Sausages. *Journal of Food Science*, **80**, M377–M384.

Warriss, P.D. (2010). Post-mortem changes in muscle and its conversion into meat. In: *Meat Science, An Introductory Text*. Pp. 65–76. Cambridge, USA: CAB International.

Wauters, J., Verplanken, K., Vercruysse, V., Ampe, B., Aluwé, M. & Vanhaecke, L. (2017). Sensory evaluation of boar meat products by trained experts. *Food Chemistry*, **237**, 516–524.

Wegener, H.C., Aarestrup, F.M., Jensen, L.B., Hammerum, A.M. & Bager, F. (1999). Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in europe. *Emerging Infectious Diseases*, **5**, 329–335.

Woraprayote, W., Malila, Y., Sorapukdee, S., Swetwiwathana, A., Benjakul, S. & Visessanguan, W. (2016). Bacteriocins from lactic acid bacteria and their applications in meat and meat products. *Meat Science*, **120**, 118–132.

Xu, Y., Li, L., Xia, W., Zang, J. & Gao, P. (2019). The role of microbes in free fatty acids release and oxidation in fermented fish paste. *LWT - Food Science and Technology*, **101**, 323–330.

Yildiz-Turp, G. & Serdaroglu, M. (2012). Partial substitution of beef fat with hazelnut oil in emulsion type sausages: Effects on chemical, physical and sensorial quality. *Journal of Food Technology*, **10**, 32–38.

Zanardi, E., Ghidini, S., Battaglia, A. & Chizzolini, R. (2004). Lipolysis and lipid oxidation in fermented sausages depending on different processing conditions and different antioxidants. *Meat Science*, **66**, 415–423.

Zeuthen, P. (2007). A Historical Perspective of Meat Fermentation. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 3–8. Iowa: USA: Blackwell Publishing Professional.

CHAPTER 3

Literature review: Food lipid and protein oxidation, nitrate and nitrite functionality in cured meats and the use of alternative natural extracts

Abstract

Lipid and protein oxidation in foodstuffs lead to impaired sensory characteristics, the possible formation of carcinogens, a reduced shelf life and nutritional value. In contrast, these deteriorative reactions are required to an extent to create desired dry-cured and fermented meat aromas and flavours. To better control oxidation in salami the oxidative process needs to be understood. In this regard, nitrate (NO_3) and nitrite (NO_2) curing salts play a pivotal role in delaying rancidity in salami. Other than combating oxidation, the cured meat colour pigment (nitrosomyoglobin) formation and potent antimicrobial effects (especially against toxin producing *Clostridia*) of NO_2 and its derivatives are reviewed. The health risks surrounding the use of NO_3/NO_2 are discussed and subsequently the use of curing alternatives in meat models, with the focus on natural extracts exhibiting antioxidant and/or antimicrobial properties. To conclude the literature study, honeybush (*Cyclopia subternata*) extract is reviewed as a potential bioactive ingredient in salami due to its attractive polyphenolic content and antioxidant capacity.

Keywords: Lipid oxidation; protein oxidation; nitrate; nitrite; cured meat; honeybush extract; *Cyclopia subternata*

3.1. Lipid and protein oxidation

Dry-cured fermented meats are associated with deteriorative lipid and protein oxidation due to the nature of the product being comminuted, high in salt, myoglobin/metmyoglobin and haem iron (even more so in the case of red meat) (Park & Xiong, 2007; Min *et al.*, 2010; Soladoye *et al.*, 2015). Interestingly, past research studies predominantly focused on lipid, rather than protein oxidation in food models. The delay in research on the latter is presumably because of the complexity of this phenomenon, a lack of understanding of the nature of the by-products formed, the subsequent methodologies required to test for protein oxidation products and perhaps a general conviction that lipid oxidation and microbial spoilage summarises the majority of the food deteriorative challenges (Estévez, 2011; Lund *et al.*, 2011).

Oxidation of unsaturated fatty acids occurs in the presence of oxygen, either enzymatically or non-enzymatically (Mariutti & Bragagnolo, 2017). The former entails the action of lipoxygenase and is not primarily responsible for lipid oxidation in meat and meat products. Non-enzymatic oxidation occurs via one of two mechanism: autoxidation; or photo-oxidation (Mariutti & Bragagnolo, 2017). Two existing types of oxygen can participate in non-enzymatic lipid oxidation, namely naturally occurring atmospheric triplet oxygen ($^3O_2$) or singlet oxygen ($^1O_2$) (Min & Boff, 2002; Choe & Min,

2009). Triplet oxygen has a radical nature and reacts with radicals in the proses known as, autoxidation (Choe & Min, 2009; Mariutti & Bragagnolo, 2017). Food lipids, like the majority of biological compounds, are generally in the singlet, non-radical state which do not readily react with $^3O_2$, however, light, heat and metal catalysts accelerate radical formation for autoxidation to occur (Min & Boff, 2002; Choe & Min, 2009).

Autoxidation is described as a radical cascade reaction and can be summarised in three steps: initiation; propagation; and termination (Frankel, 1984; Gardner, 1989; Erickson, 2002; Mariutti & Bragagnolo, 2017). During initiation, reactive oxygen species (ROS, i.e. superoxide anion, O_2^-; hydroxy (OH); peroxy (PO); alkoxy (RO); and hydroperoxyl (HOO) radicals) removes a hydrogen atom between double bonds in unsaturated hydrocarbon fatty acid chains, creating lipid free radicals (R°) (Frankel, 1984; Erickson, 2002; Choe & Min, 2005). The dissociation energy (energy required to break the chemical bond) between adjacent double bonds in polyunsaturated fatty acids (PUFAs) are much lower compared to saturated fatty acids, making them the ideal targets for ROS attack (Frankel, 1985; Gardner, 1989; Min & Boff, 2002). Lipid free radicals can in return rapidly react with naturally occurring $^3O_2$, yielding lipid peroxyl radicals (ROO°), (Frankel, 1984; Erickson, 2002). During propagation, highly unstable peroxyl radicals can further react with other unsaturated fatty acids, creating lipid hydroperoxides (ROOH) and an additional lipid free radical (R°), (Frankel, 1984; Erickson, 2002). Hydroperoxides, although harmless in their nature to food quality, are considered as primary oxidation products responsible for the off flavours and aromas when decomposed into secondary volatile and non-volatile compounds, including: aldehydes; ketones; alcohols; esters; epoxy compounds; and organic acids (Frankel *et al.*, 1981; Frankel, 1982; Erickson, 2002). Carbonyls (aldehydes and ketones) are particularly important because of their low odour thresholds contributing either to unpleasant meat aromas or desired aromas (Ramaswamy & Richards, 1982; Dirinck *et al.*, 1997; Min & Boff, 2002b). Aldehydes which contribute to undesired aromas include: nonanal; 2-pentyl-furan; trans-2-heptenal; and 2,4-decadienal, imparting fatty, oily and/or rancid aromas (Gilles, 2009). In contrast, other oxidation products may contribute positively, like 1-octen-3-ol, contributing to meat aroma imparting a mushroom flavour (Dirinck *et al.*, 1997). Finally, during termination, radicals are neutralized or stabilized by reacting with each other (Erickson, 2002).

Primary and secondary by-products of lipid oxidation may in turn initiate amino acid oxidation, in this regard cysteine and methionine are highly susceptible due to their sulphur containing centres (Estévez, 2011). As with lipid oxidation, proteins oxidise via a free radical cascade reaction, however, the latter process may be much more complex yielding a vast amount of by-products. The main protein oxidation products are aldehydes and ketones as a result of carbonylation (Estévez, 2011; Soladoye *et al.*, 2015). Amino acid structures are affected by H_2O_2, myoglobin, metmyoglobin and salt (Estévez, 2011), since salami contain all of the above-mentioned, protein oxidation is

expected to occur. Other precursors for protein oxidation include, free radicals: hydroxyl groups (OH); superoxide (O_2^-); peroxyl (PO); nitric oxide (NO); and non-radicals: hydrogen peroxide (H_2O_2); $^1O_2$; peroxynitrite ($ONOO^-$); hypochlorous acid (HOCl); and ozone (O_3), (Shacter, 2000; Soladoye *et al.*, 2015). Nitrate/nitrite chemistry leads to the formation of $ONOO^-$ which is a strong oxidizing agent (Majou & Christieans, 2018) and will be discussed in more detail later in the review. This implies that the meat curing process may initiate or contribute partially to oxidative reactions.

Dry-cured and fermented sausages are susceptible to oxidation due to processing (i.e. mincing, mixing) (Nassu *et al.*, 2003; Olivares *et al.*, 2009a). Sun *et al.* (2011) demonstrated the increase of sarcoplasmic protein oxidation during fermentation and oven-drying of Cantonese sausages. Excessive actin-myosin oxidation could lead to impaired water holding and emulsification capacity (Estévez, 2011), in turn altering salami texture. Apart from affecting sensory characteristics, the oxidation of amino acids leads to a loss thereof and hence, negatively effects the nutritional value of meat, especially when essential amino acids (i.e. lysine, arginine and threonine) are irreversibly altered (Park & Xiong, 2007; Estévez, 2011).

Although lipid and protein oxidation are generally perceived as an unwanted deteriorative phenomenon, these reactions are partially required to achieve the wanted aroma characteristics of dry-cured fermented meat (Shahidi *et al.*, 1986; Buscailhon *et al.*, 1994; Ordóñez *et al.*, 1999; Olivares *et al.*, 2009b; Chen *et al.*, 2017). An extensive amount of research has been dedicated to volatile compounds generated in salami (Berger *et al.*, 1990; Meynier *et al.*, 1999; Sunesen *et al.*, 2001; Bianchi *et al.*, 2007; Di Cagno *et al.*, 2008; Olivares *et al.*, 2009b; Corral *et al.*, 2014; Flores *et al.*, 2015). Salami volatile profiles are commonly analysed when the raw ingredients or processing methodology are adapted, for instance: with salt, nitrate and nitrite reductions; different starter culture strains are used; and with a change in salami dimensions or fermentation/ripening conditions (Montel *et al.*, 1996; Bruna *et al.*, 2001; Olesen *et al.*, 2004; Marco *et al.*, 2008; Tabanelli *et al.*, 2012; Corral *et al.*, 2013; Coloretti *et al.*, 2014; Hospital *et al.*, 2015; Montanari *et al.*, 2016, 2018; Perea-Sanz *et al.*, 2019).

Irrespective of the positive flavours created through mild lipid and protein oxidation in salami, these reactions should foremostly be controlled to prevent quality deterioration. In this regard, selected salami processing variables may influence the extent of lipid and protein oxidation, such as the amount and composition of added fat (saturated versus mono-/polyunsaturated). Fuentes *et al.* (2014) demonstrated the increase in lipid oxidation as the total lipid content increased in sausage models. The source of added fat directly influenced the fatty acid composition and in turn determined the oxidative stability of the final product. Furthermore, a significant positive relationship was found between PUFA content and hexanal values (linked to rancidity), (Fuentes *et al.*, 2014). It is noteworthy to mention the effect of using a meat source from monogastric animals (i.e. pork, or turkey) with improved fatty acid profiles with a higher PUFA content for processed meat products.

Although such meats are expected to be more susceptible to lipid oxidation due to a higher amount of unsaturated double bonds, the additional antioxidants obtained through for instance a grass or acorn diet (i.e. tocopherols) may counteract this deteriorative effect in the final meat product (Mercier *et al.*, 2001, 2004; Estévez & Cava, 2006).

The extent of lipid oxidation can be studied by measuring primary (i.e. hydroperoxides and conjugated dienes) or secondary (i.e. pentanal, hexanal and malondialdehyde, MDA) oxidation products (Gray & Monahan, 1992; Dobarganes & Velasco, 2002; Mariutti & Bragagnolo, 2017). Although many analytical methods exists for analysing lipid oxidation (Halliwell & Chirico, 1993; Ross & Smith, 2006), the peroxide value (PV, quantifying the mEq peroxides/g of sample) and the thiobarbituric reactive substances test (TBARS, quantifying the mg MDA/kg of sample) are commonly reported for meat products (Wang *et al.*, 1995; Sebranek *et al.*, 2005; Marco *et al.*, 2008; Marangoni & Moura, 2011; Lu & Yanjun, 2016; Zhang *et al.*, 2016; Liu *et al.*, 2018). As for protein oxidation, protein carbonyls are commonly measured using the 2,4-dinitrophenylhydrazine (DNPH) method expressed as nmol carbonyl/mg of protein (Cichoski *et al.*, 2011; Estévez, 2011; Fuentes *et al.*, 2014; Soladoye *et al.*, 2015). These analytical methods should ideally be coupled with sensory analysis, as the latter remains the closest approximation of consumer perception (Wąsowicz *et al.*, 2004).

Due to the known limitations of TBARS and primary oxidation product measurements, the analysis of organic volatile compounds (VOCs) linked with oxidation have received much attention (Ross & Smith, 2006). In this sense, the solid-phase micro-extraction (SPME) method has been developed for head space VOC analysis (Roberts *et al.*, 2000; Lorenzo, 2014). Certain VOCs are highlighted in literature for their association with rancidity, such as hexanal (Muguerza *et al.*, 2001; Gøtterup *et al.*, 2008; Fuentes *et al.*, 2014). These biomarkers can be semi-quantified between samples using an internal standard of known concentration, given that the analytical method remains unchanged between samples and the standard (Roberts *et al.*, 2000).

3.2. The multifunctionality of nitrate and nitrite

3.2.1. Antioxidant effect

Nitrite, nitrate and the combination thereof are applied in cured meats to ensure food safety and quality, foremostly seen by a stable, desirable pink-red colour over a long shelf life period with the absence of rancidity (Sebranek & Bacus, 2007; Sindelar & Milkowski, 2011). Due to the well-known antioxidant effect of nitrite, many commercially available synthetic antioxidants (i.e. butylated hydroxyanisole, BHA and butylated hydroxytoluene, BHT) are not commonly used in cured meats (Sindelar & Milkowski, 2011, 2012). These synthetic food additives in turn have shown to impart cytotoxic effects (Safer & Al-nughamish, 1999; Sarafian *et al.*, 2002). The antioxidant mechanism of nitrite is not fully clear, nonetheless proposed mechanisms include: the sequestering of oxygen

making it unavailable for participation in deteriorative reactions; formation of a stable complex between nitrite derivatives and haem iron (Fe^{2+}) inhibiting release of this metal catalyst; and stabilising unsaturated fatty acids by forming nitro-nitroso derivatives (Igene *et al.*, 1985; Freybler *et al.*, 1993; Honikel, 2008; Andrée *et al.*, 2010).

A much-appreciated cured meat flavour is probably achieved by the balance of naturally occurring meat sulphur volatile compounds, in combination with the suppression of oxidation products, like hexanal (Sindelar & Milkowski, 2012; Thomas *et al.*, 2013). Indeed, no single volatile compound is linked to the action of nitrate and/or nitrite to create the typical cured meat flavour (Flores, 2018), however, reducing the amount of nitrate/nitrite increased the amount of VOCs in chorizo (Hospital *et al.*, 2015). This phenomenon was explained by higher counts of gram-positive catalase positive cocci (GCC+) as a consequence of nitrate/nitrite reduction, rather than a direct effect of nitrate/nitrite on the formation of volatile compounds. Supporting this study, Perea-sanz *et al.* (2018) observed an increase in amino acid oxidation when nitrate was reduced in dry-cured fermented sausages, evident by the increased amount of volatile compounds originating from branched chain amino acids. Since many microbial strains in dry-cured fermented sausages can partake in amino acid degradation (proteolysis), the consequence of reducing nitrate most likely influenced the microbial flora in the sausage and indirectly, the volatile profile. A few other authors have found a correlation between nitrite addition and the increase of specific volatile compounds in dry-cured fermented sausages, such as, methyl ketones: 2-pentanone; 2-heptanone; 2-octanone; 2-nonanone and secondary alcohols: 2-pentanol; 2-heptanol; and 1-octen-3-ol (Marco *et al.*, 2006; Olivares *et al.*, 2009a). These ß-oxidation products originate from microbial metabolism (Gilles, 2009; Mainar *et al.*, 2017). Additionally, Marco *et al.* (2006) reported a drastic increase in lactic acid bacteria metabolic by-products (i.e. 2,3-butanediol and acetoin) in nitrite containing dry-cured sausages.

A few studies demonstrated the potent antioxidant effect of nitrite even when minute amounts were added. The typical, desirable cured meat aroma was more prominent in hams treated with 50 mg/kg (ppm) nitrite, in comparison with 0.02% BHT or 1000 mg/kg citric acid (Mac Donald *et al.*, 1980). The increase in the cured meat aroma intensity was directly correlated to the decrease in rancid, off flavours. Morrissey and Tichivangana (1985) reported a significant reduction in TBARS in a cooked meat models in the presence of 20 mg/kg nitrite and a highly significant reduction at a 50 mg/kg concentration. Other authors have also demonstrated the antioxidant effect of nitrite at 40 mg/kg in mortadella (Al-Shuibi & Al-Abdullah, 2002).

The same nitrite chemistry involved in creating the nitrosomyoglobin colour pigment (subsequently reviewed) is suggested to be involved in the antioxidant activity (Sebranek & Bacus, 2007), especially with regard to reaction with myoglobin.

3.2.2. Colour formation

Since consumers tend to directly associate meat quality with appearance (Verbeke *et al.*, 2005; Font-i-Furnols & Guerrero, 2014), the stable colour formation effect of nitrite is valuable for the meat industry. As with other curing effects (antibacterial and antioxidant) the reduction of nitrate to nitrite is a prerequisite for the colour formation to occur (Hammes, 2012). The reduction of nitrate is predominantly described in literature as a microbial enzymatic reaction, however, the possibility of chemical nitrate reduction independent of microbial metabolism is also proposed (Hammes, 2012).

Meat colour stability is rather complex (Mancini & Hunt, 2005) and the addition of nitrite to cured meat makes it even more so (Gøtterup *et al.*, 2008). The hue and chroma of fresh red meat depends on the quantity and chemical state of the haem protein, myoglobin (Mancini & Hunt, 2005; Yin *et al.*, 2011; Neethling *et al.*, 2017; Purslow *et al.*, 2020). In turn, the physical structure (i.e. the myofibril diameter and sarcomere length) and pH of the muscle influence light shattering and in turn the lightness or darkness (CIE L*) of meat (Hughes *et al.*, 2019; Purslow *et al.*, 2020). However, this review will focus on the influence of the myoglobin chemistry on meat colour as a result of nitrate/nitrite addition.

The sixth reversable bonding site and the redox state (ferrous, Fe^{2+} or ferric, Fe^{3+}) of the central iron atom in the haem ring creates the possibility of four different myoglobin chemical states in fresh meat (Mancini & Hunt, 2005). Additionally, a fifth chemical state is found in cured meats, as summarised in Table 3.1.

Table 3.1 Different myoglobin chemical states affecting meat colour[1]

Myoglobin chemical state	Reaction	Ligand in the 6th binding site	Haem iron redox state	Colour
Oxymyoglobin	Oxygenation	O_2	Fe^{+2}	Bright, cherry-red
Metmyoglobin	Oxidation	O_2	Fe^{+3}	Brown
Deoxymyoglobin	Oxidation and reduction	No ligand	Fe^{+2}	Purple-red
Carboxymyoglobin	Carboxymyoglobin formation	CO/O_2	Fe^{+2}	Bright red
Nitrosomyoglobin	Nitrosolyted	NO	Fe^{+2}	Red to pinkish

[1]Compiled from Mancini and Hunt (2005).

The typical colour of cured meat is accomplished through binding of nitric oxide (NO) in the sixth reversable binding site of the myoglobin haem iron to create the pink-red, heat stable nitrosomyoglobin (NOMb) pigment as illustrated in Table 3.1 (Skibsted, 2011; Hammes, 2012; Taormina, 2014). Upon heat exposure the protein moiety denatures, however, the porphyrin ring
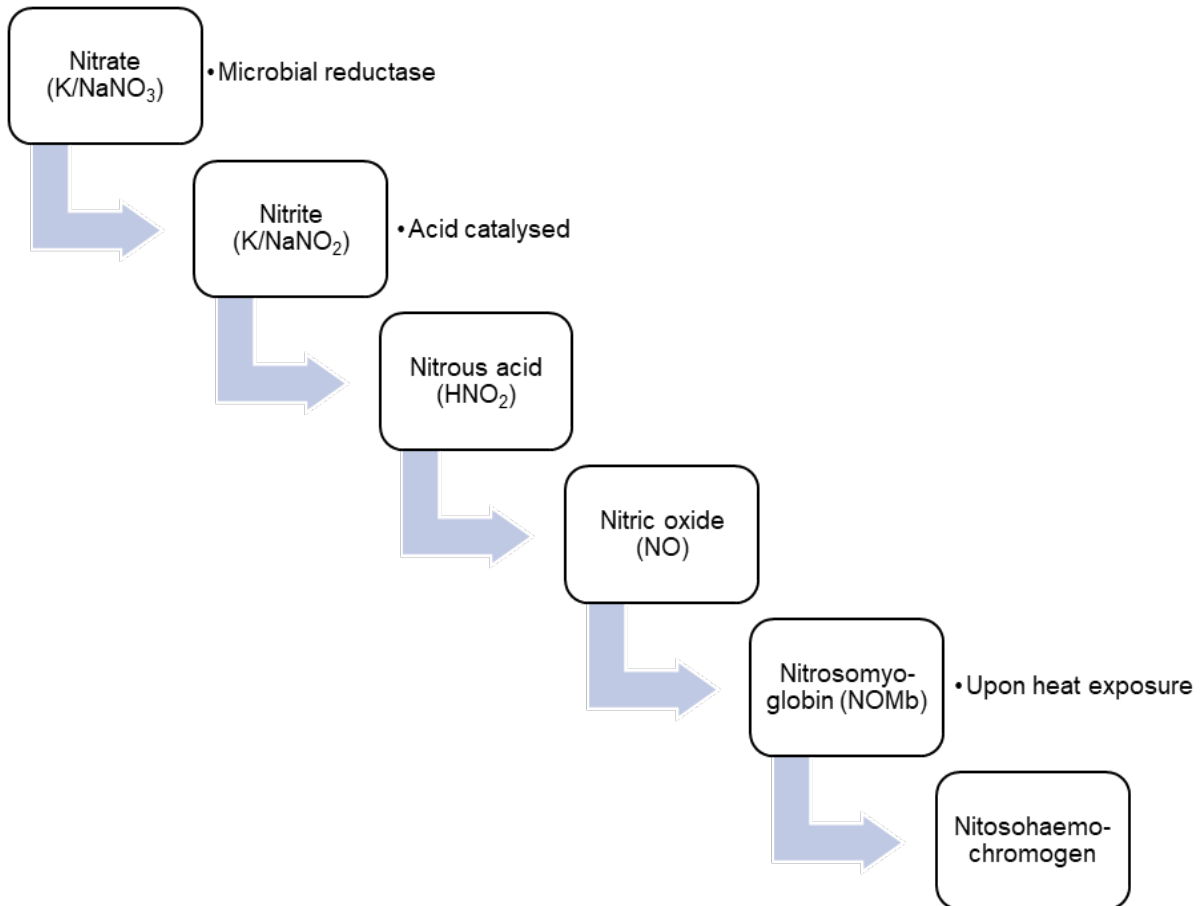
remains stable, which is now known as nitrosohemochromogen and this is more pinkish in colour as compared to NOMb (Honikel, 2008; Hammes, 2012; Taormina, 2014). After nitrate reduction to nitrite, nitrous acid is formed by an acid catalysed reaction, which decomposes into nitric oxide which is capable of binding to myoglobin, creating NOMb (Fig. 3.1), (Taormina, 2014). The formation of NOMb in salami is much quicker when nitrite is used as opposed to nitrate, reaching a maximum NOMb concentration within two days of fermentation (Gøtterup *et al.*, 2008).

Microorganisms play a pivotal role in the cured colour formation due their nitrate reductase capabilities, especially when nitrate as opposed to nitrite is added (Gøtterup *et al.*, 2008; Hammes, 2012). Coagulase negative staphylococci and members of the *Kocuria* (*Micrococcacea*) family are commonly added to salami for their nitrate reductase activity (Casaburi *et al.*, 2005; Sebranek & Bacus, 2007), however, some lactic acid bacteria strains have also exhibited nitrate reductase activity, including: *L. sakei*; *L. plantarum*; and *L. farciminis* (Hammes, 2012; Landeta *et al.*, 2013).

In the case of exclusive nitrate addition, the amount and formation rate of NOMb depends on the concentration of and nitrate reducing ability of the microbial strains in the meat (Gøtterup *et al.*, 2008; Mainar & Leroy, 2015). These bacteria may be sensitive towards processing conditions, emphasizing the importance of process optimisation to achieve successful nitrate reduction, especially in the case of limited added nitrate and/or microbial strains with nitrate reductase activity (Mainar & Leroy, 2015; Stavropoulou *et al.*, 2018). Processing factors to consider include: oxygen availability (Talon & Leroy, 2014); pH (Mainar & Leroy, 2015); and temperature (Casaburi *et al.*, 2005). When the meat environment becomes oxygen limited, the low O_2 partial pressure leads to the dissociation of O_2 in the sixth bonding site of the myoglobin haem iron, allowing NO to bind in this position. Furthermore, the limited oxygen supply forces bacteria with nitrate reductase activity to utilise nitrate as terminal acceptor as opposed to oxygen, ultimately generating nitrite (Hammes, 2012; Majou & Christieans, 2018). An acidic environment (pH = 5.0-6.0) seems to be sufficient to dissociate nitrous acid to yield NO (Sobko *et al.*, 2005; Talon & Leroy, 2014; Majou & Christieans, 2018) again highlighting the role of lactic acid bacteria in the curing process (Gøtterup *et al.*, 2008). Temperatures above 30°C have shown to promote the growth of certain CNS strains (Casaburi *et al.*, 2005), however, the nitrate reduction process can also successfully occur at lower temperatures (15-20°C), (Mainar & Leroy, 2015). Other factors may accelerate or aid in nitrosomyoglobin formation, such as a high salt (NaCl) or ascorbate (vitamin C) content, commonly associated with salami (Skibsted, 2011). Sodium chloride can react with nitrite, subsequently forming the strong nitrosalyting agent known as nitrosohypochloride (NOCl), which accelerates NOMb formation (Møller & Skibsted, 2002; Skibsted, 2011).

Very little nitrite is required to form this stable colour in cooked cured meat (2-14 mg/kg), but much higher concentrations are needed for homogenous curing and a long-lasting effect (Sofos *et al.*, 1979; Sebranek & Bacus, 2007). Likewise, as with the cured meat flavour formation (the

antioxidant effect), an added amount of nitrite between 25-50 mg/kg is likely sufficient to create a satisfactory cured meat colour (Sebranek & Bacus, 2007; Sindelar & Milkowski, 2011).

Nitrate (K/NaNO$_3$) • Microbial reductase

Nitrite (K/NaNO$_2$) • Acid catalysed

Nitrous acid (HNO$_2$)

Nitric oxide (NO)

Nitrosomyo-globin (NOMb) • Upon heat exposure

Nitosohaemo-chromogen

Figure 3.1 Summary of the nitrite chemistry involved in the colour formation of cured meats.

3.2.3. Antimicrobial effect

As with the chemistry behind the cured meat colour formation, the potent antimicrobial action of nitrite is probably not fully understood, although many different modes of action are proposed in literature. Sofos *et al.* (1979) extensively reviewed proposed antimicrobial mechanisms, however, many of these hypotheses were equivocal in literature at that time, like the inhibitory effect of nitrite on *Clostridium* spore germination and increased effect on spore heat susceptibility. Also, researchers differed over the significance of the ingoing amount of nitrite, versus the residual amount (Sofos *et al.*, 1979). Benedict (1980) subsequently reviewed a variety of proposed antimicrobial mechanisms of nitrite and nitrous acid. These included: interference with carbohydrate metabolism; destructive action towards cell membranes altering transport in and out of the cell; essential enzymatic inhibition; the deamination of nucleic acid bases; the interference of iron-sulphur proteins, metal chelating and subsequent hindering of essential co-factor uptake, to name a few. Other researchers discussed the destruction of iron-sulphur proteins (Cammack *et al.*, 1999) and DNA base removal by nitrous acid causing faulty DNA replication (Paik & Lee, 2014). The intricate, multitargeted antimicrobial action of nitrite and its derivatives are evident from these past reviews. Today, the role of nitric oxide and its derivatives in the curing process are perhaps better understood.

Peroxynitrite ($ONOO^-$) is a potent oxidant and antimicrobial agent capable of disrupting proteins, DNA and lipid structures, ultimately causing bacterial cell destruction (Denicola *et al.*, 1998; Lindemann *et al.*, 2013; Majou & Christieans, 2018). Nitric oxide can react to yield $ONOO^-$ by two distinct pathways which could co-exist *in vivo* (Majou & Christieans, 2018). The first entails the reaction of nitric oxide with neutrophil-derived superoxide anion (O_2^-) yielding $ONOO^-$ (Fig. 3.2). Nitrite can alternatively react with hydrogen peroxide (H_2O_2) to yield $ONOO^-$ (Fig. 3.3), (Denicola *et al.*, 1998; Radi, 2013; Majou & Christieans, 2018).

$$O_2^- \cdot + NO \cdot \rightarrow ONOO^-$$

Figure 3.2 The reaction between superoxide anion and nitric oxide yielding peroxynitrite.

$$NO_2^- + H_2O_2 \rightarrow ONOO^- + H_2O$$
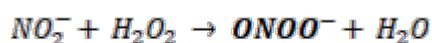
Figure 3.3 The reaction between nitrite and hydrogen peroxide yielding peroxynitrite.

The effectiveness of $ONOO^-$ is highly pH dependent, as the protonated form of peroxynitrite, known as peroxynitrous acid (ONOOH), can cross bacterial cell membranes more easily via passive diffusion (Cammack *et al.*, 1999; Majou & Christieans, 2018). A pH difference of one unit showed a large difference in membrane permeability, were ONOOH had significantly higher permeability at a pH of 5.0, as compared to a pH of 6.0 (Majou & Christieans, 2018). The oxidative stress brought about by $ONOO^-$/ONOOH is thus not exclusively dependent on the NO_2, and subsequent NO, O_2, and H_2O_2 concentrations, but also the matrix pH (Majou & Christieans, 2018).

The effectiveness of nitrite against *Clostridium botulinum* (Christiansen *et al.*, 1974; Sofos *et al.*, 1979; Benedict, 1980) is probably how it gained popularity amongst antimicrobial agents, as *C. botulinum* is a lethal and feared pathogen historically associated with processed meat products, especially sausages (Leroy *et al.*, 2013). Emile Pierre van Ermengem, a Belgian Scientist first identified *C. botulinum* after an intoxication outbreak in 1985 caused by salted ham (Leroy *et al.*, 2013; Grenda *et al.*, 2014).

Toxin producing clostridia (*C. botulinum* and *C. perfringens*) are of major concern in certain foodstuffs as toxin ingestion can cause a severe neurological disease in both humans and animals, known as botulism (Cammack *et al.*, 1999; Dahlsten *et al.*, 2008). Botulism neurotoxins are in fact the most lethal known to man, with the ingestion of as little as 30-100 ng being potentially fatal (Peck, 2009). Seven different types of botulinum toxins have been identified, namely A to G (Merialdi *et al.*, 2016), however, only four of these are relevant to human disease related strains (A, B, E and F) (Dahlsten *et al.*, 2008). The two metabolic groups involved are, proteolytic (with the ability to hydrolyse proteins) and non-proteolytic clostridia strains. Proteolytic strains can produce toxins A, B and F and non-proteolytic strains B, E or F (Dahlsten *et al.*, 2008; Merialdi *et al.*, 2016). The latter, being psychotropic, seems to be of primary concern in chilled foods, able to grow and produce toxins at refrigerated temperatures (Lynt *et al.*, 1982; Graham *et al.*, 1997; Peck *et al.*, 2008). On the other hand, proteolytic toxin production can occur in acidic environments with a pH as low as 4.2 (Smelt *et al.*, 1982). A much higher salt concentration is required to inhibit growth and toxin production of proteolytic (10%) as compared to non-proteolytic (5%) clostridia strains (Lynt *et al.*, 1982).

Based on these parameters, salami intrinsic parameters may support the growth of proteolytic or non-proteolytic toxin producing clostridia strains, having a favourable pH, salt concentration and storage temperature (either refrigerated or ambient). Although not fully appreciated by consumers, botulism is said to be "completely controlled by nitrite" (Sindelar & Milkowski, 2011). Before the industrial application of nitrite, botulism was a feared illness associated with sausages. In fact, the word "botulism" originates from the Latin word, *butulus*, meaning "sausage" (Leroy *et al.*, 2013).

The *Clostridium* genus is highly susceptible to oxidative stress (Majou & Christieans, 2018) and thus also to the effect of the free radicals originating from nitrate/nitrite. These compounds are said to interact with the iron-sulphur proteins of clostridial species causing a rapid decrease in internal adenine triphosphate (ATP) and pyruvate (Hospital *et al.*, 2016). *Clostridium botulinum* can successfully be inhibited in salami without the addition of nitrate and/or nitrite, purely as a result of the combination of multiple hurdle effects (competitive lactic acid bacteria growth, pH and a_w reduction and temperature control) (Hospital *et al.*, 2016). However, the pathogenic risk is greater when one of these hurdles are not successfully met. The germination process of *C. botulinum*

strains might take many days (Hospital *et al.*, 2016), emphasising the vital role of residual nitrite in salami over storage time.

Although the antimicrobial mechanism is not clarified, nitrite showed an inhibitory effect against *Staphylococcus aureus* (Tompkin *et al.*, 1973), *Enterobacteriaceae* (Hospital *et al.*, 2015; Cardinali *et al.*, 2018)*, Listeria* (Hospital *et al.*, 2012), *Salmonella* (Hospital *et al.*, 2014) and overall bacterial diversity (Cardinali *et al.*, 2018) in processed meats. While nitrate and/or nitrite showed no effect against other spoilage microorganisms such as *Proteus vulgaris* and *Serratia liquefaciens* (Hospital *et al.*, 2017). Lactic acid bacteria proliferation has proven to be unaffected by nitrate and/or nitrite (Hospital *et al.*, 2012, 2016; Cardinali *et al.*, 2018). The capability of some lactic acid bacteria strains to proliferate better in the presence of nitrate and/or nitrite than others is probably due to adaption of their membrane structures, amino acid sequence of their DNA or vital enzymes, as highly reactive by-products of nitrite usually cause destruction in these structures (Benedict, 1980; Paik & Lee, 2014). Alternatively, their resistance can be due to their nitrite reductase activity which is described as a detoxifying mechanism in literature (Dodds & Collins-Thompson, 1984; Wang *et al.*, 2013; Paik & Lee, 2014). Conversely, lactic acid bacteria strains with nitrite reductase activity did not necessarily proliferate the best when exposed to nitrite (Paik & Lee, 2014) and strains which proliferated best did not exhibit nitrite reductase activity (Dodds & Collins-Thompson, 1984). Other mechanisms explaining the resistance of lactic acid bacteria have been proposed based on their fermentation pathways, with homofermentative strains being more robust and less sensitive to nitrite (Dodds & Collins-Thompson, 1984). The nitrite reducing ability of moulds and yeasts probably also explain their proliferation in nitrate/nitrite added cured meats (Morton, 1956; Tachiki *et al.*, 2016). The ability of lactic acid bacteria, yeasts and moulds to proliferate in the presence of maximum permitted nitrate and/or nitrite in salami is nonetheless, highly beneficial for salami production.

The antimicrobial effect of nitrite is emphasised as part of a multiple hurdle strategy (Paik & Lee, 2014) and its role is evident when considering the many foodborne botulism outbreaks from cured meats of home-produced origin, free of nitrate and/or nitrite (Sofos *et al.*, 1979). Although nitrite and its derivatives play a crucial role in the microbial safety of salami, one cannot neglect other intrinsic and extrinsic factors such as pH, a_w and temperature to combat the growth of unwanted bacteria (Hospital *et al.*, 2017; Majou & Christieans, 2018). If latter hurdles are not met, a serious food safety risk might develop, especially at the onset of fermentation when temperature, pH and a_w favour the growth of pathogens (Hospital *et al.*, 2015, 2016). In fact, previous studies have reported the inhibitory effect of nitrite to be dependent on pH, NaCl concentration, temperature and oxygen availability, of which a sufficient pH reduction seems to be the determining factor for success (Castellani & Niven Jr, 1955; Buchanan & Solberg, 1972; Tompkin *et al.*, 1973; Sofos *et al.*, 1979; McClure *et al.*, 1991).

3.3. Nitrite and nitrate as a public concern

Ingestion of a high concentration of nitrite is known to be poisonous. Accidental ingestion of nitrite contaminated drinking water (Bradberry *et al.*, 1994) and sausages (Kennedy *et al.*, 1997) have resulted in adverse health effects. The ability of haemoglobin to bind and transport oxygen through the blood is dependent on the presence of a bound haem-group (Berg *et al.*, 2015). The iron centre in the haem group can exist in both the ferrous (Fe^{2+}) or ferric (Fe^{3+}) state, however, the latter is incapable of binding oxygen (Berg *et al.*, 2015). Nitrite has the ability to oxidize ferrous iron to the ferric state, forming methaemoglobin (MetHb) unable to bind and transport oxygen successfully throughout the body (Kohn *et al.*, 2002). This condition is especially fatal in infants and can cause what is known as the blue baby syndrome as a result of cyanosis which creates a blue discoloration of the skin due to poor oxygen transport through the body (Knobeloch *et al.*, 2000; Savino *et al.*, 2006). Nitrate on the other hand, occurs in high concentrations in a variety of plant sources (especially vegetables) and the consumption thereof is promoted for cardiovascular health benefits (Hord *et al.*, 2009; Sindelar & Milkowski, 2012; Butler, 2015; Bedale *et al.*, 2016; Colla *et al.*, 2018).

With regard to processed meats, nitrite is clearly a highly effective and multifunctional compound (as reviewed earlier) and could be irreplaceable (Sebranek & Bacus, 2007; Sindelar & Milkowski, 2011). Nonetheless, the use of curing agents have been scrutinised in an ongoing debate since the formation of carcinogenic *N*-nitrosamines (e.g. *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine) as a result of nitrite exposure under certain conditions in the early 1970s in fried bacon (Cassens, 1997; Sebranek & Bacus, 2007; De Mey *et al.*, 2014; Herrmann *et al.*, 2015). Nitrosamine formation occurs when nitrite is converted to nitric oxide and subsequently reacts with secondary amines in an acidic environment under a high temperature exposure (> 130°C), such as frying (Honikel, 2008; Sindelar & Milkowski, 2011; Hammes, 2012; Butler, 2015; Herrmann *et al.*, 2015; Bedale *et al.*, 2016).

The residual amounts of nitrate in processed meats (< 20 mg/kg; Honikel, 2008; Milkowski *et al.*, 2010; De Mey *et al.*, 2014) are generally much lower than the concentration found in plant sources (Bahadoran *et al.*, 2016). Indeed, the contribution of cured meats to dietary nitrate and nitrite in modern-day is said to be negligible (Leroy *et al.*, 2013) as compared to the vast amounts of these products consumed from eating certain plants, drinking water and by means of natural production thereof in human saliva (Archer, 2002; Sindelar & Milkowski, 2012). The mistrust in nitrates/nitrites is probably contributed to correlations found between the consumption of red and processed meats and cancer (Xu *et al.*, 2013) and the World Health Organization's (WHO) recommendation to consume preserved meats in moderation (World Health Organization, 2003). Additionally, the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) announced an increased risk of colorectal cancer with the high consumption of processed meat in 2007 (Demeyer *et al.*, 2008). However, publications used to substantiate this

statement were subjected to criticism. For instance, the results were based on dietary questionnaires which lacked differentiating between the meat products consumed, e.g. whether fermented or non-fermented, heat treated or not, or which preservatives they contained (Demeyer *et al.*, 2008). The health risks related to consuming dry-cured fermented meats specifically are thus still vague. Regardless, the mistrust in the use of curing agents in meat are reflected in the ongoing revision of the legally permitted maximum and minimum amounts of nitrate and nitrite in foodstuffs (Mortensen *et al.*, 2017b,a). Discrepancies amongst countries complicate this dilemma even further. Denmark for instance requested yet another revision of the European Union (EU) maximum permitted limits in 2007, convinced that lower amounts are sufficient to achieve food safety (Hospital *et al.*, 2014). Denmark allows a maximum permitted amount of 60 mg/kg of nitrite to the majority of processed meat products (with exceptions), in comparison to the EU (Directive 2006/52/EC) allowing a maximum of 150 mg/kg (Herrmann *et al.*, 2015). This contradictory demand to eliminate preservatives whilst wanting safe and nutritious food is not limited to nitrate and nitrite salts. This seems to be a general trend amongst modern consumers (Bedale *et al.*, 2016).

Regardless of the continuous debate surrounding curing agents in meat, consumer demand is still the predominant factor influencing new product development. Consumers are becoming increasingly aware of food additives and accordingly favour cleaner labelled products, including reduced nitrate/nitrite meat products (De Andrade *et al.*, 2017; Di Vita *et al.*, 2019). The food industry is faced with a rather contradicting situation, as consumers equally fear food additives, as they do foodborne diseases which is foremostly the reason for the use of these additives, including nitrite (Bedale *et al.*, 2016). Negative consumer perspective surrounding food additives are subscribed to a lack of knowledge or understanding their application and purpose in food products (Bedale *et al.*, 2016). Nonetheless, the effect of lowering the amount of ingoing nitrate and/or nitrite in dry-cured and fermented sausages have been studied extensively (Cenci-goga *et al.*, 2012; Hospital *et al.*, 2015; Mainar *et al.*, 2016; Cardinali *et al.*, 2018; Christieans *et al.*, 2018; Perea-Sanz *et al.*, 2019). From a food safety point of view, the benefits of the controlled use of nitrate and nitrite as food additives may overshadow the potential dangers (Cassens, 1997; Honikel, 2008).

3.4. Alternatives for nitrate and nitrite in cured meat

To eliminate nitrite from processed meats would pose a number of challenges, including: the control of unwanted microbial growth; achieving unique cured meat characteristics (colour and flavour); cost increase; and consumer dissatisfaction (Sindelar & Milkowski, 2011).

One approach to eliminating the direct addition of nitrite to meat products would be by adding a combination of nitrate reducing bacteria in combination with natural extracts high in nitrate, including: celery (Sebranek & Bacus, 2007; Magrinyà *et al.*, 2009; Alahakoon *et al.*, 2015; Posthuma *et al.*, 2018); beetroot (Sucu & Turp, 2018); and parsley (Riel *et al.*, 2017). This approach

to produce so called "naturally cured meats" is, however, a mere pseudosolution and may create additional challenges, such as amendments to labelling regulations and inconsistent amounts of added nitrate and subsequently also residual nitrite (Sebranek & Bacus, 2007).

Another interesting research focus found in literature, is the use of microbial strains with the ability to convert myoglobin to nitrosomyoglobin in the absence of added nitrate and/or nitrite. The latter can occur through inherent nitric oxide synthase (NOS) enzyme activity capable of forming nitric oxide from the amino acid, L-arginine in the presence of NAPDH (Arihara *et al.*, 1993; Mainar *et al.*, 2017; Ras *et al.*, 2018a,b). This revolutionary discovery lead to researchers investigating the possibility of these so-called reddening strains to replace nitrate and nitrite as colour fixing agents in cured meats (Morita *et al.*, 1998; Møller *et al.*, 2003; Li *et al.*, 2013; Mainar & Leroy, 2015). Furthermore, the use of bacteriocin producing staphylococci in nitrate/nitrite free salami may achieve a twofold function towards colour development and bioprotection (Mainar *et al.*, 2016).

However, the research topic receiving the most attention would be the application of plant extracts exhibiting antimicrobial and/or antioxidant capacity in meat products (Falowo *et al.*, 2014; Shah *et al.*, 2014; Aziz & Karboune, 2018; Jin *et al.*, 2018; Nikmaram *et al.*, 2018; Martínez *et al.*, 2019). Polyphenols consist of a large group of biological active compounds and are ubiquitously found in plants (e.g. fruits, vegetables, herbs and spices, seeds and hulls, tree bark, wine and teas) as secondary metabolites aiding in protection against toxins, radiation and pathogens (Moure *et al.*, 2001; Iranshahi *et al.*, 2015). The chemical structure of flavonoids (the latter forms part of the polyphenol group) is what creates their antioxidant activity, having locational double bonds, *o*-dihydroxy and hydroxyl groups in their ring structures (Tapas *et al.*, 2008). Polyphenolic compounds and ascorbic acid are acknowledged as the most important natural antioxidants in foodstuffs (Choe & Min, 2009). The proposed structure related antioxidant mechanisms of polyphenolic compounds include: chain breaking through scavenging of lipid peroxyl radicals; hydrogen donation to free radicals; singlet oxygen quenching; and pro-oxidant metal chelation (Rice-Evans *et al.*, 1996). The interest in natural extracts in meat is evident by the 22 peer-reviewed articles published in *Food Research International* scientific journal dedicated to natural antioxidants to reduce the oxidation process of meat and meat products (last updated in 2019 https://www.sciencedirect.com/journal/food-research-international/special-issue/10L4PKNM5MX). Furthermore, the authorization of rosemary extract in meat products with an official E-number (E-392) granted by the European Union (Directive 95/2/EC), illustrates the commercial feasibility of natural extracts in meat (Teruel *et al.*, 2015). Although a vast amount of literature is available on the substitution of nitrate/nitrite with natural extracts, this literature review will only highlight a few studies.

Rape (*Brassica campestris* L.) bee pollen rich in flavonoids (quercetin, rutin and kaempferol) decreased the PV and TBARS in salami (Zhang *et al.*, 2016). A combination of plant extracts and

reduced sodium nitrite showed a synergistic antibotulinal effect in a meat model (Cui *et al.*, 2010) and the addition of tomato peel resulted in acceptable textural and sensory characteristics in dry-cured fermented sausages (Calvo *et al.*, 2008). Deda *et al.* (2007) demonstrated the feasibility to reduce the ingoing amount of nitrite from 150 mg/kg to 100 mg/kg when combined with 12% tomato paste without negatively effecting the characteristics of frankfurters. Doolaege *et al.* (2012) concluded that as little as 80 mg/kg nitrite was sufficient to produce liver pâté with acceptable colour and lipid stability with the aid of rosemary extract. *Coriandrum Sativum* L. essential oil showed successful antioxidant activity in Italian salami, even more so compared to the synthetically used butylated hydroxytoluene (BHT) (Marangoni & Moura, 2011), whilst *Origanum vulgare* extract showed an equal antioxidant capacity as compared to sodium erythorbate in sheep sausages without altering the physicochemical and sensory quality of the product (Fernandes *et al.*, 2018). Furthermore, rosemary essential oil in Iberian pork frankfurters reduced TBARS, hexanal and carbonyls from protein oxidation, although only effective in high added amounts (300 and 600 mg/kg) (Estévez & Cava, 2006). Alirezalu *et al.* (2019) successfully produced nitrite-free frankfurter-type sausages with a combination of natural antimicrobial and antioxidant compounds and proposed polylysine, chitosan and a mixed extract of green tea, stinging nettle, and olive leaves as a potential nitrite replacement. Although the application of a variety of natural extracts in meat have been studied, consumers have a higher prevalence for familiar tastes associated with meat like thyme or rosemary, while green tea for instance is seen as unacceptable or unusually perceived in meat products (Hung *et al.*, 2016).

Stakeholders in a European study by Hung *et al.* (2016) agreed that the complete elimination of nitrite is not practical, especially when the desired pink-red colour is considered, while others argued that complete substitution is possible, but will be undoubtfully challenging. Perhaps a more realistic approach is the so called, multiple hurdle effect. This would imply the combination of lower levels of nitrite/nitrate combined with advanced processing steps and the addition of natural compounds exhibiting antimicrobial and/or antioxidant effects (Alahakoon *et al.*, 2015).

3.5. Honeybush (*Cyclopia subternata*)

Cyclopia subternata Vogel (Family: Fabaceae; Tribe: Podalyrieae), locally known as honeybush tea, is native to South Africa and commercially produced as a hot beverage after fermentation (Joubert *et al.*, 2008a; De Beer *et al.*, 2012). Of the 23 known *Cyclopia* species, only three have commercial importance, of which *C. subternata* is one (De Beer & Joubert, 2010; Joubert *et al.*, 2011). The characteristic sweet taste and brick-brown appearance of the tea are achieved with optimal time-temperature fermentations (e.g. 90°C, 24h for *C. subternata*) (Stepanova *et al.*, 2012; Erasmus *et al.*, 2017). Green honeybush in contrast, refers to the cut and dried plant material without the fermentation step (Joubert *et al.*, 2011). Coarse waste material with a high stem content unsuitable for commercial tea can be used for extract purposes (Joubert *et al.*, 2008a). In this sense, a non-

commercial honeybush by-product rich in antioxidants can be optimally used. Du Preez *et al.* (2016) demonstrated the feasibility to produce hesperidin-enriched *C. maculata* extract from a by-product high in stems for increase profitability of the South African honeybush industry. Recently, Joubert *et al.* (2019) compiled a 20-year milestone review regarding research on *Cyclopia* species. There the authors summarized the standard extraction procedure as 1:10 mass to solvent ratio with 30 min extraction time at 93°C (Joubert *et al.*, 2019). The quality of these extracts are regulated by determining the total polyphenol content (TP) and the total antioxidant activity (TAA) (Joubert *et al.*, 2008a, 2011). Although the polyphenolic composition differs between species, xanthones (mangiferin and isomangiferin) and a flavone (hesperidin) occur in all species (Joubert *et al.*, 2008a).

The growing demand in health promoting, antioxidant rich foods gained honeybush its international recognition and the economic importance for Southern Africa (Kokotkiewicz *et al.*, 2012). Both fermented and unfermented honeybush have proved to exhibit antioxidant capacity by means of the 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and lipid peroxidation (LPO) assays (Joubert *et al.*, 2008a; De Beer *et al.*, 2012; Magcwebeba *et al.*, 2016). Due to the detrimental effect of high temperature fermentation on certain polyphenols, the green, unfermented honeybush state is preferred for extract purposes (Joubert *et al.*, 2008b).

Cyclopia subternata, as with the other *Cyclopia* species, has proven to be rich in polyphenolics, including the flavonoids: hesperidin (major flavonoid constituent); narirutin; eriocitrin; epigallocatechin gallate; the flavones: luteolin; scolymoside; orobol; and the xanthones: mangiferin; and isomangiferin (Kamara *et al.*, 2004; De Beer *et al.*, 2009; De Beer & Joubert, 2010). More recently, a benzophenone (iriflophenone-3-C-β-glucoside); dihydrochalcone (phloretin-3',5'-di-C-β-glucoside); flavone, isorhoifolin (Kokotkiewicz *et al.*, 2012); iriflophenone-di-*O*,*C*-hexoside, (*R*)-, (*S*)-eriodictyol-di-*C*-hexoside; vicenin-2; and 3-hydroxyphloretin-3',5'-di-*C*-hexoside (De Beer *et al.*, 2012) were also identified and judging by their chemical structures, these compounds should contribute to the protective, antioxidant capacity of *C. subternata*. Subsequently, rapid methods were developed for screening of xanthone content in *C. subternata* (Joubert *et al.*, 2012)*.

Hesperidin (Hsd), first isolated by Lebreton (1827), belongs to the flavonoid polyphenolic group and is primarily associated with citrus fruits (Iranshahi *et al.*, 2015). The Hsd molecule consists of an aglycone unit called, hesperetin (Hst) and a disaccharide, rutinose, which consists of glucose and rhamnose. Hesperidin, Hst and its derivatives exhibit a variety of bioactive functions, including antimicrobial and antioxidant ability through radical scavenging, metal chelating and malondialdehyde formation inhibition (Lee *et al.*, 2003; Iranshahi *et al.*, 2015). Furthermore, mangiferin proofed to be a potent O^{2-} scavenger *in vitro* (Leiro *et al.*, 2003). These modes of

antioxidant effects are in accordance with Frei and Higdon (2003) who reviewed and summarized the potential antioxidant mechanisms of tea polyphenolics.

The three commercial species: *C. genistoides*; *C. longifolia;* and *C. subternata* share common sensory traits described as fynbos-floral and fruity-sweet, with the exception of *C. subternata* lacking in a bitter taste which could be detected in its counterparts due to faulty fermentation (Theron *et al.*, 2014; Erasmus *et al.*, 2017; Alexander *et al.*, 2019). Variation in volatile compounds between *Cyclopia* species (either qualitatively or quantitatively) are responsible for the unique sensory profiles of each species (Ntlhokwe *et al.*, 2018). Le Roux *et al.* (2012) identified a total of 183 VOCs in *C. subternata* of which the majority were terpenoids (56%). Furthermore, typical honeybush flavours were detected by the gas chromatography-olfactometry (GC-O) assessors and 37 aroma active compounds were present, of which the most important were: (*E*)-*β*-damascenone; (*R/S*)-linalool; (*E*)-*β*-damascone; geraniol; (*E*)-*β*-ionone; and (7*E*)-megastigma-5,7,9-trien-4-one (Le Roux *et al.*, 2012). Volatile compounds with an aroma diluent (AFD) factor ≥ 2 are acknowledged as odour active (Le Roux *et al.*, 2012). Four of the identified odorants in *C. subternata* with the highest AFD factors were associated with a sweet, apricot aroma, namely: (*E*)-*β*-Damascenone; (*R/S*)-linalool; geraniol; and (*E*)-*β*-ionone (Le Roux *et al.*, 2012). A more recent study identified a grand total of 283 volatile compounds in *C. subternata* using two dimensional gas chromatography coupled to time of flight mass spectroscopy (GC x GC combined with TOF-MS), highlighting the complexity of the volatile profile of these teas (Ntlhokwe *et al.*, 2018). Although the volatile compound likely responsible for the honey aroma (benzeneacetaldehyde) was shown to be present in all three species, it was primarily associated with *C. genistoides* and *C. subternata* (Ntlhokwe *et al.*, 2018)*.

The major flavonoids in *C. subternata*, Hsd and its aglycone, Hst, have shown to exhibit anti-microbial, antiviral and antifungal activities (Coetzee *et al.*, 2008; Abuelsaad *et al.*, 2013; Iranshahi *et al.*, 2015). Although their inhibitory effect seems to vary between studies (Iranshahi *et al.*, 2015). The xanthone, mangiferin, is considered as antiviral agents used in folk medicines (Yoshimi *et al.*, 2001). Mangiferin and its analogues showed inhibitory effects against bacteria (*Bacillus pumilus*, *B. cereus*, *Salmonella virchow, Staphylococcus aureus* and *Escherichia coli*) and fungal species (*Thermoascus aurantiacus*, *Aspergillus flavus* and *A. niger*) in previous studies (Singh *et al.*, 2009, 2012). Numerous other bioactivities of mangiferin (i.e. anti-inflammatory and anti-tumour) are reviewed elsewhere (Kamara *et al.*, 2004).

Previous researchers have applied rooibos, another indigenous South African tea similarly rich in polyphenolic compounds (Magcwebeba *et al.*, 2016) exhibiting radical scavenging and lipid peroxidation inhibition (Snijman *et al.*, 2009), to extend the shelf life of processed meat products. These included: ostrich salami (Cullere *et al.*, 2013); ostrich droëwors (Hoffman *et al.*, 2014); blesbok and springbok droëwors (Jones *et al*., 2015); and rabbit patties (Cullere *et al.*, 2019). Regardless of the extensive amount of research dedicated to the polyphenolic content and

antioxidant activity of various *Cyclopia* species, the application of honeybush in processed meat products remains unexplored.

3.5.1. Challenges to consider

Cutting and rolling of plant material and high temperature exposure during fermentation for tea production will lead to a loss of bioactive compounds, therefore the green, unfermented state of *C. subternata* is a better alternative for bio-active extract purposes than the oxidative fermentative counterpart (Frei & Higdon, 2003 Joubert *et al*., 2008b). Nonetheless, damaged plant cells during green *C. subternata* extract preparation may leach polyphenol oxidase, causing oxidation of previously compartmentalized antioxidant compounds (Frei & Higdon, 2003). Furthermore, yield, antioxidant capacity and subsequent stability of plant extracts depend on the nature of the extraction solvent used and the processing steps the raw material is subjected to (e.g. time, temperature and pressure combinations) (Moure *et al*., 2001; De Beer *et al*., 2012; Wang *et al*., 2013a). Garrido *et al.* (2011) reported a variety in polyphenolic composition and subsequent lipid oxidation inhibition effect of grape pomace extract due to different extraction methods. In accordance, Teruel *et al.* (2015) found a significant variation in rosemary extract parameters (TP and TAC) due to choice of solvent (acetone or methanol) and format of the extract (powder or liquid). In this regard, many techniques have been developed and improved to increase bioactive compound yield from plants (Azmir *et al.*, 2013; Adiamo *et al*., 2019). Apart from the extraction procedure, inherent plant material variation furthermore contributes greatly to quality variation of plant extracts. In an attempt to aid in quality standardisation of *C. subternata* extracts, De Beer *et al*. (2012) demonstrated a variation in TP and TAC of aqueous extracts according to season, geographical region, agricultural practices and part of the plant used. Moreover, harvest time (summer versus winter) and area (Western seaboard versus Cape Peninsula) significantly affected polyphenolic content in wild *C. genistoides* (Joubert *et al.*, 2014). For extract production, the honeybush plant material is generally not sieved to remove the stem fraction. This varying ratio of stem to leaf ratio not only influences the TP and TAC, but also the extract yield (De Beer *et al.*, 2012; Joubert *et al*., 2019). Knowing this, it is important to consider the effect of drought as a water scarcity will lead to the loss of plant leaves, altering the leaf to stem ratio (Joubert *et al.*, 2019). Environmental stress exposure in turn, may lead to an increase in polyphenolic production in plants and thus an inconsistency in raw material intended for extract purposes (Sharma *et al*., 2019; Toscano *et al*., 2019).

The challenge to produce natural extracts with standardised, high polyphenolic content was highlighted by Pauck (2016), greatly ascribed due to the high variability in phenolic composition of the raw material. Other probable hurdles to overcome when using *Cyclopia* extracts include: degradation and difficulty to work with the extract due to stickiness (Sansone *et al*., 2011); insolubility and impaired wettability (Tomás-Navarro *et al.*, 2014; De Beer *et al.*, 2018); and a

potential bitter taste in the final product in the case of adding certain *Cyclopia* species, especially *C. genistoides* (Theron *et al.*, 2014). The poor insolubility of *Cyclopia* extracts in aqueous matrixes is ascribed to the hesperidin content (Tomás-Navarro *et al.*, 2014), while the association with a bitter taste appears to be correlated mainly with the mangiferin content, amongst other flavanones (Alexander *et al.*, 2019). In the light of commercial feasibility of *Cyclopia* food extracts, a few studies have addressed potential processing challenges. Minimising the aspalathin variation in green rooibos extract production has been explored (Miller *et al.*, 2017) and the extraction yield of xanthone and benzophenone from *C. genistoides* with regard to solvent composition was optimised (Bosman *et al.*, 2017). Beelders *et al.* (2017, 2018) contributed to the understanding of the thermal stability and degradation products of xanthones and benzophenones selected from *C. genistoides.*

The conflicting pro-oxidant effect of polyphenolic compounds in high concentrations is also found in literature. Rooibos extract did not successfully inhibit lipid oxidation in ostrich droëwors, possibly due to the fatty acid profile of the meat (higher PUFAs) and long drying period during which the rooibos could have acted as a pro-oxidant over time (Hoffman *et al.*, 2014). The provoking of antioxidants to become prooxidative is explained by their ability to reduce transition metals (such as Fe^{3+}) to a catalytic active state (Fe^{2+}) capable of reacting with oxygen or hydrogen peroxide, in turn creating oxidation initiators (Moure *et al.*, 2001).

It is noteworthy to mention that flavonoids (including: flavanols; flavanones, i.e. hesperidin; and flavones, i.e. luteolin; flavonols) are generally insoluble in fat and oil, but may portray antioxidant effects in meat and fat emulsions (Choe & Min, 2009). The so called "polar paradox" explains the controversial phenomenon of hydrophilic antioxidants being more effective in protecting bulk oils at the oil-air interfaces, as compared to oil/fat soluble antioxidants in the oil phase. In contrast, lipophilic antioxidants may more effectively inhibit lipid oxidation in oil-in-water emulsions at the oil-water interface as compared to hydrophilic compounds clustered in the aqueous phase (Moure *et al.*, 2001; Di Mattia *et al.*, 2009). Salami is a high fat emulsion product, the effectiveness of honeybush extract as a natural antioxidant may foremostly depend on the lipid affinity of the bioactive compounds. The polarity of the polyphenolic compounds in honeybush extract will define their affinity to the lipid versus the protein fraction in salami. Their localisation (lipid, water or interface) will determine their protective role in the lipid oxidation chain reaction (Di Mattia *et al.*, 2009).

Finally, the characteristic chemical structures of polyphenols make these compounds highly susceptible to degradation as they are sensitive to high temperatures, light, oxygen, moisture and an unfavourable pH (Fang & Bhandari, 2011; De Beer *et al.*, 2018). Storing condition of the extract prior to use will be vital to preserve its functionality (De Beer *et al.*, 2018).

3.6. Conclusions

The food industry, in particular the processed meat industry, is challenged with the demand for less additives in foodstuffs, whilst ensuring food safety and quality. As a consequence, a lot of research has been dedicated to replacing the traditional curing agents nitrate (NO_3) and nitrite (NO_2) in cured meat products, e.g. salami. Based on the evidence supplied in this review, the multipurpose, highly effective NO_3/NO_2 preserving salts are difficult to completely replace in cured meats. Nonetheless, the vast majority of plant extracts portraying antioxidant and/or antimicrobial effects show potential in the partial replacement of NO_3/NO_2 in processed meat products. Among the latter, honeybush (*Cyclopia* spp.) as a bio-active ingredient remains unexplored in this regard.

3.7. References

Abuelsaad, A.S.A., Mohamed, I., Allam, G. & Al-solumani, A.A. (2013). Antimicrobial and immunomodulating activities of hesperidin and ellagic acid against diarrheic *Aeromonas hydrophila* in a murine model. *Life Sciences*, **93**, 714–722.

Adiamo, O.Q., Netzel, M.E., Hoffman, L.C., Sultanbawa, Y. (2019). Acacia seed proteins: Low or high quality? A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, 1-23, https://doi.org/10.1111/1541-4337.12508.

Al-Shuibi, A.M. & Al-Abdullah, B.M. (2002). Substitution of nitrite by sorbate and the effect on properties of mortadella. *Meat Science*, **62**, 473–478.

Alahakoon, A.U., Jayasena, D.D. & Jo, C. (2015). Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science and Technology*, **45**, 37–49.

Alexander, L., De Beer, D., Muller, M., Van der Rijst, M. & Joubert, E. (2019). Bitter profiling of phenolic fractions of green *Cyclopia genistoides* herbal tea. *Food Chemistry*, **276**, 626–635.

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

Andrée, S., Jira, W., Schwind, K.-H., Wagner, H. & Schwägele, F. (2010). Chemical safety of meat and meat products. *Meat Science*, **86**, 38–48.

Archer, D.L. (2002). Evidence that ingested nitrate and nitrite are beneficial to health. *Journal of Food Protection*, **65**, 872–875.

Arihara, K., Kushida, H., Kondo, Y., Itoh, M., Luchansky, J.B. & Cassens, R.G. (1993). Conversion of metmyoglobin to bright red myoglobin derivatives by *Chromobacterium violaceum*, *Kurthia* sp., and *Lactobacillus fermentum* JCM1173. *Journal of Food Science*, **58**, 38–42.

Aziz, M. & Karboune, S. (2018). Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, **58**, 486–511.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N. & Omar, A.K.M. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*, **117**, 426–436.

Bahadoran, Z., Mirmiran, P., Jeddi, S., Azizi, F., Ghasemi, A. & Hadaegh, F. (2016). Nitrate and nitrite content of vegetables, fruits, grain, legumes, dairy products, meats and processed meats. *Journal of Food Composition and Analysis*, **51**, 93–105.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, **120**, 85–92.

Beelders, T., De Beer, D., Ferreira, D., Kidd, M. & Joubert, E. (2017). Thermal stability of the functional ingredients, glucosylated benzophenones and xanthones of honeybush (*Cyclopia genistoides*) in an aqueous model solution. *Food Chemistry*, **233**, 412–421.

Beelders, T., De Beer, D., Kidd, M. & Joubert, E. (2018). Modeling of thermal degradation kinetics of the *C*-glucosyl xanthone mangiferin in an aqueous model solution as a function of pH and temperature and protective effect of honeybush extract matrix. *Food Research International*, **103**, 103–109.

Benedict, R.C. (1980). Biochemical basis for nitrite-inhibition of *Clostridium botulinum* in cured meat. *Journal of Food Protection*, **43**, 877–891.

Berg, J.M., Tymoczko, J.L., Gatto, G.J. & Stryer, L. (2015). Hemoglobin: Portrait of a Protein in Action. In: *Biochemistry* (edited by L. Schultz). Pp. 191–214. New York: W.H. Freeman & Company.

Berger, R.G., Macku, C., German, J.B. & Shibamoto, T. (1990). Isolation and identification of dry salami volatiles. *Journal of Food Science*, **55**, 1239–1242.

Bianchi, F., Cantoni, C., Careri, M., Chiesa, L., Musci, M. & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, **72**, 1552–1563.

Bosman, S.C., De Beer, D., Beelders, T., Willenburg, E.L., Malherbe, C.J., Walczak, B. & Joubert, E. (2017). Simultaneous optimisation of extraction of xanthone and benzophenone *α*-glucosidase inhibitors from *Cyclopia genistoides* and identification of superior genotypes for propagation. *Journal of Functional Foods*, **33**, 21–31.

Bradberry, S.M., Gazzard, B. & Vale, J.A. (1994). Methemoglobinemia caused by the accidental contamination of drinking water with sodium nitrite. *Journal of Toxicology: Clinical Toxicology*, **32**, 173–178.

Bruna, J.M., Hierro, E.M., De la Hoz, L., Mottram, D.S., Fernández, M. & Ordóñez, J.A. (2001). The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. *Meat Science*, **59**, 97–107.

Buchanan, R.L. & Solberg, M. (1972). Interaction of sodium nitrate, oxygen, and pH on growth of *Staphylococcus aureus*. *Journal of Food Science*, **37**, 81–85.

Buscailhon, S., Berdagué, J.L., Bousset, J., Cornet, M., Gandemer, G., Touraille, C. & Monin, G. (1994). Relations between compositional traits and sensory qualities of french dry-cured ham. *Meat Science*, **37**, 229–243.

Butler, A. (2015). Nitrites and nitrates in the human diet: Carcinogens or beneficial hypotensive agents? *Journal of Ethnopharmacology*, **167**, 105–107.

Calvo, M.M., García, M.L. & Selgas, M.D. (2008). Dry fermented sausages enriched with lycopene from tomato peel. *Meat Science*, **80**, 167–172.

Cammack, R., Joannou, C.L., Cui, X.-Y., Martinez, C.T., Maraj, S.R. & Hughes, M.N. (1999). Nitrite and nitrosyl compounds in food preservation. *Biochimica et Biophysica Acta*, **1411**, 475–488.

Cardinali, F., Milanović, V., Osimani, A., Aquilanti, L., Taccari, M., Garofalo, C., Polverigiani, S., Clementi, F., Franciosi, E., Tuohy, K., Mercuri, M.L., Altissimi, M.S. & Haouet, M.N. (2018). Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites. *International Journal of Food Microbiology*, **278**, 61–72.

Casaburi, A., Blaiotta, G., Mauriello, G., Pepe, O. & Villani, F. (2005). Technological activities of *Staphylococcus carnosus* and *Staphylococcus simulans* strains isolated from fermented sausages. *Meat Science*, **71**, 643–650.

Cassens, R.G. (1997). Composition and safety of cured meats in the USA. *Food Chemistry*, **59**, 561–566.

Castellani, A.G. & Niven Jr, C.F. (1955). Factors affecting the bacteriostatic action of sodium nitrite. *Applied Microbiology*, **3**, 154–159.

Cenci-goga, B.T., Rossitto, P. V, Sechi, P., Parmegiani, S., Cambiotti, V. & Cullor, J.S. (2012). Effect of selected dairy starter cultures on microbiological, chemical and sensory characteristics of swine and venison (*Dama dama*) nitrite-free dry-cured sausages. *Meat Science*, **3**, 599-606.

Chen, Q., Kong, B., Han, Q., Xia, X. & Xu, L. (2017). The role of bacterial fermentation in lipolysis and lipid oxidation in Harbin dry sausages and its flavour development. *LWT - Food Science and Technology*, **77**, 389–396.

Choe, E. & Min, D.B. (2005). Chemistry and reactions of reactive oxygen species in foods. *Journal of Food Science*, **70**, R142–R159.

Choe, E. & Min, D.B. (2009). Mechanisms of antioxidants in the oxidation of foods. *Comprehensive Reviews in Food Science and Food Safety*, **8**, 345–358.

Christiansen, L.N., Tompkin, R.B., Sharapis, A.B., Kueper, T. V, Johnston, R.W., Kautter, D.A. & Kolari, O.J. (1974). Effect of sodium nitrite on toxin production by *Clostridium botulinum* in bacon. *Applied Microbiology*, **27**, 733–737.

Christieans, S., Picgirard, L., Parafita, E., Lebert, A. & Gregori, T. (2018). Impact of reducing nitrate/nitrite levels on the behavior of *Salmonella Typhimurium* and *Listeria monocytogenes* in French dry fermented sausages. *Meat Science*, **137**, 160–167.

Cichoski, A.J., Cansian, R.L., De Oliveira, D., Gaio, I. & Saggirato, A.G. (2011). Lipid and protein oxidation in the internal part of italian type salami containing basil essential oil (*Ocimum basilicum* L.). *Ciência e Tecnologia de Alimentos*, **31**, 436–442.

Coetzee, G., Marx, I.J., Pengilly, M., Bushula, V.S., Joubert, E. & Bloom, M. (2008). Effect of rooibos and honeybush tea extracts against *Botrytis cinerea*. *South African Journal for Enology and Viticulture*, **29**, 33–38.

Colla, G., Kim, H., Kyriacou, M.C. & Rouphael, Y. (2018). Nitrate in fruits and vegetables. *Scientia Horticulturae*, **237**, 221–238.

Coloretti, F., Tabanelli, G., Chiavari, C., Lanciotti, R., Grazia, L., Gardini, F. & Montanari, C. (2014). Effect of wine addition on microbiological characteristics, volatile molecule profiles and biogenic amine contents in fermented sausages. *Meat Science*, **96**, 1395–1402.

Corral, S., Salvador, A. & Flores, M. (2013). Salt reduction in slow fermented sausages affects the generation of aroma active compounds. *Meat Science*, **93**, 776–785.

Corral, S., Salvador, A. & Flores, M. (2014). Elucidation of key aroma compounds in traditional dry fermented sausages using different extraction techniques. *Journal of the Science of Food and Agriculture*, **95**, 1350–1361.

Cui, H., Gabriel, A.A. & Nakano, H. (2010). Antimicrobial efficacies of plant extracts and sodium nitrite against *Clostridium botulinum*. *Food Control*, **21**, 1030–1036.

Cullere, M., Hoffman, L.C. & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products. *Meat Science*, **95**, 72–77.

Cullere, M., Tasoniero, G., Secci, G., Parisi, G., Smit, P., Hoffman, L.C. & Dalle Zotte, A (2019). Effect of the incorporation of a fermented rooibos (*Aspalathus linearis*) extract in the manufacturing of rabbit meat patties on their physical, chemical, and sensory quality during refrigeration storage. *LWT – Food Science and Technology*, **108**, 31-38.

Dahlsten, E., Korkeala, H., Somervuo, P. & Lindström, M. (2008). PCR assay for differentiating between group I (proteolytic) and group II (non proteolytic) strains of *Clostridium botulinum*. *Intemationol Journal of Food Microbiology*, **124**, 108–111.

De Andrade, J.C., Nalério, E.S., Giongo, C., De Barcellos, M.D., Ares, G. & Deliza, R. (2017). Consumer perception of dry-cured sheep meat products: Influence of process parameters under different evoked contexts. *Meat Science*, **130**, 30–37.

De Beer, D. & Joubert, E. (2010). Development of HPLC method for *Cyclopia subternata* phenolic compound analysis and application to other *Cyclopia* spp. *Journal of Food Composition and Analysis*, **23**, 289–297.

De Beer, D., Pauck, C.E., Aucamp, M., Liebenberg, W., Stieger, N., Van der Rijst, M. & Joubert, E. (2018). Phenolic and physicochemical stability of a functional beverage powder mixture during storage: Effect of the microencapsulant inulin and food ingredients. *Journal of the Science of Food and Agriculture*, **98**, 2925–2934.

De Beer, D., Schulze, A.E., Joubert, E., De Villiers, A., Malherbe, C.J. & Stander, M.A. (2012). Food ingredient extracts of *Cyclopia subternata* (honeybush): Variation in phenolic composition and antioxidant capacity. *Molecules*, **17**, 14602–14624.

Deda, M.S., Bloukas, J.G. & Fista, G.A. (2007). Effect of tomato paste and nitrite level on processing and quality characteristics of frankfurters. *Meat Science*, **76**, 501–508.

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M.C., Fraeye, I., Heyden, Y. Vander & Paelinck, H. (2014). The occurrence of *N*-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Science*, **96**, 821–828.

Demeyer, D., Honikel, K. & De Smet, S. (2008). The world cancer research fund report 2007: A challenge for the meat processing industry. *Meat Science*, **80**, 953–959.

Denicola, A., Souza, J.M. & Radi, R. (1998). Diffusion of peroxynitrite across erythrocyte membranes. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*, **95**, 3566–3571.

Di Cagno, R., Lòpez, C.C., Tofalo, R., De Gallo, G., Angelis, M., Paparella, A., Hammes, W.P. & Gobbetti, M. (2008). Comparison of the compositional, microbiological, biochemical and volatile profile characteristics of three Italian PDO fermented sausages. *Meat Science*, **79**, 224–235.

Di Mattia, C.D., Sacchetti, G., Mastrocola, D. & Pittia, P. (2009). Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions. *Food Research International*, **42**, 1163–1170.

Dirinck, P., Van Opstaele, F. & Vandendriessche, F. (1997). Flavour differences between northern and southern European cured hams. *Food Chemistry*, **59**, 511–521.

Di Vita, G., Blanc, S., Brun, F., Bracco, S. & D'Amico, M. (2019). Quality attributes and harmful components of cured meats: Exploring the attitudes of Italian consumers towards healthier cooked ham. *Meat Science*, **155**, 8–15.

Dobarganes, M.C. & Velasco, J. (2002). Analysis of lipid hydroperoxides. *European Journal of Lipid Science and Technology*, **104**, 420–428.

Dodds, K.L. & Collins-Thompson, D.L. (1984). Nitrite tolerance and nitrite reduction in lactic acid bacteria associated with cured meat products. *International Journal of Food Microbiology*, **1**, 163–170.

Donald, B. Mac, Gray, J.I., Stanley, D.W. & Usborne, W.R. (1980). Role of nitrite in cured meat flavor: Sensory analysis. *Journal of Food Science*, **45**, 885–904.

Doolaege, E.H.A., Vossen, E., Raes, K., De Meulenaer, B., Verhé, R., Paelinck, H. & De Smet, S. (2012). Effect of rosemary extract dose on lipid oxidation, colour stability and antioxidant concentrations, in reduced nitrite liver pâtés. *Meat Science*, **90**, 925–931.

Du Preez, V.P.B., De Beer, D. & Joubert, E. (2016). By-product of honeybush (*Cyclopia maculata*) tea processing as source of hesperidin-enriched nutraceutical extract. *Industrial Crops and Products*, **87**, 132–141.

Erasmus, L.M., Theron, K.A., Muller, M., Van der Rijst, M. & Joubert, E. (2017). Optimising high-temperature oxidation of *Cyclopia* species for maximum development of characteristic aroma notes of honeybush herbal tea infusions. *South African Journal of Botany*, **110**, 144–151.

Erickson, M.C. (2002). Lipid Oxidation of Muscle Foods. In: *Food Lipids: Chemistry, Nutrition, and Biotechnology* (edited by C.C. Akoh & D.B. Min). New York: Marcel Dekker, Inc.

Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science*, **89**, 259–279.

Estévez, M. & Cava, R. (2006). Effectiveness of rosemary essential oil as an inhibitor of lipid and protein oxidation: Contradictory effects in different types of frankfurters. *Meat Science*, **72**, 348–355.

Falowo, A.B., Fayemi, P.O. & Muchenje, V. (2014). Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Research International*, **64**, 171–181.

Fang, Z. & Bhandari, B. (2011). Effect of spray drying and storage on the stability of bayberry polyphenols. *Food Chemistry*, **129**, 1139–1147.

Fernandes, R.P.P., Trindade, M.A., Lorenzo, J.M. & De Melo, M.P. (2018). Assessment of the stability of sheep sausages with the addition of different concentrations of *Origanum vulgare* extract during storage. *Meat Science*, **137**, 244–257.

Flores, M. (2018). Understanding the implications of current health trends on the aroma of wet and dry cured meat products. *Meat Science*, **144**, 53–61.

Flores, M., Corral, S., Cano-garcía, L., Salvador, A. & Belloch, C. (2015). Yeast strains as potential aroma enhancers in dry fermented sausages. *International Journal of Food Microbiology*, **212**, 16–24.

Font-i-Furnols, M. & Guerrero, L. (2014). Consumer preference, behavior and perception about meat and meat products: An overview. *Meat Science*, **98**, 361–371.

Frankel, E.N. (1982). Volatile lipid oxidation products. *Progress in Lipid Research*, **22**, 1–33.

Frankel, E.N. (1984). Lipid oxidation: Mechanisms, products and biological significance. *Journal of the American Oil Chemists' Society*, **61**, 1908–1917.

Frankel, E.N. (1985). Chemistry of free radical and singlet oxidation of lipids. *Progress in Lipid Research*, **23**, 197–221.

Frankel, E.N., Neff, W.E. & Selke, E. (1981). Analysis of autoxidized fats by gas chromatography-mass spectrometry: VII. Volatile thermal decomposition products of pure hydroperoxides from autoxidized and photosensitized oxidized methyl oleate, linoleate and linolenate. *Lipids*, **16**, 279–285.

Frei, B. & Higdon, J. V. (2003). Antioxidant Activity of Tea Polyphenols *In Vivo*: Evidence from Animal Studies. In: *Proceedings of the Third International Scientific Symposium on Tea and Human Health: Role of Flavonoids in the Diet Antioxidant*. Pp. 3275–3284. Corvallis, Oregon State University.

Freybler, L.A., Gray, J.I., Asghar, A., Booren, A.M., Pearson, A.M. & Buckley, D.J. (1993). Nitrite stabilization of lipids in cured pork. *Meat Science*, **33**, 85–96.

Fuentes, V., Estévez, M., Ventanas, J. & Ventanas, S. (2014). Impact of lipid content and composition on lipid oxidation and protein carbonylation in experimental fermented sausages. *Food Chemistry*, **147**, 70–77.

Gardner, H.W. (1989). Oxygen radical chemistry of polyunsaturated fatty acids. *Free Radical Biology and Medicine*, **7**, 65–86.

Garrido, M.D., Auqui, M., Martí, N. & Linares, M.B. (2011). Effect of two different red grape pomace extracts obtained under different extraction systems on meat quality of pork burgers. *LWT - Food Science and Technology*, **44**, 2238–2243.

Gilles, G. (2009). Dry cured ham quality as related to lipid quality of raw material and lipid changes during processing: A review. *Grasas Y Aceites*, **60**, 297–307.

Gøtterup, J., Olsen, K., Knøchel, S., Tjener, K., Stahnke, L.H. & Møller, J.K.S. (2008). Colour formation in fermented sausages by meat-associated staphylococci with different nitrite- and nitrate-reductase activities. *Meat Science*, **78**, 492–501.

Graham, A.F., Mason, D.R., Maxwell, F.J. & Peck, M.W. (1997). Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperature. *Letters in Applied Microbiology*, **24**, 95–100.

Gray, J.I. & Monahan, F.J. (1992). Measurement of lipid oxidation in meat and meat products. *Trends in Food Science and Technology*, **3**, 315–319.

Grenda, T., Kukier, E. & Kwiatek, K. (2014). Methods and difficulties in detection of *Clostridium botulinum* and its toxins. *Polish Journal of Veterinary Sciences*, **17**, 195–205.

Halliwell, B. & Chirico, A. (1993). Lipid peroxidation: Its mechanism, measurement, and significance. *The American Journal of Clinical Nutrition*, **57**, 715S-725S.

Hammes, W.P. (2012). Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. *Food Microbiology*, **29**, 151–156.

Herrmann, S.S., Duedahl-Olesen, L. & Granby, K. (2015). Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, **48**, 163–169.

Hoffman, L.C., Jones, M., Muller, N., Joubert, E. & Sadie, A. (2014). Lipid and protein stability and sensory evaluation of ostrich (*Struthio camelus*) droëwors with the addition of rooibos tea extract (*Aspalathus linearis*) as a natural antioxidant. *Meat Science*, **96**, 1289–1296.

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, **78**, 68–76.

Hord, N.G., Tang, Y. & Bryan, N.S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *The American Journal of Clinical Nutrition*, **90**, 1–10.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Hospital, X.F., Hierro, E., Arnau, J., Carballo, J., Aguirre, J.S., Gratacós-Cubarsí, M. & Fernández, M. (2017). Effect of nitrate and nitrite on *Listeria* and selected spoilage bacteria inoculated in dry-cured ham. *Food Research International*, **101**, 82–87.

Hospital, X.F., Hierro, E. & Fernández, M. (2012). Survival of *Listeria innocua* in dry fermented sausages and changes in the typical microbiota and volatile profile as affected by the concentration of nitrate and nitrite. *International Journal of Food Microbiology*, **153**, 395–401.

Hospital, X.F., Hierro, E. & Fernández, M. (2014). Effect of reducing nitrate and nitrite added to dry fermented sausages on the survival of *Salmonella Typhimurium*. *Food Research International*, **62**, 410–415.

Hospital, X.F., Hierro, E., Stringer, S. & Fernández, M. (2016). A study on the toxigenesis by *Clostridium botulinum* in nitrate and nitrite-reduced dry fermented sausages. *International Journal of Food Microbiology*, **218**, 66–70.

Hughes, J., Clarke, F., Li, Y., Purslow, P. & Warner, R. (2019). Differences in light scattering between pale and dark beef *longissimus thoracis* muscles are primarily caused by differences in the myofilament lattice, myofibril and muscle fibre transverse spacings. *Meat Science*, **149**, 96-106.

Hung, Y., Verbeke, W. & De Kok, T.M. (2016). Stakeholder and consumer reactions towards innovative processed meat products: Insights from a qualitative study about nitrite reduction and phytochemical addition. *Food Control*, **60**, 690–698.

Igene, J.O., Yamauchi, K., Pearson, A.M., Gray, J.I. & Aust, S.D. (1985). Mechanisms by which nitrite inhibits the development of warmed-over flavour (WOF) in cured meat. *Food Chemistry*, **18**, 1–18.

Iranshahi, M., Rezaee, R., Parhiz, H., Roohbakhsh, A. & Soltani, F. (2015). Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin. *Life Sciences*, **137**, 125–132.

Jin, S., Choi, J.S., Yang, H., Park, T. & Yim, D. (2018). Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science*, **146**, 34–40.

Jones, M., Hoffman, L.C. & Muller, M. (2015). Effect of rooibos extract (*Aspalathus linearis*) on lipid oxidation over time and the sensory analysis of blesbok (*Damaliscus pygargus phillipsi*) and springbok (*Antidorcas marsupialis*) droëwors. *Meat Science*, **103**, 54–60.

Joubert, E., Botha, M., Maicu, C., De Beer, D. & Manley, M. (2012). Rapid screening methods for estimation of mangiferin and xanthone contents of *Cyclopia subternata* plant material. *South African Journal of Botany*, **82**, 113–122.

Joubert, E., De Beer, D., Hernández, I. & Munné-Bosch, S. (2014). Accummulation of mangiferin, isomangiferin, iriflophenone-3-C-ß-glucoside and hesperidin in honeybush leaves (*Cyclopia genistoides* Vent.) in response to harvest time, harvest interval and seed source. *Industrial Crops and Products*, **56**, 74–82.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. & Gelderblom, W.C.A. (2019). Formal honeybush tea industry reaches 20-year milestone – Progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, **127**, 58–79.

Joubert, E., Gelderblom, W.C.A., Louw, A. & De Beer, D. (2008a). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides* - A review. *Journal of Ethnopharmacology*, **119**, 376–412.

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. & De Lange, J.H. (2011). Honeybush (*Cyclopia* spp.): From local cottage industry to global markets - The catalytic and supporting role of research. *South African Journal of Botany*, **77**, 887–907.

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2008b). Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry*, **56**, 954–963.

Kamara, B.I., Brand, J.D., Brandt, Vincent, E. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391–5395.

Kennedy, N., Smith, C.P. & McWhinney, P. (1997). Faulty sausage production causing methaemoglobinaemia. *Archives of Disease in Childhood*, **76**, 367–368.

Knobeloch, L., Salna, B., Hogan, A., Postle, J. & Anderson, H. (2000). Blue babies and nitrate-contaminated well water. *Environmental Health Perspectives*, **108**, 675–678.

Kohn, M.C., Melnick, R.L., Ye, F. & Portier, C.J. (2002). Pharmacokinetics of sodium nitrite-induced methemoglobinemia in the rat. *Drug Metabolism and Disposition*, **30**, 676–683.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Landeta, G., Curiel, J.A., Carrascosa, A.V., Muñoz, R. & De las Rivas, B. (2013). Technological and safety properties of lactic acid bacteria isolated from Spanish dry-cured sausages. *Meat Science*, **95**, 272–280.

Lee, K.G., Shibamoto, T., Takeoka, G.R., Lee, S.E., Kim, J.H. & Park, B.S. (2003). Inhibitory effects of plant-derived flavonoids and phenolic acids on malonaldehyde formation from ethyl arachidonate. *Journal of Agricultural and Food Chemistry*, **51**, 7203–7207.

Leiro, J.M., Álvarez, E., Arranz, J.A., Siso, I.G. & Orallo, F. (2003). *In vitro* effects of mangiferin on superoxide concentrations and expression of the inducible nitric oxide synthase, tumour necrosis factor-alpha and transforming growth factor-beta genes. *Biochemical Pharmacology*, **65**, 1361–1371.

Leroy, F. & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology*, **15**, 67–78.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L. & Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: A historical outlook. *Trends in Food Science and Technology*, **31**, 130–137.

Le Roux, M., Cronje, J.C., Burger, B.V. & Joubert, E. (2012). Characterization of volatiles and aroma-active compounds in honeybush (*Cyclopia subternata*) by GC-MS and GC-O analysis. *Journal of Agricultural and Food Chemistry*, **60**, 2657–2664.

Li, P., Kong, B., Chen, Q., Zheng, D. & Liu, N. (2013). Formation and identification of nitrosylmyoglobin by *Staphylococcus xylosus* in raw meat batters: A potential solution for nitrite substitution in meat products. *Meat Science*, **93**, 67–72.

Lindemann, C., Lupilova, N., Müller, A., Warscheid, B., Meyer, H.E., Kuhlmann, K., Eisenacher, M. & Leichert, L.I. (2013). Redox proteomics uncovers peroxynitrite-sensitive proteins that help *Escherichia coli* to overcome nitrosative stress. *The Journal of Biological Chemistry*, **288**, 19698–19714.

Liu, M., Lampi, A.M. & Ertbjerg, P. (2018). Unsaturated fat fraction from lard increases the oxidative stability of minced pork. *Meat Science*, **143**, 87–92.

Lorenzo, J.M. (2014). Influence of the type of fiber coating and extraction time on foal dry-cured loin volatile compounds extracted by solid-phase microextraction (SPME). *Meat Science*, **96**, 179–186.

Lu, H. & Yanjun, H. (2016). Effects of combined starter cultures on quality of fermented sausage during ripening. *Journal of Food Engineering and Technology*, **5**, 38–47.

Lund, M.N., Heinonen, M., Baron, C.P. & Estévez, M. (2011). Protein oxidation in muscle foods: A review. *Molecular Nutrition and Food Research*, **55**, 83–95.

Lynt, R.K., Kautter, D.A. & Solomon, H.M. (1982). Differences and similarities among proteolytic and nonproteolytic strains of *Clostridium botulinum* types A, B, E and F: A review. *Journal of Food Protection*, **45**, 466–474.

Magcwebeba, T.U., Riedel, S., Swanevelder, S., Swart, P., De Beer, D., Joubert, E. & Gelderblom, W.C.A. (2016). The potential role of polyphenols in the modulation of skin cell viability by *Aspalathus linearis* and *Cyclopia* spp. herbal tea extracts *in vitro*. *Journal of Pharmacy and Pharmacology*, **68**, 1440–1453.

Mainar, M.S. & Leroy, F. (2015). Process-driven bacterial community dynamics are key to cured meat colour formation by coagulase-negative staphylococci via nitrate reductase or nitric oxide synthase activities. *International Journal of Food Microbiology*, **212**, 60–66.

Mainar, M.S., Stavropoulou, D.A. & Leroy, F. (2017). Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: A review. *International Journal of Food Microbiology*, **247**, 24–37.

Mainar, M.S., Xhaferi, R., Samapundo, S., Devlieghere, F. & Leroy, F. (2016). Opportunities and limitations for the production of safe fermented meats without nitrate and nitrite using an antibacterial *Staphylococcus sciuri* starter culture. *Food Control*, **69**, 267–274.

Majou, D. & Christieans, S. (2018). Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. *Meat Science*, **145**, 273–284.

Mancini, R.A. & Hunt, M.C. (2005). Current research in meat color. *Meat Science*, **71**, 100–121.

Marangoni, C. & Moura, N.F. (2011). Antioxidant activity of essential oil from *Coriandrum Sativum* L. in Italian salami. *Ciência e Tecnologia de Alimentos*, **31**, 124–128.

Marco, A., Navarro, J.L. & Flores, M. (2006). The influence of nitrite and nitrate on microbial, chemical and sensory parameters of slow dry fermented sausage. *Meat Science*, **73**, 660–673.

Marco, A., Navarro, J.L. & Flores, M. (2008). The sensory quality of dry fermented sausages as affected by fermentation stage and curing agents. *European Food Research and Technology*, **226**, 449–458.

Mariutti, L.R.B. & Bragagnolo, N. (2017). Influence of salt on lipid oxidation in meat and seafood products: A review. *Food Research International*, **94**, 90–100.

Martínez, L., Bastida, P., Castillo, J., Ros, G. & Nieto, G. (2019). Green alternatives to synthetic antioxidants, antimicrobials, nitrates, and nitrites in clean label Spanish chorizo. *Antioxidants*, **8**, 1–18.

McClure, P.J., Kelly, T.M. & Roberts, T.A. (1991). The effects of temperature, pH, sodium chloride and sodium nitrite on the growth of *Listeria monocytogenes*. *Intemationol Journal of Food Microbiology*, **14**, 77–92.

Mercier, Y., Gatellier, P. & Renerre, M. (2004). Lipid and protein oxidation *in vitro*, and antioxidant potential in meat from Charolais cows finished on pasture or mixed diet. *Meat Science*, **66**, 467–473.

Mercier, Y., Gatellier, P., Vincent, A. & Renerre, M. (2001). Lipid and protein oxidation in microsomal fraction from turkeys: Influence of dietary fat and vitamin E supplementation. *Meat Science*, **58**, 125–134.

Merialdi, G., Ramini, M., Parolari, G., Barbuti, S., Frustoli, M.A., Taddei, R., Pongolini, S., Ardigò, P. & Cozzolino, P. (2016). Study on potential *Clostridium botulinum* growth and toxin production in Parma ham. *Italian Journal of Food Safety*, **5**, 69–74.

Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E. & Gandemer, G. (1999). Volatile compounds of commercial Milano salami. *Meat Science*, **51**, 175–183.

Milkowski, A., Garg, H.K., Coughlin, J.R. & Bryan, N.S. (2010). Nutritional epidemiology in the context of nitric oxide biology: A risk-benefit evaluation for dietary nitrite and nitrate. *Nitric Oxide*, **22**, 110–119.

Miller, N., De Beer, D. & Joubert, E. (2017). Minimising variation in aspalathin content of aqueous green rooibos extract: Optimising extraction and identifying critical material attributes. *Journal of the Science of Food and Agriculture*, **97**, 4937–4942.

Min, B., Cordray, J.C. & Ahn, D.U. (2010). Effect of NaCl, myoglobin, Fe (II), and Fe (III) on lipid oxidation of raw and cooked chicken breast and beef loin. *Journal of Agricultural and Food Chemistry*, **58**, 600–605.

Min, D.B. & Boff, J.M. (2002a). Chemistry and reaction of singlet oxygen in foods. *Comprehensive Reviews in Food Science and Food Safety*, **1**, 58–72.

Min, D.B. & Boff, J.M. (2002b). Lipid Oxidation of Edible Oil. In: *Food Lipids: Chemistry, Nutrition, and Biotechnology* (edited by C.C. Akoh & D.B. Min). New York: Marcel Dekker, Inc.

Møller, J.K.S., Jensen, J.S., Skibsted, L.H. & Knöchel, S. (2003). Microbial formation of nitrite-cured pigment, nitrosylmyoglobin, from metmyoglobin in model systems and smoked fermented sausages by *Lactobacillus fermentum* strains and a commercial starter culture. *European Food Research and Technology*, **216**, 463–469.

Møller, J.K.S. & Skibsted, L.H. (2002). Nitric oxide and myoglobins. *Chemical Reviews*, **102**, 1167–1178.

Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. & Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry*, **192**, 736–744.

Montanari, C., Gatto, V., Torriani, S., Barbieri, F., Bargossi, E., Lanciotti, R., Grazia, L., Magnani, R., Tabanelli, G. & Gardini, F. (2018). Effects of the diameter on physico-chemical, microbiological and volatile profile in dry fermented sausages produced with two different starter cultures. *Food Bioscience*, **22**, 9–18.

Montel, M.-C., Reitz, J., Talon, R., Berdagué, J.-L. & Rousset-Akrim, S. (1996). Biochemical activities of *Micrococcaceae* and their effects on the aromatic profiles and odours of a dry sausage model. *Food Microbiology*, **13**, 489–499.

Morita, H., Sakata, R. & Nagat, Y. (1998). Nitric oxide complex of iron (II) myoglobin by *Staphylococcus xylosus*. *Journal of Food Science*, **63**, 352–355.

Morrissey, P.A. & Tichivangana, J.Z. (1985). The antioxidant activities of nitrite and nitrosylmyoglobin in cooked meats. *Meat Science*, **14**, 175–190.

Mortensen, A., Aguilar, F., Crebelli, R., Di Domenico, A., Dusemund, B., Frutos, M.J., Galtier, P., Gott, D., Gundert-Remy, U., Lambré, C., Leblanc, J.-C., Lindtner, O., Moldeus, P., Mosesso, P., Oskarsson, A., Parent-Massin, D., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R.A., Wright, M., Brandt, P. Van den, Fortes, C., Merino, L., Toldrá, F., Arcella, D., Christodoulidou, A., Abrahantes, J.C., Barrucci, F., Garcia, A., Pizzo, F., Battacchi, D. & Younes, M. (2017a). Re-evaluation of potassium nitrite (E249) and sodium nitrite (E250) as food additives. *European Food Safety Authority*, **15**, 1–157.

Mortensen, A., Aguilar, F., Crebelli, R., Di Domenico, A., Dusemund, B., Frutos, M.J., Galtier, P., Gott, D., Gundert-Remy, U., Lambré, C., Leblanc, J.-C., Lindtner, O., Moldeus, P., Mosesso, P., Oskarsson, A., Parent-Massin, D., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R.A., Wright, M., Brandt, P. Van Den, Fortes, C., Merino, L., Toldrá, F., Arcella, D., Christodoulidou, A., Barrucci, F., Garcia, A., Pizzo, F., Battacchi, D. & Younes, M. (2017b).

Re-evaluation of sodium nitrate (E251) and potassium nitrate (E252) as food additives. *European Food Safety Authority*, **15**, 1–123.

Morton, A.G. (1956). A study of nitrate reduction in mould fungi. *Journal of Experimental Botany*, **7**, 97–112.

Moure, A., Cruz, J.M., Franco, D., Domínguez, J.M., Sineiro, J., Domínguez, H., Núñez, M.J. & Parajó, J.C. (2001). Natural antioxidants from residual sources. *Food Chemistry*, **72**, 145–171.

Muguerza, E., Gimeno, O., Ansorena, D., Bloukas, J.G. & Astiasarán, I. (2001). Effect of replacing pork backfat with pre-emulsified olive oil on lipid fraction and sensory quality of Chorizo de Pamplona - A traditional Spanish fermented sausage. *Meat Science*, **59**, 251–258.

Nassu, R.T., Goncalves, L.A.G., da Silva, M.A.A.P. & Beserra, F.J. (2003). Oxidative stability of fermented goat meat sausage with different levels of natural antioxidant. *Meat Science*, **63**, 43–49.

Neethling, N.E., Suman, S.P., Sigge, G.O., Hoffman, L.C. & Hunt, M.C. (2017). Exogenous and endogenous factors influencing color of fresh meat from ungulates. *Meat and Muscle Biology*, **1**, 253-275.

Nikmaram, N., Budaraju, S., Barba, F.J., Lorenzo, J.M., Cox, R.B., Mallikarjunan, K. & Roohinejad, S. (2018). Application of plant extracts to improve the shelf-life, nutritional and health-related properties of ready-to-eat meat products. *Meat Science*, **145**, 245-255.

Ntlhokwe, G., Muller, M., Joubert, E., Tredoux, A.G.J. & De Villiers, A. (2018). Detailed qualitative analysis of honeybush tea (*Cyclopia* spp.) volatiles by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry and relation with sensory data. *Journal of Chromatography A*, **1536**, 137–150.

Olesen, P.T., Meyer, A.S. & Stahnke, L.H. (2004). Generation of flavour compounds in fermented sausages - The influence of curing ingredients, *Staphylococcus* starter culture and ripening time. *Meat Science*, **66**, 675–687.

Olivares, A., Navarro, J.L. & Flores, M. (2009a). Distribution of volatile compounds in lean and subcutaneous fat tissues during processing of dry fermented sausages. *Food Research International*, **42**, 1303–1308.

Olivares, A., Navarro, J.L. & Flores, M. (2009b). Establishment of the contribution of volatile compounds to the aroma of fermented sausages at different stages of processing and storage. *Food Chemistry*, **115**, 1464–1472.

Ordóñez, J.A., Hierro, E.M., Bruna, J.M. & De la Hoz, L. (1999). Changes in the components of dry-fermented sausages during ripening. *Critical Reviews in Food Science and Nutrition*, **39**, 329–367.

Paik, H.-D. & Lee, J.-Y. (2014). Investigation of reduction and tolerance capability of lactic acid bacteria isolated from kimchi against nitrate and nitrite in fermented sausage condition. *Meat Science*, **97**, 609–614.

Park, D. & Xiong, Y.L. (2007). Oxidative modification of amino acids in porcine myofibrillar protein isolates exposed to three oxidizing systems. *Food Chemistry*, **103**, 607–616.

Pauck, C.E. (2016). *Effects of formulation on the stability of green Cyclopia subternata extract during spray-drying and storage*. MSc Thesis, Stellenbosch University. URL: https://scholar.sun.ac.za/handle/10019.1/98410.

Peck, M.W. (2009). Biology and genomic analysis of *Clostridium botulinum*. *Advances in Microbial Physiology*, **55**, 184-265.

Peck, M.W., Goodburn, Kaarin, E., Betts, R.P. & Stringer, S.C. (2008). Assessment of the potential for growth and neurotoxin formation by *Clostridium botulinum* in short shelf-life commercial foods designed to be stored chilled. *Trends in Food Science and Technology*, **19**, 207–216.

Perea-sanz, L., Montero, R., Belloch, C. & Flores, M. (2018). Nitrate reduction in the fermentation process of salt reduced dry sausages: Impact on microbial and physicochemical parameters and aroma profile. *International Journal of Food Microbiology*, **282**, 84–91.

Perea-Sanz, L., Montero, R., Belloch, C. & Flores, M. (2019). Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage. *Meat Science*, **147**, 100–107.

Posthuma, J.A., Rasmussen, F.D. & Sullivan, G.A. (2018). Effects of nitrite source, reducing compounds, and holding time on cured color development in a cured meat model system. *LWT - Food Science and Technology*, **95**, 47–50.

Purslow, P.P., Warner, R.D., Clarke, F.M., Hughes, J.M. (2020). Variations in meat colour due to factors other than myoglobin chemistry; a synthesis of recent findings (invited review). *Meat Science*, **159**, 1-9.

Radi, R. (2013). Peroxynitrite, a stealthy biological oxidant. *The Journal of Biological Chemistry*, **288**, 26464–26472.

Ramaswamy, H.S. & Richards, J.F. (1982). Flavor of poultry meat - A review. *Canadian Institute of Food Science and Technology*, **15**, 7–18.

Ras, G., Bailly, X., Chacornac, J.P., Zuliani, V., Derkx, P., Seibert, T.M., Talon, R. & Leroy, S. (2018a). Contribution of nitric oxide synthase from coagulase-negative staphylococci to the

development of red myoglobin derivatives. *International Journal of Food Microbiology*, **266**, 310-316.

Ras, G., Leroy, S. & Talon, R. (2018b). Nitric oxide synthase: What is its potential role in the physiology of staphylococci in meat products? *International Journal of Food Microbiology*, **282**, 28–34.

Rice-Evans, C.A., Miller, N.J. & Paganga, G. (1996). Structure-antioxdant activity relationships of flavonoids and phenolic acids. *Free Radical and Medicine*, **20**, 933–956.

Riel, G., Boulaaba, A., Popp, J. & Klein, G. (2017). Effects of parsley extract powder as an alternative for the direct addition of sodium nitrite in the production of mortadella-type sausages - Impact on microbiological, physicochemical and sensory aspects. *Meat Science*, **131**, 166–175.

Roberts, D.D., Pollien, P. & Milo, C. (2000). Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *Journal of Agricultural and Food Chemistry*, **48**, 2430–2437.

Ross, C.F. & Smith, D.M. (2006). Use of volatiles as indicators of lipid oxidation in muscle foods. *Comprehensive Reviews in Food Science and Food Safety*, **5**, 18–25.

Safer, A.M. & Al-nughamish, A.J. (1999). Hepatotoxicity induced by the anti-oxidant food additive, butylated hydroxytoluene (BHT), in rats: An electron microscopical study. *Histology and Histopathology*, **14**, 391–406.

Sansone, F., Mencherini, T., Picerno, P., D'Amore, M., Aquino, R.P. & Lauro, M.R. (2011). Maltodextrin/pectin microparticles by spray drying as carrier for nutraceutical extracts. *Journal of Food Engineering*, **105**, 468–476.

Sarafian, T.A., Kouyoumjian, S., Tashkin, D. & Roth, M.D. (2002). Synergistic cytotoxicity of Delta (9)-tetrahydrocannabinol and butylated hydroxyanisole. *Toxicology Letters*, **133**, 171–179.

Savino, F., Maccario, S., Guidi, C., Castagno, E., Farinasso, D., Cresi, F., Silvestro, L. & Mussa, G.C. (2006). Methemoglobinemia caused by the ingestion of courgette soup given in order to resolve constipation in two formula-fed infants. *Annals of Nutrition and Metabolism*, **50**, 368–371.

Sebranek, J.G. & Bacus, J.N. (2007). Cured meat products without direct addition of nitrate or nitrite: What are the issues? *Meat Science*, **77**, 136–147.

Sebranek, J.G., Sewalt, V.J.H., Robbins, K.L. & Houser, T.A. (2005). Comparison of a natural rosemary extract and BHA/BHT for relative antioxidant effectiveness in pork sausage. *Meat Science*, **69**, 289–296.

Shacter, E. (2000). Quantification and significance of protein oxidation in biological sample. *Drug Metabolism Reviews*, **32**, 307–326.

Shah, M.A., Don Bosco, S.J. & Mir, S.A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, **98**, 21–33.

Shahidi, F., Rubin, L.J., D'Souza, L.A., Teranishi, R. & Buttery, R.G. (1986). Meat flavor volatiles: A review of the composition, techniques of analysis, and sensory evaluation. *Critical Reviews in Food Science and Nutrition*, **24**, 141–243.

Sharma, A., Shahzad, B., Rehman, A., Bhardwaj, R., Landi, M., Zheng, B. (2019). Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. *Molecules*, **24**, 1-22.

Sindelar, J.J. & Milkowski, A.L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. In: *American Meat Science Association*. Pp. 1–14.

Sindelar, J.J. & Milkowski, A.L. (2012). Nitric oxide human safety controversies surrounding nitrate and nitrite in the diet. *Nitric Oxide*, **26**, 259–266.

Singh, S., Dhyani, S.K., Kumar, S.S. & Sharma, S. (2009). Antimicrobial evaluation of mangiferin analogues. *Indian Journal of Pharmaceutical Sciences*, **71**, 328–331.

Singh, S.K., Tiwari, R.M., Sinha, S.K., Danta, C.C. & Prasad, S.K. (2012). Antimicrobial evaluation of mangiferin and its synthesized analogues. *Asian Pacific Journal of Tropical Biomedicine 2*, **2**, S884–S887.

Skibsted, L.H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, **24**, 176–183.

Smelt, J.P.P.M., Raatjes, G.J.M., Crowther, J.S. & Verrips, C.T. (1982). Growth and toxin formation by *Clostridium botulinum* at low pH values. *Journal of Applied Bacteriology*, **52**, 75–82.

Snijman, P.W., Joubert, E., Ferreira, D., Li, X.-C., Ding, Y., Green, I.R. & Gel. (2009). Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and trolox. *Journal of Agricultural and Food Chemistry*, **57**, 6678–6684.

Sobko, T., Reinders, C.I., Jansson, E. Å., Norin, E., Midtvedt, T. & Lundberg, J.. (2005). Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. *Nitric Oxide*, **13**, 272–278.

Sofos, J.N., Busta, F.F. & Allen, C.E. (1979). Botulism control by nitrite and sorbate in cured meats: A review. *Journal of Food Protection*, **42**, 739–770.

Soladoye, O.P., Juárez, M.L., Aalhus, J.L., Shand, P. & Estévez, M. (2015). Protein oxidation in processed meat: Mechanisms and potential implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, **14**, 106–122.

Stavropoulou, D.A., Filippou, P., De Smet, S., De Vuyst, L. & Leroy, F. (2018). Effect of temperature and pH on the community dynamics of coagulase-negative staphylococci during spontaneous meat fermentation in a model system. *Food Microbiology*, **76**, 180–188.

Stepanova, A. V, Kotina, E.L., Tilney, P.M. & Van Wyk, B.E. (2012). Leaf and stem anatomy of honeybush tea (*Cyclopia* species, Fabaceae). *South African Journal of Botany*, **82**, 123–128.

Sucu, C. & Turp, G.Y. (2018). The investigation of the use of beetroot powder in Turkish fermented beef sausage (sucuk) as nitrite alternative. *Meat Science*, **140**, 158–166.

Sun, W., Zhao, M., Yang, B., Zhao, H. & Cui, C. (2011). Oxidation of sarcoplasmic proteins during processing of Cantonese sausage in relation to their aggregation behaviour and *in vitro* digestibility. *Meat Science*, **88**, 462–467.

Sunesen, L.O., Dorigoni, V., Zanardi, E. & Stahnke, L. (2001). Volatile compounds released during ripening in Italian dried sausage. *Meat Science*, **58**, 93–97.

Tabanelli, G., Coloretti, F., Chiavari, C., Grazia, L., Lanciotti, R. & Gardini, F. (2012). Effects of starter cultures and fermentation climate on the properties of two types of typical Italian dry fermented sausages produced under industrial conditions. *Food Control*, **26**, 416–426.

Tachiki, T., Sakai, K., Yamamoto, K., Hatanaka, M. & Tochikura, T. (2016). Conversion of nitrite to nitrate by nitrite-resistant yeasts. *Agricultural and Biological Chemistry*, **52**, 1999–2005.

Talon, R. & Leroy, S. (2014). Fermented Foods: Fermented Meat Products and the Role of Starter Cultures. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 870-874.

Taormina, P.J. (2014). Meat and Poultry: Curing of Meat. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 501-507.

Tapas, A.R., Sakarkar, D.M. & Kakde, R.B. (2008). Flavonoids as nutraceuticals: A review. *Tropical Journal of Pharmaceutical Research*, **7**, 1089–1099.

Teruel, M.R., Garrido, M.D., Espinosa, M.C. & Linares, M.B. (2015). Effect of different format-solvent rosemary extracts (*Rosmarinus officinalis*) on frozen chicken nuggets quality. *Food Chemistry*, **172**, 40–46.

Theron, K.A., Muller, M., Van der Rijst, M., Cronje, J.C., Le Roux, M. & Joubert, E. (2014). Sensory profiling of honeybush tea (*Cyclopia* species) and the development of a honeybush sensory wheel. *Food Research International*, **66**, 12–22.

Thomas, C., Mercier, F., Tournayre, P., Martin, J. & Berdagué, J. (2013). Effect of nitrite on the odourant volatile fraction of cooked ham. *Food Chemistry*, **139**, 432–438.

Tomás-Navarro, M., Vallejo, F., Borrego, F. & Tomás-Barberán, F.A. (2014). Encapsulation and micronization effectively improve orange beverage flavanone bioavailability in humans. *Journal of Agricultural and Food Chemistry*, **62**, 9458–9462.

Tompkin, R.B., Ambrosino, J.M. & Stozek, S.K. (1973). Effect of pH, sodium chloride, and sodium nitrite on enterotoxin A production. *Applied Microbiology*, **26**, 833–837.

Toscano, S., Trivellini, A., Cocetta, G., Bulgari, R., Francini, A., Romano, D., Ferrante, A. (2019). Effect of preharvest abiotic stresses on the accumulation of bioactive compounds in horticulture practice. *Frontiers in Plant Science*, **10**, 1-17.

Verbeke, W., De Smet, S., Vackier, I., Van Oeckel, M.J., Warnants, N. & Van Kenhove, P. (2005). Role of intrinsic search cues in the formation of consumer preferences and choice for pork chops. *Meat Science*, **69**, 343–354.

Wang, F.-S., Jiang, Y.-N. & Lin, C.-W. (1995). Lipid and cholesterol oxidation in Chinese-style sausage using vacuum and modified atmosphere packaging. *Meat Science*, **40**, 93–101.

Wang, L., Wang, Z. & Li, X. (2013a). Preliminary phytochemical and biological activities study of solvent extracts from a cold-field fruit - *Malus baccata* (Linn.) Borkh. *Industrial Crops and Products*, **47**, 20–28.

Wang, X.H., Ren, H.Y., Liu, D.Y., Zhu, W.Y. & Wang, W. (2013b). Effects of inoculating *Lactobacillus sakei* starter cultures on the microbiological quality and nitrite depletion of Chinese fermented sausages. *Food Control*, **32**, 591–596.

Wąsowicz, E., Gramza, A., Hęś, M., Jeleń, H.H., Korczak, J., Małecka, M., Mildner-Szkudlarz, S., Rudzińska, M., Somatyja, U. & Zawirska-Wojtasiak, R. (2004). Oxidation of lipids in food. *Polish Journal of Food and Nutrition Sciences*, **13**, 87–100.

Xu, X., Yu, E., Gao, X., Song, N., Liu, L., Wei, X., Zang, W. & Fu, C. (2013). Red and processed meat intake and risk of colorectal adenomas: A meta-analysis of observational studies. *International Journal of Cancer*, **132**, 437–448.

Yin, S., Faustman, C., Tatiyaborwomtham, N., Ramanathan, R., Maheswarappa, N.B., Mancini, R.A., Joseph, P., Suman, S.P. & Sun, Q. (2011). Species-specific myoglobin oxidation. *Journal of Agricultural and Food Chemistry* , **59**, 12198–12203.

Yoshimi, N., Matsunaga, K., Katayama, M., Yamada, Y., Kuno, T., Qiao, Z., Hara, A., Yamahara, J. & Mori, H. (2001). The inhibitory effects of mangiferin, a naturally occurring glucosylxanthone, in bowel carcinogenesis of male F344 rats. *Cancer Letters*, **163**, 163–170.

Zhang, Y., Yang, F., Jamali, M.A. & Peng, Z. (2016). Antioxidant enzyme activities and lipid oxidation in rape (*Brassica campestris* L.) bee pollen added to salami during processing. *Molecules*, **21**, 1–13.

CHAPTER 4

Evaluation of unfermented honeybush (*Cyclopia subternata*) extract in typical Italian salami: A pilot study[1]

Abstract

This was a preliminary (pilot) study for the familiarisation with the skills required for salami making under guidance of experienced Italian salami producers. This is the first study investigating the effect of unfermented honeybush extract as a bio-active ingredient in a processed red meat product, with typical pork Italian salami chosen as the model. A total of 68 pork salami were manufactured which were subdivided into three treatments: C= no added nitrate or honeybush; N= added nitrate (100 mg/kg) and ascorbic acid; and H= added honeybush (0.5% w/w). After 35 days of controlled ripening, H salami had a higher water activity (a_w= 0.928, p ≤ 0.01), compared with the C (a_w= 0.923) and N (a_w= 0.924), also reflected in the higher moisture content of H salami (35.3%), compared to the C (33.5%; p ≤ 0.01). Final pH (5.35-5.24) was not affected by treatment. Salami with honeybush extract had less spontaneous outer surface mould growth or coverage at the end of ripening.

Keywords: Honeybush (*Cyclopia subternata*); extract; Italian salami

4.1 Introduction

Typical Italian salami is characterised as a long ripened, dry-cured and fermented sausage, usually covered in surface moulds and generally not smoked. These Mediterranean type sausages are known and appreciated for their mildly acidic taste, seldom reaching a final pH lower than 5 (Holck *et al.*, 2017). Grey-white outer surface mould growth (either as a result of deliberate inoculation or spontaneous colonisation from the equipment and/or environment) is a desirable phenomenon in these salami, contributing to product aesthetics and importantly, a particular sensory profile achieved through complex extracellular protease and lipase actions (Flores, 1997).

Despite the high value and popular status of salami, modern-day consumer demand for cleaner labelled and less processed foodstuffs is the driving force for new product development and processing innovation, not limited to, but including traditional meat products (Weiss *et al.*, 2010). Regarding dry-cured meats, an abundance of research has been aimed at investigating the possible adverse health effects of the curing agent nitrate (and more so nitrite) often used in salami

production. These additives are involved in the synthesis of *N*-nitrosamines, some of which are classified as probable human carcinogens (IARC, 1978; Herrmann *et al.*, 2015a,b; De Mey *et al.*, 2017).

Nitrite is known as a multifunctional, highly effective antimicrobial and antioxidant agent, as well as for its role in providing a stable pink-red salami colour, a compound for which there is yet to be a single replacement (Sebranek & Bacus, 2007; Honikel, 2008). Although the scientific evidence associating nitrite with adverse human health effects are equivocal (Hord *et al.*, 2009; Aschebrook-kilfoy *et al.*, 2013; Butler, 2015; Song *et al.*, 2015; Bedale *et al.*, 2016), researchers are attempting to omit or partially replace nitrate and nitrite in processed meat products by using novel, naturally-derived compounds with inherent antimicrobial and/or antioxidant effects (Gassara *et al.*, 2016; Alirezalu *et al.*, 2019).

Honeybush (*Cyclopia subternata* Vogel, Family: Fabaceae, Tribe: Podalyrieae) is an indigenous South African shrub enjoyed as hot brewed tea after fermenting the stems and leaves (Joubert *et al.*, 2008; De Beer *et al.*, 2012). *Cyclopia subternata* has proven to be rich in polyphenolic compounds, especially in the green, unfermented state compared to the high temperature exposed fermented state (Kamara *et al.*, 2004; Joubert *et al.*, 2011; Kokotkiewicz *et al.*, 2012). The potential of green, unfermented honeybush extract received attention as a possible bioactive food ingredient evident by the processing patent of a vacuum dried extract in 2002 (De Beer & Joubert, 2002). Nowadays, unfermented honeybush is not only recognised as a beverage, but also as a potential extract for functional foods, which is also the case for rooibos (*Aspalathus linearis*) (De Beer & Joubert, 2010). The latter has succesfulyl been incorporated in the production of droëwors (Jones *et al.*, 2015) and fresh meat patties (Cullere *et al.*, 2019) in attempts to combat oxidation.

There is a lack of research surrounding the potential of using honeybush in processed meats as a bio-active food ingredient. The objective of the present experiment was to study the possible application of unfermented *C. subternata* extract in the manufacturing of typical Italian type salami compared to a traditional recipe using nitrate. Salami weight loss (%) was measured over 35 ripening days and physical-chemical (water activity, pH and moisture content) and sensory characteristics were analysed on the final ready-to-eat salami product.

4.2 Materials and Methods

4.2.1 Honeybush extract

Unfermented, vacuum dried *Cyclopia subternata* hot water extract (1:10 m/v) was supplied by the Agricultural Research Council (ARC) - Infruitec Nietvoorbij, Stellenbosch University and prepared as previously described (Schulze *et al.*, 2016). In short, honeybush plant material was subjected to preheated purified water (90°C, 30 min) in a percolator-type extraction vessel. The extract was

subsequently drained, centrifuged, concentrated, vacuum dried (40°C, 24 h), (Schulze *et al.*, 2016) and frozen (-20°C) in a moisture, light and oxygen impermeable packaging. The final extract (in the form of a fine, brick-brown powder) had a pH of ~5.0 and did not exhibit buffering capacity. The extract was added as a single salami treatment (H= 0.5% w/w of added honeybush).

4.2.2 Salami production

Typical Italian pork salami were manufactured in a meat laboratory (Veneto Agricoltura, Thiene, Vicenza, Italy). Fresh pork meat and fat (collected from the shoulder and belly, ~7:3/meat:fat) was purchased from a local butcher and kept refrigerated (~4°C) until processing commenced.

The total meat and fat (33.5 kg) were minced (6 mm grinder Roberto Cavalli Meat Processing Machinery Srl, model TCS 32, Italy) and mixed using an electrical kneading machine (Roberto Cavalli Meat Processing Machinery, model IMP 50) to create an even distribution of meat and fat. The total meat and fat batter was divided into three batches (11.15 kg each) and the common ingredients were added to each, including: salt (NaCl, 2.5%, 278.75 g); dextrose (0.5%, 55.8 g); and starter culture mixture (0.02%, 1.6 g). The starter culture mixture (in a freeze-dried powder form, SA13-100M, BIOAGRO) contained a total amount of ~305 x 10^9 ± 0.5 log colony forming units (CFU)/100 kg of salami batter in approximately equal amounts of lactic acid bacteria: *Lactobacillus sakei;* and *Pediococcus pentosaceus;* and nitrate reducing bacteria: *Staphylococcus xylosus*. Each batch was subsequently divided into three treatments (3.8 kg each) including: C= without any nitrate or honeybush; N= with potassium nitrate (100 mg/kg, 0.379 g) and ascorbic acid (0.05%; 1.895 g); and H= with honeybush (0.5% w/w, 18.95 g). The meat and fat from one animal was used to eliminate variability at this level, however, simultaneously also defining the limits of this study.

Before stuffing the meat batter into casings, the pH was measured in four different locations by directly inserting a calibrated glass probe with automated thermometer (Knick, Portamess) in a dedicated piece of raw salami batter (including all treatments: C; N; and H; and batches 1-3). Salami were manually stuffed into bovine intestines (L= 9 m, ID= 60-65 mm) using a vertical bagging machine (model ID 25V, Roberto Cavalli Meat Processing Machinery Srl). Care was taken to avoid air pocket formation inside the casings; the salami were tied with a butchers knot and labelled according to the batch (1-3), treatment (C= Control; N= Nitrate; H= Honeybush) and replication (1-23). A total of 68 (n= 23 C, n= 23 N and n= 22 H) salami were produced, with an average fresh weight of ~530 g each. Salami in the present trial were not inoculated (dipped or sprayed) with a mould starter culture, as spontaneous mould growth on the salami surfaces were left to develop.

The salami were allocated in an atmosphere controlled ripening chamber (Majolo® Plus 100 Seasoning Controller, Addendum A, Fig. 4.1). The environmental conditions were as follows: 21°C ± 1°C (day-1); gradually lowered to 16°C ± 1°C (day-6), during which the relative humidity (RH) varied between 65 and 80%. The temperature was then maintained at 14°C ± 1°C at a RH of 80% ± 10%

for 29 days. The salami were relocated weekly inside the ripening chamber to aid in homogenous drying. A final weight loss of ~35% was used as the indicative end-point of ripening.

4.2.3 Physical-chemical parameters

Weight loss (%) of all salami (n= 68) were recorded over time on day-5; 14; 21; 32; and 35, whilst taking care not to disrupt the spontaneous outer surface mould growth. A centre square portion (1x1 cm^2; ~3 mm in width) of the final product salami (n= 45, including all batches:1-3; treatments: C; N; and; H; and 5 replications) were sampled using a knife and tweezers and placed in a Teflon$^®$ sample cup to prevent moisture uptake prior to measurements. Water activity was measured using a calibrated Aqualab 4TE water activity meter (Meter Group, Inc. USA). The final product pH was measured (n= 45, including all batches: 1-3; treatments: C; N; and H; and 5 replications) by inserting a glass pH electrode with built in thermometer (Knick Portamess 911, Berlin, Germany) parallel with the salami length, firstly into the salami centre (pH inner, Addendum A, Fig. 5.4 location A) and secondly underneath the casing (pH outer, Addendum A, Fig. 5.4 location B). All analyses commenced at room temperature (~21°C). The moisture content (%) of the final product salami was determined gravimetrically (AOAC, 1990) by subjecting salami samples (10 g) to heat exposure in a drying oven (24 h, 100°C).

4.2.4 Sensory analysis

The present study is the first attempt at assessing the impact of honeybush extract on salami sensory characteristics. Previous research has characterised the sensory profile of *C. subternata* prepared as a hot brewed tea as being "fynbos-floral" and "fruity-sweet" lacking in a bitter taste as opposed to other potential bitter counterparts (*C. genistoides* and *C. longifolia*) (Erasmus *et al.*, 2017; Alexander *et al.*, 2019). For this reason, it was concluded that the *Cyclopia* species could successfully be applied in the manufacturing of food products. Salami were submitted to sensory analysis of appearance and aroma to detect possible differences among the experimental treatments (C, N and H). Due to the lack of final product microbial analyses, assessors were asked to evaluate the salami only with visual and olfactive senses. The sensory analysis was performed by a six-member trained panel (Istituto per la Qualità e le Tecnologie Agroalimentari, Laboratorio Analisi Sensoriale – Veneto Agricoltura, Thiene, Vicenza, Italy) qualified as experts according to ISO 8586:2012 with sensory descriptive test experience according to ISO 13299:2016 on various food matrices.

Samples were evaluated using a 9-point continuous scale with increasing intensity (1= low intensity and 9= high intensity) for salami odour and external and internal salami colour. With regards to the colour and odour, the 1-9 scale represented the typical colour and odour expected for ripened salami (9 being the best or highest score for these attributes). The surface mould cover was classified according to colour: white; grey; yellow; and green and growth extend, where: class 1= 0%; class 2= 30%; class 3= 60%; and class 4= 100% mould growth or coverage (Addendum A, Fig.

4.2). A total of 15 salami/treatment (C, N and H) were used over 3 days of analysis (5 salami/treatment/session).

4.2.5 Statistical analysis

Data were analysed using the General Linear Model (GLM) procedures of SAS 9.3 statistical software package for Windows (Cary, NC, U.S.). Salami weight loss and physical-chemical traits were analysed, by day of analysis, through a one-way ANOVA with treatment as fixed effect and batch as block effect. A two-way ANOVA was applied to detect any treatment effect on the salami sensory scores with treatment, the assessors and their interaction as fixed effects. Least square means (LSMs) were obtained and post-hoc pairwise comparisons were performed using the Bonferroni correction. Significance was considered at 5% confidence level. Surface mould cover percentage was analysed with a one-way non-parametric ANOVA (Kruskal-Wallis test) and the mould colour evaluation frequencies were evaluated through a χ^2-test using the Maraschino procedure.

4.3 Results

4.3.1 Physical-chemical parameters

All salami reached the final desired weight loss within 30-35% after 35 ripening days with no significant difference among treatments over time (Table 4.1). Although treatment had no effect on weight loss % over time, the final product honeybush-treated salami (H) were significantly higher in moisture content and a_w (p < 0.01) compared to the other treatments (C and N, Table 4.2). The initial average pH of the raw meat batters were: 5.50 (C); 5.46 (N); and 5.51 (H), respectively (calculated as the average of four readings for each treatment) and after 35 ripening days the final pH (salami centres) was between 5.24-5.35 with no significant differences ascribable between the treatments (Table 4.2).

Table 4.1 Effect of unfermented honeybush (*Cyclopia subternata*) extract compared with nitrate inclusion in Italian type salami on the initial weight of fresh salami and weight loss after 5, 14, 21, 32 and 35 days of controlled ripening

Treatment	Control	Nitrate	Honeybush	RSD[1]	p-value
n	23	23	22		
Initial weight (g)	521	518	541	67.1	> 0.05
Weight loss (%):					
Day-5	13.3	13.5	13.4	1.39	> 0.05
Day-14	23.6	23.7	23.3	1.66	> 0.05
Day-21	29.0	28.8	28.4	1.77	> 0.05
Day-32	34.5	34.4	33.8	1.88	> 0.05
Day-35	35.7	35.5	35.0	1.92	> 0.05

[1]Residual standard deviation; Control= salami without the addition of nitrate or honeybush; Nitrate= salami with added potassium nitrate (100 mg/kg) and ascorbic acid (0.5% w/w); Honeybush= salami with added honeybush extract (0.5% w/w). p > 0.05= not significant.

Table 4.2 Effect of unfermented honeybush (*Cyclopia subternata*) compared with nitrate inclusion in Italian type salami on the pH (inner and outer), moisture content and a_w at the end of a 35-day controlled ripening period

Treatment	Control	Nitrate	Honeybush	RSD[1]	p-value
n	23	23	22		
pH-final (inner)	5.35	5.28	5.24	0.13	> 0.05
pH-final (outer)	5.63	5.55	5.54	0.13	> 0.05
Moisture (%)	33.5[a]	34.7[ab]	35.3[b]	0.37	**≤ 0.01**
a_w	0.923[a]	0.925[a]	0.928[b]	0.004	**≤ 0.01**

[1]Residual standard deviation; [a-b] Different superscripts in the same row indicate significant differences in attributes according to the respective p-values; p > 0.05= not significant; p ≤ 0.01= significant; Control= salami without the addition of nitrate or honeybush; Nitrate= salami with added potassium nitrate (100 mg/kg) and ascorbic acid (0.5% w/w); Honeybush= salami with added honeybush extract (0.5% w/w).

4.3.2 Sensory analysis

Even if the present experiment did not consider salami flavour and taste, results indicated that internal colour and odour intensity were similar for all treatments (p > 0.05; Table 4.3). However,

when examining internal colour closer there was a visible difference in some replications in the honeybush treated salami. Upon slicing the salami (H treatment), localised spots with an orange (in the salami centres) and dark-brown appearance (directly beneath the casing) were visible (Addendum A, Fig. 4.3). Results regarding mould colour characterisation (Table 4.4) indicated that white-grey moulds proliferated better on the honeybush-treated salami (H) compared to the C (p < 0.05), whereas the nitrate treated salami (N) resulted in intermediate values. On the other hand, the H salami had significantly fewer moulds compared with the N salami, with the C showing intermediate results (p < 0.05, Fig. 4.1) with the majority of the mould growth on the H salami represented in class 2 (30% coverage), compared with N and C salami with intermediate representation in this class (p < 0.05). Considering class 4 (100% coverage), the outcome was reversed with no honeybush treated salami represented in this class. Overall, the majority of the salami were either classified in class 2 (30% coverage) or class 3 (60% coverage), while class 4 (100% coverage) was poorly represented (Fig. 4.2).

Table 4.3 Effect of unfermented honeybush (*Cyclopia subternata*) compared with nitrate inclusion in Italian type salami on the sensory traits: external colour; internal colour; and odour intensity

Treatment	Control	Nitrate	Honeybush	RSD[1]	p-value
n	15	15	15		
External colour	5.34	5.80	5.42	1.35	**≤ 0.1**
Internal colour	4.93	4.92	4.88	1.05	> 0.05
Odour intensity	6.09	6.16	6.13	0.59	> 0.05

[1]Residual standard deviation; p > 0.05= not significant; p ≤ 0.05= significant; Control= salami without the addition of nitrate or honeybush; Nitrate= salami with added potassium nitrate (100 mg/kg) and ascorbic acid (0.05% w/w); Honeybush= salami with added honeybush extract (0.5% w/w); For each attribute a nine point scale ranging from 1 (low intensity) to 9 (high intensity) was used.

Table 4.4 Effect of unfermented honeybush (*Cyclopia subternata*) compared with nitrate inclusion in Italian type salami: mould colour evaluation (% of the total number of salami classified in a specific class) having at least 1 type of mould

Treatment	Control	Nitrate	Honeybush	χ^2	p-value
n	15	15	15		
Surface mould colour					
White	23.9	13.8	20.6	2.1955	> 0.05
White-grey	17.9[b]	23.1[ab]	39.7[a]	8.5130	**≤ 0.05**
White-yellow-grey	14.9	15.4	6.35	3.0680	> 0.05
White-green-grey	8.96	7.69	7.94	0.0788	> 0.05

χ^2 = Chi-square; [a,b] Different superscripts in the same row indicate a significant difference between attributes according to the respective p-values; p > 0.05= not significant; p ≤ 0.05= significant; Control= salami without the addition of nitrate or honeybush; Nitrate= salami with added potassium nitrate (100 mg/kg) and ascorbic acid (0.05% w/w); Honeybush= salami with added honeybush extract (0.5% w/w).
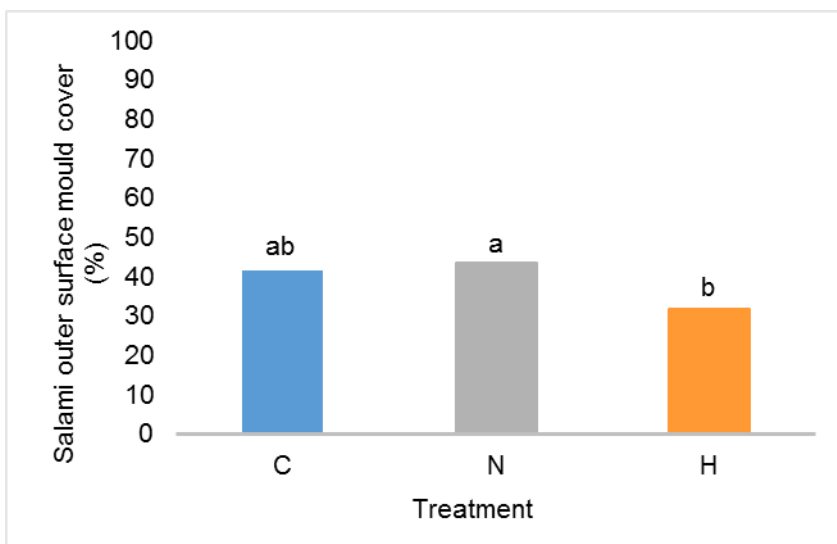
a-b Different superscripts indicate a significant difference in outer mould cover % between treatments ($p < 0.05$); C= control, salami without the addition of nitrate or honeybush; N= nitrate, salami with added potassium nitrate (100 mg/kg) and ascorbic acid (0.05% w/w); H= honeybush, salami with added honeybush extract (0.5% w/w).

Figure 4.1 Effect of unfermented honeybush (*Cyclopia subternata*) extract compared to nitrate inclusion on the spontaneous outer surface mould cover percentage of typical Italian salami.
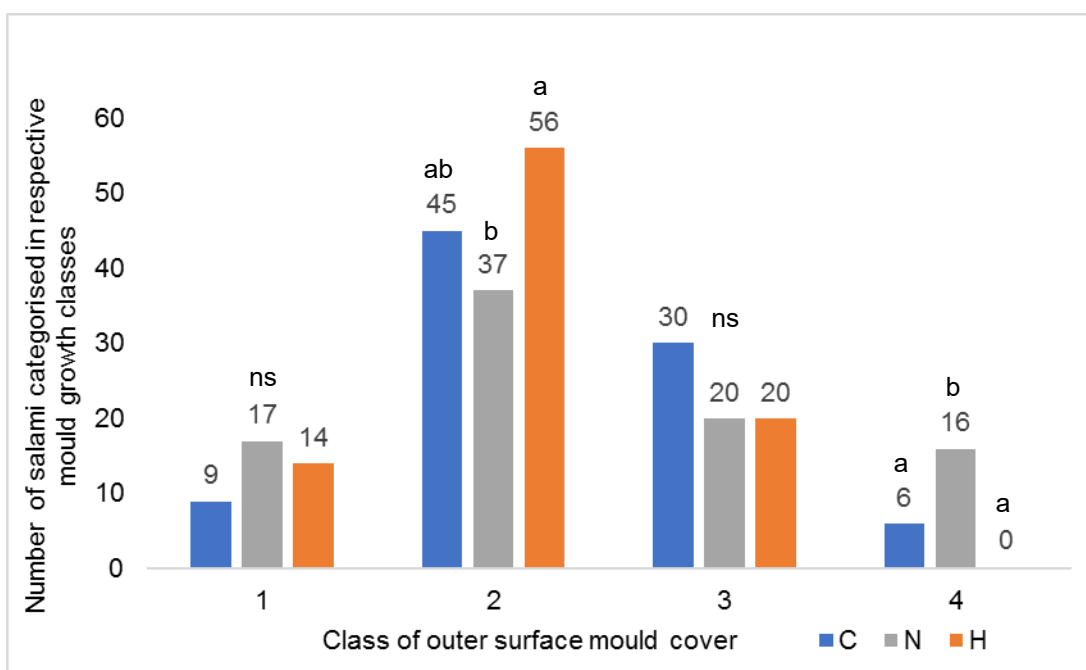
a-b Different superscript indicate significant differences in outer mould cover % between salami treatments ($p \leq 0.05$); ns= not significant ($p > 0.05$); Class 1= 0%; Class 2= 30%; Class 3= 60%; and Class 4= 100% mould coverage; C= control, salami without the addition of nitrate or honeybush; N= nitrate, salami with added potassium nitrate (100 mg/kg) and ascorbic acid (0.05% w/w); H= honeybush, salami with added honeybush extract (0.5% w/w).

Figure 4.2 Effect of unfermented honeybush (*Cyclopia subternata*) extract compared to nitrate on the spontaneous outer surface mould cover percentage of typical Italian salami.

4.4 Discussion

Salami weight loss in the present trial was satisfactory after 35 ripening days as a range of 30-35% weight loss is often used as a quality indicator check for a commercially ready-to-eat Italian type salami (Feiner, 2006; Cullere *et al.*, 2012). The salami a_w at the end of the ripening was, however, in the range between 0.923-0.928, which was slightly higher than the recommended a_w of 0.92 as the safety threshold to inhibit the growth of *Listeria monocytogenes* (Roccato *et al.*, 2017). Alternatively, a combination of a pH ≤ 5.2 and a_w ≤ 0.95 can also ensure microbial safety (Food Standards Australia New Zealand, 2017), which is similar to the salami in the present trial. Similarly, a higher salami a_w was noted when a freeze-dried rooibos extract was used in ostrich salami (Cullere *et al.*, 2013). This finding was also partially supported by moisture content results of the final product salami from the present trial, values observed for the H salami were significantly higher ($p < 0.01$) compared with the C salami, but similar to the N-treated salami (Table 4.2). This could be related to the salami pH, where a lower pH could have exerted a water retention effect. The moisture content of the present trial salami (Table 4.2) were within the range of 24.3-53% moisture, which is also reported for typical commercial Italian salami (Zanardi *et al.*, 2010). Satisfactory salami weight loss (%), final a_w and moisture content reflected a successful drying-ripening process within the controlled ripening chamber.

In general, the pH of fresh salami batter decreases during fermentation to an extent depending on the controlled environmental parameters, but also on the inoculated starter cultures, mainly due to the conversion of carbohydrates to lactic acid by lactic acid bacteria (Greco *et al.*, 2005). The pH range detected in the final product is typical of mildly acidic Mediterranean salami (Holck *et al.*, 2017) and coherent with values indicated for commercial Italian salami (5.15 ≤ pH ≤ 6.83) (Zanardi *et al.*, 2010).

Although some polyphenolic compounds present in honeybush exhibited antimicrobial properties (Singh *et al.*, 2012; Iranshahi *et al.*, 2015) the addition of 0.5% honeybush extract did not influence the final salami pH. Therefore, it is hypothesised that the extract had no effect on the lactic acid bacteria growth (not analysed) which is primarily responsible for the pH decline in this type of meat product. In this experiment the results for all treatments showed that the centre of the salami was slightly more acidic than beneath the casing. This was expected due to lactic acid metabolization and subsequent ammonia production by the moulds located on the outer salami surface (Flores, 1997). The pH gradient becomes smaller and eventually disappears as the ripening duration proceeds. The samples of the present study had 35 days of processing and ripening, which is considered rather short for fresh salami of ~530 g weight.

No differences amongst treatments in the internal salami colour indicated that the honeybush was neither more effective than the control, nor less effective than nitrate in creating a desirable, attractive internal salami colour. The only exception was regarding the external salami colour, for

which a tendency was observed towards a higher intensity for the N salami (p ≤ 0.1, resembling the typical external colour of ripened salami better). The similar internal colour of salami (Table 4.3) might be due to the role of the added coagulase-negative staphylococcus (*S. xylosus*) as part of the starter culture mixture, as extracellular bacterial nitric oxide synthase (NOS) produced by *S. xylosus* have the ability to convert metmyoglobin into the pink-red nitrosomyoglobin in the absence of added nitrate or nitrite (Li *et al.*, 2013; Ras *et al.*, 2018). This could explain why even the C and the H salami without the addition of nitrate (and subsequently reduced nitrite) were similar in internal colour intensity, as determined by the sensory panel (Table 4.3). The localised browning could be due to oxidisation of agglomerated honeybush extract upon oxygen exposure leading to discolouration underneath the casings (Addendum A, Fig. 4.3). The incorporation of freeze-dried and pulverised fermented rooibos extract (a similar product compared to honeybush extract) into different processed meat products, including: ostrich (Hoffman *et al.*, 2014); ungulates (Jones *et al.*, 2015); and rabbit (Cullere *et al.*, 2019), highlighted an effect on the sensory attributes of the respective products. Depending on the type of product, the effects were positive (mitigation of the game meat flavour), neutral (rabbit meat with 0.5% inclusion level) or negative (rabbit meat with 1% and 2% inclusion levels); in all cases a dose-dependent effect was observed.

Both yeasts and moulds may colonise the surface of salami, yeasts have a key role in the fermentation process and moulds are fundamental to provide a desirable appearance, contribute to technical functions and sensory characteristics of the final product (Spotti *et al.*, 2008). It was initially hypothesised that the lower mould cover percentage of H salami compared to N could be ascribed to the demonstrated antimicrobial effect of hesperidin, a flavone present in fairly high amounts in the honeybush extract (Iranshahi *et al.*, 2015). In contrast, the fact that C and H salami did not differ in the latter sense suggested that other mechanisms, which would require an in-depth investigation, may have played a role in salami mould coverage. An extensive variety in salami mould coverage is unwanted as it may lead to heterogeneity of physical-chemical characteristics, such as: weight loss %; a_w; pH; and volatile compounds (and possibly sensory profile), as mould growth and metabolism (i.e. lactic acid, amino acid and lipid metabolization) influence all of these parameters (Bruna *et al.*, 2001; Sunesen & Stahnke, 2003). Furthermore, in the case of spontaneous mould growth, the mycobiota may consist of numerous species of which the toxicity and antibiotic production are unknown and may contribute to unwanted bitter flavours and discolouration (i.e. yellow or black) (Canel *et al.*, 2013; Lozano-Ojalvo *et al.*, 2015).

4.5 Conclusions

Based on the results of the present research study, honeybush (*Cyclopia subternata*) extract seems promising as a natural ingredient intended for salami manufacturing, however, a more in-depth research study is required to gain information surrounding the salami microbial quality and volatile composition when adding such an extract. The use of standardised, synthetic, casings (instead of

natural casings) will aid in the production of more homogenise appearing salami. Dissolving the honeybush extract prior to use is recommended to avoid localised discolouration due to oxidation. Repeating the study with a larger pool of meat and fat to avoid pseudo-replications and incorporating honeybush in increments may provide more information with regard to the effect of honeybush on the physical-chemical product parameters. Surface mould inoculation by dipping or spraying may ensure better mould growth or coverage of the salami surfaces, as the spontaneous growth of environmental moulds were not equal within treatments. The use of synthetic casing and ensuring a more homogenous mould colonisation will in turn equalise the possible effects on salami physical-chemical characteristics (i.e. pH, weight loss % and a_w).

4.6 References

Alexander, L., De Beer, D., Muller, M., Van der Rijst, M. & Joubert, E. (2019). Bitter profiling of phenolic fractions of green *Cyclopia genistoides* herbal tea. *Food Chemistry*, **276**, 626–635.

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

AOAC, 1990. Association of Official Analytical Chemists. 15th ed. Official Methods of Analysis vol. 2. Kenneth Helrich, Arlington, VA, USA. Virginia.

Aschebrook-kilfoy, B., Shu, X., Gao, Y., Ji, B., Yang, G., Li, H.L. & Rothman, N. (2013). Thyroid cancer risk and dietary nitrate and nitrite intake in the Shanghai women's health study. *International Journal of Cancer*, **132**, 897–904.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, **120**, 85–92.

Bruna, J.M., Hierro, E.M., De la Hoz, L, Mottram, D.S., Fernández, M. & Ordóñez, J.A. (2001). The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. *Meat Science*, **59**, 97–107.

Butler, A. (2015). Nitrites and nitrates in the human diet: Carcinogens or beneficial hypotensive agents? *Journal of Ethnopharmacology*, **167**, 105–107.

Canel, R.S., Wagner, J.R., Stenglein, S.A. & Ludemann, V. (2013). Indigenous filamentous fungi on the surface of Argentinean dry fermented sausages produced in Colonia Caroya (Córdoba). *International Journal of Food Microbiology*, **164**, 81–86.

Cullere, M., Dalle Zotte, A. & Hoffman, L.C. (2012). Prelimanary study to test rooibos (*Aspalathus linearis*) as a natural antioxidant applied to ostrich meat patties and Italian type salami. In: *58th International Congress of Meat Science and Technology*. Montreal, Canada.

Cullere, M., Hoffman, L.C. & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products. *Meat Science*, **95**, 72–77.

Cullere, M., Tasoniero, G., Secci, G., Parisi, G., Smit, P., Hoffman, L.C. & Dalle Zotte, A. (2019). Effect of the incorporation of a fermented rooibos (*Aspalathus linearis*) extract in the manufacturing of rabbit meat patties on their physical, chemical, and sensory quality during refrigerated storage. *LWT - Food Science and Technology*, **108**, 31–38.

De Beer, S.W. & Joubert, E. (2002). Preparation of tea-like beverages S.A. patent number 2002/2802.

De Beer, D. & Joubert, E. (2010). Development of HPLC method for *Cyclopia subternata* phenolic compound analysis and application to other *Cyclopia* spp. *Journal of Food Composition and Analysis*, **23**, 289–297.

De Beer, D., Schulze, A.E., Joubert, E., De Villiers, A., Malherbe, C.J. & Stander, M.A. (2012). Food ingredient extracts of *Cyclopia subternata* (honeybush): Variation in phenolic composition and antioxidant capacity. *Molecules*, **17**, 14602–14624.

De Mey, E., De Maere, H., Paelinck, H. & Fraeye, I. (2017). Volatile *N*-nitrosamines in meat products: Potential precursors, influence of processing, and mitigation strategies. *Critical Reviews in Food Science and Nutrition*, **57**, 2909–2923.

Erasmus, L.M., Theron, K.A., Muller, M., Van der Rijst, M. & Joubert, E. (2017). Optimising high-temperature oxidation of *Cyclopia* species for maximum development of characteristic aroma notes of honeybush herbal tea infusions. *South African Journal of Botany*, **110**, 144–151.

Feiner, G. (2006). Raw Fermented Salami. In: *Meat Products Handbook: Practical Science and Technology*. Pp. 314–375. New York: Woodhead Publishing Limited Cambridge: England.

Flores, J. (1997). Mediterranean vs northern European meat products. Processing technologies and main differences. *Food Chemistry*, **59**, 505–510.

Food Standards Australia New Zealand (2017). Production of Uncooked Fermented Salami (UCFM), p. 10. URL: https://www.mpi.govt.nz/dmsdocument/20441/send.

Gassara, F., Kouassi, A.P., Brar, S.K. & Belkacemi, K. (2016). Green alternatives to nitrates and nitrites in meat-based products - A Review. *Critical Reviews in Food Science and Nutrition*, **56**, 2133–2148.

Greco, M., Mazzette, R., De Santis, E.P.L., Corona, A. & Cosseddu, A.M. (2005). Evolution and identification of lactic acid bacteria isolated during the ripening of Sardinian sausages. *Meat Science*, **69**, 733-739.

Herrmann, S.S., Duedahl-Olesen, L., Christensen, T., Olesen, P.T. & Granby, K. (2015a). Dietary exposure to volatile and non-volatile *N*-nitrosamines from processed meat products in Denmark. *Food and Chemical Toxicology*, **80**, 137–143.

Herrmann, S.S., Duedahl-Olesen, L. & Granby, K. (2015b). Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, **48**, 163-169.

Hoffman, L.C., Jones, M., Muller, N., Joubert, E. & Sadie, A. (2014). Lipid and protein stability and sensory evaluation of ostrich (*Struthio camelus*) droëwors with the addition of rooibos tea extract (*Aspalathus linearis*) as a natural antioxidant. *Meat Science*, **96**, 1289-1296.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M. & Heir, E. (2017). Health and safety considerations of fermented sausages. *Journal of Food Quality*, **2017**, 1–25.

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, **78**, 68–76.

Hord, N.G., Tang, Y. & Bryan, N.S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *The American Journal of Clinical Nutrition*, **90**, 1–10.

International Agency for Research on Cancer (1978). IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Some *N*-nitroso compounds,**17**, 1–349.

ISO 8586:2012. Sensory analysis - General guidance for the selection, training and monitoring of selected assessors and expert sensory assessors. URL: https://www.iso.org/standard/45352.html.

ISO 13299:2016. Sensory analysis - Methodology - General guidance for establishing a sensory profile URL: https://www.iso.org/standard/58042.html.

Iranshahi, M., Rezaee, R., Parhiz, H., Roohbakhsh, A. & Soltani, F. (2015). Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin. *Life Sciences*, **137**, 125–132.

Jones, M., Hoffman, L.C. & Muller, M. (2015). Effect of rooibos extract (*Aspalathus linearis*) on lipid oxidation over time and the sensory analysis of blesbok (*Damaliscus pygargus phillipsi*) and springbok (*Antidorcas marsupialis*) droëwors. *Meat Science*, **103**, 54–60.

Joubert, E., Gelderblom, W.C.A., Louw, A. & De Beer, D. (2008). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides* - A review. *Journal of Ethnopharmacology*, **119**, 376–412.

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. & De Lange, J.H. (2011). Honeybush (*Cyclopia* spp.): From local cottage industry to global markets - The catalytic and supporting role of research. *South African Journal of Botany*, **77**, 887–907.

Kamara, B.I., Brand, J.D., Brandt, Vincent, E. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391–5395.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Li, P., Kong, B., Chen, Q., Zheng, D. & Liu, N. (2013). Formation and identification of nitrosylmyoglobin by *Staphylococcus xylosus* in raw meat batters: A potential solution for nitrite substitution in meat products. *Meat Science*, **93**, 67–72.

Lozano-Ojalvo, D., Rodríguez, A., Cordero, M., Bernáldez, W., Reyes-Prieto, M. & Córdoba, J.J. (2015). Characterisation and detection of spoilage mould responsible for black spot in dry-cured fermented sausages. *Meat Science*, **100**, 283–290.

Ras, G., Bailly, X., Chacornac, J.P., Zuliani, V., Derkx, P., Seibert, T.M., Talon, R. & Leroy, S. (2018). Contribution of nitric oxide synthase from coagulase-negative staphylococci to the development of red myoglobin derivatives. *International Journal of Food Microbiology*, **266**, 310-316.

Roccato, A., Uyttendaele, M., Barrucci, F., Cibin, V., Favretti, M., Cereser, A., Dal, M., Pezzuto, A., Piovesana, A., Longo, A., Ramon, E., De Rui, S. & Ricci, A. (2017). Artisanal Italian salami and soppresse: Identification of control strategies to manage microbiological hazards. *Food Microbiology*, **61**, 5–13.

Schulze, A.E., De Beer, D., Mazibuko, S.E., Muller, C.J.F., Roux, C., Willenburg, E.L., Nyunaï, N., Louw, J., Manley, M. & Joubert, E. (2016). Assessing similarity analysis of chromatographic fingerprints of *Cyclopia subternata* extracts as potential screening tool for *in vitro* glucose utilisation. *Analytical and Bioanalytical Chemistry*, **408**, 639–649.

Sebranek, J.G. & Bacus, J.N. (2007). Cured meat products without direct addition of nitrate or nitrite: What are the issues? *Meat Science*, **77**, 136–147.

Song, P., Wu, L. & Guan, W. (2015). Dietary nitrates, nitrites, and nitrosamines intake and the risk of gastric cancer: A meta-analysis. *Nutrients*, **7**, 9872–9895.

Spotti, E., Berni, E. & Cacchioli, C. (2008). Characteristics and Applications of Moulds. In: *Meat Biotechnology*, (edited by F. Toldrá). Pp. 181-195. New York, USA: Springer Science+Business Media.

Sunesen, L.O. & Stahnke, L.H. (2003). Mould starter cultures for dry sausages - Selection, application and effects. *Meat Science*, **65**, 935–948.

Weiss, J., Gibis, M., Schuh, V. & Salminen, H. (2010). Advances in ingredient and processing systems for meat and meat products. *Meat Science*, **86**, 196–213.

Zanardi, E., Ghidini, S., Conter, M. & Ianieri, A. (2010). Mineral composition of Italian salami and effect of NaCl partial replacement on compositional, physico-chemical and sensory parameters. *Meat Science*, **86**, 742–747.

CHAPTER 5

The physical-chemical changes of warthog (*Phacochoerus africanus*) salami with different levels of unfermented honeybush (*Cyclopia subternata*) extract over a 40-day ripening period

Abstract

The use of curing agents, nitrate (NO_3) and nitrite (NO_2), in salami remains controversial, simultaneously, the interest in bio-active extracts in processed meats are ever growing. The objective of this study was to analyse the effect of different levels of unfermented honeybush extract on the physical-chemical characteristics of small diameter (32 mm) warthog salami with reduced added sodium nitrate (75 mg/kg), including: pH; water activity (a_w); CIE L*a*b* surface colour; texture profile analysis properties; proximate composition; titratable free fatty acids; and titratable acidity. Ten mature warthogs and pork fat from a pool of domesticated sows were used to create a total of six salami batches (29.6 kg each) which were subdivided into five treatments (C, T1-T4, where: C= 0%; T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5% w/w of added honeybush). Salami sufficiently decreased in moisture (30.1%) and were well within the recommended a_w for microbial safety, with T4 resulting in a significantly higher a_w (0.873, $p < 0.001$). Although the highest honeybush addition significantly lowered the salami pH (p= 0.004), the pH overall failed to decrease sufficiently after stuffing, resulting in a strangely high final product salami pH (6.71-7.46). The control salami had the highest redness after a 40-day ripening period (CIE a*= 11.73), although not significant as compared to salami with honeybush: T1 (CIE a*= 10.81); and T3 (CIE a*= 11.45). The retention of redness was best in salami with the highest honeybush inclusion levels (T3 and T4) after five and a half months of frozen storage (-20°C). Warthog meat which is naturally darker than domesticated pork seems suitable for the production of attractive, vivid red salami with less added sodium nitrate. The addition of honeybush extract in game salami seems promising for a colour retention effect during frozen storage.

Keywords: salami; warthog meat; sodium nitrate reduction; honeybush (*Cyclopia subternata*); natural extract.

5.1 Introduction

Italy is a country which is known for high quality salami production where locals take great pride in regional specialties, reflected in their many dry-cured fermented meats with geographical quality trademarks (Montanari *et al.*, 2016; Roccato *et al.*, 2017). One example of a regional product is wild boar (*Sus scrofa;* Family*: Suiidae*) salami produced in the Tuscan region where these animals are regarded as an agricultural pest and often culled to prevent vineyard destruction. The common warthog (*Phacochoerus africanus;* Family*: Suiidae*) is similarly regarded as an agricultural pest in certain regions in South Africa (Swanepoel *et al.*, 2016a). The physical-chemical properties of warthog meat have been studied to promote commercialisation of meat that is said to be

underrepresented in the South African game meat market (Swanepoel *et al.*, 2014, 2016c). Although salami is predominantly produced from pork meat and fat, especially in the Mediterranean regions (Demeyer *et al.*, 2000), various South African game meat species have been used for this purpose (Todorov *et al.*, 2007; Van Schalkwyk *et al.*, 2010; Chakanya *et al.*, 2018). The production of processed meat products from less desired whole warthog cuts (e.g. forequarters) was previously proposed (Swanepoel *et al.*, 2016c). This could positively contribute to the South African game meat industry through high quality products created from lower value cuts and furthermore aid in food security. Warthog meat has proven to be suitable for the production of cabanossi (Swanepoel *et al.*, 2016b; Hoffman *et al.*, 2019; Mahachi *et al.*, 2019), however, it has not yet been used in the production of salami.

Microbiologically safe salami production is reliant on a variety of microbial hurdles, including lowering of the pH and water activity (a_w), a high salt content and the use of nitrate (NO_3) and/or nitrite (NO_2) preserving salts (Holck *et al.*, 2017). Outer surface mould growth is an additional microbial hurdle and is particularly popular amongst Mediterranean salami, contributing to artisanal aesthetics and more importantly, product quality and safety (Flores, 1997; Leroy *et al.*, 2006). These preferred surface moulds outcompete potentially harmful moulds, protect the salami against oxygen exposure, aid in gradual water loss to prevent case hardening and contribute to an intricate sensory profile through lipolytic and proteolytic actions (Flores, 1997; Lücke, 2000; Sunesen & Stahnke, 2003).

Nitrate and nitrite salts, commonly added to processed meats, are regulated as food additives where the European Union (Directive 2008/1333/EC) permits a maximum of 150 mg/kg (ppm) of added sodium/potassium nitrate and 150 mg/kg of sodium/potassium nitrite ($NaNO_2$/KNO_3; E252, E251). In turn, the South African Department of Health permits a maximum of 200 mg/kg of added sodium/potassium nitrate and 160 mg/kg of added sodium/potassium nitrite (DoH, Act of 54 of 1972). Nitrate, as opposed to nitrite, is more readily associated with slow acidifying, Mediterranean type salami to allow for gradual reduction to nitrite through exogenous microbial reductase (Flores, 1997; Blaiotta *et al.*, 2004). The multifunctionality of nitrite has been emphasised repeatedly as a highly effective antimicrobial, antioxidant and partaker in creating the particular cured flavour and attractive pink-red colour associated with salami (Møller & Skibsted, 2002; Sindelar & Milkowski, 2011; Taormina, 2014; Feng *et al.*, 2016). Unfortunately, these highly effective curing agents have proven to lead to the formation of carcinogenic *N*-nitrosamines when exposed to a high temperature and an acidic environment (Hammes, 2012; Herrmann *et al.*, 2015). Concerns were raised over the regular consumption of nitrate/nitrite containing processed meats given that the necessary precursors for this reaction are available in products like salami, being amine containing compounds (such as free amino acids and biogenic amines) and available nitrite (Sindelar & Milkowski, 2011; De Mey *et al.*, 2017). Despite scientific proof promoting the health benefits of a nitrate rich diet, i.e.

consuming vegetables high therein (Hord *et al.,* 2009; Butler, 2015), there have been numerous revisions of the safety limit of these additives in cured meat products (Bedale *et al.,* 2016; Mortensen *et al.,* 2017a,b). After all, consumer demand ultimately drives new product development, recipe formulation and processing alteration (Weiss *et al.,* 2010). This has led to the latest trend of including substances for replacing or mimicking the functions of nitrate/nitrite in cured meat products, with the focus on natural extracts with microbial and/or antioxidant activity (Shah *et al.,* 2014; Alahakoon *et al.,* 2015; Gassara *et al.,* 2016; Jin *et al.,* 2018a; Alirezalu *et al.,* 2019; Ribeiro *et al.,* 2019).

Honeybush (*Cyclopia subternata* Vogel; Tribe: Podalyrieae; Family: Fabaceae) is a shrub endemic to South Africa and is enjoyed as hot brewed tea after a fermentation (oxidation) period (Joubert *et al.,* 2011, 2019). The increasing interest in dietary polyphenolic compounds urged research surrounding the potential health promoting effects of honeybush consumption. As found with rooibos (another endemic shrub enjoyed as brewed tea), honeybush has proven to be rich in polyphenolic compounds: primarily the flavone, hesperidin; and xanthones, mangiferin and isomangiferin (Kamara *et al.,* 2004; Kokotkiewicz *et al.,* 2012; Magcwebeba *et al.,* 2016). Although total phenolic composition and antioxidant capacity are variable, both fermented and unfermented (green) honeybush has proven to exhibit antioxidant capacity (Joubert *et al.,* 2008; De Beer *et al.,* 2012). However, a higher content of bioactive compounds in the latter shifted research focus towards unfermented honeybush extracts for the functional foods and nutraceutical industries (Joubert *et al.,* 2019). Fermented and unfermented rooibos extracts have been applied in a variety of processed meat products and showed promising antioxidant results (Cullere *et al.,* 2013; Hoffman *et al.,* 2014; Jones *et al.,* 2015; Cullere *et al.,* 2019). Research lacks the investigation of honeybush extract in processed meat products. The objective of this study was to investigated the effect of unfermented *C. subternata* extract added in increasing concentrations: C= 0%; T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5% in dry-cured fermented warthog salami with reduced added sodium nitrate (75 mg/kg). The maximum addition of the extract was chosen based on a preliminary study (Chapter 4) whereby 0.5% of added honeybush showed positive results in typical Italian salami in terms of sensory attributes and secondly, to prevent a possible prooxidant effect at higher concentrations (Lambert & Elias, 2010; Hoffman *et al.,* 2014).

5.2 Materials and Methods

5.2.1 Salami ingredients

5.2.1.1 Warthog meat and pork fat

Ten free roaming warthogs (nine sows and one boar) were sourced from the Limpopo province, South Africa. The total weight of frozen, deboned warthog meat for salami production equalled 125 kg. Pork fat (53 kg) from the shoulders and back from more than five domestic sows (Landrace and Large White crossbred, 2-3 years of age) was purchased and frozen (-20°C) prior to use. The pigs

predominantly received a diet containing maize, soya and canola in feeding lots in the Swartland area, Western Cape, South Africa.

5.2.1.2 Honeybush (Cyclopia subternata) extract

Unfermented, vacuum dried *Cyclopia subternata* hot water extract (1:10 m/v) was supplied by the Agricultural Research Council (ARC) - Infruitec Nietvoorbij, Stellenbosch University, and prepared as previously described (Schulze *et al.*, 2016). In short, honeybush plant material was subjected to preheated purified water (90°C, 30 min) in a percolator-type extraction vessel. The extract was subsequently drained, centrifuged, concentrated, vacuum dried (40°C, 24 h) and frozen (-20°C) in a moisture, light and oxygen impermeable packaging. The extract was added in increasing amounts according to salami treatment, where: Control= 0%; Treatment 1= 0.125%; Treatment 2= 0.25%; Treatment 3= 0.375%; and Treatment 4= 0.5% of added honeybush extract.

The minimum amount of distilled water (dH$_2$O) required to completely dissolve the maximum amount of extract required (T4= 0.5% w/w, 30.56 g of honeybush), was predetermined as 100 mL dH$_2$O. The amount of honeybush extract per respective treatment (T1-T4) in 100 mL dH$_2$O were heated on a magnetic stirrer (80°C for 5-10 min, speed setting 4-5) until visible evaporation bubbles appeared on the surface, followed by a mixing period at a lower temperature (50°C, another 5-10 min), until completely dissolved and free of lumps. The result was a dark-brown/brick coloured solution with a sweet floral or honey sent (Addendum A, Fig. 5.1).

5.2.1.3 Starter culture mixture, sodium nitrate, salt and sugar

A non-commercial cell suspension was prepared as a starter culture mixture by the Microbiology Department, Stellenbosch University. The mixture (~305 x 10^9 ± 0.5 log colony forming units per 100 kg of raw meat and fat) consisted of equal amounts of lactic acid bacteria: *Lactobacillus sakei* (AQ14); and *Pediococcus pentosaceus* (AEH4); and a nitrate reducing Micrococcus species (MC2). The cultures were suspended in a minimum amount of water to avoid excess liquid addition to the raw meat batter.

Pure sodium nitrate (NaNO$_3$) crystals were finely blended (commercial Sunbeam grinder) to prevent the addition of lumps. The latter was added to all treatments (C, T1-T4) at a concentration of 75 mg/kg (0.456 g/6.11 kg, Table 5.1). Fine, non-iodized sodium chloride (Royal Salt, 2.5%) was added as an ingredient, as well as glucose (0.2%) to act as a fermentable sugar source for the starter culture mixture.

5.2.2 Salami production

5.2.2.1 Experimental replicates and treatment formulation

The total amount of warthog meat (125 kg) and pork fat (53 kg) were randomly divided into respective batches before processing of each, in order to avoid pseudo-replications between the meat and/or fat from one animal. From the total meat and fat mixture, six batches (29.6 kg each;

meat:fat ratio of 7:3) were produced and these formed the statistical replications. Batches (i.e. replications) were further subdivided into five treatments (6.1 kg each: C= 0%; T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5% of added honeybush extract) and 10-14 salami were produced to ensure an adequate number available for all required analyses to be conducted. Other common salami ingredients included (w/w): sodium chloride (2.5%); distilled water (1.6%); glucose (0.2%); starter culture mixture (0.08%) and sodium nitrate (0.0075%), as illustrated in Table 5.1 The salami length was calculated based on the required sample size for subsequent analyses, e.g. a 2 cm salami portion was required for a single texture profile analysis (TPA) compression. The total salami length equalled ~40 cm, weighing ~400 g (fresh).

Table 5.1 Formulation of warthog (*Phacochoerus africanus*) salami with different levels of unfermented honeybush (*Cyclopia subternata*) extract

	Control		Treatment 1		Treatment 2		Treatment 3		Treatment 4	
	Mass	%	Mass	%	Mass	%	Mass	%	Mass	%
Meat and fat (kg)	5.85	95.7	5.84	95.6	5.84	95.5	5.83	95.3	5.82	95.2
Salt (NaCl, g)	150.50	2.5	150.50	2.5	150.50	2.5	150.50	2.5	150.50	2.5
dH$_2$O (ml)	100.00	1.6	100.00	1.6	100.00	1.6	100.00	1.6	100.00	1.6
Glucose (g)	11.84	0.2	11.84	0.2	11.84	0.2	11.84	0.2	11.84	0.2
Starter culture mixture (ml)	4.89	0.08	4.89	0.08	4.89	0.08	4.89	0.08	4.89	0.08
Sodium nitrate (NaNO$_3$, g)[1]	0.46	0.0075	0.46	0.0075	0.46	0.0075	0.46	0.0075	0.46	0.0075
Honeybush extract (g)	-	-	7.64	0.125	15.28	0.25	22.92	0.375	30.56	0.5
Total weight (kg)	6.11		6.11		6.11		6.11		6.11	

[1]Sodium nitrate was added in a concentration of 75 mg/kg as opposed to the maximum permitted European Union limit of 250 mg/kg expressed as NaNO$_2$/KNO$_3$ when no nitrite is added (EC 1333/2008 of 16 December 2008 for non heat treated meat products).

5.2.2.2 Production procedure

The previously frozen warthog meat was semi-defrosted (4°C, 24 h). The pork fat was kept frozen (-20°C) until processing to prevent smearing and a temperature increase of the meat batter during mixing. The frozen fat was reduced into cubes (~5 cm^3, Addendum A, Fig. 5.2) using a band saw (Crown-Okto, Crown National, South Africa). Any visible sinew was removed from the warthog meat and it was roughly cut into similar sized cubes using a knife. Half of the fat cubes were added in a bowl cutter (Mainca, CM14, Spain; 25 s, speed setting 1) before adding the meat and remaining fat

and increasing the speed (35 s, speed setting 2) to create a coarse meat and fat mixture (Addendum A, Fig. 5.3). This mixture, referred to as the salami batter, was transferred and mixed (60 s) in an industrial food mixer (Hobart, A200, FM Mixer, London), whilst adding the common ingredients and honeybush extract according to treatment. Care was taken not to over mix the batter, whilst ensuring adequate mixing to create a sticky consistency. The salami batter was stuffed into moisture permeable collagen casings (32 mm in diameter) and tagged with colour coded labels to distinguish treatments (C, T1-T4) and replications (1-6). The salami was transported to *The Flying Pig* (Darling, Western Cape, South Africa) meat processing facility and dipped in a commercial *Penicillium nalgiovense* suspension (Mould 600, 25 g/10 L water). They were randomly allocated to locations in a non-automated, artisanal-type ripening room (4 m in length x 3.5 m in width x 3.5 m in height) without circulating air flow, containing a variety of other fermented meats. Five temperature and relative humidity (RH) loggers (LogTag, SA, Trex-8) were randomly allocated in the roof between the salami to record environmental changes over time.

The ripening period was 40 days during which salami were sampled to determine physical and chemical changes over time. Three salami were dedicated to weight loss (%) measurements serving as an indication of sufficient ripening duration in the room. A total of 30 salami (representing all treatments: C; T1-T4; and 6 replicates of each) were removed from the ripening room on each predetermined sampling day (day-3; 6; 10; 15; 22; 25; 30; and 40) for measuring of the pH, a_w, surface colour measurements (CIE L*a*b*), and texture profile analysis (TPA). Since some of these analyses are sample destructive, the respective salami were discontinued from the ripening room after sampling (except for the three weight loss indication salami). The salami only had a firm enough texture for texture analysis after 10 ripening days, hence the TPA measurements were recorded from ripening day-10 onwards and the surface colour (CIEL*a*b*) from day-6 onwards to allow for slicing and blooming.

5.2.3 pH

The pH was measured by means of three slightly different methods over the ripening time. Initial measurements (ripening day-1 and day-3) were taken from homogenised salami samples. Dedicated salami pieces (n= 30 per sampling day: C; T1-T4; and replications 1-6) were homogenised (10 s, 20 000 rpm) using a sample mill (FOSS Analytical, Knifetec 1095) equipped with a high-speed rotor blade ideal for high fat sample processing. Homogenised samples (3 g in 27 ml dH_2O) were magnetically stirred (30 min, 300 rpm; Electronicrührer, VariMag®, Poly 15) and pH measurements were recorded (ACCSEN pH70 + DHS; pH electrode: AEL92T2, Italy). Differing from the initial pH measurements, localised pH measurements of whole salami pieces (ripening day-6 to day-40) were recorded by inserting a glass electrode with built in thermometer (Hanna Edge™ HI98 series; pH electrode: FC2053 FoodCare, South Africa) parallel to the salami length, perpendicular to the sliced salami centre (A) and underneath the casing (B) (Addendum A Fig. 5.4). After five and a

half months of frozen storage (-20ºC), the salami pH was similarly recorded as done initially with a slight change in sample preparation. Samples (3g in 27 ml dH_2O) were homogenised (30 s, 8000 rpm; IKA® T18 digital Ultra Turrax®) and filtered through gauze to remove cloudy meat and fat particles after which pH measurements were taken. The pH meters were calibrated according to the manufacturer's instructions before readings commenced and all pH readings were recorded in duplicate at room temperature (~21°C).

5.2.4 Water activity (aw)

A centre rectangle portion (10 x 10 x 3 mm^3) of the salami (n= 30 per sampling day: C; T1-T4; and replications 1-6) were sampled using a knife and tweezers and placed in a Teflon® sample cup to prevent moisture uptake prior to measurements. Water activity was measured using a calibrated (Standard a_w= 0.984, Aqualab, Decagon) Aqualab 4TE water activity meter (Meter Group, Inc. USA) at room temperature (~21°C).

5.2.5 Surface colour

Salami slices (4-5 mm thick) were cut at a 45 ° angle as commonly served, placed on a white background and allowed to air bloom for 30 min (Needham *et al.*, 2020). The machine was calibrated according to the manufacturer's instructions using standard black and white colours and green colour check before salami colour measurements commenced. Five instrumental colour measurements were taken (n= 30 per sampling day: C; T1-T4; and replications 1-6) according to the 3-axis CIE L* (lightness), a* (green-red) and b* (blue-yellow) measuring system using a hand held Colour-guide 45°/0° colorimeter (BYK-Gardner GmbH, Germany, Addendum A, Fig. 5.5). The a* and b* values were subsequently used to calculate the chroma value (relative saturation or intensity) and the hue-angle (colour definition).

Chroma (C*)= $\sqrt{(a^*)^2 + (b^*)^2}$

Hue-angle (°)= $\tan^{-1}(\frac{b^*}{a^*})$

5.2.6 Textural profile analysis (TPA)

Texture profile analysis was performed (~21°C) to evaluate different salami texture attributes (Szczesniak *et al.*, 1963; Bourne, 1968). A minimum of four cylindrical portions per salami (n= 30 per sampling day: C; T1-T4; and replications 1-6) of 20 mm in length (L) and ~30 mm in diameter (D) were sampled, resulting in a recommended D/L ratio of 1.5 for TPA measurements of meat products (Mittal *et al.*, 1992). The salami were not cored as done by previous authors (Van Schalkwyk *et al.*, 2010), as a uniform diameter was achieved by using standardised synthetic casings (32 mm in diameter). The sample diameter was smaller than the diameter of the probe (35 mm), ensuring proper upper compression. A Force Transducer Instron (Model 2519-107, 5000 N Capacity, SANAS accredited No. 827) equipped with a 3.5 cm diameter cylindrical probe, in

conjunction with Bluehill 3 version 3.65 Illinois Tool Works software were used. The total sample surface, with the outer casings included, was uniaxially compressed twice to 50% of the original height by means of a 1000 N load cell at a cross-head speed of 100 mm/min. A total distance of 20 mm was selected for the probe to move downwards (10 mm) and upwards (10 mm) to the original starting point. The load was balanced and the extension (mm) zeroed before every reading. A single measurement consisted of four consecutive steps: initial compression; relaxation; recompression; and final relaxation, resulting in numerical values representing texture attributes. Real time graphs (Addendum A, Fig. 5.6) were plotted as the compression tests proceeded which represented sample behaviour when placed under pressure.

The following values were generated: maximum force for the first cycle (N); energy to reach the maximum load for the first cycle (J); maximum force for the second cycle (N); energy to reach the maximum load for the second cycle (J); springiness (mm); gumminess (N); and springiness (%). Of these, the following TPA parameters were regarded applicable to the study: hardness (N), the maximum force of the first compression (Addendum A, Fig. 5.6 at position 1); springiness (mm), the ability of a sample to recover to its original shape after the deforming force was removed; cohesiveness (dimensionless ratio of A2/A1, where A1 represents the total energy for the first compression and A2 the total energy for the second compression), the extent to which the sample can be deformed prior to rapture; and chewiness (J), defined as the work required to masticate a solid sample before swallowing (hardness x cohesiveness x springiness) (Caine *et al.*, 2003; Herrero *et al.*, 2007).

5.2.7 Proximate analyses

Samples were homogenised for 10 s using a sample mill (FOSS Analytical, KnifetecTM 1095) equipped with a high-speed rotor blade (20 000 rpm), ideal for homogenising of high-fat samples prior to testing (Addendum A, Fig. 5.7). The blades and inner container were cleaned with acetone and dried with paper towel between samples. The moisture, crude protein, total lipid and ash content (%) of the raw meat batter on the day of production (day-0) and of the final product salami (day-40) were analysed in duplicate (all treatments: C; T1-T4; and replications 1-6). All tests were conducted in an Agricultural Laboratory Association of South Africa (AgriLASA) accredited laboratory, proven to perform accurate and repeatable proximate analyses.

5.2.7.1 Moisture and ash content

The moisture content (Method 934.01) was determined on 2.5 g salami samples based on evaporation and weight loss by drying (Labcon drying oven) at 100-105°C, for 24 h (AOAC, 2002a). Dried samples were subsequently used to determine total ash content (Method 942.05) by subjecting the samples to a temperature and time combination (500°C, 6 h) in a furnace (Kiln Contractors, Labofurn), (AOAC, 2002b).

5.2.7.2 Total lipid content

The total lipid content was determined as described by Lee *et al*. (1996) by defatting a 5 g sample with chloroform:methanol (2:1 v/v) ideal for samples with a fat content > 5%. The defatted and oven dried samples were homogenised using a handheld grinder (Sunbeam) before being used for protein analysis.

5.2.7.3 Crude protein content

Crude protein (Method 992.15) was evaluated according to the DUMAS combustion method, using a LECO (FP-528 Determinator) and a multiplication factor of 6.25 to determine total protein from total nitrogen content (AOAC, 2002c). Ethylenediamine tetraacetic acid (EDTA) certified reference material (EDTA LCRM®, 9.56 ± 0.07% nitrogen, Leco, Part no. 502-896, Lot no. 1000) was used as external standard to ensure accurate and reproducible nitrogen readings.

5.2.8 Free fatty acid content (FFA)

Final product (40-day ripened) salami were vacuum sealed and frozen (-20°C) for five and a half months before free fatty acid analysis commenced based on the acid base titration method as described by Egan *et al*. (1981). Salami were removed from frozen storage on the day of analysis and sample pieces were collected from frozen salami using a band saw (Crown-Okto, Crown National, South Africa). A salami section (0.5 cm) was removed from both ends of the samples to exclude oxygen exposed surfaces. The remaining sample pieces were homogenised for 10 s (Dampa bowl cutter Model CT 35N, Golasecca, Italy).

The free fatty acid content analysis entailed the addition of 30 ml of chloroform and 0.5 g of $NaSO_4$ (Sigma-Aldrich, 239313) to 10 g of the previously homogenised salami sample and this was further homogenised (30 s, 8000 rpm; IKA® T18 digital Ultra Turrax®). The mixture was filtered (Whatman no 1) and the total fat content of a 5 ml aliquot was calculated according to the percentage weight loss after heat exposure (80°C, 45 min) on a sand plate (Labcon, Protea Laboratory Services, Cape Town). Neutralised ethanol (95%) was added to the remaining filtrate in a 1:1 ratio. After the addition of three drops of phenolphthalein indicator (0.5 g in 50 ml ethanol and 50 ml dH_2O), the filtrate was titrated against 0.1 M NaOH whilst on a magnetic stirrer, until a faint pink colour appeared for at least 30 s (indicating the titration end point). The FFA% (expressed as g oleic acid/100 g of fat) was subsequently calculated as FFA% = [(S x N x F) x 28.2]/ W.

Where:

S= titration volume of NaOH (ml)

N= normality of the NaOH solution (0.1)

F= factor of the $NaSO_4$ (1)

W= fat in the sample (g)

5.2.9 Titratable acidity (TA)

Vacuum sealed, frozen (-20°C) salami pieces were sampled and homogenised as described for FFA. Homogenous salami (3 g in 27 ml dH$_2$O) were stirred for 30 min at 300 rpm (Electronicrührer, VariMag ®, Poly 15). The mixture was filtered through gauze (Addendum A, Fig. 5.8) and three drops of phenolphthalein indicator (as prepared for FFA) was added. The mixture was titrated against 0.1 M NaOH whilst on a magnetic stirrer, until a faint pink colour appeared for at least 30 s (indicating the titration end point). The TA (expressed as lactic acid/100 g sample) was subsequently calculated by using the equation below (Glass *et al.*, 1992). The FFA and TA of the experimental salami (C, T1-T4) were compared to retail purchased salami (*Ricomondo* Salami, Gastro Foods) as a benchmark (*Ricomondo* Salami ingredients list: Pork, Salt, Whey Powder, Spices, Dextrose, Red Wine, Garlic, MSG Flavour Enhancer (E621), Sodium Ascorbate Antioxidant (E301), Sodium Nitrite (E250) Curing Agent, Acidity Regulator (E500), Colourant (E127), Starter Cultures).

Lactic acid %= (N x V x M)/ (S x 10)

Where:

N= normality of the NaOH solution (0.1)

V= titration volume of NaOH (ml)

M= molecular weight of lactic acid divided by the number of hydrogen ions in the molecule (90.008)

S= sample weight (g)

5.2.10 Statistical analysis

Mixed model ANOVA were conducted using the "lmer" package in R. Treatment and time (and in the selected case of pH, position) were treated as fixed effects. The samples were treated as a random effect. For post hoc analysis, Fisher Least Significant Difference (LSD) was used. In some cases where outliers were present, further investigation showed that the outliers did not affect the outcomes of the results.

5.3 Results

The average weight loss of the salami was ~40% after 40 ripening days and salami were homogenously covered in *P. nalgiovense* mould, irrespective of treatment (Addendum A, Fig. 5.9). Table 5.2. summarises the statistical significance (p-values) of the influence of the main effects of treatment (T) and day (D) and their interaction (TxD) on salami attributes: a$_w$; surface colour; TPA; and proximate composition. Due to the potential influence of the position or location of the pH measurements taken in the salami, the statistical significance (p-values) of the influence of treatment (T), day (D) and position (P) and their interactions (TxDxP; TxD; TxP; DxP) are shown in

Table 5.3. The change in RH inside the ripening room over the 40-day ripening period (recorded by five data loggers) is depicted in Fig. 5.1.

Table 5.2 Level of statistical significance (p-values) for the main effects of treatment (T), day (D) and their interaction (TxD) on the attributes of warthog salami with different levels of honeybush (T: *Cyclopia subternata*) during fermentation and a 40-day ripening period (D)

Attributes	Interactions and main effects		
	TxD	Treatment	Day
Water activity	**< 0.001**	**< 0.001**	**< 0.001**
Surface colour			
CIE L*	**< 0.001**	0.372	**< 0.001**
CIE a*	**< 0.001**	0.195	**< 0.001**
CIE b*	**0.004**	**< 0.001**	**< 0.001**
Chroma	**< 0.001**	**0.006**	**< 0.001**
Hue	**< 0.001**	**< 0.001**	**< 0.001**
Texture profile analysis			
Hardness (N)	0.057	0.909	**< 0.001**
Springiness (mm)	0.143	**0.003**	**< 0.001**
Cohesiveness	0.670	0.758	**< 0.001**
Chewiness (J)	**0.010**	0.698	**< 0.001**
Proximate composition (%)			
Moisture	0.206	0.580	**< 0.001**
Protein	0.569	0.272	**< 0.001**
Total lipid	0.282	0.227	0.250
Ash	0.106	**0.009**	**< 0.001**
Free Fatty acid %	-	**< 0.001**	-
Titratable acidity	-	**< 0.001**	-

TxD= treatment x day; p > 0.05= not significant; p ≤ 0.05= significant; p ≤ 0.01= significant; p ≤ 0.001= highly significant.

Table 5.3 Level of statistical significance (p-values) for the main effects of treatment (T), day (D) and position (P) and their interactions (TxDxP; TxD; TxP; DxP) on the pH of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and a 40-day ripening period (D)

Attribute	Interactions and main effects						
	TxDxP	TxD	TxP	DxP	T	D	P
pH	0.529	**0.004**	0.184	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

TxDxP= treatment x day x position; p > 0.05= not significant; p ≤ 0.01= significant; p ≤ 0.001= highly significant.

5.3.1 Relative humidity and temperature

The relative humidity (%) varied considerably depending on the location in the ripening room as illustarted in Fig. 5.1. Two of the five loggers (series 3 and 4) recorded higher RH% throughout the ripening period. The RH initialy ranged between 80-93.5%, followed by an increase (96-98.2%) and finally all data loggers showed a decrease in RH ranging between 70-90%. The temperature stayed within 15-17.8°C during the total 40-day ripening period.
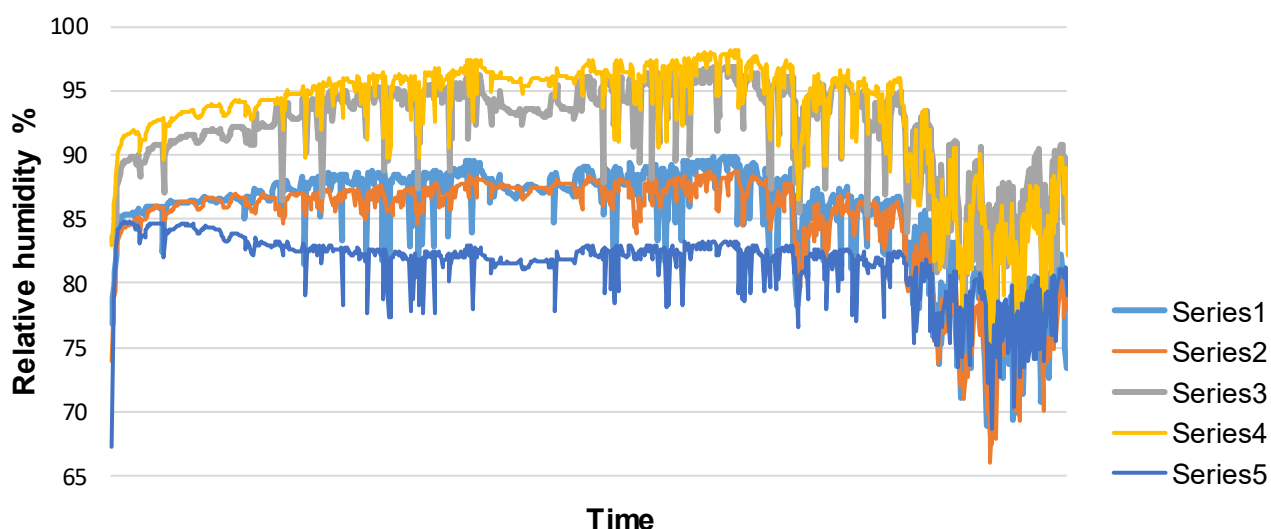
Figure 5.1 Relative humidity% recorded by 5 data loggers (LogTag, SA, Trex-8, Series 1-5) at different locations inside an artisanal-type ripening room over a 40-day ripening period.

5.3.2 pH

Influence of the interactions (TxD) and (DxP) between the main effects of treatment (T), day (D) and position (P) on salami pH are shown in Table 5.4 and Table 5.5, respectively.

After day-1 in the ripening room the salami pH was in the range of 5.46-5.72 (Table 5.4), followed by a significant increase until day-3 (pH > 6.00, p= 0.004). The pH was the lowest on ripening day-6 (pH= 5.53-5.71), although not significantly lower than initially on ripening day-1.

Following from ripening day-6 to the final ripening day (day-40), the pH further significantly increased (pH= 6.71-7.46, p= 0.004). After five and a half months of frozen storage (-20°C), the salami pH either stayed similar compared to ripening day-40 (C and T1) or increased (T2, T3 and T4, p= 0.004). There was a significant difference in treatment from ripening day-10, with T4 salami being the most acidic (pH= 5.83) and the control salami the least (pH= 6.24, p= 0.004). This trend was in fact seen on all the sampling days throughout the ripening period. Salami with the highest honeybush inclusion level (T4) had the lowest final pH (pH= 6.71) after 40 ripening days and after five and a half months of frozen storage T4 was still significantly lower in pH (pH= 7.03) compared to salami with less honeybush (C; T1; and T2, p= 0.004), although similar compared to T3.

The salami centres were significant lower in pH compared to underneath the outer casings from ripening day-6 to day-25 (p< 0.001, Table 5.6). The effect of salami position or location on the pH was negligible after 30 days in the ripening room (as seen on ripening day-30, Table 5.5).

Table 5.4 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on the pH (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40)

Treatment n= 12	Day									
	1	3	6	10	15	22	25	30	40	5½ MF
C	5.67^t±0.05	6.21^{nopq}±0.11	5.71^{st}±0.31	6.24^{nopq}±0.38	6.87^{ghijk}±0.33	7.28^{abcd}±0.29	7.23^{bcde}±0.31	7.30^{abc}±0.29	7.46^{ab}±0.18	7.51^a±0.13
T1	5.70^{st}±0.05	6.18^{nopq}±0.08	5.63^{st}±0.33	6.10^{qr}±0.33	6.76^{ijklm}±0.27	7.04^{cdefgh}±0.32	6.91^{fghijk}±0.42	7.28^{abcd}±0.49	7.30^{abcd}±0.22	7.47^{ab}±0.19
T2	5.72^{st}±0.08	6.15^q±0.09	5.68^{st}±0.35	6.20^{nopq}±0.40	6.80^{hijk}±0.23	$6.94^{efghijk}$±0.28	7.18^{bcdef}±0.38	$7.00^{defghij}$±0.34	7.12^{cdefg}±0.32	7.56^a±0.26
T3	5.70^{st}±0.05	6.17^{oq}±0.09	5.57^{st}±0.32	6.08^{qr}±0.47	6.46^{mnp}±0.44	6.79^{hijk}±0.48	6.74^{hijkl}±0.40	$7.02^{cdefghij}$±0.37	6.91^{fghijk}±0.23	7.28^{abcd}±0.23
T4	5.72^{st}±0.07	6.16^{pq}±0.10	5.53^t±0.26	5.83^{rs}±0.0.40	6.35^{nopq}±0.35	6.46^{lmno}±0.21	6.67^{klm}±0.19	6.77^{hijk}±0.30	6.71^{jklm}±0.35	$7.03^{cdefghi}$±0.24

[a-t] Different superscripts indicate significant differences between treatments and days (p= 0.004); SD= standard deviation; n=12 (6 replications of each treatment in duplicate on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush; 5½ MF= five and a half months frozen.

Table 5.5 Influence of the interaction (DxP) between the main effects of day (D) and position (P) of the measurement on the pH (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during ripening (day-6 to day-40)

Position	Day						
n= 30	6	10	15	22	25	30	40
Centre	$5.36^k{\pm}0.12$	$5.76^j{\pm}0.21$	$6.54^g{\pm}0.37$	$6.85^{ef}{\pm}0.41$	$6.90^{de}{\pm}0.41$	$7.06^{bc}{\pm}0.41$	$7.09^b{\pm}0.36$
Outer	$5.89^i{\pm}0.18$	$6.42^g{\pm}0.26$	$6.76^f{\pm}0.36$	$6.95^{cd}{\pm}0.43$	$7.00^{bc}{\pm}0.41$	$7.09^b{\pm}0.40$	$7.10^b{\pm}0.39$

[b-k] Different superscripts indicate significant differences between days and position ($p < 0.001$); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day).

5.3.3 Water activity (a_w)

Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on salami a_w is shown in Table 5.6. The a_w of C; T1; and T4 salami significantly decreased after 10 days in the ripening room, compared to the a_w of T2 and T3, which only significantly decreased after 15 days ($p < 0.001$). Treatment had a significant effect from day-25 until the end of ripening (day-40, $p < 0.001$), where the C; T1; and; T2 had a similar a_w (a_w= 0.833-0.839) and salami with the highest inclusion level of honeybush (T4) had a significantly higher a_w (0.873, $p < 0.001$).

Table 5.6 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on the water activity (a$_w$) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40)

Treatment n= 6	Day								
	1	3	6	10	15	22	25	30	40
C	0.963^a±0.001	0.961^{abcde}±0.003	0.958^{abcdef}±0.003	0.952^{efghi}±0.003	0.941^k±0.008	0.928^l±0.009	0.916^{no}±0.007	0.894^r±0.007	0.835^u±0.012
T1	0.963^a±0.002	0.961^{abcd}±0.001	0.957^{abcdef}±0.003	0.951^{fghij}±0.003	0.944^{ijk}±0.003	0.927^l±0.006	0.918^{mn}±0.008	0.897^{qr}±0.007	0.833^u±0.021
T2	0.962^{ab}±0.004	0.962^{abc}±0.002	0.956^{abcdef}±0.007	0.954^{bcdefg}±0.002	0.943^{jk}±0.007	0.925^{lm}±0.012	0.925^{lm}±0.006	0.901^{pqr}±0.004	0.839^u±0.010
T3	0.959^{abcdef}±0.004	0.960^{abcde}±0.002	0.957^{abcdef}±0.003	0.953^{defgh}±0.003	0.945^{hijk}±0.004	0.925^{lm}±0.007	0.921^{lmn}±0.007	0.904^{pq}±0.011	0.848^t±0.017
T4	0.962^{ab}±0.003	0.960^{abcde}±0.004	0.955^{abcdefg}±0.002	0.953^{cdefgh}±0.005	0.946^{ghijk}±0.005	0.928^l±0.007	0.925^{lm}±0.004	0.909^{op}±0.007	0.873^s±0.020

[a-u] Different superscripts indicate significant differences between treatments and days (p< 0.001); SD= standard deviation; n= 6 (6 replications of each treatment on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush.

5.3.4 Surface colour

5.3.4.1 CIE L* (lightness)

Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on salami lightness (CIE L*) is shown in Table 5.7. Salami lightness overall significantly decreased over time, starting from day-22 (including treatments T1-T4) and day-25 (C), respectively (p< 0.001). All the salami were significantly darker on ripening day-40 compared to day-6 (p< 0.001). After five and a half months of frozen storage, certain salami (T1 and T4) remained similar in lightness compared to ripening day-40, while others significantly increase in lightness (C; T2; and T3), although still significantly darker compared to day-6 (p < 0.001). Treatment had an effect on salami lightness from ripening day-30 to day-40. Salami with the highest inclusion level of honeybush (T4) were significantly lighter (CIE L*= 37.98) on day-40 compared to the other treatments (C; T1-T3, p < 0.001). After five and a half months of frozen storage, T3 (CIE L*= 37.92) was significantly lighter than T1 (CIE L*= 35.09, p < 0.001), although similar compared to the control, T2 and T4 salami.

5.3.4.2 CIE a* (green-red)

Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on CIE a* (redness) is shown in Table 5.8. Salami redness overall significantly decreased within the first 25 days in the ripening room, followed by a significant increase at the end of ripening (day-40, p < 0.001). Only T3 salami remained similar in redness (CIE a*= 11.45) than initially on day-6 (CIE a*= 11.92). After five and a half months of frozen storage, only the salami with the highest honeybush inclusion level (T4= 0.5%) remained similar in redness (CIE a*= 10.18) compared to the final ripening day (day-40, CIE a*= 10.44). Salami treatments differed significantly in redness from day-6 in the ripening room, with salami without any honeybush (C) and with the lowest inclusion level (T1= 0.125%) resulting in higher redness values (p < 0.001). The control salami had the highest redness value on day-40 (CIE a*= 11.73), although not significantly different from T1 and T3. Whereas T4 salami was the least red (CIE a*= 10.44), although similar than T1 and T2. Finally, T3 (CIE a*= 10.07) and T4 (CIE a*= 10.18) were significantly higher in redness after five and a half months of frozen storage compared to the other treatments (p < 0.001).

5.3.4.3 CIE b* (blue-yellow)

Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on the CIE b* value is shown in Table 5.9. There was a decreasing trend in yellowness from day-6 to day-22, followed by an increase therein until day-25. On day-25, no salami differed significantly from the initial yellowness on day-6. Thereafter, the yellowness of all salami significantly decreased until the final ripening day (day-40, p= 0.004). Five and a half months of frozen storage did not significantly affect the yellowness of T4 and T3 salami. Treatment 4 was initially more yellow on day-6 (CIE b*= 12.08, p= 0.004) compared to the rest, but similar compared to the control salami on day-10 and day-15. Treatment 4 was significantly more yellow on day-40 compared to the other treatments (CIE b*= 8.84, p= 0.004), although similar compared to T3 (CIE b*= 8.30). After five and a half

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months of frozen storage, T4 salami remained significantly more yellow compared to the other treatments (CIE b*= 8.60, p= 0.004).

5.3.4.4 Chroma (relative saturation)

Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on the chroma is shown in Table 5.10. Chroma (C*) or relative saturation of salami decreased from day-10 (C, T1 and T4) and day-15 (T2 and T3), respectively. The chroma of all salami was significantly lower on ripening day-40 compared to day-6 (p < 0.001). After five and a half months of frozen storage, the chroma significantly decreased from the final ripening day (day-40, p < 0.001), except for salami with the highest honeybush inclusion level (T4= 0.5%). Treatment had a significant effect on chroma from day-6 (p < 0.001). Initially, the control salami had the highest chroma value (CIE C*= 17.01), although only significantly higher compared to T2 (CIE C*= 16.06). Treatment 4 showed a general trend with higher CIE C* values from day-10 to day-30, however, treatment finally had no effect after 40 days in the ripening room. The effect of treatment was evident after five and a half months of frozen storage with T3 (CIE C*= 13.79) and T4 (CIE C*= 13.38) resulting in significantly higher chroma values (p < 0.001).

5.3.4.5 Hue angle

Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on the salami hue angle is shown in Table 5.11. Salami hue angle significantly decreased from day-6 to day-40 (p < 0.001). Five and a half months of frozen storage had no effect on salami hue angle compared to day-40. Initially (day-6), T4 salami were similar in hue angle (CIE hue°= 45.45) compared to T3 (CIE hue°= 43.17), but significantly higher than the other salami treatments (p < 0.001). Treatment 4 salami also had a significantly higher hue value on the final ripening day (day-40, CIE hue°= 40.27, p < 0.001) and after five and a half months of frozen storage (CIE hue°= 39.61), although similar compared to T3 (CIE hue°= 38.08).

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Table 5.7 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on surface colour (CIE L*, lightness) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40) and after 5½ months of frozen storage

Treatment n= 30	Day							
	6	10	15	22	25	30	40	5½ MF
C	44.36^{abc}±2.38	44.19^{abcde}±1.89	44.32^{abcd}±3.48	42.92^{bcdefg}±2.15	40.29^{ijklm}±1.80	37.34^{no}±2.44	34.58^{qr}±3.39	37.06^{nop}±6.19
T1	43.85^{abce}±2.48	44.04^{abce}±2.83	44.63^{ab}±3.11	42.24^{dfghik}±2.45	40.32^{jl}±3.24	38.70^{mn}±2.07	35.45^{opqr}±2.08	35.09^{pqr}±4.53
T2	44.40^{abc} ±3.13	45.21^{a}±2.54	44.16^{abcd}±3.65	42.13^{efgij}±2.65	40.52^{hklm}±3.12	40.50^{hklm}±3.51	34.84^{r}±3.38	36.63^{nopq}±4.43
T3	44.62^{ab}±2.82	45.92^{a}±2.56	44.73^{ab}±3.33	42.55^{cdefgh}±2.85	41.56^{fghijkl}±3.48	40.62^{ijklm}±2.35	34.80^{qr}±3.90	37.92^{n}±4.60
T4	45.02^{a}±1.88	45.17^{a}±2.40	44.02^{abcde}±2.94	43.00^{bcdef}±2.58	41.07^{ghijkl}±1.58	41.27^{ghijkl}±2.66	37.98^{n}±2.50	37.17^{no}±5.17

[a-r] Different superscripts indicate significant differences between treatments and days ($p < 0.001$); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush; 5½ MF= five and a half months frozen.

Table 5.8 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on surface colour (CIE a*, redness) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40) and after 5½ months of frozen storage

Treatment n= 30	Day							
	6	10	15	22	25	30	40	5½ MF
C	13.14[a]±1.43	10.80[efg]±0.83	10.32[fghijk]±1.37	10.08[hijklmn]±1.29	9.75[jklmnop]±1.43	11.58[bcd]±1.28	11.73[bc]±1.42	8.83[r]±2.68
T1	12.97[a]±0.92	11.12[bcdef]±1.36	8.94[pqr]±2.41	9.86[ijklmno]±1.14	8.88[qr]±1.00	10.76[defgh]±1.03	10.81[cdefgh]±1.37	8.82[r]±2.02
T2	11.93[b]±1.38	10.60[fghi]±0.97	9.40[nopqr]±1.39	10.14[ghijklm]±1.17	8.99[pqr]±1.41	10.19[ghijkm]±1.45	10.85[defgh]±1.75	9.47[lnopqr]±2.02
T3	11.92[b]±1.42	10.25[ghijkl]±1.40	9.47[mopqr]±1.76	10.21[ghijkln]±1.22	9.17[opqr]±1.32	10.41[fghijk]±1.10	11.45[bcde]±0.97	10.07[ghijklmn]±1.99
T4	11.86[b]±1.09	10.78[defgh]±1.29	9.91[ijklmno]±1.60	10.52[fghij]±0.97	9.70[klmnopq]±0.90	10.60[fghi]±1.24	10.44[fghij]±1.49	10.18[ghijklmn]±1.66

[a-r] Different superscripts indicate significant differences between treatments and days ($p < 0.001$); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush; 5½ MF= five and a half months frozen.

Table 5.9 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on surface colour (CIE b*, yellowness) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40) and after 5½ months of frozen storage

Treatment n= 30	Day							
	6	10	15	22	25	30	40	5½ MF
C	$10.75^{ghij}\pm1.19$	$11.51^{bcde}\pm1.04$	$11.47^{bcdef}\pm1.35$	$10.04^{kl}\pm0.86$	$10.91^{fghi}\pm1.21$	$8.77^{no}\pm0.88$	$7.57^{qr}\pm1.16$	$6.12^{t}\pm1.74$
T1	$10.75^{ghijk}\pm0.87$	$10.92^{efgh}\pm1.02$	$11.20^{cdefg}\pm1.49$	$10.19^{ijkl}\pm1.13$	$10.35^{hijkl}\pm1.24$	$9.06^{mn}\pm1.20$	$7.88^{pq}\pm1.24$	$6.39^{st}\pm1.25$
T2	$10.69^{ghijk}\pm0.80$	$11.24^{cdefg}\pm0.72$	$11.25^{cdefg}\pm0.74$	$10.12^{jkl}\pm1.50$	$10.98^{efgh}\pm0.88$	$8.82^{no}\pm0.88$	$7.88^{q}\pm0.94$	$6.89^{rs}\pm1.34$
T3	$11.15^{efg}\pm1.12$	$11.86^{abc}\pm0.97$	$11.75^{abcd}\pm1.42$	$10.29^{hijk}\pm1.44$	$11.29^{cdefg}\pm1.24$	$9.69^{lm}\pm1.14$	$8.30^{opq}\pm0.94$	$7.83^{q}\pm1.42$
T4	$12.08^{ab}\pm1.35$	$12.07^{ab}\pm0.75$	$12.11^{ab}\pm1.89$	$11.08^{defg}\pm1.14$	$12.37^{a}\pm0.98$	$10.56^{ghijk}\pm1.14$	$8.84^{no}\pm1.28$	$8.60^{nop}\pm2.26$

[a-t] Different superscripts indicate significant differences between treatments and days (p= 0.004); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush; 5½ MF= five and a half months frozen.

Table 5.10 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on surface colour (CIE C*, relative saturation) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40) and after 5½ months of frozen storage months of frozen storage

Treatment n= 30	Day							
	6	10	15	22	25	30	40	5½ MF
C	17.01^{ab}±1.51	15.83^{defg}±0.67	15.50^{defgh}±1.19	14.28^{klmnopqr}±0.86	14.71^{ijklm}±1.02	14.57^{jklmno}±1.10	13.99^{nopqr}±1.54	10.81^{u}±2.97
T1	16.86^{abc}±1.04	15.63^{defg}±1.30	14.52^{jklmno}±1.59	14.22^{lmnopqr}±1.19	13.68^{pqr}±1.13	14.11^{mnopqr}±1.06	13.40^{qrs}±1.64	10.99^{tu}±1.88
T2	16.06^{cde}±1.15	15.48^{defgi}±0.72	14.69^{hjklmn}±1.24	14.40^{klmnop}±1.17	14.24^{lmnopq}±1.07	13.52^{rs}±1.31	13.47^{rs}±1.53	11.76^{t}±2.17
T3	16.35^{abcd}±1.56	15.71^{defg}±1.39	15.17^{fghijk}±1.61	14.55^{jklmnop}±1.50	14.60^{ijklmno}±1.20	14.25^{lmnopqr}±1.33	14.16^{lmnopqr}±1.14	12.79^{s}±2.26
T4	16.96^{a}±1.28	16.23^{bcd}±0.91	15.74^{def}±1.73	15.31^{efghij}±1.18	15.74^{def}±1.04	14.99^{ghijkl}±1.40	13.71^{opqr}±1.77	13.38^{qrs}±2.55

[a-u] Different superscripts indicate significant differences between treatments and days ($p < 0.001$); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush; 5½ MF= five and a half months frozen.

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Table 5.11 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on surface colour (hue angle) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) during fermentation and ripening (day-1 to day-40) and after 5½ months of frozen storage

Treatments n= 30	Day							
	6	10	15	22	25	30	40	5½ MF
C	$39.32^{nop}\pm3.68$	$46.79^{efgh}\pm4.15$	$48.05^{defg}\pm5.65$	$45.00^{hi}\pm5.20$	$48.29^{cdef}\pm6.19$	$37.26^{pqr}\pm4.38$	$32.86^{s}\pm4.10$	$35.27^{rs}\pm6.46$
T1	$39.65^{mnop}\pm2.44$	$44.57^{hij}\pm3.98$	$51.77^{a}\pm9.47$	$45.94^{fgh}\pm4.30$	$49.28^{bcde}\pm4.73$	$40.07^{mno}\pm4.73$	$36.03^{qr}\pm3.77$	$36.39^{qr}\pm8.00$
T2	$42.00^{jklm}\pm3.99$	$46.73^{efgh}\pm3.61$	$50.34^{abcd}\pm3.78$	$44.83^{hi}\pm6.13$	$50.87^{abc}\pm5.21$	$41.07^{klmn}\pm4.63$	$36.31^{qr}\pm5.57$	$36.36^{qr}\pm5.55$
T3	$43.17^{ijk}\pm3.19$	$49.30^{abcde}\pm3.70$	$51.28^{ab}\pm6.14$	$45.14^{hi}\pm4.53$	$50.93^{abc}\pm5.33$	$42.91^{ijkl}\pm3.43$	$35.92^{qr}\pm2.91$	$38.08^{opq}\pm4.52$
T4	$5.45^{ghi}\pm3.99$	$48.34^{cdef}\pm4.13$	$50.56^{abcd}\pm6.27$	$46.44^{fgh}\pm3.47$	$51.87^{ab}\pm3.01$	$44.92^{hi}\pm3.72$	$40.27^{lmno}\pm3.61$	$39.61^{mnop}\pm5.27$

[a-s] Different superscripts indicate significant differences between treatments and days ($p < 0.001$); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush; 5½ MF= five and a half months frozen.

5.3.5 Texture profile analysis

5.3.5.1 Hardness (N)

Although there was an indication (p= 0.057, Table 5.2) that there was an interaction between treatment (T) and day (D), further analyses indicated that treatment (p= 0.909) hardly contributed to this, whilst day (p < 0.001) was the main influencer of difference in salami hardness. The latter will thus be discussed further (Table 5.12). Salami hardness significantly increased from day-10 (43.57 N) until day-15 (67.09 N, p < 0.001), however, it did not significantly change during the subsequent week in the ripening room (day-15 to day-22). From day-22 to day-25, in turn, the salami hardness increased significantly (p < 0.001) and subsequently stayed similar until day-30. Finally, the hardness increased between day-30 and day-40 in the ripening room (p < 0.001), reaching a final hardness of 168.85 N.

5.3.5.2 Springiness (mm)

There was no interaction (p= 0.143; Table 5.2) between treatment and day (TxD), although both treatment (p= 0.003) and day (p < 0.001) had an effect on salami springiness and will therefore be discussed further. Salami springiness with treatment (T) and day (D) as the main effect is shown in Table 5.13 and Table 5.12, respectively. Salami with the highest honeybush inclusion level (T4= 0.5%) had similar springiness (2.91 mm) compared to T3 (3.10 mm), however, T4 was significantly less springy compared to the other treatments (p= 0.003, Table 5.13). Initially the springiness significantly decreased from day-10 (3.71 mm) until day-15 (3.10 mm), followed by a significant increase until day-22 (3.27 mm) and finally decreased again (day-22 to day-40, 3.02 mm, p < 0.001, Table 4.12).

5.3.5.3 Cohesiveness (dimensionless)

There was no interaction (p= 0.670; Table 5.2) between treatment and day, nor did treatment (p= 0.758) influence the cohesiveness of the salami; therefore, only day (p < 0.001) will be discussed further (Table 5.12). Salami cohesiveness overall significantly increased from day-10 (1.40) to day-15 (1.57, p < 0.001), followed by a significant decrease until day-40 (1.19, p < 0.001). The salami cohesiveness was highest on day-15 and lowest on day-30 and day-40 (1.19).

5.3.5.4 Chewiness (J)

There was a significant interaction between the main effects (TxD, p= 0.01, Table 4.2) on salami chewiness. Subsequently, the influence of the interaction (TxD) between the main effects of treatment (T) and day (D) is shown in Table 5.14. Salami chewiness overall increased from day-10 to day-40, with the greatest increase seen from day-30 to day-40 (p= 0.01). All treatments (C, T1-T4) had similar chewiness from day-10 until day-30. On the final ripening day (day-40), T4 was significantly less chewy (464.55 J), compared to the other treatments (p= 0.01), ranging between 578.89 to 679.07 J (Table 5.14).

Table 5.12 The influence of the main interaction (D) on texture properties: hardness (N); springiness (mm) and cohesiveness (dimensionless) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) over ripening days (day-10 to day-40)

Attribute	Day					
n= 180	10	15	22	25	30	40
Hardness (N)	44.13[d]±14.25	68.86[c]±25.92	75.08[c]±20.72	94.98[b]±26.05	104.91[b]±19.89	170.27[a]±41.27
Springiness (mm)	3.71[a]±0.52	3.08[c]±0.53.	3.26[b]±0.32	3.04[cd]±0.26	2.96[d]±0.25	3.02[cd]±0.25
Cohesiveness	1.40[b]±0.20	1.57[a]±0.53	1.28[c]±0.15	1.23[cd]±0.09	1.19[cd]±0.04	1.19[d]±0.06

[a-d] Different superscripts in the same row indicate significant differences between days ($p < 0.001$); SD= standard deviation; n= 180 (5 treatments x 6 replications of each treatment x 6 repeated measurements per sampling day).

Table 5.13 The influence of the main interaction (T) on springiness (mm) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*)

Treatments n= 216	C	T1	T2	T3	T4
Springiness (mm)	3.33[a]±0.37	3.27[ab]±0.46	3.30[ab]±0.40	3.09[bc]±0.39	2.89[c]±0.49

[a-c] Different superscripts in the same row indicate significant differences between treatments (p= 0.003); SD= standard deviation; n= 216 (6 replications of each treatment x 6 repeated measurements x 6 sampling days); C= 0%; T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush.

Table 5.15 Influence of the interaction (TxD) between the main effects of treatment (T) and day (D) on chewiness (J) (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) as effected by day in the ripening room (day-10 to day-40)

Treatment n= 30	Day					
	10	15	22	25	30	40
C	195.82^k±87.38	302.94^{dfghij}±92.79	247.58^{hijk}±59.56	349.02^{bcdefg}±71.10	406.02^{bce}±81.73	679.07^a±135.44
T1	221.76^{ijk}±63.78	278.06^{fghijk}±85.15	299.00^{cdefghij}±56.15	355.89^{bcdefg}±104.52	349.35^{bcdefgh}±94.40	670.10^a±207.85
T2	223.65^{ijk}±39.14	305.06^{efghij}±68.26	333.70^{cdefgh}±67.49	336.29^{cdefgh}±64.95	404.68^{bcd}±69.94	660.94^a±147.64
T3	208.77^{jk}±35.78	325.31^{cdefghi}±101.95	333.11^{cdefghi}±113.70	366.7^{bcdefg}±117.93	377.59^{bcdef}±92.75	578.89^a±147.47
T4	272.06^{ghijk}±91.64	313.25^{fghijk}±72.52	329.06^{cdefghi}±67.54	346.10^{cdefgh}±94.26	313.52^{cdefghij}±50.15	464.55^b±105.64

[a-k] Different superscripts indicate significant differences between treatments and days (p= 0.01); SD= standard deviation; n= 30 (5 treatments x 6 replications on each respective sampling day); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush.

5.3.6 Proximate analysis

The interaction between treatment and day (TxD) had an insignificant effect on salami moisture (p= 0.206); protein (p= 0.569), total lipids (p= 0.282), and ash (p= 0.106, Table 5.2). Treatment also did not affect salami moisture (p= 0.580), protein (p= 0.272), and total lipid content (p= 0.227), however, it influenced the ash content (p= 0.009). Day, however, had a significant effect on salami moisture, protein, and ash (p < 0.001), but had no effect on total lipids (p= 0.250, Table 5.2). Subsequently, the effect of day on salami moisture; protein; and ash content (Table 5.15) and the effect of treatment on the ash content (Table 5.16) will be discussed further. The salami moisture (%) significantly decreased from day-0 (56.6%) to day-40 (26.6%, p < 0.001), whilst the protein and ash content significantly increased from 23.4% to 46.3% and 3.3% to 5.5%, respectively (p < 0.001, Table 5.15). Total salami lipid content was not significantly influenced over time with day-0 (17.3%) resulting in similar lipid content compared to day-40 (15.9%, Table 5.15). The control salami resulted in a significantly higher ash content (4.6%, p= 0.009), although similar compared to T2 (4.4%, Table 5.16).

Table 5.15 The influence of the main effect (D) on the proximate composition: moisture; protein; total lipid; and ash content (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*) before (day-0) and after (day-40) a 40-day ripening period

Day	Proximate composition %			
n= 60	Moisture	Protein	Total lipid	Ash
Day-0	56.6[a]±1.9	23.4[a]±5.5	17.3[a]±5.9	3.3[a] ±0.2
Day-40	26.6[b]±2.7	46.3[b]±3.8	15.9[a]±4.6	5.5[b]±0.5

[a-b] Different superscripts in the same column indicate significant differences in proximate composition attributes (p < 0.001); SD= standard deviation; Day-0= raw salami batter from the production day; Day-40= the final product after fermentation and ripening; n= 60 (5 treatments x 6 replications x duplicate).

Table 5.16 The influence of the main effect (T) on proximate ash% (Means ± SD) of warthog salami with different levels of honeybush (*Cyclopia subternata*)

Proximate composition (%)	Treatment				
n= 24	C	T1	T2	T3	T4
Ash	4.6[a]±1.4	4.3[bc]±1.1	4.4[ab]±1.2	4.3[bc]±1.1	4.1[c]±1.0

[a-c] Different superscripts indicate significant differences between treatments (p= 0.009); SD= standard deviation; n= 24 (6 replications of each treatment in duplicate over two sampling days); C= 0%; T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush.

5.3.7 Free fatty acids and titratable acidity

Free fatty acid (FFA) percentage (expressed as g oleic acid/100 g of fat) and titratable acidity (expressed as g lactic acid/100 g sample) as affected by treatment are shown in Table 5.17. Treatment 3 had the highest FFA content (0.846 g oleic acid/100 g of fat), although significantly lower compared to the retail salami (1.679 g oleic acid/100 g of fat), ($p < 0.001$). In turn, the control salami had the highest lactic acid content (0.234 g lactic acid/100 g sample), although significantly lower compared to the retail salami (0.834 g lactic acid/100 g sample), ($p < 0.001$).

Table 5.17 The influence of the main effect (T) on the free fatty acids (g oleic acid/100 g of fat) and titratable acidity (g lactic acid/ 100 g sample) of warthog salami with different levels of honeybush (*Cyclopia subternata*), compared to a retail salami (Means ± SD)

Treatment	C	T1	T2	T3	T4	Retail*
n= 12	12	12	12	12	12	2
FFA	0.642[d]±0.218	0.829[bc]±0.378	0.714[cd]±0.221	0.846[b]±0.197	0.785[bc]±0.222	1.679[a]±0.046
TA	0.234[b]±0.055	0.196[c]±0.031	0.177[c]±0.033	0.182[c]±0.026	0.170[c]±0.046	0.834[a]±0.012

[a-d] Different superscripts in the same row indicate significant differences between treatments ($p < 0.001$); SD= standard deviation; n= 12 (6 replications of each treatment in duplicate); *n= 2 (in duplicate); FFA= free fatty acids; TA= titratable acidity; C= 0%; T1= 0.125%, T2= 0.25%, T3= 0.375%, T4= 0.5% (w/w) of added honeybush.

5.4 Discussion

5.4.1 Weight loss (%), RH, temperature and mould growth

Overall, the salami had a satisfactory weight loss of ~40% after a 40-day ripening period (calculated as the average of three dedicated salami inside the ripening room) which is above the minimum recommended 25% weight loss as a guideline to reach an adequate a_w reduction of ≤ 0.92, (Roccato *et al.*, 2017). The white-grey appearance of the salami after 40 ripening days (Addendum A, Fig. 5.9) is typical of *P. nalgiovense* proliferation (Canel *et al.*, 2013) and regarded as a desired trait in high quality salami, however, the proliferation of these moulds should be controlled for reasons elaborated on later in the discussion. Dipping the salami in a mould suspension ensured homogenous mould growth and eliminated the variability in appearance as seen with spontaneous mould colonisation where salami from the same batch and treatment differed in percentage mould coverage (Chapter 4). Temperature and RH data loggers indicated a variability within the ripening room according to location, especially with regards to RH (Fig. 5.1).

5.4.2 Water activity and pH

A combined effect of a pH < 5.2 and a_w < 0.95 is said to ensure microbial safety of salami (Feiner, 2006; Food Standards Australia New Zealand, 2017). None of the salami in the present study fell

within this combination due to higher final pH values (Table 5.5). Lee and Styliadis (1996), however, highlighted the variation in recommended a_w and pH combinations to achieve microbially safe salami. The pH and a_w are easily measured and directly linked to the proliferation of pathogens and spoilage bacteria (Forsythe, 2010). However, it is ultimately the combined and synergistic effect of multiple hurdles that determine the final salami product safety (Lee & Styliadis, 1996; Thomas *et al.*, 2008) and not only the pH and a_w. To gain more information surrounding product safety, final product salami microbial analyses is required (Chapter 6).

Salami from the present trial had an unexpectedly high pH (7.46-6.71, Table 5.5) after the fermentation and ripening period. Slightly fermented salami pH commonly ranges between 5.2-6.3 (Aymerich *et al.*, 2003), but higher pH values (6.81-6.83) have been reported in Italian and game salami (Zanardi *et al.*, 2010; Cenci-Goga *et al.*, 2012). The presence of inherent sugars (additional fermentable substrates for the LAB starter cultures) in the honeybush extract might explain the lower pH of salami with a higher honeybush addition (T3 and T4). Salami with no or less honeybush (C, T1; and T2) had a final pH above 7.00, which could pose a potential microbial risk, especially if unrefrigerated (Lee & Styliadis, 1996). After frozen storage (five and a half months, -20°C) all the treatments had a pH above 7.00, exceeding the typical pH range of slightly fermented salami (Aymerich *et al.*, 2003).

A high pH phenomenon in salami can be ascribed to various contributing factors, including: competing background microflora consuming the added fermentable glucose source (Stahnke & Tjener, 2007); a rapid a_w reduction during drying hindering lactic acid bacteria (LAB) growth and subsequent organic acid production (Lücke, 1994); autochthonous**,** weak acidifying LAB outgrowing the added LAB starter culture strains (Casaburi *et al.*, 2007); lactate metabolization by surface moulds (Lücke, 1994; Flores, 1997; Sunesen & Stahnke, 2003); and ammonia production as a result from microbial proteolysis (Muthukumar *et al.*, 2014; Hospital *et al.*, 2015).

The amount of microbial organic acid production (i.e. extent of pH reduction) depends on the initial amount of available fermentable sugars (Lücke, 1994; Demeyer *et al.*, 2000). The added glucose concentration in this trial (0.2%) was in the range of proposed amounts (Lücke, 1994; Lebert *et al.*, 2007b), although slightly lower than proposed by others (Leistner, 1995; Houben & Van't Hooft, 2005). A limited amount of fermentable sugar could have retarded LAB proliferation, however, dried warthog sausages (cabanossi) without the addition of sugars or starter cultures successfully fermented to reach a pH of 5.1 (Mahachi *et al.*, 2019). The autochthonous LAB in these cabanossi were not reliant on added sugars, but on the meat glycogen storage as primary sugar source for proliferation. Meat has varying glycogen stores after slaughter and may result in different fermentation patterns (Feiner, 2006). Todorov *et al.* (2007) reported better proliferation of LAB strains in certain game salami compared to other game species used, also possibly due to glycogen content differences between the meat. Supporting this, Chakanya *et al.* (2018) found springbok salami to have the lowest pH (5.04), although Van Schalkwyk *et al.* (2010) found

springbok salami to have the highest pH (5.46) amongst different game salami. Differing from the present study, the use of commercial starter cultures and premixed additives containing a sufficient amount of fermentable sugar probably guaranteed the sufficient pH decline (pH ≤ 5.77) in both these beforementioned studies, irrespective of the initial game meat glycogen stores. Warthogs are known to be vigilant animals prone to ante mortem stress (Swanepoel *et al.*, 2016c) which could lead to a drastic decrease in glycogen stores, resulting in meat with a high ultimate pH (pHu), ultimately limiting the extent of salami acidification. Arguably, if the glycogen content was limited and the added amount of glucose (0.2%) was insufficient in the present trial, *L. sakei* (added as part of the LAB starter culture strains) should have followed alternative fermentation pathways involving nucleoside and amino acid metabolism to proliferate and successfully generate organic acids (Chaillou *et al.*, 2005; Rimaux *et al.*, 2011b,a). These genetic features make this microorganism robust and ideal for meat starter cultures, as nucleosides and free amino acids are abundant in meat. Furthermore, the total amount of LAB starter culture strains (*L. sakei* and *P. pentosaceus*) equalled ~203 x 10^9 CFU/kg of meat and fat, which exceeded the recommended amount of LAB starter cultures to successfully ferment meat (10^5-10^8 CFU/g, Elias *et al.*, 2014). It is, however, of utmost importance to predetermine the robustness of LAB starter cultures and whether the intrinsic meat environment and extrinsic climatic conditions will favour the proliferation of applied strains in salami (De Vuyst, 2000), as not all strains may perform optimally in different meat sources (i.e. game meat versus domestic pork meat, Leroy *et al.*, 2006).

Pediococci pentosaceus, on the other hand, is commonly applied in fast fermenting American style sausages with an optimal growth at higher temperatures (~40°C, Talon & Leroy, 2014). The temperature range during the present trial ripening period (15-17°C) might have retarded the growth of acidifying *P. pentosaceus* and limited subsequent acid production, however, previous researchers used a starter culture which only consisted out of *P. pentosaceus* and a successful salami pH decline was achieved (pH= 5.46-4.88) in a similar lower temperature range (18-22°C, Van Schalkwyk *et al.*, 2010). Differing from the present trial, Van Schalkwyk *et al.* (2010) did inoculate their salami with surface moulds with the ability to metabolize secreted lactate and, their smoking step to prevent excessive spontaneous mould growth might explain the lower pH achieved in the latter study (Flores, 1997).

The fact that the salami centre was initially more acidic than beneath the casing (Table 5.6), indicated that microbial organic acid production did indeed occur (Flores, 1997). A pH gradient is expected in unsmoked, mould covered salami due to mould lactic acid metabolization and subsequent ammonia production increasing the salami pH (Flores, 1997). Within 30 days of ripening the pH gradient became negligible, indicating that the centre lactic acid was almost completely metabolized. Indeed, small diameter salami with abundant mould growth may pose a greater risk of microbial spoilage as mould mycelium can penetrate more easily to the salami centre, causing a pH increase (Grazia *et al.*, 1986; Rödel *et al.*, 1993). Mould growth is especially

associated with amino acid breakdown and with the combined effect of lactic acid metabolization and subsequent ammonia accumulation over time, mould covered salami pH can drastically increase (Lücke, 1994; Flores, 1997; Bruna *et al.*, 2000, 2001b; Sunesen & Stahnke, 2003; Ockerman & Basu, 2007). The temperature and RH% in the present study were optimum for accelerated *P. nalgiovense* proliferation and mycelium invasion which have shown to exhibit an extremely fast growth rate (2 mm/day at 14°C, 90% RH). In the case of using small diameter salami casings, Rödel *et al.* (1993) advised to include surplus fermentable sugars in the salami batter to ensure LAB proliferation and a sufficient pH decrease. The salami diameter in the present study (32 mm) was considered small compared to traditional Italian salami (~50-80 mm, Demeyer *et al.*, 2000; Lebert *et al.*, 2007a; Montanari *et al.*, 2016; Roccato *et al.*, 2017). Larger diameter salami may reach a lower pH due to more controlled and gradual water loss, as opposed to smaller diameter salami which could potentially dry more rapidly and allow more oxygen penetration, inhibiting LAB growth (Lücke, 1994). Lactic acid bacteria acid production becomes negligible when salami a_w decreases to less than 0.92 (Lücke, 1994), or arguably, 0.95 (Feiner, 2006). Salami in the present trial only had an $a_w < 0.92$ after day-22 in the ripening room, at which stage the pH still ceased to decline as expected. Contradicting this, Mahachi *et al*. (2019) reported a sufficient pH decrease (pH= ~5.1) in warthog cabanossi with a small diameter (22 mm) without the addition of LAB starter cultures. In the beforementioned study, a commercially used curing mixture (not specified but assumed to contain nitrite and possibly nitrate) was applied and the sausage outer surfaces were smoked. The addition of curing agents (nitrate and nitrite) according to maximum permitted amounts could have promoted selective proliferation of naturally occurring LAB in the fresh meat of the beforementioned study (Cardinali *et al.*, 2018; Perea-Sanz *et al.*, 2019) and the smoking most likely prevented mould colonization and subsequent lactate metabolism and ammonia production (Flores, 1997), ensuring sufficient lowering of the cabanossi pH.

Apart from mould metabolism, ammonia production (causing a pH increase) could have originated from a few other possible scenarios which will be briefly discussed. The first possibility includes background, spoilage microorganisms present in the salami. Simple sugars (such as the fermentable glucose added in the present trial) are usually also preferred by spoilage microorganisms (Gill, 1983). If this sugar source becomes depleted, microorganisms have the ability to use free amino acids as an energy source and subsequently generate ammonia, ultimately rising the pH (Gill, 1983). Salami are generally known to contain an abundant amount of free amino acids ascribed to endogenous meat tissue and exogenous microbial protease activity (Montel *et al*., 1996; Toldrá, 1998; Talon & Leroy, 2014). Similarly, a high pH phenomenon in salami was ascribed to microbial protein metabolism counteracting the lowering in pH generated by LAB (Hospital *et al*., 2015; Montanari *et al*., 2018). Secondly, the alternative metabolic pathways of *L. sakei* when glucose (the preferred energy source) is depleted as mentioned earlier, involves nucleoside utilisation (adenosine deaminase and inosine hydrolase) and amino acid metabolism via the arginine deiminase pathway (Chaillou *et al*., 2005; Rimaux *et al*., 2011a, b). Nonoxidative

deamination of free amino acids, such as in the latter case, may lead to the accumulation of ammonia (Ordóñez *et al.*, 1999). Thirdly, microbial protein deamination and decarboxylation could also contribute to ammonia production (Demeyer, 2007). Contaminant spoilage microorganisms such as *Enterobacteriaceae*, lactobacilli, pediococci and enterococci, as well as beneficial LAB bacteria may exhibit decarboxylase activity, although starter culture strains are generally selectively chosen with the absence of this gene (Ordóñez *et al.*, 1999; Latorre-Moratalla *et al.*, 2012; Ravyts *et al.*, 2012; Holck *et al.*, 2017). The mixed starter culture in the present study was not tested for the presence of decarboxylase activity, hence this metabolic trait could have contributed to the rise in final product salami pH.

The salami successfully dried over the 40-day ripening period, indicated by the absence of an outer crust formation and the low a_w of the final product centre. Treatment 4 had the highest a_w (0.8726), which is, however, far below the recommended a_w of 0.91-0.92 for microbial safety (Ross & Shadbolt, 2001; Roccato *et al.*, 2017). As pH decreases, meat proteins reach their iso-electric point (zero net charge) leading to loss of binding capacity, impairing water holding capacity and promoting a_w reduction (Miralles *et al.*, 1996; Flores, 1997; Warriss, 2010). A decrease in pH is thus expected to be parallel with a decrease in a_w. This was contradicted in the present study with T4 resulting in the lowest pH and the highest a_w, in accordance with Olivares *et al.* (2010) who also found a lower salami pH correlated with a higher a_w.

5.4.3 Surface colour

Lowering the ingoing amount of nitrate and/or nitrite can have implications on salami colour, especially with regards to redness, as nitrosomyoglobin (result of nitric oxide binding to myoglobin) creates the typical, attractive red-pink colour of cured meats (Møller & Skibsted, 2002; Skibsted, 2011). Other researchers who incorporated natural extracts in processed meat products reported a retention or protection of redness (Sebranek *et al.*, 2005; Ifesan *et al.*, 2009; Colindres & Brewer, 2011). Since the chemical state of the myoglobin haem protein determines the redness of meat, a possible mechanism for the protection of redness by adding natural antioxidants includes delaying the formation of the undesired, brown metmyoglobin (Formanek *et al.*, 2003; Muthukumar *et al.*, 2014). In the case of adding honeybush in salami, both the antioxidant effect and the natural brick-brown colour could play a role in redness retention as seen in the present trial, in accordance with Ifesan *et al.* (2009) who also found a significant colour enhancing effect due to a red-brown extract added to pork meat.

The overall decrease in salami CIE L*a*b* colour measurements during the 40-day ripening period could be ascribable to deteriorative oxidation reactions, especially in the case of loss in redness (CIE a*) which indicates the formation of metmyoglobin (Muthukumar *et al.*, 2014; Ergezer *et al.*, 2018). The increase in darkness (decrease in CIE L*, Table 5.7) could be ascribed to myoglobin oxidation (Fongaro *et al.*, 2015) or water loss (Olivares *et al.*, 2010) over time. In contrast, Jin *et al.* (2018) reported salami lightness to stay similar during a month storage period.

The beforementioned study, however, used lean pork meat as opposed to game meat which is naturally higher in myoglobin content (Hoffman, 2000). A high myoglobin content in warthog meat is expected (not analysed in the present trial) due to their active nature (Swanepoel *et al.*, 2016c), which could in turn promote oxidation reactions due to a higher haem iron content (Papuc *et al.*, 2017). The higher CIE L* of T4 salami (day-40, Table 5.7) could be associated with a higher a_w as a result of using a hygroscopic tea extract, similarly motivated by Fernández-López *et al.* (2005) who found higher lightness values after applying lemon and orange extracts in meatballs. Salami pH also affects the colour, with a lower pH resulting in higher lightness (Abril *et al.*, 2001), as also seen in the present study.

The increase in salami redness during day-15 and day-22 could be due to nitrosomyoglobin generation ascribed to gradual nitrite formation over time, where a slower fermentation and higher pH favour the growth of *Micrococcaceae* and subsequent nitrate reductase activity (Ordóñez *et al.*, 1999). Nitrate reduction to nitrite and subsequent nitric oxide required for nitrosomyoglobin formation is catalysed by an acidic environment (Hammes, 2012). The salami had a favourable pH (≤ 6.87) before day-22 for *Micrococcaceae* growth, arguably the pH was too high for optimum nitric oxide formation at pH of 5.0-5.5 (Talon & Leroy, 2014). The redness of salami on day-40 (CIE a*= 10.44-11.73, Table 5.8) was similar to 3 month ripened, small diameter (38 mm) salami (CIE a*= 11.2) with a naturally derived nitrate source (Mora-Gallego *et al.*, 2014). Although the control salami had the highest CIE a* value on day-40, it did not differ from salami with 0.375% of added honeybush (T3). Furthermore, the chroma (CIE C*) did not differ between treatments, indicating that all salami were equally intense or vivid in colour (Miltenburg *et al.*, 1992). Positively, salami from the present trial had a higher redness on day-40 compared to 4 week ripened sausages with 0.01% sodium nitrite, 0.8% celery powder, 0.6% fruit extract powder, 0.45% purple sweet potato powder, 0.5% fruit and vegetable extract, 0.04% Gardenia Red and, 0.04% paprika + 0.03% blueberry powder, respectively (Jin *et al.*, 2018b). In contrast, the salami were not as red compared to those with higher amounts of added sodium nitrate (250 mg/kg; 212.5 mg/kg; and 187.5 mg/kg) resulting in a CIE a* of: 18.5; 18.4; and 17.9, respectively (Perea-Sanz *et al.*, 2019). Greek fermented sausages with added sodium nitrite also resulted in a higher redness after 26 ripening days (CIE a*= ~11.5-12.5, Baka *et al.*, 2011). After frozen storage (five and a half months, -20°C), T4 salami had the highest redness value and was the only treatment capable of conserving redness from ripening day-40. Retention of redness after frozen storage in T4 correlates with a higher CIE C* value indicating a more intense or vivid colour, less close to a grey or dull appearance (Hunt *et al.*, 1999; Choi *et al.*, 2011). A minimum added concentration of 25-50 mg/kg of nitrite might be adequate to induce an acceptable cured colour formation (Sindelar & Milkowski, 2011). The added amount of nitrate in the present trial (75 mg/kg) could have contributed significantly to the redness after being reduced to nitrite. Hue angle change over time is said to be visible to the human eye, where an increase therein indicates a colour development from red to yellow, evident by the hue angle calculation (Pogorzelska *et al.*, 2018). Indeed, as seen in the

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present trial, as the yellowness increased in T4 (probably mainly because of the natural pigment of the honeybush extract), so did the hue angle. Although honeybush significantly increased the salami yellowness (Table 5.19), the final product salami were actually less yellow compared to previous reported traditional Italian salami after a 38-day ripening period (CIE b*= 17.82, Casaburi *et al.*, 2007) and similar compared to Greek fermented sausages with an autochthonous *L. sakei* starter culture strain after 26 ripening days (CIE b*= ~7, Baka *et al.*, 2011).

5.4.4 Texture Profile Analysis

The increase in salami hardness was expected due to moisture loss over time (Serra *et al.*, 2005; Mora-Gallego *et al.*, 2014). Salami hardness was highest on day-40 (Table 5.12), corresponding with the lowest a_w (Table 5.4). Similarly, as found in dry-cured hams, the salami springiness and cohesiveness decreased over time as the a_w decreased (Serra *et al.*, 2005). Chewiness, a secondary textural property, is the product of hardness; cohesiveness; and springiness (Szczesniak *et al.*, 1963; Bourne, 1978). Treatment significantly affected springiness and subsequently also chewiness, as seen in T3 and T4 with the lowest springiness (Table 5.13) and chewiness (Table 5.14). Proteolytic and lipolytic activity due to excessive mould growth can cause a decrease in salami hardness and chewiness (Bruna *et al.*, 2001a). Previous researchers found *P. aurantiogriseum* added to salami to exhibit a softening effect (Bruna *et al.*, 2001a). The beforementioned authors reported a similar hardness (~167 N) compared to the present trial salami after 40 ripening days (Table 5.12). This could indicate that the present trial salami were similarly softer due to mould growth, however, salami also had similar hardness on ripening day-25 (~95 N) compared to salami without mould starter inoculation on ripening day-20 (~90 N, Alamprese *et al.*, 2016), contradicting this. Salami hardness and springiness were also similar to that of commercial mini-*fuets* (typical Catalan cured mini-sausages, Herrero *et al.*, 2007), although the *fuets* were slightly higher in total fat content (21.9-24.4%) and much lower in a_w (0.651-0.648). The salami chewiness in contrast, was much higher in the present study compared to the mini-*fuets*. This could be ascribed to the lower total fat content of the warthog salami (Olivares *et al.*, 2010). Texture Profile Analysis results are dependent on instrument operation and intrinsic product characteristics (e.g. pH, moisture, fat content, processing methodology, and environmental conditions during fermentation and ripening, Tabanelli *et al.*, 2012; Riel *et al.*, 2017). Results will hardly ever be fully comparable to previous research studies due to sample and instrument variation.

5.4.5 Proximate composition

Salami moisture content decreased from 56.6% to 26.6% resulting in a moisture loss of ~30% (Table 5.15) which is in accordance with typical Italian salami ranging between 24.3-53% moisture (Moretti *et al.*, 2004; Zanardi *et al.*, 2010). Moreover, a 30% moisture loss is a typical characteristic of long ripened, dry salami (Zeuthen, 2007). Although salami is characterized as a high fat meat product containing between 30-50% total fat (Colmenero, 2000), the present trial salami had a

lower total lipid content (15.89%, Table 5.15). Previous researchers reported a similar raw salami lipid content (~17%), however, differing from the present trail, the total lipid content increased to ~25-28% after ripening (Pasini *et al.*, 2018). In the latter study, however, pork as opposed to game meat was used. Lower total lipids in the raw meat batter and the final product salami in the present study could be due to the utilisation of warthog meat containing less intramuscular fat compared to traditionally used pork meat (Swanepoel *et al.*, 2016b; Mahachi *et al.*, 2019). From a previous study, however, salami similarly prepared by other researchers (with a 7:3 ratio of game meat:pork fat) increased in total fat content from 22.6% to 35.7% after drying (Chakanya *et al.*, 2018).

The protein content in the final product salami from the present trial is much higher than typical Italian salami (24.4-37.4%) (Moretti *et al.*, 2004; Zanardi *et al.*, 2010) and game salami (Van Schalkwyk *et al.*, 2010; Chakanya *et al.*, 2018), but this could be linked to the lower lipid content. Ash content (5.46%) was higher than previously produced warthog cabanossi (4.6%, Swanepoel *et al.*, 2016b) and game salami (4.3-5%, Van Schalkwyk *et al.*, 2010; Chakanya *et al.*, 2018). A higher ash content in the honeybush containing salami could have been expected due to naturally occurring minerals in the honeybush extract (not tested), (Mckay & Blumberg, 2007; Joubert *et al.*, 2011). In contrast, the control salami without honeybush resulted in the highest ash content (4.6%), eliminating the possible effect of the honeybush.

5.4.6 Free fatty acids (% oleic acid) and titratable acidity (% lactic acid)

In addition to measuring the salami pH, the acid titration method was also executed to compare experimental with retail salami. Salami free fatty acid content (% oleic acid) was similar to that reported by other researchers (Zdolec *et al.*, 2008). The control salami had less FFAs compared to T4 (Table 5.17), however, the retail salami resulted in a significantly higher FFA content, despite the addition of the antioxidants sodium ascorbate and sodium nitrite, both capable of retarding lipid oxidation. Salami from the present trial had a much lower lactic acid content in comparison to the retail salami, supported by a lower pH recorded in the retail salami compared to the experimental salami (data not shown). The lactic acid content after 40 ripening days (Table 5.17) was similar to raw sausages on the day of stuffing (pH= 5.89-5.77, Zdolec *et al.*, 2008), and less than the lowest total acidity reported for pork salchichón (0.3, Capita *et al.*, 2006), reflecting the poor lactic acid content in the present trial salami after the ripening period. The retail salami, on the other hand, was similar in titratable acidity compared to ostrich, deer and industrially produced chorizo (Castaño *et al.*, 2002; Capita *et al.*, 2006).

5.5 Conclusion

The addition of 0.5% unfermented honeybush (*Cyclopia subternata*) extract showed a protective effect of salami redness (CIE a*) during frozen storage. Treatment had no effect on salami proximate composition (moisture; protein; and total lipid content) and texture (hardness; chewiness; and cohesiveness). Adding a hygroscopic tea extract to salami might cause an increase in final product a_w. In the case of producing small diameter salami and applying a mould

inoculation, the addition of extra fermentable sugar is recommended to ensure a sufficient pH decrease for microbial safety. Warthog meat, naturally darker in colour, is a promising option for salami production with less added nitrate and/or nitrite. Warthog salami with the addition of honeybush extract could be a marketable ready-to-eat meat product based on the present consumer interest in processed meat products with added bio-active ingredients and less additives. Food safety, however, cannot be compromised, a future suggestion would be to combine honeybush with organic acids to ensure a low pH, especially in the case of surface mould growth.

5.6 References

Abril, M., Campo, M.M., Önenç, A., Sañudo, C., Albertí, P. & Negueruela, A.I. (2001). Beef colour evolution as a function of ultimate pH. *Meat Science*, **58**, 69–78.

Alahakoon, A.U., Jayasena, D.D. & Jo, C. (2015). Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science and Technology*, **45**, 37–49.

Alamprese, C., Fongaro, L. & Casiraghi, E. (2016). Effect of fresh pork meat conditioning on quality characteristics of salami. *Meat Science*, **119**, 193–198.

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

AOAC (2002a). Loss on drying (moisture) at 95-100°C for feeds. AOAC Official Method 934.01. In: *Official Methods of Analysis*, 17th ed. Arlintong, Virginia, USA: Association of Official Analytical Chemists Inc.

AOAC (2002b). Ash of animal feed. AOAC Official Method 942.05. In: *Official Methods of Analysis*, 17th ed. Arlintong, Virginia, USA: Association of Official Analytical Chemists Inc.

AOAC (2002c). Dumas combustion method. AOAC Official Method 992.15. In: *Official Methods of Analysis*, 17th ed. Arlintong, Virginia, USA: Association of Official Analytical Chemists Inc.

Aymerich, T., Martín, B., Garriga, M. & Hugas, M. (2003). Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. *Applied and Environmental Microbiology*, **69**, 4583–4594.

Baka, A.M., Papavergou, E.J., Pragalaki, T., Bloukas, J.G. & Kotzekidou, P. (2011). Effect of selected autochthonous starter cultures on processing and quality characteristics of Greek fermented sausages. *LWT - Food Science and Technology*, **44**, 54–61.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, **120**, 85–92.

Blaiotta, G., Pennacchia, C., Villani, F., Ricciardi, A., Tofalo, R. & Parente, E. (2004). Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented

sausages. *Journal of Applied Microbiology*, **97**, 271–284.

Bourne, M. (1968). Texture profile of ripening pears. *Journal of Food Science*, **33**, 223–226.

Bourne, M.C. (1978). Texture profile analysis. *Food Technology*, **32**, 62–72.

Bruna, J.M., Fernández, M., Hierro, E.M., Ordóñoz, J.A. & De la Hoz, L. (2000). Combined use of Pronase E and a fungal extract (*Penicillium aurantiogriseum*) to potentiate the sensory characteristics of dry fermented sausages. *Meat Science*, **54**, 135–145.

Bruna, J.M., Hierro, E.M., De la Hoz, L., Mottram, D.S., Fernández, M. & Ordóñez, J.A. (2001a). The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. *Meat Science*, **59**, 97–107.

Bruna, J.M., Ordóñez, J.A., Fernández, M., Herranz, B. & De la Hoz, L. (2001b). Microbial and physico-chemical changes during the ripening of dry fermented sausages superficially inoculated with or having added an intracellular cell-free extract of *Penicillium aurantiogriseum*. *Meat Science*, **59**, 87–96.

Butler, A. (2015). Nitrites and nitrates in the human diet: Carcinogens or beneficial hypotensive agents? *Journal of Ethnopharmacology*, **167**, 105–107.

Caine, W.R., Aalhus, J.L., Best, D.R., Dugan, M.E.R. & Jeremiah, L.E. (2003). Relationship of texture profile analysis and Warner-Bratzler shear force with sensory characteristics of beef rib steaks. *Meat Science*, **64**, 333–339.

Canel, R.S., Wagner, J.R., Stenglein, S.A. & Ludemann, V. (2013). Indigenous filamentous fungi on the surface of Argentinean dry fermented sausages produced in Colonia Caroya (Córdoba). *International Journal of Food Microbiology*, **164**, 81–86.

Capita, R., Llorente-Marigómez, S., Prieto, M. & Alonso-Calleja, C. (2006). Microbiological profiles, pH, and titratable acidity of chorizo and salchichón (two Spanish dry fermented sausages) manufactured with ostrich, deer, or pork meat. *Journal of Food Protection*, **69**, 1183–1189.

Cardinali, F., Milanović, V., Osimani, A., Aquilanti, L., Taccari, M., Garofalo, C., Polverigiani, S., Clementi, F., Franciosi, E., Tuohy, K., Mercuri, M.L., Altissimi, M.S. & Haouet, M.N. (2018). Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites. *International Journal of Food Microbiology*, **278**, 61–72.

Casaburi, A., Aristoy, M., Cavella, S., Di Monaco, R., Ercolini, D., Toldra, F. & Villani, F. (2007). Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (Southern Italy) as affected by the use of starter cultures. *Meat Science*, **76**, 295–307.

Castaño, A., Fontán, M.C.G., Fresno, J.M., Tornadijo, M.E. & Carballo, J. (2002). Survival of *Enterobacteriaceae* during processing of Chorizo de cebolla, a Spanish fermented sausage. *Food Control*, **13**, 107–115.

Cenci-Goga, B.T., Rossitto, P. V., Sechi, P., Parmegiani, S., Cambiotti, V. & Cullor, J.S. (2012). Effect of selected dairy starter cultures on microbiological, chemical and sensory characteristics of swine and venison (*Dama dama*) nitrite-free dry-cured sausages. *Meat Science*, **90**, 599–606.

Chaillou, S., Champomier-Vergès, M.-C., Cornet, M., Crutz-Le Coq, A.-M., Dudez, A.-M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V. & Zagorec, M. (2005). The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nature Biotechnology*, **23**, 1527–1533.

Chakanya, C., Arnaud, E., Muchenje, V. & Hoffman, L.C. (2018). Changes in the physico-chemical attributes through processing of salami made from blesbok (*Damaliscus pygargus phillipsi*), eland (*Taurotragus oryx*), fallow deer (*Dama dama*), springbok (*Antidorcas marsupialis*) and black wildebees (*Connochaetes gnou*) in comparison to pork. *Meat Science*, **146**, 87–92.

Choi, Y.-S., Choi, J.-H., Han, D.-J., Kim, H.-Y., Lee, M.-A., Kim, H.-W., Jeong, J.-Y. & Kim, C.-J. (2011). Effects of rice bran fiber on heat-induced gel prepared with pork salt-soluble meat proteins in model system. *Meat Science*, **88**, 59–66.

Colindres, P. & Brewer, M.S. (2011). Oxidative stability of cooked, frozen, reheated beef patties: Effect of antioxidants. *Journal of the Science of Food and Agriculture*, **91**, 963–968.

Colmenero, F.J. (2000). Relevant factors in strategies for fat reduction in meat products. *Trends in Food Science and Technology*, **11**, 56–66.

Cullere, M., Hoffman, L.C. & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products. *Meat Science*, **95**, 72–77.

Cullere, M., Tasoniero, G., Secci, G., Parisi, G., Smit, P., Hoffman, L.C. & Dalle Zotte, A. (2019). Effect of the incorporation of a fermented rooibos (*Aspalathus linearis*) extract in the manufacturing of rabbit meat patties on their physical, chemical, and sensory quality during refrigerated storage. *LWT - Food Science and Technology*, **108**, 31–38.

De Beer, D., Schulze, A.E., Joubert, E., De Villiers, A., Malherbe, C.J. & Stander, M.A. (2012). Food ingredient extracts of *Cyclopia subternata* (honeybush): Variation in phenolic composition and antioxidant capacity. *Molecules*, **17**, 14602–14624.

De Mey, E., De Maere, H., Paelinck, H. & Fraeye, I. (2017). Volatile *N*-nitrosamines in meat products: Potential precursors, influence of processing, and mitigation strategies. *Critical Reviews in Food Science and Nutrition*, **57**, 2909–2923.

Demeyer, D. (2007). Composition and Nutrition. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 243–256. Iowa: USA: Blackwell Publishing Professional.

Demeyer, D., Raemaekers, M., Rizzo, A., Holck, A., Smedt, A. De, Hagen, B., Montel, C., Zanardi,

E., Murbrekk, E., Leroy, F., Vandendriessche, F., Lorentsen, K., Venema, K., Sunesen, L., Stahnke, L., Vuyst, L. De, Talon, R., Chizzolini, R. & Eerola, S. (2000). Control of bioflavour and safety in fermented sausages: First results of a European project. *Food Research International*, **33**, 171–180.

Department of Health, Foodstuffs Cosmetics and Disinfectants Act 54 of 1972, Regulation on Preservatives and Additives. URL: http://www.health.gov.za/index.php/shortcodes/2015-03-29-10-42-47/2015-04-30-09-10-23/2015-04-30-09-11-35/category/181-act.

De Vuyst, L. (2000). Technology aspects related to the application of functional starter cultures. *Food Technology and Biotechnology*, **38**, 105–112.

Egan, H., Kirk, R.S. & Sawyer, R. (1981). *Pearson's Chemical Analysis of Foods*. 8th edn. New York: Longman Inc.

Elias, M., Potes, M.E., Roseiro, L.C., Santos, C., Gomes, A. & Agulheiro-Santos, A.C. (2014). The effect of starter cultures on the Portuguese traditional sausage "Paio do Alentejo" in terms of its sensory and textural characteristics and polycyclic aromatic hydrocarbons profile. *Journal of Food Research*, **3**, 45–56.

Ergezer, H., Kaya, H.İ. & Şimşek, Ö. (2018). Antioxidant and antimicrobial potential of artichoke (*Cynara scolymus* L.) extract in beef patties. *Czech Journal of Food Sciences*, **36**, 154–162.

Feiner, G. (2006). Raw Fermented Salami. In: *Meat Products Handbook: Practical Science and Technology*. Pp. 314–375. New York: Woodhead Publishing Limited Cambridge: England.

Feng, X., Li, C., Jia, X., Guo, Y., Lei, N., Hackman, R.M., Chen, L. & Zhou, G. (2016). Influence of sodium nitrite on protein oxidation and nitrosation of sausages subjected to processing and storage. *Meat Science*, **116**, 260–267.

Fernández-López, J., Zhi, N., Aleson-Carbonell, L., Pérez-Alvarez, J., Kuri, V., Hernández, M. & Beniel Km, C. (2005). Antioxidant and antibacterial activities of natural extracts: Application in beef meatballs. *Meat Science*, **69**, 371–380.

Food Standards Australia New Zealand, (2017). Production of Uncooked Fermented Salami (UCFM) p.10. URL: https://www.mpi.govt.nz/dmsdocument/20441/send.

Flores, J. (1997). Mediterranean vs northern European meat products. Processing technologies and main differences. *Food Chemistry*, **59**, 505–510.

Fongaro, L., Alamprese, C. & Casiraghi, E. (2015). Ripening of salami: Assessment of colour and aspect evolution using image analysis and multivariate image analysis. *Meat Science*, **101**, 73–77.

Formanek, Z., Lynch, A., Galvin, K., Farkas, J. & Kerry, J.P. (2003). Combined effects of irradiation and the use of natural antioxidants on the shelf-life stability of overwrapped minced beef.

Meat Science, **63**, 433–440.

Forsythe, S.J. (2010). Basic aspects. In: *The Microbiology of Safe Food*. Pp. 52–100. West Sussex, United Kingdom: Blackwell Publishing Ltd.

Gassara, F., Kouassi, A.P., Brar, S.K. & Belkacemi, K. (2016). Green alternatives to nitrates and nitrites in meat-based products – A Review. *Critical Reviews in Food Science and Nutrition*, **56**, 2133–2148.

Gill, C.O. (1983). Meat spoilage and evaluation of the potential storage life of fresh meat. *Journal of Food Protection*, **46**, 444–452.

Glass, K.A., Loeffelholz, J.M., Ford, J.P. & Doyle, M.P. (1992). Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Applied and Environmental Microbiology*, **58**, 2513–2516.

Grazia, L., Romano, P., Bagni, A., Roggiani, D. & Guglielmi, G. (1986). The role of moulds in the ripening process of salami. *Food Microbiology*, **3**, 19–25.

Hammes, W.P. (2012). Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. *Food Microbiology*, **29**, 151–156.

Herrero, A.M., Ordóñez, J.A., De Avila, R., Herranz, B., De la Hoz, L. & Cambero, M.I. (2007). Breaking strength of dry fermented sausages and their correlation with texture profile analysis (TPA) and physico-chemical characteristics. *Meat Science*, **77**, 331–338.

Herrmann, S.S., Duedahl-Olesen, L. & Granby, K. (2015). Occurrence of volatile and non-volatile *N*-nitrosamines in processed meat products and the role of heat treatment. *Food Control*, **48**, 163–169.

Hoffman, L.C. (2000). Meat quality attributes of night-cropped Impala (*Aepyceros melampus*). *South African Journal of Animal Science*, **30**, 133–137.

Hoffman, L.C., Jones, M., Muller, N., Joubert, E. & Sadie, A. (2014). Lipid and protein stability and sensory evaluation of ostrich (*Struthio camelus*) droëwors with the addition of rooibos tea extract (*Aspalathus linearis*) as a natural antioxidant. *Meat Science*, **96**, 1289–1296.

Hoffman, L.C., Schoon, K., Rudman, M., Brand, T.S., Dalle Zotte, A. & Cullere, M. (2019). Profile of cabanossi made with exotic meats and olive oil. *Meat Science*, **147**, 20–27.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M. & Heir, E. (2017). Health and safety considerations of fermented sausages. *Journal of Food Quality*, **2017**, 1–25.

Honikel, K. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Science*, **49**, 447-457.

Hord, N.G., Tang, Y. & Bryan, N.S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *The American Journal of Clinical Nutrition*, **90**, 1–10.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Houben, J.H. & Van't Hooft, B.J. (2005). Variations in product-related parameters during the standardised manufacture of a semi-dry fermented sausage. *Meat Science*, **69**, 283–287.

Hunt, M.C., Sørhelm, O. & Sllnde, E. (1999). Color and heat denaturation of myoglobin forms in ground beef. *Journal of Food Science*, **64**, 847–851.

Ifesan, B.O.T., Siripongvutikorn, S., Hutadilok-Towatana, N. & Voravuthikunchai, S.P. (2009). Evaluation of the ability of *Eleutherie americana* crude extract as natural food additive in cooked pork. *Journal of Food Science*, **74**, M352–M357.

Jin, S.-K., Choi, J.S., Yang, H.-S., Park, T.-S. & Yim, D.-G. (2018a). Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science*, **146**, 34–40.

Jones, M., Hoffman, L.C. & Muller, M. (2015). Effect of rooibos extract (*Aspalathus linearis*) on lipid oxidation over time and the sensory analysis of blesbok (*Damaliscus pygargus phillipsi*) and springbok (*Antidorcas marsupialis*) droëwors. *Meat Science*, **103**, 54–60.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. & Gelderblom, W.C.A. (2019). Formal honeybush tea industry reaches 20-year milestone - Progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, **127**, 58–79.

Joubert, E., Gelderblom, W.C.A., Louw, A. & De Beer, D. (2008). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides* - A review. *Journal of Ethnopharmacology*, **119**, 376–412.

Joubert, E., Joubert, M.E., Bester, C., De Beer, D. & De Lange, J.H. (2011). Honeybush (*Cyclopia* spp.): From local cottage industry to global markets - The catalytic and supporting role of research. *South African Journal of Botany*, **77**, 887–907.

Kamara, B.I., Brand, J.D., Brandt, Vincent, E. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391–5395.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Labadie, J. (2007). Spoilage Microorganisms: Risks and Control. In: *Handbook of Fermented Meat and Poultry*. Pp. 421–426. Iowa: USA: Blackwell Publishing Professional.

Lambert, J.D. & Elias, R.J. (2010). The antioxidant and pro-oxidant activities of green tea polyphenols: A role in cancer prevention. *Archives of Biochemistry and Biophysics*, **501**, 65–72.

Latorre-Moratalla, M.L., Bover-Cid, S., Veciana-Nogués, M.T., Vidal-Carou, M.C., Carballo, J. & Tofalo, R. (2012). Control of biogenic amines in fermented sausages: Role of starter cultures. *Frontiers in Microbiology*, **3**, article 169.

Lebert, I., Leroy, S., Giammarinaro, P., Lebert, A., Chacornac, J.P. & Bover-Cid, S. (2007a). Diversity of microorganisms in the environment and dry fermented sausages of small traditional French processing units. *Meat Science*, **76**, 112–122.

Lebert, I., Leroy, S. & Talon, R. (2007b). Microorganisms in Traditional Fermented Meats. In: *Handbook of fermented meat and poultry* (edited by F. Toldrá). Pp. 113–124. Iowa: USA: Blackwell Publishing Professional.

Lee, C.M., Trevino, B. & Chaiyawat, M. (1996). A Simple and rapid solvent extraction method for determining total fats in fish tissue. *Journal of Association of Official Analytical Chemists International*, **79**, 487–492.

Lee, M.B. & Styliadis, S. (1996). A survey of pH and water activity levels in processed salamis and sausages in metro Toronto. *Journal of Food Protection*, **59**, 1007–1011.

Leistner, L. (1995). Stable and safe fermented sausages world-wide. In: *Fermented meats* (edited by G. Campbell-Platt & P.E. Cook). Pp. 160–175. Springer Science+Business Media Dordrecht.

Leroy, F., Verluyten, J. & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology*, **106**, 270–285.

Lücke, F.-K. (1994). Fermented meat products. *Food Research International*, **27**, 299–307.

Lücke, F.-K. (2000). Utilization of microbes to process and preserve meat. *Meat Science*, **56**, 105–115.

Magcwebeba, T.U., Riedel, S., Swanevelder, S., Swart, P., De Beer, D. Joubert, E. & Gelderblom, W.C.A. (2016). The potential role of polyphenols in the modulation of skin cell viability by *Aspalathus linearis* and *Cyclopia* spp. herbal tea extracts *in vitro*. *Journal of Pharmacy and Pharmacology*, **68**, 1440–1453.

Mahachi, L.N., Rudman, M., Arnaud, E., Muchenje, V. & Hoffman, L.C. (2019). Development of semi dry sausages (cabanossi) with warthog (*Phacochoerus africanus*) meat: Physicochemical and sensory attributes. *LWT - Food Science and Technology*.

Mckay, D. & Blumberg, J.B. (2007). A review of the bioactivity of South African herbal teas: Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*). *Phytotherapy Research*,

21, 1–16.

Miltenburg, G.A., Wensing, T., Smulders, F.J.M. & Breukink, H.J. (1992). Relationship between blood hemoglobin, plasma and tissue iron, muscle heme pigment, and carcass color of veal. *Journal of Animal Science*, **70**, 2766–2772.

Miralles, M.C., Flores, J. & Perez-Martinez, G. (1996). Biochemical tests for the selection of Staphylococcus strains as potential meat starter cultures. *Food Microbiology*, **13**, 227–236.

Mittal, G.S., Nadulski, R., Barbut, S. & Negi, S.C. (1992). Textural profile analysis test conditions for meat products. *Food Research International*, **25**, 411–417.

Møller, J.K.S. & Skibsted, L.H. (2002). Nitric oxide and myoglobins. *Chemical Reviews*, **102**, 1167–1178.

Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. & Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry*, **192**, 736–744.

Montanari, C., Gatto, V., Torriani, S., Barbieri, F., Bargossi, E., Lanciotti, R., Grazia, L., Magnani, R., Tabanelli, G. & Gardini, F. (2018). Effects of the diameter on physico-chemical, microbiological and volatile profile in dry fermented sausages produced with two different starter cultures. *Food Bioscience*, **22**, 9–18.

Montel, M.-C., Reitz, J., Talon, R., Berdagué, J.-L. & Rousset-Akrim, S. (1996). Biochemical activities of Micrococcaceae and their effects on the aromatic profiles and odours of a dry sausage model. *Food Microbiology*, **13**, 489–499.

Mora-Gallego, H., Serra, X., Guàrdia, M.D. & Arnau, J. (2014). Effect of reducing and replacing pork fat on the physicochemical, instrumental and sensory characteristics throughout storage time of small caliber non-acid fermented sausages with reduced sodium content. *Meat Science*, **97**, 62–68.

Moretti, V.M., Madonia, G., Diaferia, C., Mentasti, T., Paleari, M.A., Panseri, S., Pirone, G. & Gandini, G. (2004). Chemical and microbiological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions. *Meat Science* , **66**, 845–854.

Mortensen, A., Aguilar, F., Crebelli, R., Domenico, A. Di, Dusemund, B., Frutos, M.J., Galtier, P., Gott, D., Gundert-Remy, U., Lambré, C., Leblanc, J.-C., Lindtner, O., Mouldeus, P., Mosesso, P., Oskarsson, A., Parent-Massin, D., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R.A., Wright, M., Brandt, P. Van den, Fortes, C., Merino, L., Toldrá, F., Arcella, D., Christodoulidou, A., Abrahantes, J.C., Barrucci, F., Garcia, A., Pizzo, F., Battacchi, D. & Younes, M. (2017a). Re-evaluation of potassium nitrite (E249) and sodium nitrite (E250) as food additives. *European Food Safety Authority*, **15**, 1–157.

Mortensen, A., Aguilar, F., Crebelli, R., Domenico, A. Di, Dusemund, B., Frutos, M.J., Galtier, P.,

Gott, D., Gundert-Remy, U., Lambré, C., Leblanc, J.-C., Lindtner, O., Mouldeus, P., Mosesso, P., Oskarsson, A., Parent-Massin, D., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R.A., Wright, M., Brandt, P. Van Den, Fortes, C., Merino, L., Toldrá, F., Arcella, D., Christodoulidou, A., Barrucci, F., Garcia, A., Pizzo, F., Battacchi, D. & Younes, M. (2017b). Re-evaluation of sodium nitrate (E251) and potassium nitrate (E252) as food additives. *European Food Safety Authority*, **15**, 1–123.

Muthukumar, M., Naveena, B.M., Vaithiyanathan, S., Sen, A.. & Sureshkumar, K. (2014). Effect of incorporation of *Moringa oleifera* leaves extract on quality of ground pork patties. *Journal of Food Sciece and Technology*, **51**, 3172–3180.

Needham, T., Laubser, J.G., Kotrba, R., Bureš, D. & Hoffman, L.C. (2020). Influence of ageing on the physical qualities of the *longissimus thoracis et lumborum* and *biceps femoris* muscles from male and female free-ranging common eland (*Taurotragus oryx*). *Meat Science*, **159**, 1–6.

Ockerman, H.W. & Basu, L. (2007). Production and consumption of fermented meat products. In: *Handbook of fermented meat and poultry* (edited by F. Toldra). Pp. 9–15. Iowa: USA: Blackwell Publishing Professional.

Olivares, A., Navarro, J.L., Salvador, A. & Flores, M. (2010). Sensory acceptability of slow fermented sausages based on fat content and ripening time. *Meat Science*, **86**, 251–257.

Ordóñez, J.A., Hierro, E.M., Bruna, J.M. & De la Hoz, L. (1999). Changes in the components of dry-fermented sausages during ripening. *Critical Reviews in Food Science and Nutrition*, **39**, 329–367.

Papuc, C., Goran, G. V, Predescu, C.N. & Nicorescu, V. (2017). Mechanisms of oxidative processes in meat and toxicity induced by postprandial degradation products: A review. *Comprehensive Reviews in Food Science and Food Safety*, **16**, 96–123.

Pasini, F., Soglia, F., Petracci, M., Caboni, M.F., Marziali, S., Montanari, C., Gardini, F., Grazia, L. & Tabanelli, G. (2018). Effect of fermentation with different lactic acid bacteria starter cultures on biogenic amine content and ripening patterns in dry fermented sausages. *Nutrients*, **10**, 1–16.

Perea-Sanz, L., Montero, R., Belloch, C. & Flores, M. (2019). Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage. *Meat Science*, **147**, 100–107.

Pogorzelska, E., Godziszewska, J., Brodowska, M. & Wierzbicka, A. (2018). Antioxidant potential of *Haematococcus pluvialis* extract rich in astaxanthin on colour and oxidative stability of raw ground pork meat during refrigerated storage. *Meat Science*, **135**, 54–61.

Ravyts, F., De Vuyst, L. & Leroy, F. (2012). Bacterial diversity and functionalities in food fermentations. *Engineering in Life Sciences*, **12**, 356–367.

Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. URL: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02008R1333-20170320&from=ES.

Ribeiro, J.S., Santos, M.J.M.C., Silva, L.K.R., Pereira, L.C.L., Santos, I.A., Lannes, S.C. & da Silva, M.V. (2019). Natural antioxidants used in meat products: A brief review. *Meat Science*, **148**, 181–188.

Riel, G., Boulaaba, A., Popp, J. & Klein, G. (2017). Effects of parsley extract powder as an alternative for the direct addition of sodium nitrite in the production of mortadella-type sausages – Impact on microbiological, physicochemical and sensory aspects. *Meat Science*, **131**, 166–175.

Rimaux, T., Vrancken, G., Pothakos, V., Maes, D., De Vuyst, L. & Leroy, F. (2011a). The kinetics of the arginine deiminase pathway in the meat starter culture *Lactobacillus sakei* CTC 494 are pH-dependent. *Food Microbiology*, **28**, 597–604.

Rimaux, T., Vrancken, G., Vuylsteke, B., De Vuyst, L. & Leroy, F. (2011b). The pentose moiety of adenosine and inosine is an important energy source for the fermented-meat starter culture *Lactobacillus sakei* CTC 494. *Applied and Environmental Microbiology*, **77**, 6539–6550.

Roccato, A., Uyttendaele, M., Barrucci, F., Cibin, V., Favretti, M., Cereser, A., Dal, M., Pezzuto, A., Piovesana, A., Longo, A., Ramon, E., De Rui, S. & Ricci, A. (2017). Artisanal Italian salami and soppresse: Identification of control strategies to manage microbiological hazards. *Food Microbiology*, **61**, 5–13.

Rödel, W., Stiebing, A. & Krökel, L. (1993). Ripening parameters for traditional dry sausages with a mould covering. *Fleischwirtsch*, **73**, 848–853.

Ross, T. & Shadbolt, C.T. (2001). *Predicting Escherichia coli inactivation in uncooked comminuted fermented meat products*. School of Agricultural Science University of Tasmania, North Sydney, Australia. URL: https://pdfs.semanticscholar.org/b2ef/b717ea9684b4b3ee0ca96356d3b91df54281.pdf?_ga=2.121290952.1569392497.1580474732-317260316.1528390521.

Schulze, A.E., De Beer, D., Mazibuko, S.E., Muller, C.J.F., Roux, C., Willenburg, E.L., Nyunaï, N., Louw, J., Manley, M. & Joubert, E. (2016). Assessing similarity analysis of chromatographic fingerprints of *Cyclopia subternata* extracts as potential screening tool for *in vitro* glucose utilisation. *Analytical and Bioanalytical Chemistry*, **408**, 639–649.

Sebranek, J.G., Sewalt, V.J.H., Robbins, K.L. & Houser, T.A. (2005). Comparison of a natural rosemary extract and BHA/BHT for relative antioxidant effectiveness in pork sausage. *Meat Science*, **69**, 289–296.

Serra, X., Ruiz-Ramírez, J., Arnau, J. & Gou, P. (2005). Texture parameters of dry-cured ham *m.*

biceps femoris samples dried at different levels as a function of water activity and water content. *Meat Science*, **69**, 249–254.

Shah, M.A., Don Bosco, S.J. & Mir, S.A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, **98**, 21–33.

Sindelar, J.J. & Milkowski, A.L. (2011). Sodium Nitrite in Processed Meat and Poultry Meats: A Review of Curing and Examining the Risk/Benefit of Its Use. In: *American Meat Science Association*. Pp. 1–14.

Skibsted, L.H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide*, **24**, 176–183.

Stahnke, L.H. & Tjener, K. (2007). Influence of Processing Parameters on Cultures Performance. In: *Handbook of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 187–196. Iowa: USA: Blackwell Publishing Professional.

Sunesen, L.O. & Stahnke, L.H. (2003). Mould starter cultures for dry sausages-selection, application and effects. *Meat Science*, **65**, 935–948.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2014). The influence of season and sex on the carcass yield of common warthog (*Phacochoerus africanus*). *South African Journal of Wildlife Research*, **44**, 179–188.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2016a). Farmers' perceptions of the extra-limital common warthog in the Northern Cape and free state provinces, South Africa. *Wildlife Society Bulletin*, **40**, 112–121.

Swanepoel, M., Leslie, A.J. & Hoffman, L.C. (2016b). Comparative analyses of the chemical and sensory parameters and consumer preference of a semi-dried smoked meat product (cabanossi) produced with warthog (*Phacochoerus africanus*) and domestic pork meat. *Meat Science*, **114**, 103–113.

Swanepoel, M., Leslie, A.J., Van der Rijst, M. & Hoffman, L.C. (2016c). Physical and chemical characteristics of warthog (*Phacochoerus africanus*) meat. *African Journal of Wildlife Research*, **46**, 103–120.

Szczesniak, A.S., Brandt, M.A. & Friedman, H.H. (1963). Development of standard rating scales for mechanical parameters of texture and correlation between the objective and the sensory methods of texture evaluation. *Journal of Food Science*, **28**, 397–403.

Tabanelli, G., Coloretti, F., Chiavari, C., Grazia, L., Lanciotti, R. & Gardini, F. (2012). Effects of starter cultures and fermentation climate on the properties of two types of typical Italian dry fermented sausages produced under industrial conditions. *Food Control*, **26**, 416–426.

Talon, R. & Leroy, S. (2014). Fermented Foods, Fermented Meat Products and the Role of Starter

Cultures. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 870-874.

Taormina, P.J. (2014). Meat and Poultry, Curing of Meat. *Encyclopedia of Food Microbiology* (2nd ed.). Pp. 501-507.

Thomas, R., Anjaneyulu, A.S.R. & Kondaiah, N. (2008). Development of shelf stable pork sausages using hurdle technology and their quality at ambient temperature (37±1°C) storage. *Meat Science*, **79**, 1–12.

Todorov, S.D., Koep, K.S.C., Van Reenen, C.A., Hoffman, L.C., Slinde, E. & Dicks, L.M.T. (2007). Production of salami from beef, horse, mutton, blesbok (*Damaliscus dorcas phillipsi*) and springbok (*Antidorcas marsupialis*) with bacteriocinogenic strains of *Lactobacillus plantarum* and *Lactobacillus curvatus*. *Meat Science*, **77**, 405–412.

Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Science*, **49**, S101–S110.

Van Schalkwyk, D.L., Mcmillin, K.W., Booyse, M., Witthuhn, R.C. & Hoffman, L.C. (2010). Physico-chemical, microbiological, textural and sensory attributes of matured game salami produced from springbok (*Antidorcas marsupialis*), gemsbok (*Oryx gazella*), kudu (*Tragelaphus strepsiceros*) and zebra (*Equus burchelli*) harvested in Namibia. *Meat Science*, **88**, 36–44.

Warriss, P.D. (2010). Post-mortem Changes in Muscle and its Conversion into Meat. In: *Meat Science, an introductory text*. Pp. 65–76. Cambridge, USA: CAB International.

Weiss, J., Gibis, M., Schuh, V. & Salminen, H. (2010). Advances in ingredient and processing systems for meat and meat products. *Meat Science*, **86**, 196–213.

Zanardi, E., Ghidini, S., Conter, M. & Ianieri, A. (2010). Mineral composition of Italian salami and effect of NaCl partial replacement on compositional, physico-chemical and sensory parameters. *Meat Science*, **86**, 742–747.

Zdolec, N., Hadžiosmanovic, M., Kozačinski, L., Cvrtila, Z., Filipovi, I., Škrivanko, M. & Leskovar, K. (2008). Microbial and physicochemical succession in fermented sausages produced with bacteriocinogenic culture of *Lactobacillus sakei* and semi-purified bacteriocin mesenterocin Y. *Meat Science*, **80**, 480–487.

Zeuthen, P. (2007). A Historical Perspective of Meat Fermentation. In: *Handbook of fermented meat and poultry* (edited by F. Toldrá). Pp. 3–8. Iowa: USA: Blackwell Publishing Professional.

CHAPTER 6

Microbial analyses of warthog salami with reduced added sodium nitrate (75 mg/kg) and different levels of unfermented honeybush (*Cyclopia subternata*) extract after a 40-day ripening period

Abstract

Warthog salami with different levels of honeybush (*Cyclopia subternata*) extract and reduced added sodium nitrate (75 mg/kg) were manufactured, mould ripened for 40 days and microbial analyses were performed on the final product. The treatments entailed: C= 0%; T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5% of added honeybush, with an added starter culture mixture (*Lactobacillus sakei*, *Pediococcus pentosaceus,* and a *Micrococcus* species) and a surface mould inoculation (*Penicillium nalgiovense*). All salami were free from *Escherichia coli, Listeria monocytogenes, Salmonella* spp. and *Staphylococcus aureus*. Lactic acid bacteria were lowest in the control salami (6.57 log CFU/g) and increased with increasing honeybush concentration, with T3 and T4 resulting in the highest counts (> 7.0 log CFU/g). This study proved the feasibility of producing game salami with a high pH (6.71-7.46) free from major pathogens with a 70% reduction in added sodium nitrate (75 mg/kg, as opposed to the maximum permitted amount stipulated by the European Union of 250 mg/kg in the case of no added nitrite) with the addition of a natural extract, honeybush. The honeybush addition presumably promoted the growth of wanted acidifying bacteria, with no effect with regard to bacterial proliferation inhibition.

Keywords: warthog salami; honeybush (*Cyclopia subternata*); nitrate reduction; microbial analyses

6.1 Introduction

Food safety is often achieved by means of a multiple hurdle effect (Leistner, 2000) with salami being excellent examples of such a scenario. The combined action of salting and drying; lowering of the water activity (a_w); stuffing of meat and fat into casings creating an anaerobic environment; lowering of the pH through lactic acid producing bacteria; and smoking of the outer surface have assured safe salami production for ages (Leroy *et al.*, 2013; Holck *et al.*, 2017). The accidental contamination of sodium chloride with potassium nitrate (saltpetre) lead to the revolutionary discovery of nitrite, appreciated as a potent antimicrobial agent, amongst other valuable characteristics (Honikel, 2008; Sindelar & Milkowski, 2011). Furthermore, botulism poisoning has successfully been prevented by the commercial application of nitrate and/or nitrite salts in cured meats (Sebranek & Bacus, 2007). Also, nitrite and/or nitrate have shown to partake in the inhibition of other pathogens, including: *Listeria monocytogenes*; *Salmonella* spp.; and *Staphylococcus* spp. (Hospital *et al*., 2014, 2015, 2016; Christieans *et al*., 2018). The antibacterial role of nitrate and nitrite, especially in salami with a higher pH (> 5.2) contributes significantly to food safety even at a 50% reduced concentration (75/75 mg/kg $NaNO_2$/$NaNO_3$; Hospital *et al.*, 2014). The vital role of

nitrite as part of a multiple hurdle effect in dry-cured and fermented sausages is clear from literature, especially in the first days after stuffing, as the salami a_w and pH have not yet decreased (Hospital *et al.*, 2015, 2017; Christieans *et al.*, 2018) to < 0.95 and < 5.2, respectively (Feiner, 2006; Food Standards Australia New Zealand, 2017). However, the addition of nitrite and/or nitrate curing agents can not necessarily ensure microbially safe salami if other hurdles such as temperature control, a sufficient pH and a_w reduction are not met (Hospital *et al.*, 2014).

The equivocal conclusions surrounding human adverse health effects related to nitrate and nitrite consumption from cured meats (Hord *et al.*, 2009; Bryan *et al.*, 2012; Aschebrook-kilfoy *et al.*, 2013; Song *et al.*, 2015) promoted the investigation of nitrite reduction and/or alternatives in processed meats. Many attempts have been made to replace nitrate and nitrite with natural extracts, herbs and essential oils exhibiting antimicrobial effects (Shah *et al.*, 2014; Aziz & Karboune, 2018; Jin *et al.*, 2018; Alirezalu *et al.*, 2019; Ribeiro *et al.*, 2019). The special regulatory provision for organic and naturally cured meats by the European Union (EU) reflects the demand for these type of cleaner labelled processed meat products (Hospital *et al.*, 2015). In this regard, the EU allows a maximum ingoing amount of 80 mg/kg of nitrate/nitrite, expressed as sodium or potassium salts, with a maximum residual amount of 50 mg/kg of nitrate and nitrite, respectively (European Commission, 2008), as opposed to the maximum ingoing amount of 150 mg/kg of nitrate and nitrite (Directive 2008/1333/EC).

Honeybush (*Cyclopia subternata*, Tribe: Podalyrieae; Family: Fabaceae), an endemic South African shrub, has received much attention as a potential bioactive food ingredient (in the form of a green, unfermented extract) primarily due to its rich polyphenolic content (Joubert *et al.*, 2008; Kokotkiewicz *et al.*, 2012; Schulze *et al.*, 2015; Joubert *et al.*, 2019). Coetzee *et al.* (2008) demonstrated the antifungal potential of *Cyclopia* spp. extracts against the plant pathogen, *Botrytis cinereal,* although the active compounds responsible for the inhibition lacked identification. Fermented and unfermented solvent extractions of *C. intermedia* showed antimicrobial effects against Gram-positive bacteria (i.e. *Staphylococcus aureus*) and yeasts (i.e. *Candida albicans*), although relatively weak (Dube *et al.*, 2017). Although the antioxidant capacity did not seem to relate to the antimicrobial potency of *C. intermedia* extracts (Dube *et al.*, 2017), the major polyphenolic compounds present in *Cyclopia* spp., being the xanthone: mangiferin; and flavone: hesperidin showed antibacterial, -viral and/or -fungal activities in previous studies (Singh *et al.*, 2012; Iranshahi *et al.*, 2015). *Cyclopia subternata* was previously applied in dry-cured fermented Italian (Chapter 4) and warthog salami (Chapter 5) to investigate the effect on the product physical-chemical traits. Spontaneous surface mould growth did not proliferate to the same extend on honeybush treated Italian salami (0.5% w/w of added honeybush) compared to those without honeybush and with added nitrate (Chapter 4). In turn, the lactic acid bacteria (LAB) growth appeared unaffected by the addition of honeybush in typical Italian salami (Chapter 4). An insufficient pH decrease of the warthog salami after stuffing and an unusual high final product

salami pH (6.71-7.46, Chapter 5) raised a food safety concern and questioned the functionality of the added lactic acid bacteria (LAB) starter culture strains (*Lactobacillus sakei* and *Pediococcus pentosaceus*). Therefore, the objective of this study was to conduct final product microbial analyses on warthog salami with reduced added sodium nitrate (75 mg/kg), no added nitrite and increasing honeybush (*Cyclopia subternata*) concentrations (0.25%, 0.125%, 0.375% and 0.5%) after a 40-day mould ripening period to gain insight surrounding the presence of: total viable count (TVC); *Escherichia coli/*coliforms (EC); LAB; anaerobic spore formers; and pathogens: *Listeria monocytogenes; Salmonella;* and *Staphylococcus aureus*.

6.2 Materials and Methods

6.2.1 Salami ingredients

6.2.1.1 Warthog meat and pork fat

Ten free roaming warthogs (nine sows and one boar) were sourced from the Limpopo province, South Africa. The total weight of frozen, deboned warthog meat for salami production equalled 125 kg. Pork fat (53 kg) from the shoulders and back from more than five domestic sows (Landrace and Large White crossbred, 2-3 years of age) was purchased and frozen (-20°C) prior to use. The pigs predominantly received a diet containing maize, soya and canola in feeding lots in the Swartland area, Western Cape, South Africa.

6.2.1.2 Honeybush (Cyclopia subternata) extract

Unfermented, vacuum dried *Cyclopia subternata* hot water extract (1:10 m/v) was supplied by the Agricultural Research Council (ARC) - Infruitec Nietvoorbij, Stellenbosch University, and prepared as previously described (Schulze *et al.*, 2016). In short, honeybush plant material was subjected to preheated purified water (90°C, 30 min) in a percolator-type extraction vessel. The extract was subsequently drained, centrifuged, concentrated, vacuum dried (40°C, 24 h) and frozen (-20°C) in a moisture, light and oxygen impermeable packaging. The extract was added in increasing amounts according to salami treatment, where: Control= 0%; Treatment 1= 0.125%; Treatment 2= 0.25%; Treatment 3= 0.375%; and Treatment 4= 0.5% of added honeybush extract.

The minimum amount of distilled water (dH$_2$O) required to completely dissolve the maximum amount of extract required (T4= 0.5% w/w, 30.56 g of honeybush), was predetermined as 100 mL dH$_2$O. The amount of honeybush extract per respective treatment (T1-T4) in 100 mL dH$_2$O were heated on a magnetic stirrer (80°C for 5-10 min, speed setting 4-5) until visible evaporation bubbles appeared on the surface, followed by a mixing period at a lower temperature (50°C, another 5-10 min), until completely dissolved and free of lumps. The result was a dark-brown/ brick coloured solution with a sweet floral or honey sent (Addendum A, Fig. 5.1).

6.2.1.3 Starter culture mixture, sodium nitrate, salt and sugar

A non-commercial cell suspension was prepared as a starter culture mixture by the Microbiology Department, Stellenbosch University. The mixture (~305 x 10^9 ± 0.5 log colony forming units per

100 kg of raw meat and fat) consisted of equal amounts of lactic acid bacteria: *Lactobacillus sakei* (AQ14); and *Pediococcus pentosaceus* (AEH4); and a nitrate reducing *Micrococcus* species (MC2). The total amount of LAB (*L. sakei* and *P. pentosaceus)* in the starter culture mixture corresponded to 203 x 10^9 CFU/ kg of meat and fat. The cultures were suspended in a minimum amount of water to avoid excess liquid addition to the raw meat batter.

Pure sodium nitrate (NaNO$_3$) crystals were finely blended (commercial Sunbeam grinder) to prevent the addition of lumps. The latter was added to all treatments (C, T1-T4) at a concentration of 75 mg/kg (0.456 g /6.11 kg). Fine, non-iodized sodium chloride (Royal Salt, 2.5%) was added as an ingredient, as well as glucose (0.2%) to act as a fermentable sugar source for the starter culture mixture.

6.2.2 Salami production

6.2.2.1 Experimental replicates and treatment formulation

The total amount of warthog meat (125 kg) and pork fat (53 kg) were randomly divided into respective batches before processing of each, in order to avoid pseudo-replications between the meat and/or fat from one animal. From the total meat and fat mixture, six batches (29.6 kg each; meat:fat ratio of 7:3) were produced and these formed the statistical replications. Batches (i.e. replications) were further subdivided into five treatments (6.1 kg each: C= 0%; T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5% of added honeybush extract) and 10-14 salami were produced to ensure an adequate number available for all required analyses to be conducted. Other common salami ingredients included (w/w): sodium chloride (2.5%); distilled water (1.6%); glucose (0.2%); starter culture mixture (0.08%); and sodium nitrate (0.0075%). The salami length was calculated based on the required sample size for subsequent analyses, e.g. a 2 cm salami portion was required for a single texture profile analysis (TPA) compression. The total salami length equalled ~40 cm, weighing ~400 g (fresh).

6.2.2.2 Production procedure

The previously frozen warthog meat was semi-defrosted (4°C, 24 h). The pork fat was kept frozen (-20°C) until processing to prevent smearing and a temperature increase of the meat batter during mixing. The frozen fat was reduced into cubes (~5 cm^3, Addendum A, Fig. 5.2) using a band saw (Crown-Okto, Crown National, South Africa). Any visible sinew was removed from the warthog meat and it was roughly cut into similar sized cubes using a knife. Half of the fat cubes were added in a bowl cutter (Mainca, CM14, Spain; 25 s, speed setting 1) before adding the meat and remaining fat and increasing the speed (35 s, speed setting 2) to create a coarse meat and fat mixture (Addendum A, Fig. 5.3). This mixture, referred to as the salami batter, was transferred and mixed (60 s) in an industrial food mixer (Hobart, A200, FM Mixer, London), whilst adding the common ingredients and honeybush extract according to treatment. Care was taken not to over mix the batter, whilst ensuring adequate mixing to create a sticky consistency. The salami batter was stuffed into moisture permeable collagen casings (32 mm in diameter) and tagged with colour

coded labels to distinguish treatments (C, T1-T4) and replications (1-6). The salami was transporter to *The Flying Pig* (Darling, Western Cape, South Africa) meat processing facility and dipped in a commercial *Penicillium nalgiovense* suspension (Mould 600, 25 g/10 L water). The latter were randomly allocated to locations in a non-automated, artisanal-type ripening room (4 m in length x 3.5 m in width x 3.5 m in height) without circulating air flow, containing a variety of other fermented meats. Five temperature and relative humidity (RH) loggers (LogTag, SA, Trex-8) were randomly allocated in the roof between the salami to record environmental changes over time. The ripening period was 40 days during which salami were sampled to determine physical and chemical changes over time (Chapter 5).

6.2.3 Microbial analyses

6.2.3.1 Microbial sampling procedure

Salami pieces (25 g each) of all the treatments (C, T1-T4) within replications (1-6) were aseptically sampled from the final product salami after a 40-day ripening period. Samples were vacuum sealed and frozen (-20°C) until the day of analyses. All samples were analysed with the casing intact. Bacteriological media were prepared according to the manufacturer's instructions and all media and glassware were autoclaved (121°C, 15 psi for 15 min) before used. A summary of the relevant media can be seen attached in Addendum B.

6.2.3.2 Total viable count, (TVC) E.coli/coliforms (EC) and lactic acid bacteria (LAB)

Previously frozen samples were thawed (12 h, ~4°C) for subsequent analysis. Peptone buffered water (PBW, Oxoid) was used to create a serial dilution series. Based on the sample weight (25 g), 225 ml of PBW was aseptically added to the samples to create the first 1:10 dilution (10^{-1}), (McLandsborough, 2005). The samples were homogenised in stomacher bags (Lasec, 80-400 ml) for 60 s (Interscience BagMixer®, France) at room temperature (~21°C). Petrilfims (3M™ Petrifilm™) were used to enumerate TVC, EC and LAB, respectively. The efficacy of petrifilms have shown to be satisfactory for the enumeration of these bacterial groups (Matner *et al.*, 1990; Schraft & Watterworth, 2005; Nero *et al.*, 2006; De Castilho *et al.*, 2015). The dilution series were pipetted (1 ml) in duplicate onto the respective films, gently pressed with the provided flat spreader according to instructions, left to solidify and incubated. After the respective incubation periods (TVC: 35 ± 1°C, 48 ± 3 h; EC: 35 ± 1°C, 24 ± 2 h; and LAB: 28-37°C, 48 ± 3 h) the total colony forming units per gram (CFU/g) were enumerated following the 3M Petrifilm™ interpretation guidelines (AOAC® Official Methods of Analysis 990.12, 998.08 and 991.14; AOAC® Performance Tested Method[SM], certificate 041701). The preferable counting range on 3M Petrifilm Aerobic Films is ≤ 300 colonies. If the count exceeded this range, it was estimated by calculating the average number of colonies in two squares (1 cm^2 each) and multiplying the average by 20 or 30 (the total area of TVC and EC films are 20 cm^2 and that of LAB, 30 cm^2; AOAC® Official Methods 990.12 and AOAC® Official Methods 998.08). Blue colonies associated with entrapped gas bubbles were enumerated as *E. coli* and red colonies closely associated with entrapped gas bubbles were

recorded as coliforms. The total coliform count was reported as the sum of the blue and red colonies with associated gas bubbles (AOAC® Official Methods 998.08).

6.2.3.3 *Listeria monocytogenes* (ISO 16140 N° BRD 07/4-09/98)

Additional salami pieces (25 g, including all treatments: C, T1-T4; and replications 1-6) were solely dedicated to testing for the presence of *L. monocytogenes* due to the unique primary enrichment step. Half fraser broth Plus (ISO Neogen) with supplement (Bio-Rad) was used as selective primary enrichment. Samples were homogenised as explained for TVC, EC and LAB. The stomacher bags were incubated (30°C, 24 ± 2 h) before streaking onto Rapid'*L.mono* plates (AEC-Amersham) in duplicate and incubated inversely (37°C, 24 ± 2 h) before interpretation. Chromogenic Rapid'*L.mono* agar enables one to presumptively distinguish between pathogenic and non-pathogenic *Listeria* based on the secretion of phosphatidylinositol phospholipase C (PI-PLC) and inability of *L. monocytogenes* to metabolise the monosaccharide, xylose, resulting in black or grey-blue colonies without surrounding yellow halos (Lauer *et al.*, 2005; Gorski, 2008).

6.2.3.4 *Salmonella* (EN ISO 6579/A1 02/2006)

Peptone buffered water was used as primary, non-selective enrichment. The first dilution (10^{-1}) was created as described for TVC, EC and LAB and samples were similarly homogenised in respective stomacher bags and subsequently incubated (37°C, 18 ± 2 h). Rappaport-vassiliadis soya peptone (RVS) broth (Oxoid) was used as selective secondary enrichment. In summary, 10 ml of RVS broth per sample was prepared in test tubes, autoclaved and refrigerated (~4°C) until used the following day. Xylose lysine deoxycholate (XLD) agar (Oxoid) was dissolved in a microwave, aseptically poured in petri dishes and left to solidify. Each sample (0.1 ml) was pipetted from the stomacher bag into its respective RVS test tube. The tubes were vortexed and incubated (41.5 ± 1°C, 24 ± 3 h) before streaking onto XLD-plates in duplicate and incubated inversely (37 ± 1°C, 24 ± 3 h). The XLD agar contains a Gram-positive inhibiting agent known as deoxycholate and is formulated to promote the growth of fastidious enteric pathogens such as *Salmonella* (Blom *et al.*, 1999). Ferric ammonium citrate acts as a chromogenic indicator responsible for a visible black colony formation in the presence of hydrogen sulphide, a known metabolic product of salmonellae (Rambach, 1990; Jajere, 2019).

6.2.3.5 *Staphylococcus aureus* (NF EN ISO 6888-1/A1, 01/2004)

Samples were homogenised, dilution series were created using PBW (as previously explained for *Salmonella* spp.) and Baird Parker agar with egg-yolk tellurite emulsion (Fluka Analytical) were used as diagnostic agents (Baird-Parker, 1962). Samples (0.1 ml) were pipetted from the dilution series onto the respective petri dishes and a sterile glass hockey stick was used to create spread plates in duplicate (McLandsborough, 2005). Plates were incubated inversely (37°C, 24 ± 2 h) before presumptive, black-grey *Staphylococcus aureus* colonies were subjected to the coagulase test.

6.2.3.6 Confirmation of Staphylococcus aureus

Staphylococci are tellurite resistant with the ability to reduce tellurite to telluride (Lithgow *et al.*, 2004). This reaction leads to the formation of distinctive shiny, grey-black colonies with clear halos on Baird Parker agar (Coloretti *et al.*, 2014; Oxoid Microbial Products 2019a). Presumptive black *S. aureus* colonies were isolated from the Baird Parker plates, inoculated in non-selective Tryptone Soya Broth (Oxoid) and further incubated (30-35°C, 18-24 h). *Staphylococcus aureus* is coagulase positive, this trait is one point of difference used to distinguish the bacteria within the *Staphylococcus* genus (Foster, 1996; Hanselman *et al.*, 2009; Becker *et al.*, 2014). The coagulase test was performed using lyophilised rabbit plasma with ethylenediamine tetra acetic acid (EDTA, Oxoid). The slide test method can be used (Maksimovic *et al.*, 2018), however, the test tube method was preferred, as the latter indicates both bound and free coagulase (Remel, 2014). Rehydrated coagulase plasma (0.5 ml) was inoculated with cultures from the tryptone soya broth using a sterilised metal loop and incubated in a water bath (35-37°C). The tubes were observed every 30 min within a 4 h time period for clot formation. If no clotting was observed, the tubes were incubated further (37°C, 24 h). Positive *S. aureus* tubes were confirmed if clot formation was observed within 4 h or after the additional incubation period. The enumeration of *Staphylococci* on Baird-Parker agar followed by the coagulase confirmation test is a common practice found in literature (Raus & Love, 1983; Moretti *et al.*, 2004; Cenci-Goga *et al.*, 2012; Maksimovic *et al.*, 2018; Perea-Sanz *et al.*, 2019; Shiningeni *et al.*, 2019).

6.2.3.7 Anaerobic spore count

Samples were homogenised and dilution series created using PBW as previously described for *Salmonella* spp. A first layer of reinforced clostridial agar (RCA, Oxoid) was aseptically poured in petri dishes and left to solidify. The dilution series were subjected to a heat shock by placing test tubes in a water bath (80°C, 60 s). Samples were immediately cooled to below 45°C (SANS 5761:2008). Cooled samples were pipetted on the first layer of RCA in duplicate and a second layer of RCA was aseptically poured. Double layered RCA plates were incubated inversely (37°C, 6 days) in anaerobic jars each with a gas sachet (Oxoid, AnaeroGen™ 2.5 L) for the creation of a strict anaerobic environment. After the incubation period, a Gram's stain (Thairu *et al.*, 2014) was performed to observe the microscopical cellular morphology. Additionally, colonies were exposed to 3% hydrogen peroxide for the catalase test. If no gas bubbles appeared within 10 min of exposure the organisms were classified as catalase negative (SANS 5761:2008). The anaerobic spore former analysis was repeated with a heat shock exposure at 77°C for 20 min (Craven & Blankenship, 1985). Double layer RCA plates were similarly prepared as explained earlier and incubated both strict anaerobically and facultative anaerobically (37°C, 60 h).

6.2.3.8 Confirmation of spore formers

Colonies from RCA plates previously incubated under strict anaerobic conditions and facultative anaerobic conditions (including control and honeybush treated salami) were exposed to the Gram-

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stain and catalase test. Thereafter colonies were streaked on *Bacillus cereus* agar with polymyxin B supplement (Oxoid) and incubated (37°C, 24 h). The presence of endospores and sporangia morphology were observed after the incubation period with a Schaeffer-Fulton stain (Schaeffer & Fulton, 1933). Spore and sporangium morphology and the catalase test can be used amongst other techniques to classify *Bacillus* spp. into groups (Encinas *et al.*, 1996). Finally, pure *Bacillus* strains were streaked on nutrient agar (Oxoid) and incubated (37°C, 24 h) before subjected to an analytical profile index (API) 20E in combination with API 50CH (BioMérieux, France) confirmation test (Logan & Berkeley, 1984; Alippi & Abrahamovich, 2019). After an incubation period (37°C, 24 h) an API identification code based on chromogenic results was used to determine % similarity with a database (APIWEB™).

6.3 Results

6.3.1 Total Viable Count, E.coli/coliforms, Lactic acid bacteria and major pathogens

The results of TVC, total coliforms and LAB are depicted in Table 6.1 as the average log CFU/g calculated per treatment (C, T1-T4) within six replicates (n= 30, 5 treatments x 6 replications). Treatment had an effect on the TVC and LAB, as both these bacterial groups increased with increasing amount of added honeybush. Treatment 4 was the only group with acknowledgeable amount of coliforms, however, the other honeybush treated salami were virtually free from coliforms. All salami treatments were free from: *E. coli*; *L. monocytogenes*; *Salmonella* spp.; and *S. aureus.*

Table 6.1 The total viable, coliform and lactic acid bacteria count of dry-cured and fermented 40-day mould ripened warthog salami with different levels of honeybush (*Cyclopia subternata*) expressed as the average log CFU/g

Salami treatment	Microbial analysis		
n= 30	Total viable count	Coliforms	Lactic acid bacteria
Control (C)	6.76	< 1	6.57
Treatment 1 (T1)	6.93	< 1	6.94
Treatment 2 (T2)	> 7.00	< 1	> 7.00
Treatment 3 (T3)	> 7.00	< 1	> 7.00
Treatment 4 (T4)	> 7.00	3.5	> 7.00

n= 30 (5 treatments x 6 replications); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375% and T4= 0.5% (w/w) of added honeybush; CFU/g= colony forming units per gram of sample.

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6.3.2 Enterococcus faecium and Bacillus subtilis

Initially, the anaerobic RCA plates resulted in growth too numerous to count (TNTC). The Gram stain and catalase test confirmed gram-positive, catalase-negative cocci, eliminating anaerobic spore formers. The colony morphology examined after Gram staining resembled that of enterococci grouped in strains of two, i.e. diplococci (Lorenzo *et al.*, 2010). Pure stains were identified as *Enterococcus faecium* (Merieux NutriSciences, 7 Warrington Road, Claremont, 7708, Cape Town, South Africa; Accreditation # T0050[2]). After isolating *E. faecium*, the anaerobic spore forming test was repeated with an adjustment in the heat shock step (section 6.2.3.7). *Enterococcus faecium* was again enumerated, irrespective of treatment or incubation conditions (strict anaerobic or facultative anaerobic), as shown in Table 6.2.

Table 6.2 *Enterococcus faecium* enumerated from dry-cured and fermented 40-day mould ripened warthog salami with different levels of honeybush *(Cyclopia subternata)* expressed as the average log CFU/g after an incubation at strict anaerobic and facultative anaerobic conditions

Salami treatment n= 30	Incubation condition	
	Strict anaerobic	Facultative anaerobic
Control (C)	2.19	2.38
Treatment 1 (T1)	1.43	1.72
Treatment 2 (T2)	2.67	2.45
Treatment 3 (T3)	2.65	3.05
Treatment 4 (T4)	1.93	2.68

n= 30 (5 treatments x 6 replications); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375% and T4= 0.5% of added honeybush; CFU/g= colony forming units per gram of sample.

A colour change from lime-green to turquoise confirmed the presence of a *Bacillus* sp. (Oxoid Microbial Products, 2019b) in salami treatments (C and T4), supported by a positive catalase test and rod-shaped morphology (Encinas *et al.*, 1996; Matarante *et al.*, 2004). Furthermore, the API test confirmed the presence of *Bacillus subtilis* with a 94.8% identification similarity (APIWEB[TM]).

6.4 Discussion

The TVC from the present trial salami appeared high (> 7.0, Table 6.1), however, this phenomenon is typical of non heat treated and fermented sausages (Papamanoli *et al.*, 2003; Cenci-Goga *et al.*, 2012; Hospital *et al.*, 2015) due to the addition of starter cultures. In fact, a satisfactory TVC limit is specified as "not applicable" to dry-cured and fermented meats as a high TVC count will not

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[2] https://www.merieuxnutrisciences.com/za/sites/merieux_nutrisciences_za/files/atoms/files/t0050_sanas_certificate_schedule_of_accreditation_issue_no._21_may_2016.pdf

necessarily be a poor microbial quality indication as for other product categories (Gilbert *et al.*, 2000; SANS 885:2011; Food Standards Australia New Zealand, Compendium of Microbiological Criteria for Food, 2018; Food Safety Authority of Ireland, 2019). Nonetheless, salami TVC is often analysed and reported within similar ranges of enumerated LAB (Metaxopoulos *et al.*, 1981; Cenci-Goga *et al.*, 2012; Hospital *et al.*, 2015), suggesting that the latter is mainly the dominating bacterial group in salami. Indeed, LAB is expected to dominate in salami, irrespective of the deliberate use of starter cultures and reduction or omission of nitrate/nitrite (Cardinali *et al.*, 2018). As seen in Table 6.1, the TVC most likely also predominantly resembled LAB in the present trial salami, as these enumerations were within similar ranges.

The increasing LAB count analogous with the increasing honeybush addition substantiates the effect of treatment (honeybush) on salami pH, with salami with the highest honeybush inclusion level resulting in the lowest pH (Chapter 5). The LAB starter strains were added in a higher concentration (~2 x 10^9 CFU/g) than the recommended quantity (10^5-10^8 CFU/g, Elias *et al.*, 2014). However, the LAB counts, especially of the control and T1 salami, were lower than reported for other small diameter fermented sausages without the addition of starter cultures exceeding 8.5 log CFU/g (Bover-Cid *et al.*, 2001). Similarly, traditional salami without the addition of starter cultures successfully reached a LAB count of > 8 log CFU/g (Comi *et al.*, 2005; Rantsiou *et al.*, 2005) proving the feasibility of a high LAB count even without the deliberate addition of LAB strains. This lower count of final product salami LAB counts in the present trial reflects the insufficient acid production and subsequent pH reduction (Chapter 5). In contrast, Aymerich *et al.* (2003) reported commercial *fuets* (small diameter, slightly fermented sausages) with LAB counts of 6.86 log CFU/g (within a similar range compared to the present trial) with the majority of the *fuets* resulting in LAB counts > 7 log CFU/g, rather than > 8 log CFU/g. Furthermore, the LAB counts of the present trial salami was similar to nitrite and nitrate-free game salami (7.38 log CFU/g; Cenci-Goga *et al.*, 2012) and *Fabriono*-like sausages with a similar reduction in nitrate and nitrite (75/60 mg/kg, 7.85-7.45 log CFU/g LAB; Cardinali *et al.*, 2018), free from added LAB starter cultures.

A limited quantity of added glucose (0.2%) could explain the slightly impaired LAB proliferation, however, high LAB counts in sausages with and without the addition of sugar have been reported (Bover-Cid *et al.*, 2001). It is worth highlighting that LAB dominated in *fuets* during ripening which were manufactured without the addition of starter cultures or sugar and dipped in a similar mould suspension (*P. nalgiovense,* Bover-Cid *et al.*, 2001) compared to the present trial. The lowering of the ingoing quantity of nitrate and omitting nitrite could have promoted growth of background competing or contaminant bacteria. As demonstrated by Cardinali *et al.* (2018), the absence of nitrate and nitrite resulted in a higher microbial diversity, in turn LAB proliferation increased with increasing quantities of nitrate and nitrite. Perea-Sanz *et al.* (2019) also recorded an increase in microbial counts as the ingoing amount of nitrate was reduced. On the other hand, Hospital *et al.* (2015) reported the successful proliferation of LAB starter culture strains in salami,

irrespective of the addition of nitrate and/or nitrite. Cenci-Goga *et al.* (2012) reported a LAB count > 8 log CFU/g in nitrate and nitrite free venison salami produced from deer (*Dama dama*) meat without the addition of fermentable sugars.

The presence of coliforms in T4 salami (Table 6.1) were within the acceptable limit of *Enterobacteriaceae* (including coliforms, with an unsatisfactory limit of > 10^4 in 25 g) for ready-to-eat foodstuffs, including salami (Gilbert *et al.*, 2000; Food Standards Australia New Zealand, Compendium of Microbiological Criteria for Food, 2018; Food Safety Authority of Ireland, 2019). Due to the enumeration of coliforms exclusively in T4 salami with the highest quantity of added honeybush (0.5%), it appeared as if the extract might have been the contamination source. The decimal reduction time of numerous coliform species have shown to be less than 2 min at 60°C (Denis *et al.*, 2006). Considering the extract preparation (heat exposure of 90°C for 30 min followed by vacuum drying at 40°C for 24 h, Schulze *et al.*, 2016), the processing would have most likely eliminated present coliforms. Furthermore, in the unlikely case of high coliform count in the honeybush extract after processing (not tested), the mixing step with distilled water (dH$_2$O) before introducing the extract in the salami batter (a heat exposure of 80°C for 5-10 min, followed by 50°C for 5-10 min on a magnetic stirrer) would have extensively reduced or even eliminated coliforms. Since the initial microbial load of the extract was not tested, an acceptable coliform count was not guaranteed.

The addition of honeybush extract did not result in a meaningful difference with regard to the presence of major pathogens in the salami, since all salami were free from the analysed pathogens, irrespective of treatment. *Listeria*, *E. coli*, *Salmonella* and *S. aureus* are acknowledge as associated pathogens in non heat treated meat products (Heir *et al.*, 2013; Holck *et al.*, 2017; Roccato *et al.*, 2017), hence the absence thereof in the present trial salami, irrespective of a high pH, was regarded as a positive outcome. Previous researchers also demonstrated the feasibility of producing game salami with a rather high pH (6.76-6.81) without the addition of nitrate and/or nitrite, free from *Clostridia* spp.; *Salmonella* spp.; *Listeria* spp.; *E. coli;* and *S. aureus* in the final product, possibly ascribed to antibacterial compounds from the LAB starter culture other than organic acids lowering the pH (Cenci-Goga *et al.*, 2012). However, Lee and Styliadis (1996) pointed out the limitation of end product testing, as contamination, growth and subsequent toxin accumulation during early production stages could be undetected when pathogen growth declines, as in the case of *S. aureus*. Indeed, loss of viable *S. aureus* counts could portray a possible false representation of enterotoxin risk (Halpin-Dohnalek & Marth, 1989). The prevention of high initial contamination in raw salami ingredients and the use of well adapted LAB starter cultures strains to outgrow *S. aureus* are emphasised to prevent enterotoxin formation (Metaxopoulos *et al.*, 1981). The present trial salami were at greater microbial risk due to an unexpected impaired acidification process (Chapter 5), possibly indicating ill adapted acidifying starter strains. The proliferation of *S. aureus* and subsequent toxin production during early ripening stages were not guaranteed absent.

However, pathogens (including *S. aureus*) will not be a threat in the final product salami if not introduced during the manufacturing or post processing, hence the vital importance of good hygienic practices and raw materials of high quality for salami production (Comi *et al.*, 2005; Cardinali *et al.*, 2018). The homogenous surface mould growth of all salami, irrespective of treatment (Addendum A, Fig. 5.9) contradicted the impaired mould growth found with honeybush addition in Italian salami (Chapter 4). The latter salami were, however, spontaneously colonised by moulds which originated from the processing environment as opposed to the former salami which were dipped in a *P. nalgiovense* suspension (Chapter 5).

The isolation of *E. faecium* in the final product salami within all treatments and replications (Table 6.2) was quite controversial (Franz *et al.*, 1999). The *E. faecium* count from the present trial was similar compared to Greek fermented sausages (Papamanoli *et al.*, 2003) and commercial fermented sausages (Aymerich *et al.*, 2003). Higher enterococci counts in fermented sausages (≥ 5 log CFU/g) have also been reported (Samelis *et al.*, 1998; Fontana *et al.*, 2005). *Enterococcus faecium* has been proposed as part of starter culture mixtures due to its high competitiveness and bacteriocin production (Holley *et al.*, 1988; Callewaert *et al.*, 2000; Leroy & De Vuyst, 2002). However, the isolation of this bacteria from the final product salami (Table 6.2) was regarded as a contaminant not being deliberately introduced. This scenario could be indicative of weak fermentation and limited growth of ill adapted added LAB starter strains (*L. sakei* and *P. pentosaceus*), as *E. faecium* should have been eliminated in the presence of highly competitive LAB strains (Hugas *et al.*, 2003; Jahan *et al.*, 2013). In contrast, enterococci were enumerated in fairly high counts (3-5 log CFU/g) in fermented sausages irrespective of the addition of starter cultures (Marchesini *et al.*, 1992). Furthermore, enterococci exceeding 5 log CFU/g (Cenci-Goga *et al.*, 2012) and 6 log CFU/g (Cenci-Goga *et al.*, 2008) in fermented sausages in the presence of added LAB starter cultures were found.

Enterococci are known to survive a heat exposure of 60°C for 30 min (Sherman, 1937; Foulquié Moreno *et al.*, 2006), but survival at 68°C for 30 min have been reported (Gordon & Ahmad, 1991). *Enterococcus faecium* seems to be exceptionally heat resistant, although strain dependent, with *E. faecium* P-1A resulting in a higher *D*-value (29.04min) compared to *E. faecium* E-20 (13.97min) at 66°C (Magnus *et al.*, 1988). *Enterococcus faecium* P-1A was the only isolate still viable with a *D*-value of 2.57 min at 74°C (Magnus *et al.*, 1988). The high heat resistance of *E. faecium* was confirmed in the present study, surviving a heat shock of 77°C for 20 min. Due to their robustness and ability to survive in adverse conditions typically created in dry-cured and fermented sausages (low a_w and pH, high salt), enterococci can become a food contaminant risk in salami (Giraffa, 2003; Foulquié Moreno *et al.*, 2006).

The enumeration of *E. faecium* in the final product salami possibly contributes to explaining the strangely high pH found in the majority of the salami (Chapter 5). A high pH may be caused by the accumulation of biogenic amines (not tested) as a result of background microflora

decarboxylase activity (Cenci-Goga *et al.*, 2012). Enterococci are known to exhibit this trait, however, decarboxylase activity is strain dependent (Bover-Cid *et al.*, 2001; Sarantinopoulos *et al.*, 2001; Lebert *et al.*, 2007; Komprda *et al.*, 2010). Furthermore, *E. faecium* is a poor acidifier in comparison to meat LAB starter strains (Sarantinopoulos *et al.*, 2001). If the added fermentable glucose (0.2%) was arguably insufficient for optimum LAB proliferation, contaminant *E. faecium* could have outgrown the added LAB starters leading to inadequate organic acid secretion and hence, a higher pH in the final product salami. Enterococci have showed better growth in sausages with added sugar (Bover-Cid *et al.*, 2001), thus a high initial *E. faecium* contamination could have contributed to glucose depletion and suppression of added LAB growth. Supporting this argument, Hugas *et al.* (2003) noted a higher probability of *E. faecium* survival in salami with a higher pH. Due to their high prevalence in the gastrointestinal tract of warm blooded animals and association with soil, *E. faecium* might have been introduced during slaughter of the warthogs or domestic pigs (Franz *et al.*, 1999; Maksimovic *et al.*, 2018) or in the meat processing facility (Talon *et al.*, 2007).

Bacillus subtilis was isolated from present trial salami, irrespective of treatment (Results section 6.3.2). This bacterium was previously isolated from fermented sausages (Encinas *et al.*, 1996; Matarante *et al.*, 2004; Baruzzi *et al.*, 2006). These bacteria can proliferate in a high NaCl (10%), acidic (pH 5.5-5.0) and relatively low temperature (10°C) environment, typically associated with fermented meats (Cachaldora *et al.*, 2014). *Bacillus* spp. can either be pathogenic as in the case of *B. cereus*, or potentially beneficial contributing to salami texture and organoleptic properties (Cachaldora *et al.*, 2014) by amylase and protease activities (De Boer & Diderichsen, 1991) and by inhibiting other pathogens (Caputo *et al.*, 2011). *Bacillus subtilis* TR50 survived fermentation, drying and curing of Italian salami (Baruzzi *et al.*, 2006) and lacking the *B. cereus* toxin gene, it was subsequently studied as a potential novel strain in salami to inhibit pathogens (Caputo *et al.*, 2011). *Bacillus*, having a large heterogenicity and some strains being closely related, make classification quite tedious (Rasko *et al.*, 2005; Arnesen *et al.*, 2008; Fan *et al.*, 2017). *Bacillus subtilis* is classified in Group 1 (within the small cell subgroup, lacking a swollen sporangium; Drobniewski, 1993; Standerds Unit, 2018). This bacterium is not considered as a pathogenetic threat to humans which are not immune suppressed (De Boer & Diderichsen, 1991). Food-borne risks ascribed to *Bacillus* spp. other than *B. cereus* are said to be low, although the presence of virulence genes from other species (including the strain isolated in the present trial) are not guaranteed (Matarante *et al.*, 2004; Gu *et al.*, 2019).

6.5 Conclusion

Warthog salami with reduced added sodium nitrate (75 mg/kg) and added honeybush extract (T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5%) were free from pathogens: *E.coli*; *Listeria* spp.; *Salmonella* spp.; and *S. aureus* after a 40-day mould ripening period. To better comprehend the potential pathogen inhibitory effects of honeybush extract in salami, reference strains would need to be incorporated in future studies as salami would not necessarily contain pathogens if not

introduced during processing. Honeybush addition to salami could provide additional fermentable substrates to LAB, which in turn could lead to a higher total viable count.

The reduction of nitrate does not necessarily imply a microbially unaccepted product, reflected in the absence of pathogens in the final product salami. However, the associated microbial risks increase on a large production scale particularly in the case of other hurdles not being successfully met (such as a sufficient pH decrease in this case). Emphasis remains on a multiple hurdle effect, wherein nitrate and nitrite within regulated limits have their applicable role to produce microbially safe salami.

6.6 References

Alippi, A.M. & Abrahamovich, E. (2019). HiCrome *Bacillus* agar for presumptive identification of *Bacillus* and related species isolated from honey samples. *International Journal of Food Microbiology*, **305**, 1–7.

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

AOAC Official Method 990.12. Aerobic Plate Count in Foods. Dry Rehydratable Film (Petrifilm^TM Aerobic Count Plate) Method. URL: https://www.edgeanalytical.com/wp-content/uploads/Food_AOAC-990.12.pdf.

AOAC Official Method 998.08. Confirmed *Escherichia coli* Counts in Poulltry, Meats, and Seafood. Dry Rehydratable Film Method (Petrifilm^TM *E.coli*/Coliform Count Plate). URL: https://www.scribd.com/document/353629034/AOAC-998-08-EC-en-Carnes-Mariscos-Pollo.

AOAC Official Method 991.14. Coliform and *Escherichia coli* Counts in Foods. Dry Rehydratable Film (Petrifilm^TM *E.coli*/Coliform Count Plate^TM) Methods. URL: http://edgeanalytical.com/wp-content/uploads/Food_AOAC-991.14.pdf.

AOAC Performance Tested Method^SM, certificate 041701. URL: https://multimedia.3m.com/mws/media/1385416O/3m-petrifilm-lactic-acid-bacteria-count-plate-ptm-approval-press-release.pdf.

Arnesen, L.P.S., Fagerlund, A. & Granum, P.E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, **32**, 579–606.

Aschebrook-kilfoy, B., Shu, X., Gao, Y., Ji, B., Yang, G., Li, H.L. & Rothman, N. (2013). Thyroid cancer risk and dietary nitrate and nitrite intake in the Shanghai women's health study. *International Journal of Cancer*, **132**, 897–904.

Aymerich, T., Martín, B., Garriga, M. & Hugas, M. (2003). Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. *Applied and Environmental Microbiology*, **69**, 4583–4594.

Aziz, M. & Karboune, S. (2018). Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, **58**, 486–511.

Baird-Parker, A.C. (1962). An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *Journal of Applied Bacteriology*, **25**, 12–19.

Baruzzi, F., Matarante, A., Caputo, L. & Morea, M. (2006). Molecular and physiological characterization of natural microbial communities isolated from a traditional Southern Italian processed sausage. *Meat Science*, **72**, 261–269.

Becker, K., Heilmann, C. & Peters, G. (2014). Coagulase-negative staphylococci. *Clinical Microbiology Reviews*, **27**, 870–926.

Blom, M., Meyer, A., Gerner-Smidt, P., Gaarslev, K. & Espersen, F. (1999). Evaluation of statens serum institut enteric medium for detection of enteric pathogens. *Journal of Clinical Microbiology*, **37**, 2312–2316.

Bover-Cid, S., Izquierdo-Pulido, M. & Vidal-Carou, M. (2001). Changes in biogenic amine and polyamine contents in slightly fermented sausages manufactured with and without sugar. *Meat Science*, **57**, 215–221.

Bryan, N.S., Alexander, D.D., Coughlin, J.R., Milkowski, A.L. & Boffetta, P. (2012). Ingested nitrate and nitrite and stomach cancer risk: An updated review. *Food and Chemical Toxicology*, **50**, 3646–3665.

Cachaldora, A., Fonseca, S., Gómez, M., Franco, I. & Carballo, J. (2014). Metabolic characterization of *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains isolated from traditional dry-cured sausages. *Journal of Food Protection*, **77**, 1605–1611.

Callewaert, R., Hugas, M. & De Vuyst, L. (2000). Competitiveness and bacteriocin production of enterococci in the production of Spanish-style dry fermented sausages. *International Journal of Food Microbiology*, **57**, 33–42.

Caputo, L., Quintieri, L., Morea, M. & Baruzzi, F. (2011). Antimicrobial activity of a meat-borne *Bacillus subtilis* strain against food pathogens. *European Food Research and Technology*, **232**, 183–189.

Cardinali, F., Milanović, V., Osimani, A., Aquilanti, L., Taccari, M., Garofalo, C., Polverigiani, S., Clementi, F., Franciosi, E., Tuohy, K., Mercuri, M.L., Altissimi, M.S. & Haouet, M.N. (2018). Microbial dynamics of model Fabriano-like fermented sausages as affected by starter cultures, nitrates and nitrites. *International Journal of Food Microbiology*, **278**, 61–72.

Cenci-Goga, B.T., Ranucci, D., Miraglia, D. & Cioffi, A. (2008). Use of starter cultures of dairy origin in the production of Salame nostrano, an Italian dry-cured sausage. *Meat Science*, **78**, 381–390.

Cenci-Goga, B.T., Rossitto, P. V., Sechi, P., Parmegiani, S., Cambiotti, V. & Cullor, J.S. (2012). Effect of selected dairy starter cultures on microbiological, chemical and sensory characteristics of swine and venison (*Dama dama*) nitrite-free dry-cured sausages. *Meat Science*, **90**, 599-606.

Christieans, S., Picgirard, L., Parafita, E., Lebert, A. & Gregori, T. (2018). Impact of reducing nitrate/nitrite levels on the behavior of *Salmonella Typhimurium* and *Listeria monocytogenes* in French dry fermented sausages. *Meat Science*, **137**, 160–167.

Coetzee, G., Marx, I.J., Pengilly, M., Bushula, V.S., Joubert, E. & Bloom, M. (2008). Effect of rooibos and honeybush tea extracts against *Botrytis cinerea*. *South African Journal for Enology and Viticulture*, **29**, 33–38.

Coloretti, F., Tabanelli, G., Chiavari, C., Lanciotti, R., Grazia, L., Gardini, F. & Montanari, C. (2014). Effect of wine addition on microbiological characteristics, volatile molecule profiles and biogenic amine contents in fermented sausages. *Meat Science*, **96**, 1395–1402.

Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C. & Cocolin, L. (2005). Characterisation of naturally fermented sausages produced in the north east of Italy. *Meat Science*, **69**, 381–392.

Craven, S.E. & Blankenship, L.C. (1985). Activation and injury of *Clostridium perfringens* spores by alcohols. *Applied and Environmental Microbiology*, **50**, 249–256.

De Boer, A.S. & Diderichsen, B. (1991). On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: A review. *Applied Microbiology and Biotechnology*, **36**, 1–4.

De Castilho, N.P.A., Okamura, V.T., Camargo, A.C., Pieri, F.A. & Nero, L.A. (2015). Adequacy of Petrifilm™ aerobic count plates supplemented with de Man, Rogosa & Sharpe broth and chlorophenol red for enumeration of lactic acid bacteria in salami. *Meat Science*, **110**, 253–261.

Denis, C., Cadot, P., Leguerinel, I., Thuault, D. & Sohier, D. (2006). Heat resistance of coliform species isolated from cookedham, snail flesh, and "bouchées à la reine." *Letters in Applied Microbiology*, **42**, 160–164.

Drobniewski, F.A. (1993). *Bacillus cereus* and related species. *Clinical Microbiology Reviews*, **6**, 324–338.

Dube, P., Meyer, S. & Marnewick, J.L. (2017). Antimicrobial and antioxidant activities of different solvent extracts from fermented and green honeybush (*Cyclopia intermedia*) plant material. *South African Journal of Botany*, **110**, 184–193.

Elias, M., Potes, M.E., Roseiro, L.C., Santos, C., Gomes, A. & Agulheiro-Santos, A.C. (2014). The effect of starter cultures on the Portuguese traditional sausage "Paio do Alentejo" in terms of its sensory and textural characteristics and polycyclic aromatic hydrocarbons profile. *Journal*

of Food Research, **3**, 45–56.

Encinas, J.P., Sanz-Gómez, J., García-López, M.-L., García-Armesto, M.-R. & Otero, A. (1996). Evaluation of different systems for the identification of *Bacillus* strains isolated from Spanish fermented sausages. *Meat Science*, **42**, 127–131.

Fan, B., Blom, J., Klenk, H.-P. & Borriss, R. (2017). *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Frontiers in Microbiology*, **8**, 1–15.

Feiner, G. (2006). Raw Fermented Salami. In: *Meat Products Handbook: Practical Science and Technology*. Pp. 314–375. New York: Woodhead Publishing Limited Cambridge: England.

Fontana, C., Cocconcelli, P.S. & Vignolo, G. (2005). Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *International Journal of Food Microbiology*, **103**, 131–142.

Food Safety Authority of Ireland (2019). Guidelines for the Interpretation of Results of Microbiological Testing of Ready-to-Eat Foods Placed on the Market (Revision 3). URL: https://www.fsai.ie › WorkArea › DownlaodAsset.

Food Standards Australia New Zealand, Compendium of Microbiological Criteria for Food (2018). URL: https://www.foodstandards.gov.au › Compendium_revised-Sep 2018.

Foster, T. (1996). Staphylococcus. In: *Medical Microbiology* (edited by S. Baron). Galveston: University of Texas Medical Branch.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E. & De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology*, **106**, 1–24.

Franz, C.M.A.P., Holzapfel, W.H. & Stiles, M.E. (1999). Enterococci at the crossroads of food safety? *International Journal of Food Microbiology*, **47**, 1–24.

Gilbert, R.J., De Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C.D., Richards, J., Roberts, D. & Bolton, F.J. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. *Communicable Disease and Public Health*, **3**, 163–167.

Giraffa, G. (2003). Functionality of enterococci in dairy products. *International Journal of Food Microbiology*, **88**, 215–222.

Gordon, C.L.A. & Ahmad, M.H. (1991). Thermal susceptibility of *Streptococcus faecium* strains isolated from frankfurters. *Canadian Journal of Microbiology*, **37**, 609–612.

Gorski, L. (2008). Phenotypic Identification. In: *Handbook of Listeria Monocytogenes* (edited by D. Liu). Pp. 139–168. Boca Raton: USA: CRC Press Taylor & Francis Group.

Gu, H.-J., Sun, Q.-L., Luo, J.-C., Zhang, J. & Sun, L. (2019). A first study of the virulence potential

of a *Bacillus subtilis* isolate from deep-sea hydrothermal vent. *Frontiers in Cellular and Infection Microbiology*, **9**, 1–14.

Halpin-Dohnalek, M.I. & Marth, E.H. (1989). *Staphylococcus aureus*: Production of extracellular compounds and behavior in foods - A Review. *Journal of Food Protection*, **52**, 267–282.

Hanselman, B.A., Kruth, S.A., Rousseua, J. & Weese, J.S. (2009). Coagulase positive staphylococcal colonization of humans and their household pets. *Canadian Veterinary Journal*, **50**, 954–958.

Heir, E., Holck, A.L., Omer, M.K., Alvseike, O., Måge, I., Høy, M., Rode, T.M., Sidhu, M.S. & Axelsson, L. (2013). Effects of post-processing treatments on sensory quality and Shiga toxigenic *Escherichia coli* reductions in dry-fermented sausages. *Meat Science*, **94**, 47–54.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M. & Heir, E. (2017). Health and safety considerations of fermented Sausages. *Journal of Food Quality*, **2017**, 1–25.

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, **78**, 68–76.

Hord, N.G., Tang, Y. & Bryan, N.S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *The American Journal of Clinical Nutrition*, **90**, 1–10.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Hospital, X.F., Hierro, E., Arnau, J., Carballo, J., Aguirre, J.S., Gratacós-Cubarsí, M. & Fernández, M. (2017). Effect of nitrate and nitrite on *Listeria* and selected spoilage bacteria inoculated in dry-cured ham. *Food Research International*, **101**, 82–87.

Hospital, X.F., Hierro, E. & Fernández, M. (2014). Effect of reducing nitrate and nitrite added to dry fermented sausages on the survival of *Salmonella Typhimurium*. *Food Research International*, **62**, 410–415.

Hospital, X.F., Hierro, E., Stringer, S. & Fernández, M. (2016). A study on the toxigenesis by *Clostridium botulinum* in nitrate and nitrite-reduced dry fermented sausages. *International Journal of Food Microbiology*, **218**, 66–70.

Hugas, M., Garriga, M. & Aymerich, M.T. (2003). Functionalty of enterococci in meat products. *International Journal of Food Microbiology*, **88**, 223–233.

Iranshahi, M., Rezaee, R., Parhiz, H., Roohbakhsh, A. & Soltani, F. (2015). Protective effects of flavonoids against microbes and toxins: The cases of hesperidin and hesperetin. *Life Sciences*, **137**, 125–132.

Jahan, M., Krause, D.O. & Holley, R.A. (2013). Antimicrobial resistance of *Enterococcus* species

from meat and fermented meat products isolated by a PCR-based rapid screening method. *International Journal of Food Microbiology*, **163**, 89–95.

Jajere, S.M. (2019). A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Veterinary World*, **12**, 504–521.

Jin, S., Choi, J.S., Yang, H., Park, T. & Yim, D. (2018). Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science*, **146**, 34–40.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. & Gelderblom, W.C.A. (2019). Formal honeybush tea industry reaches 20-year milestone – Progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, **127**, 58–79.

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2008). Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush Tea). *Journal of Agricultural and Food Chemistry*, **56**, 954–963.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Komprda, T., Sládková, P., Petirová, E., Dohnal, V. & Burdychová, R. (2010). Tyrosine-and histidine-decarboxylase positive lactic acid bacteria and enterococci in dry fermented sausages. *Meat Science*, **86**, 870–877.

Lauer, W.F., Facon, J.F. & Patel, A. (2005). Evaluation of a chromogenic medium for identification and differentiation of *Listeria monocytogenes* in selected foods. *Journal of AOAC International*, **88**, 511–517.

Lebert, I., Leroy, S., Giammarinaro, P., Lebert, A., Chacornac, J.P. & Bover-Cid, S. (2007). Diversity of microorganisms in the environment and dry fermented sausages of small traditional French processing units. *Meat Science*, **76**, 112–122.

Lee, M.B. & Styliadis, S. (1996). A survey of pH and water activity levels in processed salamis and sausages in Metro Toronto. *Journal of Food Protection*, **59**, 1007–1011.

Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, **55**, 181–186.

Leroy, F. & De Vuyst, L. (2002). Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. *International Journal of Food Microbiology*, **72**, 155–164.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L. & Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: A historical outlook. *Trends in Food Science and Technology*, **31**, 130–137.

Lithgow, J.K., Hayhurst, E.J., Cohen, G., Aharonowitz, Y. & Foster, S.J. (2004). Role of a cysteine synthase in *Staphylococcus aureus*. *Journal of Bacteriology*, **186**, 1579–1590.

Logan, N.A. & Berkeley, R.C.W. (1984). Identification of *Bacillus* strains using the API system. *Journal of General Microbiology*, **130**, 1871–1882.

Lorenzo, J.M., García, M.C., Cachaldora, A., Franco, I. & Carballo, J. (2010). Study of the lactic acid bacteria throughout the manufacture of dry-cured lacón (a Spanish traditional meat product). Effect of some additives. *Food Microbiology*, **27**, 229–235.

Magnus, C.A., McCurdy, A.R. & Ingledew, W.M. (1988). Further studies on the thermal resistance of *Streptococcus faecium* and *Streptococcus faecalis* in pasteurized Ham. *Canadian Institute of Food Science and Technology*, **21**, 209–212.

Maksimovic, A.Z., Zunabovic-Pichler, M., Kos, I., Mayrhofer, S., Hulak, N., Domig, K.J. & Fuka, M.M. (2018). Microbiological hazards and potential of spontaneously fermented game meat sausages: A focus on lactic acid bacteria diversity. *LWT - Food Science and Technology*, **89**, 418–426.

Marchesini, B., Bruttin, A., Romailler, N., Moreton, R.S., Stucchi, C. & Sozzi, T. (1992). Microbiological events during commercial meat fermentations. *Journal of Applied Microbiology*, **73**, 203–209.

Matarante, A., Baruzzi, F., Cocconcelli, P.S. & Morea, M. (2004). Genotyping and toxigenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages. *Applied and Environmental Microbiology*, **70**, 5168–5176.

Matner, R.R., Fox, T.L., Mciver, D.E. & Curiale, M.S. (1990). Efficacy of Petrifilm[TM] *E. coli* count plates for *E. coli* and coliform enumeration. *Journal of Food Protection*, **53**, 145–150.

McLandsborough, L.A. (2005). Fish Microflora: Basic Microbiological Techniques and Standard Plate Counts. In: *Food Microbiology Laboratory* (edited by F.M. Clydesdale). Pp. 1–13. Washington, D.C.: CRC Press.

Metaxopoulos, J., Genigeorgis, C., Fanelli, M.J., Franti, C. & Cosma, E. (1981). Production of Italian dry salami: Initiation of *Staphylococcal* growth in salami under commercial manufacturing conditions. *Journal of Food Protection*, **44**, 347–352.

Moretti, V.M., Madonia, G., Diaferia, C., Mentasti, T., Paleari, M.A., Panseri, S., Pirone, G. & Gandini, G. (2004). Chemical and microbiological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions. *Meat Science* , **66**, 845–854.

Nero, L.A., Beloti, V., Barros, M.D.A.F., Beatriz, M., Ortolani, M.B.T., Tamanini, R. & Franco, B.D.G.D.M. (2006). Comparison of Petrifilm aerobic count plates and Deman-Rogosa-Sharpe Agar for enumeration of lactic acid bacteria. *Journal of Rapid Methods and Automation in Microbiology*, **14**, 249–257.

Oxoid Microbial Products (2019a). Baird Parker Agar base. URL: http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0275&.

Oxoid Microbial Products (2019b). *Bacillus cereus* selective agar base. URL: http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0617&org=9&c=uk&lang=en.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E. & Kotzekidou, P. (2003). Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science*, **65**, 859–867.

Perea-Sanz, L., Montero, R., Belloch, C. & Flores, M. (2019). Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage. *Meat Science*, **147**, 100–107.

Rambach, A. (1990). New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. and other enteric bacteria. *Applied and Environmental Microbiology*, **56**, 301–303.

Rantsiou, K., Drosinos, E.H., Gialitaki, M., Urso, R., Krommer, J., Gasparik-Reichardt, J., Tóth, S., Metaxopoulos, I., Comi, G. & Cocolin, L. (2005). Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. *Food Microbiology*, **22**, 19–28.

Rasko, D.A., Altherr, M.R., Han, C.S. & Ravel, J. (2005). Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiology Reviews*, **29**, 303–329.

Raus, J. & Love, D.N. (1983). Characterization of coagulase-positive *Staphylococcus intermedius* and *Staphylococcus aureus* isolated from veterinary clinical specimens. *Journal of Clinical Microbiology*, **18**, 789–792.

Remel: Coagulase plasma (rabbit plasma with EDTA), (2014). R21051, Santa Fe Drive, USA.

Ribeiro, J.S., Santos, M.J.M.C., Silva, L.K.R., Pereira, L.C.L., Santos, I.A., Lannes, S.C. & da Silva, M.V. (2019). Natural antioxidants used in meat products: A brief review. *Meat Science*, **148**, 181–188.

Roccato, A., Uyttendaele, M., Barrucci, F., Cibin, V., Favretti, M., Cereser, A., Dal, M., Pezzuto, A., Piovesana, A., Longo, A., Ramon, E., De Rui, S. & Ricci, A. (2017). Artisanal Italian salami and soppresse: Identification of control strategies to manage microbiological hazards. *Food Microbiology*, **61**, 5–13.

Samelis, J., Metaxopoulos, J., Vlassi, M. & Pappa, A. (1998). Stability and safety of traditional Greek salami - A microbiological ecology study. *International Journal of Food Microbiology*, **44**, 69–82.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M.D., Rea, M.C., Lombardi, A., Cogan, T.M., Kalantzopoulos, G. & Tsakalidou, E. (2001). Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal*, **11**, 621–647.

Schaeffer, A.B. & Fulton, M.D. (1933). A simplified method of staining endospores. *Science*, **77**, 194.

Schraft, H. & Watterworth, L.A. (2005). Enumeration of heterotrophs, fecal coliforms and *Escherichia coli* in water: Comparison of 3M Petrifilm plates with standard plating procedures. *Journal of Microbiological Methods*, 335–342.

Schulze, A.E., Beelders, T., Koch, I.S., Erasmus, L.M., De Beer, D. & Joubert, E. (2015). Honeybush herbal teas (*Cyclopia* spp.) contribute to high levels of dietary exposure to xanthones, benzophenones, dihydrochalcones and other bioactive phenolics. *Journal of Food Composition and Analysis*, **44**, 139–148.

Schulze, A.E., De Beer, D., Mazibuko, S.E., Muller, C.J.F., Roux, C., Willenburg, E.L., Nyunaï, N., Louw, J., Manley, M. & Joubert, E. (2016). Assessing similarity analysis of chromatographic fingerprints of *Cyclopia subternata* extracts as potential screening tool for *in vitro* glucose utilisation. *Analytical and Bioanalytical Chemistry*, **408**, 639–649.

Sebranek, J.G. & Bacus, J.N. (2007). Cured meat products without direct addition of nitrate or nitrite: What are the issues? *Meat Science*, **77**, 136–147.

Shah, M.A., Don Bosco, S.J. & Mir, S.A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, **98**, 21–33.

Sherman, J.M. (1937). The Streptococci. *Bacteriological Reviews 1*, 3–97.

Shiningeni, D., Chimwamurombe, P., Shilangale, R. & Misihairabgwi, J. (2019). Prevalence of pathogenic bacteria in street vended ready-to-eat meats in Windhoek, Namibia. *Meat Science*, **148**, 223–228.

Sindelar, J.J. & Milkowski, A.L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. In: *American Meat Science Association*. Pp. 1–14.

Singh, S.K., Tiwari, R.M., Sinha, S.K., Danta, C.C. & Prasad, S.K. (2012). Antimicrobial evaluation of mangiferin and its synthesized analogues. *Asian Pacific Journal of Tropical Biomedicine*, S884–S887.

Song, P., Wu, L. & Guan, W. (2015). Dietary nitrates, nitrites, and nitrosamines intake and the risk

of gastric cancer: A meta-analysis. *Nutrients*, **7**, 9872–9895.

South African National Standard 5761:2008. Examination of the presence of viable spores of mesophilic *Clostridium* organisms in foods.

South African National Standard 885:2011. Processed meat products.

Talon, R., Lebert, I., Lebert, A., Leroy, S., Garriga, M., Aymerich, T., Drosinos, E.H., Zanardi, E., Ianieri, A., Fraqueza, M.J., Patarata, L. & Laukova, A. (2007). Traditional dry fermented sausages produced in small-scale processing units in Mediterranean countries and Slovakia. 1: Microbial ecosystems of processing environments. *Meat Science*, **77**, 570–579.

Thairu, Y., Nasir, I.A. & Usman, Y. (2014). Laboratory perspective of Gram staining and its significance in investigations of infectious diseases. *Sub-Saharan African Journal of Medicine*, **1**, 168–174.

CHAPTER 7

The fatty acid methyl ester composition and volatile organic compound profile of warthog (*Phacochoerus africanus*) salami with different levels of unfermented honeybush (*Cyclopia subternata*) extract

Abstract

The aim of this study was to determine the effect of honeybush extract on the fatty acid methyl ester (FAME) composition and volatile organic compound (VOC) profile of warthog salami. Honeybush significantly affected the fatty acid composition (C12:0, C14:1, C20:1, C18:3n6, C18:3n3, C20:3n3, C20:4n6, C20:5n3 and subsequently also total n-3 and the n-6/n-3 ratio). The volatile organic compound results indicated that a honeybush addition at 0.5% to warthog salami may contribute to floral, sweet and spicy aromas due to the presence of (*R/S*)-linalool, terpinene-4-ol and *α*-terpineol. Furthermore, honeybush treated salami resulted in lower amounts of rancid compounds compared to the control after a 40-day ripening period. Of these, hexanal and 1-octen-3-ol reduced the most significant in T2, T3 and T4 salami (0.25%, 0.375% and 0.5% honeybush addition, respectively) compared to the control salami ($p < 0.01$). These results indicate that honeybush might extend the shelf life of game salami with reduced added nitrate (75 mg/kg) by supressing the onset of rancidity due to unsaturated fatty acid oxidation.

7.1 Introduction

Dry-cured and fermented sausages are a valuable commodity, appreciated for their characteristic sensory profile (Leroy *et al*., 2013). However, a number of health concerns related to the frequent consumption of these sausages (e.g. salami) are evident from literature (Bolger *et al*., 2017; Holck *et al*., 2017). Salami are classified as high in fat (~20-50%) and salt (~4-6%) (Meynier *et al.*, 1999; Zanardi *et al.*, 2004; Di Cagno *et al.*, 2008; Dos Santos *et al.*, 2015). With regards to the former, saturated, hard fat is preferred to produce premium quality salami to prevent smearing during processing and to delay rancidity during shelf life (Ruiz, 2007; Yildiz-Turp & Serdaroglu, 2012; Fuentes *et al.*, 2014). High consumption of saturated fats and sodium are linked to coronary heart diseases (Hu *et al.*, 1997; Ha, 2014; Zong *et al.*, 2016). The recent sodium reduction in cured meats as stipulated by the South African Depart of Health (from a maximum permitted amount of 1300 to 1150 mg/100 g), portrays the high sodium content of cured meats (DoH, 2019). Furthermore, the common use of nitrate (NO_3) and nitrite (NO_2) salts in salami have been scrutinised due to their questionable safety. While nitrate/nitrite preserving salts serve an undeniable multifunctional role in salami (i.e. antimicrobial, antioxidant and colour fixating agents), the formation of carcinogenic *N*-nitrosamines (i.e. *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosomorpholine (NMOR)) raised concern regarding their application (IARC, 1978; De Mey *et al.*, 2017).

An abundant amount of research has been dedicated to fermented sausage reformulation to lower the amount of saturated fat (Muguerza *et al.*, 2003a; Hoz *et al.*, 2004; Rubio *et al.*, 2007; Del Nobile *et al.*, 2009) and sodium (Gelabert *et al.*, 2003; Chen *et al.*, 2019; da Silva *et al.*, 2020). Furthermore, researchers are also focussed on replacing NO_3/NO_2 with natural extracts exhibiting an antimicrobial and/or antioxidant effect (Shah *et al.*, 2014; Aziz & Karboune, 2018; Jin *et al.*, 2018; Alirezalu *et al.*, 2019; Martínez *et al.*, 2019). Amongst the botanical group, tea is a good example of plants high in antioxidants (Jiang & Xiong, 2016). The final product salami quality traits (i.e. texture, flavour and water binding capacity) are directly dependant on the raw materials, amongst other variables (Ruusunen *et al.*, 2003). In the case of new product development or processing alteration to conform to consumer demand, is it of utmost importance to evaluate their effect on product quality and safety. For instance, in order to study the contribution of salami towards dietary fat intake, the quantity and fatty acid composition of the product is analysed (Herranz *et al.*, 2008). In this regard, the daily total fat intake for adults should ideally range between 20-35% of total energy intake with the saturated fatty acid (SFA) intake not exceeding 10% (Elmadfa & Kornsteiner, 2009). The previously recommended ratio of n-6/n-3 (omega-6/omega-3) does not seem to be relevant if the individual recommended adequate intake (AI) of n-6 (2.5-9% of total energy with a minimum daily intake of linoleic acid of > 2.5%) and n-3 (0.5-2% of total energy intake with a minimum daily intake of alpha-linolenic acid of > 0.5%) are met (Woods & Fearon, 2009; FAO, 2010).

The volatile profile of salami can be studied in the head space of samples by means of gas-chromatography (Shahidi *et al.*, 1986; Roberts *et al.*, 2000). The former is commonly studied in the case of recipe alteration, for instance when the salt (Corral *et al.*, 2013), fat (Muguerza *et al.*, 2003b; Olivares *et al.*, 2011) or nitrite contents are reduced or replaced with natural alternatives (Hospital *et al.*, 2015; Aquilani *et al.*, 2018). The VOCs of salami may portray valuable information with regard to the presence of both unwanted (i.e. hexanal associated with rancidity) and wanted sausage aromas (i.e. 2-butanone associated with apricot notes) (Shahidi *et al.*, 1986; Montel *et al.*, 1996; Tabanelli *et al.*, 2012; Lorenzo *et al.*, 2013). Numerous biochemical reactions partake in aroma development in salami over the fermentation and ripening periods, such as lactic acid fermentation, protein and lipid breakdown (Ordóñez *et al.*, 1999; Montanari *et al.*, 2018). These reactions in turn, are influenced by many variables, such as the raw materials (i.e. spices and starter cultures used), processing methodology, environmental conditions; and even sausage dimensions (Montanari *et al.*, 2016). Subsequently, no single compound is responsible for the unique and complex sensory profile of salami (Marco *et al.*, 2008; Flores, 2018). The comminution of meat and fat, low water activity (a_w), high salt content, presence of metal catalysts (i.e. haem iron) and highly unsaturated phospholipids promote lipid and protein oxidation in salami (Faustman *et al.*, 2010; Min *et al.*, 2010; Jiang & Xiong, 2016). While the deteriorative process of lipid and protein oxidation are generally unwanted in foodstuffs, these reactions are necessary to an extent

for the intricate dry-cured and fermented sensory profiles of salami (Ordóñez *et al.*, 1999; Chen *et al.*, 2017).

Game meat with a free-range status and being naturally darker in colour creates a potential for the production of nitrate and/or nitrite reduced salami to suit the consumer interested in organic and "free-from" labelled meat products (Chakanya *et al.*, 2018). Warthog meat, as with other South African game meat species, has an attractive nutritional composition being low in total lipid content (< 2.2%) and high in protein (> 20%) (Swanepoel *et al.*, 2016b). Whilst a native South African shrub, locally known as honeybush (*Cyclopia subternata*, Family: Fabaceae, Tribe: Podalyrieae), has received much attention as a bio-active food ingredient in an unfermented extract form (Joubert *et al.*, 2008, 2019; De Beer & Joubert, 2010; De Beer *et al.*, 2012). Honeybush is locally enjoyed as hot brewed tea after drying and fermentation of the leaves and stems (De Beer *et al.*, 2012). *Cyclopia subternata* has proven to be rich in polyphenolics and portrays antioxidant capacity in both the fermented and unfermented state (Kamara *et al.*, 2004; De Beer & Joubert, 2010; Kokotkiewicz *et al.*, 2012). *Cyclopia subternata* is associated with fruity-sweet, rose geranium and apricot jam attributes and the complexity of it sensory profile is portrayed in the vast amount of volatiles isolated therein (Le Roux *et al.*, 2012; Theron *et al.*, 2014; Ntlhokwe *et al.*, 2018). Rooibos (*Aspalathus linearis*), similarly consumed and known for its high polyphenolic content (Magcwebeba *et al.*, 2016), showed promising results as an antioxidant when added to processed meat products (Jones *et al.*, 2015; Cullere *et al.*, 2019). However, research lacks the investigation of honeybush in this regard.

The objective of this study was to determine the fatty acid composition and volatile profile of warthog salami with reduced added nitrate (75 mg/kg) and different levels of added honeybush (*Cyclopia subternata*) extract (C= 0%; T1= 0.125%, T2= 0.25%, T3= 0.375% and T4= 0.5% of added honeybush).

7.2 Materials and Methods

7.2.1 Salami ingredients

7.2.1.1 Warthog meat and pork fat

Ten free roaming warthogs (nine sows and one boar) were sourced from the Limpopo province, South Africa. The total weight of frozen, deboned warthog meat for salami production equalled 125 kg. Pork fat (53 kg) from the shoulders and back from more than five domestic sows (Landrace and Large White crossbred, 2-3 years of age) was purchased and frozen (-20°C) prior to use. The pigs predominantly received a diet containing maize, soya and canola in feeding lots in the Swartland area, Western Cape, South Africa.

7.2.1.2 Honeybush (Cyclopia subternata) extract

Unfermented, vacuum dried *Cyclopia subternata* hot water extract (1:10 m/v) was supplied by the Agricultural Research Council (ARC) - Infruitec Nietvoorbij, Stellenbosch University, and prepared

as previously described (Schulze *et al.*, 2016). In short, honeybush plant material was subjected to preheated purified water (90°C, 30 min) in a percolator-type extraction vessel. The extract was subsequently drained, centrifuged, concentrated, vacuum dried (40°C, 24 h) and frozen (-20°C) in a moisture, light and oxygen impermeable packaging.

The minimum amount of distilled water (dH$_2$O) required to completely dissolve the maximum amount of extract required (T4= 0.5% w/w, 30.56 g of honeybush), was predetermined as 100 mL dH$_2$O. The amount of honeybush extract per respective treatment (T1-T4) in 100 mL dH$_2$O were heated on a magnetic stirrer (80°C for 5-10 min, speed setting 4-5) until visible evaporation bubbles appeared on the surface, followed by a mixing period at a lower temperature (50°C, another 5-10 min), until completely dissolved and free of lumps. The result was a dark-brown/ brick coloured solution with a sweet floral or honey scent (Addendum A, Fig. 5.1).

7.2.1.3 Starter culture mixture, sodium nitrate, salt and sugar

A non-commercial cell suspension was prepared as a starter culture mixture by the Microbiology Department, Stellenbosch University. The mixture (~305 x 10^9 ± 0.5 log colony forming units per 100 kg of raw meat and fat) consisted of equal amounts of lactic acid bacteria: *Lactobacillus sakei* (AQ14); and *Pediococcus pentosaceus* (AEH4); and a nitrate reducing *Micrococcus* species (MC2). The cultures were suspended in a minimum amount of water to avoid excess liquid addition to the raw meat batter.

Pure sodium nitrate (NaNO$_3$) crystals were finely blended (commercial Sunbeam grinder) to prevent the addition of lumps. The latter was added to all treatments (C, T1-T4) at a concentration of 75 mg/kg (0.456 g /6.11 kg). Fine, non-iodized sodium chloride (Royal Salt, 2.5%) was added as an ingredient, as well as glucose (0.2%) to act as a fermentable sugar source for the starter culture mixture.

7.2.2 Salami production

7.2.2.1 Experimental replicates and treatment formulation

The total amount of warthog meat (125 kg) and pork fat (53 kg) were randomly divided into respective batches before processing of each, in order to avoid pseudo-replications between the meat and/or fat from one animal. From the total meat and fat mixture, six batches (29.6 kg each; meat:fat ratio of 7:3) were produced and these formed the statistical replications. Batches (i.e. replications) were further subdivided into five treatments (6.1 kg each: C= 0%; T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5% of added honeybush extract) and 10-14 salami were produced to ensure an adequate number available for all required analyses to be conducted. Other common salami ingredients included (w/w): sodium chloride (2.5%); distilled water (1.6%); glucose (0.2%); starter culture mixture (0.08%); and sodium nitrate (0.0075%). The salami length was calculated based on the required sample size for subsequent analyses, e.g. a 2 cm salami portion was required for a single texture profile analysis (TPA) compression. The total salami length equalled ~40 cm, weighing ~400 g (fresh).

7.2.2.2 Production procedure

The previously frozen warthog meat was semi-defrosted (4°C, 24 h). The pork fat was kept frozen (-20°C) until processing to prevent smearing and a temperature increase of the meat batter during mixing. The frozen fat was reduced into cubes (~5 cm^3, Addendum A, Fig. 5.2) using a band saw (Crown-Okto, Crown National, South Africa). Any visible sinew was removed from the warthog meat and it was roughly cut into similar sized cubes using a knife. Half of the fat cubes were added in a bowl cutter (Mainca, CM14, Spain; 25 s, speed setting 1) before adding the meat and remaining fat and increasing the speed (35 s, speed setting 2) to create a coarse meat and fat mixture (Addendum A, Fig. 5.3). This mixture, referred to as the salami batter, was transferred and mixed (60 s) in an industrial food mixer (Hobart, A200, FM Mixer, London). Raw salami batter (6 replications, day-0) were sampled, vacuum sealed and frozen (-80°C) until volatile organic compound (VOC) analysis. The common ingredients and honeybush extract were added according to treatment. Care was taken not to over mix the batter, whilst ensuring adequate mixing to create a sticky consistency. The salami batter was stuffed into moisture permeable collagen casings (32 mm in diameter) and tagged with colour coded labels to distinguish treatments (C, T1-T4) and replications (1-6). The salami was transported to *The Flying Pig* (Darling, Western Cape, South Africa) meat processing facility and dipped in a commercial *Penicillium nalgiovense* suspension (Mould 600, 25 g/10 L water). Salami sausages were randomly allocated to locations in a non-automated, artisanal-type ripening room (4 m in length x 3.5 m in width x 3.5 m in height) without circulating air flow, containing a variety of other fermented meats. Five temperature and relative humidity (RH) loggers (LogTag, SA, Trex-8) were randomly allocated in the roof between the salami to record environmental changes over time. After a 40-day ripening period, salami sausages (including all treatments: C, T1-T4; and replications 1-6) were sampled for fatty acid methyl ester (FAME) and VOC analyses, vacuum sealed and frozen (-80°C) until analyses.

7.2.3 Fatty acid methyl ester (FAME) composition

Frozen (-80ºC) 40-day ripened salami were thawed (12 h, 4ºC) and samples (including all treatments: C, T1-T4; and replications 1-6) were roughly homogenised (10 s, 20 000 rpm, FOSS Analytical, KnifetecTM 1095). Fat extraction of samples (1 g) were executed using a 2:1 (v/v) chloroform:methanol solution (Folch *et al.*, 1957). Butylated hydroxytoluene (BHT, 20 mL 0.01%, Cat no. B-1378, Sigma Aldrich) was added as an antioxidant and heptadecanoic acid (C17:0, 5 mL, 10 mg/ml prepared in chloroform:methanol 2:1, catalogue number H3500, Sigma-Aldrich, Gauteng, South Africa) was added as internal standard. The samples were further homogenised in the extraction solvent (30 s, 10 000 rpm, IKA® T18 digital Ultra Turrax®, setting C) and a sub-sample (250 μL) of the extracted lipids was subsequently transmethylated (2 h, 70°C) using a methanol:sulphuric acid solution (2 mL, 19:1 v/v) as the transmethylating agent. After allowing the resultant mixtures to cool to room temperature (~21°C), the FAMEs were extracted with dH$_2$O and hexane (Cat no 1.04391.2500, Merck). Following separation of the dH$_2$O and FAME-containing hexane, the top hexane layer was transferred to a spotting tube and dried under nitrogen. After

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drying, hexane (100 μL) was added to each FAME sample, of which 1 μL was injected into the gas chromatograph (GC).

The FAMEs were analysed using a TRACE 1300 Thermo Scientific gas-chromatograph (Thermo Electron Corporation, Milan, Italy) equipped with a flame-ionisation detector (FID). The GC-FID system was coupled to a CTC Analytics COMBIPAL auto sampler. Separation of the FAMEs was performed on a polar Stabilwax (L= 60 m, ID= 0.25 mm, film thickness= 0.25 μm) capillary column. Helium was used as the carrier gas at a flow rate of 2 ml/min. The injector temperature was maintained at 240°C and 1 μl of the sample was injected in splitless mode. The oven temperature was programmed as follows: 50°C for 2 min, increased to 180°C for 5 min at a rate of 25°C/min; and further increased to 250°C for 2 min at a rate of 3°C/min. The FAME of each sample was identified by comparing the retention times with those of a standard FAME mixture (Supelco™ 37 Component FAME mix, Cat no. CRM47885, Supelco, USA) and semi-quantified using the internal standard (C17:0) with the results expressed as mg FAMEs/g of fat of the final product salami after 40 ripening days.

7.2.4 Volatile organic compounds

Frozen (-80ºC) 40-day ripened salami were thawed (12 h, 4ºC) and samples (including all treatments: C; T1-T4; and replications 1-6) were homogenised using a handheld grinder (Sunbeam, 30 s). The homogenised samples (2 g) were placed in 20 mL polytetrafluoroethylene (PTFE)/silicone lined screw-cap vials and 3-octanol (100 μL, 10 ppm) was added as internal standard.

Volatile analysis was performed using a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL auto sampler. Separation of the salami volatiles was performed on a Stabilwax capillary column (L= 60 m, ID= 0.25 mm and film thickness= 0.25 μm) and helium was used as the carrier gas at a flow rate of 2 ml/min with the injector temperature maintained at 250°C. Injection was performed in splitless mode by introducing a fused silica fibre covered by 50/30 μm divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS StableFlex) (Supelco, Steinheim, Germany) in the head-space. The oven temperature was programmed as follows: 40°C for 5 min, followed by a temperature increase to 240°C for 2 min at a rate of 5°C/min. The mass selective detector (MSD) was operated in a full scan mode and the source and quad temperatures were maintained at 230°C and 150°C, respectively. The transfer line temperature was maintained at 250°C. The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70eV, scanning from 35 to 550 m/z. A standard, saturated alkane mixture (100 ppm, C7 to C40 in hexane, Sigma Aldrich 49452-U) was injected to serve as an external standard. Volatile peaks were semi-quantified by computerised matching of the individual mass spectrums with the Wiley 275 mass spectral database and the Kováts indices (KI) were calculated based on the external

standard (alkane mix) retention times as seen in the equation below (Olivares *et al.*, 2011). The volatile composition was expressed as the average ratios between the peak areas of each volatile and the internal standard (3-octanol).

KI= 100 x [n + (Tu - Tn)/(TN - Tn)]

Where:

n= number of carbons in the alkane preceding the compound

Tu= retention time of the compound

Tn= retention time of the alkane preceding the compound

TN= retention time of the alkane succeeding the compound

7.2.5 Statistical analysis

To test for differences in fatty acid methyl esters and volatile organic compounds, one-way ANOVA's were done with the Fisher LSD for post hoc testing. Normal probability plots were inspected for normality and were mostly found to be acceptable. Levene's test was done to check for homogeneity of variance, and in cases where this test was significant ($p < 0.01$), the Welch test was done with Games-Howell post hoc test, not assuming equal variances and samples sizes.

7.3 Results

7.3.1 Fatty acid methyl ester (FAME) composition

Table 7.1 contains the statistical significant differences (p-values) of the influence of the honeybush treatment on the FAME composition of warthog salami after a 40-day ripening period. Besides the differences in C12:0 ($p < 0.01$), C14:1 ($p= 0.03$) and C20:1 ($p= 0.03$) content, it is evident that treatment mainly affected the polyunsaturated fatty acids (PUFAs, 18:3n6, C18:3n3, C20:3n3, C20:4n6 and C20:5n3) of 40-day ripened warthog salami (Table 7.1). The latter fatty acids will thus be discussed further (Table 7.2). Generally, as the honeybush content increased, the content of the above-mentioned PUFA decreased (Table 7.2). Treatment 1 had the highest amount of C12:0, C14:1, C20:1, C18:3n6, C18:3n3, C20:3n3, C20:4n6, C20:5n3, and n-3 fatty acids, although not significantly compared to the control or T2 in selected fatty acids (Table 7.2). In contrast, the C18:3n6 and C20:3n3 fatty acid content did not differ significantly due to honeybush treatment (Table 7.2). Treatment 4 had the highest n-6/n-3 ratio, due to the lowest n-3 PUFA content (4.19 mg/g of fat), although not significantly higher than Treatment 3 (Table 7.2). Although the effect of honeybush appeared to have significantly affected C20:3n3 (Table 7.1, p= 0.04), further analysis revealed that treatments did not differ significantly therein (Table 7.2). The total fatty acid composition of the 40-day ripened salami are shown in Addendum C, Table 7.7.

Table 7.1 Level of statistical significance (p-values) for the effect of honeybush (*Cyclopia subternata*) treatment on the total fatty acid methyl ester (FAME) composition of 40-day ripened warthog salami

Fatty acid	Common name	Systematic name	Treatment
Saturated fatty acids (SFAs)			
C12:0	Lauric acid	Dodecanoic acid	**< 0.01**[a]
C13:0	Tridecylic acid	Tridecanoic acid	0.11[a]
C14:0	Myristic acid	Tetradecanoic acid	0.27[a]
C15:0	Pentadecylic acid	Pentadecanoic acid	0.06[a]
C16:0	Palmitic acid	Hexadecanoic acid	0.15[a]
C18:0	Stearic acid	Octadecanoic acid	0.58[b]
C20:0	Arachidic acid	Eicosanoic acid	0.25[b]
Monounsaturated fatty acids (MUFAs)			
C14:1	Myristoleic acid	*cis*-Tetradec-9-enoic acid	**0.03**[b]
C16:1	Palmitoleic acid	*cis*-Hexadec-9-enoic acid	0.08[a]
C18:1n9*c*	Oleic acid	*cis*-Octadec-9-enoic acid	0.12[a]
C20:1	Gondoic acid	*cis*-Eicos-11-enoic acid	**0.03**[b]
C22:1n9	Erucic acid	*cis*-Tetracos-15-enoic acid	0.09[b]
Polyunsaturated fatty acids (PUFAs)			
C18:2n6*c*	Linoleic acid	*all-cis*-9,12-octadecadienoic acid	0.20[a]
C18:3n6	Gamma-linolenic acid	*all-cis*-6,9,12-octadecatrienoic acid	**< 0.01**[a]
C18:3n3	Alpha-linolenic acid	*all-cis*-9,12,15-octadecatrienoic acid	**< 0.01**[b]
C20:2n6	Eicosadienoic acid	*all-cis*-11,14-eicosadienoic acid	0.08[a]
C20:3n6	Dihomo-gamma-linolenic acid	*all-cis*-8,11,14-eicosatrienoic acid	0.14[b]
C20:3n3	Eicosatrienoic acid	*all-cis*-11,14,17-eicosatrienoic acid	**0.04**[a]
C20:4n6	Arachidonic acid	*all-cis*-5,8,11,14-eicosatetraenoic acid	**< 0.01**[b]
C20:5n3	Eiscosapentaenoic acid	all-cis-5,8,11,14,17-eicosapentaenoic acid	**< 0.01**[b]
Fatty acid totals			
SFA	Saturated fatty acids		0.43[b]

Table 7.1 continued

Fatty acid totals	Common name	Treatment
MUFA	Monounsaturated fatty acids	0.12[a]
PUFA	Polyunsaturated fatty acids	0.11[a]
n-6	Omega-6 fatty acids	0.16[a]
n-3	Omega-3 fatty acids	**< 0.01[b]**
Fatty acid ratios		
PUFA:SFA	Polyunsaturated to saturated fatty acids	0.20[b]
(*n*-6)/(*n*-3)	Omega-6 to omega-3 polyunsaturated fatty acids	**0.05[b]**

[a]p-values were calculated using the Welch test in combination with the Games-Howell post hoc test; [b]p-values were calculated using the F-test in combination with Fisher's least significant difference (LSD) post hoc test. $p > 0.05$= not significant, $p \leq 0.05$= significant; $p \leq 0.01$= significant; SFA= sum of C12:0, C13:0, C14:0, C15:0, C16:0, C18:0 and C20:0; MUFA= sum of C14:1, C16:1, C18:1n9 cis, C20:1 and C22:1n9; PUFA= sum of C18:2n6c, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:3n3, C20:4n6 and C20:5n3; PUFA:SFA ratio= [(sum of C18:2n6c, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:3n3, C20:4n6 and C20:5n3)/(sum of C12:0, C13:0, C14:0, C15:0, C16:0, C18:0 and C20:0)]; *n*-6:*n*-3 ratio= [(sum of C18:2n6c, C18:3n6, C20:2n6, C20:3n6 and C20:4n6)/(sum of C18:3n3, C20:3n3 and C20:5n3)].

Table 7.2 The influence of different levels of honeybush (*Cyclopia subternata*) on the total fatty acid methyl ester (FAME) composition (mg of FAME/g fat, Means ± SD) of warthog salami (n= 6) after a 40-day ripening period

Fatty acid	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Significance
C12:0	0.53[ab] ± 0.21	0.73[a] ± 0.22	0.33[ab] ± 0.10	0.22[b] ± 0.05	0.25[b] ± 0.09	**
C14:1	0.07[ab] ± 0.05	0.12[a] ± 0.04	0.05[b] ± 0.04	0.06[b] ± 0.01	0.04[b] ± 0.04	*
C20:1	1.99[ab] ± 0.67	2.32[a] ± 0.84	1.31[c] ± 0.41	1.48[bc] ± 0.32	1.31[bc] ± 0.38	*
C18:3n6	0.37[a] ± 10	0.45[ab] ± 0.15	0.28[ab] ± 0.07	0.20[b] ± 02	0.23[ab] ± 0.06	**
C18:3n3	5.76[ab] ± 2.39	7.47[a] ± 3.41	3.41[bc] ± 1.23	3.40[bc] ± 1.31	2.67[c] ± 1.99	**
C20:3n3	1.14[a] ± 0.47	1.53[a] ± 0.63	0.81[a] ± 0.29	0.70[a] ± 0.13	0.50[a] ± 0.25	**
C20:4n6	0.25[a] ± 0.10	0.31[a] ± 0.11	0.15[b] ± 0.05	0.15[b] ± 0.03	0.12[b] ± 0.05	**
C20:5n3	0.19[ab] ± 0.07	0.25[a] ± 0.10	0.13[bc] ± 0.05	0.09[c] ± 0.02	0.10[c] ± 0.04	**
n-3	7.10[ab] ± 2.88	9.24[a] ± 3.99	4.35[bc] ± 1.50	4.20[bc] ± 1.41	3.27[c] ± 2.21	**
n-6/*n*-3	2.88[b] ± 0.71	2.65[b] ± 0.52	3.14[b] ± 0.62	3.67[ab] ± 0.89	4.19[a] ± 1.29	*

[a-c] Different superscripts in the same row indicate significant differences between treatments; *= significant ($p \leq 0.05$); **= significant ($p \leq 0.01$); n= 6 (including all replications 1-6, per treatment); SD= standard deviation; C= 0%, Treatment 1= 0.125%, Treatment 2= 0.25%, Treatment 3= 0.375%, and Treatment 4= 0.5% (w/w) of added honeybush.

7.3.2 Volatile organic compound (VOC) profile

A total of 47 VOCs were detected which were mainly represented by alcohols (12) and ketones (11), followed by aldehydes (7), others (6), organic acids (4), pyrazines (3), hydrocarbons (2), and furans (2). Table 7.3 shows the statistical significant differences (p-values) of the influence of honeybush on the total VOCs detected in the raw salami batter (warthog meat and pork fat, day-0) as well as the final product 40-day ripened salami. In the case of the Levene's test for homogeneity being significant ($p < 0.01$), the p-values of selected VOCs were calculated using the Games-Howell post hoc test (Table 7.4). Addendum C, Table 7.8 compares the significance of selected VOCs between treatments (i.e. there was a significant difference in 2-butanone between the raw meat and fat (MF) and T4 as seen in column 6 and row 1, $p < 0.001$).

The total VOCs increased over the fermentation and ripening period, portrayed by the greater amount of VOCs detected in 40-day ripened salami (Table 7.3). Of the total VOCs (47), only 23 compounds were detected in the raw meat and fat. On the other hand, the latter contained certain volatiles not detected in the ripened salami, including: 2-pentanol; 1-butanol; heptanal; and butanoic (butyric) acid), as seen in Table 7.3. Of these, the meat and fat only contained a significantly higher amount of 2-pentanol (p= 0.011) and butanoic acid (p= 0.048) compared to the ripened salami, Table 7.3.

Treatment had a significant effect on selected ketones (2-butanone, 2-heptanone, 2,3-octanedione and 2-nonanone), alcohols (1-butanol-3-methyl, 1-pentanol, 4-methyl-1-pentanol, 1-octen-3-ol, terpinen-4-ol and benzeneethanol), aldehydes (hexanal and nonanal), n-undecane, 2,5-dimethylpyrazine, acids (methyl-2-hydroxy-4-methylpentanoate and 3-methylbutanoic acid), furans (2-pentylfuran and 2,3-dihydrobenzofuran) and others ((R/S)-linalool, α-terpineol and 1,2-dimethoxybenzene) as seen in Table 7.3 and Table 7.4. The affected VOCs are expressed in semi-quantitative amounts as the average ratios (Mean ± SD) between the volatile compounds and the internal standard (3-octanol) peak areas in Table 7.4 and grouped according to possible origin and odour descriptions in Table 7.5.

Treatment significantly increased 2-butanone with T4 containing a highly significant amount more than the C ($p < 0.001$) and T2 (0.003), Table 7.4. The effect of honeybush addition on benzeneethanol content was evident from a 0.375% addition (Treatment 3, p= 0.03, Table 7.4). Treatment 4 also had a significantly higher amount of 2,5-dimethylpyrazine compared to T3 (p= 0.020) and the MF (0.020), Table 7.4. Certain acids (methyl-2-hydroxy-4-methylpentanoate and 3-methylbutanoic acid) increased with increasing honeybush addition, with T4 resulting in significantly higher amounts of the former (p= 0.04) and T2-T4 of the latter ($p < 0.01$) compared to the C and MF. Furthermore, terpinen-4-ol and 2,3-dihydrobenzofuran were only detected in T2-T4. (R/S)-Linalool and α-terpineol increased with honeybush addition, with T4 resulting in the highest content of the former ($p < 0.01$), although comparable to T3 and T3 resulting in the highest content of the latter, although comparable to T4. Treatment 4 also contained a significantly higher amount

of terpinene-4-ol compared to the MF, C and T1 (p= 0.004, Table 7.4). Treatment 3 had the highest 2-heptanone and 2-nonanone (p= 0.02) content, although not significantly so compared to T1 and T2. In contrast, the 2,3-octanedione, 1-pentanol, hexanal, 2-pentylfuran and undecane content decreased as honeybush increased (Table 7.4). The C had the highest 1-octen-3-ol (p < 0.01), 4-methyl-1-pentanol (p= 0.02) and nonanal (p= 0.04) content, although the latter two was comparable to T2 and T1, respectively. Furthermore, although a slight indication, the C was also higher in n-undecane compared to T3 (p= 0.047, Table 7.4).

On the other hand, honeybush did not affect the 2,3-heptanedione, 1-butanol-3-methyl, 2,6-dimethylpyrazine, 2,5,3-trimethylpyrazine, 1,2-dimethoxybenzene, 1,3-dimethoxybenzene and butanoic (butyric) acid content between treatments (C, T1-T4). These VOCs rather increased in the ripened salami (irrespective of treatment) due to fermentation and/or ripening. The C (p= 0.045), T3 (p= 0.011) and T4 (p= 0.011) significantly increased in 2,3-heptanedione compared to the MF, Table 7.4. All treatments had a significantly higher amount of 1-butanol-3-methyl (p < 0.01) compared to the MF, Table 7.4. The C (p= 0.006) and T4 (p= 0.026) had a much higher 2,6-dimethylpyrazine content compared to the MF. Furthermore, the C (p < 0.001), T3 (0.032) and T4 (0.005) had a much higher 2,5,3-trimethylpyrazine content compared to the MF. Treatment 4 contained a significantly higher amount of 1,2-dimethoxybenzene (p= 0.009, Addendum C, Table 7.8) compared to the MF, although similar to the other treatments (C, T1-T3). Treatments (C, T1-T3) increased in 1,3-dimethoxybenzene (p= 0.03) compared to the MF, although T4 was comparable to the latter (Table 7.4).

Table 7.3 Level of statistical significance (p-values) for the effect of honeybush (*Cyclopia subternata*) treatment on the total volatile organic compound (VOCs) of the raw salami batter (warthog meat and pork fat, day-0) and final product 40-day ripened salami

Retention time (min)	VOC	Treatment						p-value
		MF	C	T1	T2	T3	T4	
		Day						
		0	40	40	40	40	40	
	Ketones (11)							
5.42	2-Butanone	✗	✗	✓	✓	✓	✓	A
7.35	3-Methyl-2-butanone	✓	✓	✓	✓	✓	✓	0.10[b]
8.48	3-Methyl-2-pentanone	✗	✓	✓	✓	✓	✓	0.20[b]
9.85	2,3-Pentanedione	✓	✓	✓	✓	✗	✗	0.07[b]
12.73	2,3-Heptanedione	✗	✓	✓	✓	✓	✓	A
13.82	2-Heptanone	✗	✓	✓	✓	✓	✓	**0.01[b]**
16.10	3-Octanone	✓	✓	✓	✓	✓	✓	0.07[b]
17.15	Acetoin	✓	✓	✓	✓	✓	✓	0.26[b]
18.14	2,3-Octanedione	✓	✓	✓	✓	✓	✓	**0.04[b]**
18.67	2-Acetyl-1-pyrroline	✗	✓	✓	✓	✓	✓	0.07[b]
20.06	2-Nonanone	✗	✓	✓	✓	✓	✓	**0.02[b]**
	Alcohols (12)							
14.93	1-Butanol-3-methyl (isoamyl alcohol)	✓	✓	✓	✓	✓	✓	**< 0.01[b]**
12.28	2-Pentanol	✓	✗	✗	✗	✗	✗	**0.011[a]**
13.00	1-Butanol	✓	✗	✗	✗	✗	✗	0.069[a]

Table 7.3 continued

Retention time (min)	Alcohols	MF	C	T1	T2	T3	T4	p-value
				Day				
		0	40	40	40	40	40	p-value
16.18	1-Pentanol	✓	✓	✓	✓	✓	✓	A
18.21	2-Heptanol	✗	✓	✗	✓	✓	✓	> 0.05[a]
19.17	1-Pentanol-4-methyl	✓	✓	✓	✓	✓	✓	**0.02[b]**
19.27	2-Propanol	✓	✗	✗	✗	✓	✗	> 0.05[a]
21.75	1-Octen-3-ol	✓	✓	✓	✓	✓	✓	**< 0.01[b]**
24.57	1-Octanol	✓	✓	✓	✓	✓	✓	0.16[b]
25.72	Terpinen-4-ol	✗	✗	✗	✓	✓	✓	A
31.88	Benzyl alcohol	✓	✓	✓	✓	✓	✓	> 0.05[a]
32.63	Benzeneethanol	✗	✓	✓	✓	✓	✓	**0.03[b]**
	Aldehydes (7)							
5.77	3-Methylbutanal	✗	✓	✓	✓	✓	✓	0.10[b]
10.54	Hexanal	✓	✓	✓	✓	✓	✗	**< 0.01[b]**
13.86	Heptanal	✓	✗	✗	✗	✗	✗	> 0.05[a]
20.17	Nonanal	✓	✓	✓	✓	✓	✓	**0.04[b]**
23.65	Benzaldehyde	✓	✓	✓	✓	✓	✓	0.09[b]
26.57	Benzeneacetaldehyde	✗	✓	✓	✓	✓	✓	> 0.05[a]
33.04	2-Phenyl-2-butenal	✗	✗	✗	✗	✓	✓	> 0.05[a]

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Table 7.3 continued

Retention time (min)		Treatment						p-value
		MF	C	T1	T2	T3	T4	
		Day						
		0	40	40	40	40	40	
	Hydrocarbons (2)							
14.1	l-Limonene	✓	✕	✓	✓	✓	✓	> 0.05[a]
17.36	n-Undecane	✕	✓	✓	✓	✕	✓	A
	Pyrazines (3)							
18.30	2,5-Dimethylpyrazine	✕	✓	✓	✓	✕	✓	A
18.46	2,6-Dimethylpyrazine	✕	✓	✓	✓	✓	✓	A
20.64	2,3,5-Trimethylpyrazine	✕	✓	✓	✓	✓	✓	A
	Organic acids (4)							
21.95	Acetic acid	✓	✓	✓	✓	✓	✓	0.19[b]
22.39	Methyl-2-hydroxy-4-methylpentanoate	✕	✓	✓	✓	✓	✓	**0.04[b]**
26.35	Butanoic (butyric) acid	✓	✕	✕	✕	✕	✕	**0.048[a]**
27.42	3-Methylbutanoic acid	✓	✓	✓	✓	✓	✓	**< 0.01[b]**
	Furans (2)							
15.29	2-Pentylfuran	✓	✓	✓	✕	✕	✕	A
41.59	2,3-Dihydrobenzofuran	✕	✕	✕	✓	✓	✓	A
	Others (6)							
13.43	α-Terpinene	✕	✕	✕	✕	✕	✓	> 0.05[a]
16.46	Cymene	✕	✕	✕	✕	✓	✓	> 0.05[a]
24.24	(*R/S*)-Linalool	✕	✓	✓	✓	✓	✓	**< 0.01[b]**

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Table 7.3 continued

Retention time (min)	*Others*	MF	C	T1	T2	T3	T4	p-value
		Treatment						
		0	40	40	40	40	40	
		Day						
27.96	α-Terpineol	✗	✗	✓	✓	✓	✓	A
28.45	1,2-Dimethoxybenzene	✗	✓	✓	✓	✓	✓	A
29.04	1,3-Dimethoxybenzene	✗	✓	✓	✓	✓	✓	**0.03**[b]

✓= present; ✗= under detection limit or absent; MF= [a] In the case of the Levene's test being highly significant, the p-values were calculated using Games-Howell post hoc test; [b] In the case of the Levene's test being insignificant, the p-values were calculated using the F-test; A= see Addendum C, Table 7.8 for the respective p-values; $p > 0.05$= not significant; $p \leq 0.05$= significant; $p \leq 0.01$= significant; MF= raw warthog meat and pork fat without honeybush (day-0); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, and T4= 0.5% of added honeybush after a 40-day ripening period.

Table 7.4 Total volatile organic compounds (VOCs) detected in raw warthog salami batter (meat and fat, day-0) and with different levels of honeybush (*Cyclopia subternata*) after a 40-day ripening period, expressed as the average ratios (Means ± SD) between the volatile and the internal standard (3-octanol) peak areas

VOC	Treatment	MF	C	T1	T2	T3	T4	p-value
	Day	0	40	40	40	40	40	
Ketones								
2-Butanone		n.d.	n.d.	0.23 ± 0.33	0.21 ± 0.15	0.68 ± 0.32	0.65 ± 0.11	A
2,3-Heptanedione		n.d.	0.08 ± 0.05	0.16 ± 0.23	0.04 ± 0.10	0.15 ± 0.06	0.16 ± 0.06	A
2-Heptanone		n.d.[c]	0.46 ± 0.19[b]	0.42 ± 0.24[abc]	0.56 ± 0.40[ab]	0.85 ± 0.54[a]	0.34 ± 0.20[bc]	**0.01**[B]
2,3-Octanedione		0.15 ± 0.04[ab]	0.22 ± 0.23[a]	0.11 ± 0.10[ab]	0.12 ± 0.08[ab]	0.03 ± 0.03[b]	0.01 ± 0.02[b]	**0.04**[B]
2-Nonanone		n.d.[c]	0.43 ± 0.32[bc]	0.38 ± 0.18[bc]	0.73 ± 0.63[ab]	1.11 ± 0.66[a]	0.56 ± 0.29[bc]	**0.02**[B]
Alcohols								
1-Butanol-3-methyl (isoamyl alcohol)		0.02 ± 0.02[b]	0.82 ± 0.24[a]	0.88 ± 0.28[a]	0.87 ± 0.21[a]	1.08 ± 0.45[a]	0.89 ± 0.27[a]	**< 0.01**[B]
2-Pentanol		0.10 ± 0.02[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	0.11
1-Pentanol		0.57 ± 0.15	0.09 ± 0.09	0.09 ± 0.02	0.08 ± 0.02	0.01 ± 0.02	0.02 ± 0.02	A
1-Pentanol-4-methyl		0.07 ± 0.02[bc]	0.14 ± 0.06[a]	0.08 ± 0.02[bc]	0.10 ± 0.06[ab]	0.07 ± 0.03[bc]	0.05 ± 0.02[c]	**0.02**[B]
1-Octen-3-ol		0.14 ± 0.04[bc]	0.32 ± 0.12[a]	0.13 ± 0.04[bc]	0.20 ± 0.10[b]	0.11 ± 0.04[c]	0.08 ± 0.03[c]	**< 0.01**[B]
Terpinen-4-ol		n.d.	n.d.	n.d.	0.04 ± 0.03	0.06 ± 0.04	0.06 ± 0.02	A
Benzeneethanol		n.d.[c]	0.10 ± 0.08[c]	0.14 ± 0.09[bc]	0.85 ± 0.99[abc]	1.63 ± 1.51[a]	1.32 ± 1.12[ab]	**0.03**[B]
Aldehydes								
Hexanal		1.32 ± 0.38[a]	0.67 ± 0.75[b]	0.19 ± 0.10[c]	0.16 ± 0.10[c]	0.06 ± 0.06[c]	n.d.[c]	**< 0.01**[B]
Nonanal		0.08 ± 0.03[b]	0.19 ± 0.10[a]	0.14 ± 0.08[ab]	0.11 ± 0.05[b]	0.11 ± 0.06[b]	0.06 ± 0.04[b]	**0.04**[B]
Hydrocarbons								
n-Undecane		n.d.	0.13 ± 0.07	0.07 ± 0.08	0.03 ± 0.03	n.d.	0.01 ± 0.03	A

Table 7.4 continued

VOC	MF	C	T1	T2	T3	T4	p-value
	0	40	40	40	40	40	
Pyrazines							
2,5-Dimethylpyrazine	n.d.	0.10 ± 0.12	0.04 ± 0.07	0.02 ± 0.06	n.d.	0.08 ± 0.04	A
2,6-Dimethylpyrazine	n.d.	0.12 ± 0.04	0.03 ± 0.06	0.04 ± 0.05	0.07 ± 0.07	0.08 ± 0.04	A
2,3,5-Trimethylpyrazine	n.d.	0.09 ± 0.01	0.07 ± 0.03	0.07 ± 0.05	0.09 ± 0.05	0.07 ± 0.02	A
Organic acids							
Pentanoicacid-2-hydroxy-4-methyl-methylester	n.d.[c]	0.20 ± 0.17[bc]	0.73 ± 1.03[abc]	0.63 ± 0.63[abc]	0.77 ± 0.51[ab]	1.15 ± 0.54[a]	**0.04**[B]
Butanoic (butyric) acid	0.46 ± 0.16[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	**0.048**
3-Methylbutanoic acid	0.11 ± 0.03[c]	0.24 ± 0.11[c]	0.35 ± 0.06[bc]	0.69 ± 0.36[ab]	0.81 ± 0.44[a]	0.83 ± 0.35[a]	**< 0.01**[B]
Furans							
2-Pentylfuran	0.008 ± 0.009	0.08 ± 0.03	0.01 ± 0.02	n.d.	n.d.	n.d.	A
2,3-Dihydrobenzofuran	n.d.	n.d.	n.d.	0.06 ± 0.03	0.12 ± 0.06	0.11 ± 0.03	A
Others							
(*R/S*)-Linalool	n.d.[d]	0.07 ± 0.06[d]	0.29 ± 0.09[cd]	0.57 ± 0.20[bc]	0.77 ± 0.29[ab]	0.95 ± 0.43[a]	**< 0.01**[B]
α-Terpineol	n.d.	n.d.	0.07 ± 0.02	0.17 ± 0.05	0.28 ± 0.12	0.26 ± 0.09	A
1,2-Dimethoxybenzene	n.d.	0.04 ± 0.03	0.02 ± 0.03	0.10 ± 0.10	0.09 ± 0.06	0.09 ± 0.03	A
1,3-Dimethoxybenzene	n.d.[b]	0.13 ± 0.07[a]	0.15 ± 0.14[a]	0.19 ± 0.09[a]	0.16 ± 0.08[a]	0.10 ± 0.07[ab]	**0.03**[B]

n.d.= not detected or absent; [A] In the case of the Levene's test being highly significant, the p-values were calculated using Games-Howell post hoc test, see Table 7.4; [B] In the case of the Levene's test being insignificant, the p-values were calculated using the F-test; [a-d] Different superscripts in the same row indicate significant differences between treatments; p > 0.05= not significant; p ≤ 0.05= significant; p ≤ 0.01= significant; SD= standard deviation; MF= raw warthog meat and pork fat without honeybush (day-0); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, and T4= 0.5% of added honeybush after a 40-day ripening period.

Table 7.5 The retention time, Kováts Index and odour descriptions of volatile compounds detected in warthog salami with different levels of honeybush (*Cyclopia subternata*) after a 40-day ripening period, grouped according to possible origin

Compound	KI[1]	Odour descriptions	Previously detected
Lipid autoxidation			
Hexanal*	1075.55	Grassy, rancid, woody, fatty[e, l, y, z]	a, b, c, e, g, h, i, j, k, l, n, o, u, v, w
2-Pentylfuran*	1224.51	Onions, savoury, rancid[e], butter, green beans[z]	a, c, e, g, i, l, n, o, u, v, aa
1-Pentanol*	1253.65	Roasted meat[e,], pungent[y]	a, b, c, e, i, k, n, o, u, v
n-Undecane	1293.02	Pork, beef, chicken[f]	a, g, m, n, o, aa
Nonanal*	1391.96	Plastic, soap[e], citrus, malty[l], fatty, paint[y], green, tallowy[z]	a, b, c, e, l, j, l, n, o, v, w
Pentanoicacid-2-hydroxy-4-methyl-methylester	1475.95	-	aa
Bacterial metabolism			
Lipid-ß-oxidation			
2,3-Octanedione	1320.64	-	f, i, j, n, o
2-Nonanone*	1387.90	Roasted, burnt[e]	e, i, o, v, aa
1-Octen-3-ol*	1451.53	Mushroom[e, h]	a, b, c, e, h, i, n, o, u, v, w
2-Heptanone*	1177.40	Medicinal, fruity[e, l]	a, c, e, l, n, o, u, v
Carbohydrate fermentation			
1-Pentanol-4-methyl	1356.44	-	x
Butanoic (butyric) acid	1636.36	Cheesy[e,p]	e, k, n, o
Amino acid degradation			
2-Butanone	878.31	Apricot notes[d], pleasant[y]	a, b, c, d, j, k, n, o
1-Butanol-3-methyl (isoamyl alcohol)	1211.51	Whiskey, malt[d]	c, d, i, n
2,5-Dimethylpyrazine	1325.27	-	a, p
2,6-Dimethylpyrazine	1330.96	Mashed potatoes[h]	h, n, o, q
3-Methylbutanoic acid*	1681.78	Cheese, feet[e]	e, k, n, u, w
Unknown			
2-Pentanol	1129.85	-	s
2,3-Heptanedione	1143.65	-	
2,3,5-Trimethylpyrazine	1409.16	-	f
(*R/S*)-Linalool*	1549.19	Floral, spice, wood, refreshing[r, w]	f, s, t, u, v, w
Terpinen-4-ol*	1609.32	Earthy, spicy[l]	l, s, u, v
α-Terpineol*	1704.24	Floral, sweet[w]	f, h, u, v, w

Table 7.5 continued

Compound	KI[1]	Odour descriptions	Previously detected
Benzene ethanol	-	-	
1,2-Dimethoxybenzene	-	-	
1,3-Dimethoxybenzene	-	-	
2,3-Dihydrobenzofuran	-	-	

[1]Kováts Index calculated for a Stabilwax column (L= 60 m, ID= 0.25 mm, film thickness= 0.25 μm); [a] (Corral *et al.*, 2013); [b] (Tabanelli *et al.*, 2012); [c] (Olivares *et al.*, 2009a); [d] (Olivares *et al.*, 2009b); [e] (Marco *et al.*, 2007); [f] (Shahidi *et al.*, 1986); [g] (Lorenzo *et al.*, 2013); [h] (Meynier *et al.*, 1999); [i] (Hospital *et al.*, 2012); [j] (Montanari *et al.*, 2018); [k] (Montanari *et al.*, 2016); [l] (Moretti *et al.*, 2004); [m] (Sunesen *et al.*, 2001); [n] (Søndergaard & Stahnke, 2002); [o] (Olivares *et al.*, 2011); [p] (Corral *et al.*, 2014); [q] (Sales & Kotrba, 2013); [r] (Furia, 1980); [s] (Berger *et al.*, 1990) [t] (Bianchi *et al.*, 2007); [u] *detected in *Cyclopia subternata* (Le Roux *et al.*, 2012); [v] *(Ntlhokwe *et al.*, 2018); [w] (Theron *et al.*, 2014); [x] (Rotsatchakul *et al.*, 2009); [y] (Morales *et al.*, 1997); [z] (Xu *et al.*, 2018); [aa] (Lorenzo *et al.*, 2013).

7.4 Discussion

The diet of monogastric animals directly influence the fatty acid composition of the meat, therefore the salami fatty acid content resembles that of the warthog and pig diets (Morel *et al.*, 2006; Herranz *et al.*, 2008; Carrapiso *et al.*, 2020). Warthogs are predominantly grazers and their nutrient intake depended on the rainfall influencing the nutrient content of available grass according to the region in Limpopo where the animals in this study originated from (Treydte *et al.*, 2006). In contrast, domestic pigs received a diet mixed with maize, soya, and canola. The most abundant PUFA in grasses and maize is C18:3n3 and C18:2n6*c* respectively, explaining why the final product salami contained the highest amount of these PUFAs (Addendum C, Table 7.7) ascribable to the diets of both the warthogs and the domestic pigs (Khan *et al.*, 2012; Marino *et al.*, 2015). Furthermore, the C18:3n3 content of T4 (2.67 mg/g fat= ~2.81%, Table 7.2) was similar to final product warthog cabanossi with 30% pork back fat (~2.40%) (Mahachi *et al.*, 2019), however, higher compared to warthog cabanossi without pork fat addition (1.01 mg/g fat), (Swanepoel *et al*., 2016a).

The most abundant fatty acid in the final product salami was oleic acid (C18:1n9*c,* Addendum C, Table 7.7), which is in accordance with European and American pork salami analysed by previous researchers (Muguerza *et al.*, 2001; Moretti *et al.*, 2004; Herranz *et al.*, 2008). Warthog cabanossi (without pork fat) also contained the highest amount of this fatty acid (Swanepoel *et al.*, 2016a). The total SFA content (Addendum C, Table 7.7, 65.15-89.88 mg/g fat, ~48-59%) was much higher compared to European (~37-41%) and American style pork salami (~36-44%, Herranz *et al.*, 2008), however, comparable to pork cabanossi (~63 mg/g SFA, Swanepoel *et al.*, 2016a). With regard to the MUFAs, the present trial salami (Addendum C, Table 7.7, 44.09-84.32 mg/g fat, ~31-41%) had a lower total MUFA content compared to European (~46-51%) and American pork salami (~42-48%) (Herranz *et al.*, 2008), although much higher compared to warthog cabanossi (13.8 and 20.02 mg/g of fat, Swanepoel *et al.*, 2016*a*). In contrast, the PUFA

content of the present trial salami (Addendum C, Table 7.7, 14.78-33.24 mg/g fat, ~10-16%) compared well to European pork salami (11.5-14.6%), American pork salami (10.92-19.89%) warthog and pork cabanossi (10-35 mg/g of fat) (Herranz *et al.*, 2008; Swanepoel *et al.*, 2016a). The *n*-6/*n*-3 ratio (Table 7.2) was much lower compared to European (9.72-14.64) and American (7.26-15.87) pork salami (Herranz *et al.*, 2008), but comparable to warthog (*n*-6/*n*-3= 2.4) and pork (*n*-6/*n*-3= 2.8) cabanossi (Swanepoel *et al.*, 2016a).

The volatile profile of the present trial 40-day ripened salami resembled that of fermented sausages studied by previous researchers, who also reported (although variable) alcohols, ketones and aldehydes as the dominating groups (Montel *et al.*, 1996; Di Cagno *et al.*, 2008; Montanari *et al.*, 2016). The majority of the VOCs detected in the present study are commonly detected in dry-cured and fermented sausages, as indicated in Table 7.5. Certain VOCs detected in the salami are associated with mould metabolism, including 1-octen-3-ol (Bruna *et al.*, 2001; Sunesen & Stahnke, 2003). In this regard, the control salami had a significantly higher content of 1-octen-3-ol (p < 0.01, Table 7.4), which correlates with the high pH of the C salami (Chapter 5) portraying the metabolism of the surface moulds known for lactic acid utilisation and rising of salami pH (Sunesen & Stahnke, 2003).

Lipid oxidation in dry-cured and fermented sausages can be evaluated by assessing the formation of certain oxidation by-products by means of GC-MS. In this sense, hexanal is frequently studied to monitor oxidation in foodstuffs, including in dry-cured and fermented sausages (Muguerza *et al.*, 2001; Gøtterup *et al.,* 2008; Elisia & Kitts, 2011; Fuentes *et al.*, 2014). According to Shahihi and Pegg (1994), the presence of hexanal can serve as a meat flavour deterioration index. Similarly, salami flavour deterioration was correlated with an increase in heptanal, nonane, undecane and hexanal (Lorenzo *et al.*, 2013). Lipid oxidation by-products mainly originate from unsaturated fatty acids. In this regard, the oxidation of linoleic acid (C18:2n6) leads to the accumulation of hexanal, 1-pentanol, 2-pentylfuran and 1-octen-3-ol and the oxidation of oleic acid (C18:1n9) to octanal and nonanal (Morales *et al.*, 1997; Elisia & Kitts, 2011; Yang *et al.*, 2017; Xu *et al.*, 2018). These compounds contribute significantly to off-flavours, having low odour thresholds easily detected by humans (Morales *et al.*, 1997; Xu *et al.*, 2018). Linoleic and oleic acid are thus expected to decrease with the increase of lipid oxidation over time. However, honeybush had no effect on linoleic or oleic acid content after 40 ripening days (Table 7.1), however, a significant decrease in their oxidation by-products was found as honeybush increased (Table 7.4). In contrast, previous researchers found the highest concentrations of alpha linolenic (C18:3n3) and linoleic acids in salami on 42-day and 63-day shelf life, which corresponded to the highest amounts of oxidation products originating from these fatty acids (Olivares *et al.*, 2011).

From the semi-quantification of the VOCs, one can interpret that honeybush supressed oxidation with a decrease in rancid compounds compared to the control salami, including: hexanal; nonanal; 1-pentanol; 1-octen-3-ol; 2-pentylfyran; and undecane (Table 7.4). Of the former

compounds, it is noteworthy to highlight that all the honeybush treated salami (T1-T4) contained significantly less hexanal and 1-octen-3-ol (p < 0.01) compared to the control salami (Table 7.4). Lipid oxidation contributes majorly to quality deterioration of dry-cured and fermented meats and limits the shelf life thereof (Ruiz, 2007). Since hexanal is associated with rancidity (Table 7.5), the delay of excessive hexanal formation could portray the extension of salami shelf life.

In contrast to the decline in rancid aromas, 2-butanone imparting a pleasant sausage aroma significantly increased in T4 compared to the control salami (p < 0.001, Addendum C, Table 7.8). Honeybush might have also contributed uniquely to the 40-day ripening salami in terms of higher (*R/S*)-linalool (p < 0.01, Table 7.4), *α*-terpineol (p= 0.005, Addendum C, Table 7.8) and terpinen-4-ol (p= 0.004, Addendum C, Table 7.8) contents compared to the control salami. Furthermore, although not significant, it is noteworthy to mention that only T3 and T4 salami contained detectable amounts of cymene (Table 7.3). (*R/S*)-Linalool is associated with a refreshing floral, spicy and woody aroma, *α*-terpineol with a floral and sweet aroma and terpinen-4-ol with earthy and spicy aromas (Table 7.5). All four these before-mentioned compounds have been detected in *Cyclopia subternata* (amongst other VOCs also present in the salami, Table 7.5).

7.5 Conclusion

According to the semi-quantification technique (GC-MS) followed to study the VOCs of 40-day ripened warthog salami with different honeybush levels, the honeybush supressed the development of rancidity. This is substantiated by the lower amount of VOCs associated with rancidity (originating mainly from linoleic and oleic fatty acids) in salami treated with honeybush as compared to the control salami (including: hexanal; nonanal; 1-pentanol; 1-octen-3-ol; 2-pentylfyran; and undecane). Of all the rancid VOCs, salami with 0.125%, 0.25%, 0.375% and 0.5% honeybush extract inclusion contained significantly lower amounts of hexanal (p < 0.01) and 1-octen-3-ol (p < 0.01) compared to the control with no honeybush addition. To better understand the effect of honeybush on oxidation reactions in a meat model, it is recommended to perform more than one analysis (i.e. peroxide value or thiobarbituric acid reactive substances TBARS test together with GC-MS) over time.

7.6 References

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

Aquilani, C., Sirtori, F., Flores, M., Bozzi, R., Lebret, B. & Pugliese, C. (2018). Effect of natural antioxidants from grape seed and chestnut in combination with hydroxytyrosol, as sodium nitrite substitutes in Cinta Senese dry-fermented sausages. *Meat Science*, **145**, 389–398.

Aziz, M. & Karboune, S. (2018). Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, **58**, 486–511.

Berger, R.G., Macku, C., German, J.B. & Shibamoto, T. (1990). Isolation and identification of dry salami volatiles. *Journal of Food Science*, **55**, 1239–1242.

Bianchi, F., Cantoni, C., Careri, M., Chiesa, L., Musci, M. & Pinna, A. (2007). Characterization of the aromatic profile for the authentication and differentiation of typical Italian dry-sausages. *Talanta*, **72**, 1552–1563.

Bolger, Z., Brunton, N.P., Lyng, J.G. & Monahan, F.J. (2017). Comminuted meat products - Consumption, composition, and approaches to healthier formulations. *Food Reviews International*, **33**, 143–166.

Bruna, J.M., Hierro, E.M., De la Hoz, L., Mottram, D.S., Fernández, M. & Ordóñez, J.A. (2001). The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. *Meat Science*, **59**, 97–107.

Carrapiso, A.I., Tejeda, J.F., Noguera, J.L., Ibáñez-Escriche, N. & Ganzález, E. (2020). Effect of the genetic line and oleic acid-enriched mixed diets on the subcutaneous fatty acid composition and sensory characteristics of dry-cured shoulders from Iberian pig. *Meat Science*, **159**, 1-6.

Chakanya, C., Arnaud, E., Muchenje, V. & Hoffman, L.C. (2018). Fermented meat sausages from game and venison: What are the opportunities and limitations? *Journal of the Science of Food and Agricultu*re, doi: 10.1002/jsfa.9053.

Chen, J., Hu, Y., Wen, R., Liu, Q., Chen, Q. & Kong, B. (2019). Effect of NaCl substitutes on the physical, microbial and sensory characteristics of Harbin dry sausage. *Meat Science*, **156**, 205–213.

Chen, Q., Kong, B., Han, Q., Xia, X. & Xu, L. (2017). The role of bacterial fermentation in lipolysis and lipid oxidation in Harbin dry sausages and its flavour development. *LWT - Food Science and Technology*, **77**, 389–396.

Corral, S., Salvador, A. & Flores, M. (2013). Salt reduction in slow fermented sausages affects the generation of aroma active compounds. *Meat Science*, **93**, 776–785.

Corral, S., Salvador, A. & Flores, M. (2014). Elucidation of key aroma compounds in traditional dry fermented sausages using different extraction techniques. *Journal of the Science of Food and Agriculture*, **95**, 1350–1361.

Cullere, M., Tasoniero, G., Secci, G., Parisi, G., Smit, P., Hoffman, L.C. & Dalle Zotte, A. (2019). Effect of the incorporation of a fermented rooibos (*Aspalathus linearis*) extract in the

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manufacturing of rabbit meat patties on their physical, chemical, and sensory quality during refrigerated storage. *LWT - Food Science and Technology*, **108**, 31–38.

da Silva, S.L., Lorenzo, J.M., Machado, J.M., Manfio, M., Cichoski, A.J., Fries, L.L.M., Morgano, M.A. & Campagnol, P.C.B. (2020). Application of arginine and histidine to improve the technological and sensory properties of low-fat and low-sodium bologna-type sausages produced with high levels of KCl. *Meat Science*, **159**, 1–5.

De Beer, D. & Joubert, E. (2010). Development of HPLC method for *Cyclopia subternata* phenolic compound analysis and application to other *Cyclopia* spp. *Journal of Food Composition and Analysis*, **23**, 289–297.

De Beer, D., Schulze, A.E., Joubert, E., De Villiers, A., Malherbe, C.J. & Stander, M.A. (2012). Food ingredient extracts of *Cyclopia subternata* (honeybush): Variation in phenolic composition and antioxidant capacity. *Molecules*, **17**, 14602–14624.

De Mey, E., De Maere, H., Paelinck, H. & Fraeye, I. (2017). Volatile *N*-nitrosamines in meat products: Potential precursors, influence of processing, and mitigation strategies. *Critical Reviews in Food Science and Nutrition*, **57**, 2909–2923.

Del Nobile, M.A., Conte, A., Incoronato, A.I., Panza, O., Sevi, A. & Marino, R. (2009). New strategies for reducing the pork back-fat content in typical Italian salami. *Meat Science*, **81**, 263–269.

Department of Health, Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972, amended by government notice 812, 2019. Reduction of total sodium (Na) in certain foodstuffs. URL: https://www.foodfocus.co.za/assets/documents/UPDATED_Notice%20214%20-Regulations%20relating%20reduction%20of%20sodium.pdf.

Di Cagno, R., Lòpez, C.C., Tofalo, R., Gallo, G., De Angelis, M., Paparella, A., Hammes, W.P. & Gobbetti, M. (2008). Comparison of the compositional, microbiological, biochemical and volatile profile characteristics of three Italian PDO fermented sausages. *Meat Science*, **79**, 224–235.

Dos Santos, B.A., Campagnol, P.C.B., Da Cruz, A.G., Galvão, M.T.E.L., Monteiro, R.A., Wagner, R. & Pollonio, M.A.R. (2015). Check all that apply and free listing to describe the sensory characteristics of low sodium dry fermented sausages: Comparison with trained panel. *Food Research International*, **76**, 725–734.

Elisia, I. & Kitts, D.D. (2011). Quantification of hexanal as an index of lipid oxidation in human milk and association with antioxidant components. *Journal of Clinical Biochemistry and Nutrition*, **49**, 147–152.

Elmadfa, I. & Kornsteiner, M. (2009). Fats and fatty acid requirements for adults. *Annals of Nutrition and Metabolism*, **55**, 56–75.

Faustman, C., Sun, Q., Mancini, R. & Suman, S.P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science*, **86**, 86–94.

Flores, M. (2018). Understanding the implications of current health trends on the aroma of wet and dry cured meat products. *Meat Science*, **144**, 53–61.

Folch J., Lees M., Sloane-Stanley G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, **226**, 495-509.

Food and Agriculture Organization of the United Nations (2010). Fats and fatty acids in human nutrition: Report of an expert consultation. *FAO Food and Nutrition Paper*, **91**, 55-62.

Fuentes, V., Estévez, M., Ventanas, J. & Ventanas, S. (2014). Impact of lipid content and composition on lipid oxidation and protein carbonylation in experimental fermented sausages. *Food Chemistry*, **147**, 70–77.

Furia, T.E. (1980). *Handbook of Food Additives*, 2nd ed. Boca Raton, Florida, CRC Press, Inc, P. 283.

Gelabert, J., Gou, P., Guerrero, L. & Arnau, J. (2003). Effect of sodium chloride replacement on some characteristics of fermented sausages. *Meat Science*, **65**, 833–839.

Gøtterup, J., Olsen, K., Knøchel, S., Tjener, K., Stahnke, L.H. & Møller, J.K.S. (2008). Colour formation in fermented sausages by meat-associated staphylococci with different nitrite- and nitrate-reductase activities. *Meat Science*, **78**, 492–501.

Ha, S.K. (2014). Dietary salt intake and hypertension. *Electrolytes and Blood Pressure*, **12**, 7–18.

Herranz, B., Ordóñez, J.A., De la Hoz, L., Hierro, E., Soto, E. & Cambero, M.I. (2008). Fatty acid composition of salami from different countries and their nutritional implications. *International Journal of Food Sciences and Nutrition*, **59**, 607–618.

Holck, A., Axelsson, L., McLeod, A., Rode, T.M. & Heir, E. (2017). Health and safety considerations of fermented sausages. *Journal of Food Quality*, **2017**, 1–25.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Hospital, X.F., Hierro, E. & Fernández, M. (2012). Survival of *Listeria innocua* in dry fermented sausages and changes in the typical microbiota and volatile profile as affected by the concentration of nitrate and nitrite. *International Journal of Food Microbiology*, **153**, 395–401.

Hoz, L., Dõarrigo, M., Cambero, I. & Ordóñez, J.A. (2004). Development of an *n*-3 fatty acid and *α*-tocopherol enriched dry fermented sausage. *Meat Science*, **67**, 485–496.

Hu, F.B., Stampfer, M.J., Manson, J.E., Rimm, E., Colditz, G.A., Rosner, B.A., Hennekens, C.H. & Willett, W.C. (1997). Dietary fat intake and the risk of coronary heart disease in women. *New England Journal of Medicine*, **337**, 1491–1499.

International Agency for Research on Cancer (1978). IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Some *N*-nitroso compounds,**17**, 1–349.

Jiang, J. & Xiong, Y.L. (2016). Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. *Meat Science*, **120**, 107–117.

Jin, S., Choi, J.S., Yang, H., Park, T. & Yim, D. (2018). Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science*, **146**, 34–40.

Jones, M., Hoffman, L.C. & Muller, M. (2015). Effect of rooibos extract (*Aspalathus linearis*) on lipid oxidation over time and the sensory analysis of blesbok (*Damaliscus pygargus phillipsi*) and springbok (*Antidorcas marsupialis*) droëwors. *Meat Science*, **103**, 54–60.

Joubert, E., De Beer, D., Malherbe, C.J., Muller, M., Louw, A. & Gelderblom, W.C.A. (2019). Formal honeybush tea industry reaches 20-year milestone – Progress of product research targeting phenolic composition, quality and bioactivity. *South African Journal of Botany*, **127**, 58–79.

Joubert, E., Richards, E.S., Van der Merwe, J.D., De Beer, D., Manley, M. & Gelderblom, W.C.A. (2008). Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush Tea). *Journal of Agricultural and Food Chemistry*, **56**, 954–963.

Kamara, B.I., Brand, J.D., Brandt, Vincent, E. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391–5395.

Khan, N.A., Cone, J.W., Fievez, V. & Hendriks, W.H. (2012). Causes of variation in fatty acid content and composition in grass and maize silages. *Animal Feed Science and Technology*, **174**, 36–45.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Le Roux, M., Cronje, J.C., Burger, B.V & Joubert, E. (2012). Characterization of volatiles and aroma-active compounds in honeybush (*Cyclopia subternata*) by GC-MS and GC-O analysis. *Journal of Agricultural and Food Chemistry*, **60**, 2657–2664.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L. & Scholliers, P. (2013). Meat fermentation at the crossroads of innovation and tradition: A historical outlook. *Trends in Food Science and Technology*, **31**, 130–137.

Lorenzo, J.M., Bedia, M. & Bañón, S. (2013). Relationship between flavour deterioration and the volatile compound profile of semi-ripened sausage. *Meat Science*, **93**, 614–620.

Magcwebeba, T.U., Riedel, S., Swanevelder, S., Swart, P., De Beer, D., Joubert, E. & Gelderblom, W.C.A. (2016). The potential role of polyphenols in the modulation of skin cell viability by *Aspalathus linearis* and *Cyclopia* spp. herbal tea extracts *in vitro*. *Journal of Pharmacy and Pharmacology*, **68**, 1440–1453.

Mahachi, L.N., Rudman, M., Arnaud, E., Muchenje, V. & Hoffman, L.C. (2019). Development of semi dry sausages (cabanossi) with warthog (*Phacochoerus africanus*) meat: Physicochemical and sensory attributes. *LWT - Food Science and Technology*, 115, https://doi.org/10.1016/j.lwt.2019.108454.

Marco, A., Navarro, J.L. & Flores, M. (2007). Quantitation of selected odor-active constituents in dry fermented sausages prepared with different curing salts. *Journal of Agricultural and Food Chemistry*, **55**, 3058–3065.

Marco, A., Navarro, J.L. & Flores, M. (2008). The sensory quality of dry fermented sausages as affected by fermentation stage and curing agents. *European Food Research and Technology*, **226**, 449–458.

Marino, R., Albenzio, M., della Malva, A., Muscio, A. & Sevi, A. (2015). Nutritional properties and consumer evaluation of donkey bresaola and salami: Comparison with conventional products. *Meat Science*, **101**, 19–24.

Martínez, L., Bastida, P., Castillo, J., Ros, G. & Nieto, G. (2019). Green alternatives to synthetic antioxidants, antimicrobials, nitrates, and nitrites in clean label spanish chorizo. *Antioxidants*, **8**, 1–18.

Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E. & Gandemer, G. (1999). Volatile compounds of commercial Milano salami. *Meat Science*, **51**, 175–183.

Min, B., Cordray, J.C. & Ahn, D.U. (2010). Effect of NaCl, myoglobin, Fe (II), and Fe (III) on lipid oxidation of raw and cooked chicken breast and beef loin. *Journal of Agricultural and Food Chemistry*, **58**, 600–605.

Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. & Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry*, **192**, 736–744.

Montanari, C., Gatto, V., Torriani, S., Barbieri, F., Bargossi, E., Lanciotti, R., Grazia, L., Magnani, R., Tabanelli, G. & Gardini, F. (2018). Effects of the diameter on physico-chemical,

microbiological and volatile profile in dry fermented sausages produced with two different starter cultures. *Food Bioscience*, **22**, 9–18.

Montel, M.-C., Reitz, J., Talon, R., Berdagué, J.-L. & Rousset-Akrim, S. (1996). Biochemical activities of *Micrococcaceae* and their effects on the aromatic profiles and odours of a dry sausage model. *Food Microbiology*, **13**, 489–499.

Morales, M.T., Rios, J.J. & Aparicio, R. (1997). Changes in the volatile composition of virgin olive oil during oxidation: Flavors and off-flavors. *Journal of Agricultural and Food Chemistry*, **45**, 2666–2673.

Morel, P.C.H., McIntosh, J.C. & Janz, J.A.M. (2006). Alteration of the fatty acid profile of pork by dietary manipulation. *Asian-Australasian Journal of Animal Sciences*, **19**, 431–437.

Moretti, V.M., Madonia, G., Diaferia, C., Mentasti, T., Paleari, M.A., Panseri, S., Pirone, G. & Gandini, G. (2004). Chemical and microbiological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions. *Meat Science* , **66**, 845–854.

Muguerza, E., Ansorena, D. & Astiasarán, I. (2003a). Improvement of nutritional properties of Chorizo de Pamplona by replacement of pork backfat with soy oil. *Meat Science*, **65**, 1361–1367.

Muguerza, E., Ansorena, D., Bloukas, J.G. & Astiasarán, I. (2003b). Effect of fat level and partial replacement of pork backfat with olive oil on the lipid oxidation and volatile compounds of greek dry fermented sausages. *Journal of Food Science*, **68**, 1531–1536.

Muguerza, E., Gimeno, O., Ansorena, D., Bloukas, J.G. & Astiasarán, I. (2001). Effect of replacing pork backfat with pre-emulsified olive oil on lipid fraction and sensory quality of Chorizo de Pamplona - A traditional Spanish fermented sausage. *Meat Science*, **59**, 251–258.

Ntlhokwe, G., Muller, M., Joubert, E., Tredoux, A.G.J. & De Villiers, A. (2018). Detailed qualitative analysis of honeybush tea (*Cyclopia* spp.) volatiles by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry and relation with sensory data. *Journal of Chromatography A*, **1536**, 137–150.

Olivares, A., Navarro, J.L. & Flores, M. (2009a). Distribution of volatile compounds in lean and subcutaneous fat tissues during processing of dry fermented sausages. *Food Research International*, **42**, 1303–1308.

Olivares, A., Navarro, J.L. & Flores, M. (2009b). Establishment of the contribution of volatile compounds to the aroma of fermented sausages at different stages of processing and storage. *Food Chemistry*, **115**, 1464–1472.

Olivares, A., Navarro, J.L. & Flores, M. (2011). Effect of fat content on aroma generation during processing of dry fermented sausages. *Meat Science*, **87**, 264–273.

Ordóñez, J.A., Hierro, E.M., Bruna, J.M. & De la Hoz, L. (1999). Changes in the components of dry-fermented sausages during ripening. *Critical Reviews in Food Science and Nutrition*, **39**, 329–367.

Roberts, D.D., Pollien, P. & Milo, C. (2000). Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *Journal of Agricultural and Food Chemistry*, **48**, 2430–2437.

Rotsatchakul, P., Visesanguan, W., Smitinont, T. & Chaiseri, S. (2009). Changes in volatile compounds during fermentation of nham (Thai fermented sausage). *International Food Research Journal*, **16**, 391-414.

Rubio, B., Martínez, B., Sánchez, M.J., García-Cachán, M.D., Rovira, J. & Jaime, I. (2007). Study of the shelf life of a dry fermented sausage "salchichon" made from raw material enriched in monounsaturated and polyunsaturated fatty acids and stored under modified atmospheres. *Meat Science*, **76**, 128–137.

Ruiz, J. (2007). Ingredients. In: *Handboof of Fermented Meat and Poultry* (edited by F. Toldrá). Pp. 59-76. Iowa: USA: Blackwell Publishing Professional.

Ruusunen, M., Vainionpää, J., Puolanne, E., Lyly, M., Lähteenmäki, L., Niemistö, M., Ahvenainen, R. (2003). Physical and sensory properties of low-salt phosphate-free frankfurters composed with various ingredients. *Meat Science*, **63**, 9-16.

Sales, J. & Kotrba, R. (2013). Meat from wild boar (*Sus scrofa* L.): A review. *Meat Science*, **94**, 187–201.

Shah, M.A., Don Bosco, S.J. & Mir, S.A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat Science*, **98**, 21–33.

Shahidi, F. & Pegg, R.B. (1994). Hexanal as an indicator of meat flavour deterioration. *Journal of Food Lipids*, **1**, 177-186.

Shahidi, F., Rubin, L.J., D'Souza, L.A., Teranishi, R. & Buttery, R.G. (1986). Meat flavor volatiles: A review of the composition, techniques of analysis, and sensory evaluation. *Critical Reviews in Food Science and Nutrition*, **24**, 141–243.

Søndergaard, A.K. & Stahnke, L.H. (2002). Growth and aroma production by *Staphylococcus xylosus*, *S. carnosus* and *S. equorum* - A comparative study in model systems. *International Journal of Food Microbiology*, **75**, 99–109.

Sunesen, L.O., Dorigoni, V., Zanardi, E. & Stahnke, L. (2001). Volatile compounds released during ripening in Italian dried sausage. *Meat Science*, **58**, 93–97.

Sunesen, L.O. & Stahnke, L.H. (2003). Mould starter cultures for dry sausages-selection, application and effects. *Meat Science*, **65**, 935–948.

Swanepoel, M., Leslie, A.J., Hoffman, L.C. (2016a). Comparative analyses of the chemical and sensory parameters and consumer preference of a semi-dried smoked meat product (cabanossi) produced with warthog (*Phacochoerus africanus*) and domestic pork meat. *Meat Science*, **114**, 103-113.

Swanepoel, M., Leslie, A.J., Van der Rijst, M. & Hoffman, L.C. (2016b). Physical and chemical characteristics of warthog (*Phacochoerus africanus*) meat. *African Journal of Wildlife Research*, **46**, 103–120.

Tabanelli, G., Coloretti, F., Chiavari, C., Grazia, L., Lanciotti, R. & Gardini, F. (2012). Effects of starter cultures and fermentation climate on the properties of two types of typical Italian dry fermented sausages produced under industrial conditions. *Food Control*, **26**, 416–426.

Theron, K.A., Muller, M., Van der Rijst, M., Cronje, J.C., Le Roux, M. & Joubert, E. (2014). Sensory profiling of honeybush tea (*Cyclopia* species) and the development of a honeybush sensory wheel. *Food Research International*, **66**, 12–22.

Treydte, A.C., Bernesconi, S.M., Kreuzer, M. & Edwards, P.J. (2006). Diet of the common warthog (*Phacochoerus africanus*) on former cattle grounds in a Tanzanian savanna. *Journal of Mammalogy*, **87**, 889–898.

Woods, V.B. & Fearon, A.M. (2009). Dietary sources of unsaturated fatty acids for animals and their transfer into meat , milk and eggs: A review. *Livestock Science*, **126**, 1–20.

Xu, L., Yu, X., Li, M., Chen, J. & Wang, X. (2018). Monitoring oxidative stability and changes in key volatile compounds in edible oils during ambient storage through HS-SPME/GC–MS. *International Journal of Food Properties*, **20**, S2926–S2938.

Yildiz-Turp, G. & Serdaroglu, M. (2012). Partial substitution of beef fat with hazelnut oil in emulsion type sausages: Effects on chemical, physical and sensorial quality. *Journal of Food Technology*, **10**, 32–38.

Zanardi, E., Ghidini, S., Battaglia, A. & Chizzolini, R. (2004). Lipolysis and lipid oxidation in fermented sausages depending on different processing conditions and different antioxidants. *Meat Science*, **66**, 415–423.

Zong, G., Li, Y., Wanders, A.J., Alssema, M., Zock, P.L., Willett, W.C., Hu, F.B. & Sun, Q. (2016). Intake of individual saturated fatty acids and risk of coronary heart disease in US men and women: Two prospective longitudinal cohort studies. *British Medical Journal*, **355**, 1–11.

CHAPTER 8

General conclusions and recommendations

The continuous research surrounding the reduction or substitution of potassium and sodium nitrite (NO_2) and nitrate (NO_3) preserving salts (E249, E250, E251 and E252) in cured meat products portrays the mistrust of the consumer therein and the demand for cleaner labelled meat products (Di Vita *et al.*, 2019). The unfortunate evidence of the formation of carcinogenic compounds in cured meats due to nitrate/nitrite addition caused an ongoing debate whether these preserving salts are added in safe limits (reflected by the numerous revisions of international acceptable limits) and whether they can be partially replaced or omitted completely (Cenci-goga *et al.*, 2012; Eskandari *et al.*, 2013; Hospital *et al.*, 2015). This creates an incredible challenge for the meat industry, as the demand for less preservatives and simultaneous fear of food pathogens contradicts each other (Bedale *et al.*, 2016). Oxidation leading to rancidity and loss in quality and the proliferation of lethal food pathogens, such as *Clostridium botulinum*, are associated with salami. To combat these beforementioned unwanted processes in salami which are successfully inhibited through the addition of nitrate/nitrite, researchers are exploring the use of natural extracts portraying an antioxidant and/or antimicrobial effect (Gassara *et al.*, 2016; Aquilani *et al.*, 2018; Aziz & Karboune, 2018; Alirezalu *et al.*, 2019; Martínez *et al.*, 2019).

Based on the above-mentioned challenges, the objective of this study was to determine the feasibility and effect of adding a natural extract indigenous to Southern Africa, namely honeybush (*Cyclopia subternata*), to salami. This main research objective was analysed in four respective research chapters. The feasibility of incorporating honeybush extract (0.5% w/w) in typical pork Italian salami (Chapter 4) was demonstrated. From this preliminary study, certain practical limitations were noticed and improved on in the following research chapter, using South African warthog meat to produce salami (Chapter 5). These limitations were considered valuable for future researchers attempting salami processing or recipe alterations. The limitations included the use of natural bovine casings. The use of synthetic casings in the following trial as opposed to natural casings ensured homogeneity in terms of salami dimensions, which in turn influences other physical-chemical traits (e.g. water and weight loss, microbial growth). Furthermore, the hygroscopic nature of the brick-brown honeybush extract created localised discoloration, presumably due to oxidation beneath the sausage casing. Dissolving the extract in a predetermined amount of distilled water ensured homogenous incorporation into the salami batter. Finally, dipping the salami in a commercial mould suspension as opposed to spontaneous mould colonisation contributed to a more homogenous end product, eliminating the effect of additional variables influencing physical-chemical traits, as mould growth effects water loss, final product pH, lipid and protein oxidation (Sunesen & Stahnke, 2003).

This study gained valuable information with regard to physical-chemical (i.e. pH, a_w, CIE L*a*b* colour, texture, proximate composition, fatty acid content and volatile organic compounds)

changes when adding honeybush extract to game (i.e. warthog) salami with reduced added sodium nitrate (75 mg/kg). A honeybush addition of 0.5% (w/w) may aid in maintaining game salami redness (CIE a*) over a frozen storage period of up to five and a half months (Chapter 5). The latter finding together with the decline in rancid volatile organic compounds (VOCs, Chapter 7) demonstrates the promising antioxidant effect of honeybush in salami. Indeed, honeybush extract rich in polyphenolics (Kamara *et al.*, 2004; Kokotkiewicz *et al.*, 2012; Schulze *et al.*, 2015) showed potential to suppress the onset of salami oxidation, seen in the decrease in certain volatile organic compounds (VOCs) associated with rancidity (Chapter 7). The decrease in unsaturated fatty acid (linoleic and oleic fatty acids) oxidation by-products as found in Chapter 7, were evident from a decline in: hexanal; nonanal; 1-pentanol; 1-octen-3-ol; 2-pentylfyran; and undecane in T4 salami (0.5% added honeybush), compared to the control salami (0% honeybush addition) after 40 ripening days. Of all the rancid VOCs, salami with 0.125%, 0.25%, 0.375% and 0.5% honeybush extract inclusion contained significantly lower amounts of hexanal and 1-octen-3-ol compared to the control with no honeybush addition. These beforementioned rancid volatiles are often analysed as oxidation bio-markers to determine the extent of oxidation in meat products (Yang *et al.*, 2017; Xu *et al.*, 2018). Of these, hexanal is popularly studied due its low odour threshold and well-known association with grassy, tallowy and rancid aromas (Morales *et al.*, 1997; Marco *et al.*, 2007; Fuentes *et al.*, 2014). As seen from the present study (Chapter 7), honeybush can contribute uniquely to game salami with floral, sweet and spicy aromas due to the significant increase in (R/S)-linalool, terpinene-4-ol and α-terpineol which are associated with these beforementioned aromas (Moretti *et al.*, 2004; Theron *et al.*, 2014). To better comprehend the effect of honeybush on oxidation reactions in a meat model, it is recommended to perform more than one analysis (i.e. peroxide value or thiobarbituric acid reactive substances TBARS test together with GC-MS) over time. Furthermore, it would be of great value to determine the consumer acceptance of warthog salami with added honeybush extract, because of the potential addition of botanical aromas not commonly associated with salami.

Honeybush (0.125%, 0.25%, 0.375% and 0.5%) can be incorporated without altering the final salami product proximate composition (moisture, protein and total lipid content) and texture (hardness, chewiness and cohesiveness), as seen in Chapter 5. However, adding this hygroscopic tea extract might cause an increase in final product salami a_w (Chapter 5). An unexpected high final product salami pH was recorded (pH= 6.71-7.46) after 40 ripening days (Chapter 5). This high pH phenomenon was hypothesised to be caused by the outgrowth of starter culture lactic acid bacteria by background microflora, in combination with surface mould (*Penicillium nalgiovense*) lactic acid metabolization (Sunesen & Stahnke, 2003). In the case of applying a mould starter culture in small diameter salami, the addition of extra fermentable sugar is highly recommended to ensure a sufficient pH decrease for microbial safety. Although the warthog salami did not sufficiently decrease in pH (to < 5.2), all salami with reduced added sodium nitrate (75 mg/kg) and added honeybush extract (T1= 0.125%; T2= 0.25%; T3= 0.375%; and T4= 0.5%) were free from

pathogens: *E.coli*; *Listeria* spp.; *Salmonella* spp.; and *S. aureus* after a 40-day mould ripening period (Chapter 6). To better comprehend the potential pathogen inhibitory effects of honeybush extract in salami, reference strains would need to be incorporated in future studies as salami would not necessarily contain pathogens if not introduced during processing. Honeybush addition, on the other hand, could presumably provide additional fermentable substrates for lactic acid bacteria as seen from the present study (Chapter 6).

From this study is evident that successful salami production is dependent on a vast amount of non-mutually exclusive physical and biochemical reactions occurring over the fermentation and ripening period. In this sense, when attempting salami processing or recipe alteration it is recommended to first familiarise oneself with the skill of salami making. Furthermore, when attempting a similar research study, it is advised to limit the number of variables to better comprehend the effect of the extract on the product physical-chemical and sensory traits. For instance, the starter culture effectiveness should be predetermined as culture strains do not necessarily proliferate with the same success in different meat sources (e.g. pork versus warthog meat).

Warthog salami with the addition of honeybush (*Cyclopia subternata*) extract could be a marketable ready-to-eat meat product based on the present consumer interest in processed meat products with added bio-active ingredients and the use of less additives (especially nitrate and nitrite preserving salts). The reduction of nitrate does not necessarily imply a microbially unaccepted product, reflected in the absence of pathogens in the final product salami with 75 mg/kg ingoing amount of sodium nitrate. However, the associated microbial risks increase on a large production scale particularly in the case of other hurdles not being successfully met (such as an insufficient pH decrease and utilisation of game meat). Food safety is non-negotiable, therefore, a future suggestion would be to combine honeybush extract with organic acids to ensure a low salami pH, especially in the case of surface mould growth on a small diameter salami. This study was the first at attempting the addition of honeybush extract in processed meat products and provided valuable baseline data for future research studies.

References

Alirezalu, K., Hesari, J., Nemati, Z., Munekata, P.E.S., Barba, F.J. & Lorenzo, J.M. (2019). Combined effect of natural antioxidants and antimicrobial compounds during refrigerated storage of nitrite-free frankfurter-type sausage. *Food Research International*, **120**, 839–850.

Aquilani, C., Sirtori, F., Flores, M., Bozzi, R., Lebret, B. & Pugliese, C. (2018). Effect of natural antioxidants from grape seed and chestnut in combination with hydroxytyrosol, as sodium nitrite substitutes in Cinta Senese dry-fermented sausages. *Meat Science*, **145**, 389–398.

Aziz, M. & Karboune, S. (2018). Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, **58**, 486–511.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, **120**, 85–92.

Cenci-goga, B.T., Rossitto, P. V, Sechi, P., Parmegiani, S., Cambiotti, V. & Cullor, J.S. (2012). Effect of selected dairy starter cultures on microbiological, chemical and sensory characteristics of swine and venison (*Dama dama*) nitrite-free dry-cured sausages. *Meat Science*, **90**, 599–606.

Di Vita, G., Blanc, S., Mancuso, T., Massaglia, S., La Via, G. & D'Amico, M. (2019). Harmful compounds and willingness to buy for reduced-additives salami. An outlook on Italian consumers. *International Journal of Environmental Research and Public Health*, **16**, 1–9.

Eskandari, M.H., Hosseinpour, S., Mesbahi, G. & Shekarforoush, S. (2013). New composite nitrite-free and low-nitrite meat-curing systems using natural colorants. *Food Science and Nutrition*, **1**, 392–401.

Fuentes, V., Estévez, M., Ventanas, J. & Ventanas, S. (2014). Impact of lipid content and composition on lipid oxidation and protein carbonylation in experimental fermented sausages. *Food Chemistry*, **147**, 70–77.

Gassara, F., Kouassi, A.P., Brar, S.K. & Belkacemi, K. (2016). Green alternatives to nitrates and nitrites in meat-based products - A Review. *Critical Reviews in Food Science and Nutrition*, **56**, 2133–2148.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M. & Hierro, E. (2015). Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile. *Food Control*, **57**, 275–281.

Kamara, B.I., Brand, J.D., Brandt, Vincent, E. & Joubert, E. (2004). Phenolic metabolites from honeybush tea (*Cyclopia subternata*). *Journal of Agricultural and Food Chemistry*, **52**, 5391–5395.

Kokotkiewicz, A., Luczkiewicz, M., Sowinski, P., Glod, D., Gorynski, K. & Bucinski, A. (2012). Isolation and structure elucidation of phenolic compounds from *Cyclopia subternata* Vogel (honeybush) intact plant and *in vitro* cultures. *Food Chemistry*, **133**, 1373–1382.

Marco, A., Navarro, J.L. & Flores, M. (2007). Quantitation of selected odor-active constituents in dry fermented sausages prepared with different curing salts. *Journal of Agricultural and Food Chemistry*, **55**, 3058–3065.

Martínez, L., Bastida, P., Castillo, J., Ros, G. & Nieto, G. (2019). Green alternatives to synthetic antioxidants, antimicrobials, nitrates, and nitrites in clean label Spanish chorizo. *Antioxidants*, **8**, 1–18.

Morales, M.T., Rios, J.J. & Aparicio, R. (1997). Changes in the volatile composition of virgin olive oil during oxidation: Flavors and off-flavors. *Journal of Agricultural and Food Chemistry*, **45**, 2666–2673.

Moretti, V.M., Madonia, G., Diaferia, C., Mentasti, T., Paleari, M.A., Panseri, S., Pirone, G. & Gandini, G. (2004). Chemical and microbiological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions. *Meat Science*, **66**, 845–854.

Schulze, A.E., Beelders, T., Koch, I.S., Erasmus, L.M., De Beer, D. & Joubert, E. (2015). Honeybush herbal teas (*Cyclopia* spp.) contribute to high levels of dietary exposure to xanthones, benzophenones, dihydrochalcones and other bioactive phenolics. *Journal of Food Composition and Analysis*, **44,** 139–148.

Sunesen, L.O. & Stahnke, L.H. (2003). Mould starter cultures for dry sausages - Selection, application and effects. *Meat Science*, **65**, 935–948.

Theron, K.A., Muller, M., Van der Rijst, M., Cronje, J.C., Le Roux, M. & Joubert, E. (2014). Sensory profiling of honeybush tea (*Cyclopia* species) and the development of a honeybush sensory wheel. *Food Research International*, **66**, 12–22.

Xu, L., Yu, X., Li, M., Chen, J. & Wang, X. (2018). Monitoring oxidative stability and changes in key volatile compounds in edible oils during ambient storage through HS-SPME/GC–MS. *International Journal of Food Properties*, **20**, S2926–S2938.

Yang, Y., Zhang, X., Wang, Y., Pan, D., Sun, Y. & Cao, J. (2017). Study on the volatile compounds generated from lipid oxidation of Chinese bacon (unsmoked) during processing. *European Journal of Lipid Science and Technology*, **119**, 1–10.

ADDENDUM A

Figure 4.1 Experimental Italian type salami in a controlled ripening chamber (Majolo® Plus 100 Seasoning Controller).

Figure 4.2 Final product salami (35-day ripened) subjected to sensory analysis evaluating outer surface mould growth, classified according to (left to right): Class 1= 0%; Class 2= 30%; Class 3= 60%; or Class 4= 100% mould growth or coverage.

Figure 4.3 Localised orange-like (centre) and dark-brown spots (beneath the casing) of 35-day ripened honeybush (*Cyclopia subternata*) treated salami (H= 0.5% w/w).
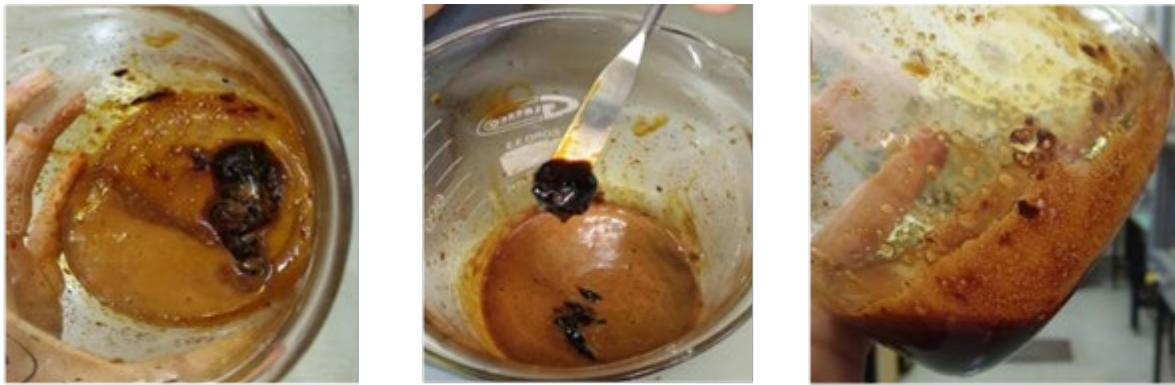
Figure 5.1 Agglomerated honeybush (*Cyclopia subternata*) extract upon water exposure (left and middle) and honeybush extract solution in distilled water (T4= 0.5% w/w; 30.56 g honeybush) prepared for salami production (right).

Figure 5.2 Size reduction of frozen pork fat cubes (~5 cm^3) created by using a band saw for salami production.


Figure 5.3 Size reduction of pork fat (left) and warthog meat (middle) to create a coarse meat and fat mixture with a bowl cutter (Mainca, Spain) for the production of warthog salami.
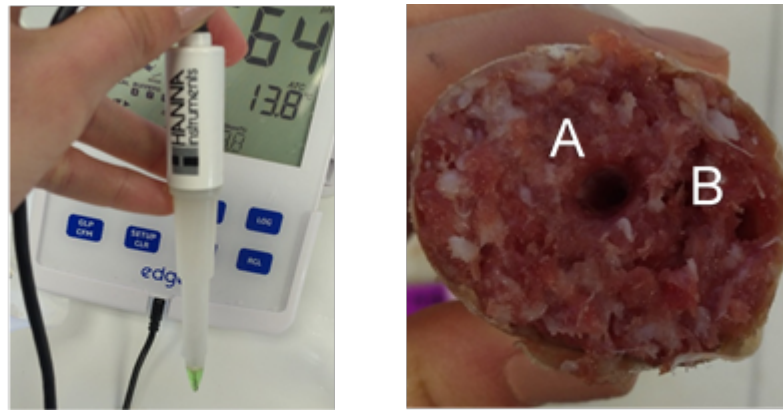
Figure 5.4 Direct pH measurements of salami by inserting a glass pH electrode parallel to the salami length and perpendicular to the sliced salami centre (A) and underneath the casing (B).

Figure 5.5 Instrumental surface colour measurements (CIE L*a*b*) of salami slices measured on a white surface background after a 30 min blooming period.
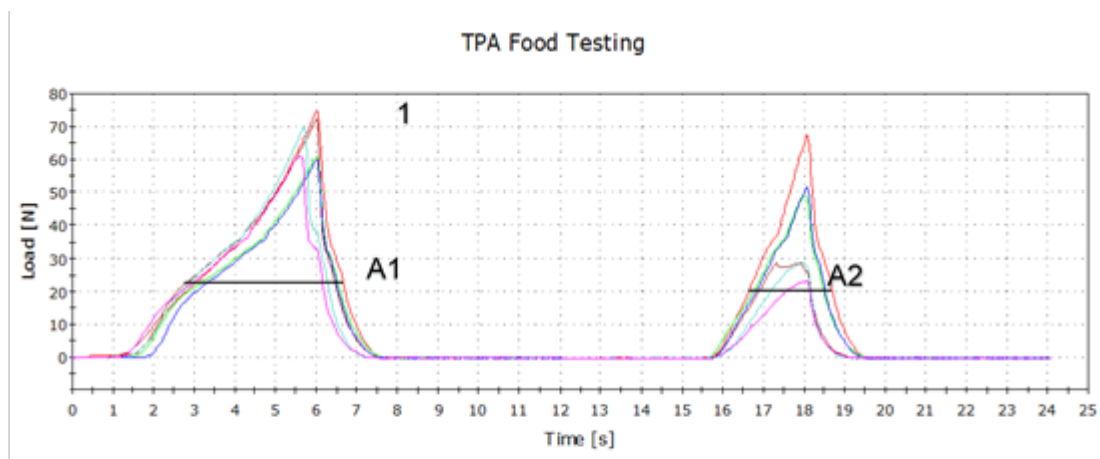
Figure 5.6 Illustration of a real time texture profile analysis graph plotted during a double compression of a cylindrical salami piece, where 1= hardness (N); A1 and A2= total energy (J) for the first and second compression, respectively.

Figure 5.7 Salami sample homogenisation (20 s, 20 000 rpm) using a sample mill (FOSS Analytical, KnifetecTM 1095) equipped with a high speed rotor blade.

Figure 5.8 Homogenised salami samples filtrated through gauze after magnetically stirred (30 min, 300 rpm) for titratable acidity analysis.

Figure 5.9 Successful white mould (*Penicillium nalgiovense*) growth on salami outer surfaces, irrespective of treatment (from left to right: C; T1; T2; T3; and T4 salami) after 40-ripening days.

ADDENDUM B

Table 6.3 Bacteriological media used during final product salami microbial analyses according to manufacturer instructions

Media	Manufacturer	Concentration
Peptone Buffered Water	Merck, Art No: HG00C134.500	2.0% m/v
Half Fraser Broth Plus	ISO Neogen, LAB211	5.5% m/v
Half Fraser Supplement	Bio-Rad, LOT: 64190748	2.25 ml reconstructed supplement in 225 ml half fraser broth
Rapid'*L.mono* plates	AEC-Amersham	-
Rappaport-Vassiliadis Soya (RVS) Peptone broth	Oxoid, CM0866	2.68% m/v
Xylose Lysine Deoxycholate (XLD) agar	Merck, Biolab Art No: HG000C21.500	5.2% m/v
Baird Parker agar	Fluka Analytical, 11705-500G	6.11% m/v
Egg-Yolk Tellurite Emulsion	Fluka Analytical, 75208	50 ml in 950 ml Baird Parker agar
Tryptone Soya Broth	Oxoid, CM0129	3% m/v
Rabbit Plasma	Oxoid, R21051	0.5 ml/test tube
Reinforced Clostridial Agar	Oxoid, CM0151	5.25% m/v
Anaerobic sachets	Oxoid, AnaeroGen™ 2.5L, AN0025A	-
Bacillus cereus Agar Base	Oxoid, CM0617	4.32% m/v
Polymyxin B Supplement	Oxoid, SR0099E	1 reconstructed vial in 475 ml *B. cereus* agar
Egg Yolk Emulsion	Oxoid, SR0047	25 ml in 475 ml *B. cereus* agar
Nutrient Agar	Merck, Biolab Art No: HG0000C1.500	3.1% m/v
API 50 CH with supplements	BioMérieux	-

ADDENDUM C

Table 7.7 The influence of different levels of honeybush (*Cyclopia subternata*) on the total fatty acid methyl ester (FAME) composition (mg of FAME/g fat, Means ± SD) of warthog salami (n= 6) after a 40-day ripening period

Fatty acid	C	T1	T2	T3	T4	Treatment
Saturated fatty acids (SFAs)						
C12:0	0.53[ab] ±0.21	0.73[a]±0.22	0.33[ab]±0.10	0.22[b]±0.05	0.25[b]±0.09	**< 0.01[A]**
C13:0	1.96[a] ±2.12	0.99[a]±0.65	0.17[a]±0.23	0.44[a]±0.34	0.36[a]±0.58	0.11[A]
C14:0	2.85[a] ±1.59	2.62[a]±0.71	2.11[a]±0.76	1.86[a]±0.34	1.77[a]±0.59	0.27[A]
C15:0	0.14[a] ±0.05	0.15[a]±0.04	0.11[a]±0.04	0.09[a]±0.01	0.10[a]±0.03	0.06[A]
C16:0	55.09[a] ±22.90	57.58[a]±17.08	46.58[a]±13.77	38.88[a]±5.16	52.12[a]±17.97	0.15[A]
C18:0	27.19[a] ±7.88	27.57[a]±8.71	23.49[a]±6.50	23.51[a]±7.73	29.69[a]±5.18	0.58[B]
C20:0	0.23[a] ±0.08	0.24[a]±0.08	0.18[a]±0.07	0.15[a]±0.03	0.23[a]±0.10	0.25[B]
Monounsaturated fatty acids (MUFAs)						
C14:1	0.07[ab]±0.05	0.12[a]±0.04	0.05[b]±0.04	0.06[b]±0.01	0.04[b]±0.04	**0.03[B]**
C16:1	4.27[a]±1.70	5.34[a]±1.71	2.78[a]±1.09	2.89[a]±0.15	2.54[a]±1.25	0.08[A]
C18:1n9*c*	63.19[a]±18.70	76.51[a]±28.44	44.34[a]±11.96	46.95[a]±2.54	40.20[a]±11.8	0.12[A]
C20:1	1.99[ab]±0.67	2.32[a]±0.84	1.31[c]±0.41	1.48[bc]±0.32	1.31[bc]±0.38	**0.03[B]**
C22:1n9	0.03[a]±0.02	0.02[ab]±.0.02	0.03[a]±0.009	0.02[ab]±0.006	0.008[b]±0.01	0.09[B]
Polyunsaturated fatty acids (PUFAs)						
C18:2n6*c*	15.37[a]±5.17	19.27[a]±7.92	10.72[a]±3.89	11.65[a]±1.52	9.31[a]±5.37	0.20[A]
C18:3n6	0.37[a]±0.10	0.45[ab]±0.15	0.27[ab]±0.07	0.20[b]±0.02	0.23[ab]±0.06	**< 0.01[A]**
C18:3n3	5.76[ab]±2.39	7.47[a]±3.41	3.41[bc]±1.23	3.40[bc]±1.31	2.67[c]±1.99	**< 0.01[B]**
C20:2n6	3.16[a]±0.98	3.74[a]±1.38	2.11[a]±0.70	2.30[a]±0.34	1.76[a]±0.73	0.08[A]
C20:3n6	0.18[a]±0.11	0.21[a]±0.11	0.09[a]±0.07	0.12[a]±0.06	0.10[a]±0.09	0.14[B]
C20:3n3	1.14[a]±0.47	1.53[a]±0.63	0.81[a]±0.29	0.70[a]±0.13	0.50[a]±0.25	0.04[A]
C20:4n6	0.25[a]±0.10	0.31[a]±0.11	0.15[b]±0.05	0.15[b]±0.03	0.12[b]±0.05	**< 0.01[B]**
C20:5n3	0.19[ab]±0.07	0.25[a]±0.10	0.13[bc]±0.05	0.09[c]±0.02	0.10[c]±0.04	**< 0.01[B]**
Fatty acid totals						
SFA	87.98[a]±33.18	89.88[a]±26.99	72.97[a]±20.93	65.15[a]±11.18	84.52[a]±22.37	0.43[B]
MUFA	69.56[a]±20.89	84.32[a]±30.83	48.52[a]±13.36	51.41[a]±2.47	44.09[a]±13.27	0.12[A]

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Table 7.7 continued

Fatty acid totals	C	T1	T2	T3	T4	Treatment
PUFA	26.43ᵃ±8.79	33.24ᵃ±13.27	17.70ᵃ±5.96	18.62ᵃ±3.00	14.78ᵃ±8.30	0.11ᴬ
n-6	19.33ᵃ±6.31	24.00ᵃ±9.60	13.35ᵃ±4.64	14.43ᵃ±1.82	11.52ᵃ±6.16	0.16ᴬ
n-3	7.10ᵃᵇ±2.88	9.24ᵃ±3.99	4.35ᵇᶜ±1.50	4.20ᵇᶜ±1.41	3.27ᶜ±2.21	**< 0.01ᴮ**
Total fatty acid content	183.97ᵃ	207.44ᵃ	139.18ᵃ	135.18ᵃ	143.40ᵃ	0.16
%SFA	47.82	43.33	52.43	48.19	58.94	-
%MUFA	37.81	40.65	34.86	38.03	30.75	-
%PUFA	14.37	16.02	12.72	13.77	10.31	-
Fatty acid ratios						
PUFA:SFA	0.33ᵃ±0.11	0.36ᵃ±0.05	0.27ᵃ±0.13	0.30ᵃ±0.08	0.20ᵃ±0.13	0.20ᴮ
(*n*-6)/(*n*-3)	2.88ᵇ±0.71	2.65ᵇ±0.52	3.14ᵇ±0.62	3.67ᵃᵇ±0.89	4.19ᵃ±1.29	**0.05ᴮ**
Total fatty acid content	183.97ᵃ	207.44ᵃ	139.18ᵃ	135.18ᵃ	143.40ᵃ	0.16

$^{a-c}$ Different superscripts in the same row indicate significant differences between treatments; ^{A} p-values were calculated using the Welch test in combination with the Games-Howell post hoc test; ^{B} p-values were calculated using the F-test in combination with Fisher's least significant difference (LSD) post hoc test; $p > 0.05$= not significant, $p \leq 0.05$= significant; $p \leq 0.01$= significant; SFA= sum of C12:0, C13:0, C14:0, C15:0, C16:0, C18:0 and C20:0; MUFA= sum of C14:1, C16:1, C18:1n9*c*, C20:1 and C22:1n9; PUFA= sum of C18:2n6*c*, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:3n3, C20:4n6 and C20:5n3; PUFA:SFA ratio= [(sum of C18:2n6*c*, C18:3n6, C18:3n3, C20:2n6, C20:3n6, C20:3n3, C20:4n6 and C20:5n3)/(sum of C12:0, C13:0, C14:0, C15:0, C16:0, C18:0 and C20:0)]; *n*-6:*n*-3 ratio= [(sum of C18:2n6*c*, C18:3n6, C20:2n6, C20:3n6 and C20:4n6)/(sum of C18:3n3, C20:3n3 and C20:5n3)].

Table 7.8 Level of statistical significance (p-values) for the effect of honeybush (*Cyclopia subternata*) on selective volatile organic compounds (VOCs) of the raw salami batter (warthog meat and pork fat day-0, n= 6) and the final product salami (day-40, n= 6)[1]

2-Butanone

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		1.000	0.740	0.117	0.234	**< 0.001**
2	C	1.000		0.740	0.117	0.234	**< 0.001**
3	T1	0.740	0.740		0.999	0.386	0.344
4	T2	0.117	0.117	0.999		0.101	**0.003**
5	T3	0.234	0.234	0.386	0.101		0.999
6	T4	**< 0.001**	**< 0.001**	0.344	**0.003**	0.999	

Table 7.8 continued

2,3-Heptanedione

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		**0.045**	0.741	0.900	**0.011**	**0.011**
2	C	**0.045**		0.976	0.920	0.294	0.219
3	T1	0.741	0.976		0.903	1.00	1.00
4	T2	0.900	0.920	0.903		0.262	0.206
5	T3	**0.011**	0.294	1.00	0.262		0.999
6	T4	**0.011**	0.219	1.00	0.206	0.999	

1-Pentanol

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		**0.022**	**0.035**	**0.034**	**0.024**	**0.025**
2	C	**0.022**		1.000	0.999	0.386	0.510
3	T1	**0.035**	1.000		0.962	**0.005**	**0.012**
4	T2	**0.034**	0.999	0.962		**0.002**	**0.010**
5	T3	**0.024**	0.386	**0.005**	**0.002**		0.951
6	T4	**0.025**	0.510	**0.012**	**0.010**	0.951	

Terpinen-4-ol

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		1.000	1.000	0.063	0.083	**0.004**
2	C	1.000		1.000	0.063	0.083	**0.004**
3	T1	1.000	1.000		0.063	0.083	**0.004**
4	T2	0.063	0.063	0.063		0.952	0.603
5	T3	0.083	0.083	0.083	0.952		0.999

Table 7.8 continued

Terpinen-4-ol

Treatment		1	2	3	4	5	6
		MF	C	T1	T2	T3	T4
6	T4	**0.004**	**0.004**	**0.004**	0.603	0.999	

n-Undecane

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		**0.047**	0.573	0.371	1.000	0.900
2	C	**0.047**		0.772	0.131	**0.047**	0.067
3	T1	0.573	0.772		0.928	0.573	0.751
4	T2	0.371	0.131	0.928		0.371	0.929
5	T3	1.000	**0.047**	0.573	0.371		0.900
6	T4	0.900	0.067	0.751	0.929	0.900	

2,5-Dimethylpyrazine

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		0.432	0.893	0.900	1.000	**0.020**
2	C	0.432		0.899	0.743	0.432	0.999
3	T1	0.893	0.899		0.999	0.893	0.834
4	T2	0.900	0.734	0.999		0.900	0.373
5	T3	1.000	0.432	0.893	0.900		**0.020**
6	T4	**0.020**	0.999	0.834	0.373	**0.020**	

Table 7.8 continued

2,6-Dimethylpyrazine

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		**0.006**	0.893	0.381	0.265	**0.026**
2	C	**0.006**		0.220	0.106	0.673	0.486
3	T1	0.893	0.220		0.998	0.889	0.686
4	T2	0.381	0.106	0.998		0.953	0.719
5	T3	0.265	0.673	0.889	0.953		0.999
6	T4	**0.026**	0.486	0.686	0.719	0.999	

2,3,5-Trimethylpyrazine

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		**< 0.001**	0.053	0.075	**0.032**	**0.005**
2	C	**< 0.001**		0.622	0.962	0.999	0.426
3	T1	0.053	0.622		0.999	0.966	1.000
4	T2	0.075	0.962	0.999		0.998	0.999
5	T3	**0.032**	0.999	0.966	0.998		0.961
6	T4	**0.005**	0.426	1.000	0.999	0.961	

2-Pentylfuran

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		**0.019**	0.981	0.599	0.599	0.599
2	C	**0.019**		**0.032**	**0.015**	**0.015**	**0.015**
3	T1	0.981	**0.032**		0.618	0.618	0.618
4	T2	0.599	**0.015**	0.618		1.000	1.000

Table 7.8 continued

2-Pentylfuran

Treatment		1 MF	**2** **C**	3 T1	4 T2	5 T3	6 T4
5	T3	0.599	**0.015**	0.618	1.000		1.000
6	T4	0.599	**0.015**	0.618	1.000	1.000	

2,3-Dihydrobenzofuran

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		1.000	1.000	**0.026**	**0.032**	**0.001**
2	C	1.000		1.000	**0.026**	**0.032**	**0.001**
3	T1	1.000	1.000		**0.026**	**0.032**	**0.001**
4	T2	**0.026**	**0.026**	**0.026**		0.349	0.130
5	T3	**0.032**	**0.032**	**0.032**	0.349		0.991
6	T4	**0.001**	**0.001**	**0.001**	0.130	0.991	

α-Terpineol

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		1.000	0.052	**0.003**	**0.015**	**0.005**
2	C	1.000		0.052	**0.003**	**0.015**	**0.005**
3	T1	0.052	0.052		**0.027**	**0.044**	**0.014**
4	T2	**0.003**	**0.003**	**0.027**		0.380	0.262
5	T3	**0.015**	**0.015**	**0.044**	0.380		0.999
6	T4	**0.005**	**0.005**	**0.014**	0.262	0.999	

Table 7.8 continued

1,2-Dimethoxybenzene

Treatment		1 MF	2 C	3 T1	4 T2	5 T3	6 T4
1	MF		0.166	0.893	0.265	0.096	**0.009**
2	C	0.166		0.905	0.690	0.504	0.143
3	T1	0.893	0.905		0.463	0.264	0.092
4	T2	0.265	0.690	0.463		0.999	0.999
5	T3	0.096	0.504	0.264	0.999		1.000
6	T4	**0.009**	0.143	0.092	0.999	1.000	

[1] The p-values were calculated by the Games-Howell test due to the Levene's test being highly significant; n= 6 referring all batches of raw salami batter (MF) and also 6 replications per treatment (C, T1-T4); p > 0.05= not significant; p ≤ 0.05= significant; p ≤ 0.01= significant; p ≤ 0.001= highly significant; SD= standard deviation; MF= raw warthog meat and pork fat without honeybush (day-0); C= 0%, T1= 0.125%, T2= 0.25%, T3= 0.375%, and T4= 0.5% of added honeybush after a 40-day ripening period.